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**Chitosan Matrices for Cell-based Bone  
Regenerative Therapies**

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...to my parents,  
Zé Rui, Miguel, David and Sofia

*I find that the harder I work, the more luck I seem to have.*

Thomas Jefferson (1743-1826)



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Current concepts of bone regenerative medicine emphasize the need for new biocompatible and bioresorbable support matrices, structurally mimicking the extracellular matrix, capable of acting as vehicles for autologous osteoprogenitor cells as well as of cell signaling molecules. Among others features, scaffolds are expected to allow anchorage, division, and differentiation of osteoprecursor cells along the osteogenic lineage, support rapid vascular ingrowth, and encourage osteoconduction with host bone. The surface properties play a major role in cell-material interactions. Surface chemistry, particularly, affects osteoblast adhesion, spreading and phenotypic expression, by influencing the adsorption and the conformation of glycoproteins involved in the cell adhesion process.

In the first part of this thesis, the research purpose was to investigate the effect of the degree of acetylation (DA) on the ability of chitosan matrices to promote cell anchorage, proliferation, and osteogenic differentiation of osteoprecursor cells, *in vitro*. For that purpose, chitosan samples with DAs in the range of 4 to 49% were prepared and characterized.

In a preliminary study, human osteoblastic cells were used to study the DA effect on cell adhesion, spreading, and short-term proliferation of osteogenic cells on chitosan films. The DA strongly influenced cell adhesion, lower DAs favoring cell adhesion. In addition, a marked effect on cell morphology and cytoskeleton organization was observed, cell spreading and cytoskeleton organization being only observed on films with DAs  $\leq 13\%$ . Moreover, the DA also influenced cell growth kinetics, cell proliferation being favored on films with a DA of 4%.

In view of the previous findings, the DA effect on cell adhesion and osteogenic differentiation of osteoprecursor cells on chitosan films was investigated, using rat bone marrow stromal cells (rBMSCs). A tendency to higher cell attachment levels on decreasing the DA was found, following the trend observed with osteoblastic cells. In addition, in time, cell cytoskeleton organization, proliferation, and osteogenic differentiation were only possible on chitosan films with a DA of 4%, while for DAs  $\geq 13\%$  cell growth was impaired. As chitosan sample with a DA of 4% showed the

highest fibronectin adsorption from serum, it is suggested that cell adhesion, cytoskeleton organization and osteogenic differentiation of rBMSCs on chitosan in the presence of serum may be mediated by the adsorbed layer of fibronectin.

Subsequently, the effect of the DA on the ability of chitosan porous scaffolds to support cell anchorage, cytoskeleton organization, activity, and proliferation of osteogenic cells, was investigated, using osteoblastic cells. Once more, cell spreading and cytoskeleton organization were only observed for DAs  $\leq 13\%$ , indicating a higher adsorption of cell adhesion glycoproteins on these substrates.

Taken together, the three studies performed revealed that DAs  $\leq 13\%$  are required for cytoskeleton organization of osteoblastic cells on chitosan films and porous scaffolds *in vitro*, and that DAs lower than 13% are required for proliferation, cytoskeleton organization and osteogenic differentiation of rBMSCs on chitosan films.

The research purpose of the second part of this thesis was to enhance the osteoconductive properties of chitosan matrices, by inducing the deposition of an apatite-like layer. For this purpose, phosphorylation of chitosan membranes was attempted, by using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{hexanol}$  reaction route, carried out at  $30^\circ\text{C}$ , with minor modifications, as an alternative to the phosphoric acid/urea/DMF phosphorylation method, which involves the use of much higher temperatures. Surface analysis by XPS, ATR-FTIR, and static contact angle measurements revealed an increase of the phosphate content with the reaction time. Moreover, EDS analysis of the cross-sections revealed that phosphorylation also occurred in the inner of the membrane, although at a lower extent.

Subsequently, the ability of the resulting phosphorylated chitosan (P-chitosan) membranes to nucleate calcium phosphates under simulated physiologic conditions was investigated. The incubation in  $\text{Ca}(\text{OH})_2$  solution led to the formation of a calcium phosphate mineral layer all over the surface of P-chitosan membranes. The subsequent immersion in Simulated Body Fluid (SBF) led to the growth of a multilayered porous mineral structure composed of partially carbonated and poorly crystalline apatite.

Finally, 8-hours phosphorylated chitosan membranes were evaluated regarding cytotoxicity towards human osteoblastic cells, no cytotoxic effect being observed.

In conclusion, the studies performed revealed that the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  reaction system is effective for the phosphorylation of chitosan membranes. 8-hours phosphorylated membranes were not cytotoxic towards osteoblastic cells, and their calcium salt was able to induce the deposition of an apatite-like layer in SBF, thus enabling further biological assays, such as the evaluation of mineralized P-chitosan membranes as substrate for osteogenic differentiation of osteoprecursor cells.



O conceito actual de medicina regenerativa do osso apela para a necessidade de novas matrizes de sustentação que, para além de serem biocompatíveis e bioreabsorvíveis, apresentem analogia estrutural com a matriz extracelular, e sejam capazes de funcionar como veículo de moléculas de sinalização celular e de células autólogas osteoprogenitoras. Entre outras propriedades, estes suportes deverão permitir a adesão, a divisão, assim como a diferenciação osteogénica de células osteoprecursoras, permitir uma rápida vascularização, e incentivar a formação do osso. As características da superfície tem uma importância preponderante nas interações célula-material. A química da superfície, em particular, influencia a adesão dos osteoblastos, a sua morfologia, e a sua expressão fenotípica, por influenciar a adsorção e a conformação de glicoproteínas envolvidas no processo de adesão celular.

A primeira parte desta tese visou a investigação do efeito do grau de acetilação (GA) na aptidão das matrizes de quitosano para promoverem a adesão, a proliferação, e a diferenciação osteogénica de células osteoprecursoras, *in vitro*. Para este fim, prepararam-se e caracterizaram-se lotes de quitosano com GAs numa gama de 4 a 49%.

Numa fase inicial, utilizaram-se células osteoblásticas humanas a fim de estudar o efeito do GA na adesão, morfologia, e proliferação de células osteogénicas a curto prazo em filmes de quitosano. O GA influenciou fortemente a adesão celular, sendo a adesão celular favorecida por GAs baixos. Para além deste facto, verificou-se um efeito marcante do GA na morfologia das células assim como na organização do respectivo citoesqueleto, já que só se observaram células espalhadas e com citoesqueleto organizado em filmes de GAs  $\leq 13\%$ . A cinética de proliferação celular também foi influenciada pelo GA, sendo favorecida em filmes com um GA de 4%.

Perante os resultados obtidos, procedeu-se ao estudo do efeito do GA na adesão e diferenciação osteogénica de células osteoprecursoras em filmes de quitosano, tendo-se utilizado para o efeito células estromais de medula óssea de rato. Verificou-se uma tendência para valores mais altos de adesão celular com a diminuição do GA, tal como previamente observado para células osteoblásticas. Para além deste facto, com o tempo, a organização do citoesqueleto, a proliferação, e a diferenciação osteogénica só foram possíveis em filmes de quitosano com um GA de 4%, tendo-se verificado

uma ausência de proliferação celular em filmes com GAs  $\geq 13\%$ . Como os filmes com um GA de 4% foram os que revelaram valores superiores de adsorção de fibronectina a partir do soro, sugere-se que a adesão, a organização do citoesqueleto, e a diferenciação osteogénica de células estromais de medula óssea de rato em quitosano na presença de soro sejam mediadas pela camada de fibronectina adsorvida à superfície.

Subsequentemente, investigou-se o efeito do GA na aptidão de esponjas de quitosano para sustentarem a adesão, a organização do citoesqueleto, a actividade, e a proliferação de células osteogénicas, utilizando para o efeito células osteoblásticas humanas. Mais uma vez, a adopção de uma morfologia espalhada e a organização do citoesqueleto só se verificaram para GAs  $\leq 13\%$ , o que sugere uma adsorção maior de glicoproteínas a estes substratos.

Em síntese, os três estudos realizados revelaram que para além de no caso de células osteoblásticas, a organização do citoesqueleto em matrizes de quitosano só ser possível para GAs  $\leq 13\%$ , no caso de células estromais de medula óssea de rato cultivadas em filmes de quitosano, a proliferação, a organização do citoesqueleto, e a diferenciação osteogénica só se verificam para GAs inferiores a este.

Na segunda parte desta tese, pretendeu-se melhorar as propriedades osteocondutoras das matrizes de quitosano, induzindo nestas a deposição de uma camada de apatite. Para este efeito, tentou-se a fosforilação de membranas de quitosano, utilizando o sistema reactivo  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{hexanol}$ , a  $30^\circ\text{C}$ , com ligeiras modificações, como alternativa ao método de fosforilação  $\text{H}_3\text{PO}_4/\text{ureia}/\text{DMF}$ , que implica o uso de temperaturas mais elevadas. A análise de superfícies por XPS, ATR-FTIR, e medição de ângulos de contacto estáticos revelou um aumento do teor de fosfatos, com a duração da reacção. Para além deste facto, a análise por EDS de cortes transversais revelou que a fosforilação também ocorreu na parte interna das membranas, embora a um nível mais reduzido.

Seguidamente, investigou-se a aptidão das membranas de quitosano fosforiladas para nuclear fosfatos de cálcio, em condições similares às fisiológicas. A incubação das membranas fosforiladas numa solução de  $\text{Ca}(\text{OH})_2$  levou à formação de uma camada mineral de fosfatos de cálcio, sobre toda a sua superfície. A imersão subsequente em

“Simulated Body Fluid” (SBF) resultou no crescimento de uma estrutura mineral porosa, composta por uma apatite pouco cristalina e parcialmente carbonatada.

Por último, as membranas de quitosano fosforiladas por um período de 8 horas foram avaliadas em termos de citotoxicidade para células humanas osteoblásticas, não se tendo verificado efeito algum citotóxico.

Em resumo, estes estudos revelaram que o sistema reactivo  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  é eficiente para a fosforilação de membranas de quitosano. As membranas de quitosano fosforiladas por um período de 8 horas não revelaram citotoxicidade para células osteoblásticas, e o seu sal de cálcio foi capaz de induzir a deposição de uma camada apatítica em SBF, o que permite a realização de mais ensaios biológicos, nomeadamente a avaliação de membranas de quitosano mineralizadas como substrato para a diferenciação osteogénica de células osteoprecursoras.



Les concepts actuels de la médecine régénérative de l'os soulignent le besoin de nouvelles matrices biocompatibles et bioresorbables ayant une structure similaire à celle de la matrice extracellulaire, capables de servir de véhicule pour les cellules ostéoprogénitrices ainsi que pour les molécules de signalisation cellulaire. Entre autres propriétés, ces supports doivent permettre à la fois l'adhésion, la prolifération et la différenciation ostéogénique de cellules précurseur afin de permettre une vascularisation rapide et stimuler la formation d'os. Les caractéristiques de surface ont une importance primordiale dans les interactions cellule-matériau. La composition chimique de la surface, en particulier, influence l'adhésion des ostéoblastes, leurs morphologies ainsi que l'expression des marqueurs phénotypiques. Ceci dérive de l'influence qu'elle exerce sur l'adsorption et sur la conformation des glycoprotéines impliquées dans l'adhésion cellulaire.

La première partie de cette thèse avait pour objectif d'étudier l'effet du degré d'acétylation (DA) sur la capacité des matrices de chitosane de promouvoir l'adhésion, la prolifération et la différenciation ostéogénique des cellules ostéoprécurseurs, *in vitro*. Dans ce but, des échantillons de chitosane avec des DAs entre 4 et 49% ont été préparés et caractérisés.

Dans une phase initiale, des ostéoblastes d'origine humaine ont été utilisés pour étudier l'effet du DA sur l'adhésion, la morphologie et la prolifération à court terme des cellules ostéogéniques en contact avec des films de chitosane. Les résultats montrent que le DA influence l'adhésion cellulaire. Ce processus est favorisé en présence de faibles DAs. De plus, un effet évident au niveau de la morphologie cellulaire et l'organisation du cytosquelette a été remarqué. En effet, des cellules allongées avec un cytosquelette organisé ont été observées en présence de films de DAs  $\leq 13\%$ . La cinétique de prolifération cellulaire a été également influencée par le DA. Un taux de division cellulaire plus élevé a été obtenu avec un DA de 4%.

Tenant compte des résultats antérieurs, l'effet du DA des films de chitosane sur l'adhésion et la différenciation ostéogénique des cellules précurseur a été analysé en utilisant des cellules stromales de la moelle osseuse du rat. Comme a été observé avec les ostéoblastes d'origine humaine, une augmentation du taux d'adhésion de cellules

stromales a été obtenue avec une diminution du DA. Ceci étant, au cours du temps, l'organisation du cytosquelette, la prolifération et la différenciation ostéogénique ont été observés uniquement avec les DA de 4%. Les films avec des DAs  $\leq 13\%$  ont montré une absence de prolifération cellulaire. Comme les films de DA de 4% ont révélé des valeurs supérieures d'adsorption de fibronectine à partir du sérum, il est suggéré que l'adhésion, l'organisation du cytosquelette et la différenciation ostéogénique des cellules stromales de la moelle sur le chitosane en présence du sérum puissent être induit par une couche de fibronectine adsorbée à la surface de celui-ci.

Par conséquent, l'influence de DA sur le potentiel des éponges de chitosane à stimuler l'adhésion, l'organisation du cytosquelette, l'activité et la prolifération des cellules ostéogéniques a été étudié. Des ostéoblastes d'origine humaine ont été utilisés. Encore une fois, l'allongement des cellules ainsi que l'organisation du cytosquelette ont été observés avec les DAs  $\leq 13\%$  suggérant une adsorption plus élevée de glycoprotéines à ce substrat.

Dans l'ensemble, ces trois études ont montré qu'*in vitro*, l'organisation du cytosquelette des ostéoblastes en matrice de chitosane, films et éponges, requière des DAs  $\leq 13\%$ . De plus, les DAs inférieurs à celui-ci sont nécessaires à la prolifération, l'organisation du cytosquelette ainsi que la différenciation des cellules stromales de la moelle osseuse du rat sur les films du chitosane.

La seconde partie de cette thèse avait pour but d'améliorer les propriétés ostéoconductrices des matrices de chitosane en induisant une déposition d'une couche d'apatite à leur surface. Pour cela, une phosphorylation des membranes de chitosane a été réalisée en utilisant un système réactif  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{hexanol}$ , à  $30^\circ\text{C}$ , avec des modifications mineures. Cette méthode est une alternative à la méthode de phosphorylation  $\text{H}_3\text{PO}_4/\text{urée}/\text{DMF}$  qui implique l'utilisation de températures plus élevées. L'analyse des surfaces par XPS, ATR-FTIR et les mesures statiques de l'angle de contact montrent une augmentation du contenu en phosphate en fonction du temps de la réaction. De plus, l'analyse des coupes transversales par EDS révèle que la phosphorylation a également eu lieu au niveau de la partie interne des membranes, malgré un niveau plus réduit.

Postérieurement, la capacité des membranes de chitosane phosphorylées à induire à une nucléation du calcium, en condition similaires aux conditions physiologiques, a été analysée. L'incubation des membranes phosphorylées dans une solution de  $\text{Ca}(\text{OH})_2$  a conduit à une formation d'une couche de minéral de phosphate de calcium sur toute leur surface. L'immersion en solution de "Simulated Body Fluid" (SBF) a donné lieu à la croissance d'une structure minérale poreuse composée par une apatite peu cristalline et partiellement carbonatée.

Finalement, l'effet cytotoxique des membranes de chitosane phosphorylées pendant une période de 8 heures a été réalisé en présence des ostéoblastes d'origine humaine. Les résultats ont montré l'absence d'effet cytotoxique de ces dernières.

En résumé, ces études ont démontré que le système réactif  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  est efficace pour la phosphorylation des membranes de chitosane. Ces membranes phosphorylées au cours d'une période de 8 heures ne montrent aucune cytotoxicité pour les cellules ostéoblastiques. De plus, leur sel de calcium a permis d'induire une déposition d'une couche d'apatite en SBF. Ceci ouvre la possibilité de réaliser des études biologiques, notamment, l'évaluation des membranes de chitosane minéralisées comme substrat destiné à stimuler la différenciation ostéogénique des cellules ostéoprécurseurs.



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# INTRODUCTION

## 1. Bone tissue engineering

### 1.1. General Aspects

Bone engineering has been practiced for centuries in the repair of fractures and, in modern medicine, for at least several decades through the implantation of dental/orthopedic devices and bone substitute materials. Recent advances in the field of regenerative medicine, and in particular of tissue engineering, promise to change profoundly medical practice.

Tissue engineering is an emerging field encompassing medicine, reactor engineering, materials science and cell and molecular biology, aiming at regenerating injured or diseased tissues instead of just repairing them. Several definitions of tissue engineering with a broader or more focused scope have been used. For instance, Langer and Vacanti<sup>1</sup> defined it as “an interdisciplinary field that applies the principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”.

According to these principles, the goal of bone tissue engineering is to restore or to augment bone tissue, which is considered as a recent approach to the long-established field of bone engineering.<sup>2</sup> The regeneration or augmentation of a tissue such as bone requires deep understanding of key aspects of bone structure and function, bone biology at cellular and molecular level, materials science and cell-substrate interactions. These aspects will be focused in the next pages.

### 1.2. Bone

Bone is a highly specialized form of connective tissue consisting of cells, an organic matrix on which minerals are deposited under cellular control, and soluble factors. Bone has several functions, including the provision of mechanical integrity for both

locomotion and protection of the internal organs, mineral homeostasis, and of an appropriate environment for hematopoiesis.

### 1.2.1. Bone structure and function

Macroscopically, bone exists as cortical bone (also called dense or compact bone) and trabecular bone (also called cancellous or spongy bone), which is mainly found at the ends of long bones, vertebral bodies and pelvic bones, interposed between cortical bone.<sup>3</sup> Cortical bone has four times the mass of trabecular bone, and presents two types of surfaces. The surface on its inner side, facing the bone marrow, is known as the endosteum, and the surface facing the surrounding soft tissues, is known as the periosteum.<sup>4</sup> At microscopic level cortical bone reveals the Haversian systems (osteons), the major structural units of cortical bone, usually oriented along the long axis of bone. The osteon is an anastomosing cylinder composed by concentrically arranged lamellae of bone matrix permeated by a variable number of osteocytes, and a central canal that contains blood vessels, lymphatics and nerves. Perpendicularly oriented to the osteons are the Volkmann canals, which provide additional vascular-lymphatic channels for the exchange of nutrients and metabolites, as well as the trafficking of cells and molecular signals, needed to sustain osseous physiology.<sup>5</sup> While cortical bone is a highly organized structure, with densely packed collagen fibrils, trabecular bone has a loosely organized open-celled structure, with porosity (percentage of void space in a solid) in the range of 50 to 90% (Fig. 1).<sup>6</sup> The morphologic differences reflect different functions, cortical bone providing the mechanical and protective functions and trabecular bone providing the metabolic functions.<sup>3</sup>

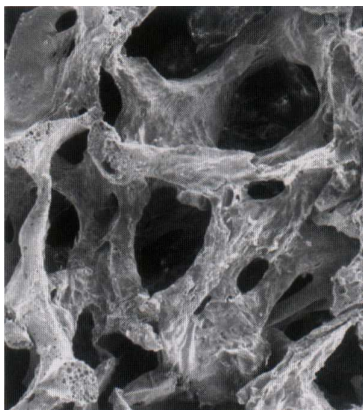


**Figure 1.** Human trabecular bone. Field width: 2.5 mm. Image courtesy of D. Baksh.

Bone houses the marrow, herein considered as a different tissue, which is located in the medulla of tubular bones, vertebrae, ribs, sternum, and flat bones. Bone marrow is the source of hematopoietic cells of erythropoietic and granulocytic lineage, as well as of undifferentiated mesenchymal stem cells that can differentiate into several connective tissue cell types including osteoblasts, chondrocytes, adipocytes, tenocytes, and myocytes.

### **1.2.2. Bone cells**

Osteoclasts are the principal bone-resorbing cells of bone, being actively involved in bone remodeling. Osteoclasts are large, multinucleated cells, arising from the fusion of mononucleated precursors, derived from the hemopoietic stem cell progeny. In cortical bone, they are found at the apex of cutting cones, tunnels which appear in cross sections as cavities. In trabecular bone, osteoclasts are generally juxtaposed to bony surfaces and often in resorption pits, known as Howship's lacunae, in the case of bone undergoing active remodeling.<sup>4</sup> When actively resorbing bone, osteoclasts present a characteristic polarized membrane with a ruffled border and a clear zone. The ruffled border is a highly infolded area of the cytoplasmatic membrane through which acid is secreted and proteolytic enzymes are released. The clear zone is an organelle-free area of the plasma membrane that serves as the point of attachment of the osteoclast to the underlying bone, whose membrane is rich in integrins, particularly  $\alpha_v\beta_3$  integrin<sup>7</sup> (Integrins are a widely expressed family of cell surface adhesion receptors, composed of heterodimers of non-covalently associated  $\alpha$  and  $\beta$  sub-units, which bind to specific amino acid sequences).<sup>8</sup> The enzyme carbonic anhydrase is known to be involved in the acidification of the resorption pit, and consequently, in the dissolution of bone mineral, by supplying protons to proton pump-bearing vacuoles.<sup>7</sup> Among the proteolytic enzymes involved in the degradation of the organic matrix are cysteine proteinases and matrix metalloproteases.<sup>9</sup> While large osteoclasts may have more than one ruffled border region, each of them resorbing at different rates, inactive osteoclasts have little or no ruffled border region. Mature osteoclasts are believed to exist for approximately 2 weeks, and can undergo several consecutive resorption cycles.<sup>7</sup>

Osteoblasts are mature, secretorily active cells responsible for the synthesis of osteoid, the protein component of bone tissue, which includes collagen I and the noncollagenous proteins of bone matrix. Osteoblasts produce osteoid at a rate of about 2-3  $\mu\text{m}$  per day, which begins to mineralize, after reaching a width of approximately 20  $\mu\text{m}$ .<sup>10</sup> Osteoblasts also produce various proteases that disrupt the osteoid layer, allowing not only the release of signaling molecules buried within it but also the access of osteoclasts to the mineralized matrix.<sup>4</sup>

Osteoblasts arise from pluripotent mesenchymal stem cells that are self-renewable, and capable of giving rise to other differentiated cell phenotypes of mesenchymal tissues including adipocytes, fibroblasts, myocytes, and chondrocytes. Ultimately, a non-dividing, mature cell is produced that is responsible for the generation of a specific tissue (Fig. 2).

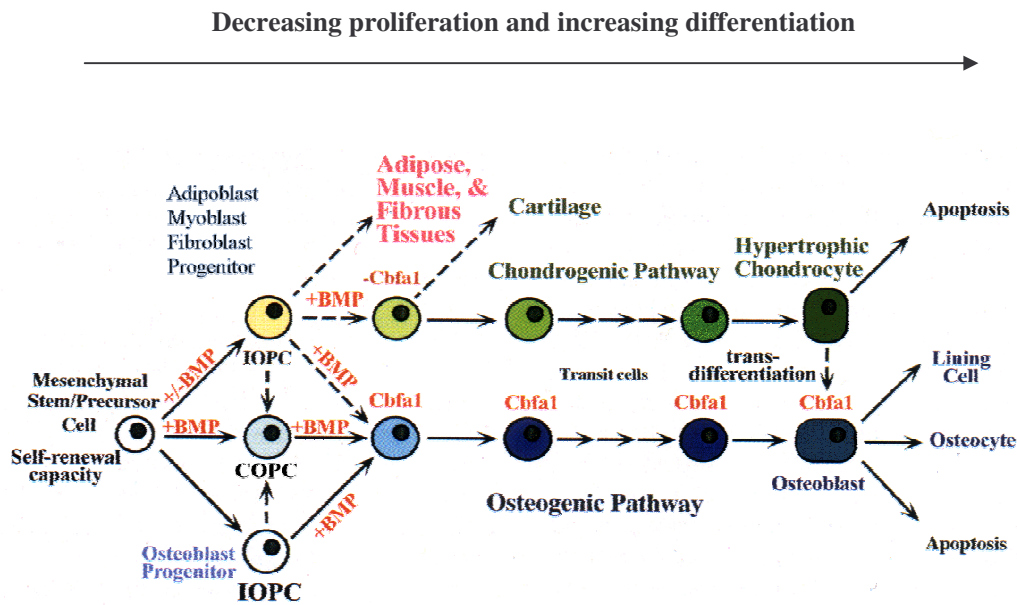


**Figure 2.** Postulated steps in osteogenic differentiation. The influence of BMPs in the early stages of cellular differentiation is depicted, together with the subsequent induction of Cbfal expression, which directs the osteogenic pathway. Osteogenic cells can also be generated by transdifferentiation of hypertrophic cartilage cells derived from the chondrogenic lineage. COPC (committed osteoprogenitor cell); IOPC (inducible osteogenic precursor cell). Adapted from Sodek et al..<sup>11</sup>

The commitment to the osteogenic lineage is believed to be controlled by a cascade of molecular events involving a combination of genetic programming and gene regulation by various hormones, cytokines, and growth factors.<sup>11</sup> Bone morphogenetic proteins (BMPs) for instance, initially characterized by their ability to promote ectopic bone and cartilage formation, are important osteoinductive signaling molecules.<sup>12</sup> These cytokines are capable of inducing differentiation of uncommitted mesenchymal cells into several cell lineages, as well as of enhancing osteoblast differentiation. Studies *in vitro* have shown that BMPs regulate core binding factor 1/osteoblast specific factor (Cbfa1/Osf2) expression in cells of the osteogenic pathway<sup>13</sup>, a transcription factor critical for osteogenesis.<sup>14</sup> Osteoprogenitor cell proliferation and differentiation are not regulated solely by soluble factors, and increasing data support the importance of various matrix molecules expressed early during the developmental sequence on progression through the osteogenic lineage.<sup>15</sup>

Lineage progression from committed osteoprogenitor cells to fully functional matrix-synthesizing osteoblasts is indicated by characteristic changes in gene expression (Fig. 3). Soon after cell plating, analysis of a population of fetal rat calvarial-derived osteoblasts shows cells under exponential growth, expressing genes associated with proliferative stages (histones), type I collagen (COL I), fibronectin (FN), as well as alkaline phosphatase (ALP) and osteopontin (OPN).<sup>15</sup> The early deposition of matrix molecules such as type I collagen and fibronectin is essential for the development of the osteoblastic phenotype, via interaction with specific cellular receptors.<sup>15-17</sup> After cell confluency, cell proliferation continues in some regions, leading to the formation of multilayered nodules. During this stage, genes associated with the synthesis of bone extracellular matrix and maturation are upregulated. This is the case of ALP, whose expression increases to allow subsequent mineralization of the collagenous matrix. At the onset of matrix mineralization, OPN is expressed at high levels, followed by bone sialoprotein and OCN, with high levels being maintained as osteoblasts differentiate further into osteocytes and lining cells. When mineralization is well progressed, the expression of ALP decreases.<sup>11,15</sup> Bone sialoprotein is transiently expressed much earlier, and then upregulated again in differentiated osteoblasts. As a result, bone matrix proteins associated with the later stages of differentiation like osteocalcin are often used as osteogenic markers in *in vitro* cultures.

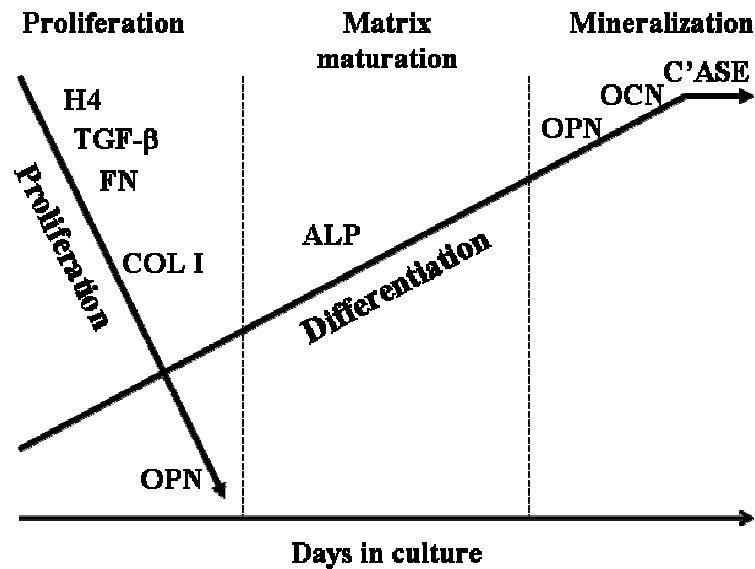


**Figure 3.** Temporal expression of cell growth and osteoblast phenotype-related genes during development of the osteoblast phenotype, in a culture of fetal rat calvarial-derived osteoblasts: H4, histone; TGF- $\beta$ , transforming growth factor beta; FN, fibronectin; COL I, type I collagen; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; C'ASE, collagenase. The principal developmental stages are shown: proliferation, extracellular matrix development and maturation, and mineralization. Usually type I collagen and histones peak during the proliferation stage and decline thereafter, alkaline phosphatase peaks during the matrix maturation stage and declines thereafter, while osteocalcin peaks during the mineralization stage. Adapted from Stein et al.<sup>18,19</sup>

The life span for human osteoblasts may range from one to ten weeks, at which time these cells may disappear by apoptosis, become lining cells, or turn into osteocytes.

Osteocytes represent the terminal step in osteoblasts differentiation. Osteocytes arise from osteoblasts that have become entrapped in the mineralized matrix representing at least 90% of all cells in bone.<sup>7</sup> However, the precise mechanism of differentiation from osteoblast to osteocyte is not known.<sup>20</sup> Each osteocyte occupies a space or lacunae within the matrix, and develops long filopodial processes through canaliculae in the matrix, to communicate via gap junctions with adjacent osteocytes, lining cells, and osteoblasts.<sup>21</sup> These dendritic cells create a highly communicative network of cells, ideally located to integrate and amplify extracellular signals, which explain why osteocytes are often called the bone's mechanosensing cell.<sup>7</sup> These cells are capable

of perilacunar matrix resorption, and are believed to be involved in the biomechanical regulation of bone mass and structure.<sup>3,20</sup>

Bone lining cells are flat, elongated and inactive cells that cover bone surfaces that are undergoing neither bone formation nor resorption<sup>3</sup>. Beneath these cells is a layer of unmineralized osteoid. Lining cells have receptors for several hormones important for initiating bone resorption and are able to respond to these substances by resorbing the layer of osteoid lying beneath them.<sup>4</sup> Lining cells communicate with the osteocytes through cell projections within canaliculae in the matrix. Lining cells are believed to play an important role in bone remodeling. Lining cells are hypothesized to activate remodeling unless restrained by an inhibitory signal produced by osteocytes in response to mechanical loading.<sup>22</sup>

### **1.2.3. Bone matrix**

Bone is composed of approximately 70% mineral, 22% protein, and 8% water.<sup>23</sup> The organic matrix is made of approximately 95% type I collagen and 5% non-collagenous proteins and proteoglycans.<sup>3</sup>

Type I collagen is the most abundant protein in vertebrates, and provides a template for the deposition of bone mineral crystals, giving bone its elastic properties. It consists of polypeptide chains arranged in a long and continuous triple helix, whose amino acid sequence consists of Gly-X-Y repeats. In about one third of the cases X is a proline and Y a hydroxyproline. Type I collagen is secreted as a propeptide, which after being cleaved by specific proteases assemble to form highly organized fibrils.<sup>24</sup> In addition to having a structural function, collagen is also important in cell attachment and differentiation, not only by providing cell-binding domains,<sup>15</sup> but also sites for binding of noncollagenous proteins, often through specific binding domains.<sup>25</sup>

The noncollagenous proteins are proteins often glycosylated that appear to regulate the organization, turnover, and mineralization of bone matrix.<sup>26</sup> Numerous noncollagenous proteins were isolated from bone. Osteocalcin, also known as Bone Gla Protein due to the presence of  $\gamma$ -carboxyglutamic acid (gla) residues, is the more abundant. Its appears to be a regulator of bone mineral formation by virtue of its binding to mineral crystals and its ability to act as a chemotactic factor for

osteoclasts.<sup>27</sup> Osteocalcin expression is commonly used as a phenotypic marker for bone cells, as there are few other cells with such high levels of expression.<sup>28</sup> Osteopontin, bone sialoprotein, osteonectin, dentin matrix protein-1, dentin sialoprotein, and matrix extracellular glycol-phosphoprotein are multifunctional phosphorylated glycoproteins with high affinity for bone mineral carrying one or more arginine-glycine-aspartate (RGD) sequences, thus capable to linking cells, mineral and matrix.<sup>26</sup> Osteopontin is the major phosphorylated glycoprotein of bone, acting as an inhibitor of mineralization as well as a chemotactic factor for osteoclasts, *in vitro*.<sup>29</sup> Bone sialoprotein accounts for approximately 15% of the human noncollagenous proteins, binds to collagen, and is usually concentrated in areas of active bone remodeling.<sup>30</sup> Osteonectin accounts for approximately 23% of the noncollagenous proteins and was reported as having a role in regulating the activities of the metalloproteases that control the degradation of the extracellular matrix.<sup>31</sup> Bone acidic glycoprotein is a phosphorylated glycoprotein with high affinity for calcium, collagen and bone mineral, with reported ability to inhibit osteoclast activity.<sup>32</sup> Other glycoproteins, namely thrombospondin, fibronectin and fibrillin are also present in the matrix. Fibronectin is one the first noncollagenous proteins to be expressed during bone formation. It is one of the most abundant extracellular matrix proteins, existing as a large dimeric protein composed of two homologous subunits. Each subunit has multiple domains that bind to cell surfaces, fibrin, heparin, and collagen.<sup>26</sup> Thrombospondin is also found in the extracellular matrix of many tissues, and its main function is though to be the regulation of angiogenesis. Fibrillin is the principal component of the microfibrillar aggregates in the skeleton, being a calcium-binding protein with a RGD domain. Of the non-collagenous proteins, osteocalcin and bone sialoprotein are essentially unique in bone while others like osteopontin and osteonectin have a broader distribution.<sup>11</sup>

Proteoglycans differ from glycoproteins for having glycosaminoglycan (GAG) side chains attached covalently to a protein core. GAGs are long chains of repeating disaccharides that are frequently sulfated. In bone they are mainly present as versican, a proteoglycan with chondroitin sulfate chains, the small leucine-rich proteoglycans (SLRPs), and the heparin sulfate proteoglycans. Versican seems to be important for matrix organization, and for promoting cell motility and growth. The SLRPs, which include decorin, biglycan, lumican, fibromodulin and osteoadherin, have ability to

bind collagen and growth factors, and were shown to regulate *in vitro* collagen fibrillogenesis. The heparin sulfate proteoglycans are present in osteoblasts, associated with cell membranes.<sup>25</sup>

Lipids are also present in bone, representing about 2-4% of the organic matrix. Lipids are of crucial importance in cell membranes and in extracellular matrix vesicles (ECMVs), in which they are present as acidic phospholipids, complexed with calcium and inorganic phosphate.<sup>26</sup> Other lipid constituents of bone were shown to be important for bone formation. Leptin for instance, produced by adipocytes, is thought to regulate bone mass metabolism, by synchronizing periods of bone growth with periods of food availability and fertility.<sup>33</sup>

Finally various hormones such as parathyroid hormone (PTH), and growth factors, such as BMPs, transforming growth factor beta (TGF- $\beta$ ), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), and vascular endothelial growth factors (VEGFs) are sequestered in bone matrix and regulate bone metabolism, function and regeneration. The growth factors (also called diffusible factors) are secreted proteins that modulate cell activity by interacting with specific receptors on the cell surface.<sup>34</sup> These soluble factors can either stimulate or inhibit chemotaxis, mitogenesis, differentiation, and apoptosis during normal growth and development, as well as in bone repair after injury.

Regarding the mineral of bone, this is a poorly crystallized and defective hydroxyapatite, the naturally occurring geologic mineral. It is accepted as a hydroxyl-deficient, calcium deficient, carbonated apatite. The process by which mineralization occurs is not completely understood. In cancellous bone, mineral deposition begins in the inner surface of ECMVs, extracellular organelles that bud off of osteoblasts. These matrix vesicles, whose membranes are rich in enzymes associated with mineralization (alkaline phosphatase, ATPase and metalloproteinases) create a protected environment where calcium and phosphate ions can accumulate and complex with acidic phospholipids forming nucleation sites for further mineralization<sup>26</sup>. In cortical bone, the mechanism of mineralization appears to be different, occurring along the collagen matrix, where there are few matrix vesicles. Mineralization begins in the region between overlapped collagen molecules, where anionic noncollagenous proteins appear to act as nucleators of initial apatite formation.<sup>26 28</sup>

#### 1.2.4. Bone remodeling

Bone is constantly being remodeled, resorbed and reformed<sup>3</sup>. Bone remodeling is a process conducted by cells, and basically consists in the localized removal of bone by osteoclasts followed by the deposition of newly formed osteoid by osteoblasts. This process enables bone to adapt to mechanical stresses and repair microdamage, and consequently, maintain its strength. The rate of cortical bone remodeling may be as high as 50% per year during the first 2 years of life, but declines to a rate of 2 to 5% per year, in the elderly. Due to its higher surface area, the rate of remodeling in trabecular bone is normally much higher than that of cortical bone, in the adult.<sup>4</sup> Under normal conditions, bone resorption is closely coupled to bone formation, resulting in no net change in bone mass.<sup>4</sup> In the elderly, the reduced number of osteogenic precursor cells and the decline in their proliferative activity contribute to the decrease of bone formation during remodeling, and consequently, to osteoporosis.

Remodeling is a surface phenomenon and occurs on periosteal, endosteal, haversian canal, and trabecular surfaces. The activation of remodeling begins by the recruitment of osteoclasts precursors. Stimuli such as PTH or mechanical strain are proposed to initiate the remodeling cycle, stimulating lining cells in cancellous bone to produce collagenase. After digestion of the thin layer of nonmineralized matrix under these cells, the mineralized matrix becomes exposed, allowing osteoclastic resorption.<sup>35</sup> The differentiation of mononucleated precursors, fusion of mononucleated osteoclasts into osteoclasts, and activation of osteoclasts requires contact with osteoblasts or pre-osteoblasts, and signaling by osteotropic factors like PTH, through specific receptors on osteoblasts/pre-osteoblasts.<sup>7</sup> After adherence to bone surface, a process mediated by integrin-binding with matrix proteins such as osteopontin, osteoclasts assemble one or more ruffled cytoplasmatic borders, and start resorbing bone, creating a resorption lacuna.<sup>7</sup> Bone resorption may last 2 to 4 weeks.<sup>35</sup> Bone formation is usually initiated after two weeks depending on chemotaxis for osteoblasts precursors,<sup>4</sup> by osteoblasts which accumulate at the base of the resorption lacuna, and subsequently fill in the resorption pit with newly formed bone. The transition from resorption to formation is called the reversal phase, due to the existence of a reversal line (also called cement line) composed by extracellular matrix rich in osteopontin, which is produced both by osteoclasts and osteoblasts.<sup>35</sup>

Besides a variety of biochemical factors bone remodeling is regulated by mechanical factors. Immobilization, microdamage produced by overloading, and disruption of blood supply following fracture, are known to activate remodeling. According to a recent theory, remodeling is maintained at a low level by strain-generated inhibitory signals produced by osteocytes under physiologic loading, inhibiting bone-lining cells from activating a new remodeling cycle.<sup>22</sup> Osteocyte death due to microdamage would necessarily reduce the transmission of inhibitory signals to nearby bone lining cells and activate a new remodeling cycle.

### **1.2.5. Bone healing**

Bone is a dynamic, highly vascularized tissue with ability to heal itself and, in most cases, namely in the case of long bone fractures, adequate mechanical stimulation is sufficient to restore bone functionality. Following injury or fracture, bone is able to restore tissue continuity, form, and function, without scar formation. All other tissues, with the exception of embryonic tissue, heal with the formation of a scar. Therefore, the term bone regeneration is often used, rather than bone repair.<sup>36</sup>

As a result of fracture or implantation, blood vessels are disrupted, and fluid, proteins, and blood cells escape to the extravascular space, with immediate formation of a hematoma or blood clot. As a consequence of hematoma, oxygen supply at the broken ends of bone fragments is discontinued, leading to local necrosis.<sup>36</sup> The blood clot consists of a fibrin matrix, in which blood cells and platelets are entrapped, together with a myriad of soluble signaling factors.<sup>36</sup> In addition to achieving hemostasis over the short-term, this fibrin-rich clot provides a temporary 3-D matrix through which cells can migrate and attach, over the longer-term.<sup>37</sup> After hematoma, inflammatory cells are recruited to the injured site for clot demolition, induced by inflammatory soluble factors entrapped within the clot, either released by activated platelets or damaged cells, or resultant from the activation of the coagulation cascade. Neutrophils appear in large numbers very soon after injury and serve to remove foreign materials from the wounded region. Macrophages appear later, as a result of differentiation of monocytes, being the main responsible for the degradation of the necrotic tissue and thrombus. The presence of foreign material in the wound, as in the case of an implant, can further stimulate and prolong inflammation, leading ultimately

to fibrous encapsulation of the implant.<sup>38</sup> Tissue regeneration is initiated by chemotactic factors such as PDGF and TGF- $\beta$  incorporated within the fibrin clot, which stimulate angiogenesis and contribute to the recruitment of mesenchymal stromal cells (MSCs). In addition, the diminishing oxygen concentration towards the centre of the wound is a chemotactic signal for endothelial and MSCs, which infiltrate through the resolving blood clot.<sup>36</sup> The initial hematoma is gradually replaced by a fibrous vascular tissue, the granulation tissue, so-called due to the presence of abundant blood vessels. The formation of the granulation tissue usually lasts for 3 weeks, before bone formation is observed by populations of differentiating osteogenic cells. Osteoid deposition and its subsequent mineralization proceed rapidly, resulting in a poorly organized bone tissue similar to the woven bone found in the embryo. With time, bone remodeling leads to the replacement of woven bone by lamellar bone including Haversian systems, which generally takes about 6 months.<sup>36,39</sup> When conditions are not optimal for bone formation, MSCs may not differentiate along the osteogenic pathway and become chondrocytes, in sites mechanically unstable and poorly vascularized, or fibroblasts, as seen next to implants that are not osteoconductive or when inflammation persists.<sup>39</sup>

### **1.3. Bone repair**

When bone injury exceeds a certain size, regeneration will not occur. Rather, a combination of bone and scar formation ensues. In the case of small defects, caused by cancer surgery, birth defects or injury, autogenous cancellous bone, both vascularized and nonvascularized, has long been considered the gold standard in clinical practice. Autogenous bone grafts include cells, matrix, and signaling molecules that are required to promote healing.<sup>40</sup> Harvest of autograft, however, results in donor site morbidity associated with infection, pain and hematoma, and increases operative time and cost. In the other hand, the use of allogeneic and xenogeneic grafts, (either mineralized or demineralized) presents several limitations, among them the possibility of acute immunogenicity and the transmission of infectious agents, especially viruses and prion proteins.<sup>41</sup> Alternatives to grafts have included different acellular bone fillers namely hydroxyapatite (HA), resorbable calcium-phosphates such as tricalcium phosphates (TCP), calcium sulfate (plaster of

Paris), and coral-derived products, particulate bioactive glasses, degradable synthetic polymers such as poly(lactide acid) (PLA), poly(glycolide acid) (PGA), poly(lactide-co-glycolide acid) (PLGA), poly(propylene fumarate) (PPF), polycaprolactone, and, poly(anhydride), biopolymers such as collagen and fibrin, composites, and bone derived materials such as demineralized bone matrix (DMB).<sup>39,42,43</sup> In the case of large open fractures or defects, metal implants made from passive alloys such as titanium alloys, cobalt chromium alloys, and stainless steel alloys are frequently used, mainly in the case of load bearing applications, but often result in implant-related osteopenia and subsequent refracture.<sup>44</sup>

#### **1.4. Bone regenerative therapies**

The most common regenerative approach in orthopedics involves the use of three-dimensional supporting structures in the form of gels or porous scaffolds to guide tissue regeneration and to position signaling molecules to locally induce vascularization or the recruitment and osteogenic differentiation of progenitor cells. This scaffold-driven regenerative therapy (also called structural tissue engineering) may or not involve an *in vitro* stage of autologous cell culture, dependent on the urgency of the treatment and on the availability of cells with osteogenic potential residing at the site of the defect.<sup>2</sup> Immunologically protected cell-matrix constructs, in which metabolically active xenogenic cells are protected by a semi-permeable membrane, are also tissue engineering products being currently investigated for bone regeneration. Other regenerative therapies also in use in orthopedics include cell transplantation and delivery of osteoinductive molecules (or of the correspondent encoding genes), which differ from the former applications in that the cells/signaling molecules here are injected or placed in the host in the absence of a carrier or a matrix.<sup>43</sup>

#### **1.5. Cell-matrix bone constructs**

Bone tissue engineering may not require, as mentioned above, cell loading with *in vitro* expanded autologous cells, prior to implantation. In fact, bone loss due to trauma requires acute bone therapies, which do not comply with the time needed to acquire patient's marrow, to isolate and amplify marrow stromal cells and to subsequently

induce an osteogenic phenotype prior to cell transplantation.<sup>5</sup> Yet, cell seeding with autologous osteogenic cells prior to implantation results in enhanced bone formation *in vivo*, as compared to the use of acellular scaffolds.<sup>45</sup> Moreover, it is known that a layer of an *in vitro* formed bonelike tissue on a scaffold accelerates bone formation *in vivo*, as compared to scaffolds loaded with osteogenic cells just before implantation.<sup>45,46</sup> Thus, the most commonly discussed bone tissue-engineered strategy aims at creating a tissue-engineered substitute for autologous bone graft, which involves isolating and expanding bone marrow stromal/mesenchymal stem cells in culture, seeding these cells onto a preformed scaffold, and culturing for a sufficient time to induce osteogenic differentiation and extracellular matrix production. The elaboration of such cell-matrix bone constructs requires cells, supporting structures and molecular signals (Fig. 4). The assembly of these components must meet the needs of the specific repair sites, and take into account patient variables of age, gender, health, systemic conditions and activity.<sup>40</sup>

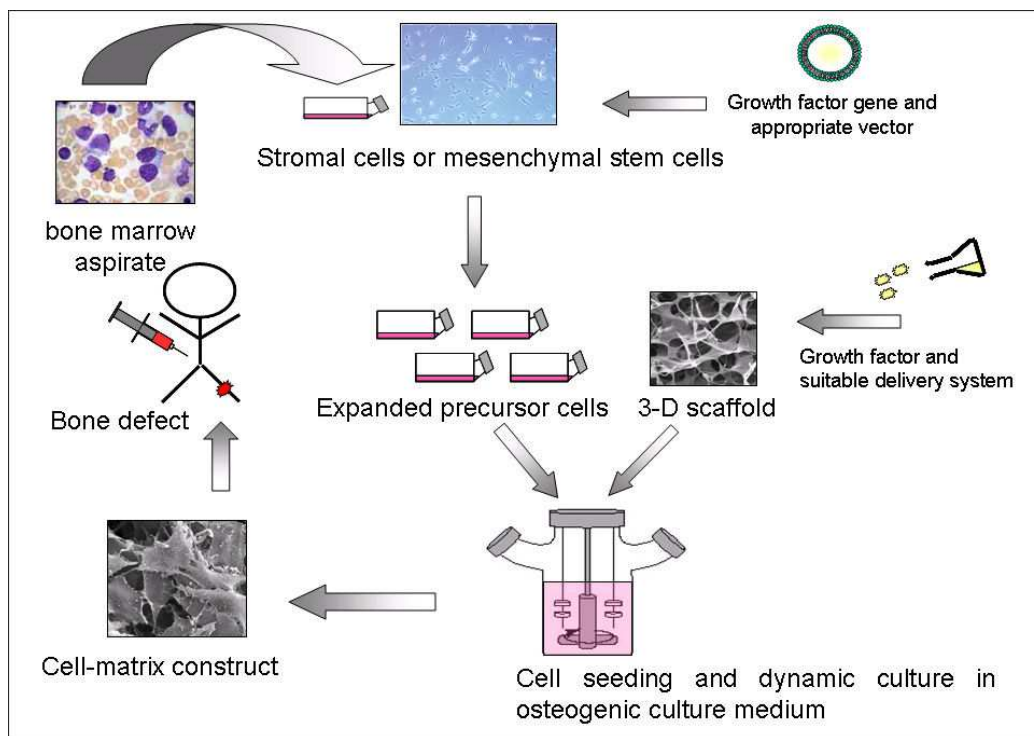


**Figure 4.** Schematic overview of a cell-based bone regenerative strategy, in which a 3-D scaffold is used as a temporary support. This approach involves harvesting of the patient's marrow, isolation and *in vitro* expansion of osteoprecursor cells, cell seeding in a 3-D

scaffold, and culturing for a sufficient time to induce osteogenic differentiation and extracellular matrix production. At the end of this period, the resulting cell-matrix construct is implanted in the same patient, leading to fully functional bone. Both bone marrow stromal cells and mesenchymal stem cells can be used as source of osteoprecursor cells. This approach may involve the use of growth factors, to encourage stem cells and inducible osteoprogenitors to progress along the osteogenic lineage. Growth factors can be delivered by transfecting cells with a growth factor gene, or by use of a controlled delivery system such as polymeric microspheres.

