

NOVEL ARTIFICIAL ACHILLES TENDON BASED ON TEXTILE STRUCTURES

PhD Thesis

Diana Raquel dos Santos Moraes

Dissertação submetida à Faculdade de Engenharia da Universidade do Porto para
obtenção do grau de Doutor em Engenharia Biomédica

Universidade do Porto
Faculdade de Engenharia
2018

This thesis was supervised by:

Professora Doutora Maria Ascensão Ferreira Silva Lopes (Supervisor)

Professora Auxiliar do Departamento de Engenharia Metalúrgica e de Materiais
da Faculdade de Engenharia da Universidade do Porto

Professor Doutor Rui Jorge Sousa Costa de Miranda Guedes (Co-supervisor)

Professor Associado do Departamento de Engenharia Mecânica
da Faculdade de Engenharia da Universidade do Porto

The host institutions of this thesis were:

- Faculdade de Engenharia da Universidade do Porto, Portugal:
 - Departamento de Engenharia Metalúrgica e Materiais;
 - Departamento de Engenharia Mecânica;
- Universidade do Minho, Portugal:
 - Departamento de Engenharia Têxtil;
 - Departamento de Física;
- Faculdade de Medicina Dentária da Universidade do Porto, Portugal;
- Lankhorst Euronete, Portugal.

The research described in this thesis was financially supported by:

Portuguese funds through FCT – Fundação para a Ciência e a Tecnologia in the framework of PhD grant SFRH/BD/88829/2012 and the Project UID/EMS/00285/2013.

Acknowledgements

Aos meus orientadores, professora Ascensão Lopes e professor Rui Guedes, agradeço a constante orientação e partilha de experiências e ensinamentos essenciais para a minha evolução profissional. A si professora, obrigada ainda pela amizade e partilha de conhecimentos não só científicos e profissionais mas também pessoais...obrigada pela sua constante preocupação relativamente à minha formação. A ambos agradeço ainda a liberdade de fazer as minhas escolhas no decorrer deste longo percurso, em que absorvi imenso conhecimento científico e me dotei de novas valências ao nível da minha postura perante qualquer desafio futuro a nível profissional ou pessoal.

Ao Dr. José Carlos Noronha e Dr. João Torres agradeço todos os conselhos, derivados da sua experiência e perspectiva médica e terapêutica, essenciais para o sucesso do trabalho desenvolvido. Agradeço ainda a outros profissionais de outras instituições de quem também recebi apoio científico e logístico, como o professor Filipe Vaz, professor Raúl Figueiro, professora Maria Helena Fernandes, Cláudia Lopes, Juliana Cruz, e Liliana Grenho. Tenho ainda que agradecer o apoio essencial a nível técnico e logístico que recebi por parte da empresa Lankhorst Euronete, Portugal. À Eng. Anabela Carrapatoso agradeço a disponibilidade e interesse pelo meu trabalho e a forma prestável como me abriu as portas para que pudesse usar vários equipamentos da empresa. Agradeço muito todo o apoio técnico e confiança em mim por parte do Eng. Nuno Lopes, e ainda toda a colaboração do Eng. Alcindo Lopes, Sr.Sérgio Morais e Eng. Pedro Sá.

Agradeço ao Miguel Rodrigues que mesmo longe consegue sempre ensinar-me algo novo e incentivar o meu espírito crítico. Agradeço à minha fiel companheira Sofia Alves, que bem perto ou bem longe, estivemos sempre em sintonia nos melhores e piores momentos. Agradeço ainda aos meus colegas de trabalho/gabinete pelo apoio e boa disposição, nomeadamente Paulo Pedrosa, Stéphanie Soares, Beatriz Vasconcelos, Patrique Fiedler, Cláudia Lopes, Sónia Simões, Cristiana Maia...

Por último, tenho que agradecer à minha principal fonte de inspiração: a minha família, a quem dedico esta tese...Obrigada pai e mãe por confiarem sempre em mim e nas minhas capacidades e pelos princípios que sempre me transmitiram e me ajudaram a ser mais forte e perseverante. Obrigada avó pelo carinho. Obrigada irmã por seres sempre capaz de me fazer rir encontrando a perspectiva mais positiva e divertida de qualquer situação. Obrigada João pelo constante carinho, apoio e incentivo e pela enooorme paciência...obrigada por me lembrares nos momentos difíceis quem sou, quem quero ser e onde quero chegar...

OBRIGADA a TODOS que me apoiaram neste caminho, que enriqueceram o meu
intelecto e espírito e me ajudaram a concretizar mais uma etapa!

Abstract

Attending to the incidence of tendon injuries worldwide affecting both young and old people, and to the fact that in some clinical cases there is not an efficient treatment approach, the present thesis focuses on the development of a biocompatible fibrous structure able to mimic the demanding *in vivo* mechanical performance of an Achilles tendon (AT) to be used as a prosthetic device to partially replace its function. For that, firstly, simple fibrous structures (braids) based on polypropylene (PP) or poly(ethylene terephthalate) (PET) multifilament yarns were developed using a textile technology-based technique. The different structures differ in yarn type, yarns number and braiding take-up rate, which consequently grant them different textile and physical properties. Those braids were then assembled in more complex textile structures (ropes) based on a core/shell architecture in order to accomplish the levels of load at failure, strain to failure, stiffness, creep and fatigue resistance observed in case of a native AT. Thus, all the structures were fully characterized by evaluation of several physical properties, such as porosity, swelling and wicking ability. Besides that, the structures mechanical behaviour was detailed evaluated by performing tensile, creep and fatigue tests.

Moreover, in order to improve the biological interaction of the optimized textile structures with the surrounding tissues after implantation, it was studied how to functionalize PP and PET surfaces in order to improve cell adhesion and proliferation. In case of PP, it was studied the immobilization of chitosan on its surface. In case of PET substrate, an ethylenediamine (EDA) based treatment was developed and optimized in order to graft (-NH₂) groups on the polymer surface. After both surface treatments, a physicochemical characterization of the surfaces was performed, besides the evaluation of the fibroblasts viability on them. Both treatments promoted a positive interaction with cells, however the EDA based treatment presented a more significant enhancement of cell adhesion and proliferation.

One of the developed ropes based on PET filaments revealed the most promising mechanical behaviour to partially replace the AT. The final prototype based on that optimized PET core/shell structure should be functional once implanted, namely its core should be bioactive to promote cell adhesion and its shell should be biopassive to prevent adhesion. So, the optimized EDA treatment was applied to the braids composing the structure core without compromising their mechanical properties, revealing a significant increase in fibroblasts adhesion and proliferation. Finally, for the biopassive purpose, a coating based on

polytetrafluoroethylene (PTFE) was performed on yarns composing the structure shell, what really prevented the cell adhesion on their surfaces, besides improving the abrasion resistance.

Resumo

Atendendo à incidência de lesões de tendões em todo o mundo, quer em jovens quer em idosos, e ao facto de em alguns casos clínicos não existir uma abordagem de tratamento eficiente, a presente tese foca o desenvolvimento de uma estrutura fibrosa biocompatível que seja capaz de mimetizar o desempenho mecânico *in vivo* de um tendão de Aquiles (TA) para ser usada como um dispositivo prótico e substituir parcialmente a sua função. Para tal, em primeiro lugar, foram desenvolvidas estruturas fibrosas simples (entrançados) à base de polipropileno (PP) ou poli (tereftalato de etileno) (PET), utilizando uma tecnologia têxtil. As diferentes estruturas diferem no tipo de fio, no número de fios e na velocidade de produção do entrançado, o que lhes confere, por conseguinte, diferentes propriedades têxteis e físicas. Esses entrançados foram depois associados em estruturas têxteis mais complexas (cordas) baseadas numa arquitetura núcleo/casca para atingir os níveis de carga/deformação à rutura, rigidez, fluência e resistência à fadiga que são observados no caso de um TA nativo. Assim, todas as estruturas foram completamente caracterizadas pela avaliação de várias propriedades físicas, tais como porosidade, inchamento e capilaridade. Além disso, o comportamento mecânico das estruturas foi avaliado detalhadamente através da realização de ensaios de tração, fluência e fadiga.

Além disso, com o objetivo de melhorar a interação biológica das estruturas têxteis otimizadas com os tecidos adjacentes após a implantação, estudou-se como funcionalizar as superfícies de PP e PET para melhorar a adesão e proliferação celular. No caso do PP estudou-se a imobilização de quitosano na sua superfície. No caso do PET desenvolveu-se um tratamento à base de etilenodiamina (EDA) para disponibilizar grupos (-NH₂) na superfície do polímero. Para ambos os tratamentos de superfície foi promovida a caracterização físico-química das superfícies, além da avaliação da viabilidade dos fibroblastos. Ambos os tratamentos promoveram uma interação positiva com as células, no entanto o tratamento baseado em EDA apresentou um aumento mais significativo da adesão e proliferação celular.

Uma das cordas desenvolvidas baseada em filamentos de PET revelou um comportamento mecânico muito promissor para substituir parcialmente o TA. O protótipo final, baseado nessa estrutura de PET otimizada, deve ser biologicamente funcional, nomeadamente o seu núcleo deve ser bioactivo para promover a adesão celular e a sua casca deve ser biopassiva para prevenir a adesão. Assim, o tratamento otimizado com EDA foi aplicado aos entrançados que compõem o núcleo da estrutura sem comprometer as suas

propriedades mecânicas, revelando um aumento significativo na adesão e proliferação dos fibroblastos. Finalmente, para o propósito biopassivo, foi realizado um revestimento à base de politetrafluoroetileno (PTFE) nos fios que compõem a casca da estrutura, o que realmente impediu a adesão celular nas suas superfícies, além de melhorar a resistência à abrasão.

Table of contents

Acknowledgements	v
Abstract.....	vii
Resumo.....	ix
Table of contents	xi
List of figures.....	xiii
List of tables.....	xix

Chapter 1

General Introduction

1.1. Motivation and objectives.....	3
1.2. Thesis outline	4
1.3. References.....	5

Chapter 2

Current approaches and future trends to promote tendon repair.....	9
2.1. Background.....	10
2.2. Structure and function of tendons	10
2.3. Tendon injuries and healing process.....	12
2.4. Current and future treatments for tendon injuries.....	15
2.5. Tendon tissue engineering enhancing strategies.....	22
2.6. Conclusion and future perspectives	24
2.7. References.....	25

Chapter 3

Characterization of polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament braided textile structures for Achilles tendon partial substitution	31
--	-----------

Chapter 4

Mechanical behaviour of ropes based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns for Achilles tendon partial substitution.....	61
---	-----------

Chapter 5

Surface functionalization of polypropylene (PP) by chitosan immobilization to enhance human fibroblasts viability	87
---	----

Chapter 6

Grafting of 1,2-ethylenediamine (EDA) on poly(ethylene terephthalate) (PET) substrates to enhance human fibroblasts viability	115
---	-----

Chapter 7

Bioactive and biopassive treatment of poly(ethylene terephthalate) (PET) multifilament textile yarns to improve/prevent fibroblasts viability	143
---	-----

Chapter 8

General discussion, conclusions and future work

8.1. General discussion and conclusions.....	177
8.2. Future work.....	183
8.3. References.....	184

List of Figures

Chapter 2

Current approaches and future trends to promote tendon repair

- Figure 2.1:** Histological images of a transverse section of a rabbit Achilles tendon. The blue spots represent tendon cells and the pink area the ground substance. (A) magnification of 40x; (B) magnification of 100x. Histological images of a longitudinal section of a rabbit Achilles tendon: (C, D) magnification of 100x 12
- Figure 2.2:** Mechanical behaviour of a tendon during the healing process..... 14

Chapter 3

Characterization of polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament braided textile structures for Achilles tendon partial substitution

- Figure 3.1:** Classification of braids attending to their architecture. 33
- Figure 3.2:** SEM images of the PP multifilament yarn: (A) 140x and (B) 2500x; and of the PET multifilament yarn: (C) 140x and (D) 2500x 37
- Figure 3.3:** X-ray diffractograms of the PP (A) and PET (B) multifilament yarns 38
- Figure 3.4:** Weight change of the PET yarn (A) and PP yarn (B) over the immersion time in water. Water height along the PP yarn or the braided structures (C) and PET yarn or the braided structures (D) over 10 min. Data are presented as mean $\pm$ SD 39
- Figure 3.5:** Optical microscopic images of PP braided structures: (A) 6YH; (B) 8YH; (C) 8YL; (D) 16YH. Optical microscopic images of PET braided structures: (E) 6YH; (F) 8YH; (G) 8YL; (H) 16YH..... 40
- Figure 3.6:** Braid angle of the textile structures based on PP yarns (A) and PET yarns (B). Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 6YH (*), 8YH (#) and 8YL (+)..... 41
- Figure 3.7:** Porosity (%) of the textile structures based on PP yarns (A) and PET yarns (B). Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 6YH (#), 8YH (+) and 8YL (•)..... 41
- Figure 3.8:** (A) Force-strain curves of the PP yarn and all braided structures. Tensile properties of the PP yarn and all braided structures: (B) stiffness, (C) load at failure and (D) strain to failure. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to yarn (*), 6YH (#), 8YH (+) and 8YL (•)..... 43

Figure 3.9: (A) Force-strain curves of the PET yarn and all braided structures. Tensile properties of the PET yarn and all braided structures: (B) stiffness, (C) load at failure and (D) strain to failure. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to yarn (*), 6YH (#), 8YH (+) and 8YL (•)	44
Figure 3.10: Representative curves of creep behaviour of PET based 16YH at different levels of force (A), in Log-Log scale (B). Representative curves of relaxation behaviour of PET based 16YH at different levels of strain (C), in Log-Log scale (D).....	45
Figure 3.11: Fatigue behaviour of PET based 16YH braid: normalized applied force (Fa=Applied Load/Failure Load) vs cycles to failure	46
 Chapter 4	
Mechanical behaviour of ropes based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns for Achilles tendon partial substitution	
Figure 4.1: The hierarchical fibrous structure of tendons	62
Figure 4.2: Optical microscopic images of braids and ropes: (A) PP_8YH braid; (B) outer part of C16B8YH_S16YL; (C) PP_16YH braid; (D) outer part of C16B16YH_S16YL; (E) PET_16YH braid; (F) outer part of C22B16YH_S16YL; (G) core/shell construction of C22B16YH_S16YL	66
Figure 4.3: Braid angle between yarns of braids and the ones composing the ropes shell. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 8YH (#), C16B8YH_S16YL (*) and C16B16YH_S16YL (+).....	67
Figure 4.4: (A) Representative force-strain curves of all PP based structures. Tensile properties of the PP 16YH braid and ropes: (B) load at failure, (C) strain to failure and (D) stiffness. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared with 8YH (#); 16YH (•); correspondent stiffness I (+); C16B8YH_S16YL (*) and stiffness II of C16B16YH_S16YL (x)	68
Figure 4.5: (A) Representative force-strain curves of the PET 16YH braid and C22B16YH_S16YL rope. Tensile properties of the PET 16YH braid and C22B16YH_S16YL rope: (B) load at failure, (C) strain to failure and (D) stiffness. Data presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared with 16YH (#); stiffness I (•); stiffness II (*) and stiffness II* (500-3500 N) (x).	69

Figure 4.6: Tensile properties of C16B16YH_S16YL after different numbers of cycles in fatigue tests: (A) load at failure and (B) strain to failure. Data are presented as mean $\pm$ SD.....	70
Figure 4.7: Tensile properties of C22B16YH_S16YL after different numbers of cycles in fatigue tests: (A) load at failure and (B) strain to failure. Data are presented as mean $\pm$ SD.....	70
Figure 4.8: Hysteresis results of PET_C22B16YH_S16YL: force-strain curves (A) and hysteresis percentage (B) after 1 and 10 cycles of sinusoidal loading between 500-3500 N and after 1, 10, 100 and 1000 cycles between 750-7500 N (data presented as mean $\pm$ SD, + $p < 0.05$ -significant difference compared with 10 cycles).....	71
Figure 4.9: (A) Representative curve of creep behaviour of PET_C22B16YH_S16YL rope over the 2000 s and the creep strain observed after reaching the required load level (B)	72
Figure 4.10: Nano-CT images of the C22B16YH_S16YL cross-section, in 2D (A) and 3D (B) mode, and a longitudinal view in 3D mode (C). Histological image of a transverse section of a rabbit AT (D).....	72
Figure 4.11: (A) Evolution of porosity percentage along 2 cm of length (layer number) of C22B16YH_S16YL. Porosity distribution (open spaces in red) inside the rope: longitudinal distribution based on data acquired from layer 1 until 124 (B) and from layer 745 until 868 (C); segmentation view (D); general view (E).....	73

Chapter 5

Surface functionalization of polypropylene (PP) by chitosan immobilization to enhance human fibroblasts viability

Figure 5.1: FTIR-ATR spectra of the untreated PP surface, Cs powder and PP samples surface after: (A) exposure to O_2 plasma over different times; (B) exposure to O_2 plasma over 5 min and treatment with 20% AAc and different Cs concentrations; (C) exposure to Ar pre-treatment over 2 min followed by O_2 plasma over 5 min and treatment with 20% AAc and two different Cs concentrations	93
Figure 5.2: Water C.A. variation on the PP surface after: (A) exposure to O_2 plasma over different times and to the Ar pre-treatment over 2 min followed by different times of O_2 ; (B) functionalization with different Cs concentrations after the O_2 plasma treatment over 5 minutes and 20% AAc grafting.....	94

Figure 5.3: Levels of acid orange absorbance: (A) for four different samples exposed to different plasma treatments and immersed in different Cs solution concentrations, as function of the AAc solution concentration; (B) for different Cs concentrations after the O ₂ plasma treatment over 5 minutes and 20% AAc grafting.....	97
Figure 5.4: SEM images of PP samples surface: (A) untreated PP; (B) PP treated with 20% AAc_0.4% Cs; (C) PP treated with 20% AAc_0.8% Cs; (D) PP treated with 20% AAc_2% CS and (E) PP treated with 20% AAc_4% CS.....	98
Figure 5.5: Representative optical profilometry images of PP samples surface and R _a values: (A) untreated PP; (B) PP treated with 20% AAc_0.4% Cs; (C) PP treated with 20% AAc_0.8% Cs; (D) PP treated with 20% AAc_2% CS and (E) PP treated with 20% AAc_4% CS.....	100
Figure 5.6: (A) Levels of resazurin fluorescence at different incubation time points after the cell seeding on untreated PP samples and PP_20% AAc_2 % Cs samples. Microscopic images of cells seeded on PP_20% AAc_2 % Cs samples after: (B) 1 day; (C) 3 days and (D) 7 days of incubation. Data are presented as mean ± SD. <i>p</i> < 0.05-significant difference comparing untreated PP over time (*); comparing PP_20% AAc_2 % Cs over time (+); comparing the untreated PP with PP_20% AAc_2 % Cs at day 3 (#).....	101
Figure 5.7: SEM images of cells seeded on PP samples surface over incubation time: (A1) untreated PP after 1 day; (A2) untreated PP after 7 days; (B1) PP treated with 20% AAc_2 % Cs after 1 day and (B2) PP treated with 20% AAc_2 % after 7 days.....	102

Chapter 6

Grafting of 1,2-ethylenediamine (EDA) on poly(ethylene terephthalate) (PET) substrates to enhance human fibroblasts viability

Figure 6.1: Reaction scheme between PET and EDA	117
Figure 6.2: FTIR-ATR spectra of the untreated PET surface and after O ₂ plasma treatment (A) or Ar plasma pre-treatment over 2 min followed by O ₂ plasma over different periods (B). FTIR-ATR spectra of the functionalized samples W5050 and E2050 previously exposed to different plasma treatments: (A) O ₂ plasma or (B) Ar plasma pre-treatment over 2 min followed by O ₂ plasma over 8 min.....	121

Figure 6.3: Water C.A. variation on the PET surface after: exposure to O ₂ plasma over different times and to the Ar pre-treatment over 2 min followed by different times of O ₂	122
Figure 6.4: (A) Levels of acid orange absorbance for untreated PET and all functionalized samples (using the previous plasma treatment based on O ₂ over 8 min). (B) Levels of acid orange absorbance for PET_W5050 and PET_E2050, using two different plasma treatments before immersion in EDA solution. Data are presented as mean ± SD. <i>p</i> < 0.05-significant difference comparing the same immersion conditions but with a previous different plasma treatment (+); comparing the plasma treatment based on O ₂ , but using different immersion conditions (*); comparing the plasma treatment based on Ar and O ₂ , but using different immersion conditions (#)	124
Figure 6.5: SEM images of PET samples surface: untreated PET at a magnification of 1000x (A1) and 5000x (A2); PET_W5050 at a magnification of 1000x (B1) and 5000x (B2); PET_E2050 at a magnification of 1000x (C1) and 5000x (C2).....	126
Figure 6.6: Representative optical profilometry images of PET samples surface before and after functionalization, as well the measured R _a values: (A) untreated PET; (B) PET_W5050; (C) PET_E2050	127
Figure 6.7: Levels of resazurin fluorescence at different incubation time points after the cell seeding on untreated PET samples and PET_W5050 samples. Data are presented as mean ± SD. <i>p</i> < 0.05-significant difference comparing untreated PET samples over time (*); comparing PET_W5050 samples over time (+); comparing the untreated and functionalized samples at each time point (#).....	128
Figure 6.8: SEM images of cells seeded on PET samples surface over incubation time: untreated PET after 1 day (A1), 3 days (A2) and 7 days (A3); (B1) PET_W5050 after 1 day (B1), 3 days (B2) and 7 days (B3).....	129

Chapter 7

Bioactive and biopassive treatment of poly(ethylene terephthalate) (PET) multifilament textile yarns to improve/prevent fibroblasts viability

Figure 7.1: Load at failure (N) (A) and strain to failure (%) (B) of all EDA grafted PET yarns. Data are presented as mean ± SD. <i>p</i> < 0.05-significant difference compared to untreated yarn (+)	151
Figure 7.2: Representative force – strain curves of EDA grafted PET yarns (A) and EDA grafted braid (B).....	152

Figure 7.3: FTIR-ATR spectra of the untreated PET yarn, EDA grafted yarns and braids (A), and PTFE coated yarns (B).....	153
Figure 7.4: SEM images of untreated PET yarns (A and B); PET yarns after the plasma activation (C); of PET yarns (that compose the braids) after EDA grafting, namely of EDA_E2050_30_Braid samples (D, E and F); PET yarns after PTFE coating, namely of PTFE_100 samples (G and H).....	154
Figure 7.5: EDS spectra of zone 1 (Z1), 2 (Z2) and 3 (Z3) assigned in SEM images of figure 7.4	155
Figure 7.6: Representative optical profilometry images of yarns filaments composing an untreated PET braid (A); an EDA_E2050_30m_Braid sample (B); and PTFE_100 sample (C)	156
Figure 7.7: XPS overview and detailed spectra of untreated and EDA treated PET braid.	158
Figure 7.8: PET_16YH braids after staining with fuchsin acid: (A) untreated 16YH braid and (B) EDA_E2050_30_16YH braid	159
Figure 7.9: Water C.A. (°) on PTFE coated yarns depending on concentration of the spraying solution. Data are presented as mean ± SD. $p < 0.05$ -significant difference compared to 30 g/L (#) and 50 g/L (*).	159
Figure 7.10: Levels of resazurin fluorescence at different incubation time points after the cell seeding on (A) non-grafted PET braids and EDA grafted PET braids; (B) on uncoated and PTFE coated yarns. Data are presented as mean ± SD. $p < 0.05$ -significant difference compared to untreated PET braid (#) at each time point.....	160
Figure 7.11: SEM images of fibroblasts seeded on EDA_E2050_30_Braid samples after 7 (A and B), 14 (D and E) and 21 (F and G) days of culture, and on untreated PET braid samples after 14 days of culture (C)	161

List of Tables

Chapter 2

Current approaches and future trends to promote tendon repair

Table 2.1: Description of the healing phases in a tendon regeneration process..... 14

Table 2.2: Commercially available products for tendon tissue regeneration..... 21

Chapter 3

Characterization of polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament braided textile structures for Achilles tendon partial substitution

Table 3.1: Production and textile parameters of the produced structures, using PP or PET multifilament yarns 34

Table 3.2: Permanent strain (ϵ_p) and recoverable strain (ϵ_r) for each creep level..... 46

Chapter 4

Mechanical behaviour of ropes based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns for Achilles tendon substitution

Table 4.1: Production and textile parameters of the produced structures, using PP or PET multifilament yarns 64

Chapter 5

Surface functionalization of polypropylene (PP) by chitosan immobilization to enhance human fibroblasts viability

Table 5.1: Samples name according to the used AAc and Cs concentrations..... 90

Table 5.2: Representative AFM micrographs and roughness parameters (R_a and R_q) of untreated PP surface and after exposure to different plasma conditions 96

Chapter 6

Grafting of 1,2-ethylenediamine (EDA) on poly(ethylene terephthalate) (PET) substrates to enhance human fibroblasts viability

Table 6.1: Samples name according to the EDA functionalization conditions 118

Table 6.2: AFM micrographs and roughness parameters (R_a and R_q) of the untreated PET and PET substrates exposed to different plasma treatments 123

Chapter 7

Bioactive and biopassive treatment of poly(ethylene terephthalate) (PET) multifilament textile yarns to improve/prevent fibroblasts viability

Table 7.1: Name of PET yarns or braids samples grafted with EDA, depending on EDA solution concentration, solvent, temperature and immersion time	147
Table 7.2: Name of PET yarns samples coated with PTFE, depending on the concentration of spraying solution	147
Table 7.3: Average roughness (R_a) values, measured by optical profilometry	156

Chapter 1

General introduction

1.1. Motivation and objectives

Tendon and ligament injuries have high incidence in an aged population, besides of course the sports people. Since their repair is technically demanding several efforts have been done to achieve reliable and efficient treatment solutions [1]. Therefore, the focus of this work goes along with the priorities of the EIP-AHA (European Innovation Partnership on Active and Healthy Ageing, European Commission) by contributing to European better quality of life, keeping the public active and mobile, reduce health care costs and enhance competitiveness to global market on the regenerative medicine by offering a solution to a current unsolved health problem [2].

Regarding age-related injuries of tendons, tendinopathy is a common clinical problem, mostly in case of rotator cuff and Achilles tendon (AT) [3, 4]. In a group of patients with shoulder pain evaluated, patients aging 65 years and older had a full-thickness rotator cuff tear prevalence of 22% and for each 10-year age increase, the tears odds significantly increases. Moreover, patients older than 60 years are at increased risk of Achilles tendonitis or even rupture [3]. Regarding sporting injuries, ranging from repetitive strain injuries to complete ruptures, are a very common health problem affecting adult population [5, 6]. In Finland, a population of 5 million experiences more than 200,000 acute sports-related tendon injuries yearly and the number of overuse injuries in the USA is estimated to be about 30–50% of all sports-related injuries [5]. Worldwide, the AT has been one of the most common sports-related injuries, a 2 in 100,000 individual tendon injury rate increased to a 12 in 100,000 individual injury rate in less than 10 years. In the USA, each year, about 11% of regular runners suffer from Achilles tendinopathy and in Europe it has been reported an incidence rate of 37.3 cases per 100,000 individuals [1, 7]. Therefore, this prevalence represents a high treatment cost to the healthcare system of each country [6].

Due to their inefficient healing ability in case of acute or chronic injury, tendons injuries are prevalent and debilitating impairments that affect life quality among adult population worldwide. In the last years, attending to the social and economic concern associated to the high prevalence of tendons injuries and the limited success of the available current treatments, such as surgical repair or grafts application, natural and synthetic scaffolds have been developed. Depending on injury extension those scaffolds to be used just as graft-augmentation devices, but in some clinical cases it may be needed to partially replace some extension of the damaged tendon. In market, there are already several commercial available products to be used as graft-augmentation devices. However, to partially replace the mechanical function of a tendon, just few development attempts have been reported [1, 8].

Therefore, the main challenge of this work is to develop a biocompatible structure with a certain architecture able to mimic the *in vivo* mechanical performance of an AT to be used as a prosthetic device to partially replace its function, allowing to recovery the locomotion and joint stability [1]. Hence, the structure must present a non-linear viscoelastic response to loading, with a good resistance to creep and fatigue for a long-term. Moreover, that structure surface must interact with local native cells in order to promote an appropriate biological integration of the implant in host body, allowing the neotissue ingrowth.

1.2. Thesis outline

The present document is a scientific article-based thesis, namely based in one article already published in a peer-reviewed international journal (*Annals of Biomedical Engineering*, DOI: 10.1007/s10439-015-1369-5 [1]) and other six articles that will be submitted for publication after the submission of a patent to the *National Institute of Intellectual Property (INPI)*. As such, the manuscript is divided into eight chapters, which are sequentially disposed according to the main completed phases to accomplish the established goals.

The present *Chapter 1* presents the motivation and main objectives of this work, as well as the thesis outline. *Chapter 2*, based on the already published article, besides reporting the state-of-the-art, it also addresses several theoretical issues essential for the work framework. Regarding the experimental results, *Chapter 3* is dedicated to the study of two different polymeric multifilament textile yarns, which compose different structures using a textile-based technology. The physical and mechanical properties of the yarns and related textile structures are fully characterized on this chapter. In *Chapter 4*, more complex textile structures based on some simple structures of the previous chapter are presented and their physical and mechanical properties are evaluated. *Chapter 5* and *6* are dedicated to the study of different bioactive surface treatments to be applied on substrates based on same polymers of yarns used in the developed structures, in order to improve the cell viability on those materials surface. In *Chapter 7*, a bioactive and another biopassive surface treatment are developed and applied to one of the used yarns. Finally, chapter 8 comprehends the general discussion, main conclusions and future work to even improve the performance of the developed prosthetic device.

1.3. References

- [1] Morais DS, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair, *Annals of Biomedical Engineering*. 2015; 43(9):2025-35.
- [2] Commission E. Innovation Union - A Europe 2020 Initiative, http://ec.europa.eu/research/innovation-union/index_en.cfm?section=active-healthy-ageing2015, accessed in December 2016.
- [3] McCarthy MM, Hannafin JA. The mature athlete aging tendon and ligament. *Sports Health: A Multidisciplinary Approach*. 2014;6:41-8.
- [4] Loeser RF. Age-related changes in the musculoskeletal system and the development of osteoarthritis. *Clinics in geriatric medicine*. 2010;26:371-86.
- [5] Maffulli N., Renstrom P., Leadbetter W. *Tendon Injuries-Basic Science and Clinical Medicine*: Springer; 2005, London.
- [6] Chen J., Xu J., Wang A., Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. *Expert review of medical devices*. 2009;6:61-73.
- [7] Hess G. W. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot & ankle specialist*. 2010;3:29-32.
- [8] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.

Chapter 2

**Current approaches and future trends to
promote tendon repair**

Current approaches and future trends to promote tendon repair

Morais D.S.^{1,2}, Torres J.^{3,4}, Guedes R.M.^{2,5}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200–319 Porto, Portugal

⁴ Hospital S. Joao, Alameda Prof. Hernâni Monteiro, 4200–319 Porto, Portugal

⁵ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

(Received 9 February 2015; accepted 16 June 2015; published online 30 June 2015)

Associate Editor Michael S. Detamore oversaw the review of this article.

Abstract

Tendons are composed by extracellular collagen fibres arranged in regular arrays and are responsible to transmit tensile forces from a muscle to a bone. Due to their poor healing ability, in some cases tendons injuries are debilitating impairments that affect life quality among adult population worldwide. In the last years, attending to the social and economic concern associated to the high prevalence of tendons injuries and the limited success of the available current treatments, several scaffolds have been developed. Some of these scaffolds are intended to be used as graft-augmentation devices and others to fully replace a damaged tendon. The synthetic ones present superior mechanical characteristics compared to biological scaffolds. However, attending to the specific tendons physiology, even the synthetic scaffolds still don't present the ideal mechanical properties to accomplish a complete and long-term functional tissue repair. Therefore, to enhance tendogenesis when using a tendon engineering approach, several methodologies have been developed to associate with scaffolds, including surface modification and cell seeding.

Key Words: Tendon injuries, tendon repair, graft-augmentation devices, prosthetic devices.

2.1. Background

Nowadays, occupational but mainly sporting injuries of tendons, ranging from repetitive strain injuries to complete ruptures, are among the most common health problem affecting adult population [4,29]. In Finland, a population of 5 million experiences more than 200,000 acute sports-related tendon injuries yearly and the number of overuse injuries in the USA is estimated to be about 30–50% of all sports-related injuries.²⁹ About 51% of people over the age of 80 years experience rotator cuff injury in the USA each year and 11% of regular runners suffer from Achilles tendinopathy [4,13]. Thus, this prevalence represents a high treatment cost to the healthcare system of each country. In Australia, direct medical costs regarding rotator cuff repair is estimated to be about \$250 million annually. In USA, treatments related with shoulder pain due to tendons injury cost to the government up to \$7 billion annually [4].

Tendon injuries are difficult to manage and although spontaneously healing can occur this often results in the formation of scar tissue, which has low functionality. In some cases, a simple suture backing together the tendon ends is done, in other cases, the used approach is a tissue replacement with auto or allografts, but these reveal disadvantages [16,38]. Therefore, driven by this market demand, several biological and synthetic scaffolds have been developed during the last 15 years. Some scaffolds are designed just to share mechanical loads with the native tissue or a biological graft, while others are developed to fully replace the tendon and gradually promote the native tissue regeneration [4,24,28]. Using a tissue engineering approach, specific cell types and growth factors can be associated to the scaffolds to improve the regeneration process and closely resembles the native tissue. The main challenge when designing scaffolds for tendon tissue engineering is to develop a structure that properly mimics the mechanical performance of the native tissue [22,27,38].

This review presents the structure and function of the tendinous tissue, the associated injuries and healing process, as well as the current treatment approaches, available commercial products, tendon tissue engineering materials under development and associated enhancing strategies.

2.2. Structure and function of tendons

Tendons are classified as dense regular connective tissues and they physically bind muscles to skeletal structures, allowing locomotion and joint stability. They are composed by numerous extracellular collagen fibres arranged in regular arrays in the extracellular matrix

(ECM) and few cells, as observed in figure 2.1 [10,18]. Two main types of fibroblasts are present in the tissue, tenoblasts (immature cells) and tenocytes (mature tenoblasts), which are responsible for the synthesis of the ECM components [22,29]. The dry weight of normal tendons generally consist of 65–80% collagen (mostly type I), and includes 1–2% elastin embedded in a ground substance.⁵¹ The ground substance is mainly composed by water and residual amounts of proteoglycans (aggrecan and decorin) and glycoproteins (fibronectin and tenascin-C), providing the structural support and relative gliding of the fibres [22,29]. Tropocollagen is the structural unit of type I collagen, being synthesized in tenoblasts as procollagen, which is then secreted and cleaved extracellularly [29,43]. Tropocollagen microfibrils assemble into fibrils, which finally assemble into a tendon fibre [29,43]. These fibres are arranged in regular parallel arrays, as observed in figures 2.1C and 2.1D, and associated in bundles, which are involved by a loose connective tissue membrane (endotenon), in accordance with the transverse sections of fibre bundles limited by a membrane observed in figures 2.1A and 2.1B. A tendon, a group of fibrous bundles, is surrounded by a thin connective tissue sheath (epitenon), which is even surrounded by other loose connective tissue layer (paratenon) [20,22,29]. Collagen fibrils present an elongated shape when they are stretched and a crimped, waveform appearance when they are not stretched, as observed in figures 2.1C and 2.1D. The fibrous crimp acts as a shock absorber along the tissue length and prevents the fibres damage when a slight longitudinal elongation occurs [21,22,29].

Tendons present a complex mechanical behaviour due to the complex hierarchical collagen fibrous structures, having as primary function the transmission of tensile forces from a muscle to a bone and acting as a buffer by absorbing external excessive forces to prevent muscle damage [22,29]. Tendons response to load is non-linear and anisotropic, presenting high mechanical strength, good flexibility and a viscoelastic behaviour, due to the viscous properties of the collagen fibres and ground substance, exhibiting force-relaxation, creep and mechanical hysteresis [22,29,36,43]. A typical stress– strain curve of an isolated tendon, in elongation-to-failure conditions, presents three different regions. In the first region, named as toe region, small forces result in a large lengthening due to the crimped collagen fibrous nature, and when the stress is released the crimped pattern and tendon length are restored [16,22,29,43]. In case of further elongation a second region, named as linear region, appears with constant and higher stiffness (curve slope), from about 2–4% of strain. After this region, if the elongation continues, collagen fibres start to fail in an unpredictable way causing tears in the tissue, leading to the total rupture [16,29,43].

The maximum force, maximum strain, stiffness and Young's modulus depend on the thickness and collagen content of the studied tendon type, patient gender and age [20,29,43]. Mechanical experiments using several tendons reported Young's modulus values in the range of 1–2 GPa; ultimate stress values in the range of 50–100 MPa; ultimate strain values of 4–10% and toughness values in the range of 1000–4500 J/kg [29,43].

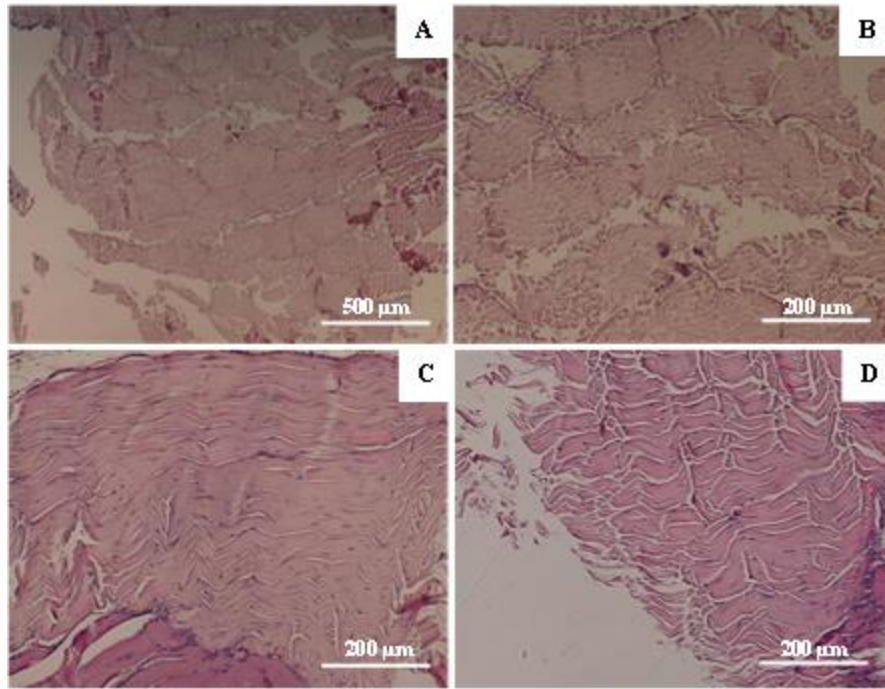


Figure 2.1: Histological images of a transverse section of a rabbit Achilles tendon. The blue spots represent tendon cells and the pink area the ground substance. (A) magnification of 40x; (B) magnification of 100x. Histological images of a longitudinal section of a rabbit Achilles tendon: (C, D) magnification of 100x.

2.3. Tendon injuries and healing process

Tendon injuries cause considerable morbidity on the damaged site, really affect the patient life quality and the consequent mechanical disability may last for several months. Tendons can present acute or chronic injuries caused by extrinsic or intrinsic causes, either alone or in combination. Acute injuries are mainly caused by extrinsic factors, while chronic injuries are mainly caused by intrinsic factors. Extrinsic factors include prescription drugs, bad nutrition, excessive loads, inadequate sports practices and environmental conditions; intrinsic factors include age, excessive body weight and biomechanical irregularities, such as muscle imbalance [14,38,43].

Tendinopathies are associated with pathological changes in a tendon caused by overload and overuse, which can cause a partial lesion or even total tissue rupture, and they

can be classified as tendinitis or tendinosis [29,38]. Tendinitis, often associated with acute injuries, consists in an inflammatory degeneration caused by micro-tears that appear when the musculotendinous unit suffers an acute overload by a tensile force too heavy and/or too sudden [38,43]. Tendinosis appears when there is a continued overuse of a tendon without time for tissue resting and healing, so they are associated with non-inflammatory tissue degeneration, consisting in degradation of collagen fibres caused by chronic overuse. Tendinosis is often clinically silent and its only manifestation may be a consequent tendon rupture [29,38,43]. Besides tendinopathies, tendons can also suffer a sudden complete rupture, which is an acute injury mainly caused by extrinsic factors, such as an excessive load or inadequate sports practice, although intrinsic factors can also contribute to that [29,43].

The most frequently damaged tendons are the ones subjected to the highest stresses, the ones most exposed to repeated strains (shear or compressive forces) and the ones with less vascularization [29,38]. Thus, the tendons that present more tendinopathies are the patellar, Achilles, rotator cuff and hand and foot flexor tendons. Patellar and Achilles tendons, for instance, are exposed to compressive loads generated in the tendon insertion in bone in case of knee flexion or ankle dorsiflexion, respectively. Shear forces are generated by the patellar tendon at various angles throughout the knee joint range, and the by the twist of Achilles tendon from proximal to distal as it inserts into the calcaneus [6,41]. Patellar tendinopathy is a very painful chronic degeneration, represents more than 30% of sports-related injuries [29,38,41]. Worldwide, Achilles tendinopathy has been described as one of the most common sports-related injuries, with an increase from 2 in 100,000 individual tendon injury rate to a 12 in 100,000 individual injury rate in less than 10 years [13]. Concerning the European community, it has been reported an incidence rate of 37.3 cases per 100,000 individuals [11]. The healing of tendons after an injury, is a very slow and inefficient process which never restores the biological and biomechanical properties completely, as observed in figure 2 [14,38]. This process requires to reestablish the tendon fibres and structure, and the gliding mechanism between the tendon and the surrounding structures.

The healing process can be divided in three main phases distinguishable by the cellular and biochemical processes, as presented in table 2.1, the inflammatory or exudative, the fibroplasia or proliferation and the remodelling or maturation phase. These phases can overlap and the efficiency and duration of each one can vary depending on the tissue loss degree involved in the injury, being expected a classic healing process for a low degree of tissue loss [14,34,38].

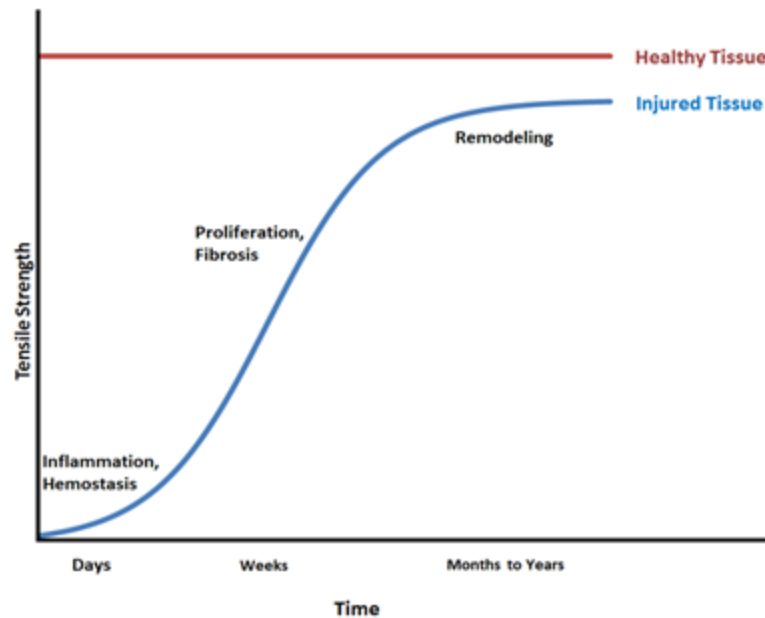


Figure 2.2: Mechanical behaviour of a tendon during the healing process.

Table 2.1: Description of the healing phases in a tendon regeneration process [13,15,33].

Healing phase	Description
Inflammatory/ exudative	• Shortly after injury, it is observed the formation of a hematoma and the consequent clot cause the release of several chemotactic factors; • Factors attract inflammatory cells, to the injury site, to resorb the clot, cellular debris and foreign body matter; • Fibroblasts are recruited to produce new collagen matrix, allowing the attachment of surrounding tissues to the damaged site, resulting in adhesion formation; • Recruited angiogenic factors promote the growth of blood vessels.
Fibroplasia/ proliferation	• It may take several weeks, starting with a reduction of the number of macrophages and mast cells; • Fibroblasts proliferate and synthesize in abundance type III collagen, proteoglycans and other ECM components, arranged in a random manner; • The new tissue is highly cellular and it contains large amounts of water.
Remodelling/m aturation	• It begins 6 or 8 weeks after injury and may take longer than 1 year; • There is a decrease in cellularity and matrix synthesis, and the synthesis of collagen type III is gradually converted to collagen type I; • Collagen fibres organize along the longitudinal tendon axis; • Over time, the interaction among the collagen structural units increases the tissue stiffness and tensile strength.

2.4. Current and future treatments for tendon injuries

Currently, tendon injuries, whether acute or chronic, are usually managed using two approaches, conservative, surgical or simultaneously both [22,38]. The conservative management, used as the first approach in some clinical cases of low degree injury to relieve the pain, involves rest, mechanical conditioning, corticosteroids injection, orthotics, ultrasound, laser or shockwave treatment [12,38]. However, due to the limited tendon ability for self-healing in some injury cases, this recovery approach requires long treatment periods, potential partial function loss and recurrent injury, failing in many cases [1,38]. So, when this approach does not result or is not appropriate attending to the extension of the lesion, such as in cases of total rupture, surgical intervention is used, suturing the injured ends together or fixing the tendon to the bone. But, in many cases this approach can also fail due to the poor healing ability of the degenerated tissue involved, which even after healing presents a loss of mechanical performance compared to the native tissue, being susceptible to rupture again and a repeated surgery is required. Thus, in order to improve the tissue repair after surgical intervention, some medical doctors have studied the injection of therapeutic cells or growth factors in the damaged tissue [12,22,38].

An important limitation of the tendons healing process, after tissue partial or total rupture and the consequent surgical procedure, is the formation of scarring and fibrous peritendinous adhesions [12,38]. Paratenon disruption, after tissue rupture and surgery, allows granulation tissue and fibroblasts from surrounding tissues to invade the damaged site [12,38]. Therefore, exogenous cells predominate over endogenous tenocytes, allowing the surrounding tissues to attach to the repairing tissue resulting in adhesions formation. Those adhesions inhibit the movement of the repairing tissue in its physiological space, which prevents the stress transmission into the tissue, impairing the collagen fibres alignment and consequently a normal tissue function [12,38]. Besides that, it is well demonstrated that the mechanical loading, inexistent in case of physical immobilization, is essential for tenocytes proliferation and differentiation during the healing process [12,34,38].

In some clinical cases, attending to the extensive damage, in spite of the several drawbacks associated to biological grafts they may be required to replace the damaged tendon. Autografts are only available in limited amounts, can induce morbidity, tissue laxity, poor tissue integration and functional disability at the donor site. Allografts and xenografts are not recommended once they may cause a harmful response from the immune system, causing rejection, and present the risk of disease transmission [2,12,22,38].

Therefore, due to the limitations of these treatment approaches, finding suitable scaffolds to promote the tissue regeneration *in vivo*, or even an artificial tendinous tissue by association of cells and growth factors to those devices *in vitro*, using a tissue engineering approach, is nowadays a clinical challenge [14,16,35,39]. In the last years, some commercial scaffolds, in the form of patches, have been used to provide some protection in case of soft tissue tears or to provide some mechanical support when associated with grafts. Besides that, to accomplish a treatment solution for extensive or total damage of the tissue, other scaffolds are being developed by researchers to be used as prosthetic devices to fully replace a tendon [24,31].

2.4.1. Scaffolds for tendons regeneration

When designing a scaffold for tissue regeneration there are some general characteristics that it must fulfill as an implantable device, as well as other essential specific requirements attending to the specific tissue intended to be engineered. First of all, a scaffold must be biocompatible and bio-functional, meaning the ability to promote cell adhesion, proliferation and differentiation, ECM components synthesis, and consequently the tissue formation and ingrowth [4,22]. Besides that, the scaffold can be biodegradable, presenting a gradually degradation to allow a controlled exposure of the new tissue to the local mechanical environment, promoting a new tissue with a structure and function more similar to the native tissue. In order to avoid a stress shielding phenomenon, the scaffold degradation rate should approximately match with the rate of the new tissue formation, allowing room for new tissue growth. For tendon tissue engineering, the scaffold ideally should mimic the fibrous crimped micro-architecture of the tissue to stimulate a good cell response and tissue mechanical performance, being capable of shielding the injured tissue from stress until the regenerated tissue is strong enough to withstand the applied stresses, promoting an appropriate integration [2,4,22,38]. Therefore, the major challenge of tendon tissue engineering is to find a material and structure that once associated with resident cells is able to mimic the stiffness, ductility, non-linearity and viscoelastic response of the native tissue [22,38]. In the last years, researchers have developed different biological, natural or synthetic scaffolds, however their mechanical properties need to be greatly improved, being the main problem the long-term failure due to fatigue, creep and wear [1,4,33].

Biological scaffolds are protein-based extracellular matrices derived from human or animal connective tissues and some commercially available are presented in “Scaffolds Available on the Market” section. Some tissues, like dermis, pericardium or small intestine

are processed to remove the tissue non-collagen components, preventing the rejection risk by the patient but retaining the natural support fibrous structure, providing a better and faster host cells integration. However, the used decellularization method may present some drawbacks, for instance, remaining chemicals that can be toxic to the host cells. Besides that, these scaffolds may present low mechanical properties and undefined degradation rate [22,28,38].

Regarding the natural scaffolds the most used polymers include collagen, chitosan, silk and fibrin. Despite their higher bio-functionality, biocompatibility and biodegradation, they present some limitations when comparing with the synthetic ones, such as the limited processability, the variety from batch to batch, the lower mechanical properties and the higher induction of antigenic and immunogenic reactions [22,27]. *Kwon et al.* produced a braided hybrid scaffold composed by collagen and silk which was tested in a rabbit model. The average tensile strength of the native tendons was about 220 ± 20 N, while the regenerated tendons presented values only of about 167 ± 30 N. Histologically, the regenerated tendons resembled the native tendons, presenting organized bundles of crimped fibres [23].

Attending to the low mechanical properties of biological and natural scaffolds, several synthetic scaffolds have been studied, being composed by several distinct polymers and co-polymers, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(ϵ -caprolactone) (PCL), poly(L-lactic acid) (PLLA) and poly(lactide-coglycolide) (PLGA). Synthetic scaffolds present several advantages comparing to the natural ones, namely the controllable and reproducible mechanical and chemical properties; the easy production of structures with different sizes, shapes and porosity levels; the predictable degradation rate and the low immunogenicity risk. Nevertheless, they also present some limitations, such as, the lack of functional chemical groups involved in cell adhesion and the possible release of acidic byproducts into the bloodstream during degradation, causing adverse reactions [2,4,22,27]. *Stoll et al.*, for instance, developed a PGA scaffold, which was inserted in an Achilles tendon defect of a rabbit model in conjugation with Achilles tenocytes seeded on it and fibrin. After 6 and 12 weeks of implantation, macroscopical scoring revealed very good results for the tenocytes seeded PGA + fibrin group when compared with the controls (untreated defects group) [46]. *Kolluru et al.* developed mineralized PLGA nanofibres for scaffolds for tendon-to-bone insertion sites, which incorporates mineralized and unmineralized tissues with different strengths and stiffnesses. These fibres revealed outstanding strain-hardening behaviour and ductility when stretched uniaxially, even in the presence of surface

mineralization [21]. In order to combine the bio-functionality of the natural polymers with the mechanical performance of the synthetic polymers researchers have also studied hybrid scaffolds composed by both types. *Xu et al.*, for instance, have recently worked with a PLLA/PCL-collagen scaffold seeded with tendon-derived stem cells for the regeneration of a patellar tendon defect in a rabbit model and the results were very promising [53].

2.4.2. Scaffolds available on the market

2.4.2.1. Graft-augmentation devices

Graft-augmentation devices are scaffolds, usually in the form of patches, used to provide immediate protection, after an injury involving tissue tears, in order to share mechanical stresses with the injured tissue, promoting better tissue regeneration. They have also been used with biological grafts until neovascularization is complete and the ingrowth tissue is already capable to withstand the forces associated to the body movement. These devices are intended to be temporary, just until the complete tissue recovery, and just to share part of the mechanical stresses. Thus, they must be degradable and it is not necessary and desirable that they maintain the mechanical performance for too long, once they must gradually transfer mechanical stress to the native tissue [28,38]. The most used commercially available products, as graft-augmentation devices, are presented in table 2.2.

Biological devices

Restore® membrane is produced from the small intestine submucosa (SIS) of pathogen-free pigs and it is an acellular scaffold composed by more than 90% of fibrillar collagen and 5–10% of lipids, carbohydrates and transforming growth factor- β (TGF- β) [4,28]. Some clinical studies have revealed this product efficacy in rotator cuff tendon repair, but other studies, such as *Sclamberg et al.* and *Walton et al.*, showed that it is not appropriate for tendon reconstruction [42,50]. The main side effect reported for Restore® is severe postoperative non-infectious edema probably due to the immune response to some residual porcine genetic material [8,15,30]. Other device produced from porcine SIS is CuffPatch®, which is an acellular scaffold composed by 97% of collagen and about 2% of elastin [4,8,28]. A mechanical study reported its physical properties as the weakest among five tested products and it has been reported as containing negligible amounts of porcine DNA [1,4,49].

TissueMend® is a single acellular layer, non-denatured collagen membrane, produced from foetal bovine dermis, with about 1 mm of thickness, and it has been reported to contain significantly higher genetic materials than Restore® and CuffPatch®, causing a higher risk of adverse effects [4,6]. BioBlanket®, another product obtained from bovine dermis, is an

acellular porous matrix composed by proprietary blend of fibrous and acid soluble collagens. It has presented a slow degradation time when compared to Restore[®], namely up to 1 year *in vivo*, which can be an advantage for the tendon healing process [4,47]. Zimmer[®] is also another acellular collagen scaffold sourced from porcine dermal tissue, mainly composed by type I collagen and residual amounts of type III collagen and elastin. This product has been successfully used for the reconstruction of soft tissues, as the abdominal wall, urinary tract and rotator cuff [4,45].

OrthoADAPT[®] is also an acellular collagen scaffold but derived from equine pericardium and its mechanical properties are very similar to CuffPatch[®], which presents, as already referred, the weakest properties among the most used commercial products [4,8]. GraftJacket[®] is an acellular dermal matrix produced from human cadaver by a technique that removes epidermal and dermal cells, retaining the native extracellular architecture and vascular channels. The scaffold is composed by type I, III, IV, and VII collagen, elastin, chondroitin, sulfate, proteoglycans and fibroblast growth factor (FGF) [4,28]. In the clinical studies using GraftJacket[®] for tendon repair no adverse effects, such as inflammatory response, edema or postoperative infection were reported. In addition, the product has revealed the strongest mechanical properties among the most used patches, contributing for tendon regeneration [1,8].

Synthetic devices

Gore-Tex[®] is a microporous patch composed by expanded polytetrafluoroethylene (ePTFE), allowing the host tissue incorporation. It has been used for different soft tissues, as rotator cuff and patellar tears reconstruction, without any adverse effect. In a clinical study, 28 rotator cuff tears were surgically repaired with this product and during the follow-up time patients presented pain relief, a good range of motion and muscle strength. SportmeshTM is a membrane composed by biodegradable polyurethane urea and it has been used for different tendons repair, such as Achilles and patellar, but mainly for the rotator cuff [4,28]. Leeds-Kuff PatchTM and Rota-LokTM are both composed by polyester and used for rotator cuff, but their structure and indications are different. The Leeds-Kuff PatchTM is a knitted polyester fabric used for reinforcement of the rotator cuff following or during repair by suture. The Rota-LokTM comprises a wide central scaffold which tapers to a dense thin cord at each end and it bridges the gap between the torn rotator cuff and the humeral head. This product is indicated for reconstruction of chronic massive, full thickness rotator cuff tears. AchilloCordPLUSTM is a densely woven flexible tubular structure with flat ends, allowing the device to be easily threaded through the eye of a probe, which is used to pull it through soft

tissue and bone tunnels. This product is mostly used in case of acute Achilles tendon ruptures during sport to promote support to the injured tissue, where an extended period of postoperative immobilization is undesirable [52].

2.4.2.2. Artificial prosthetic devices

The prosthetic devices are scaffolds developed to fully replace a tendon when any other treatment approach can not be used to repair the injured tissue. In the last years, researchers have been working to this purpose, however due to the demanding mechanical requirements of tendons there are very few devices on the market and no permanent and reliable artificial tendon exists clinically [4,38].

OrthoCouplerTM, presented in table 2.2, provides a fully functional re-attachment of a forcefully contracting muscle to prosthetic bone or living bone, in the absence of the adequate tendon. The device is composed by 16 needle-drawn bundles of several hundred to a few thousand fine poly(ethylene terephthalate) (PET) fibres. *In vivo* tests performed for the semitendinosus tendon, using goat models, revealed failure forces for the OrthoCouplerTM specimens 2.5 times higher than for the controls, in which injured tissue ends were attached with a commercial suture. Tissue integration was observed histologically without evidence of microencapsulation or compromise of vascularity [31]. Lars[®] Ligament and Leeds-Keio, presented in table 2.2, are synthetic polymeric structures mainly developed for ligaments replacement, but they have also been used for tendons replacement [4].

Lars[®] Ligament is a textile device made of PET fibres and it has been approved for a range of applications including anterior cruciate ligament (ACL) reconstruction and Achilles tendon repair by the health authorities of Canada, Europe and several other countries, but not in the USA. However, clinical studies with promising results have been mostly reported for ACL reconstruction, despite the lower mechanical strength of the device (998 ± 148 N) when compared to the native tissue (around 1246 ± 243 N). In these cases, only minor complications were observed, such as knee stiffness, severe complications such as synovitis, osteolysis and foreign body rejection have not been reported with Lars[®], revealing its good biocompatibility [4,24].

Leeds-Keio, which is also a textile structure composed by PET, was specifically designed for ACL reconstruction presenting a stiffness value of 200 N/ mm, which is similar to that of natural ACL, but it has been studied to be used as prosthesis for other ligament and tendon reconstruction. Clinical studies using Leeds-Keio for ACL reconstruction reported adverse events likes rerupture, tunnel enlargement, synovitis associated with polyester

particles, greater pivot-shift and laxity. However, positive results for ACL and some tendons replacement have also been published and it seems that the improvement of the surgical technique has allowed to achieve best results in last years [4,52].