### 1.5.1. Cells

Bone marrow stromal cells (BMSCs) remain the most widely used source of osteogenic cells in bone tissue engineering studies.<sup>2</sup> Stromal cells were initially used to provide an appropriate environment for the growth and differentiation of nonadherent hematopoietic cells, *in vitro*. They form an adherent cell layer in long-term bone marrow cultures, and consist of a heterogeneous population of non-hematopoietic cells of mesenchymal origin and fibroblastic appearance. Monocytes, macrophages and endothelial cells are also present in the adherent layer as contaminants but their presence is reduced after passaging, by lack of adherence.<sup>47</sup> The starting bone marrow cell population comprises a small number of committed osteoprogenitor cells capable of bone formation *in vitro* (i.e. <1% of the stromal cell layer in rat bone marrow cultures), and a population of inducible osteogenic precursor cells that requires the presence of differentiation stimuli to progress along the osteogenic pathway.<sup>48</sup> As the number of committed and inducible osteoprogenitor cells within bone marrow is limited, and decreases with age, their *in vitro* amplification is required prior to scaffold cell seeding. Following *in vitro* expansion, cells are seeded into a 3-D matrix and either directed *in vitro* into the osteogenic lineage prior to implantation, to accelerate bone regeneration, or transplanted into the patient in an undifferentiated state to differentiate into osteogenic cells in the presence of local signaling molecules, *in vivo*.<sup>2</sup>

Osteogenic differentiation *in vitro* is promoted by using an osteogenic culture medium, which includes appropriate levels of dexamethasone, a synthetic glucocorticoid added to enhance osteogenic differentiation of inducible osteoprecursors, fetal calf serum, a source of organic phosphate to facilitate matrix

mineralization, as well as ascorbic acid, required for the hydroxylation of proline which is required for the stabilization of collagen triple helix. Current bone marrow culture protocols are still based on the protocol developed by Maniopoulos et al.<sup>49</sup>

With the emerging of stem cell biology, mesenchymal stem cells are being increasingly used in bone tissue engineering. These are usually obtained from bone marrow aspirates after purification by density centrifugation and sorting with antibodies against cell surface markers, followed by *in vitro* expansion.<sup>50</sup> Embryonic stem cells constitute an alternative to adult mesenchymal stem cells, but their use is still limited due to ethical issues. Moreover, through gene therapy technology, cells can be genetically modified to over-express osteoinductive factors.<sup>51,52</sup>

Primary osteoblast-like cells isolated from periosteum are not presently used for bone tissue engineering. They have a reduced ability to proliferate as compared to mesenchymal stem cells as they are fully differentiated, and have a tendency to dedifferentiation in culture. Still, they constitute a valuable option for the study of cell-material interactions, together with some transformed cell lines.<sup>5</sup>

### **1.5.2. Signaling molecules**

Inclusion of growth factors/hormones within tissue engineered constructs mimics the natural tissue environment and presumably leads to improved healing. Stem cells and inducible osteoprogenitors can be encouraged to progress along the osteogenic lineage pathway if appropriate signaling molecules are available in a proper dose. Moreover, implant vascularization (by assembly of capillaries from undifferentiated endothelial cells or from the sprouting of preexisting blood vessels) can also be promoted if angiogenic factors are present. Presently, the following classes of extracellular signaling molecules are currently considered for bone tissue engineering: Transforming growth factor beta (TGF- $\beta$ ), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF) and vascular endothelial growth factors (VEGFs).<sup>34</sup> Their biological mechanisms, as well as the rationale for their use in tissue-engineered therapies were recently reviewed.<sup>34,53</sup>

Growth factors have a short active half-life (i.e. minutes). In order to insure temporal and spatial accessibility to specific cell receptors growth factors are not usually

released in a bioactive state, but sequestered within the interstitium or in circulation, associated with specific binding proteins or ECMs. Therefore, controlling the location, concentration, receptor accessibility and sequestration of a growth factor can markedly increase scaffold osteoinduction.<sup>34,52</sup> Controlled growth factor release strategies have included encapsulation within polymeric microspheres or nanoparticles, non-specific adsorption to polymeric surfaces, and entrapment within injectable gels, among others. Covalently binding of growth factors allows the establishment of spatial gradients of growth factors, which can be used to promote cell migration, a phenomenon also known as haptotaxis. As an advantage, surface-bound factors frequently exhibit higher activity when compared to their diffusible analogs.<sup>40</sup> Gene therapy is a promising alternative approach to provide a local sustained release of osteoinductive factors.<sup>51</sup> This approach involves the transduction of genes encoding signaling molecules to cells, both *in vitro* and *in vivo*, using a suitable vector.

### **1.5.3. Supports**

Bone cells are anchorage dependent cells and require the presence of a substrate to retain their ability to proliferate and perform their specific function. Scaffolds for bone regeneration serve therefore as templates for cell anchorage and apposition of bone extracellular matrix, providing the structural support to the newly formed tissue.

The ideal scaffold should fulfill a number of requirements: (1) ability to retain cells and guide bone formation namely attachment, spreading, proliferation and differentiation of osteoprogenitor cells; (2) ability to support vascular invasion; (3) large surface area to volume ratio to allow cell attachment and proliferation; (4) degradation rate in tune with the rate of bone formation; (5) biodegradability by simple hydrolysis or enzymatic degradation; (6) biocompatibility (i.e. the scaffold or its degradation by-products should not trigger an adverse inflammatory reaction or immune response once implanted); (7) mechanical properties similar to those of the bone repair site; (8) sterilizability; (9) be easily processed into three-dimensional shapes to accurately fill specific defects and, (10) ability to carry signaling molecules such as growth factors.<sup>42,54</sup> Factors such as surface properties, porosity and pore size, degradation process, all contribute to the overall scaffold performance.

Surface properties such as chemistry, roughness, and surface energy play a major role in cell-material interactions, particularly when considering an absorbable material, which is always presenting a new surface. These surface characteristics determine not only how biological molecules will adsorb to the surface, but particularly their spatial orientation upon adsorption. This is of particular importance in host-implant interactions, as the adsorption of proteins present in physiological fluids such as albumin, immunoglobulins, fibrinogen, and fibronectin, dictate the subsequent inflammatory response and the fate of the implant. *In vitro*, osteoblast adhesion to biomaterials, spreading, and gene expression are much dependent on the initial adsorbed layer of glycoproteins containing Arg-Gly-Asp (RGD) domains such as type I collagen, fibronectin (FN), and vitronectin, present in serum or secreted by the cells. Osteoblast adhesion to biomaterials involves an initial cell attachment phase, implying short-term events of physico-chemical nature established between the materials surface and the cells (such as ionic bindings and van der Waals forces), and an adhesion phase occurring in the longer term, involving the binding of cell-surface molecules namely integrins and proteoglycans to ECM proteins.<sup>55</sup> Integrin binding induces the rearrangement of F-actin fibers leading to cell anchorage and spreading,<sup>56</sup> and triggers signals that direct subsequent cellular responses, including proliferation,<sup>57</sup> and differentiation.<sup>17</sup>  $\beta_1$  integrins represent the dominant adhesion mechanism to extracellular matrix ligands, and consequently, biomaterials.<sup>58</sup> Integrin expression in bone derived cells is dependent on the cell type,<sup>59</sup> and increases with ligand surface concentration.<sup>60</sup>

Many studies point out the importance of surface chemistry to protein adsorption and conformation, and subsequent osteoblast adhesion, spreading, or differentiation.<sup>16,61,62</sup> Recent studies using self-assembled monolayers (SAMs) of alkanethiols on Au exposing different functional groups provide an insight into the relationship between surface chemistry, Fn adsorption, and osteoblast adhesion.<sup>63-65</sup> According to Keselowsky et al.,<sup>63</sup> surface chemistry influences the adsorption kinetics of Fn and alter its conformation and activity following the trend (surfaces from most to least altered):  $\text{CH}_3 > \text{NH}_2 = \text{COOH} > \text{OH}$ . In addition, SAMs exposing OH groups present a more favorable conformation for  $\alpha_5\beta_1$  integrin binding and osteoblast adhesion than SAMs exposing  $\text{CH}_3$  groups. Surface charge also influences the response of osteogenic cells, *in vitro*. Shelton et al. showed that surface charge modulates protein

adsorption from serum and the migratory morphology of osteoblasts.<sup>66</sup> In another work, Sayer et al. showed that positively charged surfaces enhance cell attachment of BMSCs, but decreases cell spreading and differentiation, when compared to negatively charged surfaces.<sup>67</sup> Surface energy is also believed to influence protein adsorption, and as a consequence, cell adhesion, the latter being usually better on moderately hydrophilic surfaces, as compared to hydrophobic ones.<sup>68</sup> Recently, Lim et al. showed that osteoblastic cells cultured on hydrophobic substrates express lower levels of  $\alpha_v$  and  $\beta_3$  integrin subunits than do cells on hydrophilic substrates, pointing out the importance of surface wettability in integrin-mediated bone cell adhesion.<sup>69</sup> However, when osteoblasts were cultured on glass surfaces modified with different functional groups, cell attachment and spreading varied with surface chemistry rather than with wettability.<sup>70</sup> Cells also react differently according to surface roughness. In the case of samples with micrometer scale topography, cell adhesion, spreading and proliferation of human bone cells are generally enhanced on smooth samples as compared to rough and less organized ones.<sup>71,72</sup> In the case of samples with nanometer scale topography, roughness is reported either to inhibit cell proliferation,<sup>73</sup> or to induce merely a delay in cell spreading.<sup>74</sup>

Due to the influence of surface properties, surface modification is often used as a tool to render polymers more suitable for *in vitro* cell attachment and proliferation of osteogenic cells. Several approaches have been used to modify the surface characteristics of biomaterials, among them the following:

(1) Surface grafting of chemical functionalities. The incorporation of chemical moieties at the surface is a well known strategy to improve cell attachment to polymeric surfaces, which are often too hydrophobic. This is the case of polystyrene substrates used for tissue culture, which are usually treated by glow discharge or exposure to sulfuric acid, to increase the number of charged groups at the surface.<sup>68</sup> Functional groups are also incorporated in order to provide chemical sites for post-reactions, namely to induce apatite nucleation, to enhance the adhesive strength of apatite coatings, or to allow the binding of bioadhesive motifs, approaches that are discussed in more detail below. Gas plasma treatments are among the more versatile techniques available, as they allow the introduction of a wide range of functional groups.<sup>75</sup> Although these

have been essentially applied to functionalize 2-D matrices, they have been also successful used for the surface modification of 3-D scaffolds.<sup>76</sup>

(2) Deposition of an apatite-like layer. The deposition of a calcium phosphate layer is often desirable to enhance osteoconductivity and bone-bonding to implants.<sup>77</sup> The presence of a bone-like mineral layer on polymeric scaffolds was shown to enhance bone regeneration,<sup>78</sup> as well as to improve mechanical integrity of the constructs.<sup>79</sup> However, the effect of CaP mineral substrates on the proliferation and osteogenic differentiation of osteoprogenitor cells vary with both the structure and the composition of the particular CaP mineral,<sup>80-84</sup> despite calcium phosphates were shown to be effective carriers for stem cell transplantation.<sup>85</sup> Several approaches have been developed to induce the deposition of such films. Tanahashi et al.<sup>86</sup> proposed a calcium phosphate coating method based on the immersion of non-mineralizing polymeric substrates wrapped with CaO-SiO<sub>2</sub>-based glass grains in simulated body fluid (SBF), with ion concentrations approximately equal to those of human blood plasma, at physiological temperatures. The grafting of negatively charged functionalities, such as phosphate groups, which are effective in inducing apatite nucleation, is also an efficient method to induce bone-mineral-like deposition in SBF.<sup>87-90</sup> Recently, Hartgerink et al. were able to direct mineralization of hydroxyapatite on a nanostructured fibrous scaffold, prepared by self-assembly of peptides, similarly to what is observed with collagen fibrils and hydroxyapatite crystals in bone.<sup>91</sup>

(3) The so-called biomimetic strategies. These are aimed at avoiding the non-specific protein adsorption, and include the precoating with adhesive proteins,<sup>92,93</sup> and the binding of bioadhesive motifs such as linear and cyclic RGDs peptides,<sup>94,95</sup> heparin-binding peptides,<sup>96</sup> collagen-mimetic peptides,<sup>97</sup> and recombinant fragments of Fn.<sup>98</sup>

In terms of structure, scaffolds for osteogenesis should mimic bone morphology and structure, in order to optimize implant integration into surrounding tissue.<sup>99</sup> Therefore, the natural open-pore geometry of trabecular bone structure is often the structure envisaged while designing scaffolds for bone tissue engineering. Porous scaffolds with uniformly distributed and interconnected pores are desirable for cellular colonization. Porosity and pore size of 3-D scaffolds play a critical role in bone

formation both *in vitro* and *in vivo*, and this was the subject of a recent review.<sup>99</sup> In this review, exact values of porosity and pore size are not suggested by the authors as a general guide for 3-D supports, due to the wide range of application sites and to the diversity of available biomaterials. Highly porous structures are desirable as they permit a spatially uniform cell distribution during cell seeding, facilitate diffusion of oxygen and nutrients, and maximize space for cell attachment, growth, ECM production, and vascularization. *In vivo*, highly porous scaffolds were shown to enhance bone ingrowth and osseointegration of the implants, although *in vitro* osteogenesis was not found to be significantly influenced by porosity. In terms of pore size, pore sizes >300  $\mu\text{m}$  are recommended, as they were shown to favor bone ingrowth and implant vascularization *in vivo*. Smaller pores can lead to osteochondral ossification, associated to poor oxygenation.<sup>99</sup> Still, the optimum pore size remains a controversial matter, and basically this is highly dependent on the material used as support. *In vitro*, the pore size may also be critical for cell migration and scaffold colonization, particularly when cells are dispensed dropwise onto the surface. Small pores prevent cellular penetration and matrix elaboration within the pores leading to pore bridging and occlusion of the surface pores by cells.<sup>2</sup> In addition to porosity and pore size, pore interconnectivity is also important for bone ingrowth throughout the implant. A highly interconnected macroporosity is desirable for cell migration. In the absence of this, bone grows preferentially along the implant-host interface.<sup>2</sup> The diameter of pore interconnections is at least as important as the effective pore size, and pore interconnections as small as 20  $\mu\text{m}$  were reported to be efficient for *in vivo* bone ingrowth.<sup>100</sup> Although increased porosity and pore size are desirable for bone ingrowth, the result is a reduction in mechanical properties which can compromise the structural integrity of the cell-matrix construct. As a consequence, a balance must be reached depending on the specific site of application, rate of degradation, and rate of bone formation.

The materials used in the fabrication of scaffolds for bone tissue engineering, include polymers (naturally-derived or synthetic), ceramics, composites of these two, and bone-derived materials. Materials used are: (1) demineralized bone matrix (DMB); (2) naturally-derived polymers such as collagen, hyaluronic acid, fibrin, silk fibroin, and chitosan; (3) synthetic polymers including the homopolymers and copolymers of poly(lactide acid), poly(glycolide acid), and poly(lactide-co-glycolide acid,

polycaprolactones, tyrosine-derived polycarbonates, polyanhydrides and poly(propylene fumarate), (4) ceramics such as hydroxyapatite, tricalcium phosphates, calcium metaphosphate, coral-derived products, Bioglass<sup>®</sup>, and glass-ceramics; and (5) composites.<sup>42,54,101</sup>

Polymers, in particular, are very versatile materials, as by altering composition and physical properties such as molecular weight, polydispersity, crystallinity, and thermal transitions, they can be prepared in a wide range of degradation rates and mechanical properties. Naturally derived polymers offer several advantages when compared to synthetic polymers, namely biocompatibility, biodegradability, and biological activity, as most of them are present in structural tissues of living organisms. However, the low mechanical strength and high rates of degradation of natural polymers often result in their use in combination with ceramics, or in subsequent cross-linking reactions to reduce the degradation rates. Synthetic polymers can be tailored to meet an absorption time requirement, potentially facilitate reproducibility and scale-up, and offer no concerns of disease transmission, which often constitute a problem with naturally occurring polymers. Among these, PLA, PGA, and PLGA, which have been used for decades as biodegradable sutures and fixation devices with long-term success, are the most common. However, scaffolds based on these synthetic polymers present some limitations, namely the burst release of high amounts of acidic degradation by-products, at later stages of their degradation. Ceramics have been extensively used in the treatment of bony defects owing to their similarity to the inorganic phase of bone. The appeal of the calcium phosphates rests largely with their biocompatibility, osteoconduction ability, as well as a remarkable ability to bind directly to bone.<sup>102</sup> However, they usually present poor degradation properties, when compared to polymers. Composites of polymers with ceramics combine the advantageous features of polymers and ceramics, and allow a readily controlled biodegradation rate.<sup>103</sup> Matrices formed directly from genetically engineered polypeptides, containing biological active motifs, are also promising materials, as they allow the scaffold to mimic the extracellular matrix.<sup>104</sup>

Various materials have been evaluated as preformed supports for the growth and differentiation of osteogenic cells, including demineralized bone matrix (DMB),<sup>105</sup> biopolymers,<sup>106,107</sup> synthetic polymers,<sup>108,109</sup> ceramics,<sup>110</sup> and composites.<sup>111</sup> Some

have been shown to result in bony union of critical-sized defects and restoration of bone function, following seeding with autologous marrow-derived cells.<sup>45,50,112</sup>

Finally, the final tissue-engineered construct should have mechanical properties comparable to those of the implantation site. However, the use of external or internal fixators and patient weight-bearing management allows a reasonably well-controlled mechanical environment, and therefore, the strength and modulus of the cell-matrix construct does not necessarily need to be equal to that of bone.<sup>113</sup>

#### **1.5.4. Culture systems: static versus dynamic**

Three-dimensional cultures in tissue engineering lead to higher cell densities, when compared to monolayer cultures, requiring very frequent changes of medium to ensure nutrient supply and elimination of metabolic products.<sup>114</sup> In conventional static culture conditions, it is difficult to sustain long-term experiments without contamination and to promote the synthesis *in vitro* of bone matrix.<sup>2</sup> Although thin (<2mm) scaffolds can be cultured in standard petri dishes in static conditions, dynamic systems that permit continuous movement of the medium throughout the scaffold during the culture period are preferred.<sup>115</sup> Dynamic culture systems that supply a constant flow of fresh culture medium provide stabilized culture conditions and reduce risks of contamination, sustaining a more viable and metabolically active cell population. Besides sterile handling, only autologous serum from the patient should be used throughout the time of *in vitro* culture to exclude any viral infections or immune responses to foreign antigens.<sup>114</sup>

#### **1.6. Available tissue engineered bone products**

The worldwide market in 2002 for bone replacement and repair including grafts (autologous, allogeneic, and xenogeneic) and synthetic bone materials was estimated at about 300 million €. <sup>116</sup> The potential of bone tissue engineered products is therefore huge. For the time being, due to competing products and not sufficiently advanced technology in bone tissue engineering, this application field is focused on niche markets such as dental or maxillofacial surgery. At present only few tissue-engineering bone products are on the market. Most of them are acellular systems

combined with osteoinductive molecules, and not cell-matrix constructs. Most often, bone defects result from trauma and require acute treatments. The impossibility of expanding autologous cells in such short period, together with the increased costs associated with the complexity of cell-based tissue engineering approaches, possibly contributes to the reduced number of cell-biomaterial constructs presently available. An overview on the companies active on this field can be found elsewhere.<sup>116</sup> Products using osteoblasts or bone marrow cells combined with several biomaterials are currently being tested in clinical trials.<sup>116</sup>

## **2. Chitosan**

### **2.1. The emergence of chitin and chitosan**

The application of carbohydrate molecules to the development of biomedical products and therapeutic compounds has had a sharply increased level of activity in recent years, leading in part to the identification of Glycomics as a legitimate new area of biotechnology. Chitin is the most abundant polysaccharide produced by biosynthesis after cellulose, with an estimated yearly production of at least  $10^{10}$  T,<sup>117</sup> the marine crustaceans being estimated to produce  $1.56 \times 10^9$  T.<sup>118</sup> Chitin occurs as the equivalent to cellulose in plants, in certain fungi and yeasts as a cell wall material, frequently complexed with other polysaccharides, and in many invertebrate groups such as mollusks and arthropods as the fibrous support of the inorganic mineral phase of their exoskeleton, occurring as an alternative to collagen.<sup>119</sup> Chitin was first discovered by Braconnot, member of the Academy in Sciences of Nancy, France, in 1811, as the alkali-resistant fraction from isolates some higher fungi. The term chitin comes later, in 1823, when Odier found a material with the same characteristics in the cuticle of the cockchafer beetle, naming it “chitin”, after the greek word “chiton”, that denotes coat of mail. The discovery of chitosan, the major derivative of chitin, is attributed to Rouget in 1859, who reported that treatment of chitin with concentrated potassium hydroxide solution under reflux rendered chitin soluble in dilute solutions of organic acids. Despite its early scientific discovery, and being the second most abundant polysaccharide after cellulose, chitin remained in the realm of naturalists and as an almost unused biomass resource until the 1950's. Several factors contributed to this

delay. In contrast to cellulose, which is readily available from trees and cotton plants, sources of chitin such as the exoskeletons of crustaceans and insects are more difficult to explore. In addition, in spite of its structural similarity with cellulose, chitin is much less reactive to many chemicals due to the presence of strong intra and inter-chain hydrogen bonds, being insoluble in most of the organic and inorganic solvents known for cellulose. With the advent of the canning industry, and in particularly of the crab and shrimp processing industry, the accumulation of the shellfish waste led to the need of exploring this new resource.<sup>117,120</sup>

## 2.2. Chitin/chitosan structure

Chitin is a linear, high molecular weight, crystalline polysaccharide, theoretically comprised entirely by *N*-acetylated D-glucosamine units, in a  $\beta$ -(1-4) linkage. However, naturally occurring chitin contains different proportions of *N*-glucosamine units, dependent on the source, and in practice this biopolymer exist as a co-polymer (with exception to chitin obtained from diatoms spines).<sup>120</sup> In chitin, the chains are arranged in sheets or stacks, the chains of each sheet having the same direction or sense and being bonded through intrasheet hydrogen bonds between two adjacent chains. Chitins are biosynthesized by chitin synthetases and have been found in three polymorphic forms,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chitin, which differ in the arrangement of chains within the crystalline regions. In  $\alpha$ -chitin, which is the one found in crab, lobster, and shrimp shells, adjacent sheets have opposite directions, and thus an antiparallel chain arrangement. In  $\beta$ -chitin, which is the one occurring in the pen of the squid *Loligo* and in pogonophore tubes, adjacent sheets have the same direction, and thus a parallel chain arrangement. In  $\gamma$ -chitin every third sheet has the opposite direction to the preceeding two sheets. In addition to intrasheet interchain hydrogen bonds,  $\alpha$ -chitin also presents intersheets hydrogen bonds between adjacent chains. In fact these intersheet bondings are responsible for the lack of swelling in water of  $\alpha$ -chitin whereas  $\beta$ -chitin swells readily in water and forms hydrates.<sup>120</sup>  $\alpha$ -Chitin is believed to be more stable than  $\beta$ -chitin, as the later converts to  $\alpha$ -chitin upon treatment with concentrated HCl, but not in the reverse way.<sup>121</sup>

The principal derivative of chitin is chitosan, usually obtained by alkaline deacetylation of chitin. Chitosan is thus a linear co-polymer of D-glucosamine and

*N*-acetyl D-glucosamine in a  $\beta 1 \rightarrow 4$  linkage (Fig. 5). Chitosan is also crystalline, but as compared to chitin, presents a longer distance between adjacent chains belonging to the same sheet, due to the removal of the N-acetyl groups during the conversion from chitin to chitosan, which hold together adjacent chains through C(2)N-H $\cdots$ O=C(7) hydrogen bonds.<sup>120</sup>

In chitin terminology, the molar fraction of *N*-acetylated units is termed the degree of acetylation (DA), expressed in percentage, or most recently, fraction of *N*-acetylated units ( $F_A$ ).<sup>122</sup> Instant differentiation between chitin and chitosan can be attained from their solubility. Chitin is soluble in *N,N*-dimethylacetamide (DMAc) in the presence of 5-10% (w/v) lithium chloride and insoluble in dilute acid solutions, while the reverse is true for chitosan.<sup>117,120</sup> As a DA around or lower than 50% is usually required for chitosan solubility in dilute acidic solutions, the term chitosan is applied both to fully deacetylated chitin and partially deacetylated chitin with DAs  $\leq 50\%$ .

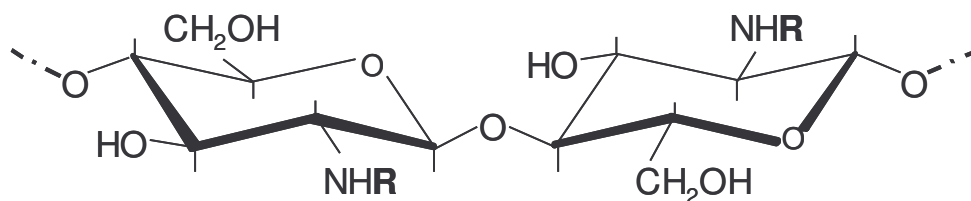


**Figure 5.** Chemical structure of chitosan. Glucosamine (R=H) is the predominant repeating unit. The acetylated repeating units, *N*-acetylglucosamine (R=COCH<sub>3</sub>) are randomly distributed.

### 2.3. Chitosan: solution properties

Chitosan is neither soluble in water nor in organic solvents. However, after protonation of amine functionalities from glucosamine units by acid, the electrostatic repulsions between  $\text{NH}_3^+$  groups lead to the destruction of interchain attractive interactions such as hydrogen bonds and hydrophobic interactions, and consequently, to chitosan solubility. At pH lower than its  $\text{pK}_a$ , which may range from  $\sim 6.5$  to  $\sim 7$ , chitosan is a polycation and at pH 4.0 and below, chitosan is completely protonated.<sup>123</sup> Chitosan solubility depends on chitosan charge density, which is tightly connected with structural parameters such as the DA, the chain length, and the

distribution of *N*-acetylated glucosamine units, as well as on environmental parameters such as pH, ionic strength, and the dielectric constant of the media.<sup>124</sup> The solubility range increases on increasing the DA, due to the increase of the steric hindrance related to the increase of the number of the acetyl groups, together with the increase of the intrinsic p*K*<sub>a</sub>. According to Sorlier et al.,<sup>123</sup> the intrinsic p*K*<sub>a</sub> of chitosan increases from 6.46 to 6.8 as the DA increases from 5 to 35%, respectively, revealing an increase of cationicity of amine functionalities on increasing the DA. In the presence of high ionic strengths, solubility is reduced. The high concentration of protons leads to the screening of the electrostatic interactions occurring between polymeric chains, with subsequent establishment of chain interactions, and polymer precipitation. As a result, chitosan is not soluble in strong acids such as hydrochloric acid solutions with molarities higher than 0.1M.<sup>124</sup>

In aqueous solutions, chitosan chains aggregate, higher DAs leading to an increased number of aggregates in solution,<sup>125</sup> which has been attributed to hydrophobic interactions occurring mainly between acetylated units.

## **2.4. Preparation of chitin and chitosan**

Currently commercially available chitin is prepared from the exoskeletons of crab, shrimp and prawn, obtained as waste from the seafood processing industry. In these, chitin is closely associated with proteins, inorganic material (mainly CaCO<sub>3</sub>), pigments and lipids. Deproteinization and demineralization are generally carried out by treatment with 1 to 2M NaOH at 70°C or higher temperature, and 1.25M HCl at room temperature, respectively, deproteinization being usually done prior to demineralization. Both treatments may lead to the cleavage of chitin polymeric chains. In this sense, a number of alternative methods have been envisaged in order to minimize the hydrolysis of glycosidic linkages during chitin extraction, including the use of proteolytic enzymes to remove protein and EDTA at alkaline pH values to remove mineral. Finally, the pigments present in the exoskeletons of crustacean (mainly carotenoids) can be extracted with ethanol, acetone, or oxidizing agents such as KMnO<sub>4</sub>.<sup>120</sup> The preparation of squid chitin although similar occurs under milder conditions, as β-chitin is composed exclusively of chitin and proteins, with only traces of metal salts.<sup>126</sup>

## 2.5. Chitin deacetylation

Deacetylation may be carried out under acid or basic conditions, but basic conditions are preferred, due to the susceptibility of chitin glycosidic linkages to acid hydrolysis. The deacetylation of  $\alpha$ -chitin is usually carried out using strong aqueous bases at 90°C-150°C for a few hours, to give chitosan with a DA between 5 to 30%.<sup>120,122,127</sup> High reaction temperatures reduce the time required for deacetylation, but result in increased hydrolysis of polymeric chains. Deacetylation of chitin proceeds rapidly in 50% (w/v) aqueous NaOH at 100°C during the first hour of alkali treatment, but extension of the reaction time results rather in chain hydrolysis than in significant deacetylation.<sup>120</sup> To obtain chitosans with low DAs (<15%), multistep alkali treatments with interspersed washing are required.<sup>128</sup> The washing with water in-between treatments induces the swelling of chitin chains, and increases the accessibility of chitin for subsequent deacetylation. In order to reduce chain cleavage during alkaline deacetylation, reactions are carried out at as low temperature as possible, under inert atmosphere such as nitrogen or argon, or in the presence of oxygen scavengers or reducing agents, such as NaBH<sub>4</sub>. As chitin is not soluble in such systems, deacetylation occurs under heterogeneous conditions. During deacetylation of  $\alpha$ -chitin, NaOH acts initially on the amorphous regions of the polymer, and only afterwards on the crystalline regions. Heterogeneous deacetylation leads therefore to a block distribution of acetylated units, rather to a random distribution of the same. As a result, the characteristic IR bands attributed to crystallization of blocks of poly [ $\beta$ -(1 $\rightarrow$ 4) D-glucosamine] are only perceptible for DAs  $\leq$  9%, due to the later deacetylation of segments originally present in the crystalline region of chitin.<sup>120,128</sup> As compared to  $\alpha$ -chitin,  $\beta$ -chitin (present in the squid pen) is much easier to deacetylate, due to the absence of intersheet hydrogen bonding, which make crystalline regions more accessible to reactives.<sup>127</sup> During deacetylation of  $\beta$ -chitin, the crystallinity decreases rapidly compared to that of  $\alpha$ -chitin, and a completely amorphous product is obtained.<sup>129</sup>

In the present thesis, squid pen chitosan was deacetylated in 50% (w/v) NaOH under N<sub>2</sub>, using successive 1h alkali treatments, three at 65°C and one at 100°C, with interspersed washing in hot water, according to Mima et al.<sup>128</sup>

## 2.6. Chitosan *N*-acetylation

The acetylation of chitin/chitosan was recently reviewed.<sup>127</sup> Chitosan *N*-acetylation is usually carried out under homogeneous conditions, which involves the dissolution of chitosan in a diluted acidic solution, prior to adding a co-solvent and the acetylating reactive. The method used in the present thesis is based on the method developed by Hirano et al.,<sup>130</sup> in which chitosan acetylation is performed by reacting chitosan amine functionalities with acetic anhydride at room temperature, in an aqueous, methanolic acetic acid solution. This reaction is carried out at room temperature, and hydrolysis of polymeric chains was not reported. Acetyl groups are randomly introduced, leading to a homogeneous product. Esterification of hydroxyl groups is also likely to occur, and these are removed by treatment with 0.5M ethanolic KOH.

*N*-acetylation is useful when chitosan samples with different DAs but same average molecular weight are required, as it is the case of studies focused on the effect of only one of the two parameters, DA or average molecular weight. Such samples are prepared from an almost fully-deacetylated chitosan, which is subsequently acetylated by adding a variable volume of acetic anhydride, so that different values of DA are obtained.<sup>131</sup> Despite being the ideal approach for such studies, this strategy involves deacetylation of the original chitosan prior to homogeneous *N*-acetylation, which besides being laborious, leads to a substantial decrease of the molecular weight.

## 2.7. Chitosan characterization

The degree of *N*-acetylation (DA) and the average molecular weight are the characteristics most frequently assessed. For pharmaceutical and medical applications, the characterization of chitosan in terms of toxic heavy metals and residual protein is also of crucial importance. As a consequence, standards for chitosan use in these applications were proposed.<sup>132</sup>

### 2.7.1. Degree of acetylation

The DA is a structural parameter influencing charge density, crystallinity, solubility, as well as chitosan propensity to enzymatic degradability. Therefore, its accurate

determination is of major importance. A number of methods have been developed for the determination of the DA of chitosan, including colloid titration with a polyanion<sup>133</sup>, dye adsorption,<sup>134</sup> and spectroscopies such as ultraviolet (UV),<sup>135</sup> infrared (IR)<sup>136</sup>, and liquid/solid state nuclear magnetic resonance (NMR).<sup>137,138</sup> The advantages and drawbacks of each technique have been discussed.<sup>139</sup> Among these, <sup>1</sup>H NMR is considered the more accurate technique for the determination of the DA of chitosan, and IR spectroscopy the most frequently used one, due to its simplicity associated with accuracy. In the present study, FT-IR spectroscopy was used instead of <sup>1</sup>H NMR, as the later requires complete dissolution of the sample prior to analysis, which was not possible in the case of one of the samples.

The use of FT-IR spectroscopy for the determination of the DA is based on the variation of an absorbance band characteristic of *N*-acetyl groups, as a function of an internal reference band. The DA value can be extrapolated from a calibration curve established using an absolute technique, such as NMR. Again, a number of different methods have been proposed, differing in terms of the analytical and reference IR bands used, as well as in term of the baselines used for the determination of the correspondent absorbance values.<sup>140</sup> One of the more frequently used is the method of Baxter et al.<sup>141</sup>, which uses the amide I band at 1655 cm<sup>-1</sup> as the analytical band (using the baseline originally proposed for use with the  $A_{1655}/A_{2867}$  ratio) and the hydroxyl band at 3450 cm<sup>-1</sup> as the internal reference band. The sample has therefore to be perfectly dry and the IR spectra immediately recorded. The DA is determined as follows:

$$DA(\%) = (A_{1655}/A_{3450}) \times 115.$$

This relation was validated by dye adsorption, for DAs comprised between 0 and 55%.

More recently, Brugnerotto et al.<sup>142</sup> proposed the use of the amide III band (C-N stretching coupled with N-H in plane deformation) at 1320 cm<sup>-1</sup> as the analytical band and the band at 1420 cm<sup>-1</sup> as the internal reference band. The authors analyzed chitin/chitosan samples covering the entire range of DAs, and used samples from different sources, including squid pen. A very good linear correlation between the ratio of absorbance bands ( $A_{1320}/A_{1420}$ ) and the experimental values obtained from

NMR was found in all the range of DA values, which could be expressed by the following relation:

$$A_{1320}/A_{1420} = 0.3822 + 0.03133 \times \text{DA} (\%).$$

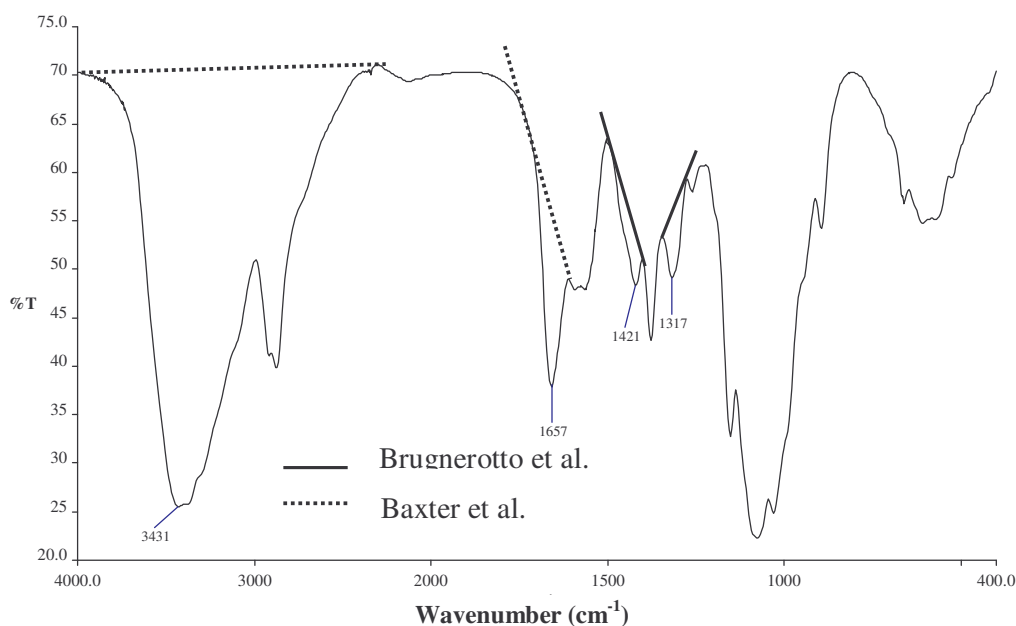


**Figure 6.** Squid pen chitosan IR spectra, showing the baselines proposed by Brugnerotto et al.<sup>142</sup> and Baxter et al.<sup>141</sup> for the determination of the  $A_{1320}/A_{1420}$  and  $A_{1655}/A_{3450}$  ratio, respectively.

Brugnerotto et al. found superior agreement between the experimental and estimated DA values using this ratio of absorbance bands than using those involving the band at  $3450\text{ cm}^{-1}$ . However, as  $\beta$ -chitin samples presenting DAs higher than 95% lied out of the linear correlation curve presented above, the authors concluded that this relation should not be used for  $\beta$ -chitin samples with DAs greater than  $\cong 60\%$ .

### 2.7.2. Molecular weight

Depending on its source and preparation procedure, chitosan molecular weight may range from 300 to over than 1 000 kD, squid pen chitosan presenting usually higher

molecular weight, as compared to chitosan obtained from the exoskeletons of crustaceans.<sup>143</sup> Several techniques can be used to determine the average molecular weight of chitosan. Among them are capillary viscometry, ultracentrifugation, and size exclusion chromatography coupled with light scattering.

Whatever the technique used, the tendency of the polymer chains to aggregate in solution constitutes a problem.<sup>120</sup> In this study, capillary viscometry was initially performed. Capillary viscometry allows the determination of the viscosity-average molecular weight, by applying the equation of Mark-Houwink:  $[\eta] = KM_v^a$ , where  $[\eta]$  stands for intrinsic viscosity and  $M_v$  for viscosity average molecular weight. The values of the coefficients  $K$  and  $a$  are previously determined from the double logarithmic plot of  $[\eta]$  values with molecular weight values measured by an absolute method. Viscometry was carried out according to Wang et al.<sup>144</sup>, using the Mark-Houwink coefficients determined by the same author, which take into account the DA of the samples.

High performance size exclusion chromatography coupled with light scattering was used as soon as it was available at INEB. Here, the analysis was carried out according to Terbojevich et al.<sup>145</sup>, using the system 0.2 M  $\text{CH}_3\text{COONa}$ /0.5 M  $\text{CH}_3\text{COOH}$  as mobile phase. The Mark-Houwink coefficients used to calibrate the system were those previously determined by the authors, and did not depend on the DA. In addition, as by light scattering, the weight-average molecular weight depends on the refractive index increment value ( $\text{dn}/\text{dC}$ ), a  $\text{dn}/\text{dC}$  value of 0.205 mL/g was used, which was the value determined by Terbojevich et al. in their study, for chitosan samples with DAs ranging from 15% to 42%, regardless the DA. However, other  $\text{dn}/\text{dC}$  values can be found in literature, including some that are dependent on the DA.<sup>146</sup>

## **2.8. Processing into films and porous scaffolds**

Chitosan is a very versatile material, being easily processed into solutions, fibers, gels, pastes, beads, films, microcrystalline powders, and sponges, without the use of toxic solvents.<sup>147</sup>

Chitosan has excellent film forming capabilities. Chitosan films are obtained from chitosan acidic solutions, by casting the resulting gel on a flat support surface followed by solvent evaporation.

Sponge-like porous matrices are usually obtained from chitosan acidic solutions by thermally-induced phase separation and subsequent sublimation of the ice crystals. During freezing, ice crystals nucleate from solution and grow along the lines of thermal gradients, resulting in a macroporous structure after sublimation of the ice crystals. By varying the freezing conditions, the pore size can be modulated from 1 to 250  $\mu\text{m}$ . Chitosan solution concentration also affects pore average diameter, but at a lesser extent, with smaller pores being formed at higher concentrations.<sup>148</sup> Recently, Ho et al.<sup>149</sup> proposed an alternative method for the preparation of chitosan scaffolds, in which freeze-drying (which is time and energy consuming) is not required. In this method, the frozen chitosan acidic solution is immersed in a sodium hydroxide aqueous solution containing ethanol, at a temperature lower than the freezing point of the polymer solution, in order to induce chitosan gelation. After gelation, the porous structures are dried at room temperature.

A rapid prototyping system was also developed. This involves the sequential dispensing of a sodium hydroxide solution and a chitosan acetic acid solution, and results in a gel-like chitosan strand.<sup>150</sup>

## 2.9. Chitosan biological properties

**Biocompatibility.** In 2001, VandeVord et al.<sup>151</sup> examined the biocompatibility of chitosan scaffolds (DA=0.08), using a mammalian implantation model. An early migration of neutrophils into the implantation area, which resolved with increasing implantation time, was reported. Besides this early accumulation of neutrophils, which are cells usually associated with acute inflammation, no evidence of other signs associated with an inflammatory response, such as erytema and edema were found. Endotoxins were not detected, and a chronic inflammatory response was not developed. In addition, a very low incidence of specific immune reactions was observed, as determined by lymphocyte proliferation assays, and antibody binding responses measured using ELISA techniques. Formation of normal granulation tissue associated with accelerated angiogenesis, the typical healing response, was observed. Taken together, the results from this study indicated that chitosan has a high degree of biocompatibility. These results are in accordance to previous studies,<sup>152</sup> and to the

study of Tomihata et al.,<sup>153</sup>, in which the biocompatibility of chitosan films with different DAs is reported, even for highly deacetylated chitin derivatives.