Table 2.2: Commercially available products for tendon tissue regeneration [4,16,25,45,47].

Device	Company	Material	Application
Restore®	DePuy Orthopedics, USA	Porcine SIS	Rotator cuff, Achilles, patellar, biceps and quadriceps tendons
CuffPatch®	Arthrotek, USA	Porcine SIS	Rotator cuff
TissueMend®	Stryker Orthopedics, USA	Foetal bovine dermis	Rotator cuff, Achilles, patellar, biceps and quadriceps tendons
Bio-Blanket®	Kensey Nash Corp., USA	Bovine dermis	Soft tissues
Zimmer®	Zimmer, USA	Porcine dermis	Soft tissues
OrthADAPT®	Pegasus Biologic Inc., USA	Equine pericardium	Rotator cuff and Achilles tendons
GraftJacket®	Wright Medical, USA	Human cadaver dermis	Foot tendons
Gore-Tex®	WL Gore and Associates, USA	Poly(tetrafluoroethylene)	Soft tissues
Sportmesh™	Biomet Sports Medicine, USA	Poly(urethane) urea	Rotator cuff
Leeds-Kuff Patch™	Neoligaments, UK	Polyester	Rotator cuff tendon
Rota-Lok™	Neoligaments, UK	Polyester	Rotator cuff tendon
AchilloCordPLUS™	Neoligaments, UK	Poly(ethylene terephthalate)	Achilles tendon
OrthoCoupler™	Surgical Energetics LLC, USA	Poly(ethylene terephthalate)	Achilles tendon
Lars Ligament®	Lars, France	Poly(ethylene terephthalate)	Anterior cruciate ligament and Achilles tendon
Leeds-Keio	Neoligaments, UK	Poly(ethylene terephthalate)	Anterior cruciate ligament and Achilles tendon

2.5. Tendon tissue engineering enhancing strategies

In order to improve the tendon regeneration results promoted by the application of a natural or synthetic scaffold, some different strategies are under research in order to be associated to the structure, such as surface modification, cellular seeding, growth factor addition and mechanical stimulation.

2.5.1. Scaffolds surface functionalization

In tissue engineering, the optimization of the cell biomaterial interactions is essential for successful tissue regeneration. The scaffold must promote a good cell adhesion and proliferation and consequent synthesis and organization of the ECM components. The materials that lack chemical cell attachment groups can be modified to covalently link small molecules/chemical groups to their surfaces [22,27]. The cell-matrix interactions are typically mediated by cell surface integrin receptors, which bind to short peptides sequences, such as Arg-Gly-Asp (RGD). Therefore, RGD has already been incorporated in some scaffolds as *Chen et al.* have done for a silk scaffold, increasing the cell density by 250% and the production of type I collagen by 410% [3]. However, the modification of substrates with RGD peptides can also reveal some drawbacks. Firstly, not all ECM proteins have an RGD-binding site, needing the presence of other peptides to adhere to the substrate. Another disadvantage may be the too much adhesion between cells and the substrate, resulting in impaired cell motility [7,44]. *Lu et al.* showed that fibronectin coating of poly(lactic-coglycolic acid) (PLGA) and poly(lactic acid) (PLLA) scaffolds improved cellular attachment [27]. A disadvantage related with long chain proteins may be their random orientation, which causes a sizeable percentage of active sites to be in a position where cellular binding may be impossible [5]. *Sahoo et al.* coated a PLGA scaffold with electro-spun PLGA nanofibres, with diameters varying from 300 to 900 nm, enhancing cellular attachment, proliferation and ECM components production [39,40]. However, the promoted coating is heterogeneous due to the wide dispersion of the nanofibres diameter, varying from 300 to 900 nm, and this technique presents a low reproducibility.

2.5.2. Cell seeding and delivery of growth factors

In order to engineer a new tissue that properly mimics the required biological and mechanical properties of the native tissue, a scaffold may be functionalized *in vitro* or *in vivo* by seeding on it therapeutic cells and delivering growth factors. The introduction of the

appropriate cells and growth factors in the injury site will stimulate the resident tissue cells to proliferate and produce and organize the ECM components [2,27,38].

In the last years different cell sources have been used to this purpose, such as autologous or allogenic tenocytes, dermal fibroblasts, adult stem cells, cord blood stem cells (CBSCs) and embryonic stem cells (ESCs) [22,27,38]. An autologous source is obviously the most recommended to avoid immune reactions or morbidity, however, autologous tenocytes present some drawbacks, such as limited availability and donor site morbidity [2,38]. Regarding ESCs (totipotent cells) and CBSCs (pluripotent cells), although much studies have been performed using these cells, there is still little evidence of their *in vitro* capacity to differentiate into tenocytes. Besides that, the embryonic cells use may be limited due to the harvesting difficulty, ethical issues and regulation concerns [2,12,38]. Adult stem cells (multipotent), like bone marrow mesenchymal stem cells (BMSCs), have shown a good ability to differentiate into the tenocyte lineage when exposed to mechanical stress, as *Doroski et al.* and *Cheng et al.* have proved [5,9]. Another potential source of adult stem cells for tendon engineering is adipose tissue (ASCs), since harvesting causes minimal donor site morbidity, minimal patient discomfort, and provides substantial enough cell number to what is desired [2,26,35].

Growth factors are responsible for the regulation of some cell responses and they are truly involved in the tissue healing process after injury, in which they can stimulate cellular proliferation, differentiation and matrix production [2,22,27]. Thus, during the healing process, the delivery of these factors, at the required dosages in a temporal and spatial pattern, can improve clinical outcomes.

In the last years, the main growth factors that have revealed potential for tendon tissue engineering are: TGF- β , vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblastic growth factor (bFGF). bFGF and PDGF have been reported as the main mitogenic agents for fibroblasts, mainly when used in combination [2,12,22,48]. VEGF is essential for neovascularization in the healing process and in a later stage is responsible to establish and maintain the vasculature present in the endotenon and epitenon [16,17,37].

2.5.3. Mechanical load application

As already discussed, mechanical stress modulates the tendons cell behaviour, their metabolism, proliferation, orientation and matrix deposition. *In vivo*, tenocytes are continuously subjected to various mechanical loads promoted by muscular contraction or

other body movement. Therefore, in last years, people working on tendon tissue engineering have studied the *in vitro* application of mechanical stimulation to the scaffolds seeded with cells, in order to better mimic the *in vivo* conditions. To this purpose, several bioreactors have been developed, with uniaxial or multi-axial applied forces, and with cyclic strain, to provide a dynamic environment to guide tissue remodelling [2,27,38].

Messenger et al. concluded that the cyclic tensile strain interferes in the collagen fibres alignment, which aligned along the strain direction, while in the control group the fibres were randomly oriented [32]. *Juncosa Melvin et al.* observed an increase in the gene expression level of type I collagen when the developed stem cell-collagen sponge constructs were subjected to mechanical stimulation in a bioreactor. Moreover, the stimulated constructs also had 2.5 times the linear stiffness and 4 times the linear modulus of nonstimulated constructs [19]. *Laurent et al.* have incubated a novel poly(L-lactide-co-e-caprolactone) (PLCL) fibrous braided scaffold seeded with BMSCs in a tension-torsion bioreactor. That dynamic culture, when compared with a static culture, presented a high cellular adhesion and proliferation in 28 days [25].

2.6. Conclusion and future perspectives

Despite the progressing in the last 10 years at an increasingly rapid pace regarding the development of new natural and synthetic scaffolds, the ideal one for tendon repair has not been reported yet. Among the several challenges associated to those scaffolds, the main one is the required mechanical performance. Thus, to closely mimic that performance it may be interesting to mimic the complex native hierarchical crimped fibrous structures, developing strength assemblies of twisted or braided nanofibres. Moreover, it has been reported that some prostheses fail due to scaffold pullout at the ends.⁴ In future researchers must also study the best sealing system to each end of the prosthetic device attending to the tissue that it will be anchored to, bone and muscle. Besides that, it would be advantageous to adapt the scaffolds degradation rate to the new tissue formation rate, providing a progressive tissue ingrowth with adequate integration and mechanical stimulation.

Scaffolds can directly promote the tissue repair *in situ* due to the interaction with resident cells, or using a tissue engineering approach they can be colonized with therapeutic cells *in vitro* and then a living substitute is integrated with native counterparts *in vivo*. However, the success of therapeutic cells hybridization on scaffolds has not been conclusive maybe due to cell source and density or scaffolds used. Even so, the potential combination of

autologous stem cells, growth factors and mechanical stimulus with scaffolds should be further explored to improve the tissue repair.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project PEst-C/EME/UI0285/2011.

2.7. References

- [1] Barber, F. A., M. A. Herbert, and D. A. Coons. Tendon augmentation grafts: biomechanical failure loads and failure patterns. *Arthroscopy* 22:534–538, 2006.
- [2] Bassetto, F., A. Volpin, and V. Vindigni. Regenerative medicine for tendon regeneration and repair: the role of bioscaffolds and mechanical loading. In: *Biomaterials Science and Engineering*, edited by R. Pignatello. Padova: InTech, 2011, pp. 370–386.
- [3] Chen, J., G. H. Altman, V. Karageorgiou, R. Horan, A. Collette, V. Volloch, T. Colabro, and D. L. Kaplan. Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. *J Biomed Mater Res A* 67:559–570, 2003.
- [4] Chen, J., J. Xu, A. Wang, and M. Zheng. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. *Expert Rev Med Devices* 6:61–73, 2009.
- [5] Cheng, M. T., C. L. Liu, T. H. Chen, and O. K. Lee. Comparison of potentials between stem cells isolated from human anterior cruciate ligament and bone marrow for ligament tissue engineering. *Tissue Eng A* 16:2237–2253, 2010.
- [6] Cook, J. L., and C. Purdam. Is compressive load a factor in the development of tendinopathy? *Br J Sports Med* 3:54–58, 2011.
- [7] Cox, C. S. *Progenitor Cell Therapy for Neurological Injury*. New York: Springer, 2011.
- [8] Derwin, K. A., A. R. Baker, R. K. Spragg, D. R. Leigh, and J. P. Iannotti. Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, bio chemical, and cellular properties. *J Bone Joint Surg Am* 88:2665–2672, 2006.
- [9] Doroski, D. M., M. E. Levenston, and J. S. Temenoff. Cyclic tensile culture promotes fibroblastic differentiation of marrow stromal cells encapsulated in poly(ethylene glycol)-based hydrogels. *Tissue Eng A* 16:3457–3466, 2010.
- [10] Dudek, R. W. *High-Yield Histology* (2nd ed.). Philadelphia: Lippincott Williams & Wilkins, 2000.
- [11] European Agency for Safety and Health at Work. *OSH in Figures: Work-Related Musculoskeletal Disorders in the EU—Facts and Figures*. Luxembourg: Publications Office of the European Union, 2010.

- [12] Hampson K., N. Forsyth, A. Haj, and N. Maffulli. Tendon Tissue Engineering. In: Topics in Tissue Engineering UK, edited by N. Ashammakhi, R. Reis, and F. Chiellini, 2008.
- [13] Hess, G. W. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot Ankle Spec* 3:29–32, 2010.
- [14] Hoffmann, A., and G. Gross. Tendon and ligament engineering: from cell biology to *in vivo* application. *Regen Med* 1:563–574, 2006.
- [15] Iannotti, J. P., M. J. Codsi, Y. W. Kwon, K. Derwin, J. Ciccone, and J. J. Brems. Porcine small intestine submucosa augmentation of surgical repair of chronic two-tendon rotator cuff tears. A randomized, controlled trial. *J Bone Joint Surg Am* 88:1238–1244, 2006.
- [16] James, R., G. Kesturu, G. Balian, and A. B. Chhabra. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *J Hand Surg* 33:102–112, 2008.
- [17] Jenner, J. M., F. van Eijk, D. B. Saris, W. J. Willems, W. J. Dhert, and L. B. Creemers. Effect of transforming growth factor-beta and growth differentiation factor-5 on proliferation and matrix production by human bone marrow stromal cells cultured on braided poly lactic-co-glycolic acid scaffolds for ligament tissue engineering. *Tissue Eng* 13:1573–1582, 2007.
- [18] Johnson, K. E. *Histology and Cell Biology* (2nd ed.). Baltimore: Williams & Wilkins, 1991.
- [19] Juncosa-Melvin, N., K. S. Matlin, R. W. Holdcraft, V. S. Nirmalanandhan, and D. L. Butler. Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair. *Tissue Eng* 13:1219–1226, 2007.
- [20] Jung, H., M. Fisher, and S. Woo. Role of biomechanics in the understanding of normal, injured, and healing ligaments and tendons. *Sports Med Arthrosc Rehabil Ther Technol* 1:1–17, 2009.
- [21] Kolluru, P. V., J. Lipner, W. Liu, Y. Xia, S. Thomopoulos, G. M. Genin, and I. Chasiotis. Strong and tough mineralized PLGA nanofibers for tendon-to-bone scaffolds. *Acta Biomater* 9:9442–9450, 2013.
- [22] Kuo, C. K., J. E. Marturano, and R. S. Tuan. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports Med Arthrosc Rehabil Ther Technol* 2:20, 2010.
- [23] Kwon, S. Y., J. W. Chung, H. J. Park, Y. Y. Jiang, J. K. Park, and Y. K. Seo. Silk and collagen scaffolds for tendon reconstruction. *Proc Inst Mech Eng H* 228:388–396, 2014.
- [24] Lars®, 2013. Lars-ligaments, www.lars-ligaments.com, 25th October 2014.
- [25] Laurent, C., C. Vaquette, C. Martin, E. Guedon, X. Wu, A. Delconte, D. Dumas, S. Hupont, N. De Isla, R. Rahouadj, and X. Wang. Towards a tissue-engineered ligament: design and preliminary evaluation of a dedicated multi-chamber tension-torsion. *Bioreact Process* 2:167–179, 2014.
- [26] Little, D., F. Guilak, and D. S. Ruch. Ligament-derived matrix stimulates a ligamentous phenotype in human adipose-derived stem cells. *Tissue Eng A* 16:2307–2319, 2010.
- [27] Liu, Y., H. Ramanath, and D. Wang. Tendon tissue engineering using scaffold enhancing strategies. *Trends Biotechnol* 24:201–209, 2008.

- [28] Longo, U. G., A. Lamberti, N. Maffulli, and V. Denaro. Tendon augmentation grafts: a systematic review. *Br Med Bull* 94:165–188, 2010.
- [29] Maffulli, N., P. Renstrom, and W. Leadbetter. *Tendon Injuries-Basic Science and Clinical Medicine*. London: Springer, 2005.
- [30] Malcarney, H. L., F. Bonar, and G. A. Murrell. Early inflammatory reaction after rotator cuff repair with a porcine small intestine submucosal implant: a report of 4 cases. *Am J Sports Med* 33:907–911, 2005.
- [31] Melvin, A., A. Litsky, J. Mayerson, D. Witte, D. Melvin, and N. Juncosa-Melvin. An artificial tendon with durable muscle interface. *J Orthop Res* 28:218–224, 2010.
- [32] Messenger, M. P., M. Raifel, B. B. Seedhom, and S. J. Brookes. Enamel matrix derivative enhances tissue formation around scaffolds used for tissue engineering of ligaments. *J Tissue Eng and Regen Med* 4:96–104, 2010.
- [33] Meyer, I., T. Meyer, J. Handschel, and H. P. Wiesmann. *Fundamentals of Tissue Engineering and Regenerative Medicine*. Berlin: Springer, pp. 255–271, 2009.
- [34] Moshiri, A., and A. Oryan. Role of tissue engineering in tendon reconstructive surgery and regenerative medicine: current concepts, approaches and concerns. *Hard Tissue* 1:11, 2012.
- [35] Park, A., M. V. Hogan, G. S. Kesturu, R. James, G. Balian, and A. B. Chhabra. Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendonspecific markers. *Tissue Eng A* 16:2941–2951, 2010.
- [36] Peltonen, J., N. J. Cronin, L. Stenroth, T. Finni, and J. Avela. Viscoelastic properties of the Achilles tendon *in vivo*. *SpringerPlus* 2:212–220, 2013.
- [37] Pryce, B. A., S. S. Watson, N. D. Murchison, J. A. Staverosky, N. Dünker, and R. Schweitzer. Recruitment and maintenance of tendon progenitors by TGFbeta signalling are essential for tendon formation. *Development* 136:1351–1361, 2009.
- [38] Rodrigues, M. T., R. L. Reis, and M. E. Gomes. Engineering tendon and ligament tissues: present developments towards successful clinical products. *J Tissue Eng Regen Med* 7:673–686, 2013.
- [39] Sahoo, S., L. Ang, J. Goh, and S. Toh. Bioactive nanofibers for fibroblastic differentiation of mesenchymal precursor cells for ligament/tendon tissue engineering applications. *Differentiation* 79:102–110, 2010.
- [40] Sahoo, S., H. Ouyang, J. C. Goh, T. E. Tay, and S. L. Toh. Characterization of a novel polymeric scaffold for potential application in tendon/ligament tissue engineering. *Tissue Eng* 12:91–99, 2006.
- [41] Schindler, O. S. Basic kinematics and biomechanics of the patella femoral joint Part 2: the patella in total knee arthroplasty. *Acta Orthop Belg* 78:11–29, 2012.
- [42] Sclamberg, S. G., J. E. Tibone, J. M. Itamura, and S. Kasraeian. Six-month magnetic resonance imaging followup of large and massive rotator cuff repairs reinforced with porcine small intestinal submucosa. *J Shoulder Elbow Surg* 13:538–541, 2004.

- [43] Sharma, P., and N. Maffulli. Biology of tendon injury: healing, modeling and remodeling. *J Musculoskelet Neuronal Interact* 6:181–190, 2006.
- [44] Siebers, M., P. Brugge, X. F. Walboomers, and J. A. Jansen. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. *Biomaterials* 26:137–146, 2004.
- [45] Soler, J. A., S. Gidwani, and M. J. Curtis. Early complications from the use of porcine dermal collagen implants (Permacol) as bridging constructs in the repair of massive rotator cuff tears. A report of 4 cases. *Acta Orthop Belg* 73:432–436, 2007.
- [46] Stoll, C., T. John, C. Conrad, A. Lohan, S. Hondke, W. Ertel, C. Kaps, M. Endres, M. Sittinger, J. Ringe, and G. Schulze-Tanzil. Healing parameters in a rabbit partial tendon defect following tenocyte/biomaterial implantation. *Biomaterials* 32:4806–4815, 2011.
- [47] Sullivan, E. K., D. A. Kamstock, A. S. Turner, S. M. Goldman, and R. T. Kronengold. Evaluation of a flexible collagen surgical patch for reinforcement of a fascial defect: experimental study in a sheep model. *J Biomed Mater Res B* 87:88–94, 2008.
- [48] Thomopoulos, S., M. Zaegel, R. Das, F. L. Harwood, M. J. Silva, D. Amiel, S. Sakiyama-Elbert, and R. H. Gelberman. PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling. *J Orthop Res* 25:1358–1368, 2007.
- [49] Valentin, J. E., J. S. Badylak, G. P. McCabe, and S. F. Badylak. Extracellular matrix bioscaffolds for orthopaedic applications. A comparative histologic study. *J Bone Joint Surg Am* 88:2673–2686, 2006.
- [50] Walton, J. R., N. K. Bowman, Y. Khatib, J. Linklater, and G. A. Murrell. Restore[®] orthobiologic implant: not recommended for augmentation of rotator cuff repairs. *J Bone Joint Surg Am* 89:786–791, 2007.
- [51] Weinreb, J. H., C. Sheth, J. Apostolakis, M. McCarthy, B. Barden, M. P. Cote, and A. D. Mazzocca. Tendon structure, disease, and imaging. *MLTJ* 4(1):66–73, 2014.
- [52] Xiros, 2011. Neoligaments, www.neoligaments.com, 12th October 2014.
- [53] Xu, Y., S. Dong, Q. Zhou, X. Mo, L. Song, T. Hou, J. Wu, S. Li, Y. Li, P. Li, Y. Gan, and J. Xu. The effect of mechanical stimulation on the maturation of TDSCspoly(L-lactide-co-ε-caprolactone)/collagen scaffold constructs for tendon tissue engineering. *Biomaterials* 35:2760–2772, 2014.

Chapter 3

**Characterization of polypropylene (PP)
and poly(ethylene terephthalate) (PET)
multifilament braided textile structures for
Achilles tendon partial substitution**

Characterization of polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament braided textile structures for Achilles tendon partial substitution

Morais D.S.^{1,2}, Cruz J.³, Fangueiro R.³, Guedes R.M.^{2,4}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr.Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Centre for Textile Science and Technology, University of Minho, Portugal

⁴ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr.Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

Abstract

When tendon injuries present a too large damage extension, the conventional treatment options do not result, so a new prosthetic device needs to be developed to partially replace a tendon. Therefore, in this study, different braided textile structures based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns were developed, using a textile technology-based technique, in order to mimic the fibrous structure of tendons. Structures with different architectures were developed using different PP or PET yarns number and different braiding take-up rates, which consequently leads to different braid angles. The braids architecture influenced the porosity level, swelling profile, wicking ability and mostly their mechanical behaviour. It was observed that the load at failure of the braids was mainly controlled by the number of yarns, but the strain level was mostly influenced by the take-up rate and consequently by the braid angle. Regarding the stiffness level, it results from a combination of the yarns number and braid angle. The structure based on 16 PET yarns, produced with the highest take-up rate, revealed a very promising creep and force-relaxation behaviour for the final application, as well as a very interesting fatigue resistance.

Keywords: polypropylene, PP, poly(ethylene terephthalate), PET, braided structure, textile, tendon.

3.1. Introduction

Tendons are classified as dense regular connective tissues and they physically bind muscles to skeletal structures, allowing locomotion and joint stability. Nowadays, occupational but mainly sporting injuries of tendons, ranging from repetitive strain injuries to complete ruptures, are among the most common health problem affecting adult population. The number of overuse injuries in the USA, for instance, is estimated to be about 30% to 50% of all sports-related injuries, besides 51% of people over 80 years experiencing rotator cuff injury and 11% of regular runners suffer from tendinopathy in Achilles tendon (AT) [1-3]. In some cases, in which the extension of tendon damage is too large, the conventional treatments options, such as repair surgery or biografts, may be not efficient to recover the tissue functionality. Therefore, to accomplish a treatment solution for extensive damage of the tissue, other scaffolds are being developed by researchers to be used as prosthetic devices to partially replace a tendon [4].

As a connective tissue, tendons are minority composed by cells and mainly composed by extracellular matrix (ECM), which has high density of type I collagen fibres. The complex hierarchical fibrous structures of collagen are responsible for the very complex tissue mechanical properties. Tropocollagen molecules, which are the structural unit of type I collagen, are assembled into microfibrils, which then assemble to form fibrils, which finally assemble into a tendon fibre. These fibres are arranged in regular patterns, namely in parallel arrays [3, 5, 6]. Therefore, when designing a scaffold for tendon substitution it must be taken into account this complex fibrous micro-architecture of the tissue and the consequent mechanical performance.

A simple tubular braid is a textile structure formed by crossing a number of yarns/strands diagonally in such a way that each group of yarns/strands pass alternately over and under a group of yarns/strands laid up in the opposite direction [7-9]. Due to its structural integrity, durability, design flexibility and precision, braided structures have been used for different critical applications [7, 9]. Regarding the braiding pattern, which consists of the intersection repeat of the yarn groups, as presented in figure 3.1, these structures may be classified as diamond (1/1 repeat), regular (2/2 repeat), which is the most used, or Hercules (3/3 repeat). Besides that, the braided structures can even be categorized as biaxial or triaxial, according to the orientation of the constituent yarns/strands, as observed in figure 3.1. In general, both types of braids have two sets of braider yarns/strands placed in the clockwise and counter-clockwise directions (typically each yarn/strand aligned in the bias direction), whereas triaxial braids also have an additional set of strands aligned in the direction of braid

axis [8-10]. In general, the tensile properties of braided structures are strongly dependent on their architecture and the kinematics and mechanics involved in the braid formation [7, 8].

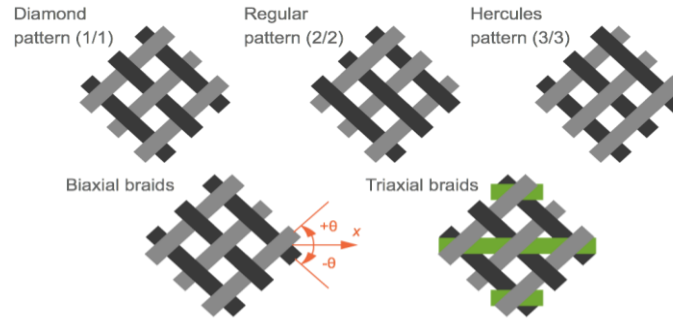


Figure 3.1: Classification of braids attending to their architecture [11].

As base materials for scaffolds for tendons repair substitution, different natural and synthetic polymers have been studied [4, 12]. The natural polymers, in spite of may present a better biocompatibility and bioactivity than the synthetic polymers, they usually fail due to their inappropriate mechanical performance [4, 13]. Therefore, in this study different braided textile structures, based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns, were produced using a textile technology-based technique. Those fibrous structures will afterwards be associated and arranged to form a more complex structure and mimic the complex fibrous micro-architecture of tendons and consequently their mechanical behaviour. The used synthetic fibres are among the most popular in textiles for different applications, since the 1950s, and they present an interesting force-strain curve shape for the purposed application [14]. Moreover, both polymers have been used in different medical devices, such as abdominal meshes biomaterials, presenting a good biocompatibility level [15, 16].

3.2. Materials and methods

3.2.1. Textile structures production

PP and PET multifilament yarns, with a linear density of 1200 and 1112 dtex respectively, were purchased from *Sarla Europe, LDA* (Portugal) to produce different biaxial braided structures on a vertical braiding machine with 16 carriers, under controlled process conditions. For each yarn type, four different braided structures were produced, using always the same pattern (1/1) but different yarns number (6, 8 and 16) and/or take-up rate (H and L), as presented in table 3.1. The take-up rate was changed by using change gears and two

different levels were used, the higher level (H) was of 3.94 cm/s and the lowest level (L) was of 1.44 cm/s.

Table 3.1.: Production and textile parameters of the produced structures, using PP or PET multifilament yarns.

Polymeric material	Textile structure	Number of filaments (yarn)/ yarns (braid)	Take-up rate	Braids/cm	Linear density (tex)	Tenacity (N/tex)
PP	Yarn	137 ± 4	---	---	120	0.62
	6YH	6	H	---	679 ± 5	0.60
	8YH	8	H	1.2 ± 0.1	1103 ± 4	0.51
	8YL	8	L	3.2 ± 0.2	1183 ± 5	0.48
	16YH	16	H	2.2 ± 0.1	1862 ± 6	0.58
PET	Yarn	162 ± 4	---	---	111	0.66
	6YH	6	H	---	682 ± 3	0.56
	8YH	8	H	1.4 ± 0.1	903 ± 5	0.65
	8YL	8	L	3.5 ± 0.2	943 ± 7	0.59
	16YH	16	H	2.4 ± 0.1	1835 ± 5	0.64

3.2.2. Physicochemical characterization

Scanning electron microscopy (SEM) analysis was performed, using a *FEI Quanta 400 FEG ESEM* microscope, in order to analyze the surface morphology of PP and PET yarn filaments and estimate the yarns diameter. The SEM images were acquired using the secondary electrons mode and the filaments elemental composition was evaluated by energy dispersive X-ray spectroscopy (EDS).

The X-ray diffraction (XRD) analysis of the PP and PET yarns was performed using a *Bruker AXS D8 Discover* diffractometer, in mode θ -2 θ , using a step size of 0.04 ° and an integration time of 1 s. For the identification of each crystalline phase and estimation of the crystallinity percentage ($\%x_c$), it was used the *EVA* software using the equation: $\%x_c = \left(\frac{A_c}{A_c + A_a} \right) \times 100$, where A_c is the area of the crystalline phase and A_a is the area of the amorphous phase of the diffractograms.

The swelling profile of the PP and PET yarns was evaluated by immersion of the yarns in phosphate-buffered saline solution (PBS, pH 7.4), using 5 samples of each. Firstly, each sample was weighed before immersion (W_o), about 0.1 g. Afterwards, the samples were placed in a mesh and immersed in the PBS solution and incubated at 37 °C, for 15 or 30 min.

The mesh was then removed and left 5 min air drying before weighting (W_f) the PP or PET yarn. The weight change (ΔW) of each sample represents the water absorption and was calculated using the equation: $\Delta W (\%) = \frac{W_f - W_o}{W_o} \times 100$. The vertical wicking ability of the PP and PET yarns and braided structures was evaluated, according to *Fangueiro R. et al*, using 5 samples of each polymer of 20 cm length, which were suspended vertically with its bottom end dipped in a reservoir of distilled water with a color agent [17]. The bottom end of each specimen was clamped with a clip to ensure that it could be immersed vertically at a depth of 3 cm into the colored water. The wicking heights, measured every minute over 10 min, were recorded.

The yarn-on-yarn abrasion was evaluated, according to CY 1503-00 from *Cordage Institute International Standard* [18], in a system where two portions of one yarn are intertwined (3 intertwisting wraps), they rub against one another under controlled tension due to the cyclic motion (72 rpm) imposed by rotation of a crank to which the yarn is attached. The tension is imposed by a weight of 2% of the yarn tensile maximum force and the number of cycles until the yarn failure was recorded for 5 samples.

All the textile structures were observed in triplicate by optical microscopy in order to estimate their diameter and their braid angle through the images treatment by the software *J image*. In order to assess the total porosity volume of each textile structure, 3 samples with 20 cm length were cut and the geometrical volume (V_g) was calculated considering each sample as a cylinder. Each sample was weighted (m) to determine the theoretical volume (V_t) if the cylinder was composed by 100% of PP or PET ($V_t = \frac{m}{\rho}$). Thus, the porosity percentage (P) can be calculated in accordance with the equation: $P (\%) = \frac{V_g - V_t}{V_g} \times 100$.

3.2.3. Static mechanical behaviour

The tensile properties of the yarns and braids were evaluated by performing uniaxial tensile tests (5 samples of each) on *Lloyd instruments LR 30K* equipment, in displacement control, up to rupture. The initial distance between grips was defined as 235 mm and the displacement rate as 8.5 mm/s. Mechanical properties such as stiffness (N/mm), load at failure (N) and strain to failure (ϵ , %) of all structures were measured.

The tensile properties of the PP and PET yarns were also evaluated after possible degradation by hydrolysis. For that, yarn samples were immersed in 4 mL of PBS (pH 7.4) (5 samples of each) and their initial weight was previously recorded. All samples were incubated at 37 °C and under 60 rpm agitation. After 15 days, 1, 2, 4, 8, 12 and 18 months,

the PP and PET yarn samples were removed, dried at room temperature, weighted and subjected to a tensile test with the parameters indicated before.

3.2.4. Time-dependent mechanical behaviour

3.2.4.1. Creep and relaxation tests

In creep tests, PET 16YH braid samples were uniaxially loaded to 30, 50 or 70% of their load at failure in 20 s and kept at this level for about 1000 s. After this holding phase, they were ramped down reaching the 0 N force after another 1000 s. In case of relaxation tests, PET 16YH braid samples were stretched to 30, 50 or 70% of their strain to failure at failure in 20 s and then kept at this level for about 1000 s. After this holding phase, they were ramped down reaching the 0% strain after another 1000 seconds. Both tests were performed in triplicate for each percentage on *Instron ElectroPuls E1000* equipment with a load cell of 2 kN and the initial distance between grips was defined as 235 mm.

3.2.4.2. Monotonic cyclic tests

In fatigue tests, PET 16YH braid samples, constantly being hydrated with PBS (pH 7.4), were exposed to a sinusoidal load until failure or in limit up to 1 million cycles. The applied sinusoidal load, at a frequency of 5 Hz, was defined using a maximum load corresponding to 30, 50, 65 or 70% of their load at failure and a minimum load corresponding to 10% of the load at failure being used. The tests were performed in triplicate for each percentage on *Instron ElectroPuls E1000* equipment with a load cell of 2 kN and the initial distance between grips was defined as 235 mm.

3.2.5. Statistical analysis

Experimental data is presented as mean $\pm$ SD (standard deviation). Statistical analysis of data was performed using the one-way ANOVA test with Bonferroni post hoc analysis, using the software SigmaStat 3.5. The differences were considered to be significant at a level of $p < 0.05$.

3.3. Results

3.3.1. Physicochemical characterization

3.3.1.1. SEM analysis

In figures 3.2.A/3.2.B and 3.2.C/3.2.D it is possible to observe, by SEM analysis at two different magnifications (140x and 2500x), that the PP and PET yarns are composed by several

filaments, which are all aligned in parallel. In figures 3.2.B and 3.2.D, it is possible to observe the surface morphology of the PP and PET filaments, which is smooth and homogeneous, being detectable only some punctual defects. Besides that, as expected, the acquired EDS spectra revealed the high level of C content in PP filaments and in case of PET filaments the presence of C and O (data not showed). Based on SEM images the yarns diameter was also estimated, being about $989.0 \pm 1.7 \mu\text{m}$ in case of PP and about $830.8 \pm 1.6 \mu\text{m}$ in case of PET.

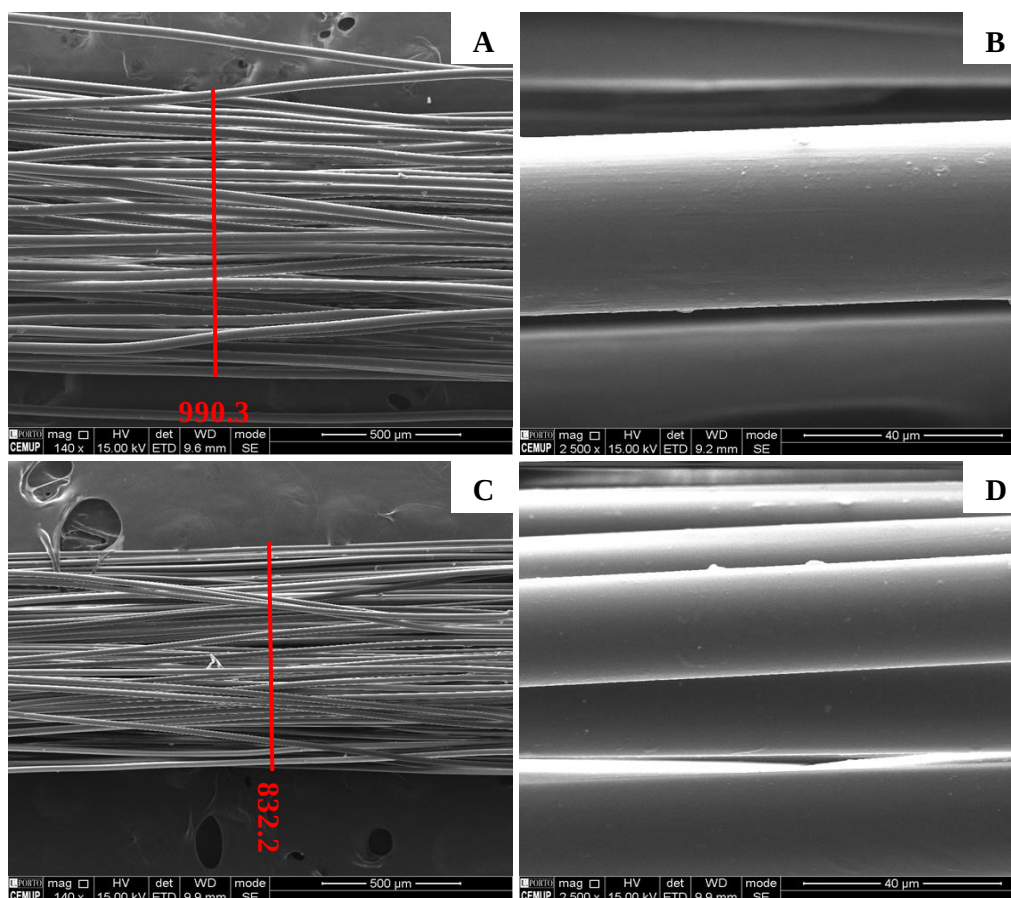


Figure 3.2: SEM images of the PP multifilament yarn: (A) 140x and (B) 2500x; and of the PET multifilament yarn: (C) 140x and (D) 2500x.

3.3.1.2. XRD analysis

In figure 3.3.A, it is presented the XRD spectrum of the PP yarn, which presents peaks at positions 2θ of about 14° , 17° , 18.5° and 21.8° . The crystallinity percentage ($\%x_c$) for the PP yarns according to the XRD data was estimated as about $61.2\% \pm 1.1$.

Figure 3.3.B it is presented the XRD spectrum of the PET yarn, which presents peaks at positions 2θ of about 17.6° , 22.8° and 25.6° . The $\%x_c$ for the PET yarns according to the XRD data was estimated as about $55.0\% \pm 3.2$.

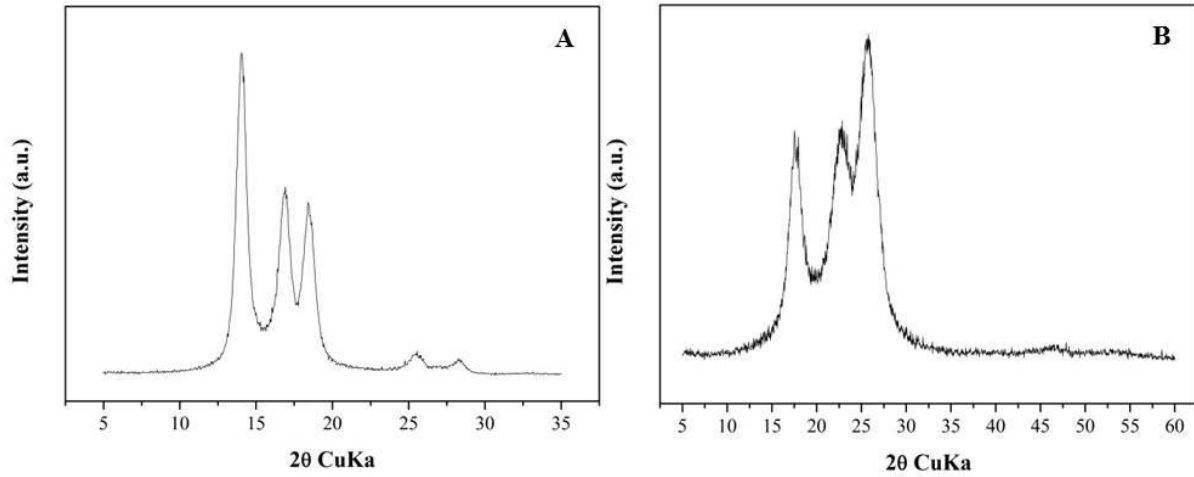


Figure 3.3: X-ray diffractograms of the PP (A) and PET (B) multifilament yarns.

3.3.1.3. Yarns swelling profile

Figures 3.4.A and 3.4.B show the weight change of the PP and PET yarn respectively, after immersion in water over 30 min. As observed, after the first 15 min there is an increase of the yarn weight of about 270% in case of PP and about 135% in case of PET. After 30 min of immersion there is no significant change in weight for both yarns.

3.3.1.4. Yarn-on-Yarn abrasion test

Regarding the abrasion resistance of the used multifilament yarns, PP yarns were able to withstand about 959 ± 160 cycles and PET yarns a much greater number of cycles, namely about 14742 ± 1142 .

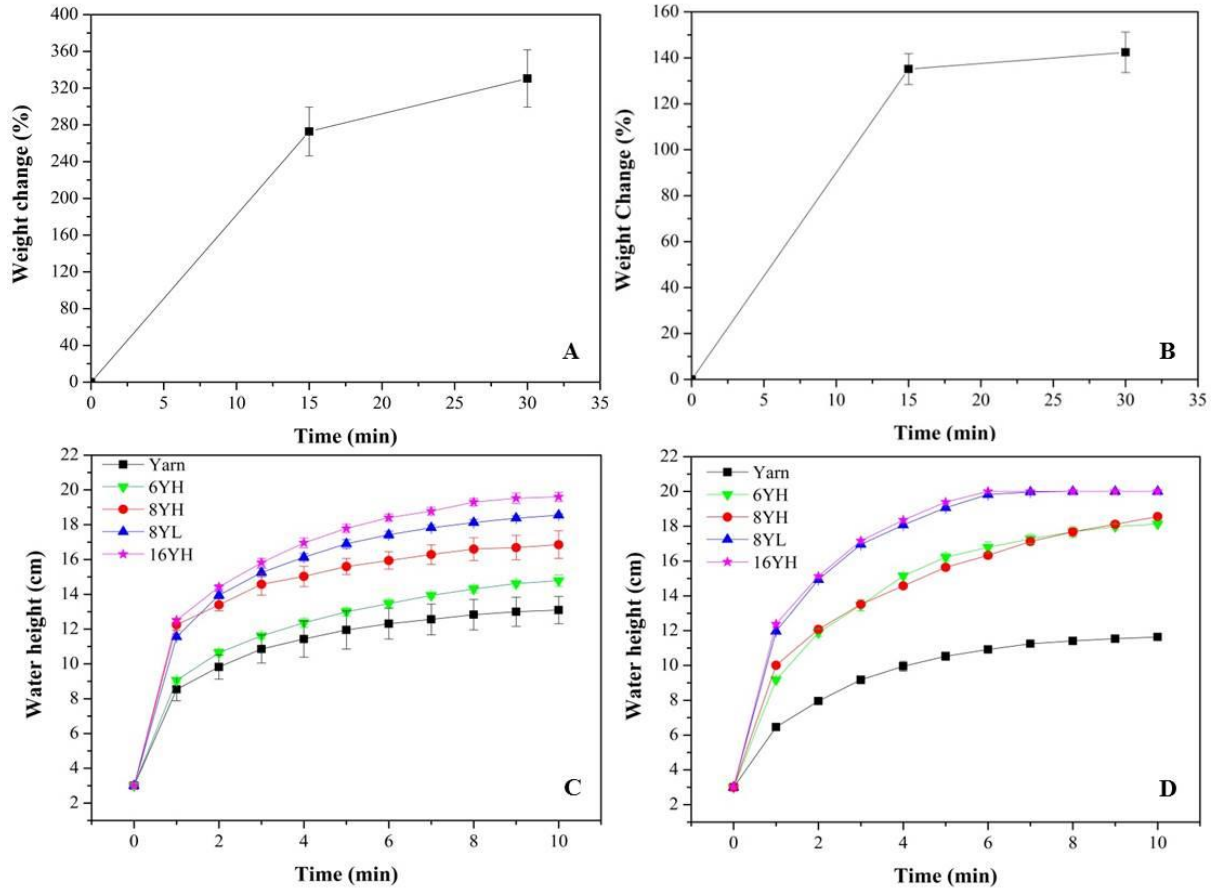


Figure 3.4: Weight change of the PET yarn (A) and PP yarn (B) over the immersion time in water. Water height along the PP yarn or the braided structures (C) and PET yarn or the braided structures (D) over 10 min. Data are presented as mean $\pm$ SD.

3.3.1.5. Optical microscopy

Figure 3.5 presents the optical microscopic images of all braided structures based on PP (figures 3.5.A, 3.5.B, 3.5.C and 3.5.D) or PET yarns (figures 3.5.E, 3.5.F, 3.5.G and 3.5.H), which may be classified as biaxial attending to the orientation of the constituent yarns and as diamond, in case of braids with 6 and 8 yarns, and as regular, when using 16 yarns, attending to the braiding pattern. In this figure 3.5 it is possible observe the braids architecture and how the multifilament yarns are arranged among them. Using these images the braids diameter, presented in figure 3.5, and braids/cm, presented in table 3.1, was estimated. As presented, for the same yarn and take-up rate, both parameters tend to increase as the number of yarns in the structure increases. Besides that, comparing the same braided structure but with different yarn type, the diameter is higher when using the PP yarn. Regarding the braids/cm, when using the same yarns number, it is higher when the take-up rate is lower.

Moreover, also based on optical microscopic images, the braid angle was calculated, which represents the acute angle that each yarn makes with the braid longitudinal line, as marked with red lines. As observed in figure 3.6, for the same take-up rate, as the number of yarns increases the braid angle also increase using any one of the yarns, however for the same structure the braid angle is higher when using the PP yarn. In case of PP (figure 3.6.A), when the yarns number increases from 6 to 16 the angle rises from about $6.9^\circ \pm 0.4$ to $16^\circ \pm 1.6$. In case of PET (figure 3.6.B), when the yarns number increases from 6 to 16 the angle rises from about $4.6^\circ \pm 0.4$ to $13.4^\circ \pm 0.9$. When using 8 yarns of any one of the materials, the braid angle is significantly higher when the take-up rate is lower (8YL).

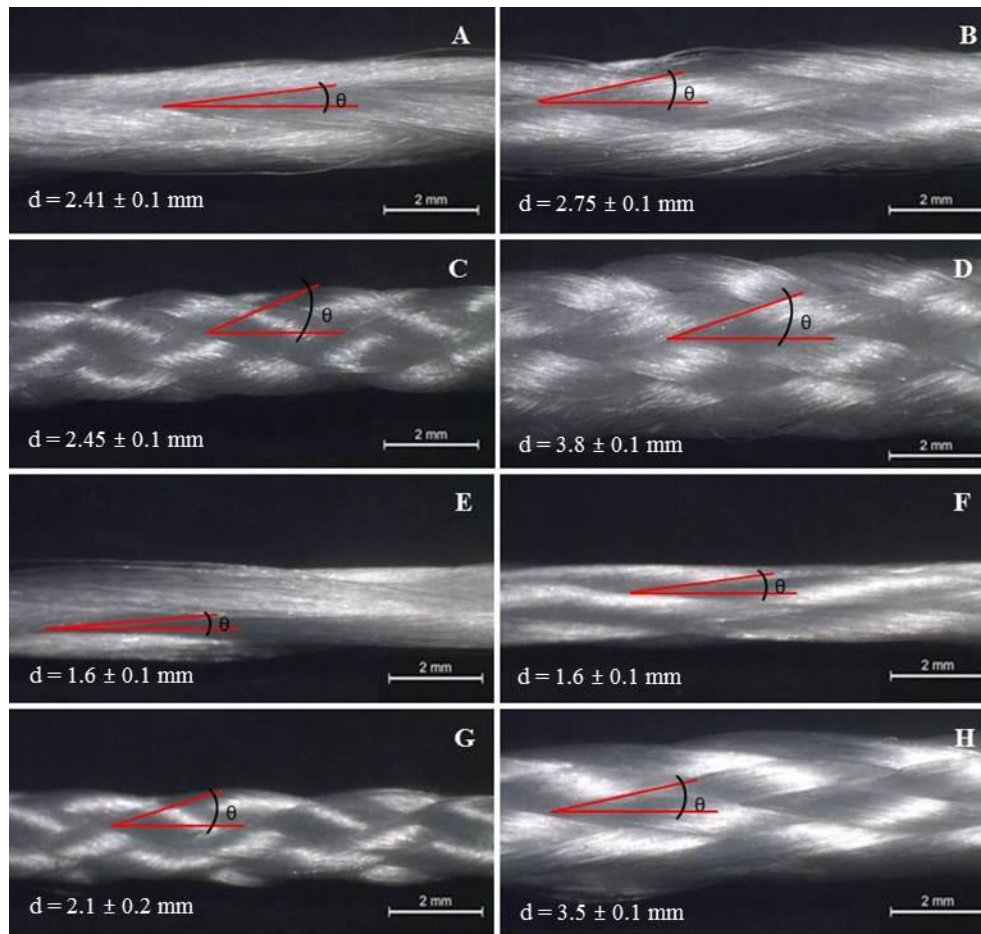


Figure 3.5: Optical microscopic images of PP braided structures: (A) 6YH; (B) 8YH; (C) 8YL; (D) 16YH. Optical microscopic images of PET braided structures: (E) 6YH; (F) 8YH; (G) 8YL; (H) 16YH.

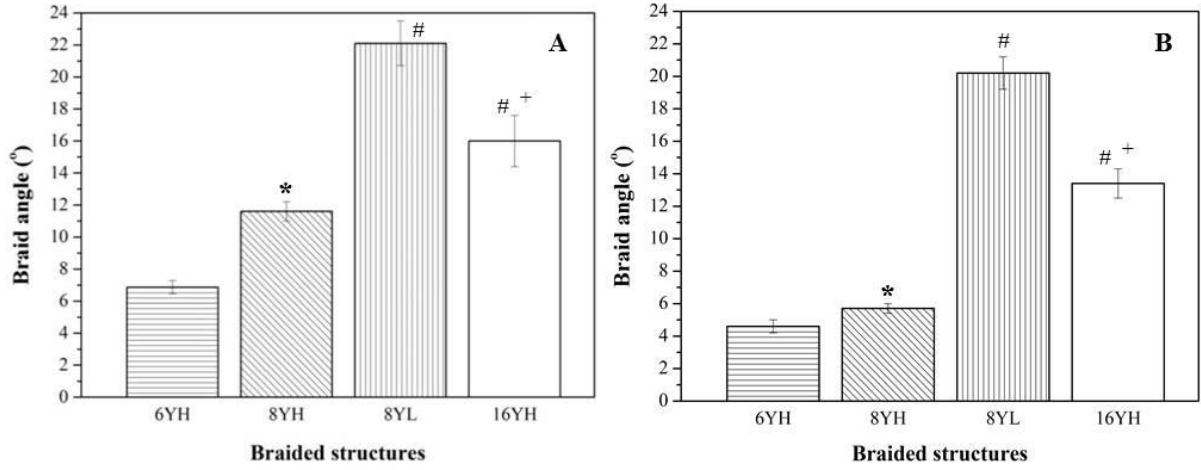


Figure 3.6: Braid angle of the textile structures based on PP yarns (A) and PET yarns (B). Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 6YH (*), 8YH (#) and 8YL (+).

3.3.1.6. Braids porosity

In figure 3.7 is presented the porosity level of each braided structure using the PP (figure 3.7.A) or PET yarns (figure 3.7.B). As observed, for both yarns, the porosity level does not present a significant change as the number of yarns or take-up rate changes. Even so, using each one of the yarns, the highest porosity level is observed for the 16YH structure, which is about 88% in case of PP and 85% in case of PET.

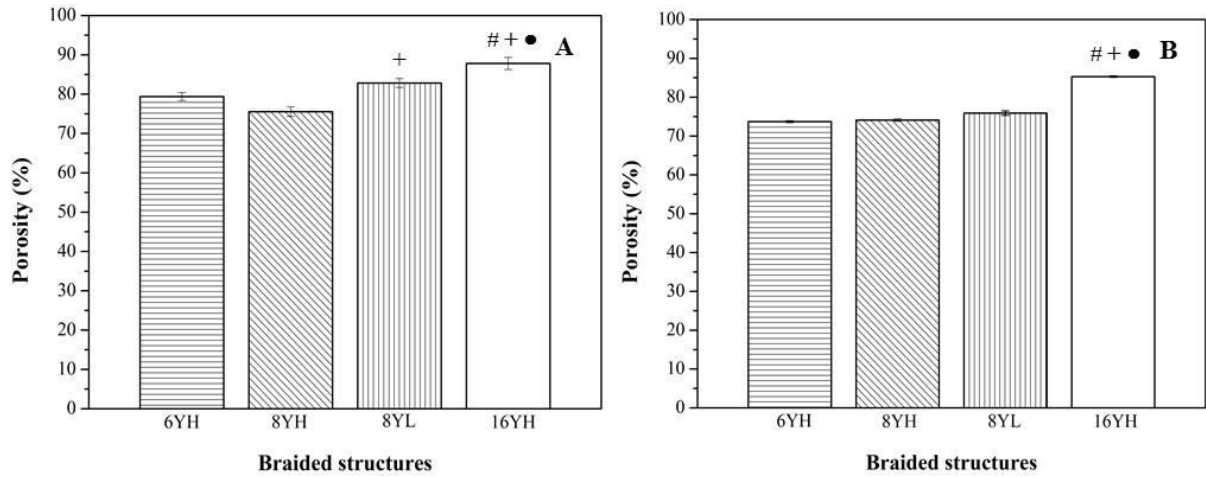


Figure 3.7: Porosity (%) of the textile structures based on PP yarns (A) and PET yarns (B). Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 6YH (#), 8YH (+) and 8YL (•).

3.3.1.7. Wicking profile

In order to evaluate the yarns and braids wicking profile, in figures 3.4.C and 3.4.D is presented the water height level along the yarns or braids, which increases over time for all PP and PET based textile structures. In case of PP, at the first minute, the yarn and the 6YH braid present a water height of about 8 cm, which is lower than the height in case of 8YH, 8YL and 16YH braids, which present a similar value of about 12 cm. Until about 4 min, the yarn and the 6YH braid present always a very similar height and the other 3 braids present similar values among them. However, after those 4 min the water height becomes significantly different among the yarn and all braids, being observed the highest water height for the 16YH braid, then for 8YL, 8YH, 6YH and finally for the yarn. Regarding the PET structures, at the first minute, the yarn presents the lowest water height of about 6 cm, then the 6YH and 8YH braids present a value of about 10 cm and the highest value of about 12 cm is observed for 8YL and 16YH. After the first minute, over time, the water height relation among the yarn and braids remains the same, the yarn presents always the lowest value, the 6YH and 8YL present a middle value and the 8YL and 16YH present the highest height value.

3.3.2. Static mechanical behaviour

Figure 3.8.A represents the force-strain curves of tensile tests of the PP yarn and braids, where is possible to observe that all braids present curves with a similar shape. However, they present different values of load at failure, as observed in figure 3.8.C, and strain to failure, as observed in figure 3.8.D. The yarn presents the lowest force at failure and regarding the braids, for the same take-up rate, the force increases as the number of yarns in braid increases (6YH, 8YH and 16YH). Comparing the braids with the same number of yarns with different take-up rate (8YH and 8YL), the load at failure is very similar between them. Regarding the strain at failure, when the yarns number increases for the same take-up rate there is no significant difference among the braids. However, between 8YH and 8YL there is a significant difference in strain to failure, being higher in case of 8YL. Moreover, in figure 3.8.B it is possible to observe the stiffness difference among the braids, which increases when the number of yarns increases, but decreases when the take-up rate is lower for the same yarns number.

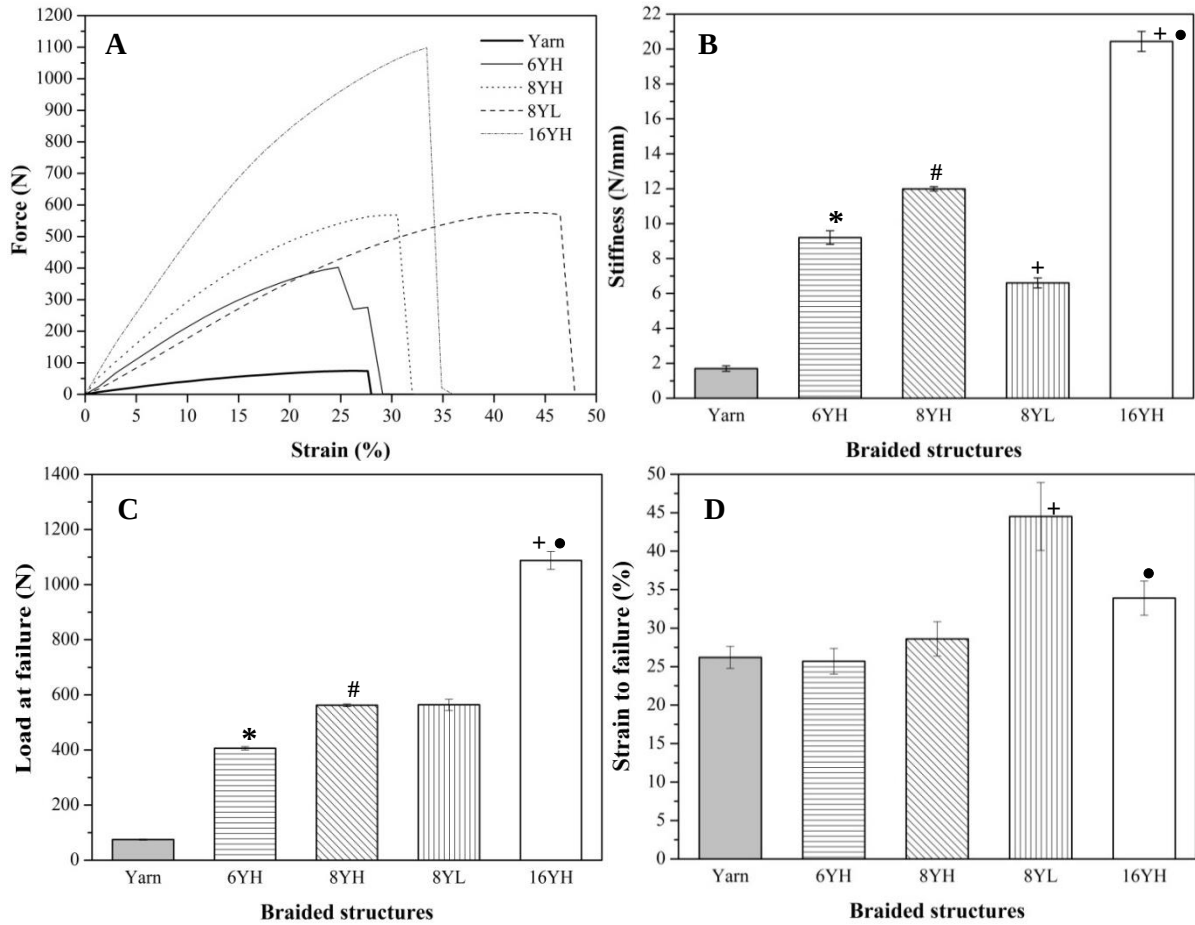


Figure 3.8: (A) Force-strain curves of the PP yarn and all braided structures. Tensile properties of the PP yarn and all braided structures: (B) stiffness, (C) load at failure and (D) strain to failure. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to yarn (*), 6YH (#), 8YH (+) and 8YL (•).

Regarding PET braids, the force-strain curves are presented in figure 3.9.A. It is possible to observe that all braids present curves with a similar shape, but with different values of load at failure, as observed in figure 3.9.C, and strain to failure, as observed in figure 3.9.D. The yarn presents the lowest force at failure and regarding the braids, for the same take-up rate, the load at failure increases as the number of yarns in braid increases (6YH, 8YH and 16YH). Comparing the braids with the same number of yarns but with different take-up rate (8YH and 8YL), the load at failure is very similar between them. Regarding the strain at failure, when the yarns number is the same but using a different take-up rate, there is no significant difference among the braids. Moreover, in figure 3.9.B it is possible to observe the stiffness difference among the braids, which increases with the number of yarns and the take-up rate.

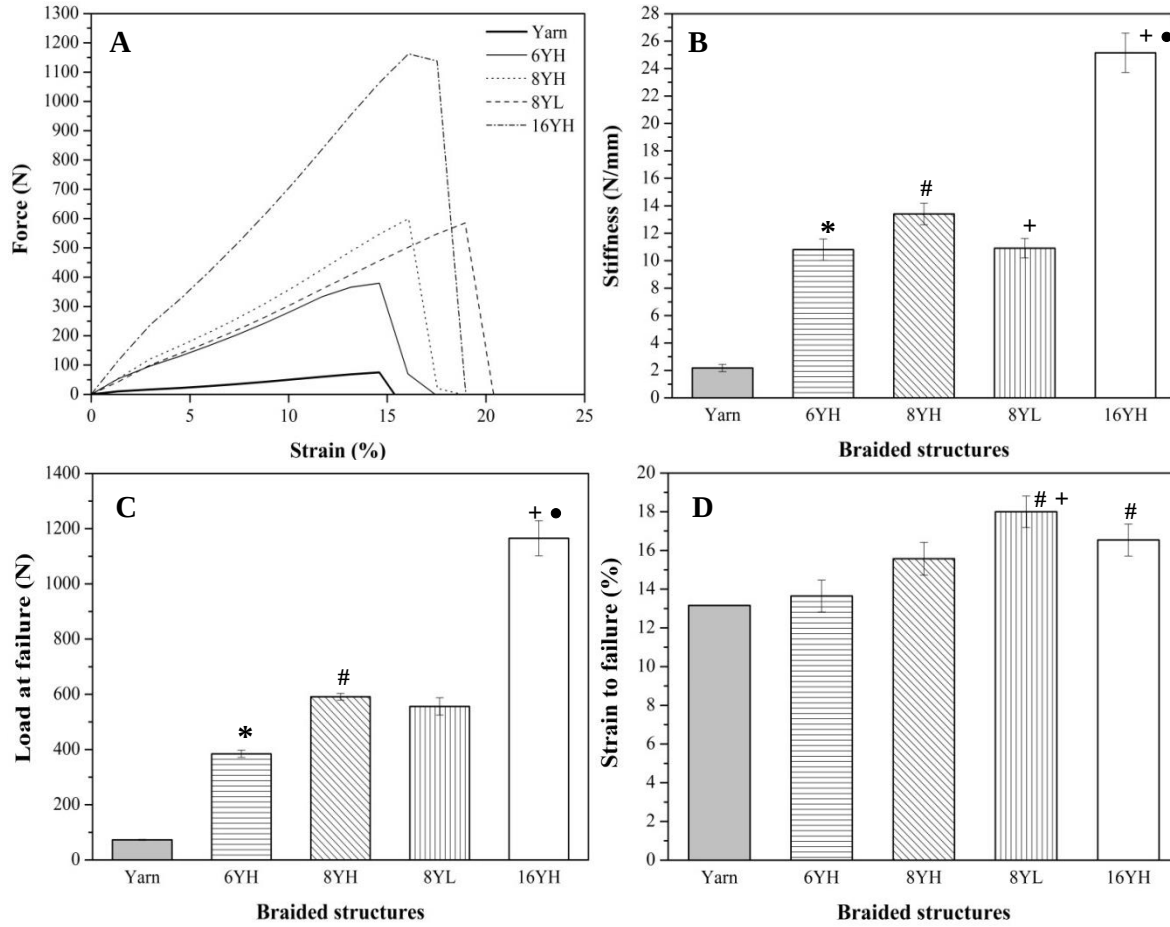


Figure 3.9: (A) Force-strain curves of the PET yarn and all braided structures. Tensile properties of the PET yarn and all braided structures: (B) stiffness, (C) load at failure and (D) strain to failure. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to yarn (*), 6YH (#), 8YH (+) and 8YL (●).

It should also be referred that after immersion in PBS, for degradation evaluation, the yarns samples do not revealed significant weight change and their tensile properties remained constant (data not show).

3.3.3. Time-dependent mechanical behaviour

3.3.3.1. Creep and relaxation tests

Figures 3.10.A and 3.10.C show the results of creep and relaxation behaviour of the 16YH structure composed by PET yarns. As observed in figure 3.10.A, as higher is the applied force on the structure higher is the reached strain level, which slightly increases over the 1000 s of loading. This behaviour is evident when plotting the most linear region of the different creep curves in a Log-Log scale, where is possible to observe the very low and similar creep rate for all force levels. The values of the permanent strain (ϵ_p), which is very

similar for all force levels, and the recoverable strain (ϵ_r), which increases when the force level increases, are presented in table 3.2.

Regarding the relaxation behaviour, as observed in figure 3.10.C, as higher is the applied force on the structure higher is the reached load level, which slightly decreases over the 1000 s. This behaviour is evident when plotting the most linear region of the different creep curves in a Log-Log scale, where is possible to observe the low and similar relaxation rate for all strain levels.

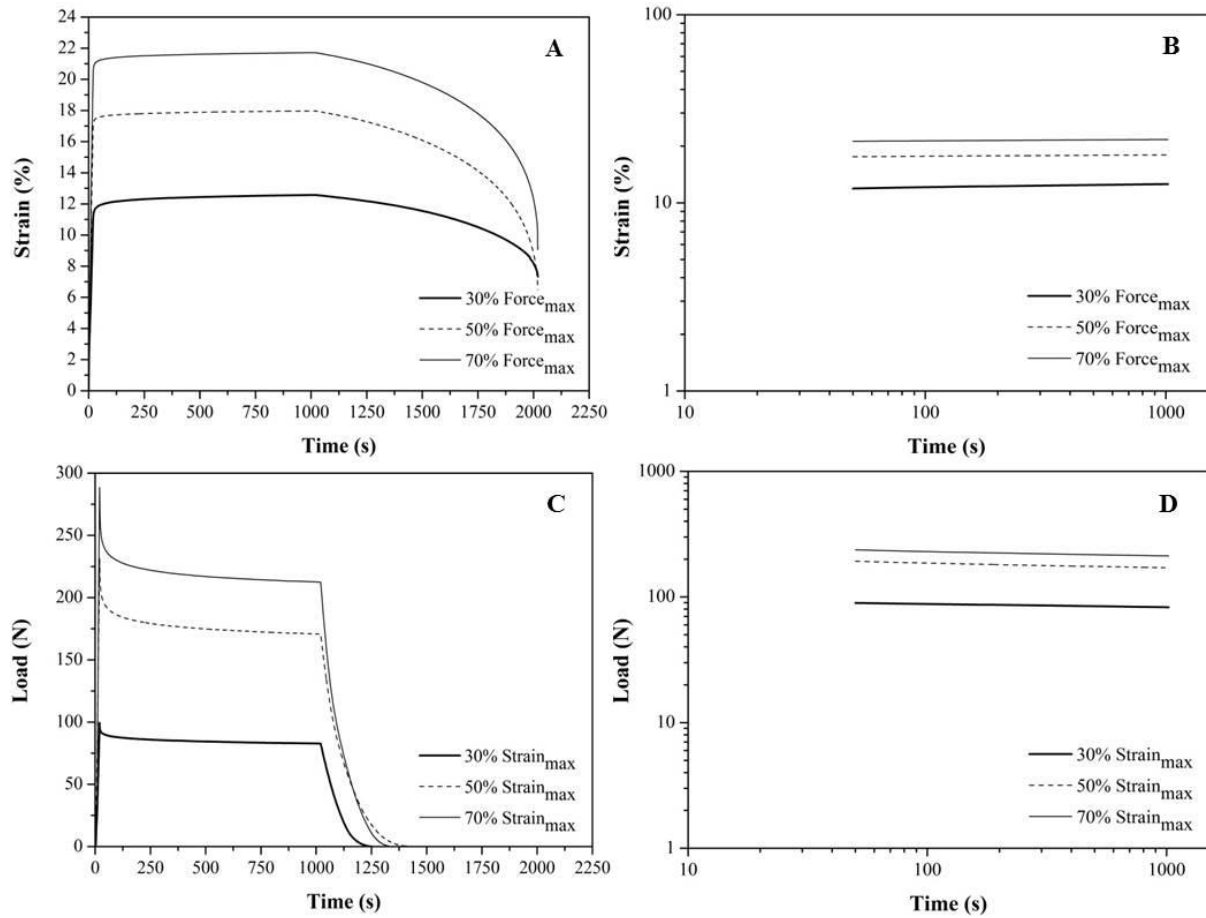


Figure 3.10: Representative curves of creep behaviour of PET based 16YH at different levels of force (A), in Log-Log scale (B). Representative curves of relaxation behaviour of PET based 16YH at different levels of strain (C), in Log-Log scale (D).

Table 3.2: Permanent strain (ε_p) and recoverable strain (ε_r) for each creep level.

	Creep level (%)		
Strain (%)	30	50	70
$\varepsilon_p = \varepsilon_t \approx 2000 \text{ s}$	10.6 ± 1.5	13.5 ± 0.8	11.0 ± 1.5
$\varepsilon_{max} = \varepsilon_t \approx 1000 \text{ s}$	14.4 ± 2.3	19.4 ± 0.7	21.6 ± 0.6
$\varepsilon_r = \varepsilon_{max} - \varepsilon_p$	3.8 ± 1.2	6.5 ± 0.4	9.6 ± 0.7

3.3.3.2. Monotonic cyclic test

The fatigue behaviour of the PET based 16YH braid is presented in figure 3.11, where is possible to observe that as the load level increases the number of cycles that the structure is able to withstand decreases and a linear relation on the semi-logarithmic scale exists between the applied forces and the number of cycles to failure.

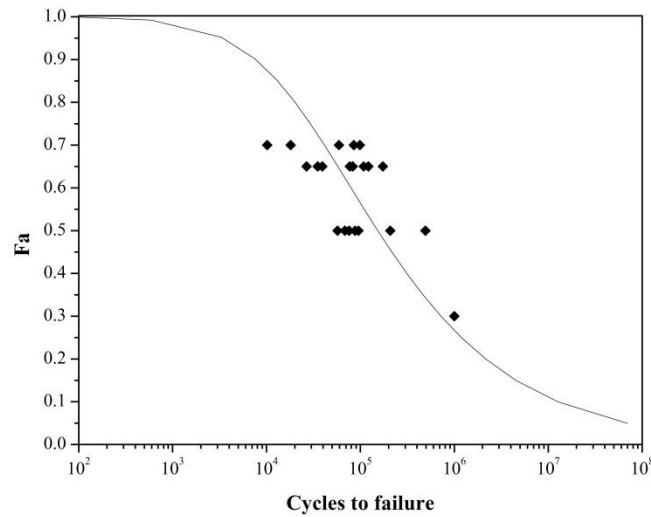


Figure 3.11: Fatigue behaviour of PET based 16YH braid: normalized applied force (F_a =Applied Load/Failure Load) vs cycles to failure.

3.4. Discussion

In some cases, in which the extension of tendon injury is too large, the conventional treatments options, such as repair surgery or usage of biografts, are not efficient to regenerate the tissue and recover its functionality. Therefore, some researchers are working in the development of prosthetic devices to replace a tendon. However due to the demanding mechanical requirements of tendons there are very few devices on the market and no permanent and reliable artificial tendon exists clinically [4]. In order to develop a new device with those mechanical requirements, the authors have developed and characterized simple fibrous textile structures that afterwards will be associated and arranged to form a more complex structure and mimic the complex fibrous micro-architecture of tendons. For these structures multifilament yarns were selected, which are very small, in the order of one millimetre or less and may contain up to several hundred tiny filaments of 10 to 50 μm diameter [19]. Multifilament synthetic yarns present lower flexural rigidity than monofilaments, allowing to produce more flexible and geometric adaptable structures. Besides that, the multifilaments also present higher permeability, swelling and wicking ability, and surface area than monofilament yarns, which are important features in a tissue regeneration process, as will be discussed in this section [20, 21]. Moreover, the multifilament yarns may present a lower failure probability due to the phenomenon of size effect on the structural strength. In a monofilament yarn, when the load reaches a certain level, the break initiates from a flaw, which propagates across the fibre and it opens, owing to the drawing (high plastic extension) of the fibre remainder, until a final multiple crack zone completes the yarn failure. In a multifilament yarn, when the load reaches a certain level, smaller defects appear on their several tiny filaments, which propagate in a controlled manner. Even if some yarns filaments disrupt, other ones may keep the yarn cohesion and still withstand some loading [14, 22, 23]. Therefore, among different synthetic multifilament yarns, after tensile testing them and attending to the tenacity and stiffness levels, two multifilament yarns based on PET and PP, which have been used in several biomedical applications as biocompatible materials [15, 24, 25], were selected.

First of all, SEM analysis showed the smooth and homogeneous surface of the yarns filaments and that they are all aligned in parallel, what grants to the yarn a stiffness higher than if the filaments were not so well organized and longitudinally parallel. This alignment of all filaments with the loading axis, when the yarn is loaded in its longitudinal direction, leads to a higher stiffness. Moreover, the SEM images were also used to measure the yarns diameter, being the average presented in figure 3.2. As described in table 3.1 the PP yarn,

which has a linear density of about 1200 dtex, presents a diameter higher than PET yarn (about 989.0 against 830.8 μm), but the number of filaments is lower than in case of PET yarn (about 137 against 162 filaments). Thus, the PET filaments must be finer than PP filaments, as is possible to check in figures 1.B and 1.D.

The XRD analysis was performed to evaluate the yarns crystallinity level, because it will influence their mechanical performance and biodegradability [26]. The analysis indicated that both polymeric yarns are semi-crystalline. In case of PP yarn, its XRD spectrum shows characteristic peaks at positions 2θ of about 14° , 17° and 18.5° , which present a correlation with the ICDD sheet of PP (ICDD # 00-054-1936). However, another peak at 21.8° , which corresponds to the plane (140) and should have the second highest intensity, should also be observed. The lack of this plane may be due to the preferential filaments orientation in the plane (110), which may have occurred during the yarns processing [27]. The XRD pattern of the PET yarn shows characteristic reflections of crystallinity relative to planes (010), (110) and (1000), for scattering angles 2θ of about 17.6° , 22.8° and 25.6° , respectively [28, 29]. The crystallinity level for the PP and PET yarns, according to the XRD data, was estimated as $61.2\% \pm 1.1$ and $55.0\% \pm 3.2$ respectively [30]. The amorphous regions in the filaments matrix act like a rubber to give extensibility to them, while the crystallites hold the structure together and limit the extensibility. When the degree of molecular orientation (crystallinity) increases, the strength of the filaments also increases, but there is a limit to how much draw can be applied without breaking the filaments [14, 30]. So, the yarns crystallinity level will influence the stiffness (N/mm) of the yarns, i.e. the load level necessary to extend the yarn 1 mm.

When a textile structure is mechanically repeatedly requested, it may fail due to the breakage of the yarns/filaments by internal abrasion. Abrasion is the physical destruction of fibres, yarns, and fabrics, resulting from the rubbing of a textile surface over another surface. In case of internal abrasion, also called as yarn-on-yarn abrasion, there is the rubbing of one yarn surface with another yarn surface [19, 31]. PET yarns have revealed a significant higher abrasion resistance than PP yarns, once the average number of loading cycles was about 15 times higher than in case of PP. Many parameters may influence the abrasion resistance, namely the filament type, fineness, roughness, length and mechanical properties. In general, polyester based fibres present a higher abrasion resistance than PP based fibres [31, 32]. Moreover, the use of finer filaments in the production of yarns increments the number of filaments in the cross-section, leading to a higher cohesion which results in better abrasion

resistance as happens in case of PET yarns that have finer and more filaments than PP yarn [31].

For each one of the yarns, 4 different braided structures were produced, as presented in table 3.1 and observed in figure 3.5. In general, for both yarns, for the same take-up rate (H), there is a trend, when the yarns number increases the number of braids/cm tends to increase, as well as the diameter and linear density of the structure. The increase in diameter is easy to understand due to the increase of the cross-sectional area and the linear density increase was already expected due to the presence of more polymeric matter per structure length unity. Regarding the number of braids/cm, it is influenced by the number of yarns due to their different arrangement in braiding process. In case of using the same number of yarns but different take-up rates (8YH and 8YL), the number of braids/cm is higher for 8YL, as presented in table 3.1, because the number of braiding points/s is higher when the braiding take-up rate is lower. In spite of having the same yarns number, the 8YL structures diameter is higher than in case of 8YH structures due to the different yarns arrangement and compaction caused by the different number of braiding points. As consequence, the linear density is higher in case of 8YL braid than in case of 8YH, due to the higher number of braids/cm, meaning more polymeric material/cm.

During the braiding process the yarns interlace diagonally, meaning that each yarn makes an angle with the structure longitudinal axis, as assigned in figure 3.5 with red lines, which can be between 1° and 89° but is usually in the range of 30° – 80° . This angle is called the braid angle and is the most important geometrical parameter of braided structures [7, 9]. As observed in figure 3.6, the braid angle increases when the number of yarns increases or when the take-up rate decreases. The braid angle of a structure is of course related with the number of braiding points/cm. Therefore, as already discussed, when the yarns number increases or the take-up rate decreases, the number of braids/cm tends to increase leading to a higher braid angle. These results are in accordance with literature that describes the same effect of the take-up rate and number of yarns on the braid angle [7, 9, 33] Besides that, comparing the same braided structure but with a different yarn type, PP_{16YH} and PET_{16YH}, the diameter and braid angle is slightly higher when using the PP yarn than the PET yarn, but the number of braids/cm tends to be higher when using the PET yarn. This may be related with the PP yarn higher diameter and its flattening/enlargement in the structure.

The porosity of a scaffold for living tissues regeneration can modulate the functionality and cellular response to the implant. Pore interconnectivity extending through an implant

increases the overall surface area for cell attachment, enhancing its regenerative properties by allowing the tissue ingrowth into the structure matrix [34, 35]. Therefore, the porosity level of all produced structures was evaluated and it increased when the yarns number increased to 16, being about 88% in case of PP and 85% in case of PET. The porosity of the textile structures is mainly due to the open spaces among the yarns, but also due to smaller spaces among filaments composing each yarn, so when increasing the yarns number it would be expected to have more open spaces. However, when increasing the number of yarns and keeping all other braiding parameters, the compaction among them is different, what is proved by the very similar diameter when using 6 or 8 yarns.

One of the scaffolds limitations is the ability to promote cell infiltration, oxygen and nutrients diffusion which is important to ensure cell attachment, proliferation and differentiation. The scaffold design is a way to overcome these limitations and it has been proved that a high wicking ability increases fluid intake and enhance cells infiltration and distribution [36-38]. In textile structures, the liquid transfer mechanisms include water diffusion and capillary wicking, which are mainly determined by effective capillary pore distribution, pathways and surface tension. Wicking is the spontaneous flow of a liquid in a porous substance, driven by the capillary forces. The flow and retention of water will be affected by the form and alignment of the open spaces in the structure, which depend on how the fibres are packed together. As capillary forces are caused by wetting, wicking is a result of spontaneous wetting in a capillary system. Besides that, wetting may cause the fabric swelling depending on yarns type, changing the capillary space position [14, 17, 39]. Therefore, in order to understand and evaluate this functionality, the swelling profile of the used yarns and wicking behaviour of the yarns and braids were evaluated.

The water uptake (swelling) by the yarns occurs as a result of water entering into yarn structure once that the osmotic pressure is greater than the forces of the crosslinking bonds that maintain the structure of the polymeric structure. Water molecules diffuse into the amorphous regions of the yarn and break inter-molecular hydrogen bonds, allowing an increase in the intermolecular distance of the polymer chains, which causes swelling. As a result, swelling increases the amorphous regions of the yarn [40-42]. Since the PP and PET yarns weight significantly increased after 15 min immersed in water, as observed in figures 3.4.A and 3.4.B, it means that yarns swelling occurred due to the water absorption into the structure. However, after the 15 min until the 30 min the swelling rate significantly decreased, indicating a saturation state. When swelling increases over time it means that the void spaces of the yarns are still being filled with water until it reaches a saturation point,

where an equilibrium state with aqueous medium is reached, meaning that the osmotic pressure is balanced in and out of the structure by the forces of the crosslinking bonds [40, 42, 43]. In addition, it must be noticed that the swelling level for the PET yarn is always higher than for PP yarn, what may be explained by the lower water contact angle in case of PET than in case of PP, allowing an easier water entrance.

Capillary rise within a textile is influenced by the structure architecture, namely by its porosity, yarns content and braid angle, which will influence the inter- and intra- yarns pores dimension and distribution (pathways) along the structure and consequently the quality of the capillary channels. A structure with more capillary spaces will have a high capillary rise. In figures 3.4.C and 3.4.D, it is possible to observe that as the number of yarns increases, for the same take-up rate, the water height at each time point also increases. When comparing the 4 braids with the respective yarn, the significant higher water height is explained by the significant increase of inter- and intra- yarns pores. Comparing the 16YH with all other structures, this phenomenon could also be explained by the amount of inter- and intra- yarns pores, which is slightly higher than in other braids (figure 3.7). However, as already discussed, for both materials types, when the yarns number changes from 6 to 8 there is no difference in porosity level. So, when using PP yarns, the wicking difference between these two structures may be due to the different pores distribution. Regarding the structures produced with 8 yarns, the 8YH presented a lower capillary rise than the 8YL. However, once since 8YH and 8YL present a porosity level very similar, it means the braiding angle and consequently the braid architecture may have a high importance on the capillarity. The different architecture may create pores with different size and distribution, creating capillary channels with a lower or higher capillary pressure [37]. Moreover, with increasing time, namely after about 6 min, the water height tends to stabilize, what may be explained by the saturation state already discussed. During the diffusion of the liquid into the yarn the capillary action and diffusion mechanisms are coupled, and the swelling generally reduces the dimensions of the yarn pore space, decreasing the rate of absorption over time. In an extreme case, some kind of *blocking* occurs in which swelling of the material completely closes the wicking channels, preventing further wicking and limiting liquid transport by a diffusion process [40].

Once the produced braids are intended to be used as part of a more complex structure for partial tendons substitution, which requires a certain force, strain and stiffness level, the tensile mechanical properties of PP and PET yarns and braided structures were evaluated. From figures 3.8.C and 3.9.C, it is possible to observe that as the number of yarns increases,

for the same take-up rate, the load at failure also increases. This happens because there are more yarns available to withstand the applied load. Changing the take-up rate does not influence the load at failure once the yarns number is the same (8YH and 8YL). However, the tenacity level, which is an usual measure of the specific stress at break in textile structures, is higher in case of 8YH, once the two structures present a very similar load at failure but the 8YH presents a lower tex value [7, 14].

Regarding the strain to failure, it also increases as the number of yarns increases. This phenomenon may be explained by the increase of the braid angle with yarns number, what makes the yarns in structure take more time to align with the braid longitudinal axis and consequently with tensile load direction. For the same reason, when comparing the 8YH and the 8YL, the strain to failure increases when the take-up rate decreases due to the increase in braid angle [7, 8]. Therefore, the structures stiffness, for both materials using a specific take-up rate, increases as the yarns number increases due to the force and strain levels relation. When the take-up rate decreases, for the same yarns number, the stiffness decreases due to the strain level increase. Moreover, comparing the two 16YH structures, the stiffness is higher when using the PET yarn, what is explained by the higher force and lower strain than when using the PP yarn. This is in accordance with the tensile properties when comparing the isolated yarns, being the PET yarn the one that presents the higher stiffness [14]. Besides that, the higher strain of PP_16YH braid is also related with the higher braid angle than in case of PET_16YH, what may be explained by the higher diameter of PP yarn ($989.0 \pm 1.7 \mu\text{m}$) comparing to PET yarn ($830.8 \pm 1.6 \mu\text{m}$). After evaluation of the braids tensile properties, the PET 16YH structure was the selected one for further mechanical characterization once it presents the higher stiffness level, which is a demanding requirement for tendons substitution. Moreover, it has been reported a higher fatigue resistance when using multifilament PET yarns than PP yarns in ropes for marine energy devices, mostly due to the higher abrasion resistance [32].

The viscoelastic tendons behaviour, means a time dependent mechanical behaviour, due to the viscous properties of the collagen fibres and ground substance, exhibiting creep, force-relaxation and mechanical hysteresis (energy dissipation). The relation between tendons force and strain is not constant, it depends of load level and time of displacement [44, 45]. Therefore, considering this viscoelastic behaviour of tendons, it is also crucial to understand how the PET structures mechanical behaviour is time dependent.

Creep phenomenon is characterized by an increasing deformation under constant load, typically comprising three stages: a) primary stage occurring in the initial stage of loading

when the creep rate decreases rapidly with time; b) secondary stage when creep rate reaches a steady state; c) tertiary stage characterized by a rapid creep rate increase leading to creep failure. This contrasts with an elastic material, which does not exhibit increase of deformation no matter how long the load is applied. Creep deformations, which occur at lower forces, are recoverable, after load have been removed, given enough time. The major effect at low forces is a progressive straightening of molecules, often with a change in the links between molecular segments; this is usually recoverable and does not affect strength [30, 46, 47]. Creep deformations, at higher forces, are not totally recoverable. At those high forces, whole molecules slide past one another and this leads, eventually, to rupture. In general, during creep phenomenon, both recoverable and permanent deformations occur simultaneously. In force-relaxation phenomenon, the force withstand by the structure will decrease at a constant strain level. The stress required to maintain the constant strain value is decreased with time, because the molecules of polymer get relaxed with time, and to maintain the level of strain, somewhat lower value of stress is sufficient [14, 30, 47].

The PET_{16YH} braid revealed a very good resistance to creep, once its deformation remains almost the same over time, meaning a very low creep rate (creep curve slope) for all force levels (for instance, about $0.4 \pm 0.2\%$ for the 70% level) as observed in figure 3.10.A, being more evident in figure 3.10.B. The observed recoverable strain (ϵ_r), which is due to the elastic recoverable deformation of yarns but mainly of the structure by itself, tends to increase when the load level increases (table 3.2) as already expected once the ϵ_{max} also increases [14, 47]. The permanent strain, which is due to some plastic deformation of polymeric yarns, is very low and similar for all load levels, even at 70% of the load at failure. This is a very positive creep behaviour attending to the final application of the structure, in which it will be exposed to different forces over long time periods. *Hawkins et al.*, for instance, reported a strain increase of about 3.5% after 7 minutes of AT stimulation, which is really above the creep rate observed for these structures [48]. Furthermore, the PET_{16YH} structure revealed a low and similar relaxation rate for all strain levels as presented in figure 3.10.C and more evident in figure 3.10.D, meaning that when it is extended until different lengths it will be able to withstand the consequent load over long time periods without getting relaxed.

Attending to the final application, the developed structures will be under long-term and repeated loading and they must remain working for a long time period. Thus, the fatigue resistance, which is one of the major reasons for failure in structural materials, of PET_{16YH} structure was evaluated by exposure to different cyclic force levels. As

observed in figure 3.11, the structure lifetime tends to decrease when the force level increases, as also reported for most materials. However, it must be noticed that for the level of 30% of the load at failure the structure was able to withstand 1 million cycles even at frequency of 5 Hz, which is really above the frequency of 1.37 Hz experienced by the AT for instance in a marathon of 2 h 14 min at 19 km/h [49].

This creep, relaxation and fatigue behaviour of PET structure was already expected once that PET fibres are reported as being very interesting for different technical applications with demanding mechanical requirements and repeated use for long time periods [46].

3.5. Conclusions

After the promoted characterization of all produced braids, it is possible to conclude that the braids architecture actually define their physical and mechanical behaviour, besides of course the intrinsic physical properties of the yarns that compose them. The number of yarns in the structure and the braiding take-up rate are the main parameters that can be adjusted to construct structures with different architectures, namely with a different braids/cm, diameter, linear density, tenacity, braid angle and porosity level. The wicking ability of braids was dependent on structure pores amount but also on how those pores communicate, which also depends on the architecture, namely how the yarns are arranged and packed in the structure.

The load failure level of the braids is mainly controlled by the number of yarns, but the strain level is mostly influenced by the take-up rate and consequently by the braid angle. Regarding the stiffness level, it results from a combination of the yarns number and braid angle. The PET_{16YH} structure revealed a very promising creep and force-relaxation behaviour for the final application, as well as a very interesting fatigue resistance. Therefore, PET_{16YH} braids may be interesting structures to be used as elementary parts of a more complex textile structure in order to reach the real level of load at failure, strain to failure and stiffness of different tendons.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project PEst-C/EME/UI0285/2011.

3.6. References

- [1] Chen J., Xu J., Wang A., Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. *Expert review of medical devices*. 2009;6:61-73.
- [2] Hess G. W. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot & ankle specialist*. 2010;3:29-32.
- [3] Maffulli N., Renstrom P., Leadbetter W. *Tendon Injuries-Basic Science and Clinical Medicine*: Springer; 2005, London.
- [4] Morais DS, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair, *Annals of Biomedical Engineering*. 2015; 43(9):2025-35.
- [5] Kuo C. K., Marturano J. E., Tuan R. S. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2010;2:20.
- [6] Sharma P., Maffulli N. Biology of tendon injury: healing, modeling and remodeling. *Journal of musculoskeletal & neuronal interactions*. 2006;6:181-90.
- [7] Omeroglu S. The effect of braiding parameters on the mechanical properties of braided ropes. *Fibres and Textiles in Eastern Europe*. 2006;14:53.
- [8] Rawal A, Saraswat H, Sibal A. Tensile response of braided structures: a review. *Textile Research Journal*. 2015;85:2083-96.
- [9] Kyosev Y. *Braiding technology for textiles: Principles, design and processes*: Elsevier; 2014, United Kingdom.
- [10] Schwartz P. *Structure and mechanics of textile fibre assemblies*: Elsevier; 2008, USA.
- [11] Lei X. Braided Textile Composites, <http://www.xcomposites.com/braided.html>, accessed in August 2016.
- [12] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.
- [13] Longo U. G., Lamberti A., Maffulli N., Denaro V. Tendon augmentation grafts: a systematic review. *British medical bulletin*. 2010;94:165-88.
- [14] Hearle JW, Morton WE. *Physical properties of textile fibres*: Elsevier; 2008, USA.
- [15] Saxena S, Ray AR, Kapil A, Pavon-Djavid G, Letourneur D, Gupta B, et al. Development of a new polypropylene-based suture: plasma grafting, surface treatment, characterization, and biocompatibility studies. *Macromolecular bioscience*. 2011;11:373-82.
- [16] Veleirinho B, Coelho DS, Dias PF, Maraschin M, Pinto R, Cargnin-Ferreira E, et al. Foreign body reaction associated with PET and PET/chitosan electrospun nanofibrous abdominal meshes. *PloS one*. 2014;9:e95293.

- [17] Fangueiro R, Filgueiras A, Soutinho F, Meidi X. Wicking behavior and drying capability of functional knitted fabrics. *Textile Research Journal*. 2010;80:1522-30.
- [18] Cordage Institute International Standard, Test method for yarn-on-yarn abrasion, CI 1503-00. August, 2001.
- [19] McKenna HA, Hearle JW, O'Hear N. *Handbook of fibre rope technology*: Elsevier; 2004, USA.
- [20] Alagirusamy R, Das A. *Technical textile yarns*: Elsevier; 2010, USA.
- [21] Potic M, Ignjatovic I, Savic V, Djekic P, Radenkovic G. Mechanical properties and tissue reinforcement of polypropylene grafts used for pelvic floor repair—an experimental study. *Hernia*. 2011;15:685-90.
- [22] Bažant ZP. Size effect on structural strength: a review. *Archive of applied mechanics*. 1999;69:703-25.
- [23] Vořechovský M, Chudoba R. Stochastic modeling of multi-filament yarns: II. Random properties over the length and size effect. *International journal of Solids and Structures*. 2006;43:435-58.
- [24] Modjarrad K, Ebnesajjad S. *Handbook of Polymer Applications in Medicine and Medical Devices*: Elsevier; 2013, USA.
- [25] Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. *Biomaterials science: an introduction to materials in medicine*: Academic press; 2004, Canada.
- [26] Sarasua J, Lopez-Rodriguez N, Zuza E, Petisco S, Castro B, Del Olmo M, et al. Crystallinity assessment and in vitro cytotoxicity of polylactide scaffolds for biomedical applications. *Journal of Materials Science: Materials in Medicine*. 2011;22:2513-23.
- [27] Favaro MM, Branciforti MC, Bretas RES. A X-ray study of β -phase and molecular orientation in nucleated and non-nucleated injection molded polypropylene resins. *Materials Research*. 2009;12:455-64.
- [28] Huang Z, Bi L, Zhang Z, Han Y. Effects of dimethylolpropionic acid modification on the characteristics of polyethylene terephthalate fibers. *Molecular medicine reports*. 2012;6:709-15.
- [29] Nunes RAX, Costa VC, Calado VMdA, Branco JRT. Wear, friction, and microhardness of a thermal sprayed PET: poly (ethylene terephthalate) coating. *Materials Research*. 2009;12:121-5.
- [30] Balani K, Verma V, Agarwal A, Narayan R. *Biosurfaces: A Materials Science and Engineering Perspective*: John Wiley & Sons; 2015, Canada.
- [31] Özdil N, Kayseri GÖ, Mengüç GS. Analysis of abrasion characteristics in textiles. Edited by Marcin Adamiak. 2012:119, Turkey.
- [32] Weller S, Davies P, Johanning L, Banfield S. Merific, Marine Energy in Far Peripheral and Island Communities, Guidance on the use of synthetic fibre ropes for marine energy devices. 2013.
- [33] Bicking AM, Oxenham W. Variables and methods for aesthetic braid design. *Journal of Textile and Apparel, Technology and Management*. 2012;7.

- [34] Cooper JA, Lu HH, Ko FK, Freeman JW, Laurencin CT. Fiber-based tissue-engineered scaffold for ligament replacement: design considerations and in vitro evaluation. *Biomaterials*. 2005;26:1523-32.
- [35] Laurencin CT, Freeman JW. Ligament tissue engineering: an evolutionary materials science approach. *Biomaterials*. 2005;26:7530-6.
- [36] Tabbaa S, Burg KJ. The effect of wicking fibres in tissue-engineered bone scaffolds. *Journal of tissue engineering and regenerative medicine*. 2015;9:469-72.
- [37] Oh DS, Kim YJ, Hong M-H, Han M-H, Kim K. Effect of capillary action on bone regeneration in micro-channeled ceramic scaffolds. *Ceramics International*. 2014;40:9583-9.
- [38] Tabbaa S, Mlynarczyk A, Burg KJ. Improved Cellular Recruitment and Mass Transport for Large Bone Defect Regeneration, Clemson and Kansas State University.
- [39] Benltoufa S, Fayala F, Nasrallah SB. Determination of yarn and fiber diameters after swelling using a capillary rise method. *Journal of The Textile Institute*. 2012;103:517-22.
- [40] Akinli-Kogak S. The influence of fiber swelling on paper wetting: Citeseer; 2001.
- [41] Adanur S. Wellington Sears Handbook of industrial textiles: CRC Press; 1995, England.
- [42] Morais DS, Rodrigues M, Silva T, Lopes M, Santos M, Santos J, et al. Development and characterization of novel alginate-based hydrogels as vehicles for bone substitutes. *Carbohydrate polymers*. 2013;95:134-42.
- [43] De SK, Aluru N, Johnson B, Crone W, Beebe DJ, Moore J. Equilibrium swelling and kinetics of pH-responsive hydrogels: Models, experiments, and simulations. *Journal of Microelectromechanical Systems*. 2002;11:544-55.
- [44] Peltonen J, Cronin NJ, Avela J, Finni T. In vivo mechanical response of human Achilles tendon to a single bout of hopping exercise. *Journal of Experimental Biology*. 2010;213:1259-65.
- [45] James R., Kesturu G., Balian G., Chhabra A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery*. 2008;33:102-12.
- [46] Lechat C, Bunsell AR, Davies P. Tensile and creep behaviour of polyethylene terephthalate and polyethylene naphthalate fibres. *Journal of Materials Science*. 2011;46:528-33.
- [47] DNV-GL. Design, testing and analysis of offshore fibre ropes, DNVGL-RP-E305. September, 2015.
- [48] Hawkins D, Lum C, Gaydos D, Dunning R. Dynamic creep and pre-conditioning of the Achilles tendon in-vivo. *Journal of biomechanics*. 2009;42:2813-7.
- [49] De Zee M, Bojsen-Møller F, Voigt M. Dynamic viscoelastic behavior of lower extremity tendons during simulated running. *Journal of Applied Physiology*. 2000;89:1352-9.

Chapter 4

**Mechanical behaviour of ropes based on
polypropylene (PP) and poly(ethylene terephthalate)
(PET) multifilament yarns for Achilles tendon
partial substitution**

Mechanical behaviour of ropes based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns for Achilles tendon partial substitution

Morais D.S.^{1,2}, Cruz J.³, Fangueiro R.³, Lopes H.⁴, Guedes R.M.^{2,5}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Centre for Textile Science and Technology, University of Minho, Portugal

⁴ DEM, ISEP, Instituto Politécnico do Porto, Portugal

⁵ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

Abstract

In some clinical cases, attending to the type and extension of the Achilles tendon injury, a partial replacement of the tissue may be needed. Driven by this, the authors developed different textile structures based on polypropylene (PP) and poly(ethylene terephthalate) (PET) multifilament yarns, using a textile technology-based technique, in order to mimic the fibrous structure of tendons. Those structures are based on a core/shell system, being the core composed by several sub-components (braids) and the shell based on braided yarns enclosing the core. It was observed that the load at failure of ropes is mainly defined by the number of yarns that compose it, but the strain level is mostly influenced by the production take-up rate of the core braids and consequently by the braid angle. The ropes stiffness level results from a combination of the yarns number and braid angle of the core braids. The structure based on PET yarns revealed a non-linear force-strain curve similar to a typical curve of a natural Achilles tendon (AT), presenting a load at failure, strain to failure and stiffness levels very promising for AT replacement. Moreover, the PET rope also revealed a very promising fatigue and appropriate creep resistance for the final application, being in accordance to what has been reported for native AT.

Keywords: polypropylene, PP, poly(ethylene terephthalate), PET, braid, rope, Achilles tendon.

4.1. Introduction

Tendons are components of the skeletal system structure that play an important role to maintain the static and dynamic balance of the human body, allowing locomotion, load transfer and joint stability. Tendons attach physically muscle to bone, being a connective tissue composed by fibroblastic cells and an extracellular matrix mainly composed by collagen fibers, which are assembled in fascicles that are surrounded by a loose connective tissue layer named *paratenon*, as observed in figure 4.1 [1-3]. The complex hierarchical fibrous structures of collagen are responsible for the complex mechanical properties of tendons. Tendons present a non-linear viscoelastic and

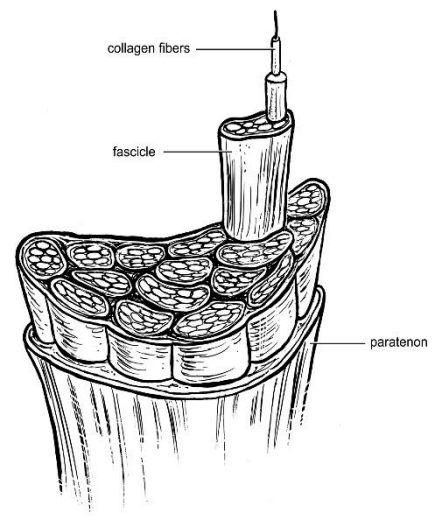


Figure 4.1: The hierarchical fibrous structure of tendons.

anisotropic behaviour in response to loading. A typical stress-strain curve of an isolated tendon, in elongation-to-failure conditions, presents 3 different regions, the *toe* region, *linear* region and finally the rupture point [1, 4]. When stressed tendons don't behave perfectly elastically, they present a viscoelastic behaviour, due to the viscous properties of the collagen fibres and ground substance, exhibiting force-relaxation, creep, and mechanical hysteresis [1, 5, 6].

Tendons partial injuries or even total ruptures are common and affect athletes and nonathletes alike. The cause is multifactorial and controversial involving biological, anatomical and mechanical factors [1, 7, 8]. It is estimated that the global market for Orthopaedic Soft Tissue Repair, including ligaments and tendons, will be about EUR 7 billion by the end of 2019 [9]. Achilles tendon tendinopathy, for instance, affects not only competitive and recreational athletes but also people who are not active, mostly with increasing age [10, 11]. However, over 80 percent of ruptures occur during recreational sports and about 10 percent of patients who sustain an AT rupture had preexisting AT problems. Observational data suggest that competitive athletes have a lifetime incidence of Achilles tendinopathy of 24 percent, with 18 percent sustained by athletes younger than 45 years. Tendon rupture occurs in 8.3 percent. Among competitive runners, the lifetime incidence of Achilles tendinopathy may be as high as 40 to 50 percent [11].

The complex mechanical behaviour makes tendons injuries difficult to manage and although spontaneous healing can occur this often results in the formation of scar tissue with low functionality [1]. In some cases, a simple suture backing together the tendon ends is

done, but this repair practice is often ineffective. In other cases, the used approach is a tissue replacement with auto or allografts, but these reveal disadvantages, as rejection risk [12, 13]. Thus, in order to develop a new treatment approach, several grafts intended to be used just as graft augmentation devices or to fully replace a damaged tendon (prostheses) have been proposed. However, due to the demanding mechanical requirements of tendons, there is not an efficient device in the market to fully replace a damaged tendon due to the long term failure. The devices failure is usually caused by lack of either mechanical performance or biocompatibility. Mechanical failures include the rupture caused by wear, fatigue or severe loading, laxity in the joint after creep of the device or loosening of the fixation element in the bone end [1, 6, 13].

Ropes with a hierarchical fibrous structure are produced by textile techniques. The ropes structure contains and must control synthetic or natural yarns which are assembled according to a certain architecture in order to accomplish a required breaking strength and extension, using the minimum amount of yarns. The yarns may be just aligned among them or either laying (twisting) or interlacing (braiding) techniques may be used for their assemblage as rope sub-components, which may be just aligned or even arranged in a certain way. Depending on the architecture, some ropes may require enclosure techniques of braided or extruded jackets to contain the enclosed yarns or sub-components (as twists or braids) [14, 15]. The appearance and mechanical performance of a rope is influenced by different factors, namely by the yarn type and diameter, by the twisting level or braiding angle of the sub-components and of course their diameter and number, by the tension applied on yarns and sub-components during the rope production, and by possible post-production treatments [15].

Therefore, in order to mimic the hierarchical fibrous structure of tendons, namely of the AT, and consequently its mechanical performance, the main purpose of this work is to develop complex fibrous structures using a textile technology-based technique. The structures are based on a core/shell system as used in ropes design and they must exhibit nonlinear viscoelastic behaviour under physiologic loading.

4.2. Materials and methods

4.2.1. Ropes production

Different textile structures based on a core/shell architecture, using PP or PET yarns, were produced on a vertical braiding machine with 32 carriers. The core is composed by several braids based on PP or PET multifilament yarns, and the shell is also composed by

braided PP or PET multifilament yarns. In table 4.1 are presented the construction and textile properties of each structure. The braids that compose the structures core were produced with a braiding take-up rate of 3.94 cm/s (H) using 8 (8YH) or 16 yarns (16YH), using a vertical braiding machine with 16 carriers. The yarns of shell were braided using a take-up rate of 1.44 cm/s (L). PP and PET multifilament yarns, with a linear density of 1200 and 1112 dtex respectively, were purchased from *Sarla Europe, LDA* (Portugal).

Table 4.1: Production and textile parameters of the produced structures, using PP or PET multifilament yarns.

Polymeric material	Textile structure: Braids	Number of filaments (yarn)/ yarns (braid)	Take-up rate	Braids/cm	Linear density (tex)	Tenacity (N/tex)
PP	8YH	8	H	1.2 ± 0.1	1103 ± 4	0.51
	16YH	16	H	2.2 ± 0.1	1862 ± 6	0.58
PET	16YH	16	H	2.4 ± 0.1	1835 ± 5	0.64
	Textile structure: Ropes	Braids number/type in core	Yarns number in shell	Braids/cm	Linear density (tex)	Tenacity (N/tex)
PP	C16B8YH_S16YL	16 / 8YH	16	4.2 ± 0.1	19566 ± 7	0.43
	C16B16YH_S16YL	16 / 16YH	16	4.0 ± 0.1	31716 ± 4	0.52
PET	C22B16YH_S16YL	22 / 16YH	16	4.3 ± 0.1	42143 ± 8	0.45

4.2.2. Optical microscopy

All the textile structures, braids and ropes, were observed in triplicate by optical microscopy in order to estimate their diameter and the braid or rope shell braiding angle through the images treatment by the software *J image*.

4.2.3. Static mechanical behaviour

Stiffness (N/mm), load at failure (N) and strain to failure (ϵ , %) of the three developed ropes were measured by performing uniaxial tensile tests in triplicate on *Zwick/Roell Z050* equipment with a 3000 kgf load cell. The initial distance between grips was defined as 235 mm and the displacement rate as 8.5 mm/s.

4.2.4. Time-dependent mechanical behaviour

4.2.4.1. Monotonic cyclic tests

The fatigue behaviour of PP_C16B16YH_S16YL and PET_C22B16YH_S16YL ropes was evaluated by monotonic cyclic tests, namely by exposure to a sinusoidal load up to 10, 200, 500, 1000, 2000 or 3000 cycles. The applied sinusoidal load, at a frequency of 0.1 Hz, was defined using a maximum load of 7500 N and a minimum load of 750 N. The tests were performed in triplicate on *Zwick/Roell Z050* equipment with a 3000 kgf load cell and the initial distance between grips was defined as 235 mm. After each number of cycles the samples were tensile tested according to the parameters already described in 2.3.

Moreover, using the same equipment, the PET rope was also exposed to a sinusoidal load up to 10 cycles, at a frequency of 0.1 Hz, using a maximum load of 3500 N and a minimum load of 500 N.

4.2.4.2. Creep tests

In creep tests, which were performed only for the PET rope, the samples were uniaxially loaded until to 7500 N in 30 s and kept at this level for about 2000 s. After this holding phase, they were ramped down reaching the 0 N force. The test was performed in triplicate on *Zwick/Roell Z050* equipment with a 3000 kgf load cell and the initial distance between grips was defined as 235 mm.

4.2.5. Nano-CT analysis

The scans acquirement was performed on *Zeiss Xradia 510*, at a voltage of 50 kV and a power of 4 W. Resolution of the overview pictures was 4.6 μm . The detail scans (high-density scans) were done with 800 nm resolution. The object was fixated on a turnable stand and rotated, while the detector is fixed.

4.2.6. Statistical analysis

Experimental data is presented as mean $\pm$ SD (standard deviation). Statistical analysis of data was performed using the one-way ANOVA test with Bonferroni post hoc analysis, using the software SigmaStat 3.5. The differences were considered to be significant at a level of $p < 0.05$.

4.3. Results

4.3.1. Optical microscopy, braid angle and porosity

Figure 4.2 presents the optical microscopic images of the braided structures based on PP or PET yarns, and the respective diameters. Figures 4.2.A, 4.2.C and 4.2.E present the braids that compose the core of each one of the produced ropes, which are presented in figures 4.2.B, 4.2.D and 4.2.F, respectively. As observed in figures 4.2 and 4.3, for the same yarn (PP) and take-up rate, the diameter and braiding angle is higher when using more yarns in the braid (16). Regarding the braids/cm, when using the same yarn and take-up rate, it increases with the yarns number. The PP rope (figure 4.2.D) that uses the 16YH braids (C16B16YH_S16YL) presents a higher diameter (about 8.5 mm) and higher braid angle (about 52°) than the PP rope that uses the 8YH braids (C16B8YH_S16YL) (figure 4.2.B). Besides that, comparing the same braided structure but with a different yarn type, PP_16YH (figure 4.2.C) and PET_16YH (figure 4.2.E), the diameter and braid angle is slightly higher when using the PP yarn than the PET yarn. The C22B16YH_S16YL rope presents a lower diameter than the C16B16YH_S16YL rope but a higher braid angle and number of braids/cm (table 4.1). In figure 4.2.G it is possible to observe the core/shell system used for the three ropes.

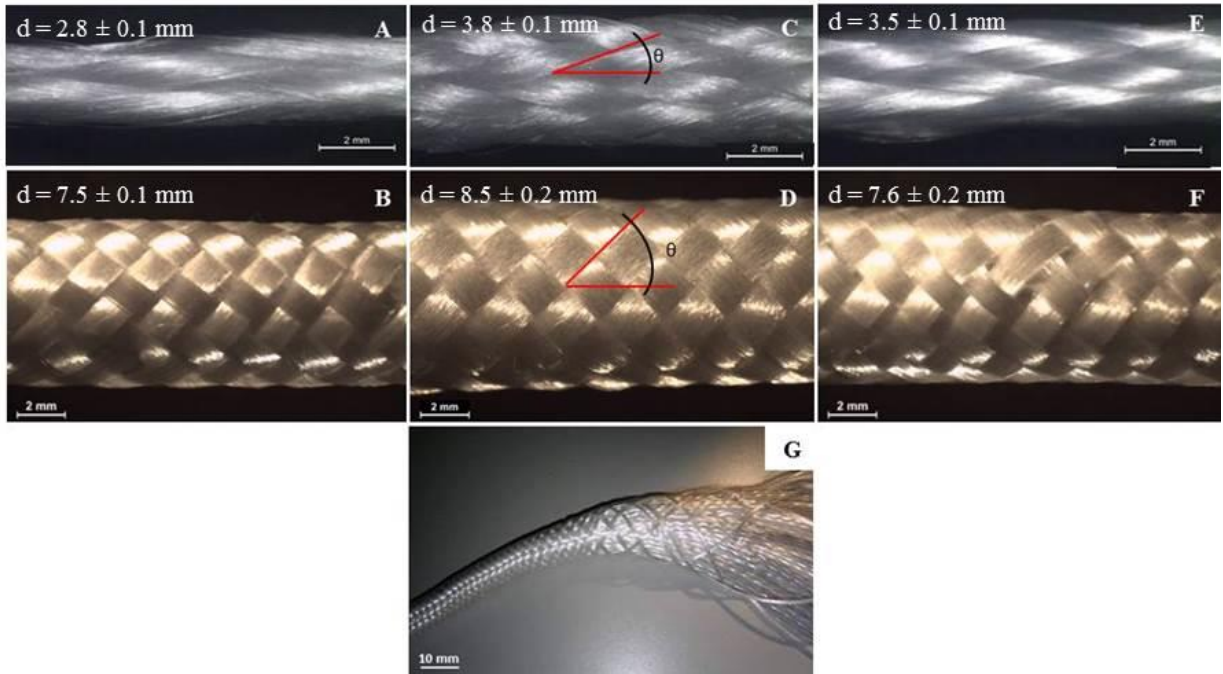


Figure 4.2: Optical microscopic images of braids and ropes: (A) PP_8YH braid; (B) outer part of C16B8YH_S16YL; (C) PP_16YH braid; (D) outer part of C16B16YH_S16YL; (E) PET_16YH braid; (F) outer part of C22B16YH_S16YL; (G) core/shell construction of C22B16YH_S16YL.

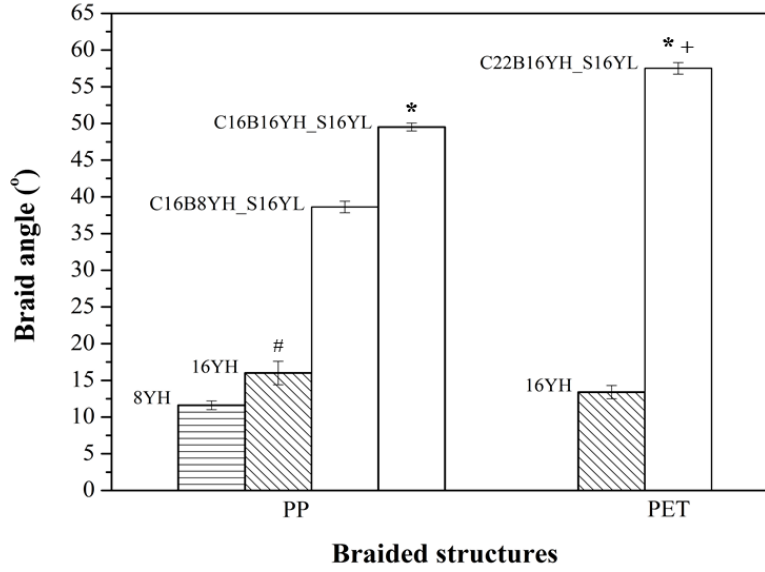


Figure 4.3: Braid angle between yarns of braids and the ones composing the ropes shell. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 8YH (#), C16B8YH_S16YL (*) and C16B16YH_S16YL (+).

4.3.2. Static mechanical behaviour

Figure 4.4.A presents the force-strain curves of all PP based structures, namely of 8YH and 16YH braids and C16B8YH_S16YL and C16B16YH_S16YL ropes. The ropes sub-components present a load at failure of 591.0 ± 12.1 N (8YH) and 1087.6 ± 32.4 N (16YH), and ropes of 8356.1 ± 279.7 N (C16B8YH_S16YL) and 16626.0 ± 217.0 N (C16B16YH_S16YL), as observed in figure 4.4.B. The strain to failure of braids is $28.6 \pm 2.2\%$ (8YH) and $33.9 \pm 2.2\%$ (16YH), and of ropes is $58.4 \pm 3.3\%$ (C16B8YH_S16YL) and $80.3 \pm 1.8\%$ (C16B16YH_S16YL), as presented in figure 4.4.C. In figure 4.4.A, it is observed that both ropes present a curve shape different from the braids, namely in the beginning of the curve, being possible to identify two distinct slopes in case of ropes, represented in figure 4.4.D as stiffness I and II. Regarding stiffness I, the C16B8YH_S16YL rope presents a value of about 26.3 ± 5.8 N/mm and the C16B16YH_S16YL rope presents a value of about 19.5 ± 2.3 N/mm. Regarding stiffness II, the C16B8YH_S16YL rope presents a value of about 73.0 ± 1.1 N/mm and the C16B16YH_S16YL rope presents a value of about 108.6 ± 6.0 N/mm. However, when the C16B16YH_S16YL rope is exposed to 10 cycles of accommodation by application of a sinusoidal load, between 750 and 7500 N, the stiffness II increases to about 178.8 ± 4.0 N/mm.

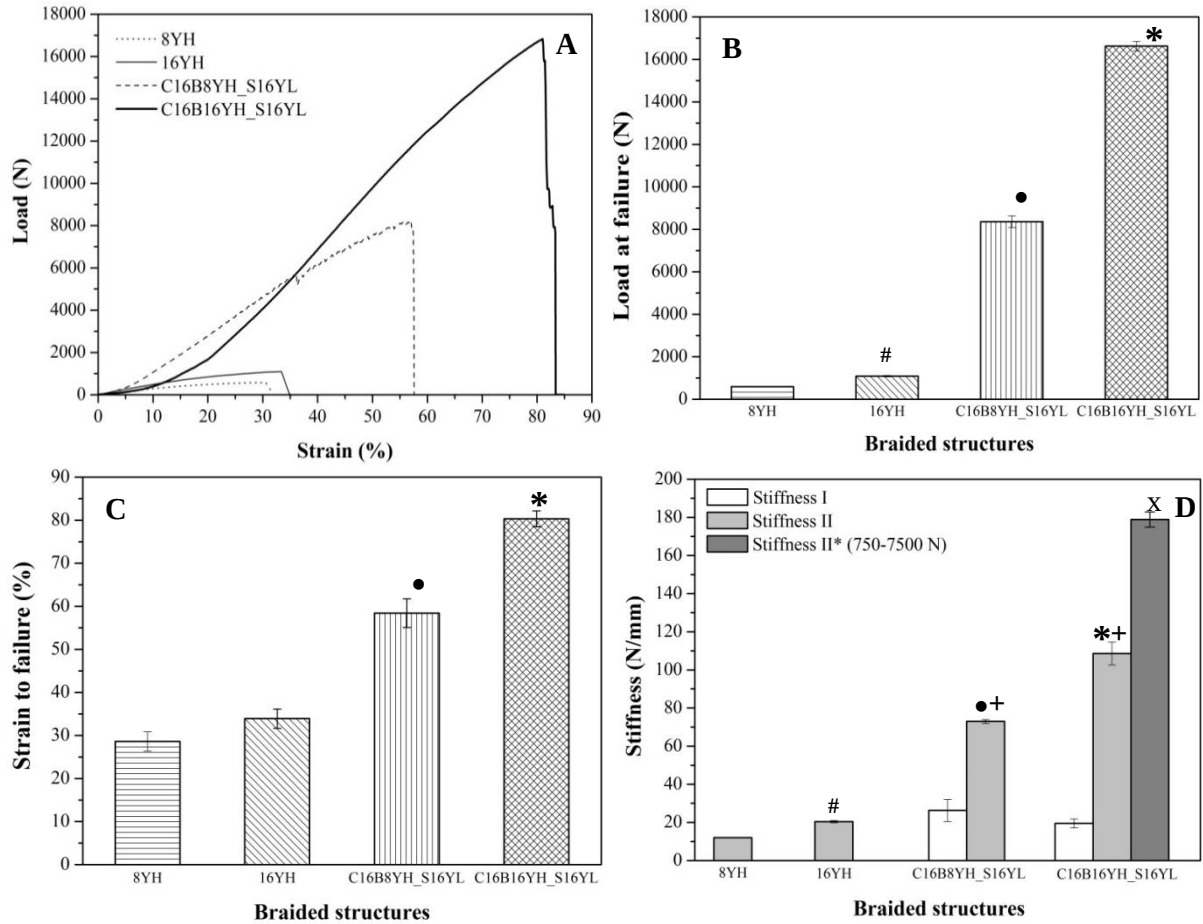


Figure 4.4: (A) Representative force-strain curves of all PP based structures. Tensile properties of the PP 16YH braid and ropes: (B) load at failure, (C) strain to failure and (D) stiffness. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared with 8YH (#); 16YH (•); correspondent stiffness I (+); C16B8YH_S16YL (*) and stiffness II of C16B16YH_S16YL (x).