**Cytocompatibility.** A wide number of cells have been successfully cultured in/on chitosan matrices, among them keratinocytes,<sup>154</sup> chondrocytes,<sup>155</sup> osteoblasts,<sup>156</sup> endothelial cells?, hepatocytes,<sup>157</sup> and Schwann cells,<sup>158</sup> indicating the cytocompatibility of chitosan towards these cells. In addition, no cytotoxic products are released from chitosan matrices, as shown by the maintenance of cell metabolic activity.

**Bacteriostatic and fungostatic properties.** Due to electrostatic interactions of chitosan cationic amino groups with anions on the bacterial wall, chitosan has an intrinsic antibacterial activity, inhibiting their proliferation.<sup>159</sup>

**Enzymatic degradation.** Chitosan is susceptible to hydrolytic action of lysozyme and *N*-acetyl- $\beta$ -D-glucosaminidase *in vivo*, being mainly depolymerized by lysozyme in human serum.<sup>160</sup> Lysozyme is present in the human serum, saliva, and other fluids, including that of surrounding cartilage, where it acts as an endohydrolase, catalysing the hydrolysis of  $\beta$ -(1-4) glycosidic linkages between *N*-acetylglucosamine units. The oligomers released activate macrophages and monocytes, inducing the production of diffusible molecules such as interferon, TNF- $\alpha$ , and interleukin-1, as well as the production of *N*-acetyl- $\beta$ -D-glucosaminidase, which further catalyzes the depolymerization of chitosan into monomeric units. These monomers can later be incorporated in hyaluronate, keratan sulphate and chondroitin sulphate.<sup>161</sup> The degradation rate increases on increasing the degree of acetylation, both *in vitro* and *in vivo*,<sup>153,162</sup> and highly deacetylated forms may last several months *in vivo*.<sup>151</sup> According to Aiba et al.,<sup>163</sup> three consecutive *N*-acetyl-D-glucosamine residues are apparently required for chitosan lysozymic hydrolysis.

**Immunoadjuvancy.** Chitosan is chemotatic for neutrophils, which has been attributed to specific interactions of chitosan or its oligosaccharides with neutrophils receptors such as selectins.<sup>151</sup> In addition to being chemotatic to neutrophils, chitosan shows a biological aptitude for activating macrophages for tumouricidal activity and for production of interleukin-1.<sup>164</sup> as well nitric oxide.<sup>165</sup> The immunoadjuvant properties of chitosan have been attributed to the *N*-acetylglucosamine (NAGA) units of the chitosan, rather than to the glucosamine units. Macrophage activation appears

to be dependent on binding of NAGA to specific cell membrane receptors, namely involved in the binding of mannose- and NAGA-glycoproteins.<sup>165</sup> Therefore, chitosan has been proposed to be used as an immunoadjuvant, to be administered to hosts whose immunity is depressed.

**Hemostatic and blood clotting properties.** Chitosan is a powerful hemostatic agent<sup>152</sup> and induces blood clotting, even in the presence of extensive anticoagulation therapy.<sup>166</sup> Blood clotting was suggested to be related to the possible formation of polyelectrolyte complexes between chitosan amino functionalities and negatively charged acidic groups present at the surface of erythrocytes. Recently, Benesch et al.<sup>167</sup> suggested that the procoagulant activity of chitosan could be related to the ability of chitosan to bind fibrinogen. In contrast, chitin displays anticoagulant properties, increasing upon *O*-sulfation,<sup>168</sup> which is attributed to its similarity to heparin, a naturally occurring sulfated glycosaminoglycan used as anticoagulant agent in clinic.

**Cell-binding properties.** Chitosan is able to agglutinate a variety of mammalian cells in suspension. Cell adhesion to chitosan is attributed to non-specific electrostatic interactions occurring directly between protonated amine groups from glucosamine units and negatively charged carboxylate and sulphate groups found in cell surface proteoglycans.<sup>154,169</sup> Close to the physiologic pH, the majority of chitosan ammonium groups are dissociated and subsequently uncharged. Still, the presence of a small amount of non-dissociated ammonium groups in chitosan chains is sufficient to provide enough cationic sites and allow the establishment of electrostatic interactions.<sup>155</sup>

**Wound healing properties.** Chitin-based products are well known for their ability to improve ordered regeneration of soft connective tissues.<sup>170</sup> Chitosan have been found to accelerate dermal wound healing and inhibit fibroplasia, leading to the attainment of a good-looking healing skin surface. This behavior has been related to chitosan ability to activate macrophages for cytokine production (TGF- $\beta$ 1, FGF, PDGF), upon hydrolysis by lysozyme.<sup>171</sup> Moreover, Howling et al.<sup>172</sup> suggested that chitosan may interact with growth factors present in serum potentiating their effect, as chitosan has a stimulatory effect on dermal fibroblasts proliferation when added to serum-containing culture medium, highly deacetylated chitosans producing a stronger mitogenic response, as compared to samples with lower levels of deacetylation.

**Bone regeneration.** The osteogenic potential of chitosan was first reported by Borah et al. in 1992.<sup>173</sup> In this study, chitosan was used to treat bone defects, made in the endochondral long bones of the rabbit. Chitosan reportedly stimulated osteogenesis with closure of the critical size bone defects, as compared to controls, in 8-12 weeks. As time progressed, the possible osteogenic, osteoconducting, and osteoinducting properties became the subject of investigation. The most significant works demonstrating the osteoconductive properties of chitosan are possibly the studies carried out by Muzarelli et al.,<sup>174,175</sup> as shown by the innumerable citations in literature concerning this subject. On the first work, Muzzarelli et al.<sup>174</sup> treated bone defects made in the tibiae of rabbits with freeze-dried methyl pyrrolidinone chitosan. The experimental sites showed signs of neoformed bone tissue, as opposed to controls, originating from the pre-existing bone as well as from the periosteum. Subsequently, Muzzarelli et al. prepared a modified chitosan (DA 8%) carrying imidazole groups, to treat bone defects made in the femoral condyle of sheep. Within 40 d after surgery, the neo-formed tissue occluded the surgical hole and assumed a trabecular structure in the peripheral area of the lesion. In the control, no sign of osteogenesis or reparative process was observed and bone marrow was rich in adipocytes. The modified chitosan was reported to have a stimulatory effect on bone formation. Although several works have demonstrated the osteoconductive properties of chitosan, in these works, chitosan is often combined with therapeutic molecules, growth factors, or calcium phosphates. The bone regenerative properties of unmodified chitosan are reported in the studies of Park et al.<sup>176</sup> In this study, the application of unmodified chitosan sponges to rat calvarial defects led to a significant increase of new bone formation, as compared to untreated defects, which became completely filled with fibrous connective tissue. In addition, a markedly increase of bone formation and mineralization was observed, in the presence of PDGF, as expected. The subsequent studies of Lee et al.<sup>177</sup> supported these results. The effect of the DA on osteogenesis was addressed by Hidaka et al.,<sup>178</sup> who implanted subperiosteally over the calvaria of rats membranes prepared from hydroxyapatite and chitosan, chitosan with DAs of 0, 6, 20, 30 and 35%, being used. The authors reported for DAs  $\leq 20\%$  a marked inflammatory reaction, followed by accumulation of osteocalcin positive cells at the site of implantation, while for lower DAs, a mild inflammation with minimal osteogenesis was observed. The DA effect on osteogenesis apparently should be

further investigated, as in this study chitosan was not used alone, but in combination with HA. Furthermore the method used to characterize the DA is not mentioned.

***In vitro* enhancement of osteogenic differentiation.** Klokkevold et al.<sup>179</sup> reported that chitosan potentiates the differentiation of mesenchymal stem cells from fetal mice calvaria, *in vitro*. In that study, culture plates previously coated with a 0.2% (w/v) chitosan acetic acid solution were used. However, the DA and the molecular weight of the chitosan used were not reported. In addition, the authors reported that chitosan was randomly distributed in the wells, which was probably due to the low chitosan concentration used to coat the wells. Finally, it is to blame that only one method was used to follow cell differentiation: the number of bone forming colonies stained dark by Von-Kossa stain at day 14. Subsequently, and supporting partially these results, Cai et al.<sup>180</sup> showed that coating of PDLLA films with chitosan (DA 15%) promoted osteogenic differentiation of neonatal rat calvaria osteoblasts, as shown by ALP activity, as compared to unmodified PDLLA films.

## **2.10. Biomedical and pharmaceutical applications**

Chitosan is being evaluated for a number of biomedical and pharmaceutical applications. Due to its wound healing properties, adhesive properties, exudate absorbability, and film forming properties, chitosan has been explored for burn and wound dressings. Other potential applications in external medical devices include contact lenses, dialysis membranes, hemostatic agents, and coatings of the inner lumen of blood-contacting polymeric tubes, onto which an antithrombotic agent is deposited. Internal medical applications include fibers for absorbable sutures, orthopedic implants such as bone pins and plates, bone fillers such as cements, and scaffolds for tissue engineering.<sup>117,181,182</sup> In this sense, a number of anchorage dependent cells have been expanded on 2D and 3D chitosan matrices, including chondrocytes and osteoblasts, envisaging cell-based regenerative therapies.<sup>156,183</sup> Chitosan is particularly interesting for cartilage tissue engineering, due to its structural similarity with hyaluronic acid, a GAG abundant in the ECM of cartilage.<sup>184</sup>

Pharmaceutical applications include hypocholesteremic agents (taking advantage of chitosan ability to bind to acidic lipids), liposome stabilization agents, and controlled release systems.<sup>182</sup> Due to the ability of chitosan to be hydrolyzed by lysozyme,

chitosan beads incorporating drugs/enzymes can be used as a sustained delivery system.<sup>185,186</sup> In addition, its cationic nature and high charge density in solution allows chitosan to form polyelectrolyte complexes with a wide variety of water-soluble anionic biomolecules, such as GAGs<sup>155,187</sup> and nucleic acids.<sup>188,189</sup> This ability has been explored to prepare hybrid materials for tissue engineering, as well to prepare non-viral vectors for gene delivery.

## **2.11. Chitosan in orthopedics**

### **2.11.1. Chitosan as a bone filler**

In orthopedics, chitosan has often been used in combination with ceramics such as HA and other calcium containing ceramics to produce composites. These have been investigated as bone fillers, and many were found suitable as bone filling materials.<sup>190,191</sup> The advantage of this approach is to develop bone substitute materials which combine the biodegradability, strength, and flexibility of chitosan with the osteogenic potential and hardness of the mineral filler. In addition, the chitosan matrix acts as a binder, preventing post-operative migration of the mineral phase.<sup>192,193</sup> Chitosan has also been explored as an adjuvant additive, to render calcium phosphate cements injectable and enable their use in minimally-invasive surgical procedures.<sup>194</sup> An injectable bone filler combining a bioabsorbable polymeric mesh with a calcium phosphate cement incorporating chitosan was also developed, envisaging the formation of interconnected macropores, *in vivo*, as the polymeric mesh degrades.<sup>195</sup> Other bone cements combining chitosan, HA, and PMMA were also developed, and reported to provide porous spaces for osteoconduction, after chitosan degradation.<sup>196</sup> Finally, the association of growth factors with chitosan-based bone fillers has also been investigated, as a combinative strategy of controlled local drug delivery and bone regenerative therapy.<sup>197</sup>

Besides being used as a filler material, chitosan has also been used as a coating material, in order to improve titanium and HA implants osteointegration.<sup>198-200</sup> Envisaging to improve the biocompatibility of electrolytically deposited apatite coatings on Ti alloys, Wang et al.<sup>201</sup> prepared a hybrid calcium phosphate/chitosan

coating. When compared to apatite coatings, this hybrid coating revealed to be more cytocompatible toward bone marrow stromal cells.

### **2.11.2. Bone tissue engineering applications**

In recent years, considerable attention has been given to the application of chitosan-based materials in the field of orthopedic tissue engineering. Interesting characteristics that render chitosan suitable for this purpose are its biocompatibility/biodegradability, structural similarity to extracellular matrix glycosaminoglycans, intrinsic antibacterial nature, ability to bind anionic molecules such as growth factors, glycosaminoglycans (GAGs), and DNA, as well as its ability to be molded into porous structures suitable for cell ingrowth and osteoconduction.<sup>202</sup> In this sense, a wide number of support matrices in the form of injectable gels or porous scaffolds have been developed for bone tissue engineering applications.

Most often, chitosan is used in combination with a variety of materials such as ceramics, PLLA, gelatin, glycosaminoglycans, as well as growth factors, in an attempt to improve its mechanical properties, osteoconduction and ability to induce bone regeneration. Binding with cell adhesive motifs has also been explored, in order to promote cell adhesion. A compilation of the current applications of chitosan as a preformed scaffold for bone tissue engineering is presented in Table 1.

Besides its application as a support matrix for cell ingrowth, chitosan, due to its gelling properties, has also been used for the local delivery of growth factors, in a controlled fashion. Finally, chitosan has also been explored as a non-viral vector for gene delivery, to be used in bone regenerative therapies.<sup>188</sup>

**Table 1.** Current applications of chitosan as a preformed scaffold for bone tissue engineering.

| Scaffold                                                                      | DA    | Pore size (µm) | Porosity (%) | Performed biological studies                                                                                       | References |
|-------------------------------------------------------------------------------|-------|----------------|--------------|--------------------------------------------------------------------------------------------------------------------|------------|
| Chitosan/TCP scaffolds                                                        | n. m. | 100            | n. m.        | Fetal rat calvarial osteoblastic cells proliferation, viability, and differentiation                               | 203        |
| Chitosan and PDGF-BB loaded chitosan scaffolds                                | n. m. | 100            | n. m.        | Rat calvarial osteoblastic cells adhesion and proliferation<br>Bone regeneration ability in a rat calvarial defect | 176        |
| Chitosan scaffolds and chitosan scaffolds reinforced with β-TCP and/or glass  | <15%  | n. m.          | 89<br>76-90  | Cytocompatibility towards human osteoblast-like MG-63 cells                                                        | 204,205    |
| Chitosan and chitosan/chondroitin sulphate scaffolds                          | 30%   | 100-150        | n. m.        | Rat calvarial osteoblastic cells adhesion and proliferation<br>Bone regeneration ability in a rat calvarial defect | 177        |
| Chitosan/PLLA scaffolds                                                       |       | 150-200        |              |                                                                                                                    |            |
| Chitosan coated PLLA scaffolds                                                |       | 100-150        |              |                                                                                                                    |            |
| (All loaded or not with PDGF-BB)                                              |       |                |              |                                                                                                                    |            |
| HA/chitosan/gelatin scaffolds                                                 | n. m. | 300-500        | 85-96        | Rat calvarial osteoblastic cells adhesion, proliferation, and differentiation                                      | 111        |
| Chitosan/gelatin and chitosan/gelatin/β-TCP macroporous scaffolds             | ~10   | 322<br>185-420 | n. m.        | Biocompatibility evaluation (Subcutaneous implantation in a rabbit model)                                          | 206        |
| Chitosan scaffolds                                                            | n. m. | 100-200        | n. m.        | Rat calvarial osteoblastic cells proliferation and differentiation                                                 | 207        |
| Chitosan/HA and Chitosan/HA/glass scaffolds<br>Chitosan-coated HA scaffolds   | <15%  | ~100           | n. m.        | Human osteoblast-like MG-63 cells viability, proliferation, and differentiation                                    | 208,209    |
| Chitosan scaffolds prepared by freeze-gelation                                | n. m. | 60-150         | > 80%        | Rat osteoblast-like cells (ROS 17/2.8) proliferation                                                               | 149        |
| Chitosan scaffolds and chitosan scaffolds modified with RGDS or RGEs peptides | 15%   | n. m.          | n. m.        | Rat osteoblast-like cells (ROS 17/2.8) adhesion, proliferation and differentiation                                 | 94         |
| Fast-setting calcium phosphate/chitosan/mannitol scaffolds                    | n. m. | n. m.          | 42-80%       | Osteoblast mouse cells (MCT3-E1) adhesion, proliferation, and viability                                            | 210        |

n. m., not mentioned.

## **2.12. Influence of the DA on cell adhesion and proliferation, *in vitro***

The DA was reported to influence cell adhesion in the case of baby hamster kidney cells, fibroblast-like cells,<sup>211</sup> keratinocytes, fibroblasts,<sup>154</sup> and breast cancer cells,<sup>212</sup> with lower DAs favoring cell adhesion. According to these studies, cell proliferation has shown to follow a similar tendency, with the exception of fibroblasts, which independently of the DA, did not proliferate. Recently, Mao et al.<sup>213</sup> investigated the effect of the DA on fibroblast adhesion and proliferation (L929 fibroblasts), obtaining similar results, namely a tendency to higher cell adhesion on decreasing the DA. The authors also showed that chitosan inhibited most of the cells entering the cell cycle, and thus cell proliferation, regardless the DA, as shown by cell cycle analysis. This effect was attributed to chitosan high positive charge density, as blending with gelatin reversed this effect. Taken together, these results show that the DA effect is cell-type dependent, and highlight the importance of a careful selection of the DA while developing chitosan scaffolds for tissue engineering. However, the influence of the DA on the adhesion and proliferation of osteogenic cells has not been addressed.

## **2.13. Phosphorylation of chitin/chitosan**

### **2.13.1. Phosphorylation methods**

The interest in phosphate esters of chitin and chitosan has increased in the early eighties, mainly because of their metal ion binding capabilities.<sup>120</sup> Two preparative techniques have developed.

The first is based on a method for preparing cellulose phosphates, by heating chitin or chitosan with phosphoric acid and urea in DMF. Chitin and chitosan phosphates of low DS (degree of substitution) were initially prepared by Sakaguchi et al.<sup>214</sup> through this method. The authors investigated their metal binding ability. These derivatives were found to adsorb particularly high amounts of uranium, when compared to the other metals. Subsequently Sakairi et al.<sup>215</sup> improved this same method obtaining chitin phosphates with degrees of substitution up to 1.4, by varying the ratio of urea to DMF.

The second method was developed by Nishi et al.<sup>216</sup>, and involves the reaction of phosphorus pentoxide with chitin dissolved in methanesulphonic acid, at low temperature (0-5°C). Highly phosphorylated derivatives of chitin and chitosan were obtained, leading to nearly fully substituted products. Chitin phosphates were water-soluble, regardless the DS, while chitosan phosphates of high DS were insoluble in water, which was attributed to the formation of inter- or intramolecular salt linkages between protonated amines and phosphate groups. According to the same study the hydroxyl groups at C-3 and C-6 positions are equally substituted with the phosphate group, being equally reactive.<sup>216</sup> Subsequently Nishi et al.<sup>217</sup> insolubilized these derivatives, in order to study their metal binding ability. Alkaline-earth metals and  $Mn^{2+}$  ions were found to adsorb more strongly to these derivatives as compared to unmodified chitin/chitosan. The pH dependence for the  $Ca^{2+}$  adsorption to these materials showed that the amount of adsorbed  $Ca^{2+}$  increased with a rise in pH, suggesting a large contribution of the phosphoryl group. The metal binding ability was attributed to the cooperative action of different functional groups, besides the phosphoryl group, including the hydroxyl and the acetylamino groups.<sup>217</sup>

### **2.13.2. Water soluble phosphorylated chitin/chitosan**

Water soluble phosphorylated chitin/chitosan has been investigated for a wide number of applications, including biomedical applications. Basically these are prepared using one of the two phosphorylation methods mentioned above, the phosphorus pentoxide/methanesulphonic acid method being the most frequently used. Among other properties, water soluble phosphorylated chitin was shown to be useful as an adsorbent support for affinity-chromatography, after cross-linking,<sup>218</sup> to inhibit calcium phosphate crystallization from a supersaturated solution,<sup>219</sup> to behave as an anti-inflammatory agent,<sup>220,221</sup> to induce cell aggregation and earlier osteogenic differentiation of rat calvaria osteoblasts, when used as a substrate for cell culture upon complexation with chitosan,<sup>222</sup> and to enhance the mechanical strength of calcium phosphate cements, leading to biocompatible, bioabsorbable, and osteoconductive bone-fillers.<sup>223,224</sup> Water soluble phosphorylated chitosan was reported to inhibit the initial adherence of oral bacteria onto human teeth surfaces,<sup>225</sup> to increase the setting time and the mechanical properties of calcium phosphate cements, resulting in

biocompatible, bioabsorbable, and osteoconductive bone-fillers,<sup>226,227</sup> and to be cytocompatible with rat osteoblasts.<sup>228</sup>

### **2.13.3. Phosphorylated chitin/chitosan fibers, films, and sponges**

Lee et al. was the first to attempt the phosphorylation of chitosan membranes, to the best of my knowledge, to investigate their ability to separate water from aqueous ethanol solutions.<sup>229</sup> Phosphorylated membranes were prepared by the phosphoric acid/urea/DMF method, conducted at 70°C. The phosphorus amount in the membranes varied from 1-80 mg/m<sup>2</sup>, depending on the reaction period. As compared to sulfonated and carboxymethylated chitosan membranes, permeate flux through phosphorylated membranes showed a four-fold increase without the loss of selectivity towards water. Subsequently, chitin/chitosan matrices such as fibers, films, and sponges were phosphorylated using the same reaction method at temperatures ranging from 120 to 155°, to induce the deposition of an apatite-like layer, due to the ability of phosphate groups to chelate calcium ions from solution.<sup>89,230-232</sup> The ICP measured phosphorus content of phosphorylated chitosan sponges was found to be at ~6-7 wt%,<sup>232</sup> and higher than that of phosphorylated chitin fibers (2.95 wt%),<sup>89,230</sup> and that of phosphorylated chitosan films (0.1-0.2 wt%).<sup>231</sup> During the phosphorylation of chitin fibers and chitosan sponges, crystals of ammonium phosphate are also formed, as by-products.<sup>89,232</sup> After soaked in a saturated Ca(OH)<sub>2</sub> solution at ambient temperature, all phosphorylated substrates showed the ability to induce the formation of a calcium deficient apatite layer after incubation in 1.5 × simulated body fluid solution (SBF). The pre-incubation in a saturated Ca(OH)<sub>2</sub> solution was required in order to produce calcium phosphate precursor sites for further calcium phosphate deposition during the SBF immersion stage.<sup>89,230-232</sup> Yokogawa et al.<sup>232</sup> suggested that the mechanism of formation of calcium phosphates during the Ca(OH)<sub>2</sub> treatment involves the diffusion of phosphate ions as well as the hydrolysis of phosphate functionalities, which lead to a high local saturation of ions, and as a consequence, to the precipitation of calcium phosphate. The hybrid materials were proposed to be potentially suitable as virus filters,<sup>89</sup> given the adsorptive properties of hydroxyapatite, as well as biomaterials,<sup>232</sup> due to the close resemblance with natural tissues.

### 3. The $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4$ /hexanol phosphorylation method

Granja et al.<sup>233</sup> prepared highly phosphorylated cellulose derivatives using the  $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4$ /hexanol reaction route. This method was originally proposed by Touey and Kingsport in 1956,<sup>234</sup> for the synthesis of water-soluble and non-degraded cellulose phosphates. After optimizing the reaction parameters using microcrystalline cellulose, Granja et al.<sup>233</sup> reported for the first time the synthesis of cellulose triphosphates. This method is an interesting alternative to the more widely used techniques for the phosphorylation of cellulose. Besides leading to highly substituted products, it is carried out at ambient temperature (30°), thus resulting in low degradation of the polymer. Additionally, phosphorylated microcrystalline cellulose revealed no cytotoxicity towards fibroblasts and osteoblasts.<sup>235</sup>

This method was applied to the surface modification of regenerated cellulose hydrogels and sponges, in order to enhance the osteointegration of regenerated cellulose implants.<sup>236</sup> Once implanted, phosphorylated cellulose could promote the formation of calcium phosphates, and ensure a satisfactory bonding between hard tissue and the biomaterial. These derivatives induced mineralization in SBF, after incubation in a calcium-containing solution.<sup>90</sup> The minerals formed revealed an apatitic carbonated and poorly crystalline structure, resembling that of carbonated hydroxyapatite found in bone mineral. Upon implantation in rabbit bone defects, phosphorylated cellulose hydrogels induced no inflammatory response, and showed osteointegration in the surrounding tissue.<sup>237</sup> The application of this chemical modification route to other polysaccharides has not been reported yet.<sup>237</sup>

### 4. Aim and structure of the thesis

The degree of acetylation (DA) was reported to influence cell adhesion of a few anchorage dependent cells including keratinocytes, fibroblasts,<sup>154</sup> and breast cancer cells,<sup>212</sup> with lower DAs favoring cell adhesion. According to these studies, cell proliferation has shown to follow a similar tendency, with the exception of fibroblasts, which independently of the DA, did not proliferate. However, in what concerns osteogenic cells, the influence of this parameter has not been reported yet. As the DA

effect appears to be cell-specific, this study should be addressed while developing chitosan-based templates for cell-based bone regenerative therapies.

The research purpose of the first part of this thesis (**Chapters II, III, and IV**) was to investigate the effect of the degree of acetylation (DA) on the ability of chitosan matrices to support cell anchorage, proliferation, and osteogenic differentiation of osteoprecursor cells, *in vitro*, envisaging its application in cell-matrix constructs for bone tissue engineering therapies. For that purpose, chitosan samples with DAs in the range of 4 to 49% were initially prepared to produce chitosan films and sponges. Films were firstly used in the biological assays, for allowing the study of interfacial phenomena (occurring at the interface between the biological milieu and the biomaterial) such as protein adsorption onto surfaces, which are known to play a key role in the cell adhesion process.

In **Chapter II**, a preliminary study on the DA effect on cell adhesion, spreading, and short-term proliferation of osteogenic cells on chitosan films was performed using human osteoblastic cells (MG-63 osteoblast-like cells). Chitosan films were characterized in terms of surface morphology and roughness, as well as in terms of surface charge. Cell attachment was assessed after 3 and 24 hours of cell culture, while cell growth kinetics was evaluated over a 5 day period. Taken together, the results obtained showed that the DA affects notably cell adhesion, spreading and proliferation of osteoblast-like cells, differences in the DA as small as 9% being critical in terms of osteoblast response to chitosan films.

In view of the previous findings, the DA effect on cell adhesion and osteogenic differentiation of osteoprecursor cells on chitosan films was investigated, using rat bone marrow stromal cells (**Chapter III**). In this study, chitosan films were further characterized in terms of wettability, as well as in terms of their ability to adsorb fibronectin (chosen as a model of cell adhesive molecule), both from single protein solution and from diluted serum. Cell attachment, cytoskeletal organization, proliferation, and osteogenic differentiation over a 21 day period were assessed. Briefly, the results showed that while chitosan films with a  $DA \geq 13\%$  are not able to support cell adhesion for more than 24 h, rBMSCs cultured on chitosan with a DA of 4% are able to spread, proliferate and differentiate, reaching a higher level of osteogenic differentiation than on the tissue culture polystyrene used as control. As chitosan sample with a DA of 4% showed the highest FN adsorption from serum, it is

suggested that cell adhesion, cytoskeletal organization, and osteogenic differentiation of rBMSCs on chitosan in the presence of serum may be mediated by the adsorbed layer of fibronectin.

In **Chapter IV**, the effect of the DA on the ability of chitosan porous scaffolds to support cell anchorage, cytoskeletal organization, activity, viability, and proliferation of osteogenic cells was investigated, using human osteoblastic cells (MG-63 osteoblast-like cells). Chitosan porous scaffolds were first characterized in terms of microstructure, porosity and pore size. Collagen sponges were equally prepared and used as 3-D positive control. Results showed that in terms of cell proliferation, alkaline phosphatase activity and cell viability, cells cultured in chitosan sponges performed as well as in the 3-D control regardless the DA, except for the highest DA used, where an inhibitory effect on cell proliferation was found. However, a marked effect of the DA on cell cytoskeletal organization was found, suggesting that the DA plays a key role in determining the affinity of osteoblastic cells towards chitosan matrices, possibly by influencing the nature of the initial adsorbed protein layer.

In the second part of this thesis (**Chapters V, VI, and VII**), the research purpose was to enhance the osteoconductive properties of chitosan matrices by inducing the deposition of an apatite-like layer. The combination of chitosan with ceramics is often attempted to enhance both the mechanical and osteoconductive properties of chitosan scaffolds. As previously mentioned, grafting of negatively charged groups such as phosphates is a well-known strategy to induce the deposition of apatite-like layers under simulated physiological conditions. In this study, phosphate groups were incorporated into chitosan films by applying the  $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4$ /hexanol phosphorylation method,<sup>233</sup> as an alternative to the phosphoric acid/urea/DMF phosphorylation method, which involves the use of much higher temperatures.

In **Chapter V**, the phosphorylation of chitosan membranes was attempted using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5$ /hexanol reaction route,<sup>233</sup> with minor modifications. Surface characterization was performed using complementary spectroscopic techniques. In addition, cross-sections of the modified membranes were also observed by scanning electron microscopy, fitted with an energy dispersive X-ray analysis system, in order to evaluate the extent of phosphorylation along the membrane thickness. Briefly, the results showed an increase of the phosphate content with the reaction time, as shown

by XPS and ATR-FTIR, a maximum of 4.7 at.% P being obtained after 48 h of treatment.

In **Chapter VI**, the resulting phosphorylated chitosan membranes were immersed in  $\text{Ca}(\text{OH})_2$  or NaOH solutions, in order to obtain the Na or the Ca salts, respectively, and investigated regarding their ability to nucleate calcium phosphates, under simulated physiologic conditions. SEM-EDS studies revealed the presence of a calcium phosphate mineral layer all over the surface of phosphorylated membranes, after incubation in  $\text{Ca}(\text{OH})_2$  solution. The subsequent immersion in simulated body fluid led to the growth of a multilayer porous mineral structure composed of partially carbonated and poorly crystalline apatite, as shown by EDS and ATR-FTIR analysis. The sodium salt of P-chitosan and the unmodified chitosan membranes did not mineralise under the same conditions.

In **Chapter VII**, phosphorylated chitosan membranes were further characterized in terms of surface morphology, roughness, as well as in terms of surface free energy. These membranes were also evaluated regarding cytotoxicity towards bone cells, envisaging their use together with mineralized membranes, in further biological assays.

In **Chapter VIII**, a short general discussion of the results is presented, since a detailed discussion is provided in each of the preceding chapters. Future work is also proposed.

**Papers resulting from this thesis:**

**Amaral IF**, Cordeiro A, Sampaio P, Barbosa MA. Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation. *Journal of Biomaterials Science: Polymer Edition*, submitted. **(Chapter II).**

**Amaral IF**, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cells osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. *Journal of Biomedical Materials Research Part A* 2005;75A(2):387-397. **(Chapter III).**

**Amaral IF**, Sampaio P, Barbosa MA. Three-dimensional culture of human osteoblastic cells in chitosan sponges: The effect of the degree of acetylation. *Journal of Biomedical Materials Research Part A* 2006;76A(2):335-346. **(Chapter IV).**

**Amaral IF**, Granja PL, Barbosa MA. Chemical modification of chitosan via phosphorylation. An XPS, FT-IR and SEM study. *Journal of Biomaterials Science: Polymer Edition* 2005;16(12):1575-1593. **(Chapter V).**

**Amaral IF**, Granja PL, Barbosa MA. *In vitro* Mineralisation of Chitosan Membranes Carrying Phosphate Functionalities. *Key Engineering Materials* 2004;254-256:577-580. **(Chapter VI).**

**Amaral IF**, Granja PL, Melo LV, Saramago B, Barbosa MA. Functionalization of chitosan membranes through phosphorylation: AFM, wettability and cytotoxicity studies. *Journal of Applied Polymer Science*, In Press. **(Chapter VII).**

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### **Attachment, spreading and short-term proliferation of human osteoblastic cells cultured on chitosan films with different degrees of acetylation**

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#### **ABSTRACT**

Chitosan (Ch) is being actively investigated as a non-protein template for the growth of an increasing number of anchorage dependent cells, including chondrocytes and bone cells. In the present work, Ch films with degrees of *N*-acetylation (DAs) in the range of 4 to 49% were evaluated regarding the attachment, spreading and short-term proliferation of osteoblasts, using human osteoblastic MG-63 cells. The films were characterized in terms of surface morphology and surface charge by atomic force microscopy and streaming potential measurements, respectively. Cell attachment was assessed after 3 and 24 hours of cell culture. After 24 h of incubation, a clear effect of the DA on cell attachment was observed, lower DAs favoring cell adhesion. With time, cell spreading and cytoskeleton organization were only attained for DAs  $\leq 13\%$ . Regarding cell proliferation, cells grown on films with the lowest DA (4%) revealed a higher specific growth rate as compared to those grown on films with higher DAs. Films with a DA of 49% failed to sustain cell proliferation. In addition, a longer lag-phase was observed on Ch, as compared to TCPS, in accordance to an observed delay of cell spreading. The present findings revealed that differences in the DA as small as

9% may be critical in terms of the osteoblast response to two-dimensional Ch-based matrices.

## INTRODUCTION

As the demand for biodegradable support matrices to be used in cell-based bone regenerative therapies develops, the former are being designed to encourage cell anchorage, spreading, proliferation and the maintenance of cell-specific functions, if not differentiation into a particular phenotype.<sup>1</sup> Desirable features are ability to support osteoconduction and vascular invasion, degradation kinetics matching timely bone formation, resorbability, sterilizability, as well as handling properties and mechanical stability adequate to the clinical application in view.<sup>2,3</sup>

Chitosan (Ch), a hetero-polysaccharide containing *N*-acetyl glucosamine, is being actively investigated as a non-protein support matrix for tissue engineered constructs.<sup>4,5</sup> The interest of Ch stems from its structural analogy to extracellular matrix glycosaminoglycans, enzymatic degradability, osteoconduction and wound-healing properties, resulting from the immunoadjuvancy of Ch oligomers.<sup>6,7</sup> In addition, its versatility to be processed into injectable, porous and membranar forms without use of toxic solvents makes it an interesting biopolymer for bone regeneration.<sup>8</sup> In this sense, an increasing number of anchorage dependent cells, including chondrocytes and bone cells, are being cultured on 2-D and 3-D Ch matrices.<sup>5,6,9-11</sup> Ch is usually obtained by heterogeneous deacetylation of chitin, a naturally occurring polysaccharide present in the exoskeleton of marine arthropods. Ch is a copolymer of *N*-acetyl glucosamine and glucosamine units in a  $\beta 1 \rightarrow 4$  linkage in which the molar fraction of *N*-acetylated units is known as the degree of acetylation (DA). Depending on the extent of deacetylation, the DA of Ch may range from 0 to 50%, which is the upper limit of solubility in dilute acidic solutions. The DA is a structural parameter affecting solubility, crystallinity, charge density, and the propensity to enzymatic degradability (higher DAs leading to faster degradation rates).<sup>12,13</sup> In addition, the number of primary available amines provided by the glucosamine units is determinant for the surface chemistry and wettability of Ch scaffolds, wettability increasing on decreasing the DA.<sup>13,14</sup> As these features constitute key parameters influencing the adsorption and conformation of proteins, cell adhesion

and the expression of a cellular phenotype are consequently affected.<sup>15</sup> In fact, the DA was reported to influence cell adhesion in the case of a few cells including fibroblast-like cells,<sup>16</sup> fibroblasts, keratinocytes,<sup>17</sup> and breast cancer cells,<sup>18</sup> with lower DAs favoring cell adhesion. According to these studies, cell proliferation has shown to follow a similar tendency, with the exception of fibroblasts, which independently of the DA, did not proliferate. However, the effect of the DA on osteoblasts cell adhesion has not been reported yet, at the best of our knowledge. As the DA effect appears to be cell specific, this study should be addressed while developing chitosan templates for *in vitro* cell culture. In the present work, Ch films with different DAs were characterized in terms of morphology and surface charge and then evaluated regarding the attachment, spreading and short-term proliferation of osteoblasts, using human osteoblastic cells.

## MATERIALS AND METHODS

### Preparation and characterization of Chs with different degrees of acetylation

Ch powder from squid pens (Chitosan 123) was kindly supplied by France Chitine (France) and further purified by filtration of Ch acidic solution and subsequent alkali precipitation. The purified Ch was then characterized in terms of DA by infrared spectroscopy in KBr pellets using a FT-IR system 2000 from Perkin-Elmer, using the amide III band at  $1320\text{ cm}^{-1}$  as the analytical band and the band at  $1420\text{ cm}^{-1}$  as the internal reference band, according to Brugnerotto et al..<sup>19</sup> A DA of approximately 30% was found. Chs with lower DAs were prepared by heterogeneous deacetylation of the purified Ch using successive 1 hr alkali treatments with intermittent washing in water, as described by Mima et al..<sup>20</sup> A sample with higher DA was obtained by *N*-acetylation of the purified Ch in aqueous, methanolic acetic acid solution, using acetic anhydride as reactive, following the method of Aiba.<sup>21</sup> The physicochemical characteristics of the resultant chitosans in terms of DA and average molecular weight can be depicted from Table 1. For DAs below 40%, the DA was determined as above mentioned. For higher DAs, the DA was determined according to Baxter et al.,<sup>14</sup> using the amide I band at  $1655\text{ cm}^{-1}$  as the analytical band and the band at  $3450\text{ cm}^{-1}$  as the internal reference band. The results presented correspond to the average ( $\pm$  SD) of 4 measurements. Weight and number average molecular weight ( $M_w$  and  $M_n$ ,

respectively) were assessed by high performance size exclusion chromatography using the system 0.2 M  $\text{CH}_3\text{COONa}$ /0.5 M  $\text{CH}_3\text{COOH}$  as mobile phase, according to Terbojevich et al..<sup>22</sup>

**Table 1.**

Chitosan characterization

| Preparation method          | DA (%)          | $M_w \times 10^5$ | $M_n \times 10^5$ |
|-----------------------------|-----------------|-------------------|-------------------|
| <i>N</i> -acetylation (1 h) | $49.1 \pm 1.54$ | n.d.              | n.d.              |
| As received                 | $29.8 \pm 1.21$ | $5.4 \pm 0.3$     | $1.3 \pm 0.2$     |
| Deacetylation (0.5 h)       | $22.1 \pm 1.30$ | $3.5 \pm 0.1$     | $1.4 \pm 0.1$     |
| Deacetylation (1 h)         | $12.9 \pm 1.31$ | $2.8 \pm 0.1$     | $1.2 \pm 0.1$     |
| Deacetylation (3 h)         | $4.23 \pm 0.46$ | $2.1 \pm 0.1$     | $1.0 \pm 0.1$     |

n.d., not determined.

### Preparation of Ch films

Ch films were obtained by solvent casting from 1% w/v Ch solutions in 0.2 M acetic acid. The polymer was allowed to dissolve for 24 h, centrifuged for degassing and poured onto the wells of 24-well tissue culture polystyrene (TCPS) plates from TPP®. A volume of 200  $\mu\text{L}$  was poured per well. After solvent evaporation, the resulting films were finally deprotonated in 0.5 M NaOH and thoroughly washed with distilled and deionized water until neutrality. Prior to cell seeding, the samples were sterilized with 70% v/v ethanol for 30 min, equilibrated in sterile phosphate buffered saline (PBS, Sigma) (10 min; 2 $\times$ ) followed by standard culture medium (30 min; 2 $\times$ ), which consisted of  $\alpha$ -minimal essential medium supplemented with 10% v/v fetal bovine serum, 50  $\mu\text{g/mL}$  gentamicin sulfate and 2.5  $\mu\text{g/mL}$  amphotericin B (all from Gibco). For characterization purposes, films were prepared on TCPS (TPP®) Petri dishes previously cut into pieces of approximately  $5.0 \times 3.0 \text{ cm}^2$ , using the same ratio of chitosan gel volume per unit area (100  $\mu\text{L}/\text{cm}^2$ ). After the neutralization step and extensive rinsing, the films were used immediately for streaming potential measurements or dried at 30°C for 24 h in a vacuum oven, for AFM surface analysis.

### Surface characterization of the films

Surface morphology was assessed by atomic force microscopy (AFM), using a PicoPlus scanning probe microscope from Molecular Imaging (USA). Imaging was performed in Tapping™ mode, using standard Si tips (Spring Constant  $\cong 1.2\text{--}3.5$  N/m). The samples were analyzed in air at room-temperature. The root mean square of the deviations from the centre  $x$ - $y$  plane, RMS roughness, was determined under a scan range of  $90 \times 90 \mu\text{m}^2$ , using the SPIP™ Version 3.0 software. Measurements were made in at least 4 randomly chosen areas, in two different samples.

Electrokinetic measurements were performed using an ElectroKinetic Analyzer (Anton Paar GmbH, Austria), based on the streaming potential method. Prior to analysis, the films were equilibrated in 1 mM KCl for 2 h.  $\zeta$ -potential measurements were performed with the asymmetric clamping cell, using 1 mM KCl (pH 5.5) as electrolyte solution. For each DA, a total of twelve measurements were performed with alternating flow direction, in two different samples. The  $\zeta$ -potential was calculated according to the modified Smoluchowski-Helmholtz equation,<sup>23</sup> using a poly(methyl metacrylate) (PMMA) plate as reference, as follows:

$$\zeta_{Test} = 2\zeta_{Total} - \zeta_{Spacer}, \quad (1)$$

Where  $\zeta_{Test}$  is the  $\zeta$  potential of the test surface,  $\zeta_{Total}$  the measured  $\zeta$  potential, and  $\zeta_{Spacer}$  the  $\zeta$  potential of the PMMA grooved spacer.

### Cell culture

MG-63 osteoblast-like cells from human osteosarcoma were obtained from the American Type Culture Collection (ATCC), and routinely cultured in standard culture medium. Cultures were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Prior to subculture, the osteoblastic phenotype of MG-63 cells was checked in terms of synthesis of osteocalcin, 72 h after stimulation by 1,25(OH)<sub>2</sub>vitaminD<sub>3</sub>.<sup>24</sup> For subculture, cells were trypsinized, stained with trypan blue, and those which excluded the dye counted with a Neubauer improved chamber. Cells were suspended in the culture medium for reseeding.

**Cell attachment assay**

Cells were seeded on Ch coated wells or directly on uncoated polystyrene (used as control), at a cell seeding density of  $2 \times 10^4$  viable cells/cm<sup>2</sup>. Cells were allowed to attach in the humidified incubator for two different time periods, 3 and 24 h. At the end of each culture period, the cultures were rinsed twice with PBS to remove the unattached cells and the number of viable cells was estimated using the viability/proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.<sup>25</sup> Briefly, the cells were incubated with MTT solution (0.5 mg/mL in Dulbecco's medium without phenol red) at 37°C for 3 h. At the end of this period the supernatant was discarded and 400 µL of DMSO were added to dissolve the formazan salts. After complete dissolution the dye solution was centrifuged and the optical density of the supernatant was read at 540 nm with reference to 620 nm (OD<sub>540/620</sub>), using a microplate spectrophotometer.

**Short-term proliferation assay**

For short-term proliferation assays, cells were seeded at a cell seeding density of  $10^4$  viable cells/cm<sup>2</sup> and cultured for periods up to 5 days, the culture medium being refreshed at day 3. Cell growth kinetics was evaluated by estimating the number of viable cells at 4 h and then each 24 h after plating, sacrificing 6 cultures per type of substrate, using the MTT assay as previously described. The specific growth rate ( $\mu$ ) was determined by fitting the absorbance data correspondent to the exponential phase to the following equation:

$$\ln \left[ \frac{A}{A_0} \right] = \mu (t - t_0) \quad (2)$$

Where  $A_0$  is the absorbance at  $t_0$ ,  $A$  the absorbance at  $t$ ,  $\mu$  the specific growth rate (h<sup>-1</sup>),  $t$  the time (h), and  $t_0$  the time associated with attachment and adjustment of cells to the substrate (h). Mean and standard error for  $\mu$  were determined for each type of substrate from linear regression analysis of culture data. Doubling time was calculated using Equation 2.

## Cell morphology and cytoskeletal organization

The morphology of cells was followed daily by contrast phase optical microscopy. Cytoskeletal organization was followed by F-actin /DNA staining. Briefly, cells were rinsed twice with PBS, fixed in 3.7% methanol-free formaldehyde, permeabilized in 0.1% buffered Triton 0.1% X-100 and incubated with 1 µg/mL RNase and 1% w/v bovine serum albumin for 1 h. Cell cytoskeletal filamentous actin (F-actin) was visualized treating the cells with 5 U/mL Alexa Fluor® 488 phalloidin (Molecular Probes) for 20 min. Cell nuclei were counterstained with 15 µM propidium iodide (Sigma) for 5 min. Samples were finally mounted in Vectashield® (Vector) and examined with a Cell Observer System (Carl Zeiss, Germany).

## Statistical analysis

Roughness and  $\zeta$ -potential data were analysed using the Mann-Whitney  $U$  test. Data concerning biological assays were analysed by one-way analysis of variance using the Bonferroni's  $t$  test for multiple comparisons. Results were considered statistically significant when  $p \leq 0.05$ . Pearson correlation coefficient was used to determine the linear association between two variables. Calculations were performed using SPSS® software (version 12.0).

## RESULTS

### Surface characterization of the films

#### *AFM imaging and roughness analysis.*

AFM amplitude images representative of TCPS and of Ch films with DAs of 4, 13, 22 and 49%, are shown in Figure 1, together with the correspondent transverse topography cross-section lines. Amplitude images are presented instead of topography ones, as surface features are more perceptible on the former. TCPS revealed a fibrous-like structured surface [Fig. 1(a)] in accordance to literature.<sup>26</sup> Films with DAs ranging from 4 to 30% showed mainly a smooth surface, with the presence of globular features

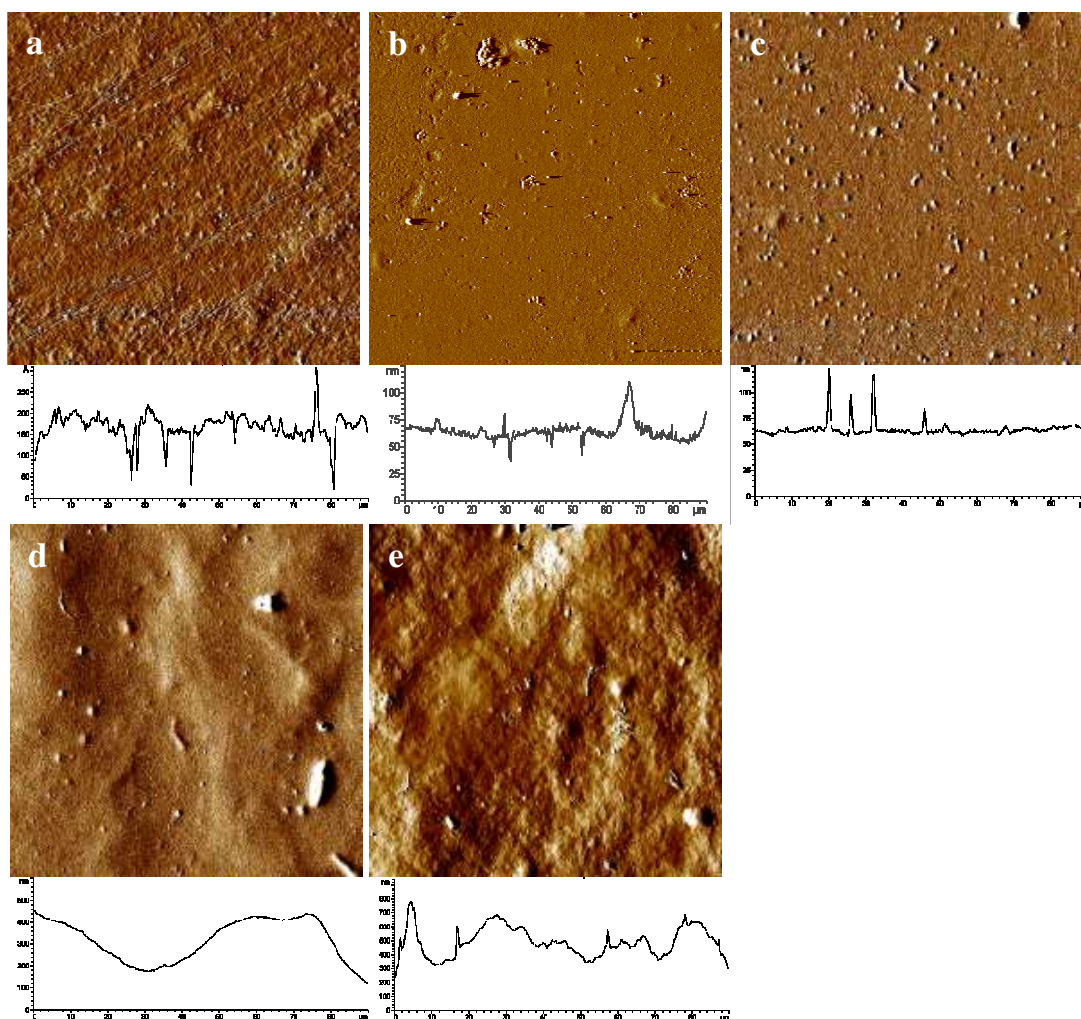


**Figure 1.** Tapping™ mode atomic force microscopy amplitude images and topography transverse cross-section lines of: (a) TCPS and (b to d) Ch films prepared from Chs with different DAs: (b) 13%; (c) 22% and (d) 49%. Data were collected from a  $90 \times 90 \mu\text{m}^2$  scan range, under ambient conditions.

measuring 2 or more micrometers in diameter [Fig. 1(b) and (c)], assigned to insoluble fibrillar aggregates. Films with a DA of 49% revealed a much more irregular surface morphology, indicating the presence of a higher amount of insoluble polymer [Fig. 1(d)]. The transverse topography cross-section lines and roughness analysis revealed for Ch films an increase of surface roughness on increasing the DA (Table 2). Mean values of RMS increasing from approximately 58 to 95 nm were found, as the DA increased from 4 to 30%, respectively. The sample with the highest DA showed a significantly higher RMS (about 371 nm), when compared to the others. The

increasing number of globular features assigned to polymer aggregates found on increasing the DA was attributed to the correspondent decrease of the Ch charge density in acidic solutions as well as to the longer length of the Ch chains.<sup>12</sup>

**Table 2.**

RMS roughness and  $\zeta$ -potential of Ch membranes, as a function of DA, determined by atomic force microscopy and streaming potential measurements, respectively. The RMS was determined in air over a scan range of  $90 \times 90 \mu\text{m}^2$ . The streaming potential was determined using 1 mM KCl (pH 5.5) as the electrolyte solution. Values reported are the average  $\pm$  SD. Ch samples were compared between them. § indicates a significant difference when compared to any of the other Ch samples; \* indicates a significant difference from films with a DA of 4%, and † indicates a significant difference from films with a DA of 13% ( $p \leq 0.05$ ).

| Surface | RMS (nm)            | $\zeta$ -potential (mV) |
|---------|---------------------|-------------------------|
| TCPS    | $31.3 \pm 11.5$     | $-18.32 \pm 0.43$       |
| DA 4%   | $57.9 \pm 21.5$     | $25.92 \pm 0.21$ §      |
| DA 13%  | $72.3 \pm 30.3$     | $17.69 \pm 0.22$        |
| DA 22%  | $88.2 \pm 7.7$ *    | $17.18 \pm 0.21$        |
| DA 30%  | $95.0 \pm 25.1$ *   | $16.99 \pm 0.26$ †      |
| DA 49%  | $371.3 \pm 114.7$ § | $14.67 \pm 0.42$ §      |

#### *Streaming potential measurements*

The  $\zeta$ -potentials of Ch films and TCPS are presented in Table 2. Ch films with a DA of 4% revealed a  $\zeta$ -potential of  $25.92 \pm 0.21$  mV, whereas films with higher DAs revealed  $\zeta$ -potentials in the range of  $17.69 \pm 0.22$  mV to  $14.67 \pm 0.42$  mV, as the DA increased from 13 to 49%, respectively. The decrease of the surface charge on increasing the DA was attributed to the lower amount of amine groups at the surface, which are expected to be positively charged at pH 5.5. In solution, the degree of dissociation ( $\alpha$ ) of protonated amines in Ch chains depends both on the pH, DA and ionic strength of the media. According to Sorlier et al.,<sup>27</sup> for pH 5.5,  $\alpha$  is of approximately 0.2 for a DA of 5.2% being lower for higher DAs. Therefore, although a higher ionic strength was used in that study, the majority of chitosan amine groups

are expected to remain in the ionized form ( $\text{NH}_3^+$ ) at pH 5.5. In the present study a linear dependence of the  $\zeta$ -potential with the DA was not observed, as films with DAs comprised between 13 and 30% revealed minimal differences in terms of  $\zeta$ -potential. It is known that polymeric surfaces react to environmental changes minimizing their interfacial tension.<sup>28</sup> In this sense, molecular reorientations might have occurred at the chitosan/air interface during the preparation of the Ch films, leading to the reorientation of polar groups like amines toward the inner part of the film and, consequently, to a lower surface concentration of amine groups, even after hydration. TCPS has revealed a  $\zeta$ -potential of  $-18.32 \pm 0.43$  mV, which is in good agreement to the literature,<sup>29</sup> and is attributed to the presence of acidic groups present at the surface.

### Cell attachment

The results regarding cell attachment at 3 and 24 h assessed by the MTT assay are presented in Figure 2. MTT is reduced to formazan crystals by mitochondrial enzymes in living cells and reading of the correspondent absorbance at 540 nm allows the estimation of the number of viable cells.<sup>25</sup> After 3 h of incubation, cell attachment for DAs in the range of 4 to 30% varied between 81 and 92% of the control, and no significant differences were found among Ch samples. However, for the highest DA (49%), a significantly lower cell attachment was already observed.

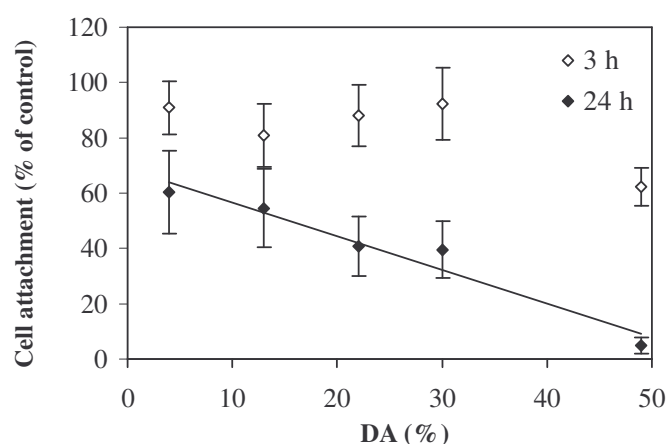


**Figure 2.** Cell attachment of MG-63 cells cultured on Ch films with different DAs, assessed at 3 and 24 h by the MTT assay. Values reported are the average  $\pm$  SD of 6 cultures, expressed as a function of the control, TCPS.

After 24 h of incubation, the percentage of cells that remained attached to the substrate clearly depended on the DA, decreasing from 60 to 5% of the control, as the DA increased from 4 to 49%. Within this range of DAs, a statistically significant Pearson correlation coefficient of  $-0.958$  ( $p \leq 0.01$ ) was found between the formazan absorbance and the DA. Therefore, a linear association between these two variables was found, with higher DAs leading to lower cell numbers. The repetition of the experiment drove to similar results.

### Cell proliferation

Cell growth kinetics, as determined by the MTT assay, is shown in Figure 3. For DAs  $\leq 30\%$  all Ch films showed significant cell proliferation between 24 and 120 h of cell culture. During this incubation period, the cell number followed generally the trend: DA 30% = DA 22% < DA 13% < DA 4%, in accordance with the initial cell adhesion levels observed after 24 h of cell culture. When comparing with the control, on which cell proliferation was observed after 4 hours of cell culture, cell growth on chitosan films revealed a longer lag-phase, of about 24 h. At different time frames, the cell number on chitosan films was significantly lower than on the control, regardless of the DA, with exception of the 4 h incubation period, where no statistical difference between the control and DAs 4%, 30% and 49% was found.

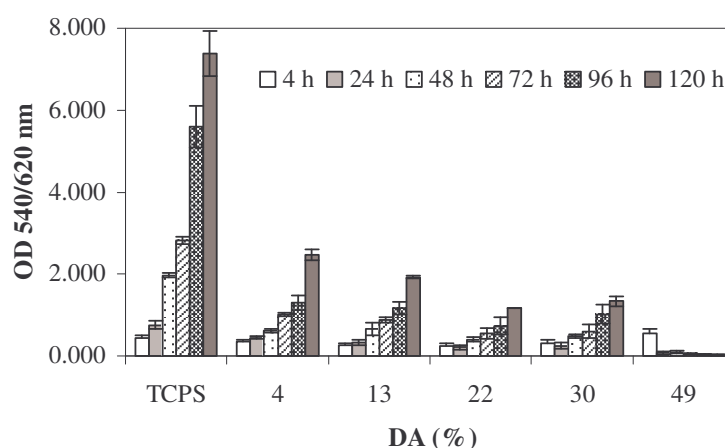


**Figure 3.** Cell growth kinetics of MG-63 cells cultured on Ch films with different DAs, as determined by the MTT assay. TCPS was used as control. Values are the average ( $\pm$  SD) of 6 cultures.

These results are in agreement with the lower cell adhesion observed for Ch samples after a 24 h incubation period, when compared to TCPS. On samples with the highest DA (49%) cell proliferation was impaired, as shown by the minimum levels of formazan absorbance observed after a 48 h incubation period.

The specific growth rates found for cells cultured on Ch films as a function of the DA, are depicted in Table 3. No cell growth was detected for a DA of 49%. The negative rate shown represents a net loss of cells due to poor cell attachment. On films with lower DAs, MG-63 cells exhibited a reduction in specific growth rate when compared to the control, of approximately 29% for the DA of 4%, and of about 44% for higher DAs. When compared among them, cells grown on Ch films with DAs in the range of 13 to 30% revealed no major differences in terms of growth rate and correspondent doubling time, cells dividing approximately every 48 h. However, for the lowest DA, a higher specific growth rate and consequent shorter doubling time were found, cells dividing approximately every 38 hours. Repetition of the experiment led to values of specific growth rates in the range of the values described.

**Table 3.**

Specific growth rate of MG-63 cells cultured on Ch films as a function of DA, assessed by the MTT assay. TCPS was used as positive control. Values reported are the average and standard error determined from linear regression of culture data. No cell growth was detected on the sample correspondent to a DA of 49%.

| Surface | Specific growth rate ( $\text{h}^{-1}$ ) | Doubling time (hours) |
|---------|------------------------------------------|-----------------------|
| TCPS    | $0.026 \pm 0.001$                        | $26.5 \pm 1.0$        |
| DA 4%   | $0.019 \pm 0.001$                        | $37.5 \pm 2.0$        |
| DA 13%  | $0.015 \pm 0.001$                        | $46.8 \pm 3.2$        |
| DA 22%  | $0.015 \pm 0.002$                        | $47.8 \pm 6.6$        |
| DA 30%  | $0.015 \pm 0.002$                        | $47.2 \pm 6.4$        |
| DA 49%  | $-0.014 \pm 0.002$                       | n.a.                  |

n.a., not applicable.

### Cell morphology and cytoskeletal organization

After 3 h of incubation, analysis of the cultures by optical microscopy revealed for the majority of cells cultured on chitosan films a round morphology, while on the control cells were already beginning to spread (figures not shown). Phase photomicrographs taken after 24 and 72 h of incubation are shown in Fig. 4. At 24 h, cell spreading was observed on films with DAs  $\leq 13\%$  (although to a lesser extent than those on the control), while for higher DAs, cells retained a spherical morphology [Fig. 4(a), (c), (e), (g), (i) and (k)].

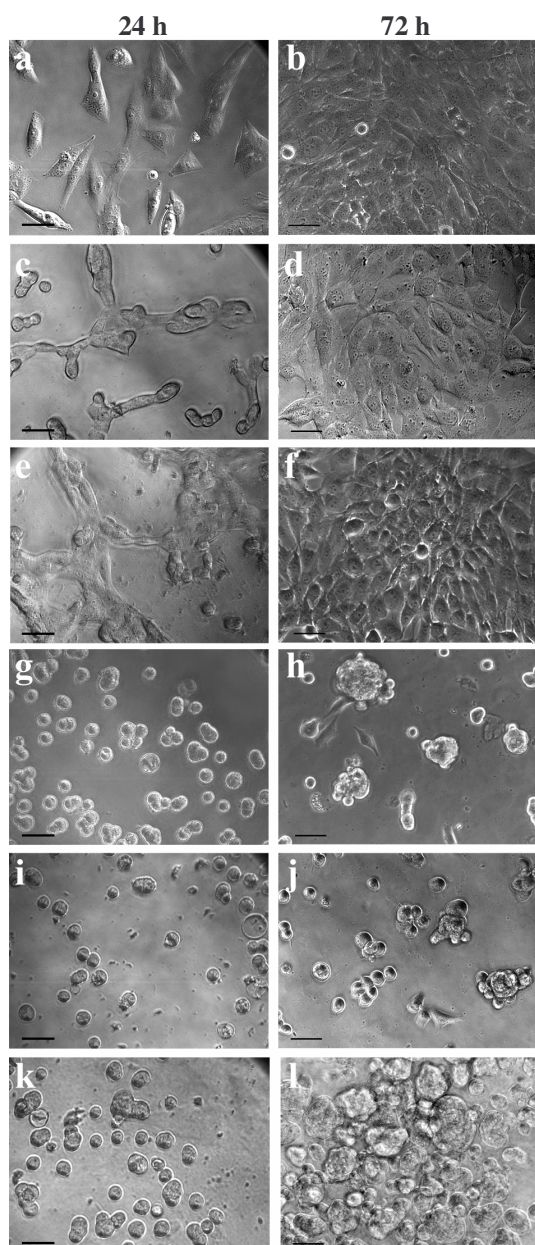


**Figure 4** Cell morphology of MG-63 cells cultured on: (a and b) TCPS and (c to l) Ch films with different DAs: (c and d) 4%; (e and f) 13%; (g and h) 22%; (i and j) 30% and (k and l) 49%. Images obtained by contrast phase optical inverted microscopy, 24 and 72 h after cell seeding. Scale bar = 50  $\mu\text{m}$ .

After 72 h of cell seeding, a clear effect of the DA on cell morphology was observed. On films with DAs  $\leq 13\%$  cells were well spread, exhibiting a polygonal or fusiform shaped morphology, already reaching confluency, as observed on the control [Fig. 4(b), (d) and (f)]. Cell spreading was progressively reduced with the increase of the DA [Fig. 4(h) and (j)]. On films with the highest DA (49%) exclusively spherically shaped cells were observed, most of them clustered into spheroid-like cellular aggregates [Fig. 4(l)].

Cytoskeleton organization was progressively higher on decreasing the DA, as shown by the increasing densification of cortical actin filaments, observed after 72 h of incubation (Fig. 5). At this time of incubation, for DAs  $\leq 13\%$ , cells displayed a well organized F-actin organization and oval nuclei, as observed for the control, revealing distinct actin filaments as well as bundles of F-actin delimiting the cytoplasm, that are better visible at higher magnification [Fig. 5(a) to (f)]. Cell-to-cell contacts and numerous cytoplasmatic processes correspondent to cell filopodia were also observed. For DAs in the range of 22 to 30%, F-actin staining was less evident, although some radially oriented filaments were observed within the cortical actin network of the cells. Herein, cells were found mainly clustered into spheroids, displaying a spherical morphology [Fig. 5(g) and (h)]. For the DA of 49%, the scarce cells observed displayed faint F-actin staining, with no discernable actin filament formation [Fig. 5(i)].

## DISCUSSION AND CONCLUSIONS

The purpose of this investigation was to assess the effect of Ch films with DAs ranging from 4 to 49% on the adhesion, spreading and short-term proliferation of osteoblast-like cells. To achieve this, MG-63 cells were used, for being reported suitable in the study of cell adhesion of bone derived cells.<sup>24</sup>

Our results showed that for a short-term incubation period (3 h) cell attachment was similar among films with DAs in the range of 4 to 30%. This initial cell attachment was mainly attributed to non-specific interactions including electrostatic interactions occurring between protonated Ch amine groups and negatively charged cell surface proteoglycans, as suggested by Chatelet et al..<sup>17</sup> Lower cell attachment was observed on films carrying the lowest  $\zeta$ -potential (DA 49%), which is in agreement to this

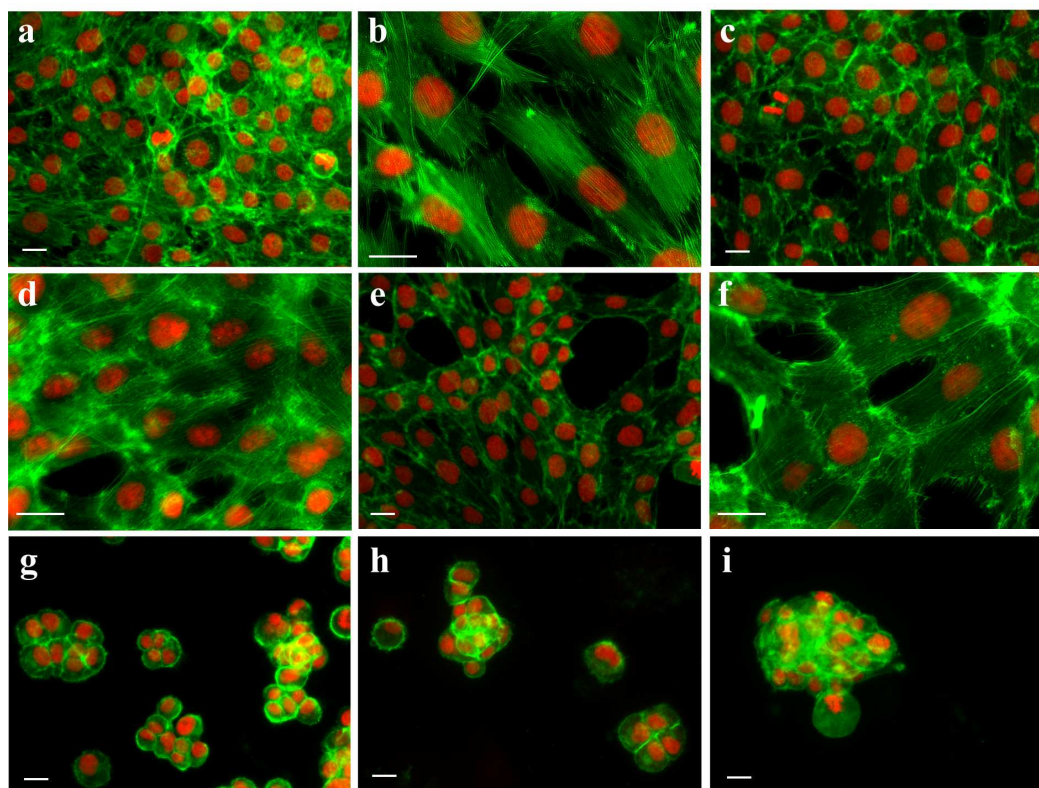


**Figure 5.** Fluorescent labeling of F-actin (green) and DNA (red) of MG-63 cells cultured on: (a and b) TCPS and (c to i) Ch films with different DAs: (c and d) 4%; (e and f) 13%; (g) 22%; (h) 30% and (i) 49%. Images obtained by fluorescent microscopy, 72 h after cell seeding.

assumption. Close to the intrinsic  $pK_a$  of Ch, which is reported to vary between 6.46 and 6.8 for DAs ranging from 5 to 35%, respectively,<sup>27</sup> the majority of Ch ammonium groups are dissociated and subsequently uncharged. Still, the presence of a small amount of non-dissociated ammonium groups in chitosan chains is sufficient to provide enough cationic sites and allow the establishment of electrostatic interactions.<sup>30</sup> After 24 h of incubation, the percentage of cells remaining attached to the films clearly depended on the DA. The cell percentage decreased from 60 to 5% of the control, as the DA increased from 4 to 49%, respectively (See Fig. 2). Therefore, cell adhesion of osteoblast-like cells to Ch followed a similar trend as the one reported for other anchorage dependent cells, revealing a greater cell affinity towards lower DAs. In the present study, the values of cell attachment as function of the control were found to be lower than those reported in the literature for other anchorage dependent

cells cultured on Ch.<sup>17,18</sup> In the mentioned studies, cell adhesion was assessed after incubation periods not longer than 1 hr. In this study, we have also observed higher cell attachment rates, for the shortest incubation period (3 h). The examination of cell morphology at 24 h also suggests a higher cell attachment strength onto films with lower DAs, as cell spreading was only observed on samples with DAs  $\leq 13\%$ . By using phalloidin staining, we investigated the cytoskeleton architecture of filamentous actin, since this is essential to mechanical coupling to the underlying substratum.<sup>15</sup> With time, cell spreading and cytoskeleton filamentous organization were also only attained on films with DAs  $\leq 13\%$ , while on films with higher DAs, cells were found mainly clustered into spheroid-like cellular aggregates, displaying faint F-actin staining. A clear effect of the DA on cell morphology was observed, which may explain why osteoblasts cultured on Ch films are reported to retain either a spherical morphology,<sup>10</sup> or the characteristic flattened appearance of migrating osteoblasts.<sup>31,32</sup> Regarding cell proliferation, the cells on films with the lowest DA (4%) proliferated at a higher rate comparing to those cultured on films with higher DAs. Films with a DA of 49% failed to sustain cell proliferation, concomitantly to the lowest cell attachment rate found for these films, after 24 h of cell culture. However, films with DAs in the range of 13 to 30% have shown similar specific growth rates among samples, despite the different cell attachment rates revealed after 24 h of incubation. The possible causes for the cellular responses described above are discussed below.

Osteoblast adhesion to biomaterials at a longer-term is modulated differentially by the type and quantity of adsorbed specific extracellular matrix glycoproteins as well as by their conformation upon adsorption. Integrin binding to glycoproteins containing an RGD sequence like fibronectin (Fn) induces the rearrangement of F-actin fibers leading to cell anchorage and spreading, and triggers signals that direct subsequent cellular responses, including proliferation and differentiation.<sup>15</sup> According to the literature, self-assembled monolayers (SAMs) exposing amine groups adsorb higher levels of Fn from solution as compared to methyl groups (which are present in chitosan acetylated units).<sup>33</sup> Moreover, upon adsorption on SAMs exposing amine groups, Fn was reported to present a more favorable conformation for  $\alpha_1$  integrin binding and osteoblast adhesion.<sup>34</sup> According to the work of Cuy et al.,<sup>35</sup> Ch films have the ability to adsorb both Fn and laminin from single protein solutions, although in those studies a single Ch with a DA  $< 20\%$  was used. Therefore, the higher levels of

cell adhesion and spreading observed on decreasing the DA are possibly related to the ability of amine groups in Ch glucosamine units to adsorb Fn from serum, and not merely to the higher cationicity and hydrophilicity of the films. The fact that among Ch samples, the sample exhibiting the highest  $\zeta$ -potential (DA 4%), revealing the highest surface concentration of amine groups, led to the higher rate of cell proliferation, a cellular response triggered by integrin-binding, is in agreement to this assumption. MG-63 cells express several Fn receptors. Among these receptors are the  $\alpha_5\beta_1$  integrin, a high affinity receptor for Fn and the  $\alpha_v$  subunit which, together with the  $\beta_1$  subunit forms a receptor for vitronectin.<sup>24</sup> On TCPS, the cell attachment observed was attributed to its ability to preferentially adsorb vitronectin from serum.<sup>36</sup> Cell adhesion to Ch films with higher DAs was possibly mainly governed by non-specific electrostatic interactions. Positively charged substrates like polylysine are reported to promote the attachment of osteoblasts in the absence of serum, although in time, cell proliferation on these surfaces is lower than on Fn coated surfaces.<sup>37</sup> The cell spreading observed for a DA of 13% suggests that in spite of the lower  $\zeta$ -potential, cell attachment strength was enough to allow the synthesis of endogenous ECM molecules and consequently cell spreading. On films with DAs of 22 and 30%, despite the spherical-shaped morphology, cells were able to proliferate within multicellular spheroids, at a similar growth rate as those cultured on films with a DA of 13%. Although in the case of osteoblasts, cell proliferation is usually inhibited when cells are poorly attached to the substrate, cells from osteosarcoma may show, as other transformed cells, an anchorage-independent growth.<sup>38</sup> Finally, we observed a longer lag-phase on Ch, as compared to TCPS, in accordance to the observed delay of cell spreading. This delay suggests that integrin-mediated cell adhesion on Ch films was lower when compared to TCPS, as a delay in cell proliferation is also observed when osteoblasts are cultured in serum depleted media.<sup>37</sup>

Cell adhesion, spreading and proliferation of human bone cells are influenced by surface roughness, generally being enhanced on smooth samples as compared to rough and less organized ones, in the case of samples with micrometer scale topography.<sup>39,40</sup> In this study, AFM investigations of Ch films revealed a smooth surface with roughness values within the nanometric range ( $< 100$  nm), while films with the highest DA showed a significantly higher surface roughness. In the case of samples with nanometer scale topography, roughness has been reported to induce merely a delay in

cell spreading.<sup>41</sup> Therefore, the significantly higher surface roughness together with the higher hydrophobicity and lower  $\zeta$ -potential observed for films with a DA of 49% may have contributed to hamper of cell adhesion on this substrate.

In summary, the DA greatly affects cell adhesion, spreading and proliferation of osteoblast-like cells on Ch films, possibly by influencing the nature of the initial adsorbed protein layer. While cell spreading was only possible on films with DAs  $\leq$  13%, cell proliferation was favored on films with a lower DA. Differences in the DA as small as 9% were found to be critical in terms of osteoblast response. For this reason, the DA should be carefully considered while choosing chitosan-based templates for *in vitro* expansion of bone cells.

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### **Rat bone marrow stromal cells osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation**

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#### **ABSTRACT**

Cell adhesion and proliferation of a few anchorage dependent cells cultured on chitosan (Ch) matrices are influenced by the degree of *N*-acetylation (DA) of Ch. In the present work, we examined the influence of the DA on the attachment, spreading, proliferation and osteogenic differentiation of rat bone marrow stromal cells (rBMSCs). Ch membranes were characterized in terms of surface morphology, roughness and wettability, and in terms of adsorption of an adhesive protein, fibronectin (Fn). Chs with DAs in the range of 4 to 49% were used. Among the chitosan samples, the DA of 4% led to the highest Fn surface concentration, both from single protein solution and from diluted serum. Furthermore, the levels of Fn adsorbed from serum found for this DA were threefold higher than for the tissue culture polystyrene control, indicating that in the presence of competitive proteins Ch is more specific towards Fn adsorption than tissue culture polystyrene. rBMSCs cultured on Ch carrying a DA of 4% were able to spread, proliferate and differentiate, reaching a

higher level of osteogenic differentiation than on the control, despite the lower cell attachment observed for all Ch samples. Because the chitosan sample with a DA of 4% showed the highest Fn adsorption from serum, we suggest that cell adhesion, spreading and osteogenic differentiation of rBMSCs on Ch may be mediated by the adsorbed layer of Fn.

## INTRODUCTION

Chitosan (Ch), a biocompatible and bioresorbable biopolymer obtained by *N*-deacetylation of chitin under alkaline conditions, is presently under investigation for a wide number of biomedical applications, including wound dressings and drug and gene delivery.<sup>1</sup> Similarly to most polysaccharides, Ch has the ability to elicit specific cellular functions, namely as immunoadjuvant, by inducing the release of cytokines which favor the histoarchitectural organization of connective tissues.<sup>2,3</sup> When applied to bone defects in animal models, Ch was found to improve osteogenesis and angiogenic activity.<sup>4,5</sup> As a two- and three-dimensional support matrix, Ch has been explored for the grow of a number of anchorage-dependent cells including bone cells.<sup>6,7</sup> Moreover in what concerns osteogenic differentiation, Klokkevold et al.<sup>8</sup> reported an enhancement of fetal mice calvariae cell differentiation, when cultured on Ch membranes.

Ch is a linear copolymer of glucosamine and *N*-acetyl glucosamine in a  $\beta 1 \rightarrow 4$  linkage, and the degree of *N*-acetylation (DA) together with the molecular weight are the most important parameters for its characterization. The DA, which is by definition the molar fraction of *N*-acetylated units, is a structural parameter influencing charge density, crystallinity and solubility, including the propensity to enzymatic degradation, with higher degrees of acetylation (DAs) leading to faster biodegradation rates.<sup>9</sup>

The DA was reported to influence cell adhesion of a few anchorage-dependent cells including keratinocytes and fibroblast-like cells, lower DAs favoring cell adhesion.<sup>10-12</sup> Moreover, cell proliferation was also reported to be influenced by the DA, dependent on the cell type, according to the same studies. However, in what concerns osteogenic cells namely bone marrow stromal cells (BMSCs), the influence of this parameter has not been reported yet, to the best of our knowledge. The use of BMSCs is gaining increasing interest for bone-regenerative cell-based therapies, since

they constitute the main source of autologous inducible and determined osteoprogenitor cells committed to osteogenesis presently available.

Osteoblast adhesion to biomaterials and gene expression are mediated by the conditioning layer of adsorbed bone glycoproteins containing Arg-Gly-Asp (RGD) domains, such as type I collagen, fibronectin (Fn), and vitronectin. Cell adhesion to cationic surfaces carrying amine functionalities was reported to be mediated also through these adhesive proteins and not by non-specific electrostatic interactions.<sup>13,14</sup> However, only a few works report the adsorption of adhesive proteins onto Ch and in those a single DA was used.<sup>15-17</sup> Recently, Benesh and Tengvall<sup>18</sup> reported fibrinogen adsorption onto chitosan but not onto a fully acetylated chitosan suggesting that the DA may influence protein adsorption.

Therefore, we hypothesize that the DA may influence osteoblast differentiation, *in vitro*, by influencing the adsorbed layer of adhesive proteins and consequently, cell attachment, spreading, cytoskeletal reorganization and gene transcription. To test our premise, Ch membranes prepared from Chs with different DAs were characterized and evaluated regarding their ability to promote the attachment, spreading, proliferation and osteogenic differentiation of rat BMSCs (rBMSCs). In addition, the various membranes were evaluated regarding their ability to adsorb Fn, chosen as a model of cell adhesive molecule, both from single protein solution and from diluted serum.

## MATERIALS AND METHODS

### Preparation and characterization of Chs with different DAs

Ch powder from squid pens was kindly supplied by France Chitine (France) and further purified by filtration of Ch acidic solution and subsequent alkali precipitation. The DA was then determined, and found to be approximately 30%, using infrared spectroscopy (FT-IR system 2000 from Perkin-Elmer) in KBr pellets using the amide III band at  $1320\text{ cm}^{-1}$  as the analytical band and the band at  $1420\text{ cm}^{-1}$  as the internal reference band, according to Brugnerotto et al.<sup>19</sup> Chs with lower DAs were prepared by heterogeneous deacetylation of the purified Ch using successive 1-h alkali treatments with intermittent washing in water, according to Mima et al.<sup>20</sup>. A sample with higher DA was obtained by *N*-acetylation of the purified Ch in aqueous,

methanolic acetic acid solution, using acetic anhydride as reactive, following the method of Aiba.<sup>21</sup> Table 1 presents the physicochemical characteristics of the resultant Chs in terms of DA and average molecular weight. The DA was determined as above mentioned, for DAs < 40%. For higher DAs, the DA was determined according to Baxter et al.,<sup>22</sup> using the amide I band at  $1655\text{ cm}^{-1}$  as the analytical band and the band at  $3450\text{ cm}^{-1}$  as the internal reference band. Results presented are the average ( $\pm$  SD) of four measurements. Weight- and number-average molecular weight ( $M_w$  and  $M_n$ , respectively), as well as polydispersity index (*PDI*) were assessed by high-performance size exclusion chromatography using the system 0.2 M  $\text{CH}_3\text{COONa}$ /0.5 M  $\text{CH}_3\text{COOH}$  as mobile phase, according to Terbojevich et al..<sup>23</sup>

**Table 1.**  
Chitosan characterization

| Sample | Preparation method          | DA (%)          | $M_w \times 10^5$ | <i>PDI</i>    |
|--------|-----------------------------|-----------------|-------------------|---------------|
| Ch I   | <i>N</i> -acetylation (1 h) | $49.1 \pm 1.54$ | n.d.              | n.d.          |
| Ch II  | As received                 | $29.8 \pm 1.21$ | $5.4 \pm 0.3$     | $3.9 \pm 0.6$ |
| Ch III | Deacetylation (0.5 h)       | $22.1 \pm 1.30$ | $3.5 \pm 0.1$     | $2.6 \pm 0.2$ |
| Ch IV  | Deacetylation (1 h)         | $12.9 \pm 1.31$ | $2.8 \pm 0.1$     | $2.3 \pm 0.1$ |
| Ch V   | Deacetylation (3 h)         | $4.23 \pm 0.46$ | $2.1 \pm 0.1$     | $2.1 \pm 0.2$ |

n.d., not determined.

### Preparation of Ch membranes

Membranes were prepared by solvent casting, from 1% w/v Ch solutions in 0.2 M acetic acid. The polymer was allowed to dissolve for 24 h, centrifuged for degassing and poured onto the wells of 24-well tissue culture polystyrene (TCPS) plates from TPP®, 200  $\mu\text{L}$  per well. After solvent evaporation at ambient temperature, the resulting membranes were deprotonated in 0.5 M aqueous NaOH and thoroughly washed with distilled and deionized water until neutrality.

For characterization purposes the membranes were prepared in six-well TCPS plates using the same ratio of Ch gel volume per unit area ( $100\text{ }\mu\text{L}/\text{cm}^2$ ), in the same manner as described above. After the neutralization step and extensive rinsing, the membranes

were dried at 30°C for 24 h in a vacuum oven, and stored in a desiccator until surface analysis.

### **Surface analysis of Ch membranes**

Atomic force microscopy imaging was performed with a PicoPlus scanning probe microscope interfaced with a Picoscan Controller 2500 (both from Molecular Imaging). Imaging was performed in Tapping™ mode, using standard Si tips. The samples were analyzed in air at room-temperature. The average roughness ( $R_a$ ), the arithmetic average of the deviations from the centre  $x$ - $y$  plane (the centre plane, is a plane such that the volumes enclosed by the image surface above and below are equal),<sup>24</sup> was determined under a scan range of  $90 \times 90 \mu\text{m}^2$ , using the SPIP™ Version 3.0 software. Measurements were made in at least five randomly chosen areas, in two different samples.

Wettability studies were performed using a video-based optical contact angle measurement device OCA 15 plus, provided with an electronic syringe unit (Dataphysics Instruments GmbH, Germany). Distilled and deionized water, presenting conductivity not higher than  $1 \mu\text{S}/\text{cm}$ , was used. Static contact angles were measured at 25°C by the sessile drop method, depositing a water droplet of  $4 \mu\text{L}$  on the membrane surface. Because of the swelling nature of the membranes, time-dependent measurements were made for a period of 300 s, and the experimental contact angles extrapolated to time zero. Contact angles were calculated by SCA20 software (Dataphysics, version 2.0), fitting the drop profiles to the Young-Laplace equation. Results are the average of at least six measurements, made in two different samples.

### **Fn adsorption studies**

Protein adsorption studies were run in parallel using two different solutions containing radiolabeled Fn ( $^{125}\text{I}$ -Fn): a solution containing serum in the same amount as the one used to equilibrate the membranes prior to the cell culture experiments (10% v/v), and a single Fn solution, prepared at a concentration such as to provide the same amount of Fn present in the former. The serum solution was prepared by diluting fetal bovine serum (Gibco) in degassed phosphate buffered saline (PBS) with 0.01 M NaI -

iodinated PBS (PBSI). The single Fn solution was prepared dissolving human plasma Fn (Sigma) in degassed PBSI, at a concentration of 20 µg/mL, given that the concentration reported for Fn in human serum is about 200 µg/mL.<sup>25</sup> Buffer with iodide (PBSI) was used in order to inhibit adsorption of free <sup>125</sup>I ions, present in trace amounts during protein adsorption experiments.<sup>26</sup> Fn was labeled with <sup>125</sup>I using the Iodogen method.<sup>27,28</sup> Purification of the <sup>125</sup>I-Fn was performed using a Sephadex G-25 M column (PD-10 column, from Amersham Pharmacia Biotech) to remove unbound <sup>125</sup>I. The yield of iodination found for the fraction used was of 98.4%. To assess if preferential adsorption of <sup>125</sup>I-labeled Fn occurred, a series of control experiments were performed varying the ratio of labeled to unlabeled Fn (10-50%). Labeled protein solutions were prepared by adding <sup>125</sup>I-Fn to unlabeled solutions in order to obtain a final activity of ~10<sup>8</sup> cpm/mg. Before being used for adsorption tests, the hydrated Ch membranes were carefully detached from the TCPS substrate and cut into 5×5 mm<sup>2</sup> strips, the edges of the membranes being discarded. Tissue culture coverslips of 13-mm diameter (SARSTEDT) were also used. The samples were sterilized in 70% v/v ethanol, hydrated in degassed PBSI overnight, and finally incubated in the labeled protein solution using at least, three replicates of each material. Adsorption tests were performed at 25°C, over a 60-min period. After protein adsorption, the membranes were rinsed three times with PBS, and then transferred to radioimmunoassay tubes, for gamma activity counting.

### **Cell culture**

Primary rBMSCs were obtained according to the method developed by Maniopoulos et al.,<sup>29</sup> under consent of the IBMC/INEB ethic committee. Briefly, the femurs and tibias of Wistar rats (male, 4 weeks old) were aseptically excised from the hind limbs, the epiphyses cut off, and the marrow flushed with standard culture medium, which consisted of α-minimal essential medium supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin sulphate, and 2.5 µg/mL amphotericin B (all from Gibco). Cells were seeded in 75-cm<sup>2</sup> plastic culture flasks, and incubated in a humidified incubator (37°C and 5% CO<sub>2</sub>). The medium was changed after the first 24 hours to remove nonadherent cells. Subsequently, the adherent cells were cultured for 10 days, the medium being renewed every 3 days. Cells from the first passage were used.

### Cell seeding on the membranes

Before cell seeding, the samples were sterilized with 70% v/v ethanol for 30 min and equilibrated in sterile PBS (10 min; two times) followed by standard medium (30 min; two times). rBMSCs were seeded on Ch membranes or directly on uncoated polystyrene, used as control, at a cell seeding density of  $1.2 \times 10^4$  viable cells/cm<sup>2</sup>. Forty-two samples of each substrate were seeded. The osteogenic culture medium consisted on standard medium supplemented with 0.2 mM ascorbic acid, 10 mM  $\beta$ -glycerophosphate and 10 nM dexamethasone. Cells were cultured for periods of 1, 4, 7, 14 and 21 days, the medium being renewed every 3 days. For each material and culture period, two samples without cells were equally incubated and used as blanks in biochemical assays.

### rBMSCs attachment, proliferation, and osteogenic differentiation

rBMSCs attachment and proliferation was monitored by quantifying nucleic acid content. Osteogenic differentiation was followed by assaying alkaline phosphatase (ALP) activity, an early marker of osteoblastic differentiation and the synthesis of osteocalcin (OCN), a specific osteogenic marker. At the end of each culture period, the cell layers were rinsed twice in PBS and stored at -20°C. The cell lysates, obtained after treatment of the cultures with 1% Triton X-100 at 5°C and brief sonication at 0°C, were subsequently assayed for DNA, ALP activity and total protein amount, as described below.

#### *DNA quantification.*

DNA was quantified using a commercially available kit (DNA-QF, Sigma) based on the binding of AT sequences to the fluorescent dye bisBenzimide (Hoechst 33258, Sigma), and using calf thymus DNA as a standard.

#### *ALP activity measurement and histochemical staining.*

For the measurement of ALP activity the cell lysates were incubated in a bicarbonate buffer solution (pH 10) containing 2mM *p*-nitrophenyl phosphate and 4mM MgCl<sub>2</sub> for 60 min at 37°C.<sup>30</sup> After stopping the reaction with 1M NaOH, hydrolysis of substrate

to *p*-nitrophenol (*p*-NP) was measured spectrophotometrically at 405 nm and compared with the values of a series of (*p*-NP) standards. Activities were normalized to cell protein content, as measured by the bicinchoninic acid method (Micro/Macro BCA, Pierce, Rockford). For ALP staining, the cell layers were rinsed twice with PBS, fixed in 3.7% formaldehyde, washed with water and stained in a diluted alkaline solution of Naphthol AS-MX phosphate containing the Fast Violet B salt, both from Sigma, for 45 min in the dark. After staining, the samples were rinsed with tap water and observed under a stereomicroscope system (Olympus). Cells expressing ALP on their surface developed a red stain after the incubation period.

#### *OCN synthesis.*

At days 14 and 21 of culture, the media was removed and assayed for OCN synthesis using a commercially available kit (Rat-MID Osteocalcin ELISA, Osteometer BioTech A/S, Denmark).

#### **Cell morphology and cytoskeletal filamentous actin organization**

The morphology of cells was followed daily by contrast phase optical microscopy. Cytoskeleton organization was assessed at day 14 of culture. Briefly, the cell layers were rinsed twice with PBS, fixed in 3.7% methanol-free formaldehyde, permeabilized in 0.1% buffered Triton X-100 and incubated with 1 µg/mL RNase and 1% w/v bovine serum albumin for 1 h. Cell cytoskeletal filamentous actin (F-actin) was visualized treating the cells with 5 U/mL Alexa Fluor<sup>®</sup> 488 phalloidin (Molecular Probes) for 20 min. Cell nuclei were counterstained with 15 µM propidium iodide (Sigma) for 5 min. Samples were finally mounted in Vectashield<sup>®</sup> (Vector) and examined with a Cell Observer System (Carl Zeiss, Germany). At day 21 of culture, the cell layers were treated for scanning electron microscopy (SEM) examination. The cell layers were rinsed twice with PBS, fixed with 1.5% v/v glutaraldehyde in 0.14 M sodium cacodylate (pH 7.4), and dehydrated in serial diluted ethanol solutions of 50, 60, 70, 80, 90 and 99% v/v. Finally, they were dried using a critical point drier CPD 020 (Balzers Union), sputtered-coated with gold, and examined by SEM (JEOL JSM-6301F).

## Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney  $U$  test, two-tailed asymptotic significance levels being considered. Results were considered statistically significant when  $p \leq 0.05$ . Calculations were performed using SPSS<sup>®</sup> software for Windows (version 12.0).

## RESULTS

### Surface analysis of Ch membranes

Atomic force microscopy images of Ch membranes for DAs ranging from 4 to 30% revealed basically a smooth surface, in accordance to similar reports [Fig. 1(a)].<sup>31</sup> Larger features, measuring about 2  $\mu\text{m}$  in diameter, were also found at the surface [Fig. 1(b)]. For a DA of 49%, Ch membranes showed a much more irregular surface morphology [Fig. 1(c) and (d)]. TCPS revealed a fibrous-like structured surface, as described elsewhere.<sup>32</sup> The results concerning roughness analysis corresponding to a  $90 \times 90 \mu\text{m}^2$  scanned area are shown in Figure 2. These studies revealed an increase of the mean values of average roughness ( $R_a$ ) with the DA from 36.1 to 75.4 nm, as the DA increased from 4 to 30%, respectively. For a DA of 49%, a significantly higher mean value of  $R_a$  was found, when compared to all the others, of about 283.7 nm. The  $R_a$  was found to be dependent on the DA, as shown by the statistical significant positive Pearson correlation coefficient found between these two parameters.