Figure 4.5.A presents the force-strain curves of both PET based structures, namely of the 16YH braid and the C22B16YH_S16YL rope. Regarding the load at failure and the strain to failure, presented in figures 4.5.B and 4.5.C, the PET braid presents a load of 1165.5 ± 63.4 N and a strain of $16.5 \pm 0.8\%$, and the rope a load of about 18359.7 ± 522.4 N and a strain of $53.9 \pm 2.2\%$. As observed in figure 4.5.A, in the beginning of curves, the rope presents a curve shape different from the braid, being possible to identify two distinct slopes referred in figure 4.5.D as stiffness I and II. Regarding stiffness I, the rope presents a value of about 28.0 ± 0.55 N/mm. Regarding stiffness II, the rope presents a value of about 208.0 ± 12.5 N/mm and the braid of about 25.1 ± 1.4 N/mm. When the rope is exposed to 10 cycles of accommodation using a load range between 500-3500 N or 750-7500 N, its stiffness II* increases to about 317.3 ± 6.2 N/mm and 397.0 ± 29.8 N/mm respectively.

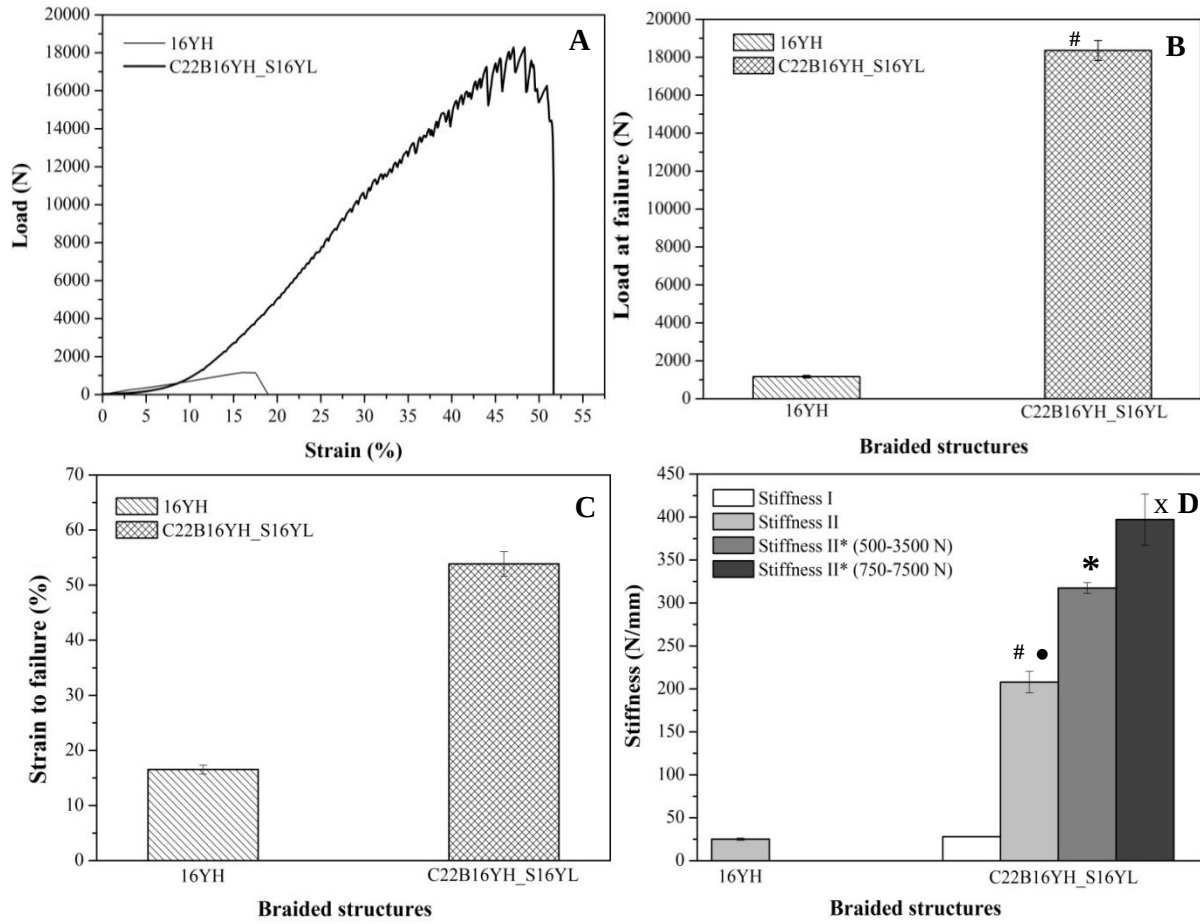


Figure 4.5: (A) Representative force-strain curves of the PET 16YH braid and C22B16YH_S16YL rope. Tensile properties of the PET 16YH braid and C22B16YH_S16YL rope: (B) load at failure, (C) strain to failure and (D) stiffness. Data presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared with 16YH (#); stiffness I (•); stiffness II (*) and stiffness II* (500-3500 N) (x).

4.3.3. Time-dependent mechanical behaviour

4.3.3.1. Monotonic cyclic tests

In figure 4.6 are presented the tensile tests results of the C16B16YH_S16YL rope after the monotonic cyclic tests. As observed, after 500 cycles of exposure to the sinusoidal load, when the rope is tensile tested there is a very significant decrease of the load at failure as observed in figure 4.6.A, from 16625.7 ± 216.7 N to 15116.7 ± 94.5 N. The strain to failure, as observed in figure 4.6.B, also presents a significant decrease after 500 cycles, from about 80.3 ± 1.9 % to 36.4 ± 1.3 %. After the 500 cycles, the failure load and strain tend to stabilize with the increasing of cycles number.

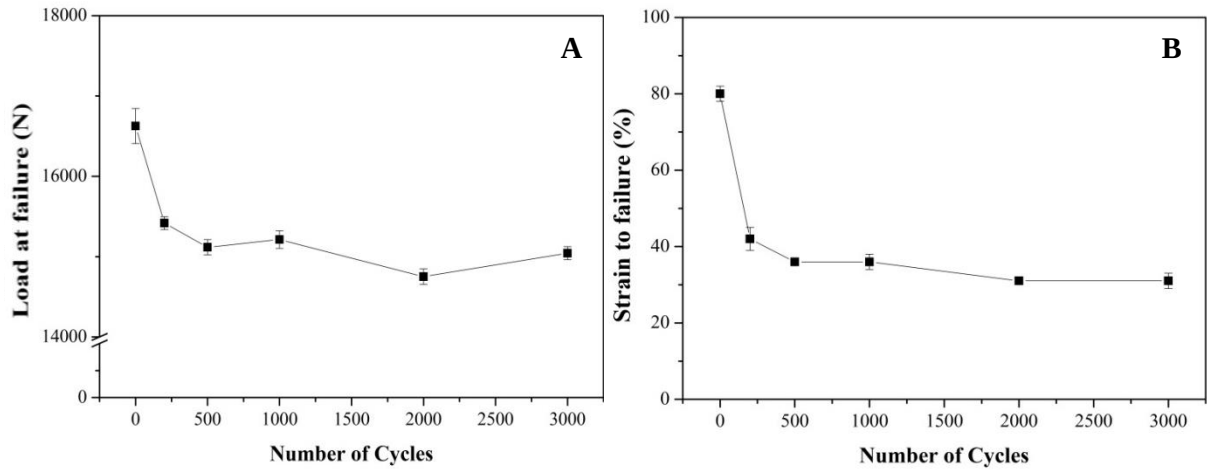


Figure 4.6: Tensile properties of C16B16YH_S16YL after different numbers of cycles in fatigue tests: (A) load at failure and (B) strain to failure. Data are presented as mean $\pm$ SD.

In figure 4.7 are presented the tensile tests results of the C22B16YH_S16YL rope after the monotonic cyclic tests. After several cycles of exposure to the sinusoidal load, when the rope is tensile tested there is not a significant change of the load at failure, it remains almost constant (figure 4.7.A.). Regarding the strain to failure (figure 4.7.B), it also remains almost constant when increasing the number of sinusoidal cycles.

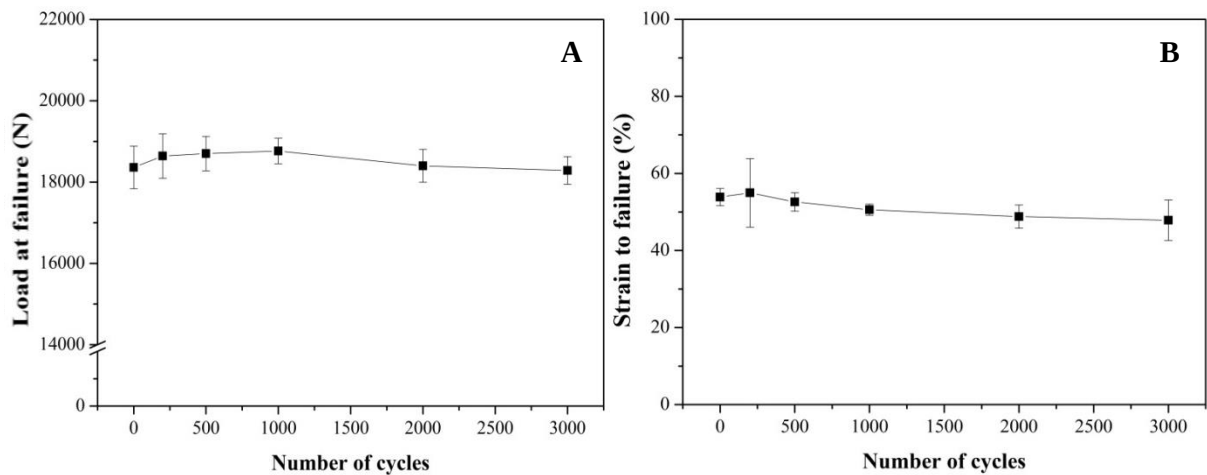


Figure 4.7: Tensile properties of C22B16YH_S16YL after different numbers of cycles in fatigue tests: (A) load at failure and (B) strain to failure. Data are presented as mean $\pm$ SD.

Figure 4.8.A reveals the force-strain curves of C22B16YH_S16YL after different numbers of cycles of an imposed sinusoidal load between 500-3500 N and 750-7500 N. After 10 cycles, for both load ranges, the area between the load and unload curves, representing hysteresis, significantly decreases, reaching a plateau after 100 cycles. Its evolution is

presented in figure 4.8.B, where is observed a significant decrease from the 10th cycle to the 100th cycle.

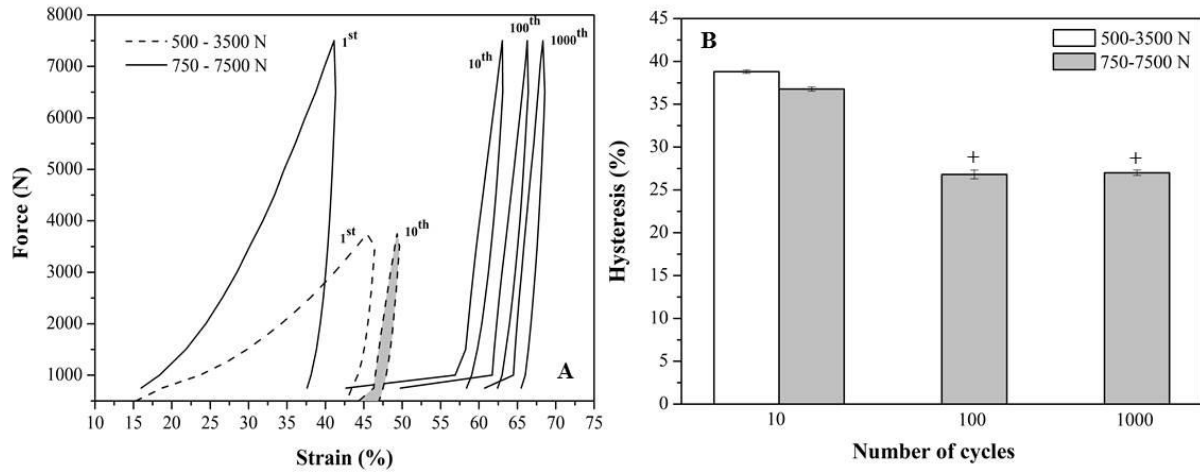


Figure 4.8: Hysteresis results of PET_C22B16YH_S16YL: force-strain curves (A) and hysteresis percentage (B) after 1 and 10 cycles of sinusoidal loading between 500-3500 N and after 1, 10, 100 and 1000 cycles between 750-7500 N (data presented as mean $\pm$ SD, + $p < 0.05$ -significant difference compared with 10 cycles).

4.3.3.2. Creep tests

Figure 4.9 shows the results of creep behaviour of the C22B16YH_S16YL rope. As observed in figure 4.9.A, over the 2000 s of loading the strain level just slightly increases, what is better observed in figure 4.9.B. The rope presents creep at a very low rate, reaching about 1.5% of creep strain after 2000s, corresponding to less than 3% of total strain.

4.3.4. Nano-CT analysis

In figures 4.10.A and 4.10.B it is possible to observe the cross-section of the C22B16YH_S16YL rope in a 2D and 3D nano-CT analysis version, respectively. The open spaces among the braids and yarns that compose the structure as well as the sub-components of the rope (the braids) and the shell yarns that are enclosing the braids are clearly seen. In figure 4.10.C is even possible to observe the arrangement of the yarns that compose the rope shell in a nano-scale. Moreover, in figure 4.10.D is presented an histological image of a transverse section of a rabbit AT, acquired by the authors in a previous work [1], in which the collagen fibre bundles are limited by a membrane, is shown. In figure 4.11 are presented the porosity results of the C22B16YH_S16YL rope after treatment of Nano-CT data. In figure 4.11.A it is presented how the porosity percentage, which was calculated for each acquired layer along the length, evolves along a sample with 2 cm of length. As observed, the porosity

level is very similar along the length, being the mean value of about 37.8 ± 0.7 %. Moreover, figures 4.11. B and 4.11.C, based on data acquired from different layers ranges, show the porosity homogeneity in rope and the interconnectivity among the structure open spaces (pores networks), forming several longitudinal thin and large channels (in red) along the rope length. In figure 4.11.D is even possible to observe how the pores are distributed in cross-sectional planes along the length and figure 4.11.E gives a general perspective of pores networks.

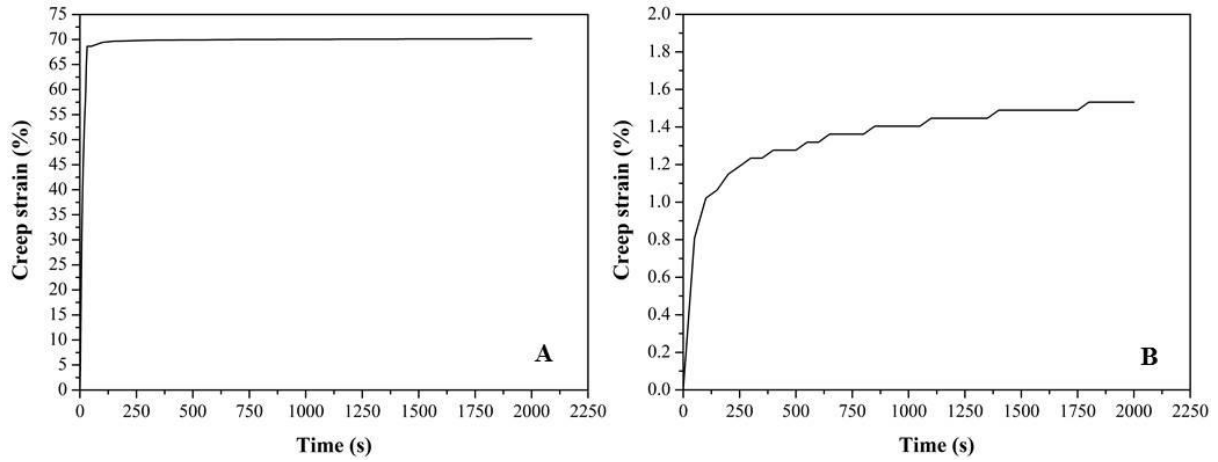


Figure 4.9: (A) Representative curve of creep behaviour of PET_C22B16YH_S16YL rope over the 2000 s and the creep strain observed after reaching the required load level (B).

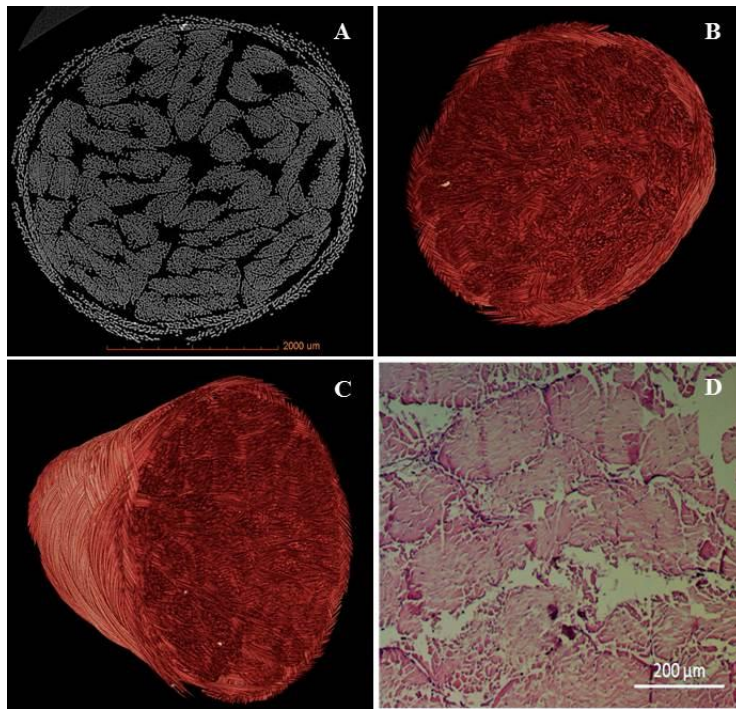


Figure 4.10: Nano-CT images of the C22B16YH_S16YL cross-section, in 2D (A) and 3D (B) mode, and a longitudinal view in 3D mode (C). Histological image of a transverse section of a rabbit AT (D) [1].

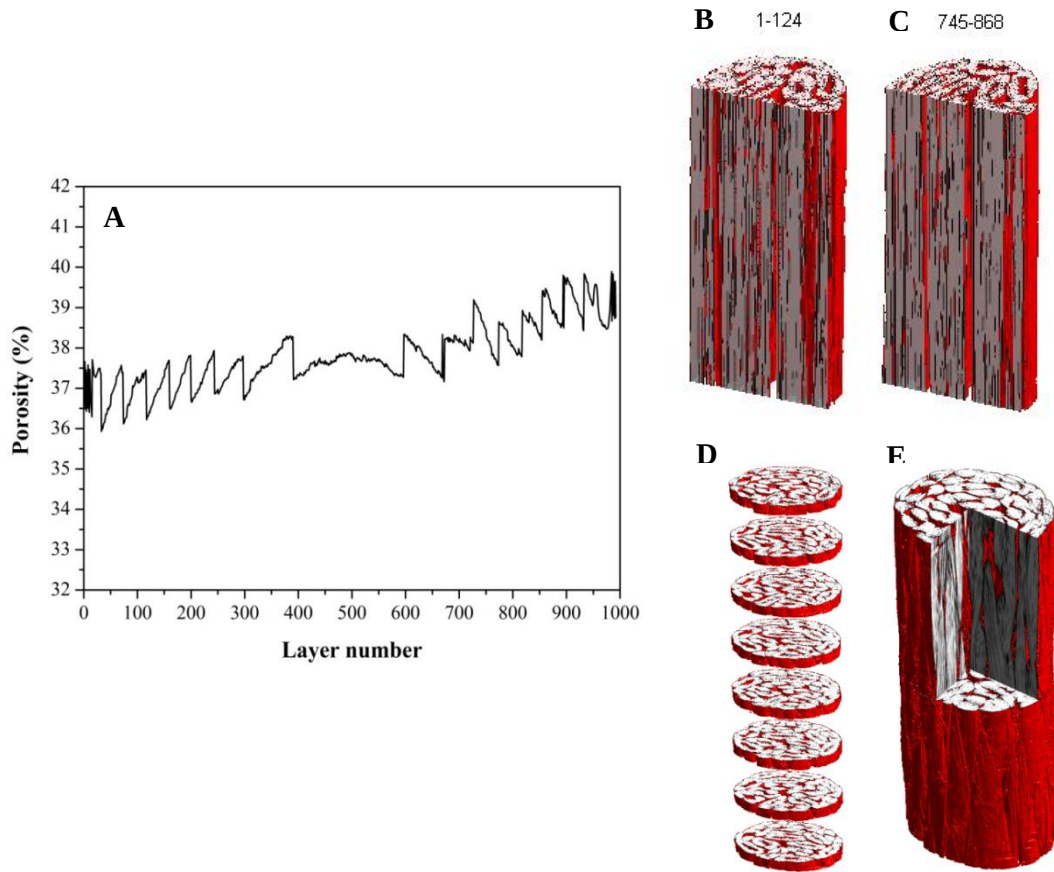


Figure 4.11: (A) Evolution of porosity percentage along 2 cm of length (layer number) of C22B16YH_S16YL. Porosity distribution (open spaces in red) inside the rope: longitudinal distribution based on data acquired from layer 1 until 124 (B) and from layer 745 until 868 (C); segmentation view (D); general view (E).

4.4. Discussion of results

The most frequently damaged tendons are the ones subjected to the highest stresses, the ones most exposed to repeated strains (shear or compressive forces) and the ones with less vascularization. By this way, the tendons that present more tendinopathies are patellar, AT, rotator cuff and hand and foot flexor tendons [8, 13]. Worldwide, the AT has been one of the most common sports-related injuries, a 2 in 100,000 individual tendon injury rate increased to a 12 in 100,000 individual injury rate in less than 10 years. In Europe, for instance, it has been reported an incidence rate of 37.3 cases per 100,000 individuals [1, 10]. Tendinopathies are associated with pathological changes in a tendon caused by overload and overuse, which can cause a partial lesion or even total tissue rupture [1]. So, in order to develop a device able to partially replace the AT, what may be necessary in some clinical cases due to the injury extension, the authors have developed and characterized simple fibrous textile structures,

called braids, that were associated and arranged as sub-components to form a more complex structure, commonly known as rope, and try to mimic the complex fibrous micro-architecture of tendons.

First of all, in figures 4.2.A, 4.2.C and 4.2.E are presented the braids based on PP or PET multifilament yarns that were previously produced, which are classified as biaxial attending to the two different orientations of the constituent yarns, and as diamond (1/1 repeat) in case of PP_8YH, and regular (2/2 repeat) in case of both 16YH braids attending to the braiding pattern. Comparing the two braids based on PP yarns, when the number of yarns increases from 8 to 16 there is an increase in diameter and of course in linear density. In braiding process, the yarns interlace diagonally, so each yarn makes an angle with the structure longitudinal axis, as assigned in figure 4.2 with red lines, which is called braid angle. The braid angle is the most important geometrical parameter of a braided structure, once it highly influences its mechanical profile [14, 16]. As observed in figure 4.3, the braid angle increases when the number of yarns increases. The braid angle of a braided structure is related with the number of braiding points/cm, when the yarns number increases the number of braids/cm tends to increase, as observed in table 4.1, leading to a higher braid angle. These results are in accordance with literature that describes the same effect of the yarns number on the braid angle [14, 16, 17]. The tenacity level, presented in table 4.1, also increases when the PP yarns number increases, in spite of the higher density, what is easily understood once there are more yarns in the structure able to withstand the tensile load applied to the whole structure. In figure 4.4.B is possible to observe the higher load at failure in case of 16YH braid.

Comparing the two 16YH braids, despite using the same yarns number, according to figure 4.2, when using PP yarns the diameter is higher than using PET yarns, what is explained by the higher diameter of PP yarn ($989.0 \pm 1.7 \mu\text{m}$) comparing to PET yarn ($830.8 \pm 1.6 \mu\text{m}$). The same happens with the linear density, which is higher when using PP yarns once they present a higher linear density (1200 dtex) than PET yarns (1112 dtex). Regarding the load at failure, the two braids present similar values (about 74 N), as observed in figures 4.4.B and 4.5.B, however the PET_16YH braid presents a higher tenacity than PP_16YH braid due to the lower linear density. Moreover, it should be noticed that when using the PP yarn the braid angle is slightly higher, what may be explained by the higher yarn diameter. Attending to this higher braid angle, but also on polymeric nature of PP yarns, the PP_16YH braids present a higher strain to failure than the PET_16YH braids [16, 18].

Each one of the braids was then used as sub-component of a rope based on a core/shell system as presented in figure 4.2.G. Each polymeric rope, all presented in figures 4.2.B, 4.2.D and 4.2.F, has a core composed by 16 or 22 braids of 8 or 16 yarns and a shell to enclose those braids composed by 16 braided yarns. Comparing the two ropes based on PP yarns, which are presented in figures 4.2.B and 4.2.D, both use 16 braids in the core but the C16B8YH_S16YL rope uses 8YH braids as sub-components and the C16B16YH_S16YL rope uses 16YH braids. Therefore, the C16B16YH_S16YL rope has more yarns in its core and consequently has a higher diameter and linear density than the C16B8YH_S16YL rope, as presented in table 4.1. Besides that, the braid angle between the shell yarns is also related with the number of braids in the rope core (and consequent diameter), being slightly higher in case of C16B16YH_S16YL (figure 4.3) once that the same 16 shell yarns have to enclose a higher volume in that rope than in case of C16B8YH_S16YL. However, the number of braids/cm slightly decreases when the rope diameter increases, namely when using 16 braids of 16YH instead of 16 braids of 8YH, as observed in table 4.1, what is in accordance with other reported studies [19]. This may be explained by the flattening/enlargement of the shell yarns when they have to enclose the 16YH braids (higher diameter). Comparing the PP_C16B16YH_S16YL and the PET_C22B16YH_S16YL ropes, the second one uses more 16YH braids than the first one and it has a higher linear density. In spite of using more braids in core, the PET rope presents a lower diameter, what may be explained by the lower diameter of each PET_16YH braid than in case of PP_16YH braid, leading to a different arrangement among the braids when they are enclosed by the shell. Moreover, the braid angle between the shell yarns and the number of braids/cm are higher for PET_C22B16YH_S16YL than for PP_C16B8YH_S16YL, in spite of having a very close diameter. This may be related with the different diameter between the PET and PP yarns composing the ropes shell. It must be noted that the shell yarns for the three ropes are also biaxially oriented and the braiding pattern is classified as diamond.

Tendons present a non-linear viscoelastic behaviour in response to loading, presenting a typical force-strain curve, in elongation-to-failure conditions, with three different regions. The first region, named as toe region, presents a gradual increase in curve slope until a strain of about 2% - 4%, which represents the stiffness I. In this region the load elongates the tendon by reduction of the collagen fibres crimp angle, however those forces do not cause significant fibre stretching. So, in this region the tendon elastic limit is not exceeded and when the stress is released the crimped pattern and tendon length are restored. The elastin fibres are probably the main responsible for bringing the stretched collagen bundles back to

the resting stage [4, 20, 21]. In case of further elongation a second region with constant stiffness (stiffness II) appears, named as linear region, in which tissue elongation happens due to the stretching imposed by the load. In this region the collagen bundles are no longer crimped, they are stretched, but when unloading they restore their initial length until a certain stress level. The force-strain curve of a tendon is classified as non-linear, the slope of the toe region is different, namely lower, from the linear region slope. Since it is easier to stretch out the crimp of the collagen fibrils, stiffness I is lower than stiffness II. If elongation continues, a third region appears in which collagen fibres fail in an unpredictable way causing tears tissue, leading to the total rupture [4, 21].

In figures 4.4.A and 4.5.A it is possible to observe the force-strain curves of each braid and each rope. PP_C16B8YH_S16YL, PP_C16B16YH_S16YL and PET_C22B16YH_S16YL ropes also present non-linear force-strain curves, being also possible to identify three regions. Comparing the two ropes based on PP yarns, the PP_C16B16YH_S16YL rope presents a load at failure higher than the PP_C16B8YH_S16YL rope as observed in figure 4.4.B. This is easily understood by the fact that the first rope has more yarns on the core to withstand the applied load, being its load at failure more or less proportional to the number of 16YH braids. Regarding the strain to failure, the PP_C16B16YH_S16YL rope also presents a higher value than the PP_C16B8YH_S16YL rope about $(16626 \pm 217 \text{ N})$. This is related with the braid angle of sub-components of each rope, a higher braid angle makes that the yarns in structure take more time to align with the braid/rope longitudinal axis and consequently with tensile load direction [15, 16]. The PET_C22B16YH_S16YL rope presents a load at failure (about $18359 \pm 222 \text{ N}$) higher than PP_C16B16YH_S16YL rope, as observed in figures 4.4.B and 4.5.B, once the PET rope has more 16YH braids with a load at failure similar to PP_16YH braids. However, the PET rope tenacity is slightly lower than in case of PP_C16B16YH_S16YL rope, once the PET rope has a higher linear density. The load at failure of PET rope is lower than expected, the force is not proportional to the number of PET_16YH braids present in core. It is believed that this phenomenon is due to the grips of the used tensile machine, they may not be the most appropriate ones to the larger diameter of PET rope. Regarding the strain to failure, the PET_C22B16YH_S16YL rope presents a lower value due to the lower strain level associated to the PET_16YH braids than in case of PP_16YH braids.

The load at failure, strain to failure and stiffness of a native AT depend on thickness and collagen content, the patient gender, age and physical activity [8, 20, 21]. *Arampatzis et al.*, for instance, have reported a load at failure of about $3597 \pm 447 \text{ N}$ with a correspondent

strain of about 5.1 ± 1.0 % before exercise and about 4019 ± 501 N with a correspondent strain to failure of about 5.8 ± 1.1 % [22]. *Lichtwark et al.* reported a load of about 5000 N for an elongation of about 25 mm [23]. *Peltonen et al.*, for instance, reported a force of about 3000 N for an elongation of about 13 mm [24]. So, the forces that PP_C16B16YH_S16YL and PET_C22B16YH_S16YL ropes are able to withstand are significantly higher than the force levels that a native AT may experience. Regarding the ropes strain level, when the rope is just loaded until the rupture at once, it is higher than in native AT, but it will decrease when the ropes are continually mechanically requested as happens in native AT working *in vivo* under loading over time.

Thus, the most demanding static requirement for the structure intended to be developed in this work is to get close to the force-strain curve slope (force/elongation relation), namely the stiffness II value of native tissue. In case of PP_C16B8YH_S16YL rope (figure 4.4.A), it is possible to identify a first region until 6% of strain with a stiffness I of about 26.3 ± 5.8 N/mm, then a second region in which the stiffness II is about 73.0 ± 1.1 N/mm, followed by rupture. In case of PP_C16B16YH_S16YL rope (figure 4.4.A), it is possible to identify a first region until 20% of strain with a stiffness I value of about 19.5 ± 2.3 N/mm, then a second region in which the curve slope (stiffness II) significantly increases to about 108.6 ± 6.0 N/mm, and finally the rupture happens. The difference in strain value at which the curve slope of the two ropes changes is mainly related with rope accommodation to the tensile machine grips. Once that the PP_C16B16YH_S16YL rope has a higher diameter than the PP_C16B8YH_S16YL rope (more yarns in core) and the core braids have a higher braid angle, the alignment of all filaments with the tensile direction will take more time. In case of PET_C22B16YH_S16YL rope (figure 4.5.A), it is also possible to identify a first region until about 8% of strain with a stiffness I value of about 27.7 ± 1.4 N/mm, then a second region appears in which the curve slope (stiffness II) significantly increases to about 208.0 ± 12.5 N/mm, and finally the rupture happens.

Tendons stiffness is the most frequently used parameter to study tendons adaptation according to several factors related with the patient life, such as the tissue maturation, aging, training, inactivity and disease [24]. Therefore, in order to better simulate a real application of the implant under development, the PP_C16B16YH_S16YL and PET_C22B16YH_S16YL ropes were exposed to a sinusoidal load over 10 cycles and the stiffness II was evaluated. The sinusoidal load was defined between 750-7500 N, once it is a load range more demanding than the ones that native AT usually experiences [23]. As observed in figures 4.4.D and 4.5.D, after those 10 cycles, the stiffness II of

PP_C16B16YH_S16YL rope increases from 108.6 ± 6.0 N/mm to 178.8 ± 4.0 N/mm, and the stiffness II of PET_C22B16YH_S16YL increases from 208 ± 12.5 N/mm to 317.3 ± 6.2 N/mm (for the load range of 500-3500 N) and to 397.0 ± 29.8 N/mm (for the load range of 750-7500 N). In literature, several values of native AT stiffness II have been reported depending on individual features, as already mentioned. *Peltonen et al.*(2013), for instance, have reported a stiffness between 193 N/mm for a fast loading rate and 207 N/mm for a slow loading rate [24]. In order to evaluate how the exercise activities influence native AT stiffness, *Peltonen et al.*(2010) performed another study in which after a single bout of hopping exercise they reported a value of about 390 ± 190 N/mm compared to 430 ± 200 N/mm before the exercise [5]. *Lichtwark et al.*, who also evaluated the AT stiffness of ten individuals, during one-legged hopping, reported a mean value of about 188 ± 43.2 N/mm [23]. *Arampatzis et al.* also studied the stiffness level before and after exercise but using two different strain rates: for the lower rate the values before and after exercise were 275.5 ± 53.0 N/mm and 261.1 ± 55.8 N/mm; for the higher rate the values before and after exercise were 257.8 ± 50.7 N/mm and 301.9 ± 56.8 N/mm [22].

Attending to the final application, the developed structures will be under long-term and repeated loading and they must be reliable for a long time period, which is one of the major reasons for failure in structural materials. Thus, the fatigue resistance of PP_C16B16YH_S16YL and PET_C22B16YH_S16YL ropes, which revealed the more interesting stiffness value, was evaluated by exposure to several force cycles. Once again, it was used a demanding force range between 750-7500 N. Both ropes were cyclically requested and then tensile tested to measure their failure load and strain. However, before the tensile test the ropes were allowed to rest for about 24 h, once that when a previously tensioned rope is allowed to rest unloaded, it will continue to contract until equilibrium is reached over few hours [15]. This contraction is associated to a recoverable elongation, which is due to the rope structure accommodation and compaction, and some elastic elongation of filaments. The remaining elongation may not be recovered, because it involves the plastic elongation of filaments, being designated as permanent elongation [15, 18]. In case of PP_C16B16YH_S16YL rope, as observed in figure 4.6.A, when the number of cycles increases the load at failure significantly decreases after 200 cycles from 16626 ± 217 N to 15417 ± 81 N. After that, as the number of cycles increases the load at failure slightly decreases tending to stabilize. Regarding the strain to failure, as observed in figure 4.6.B, when the number of cycles increases it also significantly decreases after 200 cycles from 80 ± 2 % to 42 ± 3 %. After that, as the number of cycles

increases the strain level also slightly decreases tending to stabilize. This significant decrease in strain to failure, only after 200 cycles, indicates that with repeated loading there is an increase of the permanent strain of PP_C16B16YH_S16YL rope. This reveals that there is a plastic elongation of filaments besides the structure compaction, or even the damage and rupture of some filaments, what will cause a decrease in rope lifetime.

In case of PET_C22B16YH_S16YL rope, as observed in figure 4.7, when the number of cycles increases until 3000, the failure load and strain keep always almost constant. Besides that, it must be referred that fatigue tests were also performed using the PET_16YH braids for a load range between 35-350 N (data not showed). The braids were able to withstand 1 million cycles even at a frequency of 5 Hz, which is really above the frequency of 1.37 Hz experienced by the AT for instance in a marathon of 2 h 14 min at 19 km/h [25]. This fatigue behaviour of PET braids that compose the PET rope core is also a very promising indicator about the rope lifetime.

The non-linear viscoelastic profile of tendons indicates that their mechanical behaviour is time and stress dependent [24-26]. Thus, the relationship between stress and strain is not constant, it depends on time of displacement or load, giving rise to a creep or force-relaxation phenomenon. Creep phenomenon is characterized by an increasing deformation under constant load. This contrasts with an elastic material, which does not exhibit increase of deformation no matter how long the load is applied [27-29]. In force-relaxation phenomenon, the force withstand by the structure will decrease when it is exposed to a constant strain level. The stress required to maintain the constant strain value is decreased with time because the molecules of polymer get relaxed with time, and to maintain the level of strain somewhat lower value of stress is enough [18, 28, 29].

Based on stiffness II and fatigue resistance values obtained for the different structures, the PET rope was chosen to evaluate the creep behaviour by exposing it to a cyclic loading between 750-7500 N over 10 cycles and then it was loaded again until the 7500 N and this force level was kept over 2000 s. As observed in figure 4.9, over the 2000 s the rope strain level only increased about 1.5%, revealing a very good creep resistance when compared to the creep behaviour of a native AT working *in vivo*. Hawkins *et al.*, for instance, reported a strain increase of about 3.5% after 7 minutes of stimulation [30]. Regarding the force-relaxation phenomenon, PET_16YH braids that compose the rope core were evaluated for different strain levels (data not showed) and the relaxation rate was very low. So, based on relaxation behaviour of rope sub-components and also on rope creep behaviour, it is possible to expect a very low rope force-relaxation. This means that when the rope is extended until

different lengths it will be able to withstand the consequent load over long time periods without getting relaxed.

As viscoelastic materials, tendons hysteresis is important because it reflects how much stored elastic energy is dissipated as heat during recoil of a tendon and may also affect muscle fibres strain [31]. Hysteresis (%) was calculated as the area of the loop formed between the ramp-up and relaxation force-strain curves, expressed as a percentage of the area below the ramp-up curve, as presented in figure 4.8.A by the grey area assigned for instance for the 10th for 500-3500 N, which represents the amount of energy that is absorbed/dissipated by the material/structure in form of viscoelastic deformation, by different friction and fabric mechanisms [15, 32]. Like other unidirectional fibre-reinforced composites, the parallel arrangement of collagen fibres acts to resist tension, so that contractile energy is not lost during load transmission. In most everyday activities, the loads observed in tendons are certainly well below their ultimate tensile strength. However, in quick eccentric movements of a native AT, more significant stresses can be observed exceeding the often reported failure strength of the tendon [33]. Therefore, based on monotonic cyclic (fatigue) tests, the authors studied the hysteresis behaviour of PET rope over loading. The dissipated energy mainly results from the fact of the polymeric filaments being viscoelastic, so some of the elongation energy is dissipated as heat due to internal friction. In addition, the phenomenon of friction between filaments during the cyclic solicitations also leads to some energy release as heat [15, 32].

For this study, two different load ranges were used, namely a lower range of 500-3500 N to mimic a moderate physical activity, and a higher range of 750-7500 N to mimic a more severe activity of a native AT. As observed in figure 4.8.A, for both load ranges comparing the 1st and 10th cycle, the area between the loading and unloading curves significantly decreases. At the 10th cycle, the hysteresis value is very similar for both load ranges. In case of 750-7500 N range is also possible to observe that the area tends to stabilize after the 100th cycle, as observed in figure 4.8.B, being the hysteresis value of about 27%. The decrease and stabilization of the rope hysteresis after some cycles, which was also reported by other authors, is related with the textile structure accommodation. After some load conditioning the filaments are already stretched in the tensile direction and parallel to the axis of braids in rope core, decreasing the dissipated energy [15, 32]. Moreover, in figure 4.8.A is possible to observed the already mentioned increase of stiffness II of PET rope from the 1st cycle (208 ± 12.5 N/mm) to the 10th cycle (397.0 ± 29.8 N/mm), stabilizing after that (loading phase between 750-7500 N). But in case of the natural tissue, a reduction in AT stiffness may occur

after just 1000 loading cycles, or even before that, at physiological stress levels [5, 34]. It is even important to mention that the strain point separating the toe and linear region after 10 cycles is reduced from about 8% to about 1.5 - 2%, as observed in figure 4.8.A.

In case of native AT, *Tishya et al.* reported an increasing area of the hysteresis loop during the cyclic tests, reflecting progressive extension of the toe region, reduction of stiffness and increasing of cyclic energy dissipation [34]. Several authors have reported hysteresis percentages ranging from 17 to about 35%, *Lichtwark et al.* for instance recorded an hysteresis average value of about 26%, however it depends on individual physical features and activity [23, 24]. It should also be referred that ultrasonography and magnetic resonance imaging studies have recently reported strains of the native AT ranging from 5–10% during isometric contractions at forces below the load at failure during dynamic movements [23]. The strain difference during loading and unloading at and after the 10th cycle, is about 5% in case of range 750-7500 N and about 4% in case of 500-3500 N, as observed in figure 4.8.A.

Therefore, these results of hysteresis, as well as the stiffness difference already discussed before and after some loading cycles, indicate the importance of performing a laboratorial preconditioning of the developed PET device before AT reconstruction. This interest of preconditioning was also referred by *Elmarzougui et al.* and *Graf et al.*, once it has been proved that after exposure of a tendon to several loading and unloading cycles, its force–strain curve becomes stabilised and repeatable [32, 35].

The pores amount and their interconnectivity extending through an implant enhance its regenerative properties by the increase of the overall surface area for cell attachment and by allowing the tissue ingrowth into the structure matrix [36, 37]. Therefore, the nano-CT analysis was performed in order to analyse the arrangement of yarns inside the PET rope and its porosity level. As observed, in figures 4.10 and 4.11, the rope porosity level of about $37.8 \pm 0.8\%$ is due to smaller open spaces among the yarns composing the braids and also due to bigger open spaces among the braids in core. The braids inside the core are parallel aligned and there is a high pore interconnectivity, creating channels along the longitudinal rope axis. It must be noticed the homogeneity of pores distribution in both planes, transverse and longitudinal, as observed in figure 4.11. So, this homogeneous interconnectivity and porosity level of PET rope may be very promising to promote the injured tissue regeneration. This will allow a better cell migration into the implant, higher level of cell adhesion due to the higher surface area and more efficient exchange of nutrients and oxygen with medium, enhancing the tissue and blood vessels ingrowth [36, 37]. Moreover, comparing the rope fibrous

architecture with a native tendon, the rope sub-components (16YH braids) can be compared with the collagen fibre bundles limited by a membrane that are observed in figure 4.10.D.

4.5. Conclusions

The three different ropes presented a non-linear force-strain curve with three different regions as also reported in case of native AT tensile curve. The load at failure level of ropes is mainly controlled by the number of yarns/braids in core, but the strain to failure level is mostly influenced by the take-up rate and consequent braid angle of braids that compose the core. Therefore, the ropes stiffness level results from a combination of the yarns/braids number in core and the associated braid angle. The PET_C22B16YH_S16YL rope presented a load at failure, strain and stiffness II level appropriate for the AT substitution, according to data reported in literature.

Moreover, the PET_C22B16YH_S16YL rope revealed a very promising fatigue and creep resistance according to the demanding requirements for the final application. The high porosity of the PET rope is also a very important feature of this structure to allow a better cell migration and adhesion, tissue and blood vessels ingrowth into the fibrous structure and promote successful implant integration *in vivo*.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project UID/EMS/00285/2013.

4.6. References

- [1] Morais DS, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair, *Annals of Biomedical Engineering*. 2015; 43(9):2025-35.
- [2] Dudek R.W. High-Yield Histology. 2nd ed. USA: Lippincott Williams & Wilkins; 2000.
- [3] Johnson K.E. histology and cell biology. 2nd ed. USA: Williams & Wilkins; 1991.
- [4] James R., Kesturu G., Balian G., Chhabra A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery*. 2008;33:102-12.
- [5] Peltonen J, Cronin NJ, Avela J, Finni T. In vivo mechanical response of human Achilles tendon to a single bout of hopping exercise. *Journal of Experimental Biology*. 2010;213:1259-65.

- [6] Kuo C. K., Marturano J. E., Tuan R. S. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2010;2:20.
- [7] Uquillas CA, Guss MS, Ryan DJ, Jazrawi LM, Strauss EJ. Everything Achilles: knowledge update and current concepts in management. *J Bone Joint Surg Am*. 2015;97:1187-95.
- [8] Maffulli N., Renstrom P., Leadbetter W. *Tendon Injuries-Basic Science and Clinical Medicine*: Springer; 2005, London.
- [9] Online MD. Orthopedic Soft Tissue Repair Market Is Expected To Reach USD 8.5 Billion Globally In 2019: Transparency Market Research. 2014.
- [10] Hess G. W. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot & ankle specialist*. 2010;3:29-32.
- [11] Ham P, Maughan KL. Achilles tendinopathy and tendon rupture. UpToDate, Fields, K (Ed), UpToDate, Waltham, MA. 2012.
- [12] Bassetto F., Volpin A., Vindigni V. Regenerative Medicine for Tendon Regeneration and Repair: The Role of Bioscaffolds and Mechanical Loading. In: R. P, editor. *Biomaterials Science and Engineering*: InTech; 2011: 370-86, Italy.
- [13] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.
- [14] Kyosev Y. *Braiding technology for textiles: Principles, design and processes*: Elsevier; 2014, United Kingdom.
- [15] McKenna HA, Hearle JW, O'Hear N. *Handbook of fibre rope technology*: Elsevier; 2004, USA.
- [16] Omeroglu S. The effect of braiding parameters on the mechanical properties of braided ropes. *Fibres and Textiles in Eastern Europe*. 2006;14:53.
- [17] Bicking AM, Oxenham W. Variables and methods for aesthetic braid design. *Journal of Textile and Apparel, Technology and Management*. 2012;7.
- [18] Hearle JW, Morton WE. *Physical properties of textile fibres*: Elsevier; 2008, USA.
- [19] Kyosev Y. *Recent Developments in Braiding and Narrow Weaving*: Springer; 2016, Switzerland.
- [20] Jung H., Fisher M., Woo S. Role of biomechanics in the understanding of normal, injured, and healing ligaments and tendons. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2009;1:1-17.
- [21] Sharma P., Maffulli N. Biology of tendon injury: healing, modeling and remodeling. *Journal of musculoskeletal & neuronal interactions*. 2006;6:181-90.
- [22] Arampatzis A., Peper A., Bierbaum S., Albracht K. Plasticity of human Achilles tendon mechanical and morphological properties in response to cyclic strain. *Journal of biomechanics*. 2010;43:3073-9.

- [23] Lichtwark GA, Wilson A. In vivo mechanical properties of the human Achilles tendon during one-legged hopping. *Journal of Experimental Biology*. 2005;208:4715-25.
- [24] Peltonen J., Cronin N. J., Stenroth L., Finni T., Avela J. Viscoelastic properties of the Achilles tendon in vivo. *SpringerPlus*. 2013;2:212-20.
- [25] De Zee M, Bojsen-Møller F, Voigt M. Dynamic viscoelastic behavior of lower extremity tendons during simulated running. *Journal of Applied Physiology*. 2000;89:1352-9.
- [26] Buckley CP, Salisbury SS, Zavatsky AB. Viscoelasticity of Tendons Under Transverse Compression. *Journal of biomechanical engineering*. 2016;138:101004.
- [27] Lechat C, Bunsell AR, Davies P. Tensile and creep behaviour of polyethylene terephthalate and polyethylene naphthalate fibres. *Journal of Materials Science*. 2011;46:528-33.
- [28] Balani K, Verma V, Agarwal A, Narayan R. *Biosurfaces: A Materials Science and Engineering Perspective*: John Wiley & Sons; 2015, Canada.
- [29] DNV-GL. Design, testing and analysis of offshore fibre ropes, DNVGL-RP-E305. September, 2015.
- [30] Hawkins D, Lum C, Gaydos D, Dunning R. Dynamic creep and pre-conditioning of the Achilles tendon in-vivo. *Journal of biomechanics*. 2009;42:2813-7.
- [31] Houghton L, Dawson B, Rubenson J. Achilles tendon mechanical properties after both prolonged continuous running and prolonged intermittent shuttle running in cricket batting. *J Appl Biomech*. 2013;29:453-62.
- [32] Elmarzougui S, Ben Abdesslem S, Sakli F. Hysteresis measurement for characterising the dynamic fatigue of textile artificial ligaments. *The Journal of the Textile Institute*. 2011;102:109-13.
- [33] Shepherd JH, Screen HR. Fatigue loading of tendon. *International journal of experimental pathology*. 2013;94:260-70.
- [34] Wren TA, Lindsey DP, Beaupré GS, Carter DR. Effects of creep and cyclic loading on the mechanical properties and failure of human Achilles tendons. *Annals of biomedical engineering*. 2003;31:710-7.
- [35] Graf BK, Vanderby R, Ulm MJ, Rogalski RP, Thielke RJ. Effect of preconditioning on the viscoelastic response of primate patellar tendon. *Arthroscopy: The Journal of Arthroscopic & Related Surgery*. 1994;10:90-6.
- [36] Cooper JA, Lu HH, Ko FK, Freeman JW, Laurencin CT. Fiber-based tissue-engineered scaffold for ligament replacement: design considerations and in vitro evaluation. *Biomaterials*. 2005;26:1523-32.
- [37] Laurencin CT, Freeman JW. Ligament tissue engineering: an evolutionary materials science approach. *Biomaterials*. 2005;26:7530-6.

Chapter 5

**Surface functionalization of
polypropylene (PP) by chitosan
immobilization to enhance human
fibroblasts viability**

Surface functionalization of polypropylene (PP) by chitosan immobilization to enhance human fibroblasts viability

Morais D.S.^{1,2}, Ávila B.¹, Lopes C.³, F. Vaz³, Machado A.V.⁴, Fernandes M.H.⁵,
Guedes R.M.^{2,6}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Centro de Física, Universidade do Minho, 4710-057 Braga, Portugal

⁴ IPC – Institute for Polymers and Composites, Universidade do Minho, Campus de Azurém, 4800-058 Guimarães, Portugal

⁵ Faculdade de Medicina Dentária, Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, Porto, Portugal.

⁶ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

Abstract

Driven by the market demand, several synthetic and natural grafts have been proposed during the last years for tendons regeneration. The synthetic grafts, which present a better mechanical performance than the natural ones, usually fail due to the lack of biocompatibility and bioactivity. Thus, chitosan (Cs) was immobilized on polypropylene (PP) surface, previously activated by plasma treatment, in order to improve the fibroblasts adhesion and proliferation on it. The *Orange II* dye method and FTIR-ATR analysis proved the successful Cs immobilization on PP surface. It was observed by SEM and optical profilometry analysis that the Cs concentration increase leads to a surface roughness (R_a value) increase, as well as to water contact angle (C.A) decrease at least until 2% (w/V) of Cs. Using the 2% (V7V) Cs solution concentration, according to SEM analysis and resazurin assay, the developed functionalization was well succeeded in improving fibroblasts adhesion and proliferation on PP substrates surface over 7 days of culture.

Key Words: polypropylene, PP, chitosan, surface functionalization, fibroblasts, tendon repair.

5.1. Introduction

The population aging will have a real impact on musculoskeletal health in the coming decades. In fact musculoskeletal disorders already affect more than 100 million Europeans and this number will soon increase grounding a societal need for a regenerative medicine approach [1]. Among these disorders, tendon injuries are the most difficult to manage and although spontaneously healing can occur in some situations this often results in the formation of scar tissue, which has low functionality and consequently the tendon rupture can happen. In some cases, a simple suture backing together the tendon ends is done, but this repair practice is often ineffective. In other cases, the used approach is a tissue replacement with auto or allografts, but these reveal disadvantages, as donor site morbidity, not widely availability and rejection risk [2, 3]. Driven by the market demand several synthetic and natural grafts, intended to be used as graft augmentation devices (patches) or to fully replace a damaged tendon (prostheses), have been proposed during the last years. The synthetic grafts in spite of presenting a better mechanical performance than the natural ones, they usually fail due to the lack of biocompatibility and bioactivity [4, 5].

Polypropylene (PP) is a non-polar, semi-crystalline and thermoplastic polymer used for different applications, such as packaging, textiles or even for medical purposes [6]. It has been very used in hernia repair, wound dressings, sutures, blood contacting surfaces, and thermo-responsive materials [7]. PP offers low level of tissue reaction in contact with living tissues when compared to other synthetic polymeric materials, however, it presents an hydrophobic profile due to the low surface energy, what may be harmful in cell adhesion process [8-10]. Many techniques such as corona discharges, ultraviolet irradiation and plasma treatments have been used to increase its wettability [6, 8, 11, 12]. These techniques increase the surface-free energy by incorporating oxygen-containing polar functional groups on it [8, 10]. In case of plasma treatments, the electrical energy applied from the plasma reactor dissociates the inert gas into electrons, free radicals, ions, photons, and metastable (excited) species. Those free radicals will collide with the material surface and break the covalent bonds. At that moment, the free radicals created on the material surface can combine with the air oxygen and moisture to produce thermodynamically stable functional groups on the surface. Once the plasma surface treatment affects only a limited depth (several molecular layers), the bulk properties of the material remain unchanged [8, 13].

Moreover, after the plasma activation, in order to anchor active functional groups on PP surface, specific biomolecules can be immobilized on the polymer surface to improve the device bioactivity and integration with the living tissues [14-16]. Natural polysaccharides are

very promising molecules for tissue engineering applications, since they present high biocompatibility and resemble glycosaminoglycans of the extracellular matrix (ECM). These polymers have shown a lower stimulation of immunological reactions than some synthetic polymers [17]. Chitosan (Cs), for instance, which is a biodegradable polycation composed by the monomers *D-glucosamine* and *N-acetyl-D-glucosamine* linked by $\beta(1-4)$ -glycosidic bond may be a good option to biofunctionalize PP substrates surface [14]. Cs properties, such as biocompatibility, low toxicity, biodegradability and promotion of cell adhesion, proliferation and differentiation, made it become a very appealing material for biomedical applications [17, 18].

Therefore, the purpose of this study was to functionalize PP substrates by the immobilization of Cs on their surfaces, in order to improve the interaction of those substrates with fibroblasts for tendon tissue regeneration, or even for ligaments regeneration.

5.2. Materials and methods

5.2.1. Materials

The polypropylene (PP) sheets, with a thickness of 0.1 mm, were purchased from *Goodfellow*, acrylic acid (AAc) was supplied by *Merck India Ltd.* and the chitosan (MW: 100.000-300.000) and the acid orange II (99%) were purchased from *Acros Organics*.

5.2.2. Methods

5.2.2.1. PP surface plasma treatment and acrylic acid (AAc) grafting

Circular PP samples with 0.1 mm thickness and 25 mm diameter were prepared and cleaned with ethanol to be exposed to different plasma activation treatments. A *Zepto* laboratory-sized plasma system from *Diener Electronics* ($\varnothing = 105$ mm, L = 300 mm, V = 2.6 L) was used for the plasma activation of the samples. The following plasma treatments were performed according to the used gas and the plasma treatment time, using a power of 100 W, a $pressure_{base}$ of 10 Pa and a $pressure_{work}$ of 80 Pa:

- 1) O_2 plasma over 2, 5, 8 and 10 min;
- 2) Ar plasma pre-treatment over 2 min followed by O_2 plasma over 2, 5, 8 and 10 min.

Subsequently, the plasma treated samples were immersed in different acrylic acid (AAc) aqueous solutions (10 % (V/V), 20 % (V/V) and 40 % (V/V)), at room temperature over 4 hours. After the grafting reaction, to remove any non-grafted acrylic acid (AAc) distilled water was used and the samples were dried in the room temperature.

5.2.2.2. Chitosan (Cs) immobilization on PP surface

The AAc treated PP samples were immersed in 10 mL of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) aqueous solution (concentration 10 mg/mL), buffered to pH 4.8 (in sodium citrate) at 4 °C for 30 min. EDC was used to activate carboxyl groups on AAc grafted samples. The EDC-coupled samples were then immersed in 3 mL of Cs solutions, of 5 different concentrations (w/V): 0.4 %, 0.8 %, 2 %, 4 % and 6% and buffered to pH 4.6 using acetic acid-buffered solutions at 4 °C for 24 h. According to the combination between the AAc and Cs concentrations different samples were prepared, as described in table 5.1. After the Cs immobilization, the samples were washed with acidified water at 4 °C for 30 min and dried at room temperature.

Table 5.1: Samples name according to the used AAc and Cs concentrations.

Samples name	AAc concentration % (V/V)	Cs concentration % (w/V)
PP_10% AAc_0.4% Cs	10	0.4
PP_10% AAc_0.8% Cs		0.8
PP_20% AAc_0.4% Cs	20	0.4
PP_20% AAc_0.8% Cs		0.8
PP_20% AAc_2% Cs		2
PP_20% AAc_4% Cs		4
PP_20% AAc_6% Cs		6
PP_40% AAc_0.4% Cs	40	0.4
PP_40% AAc_0.8% Cs		0.8

5.2.2.3. Physicochemical characterization

Fourier Transform Infrared Spectroscopy (FTIR) in Attenuated Total Reflectance (ATR) mode was performed on untreated PP samples, PP samples after each plasma treatment and after immersion in different Cs solutions (in triplicate), using the spectrometer *Jasco FT/IR 4100* system (64 scans and 8 cm⁻¹ nominal resolution) equipped with a Specac MkII Golden Gate single reflection ZnSe ATR crystal.

The wettability of PP samples surface after each plasma treatment and after immersion in Cs solutions was assessed by the sessile drop method (3 µl drop), measuring the water contact angle (C.A.) at room temperature, using a *OCA 20* unit from *Dataphysics*. A minimum of 9 contact angle measurements were performed for each condition, using at least 3 samples.

The surface topography, average roughness (R_a) and surface height standard deviation (R_q) of PP substrates (2 samples of each condition) were assessed before and after the plasma activation using a Multimode Atomic Force Microscope (AFM) from *Digital Instruments* using the tapping mode ($5 \times 5 \mu\text{m}^2$ scan size and 1 Hz scan rate). A Nanoscope III controller and TESP AFM tips from *Bruker* were also used.

The amount of exposed amino groups on PP surfaces after Cs immobilization was assessed by the *Orange II* dye method, using 3 samples for each type of Cs immobilization. The samples were immersed in 3 mL of dye acidic solution (14 mg/mL) (pH 3) and shaken for 5 h at 30 °C. The samples were then thoroughly rinsed three times with water acidic solution (pH 3) to remove the unbound dye. Once air-dried, the colored samples were immersed in 2 mL of water basic solution (pH 12) and shaken for 15 min at room temperature, measuring the absorbance of the solution at 485 nm.

Scanning electron microscopy (SEM) analysis was performed, using a *FEI Quanta 400 FEG ESEM* microscope, to analyze the microstructure and morphology of at least 2 samples of each Cs immobilized PP surface. The SEM images were acquired using either the secondary or the back-scattered electrons mode and the elemental composition was evaluated by energy dispersive X-ray spectroscopy (EDS). Moreover, after the Cs immobilization, the samples surface was analysed by optical profilometry using a *Bruker NPFLEX* equipment.