The water contact angle ( $\theta_{\text{water}}$ ) values determined as a function of DA are shown in Figure 2. A decrease of the  $\theta_{\text{water}}$  from  $\cong 98.5^\circ$  to  $\cong 76.4^\circ$ , as the DA decreased from 30 to 4%, respectively, was observed. Within this range of DAs, a Pearson correlation coefficient of 0.965, significant at the 0.001 level was determined between the  $\theta_{\text{water}}$  and the DA. The  $\theta_{\text{water}}$  found for the highest DA did not follow this tendency ( $\theta_{\text{water}} \cong 92^\circ$ ). However, this value has to be carefully considered, since it regards a much rougher surface when compared to all other membranes.

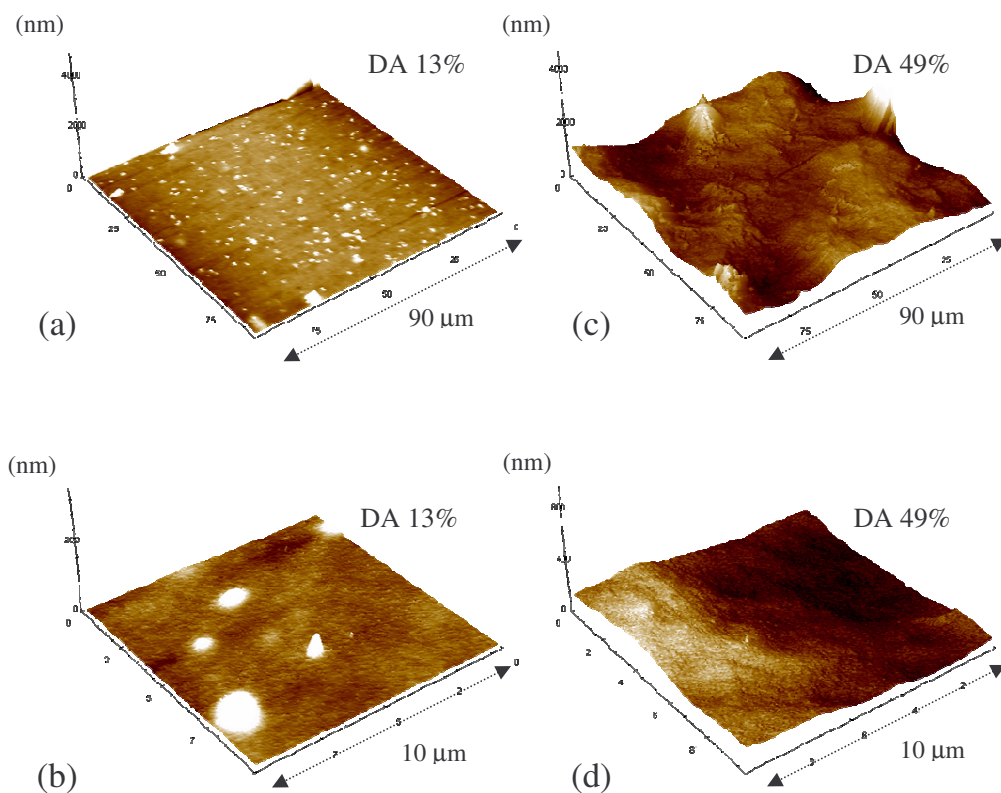


**Figure 1.** Tapping™ mode atomic force microscopy 3D images of chitosan membranes prepared from Chs with different DAs: (a and b) 13%; (c and d) 49%. Data were collected from two different scan ranges,  $90 \times 90 \mu\text{m}^2$  and  $10 \times 10 \mu\text{m}^2$ , under ambient conditions.

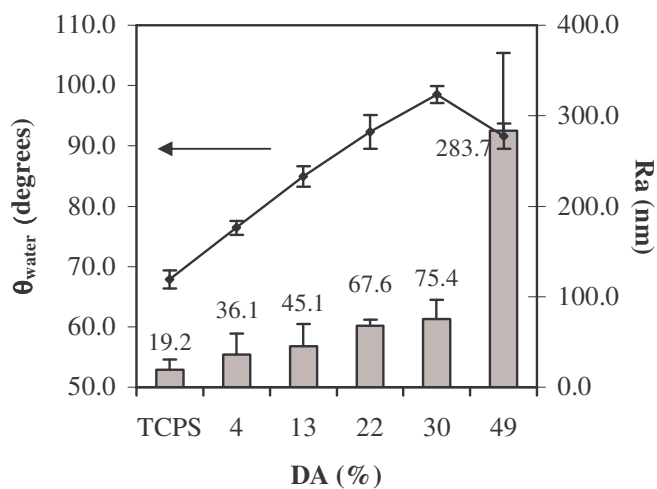


**Figure 2.** Average roughness ( $R_a$ ) and static water contact angle ( $\theta_{\text{water}}$ ) of chitosan membranes, as a function of DA, determined by atomic force microscopy and by the sessile drop method, respectively. The  $R_a$  was determined in air under a scan range of  $90 \times 90 \mu\text{m}^2$ . Values reported are the average  $\pm$  SD.

### Fn adsorption studies

Fn adsorption from a pure Fn solution is shown in [Figure 3(a)]. For DAs in the range of 13 to 49% Fn adsorption was poor, and no significant differences were found among samples, regardless the DA. In contrast, for a DA of 4%, a significantly higher amount of adsorbed Fn was found when compared to any of the other Ch samples, of about 0.6 mg/m<sup>2</sup>. TCPS led to a much higher Fn adsorption, of about 3.2 mg/m<sup>2</sup>. Fn adsorption from a serum solution followed the pattern described for a pure Fn solution [Fig 3(b)]. Again, a significantly higher Fn adsorption was found for a DA of 4%, when compared to all the others. Fn adsorption from serum was about 2% and 33% of the amount adsorbed from a pure Fn solution, for TCPS and Ch membranes, respectively.

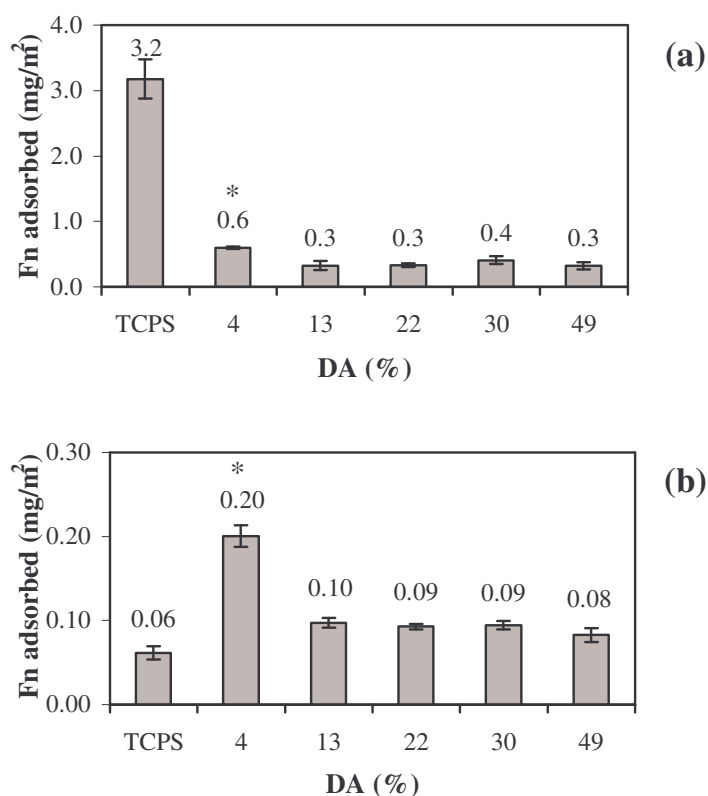


**Figure 3.** Adsorption of Fn radiolabeled with <sup>125</sup>I-Fn on Ch membranes as a function of DA (25°C; 60 min): (a) adsorption from a pure Fn solution (20 µg/mL in PBSI) and (b) adsorption from a 10% v/v solution of fetal bovine serum. Values reported are the average ± SD. Ch samples were compared between them. Asterisks indicate a significant difference when compared to any of the other Ch samples ( $p \leq 0.05$ ).

### rBMSCs attachment, proliferation, and osteogenic differentiation

rBMSCs attachment after 24 h of incubation was estimated by determining DNA content (Fig. 4). Cell attachment to Ch samples with DAs in the range of 4 to 30% was up to <19% of the control. On Ch samples with a DA of 49%, DNA was not detected. Although statistical differences were not found among all Ch samples, the negative Pearson correlation coefficient found between DNA amount and the DA, significant at the 0.05 level indicates a tendency to higher cell attachment levels on decreasing the DA.

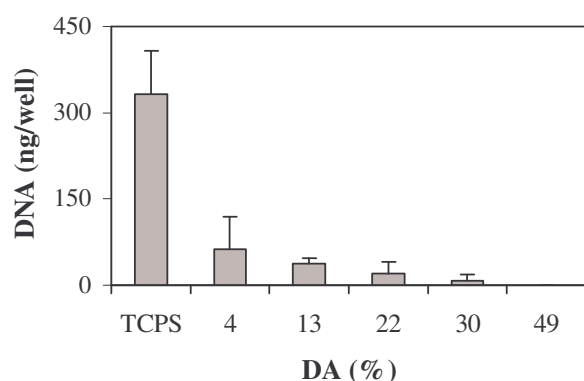


**Figure 4.** Cell attachment of rBMSCs cultured on Ch membranes with different DAs after 24 h of incubation, determined by DNA content. TCPS was used as control. Values reported are the average  $\pm$  SD of 6 cultures.

The proliferation of rBMSCs on TCPS and on Ch-coated wells with a DA of 4%, is depicted in Figure 5. On the control, the DNA content increased gradually from day 1 to day 14, reaching a plateau at this time point. On Ch with a DA of 4%, cell proliferation was poor during the first 7 days of culture. However, after this time

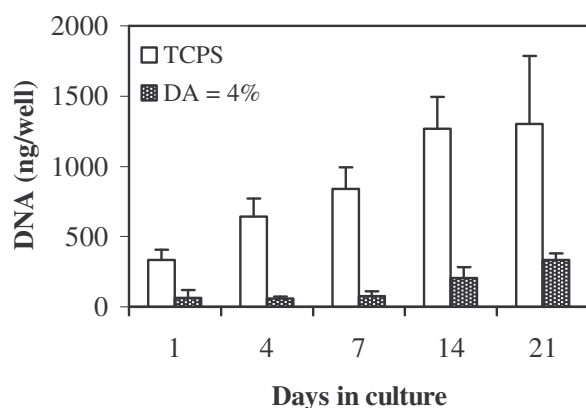


**Figure 5.** Cell growth kinetics of rBMSCs cultured on Ch membranes with a DA of 4%, as determined by DNA content. TCPS was used as control. Values are the average ( $\pm$  SD) of 6 cultures.

point, cells proliferated consistently until the end of the assay. During the whole incubation period the DNA amount increased approximately 7.6-fold and 3.9-fold in relation to day 1, on Ch and the control, respectively. The results regarding higher DAs are not shown, since minimum amounts of DNA were found for these samples throughout the incubation period.

The results regarding specific activity of ALP, are presented in Figure 6. On the control the ALP activity reached a maximum level at day 14 of culture. On chitosan with a DA of 4%, the ALP activity followed the same pattern. However, at days 14 and 21, a significantly higher level of enzyme activity was found, when compared with control. The results from activity staining of ALP at day 21 of culture revealed for both substrates histochemical reaction for ALP in most of the cells, together with the presence of clusters of cells with high catalytic activity of the enzyme (Fig. 7). When compared to the control, cells grown on Ch showed a more intense staining for the majority of cells despite the poorer cell density, in agreement with the findings obtained for the biochemical quantification of the enzyme.

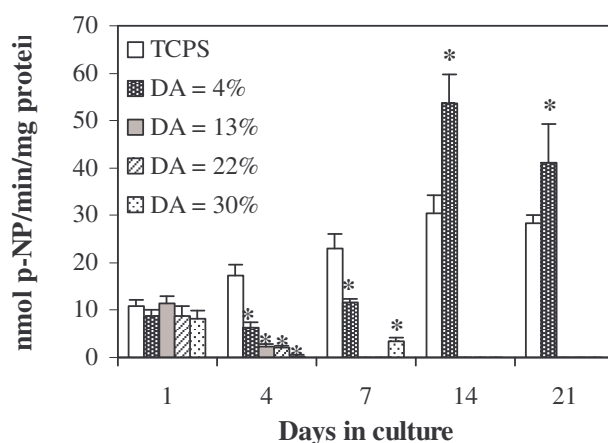


**Figure 6.** ALP activity of rBMSCs cultured on Ch membranes with different DAs, as a function of time. TCPS was used as control. ALP activity was normalized for protein content. Values are the average ( $\pm$  SD) of six cultures. Asterisks indicate a significant difference from the control, at the correspondent time point ( $p \leq 0.05$ ).

The OCN results revealed significantly higher levels of OCN synthesis for cells cultured on Ch (DA 4%), the values found being approximately sixfold and fourfold higher than the control at days 14 and 21, respectively (Fig. 8).

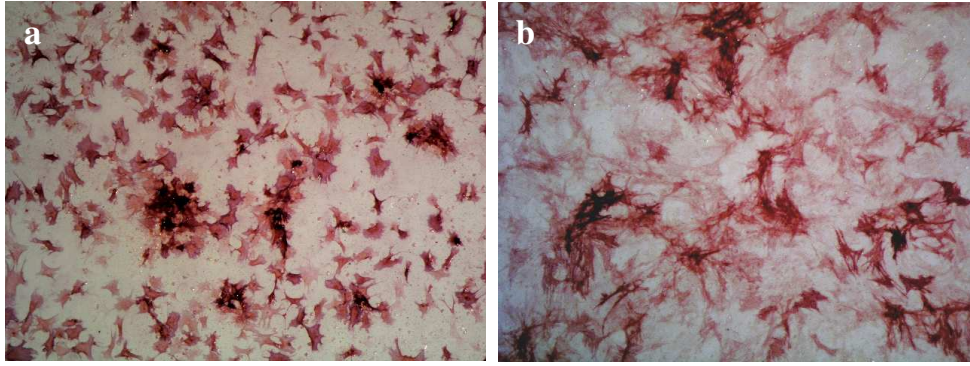


**Figure 7.** rBMSC cultures histochemically stained for ALP at day 21 of culture. Red areas are positive for ALP. (a) and (b) indicate cell culture on TCPS and on Ch membranes with a DA of 4%, respectively. Original magnification  $\times 50$ .

### rBMSCs morphology and cytoskeletal organization

Optical micrographs taken after 3 h of incubation revealed for Ch a high number of attached cells, displaying a spherical morphology (data not shown). However, after 24 h of cell incubation, most of them detached from the surface, in accordance to the DNA measurements at this time point, the remaining cells presenting a spherical-shaped morphology. Phase photomicrographs taken by day 3 of culture revealed for a DA of 4%, exclusively elongated cells with fusiform fibroblastic appearance, as on the control, as expected for BMSCs under the proliferative state [Fig. 9(a) and (b)]. On samples with higher DAs, sparse spherical cells, occasionally clustered into spheroids, were observed, although on samples with a DA of 13% spindle-like-shaped cells located at the periphery of the spheroids were also present [Fig. 9(c) to (e)]. By day 14

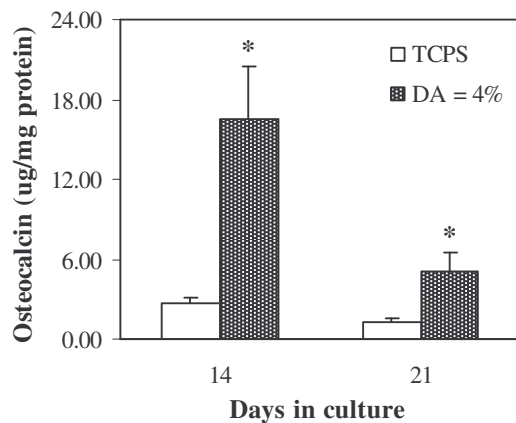


**Figure 8.** OCN synthesis of rBMSCs cultured on Ch membranes with a DA of 4%, assessed at days 14 and 21 of culture. TCPS was used as control. OCN levels were normalized for protein content. Values are the average ( $\pm$  SD) of three cultures. Asterisks indicate a significant difference from the control, at the correspondent time point ( $p \leq 0.05$ ).

of culture, F-actin/DNA labeling of cells cultured on chitosan with a DA 4% revealed polygonal-shaped cells with round nuclei, displaying a well organized F-actin cytoskeleton and numerous cell filopodia as well as cell-to-cell contacts (Fig. 10). On the control, cells showed a more fusiform appearance and oval nuclei, already at confluency. At day 21 of culture, on Ch, the SEM examination of the cell layers revealed cells with a completely flattened and spread morphology and rough dorsal surfaces, a characteristic of active cells, forming a confluent cell monolayer (Fig. 11). Fibrillar extracellular matrix assigned to collagen fibers and globular accretions with diameter of about 1  $\mu\text{m}$ , more visible at higher magnification, were also observed. No marked differences were observed in the SEM appearance of cultures compared to control, besides the presence of multilayers of cells on the control.

## DISCUSSION

The present investigation examines the effect of the DA of Ch, used as a two-dimensional support, on the attachment, spreading, proliferation and osteogenic differentiation of rBMSCs. Chs with DAs in the range of 4 to 49% were used.

Considerable efforts are being made in understanding how surface properties affect the adsorption and conformation of proteins and subsequently cell adhesion, spreading, and expression of a cellular phenotype. The Ch membranes prepared in this investigation were characterized in terms of surface roughness and wettability which, together with surface chemistry, constitute key factors influencing the protein adsorption process.<sup>33</sup>

The results obtained revealed an increase of surface roughness with the DA, indicating the presence of an increased number of fibrillar aggregates. Both charge density, which depends on DA, and chain length affect Ch solubility.<sup>34</sup> The formation of supramolecular structures in Ch acidic solutions is well documented, and particularly evident for high-molecular weight Ch.<sup>35,36</sup> In addition, because of the chemical heterogeneity of the present Ch samples, small fractions with high molecular weight and higher DA are likely to form aggregates. Therefore, the observed increase of surface roughness is probably related to the decrease of Ch charge density in the acidic solutions on increasing the DA, as well as to the higher average molecular weight of the samples.

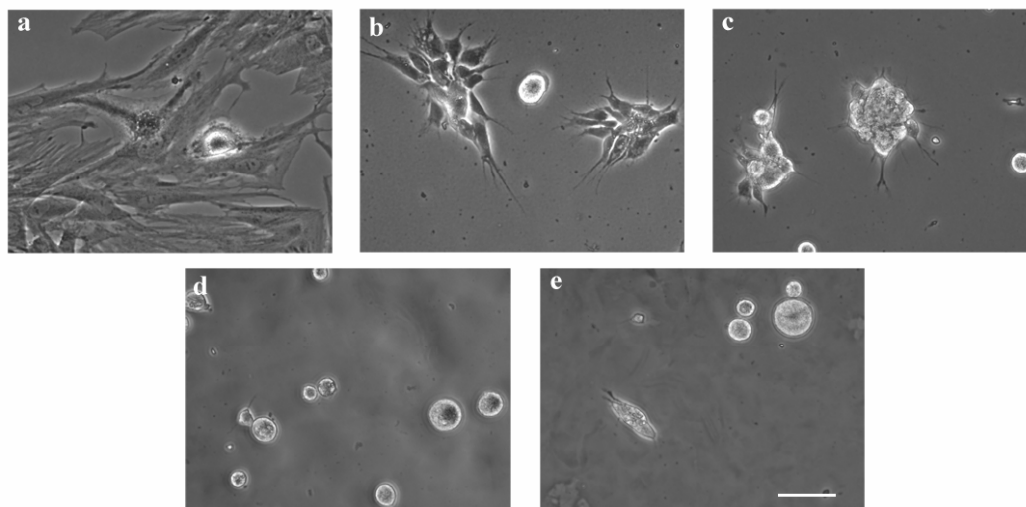


**Figure 9.** Cell morphology of rBMSCs cultured on: (a) TCPS and (b to e) Ch membranes prepared from Chs with different DAs: (b) 4%; (c) 13%; (d) 22% and (e) 30%. Images obtained by contrast phase optical inverted microscopy, 3 days after cell seeding. Scale bar = 50  $\mu\text{m}$ .

In what concerns wettability, a decrease of the water contact angle with the decrease of DA was observed for DAs  $\leq 30\%$ . This result may be explained based on experiments with self-assembling monolayers (SAMs) exposing methyl and amine groups. According to the literature, SAMs of alkanethiols on Au carrying methyl groups (present in acetylated chitosan residues) produce hydrophobic surfaces ( $\theta_{\text{water}} = 107^\circ$ ), whereas SAMs carrying amine groups form moderately wettable surfaces ( $\theta_{\text{water}} = 43^\circ$ ).<sup>37</sup> Therefore, the decrease of  $\theta_{\text{water}}$  observed on decreasing the DA may be attributed to the increase of amine functionalities at the surface, provided by glucosamine residues. Although contact angle measurements are known to be strongly affected by surface physical heterogeneities (the advancing contact angle usually increases with increasing roughness) for asperities lower than 0.1-0.5  $\mu\text{m}$  this effect is negligible.<sup>38</sup>

Regarding Fn adsorption on chitosan from a single protein solution, the highest adsorption was found for the lowest DA (4%). Although many studies report the influence of surface free energy and physical heterogeneities on the protein adsorption process, recent studies using SAMs of alkanethiols on Au carrying different functional groups allow a more accurate interpretation of the effects of surface chemistry on

protein adsorption. SAMs carrying amine groups were reported to adsorb higher levels of Fn from solution than methyl groups for a concentration of 20  $\mu\text{g/mL}$ .<sup>37</sup> Furthermore, higher retention of functional activity upon adsorption was reported, suggesting a lower degree of protein unfolding.<sup>37</sup> This indicates that the higher levels of adsorbed Fn found for a DA of 4% can be related to the higher surface concentration of amine functionalities. However, there was no correlation between the amount of Fn adsorption and the DA, suggesting that a minimum surface concentration of amine groups is required to promote Fn adsorption. In the presence of serum, Fn adsorption was lower than from a single Fn solution, which is attributable to the presence of competitive proteins. However, a DA of 4% led also to the highest Fn surface concentration. In addition, the levels of adsorbed Fn found for this DA were threefold higher than for the TCPS control, indicating that in the presence of other competitive proteins Ch membranes with a DA of 4% were more specific toward Fn adsorption than TCPS. Fn adsorption from serum on TCPS is reported to be lower as compared to other adhesive proteins, namely, vitronectin, and is attributed to the inability of Fn to adsorb in a competitive protein environment.<sup>39</sup> A study by Benesh and Tengvall<sup>18</sup> revealed larger amounts of adsorbed protein from heparinized human plasma, including fibrinogen, on Ch films carrying a DA of 15% than on fully acetylated Ch films. The present data are in agreement with this observation.

Osteoblast adhesion to biomaterials involves the initial cell attachment phase, implying short-term events of physico-chemical nature established between the

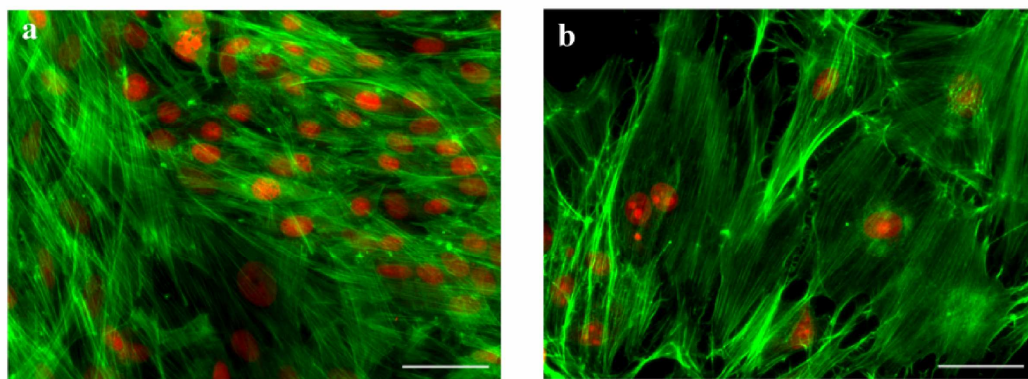


**Figure 10.** Fluorescent labeling of F-actin (green) and DNA (red) of rBMSCs cultured on: (a) tissue culture polystyrene and (b) Ch membranes prepared from Ch with a DA of 4%. Images obtained by fluorescent microscopy, 14 days after cell seeding. Scale bar = 50  $\mu\text{m}$ .

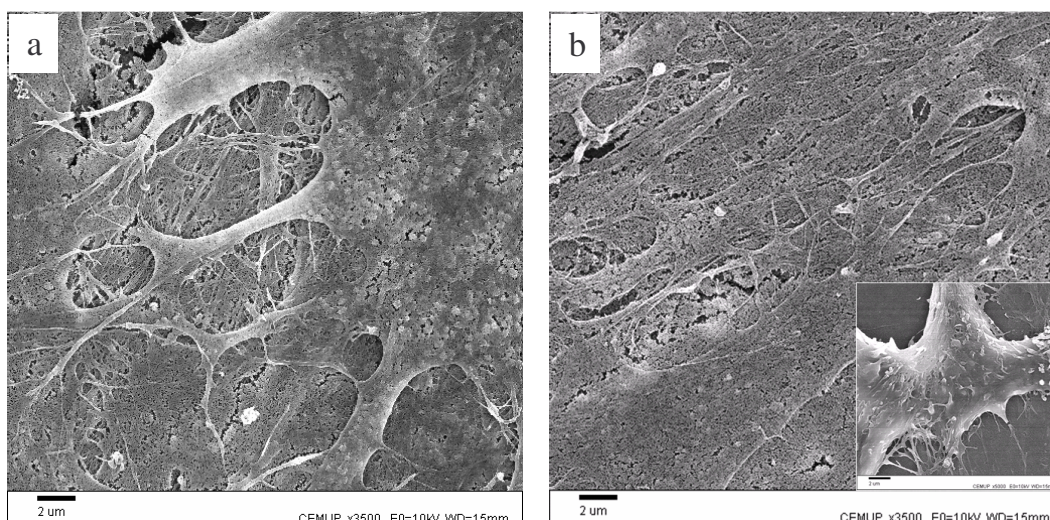


**Figure 11.** Cell morphology of rBMSCs cultured for 21 days on: (a) TCPS and (b) Ch membranes with a DA of 4%. Photographs obtained by SEM.

material's surface and the cells, and the adhesion phase occurring in the longer term, involving the binding of cell-surface molecules, namely, integrins and proteoglycans to extracellular matrix glycoproteins.

The short-term cell adhesion observed at 3 h (data not shown) can be mainly attributed to non-specific electrostatic interactions occurring directly between protonated Ch amine groups and negatively charged cell-surface proteoglycans, as suggested elsewhere.<sup>10</sup> After 24 h, the percentage of cells remaining attached was less than 19% of the control, independently of the DA, as shown by DNA amount (Fig. 4). These findings indicate for Ch a low cell attachment strength, regardless the DA, when compared to the control. The spherical morphology displayed by the cells at this time point also suggests low attachment strength on Ch, regardless the DA, in accordance to DNA results. With time, however, rBMSCs spreading and cytoskeleton organization were observed, for the lowest DA (4%) (Figs. 9 and 10).

Fibronectin, a large extracellular matrix glycoprotein existing as a soluble dimeric form in plasma, contains several cell adhesion domains among them the RGD sequence, which acts as ligand to integrins leading to the establishment of focal adhesion contacts. Cell spreading, F-actin cytoskeletal organization, and gene transcription are mediated by these specific receptor/ligand interactions.<sup>40</sup> However, a minimum functional Fn density is required for cell spreading and focal adhesion assembly.<sup>37,41</sup>

In our investigation, cell spreading and cytoskeleton filamentous organization observed for the DA of 4% correlated well with the significantly higher levels of Fn adsorption from serum. Fn-coated substrates were reported to enhance hBMSCs cell attachment, when compared to vitronectin-coated ones.<sup>42</sup> In the case of these cells, cell binding to Fn is mediated by multiple  $\beta 1$  integrins.<sup>42,43</sup> Therefore, these results suggest that, in the presence of serum, cell adhesion to Ch is mainly a specific process mediated by the adsorbed layer of Fn. According to Gong et al.,<sup>17</sup> the structure of Fn is preserved, including its epitopes, upon adsorption onto Ch with a DA lower than 15%. Although surface wettability might have contributed to greater cell affinity towards the lowest DA, it is known that for osteoblasts, cell attachment and spreading vary with surface chemistry rather than with wettability.<sup>44</sup> The higher cell attachment found for TCPS, despite the lower Fn adsorption from serum, may be associated to its ability to preferentially adsorb vitronectin, and to induce, upon adsorption, a conformation favorable for cell adhesion.<sup>39</sup> Several vitronectin receptors were reported for hBMSCs including the  $\alpha_v\beta_5$ , which probably contributes to cell adhesion on TCPS.

Regarding the effect of the DA on cell proliferation, whereas for a DA of 4%, cell growth was observed, for DAs  $\geq 13\%$ , cell growth was impaired. The inhibition of cell proliferation may be attributed to the low cell attachment strength revealed for DAs  $\geq 13\%$ , as suggested by the round morphology displayed by the cells at day 3 of culture. In the case of osteoblasts, cell proliferation is usually inhibited when cells are poorly attached to the substrate. Cell growth on Ch with a DA of 4% revealed a longer lag phase, when compared to control, in accordance to the observed delay of cell spreading. However, cell proliferated equally on the control and Ch with a DA of 4%, as shown by the increases of DNA amount found at day 21 in relation to day 1 for both substrates, of about 7.6-fold for Ch and 3.9-fold for the control.

The osteogenic differentiation of rBMS cells was followed by assaying ALP activity and staining, and OCN synthesis. On both substrates (Ch with a DA of 4% and TCPS), ALP activity attained maximum levels at day 14, indicating a shift of the cells into a more differentiated state along the osteogenic lineage, correspondent to the stage of matrix maturation.<sup>45</sup> The significantly higher ALP activity levels found for cells grown on Ch at days 14 and 21, suggest the presence of a higher amount of cells undergoing osteogenic differentiation. At day 21, despite the lower cell density, cells grown on Ch membranes showed, for the majority of them, a more intense ALP

histochemical staining, when compared to control. Moreover, the levels of OCN found for chitosan were sixfold and fourfold higher than the control at days 14 and 21, respectively. Because OCN is a specific osteoblastic marker secreted after the onset of active extracellular matrix mineralization, these results indicate that rBMSCs cultured on chitosan with a DA of 4% were able to differentiate into osteoblasts, reaching a higher level of osteogenic differentiation than on the TCPS, consistently with the higher levels of ALP specific activity observed for cells cultured on this substrate.<sup>45,46</sup> The higher levels of Fn adsorption from serum, as compared to TCPS, possibly contributed to the osteogenic differentiation of rBMSCs on this substrate. Integrin binding to Fn is required for the expression of the osteoblastic phenotype.<sup>47,48</sup> These results highlight the importance of the composition of the support material in stem cell differentiation. In the present investigation, rBMSCs attachment, spreading, and osteogenic differentiation was only promoted on a two-dimensional Ch matrix with a DA of 4%. However, because three-dimensional matrices provide a more favorable ambient for cell adhesion, rBMSC response to porous Ch matrices with different DAs is presently under investigation.

## CONCLUSIONS

Fn adsorption onto Ch membranes was dependent on the DA, a DA of 4% revealing the highest Fn adsorption both from single Fn and serum solutions. Furthermore, the levels of Fn adsorbed from serum found for this DA were threefold higher than for the TCPS control, indicating that in the presence of competitive proteins Ch is more specific toward Fn adsorption than TCPS. rBMSCs cultured on Ch carrying a DA of 4% were able to spread, proliferate, and differentiate, reaching a higher level of osteogenic differentiation than on the control, despite the lower cell attachment observed for all Ch samples. Because the Ch sample with a DA of 4% showed the highest Fn adsorption from serum, we suggest that cell adhesion, spreading, and osteogenic differentiation of rBMSCs on Ch in the presence of serum may be mediated by the adsorbed layer of Fn.

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### **Thee-dimensional culture of human osteoblastic cells in chitosan sponges: The effect of the degree of acetylation**

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#### **ABSTRACT**

In this investigation, the effect of the degree of acetylation (DA) of chitosan (Ch) on the behavior of human osteoblastic MG-63 cells cultured in thee-dimensional Ch matrices was assessed. Ch sponges with DAs in the range of 4 to 49% were prepared and characterized in terms of microstructure, porosity and pore size. Collagen sponges were used as 3D control. Cell proliferation was determined using the MTT assay while the retention of the osteoblastic phenotype was monitored by assaying alkaline phosphatase activity. Cell morphology, cytoskeletal organization and viability were assessed using different microscopy techniques. Ch sponges showed a similar microstructure regardless the DA, except for the highest DA used, where a more heterogeneous pore distribution was observed. In terms of cell proliferation, alkaline phosphatase activity and cell viability, cells cultured in Ch scaffolds performed as well as in the 3D control regardless the DA, except for the highest DA used, where an inhibitory effect on cell proliferation was found. However, while in sponges with DAs  $\leq 13\%$  cells attached and spread displaying long cell filopodia and numerous cell-to-cell contacts, in sponges with higher DAs cells tended to remain spherical and grow into spheroid-like cellular aggregates. In the present study, the DA played a key role in

determining the affinity of osteoblastic cells towards the substrates, possibly by influencing the nature of the initial adsorbed protein layer.

## INTRODUCTION

Current concepts of bone regenerative medicine emphasize the need for new biocompatible and bioresorbable filling materials, structurally mimicking the extracellular matrix, capable of acting as vehicles for autologous osteoprogenitor cells and/or cell signaling molecules. Among others features, scaffolds are expected to allow loading, division and retention of progenitor cells, support rapid vascular ingrowth and encourage osteoconduction with host bone.<sup>1</sup> In this sense, considerable efforts are being directed towards designing materials with appropriate surface chemistry and porous microarchitecture, to improve cell anchorage and scaffold colonization. Particularly, surface chemistry is an important parameter affecting osteoblast adhesion, spreading and the expression of a cellular phenotype since it affects the adsorption and conformation of glycoproteins involved in the cell adhesion process.<sup>2</sup>

Chitosan (Ch), a natural polyheterosaccharide derived from chitin by *N*-deacetylation, is a biodegradable polymer with well established biocompatibility presently under investigation for a wide number of biomedical applications, including bone repair.<sup>3,4</sup> Ch is a linear copolymer of glucosamine and *N*-acetyl glucosamine in a  $\beta 1 \rightarrow 4$  linkage. The degree of *N*-acetylation (DA), which is by definition the molar fraction of *N*-acetylated units, is, together with the molecular weight, the most important parameter used to characterize Ch. The DA is a structural parameter influencing solubility, crystallinity and charge density, and also the susceptibility to enzymatic degradation, with higher DAs leading to faster biodegradation rates.<sup>5</sup> *N*-acetylglucosamine is present in glycosaminoglycans, components of the extracellular matrix (ECM) and of cell surface, usually linked to core proteins as proteoglycans. Ch can thus provide a non-protein matrix for 3D cell ingrowth, structurally similar to extracellular proteoglycans. In addition, its versatility to be processed into injectable, porous or membranar forms without use of toxic solvents, together with its sterilizability, makes it an attractive biopolymer for bone tissue repair.<sup>6</sup>

The bone regenerative properties of Ch were investigated in several animal models. Its ability to enhance osteogenesis and angiogenic activity, as well as to inhibit fibrous tissue invasion, were reported.<sup>7,8</sup> The association of Ch with calcium phosphates and biologically active biomolecules was also reported, in an attempt to improve its osteoconduction and bone regeneration induction.<sup>8,9</sup> At present, Ch is being investigated as a temporary support for the growth of a number of anchorage dependent cells, including osteoblasts, for tissue engineering applications.<sup>8,10,11</sup> The DA was reported to influence cell adhesion in the case of baby hamster kidney cells, fibroblast-like cells,<sup>12</sup> keratinocytes, fibroblasts,<sup>13</sup> and breast cancer cells,<sup>14</sup> with lower DAs favoring cell adhesion. Recently, we reported the effect of the DA of Ch on the osteogenic differentiation of bone marrow stromal cells (BMSCs), in a 2D culture. We found that BMSCs were able to spread, proliferate and differentiate along the osteogenic lineage on films with a DA of 4% but not on films with DAs  $\geq 13\%$ .<sup>15</sup> To the best of our knowledge, the study of the DA effect on osteoblasts behavior in a 3D culture system was not addressed yet. When compared to 2D matrices, 3D matrices are reported to provide a more favorable ambient for cell adhesion.<sup>16</sup> In addition, as most of the Ch-based templates developed envisaging bone regeneration *in situ* and cell-based bone regeneration are porous, the study of the DA effect in 3D scaffolds is of paramount importance.

In this study, Ch sponges prepared from Chs with different DAs were characterized and evaluated, regarding their ability to support the attachment, spreading and proliferation of human osteoblastic cells. Collagen sponges were prepared and used as 3D positive control. Cell growth kinetics was followed using the MTT assay, while the retention of the osteoblastic phenotype was investigated by assaying the expression of alkaline phosphatase (ALP), an osteogenic marker. Cell morphology, cytoskeletal filamentous actin organization and viability within the scaffolds were examined using different microscopy techniques.

## MATERIALS AND METHODS

### Materials

Squid pen chitosan (Chitosan 123, France Chitine), generously supplied by France

Chitine, and type I insoluble collagen, derived from bovine achilles tendon (C-9879, Sigma-Aldrich), were used. All reagents used were of analytical grade.

### **Preparation and characterization of Chs carrying different DAs**

Purified Ch was prepared by filtration of Ch acidic solution and subsequent alkali precipitation. The degree of *N*-acetylation (DA) was further determined, and found to be approximately 30%, as determined by infrared spectroscopy (FT-IR system 2000, Perkin-Elmer) in KBr pellets, according to Brugnerotto et al.,<sup>17</sup> using the amide III band (C-N stretching coupled with N-H in plane deformation) at 1320 cm<sup>-1</sup> as the analytical band and the band at 1420 cm<sup>-1</sup> as the internal reference band, as follows:

$$A_{1320}/A_{1420} = 0.3822 + 0.03133 \times \text{DA} (\%) \quad (1)$$

Chs with lower degrees of *N*-acetylation were prepared by heterogeneous deacetylation of the purified Ch in 50% (w/v) NaOH under N<sub>2</sub>, using successive 1-h alkali treatments, three at 65°C and one at 100°C, with intermittent washing in water, according to Mima et al..<sup>18</sup> A Ch sample with a higher DA was obtained by *N*-acetylation of the purified Ch at room temperature, in aqueous, methanolic acetic acid solution, with 0.86 mol equivalent of acetic anhydride per glucosamine residue during 1 h, following the method of Aiba.<sup>19,20</sup> The DA of the resultant Chs was determined as above described, for DAs < 40%. For higher DAs, the DA was determined according to Baxter et al.,<sup>21</sup> using the amide I band at 1655 cm<sup>-1</sup> as the analytical band and the band at 3450 cm<sup>-1</sup> as the internal reference band. Results presented are the average ( $\pm$  SD) of 4 measurements.

Weight and number average molecular weight ( $M_w$  and  $M_n$ , respectively), polydispersity index (*PDI*) and intrinsic viscosity ( $[\eta]$ ) were determined by high performance size exclusion chromatography, using the system 0.2 M CH<sub>3</sub>COONa/ 0.5 M CH<sub>3</sub>COOH as mobile phase, according to Terbojevich et al..<sup>22</sup> Separations were performed at room temperature, in a set of aquagel-OH mixed columns (Polymer Laboratories, UK) at a flow-rate of 1.0 mL/min. Samples were dissolved for 24 h in the mobile phase (0.05% w/v), centrifuged at 19000 g for 1h, filtered through 0.45  $\mu$ m cellulose acetate membrane (Millipore), and finally injected using a manual injection

valve equipped with a 116  $\mu\text{L}$  loop. A chromatographic system with a viscometer/right angle laser light scattering (RALLS) dual detector (T60 Viscotek), and a refractive index detector (K-5002 Knaeur) operating at the same wavelength as the RALLS detector (670 nm), was used.

### **Preparation of Ch scaffolds**

Porous scaffolds were prepared from 2% w/v Ch solutions in 0.2 M acetic acid. The polymer was allowed to dissolve for 24 h, centrifuged for degassing, and poured onto the wells of 24-well tissue culture polystyrene (TCPS) plates, 400  $\mu\text{L}$  per well. The Ch samples were then frozen at  $-78^{\circ}\text{C}$  and lyophilized for 24 h, resulting in sponges of 15 mm diameter and 2 mm height.

### **Preparation of collagen scaffolds**

A 2% w/v collagen suspension was prepared swelling 1 g of collagen overnight in 25 mL of 0.52 M acetic acid at  $5^{\circ}\text{C}$ , and further adding 25 mL of ice-cooled water. The suspension was further homogenized at  $5^{\circ}\text{C}$  using a Polytron PT 10/35 (Kinematica), degassed and poured into the wells of 24-well TCPS plates, 400  $\mu\text{L}$  per well. The collagen samples were then frozen and lyophilized, as described for Ch sponges. The resulting collagen sponges were finally dehydrothermally crosslinked in a vacuum oven at  $140^{\circ}\text{C}$  for 24 h. The sponges obtained presented approximately the same dimensions as those described for Ch sponges.

### **Scaffolds characterization**

The surface morphology of the lyophilized sponges was examined by scanning electron microscopy (SEM). The samples were sputtered-coated with gold (JEOL JFC 1100) and observed using a JEOL JSM-6301F scanning electron microscope.

Sponge bulk density and porosity were determined by liquid displacement, according to Zhang et al.,<sup>23</sup> immersing 5 sponges per scaffold in graduated cylinders, using ethanol as the displacement liquid. Additionally, a sample of each scaffold was

analyzed by mercury intrusion, on a Mercury Intrusion Porosimeter PoreSizer 9320 (Micromeritics Norcross, G.A). The pore size of the hydrated scaffolds was determined by widefield microscopy. The sponges were sectioned longitudinally to the sample with a razor blade and observed with a Cell Observer System (Carl Zeiss, Germany). The average pore diameter was calculated using the relation:

$$d = \sqrt{l \cdot h} \quad (2)$$

where  $l$  and  $h$  are the maximum and the minimum pore lengths, respectively. Results presented are the average ( $\pm$  SD) of 20 measurements.

### **Cell culture**

MG-63 osteoblast-like cells from human osteosarcoma were obtained from the American Type Culture Collection (ATCC), and routinely cultured in standard culture medium, which consisted of minimal essential medium supplemented with 10% fetal bovine serum, 50  $\mu\text{g/mL}$  gentamicin sulfate and 2.5  $\mu\text{g/mL}$  amphotericin B, the medium being renewed every 3 days. Cultures were maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C. For subculture cells were trypsinized and suspended in the culture medium at  $1.25 \times 10^6$  viable cells/mL.

### **Cell seeding on scaffolds**

Prior to cell seeding, the lyophilized scaffolds were cut into pieces of c.a. 0.5  $\text{cm}^2$  each, with the help of a razor blade, immersed in absolute ethanol, and then subjected to a brief vacuum treatment in order to remove all the entrapped gas bubbles. Sterilization and hydration was accomplished by sequential immersion in serially diluted ethanol solutions of 70, 50 and 25% v/v, during periods of 30 min. Scaffolds were finally equilibrated in phosphate buffered saline (PBS) followed by standard culture medium (30 min; 3 $\times$ ), and then placed in 24-well flat suspension tissue culture plates (SARSTEDT), with the airside surface of the sponges facing upwards. 30  $\mu\text{L}$  of cell suspension were injected into the scaffolds, with a pipette tip. In order to monitor the culture the same number of cells was seeded on tissue culture coverslips of 13 mm

diameter (SARSTEDT). For each material, forty samples were seeded. Scaffolds were incubated at 37°C for 4 h, after which 600 µL of culture medium were added. Cells were cultured for periods up to 5 days, the medium being renewed on the third day.

### **Cell growth kinetics**

The number of viable cells was evaluated after 12, 48, 72, 96 and 120 h after seeding, using the viability/proliferation MTT assay.<sup>24</sup> Briefly, cultures were rinsed twice with PBS and then incubated with MTT solution (0.5 mg/mL in Dulbecco's medium without phenol red) at 37°C for 3 h. At the end of this period the supernatant was discarded, the scaffolds transferred to new well plates and 200 µL of acidic isopropanol (0.04 M HCl in isopropanol) were added to each well to dissolve the formazan crystals. After complete dissolution the dye solution was centrifuged and the optical density of the supernatant was read at 540 nm with reference to 620 nm, using a microplate spectrophotometer (SLT Labsinstruments-Austria). Cell number was determined using the linear region of a standard curve where absorbance was plotted against known number of viable cells counted using a Neubauer improved chamber. Reported values are the average ( $\pm$  SD) of 5 replicates. For each material, 2 samples without cells were equally incubated, and used as blank.

### **Alkaline phosphatase (ALP) activity**

ALP activity was determined in cell lysates 72 and 120 h after cell seeding. At the end of each culture period, the cell sponges were rinsed twice in PBS and stored at -20°C. After thawing, cell lysates were obtained after brief sonication at 0° of the cultures with 1% Triton X-100. ALP activity was assayed by incubating cell lysates with a working solution containing equal parts of 1.5 M 2-amino-2-methyl-1-propanol, 20 mM *p*-nitrophenyl phosphate and 1 mM MgCl<sub>2</sub> for 60 min at 37°C.<sup>25</sup> After stopping the reaction with 1M NaOH, hydrolysis of substrate to *p*-nitrophenol (*p*-NP) was measured spectrophotometrically at 405 nm and compared with the values of a series of (*p*-NP) standards. Results are the average ( $\pm$  SD) of 4 replicates, expressed in nanomoles of *p*-NP, produced per minute per 10<sup>6</sup> cells. For each material, four samples without cells were equally incubated, and used as blank.

### **Cell morphology and cytoskeleton organization**

Cell morphology was assessed 72 h after cell seeding, by SEM. Cell loaded materials were rinsed twice with PBS, fixed with 1.5% v/v glutaraldehyde in 0.14 M cacodylic acid buffer, and dehydrated in serial diluted ethanol solutions of 50, 60, 70, 80, 90 and 99% v/v. Finally they were dried using a critical point drier CPD 020 (Balzers Union), sputtered-coated with gold, and examined by SEM.

Cytoskeleton organization was assessed 96 h after cell seeding. Cells were rinsed twice with PBS, fixed in 3.7% methanol-free formaldehyde, permeabilized in 0.1% buffered Triton X-100 and incubated with 1 µg/mL RNase and 1% w/v bovine serum albumin for 1 h. Cell cytoskeletal filamentous actin (F-actin) was visualized treating the cells with 5 U/mL Alexa Fluor<sup>®</sup> 488 phalloidin (Molecular Probes) for 1 h. Cell nuclei were counterstained with 15 µM propidium iodide (Sigma) for 5 min. Samples were finally mounted in Vectashield<sup>®</sup> (Vector) and examined with a Cell Observer System (Carl Zeiss, Germany).

### **Live/Dead assay**

Cell viability in the scaffolds was assessed 96 h after cell seeding, using the fluorogenic substrate Calcein AM (Molecular Probes). Calcein AM freely diffuses into the cells, and is hydrolyzed by non-specific esterases into fluorescent products that are retained by cells with intact plasma membranes. Propidium iodide (PI) was used as counterstain, to identify non-viable cells. PI readily enters and stains cells with compromised membranes but cannot cross the membranes of viable cells. Cells were rinsed twice with PBS and loaded with 1 µM Calcein AM for 20 min. Subsequently, cells were incubated with 15 µM propidium iodide for 5 min, rinsed with PBS and visualized by a Leica SP2 AOBS laser scanning confocal microscope (Leica Microsystems, Germany). Serial image pairs were collected at 2 µm vertical intervals.

### **Statistical analysis**

Average pore diameter data were analyzed using ANOVA. When significant

differences were found, homogeneity of variances was checked and the Bonferroni's  $t$  test was used for multiple comparisons. Data regarding cell number and ALP activity were analyzed using the Mann-Whitney  $U$  test. Results were considered statistically significant when  $p \leq 0.05$ . Pearson correlation coefficient was used to determine the linear association between two variables. Calculations were performed using SPSS® software for Windows (version 12.0).

## RESULTS

### Ch characterization

The physicochemical characterization of Chs in terms of DA,  $M_w$ ,  $PDI$  and  $[\eta]$  is presented in Table 1. The DA found for the Chs prepared ranged from 4 to 49%. The temperatures used during the heterogeneous deacetylation of Ch were high enough to prepare an almost completely deacetylated and colorless Ch (DA 4%). Squid pen Ch is derived from  $\beta$ -chitin, which has a less tight molecular packing than  $\alpha$ -chitin, the chitin present in crab and shrimp shells. Hence, low temperatures are required for its deacetylation, when compared to  $\alpha$ -chitin.<sup>26</sup> The  $M_w$  of Ch after acetylation is not shown, since traces of insoluble parts were observed in the solution. However, no appreciable degradation is expected during acetylation of Ch.<sup>26</sup> As a result of the scission of glycosidic linkages and end-group peeling, alkaline treatment led to a decrease of the weight average molecular weight towards c.a. 40% of its initial value,

**Table 1.**

Ch characterization

| Preparation method          | DA (%)      | $M_w \times 10^5$ | $PDI$     | $[\eta]$<br>(dL·g <sup>-1</sup> ) |
|-----------------------------|-------------|-------------------|-----------|-----------------------------------|
| <i>N</i> -acetylation (1 h) | 49.1 ± 1.54 | n.d.              | n.d.      | n.d.                              |
| As received                 | 29.8 ± 1.21 | 5.4 ± 0.3         | 3.9 ± 0.6 | 12.6 ± 0.37                       |
| Deacetylation (0.5 h)       | 22.1 ± 1.30 | 3.5 ± 0.1         | 2.6 ± 0.2 | 10.8 ± 0.42                       |
| Deacetylation (1 h)         | 12.9 ± 1.31 | 2.8 ± 0.1         | 2.3 ± 0.1 | 10.1 ± 0.18                       |
| Deacetylation (3 h)         | 4.23 ± 0.46 | 2.1 ± 0.1         | 2.1 ± 0.2 | 8.20 ± 0.29                       |

n.d., not determined.

together with the decrease of the intrinsic viscosity, in the range of previous findings.<sup>27</sup> Over the same period of time the ratio  $M_w/M_n$  (*PDI*) decreased as well, from 3.9 to 2.1, as expected from the random scission of polymer chain occurring during alkaline deacetylation.<sup>28</sup>

### **Scaffolds structure**

SEM micrographs of the seeding surface of lyophilized sponges revealed for collagen sponges a highly porous microstructure with a high degree of interconnectivity, together with the presence of thin fibers, in accordance to literature [Fig. 1(a)].<sup>29</sup> Ch sponges with DAs  $\leq 30\%$  presented a similar microstructure, displaying a porous structure with interconnected pores, yet showing a more homogeneous pore distribution and the absence of fibers, when compared to collagen ones [Fig. 1(b) to (e)]. All pores were perpendicularly oriented to the surface plane and thin walled, presenting a polygonal cross section. Ch sponges with a DA of 49% showed a more heterogeneous pore distribution with the presence of fibers [Fig. 1(f)]. The characterization of the scaffolds in terms of porosity, bulk density and average pore size, is presented in Table 2. The bulk density values determined for Ch scaffolds were in the range of 0.028 to 0.057, no significant correlation being found between this structural parameter and the DA. The porosity values of Ch scaffolds determined by liquid displacement ranged from 93.0 to 97.0%, the highest porosity value being found for the DA of 49%. Also, no significant correlation was found between sponge porosity and the DA. Mercury intrusion porosimetry led to values of bulk density and porosity similar to those obtained by liquid displacement. In which concerns the average pore diameter of scaffolds in the hydrated state, a value of  $64 \pm 16 \mu\text{m}$  was found for collagen sponges, which is within the range of the values expected for sponges frozen under similar conditions.<sup>30</sup> Ch scaffolds presented average values ranging from 61 to 87  $\mu\text{m}$ , with few pores outside the 47 to 102  $\mu\text{m}$  range. Although significant differences were found among them, no significant correlation was found between average pore diameter and the DA. When compared to collagen sponges, Ch sponges with DAs of 22% and 13% presented significantly higher values of average pore diameter.

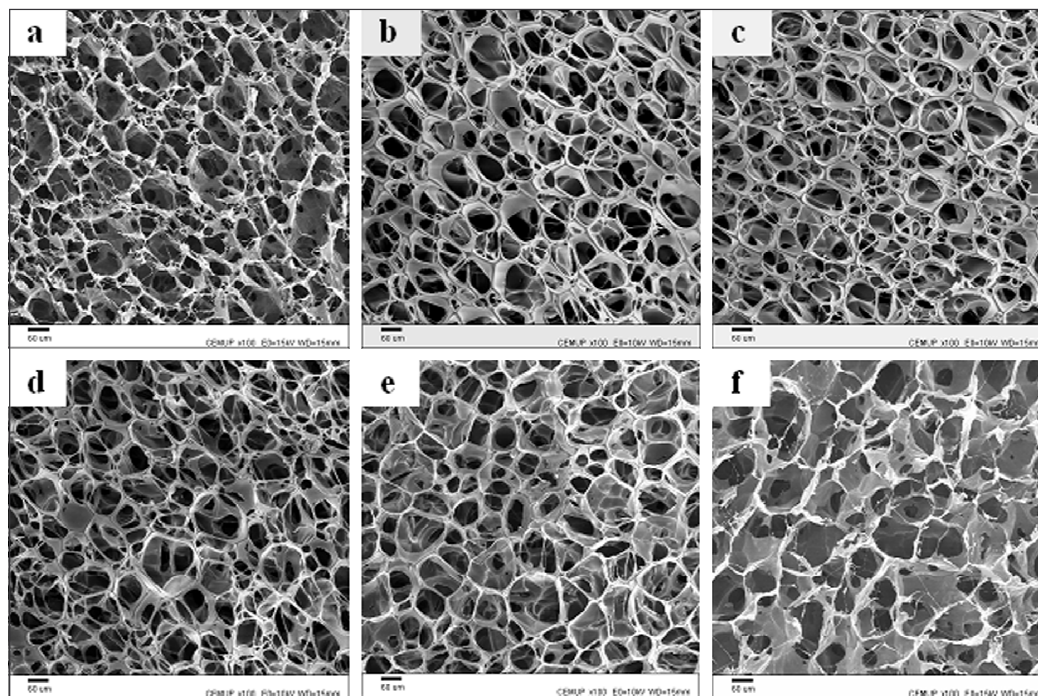


**Figure 1.** SEM photographs of the air-side surface of lyophilized sponges prepared from (a) collagen; (b to f) Chs with different DAs: (b) 4%; (c) 13%; (d) 22%; (e) 30% and (f) 49%.

**Table 2.**

Scaffolds properties

| Scaffold    | Bulk density (g·mL) |       | Porosity (%) |      | Pore average diameter (μm) |
|-------------|---------------------|-------|--------------|------|----------------------------|
|             | Liq. Disp           | M.I.  | Liq. Disp.   | M.I. |                            |
| Collagen    | 0.029               | 0.034 | 98.0         | 85.5 | 64 ± 16                    |
| Ch (DA 4%)  | 0.034               | 0.055 | 93.0         | 93.7 | 71 ± 16                    |
| Ch (DA 13%) | 0.030               | 0.040 | 95.0         | 95.1 | 85 ± 14*                   |
| Ch (DA 22%) | 0.030               | 0.035 | 95.9         | 94.9 | 87 ± 15*                   |
| Ch (DA 30%) | 0.057               | 0.030 | 93.8         | 95.4 | 61 ± 14                    |
| Ch (DA 49%) | 0.033               | 0.028 | 97.0         | 96.6 | 74 ± 22                    |

Ch, Ch; Liq. Disp., liquid displacement; M.I., mercury intrusion.

Asterisks indicate a statistical significant difference from collagen sponges. (\*  $p \leq 0.05$ ).

## Osteoblast culture within scaffolds

### Cell growth kinetics

Figure 2 shows the cell number per well/scaffold, as a function of time. The number of viable cells determined after 12 h exceeded the number of seeded cells in all the scaffolds, pointing out that the cells kept in the matrices retained their viability, regardless the DA. On the 3D control, cells proliferated all over the culture period although significantly less than on TCPS. Cell proliferation was observed on all Ch scaffolds, regardless the DA. Still, on sponges prepared from highly acetylated Ch (DA 49%) cell proliferation rate was poorer, as shown by the significantly lower cell number found from 72 h on, when compared to collagen sponges. Furthermore, a statistically significant negative correlation ( $p \leq 0.01$ ) was found between the cell number/scaffold and the DA of Ch from 72 h until the end of the assay, pointing out a tendency to lower DAs leading to higher cell numbers.

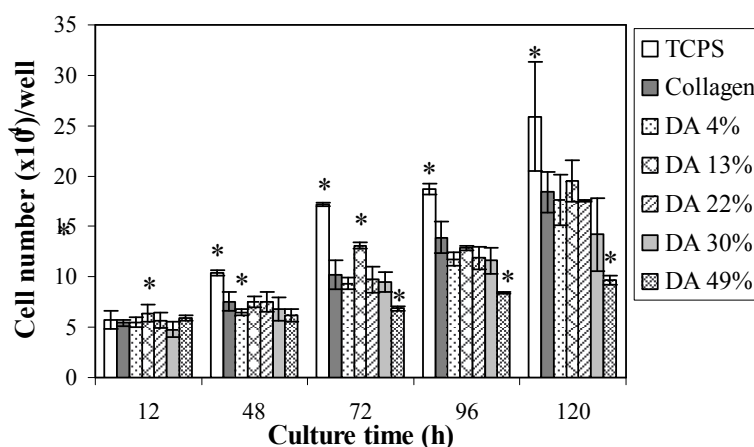


**Figure 2.** Proliferation profile of MG-63 cells cultured in Ch sponges with different DAs, as a function of time. Collagen sponges were used as 3D control, while TCPS was used to monitor the culture. Asterisks indicate a significant difference from collagen sponges, at the correspondent time point ( $p \leq 0.05$ ).

### ALP activity

Figure 3 shows cells ALP activity determined after 72 and 120 h of cell seeding. The expression of ALP activity of MG-63 cells cultured on TCPS coverslips did not vary between the time points studied. Regarding 3D matrices, similar ALP activity levels were observed among all groups at 72 h, no significant differences being found between any of the Ch groups and the collagen sponges. Yet at 120 h, cells seeded on collagen sponges revealed lower ALP activity levels than those cultured on TCPS. At this time point again, no significant differences were found between any of the Ch groups and the collagen sponges, regardless the DA. In addition, no linear dependence was found for ALP activity and Ch DA, at any time point studied.

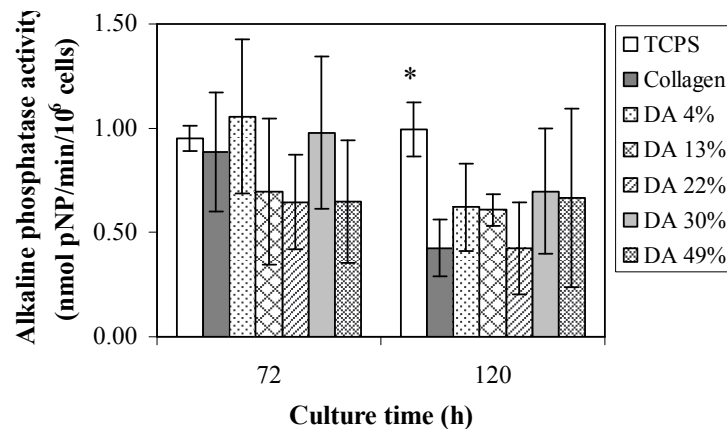


**Figure 3.** Alkaline phosphatase (ALP) activity of MG-63 cells cultured in Ch sponges with different DAs, as a function of time. Collagen sponges were used as 3D control, while TCPS was used to monitor the culture. Asterisks indicate a significant difference from collagen sponges, at the correspondent time point ( $p \leq 0.05$ ).

### *Cell morphology*

Figure 4 shows SEM micrographs of cell loaded scaffolds taken 72 h after cell seeding. Cells cultured on TCPS were spindle-like or polygonal shaped and presented a completely spread morphology, as expected. At this time point, cell layers reaching confluence could already be observed [Fig. 4(a)]. On collagen sponges, a large amount of the scaffold surface was covered with well spread stellate shaped cells, bridging between the pores, with rough dorsal surfaces, a characteristic of active

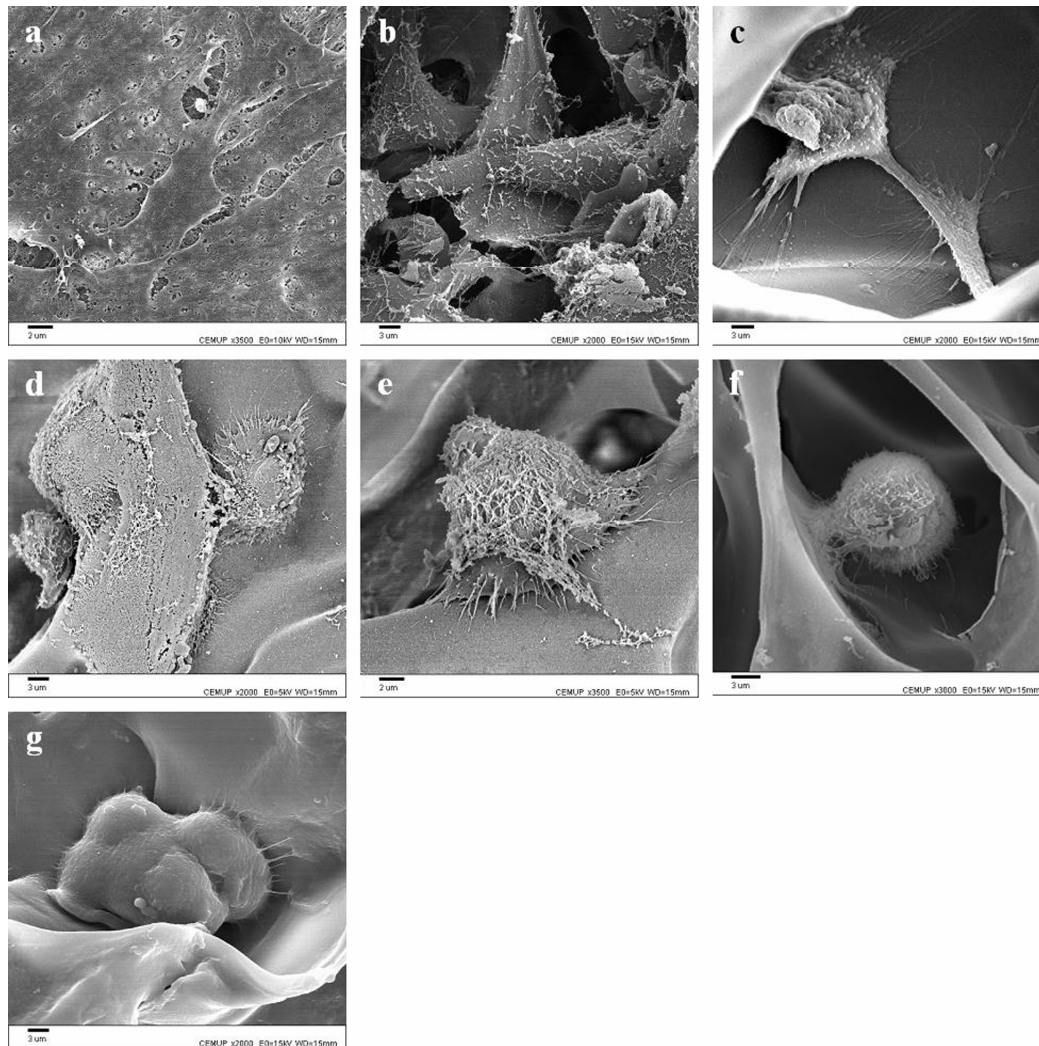


**Figure 4.** Cell morphology of MG-63 cells cultured on (a) TCPS; (b) collagen sponges and (c to g) porous scaffolds prepared from Chs with different DAs: (c) 4%; (d) 13%; (e) 22%; (f) 30% and (g) 49%. Photographs obtained by SEM, 72 h after cell seeding. Seeding surface is shown.

osteoblasts [Fig. 4(b)]. Regarding Ch scaffolds, on sponges with DAs  $\leq 13\%$  cells displayed a well spread and polygonal morphology, long cytoplasmatic processes (filopodia) and rough dorsal surfaces. Cell projections between interconnected pores were observed [Fig. 4(c) and (d)]. With the increase of DA, cell spreading was progressively lower [Fig. 4(e) to (g)]. On sponges with a DA of 30% cells displayed a spherical morphology and rough dorsal surface, being attached to the pores walls though short filopodia [Fig. 4(f)]. Sponges with a DA of 49% revealed exclusively

spherically shaped cells, most of them clustered into spheroid-like aggregates [Fig. 4(g)]. Fibrilar extracellular matrix assigned to collagen fibers was seen in all samples but to a low extent, possibly due to the low cell seeding density used in the assay.

#### *Cytoskeletal organization*

Figure 5 shows fluorescent micrographs of cell loaded scaffolds, labeled 96 h after cell seeding. Cells on TCPS presented a complex network of actin stress fibers all over the cytoplasm oriented parallel to one another and to the long axis of the cells. Bundles of actin filaments delimiting the cytoplasm were clearly visible together with numerous filamentous extensions correspondent to cell filopodia. The micrographs

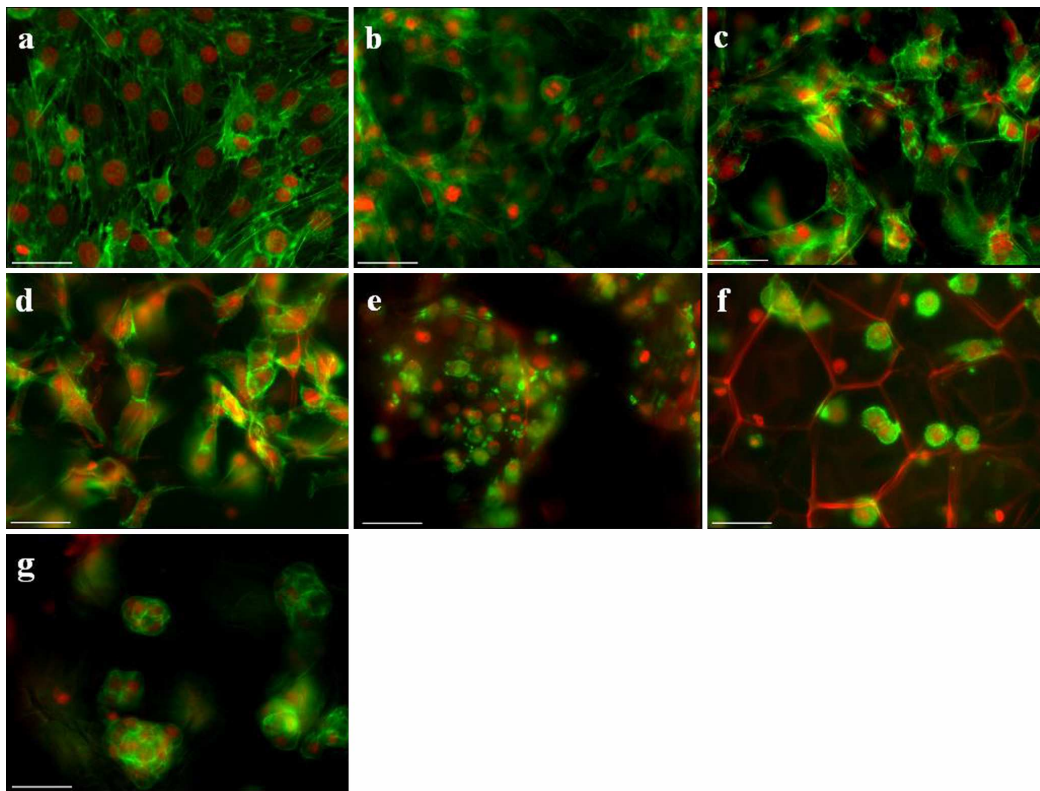


**Figure 5.** Fluorescent labeling of F-actin (green) and DNA (red) of MG-63 cells cultured on (a) TCPS, (b) collagen sponges and (c to g) porous scaffolds prepared from Chs with different DAs: (c) 4%; (d) 13%; (e) 22%; (f) 30% and (g) 49%. Images obtained by fluorescent microscopy, 96 h after cell seeding. Scale bar = 50  $\mu$ m.

reveal stellate and spindle-like shaped cells as in SEM studies, and round/oval nuclei [Fig. 5(a)]. On collagen sponges cells displayed a similar cytoskeletal organization with numerous cell-to-cell contacts and often evidence of mitosis. Cells were found at different depths of the scaffold, displaying long actin filaments often aligned along the pores walls, revealing cell growth both inside and over the pores [Fig. 5(b)]. Regarding Ch scaffolds, cell spreading followed the pattern observed for SEM images, being progressively higher with the decrease of the DA, as shown by the increasing densification of cortical actin filaments. On sponges with DAs  $\leq 13\%$  cells displayed distinct actin filaments with long F-actin fibers often parallel oriented with the pore walls, as observed for collagen sponges, with numerous cell-to-cell contacts. Cell projections between interconnected pores and cells undergoing cell division were also observed, suggesting cell colonization of the scaffolds [Fig. 5(c) and (d)]. On Ch scaffolds with  $13\% < \text{DAs} < 49\%$  F-actin staining was less evident, although some radially oriented filaments were observed within the cortical actin network. Cells displayed preferentially a spherical shape and were uniformly distributed among the pores, by that time point [Fig. 5(e) and (f)]. On scaffolds with a DA of 49% cells displayed faint F-actin staining, with no discernable actin filament formation [Fig. 5(g)].

#### *Cell viability*

Figure 6 shows 2D projections of 3D stack images of cell loaded matrices collected by CLSM, 96 h after cell seeding. On TCPS and collagen sponges, more than 95% of cells stained green, indicating that on both controls, cells remained viable at this time point [Fig. 6(a) and (b)]. Regarding collagen sponges, a uniform distribution of viable cells reaching confluence was possible to be observed until a deep of 102  $\mu\text{m}$ . In Ch scaffolds, no obvious differences were found between any of the Ch groups and the 3D control, in which concerns the percentage of cells stained green, suggesting that cell viability was not affected by the DA of Ch [Fig. 6(b) to (g)]. In which concerns cell distribution, while on scaffolds with DAs  $\leq$  of 13% a uniform distribution of viable cells was observed, similarly to what was observed for collagen sponges, on Ch scaffolds with DAs  $\geq 22\%$  cells were clustered.

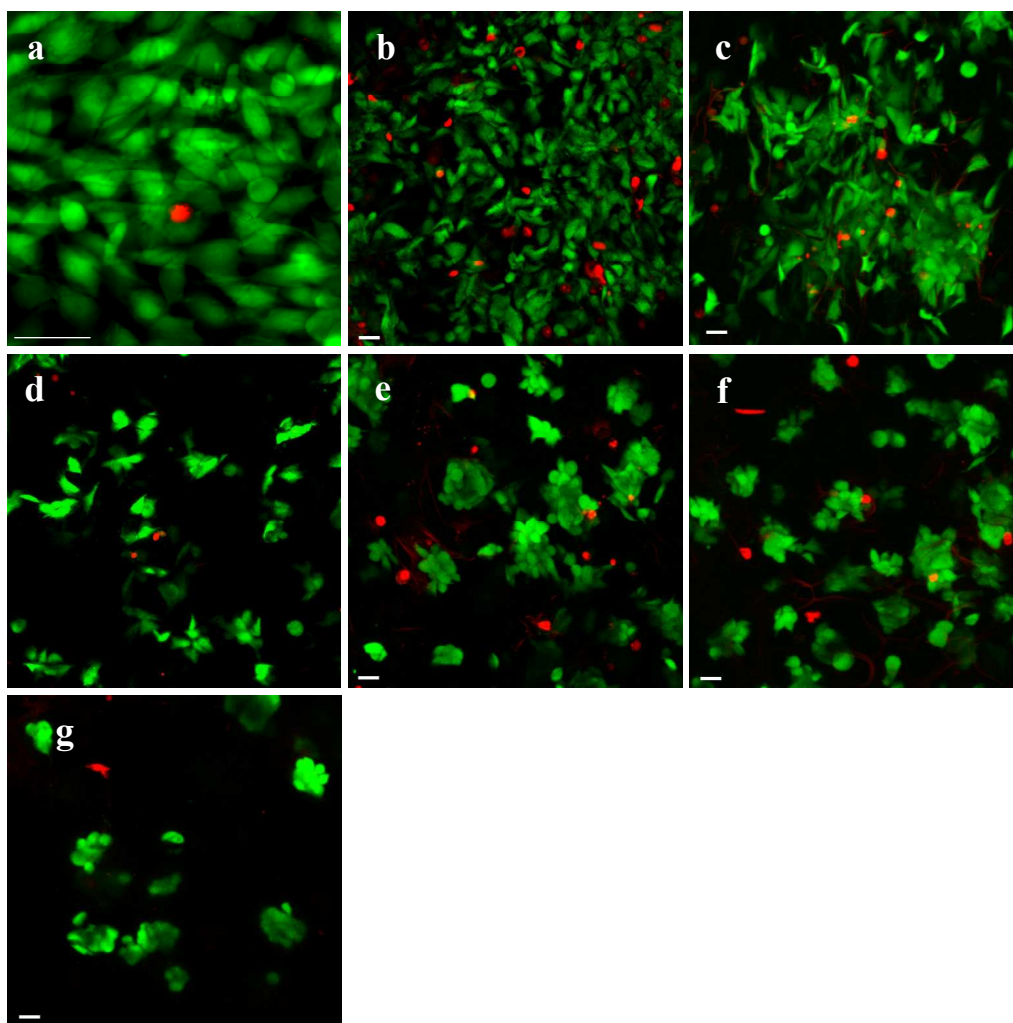


**Figure 6.** Cell viability of MG-63 cells cultured on (a) TCPS, (b) collagen sponges and (c to g) porous scaffolds prepared from Chs with different DAs: (c) 4%; (d) 13%; (e) 22%; (f) 30% and (g) 49%. Cells were labeled with calcein AM and propidium iodide. Viable cells fluoresce green, dead cells fluoresce red. 3D stack images covering a depth of (a) 171; (b) 308; (c) 329; (d) 153; (e) 147; (f) 102 and (g) 127  $\mu\text{m}$ , respectively, were collected 96 h after cell seeding by CLSM and 2D projected. Scale bar = 50  $\mu\text{m}$ .

## DISCUSSION

The aim of this investigation was to assess the effect of the DA of Ch on osteoblast morphology, proliferation and viability, in a 3D culture system.

The 3D scaffolds were characterized in terms of porosity, pore size, pore shape and interconnectivity, since these are determinant for cellular colonization, both *in vitro*

and *in vivo*. For the culture of MG-63 cells, according to Zinger et al., the minimal pore width for cell migration is around 30  $\mu\text{m}$ , the size of spread MG-63 cells, since cells need cavities equivalent or larger than their size to be able to migrate in and colonize them.<sup>31</sup> For bone tissue ingrowth larger pores are generally required, with values of pore diameter  $\geq 150 \mu\text{m}$ , even though the optimum pore size is still a controversial matter and highly dependent on the material used as support. The diameter of pore interconnections for instance, was reported to be more important than the effective pore size, pore interconnections as small as 20  $\mu\text{m}$  being reported to be efficient for *in vivo* bone ingrowth.<sup>32</sup>

In the present work, planar 3D scaffolds were prepared from Ch acidic solutions via thermally induced phase separation and subsequent sublimation of the solvent, the most commonly used method for the preparation of Ch sponges.<sup>33</sup> During freezing, ice crystals nucleate from solution and grow along the lines of thermal gradients, resulting in a macroporous structure after sublimation of the ice crystals. The Ch scaffolds obtained, independently of the DA of the Ch used, exhibited a porous honeycomb-like structure consisting of perpendicularly oriented and interconnected macropores, as reported by Nettles et al. for Ch sponges prepared in similar conditions.<sup>10</sup> The pore structure observed indicates the establishment of two thermal gradients during freezing, namely perpendicularly and radially oriented, the second contributing to interconnectivity. In terms of bulk density and average pore diameter, the values obtained were as well in the range of the values found in literature.<sup>10,34,35</sup> However, in terms of porosity, the values found, namely in the range of 93 to 97%, are higher than those reported for similar scaffolds, c.a. 80 to 90%. The high porosity found may be related to the lower thickness of the prepared scaffolds. While the porosity values here presented correspond to scaffolds 2 mm thick, the values reported by other authors regard scaffolds c.a. 4 to 5 mm thick.<sup>10,35</sup> Porosity and pore diameter were initially expected to vary with the DA. During alkaline deacetylation breakage of Ch molecular weight occurred, leading to less viscous Ch acidic solutions. According to the work of Chung et al.,<sup>36</sup> porosity and pore size of Ch sponges are dependent on the molecular weight, both decreasing with the increase of the molecular weight, although, in the same study, absolute molecular weights were not reported. In our study, this dependence was not observed, probably due to the fact that the more deacetylated sample used (DA 4%) was still a high molecular weight

Ch. The slightly more heterogeneous pore distribution, as well as the presence of fibers observed in the case of Ch sponges with the highest DA (49%), was attributed to existence of an insoluble fraction.

Osteoblasts cultured in collagen sponges attached, spread and proliferated while retaining the osteoblastic phenotype as expected, since collagen type I 3D matrices are known to induce adhesion, proliferation and osteoblastic differentiation of both osteoblastic cell lines and osteoprogenitor cells.<sup>37,38</sup> Its similarity with Ch sponges in terms of structure, average pore diameter, density and morphology allowed their use as 3D control in the present study.

All Ch scaffolds supported cell anchorage and ingrowth, cell number increasing consistently with the cell culture time, regardless the DA. In terms of cell proliferation, ALP activity and cell viability, cells cultured in Ch scaffolds performed as well as in the 3D controls, except in that correspondent to the highest DA (49%), for which a significantly lower cell number was found from 72 h until the end of the assay, when compared to collagen sponges. In fact, a tendency to higher DAs leading to lower cell numbers was found, which can be better explained taking into account the morphology and the cytoskeletal organization observed for the attached cells on the various substrates. While on sponges with DAs  $\leq 13\%$  cells attached and spread similarly to collagen sponges, displaying long cell filopodia and numerous cell-to-cell contacts, spreading out of the pores, on sponges with higher DAs cells tended to remain spherical with no discernable actin filament formation, and grow into spheroid-like cellular aggregates, as shown by SEM studies and F-actin staining. Cytoskeleton organization is essential to the maintenance of cell morphology and mechanical coupling to the underlying substrate.<sup>2</sup> Thereby, the impairment of cell spreading and actin cytoskeleton organization observed for Ch scaffolds with DAs higher than 13% suggests low attachment strength on these substrates.

Concomitant to our findings, when Lee et al.<sup>8</sup> cultured rat calvarial osteoblasts on porous Ch matrices with a DA of 30%, cells were reported to attach but to retain a round shape. On the other hand, Zhang et al.<sup>11</sup> reported MG-63 cells to display a spindle-like shape morphology after 5 days of incubation, when cultured on Ch scaffolds with a DA  $< 15\%$ , which also is in accordance with our findings.

Hence, the chemical composition of Ch scaffolds influenced the spreading of MG-63 cells, since in terms of microstructure no significant differences were found among

them. The surface concentration of amine groups is expected to increase progressively with the decrease of the DA, leading to the observed cell behavior. During the solid-liquid phase separation occurring during freeze-drying, Ch chains will tend to interact favorably with the ice crystals, presenting a conformation in which polar groups, like amine ones, are exposed at the interface. This configuration will remain after sublimation of the water, resulting in surfaces with an amine group concentration basically dependent on the DA used. Therefore, the effect of the DA on cell spreading was attributed to the different amount of amine groups present at the surface.

Cell adhesion to substrates carrying amine functionalities has been often attributed to non-specific electrostatic interactions occurring directly between protonated amine groups and negatively charged carboxylate and sulphate groups found in cell surface proteoglycans. The same mechanism was hypothesized for cell adhesion to Ch surfaces.<sup>13,39</sup> However, in biological systems, cells do not contact to naked surfaces, and protein adsorption phenomena must be taken in consideration. Osteoblast adhesion to biomaterials involves the initial cell attachment phase, implying short-term events of physico-chemical nature established between the materials surface and the cells, and the adhesion phase occurring in the longer term, involving the binding of cell-surface molecules namely integrins and proteoglycans to extracellular matrix glycoproteins. Cell spreading, F-actin cytoskeletal organization and cytoplasmatic signaling pathways are mediated by the conditioning layer of adsorbed glycoproteins containing an Arg-Gly-Asp (RGD) sequence, such as fibronectin (Fn), collagen type I and vitronectin.<sup>40</sup> In a previous study, we showed that Ch films with a DA of 4% adsorb significantly higher levels of Fn from serum, as compared to TCPS and Ch samples with higher DAs<sup>15</sup>. Cuy et al.<sup>41</sup> also reported that besides Fn, laminin adsorbs onto Ch films with a DA < 20%. Moreover, it is known that Fn presents a more favorable conformation for  $\alpha_5\beta_1$  integrin binding and osteoblast adhesion upon adsorption on model surfaces exposing amine functionalities, as compared to surfaces exposing methyl groups (present in Ch acetylated units).<sup>42</sup> Thereby, the impairment of cell spreading and actin cytoskeleton organization observed for Ch scaffolds with a DA higher than 13% was possibly associated to a reduced adsorption of cell adhesion peptides on these substrates. Although surface wettability might have also contributed to higher cell attachment on decreasing the DA, it is known that for osteoblasts, cell attachment and spreading vary with surface chemistry rather than with wettability.<sup>43</sup>

In addition, it is known that cell adhesion, spreading and proliferation of human bone cells are influenced by surface roughness, generally being enhanced on smooth samples as compared to rough and less organized ones.<sup>44</sup> As on increasing the DA and Ch molecular weight chain aggregation in acidic solutions is expected to occur,<sup>45</sup> we cannot exclude the possibility of these aggregates to contribute to an increase of surface roughness in the resulting Ch sponges, which would consequently lead to a lower cell adhesion on Ch samples with higher DAs.

In the present study, the DA played a key role in determining the affinity of osteoblasts-like cells towards the 3D substrates, possibly by influencing the nature of the initial adsorbed protein layer. These results suggest that Ch sponges with a DA  $\leq$  13% may be useful as 3D matrices for cell transplantation or for *in vitro* bone tissue engineering, for the repair of osseous defects. Moreover, the obtained results highlight the importance of the careful selection of the scaffold in terms of DA, envisaging the culture of bone cells, *in vitro*. The DA should also be considered if strategies to enhance bone formation on Ch are to be used, for instance, by the incorporation of growth factors or binding of cell adhesion peptides.

## CONCLUSIONS

3D porous matrices formulated from Chs with DAs ranging from 4 to 49% revealed a similar microstructure regardless the DA, except for the highest DA used, where a more heterogeneous pore distribution was observed. All scaffolds supported cell anchorage, proliferation, expression of ALP and viability of MG-63 osteoblasts-like cells, confirming the biocompatibility of Ch-based matrices, despite an inhibitory effect on cell proliferation observed for a DA of 49%, when compared to the 3D control. In addition, while on sponges with DAs  $\leq$  13% cells attached and spread, displaying long cell filopodia and numerous cell-to-cell contacts, on sponges with higher DAs cells tended to remain spherical and grow into spheroid-like aggregates. The DA played here a key role in determining the affinity of cells towards the 3D substrates, possibly by influencing the nature of the initial adsorbed protein layer.

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### **Chemical modification of chitosan via phosphorylation. An XPS, FT-IR and SEM study**

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#### **ABSTRACT**

In the present work, the surface of chitosan membranes was modified using a phosphorylation method carried out at room temperature. Phosphorylation may be of particular interest in materials for orthopedic applications, due to the cation exchange properties of phosphate functionalities. Phosphate groups chelate calcium ions, thus inducing the deposition of an apatite-like layer, known to improve the osteoconduction of polymer-based implants. Additionally, the negatively charged phosphate functionalities, together with the positively charged amine groups from chitosan, are expected to provide chitosan with an amphoteric character, which may be useful as a combinatorial therapeutic strategy, by simultaneously allowing the immobilization of signaling molecules like growth factors.

Phosphorylation was carried out at room temperature, by the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5$ /butanol method. Surface characterization was performed by XPS, ATR-FTIR, and SEM. Cross-sections were analyzed by SEM fitted with EDS. The phosphate content increased with the reaction time, as shown by XPS and ATR-FTIR, an atomic ratio of 0.73 P/N being obtained after 48 h of treatment. High resolution XPS spectra regarding  $\text{C}_{1s}$ ,  $\text{O}_{1s}$ ,  $\text{N}_{1s}$  and  $\text{P}_{2p}$  are discussed. The introduction of a neutralization step lead to a reduction of P content, which pointed out to the presence of phosphates ionically bound to protonated amines, in addition to phosphate esters.

EDS analysis of cross-sections revealed a gradual P reduction up to 50% towards the inner part of the membrane.

## INTRODUCTION

Chitosan is a linear co-polymer of glucosamine and *N*-acetyl glucosamine in a  $\beta 1 \rightarrow 4$  linkage obtained by *N*-deacetylation of chitin. It is a biodegradable polysaccharide with well-established biocompatibility, presently under investigation for a wide range of biomedical applications such as sutures, wound dressings, bone substitutes, tissue engineering and gene- and drug-delivery vehicles.<sup>1,2</sup>

In orthopedics, its degradability associated to its structural similarity to extra cellular matrix glycosaminoglycans makes it an attractive bio-polymer for bone tissue repair. However, despite leading to satisfactory results when applied to bone defects in animal models, namely increased osteogenesis and angiogenic activity without fibrous tissue invasion, chitosan is not sufficiently osteogenic to induce the desired rapid bone regeneration at the initial stage of bone healing.<sup>3,4</sup> Hence, chitosan has been frequently associated with calcium phosphates, not only due to the well-established osteoconduction of calcium phosphates, but also based on its analogy to collagen, the organic matrix of bone on which the inorganic phase mineralizes.<sup>5,6</sup>

Phosphorylation of chitosan seems to be of particular interest for orthopaedic applications, due to the cation exchange properties of phosphate functionalities. Phosphate groups bind calcium ions, which may then induce the formation of a calcium phosphate layer known to promote the osteoconduction of polymer-based implants.<sup>7</sup> Thus, coating chitosan membranes with a porous apatite-like layer is expected to improve cell attachment and migration, allowing its use as membranes for guided bone regeneration.

Phosphorylated chitosan membranes were reported to induce the biomimetic deposition of an apatite-like layer under simulated physiological conditions, after pre-incubation in calcium-containing solutions, due to chelation of calcium ions and partial hydrolysis of phosphate functionalities.<sup>8,9</sup>

The incorporation of phosphate functionalities on chitosan substrates could also be of interest for other biomedical applications like tissue engineering. Surface binding of signalling molecules onto resorbable scaffolds is a concept which is gaining

increasing interest in regenerative medicine, by contrast to the use of diffusible growth factors.<sup>10</sup> Phosphate groups, due to their ion exchange properties, constitute adequate functionalities for specifically binding biologically active species.<sup>11,12</sup> Phosphate carriers are cation exchangers, based on their ability to exchange ionically bound cations (e.g.  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ) with cationic residues of biomolecules in solution. Phosphorylation of porous chitosan matrices can be used to immobilize signalling biomolecules, such as growth factors promoting tissue regeneration. The ionic character of these chemical bindings, together with the mild conditions for immobilization used, usually leads to minor conformational changes of the immobilized biomolecule with subsequent retention of activity.<sup>13</sup>

Phosphorylated chitosan membranes may also be of interest for the culture of certain anchorage dependent cells. Cell attachment to chitosan is mainly attributed to electrostatic interactions between the chitosan cationic sites, provided by protonated amine groups from glucosamine units, and the negatively charged carboxylate and sulphate groups found in cell-surface proteoglycans.<sup>14</sup> However, strong attachment to chitosan substrates may hinder cell spreading and migration, as reported for fibroblasts.<sup>14</sup> Hence, the grafting of anionic groups on chitosan can be useful to tailor surface charge, thus balancing strong electrostatic interactions affecting cell behaviour.

Phosphorylation of cellulose under mild conditions has recently been reported.<sup>15</sup> This method has the advantages of leading to highly substituted products, as well as of being carried out at room temperature, resulting in low degradation of the polymer. Additionally, it has been shown to provide *in vitro* cytocompatibility and *in vivo* integration in bone tissue.<sup>16</sup> However, the application of this chemical modification route to other polysaccharides has not been reported yet.

Phosphate esters of chitin and chitosan were initially prepared due to their metal-ion-binding capabilities and basically two preparation techniques were used. The first one is based on the synthesis route of cellulose phosphates, consisting in heating chitin/chitosan at high temperature (usually 150°C) with phosphoric acid and urea in dimethylformamide.<sup>17,18</sup> The second method reported was developed by Nishi et al.,<sup>19</sup> and involves the reaction of chitin/chitosan dissolved in methanesulphonic acid with phosphorus pentoxide, at low temperature (0-5°C).

In the present work, the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5$ /hexanol method<sup>15</sup> was applied to the phosphorylation of squid chitosan membranes for different time periods. The amount

of covalently bound phosphates introduced through this phosphorylation method has been estimated. Surface characterization was performed using complementary spectroscopic techniques, namely X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR). Cross-sections of the modified membranes were also observed by scanning electron microscopy (SEM), fitted with an energy dispersive X-ray analysis system (EDS), in order to evaluate the extent of phosphorylation along the membrane thickness.

## **MATERIALS AND METHODS**

### **Materials**

Chitosan powder from squid pens was kindly donated by France Chitine (France). All reagents used were of analytical grade.

### **Purification of chitosan**

Chitosan was purified by the reprecipitation method. Briefly, chitosan was dissolved in 3% (v/v) aqueous acetic acid overnight and, after complete dissolution, the resulting gel was filtered through a No. 1 sintered glass funnel, to remove undissolved or gelatinous particles. Chitosan was then precipitated through the dropwise addition of 1 M aqueous NaOH, while stirring. Finally, the regenerated chitosan was thoroughly washed with distilled and dionized water (DDW) until neutrality, freeze-dried and grounded in a laboratory mill to yield a fine powder (40-75  $\mu\text{m}$ ).