5.2.2.4. *In vitro* biological characterization

5.2.2.4.1. Resazurin assay

L929 Fibroblasts (ATCC CCL-1TM) were cultured in α -MEM (*Gibco*) supplemented with 10% (V/V) fetal bovine serum, 1% penicillin and streptomycin solution (*Gibco*) and 2.5 $\mu\text{g/mL}$ of fungizone (*Gibco*). Cells were seeded at 2×10^4 cells per well (24-well culture plates) on the samples surface (untreated PP substrate and Cs functionalized PP substrate) for 1, 3 and 7 days at 37 °C, in a 5 % CO_2 humidified atmosphere. After each time point, 50 μl of resazurin (*Sigma-Aldrich*) at 37 °C was added to each well and the plates were incubated for 3 hours at 37 °C in a 5 % CO_2 humidified atmosphere. Then, the fluorescence (excitation: 530 nm; emission: 590 nm) was measured in a microplate reader (*Synergy HT, Biotek*). This experiment was performed in triplicate for each different material and at each time point. Moreover, for observation of cells after each time point by optical microscopy, cells also seeded on other untreated and treated samples were colored using the MTT reagent (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) (*Sigma-Aldrich*), according to the manufacture instructions.

5.2.2.4.2. SEM analysis

The morphology of L929 fibroblasts seeded on the surface of untreated PP samples and Cs functionalized PP samples, after each time point, was analysed in triplicate by SEM using a *FEI Quanta 400 FEG ESEM* microscope. Briefly, samples with cells were firstly washed twice with PBS and fixed with 1.5 % (m/V) glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4) over 15 min. Afterwards, samples were dehydrated using graded ethanol solutions from 50 % (V/V) to 100 % (V/V). The samples lasted 10 min in 70 and 80% ethanol solutions, 20 min in 90% ethanol solution and overnight in 100 % ethanol solution. Finally, samples were sputter-coated (*SPI-Module*) with a thin gold/palladium film and visualized.

5.2.2.5. Statistical analysis

Experimental data is presented as mean $\pm$ SD (standard deviation). Statistical analysis of data was performed using the one-way ANOVA test with Bonferroni post hoc analysis, using the software SigmaStat 3.5. The differences were considered to be significant at a level of $p < 0.05$.

5.3. Results

5.3.1. PP surface plasma treatment

5.3.1.1. FTIR-ATR analysis

In order to evaluate the chemical groups presented on surface of untreated PP samples and PP samples exposed to different times of plasma O_2 plasma, FTIR-ATR analysis was performed, as presented in figure 5.1.A. Using this technique no detectable changes were observed among spectra before and after exposure to the different plasma treatments. For all samples, it is possible to observe characteristic peaks of PP in the region $3000\text{--}2800\text{ cm}^{-1}$, namely at 2951 cm^{-1} , 2916 cm^{-1} , 2871 cm^{-1} and 2836 cm^{-1} . Moreover, two other intense peaks were observed at 1454 cm^{-1} and 1375 cm^{-1} . Regarding the Ar plasma pre-treatment over 2 min followed by O_2 plasma over different times, the FTIR-ATR analysis was also performed (data not showed) and also no detectable changes were observed before and after plasma exposure.

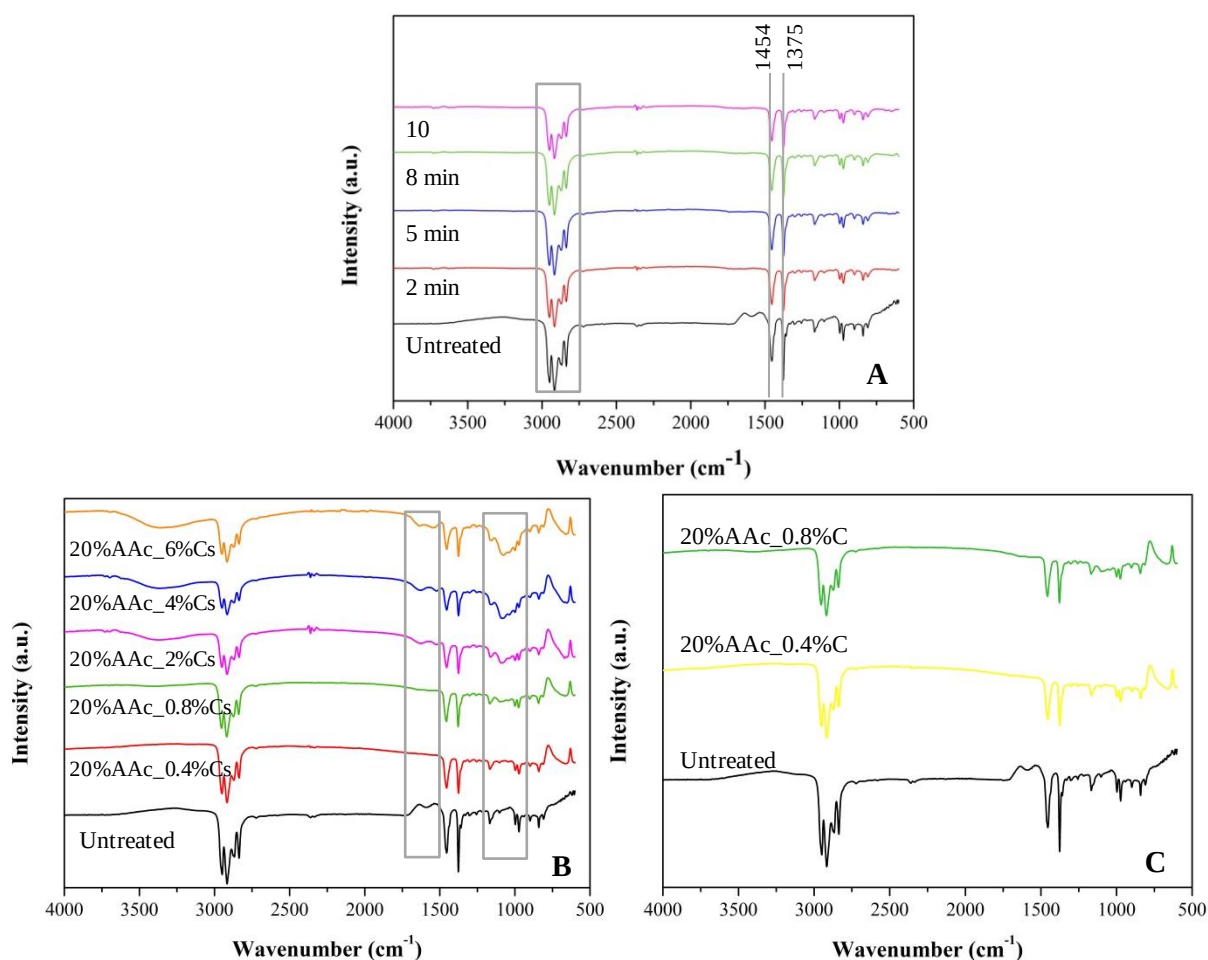


Figure 5.1: FTIR-ATR spectra of the untreated PP surface, Cs powder and PP samples surface after: (A) exposure to O_2 plasma over different times; (B) exposure to O_2 plasma over 5 min and treatment with 20% AAc and different Cs concentrations; (C) exposure to Ar pre-treatment over 2 min followed by O_2 plasma over 5 min and treatment with 20% AAc and two different Cs concentrations.

5.3.1.2. Wettability evaluation

In figure 5.2.A it is possible to observe the water C.A. variation after exposure to O_2 plasma over different times and to the Ar pre-treatment over 2 min followed by different times of O_2 . The presented results show for both plasma compositions (O_2 and Ar pre-treatment followed by O_2) a significant C.A. decrease after 2 min of plasma exposure. However, C.A. values reached the lowest levels after 5 min of treatment, being observed a decrease from $89.8^\circ \pm 0.2$ (untreated PP) to about $55.5^\circ \pm 2.6$ in case of O_2 plasma treatment over 5 min and $52.5^\circ \pm 2.9$ for the Ar pre-treatment over 2 min followed by O_2 plasma treatment over 5 min, representing for both cases a decrease of about 41%. But after the 5 min the water C.A. slightly increases again.

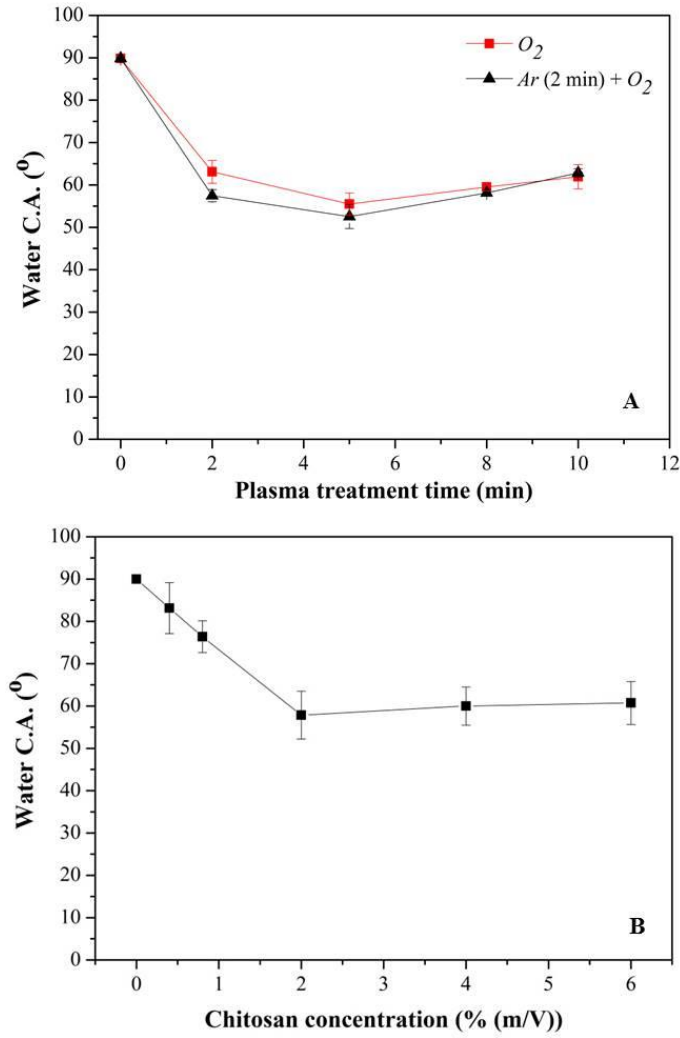


Figure 5.2: Water C.A. variation on the PP surface after: **(A)** exposure to O_2 plasma over different times and to the Ar pre-treatment over 2 min followed by different times of O_2 ; **(B)** functionalization with different Cs concentrations after the O_2 plasma treatment over 5 minutes and 20% AAc grafting.

5.3.1.3. AFM analysis

AFM analysis was performed in order to evaluate the effect of plasma treatment on PP surface roughness. Table 5.2 shows the average roughness (R_a), root mean square roughness (R_q) and representative 3D height images for untreated PP, after the O_2 plasma treatment over 5 min and after the Ar pre-treatment over 2 min followed by O_2 plasma over 5 min. As observed, the untreated PP sample surface shows a heterogeneous rough surface with high protuberances. After the O_2 plasma a non-uniform rough topography is still observed. However, the Ar (2min) + O_2 (5min) treatment caused a very less rough and more homogeneous surface, which presents similar peaks and valleys of low amplitude uniformly distributed. Moreover, the quantification of the roughness values indicates that when the PP

surface is exposed only to O_2 plasma, the R_a value decreases from 21.5 nm to 17.7 nm and when it is exposed to Ar (2min) + O_2 (5min) the R_a value decreases to 11.7 nm. As presented the R_q value also decreases after both plasma treatments, namely from about 30.5 nm to about 23.1 and 15.1 nm. According to the representative images, the topography of the plasma treated surfaces seems to be more homogenous, presenting similar peaks and valleys uniformly distributed on the surface.

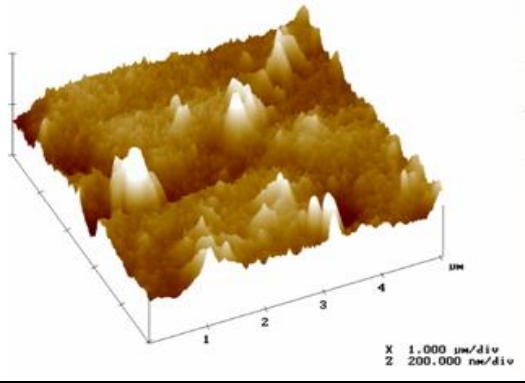
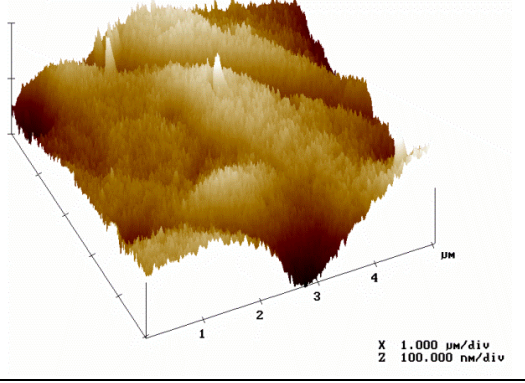
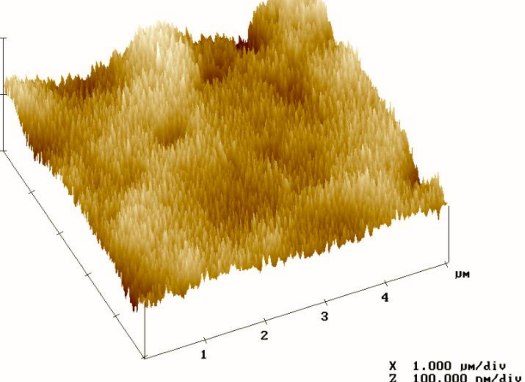
5.3.2. CS immobilization on PP surface

5.3.2.1. Physicochemical characterization

5.3.2.1.1. Orange II dye method

Figure 5.3.A presents the acid orange absorbance levels of PP samples previously exposed to different plasma compositions and subsequently immersed in AAc solutions with different concentrations and finally immersed in two distinct Cs solutions. When a plasma treatment only based on O_2 is performed, the absorbance level tends to increase as function of AAc concentration until the 20% AAc, after that it stabilizes. When using Ar plasma pre-treatment followed by O_2 plasma, the absorbance level also tends to increase as function of AAc concentration until the 20% AAc, but after that there is an absorbance decrease. For a specific plasma treatment and specific AAc concentration, for instance at 20% (V/V) where the absorbance level of all conditions reaches its maximum, the absorbance is the highest when using the highest Cs concentration (0.8%). When comparing the maximum absorbance achieved using a specific AAc and Cs concentration, but using different plasma treatments, no significant difference is observed. Figure 5.3.B shows the acid orange absorbance of PP samples after exposure to O_2 plasma over 5 min and subsequent treatment with 20% AAc and different Cs concentrations. It is possible to observe that the absorbance level rises as the Cs concentration increases, however, the increment is less significant after the 2% Cs.

Table 5.2: Representative AFM micrographs and roughness parameters (R_a and R_q) of untreated PP surface and after exposure to different plasma conditions.

Sample	Surface micrograph	R_a (nm)	R_q (nm)
Untreated PP		21.5 ± 1.3	30.5 ± 1.6
O_2 plasma over 5 min		17.7 ± 1.8	23.1 ± 2.1
Ar plasma pre-treatment over 2 min followed by O_2 plasma over 5 min		11.7 ± 1.8	15.1 ± 2.5

5.3.2.1.2. FTIR–ATR analysis

In figures 5.1.B and 5.1.C, it is presented the FTIR-ATR spectra of untreated PP and PP samples after Cs immobilization using different concentrations. It must be referred that the Cs powder spectrum was also acquired to identify the typical peaks, however the spectrum is not showed. Figure 5.1.B concerns to samples activated with O_2 plasma over 5 min and grafted with 20% AAc and figure 5.1.C concerns to samples subjected to the Ar pre-treatment over 2 min followed by O_2 plasma over 5 min and grafted with 20% AAc. revealed its typical broad

peaks with a very low intensity, being difficult to identify them with the used graph scale. When using the Ar pre-treatment followed by O₂, there is any visible change in spectra comparing the untreated PP with Cs treated PP. When using only O₂ plasma, for the higher Cs concentrations (2%, 4% and 6% (w/V)) a new broad peak of very low intensity is visible between 3000 cm⁻¹ and 3600 cm⁻¹ and other two peaks of low intensity appear around 1637 cm⁻¹ and 1164 cm⁻¹ (as assigned in figure 5.1.B).

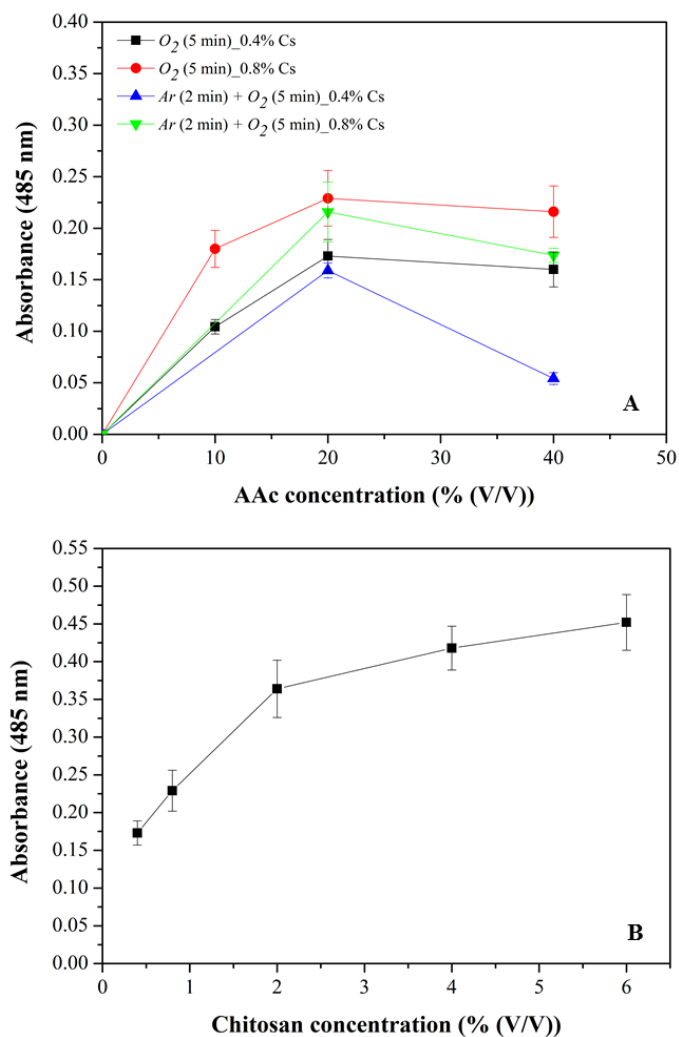


Figure 5.3: Levels of acid orange absorbance: (A) for four different samples exposed to different plasma treatments and immersed in different Cs solution concentrations, as function of the AAc solution concentration; (B) for different Cs concentrations after the O₂ plasma treatment over 5 minutes and 20% AAc grafting.

5.3.2.1.3. SEM analysis

SEM analysis was performed in order to evaluate the PP surface morphology before and after Cs immobilization on it, using different Cs concentrations, after exposure to O_2 plasma over 5 min and 20% AAc grafting. The untreated PP sample (figure 5.4.A) revealed an uniform surface, being possible to observe some crystallite boundaries. When the 0.4% Cs is immobilized on PP surface (figure 5.4.B) those boundaries are less visible, getting unnoticeable when using 0.8% Cs (figure 5.4.C), where some kind of film seems to be deposited on the material surface. Moreover, for the two highest Cs concentrations (figures 5.4.D and 5.4.E) the boundaries are also completely unnoticeable, but the surface seems rougher, mostly in case of 4% Cs, where some agglomerates are observed.

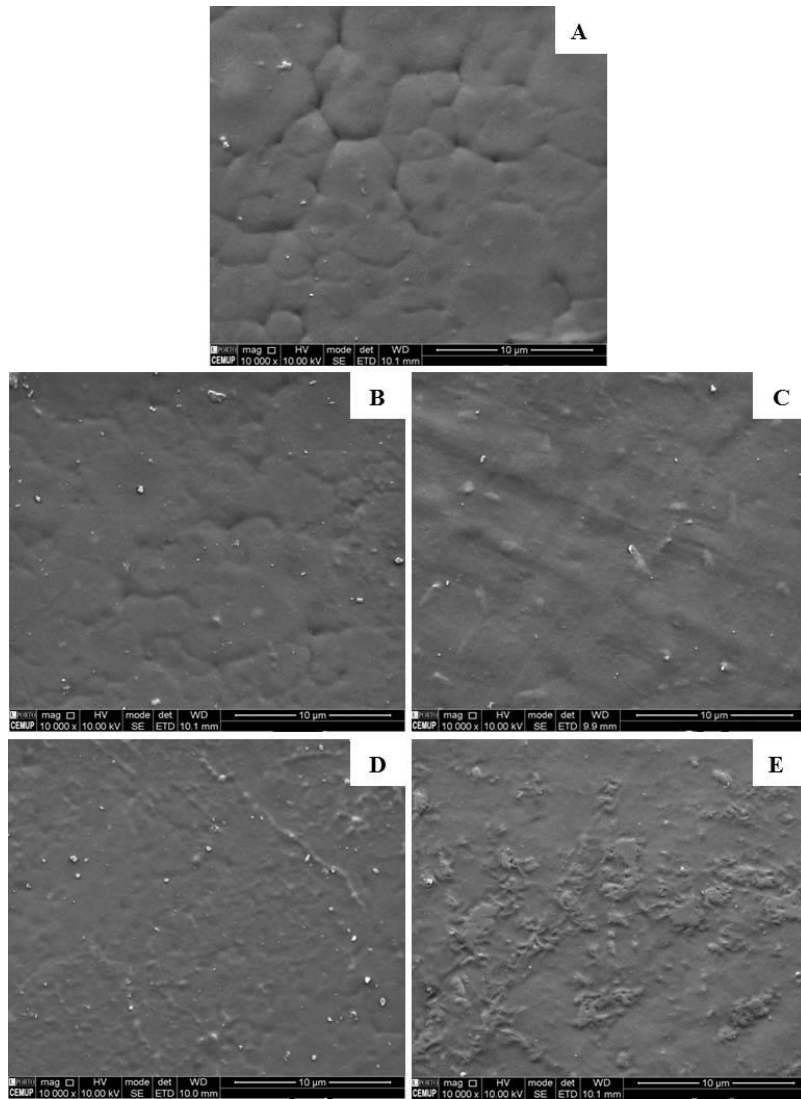


Figure 5.4: SEM images of PP samples surface: (A) untreated PP; (B) PP treated with 20% AAc_0.4% Cs; (C) PP treated with 20% AAc_0.8% Cs; (D) PP treated with 20% AAc_2% CS and (E) PP treated with 20% AAc_4% CS.

5.3.2.1.4. Optical profilometry analysis

The surface topography of PP samples functionalized with different Cs concentrations (after O_2 plasma and 20% AAc grafting) was investigated by optical profilometry (figure 5.5). It might be noticed that the Cs functionalized surfaces were washed under acidic conditions to remove any physically trapped CS on the sample surface. The untreated PP surface (figure 5.5.A) shows a surface with occasional protuberances, presenting a R_a value of about 54 nm. After Cs immobilization, the surface roughness of PP_20% AAc_0.4% Cs and PP_20% AAc_0.8% Cs (figures 5.5.B and 5.5.C), does not suffer a significant change, presenting R_a values of about 48 and 55 nm, respectively. When the Cs concentration increases to 2% and 4% (w/V) more protuberances appear on the surface, what is reflected in an increase of the R_a value to about 99 and 135 nm, respectively.

5.3.2.1.5. Wettability evaluation

In figure 5.2.B it is possible to observe the water C.A. variation with Cs concentration, using 20% AAc, where is observed that the C.A. value decreases as the Cs concentration increases until 2% (V/V). Comparing the water C.A. of the untreated PP surface with the PP_20% AAc_2% Cs there is a significant decrease of about 36.5%, from about $89.8^\circ \pm 0.2$ to $57^\circ \pm 6$. For Cs concentrations higher than 2% (V/V) there is a stabilization of the C.A. value.

5.3.2.2. *In vitro* biological characterization

5.3.2.2.1. Resazurin assay

The metabolic activity of fibroblasts seeded on the untreated PP samples and PP samples treated with 20% AAc_2% Cs (pre-treated with O_2 plasma) was evaluate and compared by the resazurin assay over 7 days of culture. As observed in figure 5.6.A, in case of untreated PP, the resazurin fluorescence increases over time. In case of treated PP, there is an increase in fluorescence from day 1 to day 3, being the values at both time points higher than in case of untreated PP. However, at day 7 the fluorescence decreases being the value lower than for the untreated samples.

In figures 5.6.B, 5.6.C and 5.6.D, using the MTT reagent, it is possible to identify the cells (blue spots) seeded on the treated PP surface over time by optical microscopy. From day 1 (figure 5.6.B) to day 3 (figure 5.6.C) it is observed an increase in number of cells, but at day 7 it seems that a part of the cell layer detached from the material surface.

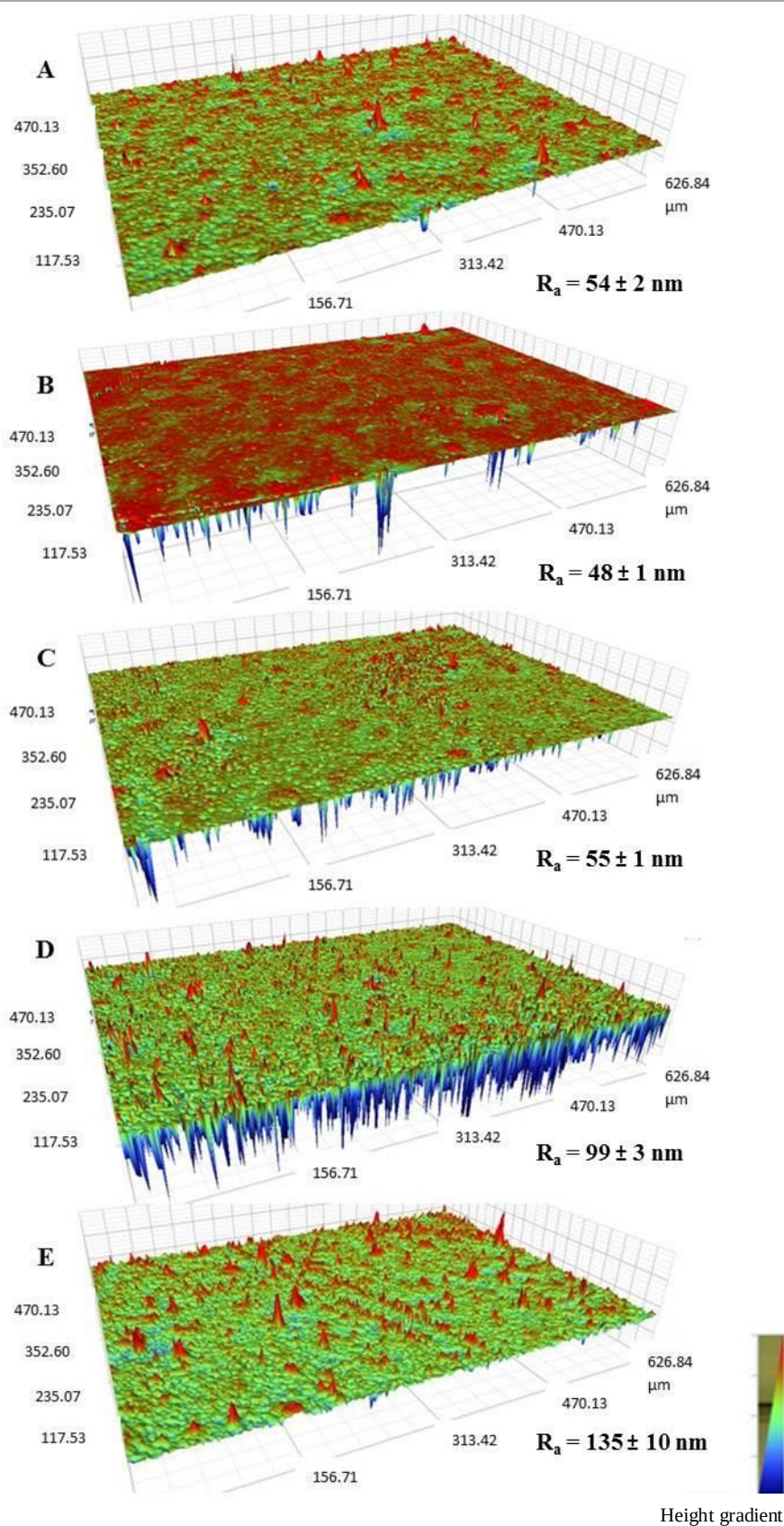


Figure 5.5: Representative optical profilometry images of PP samples surface and R_a values: **(A)** untreated PP; **(B)** PP treated with 20% AAc_0.4% Cs; **(C)** PP treated with 20% AAc_0.8% Cs; **(D)** PP treated with 20% AAc_2% CS and **(E)** PP treated with 20% AAc_4% CS.

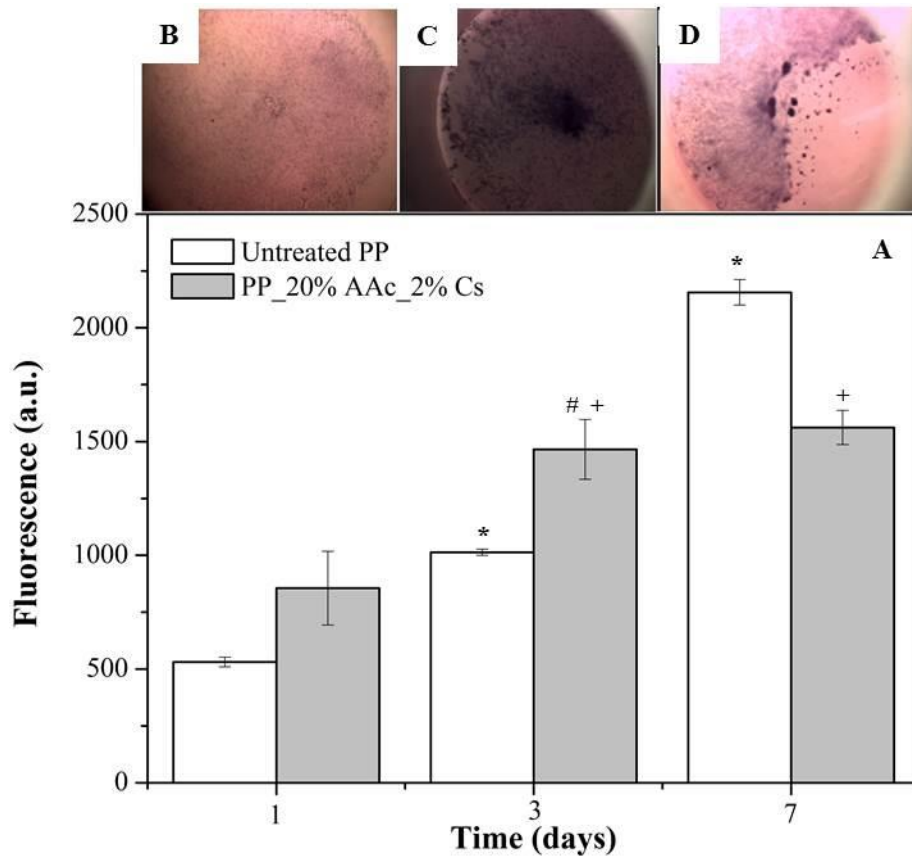


Figure 5.6: Levels of resazurin fluorescence at different incubation time points after the cell seeding on untreated PP samples and PP_20% AAc_2% Cs samples (A). Microscopic images of cells seeded on PP_20% AAc_2% Cs samples after: (B) 1 day; (C) 3 days and (D) 7 days of incubation. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference comparing untreated PP over time (*); comparing PP_20% AAc_2% Cs over time (+); comparing the untreated PP with PP_20% AAc_2% Cs at day 3 (#).

5.3.2.2.2 SEM analysis

In figure 5.7 it is possible to observe the cells seeded on the untreated PP surface (figure 5.7.A1, 5.7.A2) and on the PP surface treated with 20% AAc_2% Cs (pre-treated with O_2 plasma) (figures 7.B1, 7.B2) after 1 and 7 days of culture. After 1 day of incubation, it is possible to observe that fibroblasts adhered to the untreated and treated samples surfaces, even though the cells present different morphologies in each material, as observed in figures 5.7.A1 and 5.7.B1. On the untreated surface some cells present a spherical morphology, while other cells present a more spread morphology. On the treated samples surface, besides the higher number of cells, all of them already present a spread shape with developed filopodia. After 7 days of incubation, figures 5.7.A2 and 5.7.B2 illustrate an increase in number of cells adhered on the materials surface comparing to day 1. Both materials surface

is completely covered by an abundant cell layer, where all cells are completely spread and interconnected as a whole.

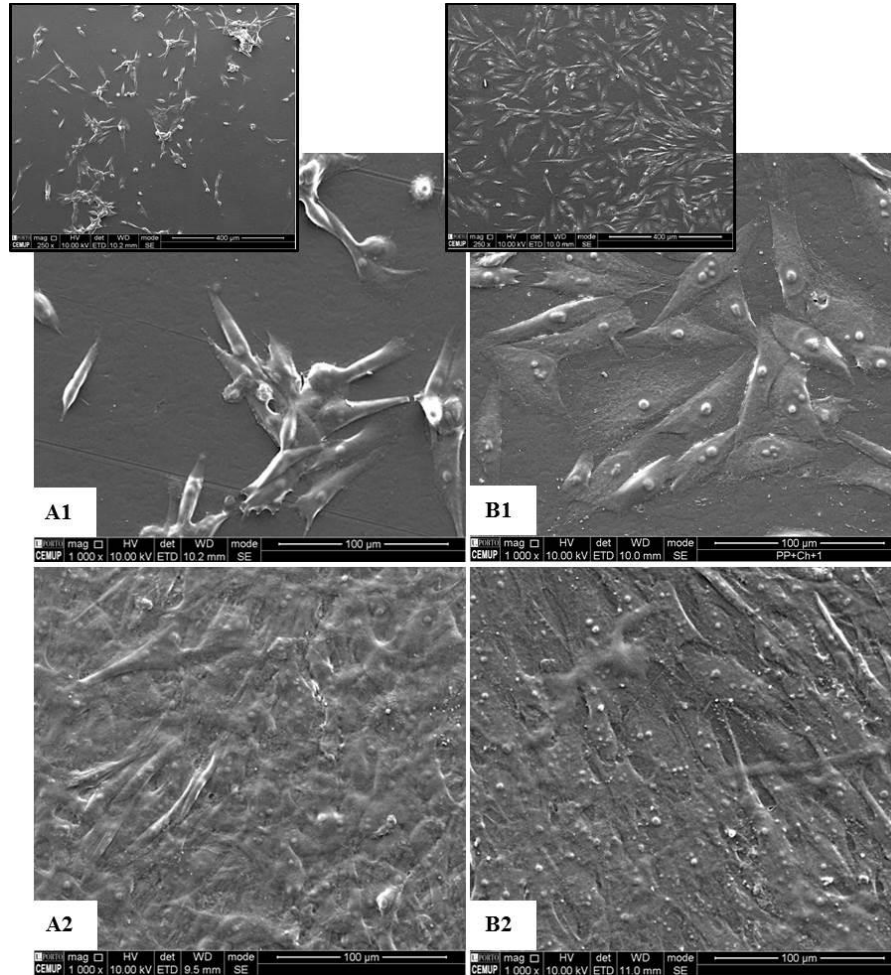


Figure 5.7: SEM images of cells seeded on PP samples surface over incubation time: (**A1**) untreated PP after 1 day; (**A2**) untreated PP after 7 days; (**B1**) PP treated with 20% AAc_2 % Cs after 1 day and (**B2**) PP treated with 20% AAc_2 % after 7 days.

5.4. Discussion of results

In order to improve the fibroblasts interaction with PP substrates for tendons regeneration, an efficient protocol was developed and optimized to functionalize the PP surface by Cs immobilization on it. In order to promote a strong bond (covalent bond) between the immobilized Cs and the PP surface, the substrates were firstly exposed to plasma treatment and acrylic acid (AAc) grafting. First of all, the activation of the PP surface with oxygen containing plasma generates oxygenated species on the surface, within a very short exposure time [13, 15, 19]. After that, the grafting of AAc may be initiated, but the plasma

treatment parameters and reaction conditions significantly influence the AAc grafting degree, what subsequently define the physicochemical features at the surface [7]. The AAc grafting introduces carboxyl groups (-COOH) on samples surface for the binding of Cs through a dehydration reaction mediated by EDC, promoting a covalent amide bonding (RCONHR'6 ring) between amino groups (-NH₂) of Cs and carboxyl groups grafted on PP surface [7].

Based on previous studies (not yet published), the studied plasma treatments included only O₂ plasma, which is essential to activate the surface, improve its hydrophilicity and AAc grafting, or Ar followed by O₂, once that Ar can eliminate possible contaminations from the surface and homogenise it before the treatment, improving the activation [6, 20]. To optimize the parameters of the surface activation process, several treatments using those two plasma compositions were carried out at different exposure times. In figure 5.1.A, it is possible to observe the fingerprinting peaks of untreated PP. The peaks at 2951 cm⁻¹ and 2871 cm⁻¹ are due to the asymmetric and symmetric stretching vibrations of methyl group (-CH₃), respectively. The peaks at 2916 cm⁻¹ and 2836 cm⁻¹ may be ascribed to the -CH₂ asymmetric and symmetric stretching vibrations, respectively [7]. Moreover, the two peaks at 1454 cm⁻¹ and 1375 cm⁻¹ are related with the asymmetric and symmetric bending vibrations of -CH₃, respectively [21]. After the different plasma treatments no new peaks were detected by the FTIR-ATR analysis.

The plasma activation of the PP surface was mainly performed to improve its wettability and consequently allow a better diffusion of the AAc water solution, achieving a better grafting degree, by introducing on PP surface new functional groups, such as carboxyl (-COOH) and hydroxyl (-OH), which may increase its surface hydrophilicity [8, 10, 21]. However, these new groups may not have been detected in FTIR-ATR analysis due to their low quantity on surface be under the minimum limit of detection. According to the results in figure 5.2.A, the water C.A. significantly decrease as the exposure time to both plasma compositions increase, at least until 5 min, but then it slightly increases again. The *Wenzel's model* ($\cos(\theta_W) = r \cos(\theta_Y)$) describes the relation between the apparent C.A. on a rough surface (θ_W), the surface average roughness ratio (r , ratio between the real and geometric area) and the C.A. on a chemically similar smooth surface (θ_Y). [6, 22] The wettability of surfaces is simultaneously related with the chemical and topographic changes promoted by the plasma treatments. According to the model and as reported by other authors, the wettability increase is usually associated to a R_a value increase due to a surface area increase (considering that no chemical changes happened) [6, 23]. However, observing figure 5.2.A and table 5.2, in this study the PP surface wettability increases when the R_a value decreases.

Therefore, taking the *Wenzel's* model in consideration, the water C.A. decrease may eventually be accomplished by chemical changes on the surface promoted by the plasma treatments, namely by the introduction of new chemical polar groups, as other authors have already reported [15, 21, 23]. Those polar groups, namely oxygen-containing polar functional groups will also allow the AAc grafting. As the exposure time to O_2 increases the surface concentration of polar functional groups also increases, leading to an improvement of surface hydrophilicity. However, after some exposure time (in this case after 5 min) an etching phenomenon may happen, leading to the removal of some weakly bonded oxide compounds or breakage over-layers on the PP surface, resulting in a decrease of polar groups present on the surface and of course of its hydrophilicity [8]. Even regarding the surfaces roughness after plasma treatment, besides the decrease of the average roughness (R_a), it must be noticed the R_q value decrease, what indicates that the surface became more homogeneous in accordance to what is observed in images presented in table 5.2 [24].

As observed in table 5.2, after O_2 plasma treatment a heterogeneous rough topography was observed, being evident a hill/valley structure with a R_a value slightly lower than in untreated PP, as observed by other authors [25, 26]. But when Ar (2min) + O_2 (5min) treatment is performed, the surface becomes less rough and more homogeneous. The roughness change is mostly promoted by the plasma physical etching on the surface. Therefore, the different topography between the two used plasmas may be explained by the atoms (Ar and O_2) radius difference.

In order to evaluate the Cs immobilization degree on PP surface, depending on plasma composition (the 5 min O_2 plasma activation was chosen due to the low water C.A.), AAc grafting and Cs concentration, the *Orange II* dye method was performed, being the acid orange absorbance proportional to the $-NH_2$ groups density on the functionalized surface [27]. As presented in figure 5.3.A, until 20% (V/V), the AAc concentration increase leads to an increment of $-NH_2$ groups number for both plasmas and Cs concentrations. After plasma activation, the AAc grafting degree will be as higher as the AAc solution concentration. A higher grafting degree means a higher number of $-COOH$ groups on PP surface available to react with $-NH_2$ groups, allowing the immobilization of more Cs. For a specific plasma treatment and AAc concentration, the $-NH_2$ groups density increases as Cs concentration increases (from 0.4% to 0.8%), indicating that with 0.4% Cs several $-COOH$ groups were still available to receive more $-NH_2$ groups. Thus, when the Cs concentration goes up to 0.8%, more Cs is immobilized on the PP surface.

However, when using the O_2 plasma treatment the absorbance level remains constant for AAc concentrations higher than 20 % (V/V). This is the indication that the interaction of $-NH_2$ groups of Cs with $-COOH$ groups may be saturated, which may be due to the occupation of all $-COOH$ groups or due to the limited Cs chains accessibility to the AAc grafted layer. According to Saxena *et al.*, as the $-COOH$ content increases the amide fraction decreases revealing that the interaction of Cs molecules with the AAc grafted chains becomes difficult as the graft level increases. This phenomenon may be understood by the fact that Cs is a long chain polysaccharide and its mobility within the AAc grafted domain on the sample surface would be a key factor in its bonding ability with $-COOH$ groups. The grafted AAc chains are hydrophilic in nature and tend to swell when the sample is immersed in the Cs solution and at low AAc graft levels the chains are shorter but for higher graft levels the chains become longer, which may difficult the Cs chains movement and hence restrict its interaction with the $-COOH$ groups.

When an Ar pre-treatment is performed for the PP surface activation, for AAc concentrations higher than 20 % (V/V) the amines density is lower than those observed when using only O_2 plasma treatment. This lower density may happen because the O_2 reactive plasma concentration in the chamber will be lower due to the space occupied by the residual Ar particles and to the higher molecular weight of Ar, which may hamper the O_2 entrance in the next 5 min into the chamber, being present in less quantity. Moreover, O_2 atoms are quite reactive and withdraw electrons from other gases on account of their relatively high electronegativity. Thus, in presence of Ar atoms the O_2 plasma density becomes low because O_2 acts as an electron and radical scavenger. Some of the electrons and free radicals lose their energy for surface activation because energy transfer, such as the adsorption, collision, and excitation, occurs through a reaction between O_2 and other electrons and free radicals [8]. As consequence, the number of activated sites on the surface, for later AAc grafting, is lower than using only O_2 plasma, what consequently decreases the number of carboxylic groups available to react with Cs amino groups.

According to these results, the 20% AAc grafting and the plasma composed only by O_2 were chosen to study if the Cs immobilization degree could be improved by increasing Cs solution concentration, as presented in figure 5.3.B. As already discussed, the increase in acid orange absorbance, from the 0.8% Cs to 2% Cs, reveals that more Cs is immobilized on PP surface, meaning that the grafted surface still have $-COOH$ groups available to react with $-NH_2$. However, when the Cs concentration increases to 4% and 6%, the absorbance increase is not significant. This may be explained by the fact that there are no available $-COOH$

groups to interact with all Cs in solution or the Cs mobility may become more difficult attending to the increase of Cs solution viscosity.

In accordance with the acid orange absorbance results, the FTIR-ATR spectra of the PP surface after Cs immobilization (figures 5.1.B and 5.1.C), described new peaks when using O₂ plasma treatment and for the higher Cs concentrations. The new broad peak of very low intensity between 3000 cm⁻¹ and 3600 cm⁻¹ may represent the hydrogen-bonded OH stretching from the carboxyl groups [28, 29]. This may prove the presence of -COOH groups on PP surface due to AAc grafting. The appearance of this peak only for higher concentrations may be explained, as already mentioned, by the difficulty of Cs chains movement, leaving some -COOH groups unoccupied. The other two peaks observed around 1637 cm⁻¹ and 1164 cm⁻¹ should be related with the C=O stretching vibration of amide (RCONHR'6 ring) and C-N stretching vibration of amine I (R-NH₂) respectively, which may not be detectable by FTIR analysis under a certain density. The amide characteristic peak confirms the presence of -CONH- bonds on the PP surface, indicating that the Cs (-NH₂ groups) was successfully linked to the AAc grafted PP surface (-COOH groups). Regarding the amine peak, when the Cs is immobilized on PP surface some of its -NH₂ groups are involved in -CONH- bonds forming amides, but other ones are kept in their amine state, as identified by the FTIR analysis. The absence of these new peaks when an Ar pre-treatment is performed is probably due to a lower density of functional groups, being not possible to detect them by FTIR analysis.

Attending to the previous results, a further characterization by SEM, optical profilometry, and wettability analysis was performed only for samples in which the surface activation was promoted using a plasma treatment composed by O₂ followed by an AAc grafting of 20% (V/V). In figure 5.4 it is possible to observe that the Cs immobilization on PP surface changed its morphology. For lower Cs concentrations (figures 5.4.B and 5.4.C), the crystallite boundaries observed on untreated PP surface became almost unnoticeable, indicating that a kind of continuous, uniform and smooth film may be above it, what may also be observed in figures 5.5.B and 5.5.C of profilometry analysis. In more detail for 0.8% Cs, in figure 5.4.C, the boundaries really disappeared, what may indicate an increase in that film thickness comparing to 0.4% Cs, achieved by more Cs immobilized on the surface. As indicated by profilometry analysis, the PP surface roughness (R_a value) for these two concentrations is almost the same as in case of untreated PP. However, when the Cs concentration increases (2%, 4% and 6% (w/V)), the R_a value also increases, being possible to observe a more rough surface with more protuberances. This is in accordance to what is

possible to observe in figures 5.4.D and 5.4.E, where some agglomerates are observed above such film, mainly for the highest Cs concentration, probably being Cs based agglomerates. Saxena *et al.* also observed similar changes on PP surface morphology when increasing the Cs content immobilized on it [7]. Moreover, these results are in accordance with the acid orange and FTIR-ATR results, namely the fact that using a higher Cs solution concentration leads to more Cs immobilized on PP surface.

Regarding the wettability of Cs treated PP, as presented in figure 5.2.B, the biggest water C.A. decrease (about 36.5%) attained for PP_20% AAc_2% Cs, may be mainly explained by the presence of –OH polar groups from Cs on the PP surface [16]. In addition, the –NH₂ groups from Cs that may be present on the surface also have an hydrophilic character [30]. When the Cs is immobilized on PP surface some of its –NH₂ groups are involved in –CONH– bonds forming amides, but other ones still in their amine state, creating a more hydrophilic surface as previously mentioned. Therefore, it would be expected that the samples treated with higher Cs solution concentrations showed more –OH and –NH₂ groups on their surface and consequently lower water C.A. values. However, for Cs concentrations higher than 2% (w/V) the water C.A. were kept the same, meaning that the quantity of Cs immobilized on the surface did not increased in proportion to the solution concentration. But this phenomenon is in accordance to what was already discussed for the acid orange absorbance results. Besides that, once again it must be noticed that the increase in surface roughness when the Cs is immobilized on PP surface may also be responsible for the hydrophobicity decrease, according to the *Wenzel's model*.

Attending to the main purpose of this work, namely to improve the bioactivity of PP surfaces, the viability and morphology of cells seeded on untreated PP and PP_20% AAc_2% Cs were evaluated. Cell attachment, adhesion and spreading are very important steps for a good cell/material interaction. The way these phenomena occur influences the cell's ability to proliferate and to differentiate, which is essential for the formation of an implant/tendon successful interface. Therefore, SEM analysis was performed in order to observe cell adhesion to the Cs treated surface as well as their morphology. As observed in figure 5.7, the cell adhesion occurred and the fibroblasts number increased over time for both tested materials. After 1 day, the cells presented different morphologies comparing the untreated with the treated samples. In case of Cs treated PP samples (figure 5.7.A1), cells are more spread than in case of untreated PP (figure 5.7.A1). After 7 days both materials surface was completely covered by a cell layer (figure 5.7.A2 and 5.7.B2), being all cells completely spread and interconnected as a whole surrounded by ECM, but in case of untreated PP it is

still possible to observe few cells with a shape not so well spread. At the beginning of the cell adhesion process, if cells present a spherical shape it indicates an initial stage of adhesion or that they are not so well adapted whereas a spread shape reveals a better adaptation and a more advanced adhesion stage [17, 31]. In a cell adhesion process, the material interacts with different cell surface receptors, establishing several sites of adhesion between the cells and the material surface [32, 33]. During the establishment of those sites, there is an organization and/or production of the cytoskeleton filamentous proteins, namely actin filaments, which tend to assemble in long bundles, leading to a spread shape with protrusions (filopodia) of the plasma membrane [17, 34, 35].

Resazurin is an indicator of cell viability since the living cells are able to reduce the resazurin in mitochondria. Therefore the fluorescence results presented in figure 5.6 are proportional to cell metabolism and therefore cell proliferation and viability [36]. After 1 and 3 days of culture, the treated samples present a higher metabolic activity than the untreated ones. However, after 7 days the fluorescence level of the treated samples decrease, probably due to the lack of space for cell proliferation, leading to the detachment of part of the cell layer as proved by optical microscopy (figure 5.6.D). The cell shape, which is controlled by the cytoskeleton organization, can also be related with cell growth and function once the cytoskeleton proteins are involved in signal transduction. So, when cells try to adhere to a material surface, the mitochondrial activity increases by the production of the necessary energy to synthesize the above-mentioned proteins [17, 31, 37]. Therefore, a cell shape more spread and a high number of cells is usually associated to a high metabolic activity, as verified when comparing the SEM images and resazurin fluorescence levels of tested materials after 1 day of culture.

The biomaterial surface characteristics, such as morphology and physical and chemical features influence the cell growth and function [38]. For instance, the materials surface roughness and wettability can influence the type and the adsorption kinetics of the serum proteins to the material surface. The adsorbed protein layer has an essential role in the cell adhesion, morphology and migration, because the charged cell membrane interact with surfaces through this protein layer [31, 39]. It has been reported in several studies that rough, enough hydrophilic and functionalized surfaces favour the adsorption of that protein layer and consequently the cell adhesion [10, 16, 27]. Thus, the low cell adhesion and metabolic levels in case of untreated PP may be explained by the lack of functional groups, low wettability and flat surface. In case of Cs treated samples, the high cell adhesion and activity may be mostly explained by the performed amine functionalization. The several $-NH_2$ groups

present on the material surface present positive charges which are able to establish electrostatic interactions with negative charges of the cell-surface proteins, promoting the adhesion of cells to the material [16, 27]. Besides that, the cells anchorage may also be improved by the wettability, roughness and topography of the Cs treated PP surface.

5.5. Conclusions

Firstly, it was verified that the plasma treatments performed on PP surface improved its wettability and activated the surface, allowing a successful AAc grafting and consequent Cs immobilization. This achievement was proved by the *Orange II* dye method and by the appearance of new amine and amide peaks on FTIR-ATR spectra when using O_2 plasma. It must be noticed, that as the Cs solution concentration increase the quantity of Cs immobilized on the surface also increases as expect, however after 2% (V/V) it remains constant. Due to the different results obtained in acid orange absorbance test and FTIR-ATR analysis, the plasma using only O_2 and the AAc concentration of 20% were chosen for the further characterization. Based on SEM, optical profilometry and water C.A. analysis, it was possible to observe that the Cs concentration increase leads to a surface roughness (R_a value) increase, as well as to water contact angle (C.A) decrease at least until 2% (w/V). Finally, the substrate PP_20% AAc_2% Cs was chosen to study the cellular response to the developed Cs immobilized PP surfaces. Thus, resazurin and SEM analysis were performed in order to analyse cell adhesion, proliferation and morphology. The Cs treated PP revealed better results than the untreated PP, promoting higher cell metabolic activity than the untreated samples. Moreover, SEM results corroborate this conclusion, since cells were spread through the surface indicating a good adhesion and cell growth occurred being the surface all covered by cells at day 7.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project UID/EMS/00285/2013.

5.6. References

- [1] Lidgren L, Gomez-Barrena E, Duda G, Puhl W, Carr A. European musculoskeletal health and mobility in Horizon 2020. *Bone and Joint Research*. 2014;3:48-50.
- [2] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.
- [3] James R., Kesturu G., Balian G., Chhabra A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery*. 2008;33:102-12.
- [4] Longo U. G., Lamberti A., Maffulli N., Denaro V. Tendon augmentation grafts: a systematic review. *British medical bulletin*. 2010;94:165-88.
- [5] Chen J., Xu J., Wang A., Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. *Expert review of medical devices*. 2009;6:61-73.
- [6] Pedrosa P, Chappé JM, Fonseca C, Machado AV, Nóbrega JM, Vaz F. Plasma Surface Modification of Polycarbonate and Poly (propylene) Substrates for Biomedical Electrodes. *Plasma Processes and Polymers*. 2010;7:676-86.
- [7] Saxena S, Ray AR, Kapil A, Pavon-Djavid G, Letourneur D, Gupta B, et al. Development of a new polypropylene-based suture: plasma grafting, surface treatment, characterization, and biocompatibility studies. *Macromolecular bioscience*. 2011;11:373-82.
- [8] Kwon O-J, Myung S-W, Lee C-S, Choi H-S. Comparison of the surface characteristics of polypropylene films treated by Ar and mixed gas (Ar/O₂) atmospheric pressure plasma. *Journal of colloid and interface science*. 2006;295:409-16.
- [9] Tang N, Jia Q, Zhang H, Li J, Cao S. Preparation and morphological characterization of narrow pore size distributed polypropylene hydrophobic membranes for vacuum membrane distillation via thermally induced phase separation. *Desalination*. 2010;256:27-36.
- [10] Alves P, Pinto S, de Sousa HC, Gil MH. Surface modification of a thermoplastic polyurethane by low-pressure plasma treatment to improve hydrophilicity. *Journal of applied polymer science*. 2011;122:2302-8.
- [11] Zhou X, Wang F, Ji Y, Chen W, Wei J. Fabrication of hydrophilic and hydrophobic sites on polypropylene nonwoven for oil spill cleanup: Two dilemmas affecting oil sorption. *Environmental science & technology*. 2016;50:3860-5.
- [12] Labay C, Canal C, García-Celma M. Influence of corona plasma treatment on polypropylene and polyamide 6.6 on the release of a model drug. *Plasma Chemistry and Plasma Processing*. 2010;30:885-96.
- [13] Grace JM, Gerenser LJ. Plasma treatment of polymers. *Journal of dispersion science and technology*. 2003;24:305-41.

- [14] Custódio CA, Alves C, Reis R, Mano J. Immobilization of fibronectin in chitosan substrates improves cell adhesion and proliferation. *Journal of tissue engineering and regenerative medicine*. 2010;4:316-23.
- [15] Siow KS, Britcher L, Kumar S, Griesser HJ. Plasma Methods for the Generation of Chemically Reactive Surfaces for Biomolecule Immobilization and Cell Colonization-A Review. *Plasma Processes and Polymers*. 2006;3:392-418.
- [16] Tang L, Thevenot P, Hu W. Surface chemistry influences implant biocompatibility. *Current topics in medicinal chemistry*. 2008;8:270-80.
- [17] Morais DS, Rodrigues M, Lopes M, Coelho M, Maurício AC, Gomes R, et al. Biological evaluation of alginate-based hydrogels, with antimicrobial features by Ce (III) incorporation, as vehicles for a bone substitute. *Journal of Materials Science: Materials in Medicine*. 2013;24:2145-55.
- [18] Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced drug delivery reviews*. 2010;62:83-99.
- [19] Ugbohue SC. *Polyolefin fibres: industrial and medical applications*: Elsevier; 2009, United Kingdom.
- [20] Leroux F, Perwuelz A, Campagne C, Behary N. Atmospheric air-plasma treatments of polyester textile structures. *Journal of adhesion science and technology*. 2006;20:939-57.
- [21] Morent R, De Geyter N, Leys C, Gengembre L, Payen E. Comparison between XPS-and FTIR-analysis of plasma-treated polypropylene film surfaces. *Surface and Interface Analysis*. 2008;40:597-600.
- [22] Wolansky G, Marmur A. Apparent contact angles on rough surfaces: the Wenzel equation revisited. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 1999;156:381-8.
- [23] Pedrosa P, Fiedler P, Lopes C, Alves E, Barradas NP, Haueisen J, et al. Ag: TiN-Coated Polyurethane for Dry Biopotential Electrodes: From Polymer Plasma Interface Activation to the First EEG Measurements. *Plasma Processes and Polymers*. 2015;13(3): 341-354.
- [24] De Oliveira R, Albuquerque D, Leite F, Yamaji F, Cruz T. Measurement of the nanoscale roughness by atomic force microscopy: basic principles and applications: INTECH Open Access Publisher; 2012, Brazil.
- [25] Saxena S, Ray AR, Gupta B. Graft polymerization of acrylic acid onto polypropylene monofilament by RF plasma. *Journal of applied polymer science*. 2010;116:2884-92.
- [26] Anjum S, Gupta A, Sharma D, Kumari S, Sahariah P, Bora J, et al. Antimicrobial nature and healing behavior of plasma functionalized polyester sutures. *Journal of Bioactive and Compatible Polymers*. 2016:0883911516668665.
- [27] Li H-L, Zhang H, Huang H, Liu Z-Q, Li Y-B, Yu H, et al. The effect of amino density on the attachment, migration, and differentiation of rat neural stem cells In Vitro. *Molecules and cells*. 2013;35:436-43.