### **Characterization of chitosan**

Chitosan was characterized in terms of degree of *N*-acetylation and average molecular weight. The degree of *N*-acetylation (DA) was determined by FT-IR spectroscopy, using the KBr technique. Each pellet was prepared by blending 2 mg of purified chitosan powder, previously dried overnight at 60°C under reduced pressure, with 100 mg of KBr, previously dried at 105°C for 24 h. The Infrared spectra were immediately recorded with a FT-IR system 2000 from Perkin-Elmer, by accumulation of 200

interferograms, at a  $4\text{ cm}^{-1}$  spectral resolution. The Amide I band ( $\nu = 1655\text{ cm}^{-1}$ ) was used as the analytical band and the hydroxyl band ( $\nu = 3450\text{ cm}^{-1}$ ) as the internal reference band. The DA was calculated according to the method proposed by Baxter et al.,<sup>20</sup> as follows:

$$\text{DA (\%)} = (A_{1655}/A_{3450}) \times 115. \quad (1)$$

Average molecular weight was determined by viscometry, in a solvent system of 0.1 M  $\text{CH}_3\text{COONa}$ /0.2 M  $\text{CH}_3\text{COOH}$ . Flow times of sample solutions were measured immediately, to avoid chain depolymerization, using an Ubbelohde viscometer 0c (Shott Geräte) at  $30 \pm 0.1^\circ\text{C}$ , with an automated viscosity measuring system (LAUDA mgw) and an extra temperature controller (Comarck). Intrinsic viscosity ( $[\eta]$ ) was obtained from linear plots of reduced viscosity ( $\eta_{\text{sp}}/C$ ) against concentration ( $C$ ), extrapolating to zero concentration. Viscosity average molecular weight ( $M_v$ ) was calculated based on the Mark-Houwink equation, as follows:

$$[\eta] = KM_v^a, \quad (2)$$

where, according to chitosans in this system,  $K = 1.64 \cdot 10^{-30} \times \text{DD}^{14}$  and  $a = -1.02 \times 10^{-2} \times \text{DD} + 1.82$ ;<sup>21</sup> DD is the degree of *N*-deacetylation (%) of chitosan, which is the inverse of the DA.

### Preparation of chitosan membranes

Membranes were prepared by solvent casting, from 1% (w/v) solutions of purified chitosan in 1% (v/v) aqueous acetic acid. After complete dissolution, the resulting gel was filtered, kept under reduced pressure in order to clear all the entrapped gas bubbles, and poured into  $120 \times 120\text{ mm}^2$  polystyrene petri dishes (60 g per plate). The gel was allowed to settle for 48 h at room temperature in a laminar flow hood. The resulting membranes were deprotonated in 0.5 M aqueous NaOH for 1 h and thoroughly washed with DDW. Finally, they were cut into  $30 \times 60\text{ mm}^2$  strips, and kept in absolute ethanol prior to chemical modification. For characterization purposes, chitosan membranes were dried at  $30^\circ\text{C}$  for 24 h in a vacuum oven, and stored in a

desiccator until further analysis. Membrane thickness was measured with a Mitutoyo dial thickness gauge No. 7301.

### **Acetylation of chitosan**

Chitosan membranes were acetylated at room temperature.<sup>22,23</sup> The membranes were steeped in an aqueous methanolic acetic acid solution (water/methanol/acetic acid = 50:200:1, v/v) and acetic anhydride (5.0 mol per glucosamine residue) was added. After 12 h under stirring the membranes were rinsed in methanol followed by ethanol, and then steeped in 0.5 M ethanolic KOH overnight to remove any ester groups. The membranes were finally washed with ethanol, followed by DDW, and then dried at 30°C for 24 h in a vacuum oven. The correspondent degree of *N*-acetylation was estimated according to Shigemasa et al.,<sup>24</sup> using the Amide II band ( $\nu = 1560\text{ cm}^{-1}$ ) as the analytical band and the C-O stretching band ( $\nu = 1070\text{ cm}^{-1}$ ) as the internal reference band. In ATR-FT-IR spectra, the internal reference band at  $3450\text{ cm}^{-1}$ , previously used for determining the DA of chitosan powder, presents low absorbance values when compared to transmission spectra.

### **Phosphorylation of chitosan**

Surface phosphorylation was performed using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{hexanol}$  method,<sup>15</sup> substituting hexanol by 2-butanol, which has a higher water solubility. Briefly, 1 g chitosan membrane strips was suspended in 40 mL 2-butanol in a round-bottomed flask. The reaction mixture was prepared by adding 43 mL of  $\text{H}_3\text{PO}_4$  to 37 mL of  $\text{Et}_3\text{PO}_4$ , followed by step-by-step addition of  $\text{P}_2\text{O}_5$ , under constant stirring. The resulting translucent solution was then gradually added to the flask. Reaction proceeded under  $\text{N}_2$  atmosphere, at 30°C, with constant stirring in a thermostated orbital shaker. Phosphorylation was carried out during different time periods, up to 48h. After chemical treatment, membranes were rinsed with ethanol and suspended twice in this solvent for 30 min. Finally, they were dialyzed against DDW for 24 h to remove free inorganic phosphate, and dried at 30°C for 24 h in a vacuum oven. Acetylated chitosan membranes, in which amines are absent, were also phosphorylated for a period of 8 h. Alternatively, acetylated and original chitosan

membranes which were phosphorylated for 8 h were both subjected to neutralization, after the ethanol rinsing step. The neutralization step was used in order to convert chitosan ammonium groups into uncharged amine groups, with consequent release of ionically bound phosphates. The strips were incubated in 0.5 M aqueous NaOH at 25°C for 30 min under stirring, thoroughly washed with DDW until neutral pH, and finally dialyzed and dried as previously described.

## Surface characterization

### *X-ray photoelectron spectroscopy (XPS)*

Spectra were obtained on a VG Scientific ESCALAB 200 A, using a Mg K $\alpha$  X-ray radiation as the excitation source (15 kV), operated at 300 W. The analyzer was run in the constant analyzer transmission mode. The emitted photoelectrons were analyzed at a 90° take-off angle from the horizontal surface plane, for all analysis. Survey spectra were acquired over a binding energy range of 0 to 1100 eV, using a pass energy of 50 eV. High-resolution spectra for the C<sub>1s</sub>, O<sub>1s</sub>, N<sub>1s</sub> and P<sub>2p</sub> regions were obtained, using a pass energy of 20 eV. Due to their insulating nature, chitosan membrane surfaces became positively charged after the emission of photoelectrons, resulting in a broadening of the spectral lines, and a drift toward higher binding energy.<sup>25</sup> The binding energies of the photoelectron peaks were therefore calibrated, assigning a binding energy of 285.0 eV to the aliphatic carbon (-CH<sub>2</sub>-) C<sub>1s</sub> peak, present as a carbon surface contaminant.<sup>25</sup> The deconvolution of the high resolution spectra was made by means of a least-squares peak analysis software, XPSPEAK version 4.1, using the Gaussian/Lorentzian sum function. A linear background was used for carbon and phosphorous regions, while an integral background based on the Shirley method was used for the oxygen and nitrogen regions.<sup>26</sup> Initial assumptions on the possible species were derived from chitosan stoichiometry and chemical bonds. Element atomic percentages were calculated from the integrated intensities of the XPS peaks, taking into account the atomic sensitivity factors of the instrument data system.

*Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FT-IR)*

The Infrared spectra were recorded with a FT-IR system 2000 from Perkin-Elmer, using the SplitPea<sup>TM</sup> accessory (Harrick Scientific), provided with a silicon internal reflection element and configured for external reflectance mode. The membranes were previously dried at 60°C in a vacuum oven for 12 h. The spectra were obtained from a 200  $\mu\text{m}$  diameter sampling area, after accumulation of 200 interferograms, at a 4  $\text{cm}^{-1}$  spectral resolution. All spectra were corrected for the ATR characteristic progressive increase in the absorbance at lower wave numbers, using the equipment software. Peak identification was obtained from the correspondent second-derivative spectra in the range between 1250 and 800  $\text{cm}^{-1}$ , where the characteristic vibrations from phosphates were expected to occur.

*Scanning electron microscopy (SEM) with energy-dispersive X-ray analysis (EDS)*

Observation was performed with a JEOL JSM-6301F scanning electron microscope, equipped with a Noran-Voyager energy dispersive spectroscope. In order to obtain a brittle fracture, the membranes were cryofractured in liquid  $\text{N}_2$ , followed by drying under vacuum. For SEM, the samples were gold sputtered and observed at an accelerating voltage of 5 kV, in order to avoid membrane degradation. For EDS analysis, samples were carbon sputtered and the correspondent cross-sections were analyzed at an accelerating voltage of 10 kV. EDS quantification of phosphorous/carbon intensity ratio was normalized to 100% at the surface, for each sample.

**RESULTS AND DISCUSSION****Characterization of chitosan**

Regenerated chitosan presented a degree of *N*-acetylation of 39% (molar fraction of *N*-acetylated units = 0.39), and an average viscosity molecular weight of 2480 kDa, which is in the range of the molecular weights reported for squid chitosan.<sup>27</sup>

The membranes obtained were clear and transparent, with a smooth shiny surface, in accordance with reports using the same chitosan animal source.<sup>28</sup> The membranes were flexible, presenting an average film thickness of  $53 \pm 3 \mu\text{m}$ .

After acetylation, the ATR-FT-IR spectra of chitosan membranes revealed the absence of the peak at  $3360 \text{ cm}^{-1}$ , assigned to the  $\text{NH}_2$  asymmetric stretching, suggesting full acetylation of chitosan amine groups. In accordance, a DA of *ca.* 100% was found.

### **Preparation and characterization of phosphorylated chitosan membranes**

#### *Physical appearance*

During reaction, chitosan membranes retained their original form and did not stick to each other. After drying, the modified membranes seemed similar to unmodified membranes, at the macroscopic level, being transparent and flexible.

#### *X-ray photoelectron spectroscopy (XPS)*

The survey spectra of chitosan membranes confirmed the presence of carbon, oxygen, and nitrogen. After phosphorylation, phosphorus was easily detected on all membranes, irrespective of the reaction time used. The possible phosphorylated products are illustrated in Figure 1. Both primary and secondary chitosan hydroxyl groups, namely at the C-6 and C-3 positions of the glucosamine units, are likely to be esterified by phosphorylation. In a recent work, Wang et al.<sup>29</sup> reported a preferential substitution of the hydroxyl group at the C-6 position, for low degrees of substitution. Due to the acidic nature of the reaction medium, chitosan amine groups were ionized, and thus probably involved in ionic bindings with free phosphate ions ( $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$ ) (product a).<sup>30</sup> In addition, since chitosan has a considerable amount of amine groups, inter or intrachain ionic bonds – salt linkages - between phosphate groups and protonated amine groups are likely to be formed (product b).<sup>19</sup> The formation of pyrophosphates from condensation of phosphates in chitosan chains, together with condensation of phosphates with incoming phosphoric acid from the reaction media, may also occur (product c).<sup>30</sup> Lee and co-workers<sup>30</sup> suggested the formation of

pyrophosphates and polyphosphates when chitosan membranes were reacted with orthophosphoric acid and urea. However, when the present method was applied to cellulose, pyrophosphates were not found.<sup>15</sup>

Regarding element atomic percentages, chitosan membranes revealed more carbon and less oxygen and nitrogen, when compared to the expected values for chitosan presenting a DA of 39%, which may be due to carbon surface contamination (Table 1). Nevertheless, when the O/N atomic ratio of unmodified chitosan was calculated, a value of 4.41 was found, which is close to the expected one (4.39). The phosphorus atomic percentage increased logarithmically with reaction time, from approximately 1.0 to 4.7, as the membrane was reacted from 1 to 48 h. If expressed as a function of nitrogen, an atomic ratio of 0.73 P/N was obtained after 48 h (Fig. 2), which can be correlated to the degree of substitution at the surface, given that one nitrogen atom is present in each chitosan monomeric unit.

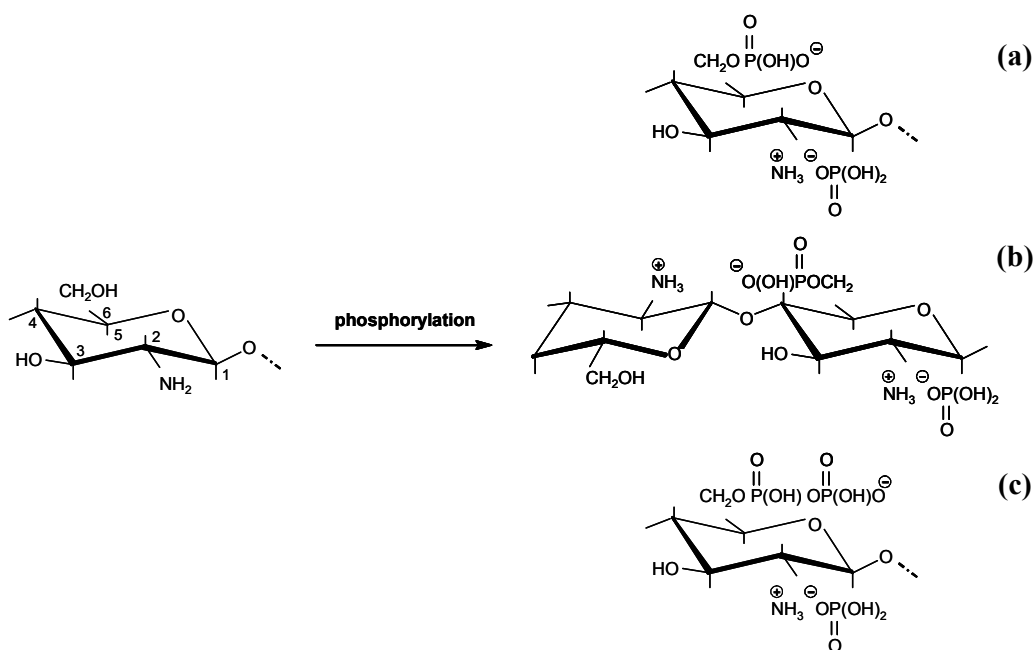


**Figure 1.** Chemical structure of possible phosphorylation products, in which chitosan amine groups are probably involved in ionic bindings with free phosphate ions,  $\text{H}_2\text{PO}_4^-$  or  $\text{HPO}_4^{2-}$ . (a) Chitosan phosphate, esterified at the hydroxyl group at the C-6 position; (b) Formation of inter- or intra-chain ionic bonds between chitosan phosphate groups and protonated amine groups from adjacent chitosan chains; (c) Formation of pyrophosphates from condensation of phosphates in chitosan chains, together with condensation of phosphates with incoming phosphoric acid from the reaction media.

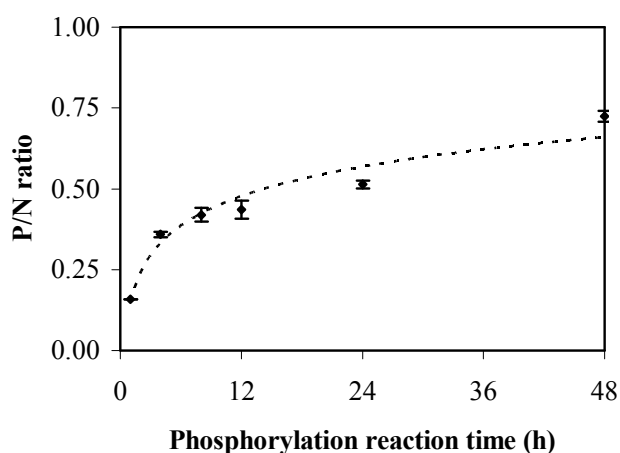
**Table 1.**

XPS element analysis (atomic %), as a function of phosphorylation reaction time.

| Reaction time (h) | C <sub>1s</sub>   | O <sub>1s</sub>   | N <sub>1s</sub>   | P <sub>2p</sub>   |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| 0                 | 62.2 [60.9; 64.1] | 31.2 [29.2; 32.4] | 7.08 [6.49; 7.79] | 0.00              |
| 1                 | 60.7 [59.3; 62.0] | 31.6 [30.0; 33.0] | 6.71 [6.61; 6.81] | 1.06 [1.04; 1.08] |
| 4                 | 56.3 [55.0; 57.7] | 35.0 [34.0; 36.3] | 6.42 [6.22; 6.53] | 2.31 [2.11; 2.50] |
| 8                 | 53.8 [53.3; 54.1] | 36.5 [36.3; 36.8] | 6.84 [6.65; 6.99] | 2.87 [2.73; 2.99] |
| 12                | 53.3 [52.5; 54.4] | 37.0 [35.9; 37.5] | 6.80 [6.73; 6.90] | 2.96 [2.86; 3.08] |
| 24                | 52.0 [50.8; 53.1] | 37.9 [37.0; 38.7] | 6.68 [6.48; 6.98] | 3.42 [3.34; 3.49] |
| 48                | 52.3 [51.4; 53.2] | 36.5 [35.9; 37.1] | 6.50 [6.37; 6.64] | 4.71 [4.54; 4.89] |

Results presented are the average [min; max] of three independent measurements.

Phosphorylation was more effective in terms of grafted phosphate groups during the first 4 h, becoming less effective after this period, probably due to the consumption of reactive hydroxyl groups. In addition, as a result of increasing reaction time, simultaneous hydrolysis of phosphate esters may have occurred, as reported for cellulose phosphates.<sup>15</sup>

**Figure 2.** P/N atomic percentages ratio determined by XPS, as a function of phosphorylation reaction time.

The oxygen amount also increased with reaction time, as expected. For each grafted phosphate group, three new oxygen atoms are introduced. The observed O/P ratios were plotted against reaction time and compared to the expected values calculated

using equation (3), by normalizing the element surface concentrations to nitrogen content (Fig. 3).

$$O/P = \frac{4.39 + (3 \times P/N)}{P/N}, \quad (3)$$

where 4.39 is the O/N atomic ratio in unmodified chitosan membranes.

As shown in Figure 3, the O/P ratios are in agreement with the expected values, for all reaction times experimented. The effect of the NaOH treatment is presented in Table 2. As previously stated, NaOH treatment was carried out in order to convert positively charged chitosan ammonium groups into uncharged amines. The NaOH neutralization step led to a decrease of surface phosphorus amount in the range of 50%, for the 8 h phosphorylated chitosan membranes, which was attributed to the release of phosphate ions, ionically bound to protonated amines.

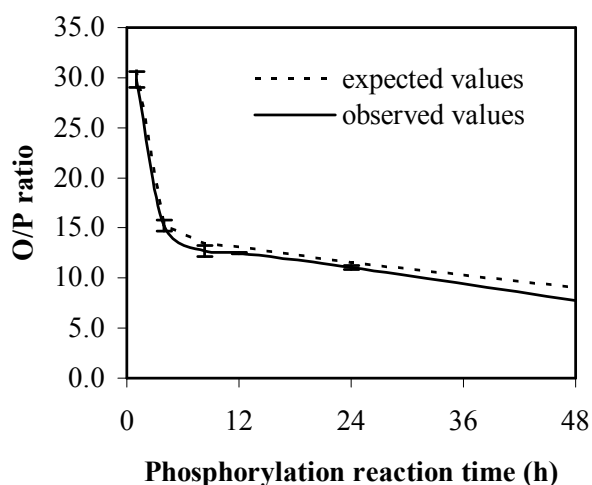


**Figure 3.** O/P atomic percentages ratio determined by XPS, as a function of phosphorylation reaction time.

Pyrophosphates, if present, were possibly hydrolyzed upon NaOH treatment, contributing to the reduction of the amount of P.<sup>31</sup> The remaining phosphorus probably corresponds to the covalently bound phosphates (chitosan phosphate esters), as phosphate monoesters of polysaccharides were shown to be remarkably stable to base-catalyzed hydrolysis.<sup>32</sup> The alkaline stability of phosphate esters has been known for a long time, and explained as being due to electrostatic repulsion by the phosphate dianion and the base hydroxyl anion.<sup>32</sup> For the same phosphorylation period, acetylated membranes revealed a lower surface phosphorus amount, namely of 1.17.

**Table 2.**

XPS phosphorus atomic percentages of unmodified and fully acetylated chitosan membranes after an 8h phosphorylation treatment, with or without subsequent neutralization in 0.5 M aqueous NaOH.

|                         | Unmodified chitosan<br>DA=39% | Acetylated chitosan<br>DA=100% |
|-------------------------|-------------------------------|--------------------------------|
| P-chitosan              | 2.79                          | 1.17                           |
| NaOH treated P-chitosan | 1.46                          | 1.08                           |

DA, degree of *N*-acetylation of chitosan; P-chitosan, phosphorylated chitosan.

In acetylated membranes, hydrogen bonding is stronger than in chitosan membranes, resulting in less hydroxyl groups available for phosphorylation.<sup>33</sup> After the NaOH treatment, an almost complete retention of P amount was observed, as expected. Acetyl groups, in contrast to amino groups, remain uncharged during phosphorylation. As a result, incorporation of phosphates through ionic binding with positively charged amines is not possible. It is worth mentioning that ionically bound phosphates were equally present, in an uncertain amount, when the phosphorylation of chitosan membranes by the phosphoric acid/urea/DMF method was reported.<sup>30</sup> The peak deconvolution of the high resolution spectra provided the chemical composition of the membranes at the surface, as indicated below.

*C<sub>1s</sub> spectra.* The resolved C<sub>1s</sub> spectrum regarding chitosan membranes revealed three peaks (figure not shown). The C<sub>1s</sub> peak at 285.0 eV was mainly assigned to the carbon surface contaminant -CH<sub>2</sub>-, but also to C-NH<sub>2</sub> chemical bindings, given that amines are reported to induce small chemical shifts, namely of 0.6 eV.<sup>34</sup> The peak at 286.6 eV was assigned to C-O, C-OH and C-N-C=O and the peak at 288.3 eV to O-C-O and N-C=O chemical bindings.<sup>34-36</sup> Spectra from phosphorylated samples did not reveal significant changes, regardless of the reaction time. The C<sub>1s</sub> peak correspondent to C-O-P chemical bindings, expected to be centered at 286.4 eV,<sup>35,37</sup> was probably overlapped by chitosan C-OH chemical bindings, observed at 286.6 eV.

*O<sub>1s</sub> spectra.* O<sub>1s</sub> levels are rather broad in nature (2.0 eV). In addition, only few works are available concerning deconvolution of O<sub>1s</sub> region of phosphate esters, to allow a

reliable data interpretation.<sup>37</sup> Nevertheless, the results of the fit assumptions are summarized in Fig. 4. Regarding the spectrum of unmodified chitosan membranes [Fig. 4(a)], three peaks were identified. The peak at 531.6 eV was assigned to  $\text{N-C=O}$  chemical bindings in *N*-acetylated-glucosamine units.<sup>33,35</sup> The peaks at 532.9 eV and 533.5 eV were assigned to  $\text{C-OH}$  and  $\text{O-C-O}$  chemical bindings, respectively.<sup>35</sup> Spectra from phosphorylated samples presented a new peak at 531.0 eV [Figs. 4(c) and (e)], which intensity increased with reaction time (Table 3). This peak was assigned to physisorbed water, possibly associated with the presence of phosphorous functionalities,<sup>38</sup> as well as to  $\text{HPO}_4^{2-}$  groups, ionically bound to protonated amine groups.<sup>39,40</sup>

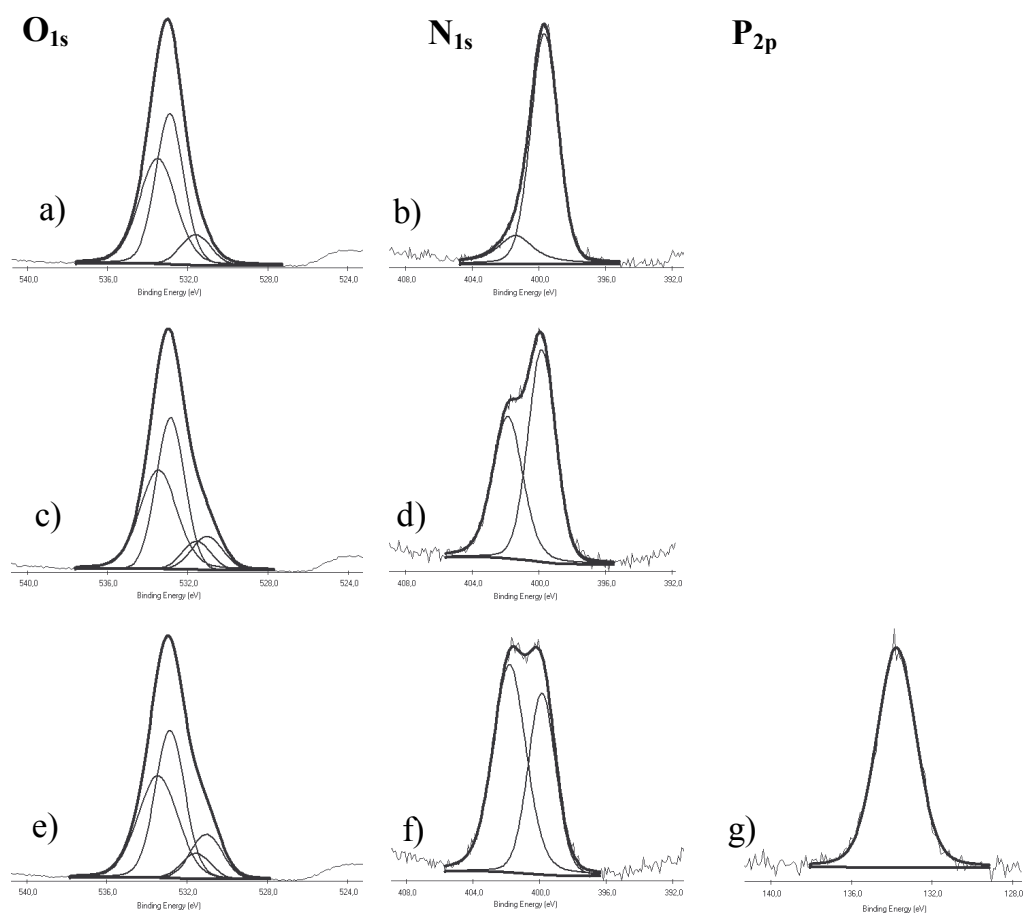


**Figure 4.** XPS high resolution spectra of: (a, b) unmodified chitosan membrane; (c, d) 8 h phosphorylated chitosan membrane; and (e to g) 48 h phosphorylated chitosan membrane.  $\text{O}_{1s}$ ,  $\text{N}_{1s}$  and  $\text{P}_{2p}$  regions are shown. The  $\text{P}_{2p}$  spectra of 8 h phosphorylated chitosan membrane is not shown, since the spectra obtained was similar to that of the membranes phosphorylated for 48 h.

**Table 3.**XPS component intensity ratios of O<sub>1s</sub> and N<sub>1s</sub>, as a function of phosphorylation reaction time.

| Reaction time (h) | O <sub>1s</sub> (531.0 eV)/O <sub>1s</sub> | N <sub>1s</sub> ( <u>N</u> H-C=O, <u>N</u> H <sub>2</sub> )/N <sub>1s</sub> |
|-------------------|--------------------------------------------|-----------------------------------------------------------------------------|
| 0                 | —                                          | 0.84 ± 0.03                                                                 |
| 8                 | 0.09 ± 0.01                                | 0.56 ± 0.01                                                                 |
| 48                | 0.14 ± 0.01                                | 0.42 ± 0.01                                                                 |

According to Nishi and co-workers,<sup>19</sup> phosphate esters present two pK<sub>a</sub> values for the phosphate group, namely pK<sub>a1</sub> approx. 3.7 and pK<sub>a2</sub> approx. 8.5. Thus, at the pH of distilled water (approx. 5.3) phosphate functionalities should be in the form of monohydrogen phosphates ( $\text{HPO}_4^-$ ). The O<sub>1s</sub> peaks corresponding to these groups and to  $\text{H}_2\text{PO}_4^-$  ionic species are expected to be centered at 531.8 eV<sup>40,41</sup> and were probably overlapped by chitosan chemical bindings. In addition, the peaks regarding oxygen atoms from C-O-P chemical bindings, reported to be centered at 533.5 eV in phosphate esters, could not be observed, probably due to overlapping by chitosan O-C-O chemical bindings, observed at 533.5 eV.<sup>37</sup> NaOH treatment lead to a decrease of the intensity of the O<sub>1s</sub> component centered at 531.0 eV (previously assigned to  $\text{HPO}_4^{2-}$  ionic species and to physisorbed water associated with phosphates), in agreement with the lower phosphorus amount observed, after the release of ionically bound phosphates (figure not shown).

*N<sub>1s</sub> spectra.* The resolved N<sub>1s</sub> spectra of chitosan membranes is shown in Fig. 4(b). Two peaks were identified. The peak at 399.7 eV was assigned to N-C=O and NH<sub>2</sub> chemical bindings,<sup>40</sup> while the peak at 401.4 eV was assigned to amino groups in the ammonium form ( $\text{NH}_3^+$ ).<sup>42</sup> Both forms, NH<sub>2</sub> and  $\text{NH}_3^+$ , were likely to be present in chitosan membranes, taking into account the pK<sub>a</sub> of chitosan amine groups (ca. 6.5),<sup>43</sup> and the fact that chitosan membranes were immersed in distilled water before being dried. Phosphorylation led to an increased number of nitrogen atoms in the ammonium form and, simultaneously, to a decrease of the intensity of the N<sub>1s</sub> component centered at 399.8 eV [Figs. 4(d) and (f)], assigned to N-C=O and NH<sub>2</sub> chemical bindings, possibly due to the acidic nature of the reaction medium. After 48 h treatment, the N<sub>1s</sub> component at 399.8 eV was reduced to 42% of total nitrogen

peak intensity (Table 3), meaning that the nitrogen atoms that contributed to this peak were essentially from *N*-acetylated chitosan units (DA=39%). The upward shift of the electron binding energy of the peak assigned to  $\text{NH}_3^+$  groups to 401.8 eV suggests the presence of protonated amines in the form of ammonium salts<sup>37</sup> as it was expected from ionic bindings, either with free phosphates or with phosphate groups in chitosan chains. After NaOH treatment, the intensity of the peak assigned to  $\text{NH}_3^+$  groups was reduced, if compared to the non-treated membranes, as expected (figure not shown). Nevertheless, this same peak was centered at 401.6 eV, a higher electron binding energy compared to the correspondent one in chitosan membranes, which suggests the presence of salt linkages established between protonated amine groups and phosphate groups in chitosan.

*P<sub>2p</sub> spectra.* All spectra from phosphorylated samples revealed a single peak centered at 133.8 eV [Fig. 4(g)]. The correspondent full-width at half-maximum (FWHM) is wide (2.3 eV), as is usual in the case of  $\text{P}_{2p}$ , since the resulting line observed corresponds to  $\text{P}_{2p1/2}$  and  $\text{P}_{2p3/2}$  core-line doublets. This peak was assigned to phosphate species, and may arise from two contributions: (a) from  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  ionic species,<sup>39,40</sup> and (b) from  $-\text{HPO}_4^-$  groups in chitosan. In a similar work, cellulose phosphates presented a single  $\text{P}_{2p}$  peak at 133.7 eV, as well.<sup>44</sup> The NaOH treatment of phosphorylated membranes did not affect the electron binding energy of  $\text{P}_{2p}$  peak.

In terms of reaction yield, it is difficult to compare the present method to the  $\text{H}_3\text{PO}_4$ /urea/DMF reaction system, the most widely used for the phosphorylation of chitosan matrices.<sup>8,9,30</sup> The amounts of phosphorus reported here were determined on surfaces and thus cannot be compared to those obtained through other phosphorylation routes, in which XPS studies were not performed.<sup>9,30</sup>

#### *ATR-FTIR spectroscopy*

The ATR-FT-IR spectra of unmodified and phosphorylated chitosan membranes as a function of reaction time are shown in Fig. 5. ATR-FT-IR spectra of unmodified and of 48h phosphorylated chitosan membranes are presented in Fig. 6, in a narrower spectral range ( $1250\text{--}800\text{ cm}^{-1}$ ). The major spectral peaks observed in this range, as well as the correspondent peak assignments are shown in Table 4.

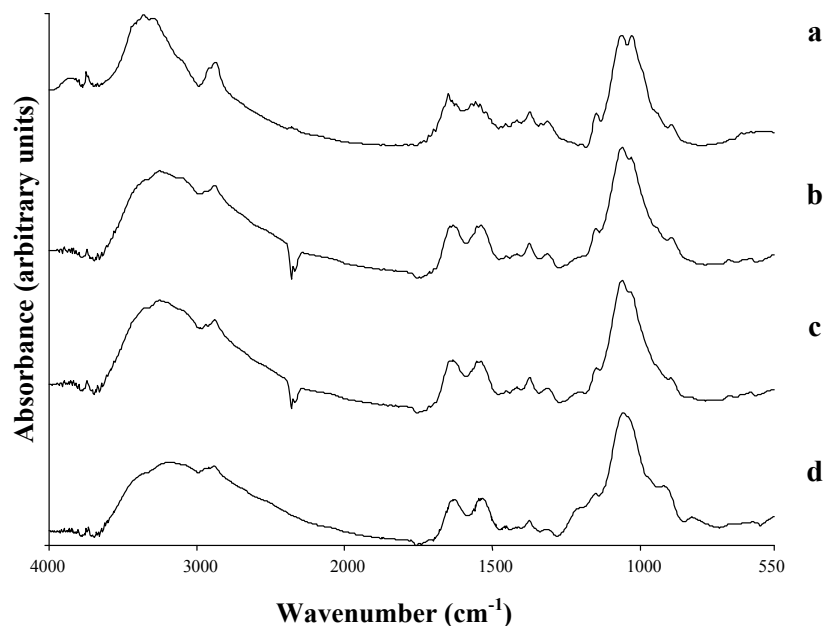


**Figure 5.** ATR-FT-IR spectra of: (a) unmodified chitosan membrane; and chitosan membranes phosphorylated for (b) 4 h, (c) 24 h and (d) 48 h.

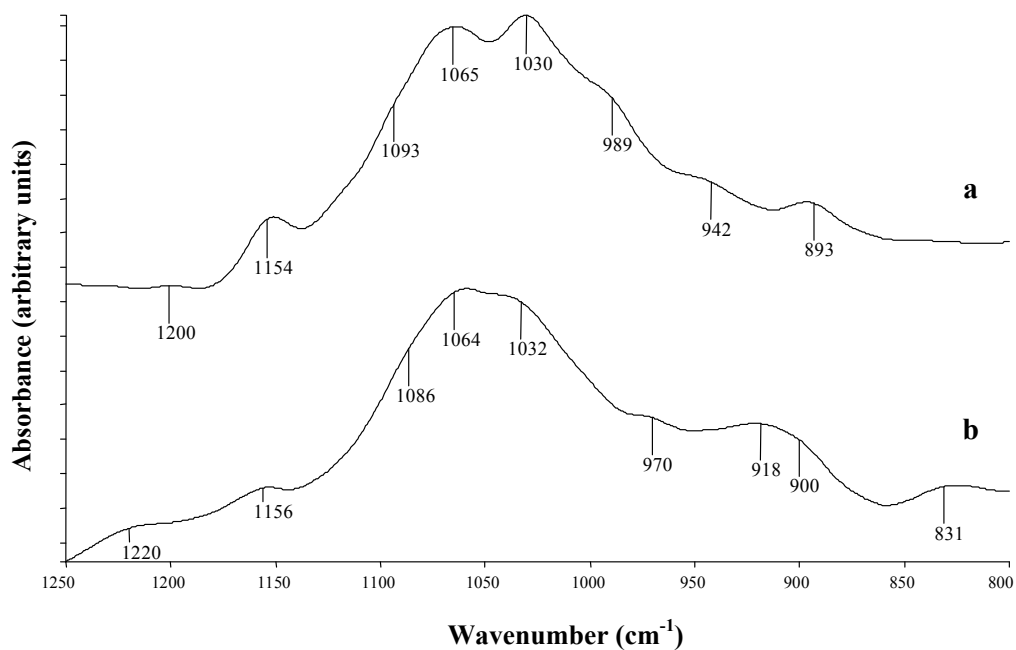


**Figure 6.** ATR-FT-IR spectra ( $1250\text{--}800\text{ cm}^{-1}$ ) of: (a) unmodified chitosan membrane and (b) 48 h phosphorylated chitosan membrane.

**Table 4.**

ATR-FT-IR spectra peak assignments of unmodified and 48 h phosphorylated chitosan membranes.

| Peak frequency (cm <sup>-1</sup> ) |                | Peak assignment                        | Ref.     |
|------------------------------------|----------------|----------------------------------------|----------|
| Chitosan                           | 48h P-chitosan |                                        |          |
| —                                  | 1220           | P=O asymmetric stretching              | 17,19,48 |
| 1200                               | —              | Amine rocking                          | 45       |
| 1154                               | 1156           | Asymmetric bridge oxygen stretching    | 46       |
| 1065                               | 1064           | -C-O stretching in ether groups        | 24,46    |
|                                    |                | C-O-P stretching in phosphate esters   | 49       |
| 1030                               | 1032           | -C-O stretching in ether groups        | 24,46    |
| 989                                | —              | CH <sub>3</sub> rocking                | 45       |
| —                                  | 970            | P-OH stretching                        | 50       |
| 942                                | —              | CH <sub>3</sub> rocking                | 45       |
| —                                  | 918            | P-OH stretching                        | 17,19,50 |
| 893                                | 900            | C-O ring stretching                    | 46       |
| —                                  | 831            | P-O stretching in phosphate monoesters | 51       |

P-chitosan: phosphorylated chitosan.

The spectrum of unmodified chitosan membranes showed characteristic peaks of amide I at 1650 cm<sup>-1</sup> (C=O stretching), amide II at 1558 cm<sup>-1</sup> (N-H in plane deformation coupled with C-N stretching), amide III (C-N stretching coupled with NH in plane deformation) and CH<sub>2</sub> wagging coupled with OH in plane deformation at 1317 cm<sup>-1</sup>.<sup>45,46</sup> The broad peak observed at 3200-3450 cm<sup>-1</sup> has the contribution of different vibrations, namely the hydrogen bonded OH stretching at 3426 cm<sup>-1</sup>, the NH<sub>2</sub> asymmetric stretching at 3360 cm<sup>-1</sup> and the NH stretching in interchain NH...O=C bonding at 3293 cm<sup>-1</sup>.<sup>45,47</sup> The other peaks at 2873 cm<sup>-1</sup> and 1375 cm<sup>-1</sup> were assigned to CH stretching and CH<sub>3</sub> symmetric deformation, respectively.<sup>45,46</sup>

Phosphorylation led to an emerging shoulder at 1220 cm<sup>-1</sup>, which can be attributed to the P=O asymmetric stretching from phosphates, as it is found in polynucleotides

between 1200 and 1230  $\text{cm}^{-1}$ .<sup>48</sup> Similar results were reported for phosphorylated chitin.<sup>17,19</sup> An increase in the absorbance of the peak at 1064  $\text{cm}^{-1}$  was also observed, and was assigned to the C-O-P stretching in phosphate esters, overlapping the C-O stretching vibrations in chitosan ether groups, in accordance to the substitution of an hydroxyl group by a phosphate one.<sup>49</sup> Furthermore, the peaks detectable in the second derivative spectra, namely at 970 and 918  $\text{cm}^{-1}$ , overlapping those of chitosan itself, as well as the peak at 831  $\text{cm}^{-1}$ , point out the presence of phosphate functionalities.<sup>17,19,50,51</sup> The decrease in the absorbance of the peak at 1317  $\text{cm}^{-1}$  also suggests phosphorylation of hydroxyl groups. Protonation of chitosan amine functionalities is suggested by the presence of two peaks, both attributed to  $\text{NH}_3^+$  groups, namely the antisymmetrical deformation at 1630  $\text{cm}^{-1}$  and the symmetric deformation at 1533  $\text{cm}^{-1}$ .<sup>46</sup> The initial amide bands I and -II bands were possibly overlapped by these vibrations. Concerning the region between 3450 and 2800  $\text{cm}^{-1}$ , only a smoothing of the spectra was observed, which can be associated with a decrease in cristallinity.<sup>44</sup> In fact, in the case of phosphoric acid monoesters, the hydrogen bonded OH groups may not appear significantly different from other strongly hydrogen bonded hydroxyl groups.<sup>51</sup> The characteristic peak near 1440  $\text{cm}^{-1}$ , indicating the presence of ammonium phosphates, was not observed, regardless of the treatments used.

### SEM/EDS

Unmodified, as well as phosphorylated chitosan membranes revealed a smooth and homogeneous film surface. In the present work blisters or bubbles were not observed after chemical treatment, as opposite to the reported in literature when the  $\text{H}_3\text{PO}_4$ /urea/DMF method was used for the same purpose.<sup>9</sup>

The cross-section of chitosan membranes (DA=39%) revealed layers of fibrils measuring 0.15-0.35  $\mu\text{m}$  in diameter, clearly oriented parallel to the surface. While chitosan membranes prepared from fully deacetylated chitosan are known to present rough cross-sectional ultrastructures, in which fibrils are absent, complete *N*-acetylation of the former was reported to lead to the formation of disorderly arranged fibrils.<sup>52</sup> Therefore, the observed fibrils were associated to the presence of partially *N*-acetylated chitosan chains, later compressed during the air-drying phase to form the

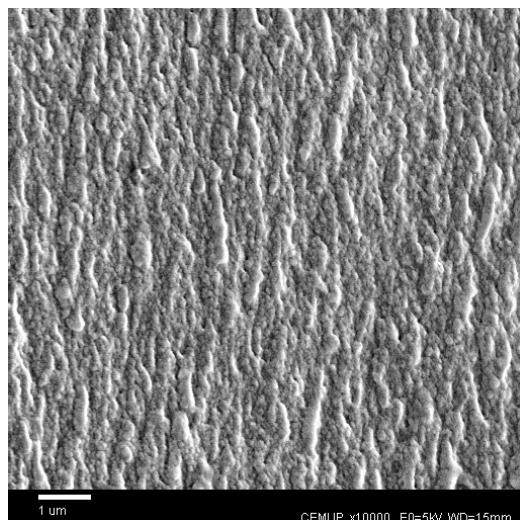


**Figure 7.** Scanning electron micrograph of the cross-section of N<sub>2</sub> fractured 8 h phosphorylated chitosan membrane.

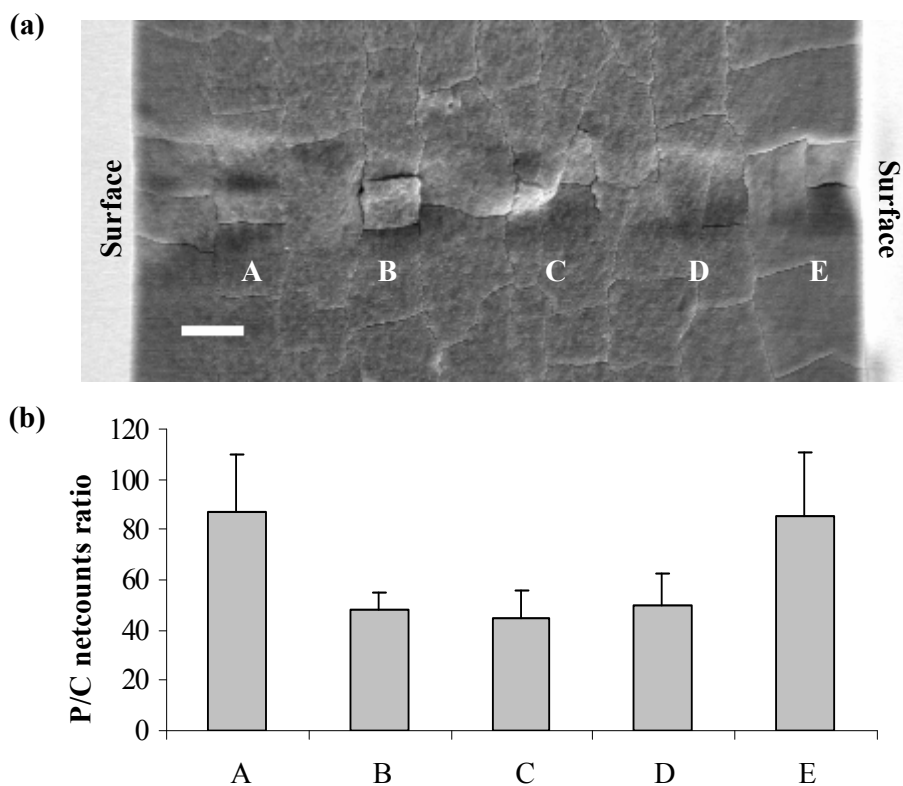


**Figure 8.** Profile of P/C intensity ratio along the cross-section of a N<sub>2</sub> fractured 8 h phosphorylated chitosan membrane: (a) SEM image showing the areas (ca.  $2 \times 2 \mu\text{m}^2$ ) where EDS analysis was performed (A to E); and (b) respective P/C intensity ratios. Intensity ratio was normalized to 100% at the surface ( $n = 4$ ).

membranes.<sup>52</sup> Figure 7 shows the cross-section of phosphorylated chitosan membranes, revealing essentially the same ultrastructure, regardless of the chemical treatment. Figure 8 shows P/C intensity ratio profile along the cross-section of 8 h phosphorylated chitosan membranes, as determined by EDS. Analysis revealed higher levels of phosphorus at the surface, as expected, and a gradual P reduction up to 50% to the inner of the membrane.

## CONCLUSIONS

Surface phosphorylation of chitosan membranes was achieved using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  reaction system, at room temperature. The surface phosphorous concentration increased with the reaction time, a maximum of 4.7 at.% P being reached after 48 h of treatment. Phosphorylation also occurred in the inner of the membrane, although to a lower extent. Furthermore, phosphorylated membranes presented a considerably higher amount of ammonium ions ( $-\text{NH}_3^+$ ) compared to unmodified membranes, as shown by XPS  $\text{N}_{1s}$  core level scan spectra. Chitosan-based surfaces carrying an amphoteric character were thus obtained, which may be used as a combinatorial strategy, to promote cell attachment and proliferation, while immobilizing signaling molecules. These materials can also be used to promote the deposition of an apatite-like layer on chitosan, to improve its osteoconduction.