- [28] Sheppard N. The historical development of experimental techniques in vibrational spectroscopy: Wiley Online Library; 2002.
- [29] Sun H-X, Zhang L, Chai H, Chen H-L. Surface modification of poly (tetrafluoroethylene) films via plasma treatment and graft copolymerization of acrylic acid. *Desalination*. 2006;192:271-9.
- [30] Hamerli P, Weigel T, Groth T, Paul D. Surface properties of and cell adhesion onto allylamine-plasma-coated polyethyleneterephthalat membranes. *Biomaterials*. 2003;24:3989-99.
- [31] Morais DS, Fernandes S, Gomes P, Fernandes M, Sampaio P, Ferraz M, et al. Novel cerium doped glass-reinforced hydroxyapatite with antibacterial and osteoconductive properties for bone tissue regeneration. *Biomedical Materials*. 2015;10:055008.
- [32] Zaleski KJ, Kolodka T, Cywes-Bentley C, McLoughlin RM, Delaney ML, Charlton BT, et al. Hyaluronic acid binding peptides prevent experimental staphylococcal wound infection. *Antimicrobial agents and chemotherapy*. 2006;50:3856-60.
- [33] Lei Y, Gojgini S, Lam J, Segura T. The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels. *Biomaterials*. 2011;32:39-47.
- [34] Hempel U, Hefti T, Dieter P, Schlottig F. Response of human bone marrow stromal cells, MG-63, and SaOS-2 to titanium-based dental implant surfaces with different topography and surface energy. *Clinical oral implants research*. 2013;24:174-82.
- [35] Wutticharoenmongkol P, Pavasant P, Supaphol P. Osteoblastic phenotype expression of MC3T3-E1 cultured on electrospun polycaprolactone fiber mats filled with hydroxyapatite nanoparticles. *Biomacromolecules*. 2007;8:2602-10.
- [36] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*. 2000;267:5421-6.
- [37] Morais DS, Coelho J, Ferraz MP, Gomes PS, Fernandes MH, Hussain NS, et al. Samarium doped glass-reinforced hydroxyapatite with enhanced osteoblastic performance and antibacterial properties for bone tissue regeneration. *Journal of Materials Chemistry B*. 2014;2:5872-81.
- [38] Vladkova TG. Surface engineered polymeric biomaterials with improved biocontact properties. *International Journal of polymer science*. 2010.
- [39] Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue engineering*. 2005;11:1-18.

Chapter 6

**Grafting of 1,2-ethylenediamine (EDA) on
poly(ethylene terephthalate) (PET) substrates to
enhance human fibroblasts viability**

Grafting of 1,2-ethylenediamine (EDA) on poly(ethylene terephthalate) (PET) substrates to enhance human fibroblasts viability

Morais D.S.^{1,2}, Brandão A.¹, Lopes C.³, Vaz F.³, Machado A.V.⁴, Fernandes M.H.⁵,
Guedes R.M.^{2,6}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Centro de Física, Universidade do Minho, 4710-057 Braga, Portugal

⁴IPC – Institute for Polymers and Composites, Universidade do Minho, Campus de Azurém, 4800-058 Guimarães, Portugal

⁵ Faculdade de Medicina Dentária, Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, Porto, Portugal.

⁶ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

Abstract

The last developed synthetic grafts for tendons regeneration, in spite of presenting a better mechanical performance than the natural ones, usually fail due to the lack of biocompatibility and bioactivity. To overcome this limitation, amino groups (-NH₂) were grafted on poly(ethylene terephthalate) (PET) substrates by immersion, being previously activated by plasma treatment, in order to enhance fibroblasts adhesion and proliferation on it. Different samples were prepared, using ethylenediamine (EDA) as the source of (-NH₂) groups, but using different grafting conditions, namely regarding the EDA concentration, solvent and temperature. The physicochemical features and morphology of all samples surface were fully characterized. The *Orange II* dye method and FTIR-ATR analysis proved the higher grafting level on sample W5050 (50% (V/V) EDA in water at 50 °C). Based on resazurin assay and SEM analysis after cell seeding on this sample, it was proved that the performed grafting significantly improved the cell adhesion and proliferation, comparing to an untreated PET substrate.

Key Words: poly(ethylene terephthalate), PET, ethylenediamine, EDA, surface functionalization, fibroblasts, tendon repair.

6.1. Introduction

Nowadays, occupational but mainly sporting injuries of tendons, ranging from repetitive strain injuries to complete ruptures, are among the most common health problem affecting adult population [1-3]. This prevalence represents a high treatment cost to the healthcare system of each country. Currently, tendon injuries are usually managed using two approaches, conservative, surgical or simultaneously both [3-5]. The conservative management involves rest, mechanical conditioning, corticosteroids injection, orthotics, ultrasound, laser or shockwave treatment [4, 6]. However, due to the limited tendon ability for self-healing, in same injury cases this recovery approach requires long treatment periods, potential partial function loss and recurrent injury [3, 4, 7]. When this approach does not result due to the injury extension surgical intervention is used, suturing the injured ends together. But, in many cases this can also fail attending to the poor healing ability of the degenerated tissue involved, which is susceptible to rupture again. In those cases, a tissue replacement can be performed using auto or allografts, which also reveal some disadvantages [3, 8].

Therefore, due to the limitations of all these treatment approaches, several biological and synthetic scaffolds have been developed during the last 15 years [1, 5]. Synthetic scaffolds present several advantages comparing to the biological ones, namely better mechanical performance; controllable and reproducible mechanical and chemical properties; predictable degradation rate and low immunogenicity risk [3, 9, 10]. Nevertheless, they may also present some limitations comparing to biological scaffolds, namely regarding their surface interaction with resident cells, due to the surface hydrophobicity and the lack of specific functional chemical groups involved in cell adhesion and proliferation [1, 5].

Polyethylene terephthalate (PET) is a linear aromatic polymer, with excellent bulk properties, such as high specific strength, good resistance to corrosion and it is relatively inexpensive to produce [10, 11]. It is used for different biomedical applications, for instance, as yarns for suture, as scaffolds for artificial blood vessels, or even in form of meshes for tendons/ligaments regeneration or hernias repair [3, 4, 12, 13]. Though, PET substrates surfaces present poor wettability due to the low surface energy and lacks active functional groups involved in cell adhesion process [10, 11]. In order to overcome these limitations, several physical and chemical approaches have been developed to modify PET scaffolds surface to promote cell adhesion and proliferation. The chemical grafting of specific functional groups on PET scaffolds surface, namely of amino groups (NH_2), has been studied based on a chemical etching in the form of aminolysis by a condensation reaction using

diamines, such as ethylenediamine (EDA) [14]. Aminolysis has been presented as effective to modify polymeric scaffolds for tissue engineering applications, where the free amino groups were used as a chemical linker to immobilize macromolecules, such as gelatin, chitosan and collagen, or they can directly interact with the extracellular matrix (ECM) [15, 16]. The (-NH₂) groups from EDA can be chemisorbed on PET substrates via (-C(O)NH) bonds, which result from the reaction between an ester group from PET and one amine side only from EDA, leading to amide formation, as presented in figure 6.1 [14]. The other amine side is available to interact with the ECM molecules, improving the interface between the scaffolds surface and surrounding cells [17, 18]. Therefore, the purpose of this study was to functionalize PET substrates by the grafting of EDA on their surfaces, in order to improve the interaction of those substrates with fibroblasts for tendon tissue regeneration, or even ligaments regeneration.

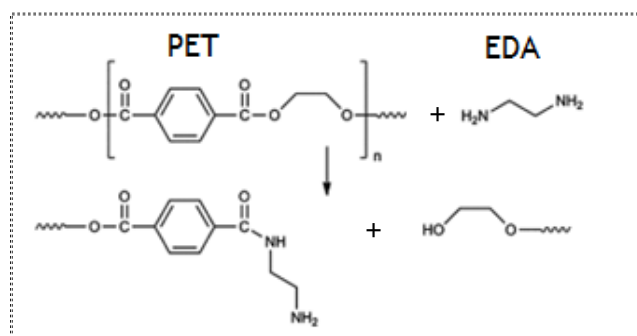


Figure 6.1: Reaction scheme between PET and EDA [14].

6.2. Materials and methods

6.2.1. Materials

The poly(ethylene terephthalate) (PET) sheet used in this study, with a thickness of 0.1 mm was purchased from *Goodfellow*, ethylenediamine (EDA) (99%) was purchased from *Sigma-Aldrich* and the acid orange II (99%) was purchased from *Acros Organics*.

6.2.2. Methods

6.2.2.1. PET surface plasma treatment

Circular PET samples with 0.1 mm thickness and 25 mm diameter were prepared and cleaned with ethanol to be exposed to different plasma activation treatments. A *Zepto* laboratory-sized plasma system from *Diener Electronics* ($\varnothing = 105$ mm, L = 300 mm, V = 2.6 L) was used for the plasma activation of the samples. Several plasma treatments were

performed according to the used gas and the plasma treatment time, using a power of 100 W, a p_{base} of 10 Pa and a p_{work} of 80 Pa:

- 1) O_2 plasma over 2, 5, 8 and 10 min;
- 2) Ar plasma pre-treatment over 2 min followed by O_2 plasma over 2, 5, 8 and 10 min.

6.2.2.2. EDA grafting on plasma treated PET surface

Immediately after each plasma treatment, PET samples were immersed in different EDA solutions, as shown in table 6.1, using water or ethanol as solvent, and incubated at different temperatures at 100 rpm orbital agitation for 24 h. After the immersion time, all samples were removed from the reactive solutions, rinsed in distilled water, then in isopropanol and again in distilled water. Finally, the grafted samples were dried under vacuum condition at room temperature before analysis.

Table 6.1: Samples name according to the EDA functionalization conditions.

Samples name	Solvent	Temperature (°C)	EDA concentration (V/V)
PET_W2025	Water	20	25%
PET_W2050			50%
PET_W5050		50	50%
PET_E2025	Ethanol	20	25%
PET_E2050			50%
PET_E5050		50	50%

6.2.2.3. Physicochemical characterization

Fourier Transform Infrared Spectroscopy (FTIR) in Attenuated Total Reflectance (ATR) mode was performed for untreated PET samples, for PET samples after each plasma treatment and after different EDA grafting conditions (in triplicate), using the spectrometer *Jasco FT/IR 4100* system (64 scans and 8 cm^{-1} nominal resolution) equipped with a Specac MkII Golden Gate single reflection ZnSe ATR crystal.

The wettability of PET samples surface after each plasma treatment and after different EDA grafting conditions was assessed by the sessile drop method (3 μl drop), measuring the water contact angle (C.A.) at room temperature, using a *OCA 20* unit from *Dataphysics*. A minimum of 9 contact angle measurements were performed for each condition, using at least 3 samples.

The surface topography, average roughness (R_a) and surface height standard deviation (R_q) of the PET substrates (2 samples of each condition) were assessed before and after the plasma activation using a Multimode Atomic Force Microscope (AFM) from *Digital Instruments* using the tapping mode (5x5 μm^2 scan size and 1 Hz scan rate). A Nanoscope III controller and TESP AFM tips from *Bruker* were also used.

The amount of exposed primary amino groups on PET surface after EDA grafting was assessed by the *Orange II* dye method, using 3 samples for each EDA grafting. The grafted PET samples were immersed in 3 mL of dye acidic solution (14 mg/mL) (pH 3) and shaken for 1 h at 40 °C. The samples were then thoroughly rinsed three times with water acidic solution (pH 3) to remove the unbound dye. Once air-dried, the colored samples were immersed in 2 mL of water basic solution (pH 12) and shaken for 15 min at room temperature, measuring the absorbance of the solution at 485 nm.

Scanning electron microscopy (SEM) analysis was performed, using a *FEI Quanta 400 FEG ESEM* microscope, to analyze the microstructure and morphology of the EDA functionalized PET samples surface (at least 2 samples of each). The SEM images were acquired using either the secondary or the back-scattered electrons mode and the elemental composition was evaluated by energy dispersive X-ray spectroscopy (EDS). Moreover, the grafted samples surface was analysed by optical profilometry using a *Bruker NPFLEX* equipment.

6.2.2.4. *In vitro* biological characterization

6.2.2.4.1. Resazurin assay

L929 cell line was cultured in α -MEM (*Gibco*) supplemented with 10% (V/V) fetal bovine serum, 1% penicillin and streptomycin solution (*Gibco*) and 2.5 $\mu\text{g/mL}$ of fungizone (*Gibco*). Cells were seeded at 2×10^4 cells per well (24-well culture plates) on the samples surface (untreated PET substrate and PET_W5050 substrate) for 1, 3 and 7 days at 37 °C, in a 5 % CO_2 humidified atmosphere. After each time point, 50 μl of resazurin (*Sigma-Aldrich*) at 37 °C were added to each well and the plates were incubated for 3 hours at 37 °C in a 5 % CO_2 humidified atmosphere. Then, the fluorescence (excitation: 530 nm; emission: 590 nm)

was measured in a microplate reader (*Synergy HT*, *Biotek*). This experiment was performed in triplicate for each time point, either for the untreated and treated samples.

6.2.2.4.2. SEM analysis

The morphology of L929 fibroblasts seeded on the surface of untreated PET samples and W5050 PET samples, after each time point, was analysed in triplicate by SEM using a *FEI Quanta 400 FEG ESEM* microscope. Briefly, samples with cells were firstly washed twice with PBS and fixed with 1.5 % (m/V) glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4) over 15 min. Afterwards, samples were dehydrated using graded ethanol solutions from 50 % (V/V) to 100 % (V/V). The samples lasted 10 min in 70 and 80% ethanol solutions, 20 min in 90% ethanol solution and overnight in 100 % ethanol solution. Finally, samples were sputter-coated (*SPI-Module*) with a thin gold/palladium film and visualized.

6.2.2.5. Statistical analysis

Experimental data is presented as mean $\pm$ SD (standard deviation). Statistical analysis of data was performed using the one-way ANOVA test with Bonferroni post hoc analysis, using the software SigmaStat 3.5. The differences were considered to be significant at a level of $p < 0.05$.

6.3. Results

6.3.1. Analysis of PET surface plasma treatment

6.3.1.1. FTIR-ATR analysis

In order to evaluate the chemical groups presented on surface of untreated PET samples and PET samples exposed to different plasma treatments, FTIR-ATR analysis was performed, as presented in figures 6.2.A (O_2 plasma) and 6.2.B (Ar plasma pre-treatment over 2 min followed by O_2 plasma). Using this technique no detectable changes were observed among spectra before and after exposure to the different plasma treatments. For all samples, it is possible to observe very strong peaks at 1709 cm^{-1} , 1244 cm^{-1} , 1097 cm^{-1} and 721 cm^{-1} , and some less strong peaks at 1409 cm^{-1} , 1337 cm^{-1} , 1016 cm^{-1} and 872 cm^{-1} .

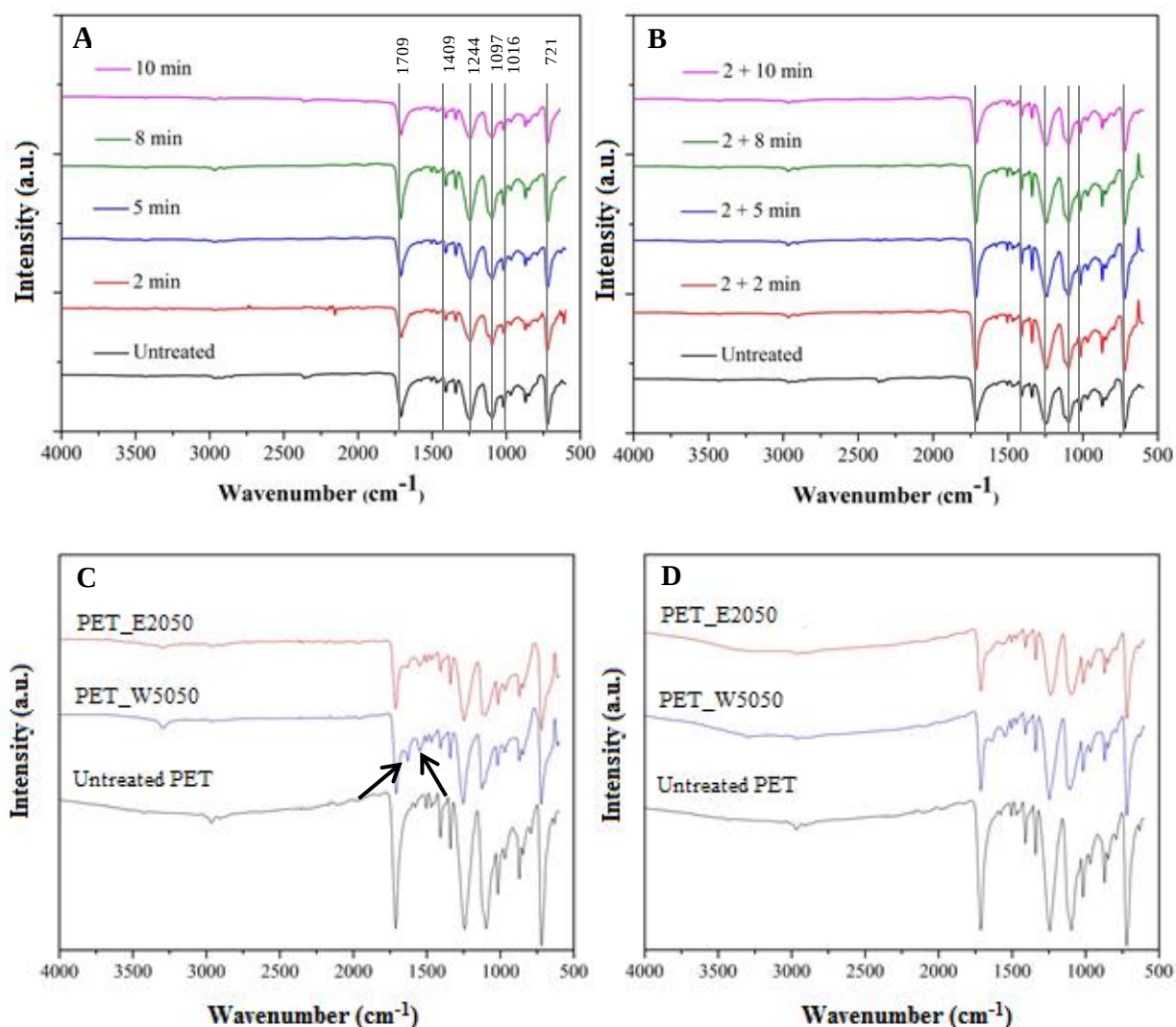


Figure 6.2: FTIR-ATR spectra of the untreated PET surface and after O_2 plasma treatment (A) or Ar plasma pre-treatment over 2 min followed by O_2 plasma over different periods (B). FTIR-ATR spectra of the functionalized samples W5050 and E2050 previously exposed to different plasma treatments: (C) O_2 plasma or (D) Ar plasma pre-treatment over 2 min followed by O_2 plasma over 8 min.

6.3.1.2. Wettability evaluation

In figure 6.3 it is possible to observe the water C.A. variation as function of each plasma treatment conditions. The presented results show for both plasma compositions (O_2 and Ar pre-treatment followed by O_2) a significant C.A. decrease after 2 min of plasma exposure. However, C.A. values reached the lowest levels after the 8 min treatment, being observed a decrease from 74.7° (untreated PET) to 10.7° in case of O_2 plasma treatment, and

10.1 ° for Ar plasma pre-treatment over 2 min followed by O₂ plasma treatment, representing in both cases a decrease of about 86%.

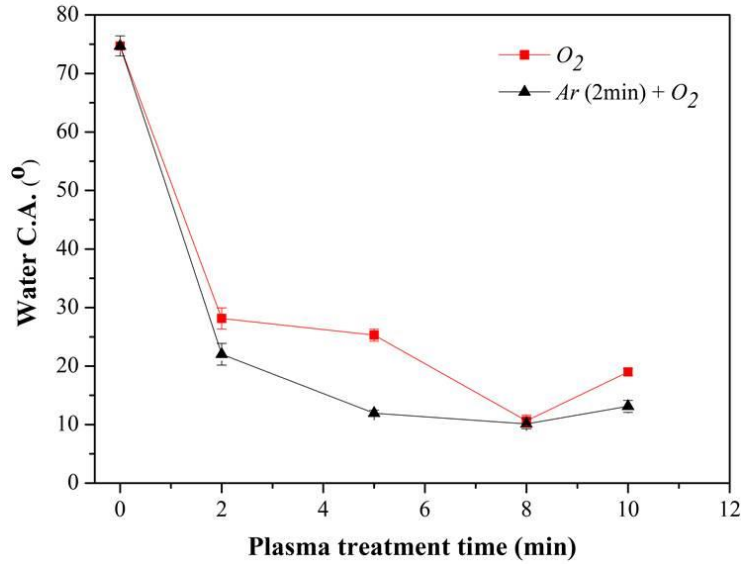
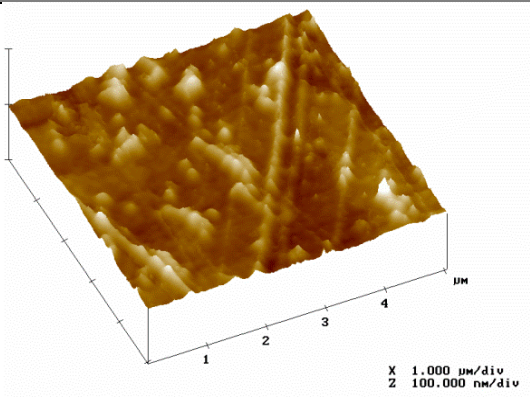
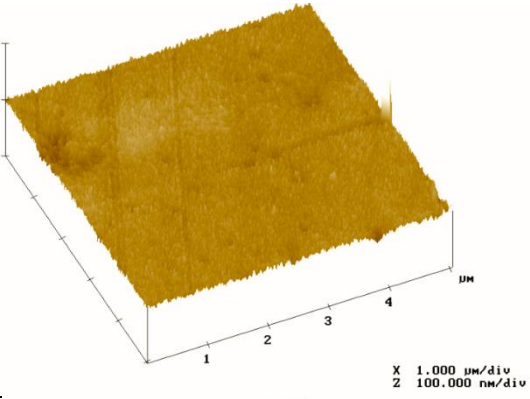
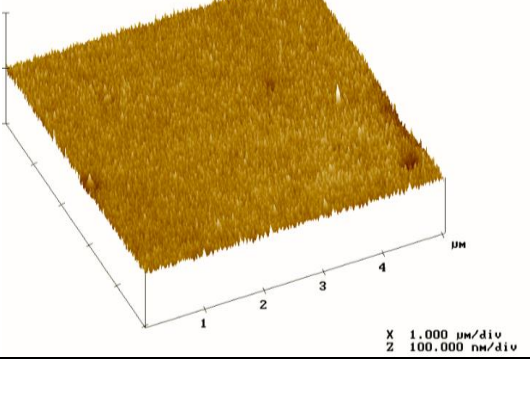


Figure 6.3: Water C.A. variation on the PET surface after: exposure to O₂ plasma over different times and to the Ar pre-treatment over 2 min followed by different times of O₂.

6.3.1.3. AFM analysis

AFM analysis was performed in order to evaluate the effect of plasma treatment on PET surface roughness. Table 6.2 shows the average roughness (R_a), root mean square roughness (R_q) and representative 3D height images for untreated PET and for the two plasma treatment conditions that presented the lower C.A.. As presented in table 6.2, when the PET surface is exposed to each plasma, the R_a value slightly decreases from about 5.1 nm to about 2.9 nm in case of O₂ plasma, and to about 3.3 nm in case Ar followed by O₂. As presented the R_q value also decreases after both plasma treatments, namely from about 7.1 nm to about 4.4 and 4.6 nm. According to the representative images, the topography of the plasma treated surfaces seems to be more homogenous, presenting similar peaks and valleys uniformly distributed on the surface.

Table 6.2: AFM micrographs and roughness parameters (R_a and R_q) of the untreated PET and PET substrates exposed to different plasma treatments.

Sample	Surface micrograph	R_a (nm)	R_q (nm)
Untreated PET		5.1 ± 1.1	7.1 ± 1.8
O ₂ plasma over 8min		2.9 ± 0.6	4.4 ± 0.9
Ar plasma pre-treatment over 2 min followed by O ₂ plasma over 8 min		3.3 ± 0.6	4.6 ± 1.0

6.3.2. Analysis of PET surface functionalized with EDA

6.3.2.1. Physicochemical characterization

6.3.2.1.1. Orange II dye method

In figure 6.4.A are presented the acid orange absorbance levels after functionalization using the different tested conditions, after exposing the PET substrates surface to O₂ plasma over 8 min. With no doubts, the highest levels are observed for samples PET_W5050 and PET_E2050. Thus, in figure 6.4.B is possible to compare the acid orange absorbance levels

also for those two samples but using different plasma treatments before the EDA immersion. When comparing the two plasma treatments for the same immersion conditions, when using O_2 plasma the acid orange absorbance is higher than using Ar plasma pre-treatment followed by O_2 plasma. When comparing the EDA grafting conditions for the same plasma treatment conditions, W5050 presents a higher absorbance than E2050, being 3.8 times higher in case of using the O_2 plasma and 3 times higher using the Ar plasma pre-treatment followed by O_2 plasma. However, according to figure 6.4.A, for the same plasma treatment and EDA concentration at 20 °C, the absorbance is higher when using ethanol as solvent (W2025 and E2025). Also, for the same EDA concentration and using water as solvent the highest temperature led to the highest absorbance (W2050 and W5050 samples).

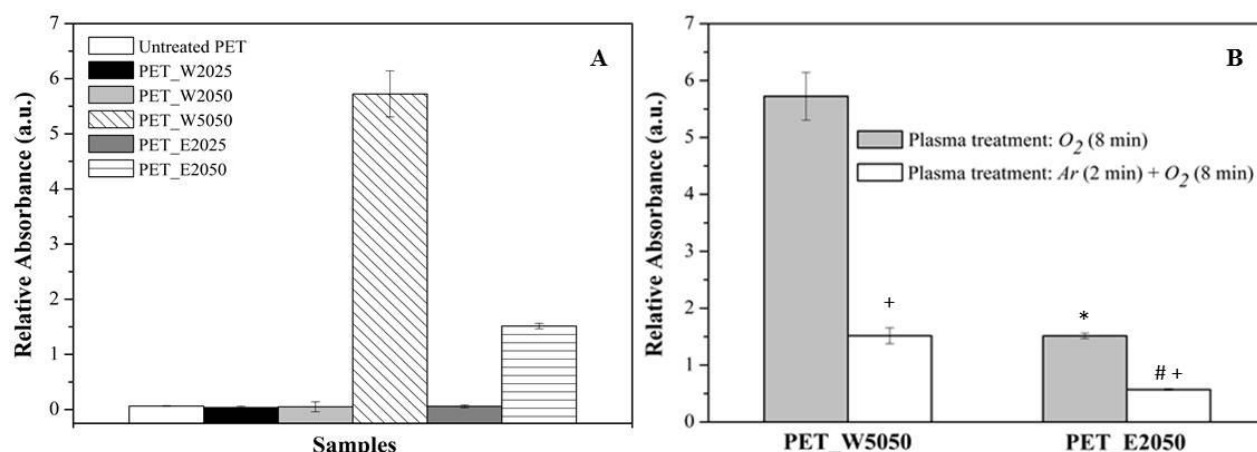


Figure 6.4: (A) Levels of acid orange absorbance for untreated PET and all functionalized samples (using the previous plasma treatment based on O_2 over 8 min). (B) Levels of acid orange absorbance for PET_W5050 and PET_E2050, using two different plasma treatments before immersion in EDA solution. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference comparing the same immersion conditions but with a previous different plasma treatment (+); comparing the plasma treatment based on O_2 , but using different immersion conditions (*); comparing the plasma treatment based on Ar and O_2 , but using different immersion conditions (#).

6.3.2.1.2. FTIR-ATR analysis

Figure 6.2.C and 6.2.D show the FTIR-ATR spectra of untreated PET and of EDA grafting W5050 and E2050 on PET surface, using O_2 plasma over 8 min and Ar plasma pre-treatment over 2 min followed by 8 min of O_2 plasma, respectively. In figure 6.2.C, comparing the untreated with EDA treated PET samples, the treated ones present new peaks at 1630 cm^{-1} and 1546 cm^{-1} , besides a new very weak wide band around $3500\text{--}2250\text{ cm}^{-1}$. In figure 6.2.D it is possible to observe the same new peaks in the region $1750\text{--}1550\text{ cm}^{-1}$, but

they are less strong and the band around $3500\text{--}2250\text{ cm}^{-1}$ barely appears. For both plasma treatment conditions (figure 6.2.C and 6.2.D), all the fingerprinting PET peaks already identified in point 6.3.1.1 are less intense in EDA treated samples than in the untreated PET. Moreover, when comparing PET samples grafted with W5050 and E2050 using the same plasma treatment, besides they present the same new peaks, those peaks signal is more intense in case of W5050.

6.3.2.1.3. SEM analysis

SEM analysis was performed in order to evaluate the PET surface morphology before and after EDA grafting in different conditions (W5050 and E2050) after 8 min of O_2 plasma. The untreated PET samples (figure 6.5.A1 and 6.5.A2) revealed an uniform and smooth surface without any relevant imperfections. The SEM images corresponding to the EDA functionalized PET samples (figure 6.5.B1, 6.5.B2, 6.5.C1 and 6.5.C2) present a completely different surface morphology with aligned topographic features, as like *strips*, in which the EDS analysis identifies the presence of the N element besides C and O (data not showed). In case of W5050, the *strips* are in a very uniform pattern through all the sample, being parallel and presenting a very regular thickness.

6.3.2.1.4. Optical profilometry analysis

In order to evaluate the PET surface topography and roughness after the EDA grafting, the treated samples were analysed by optical profilometry. Figure 6.6 shows the representative 3D height images for the untreated PET, and after the EDA graftings W5050 and E2050. As observed, both EDA treatments present a very distinct topography comparing to the untreated PET surface, revealing an increase of the R_a value from 9 nm to 508 nm in case of W5050 and to 168 nm in case of E2050. Moreover, the W5050 topography seems to be more regular and uniform than in case of E2050, presenting similar peaks and valleys through the whole surface.

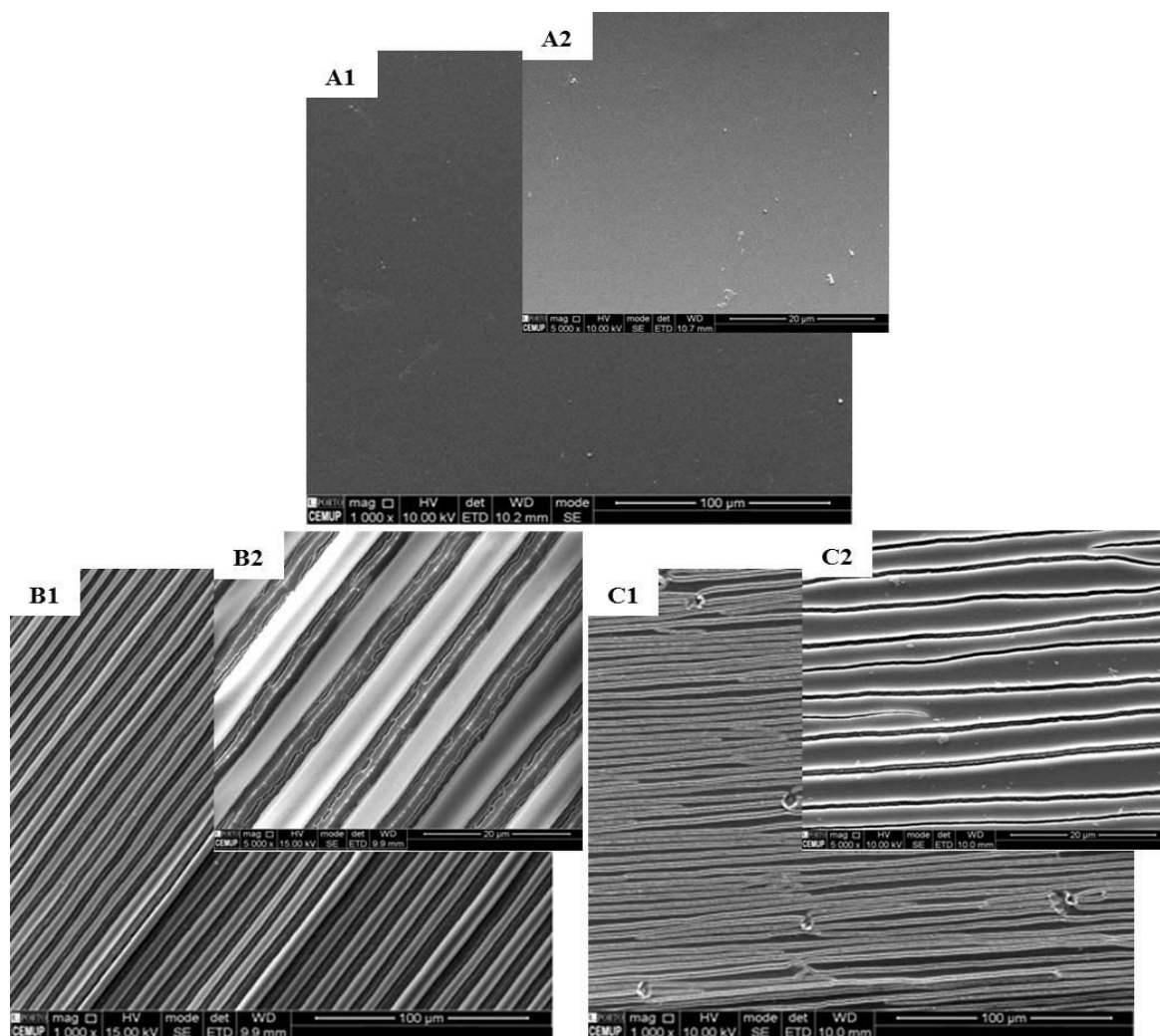


Figure 6.5: SEM images of PET samples surface: untreated PET at a magnification of 1000x (A1) and 5000x (A2); PET_W5050 at a magnification of 1000x (B1) and 5000x (B2); PET_E2050 at a magnification of 1000x (C1) and 5000x (C2).

6.3.2.1.5. Wettability evaluation

The values of water C.A. for untreated PET surface and after EDA grafting, using the W5050 and E2050 conditions after exposure to 8 min of O_2 plasma caused a statistically significant decrease of C.A. comparing to the untreated surface, namely from $74.7^\circ \pm 1.7$ to $65.9^\circ \pm 3.8$ in case of E2050 and to $61.0^\circ \pm 0.5$ in case of W5050.

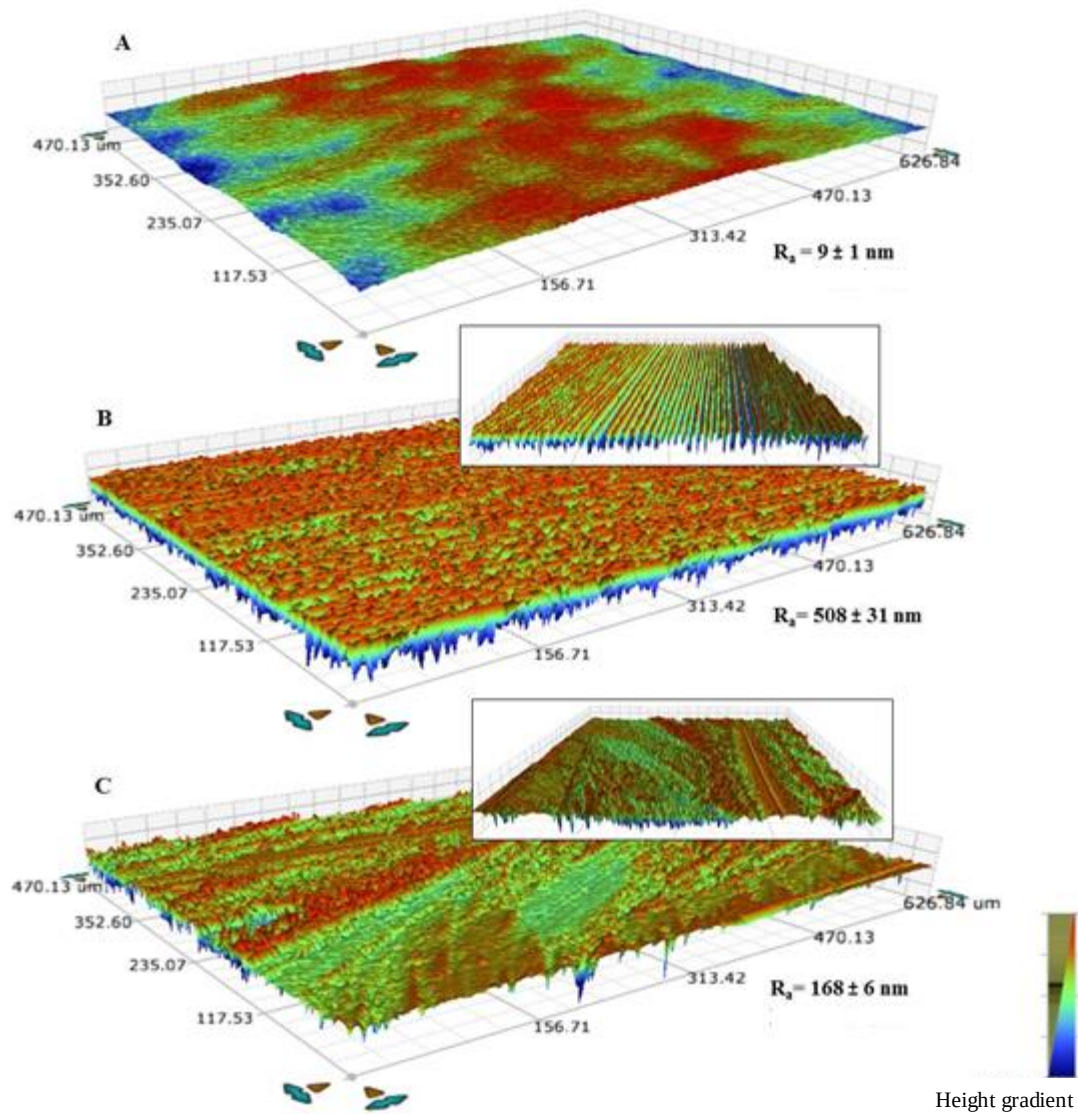


Figure 6.6: Representative optical profilometry images of PET samples surface before and after functionalization, as well the measured R_a values: (A) untreated PET; (B) PET_W5050; (C) PET_E2050.

6.3.2.2. *In vitro* biological characterization

6.3.2.2.1. Resazurin assay

The metabolic activity of the fibroblasts seeded on the untreated PET samples and W5050 grafted PET samples was evaluated and compared by the resazurin assay over 7 days of culture. As observed in figure 6.7, for both materials the resazurin fluorescence increases over time, but the grafted samples present higher fluorescence values at all time points comparing to the untreated samples.

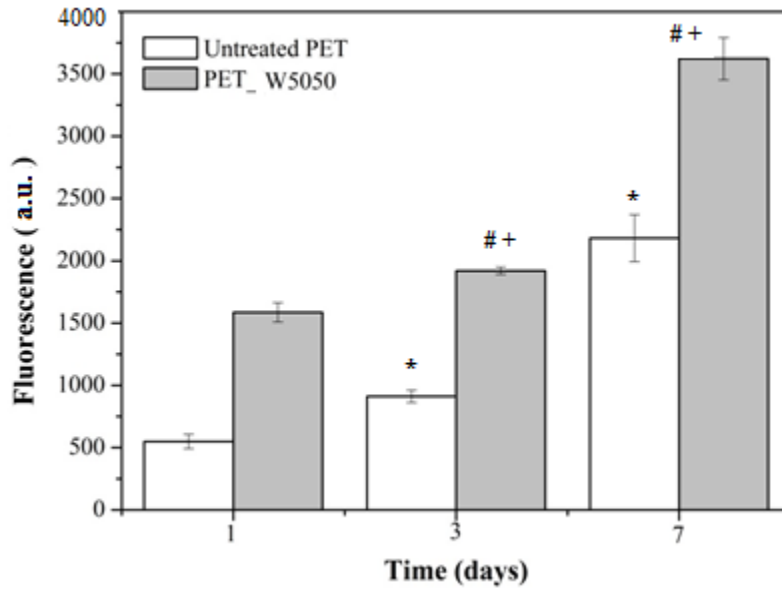


Figure 6.7: Levels of resazurin fluorescence at different incubation time points after the cell seeding on untreated PET samples and PET_W5050 samples. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference comparing untreated PET samples over time (*); comparing PET_W5050 samples over time (+); comparing the untreated and functionalized samples at each time point (#).

6.3.2.2.2. SEM analysis

In figure 6.8 it is possible to observe the cells seeded on the untreated PET surface (8.A1, 8.A2, 8.A3) and on the W5050 grafted PET surface (8.B1, 8.B2, 8.B3) at the different studied time points. After 1 day of incubation, it is possible to observe that fibroblasts adhered to the untreated and W5050 grafted samples surfaces, even though the cells present different morphologies in each material, as observed in figure 6.8.A1 and 6.8.B1. On the untreated surface some cells present a spread morphology but other ones are not so well spread. On the treated samples surface all cells already present a spread shape with developed filopodia. After 3 and 7 days of incubation, figure 6.8.A2, 6.8.B2, 6.8.A3 and 6.8.B3 illustrate an increase in the number of cells adhered on the materials surface during the culture time. Regarding the treated PET, at day 3, cells are already organized in interconnected groups getting oriented with the material's surface topographic features. At day 7, the material's surface is completely covered by an abundant cell layer, where all cells are completely spread and interconnected. On the untreated PET, after 3 and 7 days it is still possible to observe some cells not completely spread and other ones more spread, however they are not so well interconnected as a whole.

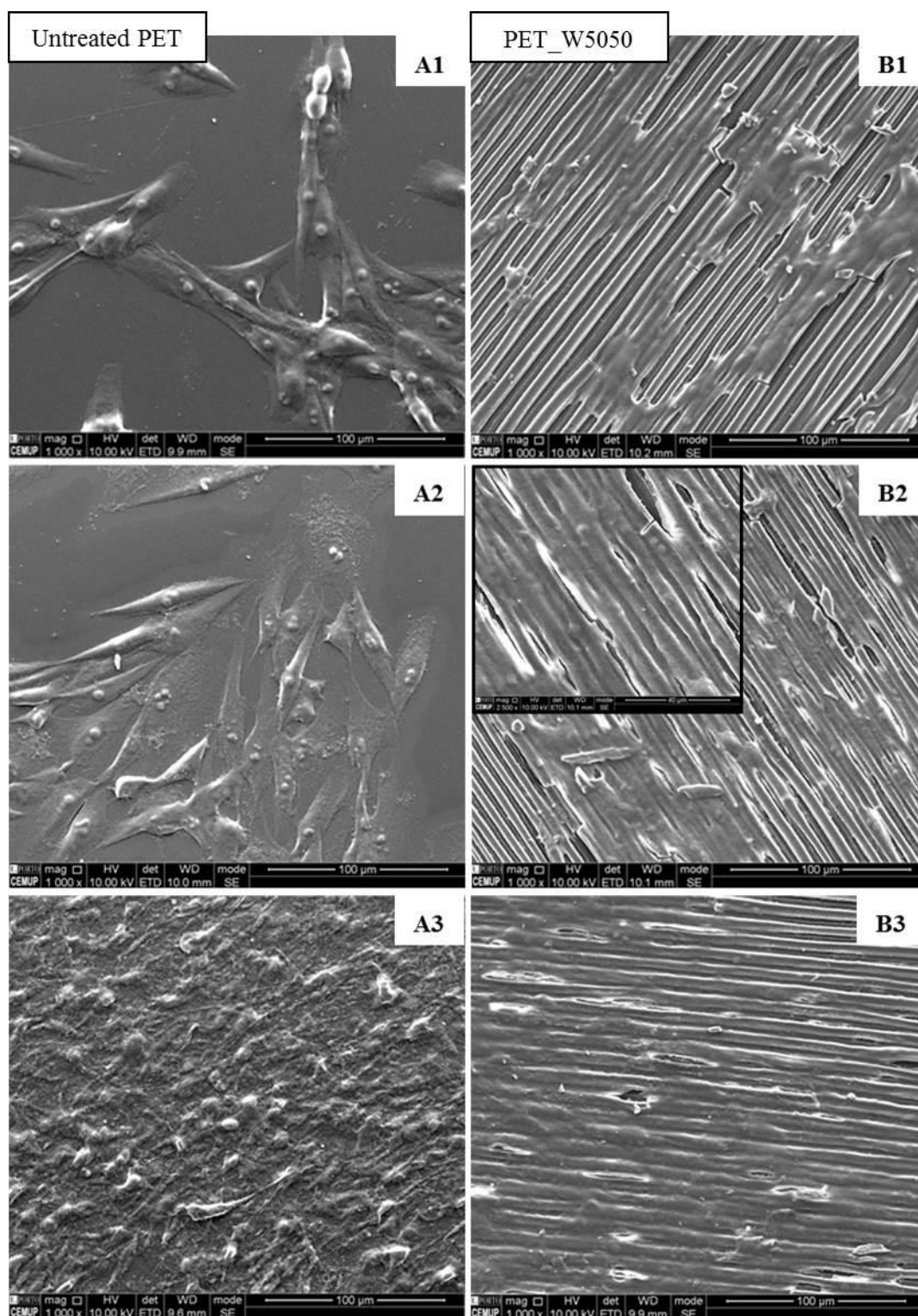


Figure 6.8: SEM images of cells seeded on PET samples surface over incubation time: untreated PET after 1 day (A1), 3 days (A2) and 7 days (A3); (B1) PET_W5050 after 1 day (B1), 3 days (B2) and 7 days (B3).

6.4. Discussion of results

Attending to the main purpose of this study, namely to improve the fibroblasts interaction with PET substrates for tendons regeneration, the grafting of functional amino groups ($-NH_2$) was performed on PET substrates based on a chemical etching in form of aminolysis by a condensation reaction using a diamine, namely the EDA. The ($-NH_2$) groups from EDA can be chemisorbed on PET substrates by reaction with one amine side only from EDA, forming amide groups, leaving the other side available to react with the ECM macromolecules [17]. The covalent conjugation between EDA and PET provides a long-term stability by avoiding the molecule detachment [18, 19]. Before the immersion in EDA, the PET substrates were exposed to different plasma treatments that may create new functional groups on the surface, such as carboxyl ($-COOH$) and hydroxyl ($-OH$), which increase the PET surface hydrophilicity to improve the contact with the EDA solution [11, 20]. Moreover, the new ($-COOH$) groups may be new points of reaction to anchorage more EDA molecules.

The studied plasma treatments included only O_2 plasma, which is essential to activate the surface and create the new groups, or Ar followed by O_2 , once that Ar can eliminate possible contaminations from the surface and homogenise it before the treatment, improving the activation [14, 21]. To optimize the surface activation process parameters, several treatments using those two plasma compositions were carried out at different exposition times. In figures 6.2.A and 6.2.B, for all plasma treatments, it is possible to observe the fingerprinting peaks of PET by FTIR-ATR analysis. Many of the peaks constitute gauche-trans pairs arising from vibrations of the glycol moiety: the peak at 1408 cm^{-1} may be attributed to methylene (CH_2) bending and the peak at 1337 cm^{-1} corresponds to CH_2 wagging [22, 23]. Other peaks are related with the vibrations of the benzene ring, namely the peak at 1016 cm^{-1} attributed to in-plane vibrations and peaks at 872 cm^{-1} and 721 cm^{-1} attributed to the out-of-plane vibrations. The peaks at 1244 cm^{-1} and 1097 cm^{-1} are mainly related with vibrations of the ester group [22, 23]. The strong peak at 1709 cm^{-1} may be related with the ester carbonyl ($C=O$) stretching or with the carboxylic acid $C=O$ stretching [23, 24]. According to the literature, after the treatment with an oxidizing plasma, it is expected the creation of new functional groups on PET surface, as already discussed, however no new peaks could be detected in FTIR-ATR spectra. This phenomenon may be explained by the overlapping of ester $C=O$ stretching bands with $C=O$ stretching bands of $COOH$ groups expected to be generated by an oxidizing plasma. There is a slight difference in position of $C=O$ stretching band of ester (at $1750\text{-}1735\text{ cm}^{-1}$) and carboxyl groups (at $1780\text{-}1710\text{ cm}^{-1}$) [24].

Figure 6.3 reveals that all the performed plasma treatments lead to a water C.A. decrease, namely from 74.7 ° to 10.7 ° and 10.1 ° after exposure to O₂ plasma (8min) and Ar pre-treatment (2 min) followed by O₂ plasma (8min). This decrease means an improvement of surface hydrophilicity as reported by other authors [14, 25, 26]. This surface wettability increase will improve the surface contact with aqueous solutions that can be used to promote some specific coatings. The *Wenzel's model* ($\cos(\theta_W) = r \cos(\theta_Y)$) describes the relation between the apparent C.A. on a rough surface (θ_W), the surface average roughness ratio (r , ratio between the real and geometric area) and the C.A. on a chemically similar smooth surface (θ_Y) [21, 27]. Hence, the wettability of surfaces is simultaneously related with the chemical and topographic changes promoted by the plasma activation treatments. According to this model and as reported by other authors, the wettability increase is usually associated to a R_a value increase due to a surface area increase. However, observing figure 6.3 and table 6.2, in this study the PET surface wettability increases when a slight decrease of the R_a value is observed, from 5.1 nm to 2.9 nm in case of O₂ plasma (8min) and 3.3 nm in case of Ar pre-treatment (2 min) followed by O₂ plasma (8min). Therefore, by the *Wenzel's model*, if the R_a value does not present a significant change, the great decrease of the rough surface C.A. must be related with the chemical changes promoted by the plasma treatments, namely by the introduction of new chemical polar groups, as other authors have already reported [26, 28, 29]. This reveals that the intended creation of new functional groups on the PET surface, such as carboxyl and hydroxyl, may have been successfully achieved despite not being confirmed by FTIR-ATR analysis. Even regarding the surfaces roughness after plasma treatment, besides the decrease of the average roughness (R_a), it must be noticed the R_q value decrease, what indicates that the surface became more homogeneous in accordance to what is observed in images presented in table 6.2 [30].

After plasma treatment of PET substrates using the two conditions that presented the lowest C.A., the samples were subjected to the different EDA treatments (table 6.1). For all the grafting conditions there was an increase of the acid orange absorbance comparing to the untreated samples, which is proportional to the amino groups density [31]. For the same plasma treatment (figure 6.4.A), maintaining the temperature and EDA concentration, the acid orange absorbance is higher in solutions using ethanol as solvent instead of water (W2050 and E2050 samples), meaning a higher density of amino groups. For the same EDA concentration and using water as solvent (W2050 and W5050 samples), the highest temperature led to the highest amino groups density, which indicates that temperature may favour the reaction process improving the covalent linking between PET surface and EDA.

All these phenomena are in accordance with results obtained by other authors, such as Cooper et al. [17]. It should be referred that the acid orange absorbance data for the grafting condition E5050 is not presented in figure 6.4.A because it caused the substrate degradation.

Regarding the influence of the plasma treatment conditions on grafting effectiveness, using the conditions with highest levels of absorbance (W5050 and E2050), after the O_2 plasma treatment the samples presented a higher amines density than performing the *Ar* pre-treatment before the reactive plasma (figure 6.4.B). This decrease of functional groups density when the pre-treatment is performed may happen because the O_2 reactive plasma concentration in the chamber will be lower due to the space occupied by the residual *Ar* particles and to the higher molecular weight of *Ar* that may hamper the O_2 entrance.

In the FTIR-ATR spectra of the PET surface after EDA grafting, according to the reaction shown in figure 6.1, new bands characteristic for amine, amide and hydroxyl groups were expected. As observed in figures 6.2.C and 6.2.D, using both plasma treatments and the grafting conditions W5050 and E2050, which presented the best levels of acid orange absorbance, the grafted PET samples present a new very weak wide band around 3500-2250 cm^{-1} that corresponds to the stretching vibration of primary amines and amides related with EDA presence [24]. Moreover, the region 1750–1500 cm^{-1} is also rich in new peaks, being the 1630 cm^{-1} and 1546 cm^{-1} peaks related with the stretching vibration of amide I and II respectively [24]. The presence of amides II is related with -CONH bonds, indicating that EDA has been successfully linked to the PET surface. When comparing the FTIR-ATR spectra using the different plasma treatments for the same EDA grafting conditions, although both treatments present the same new peaks already described, when the *Ar* pre-treatment is performed the peaks between 1750-1550 cm^{-1} are less strong and the band around 3500-2250 cm^{-1} barely appears, indicating less quantity of amino groups and -CONH bonds. Moreover, for each plasma treatment, when comparing the solvent used for the grafting (W5050 and E2050), the signal of the referred peaks is more intense for the W5050 sample, indicating a more efficient grafting by the presence of more amino groups and -CONH bonds. All these results are in accordance with the acid orange results already discussed, indicating the O_2 plasma treatment followed by the W5050 grafting as the best approach to accomplish a more efficient EDA grafting on PET surface.

Since the O_2 plasma treatment over 8 min revealed to be more interesting for amino functionalization than the *Ar* pre-treatment followed by the O_2 plasma, the water C.A., SEM and profilometry analysis were only performed on samples treated with O_2 . The surface morphology of EDA functionalized samples W5050 and E2050 (figures 6.5.B1, 6.5.B2,

6.5.C1 and 6.5.C2), based on aligned salient topographic features, may be explained because EDA preferentially etches the amorphous regions between the crystallites, remaining the polymer crystalline structure [32]. It must be referred that the used PET sheets are semi-crystalline substrates, what was proved by differential scanning calorimetry (DSC) analysis (data not showed). Also, the alignment of the *strips* may be explained because this orientation corresponds to the membrane longitudinal production, since *Goodfellow* PET sheet is biaxial oriented. By the EDS analysis, it was proved the presence of the N element mostly on those strips besides C and O elements, that as known make part of PET chemical structure. This N element presence may be in form of amino and/or amide groups on PET surface, indicating the functionalization success. When comparing the morphology of W5050 and E2050 surfaces, besides the *strips* being in a more uniform pattern in W5050, the thickness of the grafting layer seems to be thinner in case of E2050 samples because it was possible to observe some imperfections of the untreated PET surface. Moreover, this thickness difference may be related with FTIR-ATR analysis and acid orange test results, once in samples with a thinner grafting layer (E2050) the amino group peaks are less intense and their density is lower than in samples with a thicker grafting layer (W5050). Regarding profilometry analysis (figure 6.6), the topography images are in accordance to what was observed in SEM images, revealing a more uniform and regular topography in case of W5050. Moreover, the higher R_a value and the higher height of peaks in case of W5050 (508 nm) are also hint of a higher thickness of the grafting layer than in case of E2050 samples (168 nm).

Regarding wettability, both EDA graftings result in a statistical significant C.A. decrease from 74.7° in case of untreated PET to 65.9° and 61.0° in case of E2050 and W5050 samples, respectively. It has been found that when using the EDA solution with water as solvent, besides aminolysis other reactions like hydrolysis may happen, creating more functional groups that easily react with EDA to produce $-\text{COO}^-\cdot\text{NH}_3^+$, turning the PET surface more hydrophilic. On the other hand, when using ethanol as solvent, the PET ester groups are not subjected to hydrolysis, being the PET surface more hydrophobic than the ones treated in water [17, 33]. Moreover, the surface wettability is also related with the amino groups density due to their hydrophilic character [34]. Therefore, it would be expected that the W5050 samples, which presented more strong peaks concerning amines, amides and of $-\text{CONH}$ bonds and high levels of acid orange absorbance, present the lowest contact angle.

Attending to the main purpose of this work, namely to improve the bioactivity of PET surfaces by the enhancement of fibroblasts adhesion and proliferation, the viability and morphology of cells seeded on untreated PET and EDA grafted PET (W5050) were

evaluated. Cell attachment, adhesion and spreading are very important steps for a good cell/material interaction. The way these phenomena occur influences the cells ability to proliferate and to differentiate, which is essential for the formation of an implant/tendon functional successful interface. Therefore, SEM analysis was performed in order to observe cell adhesion to the EDA functionalized surfaces as well as their morphology. Observing figure 6.8 it is possible to see that cell adhesion occurred and the fibroblasts number increased over time for both tested materials, even though the cells presented different morphologies comparing the non-grafted to the grafted samples. At the beginning of the cell adhesion process, if cells present a not so well spread shape it indicates an initial stage of adhesion, a more spread shape reveals a more advanced adhesion stage [35, 36]. In a cell adhesion process, the material interacts with different cell surface receptors, establishing several sites of adhesion between the cells and the material surface [37, 38]. During the establishment of those sites, there is an organization and/or production of the cytoskeleton filamentous proteins, namely actin filaments, which tend to assemble in long bundles leading to a more spread shape with protrusions (filopodia) of the plasma membrane [35, 39, 40]. In case of untreated PET, even with increasing incubation time and available space on the surface for cells occupation, it is still possible to identify cells with a spherical shape at the three evaluated time points. In case of W5050 samples from 3 days on all cells present a spread shape and are perfectly adapted to the surface topography, revealing a more efficient adhesion process.

Resazurin has been generally used as an indicator of cell viability and its reduction is correlated with cell activity, since living cells are able to reduce the resazurin in mitochondria. Therefore the fluorescence results presented in figure 6.7 are proportional to cell metabolism and therefore cell proliferation and viability [41]. Among the tested samples, the EDA grafted samples present the highest metabolic activity for all time points. Cell morphology is controlled by the cytoskeleton organization and once its proteins are involved in signal transduction, the cell shape can also be related with cell growth and function. In fact, when cells try to adhere to a material surface, the mitochondrial activity increases by the production of the necessary energy to synthesize the above-mentioned proteins that are crucial to cell adhesion [35, 36, 42]. Therefore, a cell shape more spread and of course a higher number of cells is usually associated to a higher metabolic activity, as happened when comparing both tested materials. W5050 samples present more spread cells, as observed by SEM analysis, and consequently higher levels of metabolic activity than the untreated PET. Moreover, after 7 days of culture in W5050 samples surface the cells are completely

interconnected by abundant ECM produced by them, also proving the high level of cell viability.

Cell growth and function are influenced by the biomaterial surface characteristics, such as morphology and physical and chemical features. For instance, the materials' surface roughness and wettability can influence the type and the adsorption kinetics of the serum proteins to the material surface. The adsorbed protein layer has an essential role in the cell adhesion, morphology and migration, because the charged cell membrane interact with surfaces through this protein layer [36, 43]. It has been reported in several studies that a certain level of roughness and hydrophilicity, as well as the functionalization of surfaces with specific functional groups, such as (-OH) and (-NH₂), favour the adsorption of that protein layer and consequently the cell adhesion [18, 31]. However, some reported studies revealed preferable adhesion, growth and ECM formation of fibroblasts on (-NH₂) surfaces compared with other functional coatings [18]. Thus, the low cell adhesion and metabolic levels in case of untreated PET may be explained by the lack of functional groups, low wettability and flat surface [10, 11]. In case of EDA functionalized samples, the higher cell adhesion and activity may be mostly explained by the performed amine functionalization. The several amino groups presented on the material surface present positive charges which are able to establish electrostatic interactions with the negative charges of cell-surface proteins, promoting the adhesion of cells to the material [31]. Moreover, the EDA functionalized samples surface wettability, roughness and topography may also have favoured the cell anchorage.

6.5. Conclusions

FTIR-ATR analysis and acid orange results allowed to choose the conditions with the best amino functionalization, which were W5050 and E2050. Besides that, based on other techniques, such as water contact angle measurement, SEM analysis and optical profilometry the PET_W5050 sample was chosen to evaluate the cell interaction with it, since it presented the highest amino groups, the lowest water contact angle and the highest grafting thickness and roughness. Thus, resazurin and SEM analysis were performed in order to analyse cell adhesion, proliferation and morphology. The EDA functionalized samples (PET_W5050) was the approach that showed the best results, since it demonstrated higher cell metabolic activity than the untreated samples. Moreover, SEM results corroborate this conclusion, since cells were spread through the surface indicating a good adhesion and cell growth occurred being the surface all covered by cells at day 7.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project UID/EMS/00285/2013.

6.6. Bibliography

- [1] Chen J., Xu J., Wang A., Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. *Expert review of medical devices*. 2009;6:61-73.
- [2] Maffulli N., Renstrom P., Leadbetter W. *Tendon Injuries-Basic Science and Clinical Medicine*: Springer; 2005, London.
- [3] Morais DS, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair, *Annals of Biomedical Engineering*. 2015; 43(9):2025-35.
- [4] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.
- [5] Kuo C. K., Marturano J. E., Tuan R. S. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2010;2:20.
- [6] Hampson K., Forsyth N., Haj A., Maffulli N. *Tendon Tissue Engineering*. In: Ashammakhi N., Reis R., Chiellini F., editors. *Topics in Tissue Engineering*; 2008, United Kingdom.
- [7] Barber F. A., Herbert M. A., Coons D. A. Tendon augmentation grafts: biomechanical failure loads and failure patterns. *Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association*. 2006; 22:534-8.
- [8] James R., Kesturu G., Balian G., Chhabra A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery*. 2008;33:102-12.
- [9] Bassetto F., Volpin A., Vindigni V. *Regenerative Medicine for Tendon Regeneration and Repair: The Role of Bioscaffolds and Mechanical Loading*. In: R. P, editor. *Biomaterials Science and Engineering*: InTech; 2011: 370-86, Italy.
- [10] Liu Y., Ramanath H., Wang D. Tendon tissue engineering using scaffold enhancing strategies. *Trends in Biotechnology*. 2008;24:201-9.
- [11] Chen H, Truckenmuller DRK, Blitterswijk PdCAv, Moroni L. Fabrication of nanofibrous scaffolds for tissue engineering applications. In: Gaharwar AK, Sant S, Hancock MJ, Hacking AA, editors. *Nanomaterials in tissue engineering: Fabrication and applications*: Woodhead Publishing; 2013; 158-82.
- [12] Bartels V. *Handbook of medical textiles*: Elsevier; 2011, United Kingdom.

- [13] Veleirinho B, Coelho DS, Dias PF, Maraschin M, Pinto R, Cargnin-Ferreira E, et al. Foreign body reaction associated with PET and PET/chitosan electrospun nanofibrous abdominal meshes. *PloS one*. 2014;9:e95293.
- [14] Leroux F, Perwuelz A, Campagne C, Behary N. Atmospheric air-plasma treatments of polyester textile structures. *Journal of adhesion science and technology*. 2006;20:939-57.
- [15] Keen I, Broota P, Rintoul L, Fredericks P, Trau M, Grøndahl L. Introducing amine functionalities on a poly (3-hydroxybutyrate-co-3-hydroxyvalerate) surface: comparing the use of ammonia plasma treatment and ethylenediamine aminolysis. *Biomacromolecules*. 2006;7:427-34.
- [16] Drobota M, Persin Z, Zemljic L, Mohan T, Stana-Kleinschek K, Doliska A, et al. Chemical modification and characterization of poly (ethylene terephthalate) surfaces for collagen immobilization. *Open Chemistry*. 2013;11:1786-98.
- [17] Xue L, Lu Y. Electroless copper plating on 1, 2-ethylenediamine grafted poly (ethyleneterephthalate) for the fabrication of flexible copper clad laminate. *Journal of Materials Science: Materials in Electronics*. 2013;24:2211-7.
- [18] Tang L, Thevenot P, Hu W. Surface chemistry influences implant biocompatibility. *Current topics in medicinal chemistry*. 2008;8:270-80.
- [19] Williams R. *Surface modification of biomaterials: Methods analysis and applications*: Elsevier; 2010, United Kingdom.
- [20] Vesel A, Mozetic M. Surface functionalization of organic materials by weakly ionized highly dissociated oxygen plasma. *Journal of Physics: conference series*: IOP Publishing; 2009, 012015.
- [21] Pedrosa P, Chappé JM, Fonseca C, Machado AV, Nóbrega JM, Vaz F. Plasma Surface Modification of Polycarbonate and Poly (propylene) Substrates for Biomedical Electrodes. *Plasma Processes and Polymers*. 2010;7:676-86.
- [22] Andanson JM, Kazarian SG. In situ ATR-FTIR Spectroscopy of Poly (ethylene terephthalate) Subjected to High-Temperature Methanol. *Macromolecular symposia*: Wiley Online Library; 2008; 195-204.
- [23] Cole KC, Aji A, Pellerin E. New insights into the development of ordered structure in poly (ethylene terephthalate). 1. Results from external reflection infrared spectroscopy. *Macromolecules*. 2002;35:770-84.
- [24] Sheppard N. *The historical development of experimental techniques in vibrational spectroscopy*: Wiley Online Library; 2002.
- [25] Junkar I, Vesel A, Cvelbar U, Mozetič M, Strnad S. Influence of oxygen and nitrogen plasma treatment on polyethylene terephthalate (PET) polymers. *Vacuum*. 2009;84:83-5.
- [26] Gotoh K, Yasukawa A, Kobayashi Y. Wettability characteristics of poly (ethylene terephthalate) films treated by atmospheric pressure plasma and ultraviolet excimer light. *Polymer journal*. 2011;43:545-51.

- [27] Wolansky G, Marmur A. Apparent contact angles on rough surfaces: the Wenzel equation revisited. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 1999;156:381-8.
- [28] Pedrosa P, Fiedler P, Lopes C, Alves E, Barradas NP, Haueisen J, et al. Ag: TiN-Coated Polyurethane for Dry Biopotential Electrodes: From Polymer Plasma Interface Activation to the First EEG Measurements. *Plasma Processes and Polymers*. 2015;13(3): 341-354.
- [29] Siow KS, Britcher L, Kumar S, Griesser HJ. Plasma Methods for the Generation of Chemically Reactive Surfaces for Biomolecule Immobilization and Cell Colonization-A Review. *Plasma Processes and Polymers*. 2006;3:392-418.
- [30] De Oliveira R, Albuquerque D, Leite F, Yamaji F, Cruz T. Measurement of the nanoscale roughness by atomic force microscopy: basic principles and applications: INTECH Open Access Publisher; 2012, Brazil.
- [31] Li H-L, Zhang H, Huang H, Liu Z-Q, Li Y-B, Yu H, et al. The effect of amino density on the attachment, migration, and differentiation of rat neural stem cells In Vitro. *Molecules and cells*. 2013;35:436-43.
- [32] Aflori M. DM, Țîmpu D., Bărboiu V. . Amine functionality of poly(ethylene terephthalate) films surfaces induced by chemical and RF plasma treatments 28th ICPIG. 2007:727-30.
- [33] Irena G, Jolanta B, Karolina Z. Chemical modification of poly (ethylene terephthalate) and immobilization of the selected enzymes on the modified film. *Applied Surface Science*. 2009;255:8293-8.
- [34] Hamerli P, Weigel T, Groth T, Paul D. Surface properties of and cell adhesion onto allylamine-plasma-coated polyethyleneterephthalat membranes. *Biomaterials*. 2003;24:3989-99.
- [35] Morais DS, Rodrigues M, Lopes M, Coelho M, Maurício AC, Gomes R, et al. Biological evaluation of alginate-based hydrogels, with antimicrobial features by Ce (III) incorporation, as vehicles for a bone substitute. *Journal of Materials Science: Materials in Medicine*. 2013;24:2145-55.
- [36] Morais DS, Fernandes S, Gomes P, Fernandes M, Sampaio P, Ferraz M, et al. Novel cerium doped glass-reinforced hydroxyapatite with antibacterial and osteoconductive properties for bone tissue regeneration. *Biomedical Materials*. 2015;10:055008.
- [37] Zaleski KJ, Kolodka T, Cywes-Bentley C, McLoughlin RM, Delaney ML, Charlton BT, et al. Hyaluronic acid binding peptides prevent experimental staphylococcal wound infection. *Antimicrobial agents and chemotherapy*. 2006;50:3856-60.
- [38] Lei Y, Gojgini S, Lam J, Segura T. The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels. *Biomaterials*. 2011;32:39-47.
- [39] Hempel U, Hefti T, Dieter P, Schlottig F. Response of human bone marrow stromal cells, MG-63, and SaOS-2 to titanium-based dental implant surfaces with different topography and surface energy. *Clinical oral implants research*. 2013;24:174-82.

- [40] Wutticharoenmongkol P, Pavasant P, Supaphol P. Osteoblastic phenotype expression of MC3T3-E1 cultured on electrospun polycaprolactone fiber mats filled with hydroxyapatite nanoparticles. *Biomacromolecules*. 2007;8:2602-10.
- [41] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*. 2000;267:5421-6.
- [42] Morais DS, Coelho J, Ferraz MP, Gomes PS, Fernandes MH, Hussain NS, et al. Samarium doped glass-reinforced hydroxyapatite with enhanced osteoblastic performance and antibacterial properties for bone tissue regeneration. *Journal of Materials Chemistry B*. 2014;2:5872-81.
- [43] Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue engineering*. 2005;11:1-18.

Chapter 7

**Bioactive and biopassive treatment of
poly(ethylene terephthalate) (PET)
multifilament textile yarns to
improve/prevent fibroblasts viability**

Bioactive and biopassive treatment of poly(ethylene terephthalate) (PET) multifilament textile yarns to improve/prevent fibroblasts viability

Morais D.S.^{1,2}, Lopes C.³, F. Vaz³, Grenho L.⁴, Fernandes M.H.⁴,

Guedes R.M.^{2,5}, Lopes M.A.^{1,*}

¹ CEMUC, Departamento de Engenharia Metalúrgica e Materiais, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

² INEGI-Instituto de Engenharia Mecânica e Gestão Industrial, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

³ Centro de Física, Universidade do Minho, 4710-057 Braga, Portugal

⁴ Faculdade de Medicina Dentária, Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, Porto, Portugal.

⁵ Departamento de Engenharia Mecânica Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

* Corresponding author: malopes@fe.up.pt

Abstract

In order to modulate the physicochemical features of poly(ethylene terephthalate) (PET) multifilaments surface composing a complex textile structure, intended to be used as an implant for high extension injuries of the Achilles tendon (AT) or even for its total replacement, two different surface treatments with different purposes (bioactive and biopassive) were studied. The structure is based on a core and shell system and one treatment, based on amino groups grafting using ethylenediamine (EDA) molecules to be applied in the structure core to allow the improvement of cell adhesion and proliferation, and other treatment, based on polytetrafluoroethylene (PTFE) coating to be applied in the structure shell to avoid the cell adhesion and even to decrease the coefficient of friction of filaments. Both treatments were optimized in order to reach their purposed goals but without harm the tensile properties of PET yarns, which was evaluated by static tensile tests. The resazurin assay and SEM analysis proved that the purposed goals related with cell adhesion were achieved for both treatments and in case of PTFE coating the abrasion resistance was also improved according to the yarn-on-yarn abrasion tests.

Keywords: poly(ethylene terephthalate), PET, ethylenediamine, EDA, polytetrafluoroethylene, PTFE, multifilament yarn, braid.

7.1. Introduction

In last years, due to the high incidence of tendons injuries, several structures have been developed to be used as patches to provide some protection in case of tissue tears or as implants to partially or fully replace an injured tendon. When designing a scaffold for tissue regeneration there are some general characteristics that it must fulfil as an implantable device, as well as other essential specific requirements attending to the specific tissue intended to be engineered. First of all, it must be biocompatible, not elicit an immunological response and be bio-functional, meaning the ability to promote cell adhesion, proliferation and differentiation, extracellular matrix (ECM) components synthesis, and consequently the new tissue formation and ingrowth. Besides that, a scaffold for tendon tissue engineering must ideally mimic its mechanical performance, what may be achieved by having a fibrous micro-architecture similar to the native tissue [1-4].

The main advantage of using polymeric based structures for biomaterials development is the fact that their properties can be tailored according to the requirements of the final application. In general, the modification of material surface properties (including chemistry, wettability and morphology) has been shown to influence protein adsorption and subsequent cellular responses to the biomaterial. Therefore, different techniques have been used to tailor the properties of scaffolds surface, including plasma modification/polymerization, sputtering deposition and chemical grafting [5-7].

The authors have already developed a fibrous structure with a specific architecture, based on poly(ethylene terephthalate) (PET) multifilament yarns, with a fibrous micro-architecture similar to the Achilles tendon (AT) and able to mimic its mechanical performance. However, PET substrates surfaces present poor wettability due to the low surface energy and lacks active functional groups involved in cell adhesion process [8, 9]. The introduction of new functional groups by chemical modification reactions is one possible approach to modify the physical and chemical nature of the filaments surface. Chemical graft modification has been employed to chemically immobilize compounds onto a surface, activating the surface with specific reactive groups granting to it the desired functionality. This method usually involves covalent conjugation of a molecule to the surface, providing a long-term stability by avoiding the molecule detachment [5, 6]. The chemical grafting of amino groups ($-NH_2$) on PET surface, for instance, has been studied based on a chemical etching in the form of aminolysis using diamines. By choosing the proper grafting conditions, it should be possible to restrict the chemical modification to the surface, leaving the filaments bulk structure unchanged as well as their mechanical properties [10, 11]. In case of

ethylenediamine (EDA), the (-NH₂) groups can be chemisorbed on PET substrates via (-C(O)NH) bonds by EDA modification in water solutions, which couples with PET surface by one amine side, leaving the other available to react with the ECM macromolecules, such as collagen [12]. The (-NH₂) groups in aqueous environment are able to electrostatically interact with the cell-surface proteins, promoting the adhesion of cells to the grafted material surface, thus performing a bioactive effect [6, 13]. Some reported studies revealed preferable adhesion, growth, and ECM formation of fibroblasts on (-NH₂) surfaces compared with other functional coatings. Further analysis showed that within 45 minutes, cells cultured on (-NH₂) surfaces had already begun the formation of adhesion plaques, related with increased cell spreading on the surfaces [6].

The fibrous structure of a natural AT is protected and surrounded by a membrane, named as paratenon, which is a loose connective tissue layer and its rupture allows granulation tissue and fibroblasts from surrounding tissues to invade the damaged site. Therefore, in order to also mimic that membrane, a biopassive polytetrafluoroethylene (PTFE) based coating was studied to be applied on the outer part of the structure as an attempt to avoid the cell adhesion. PTFE has a simple linear chain molecular structure, having a wide range of applications in last years in several fields such as biomedical devices and for non-stick and anti-adhesive coatings. The coatings based on this polymer combine heat resistance with almost total chemical inertness, low surface energy (hydrophobic profile), good dielectric stability and lubrication properties due to the low coefficient of friction [14-16]. Due to its low coefficient of friction and self-lubricant features, PTFE plays an important role as bearing material for more than 50 years in several applications [17]. The unique properties of PTFE are a result of the structure of the polymer units and the strength and stability of the carbon to fluorine bonds in the polymer [18, 19]. Different physical techniques have been used for the deposition of PTFE films on different substrates, such as radio frequency (RF) sputtering and air-atomized spray technique [14, 16].

Therefore, in this study two different treatments were developed to tailor the physicochemical features of multifilaments surface and consequently their interaction with the surrounding native tissues depending on their localization in the fibrous structure. Namely, a bioactive treatment using EDA to promote cell adhesion, which will be applied on filaments surface composing the inner part of the fibrous structure, and a biopassive treatment based on PTFE with main purpose of avoiding cell adhesion, which will be applied on filaments surface composing the outer part of the structure.

7.2. Materials and methods

7.2.1. Materials

PET multifilament yarns, with a linear density of 1112 dtex purchased from Sarla Europe, LDA (Portugal), were used to produce biaxial braided structures with 16 yarns on a vertical braiding machine with 16 carriers, under controlled process conditions. Ethylenediamine (EDA) (99%) was purchased from *Sigma-Aldrich* (Germany) and the polytetrafluoroethylene (PTFE) water based suspension was kindly supplied by *Impocolor* (Portugal).

7.2.2. Methods

7.2.2.1. EDA grafting on PET multifilament yarns and braids

The EDA grafting was performed on multifilament PET yarns surface and also on braided structures based on same yarns, namely 16 yarns. Firstly, the yarns or braids, with a length of about 50 cm, were exposed to O_2 plasma activation treatment over 8 minutes. A *Zepto* laboratory-sized plasma system from *Diener Electronics* ($\varnothing = 105$ mm, $L = 300$ mm, $V = 2.6$ L) was used for the plasma activation of the samples, using a power of 100 W, a $pressure_{base}$ of 10 Pa and a $pressure_{work}$ of 80 Pa.

Immediately after the plasma treatment, the yarns or braids were immersed in EDA solutions with different concentrations, as shown in table 7.1, using water or ethanol as solvent, and incubated at different temperatures for different times, at 100 rpm orbital. After the immersion time, all samples were removed from the reactive solutions, rinsed in distilled water, then in isopropanol and again in distilled water. Finally, the grafted samples were dried under vacuum condition at room temperature before analysis.

7.2.2.2. PTFE coating of PET multifilament yarns

The coating of PTFE was performed only on PET yarns by air-atomized spray technique using water based PTFE solutions with different concentrations, as presented in table 7.2. Samples with a length of about 50 cm were coated and vertically suspended in a system with continuous rotation for the spray exposure. After that, the samples were kept in oven over 30 minutes at 60 °C.

Table 7.1: Name of PET yarns or braids samples grafted with EDA, depending on EDA solution concentration, solvent, temperature and immersion time.

Sample name	Solvent	Temperature (°C)	EDA concentration % (V/V)	Immersion time
EDA_W5050_24h_Yarn	Water	50	50	24 hours
EDA_W5050_12h_Yarn				12 hours
EDA_W5050_20m_Yarn				20 minutes
EDA_E2050_20m_Yarn	Ethanol	20	50	20 minutes
EDA_E2025_20m_Yarn			25	
EDA_E2050_30m_Yarn			50	30 minutes
EDA_E2050_30m_Braid				60 minutes
EDA_E2050_60m_Yarn				

Table 7.2: Name of PET yarns samples coated with PTFE, depending on the concentration of spraying solution.

Samples name	PTFE concentration (g/L)
PTFE_30_Yarn	30
PTFE_50_Yarn	50
PTFE_100_Yarn	100
PTFE_200_Yarn	200

7.2.2.3. Mechanical and physicochemical characterization

The EDA grafted yarns and braids and the PTFE coated yarns were tensile tested (5 samples of each) on *Lloyd instruments LR 30K* equipment, in displacement control, up to rupture. The initial distance between grips was defined as 235 mm and the displacement rate as 8.5 mm/s. After all the tests, mechanical properties such as stiffness (N/mm), load at failure (N) and strain to failure (%) of all structures were measured.

Fourier Transform Infrared Spectroscopy (FTIR) in Attenuated Total Reflectance (ATR) mode was performed, in triplicate, for untreated PET yarns, for EDA grafted yarns and braids and for PTFE coated yarns, using the spectrometer *Jasco FT/IR 4100* system (64

scans and 8 cm^{-1} nominal resolution) equipped with a Specac MkII Golden Gate single reflection ZnSe ATR crystal.

Scanning electron microscopy (SEM) analysis was performed in triplicate, using a *FEI Quanta 400 FEG ESEM* microscope, to analyze the surface microstructure and morphology of braids/yarns treated with EDA or PTFE. The SEM images were acquired using either the secondary or the back-scattered electrons mode and the elemental composition was evaluated by energy dispersive X-ray spectroscopy (EDS). Moreover, the surface of treated braids/yarns was also analysed by optical profilometry using a *NPFLEX Bruker* equipment.

The yarn-on-yarn abrasion, for the untreated PET yarns, EDA grafted yarns and PTFE coated yarns, was evaluated according to CY 1503-00 from *Cordage Institute International Standard* [20]. The test is based on a system where two portions of one yarn are intertwined (3 intertwisting wraps), they rub against one another, under controlled tension, due to the cyclic motion (72 rpm) imposed by rotation of a crank to which the yarn is attached. The tension is imposed by a weight of 2% of the yarn tensile load at failure and the number of cycles until the yarn failure was recorded for 5 samples of each yarn type.

In case of EDA grafted yarns/braids, the surface chemistry was also evaluated by X-ray Photoelectron Spectroscopy (XPS), performed using a *Kratos Axis Ultra* spectrometer with the VISION software for data acquisition. The analysis was carried out with a monochromatic Al $K\alpha$ x-ray source (1486.7 eV), operating at 15 kV (90 W) in fixed analyser transmission (FAT) mode, with a pass energy of 40 eV for regions of interest and 80 eV for the overall surveys. Data acquisition was performed with a pressure lower than 1×10^{-6} Pa and a charge neutralization system was used. Analysis of the data was carried out with CasaXPS software. A linear background was used in curve-fitting along with a GL(30) lineshape (70% Gaussian, 30% Lorentzian using the Gaussian/Lorentzian product form) for the C 1s and N 1s spectra. An exponential tail GL(55)T(2) lineshape was used for fitting the O 1s spectra as there appeared to be some intrinsic asymmetry toward higher binding energy (BE). Samples were referenced to the C 1s emission by adventitious hydrocarbon contamination at 285 eV. Peak widths were not fixed, to account for variation between different chemical states.

The untreated PET_16YH braids and EDA grafted 16YH braids were even stained with fuchsin acid, namely 3 samples of each one were kept under constant agitation in 0.5% fuchsin acid in pH 2.7 buffer solution for 90 min at room temperature. The stained specimens were rinsed with a pH 2.7 buffer solution for one minute to remove the non-linked dye.

The wettability of PTFE coated yarns using each solution concentration was assessed by the sessile drop method (3 μl drop), measuring the water contact angle (C.A.) at room

temperature, using a *OCA 20* unit from *Dataphysics*. A minimum of 9 contact angle measurements were performed using at least 3 samples of each different PTFE coating.

7.2.2.4. *In vitro* biological characterization

7.2.2.4.1. Resazurin assay

Human dermal fibroblasts were cultured in Minimal Essential Medium, alpha modification (α -MEM, *Gibco*) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin and 100 μ g/mL streptomycin (*Gibco*), and 2.5 μ g/mL amphotericin B (*Gibco*), at 37 °C in a 5% CO₂ humidified atmosphere. In case of EDA grafted PET braids, samples with 6 cm of length were used, having as control non-grated braids with the same length. All braids were sterilized with ethylene oxide. Prior to cell seeding, braids were positioned horizontally in Petri dishes and were moistened with supplemented α -MEM. The cells were then seeded in the middle of each sample length, 3×10^4 cells in 50 μ L medium, and pre-incubated for 3 hours. The braids, still positioned horizontally, were covered with medium and incubated for 24 hours at 37 °C in a 5% CO₂ humidified atmosphere. Then, the braids were vertically suspended in flasks full with medium and incubated, in the same position, over 21 days. Resazurin assay was performed for each braid individually after the first 24 hours incubation, before being vertically positioned, and throughout the culture time, at days 7, 14 and 21. Briefly, the samples were incubated with medium containing 10% of resazurin (*Sigma-Aldrich*) for 3 hours at 37 °C, after which the fluorescence (excitation: 530 nm; emission: 590 nm) was measured in a microplate reader (*Synergy HT*, *Biotek*). measured at 595 nm in a microplate reader (*Synergy HT*, *Biotek*). This experiment was performed in triplicate for each time point, either for the non-grafted and grafted braids.

In case of PTFE coated yarns, samples with 1 cm of length were used, having as control uncoated braids with the same length. The yarns were sterilized with ethylene oxide and placed in 24-well culture plates. Fibroblast cells were seeded on the yarns at 10^4 cells/cm² and incubated for 1, 4 and 7 days at 37 °C in a 5 % CO₂ humidified atmosphere. After each time point, resazurin assay was performed, as previously described. This experiment was performed in triplicate for each time point, either for the uncoated and coated braids.

7.2.2.4.2. SEM analysis

The morphology of fibroblasts seeded on the surface of the EDA grafted braids and PTFE coated yarns, after different time points, was analysed by SEM using a *FEI Quanta 400 FEG ESEM* microscope. Briefly, samples with cells were fixed in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, over 15 minutes; dehydrated using graded ethanol solutions from 50% to 100% (V/V) and critical-point dried (CPD 7501, Polaron Range). Lastly, samples were sputter-coated (*SPI-Module*) with a thin gold/palladium film and visualized.

7.2.2.4. Statistical analysis

Experimental data is presented as mean $\pm$ SD (standard deviation). Statistical analysis of data was performed using the one-way ANOVA test with Bonferroni post hoc analysis, using the software SigmaStat 3.5. The differences were considered to be significant at a level of $p < 0.05$.

7.3. Results

7.3.1. Mechanical and physicochemical characterization

7.3.1.1. Tensile tests

Based on results of the performed tensile tests, in figure 7.1 are presented the average values of the load at failure and strain to failure of EDA grafted yarns and braids using the different immersion conditions. As observed, for the EDA_W5050_24h_Yarn sample there is a big decrease in failure load and strain values comparing to the untreated PET yarn, but decreasing the immersion time, the impact on failure load and strain is lower. When using ethanol as solvent, the same behaviour is observed, there is a decrease of failure load and strain comparing to the untreated PET yarn, being the decrease as higher as the immersion time. For the EDA_E2050_30m_Braid sample the same behaviour was seen. Regarding the PTFE coated yarns, the tensile tests (data not showed) did not reveal any change of the levels of load at failure and strain to failure, comparing to the untreated PET yarn. Tensile tests were also performed for PET yarns just after the plasma treatment (data not showed) and also no changes were observed comparing to the untreated PET yarns.

Representative force-strain curves for some conditions are presented in figure 7.2, on yarn level (figure 7.2.A) and braid level (figure 7.2.B). It is possible to observe that in spite of changes in the level of load at failure and strain to failure, the shape of all force-strain curves is very similar among all samples even comparing with the untreated PET yarn.

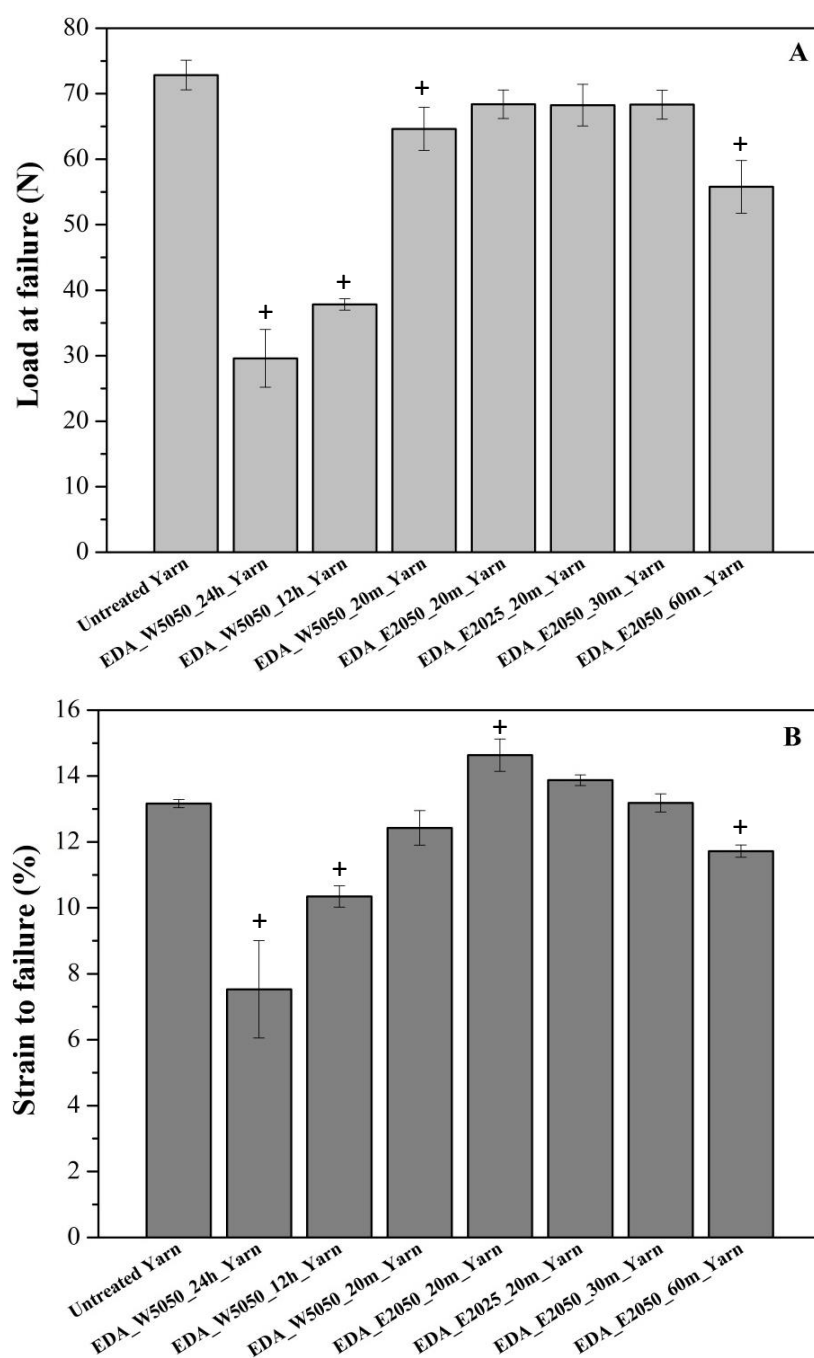


Figure 7.1: Load at failure (N) (A) and strain to failure (%) (B) of all EDA grafted PET yarns. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to untreated yarn (+).

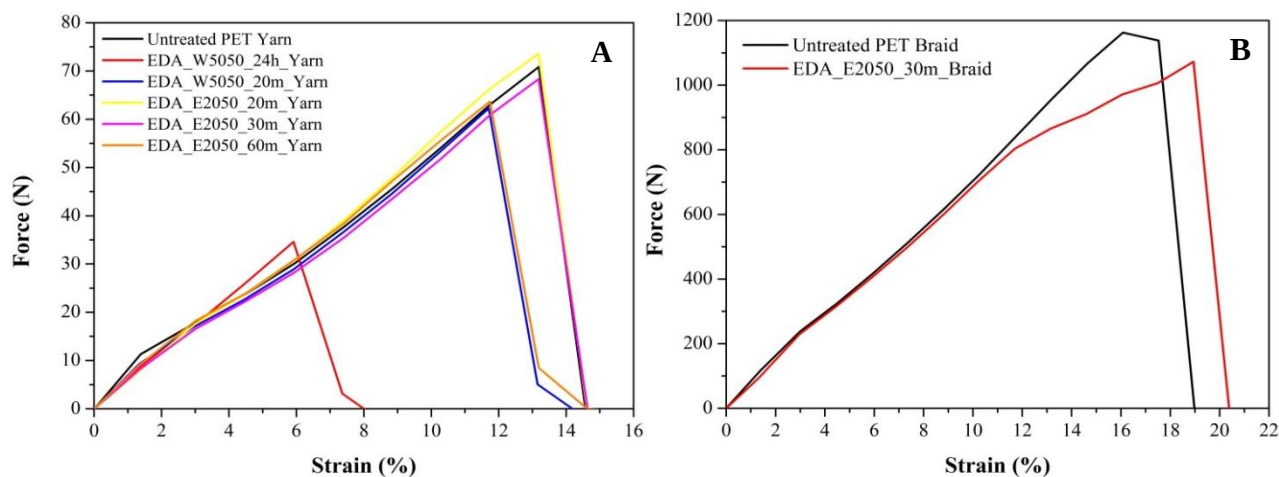


Figure 7.2: Representative force – strain curves of EDA grafted PET yarns (A) and EDA grafted braid (B).

7.3.1.2. FTIR-ATR analysis

In order to evaluate the new chemical groups presented on surface of EDA grafted PET samples and PTFE coated PET samples, the FTIR-ATR analysis was performed. In figure 7.3.A are presented the FTIR spectra of the untreated PET yarn, of PET yarn after the EDA grafting using the conditions EDA_E2050_30 and EDA_E2050_60, and also of the PET_16YH braid after the grafting EDA_E2050_30. After the EDA grafting new peaks appeared in spectra, either on yarn or braid, namely at about 1564 cm^{-1} and 1490 cm^{-1} , and a very weak wide band around $3500\text{--}3000\text{ cm}^{-1}$.

Regarding the PTFE coated yarns, the FTIR-ATR spectra for all used concentrations are presented in figure 7.3.B, and no new peaks were detected after coating.

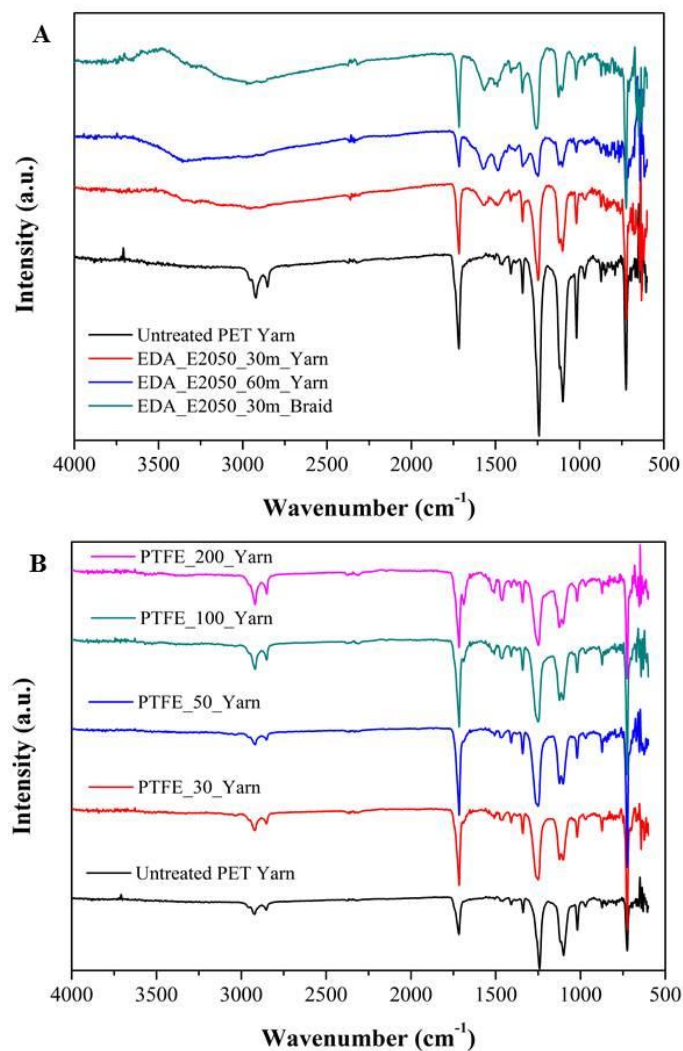


Figure 7.3: FTIR-ATR spectra of the untreated PET yarn, EDA grafted yarns and braids (A), and PTFE coated yarns (B).

7.3.1.3. SEM and EDS analysis

In order to evaluate the surface morphology of filaments composing the yarns of EDA grafted and PTFE coated samples, the SEM analysis was performed as presented in figure 7.4. Figures 7.4.A and 7.4.B reveal the surface morphology of filaments composing untreated yarns at two different magnifications and figure 7.4.C presents the surface of filaments composing the PET yarns immediately after exposing to the plasma treatment. As observed, the untreated PET filaments present a surface with some small defects, but after the plasma treatment the surface morphology becomes irregular and rougher. After the EDA grafting, namely in case of the EDA_E2050_30_Braid samples, the morphology of the composing filaments becomes completely different (figure 7.4.D), being possible to identify a smooth and homogeneous surface (figure 7.4.F) with some agglomerates (figure 7.4.E). In figure 7.5

are presented the EDS spectra of zones 1 and 2 assigned in figure 7.4.E, where is possible to identify the N element besides the O and C elements, being the N peak more intense for zone 2. After the PTFE coating, namely in case of PTFE_100 samples, the surface of filaments seems smoother comparing to the untreated PET filaments when observing the magnification of 10000 x (figures 7.4.B and 7.4.H). The EDS spectrum of zone 3, assigned in figure 7.4.H, is presented in figure 7.5, being possible to identify the F element besides the O and C elements.

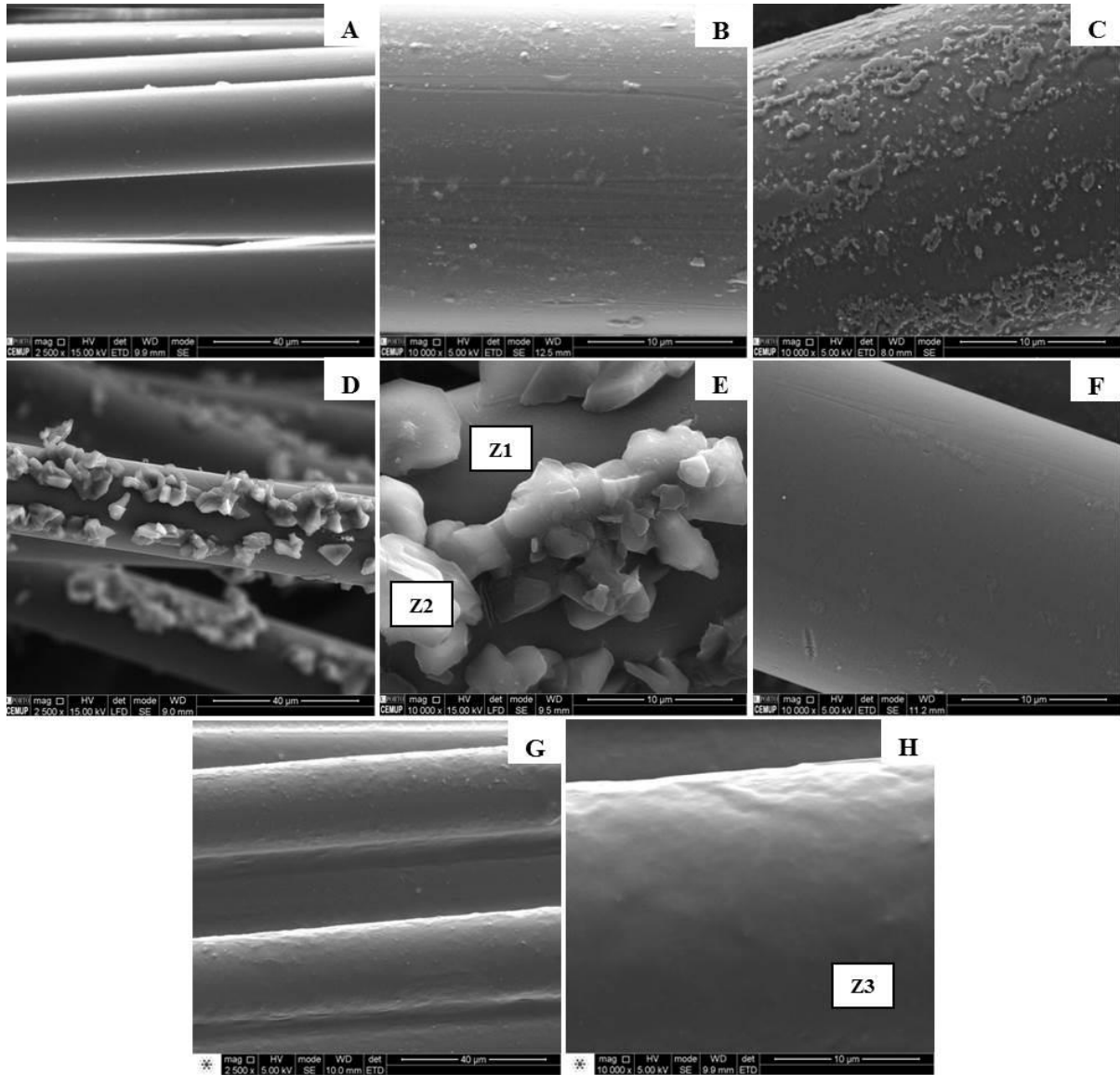


Figure 7.4: SEM images of untreated PET yarns (**A** and **B**); PET yarns after the plasma activation (**C**); of PET yarns (that compose the braids) after EDA grafting, namely of EDA_E2050_30_Braid samples (**D**, **E** and **F**); PET yarns after PTFE coating, namely of PTFE_100 samples (**G** and **H**).

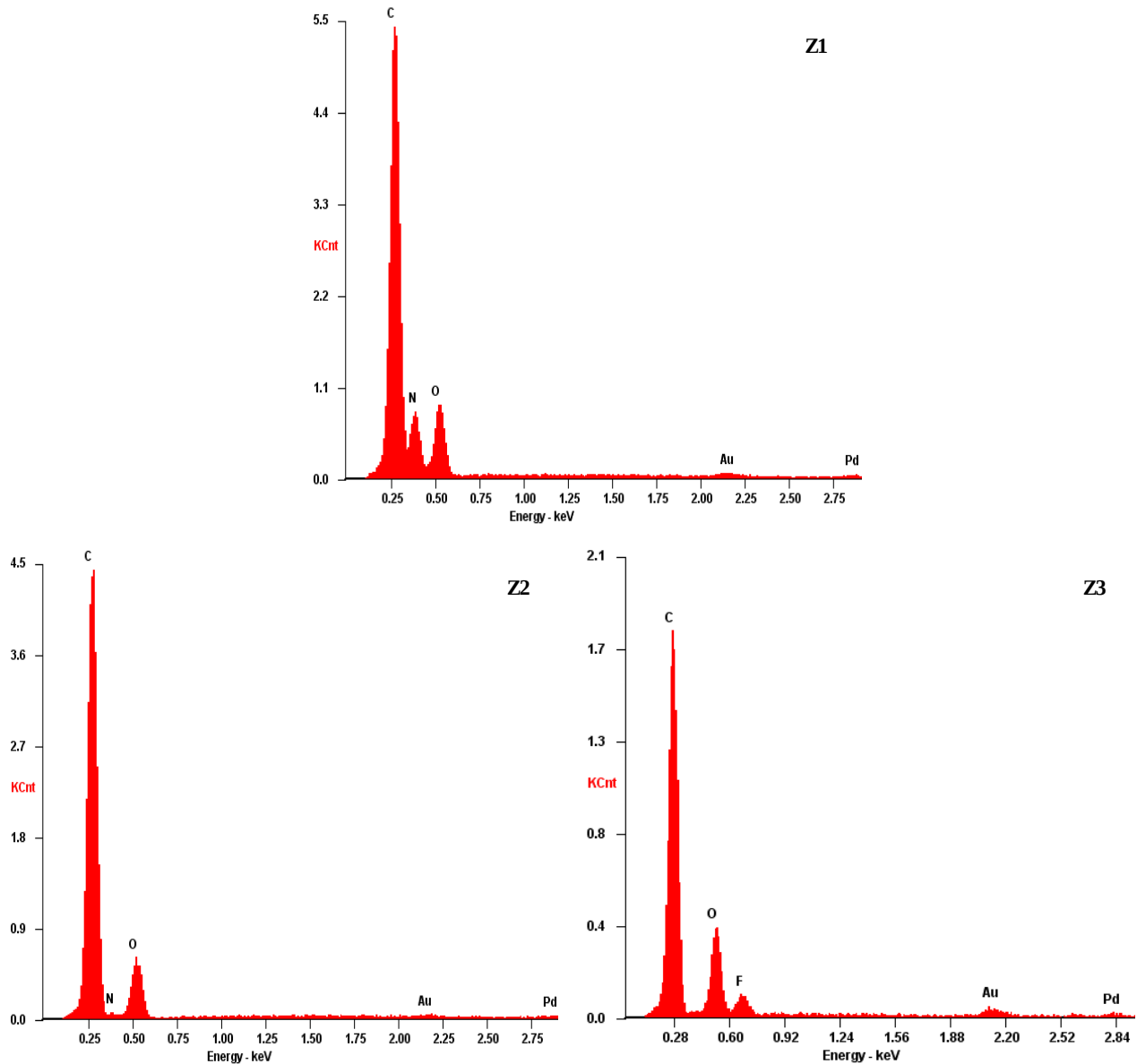


Figure 7.5: EDS spectra of zone 1 (Z1), 2 (Z2) and 3 (Z3) assigned in SEM images of figure 7.4.

7.3.1.4. Optical profilometry analysis

Based on optical profilometry analysis, the surface average roughness (R_a) of PET monofilaments composing the yarns of two different PTFE coated samples or even composing the EDA_E2050_30_Braid sample, was measured as presented in table 7.3. As control, the R_a of monofilaments composing the untreated yarns was also measured. After the EDA grafting, the R_a value slightly increases comparing to the untreated monofilaments. However, after the PTFE coating on yarns, the R_a value tends to slightly lower values than in case of the untreated monofilaments. These both phenomenon are qualitatively observed on the optical profilometry images presented in figure 7.6, where is possible to observe the surface topography of one untreated PET monofilament (figure 7.6.A), of monofilaments

composing an EDA_E2050_30_Braid sample and a PTFE_100_Yarn sample (figures 7.6.B and 7.6.C).

Table 7.3: Average roughness (R_a) values, measured by optical profilometry.

Sample	R_a (μm)
Untreated PET monofilament	0.05 ± 0.01
PET monofilament of EDA_E2050_30_Braid	0.11 ± 0.09
PET monofilament of PTFE_100_Yarn	0.03 ± 0.01
PET monofilament of PTFE_200_Yarn	0.03 ± 0.00

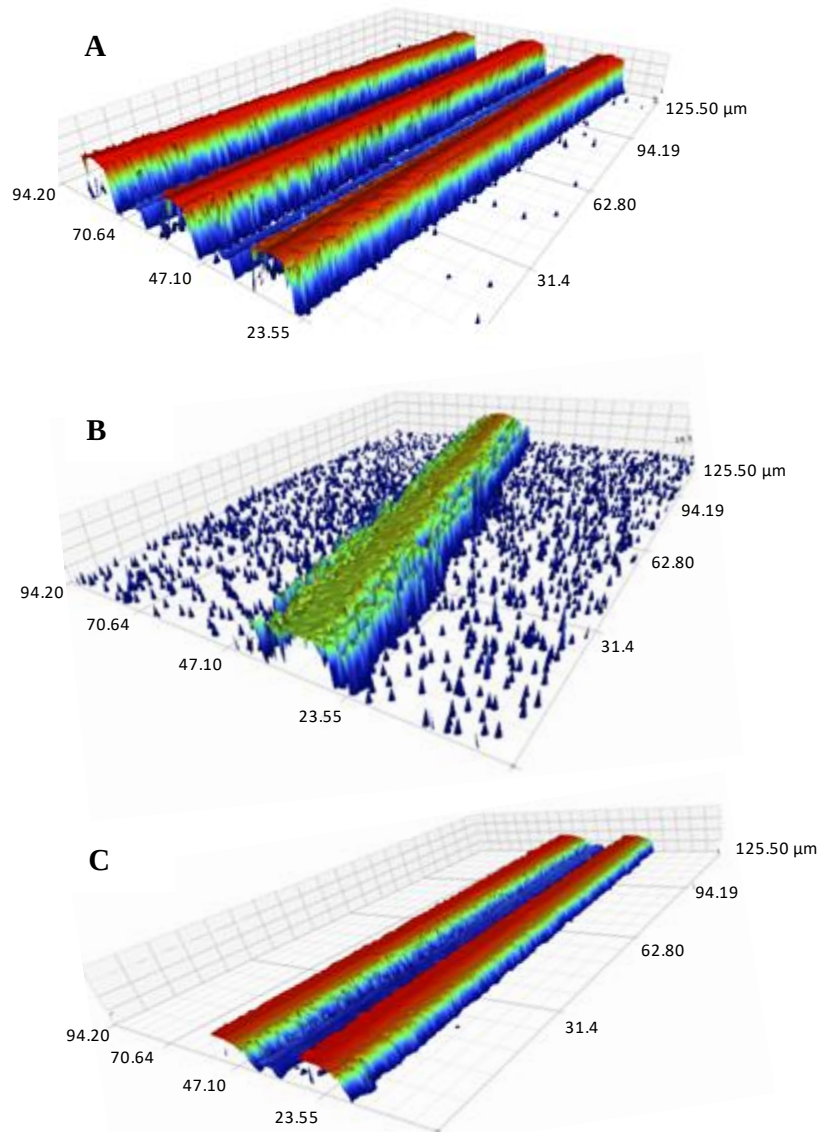


Figure 7.6: Representative optical profilometry images of yarns filaments composing an untreated PET braid (A); an EDA_E2050_30m_Braid sample (B); and PTFE_100 sample (C).

7.3.1.5. Yarn-on-Yarn abrasion test

In abrasion tests, the untreated PET yarns were able to withstand a number of cycles of about 14742 ± 1142 until rupture. The EDA_E2050_30 samples were able to withstand about 12875 ± 1273 cycles and the PTFE_100 samples about 18423 ± 1054 cycles.

7.3.1.6. XPS analysis

Figure 7.7 presents the XPS overview and detailed spectra of the untreated PET braid samples and EDA_E2050_30_Braid samples. For untreated PET, in the C1s spectrum three different peaks are assigned, namely at BE of 285.0 eV, 286.5 eV and 289.1 eV. In the O1s spectrum it is possible to observe two peaks assigned at BE of 533.0 eV and 532.2 eV. In case of EDA functionalized braids, in the N1s spectrum is possible to identify two peaks at BE of 399.3 eV and 400.5 eV. Besides that, in the O1s spectrum a new peak is assigned at BE of 530.8 eV. It is important to refer that this XPS analysis was reproducible in several analysed points of samples, besides no contaminations have been detected.

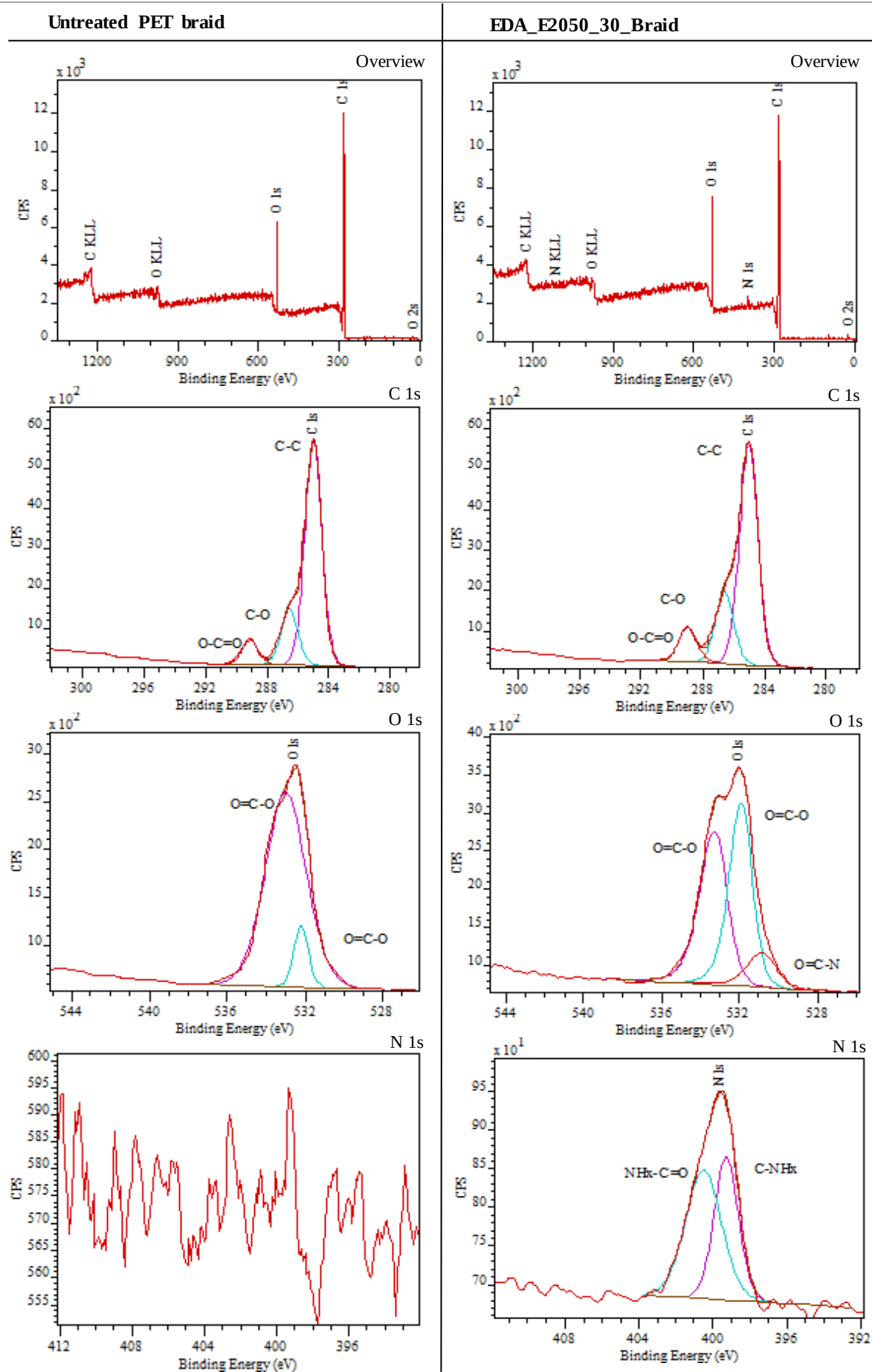


Figure 7.7: XPS overview and detailed spectra of untreated and EDA treated PET braid.

7.3.1.7. Fuchsin acid staining

In order to evaluate the homogeneity of EDA grafting on 16YH braids, they were stained with fuchsin acid and the results are presented in figure 7.8. After staining, the untreated 16YH braid stayed uncolored (figure 7.8.A) in opposition to the EDA_E2050_30_16YH braid (figure 7.8.B) which became purple. It must be noticed that the color of the grafted braid is homogeneous throughout the sample.

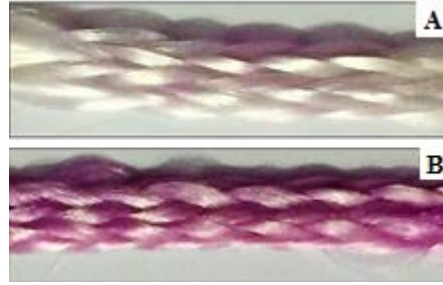


Figure 7.8: PET_16YH braids after staining with fuchsin acid: (A) untreated 16YH braid and (B) EDA_E2050_30_16YH braid.

7.3.1.8. Wettability of PTFE coated yarns

In figure 7.9 are presented the water C.A. values measured on surface of PTFE coated yarns with different concentrations in spray solution. As observed, the water C.A. significantly increases from 30g/L until 100 g/L. When increasing the concentration for more than the 100 g/L, the C.A. remains almost the same.

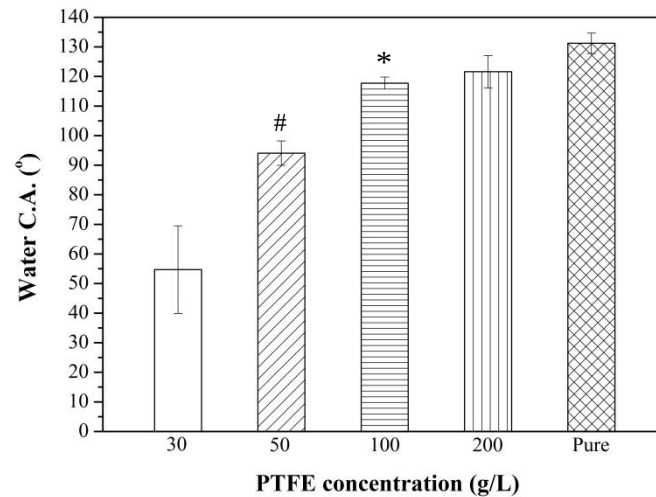


Figure 7.9: Water C.A. (°) on PTFE coated yarns depending on concentration of the spraying solution. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to 30 g/L (#) and 50 g/L (*).

7.3.2. *In vitro* biological characterization

7.3.2.1. Resazurin assay

The metabolic activity of fibroblasts seeded on the EDA grafted braids and PTFE coated yarns was evaluated by the resazurin assay over 21 and 7 days of culture, respectively, and the fluorescence values are presented in figures 7.10. In figure 7.10.A, the fluorescence values of the untreated braids remain almost constant over time. In case of EDA grafted braids the fluorescence level significantly increases over time, being the values much higher than for the untreated braids from day 7 until day 21. For the PTFE coated yarns (figure 7.10.B), the fluorescence value significantly decreases from day 1 to day 4, in which it is about 0, and remains the same at day 7. For all time points, the fluorescence is lower for the coated yarns than for the untreated PET yarns.

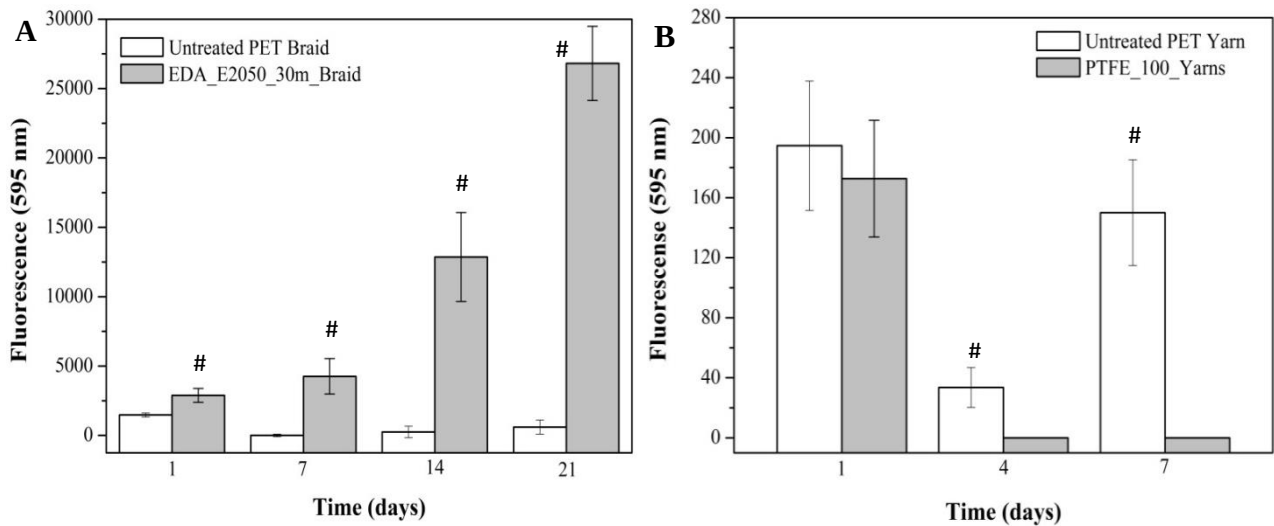


Figure 7.10: Levels of resazurin fluorescence at different incubation time points after the cell seeding on (A) non-grafted PET braids and EDA grafted PET braids; (B) on uncoated and PTFE coated yarns. Data are presented as mean $\pm$ SD. $p < 0.05$ -significant difference compared to untreated PET braid (#) at each time point.

7.3.2.2. SEM analysis

In figure 7.11 is possible to observe that the cells adhered on filaments composing the yarns of EDA_E2050_30_Braid samples after different time points until 21 days of incubation (at two different magnifications). After 7 days of incubation (figures 7.11.A and 7.11.B) it is already possible to observe some fibroblasts adhered on filaments surface. After 14 days the number of cells increases and all of them present a spread shape with developed filopodia (figures 7.11.D and 7.11.E). After the 21 days of incubation (figures 7.11.F and

7.11.G), the cells number significantly increased and the yarns surface is completely covered by an abundant cell layer, where all cells are completely spread and interconnected as a whole. Regarding untreated PET braids surface, it was very difficult to observe cells adhered at any time point, being presented in figure 7.11.C a representative image after 14 days of incubation as an example. Moreover, the SEM analysis was also performed on untreated PET yarns and PTFE_100_Yarn samples surfaces, however almost no adhered cells were observed (data not showed).

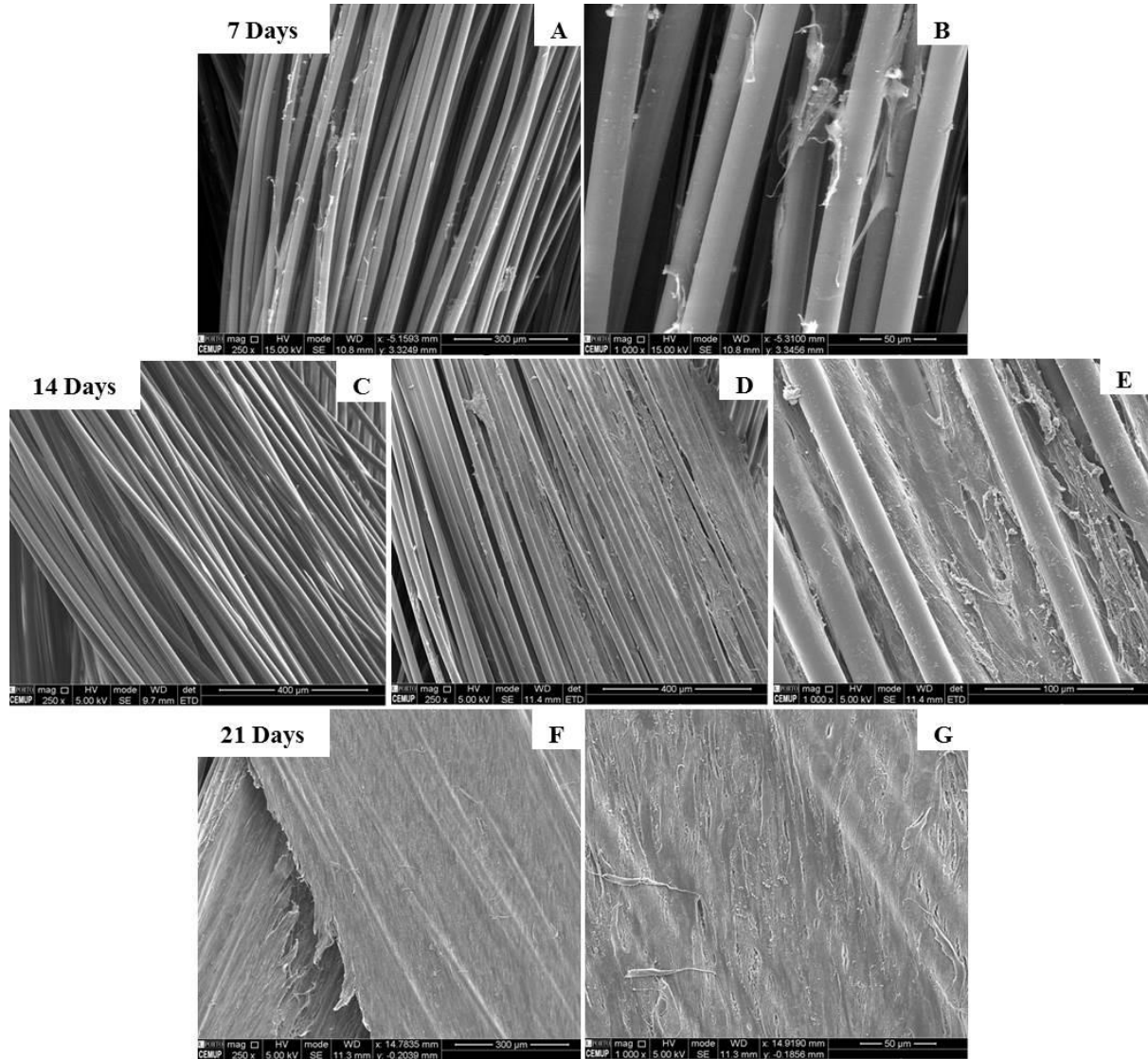


Figure 7.11: SEM images of fibroblasts seeded on EDA_E2050_30_Braid samples after 7 (A and B), 14 (D and E) and 21 (F and G) days of culture, and on untreated PET braid samples after 14 days of culture (C).

4. Discussion of results

The main goal of this work was to develop two different treatments for PET multifilament yarns, one treatment to enhance the cell adhesion and proliferation (bioactive treatment) and other one to avoid the cell adhesion (biopassive treatment) on yarns surface. These two treatments will be applied on different parts of a PET fibrous structure, which was produced on a vertical braiding textile machine with 32 carriers, intended to be used as an implant that totally or partially may substitute a damaged AT. The architecture of the structure is based on a core/shell system. The core is composed by several braids based on 16 PET multifilament yarns and the shell is also composed by braided PET multifilament yarns. The bioactive treatment will be applied to the inner part of the structure, namely in braids composing the core, in order to allow endogenous tenocytes adhesion and proliferation to promote the regeneration of the injured tendon. These endogenous tenocytes must migrate to the structure core coming from tendon tissue ends that remain in physiological space even after the rupture.

However, there is an important limitation of tendons healing process, which is the formation of scarring and fibrous peritendinous adhesions. After a tendon partial or total rupture and consequent surgical procedure, the membrane that surrounds tendon disrupts. This membrane, named as paratenon, is a loose connective tissue layer and its rupture allows granulation tissue and fibroblasts from surrounding tissues to invade the damaged site. Therefore, exogenous cells will predominate over endogenous tenocytes allowing the surrounding tissues to attach to the damaged tissue resulting in adhesions formation. Those adhesions inhibit the movement of that tissue in its physiological space, what prevents the stress transmission into it, impairing the collagen fibres alignment and consequently a normal tissue function. Moreover, it has been reported that the mechanical loading, inexistent in case of physical immobilization, is essential for tenocytes proliferation and differentiation during the healing process.[2, 13,17, 18] Therefore, in the outer part of the implant, a biopassive PTFE based coating, with a low friction level, was applied to the shell yarns to avoid the cell adhesion and consequent formation of adhesions and allow a good relative movement of the structure with the surrounding tissues.

For the bioactive purpose, the grafting of functional amino groups ($-NH_2$) was performed on PET fibrous substrates based on a chemical etching in form of aminolysis by a condensation reaction using a diamine, namely the EDA. The ($-NH_2$) groups from EDA can be chemisorbed on PET substrates via ($-C(O)NH$) bonds, which result from the reaction between an ester group from PET and one amine side only from EDA, leaving the other

available to react with the ECM macromolecules.[12] In order to enhance the chemical bonding between the PET yarns and EDA molecules, before the chemical grafting the substrates were exposed to a plasma treatment based on O_2 . This plasma treatment can create new functional groups on the PET surface, such as carboxyl ($-COOH$) groups and hydroxyl ($-OH$) groups, which increase the PET surface hydrophilicity to improve the contact with the EDA solution [8, 21]. Moreover, the new ($-COOH$) groups are new points of reaction, in which the aminolysis process (amide formation) may happen more easily than with ester group of PET.

Once that the mechanical performance of the previously optimized PET structure should not be affected by the treatment applied on core braids or on shell yarns, first of all the mechanical properties of EDA grafted yarns and PTFE coated yarns were evaluated. As referred in section 7.3.1.1., the PTFE coating did not cause any significant change of the mechanical properties of multifilament PET yarns (data not showed). However, as observed in figure 7.1, the EDA grafting conditions may significantly decrease the load at failure and strain to failure of the yarns. The first prepared and tensile tested sample was the EDA_W5050_24h_Yarn, which presented a significant decrease of the failure load and strain from 72.8 ± 2.3 N to 29.6 ± 4.4 N and from 13.2 ± 0.1 % to 7.5 ± 1.5 %, respectively. It should be referred that the grafting conditions of this sample were defined based on a previous study performed by the authors (data not shown), in which this condition allowed the grafting of more ($-NH_2$) groups on PET sheets surface. Attending to the results, it was tried to decrease the immersion time from 24 h to 12 h (EDA_W5050_12h_Yarn) and 20 min (EDA_W5050_20m_Yarn), keeping the other variables. The decrease of immersion time led to less decrease of the failure load and strain level, but it still needs to be minimized.

So for the immersion time of 20 min it was even tried to use ethanol as solvent, keeping the EDA solution concentration but using the temperature of 20 °C (EDA_E2025_20m_Yarn). Once again the choice of ethanol at 20 °C was based on results obtained in a previous study performed by the authors (data not shown). In that study it was observed that was not possible to use ethanol at 50 °C, because it caused the PET substrate degradation. It was only possible to use ethanol at 20 °C, however for the same solution concentration the grafting level was lower than using water at 50 °C. As observed in figure 7.1, in case of EDA_E2025_20m_Yarn, in spite of also happening a slight decrease of the load at failure compared to the untreated PET, that decrease is even lower than in case of EDA_W5050_20m_Yarn. Moreover, once it has been reported that the number of ($-NH_2$) groups grafted on the polymer surface is dependent with the immersion time, it was even

tried to increase the time using ethanol at 20 °C, namely to 30 and 60 min [22]. In case of 60 min (EDA_E2050_60m_Yarn), there is a significant decrease of the failure load and strain. In case of using 30 min (EDA_E2050_30m_Yarn), the mechanical performance of the grafted yarns is very similar to when using 20 min. The decrease of the load at failure is just about from 72.8 ± 2.3 N to 68.3 ± 2.2 N and the strain to failure keeps constant comparing to the untreated PET yarn. Moreover, it should be noticed, as observed in figure 7.2, that the force-strain curve shape of EDA_E2050_30m_Yarn sample is very similar to the curve shape of the untreated yarn, being the stiffness (curve slope) also very similar.

The reaction of an amine from EDA with an ester group of PET leads to chain scission at the reaction site associated to the amide formation. The amino based molecules in solution may diffuse into the PET filaments leading to the aminolysis phenomenon throughout the bulk filament, leading to its degradation in some extent. Therefore, due to the chains scission not only at the surface level, the brittleness, flexural fatigue, and ultimate tensile properties of filaments may be drastically affected [10]. So, the decrease of the failure load and strain of the grafted yarns is related with the degradation of the filaments in different extensions depending on the conditions, due to the aminolysis reaction. However, the fact of the grafted yarns keep the stiffness level very similar to the untreated PET yarns may indicate that the degradation of filaments happens homogeneously from the outside to inside along all the filament length. So, the diffusion of the reacting amines into the PET filaments should be minimized to restrict the reaction only to the most superficial layer of the filaments. That may be accomplished by controlling the reaction time, but also by using amino based molecules with a higher molecular weight once the diffusion into the PET filament will be more difficult, restricting the reaction just to the surface level [10].

After the study and optimization of the EDA grafting conditions on yarns, the grafting was performed on PET braids using the same conditions that were used in EDA_E2050_30m_Yarn, once the 30 min in ethanol was the highest possible immersion time, what is believed to be proportional to the number of amines grafted on the surface, that caused the lowest load at failure decrease, keeping the stiffness almost constant. As presented in figure 7.1, those grafting conditions on braids (EDA_E2050_30m_Braid) caused a statistically small decrease of the load at failure and increase of the strain comparing to an untreated braid. However, as observed in figure 7.2, in the linear region the curve slope (stiffness) of the EDA_E2050_30m_Braid is very similar to the untreated braid.

In order to evaluate how the immersion time can influence the grafting level on PET yarns, the FTIR-ATR analysis was performed for the samples EDA_E2050_30m_Yarn and

EDA_E2050_60m_Yarn, as presented in figure 7.3. Regarding the spectrum of the untreated PET yarns, several fingerprinting peaks may be identified. Many of the peaks constitute gauche-trans pairs arising from vibrations of the glycol moiety: the peak at 1408 cm^{-1} may be attributed to methylene (CH_2) bending and the peak at 1339 cm^{-1} corresponds to CH_2 wagging [23, 24]. Other peaks are related with the vibrations of the benzene ring, namely the peak at 1018 cm^{-1} attributed to in-plane vibrations and the peak at 873 cm^{-1} attributed to the out-of-plane vibrations. The peaks at 1241 cm^{-1} and 1100 cm^{-1} may be mainly related with vibrations of the ester group [23, 24]. The very strong peak at about 1717 cm^{-1} may be related with the ester carbonyl ($\text{C}=\text{O}$) stretching [24].

Regarding the grafted PET yarns, they present a new very weak wide band around $3500\text{-}3000\text{ cm}^{-1}$ that may correspond to the stretching vibration of primary amines or amides present on EDA molecules [25]. Moreover, a new peak at 1564 cm^{-1} is observed, which is related with the vibration of NH in secondary amides [22, 25]. The presence of amides II is related with ($-\text{CONH}$) bonds, indicating that EDA has been successfully linked to the PET surface. The new peak also observed at about 1490 cm^{-1} , according to literature may be related with stretching of benzene ring, which mobility may have been changed due to the PET chain scission caused by the aminolysis reaction [25]. When comparing the FTIR-ATR spectra after 30 and 60 min of immersion, although both treatments present the same new peaks already described, after 60 min of immersion the peak at 1564 cm^{-1} is slightly stronger than in case of 30 min, and the wide band around $3500\text{-}3000\text{ cm}^{-1}$ is also slightly stronger, what may indicate a higher quantity of amines and amides on the surface. When performing the grafting on braid the same new peaks are observed in respective spectrum, indicating the successful linkage of EDA on the braid yarns surface. Moreover, after the EDA grafting either on yarn or braid, all the pre-existing peaks of PET become attenuated, confirming the presence of a possible amino layer grafted on substrates surface.

After the EDA grafting of braids (EDA_E2050_30m_Braid) and PTFE coating of yarns, the morphology and roughness of the yarns filaments surface was evaluated by SEM (figure 7.4) and optical profilometry analysis (figure 7.6). Firstly, the morphology of untreated filaments (figure 7.4.A and 7.4.B) and just after exposure to the plasma was evaluated, as observed in figure 7.4.C. The change in surface morphology, after the plasma treatment, is mostly promoted by the plasma physical etching on the surface [26]. In case of EDA_E2050_30_Braid samples (figures 7.4.D, 7.4.E and 7.4.F), the morphology of the composing filaments becomes completely different. The whole surface has in its composition

the N element, as proved by the EDS analysis, however along the surface there are some material agglomerates richer in N than the smoother parts of the surface.

In case of PTFE₁₀₀ samples, there is the deposition of a kind of continuous and homogeneous polymeric film above the filaments surface, which seems smoother comparing to the untreated PET filaments, as observed by SEM analysis at a magnification of 10000x. The EDS analysis identified the F element on coated surface in several points, besides the O and C elements, proving the presence of PTFE on filaments surface. Moreover, the coating seems to cause a slight reduction of the average roughness level of the filaments surface comparing to the untreated filaments, as proved by the R_a values presented in table 7.3 and observed in figure 7.6. However, as already referred, the FTIR-ATR spectra of PTFE coated yarns did not reveal any new peak. Thus, besides inferring that the PTFE coating thickness is very reduced for detection by the FTIR-analysis, it is believed that the adhesion of the coating to the PET filaments surface is just based on physical interactions. The bonding structure of PTFE is very stable, it is not very reactive to chemical agents because of the stability of the fluorine to carbon bonds [18, 19].

The abrasion is related with the rubbing of a textile surface over another surface, and a low abrasion resistance is usually related with a high roughness and coefficient of friction associated to that textile surface [27, 28]. Therefore, once the PTFE coating should decrease the phenomenon of friction on PET yarns surface to allow the implant slipping, the yarn-on-yarn abrasion, was evaluated [27, 29]. As referred in section 7.3.1.5., after the PTFE (100 g/L) coating the number of withstand rubbing cycles increased from about 14742 ± 1142 to 18423 ± 1054 cycles. This phenomenon was already expected once that the PTFE coating is used in several applications to decrease the coefficient of friction between materials surfaces when they are exposed to sliding [14, 16]. This is related with the polymer units structure and the strength and stability of the carbon to fluorine bonds in the polymer. At the start of sliding, the surface of the PTFE fractures, and lumps are transferred to whatever it is sliding against. That means that the PTFE surface tends to wear away. As sliding continues, the lumps are spread out to a thin film. At the same time the surface of the PTFE is dragged out into an organised layer. Then, the two surfaces in contact both have well organised PTFE molecules which can slide over each other [18, 19]. Moreover, as already referred, once that a rough surface usually presents a higher coefficient of friction than a smooth surface, the slight decrease of roughness after the PTFE coating may slightly reduce the coefficient of friction and improve the yarns abrasion resistance. Besides that, the cohesion among PET filaments, promoted by the PTFE coating, may also lead to a better abrasion resistance

according to some reported results [27]. This improvement in abrasion may indicate a good adhesion of PTFE to the filaments surface. The abrasion resistance of EDA_E2050_30_Yarn samples was also evaluated, being the number of cycles lower than in case of untreated PET yarns, probably due to the higher roughness observed in case of the grafted yarn samples. Moreover, this also happens because the EDA grafting did not grant the cohesion among filaments.

Regarding XPS analysis, presented in figure 7.7, it was performed in order to identify the presence of amine and amide groups after the EDA functionalization. The C1s and O1s detailed spectra of untreated PET braids show the contributions of chemical bonds that are typical for PET [30]. In the C1s spectrum peaks of three different chemical environments, namely C-C, C-O and O-C=O (BE: 285.0 eV; 286.5 eV; 289.1 eV) are assigned, and in the O1s spectrum the peaks identified correspond to O=C-O and O=C-O bonds (BE: 533.0 eV; 532.2 eV) [30]. The XPS data of EDA functionalized braids indicated the presence of N, and in the N1s spectrum two peaks are identified and assigned as amine (BE: 399.3 eV) and amide (BE: 400.5 eV) linkages [31]. Besides that, in the O1s spectra a new peak is assigned to O=C-N linkage (BE: 530.8 eV) [31]. Moreover, it is possible to conclude that the N presence on braids surface is related with about 43% of amines and about 57% of amides.

The presence of amine groups in PET can be also detected by selective staining to yield qualitative information about their location and concentration in the filaments. When staining with fuchsin acid at pH 2.7, all amine groups are protonated and are able, by an anion exchange process, to electrostatically interact with the acid dye anion [10]. Figure 7.8 presents an untreated braid sample and EDA_E2050_30m_Braid sample stained with fuccin acid. As observed, only the EDA grafted sample became purple due to the reaction of the present amino groups with the dye. Besides that, the color homogeneity across the whole surface indicates that the functional amino groups are homogeneously distributed across the whole surface. It shall be referred that several EDA grafted braids were stained and the color homogeneity across the surface was observed for all samples.

Being the PTFE polymer an hydrophobic material, consisting of carbon and fluorine, [15], it was already expected the increase of the water C.A. on yarns surface after performing the PTFE coating, as reported in literature [16, 32]. As observed in figure 7.9, the decrease is proportional to the PTFE solution concentration just until 100 g/L, after that the water C.A. remains almost constant. This probably happens because above a certain concentration, using the same spray rate, all filaments surface is already homogeneously covered with a PTFE film. Regarding the EDA grafted yarns or braids, the water C.A. was not possible to measure

by the same method due to the very fast absorbance of the water drop by the yarns. However, it is believed that after the EDA grafting the filaments surface become more hydrophilic, due to the amino groups hydrophilic character [33].

Attending to the main goals proposed for the two surface treatments studied in this work, the viability and morphology of cells seeded on EDA grafted braids and PTFE coated filaments were evaluated by the resazurin assay and SEM analysis, respectively. Cell attachment, adhesion and spreading are very important steps for a good cell/material interaction. The way these phenomena occur influences the cells ability to proliferate and to differentiate, which is essential for the formation of an implant/tendon successful interface. Therefore, for the biopassive treatment, the sample PTFE_100 was selected to evaluate the cell adhesion on it mainly due to its level of roughness and hydrophobicity without compromising the mechanical performance of PET yarns. Regarding the bioactive treatment, the EDA_E2050_30_Braid sample was mainly selected due to the successful grafting of (-NH₂) groups on its surface without compromising the mechanical performance of yarns.

The SEM analysis was performed after different incubation times on PTFE_100 samples, however almost no cells were observed on the surface. In case of EDA_E2050_30m_Braid samples, in figure 7.11, it is possible to observe the cells adhered well and after 14 days the fibroblasts number significantly increased presenting a spread shape with developed filopodia. After 21 days, the number of cells continued increasing and the braids surface was completely covered by an abundant cell layer, where all cells are completely spread and interconnected as a whole surrounded by ECM. This spread shape and interconnection among cells reveals a good affinity to the EDA coated surface [34, 35]. In opposition, in filaments of untreated PET braids almost no cells were observed, as proved by figure 7.11.C after 14 days of incubation. In a cell adhesion process the surface functional groups can influence the cell adhesion and growth, likely due to the fact that surface chemical functionality affects adsorbed protein and subsequent protein-cell interactions [6, 7]. The material interacts with different cell surface receptors, establishing several sites of adhesion between the cells and the material surface [36, 37]. The adsorption is generally driven by a combination of van der Waals interactions, hydrophobic interactions, hydration effects, and electrostatic interactions between the surfaces and proteins or domains of these [7]. During the establishment of those sites, there is an organization and/or production of the cytoskeleton filamentous proteins, namely actin filaments, which tend to assemble in long bundles, leading to the spread shape with protrusions (filopodia) of the plasma membrane [34, 35, 38, 39].

Resazurin is an indicator of cell viability since the living cells are able to reduce the resazurin in mitochondria. Therefore, the fluorescence results presented in figure 7.10 are proportional to cell metabolism and therefore to cell proliferation and viability [40]. In case of EDA grafted braids the fluorescence level significantly increased over time, being much higher than for the untreated braids from day 7 until day 21, meaning a higher cell viability and proliferation. The cell shape, which is controlled by the cytoskeleton organization, can also be related with cell growth and function once the cytoskeleton proteins are involved in signal transduction [34, 35]. When cells adhere to a material surface, the mitochondrial activity increases by the production of the necessary energy to synthesize the above-mentioned proteins. So, a spread cell shape and a high number of cells are usually associated to a higher metabolic activity [34, 35, 41]. This is in accordance with the obtained results, once that the EDA_E2050_30_Braid samples, which presented much more cells on their surfaces than the untreated braids also presented a higher metabolic activity. Besides that, in case of PTFE_100 samples, in which surfaces no cells were observed, the resazurin fluorescence level was accordingly almost null.

The extreme hydrophobic profile of the PTFE coated yarns, namely of PTFE_100, is related with the non-adherence and non-viability of fibroblasts to the filaments surface, once it has been reported that cells do not have a good affinity with extremely hydrophobic or hydrophilic surfaces. *Dowling D.P. et al.*, for instance, which performed different surface treatments on polystyrene surface, observed that the highly hydrophilic or hydrophobic (110 °-155 °) surfaces led to a progressive reduction in the level of cell adhesion [42]. Moreover, the low R_a values observed for filaments composing the PTFE_100 samples may be another reason for the non-adherence of fibroblasts. Several authors have reported that the biomaterial surface characteristics, such as morphology and physicochemical features influence the cell growth and function [43]. The surface roughness and wettability can influence the type and the adsorption kinetics of the serum proteins to the material surface. The adsorbed protein layer has an essential role in the cell adhesion, morphology and migration, once the charged cell membrane interact with surfaces through this protein layer [35, 44]. It has been reported in several studies that rough, enough hydrophilic and functionalized surfaces favour the adsorption of that protein layer and consequently the cell adhesion [6, 13, 42, 45].

In case of EDA_E2050_30_Braid samples, the high cell adhesion and activity may be mostly explained by the performed amine functionalization. As already discussed, in the reaction of EDA molecules with PET, only one of the amine groups is consumed in the

reaction, being converted to an amide group, while the other side amine group is available to interact with the surrounding medium. The several (-NH₂) groups present on the material surface, when exposed to an aqueous environment present positive charges which are able to establish electrostatic interactions with negative charges of cell-surface proteins, promoting the adhesion of cells to the material [6, 13]. Besides that, the cells anchorage may also have been improved by the different roughness (slightly increased R_a value comparing to untreated PET) and topography of the EDA grafted surfaces. In addition, the incorporation of hydrophilic amine groups at the PET surface changed its surface energy as confirmed by the C.A. measurements, what influences the cell adhesion to these surfaces.

7.5. Conclusions

In both developed treatments, one to be applied on core braids or one to be applied on shell yarns, the initially purposed goals, related with the cell adhesion level and allowance of relative movement, were successfully achieved.

Regarding the conditions for the amino groups grafting on PET filaments, namely the EDA concentration, immersion time, solvent type and reaction temperature, a good balance was found in order to have an efficient amino groups grafting level on substrate surface, as proved by FTIR and XPS analysis, without compromising the filaments mechanical properties. That amount of amino groups present on EDA_E2050_30_Braid samples allowed to significantly improve the interaction between cells and the braids, increasing the cell adhesion and proliferation, as proved by the performed resazurin assay and SEM analysis. Besides that, the used conditions caused a non-significant impact on mechanical properties of the grafted braids comparing to the non-grafted ones. Regarding the PTFE coating, it was also determined an appropriate spraying solution concentration, corresponding to PTFE_100 samples that simultaneously allowed to increase the abrasion resistance of the PET filaments surface without affecting the tensile properties. Moreover, the physicochemical properties of the PTFE_100 samples surface prevented the cell adhesion on the PET yarns surface.

Acknowledgements

Authors acknowledge the financial support from FCT (Fundação para a Ciência e a Tecnologia), through the Grant SFRH/BD/88829/2012 and the Project UID/EMS/00285/2013.

7.6. References

- [1] Kuo C. K., Marturano J. E., Tuan R. S. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2010;2:20.
- [2] Rodrigues M. T., Reis R. L., Gomes M. E. Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine*. 2013;7:673-86.
- [3] Bassetto F., Volpin A., Vindigni V. Regenerative Medicine for Tendon Regeneration and Repair: The Role of Bioscaffolds and Mechanical Loading. In: R. P, editor. *Biomaterials Science and Engineering*: InTech; 2011: 370-86, Italy.
- [4] Morais DS, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair, *Annals of Biomedical Engineering*. 2015; 43(9):2025-35.
- [5] Williams R. Surface modification of biomaterials: Methods analysis and applications: Elsevier; 2010, United Kingdom.
- [6] Tang L, Thevenot P, Hu W. Surface chemistry influences implant biocompatibility. *Current topics in medicinal chemistry*. 2008;8:270-80.
- [7] J. GH. Thin Film Coatings for Biomaterials and Biomedical Applications, 1st Edition. 1st edition ed: Elsevier; 2016, United Kingdom.
- [8] Chen H, Truckenmuller DRK, Blitterswijk PdCAv, Moroni L. Fabrication of nanofibrous scaffolds for tissue engineering applications. In: Gaharwar AK, Sant S, Hancock MJ, Hacking AA, editors. *Nanomaterials in tissue engineering: Fabrication and applications*: Woodhead Publishing; 2013: 158-82.
- [9] Liu Y., Ramanath H., Wang D. Tendon tissue engineering using scaffold enhancing strategies. *Trends in Biotechnology*. 2008;24:201-9.
- [10] Avny Y, Rebenfeld L. Chemical modification of polyester fiber surfaces by amination reactions with multifunctional amines. *Journal of applied polymer science*. 1986;32:4009-25.
- [11] Bech L, Meylheuc T, Lepoittevin B, Roger P. Chemical surface modification of poly (ethylene terephthalate) fibers by aminolysis and grafting of carbohydrates. *Journal of Polymer Science Part A: Polymer Chemistry*. 2007;45:2172-83.
- [12] Xue L, Lu Y. Electroless copper plating on 1, 2-ethylenediamine grafted poly (ethyleneterephthalate) for the fabrication of flexible copper clad laminate. *Journal of Materials Science: Materials in Electronics*. 2013;24:2211-7.
- [13] Li H-L, Zhang H, Huang H, Liu Z-Q, Li Y-B, Yu H, et al. The effect of amino density on the attachment, migration, and differentiation of rat neural stem cells In Vitro. *Molecules and cells*. 2013;35:436-43.
- [14] Industries O. Teflon® & Non-Stick Coatings, <http://www.orioncoat.com/coatings/ptfe-and-non-stick/2016>, accessed in November 2016.

- [15] Hou X, Deem PT, Choy K-L. Hydrophobicity study of polytetrafluoroethylene nanocomposite films. *Thin Solid Films*. 2012;520:4916-20.
- [16] Bodas DS, Mandale A, Gangal S. Deposition of PTFE thin films by RF plasma sputtering on< 100> silicon substrates. *Applied Surface Science*. 2005;245:202-7.
- [17] Biswas S, Vijayan K. Friction and wear of PTFE—a review. *Wear*. 1992;158:193-211.
- [18] Clark J. Intermolecular bonding and the physical properties of PTFE - Questions and Comments, <http://www.chemguide.co.uk/qandc/ptfe.html>2007, accessed in November 2016.
- [19] Plastics P-STISEfRa. Teflon (PTFE): PTFE Teflon Properties, <http://www.polymer-search.com/teflon.html>2009, accessed in October 2016.
- [20] Cordage Institute International Standard, Test method for yarn-on-yarn abrasion, CI 1503-00. August, 2001.
- [21] Vesel A, Mozetic M. Surface functionalization of organic materials by weakly ionized highly dissociated oxygen plasma. *Journal of Physics: conference series: IOP Publishing*; 2009: 012015.
- [22] Drobota M, Persin Z, Zemljic L, Mohan T, Stana-Kleinschek K, Doliska A, et al. Chemical modification and characterization of poly (ethylene terephthalate) surfaces for collagen immobilization. *Open Chemistry*. 2013;11:1786-98.
- [23] Andanson JM, Kazarian SG. In situ ATR-FTIR Spectroscopy of Poly (ethylene terephthalate) Subjected to High-Temperature Methanol. *Macromolecular symposia: Wiley Online Library*; 2008: 195-204.
- [24] Cole KC, Aji A, Pellerin E. New insights into the development of ordered structure in poly (ethylene terephthalate). 1. Results from external reflection infrared spectroscopy. *Macromolecules*. 2002;35:770-84.
- [25] Sheppard N. The historical development of experimental techniques in vibrational spectroscopy: *Wiley Online Library*; 2002.
- [26] Zia AW, WANG YQ, Lee S. Effect of Physical and Chemical Plasma Etching on Surface Wettability of Carbon Fiber–Reinforced Polymer Composites for Bone Plate Applications. *Advances in Polymer Technology*. 2015;34.
- [27] Özdil N, Kayseri GÖ, Mengüç GS. Analysis of abrasion characteristics in textiles. Edited by Marcin Adamiak. 2012:119, Turkey.
- [28] Kalin M, Kogovšek J. Surface roughness and its effects in tribology. 1st BioTiNet Workshop, in Ljubljana, Slovenia, 2011.
- [29] McKenna HA, Hearle JW, O'Hear N. *Handbook of fibre rope technology*: Elsevier; 2004, USA.
- [30] Girardeaux C, Zammattéo N, Art M, Gillon B, Pireaux J, Caudano R. Amination of poly (ethylene-terephthalate) polymer surface for biochemical applications. *Plasmas and Polymers*. 1996;1:327-46.

- [31] Stevens JS, Luca AC, Pelendritis M, Terenghi G, Downes S, Schroeder SL. Quantitative analysis of complex amino acids and RGD peptides by X-ray photoelectron spectroscopy (XPS). *Surface and Interface Analysis*. 2013;45:1238-46.
- [32] Liu Y, Chen X, Xin J. Can superhydrophobic surfaces repel hot water? *Journal of Materials Chemistry*. 2009;19:5602-11.
- [33] Hamerli P, Weigel T, Groth T, Paul D. Surface properties of and cell adhesion onto allylamine-plasma-coated polyethyleneterephthalat membranes. *Biomaterials*. 2003;24:3989-99.
- [34] Morais DS, Rodrigues M, Lopes M, Coelho M, Maurício AC, Gomes R, et al. Biological evaluation of alginate-based hydrogels, with antimicrobial features by Ce (III) incorporation, as vehicles for a bone substitute. *Journal of Materials Science: Materials in Medicine*. 2013;24:2145-55.
- [35] Morais DS, Fernandes S, Gomes P, Fernandes M, Sampaio P, Ferraz M, et al. Novel cerium doped glass-reinforced hydroxyapatite with antibacterial and osteoconductive properties for bone tissue regeneration. *Biomedical Materials*. 2015;10:055008.
- [36] Zaleski KJ, Kolodka T, Cywes-Bentley C, McLoughlin RM, Delaney ML, Charlton BT, et al. Hyaluronic acid binding peptides prevent experimental staphylococcal wound infection. *Antimicrobial agents and chemotherapy*. 2006;50:3856-60.
- [37] Lei Y, Gojini S, Lam J, Segura T. The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels. *Biomaterials*. 2011;32:39-47.
- [38] Hempel U, Hefti T, Dieter P, Schlottig F. Response of human bone marrow stromal cells, MG-63, and SaOS-2 to titanium-based dental implant surfaces with different topography and surface energy. *Clinical oral implants research*. 2013;24:174-82.
- [39] Wutticharoenmongkol P, Pavasant P, Supaphol P. Osteoblastic phenotype expression of MC3T3-E1 cultured on electrospun polycaprolactone fiber mats filled with hydroxyapatite nanoparticles. *Biomacromolecules*. 2007;8:2602-10.
- [40] O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*. 2000;267:5421-6.
- [41] Morais DS, Coelho J, Ferraz MP, Gomes PS, Fernandes MH, Hussain NS, et al. Samarium doped glass-reinforced hydroxyapatite with enhanced osteoblastic performance and antibacterial properties for bone tissue regeneration. *Journal of Materials Chemistry B*. 2014;2:5872-81.
- [42] Dowling DP, Miller IS, Ardhaoui M, Gallagher WM. Effect of surface wettability and topography on the adhesion of osteosarcoma cells on plasma-modified polystyrene. *Journal of biomaterials applications*. 2010.
- [43] Vladkova TG. Surface engineered polymeric biomaterials with improved biocontact properties. *International Journal of polymer science*. 2010.
- [44] Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue engineering*. 2005;11:1-18.

[45] Alves P, Pinto S, de Sousa HC, Gil MH. Surface modification of a thermoplastic polyurethane by low-pressure plasma treatment to improve hydrophilicity. *Journal of applied polymer science*. 2011;122:2302-8.

Chapter 8

General discussion, conclusions and future work

8.1. General discussion and main conclusions

The present section has the purpose of bringing together all major results that were obtained in the individual chapters, once that all chapters are related and were essential to accomplish the initially proposed goals for this work. Due to tendons inefficient healing ability in case of acute or chronic injury, tendons injuries are prevalent and debilitating impairments that affect life quality among adult population worldwide. In the last years, attending to the social and economic concern associated to the high prevalence of tendons injuries and the limited success of the available current treatments, such as surgical repair or grafts application, several scaffolds have been developed. Depending on injury extension, those scaffolds may be needed to be used just as graft-augmentation devices, but in some clinical cases it may be needed to partially or even fully replace the damaged tendon. In the market, there are already several commercial available products to be used as graft-augmentation devices. However, to replace the mechanical function of a tendon, just few development attempts have been reported [1]. Therefore, the main challenge of this work was to develop a structure with a certain architecture able to mimic the native mechanical performance of an Achilles tendon (AT). Besides that, the structure surface must favourably interact with local native cells in order to promote an appropriate biological integration of the implant.

In order to mimic the complex fibrous architecture of a tendon and consequent mechanical performance, a fibrous textile structure was intended to be developed. In any fibrous structure, the type of yarns composing it and the arrangement among them will define the mechanical performance of the structure. Thus, the first step was to choose the most appropriate textile yarns to incorporate in the structure, if natural or synthetic and if mono or multifilament. Despite natural polymers present higher bio-functionality and biodegradation, normally the natural scaffolds present some limitations when comparing with the synthetic ones, such as the limited processability, the variety from batch to batch, the lower mechanical properties and the higher induction of antigenic and immunogenic reactions [2, 3]. On the other hand, multifilament synthetic yarns present higher flexibility, permeability, swelling and wicking ability, and surface area than monofilament yarns [4, 5]. Moreover, the multifilament yarns may present a lower failure probability due to the phenomenon of size effect on the structural strength [6, 7]. Therefore, among different synthetic multifilament yarns, after tensile testing them and attending to the tenacity and stiffness levels, two multifilament yarns based on poly(ethylene terephthalate) (PET) and polypropylene (PP), which have been used in several biomedical applications as biocompatible materials [8-10],

were selected to be incorporated in the textile braided structures, which were then used as elementary parts of a more complex textile structure in order to reach the real level of load at failure, strain to failure and stiffness of a native AT.

In *Chapter 3*, the physicochemical and mechanical properties of the two selected yarns were evaluated and they were used to produce four different braided structures depending on the number of yarns in the structure and the braiding take-up rate, as presented in table 3.1 and observed in figure 3.4. The number of yarns in the structure and the braiding take-up rate are the main parameters that can be adjusted to construct structures with different architectures, namely with a different number of braids/cm, diameter, linear density, tenacity, braid angle and porosity level. After the performed characterization of all produced braids, namely the porosity level, the wicking ability and mechanical performance, it was possible to conclude that the braids architecture actually defines their physical and mechanical behaviour, besides of course the intrinsic physical properties of the yarns that compose them. The wicking ability of braids was dependent on structure pores amount but also on how those pores communicate, which depends on the architecture, namely how the yarns are arranged and packed in the structure. Regarding, the level of load at failure of braids, PP_16YH and PET_16YH revealed the higher values, and it was concluded that it is mainly controlled by the number of yarns, but the strain level is mostly influenced by the take-up rate and consequently by the braid angle. Regarding the stiffness level, it was concluded that it results from a combination of the yarns number and braid angle. Furthermore, it should be referred that the PET_16YH structure revealed a very promising creep and force-relaxation behaviour for the final application, as well as a very interesting fatigue resistance.

Tendons present non-linear viscoelastic behaviour with a typical force-strain curve, in elongation-to-failure conditions, presenting three different regions. The first region, named as toe region, presents a gradual increase in curve slope, which represents the stiffness I. In this region the tendon elastic limit is not exceeded and when the stress is released the tendon length is restored. In case of further elongation a second region with constant stiffness (stiffness II) appears, named as linear region, in which tissue elongation happens due to the stretching imposed by the load. If elongation continues, a third region appears in which collagen fibres fail in an unpredictable way causing tears tissue, leading to the total rupture [1, 11, 12].

Two braids (PP_16YH and PET_16YH) were selected to be associated and arranged as sub-components to form a more complex structure based on a core/shell system, commonly known as rope, to mimic the complex fibrous micro-architecture of tendons. The three

different developed ropes, presented in *Chapter 4*, namely in figure 4.2, revealed a force-strain curve with three different regions, as observed in figures 4.4 and 4.5, as also reported in case of a native AT curve. Similar to what discussed about the mechanical properties of braids, it was concluded that the level of load at failure of the ropes is mainly controlled by the number of yarns/braids in core, but the strain to failure level is mostly influenced by the take-up rate and consequent braid angle of braids that compose the core. Therefore, the ropes stiffness level results from a combination of the yarns/braids number in core and the associated braid angle. The PET_C22B16YH_S16YL rope presented a level of failure load and strain, and stiffness II appropriate for the AT substitution, according to data reported on several studies on literature. Moreover, the PET_C22B16YH_S16YL rope revealed a viscoelastic behaviour with very promising fatigue and creep resistance according to the demanding requirements for the final application. The homogeneous and high level of porosity of the PET rope, as observed by nano-CT analysis (figures 4.10 and 4.11), is also a very promising feature of this structure to allow a better cell migration and tissue and blood vessels ingrowth into the fibrous structure, what consequently promotes a successful implant integration *in vivo*.

Synthetic polymers present some advantages comparing to the natural ones, namely better reproducible mechanical properties, the predictable degradation rate and the lower immunogenicity risk. Nevertheless, they also present some limitations such as the lack of functional chemical groups involved in cell adhesion process [3, 13]. Therefore, in parallel with the structures development, a surface treatment for each yarn polymer was studied using PP and PET sheets as substrates. The main goal of each treatment was to improve the cell adhesion and proliferation on each polymer surface by grafting on it some functional groups, to consequently enhance the structure integration with local native tissues. The cell adsorption to a surface is generally driven by a combination of van der Waals interactions, hydrophobic interactions, hydration effects and electrostatic interactions between materials surface and cell membrane proteins or domains of these. In relation to protein/surface interactions, adsorption depends on a range of factors, including the protein, surface net charge and charge distribution, the occurrence of hydrophobic/hydrophilic domains in the protein or surface, the surface roughness and the size of involved proteins [14-16].

The most common functionalities investigated with relation to biomaterial interactions are the carboxyl (-COOH), hydroxyl (-OH) and amino (-NH₂) groups. However, some reported studies revealed preferable adhesion, growth, and extracellular matrix (ECM)

formation of fibroblasts on (-NH₂) rich surfaces compared with other functional coatings. The (-NH₂) groups functionality displays a positive charge to the biomaterial surface, what favour a preferable adhesion, growth, and matrix formation of fibroblasts compared with other coatings [15]. The several (-NH₂) groups present on the material surface, when exposed to the human body aqueous environment present positive charges (-NH₃⁺) which are able to establish electrostatic interactions with negative charges of cell-surface proteins, promoting the adhesion of cells to the material [15, 17].

In case of PP substrate, it was studied the immobilization of chitosan (Cs) on its surface, which grant it (-NH₂) groups, as presented in *Chapter 5*. Some properties of this natural polysaccharide, such as biocompatibility, low toxicity, biodegradability and promotion of cell adhesion, proliferation and differentiation, made it become a very appealing material for biomedical applications [17, 18]. Before the Cs immobilization, it was studied the activation of the PP surface by exposure to different plasma treatments, what could improve the acrylic acid (AAc) grafting and consequent Cs immobilization. For the immobilization, different concentrations of the AAc and Cs solutions were studied, namely by the FTIR-ATR analysis (figure 5.1) and the *Orange II* dye method (figure 5.3). The *Orange II* results and the appearance of new amine and amide peaks on FTIR-ATR spectra, when using O₂ plasma activation, proved the successful immobilization in case of sample using the AAc solution concentration of 20% (V/V) and Cs solution concentration of 2% (w/V), named as PP_20% AAc_2% Cs. Regarding the cell response, it was evaluated by the resazurin assay and SEM analysis, after seeding fibroblasts on samples PP_20% AAc_2% Cs. The Cs treated PP revealed better results than the untreated PP, promoting higher cell metabolic activity than the untreated samples (figure 5.6). Moreover, SEM results (figure 5.7) corroborate this conclusion, since cells were spread through the surface indicating a good cell adhesion and growth. This improvement regarding the cell interaction with the substrate may be explained by the presence of (-NH₂) groups on the surface, besides the different wettability, roughness and morphology of the Cs treated PP surface.

In case of PET substrate, an ethylenediamine (EDA) based treatment was developed and optimized in order to graft (-NH₂) groups on the polymer surface, as described in *Chapter 6*. Before the EDA functionalization, it was also studied the activation of PET surface by exposure to different plasma treatments. Then, samples were immersed in EDA solution using different conditions, namely regarding the EDA concentration, immersion time, solvent type and reaction temperature. According to the FTIR-ATR analysis (figure 6.2) and *Orange II* results (figure 6.4), the plasma treatment and immersion conditions that

allowed the best amino functionalization were the ones used in samples PET_W5050 and PET_E2050. Both were previously exposed to O_2 plasma activation, but in case of W5050 the immersion was performed in a 50% (V/V) EDA solution using water as solvent, with a temperature of 50 °C; in case of E2050 the immersion was performed in a 50% (V/V) EDA solution using ethanol as solvent, with a temperature of 20 °C. However, the PET_W5050 sample was the one chosen to evaluate the cell response, once it presented the highest amount of (-NH₂) groups, the lowest water contact angle (C.A.) and roughness. According to the resazurin assay (figure 6.7), at all time points, the cell metabolic activity is significantly higher in case of PET_W5050 samples than in case of untreated PET samples. Moreover, in SEM images (figure 6.8) was possible to observe that cells were spread through the entire surface, indicating a good cell adhesion and growth. It is believed that this improvement in cell behaviour is due to the presence of (-NH₂) groups, but it is also influenced by the roughness and topography of the EDA functionalized surfaces. In addition, the incorporation of the hydrophilic (-NH₂) groups on the PET surface also changed the surface energy, what may also influence the cell adhesion.

After the development and complete characterization of the mechanical properties of ropes presented in *Chapter 4*, once the C22B16YH_S16YL structure was selected as the appropriate for the AT substitution, the EDA treatment already studied using PET sheets was then applied to the PET yarns, as presented in *Chapter 7*. Regarding the conditions for the (-NH₂) groups grafting on PET filaments, namely the EDA concentration, immersion time, solvent type and reaction temperature, a good balance was found in order to have an efficient grafting level on substrate surface without compromising the filaments mechanical properties. After optimizing the grafting on PET yarns, it was promoted on PET_16YH braids already developed in *Chapter 4*, named as EDA_E2050_30_Braid, in which it was used an 50% (V/V) EDA solution using ethanol as solvent and the immersion was promoted at 20 °C over 30 minutes. The (-NH₂) groups grafting on braids was successfully achieved, as proved by FTIR-ATR (figure 7.3) and XPS (figure 7.7) analysis and fuchsin acid staining (figure 7.8), without compromising their mechanical behaviour (figure 7.1 and 7.2). Regarding the biological assessment, the EDA_E2050_30_Braid samples presented a significantly improved interaction with cells than untreated braids, presenting a higher cell metabolic activity and better adhesion and proliferation than the untreated braids, as proved by the performed resazurin assay (figure 7.10.A) and SEM analysis (figure 7.11). This bioactive treatment will be applied to braids composing the C22B16YH_S16YL structure core in order to allow the cell (endogenous tenocytes) adhesion and proliferation to promote

the regeneration of the injured tendon. These endogenous tenocytes must migrate to the structure core coming from tendon tissue ends that remain in physiological space.

After tendon partial or total rupture, the membrane that surrounds tendons disrupts, which is named as paratenon, and its rupture allows granulation tissue and fibroblasts from surrounding tissues (exogenous tenocytes) to invade and to attach to the damaged site, resulting in scarring and fibrous adhesions formation. Those adhesions inhibit the movement of that tissue in its physiological space, what prevents the stress transmission into it, impairing the collagen fibres alignment and consequently a normal tissue function [1].

Therefore, in *Chapter 7* is even presented the development of a polytetrafluoroethylene (PTFE) coating intended to be applied on shell yarns of the C22B16YH_S16YL structure to mimic the paratenon membrane. The PTFE polymer is an excellent hydrophobic material, consisting of carbon and fluorine, and presents a very low coefficient of friction. Hence, it was selected to avoid the cell adhesion and consequent adhesions formation and allow the relative movement of the implant next to the surrounding tissues. In case of PTFE_100 samples a concentration of 100 g/L was used, significantly decreasing the water C.A. (figure 7.9) and increasing the abrasion resistance of PET filaments surface. Moreover, as intended, the physicochemical properties of the PTFE_100 samples surface prevented the cell adhesion on PET yarns surface (figure 7.10.B).

After the presentation and discussion of all these results, it is possible to say that the main objectives proposed for this work were successfully accomplished. Firstly, and faced as the main challenge of this work, a structure with a fibrous hierarchical architecture, namely based on a core/shell system, presenting a mechanical performance appropriate to substitute a native AT was developed. Moreover, regarding the appropriate interaction of the structure with cells, two distinct and selective surface treatments were developed, a bioactive one to be applied on structure core and a biopassive one to be applied on its shell. The bioactive functionalization, based on (-NH₂) groups, will allow the adhesion and proliferation of endogenous tenocytes in implant core for new tissue ingrowth, and the biopassive treatment will prevent the adhesion of exogenous tenocytes on the implant shell, allowing the relative movement of the implant. A patent protecting a prototype based on the architecture of the C22B16YH_S16YL structure associated to the two selective surface treatments, is being written to submit to the *National Institute of Intellectual Property (INPI)*.

Depending on the tendon injury extension, this prototype may be used just to partially replace the tendon, being inserted between two tendon ends or a tendon end and muscle end, or in more extreme and rare cases it may be needed to fully replace the tendon linking a

muscle to a bone. In next section are suggested some approaches that may be studied in future regarding the structures clinical application.

Finally, it must be referred that all knowledge generated in this work regarding the architecture and consequent physical and mechanical properties of the developed textile structures may also be used and adapted to develop other similar structures suitable for any human body tendon or even ligament, namely for the Anterior Cruciate Ligament (ACL). In addition, the different optimized surface treatments, for PP or PET substrates, may also be used with the bioactive or biopassive purpose in several other applications to control the interaction of the materials surface with fibroblasts or even with other possible cell types.

8.2. Future work

As future work, regarding the developed textile structures, it would be interesting to study the development of hybrid biodegradable structures. Once that most of biodegradable (monofilament and multifilament) yarns present a tenacity lower than non-biodegradable yarns, in order to accomplish a structure partially biodegradable, the combination of both types of filaments in the same structure may be studied. In this work, some biodegradable monofilaments, such as polydioxanone (PDO), poly(glycolic-co-caprolactone) (PGCL, 75/25), poly(glycolic-co-lactic acid) (PGLA, 90/10), and multifilament yarns, such as (poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA, 70/30) were tensile tested. The second ones presented a mechanical performance more close to the used PET yarns, so their biodegradability is being studied in order to select the yarn with longer stability. Then, the selected yarn will be inserted in braids 16YH in combination with PET yarns, using different proportions. However the number of PET yarns must always be higher than the number of the biodegradable yarns in order to keep the braid mechanical performance as long as possible.

Regarding the yarns surface treatments to improve the cell adhesion and proliferation and consequently the structure integration with the surrounding native tissues, there are some other treatments that could be studied and compared with the one performed using EDA as source of amino groups. Collagen, for instance, being present in native tendons ECM, has been immobilized on surfaces where tenocytes may selectively attach and elongate, significantly increasing their proliferation rate [18]. Silk fibroin has also been used to modify the surface of PET scaffolds for ligaments reconstruction to enhance fibroblasts attachment, proliferation and matrix production [19]. Fibronectin has also been used in resorbable polymer fibre-based scaffolds to enhance fibroblasts attachment and long term matrix

production [14]. However, besides the variety from batch to batch and the probability of antigenic and immunogenic reactions, the cost of these natural compounds tends to be higher than in case of EDA.

Regarding the clinical application of the prototype, a suture is the best option to anchor the structure to a muscle and/or tendon end. Therefore, knitted/woven assemblies and a system based in a group of needles, where fibres bundles are swaged into muscle, could be studied for that purpose. For the anchorage to bone, if a loop is incorporated in the structure end, it may be fixed using polymeric screws.

Moreover, the developed C22B16YH_S16YL structure is intended to be implanted inducing self-regeneration of the tissue *in vivo*, but a functional tissue could be implanted by *in vitro* host stem cell seeding on the scaffold, what could decrease the recovery time [20-22]. Mesenchymal stem cells (MSCs) are an example of such cells, which can differentiate into fibroblasts [20, 23]. Human adipose tissue is ubiquitous and easily obtainable in large quantities under local anesthesia with little patient discomfort. So it could be a potential source of stem cells from the own patient being treated with a very low rejection risk [24, 25]. However, the cost and time associated to the cells collecting and multiplication procedure, as well to the *in vitro* pre-seeding process on the structure, may be a disadvantage. Finally, it would be necessary to evaluate the performance of the developed functional prototype *in vivo* using an animal model, such as canine and porcine models. After the implantation surgery, the animals must be allowed to free cage activity. At different time points, samples for histology analysis must be harvested, the implantation sites must be analysed by ultrasound analysis and the walking behaviour evolution qualitatively and quantitatively evaluated.

8.3. References

- [1] Morais D, Torres J, Guedes R, Lopes M. Current Approaches and Future Trends to Promote Tendon Repair. *Annals of biomedical engineering*. 2015;1-11.
- [2] Liu Y., Ramanath H., Wang D. Tendon tissue engineering using scaffold enhancing strategies. *Trends in Biotechnology*. 2008;24:201-9.
- [3] Kuo C. K., Marturano J. E., Tuan R. S. Novel strategies in tendon and ligament tissue engineering: advanced biomaterials and regeneration motifs. *Sports medicine, arthroscopy, rehabilitation, therapy & technology : SMARTT*. 2010;2:20.

- [4] Potic M, Ignjatovic I, Savic V, Djekic P, Radenkovic G. Mechanical properties and tissue reinforcement of polypropylene grafts used for pelvic floor repair—an experimental study. *Hernia*. 2011;15:685-90.
- [5] Alagirusamy R, Das A. *Technical textile yarns*: Elsevier; 2010, USA.
- [6] Bažant ZP. Size effect on structural strength: a review. *Archive of applied mechanics*. 1999;69:703-25.
- [7] Hearle JW, Morton WE. *Physical properties of textile fibres*: Elsevier; 2008, USA.
- [8] Modjarrad K, Ebnesajjad S. *Handbook of Polymer Applications in Medicine and Medical Devices*: Elsevier; 2013, USA.
- [9] Ratner BD, Hoffman AS, Schoen FJ, Lemons JE. *Biomaterials science: an introduction to materials in medicine*: Academic press; 2004, Canada.
- [10] Mankodi HR. Studies on different type of sutures using Aloe Vera gel coating. *International Journal of Textile and Fashion Technology (IJTFT)*.1:11-6.
- [11] James R., Kesturu G., Balian G., Chhabra A. B. Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options. *The Journal of hand surgery*. 2008;33:102-12.
- [12] Kahn C. J., Wang X., Rahouadj R. Nonlinear model for viscoelastic behavior of Achilles tendon. *Journal of biomechanical engineering*. 2010;132:111002.
- [13] Bassetto F., Volpin A., Vindigni V. Regenerative Medicine for Tendon Regeneration and Repair: The Role of Bioscaffolds and Mechanical Loading. In: R. P, editor. *Biomaterials Science and Engineering*: InTech; 2011: 370-86, Italy.
- [14] Williams R. *Surface modification of biomaterials: Methods analysis and applications*: Elsevier; 2010, United Kingdom.
- [15] Tang L, Thevenot P, Hu W. Surface chemistry influences implant biocompatibility. *Current topics in medicinal chemistry*. 2008;8:270-80.
- [16] J. GH. *Thin Film Coatings for Biomaterials and Biomedical Applications*, 1st Edition. 1st edition ed: Elsevier; 2016, United Kingdom.
- [17] Li H-L, Zhang H, Huang H, Liu Z-Q, Li Y-B, Yu H, et al. The effect of amino density on the attachment, migration, and differentiation of rat neural stem cells In Vitro. *Molecules and cells*. 2013;35:436-43.
- [18] Chen X, Wang Z, Qin T-W, Liu C-J, Yang Z-M. Effects of micropatterned surfaces coated with type I collagen on the proliferation and morphology of tenocytes. *Applied Surface Science*. 2008;255:368-70.
- [19] Jiang J, Ai C, Zhan Z, Zhang P, Wan F, Chen J, et al. Enhanced Fibroblast Cellular Ligamentization Process to Polyethylene Terephthalate Artificial Ligament by Silk Fibroin Coating. *Artificial organs*. 2015.
- [20] Yates E, Rupani A, Foley G, Khan W, Cartmell S, Anand S. Ligament tissue engineering and its potential role in anterior cruciate ligament reconstruction. *Stem cells international*. 2011;2012.

- [21] Hampson K., Forsyth N., Haj A., Maffulli N. Tendon Tissue Engineering. In: Ashammakhi N., Reis R., Chiellini F., editors. Topics in Tissue Engineering, 2008, United Kingdom.
- [22] Chen J., Xu J., Wang A., Zheng M. Scaffolds for tendon and ligament repair: review of the efficacy of commercial products. Expert review of medical devices. 2009;6:61-73.
- [23] Hoffmann A., Gross G. Tendon and ligament engineering: from cell biology to in vivo application. Regenerative medicine. 2006;1:563-74.
- [24] Mizuno H. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. Journal of Nippon Medical School. 2009;76:56-66.
- [25] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue engineering. 2001;7:211-28.