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### ***In vitro* Mineralization of Chitosan Membranes Carrying Phosphate Functionalities**

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#### **ABSTRACT**

Squid chitosan membranes were phosphorylated through the  $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4/\text{butanol}$  method. P-chitosan membranes were immersed in  $\text{Ca}(\text{OH})_2$  or NaOH solutions, in order to obtain the Na or the Ca salts, respectively. These materials were investigated regarding their ability to nucleate calcium phosphates, under simulated physiologic conditions. SEM-EDS studies revealed the presence of a calcium phosphate mineral layer all over the surface of P-chitosan membranes, after incubation in  $\text{Ca}(\text{OH})_2$  solution. The release of ionically bound phosphate functionalities under alkaline conditions, possibly contributed to the formation of calcium phosphate precursor sites, due to the chelation of calcium ions from solution. During the immersion in Simulated Body Fluid (SBF), a multilayer porous mineral structure composed of poorly crystalline carbonated apatite was formed on the surface of these membranes, as shown by EDS and ATR-FTIR analysis. Unmodified membranes and P-chitosan membranes pre-incubated in NaOH solution did not mineralize.

## INTRODUCTION

The deposition of calcium compounds on polymeric biomaterials may be desirable or not, depending on the application in envisaged. In orthopedics, the presence of a calcium phosphate interface is often desirable, not only to ensure bone bonding to the implant, but also to be used as a sustained drug delivery system. Therefore, several techniques have been developed in order to turn polymeric biomaterials with limited or no tendency to mineralize into mineralizing ones. Blends with bioactive ceramics and biomimetic coatings are among the most frequent ones. The grafting of negatively charged groups, such as phosphates, is another well-known strategy to induce the formation of apatite layers in simulated plasma solutions. This approach has been applied to several natural polymers including chitin and chitosan.

Chitosan, obtained by *N*-deacetylation of chitin, is a biodegradable polysaccharide presently considered for a wide number of biomedical applications, ranging from 3-D matrices for tissue engineering to sutures, wound dressings and gene and drug delivery vehicles. Phosphorylation of chitin and chitosan is described as an effective way of inducing mineralization, after pre-incubation in calcium-containing solutions.<sup>1</sup> In the present work, an alternative phosphorylation method was used to functionalize chitosan membranes.<sup>2</sup> The ability of the resulting phosphorylated membranes to nucleate calcium phosphates under simulated physiologic conditions was investigated.

## MATERIALS AND METHODS

### Materials

Chitosan powder from squid pens was kindly provided by France Chitine (France), and subsequently purified by the reprecipitation method. The regenerated chitosan presented a molar fraction of *N*-acetylated units of 0.39, as determined by FT-IR following the method of Baxter et al.,<sup>3</sup> and an average viscosity molecular weight ( $M_v$ ) of  $2.48 \times 10^6$  Da, estimated from the intrinsic viscosity according to Wang et al..<sup>4</sup> All other reagents were of analytical grade.

## **Preparation of membranes**

Clear and transparent membranes of ca. 50  $\mu\text{m}$  thick were prepared by solvent casting, from 1% (w/v) solutions of purified chitosan in 1% (v/v) aqueous  $\text{CH}_3\text{COOH}$ . The resulting membranes were deprotonated in 0.5 M NaOH and thoroughly washed with distilled and deionized water (DDW). Finally, they were cut into  $20 \times 60 \text{ mm}^2$  strips and kept in absolute ethanol prior to chemical modification.

## **Chemical modification**

Surface phosphorylation was carried out at  $30^\circ\text{C}$  by the  $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4/\text{hexanol}$  method with minor modifications,<sup>2</sup> for a period of 8 h. After chemical treatment, membranes were rinsed with ethanol and suspended twice in this solvent for 30 min. Finally, they were dialyzed against DDW for 24 h. The surface P at.% was estimated by XPS spectroscopy.

## ***In vitro* mineralization studies**

Unmodified and P-chitosan membranes were sterilized in 70% EtOH before further use. Prior to mineralization assays, P-chitosan membranes were immersed in saturated  $\text{Ca}(\text{OH})_2$  or in 23 mM NaOH solutions for 3 days, solutions being renewed every 24 h in order to obtain the Na or the Ca salts, respectively. Chitosan membranes were equally immersed in  $\text{Ca}(\text{OH})_2$ , since unmodified chitosan is reported to absorb minute amounts of calcium.<sup>5</sup> Subsequently, each sample was immersed in 40 mL of sterile SBF at  $37^\circ\text{C}$ , for periods up to 28 days, SBF being renewed every 2 days. Polyethylene screwtop flasks were used. At the end of each immersion period, the strips were washed twice with DDW and dried under reduced pressure at  $60^\circ\text{C}$ . The calcium concentration in the supernatants was analysed by atomic absorption spectroscopy (AAS), using a nitrous oxide-acetylene flame and an ionisation buffer (2000 ppm KCl) in order to remove chemical interferences.

## Surface characterization

Surface mineralization was assessed by Scanning Electron Microscopy (SEM) equipped with an energy dispersive spectroscope (EDS) microanalysis system, Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy, using the SplitPea<sup>TM</sup> accessory, and X-ray diffraction (XRD).

## RESULTS AND DISCUSSION

Phosphorylated membranes presented a  $2.87 \pm 0.13$  P at.%, as shown by XPS studies. AAS analysis of the supernatants showed that P-chitosan membranes significantly adsorbed more calcium than unmodified membranes, during incubation in  $\text{Ca}(\text{OH})_2$  solution; values of 0.617 and 0.099  $\text{mg}\cdot\text{cm}^{-2}$  were found, respectively. These results are in accordance with the expected, since P-chitosan is known to be a better calcium ion chelator than chitosan.<sup>5</sup> SEM studies showed the presence of a mineral layer all over the surface of P-chitosan membranes, after incubation in  $\text{Ca}(\text{OH})_2$  solution [Fig. 1(a)]. EDS analysis revealed a high average Ca/P ratio, namely of  $2.42 \pm 0.18$ , probably due to the presence of  $\text{Ca}(\text{OH})_2$ . The present phosphorylated membranes have phosphate groups ionically bound to protonated chitosan amine functionalities, besides having covalently bound phosphates.<sup>6</sup> Under alkaline conditions, these ions are released and, together with the presence of covalently bound phosphates, possibly contribute to the anchorage of calcium ions, and consequently to the formation of calcium phosphate precursor sites. After immersion in SBF, several mineral layers were observed in samples immersed for 1 day, presenting an average Ca/P ratio of  $1.73 \pm 0.01$ . Samples corresponding to longer immersion periods presented thicker porous layers [Fig. 1(b)], with average Ca/P ratios in the range typical of apatites, namely 1.65, independently of the immersion time. After 7 days of immersion in SBF, the mineral layer presented a thickness of about 6  $\mu\text{m}$ , as observed in cross-sections (Fig. 2). In addition, Ca was also found in the inner of the membrane, as expected, since the inner of the membrane is also phosphorylated. No mineral formation could be observed on unmodified chitosan membranes, nor on NaOH-treated P-chitosan membranes, suggesting that Na ions were not exchanged by Ca ions from SBF.

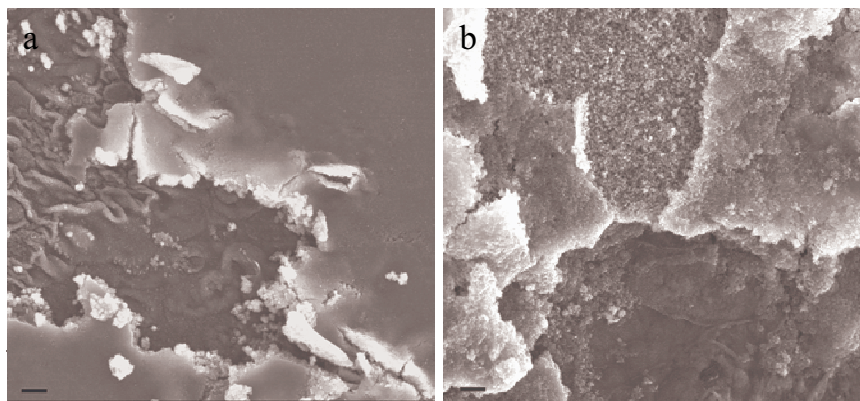


**Fig. 1.** SEM micrograph of P-chitosan membrane: (a) after pre-incubation in  $\text{Ca(OH)}_2$  and (b) after pre-incubation in  $\text{Ca(OH)}_2$  and subsequent immersion in SBF for 14 days, showing different mineral layers. The bar corresponds to 6  $\mu\text{m}$ .

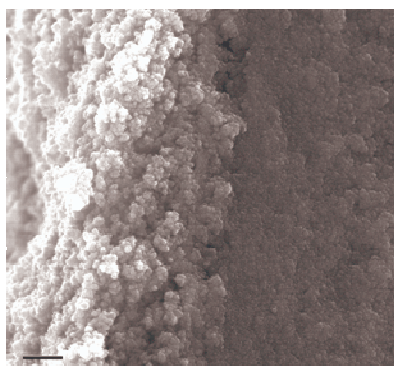


**Fig. 2** SEM micrograph of the cross-section of P-chitosan membrane after pre-incubation in  $\text{Ca(OH)}_2$  and subsequent immersion in SBF for 14 days, showing a mineral layer with a thickness of about 6  $\mu\text{m}$ . The bar corresponds to 2  $\mu\text{m}$ .

AAS analyses of the supernatants agree with the SEM results, revealing practically no calcium uptake for all materials, except for P-chitosan membranes pre-incubated in  $\text{Ca(OH)}_2$ , which presented an uptake of  $0.818 \text{ mg}\cdot\text{cm}^{-2}$  after 28 days of immersion in SBF (Fig. 3). In the case of chitosan pre-incubated in  $\text{Ca(OH)}_2$ , a release of calcium was observed, which suggests that the few anchored calcium ions were probably exchanged with other ions in solution.

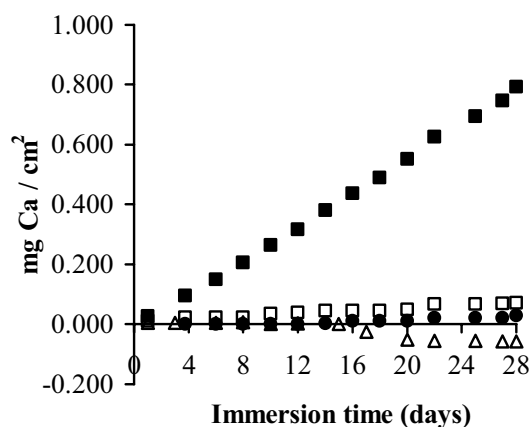


**Fig. 3.** Cumulative calcium uptake as determined by AAS, during immersion in SBF of the samples: (●) chitosan; (Δ) chitosan pre-incubated in  $\text{Ca}(\text{OH})_2$ ; (□) P-chitosan pre-incubated in NaOH; (■) P-chitosan pre-incubated in  $\text{Ca}(\text{OH})_2$ .

The ATR-FTIR spectra of P-chitosan revealed the presence of a new peak at  $1220\text{ cm}^{-1}$ , assigned to  $\text{P}=\text{O}$  asymmetric stretching, due to phosphorylation. An increase in the absorbance of the peak at  $1064\text{ cm}^{-1}$  was also observed and was assigned to the  $\text{C}-\text{O}-\text{P}$  stretching in phosphate esters, overlapping the  $\text{C}-\text{O}$  stretching vibrations in chitosan ether groups. Protonation of chitosan amine functionalities is suggested by the presence of two peaks, both attributed to  $\text{NH}_3^+$  groups, namely the antisymmetrical deformation at  $1630\text{ cm}^{-1}$  and the symmetric deformation at  $1533\text{ cm}^{-1}$ .

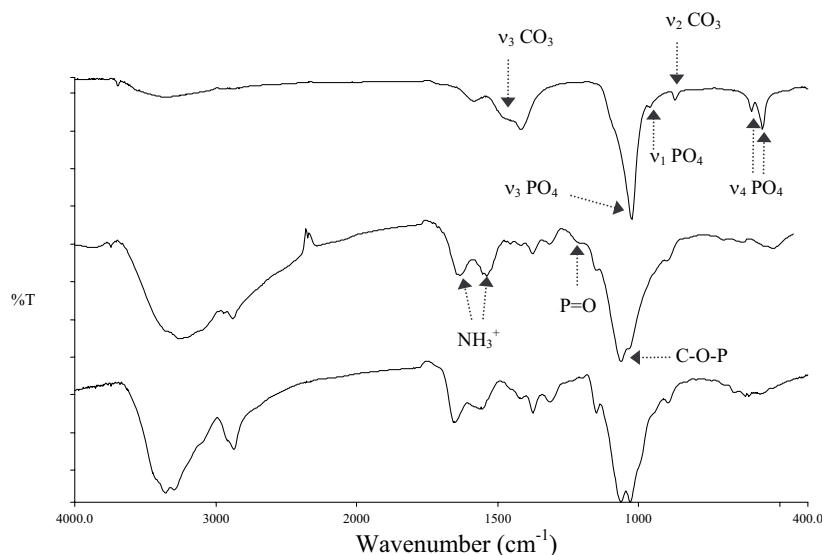


**Fig. 4.** ATR-FTIR spectra of: (a) chitosan membrane; (b) P-chitosan membrane and (c) P-chitosan membrane after pre-incubation in  $\text{Ca}(\text{OH})_2$ .

After incubation in  $\text{Ca}(\text{OH})_2$ , the ATR-FTIR spectra revealed the apatitic nature of the mineral layer previously observed by SEM. The spectra of these samples evidenced peaks characteristic of phosphate vibrations in apatitic structures, overlaying those of the phosphorylated substrate. The intense band at  $1026\text{ cm}^{-1}$  was assigned to the  $\nu_3$   $\text{PO}_4$  vibration, the peak at  $962\text{ cm}^{-1}$  was assigned to the  $\nu_1$   $\text{PO}_4$  vibration, and the peaks at  $601$  and  $562\text{ cm}^{-1}$  to the  $\nu_4$   $\text{PO}_4$  vibration (Fig. 4). In addition, peaks at  $1417$  and  $873\text{ cm}^{-1}$  were also observed, and were assigned to the  $\nu_3$  and  $\nu_2$   $\text{CO}_3$  vibration of carbonate groups, respectively, suggesting the presence of a carbonated apatite. The presence of carbonate groups certainly contributed to the high Ca/P ratio found for these mineralized samples.

Figure 5 shows the spectra obtained for the different materials after 28 days of immersion in SBF, compared to a standard hydroxyapatite sample. The characteristic peaks of phosphate vibrations were only observed in mineralized samples, in accordance to SEM-EDS and AAS studies. The presence of band splitting in the  $\nu_4$   $\text{PO}_4$  region indicates that the minerals formed were not amorphous. However, the characteristic hydroxyapatite peak assigned to OH groups was not observed. Peaks assigned to carbonate species vibrations were also present, pointing out the growth of a carbonated apatite, *in vitro*.

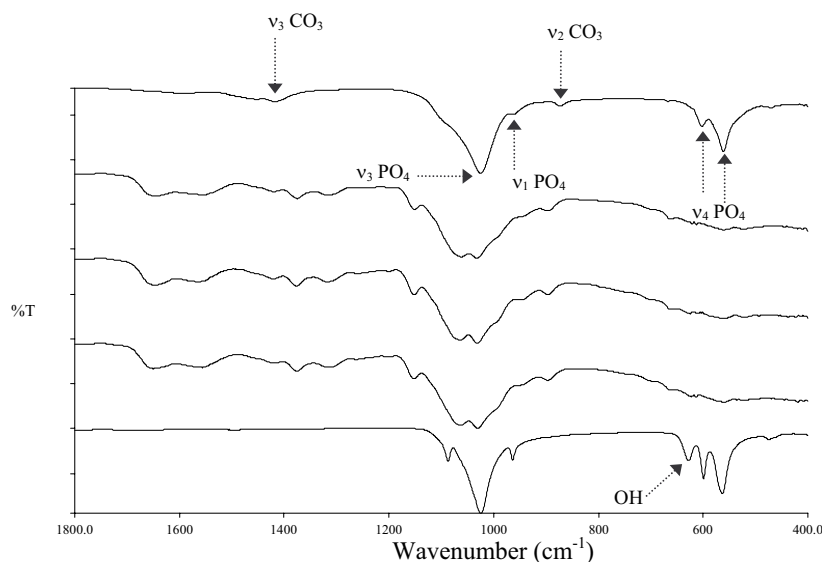


**Fig. 5.** ATR-FTIR spectra of samples, after immersion in SBF for 28 days: (a) control hydroxyapatite; (b) chitosan membrane (c) chitosan membrane pre-incubated in  $\text{Ca}(\text{OH})_2$ ; (d) P-chitosan membrane pre-incubated in NaOH and (e) P-chitosan membrane pre-incubated in  $\text{Ca}(\text{OH})_2$ .

The XRD pattern of the mineral formed on P-chitosan pre-incubated in  $\text{Ca(OH)}_2$  revealed the presence of the main characteristic peaks of crystalline HAp (Fig. 6), although less defined, indicating a poorly crystalline apatite.

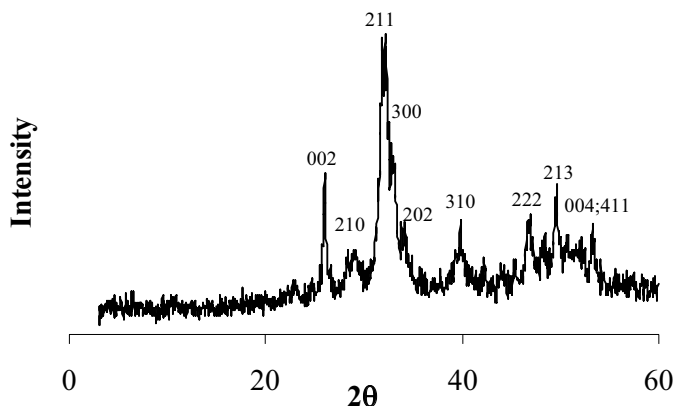


**Fig. 6.** X-ray diffraction pattern of powder formed on P-chitosan membranes pre-incubated in  $\text{Ca(OH)}_2$ , and immersed in SBF for 28 days.

## CONCLUSIONS

A Ca-P mineral was formed on phosphorylated chitosan membranes prepared by the  $\text{H}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{Et}_3\text{PO}_4/\text{hexanol}$  method, after soaking in  $\text{Ca(OH)}_2$ . The subsequent immersion in SBF led to the growth of a partially carbonated and poorly crystalline apatite. The sodium salt of P-chitosan and the unmodified chitosan membranes did not mineralise under the same conditions.

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### **Functionalization of chitosan membranes through phosphorylation: AFM, wettability and cytotoxicity studies**

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#### **ABSTRACT**

Grafting of negatively charged groups such as phosphates is a well-known strategy to induce the deposition of apatite-like layers under simulated physiological conditions. In the present investigation, chitosan was phosphorylated, in an attempt to enhance its osteoconduction. Chitosan membranes were phosphorylated at 30°C using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  reaction system, for periods up to 48 h. This method is an alternative to the phosphoric acid/urea/DMF phosphorylation method, which involves the use of much higher temperatures. In the present study, the effect of phosphorylation reaction time on the surface morphology and surface free energy of phosphorylated membranes was investigated, using atomic force microscopy and static contact angle measurements, respectively. In addition, the modified membranes were evaluated regarding cytotoxicity towards bone cells, by incubating human osteoblastic cells with extracts of the materials for two different time periods, namely 24 and 120 h. The results revealed a decrease of the average surface roughness at the nanometric scale with increasing phosphorylation reaction time. Wettability studies

showed an increase of the polar component of the surface free energy with increasing reaction time, as a consequence of the increase in phosphate surface concentration. Cytotoxicity studies revealed no cytotoxic effect of phosphorylated membranes on osteoblastic cells, regardless of the incubation period.

## INTRODUCTION

Chitosan, a biodegradable polysaccharide usually obtained by alkaline treatment of chitin, is a naturally occurring and renewable polysaccharide present in the exoskeleton of marine arthropods. With well established biocompatibility, it is presently under investigation for a wide range of therapeutic applications, such as burn and wound dressings, sutures, bone fillers, tissue engineered scaffolds and drug and gene delivery vehicles.<sup>1-3</sup> Chitosan is a linear copolymer of glucosamine and *N*-acetyl glucosamine in a  $\beta 1 \rightarrow 4$  linkage, structurally similar to extracellular matrix glycosaminoglycans. In chitosan, the molar fraction of *N*-acetylated units is known as the degree of *N*-acetylation (DA) which, depending on the extent of deacetylation, may range from 0 to 50%. The DA is a structural parameter affecting charge density, solubility and the propensity to enzymatic degradation, with higher DAs leading to faster biodegradation rates.<sup>1</sup>

Similarly to most polysaccharides, chitosan has the ability to elicit specific cellular functions, namely as an immunoadjuvant (due to the ability of *N*-acetyl-glucosamine residues to attract and activate polymorphonuclear leucocytes), inducing the production of cytokines and the subsequent regeneration of connective tissues<sup>4-6</sup>. When applied to bone defects chitosan and its derivatives were shown to enhance osteogenesis and angiogenic activity.<sup>7,8</sup> In addition, since it can be readily processed into films and porous forms without the use of toxic solvents,<sup>9</sup> an increasing number of chitosan-based scaffolds have been developed to be used as temporary templates for *in situ* bone regeneration and for cell-based bone therapies.<sup>10-12</sup> Most often, chitosan is used in combination with calcium phosphates,<sup>10,13</sup> growth factors,<sup>14</sup> or modified with cell adhesive peptide motifs,<sup>15</sup> in an attempt to improve its mechanical properties, osteoconduction and ability to induce bone regeneration.

The response of osteoblasts and bone marrow stromal cells (BMSC) to chitosan films is a matter of research interest. Osteoblast adhesion to chitosan films is dependent on

the DA, lower DAs favoring cell adhesion,<sup>16</sup> as reported for other anchorage dependent cells.<sup>17</sup> Cell adhesion of BMSCs to chitosan films follows the same trend: it is poor even for a DA as low as 4% and osteogenic differentiation is attained only on films with a DA of 4%.<sup>12</sup> However, as both enzymatic degradation and chitosan biological aptitude to enhance tissue regeneration are dependent on the DA,<sup>18</sup> the use of such low DAs may not always be desirable. As a consequence, alternative strategies to promote bone cells adhesion and differentiation on chitosan have to be sought.

In polymeric implants used in orthopedics, the presence of a calcium phosphate overlayer is often desirable in order to promote osteoconduction and to ensure bone bonding. Grafting of negatively charged functionalities, such as phosphates, is a well-known strategy to induce the deposition of apatite-like layers under simulated physiological conditions.<sup>19-22</sup> Recently, we have applied this approach to chitosan membranes using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  phosphorylation reaction system, at 30°C, for periods up to 48 h.<sup>23</sup> The phosphate content was found to increase with the reaction time, as shown by XPS and ATR-FTIR, an atomic ratio of 0.73 P/N being obtained after 48 h of treatment. The latter was correlated to the degree of substitution at the surface, given that one nitrogen atom is present in each chitosan monomeric unit. The subsequent immersion of phosphorylated membranes (P-chitosan) in a  $\text{Ca}(\text{OH})_2$  solution, followed by incubation in a simulated plasma solution, resulted in the deposition of a multilayered and poorly crystalline porous mineral structure similar to bone mineral in terms of composition.<sup>24</sup>

The phosphorylation method used is based on the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{hexanol}$  reaction route, optimized by Granja et al.<sup>25</sup> for the synthesis of highly phosphorylated cellulose derivatives, and originally proposed by Touey and Kingsport, in 1956,<sup>26</sup> for the synthesis of water-soluble and non-degraded cellulose phosphates. This method has the advantages of being carried out at room temperature, resulting in low degradation of the polymer. Additionally, upon implantation in rabbit bone defects, phosphorylated cellulose hydrogels induced no inflammatory response and showed osteointegration in the surrounding tissue.<sup>27</sup> The  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  method is an alternative to the  $\text{H}_3\text{PO}_4/\text{urea}/\text{DMF}$  method described for the phosphorylation of chitin and chitosan,<sup>28,29</sup> which is the phosphorylation pathway usually followed to introduce phosphate functionalities in chitin fibers and chitosan films/sponges, and that involves the use of high temperatures, typically higher than 120°C.<sup>21,30-32</sup>

In the present study, chitosan membranes phosphorylated for periods up to 48 h were characterized in terms of surface morphology and surface free energy which, together with surface chemistry, constitute the main determinant features affecting the initial osteoblast response at the cell-material interface directly, or indirectly, by influencing the adsorption of adhesive glycoproteins.<sup>33</sup> In addition, P-chitosan was evaluated in terms of cytotoxicity, which constitutes the initial step in a sequenced program of tests for assessment of biocompatibility.<sup>34</sup>

## **MATERIALS AND METHODS**

### **Materials**

Squid pen chitosan (Chitosan 123), was kindly supplied by France Chitine (France). All reagents used were of analytical grade.

### **Purification and characterization of chitosan**

Purified chitosan was prepared by filtration of chitosan acidic solution and subsequent alkali precipitation. The physicochemical characterization of the regenerated chitosan in terms of degree of *N*-acetylation (DA) and average molecular weight is shown in Table 1. The DA was determined by infrared spectroscopy (FT-IR system 2000 from Perkin-Elmer) in KBr pellets, using the amide III band at  $1320\text{ cm}^{-1}$  as the analytical band and the band at  $1420\text{ cm}^{-1}$  as the internal reference band, according to Brugnerotto et al..<sup>35</sup> Weight and number average molecular weight ( $M_w$  and  $M_n$ , respectively) as well as polydispersity index (*PDI*) were assessed by high performance size exclusion chromatography using the system 0.2 M  $\text{CH}_3\text{COONa}$ /0.5 M  $\text{CH}_3\text{COOH}$  as mobile phase, according to Terbojevich et al..<sup>36</sup>

### **Preparation of chitosan membranes**

Chitosan membranes were obtained by solvent casting, from 1% (w/v) chitosan solutions in 1% (v/v) acetic acid. After complete dissolution, the resulting gel was filtered, kept under reduced pressure in order to clear all the entrapped gas bubbles,

and poured into  $120 \times 120 \text{ mm}^2$  polystyrene petri dishes (60 g per plate). After solvent evaporation the resulting membranes were deprotonated in 0.5 M aqueous NaOH and thoroughly washed with distilled and deionized water (DDW). Finally, they were cut into  $30 \times 60 \text{ mm}^2$  strips, and kept in absolute ethanol prior to chemical modification. For characterization purposes, chitosan membranes were dried at 30°C for 24 h in a vacuum oven, and stored in a desiccator until further analysis.

**Table 1.**

Chitosan characterization in terms of degree of *N*-acetylation (DA), weight and number average molecular weight ( $M_w$  and  $M_n$ , respectively), and polydispersity index (*PDI*). Results listed are the average ( $\pm$  SD). 4 independent measurements and 8 measurements made in two independent samples were used for DA and average molecular weight determination, respectively.

| DA (%)          | $M_w \times 10^5$ | $M_n \times 10^5$ | <i>PDI</i>    |
|-----------------|-------------------|-------------------|---------------|
| $29.8 \pm 1.21$ | $5.4 \pm 0.3$     | $1.3 \pm 0.2$     | $3.9 \pm 0.6$ |

### Phosphorylation of chitosan membranes

Surface phosphorylation was performed using the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  reaction system.<sup>23</sup> Briefly, 1 g of chitosan membrane strips were suspended in 40 mL of 2-butanol in a round-bottomed flask. The reaction mixture was prepared by adding 43 mL of  $\text{H}_3\text{PO}_4$  to 37 mL of  $\text{Et}_3\text{PO}_4$  followed by dropwise addition of  $\text{P}_2\text{O}_5$ , under constant stirring. Reaction proceeded under  $\text{N}_2$  atmosphere, at 30°C, with constant stirring in a thermostated orbital shaker. The reaction was carried out for different time periods, up to 48 h. After chemical treatment, membranes were rinsed with ethanol and suspended twice in this solvent for 30 min. Finally, they were dialyzed against DDW for 24 h to remove free inorganic phosphate and used for the biological assays or dried as described above, for characterization purposes.

## Surface characterization

### *Atomic force microscopy (AFM) imaging and roughness analysis*

A Dimension™ 3100 AFM, with a Nanoscope® IIIa Controller and equipped with an Extender™ Electronics Module for phase-lag detection (Digital Instruments, USA), was used for the analysis. Imaging was performed in TappingMode™, using standard TappingMode™ Si tips. The samples were mounted on a magnetic puck, using a double-sided tape, and analyzed in ambient air at room temperature. The surface roughness was determined under two different scan ranges, namely  $1 \times 1 \mu\text{m}^2$  and  $400 \times 400 \text{ nm}^2$ , using Digital Instruments roughness analysis software. The following roughness height parameters were calculated:  $R_a$ , average roughness (the arithmetic average of the deviations from the centre  $x$ - $y$  plane),  $R_q$  (the root mean square of the deviations from the centre  $x$ - $y$  plane) and  $R_{\text{max}}$  (the sum of the height of the maximum peak with the depth of the maximum valley). The centre plane is a plane such as the volumes enclosed by the image surface above and below are equal.<sup>37</sup> Measurements were made in three randomly chosen areas per sample type, in at least two different samples.

### *X-ray photoelectron spectroscopy (XPS)*

Survey spectra and high-resolution spectra for the  $\text{C}_{1s}$ ,  $\text{O}_{1s}$ ,  $\text{N}_{1s}$ , and  $\text{P}_{2p}$  regions were obtained with a VG Scientific ESCALAB 200 A (UK), using a  $\text{MgK}\alpha$  X-ray radiation as the excitation source. The emitted photoelectrons were analyzed at a  $90^\circ$  take-off angle from the horizontal surface plane. Element atomic percentages were calculated from the integrated intensities of the XPS peaks, taking into account the atomic sensitivity factors of the instrument data system.

### *Surface free energy determination*

Wettability studies were performed using a video-based optical contact angle measurement device OCA 15 plus, provided with an electronic syringe unit (Dataphysics, Germany). DDW, with conductivity lower than  $1 \mu\text{S}/\text{cm}$ , and

diiodomethane (> 99%, Sigma-Aldrich), were used. The surface tension of the liquids was determined by the pendent drop method. Drops of 24  $\mu\text{L}$  or 1.8  $\mu\text{L}$  were generated from the tip of 1.65 mm or 0.52 mm needles, respectively. Measurements were carried out at 25°C, inside a thermostatted environmental chamber, previously saturated with a pool of the liquid sample. The liquid densities at 25°C were determined using a density meter (Anton Paar, Germany). The polar and dispersive components of the surface tension of the liquids at 25°C were estimated from the correspondent values at 20°C, obtained from the literature.<sup>38</sup> The estimation was based on the assumption that the ratio between the dispersive and the polar components of the surface tension did not vary with the temperature.

Samples used for wettability studies were immediately analyzed after drying. The strips were cut into squares ( $20 \times 20 \text{ mm}^2$ ), and nitrogen was used to eliminate eventual dust particles from the surface. Static contact angles were measured at 25°C by the sessile drop method, using droplets of 4  $\mu\text{L}$  for water and of 1  $\mu\text{L}$  for diiodomethane. Due to the swelling nature of the membranes and to the eventual reorientation of surface groups,<sup>39</sup> time-dependent measurements were taken for a period of 300 s, and the experimental contact angles extrapolated to time zero. A minimum of 10 drops were made per sample type in at least three different samples. Contact angles, surface tensions and surface free energies were calculated by SCA20 software (Dataphysics, version 2.0), fitting the drop profiles to the Young-Laplace equation. The polar and dispersive contributions of the surface energies were calculated according to the method of Owens and Wendt.<sup>40</sup>

### **Cytotoxicity evaluation**

Unmodified as well as P-chitosan were tested for cytotoxicity according to ISO standards (10993-5, 1992)<sup>34</sup>, using the extract method. The modified membranes assayed for cytotoxicity were the 8-h phosphorylated chitosan membranes, since they have shown ability to mineralize in simulated physiologic conditions, in a previous study.<sup>24</sup> Cytotoxicity was evaluated by measuring cell viability after cell incubation with the material's extracts for two different time periods, namely 24 and 120 h. The longer period was used in order to study the effect of leachable products on cell proliferation.

*Cell culture*

MG-63 osteoblast-like cells from human osteosarcoma were obtained from the American Type Culture Collection (ATCC) and routinely cultured in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 50  $\mu$ g/mL gentamicin sulfate and 2.5  $\mu$ g/mL amphotericin B (all from Gibco). Cultures were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. When cells reached confluency, they were trypsinized, stained with trypan blue, and those which excluded the dye counted with a hemocytometer. Cells were seeded at a density of 10<sup>4</sup> viable cells/cm<sup>2</sup> in 96-well tissue culture polystyrene plates (TPP®), and incubated under the same conditions. The 24 h assay was performed when a subconfluent monolayer was obtained. The medium was then removed and replaced by the extracts. For the 120 h assay, the medium was removed and replaced by the extracts after a 4 h incubation period.

*Extracts preparation*

Chitosan and 8-h phosphorylated chitosan membranes were used after sterilization in 70% ethanol for 30 min. The acidic groups of P-chitosan were neutralized by immersion in 20 mM NaOH during 60 min, rinsed twice in DDW, and then equilibrated in  $\alpha$ -MEM for 60 min, exchanging the medium every 15 min. The extracts were prepared by incubating the membranes in  $\alpha$ -MEM (5cm<sup>2</sup>/mL) supplemented with 50  $\mu$ g/mL gentamicin sulfate and 2.5  $\mu$ g/mL amphotericin B in an orbital incubator at 37°C, during 120 h at 100 rpm. A sample of the extract vehicle subjected to the same extraction conditions was used as control. At the end of this period, the membranes were removed and the so-called extracts obtained. Prior to being used, the extracts were serially diluted in culture medium used as control, supplemented with 10% (v/v) FBS and sterilized by filtration (0.22  $\mu$ m). Fresh culture medium was used as negative control, while a solution of phenol prepared in fresh culture medium (64 g/L) was used as positive control.

*Cell viability evaluation*

At the end of each culture period, the number of viable cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay is a colorimetric method of determining cell viability based on the reduction of the yellow tetrazolium salt to insoluble blue formazan crystals by mitochondrial enzymes in viable cells.<sup>41,42</sup> Given that the amount of formazan generated is directly proportional to the cell metabolic activity, the correspondent absorbance at 540 nm allows an estimation of the number of viable cells.<sup>42</sup> Briefly, at the end of the incubation period the extracts were removed, and the cell layers rinsed with phosphate buffered saline. Cells were then incubated with MTT (0.5 mg/mL in Dulbecco's medium without phenol red) at 37°C for 4 h. At the end of this period the supernatant was discarded and 100 µL of DMSO were added to dissolve the formazan crystals. After complete dissolution the optical density of the supernatant was read at 540 nm with reference to 620 nm, using a microplate spectrophotometer (SLT Labsinstruments, Austria). Both assays were repeated three times. Reported values are the average of 8 replicates, expressed as a percentage of the negative control.

### **Statistical analysis**

Data were analyzed using the nonparametric Mann-Whitney *U* test, two-tailed asymptotic significance levels being considered. Results were considered statistically significant when  $p \leq 0.05$ . Pearson correlation coefficient was used to determine the linear association between two variables. Calculations were performed using SPSS® software (version 12.0).

## **RESULTS AND DISCUSSION**

### **Surface characterization**

#### *Atomic force microscopy imaging and roughness analysis*

AFM 3D images of unmodified and 48-h phosphorylated chitosan membranes, taken at a scan range of  $1 \times 1 \mu\text{m}^2$ , are shown in Figure 1. Chitosan membranes revealed a predominantly hill-valley structured surface of nanoscaled topography [Fig. 1(a)], in

accordance to other reports.<sup>43</sup> Phosphorylated samples presented similar surface morphology, regardless of the reaction time used [Fig. 1(b)].

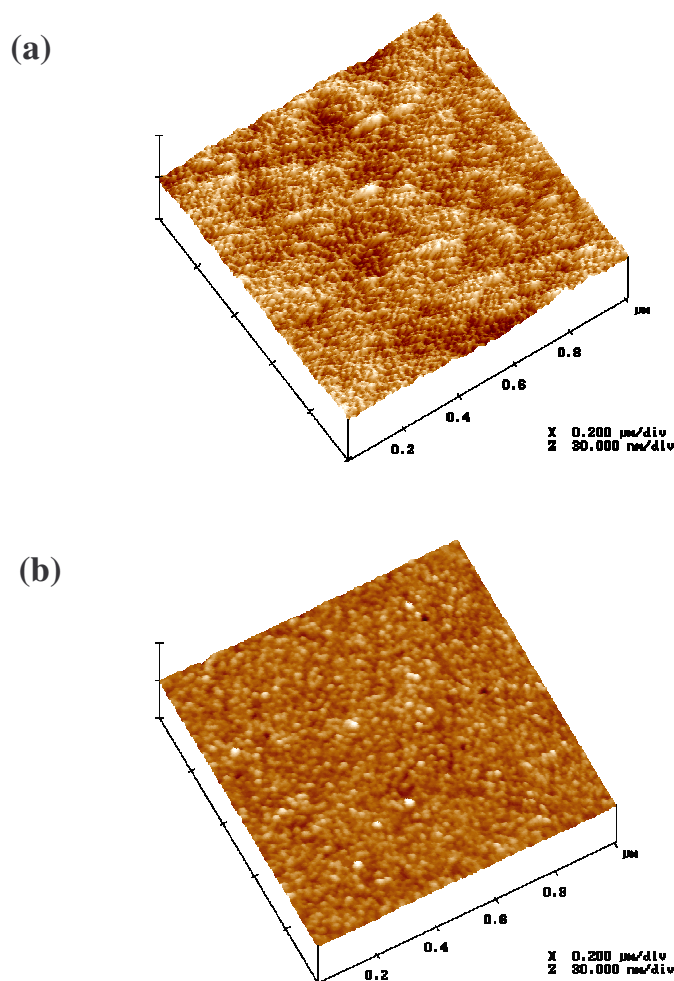


**Figure 1.** TappingMode™ atomic force microscopy 3D images of sample surfaces: (a) chitosan membrane; (b) 48 h phosphorylated chitosan membrane. Data collected from a  $1 \times 1 \mu\text{m}^2$  scan range, under ambient conditions.

The results concerning roughness analysis are shown in Table 2 and correspond to a  $1 \times 1 \mu\text{m}^2$  scanned area. Unmodified chitosan membranes revealed an average roughness ( $R_a$ ) of 1.50 nm and 1.27 nm for scanned areas of  $1 \times 1 \mu\text{m}^2$  and  $400 \times 400 \text{ nm}^2$ , respectively, indicating roughness at the nanometric scale. The maximum peak to valley differences determined in each of those measured areas were of 17.5 nm and

**Table 2.**

Surface roughness parameters of unmodified and P-chitosan, as a function of phosphorylation reaction time. Roughness was determined over a scanned area of  $1 \times 1 \mu\text{m}^2$ . Values listed are the average [min; max] of three measurements. Asterisks indicate a statistical significant difference comparing to the unmodified chitosan membranes. (\*  $p \leq 0.05$ ).

| Phosphorylation<br>reaction time (h) | $R_a$<br>(nm)          | $R_q$<br>(nm)          | $R_{\text{max}}$<br>(nm) |
|--------------------------------------|------------------------|------------------------|--------------------------|
| 0                                    | 1.50 [1.25; 1.81]      | 2.01 [1.61; 2.60]      | 17.5 [13.1; 26.0]        |
| 1                                    | 1.14 [0.950; 1.33]     | 1.84 [1.39; 2.28]      | 24.8 [19.3; 30.4]        |
| 4                                    | 0.949 [0.896; 0.979] * | 1.23 [1.17; 1.27] *    | 10.8 [9.64; 13.0] *      |
| 8                                    | 1.23 [1.15; 1.36]      | 1.58 [1.44; 2.28]      | 11.8 [11.0; 13.4]        |
| 12                                   | 0.894 [0.888; 0.902] * | 1.10 [1.04; 1.13] *    | 8.89 [7.58; 9.88] *      |
| 24                                   | 0.833 [0.381; 1.11] *  | 1.17 [0.485; 1.63]     | 9.99 [3.99; 13.7]        |
| 48                                   | 0.345 [0.276; 0.388] * | 0.446 [0.352; 0.501] * | 5.49[3.22; 7.82] *       |

$R_a$ , average roughness;  $R_q$ , root mean square of the deviations from the centre  $x$ - $y$  plane;  $R_{\text{max}}$ , sum of the height of the maximum peak with the depth of the maximum valley.

12.1 nm, respectively, and correspond to the larger features imaged. Analysis of the substrates at different scan ranges resulted in different roughness values, higher values being found when larger areas were scanned. This fact is frequently described, since  $R_a$  is the arithmetic average of the deviations from the centre  $x$ - $y$  plane, as previously defined, and therefore is a function of the scan size.<sup>44,45</sup> Phosphorylated samples presented lower  $R_a$  values, when compared to untreated membranes, although statistical significant differences were not found for all groups. However, a Pearson correlation coefficient of  $-0.802$  was found between phosphorylation reaction time and  $R_a$ , significant at the 0.001 level, suggesting that phosphorylation contributed to the smoothing of the membranes. The  $R_a$  data collected from a  $400 \times 400 \text{ nm}^2$  scanned area showed a similar trend with reaction time. Both chitin and chitosan are susceptible to acid hydrolysis, although chitosan is more resistant due to the protective effect of the protonated amine groups, which shield the glycosidic oxygen from protonation and, consequently, from hydrolysis.<sup>46</sup> The smoothing of chitosan

membranes during chemical modification may thus be related to the acidic nature of the present reaction medium.

#### *X-ray photoelectron spectroscopy (XPS)*

The survey spectra of unmodified chitosan membranes confirmed the presence of carbon, oxygen, and nitrogen. XPS results showed an increase of the surface phosphorus concentration with phosphorylation reaction time, a maximum of 4.7 at.% P being reached after 48 h of treatment, in accordance to previous results<sup>23</sup> (Table 3).

#### *Surface free energy determination*

The calculated surface tensions of water and diiodomethane were  $71.5 \pm 0.1 \text{ mJ}\cdot\text{m}^{-2}$  and  $50.5 \pm 0.1 \text{ mJ}\cdot\text{m}^{-2}$ , respectively. The values found at 25°C were lower than the values reported in literature for the same liquids at 20°C, as expected. Surface tension of most liquids decreases almost linearly with increasing temperature, if the temperature is far below the boiling point of the liquid.<sup>38</sup>

The contact angle values determined for both unmodified and P-chitosan, as a function of reaction time, are shown in Figure 2. Chitosan membranes presented a high water contact angle ( $\theta_{\text{water}} \cong 87.2^\circ$ ), and a surface free energy of  $35.4 \text{ mJ}\cdot\text{m}^{-2}$ , entirely dominated by the dispersive component (Table 3). Tomihata et al.<sup>1</sup> reported considerably lower water contact angle values ( $\theta_{\text{water}} \cong 58^\circ$ ) for chitosan films prepared from chitosan with a similar DA (31%), although these results cannot be fairly compared, since the latter regards static water contact angle measurements performed on water-swollen chitosan films. The present results revealed that chitosan membranes have a hydrophobic solid-air interface, in spite of carrying polar functional groups, namely hydroxyl and amine groups. Polymer surfaces are known to react to environmental changes in order to minimize their interfacial tension.<sup>39</sup> In aqueous environment, the polar components tend to dominate, while in equilibrium with air or vacuum the non-polar components (dispersive components) tend to dominate the interface.<sup>39,47</sup> In this sense, the hydrophobic surface exhibited by chitosan membranes was probably a consequence of molecular reorientations occurred during the vacuum drying process.

Phosphorylation improved the wettability of chitosan membranes, as expected. A water contact angle of  $53.5^\circ$  was obtained after 48 h of treatment. The change in water contact angle is not attributed to surface physical heterogeneities, since the chemical modification induced minimal changes in terms of surface morphology, as shown by AFM roughness analyses. It is generally accepted that asperities in the range of 0.1 to  $0.5\ \mu\text{m}$  or below do not lead to significant hysteresis.<sup>39,48</sup> Hence, the decrease in the water contact angle is mainly attributed to differences in surface chemical composition, i. e., to phosphate functionalities. The hydrophilicity of phosphate groups is well-established.<sup>20,49</sup> Surfaces carrying phosphate functionalities have high affinity to polar liquids like water, due to the ionic nature of phosphate groups in aqueous environment. On the other hand, the contact angles determined using diiodomethane as testing liquid did not vary with reaction time (Fig. 2). Diiodomethane, due to its apolar nature, is supposed to interact exclusively with the dispersive components of the solid. Since in the present case only polar functionalities were introduced, the contact angle remained practically unaltered ( $\theta_{\text{diiodomethane}} \cong 52^\circ$ ).

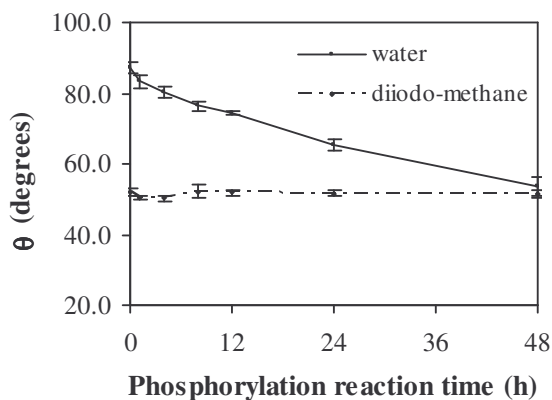


**Figure 2.** Static contact angle ( $\theta$ ) of water and diiodomethane on unmodified and P-chitosan, as a function of phosphorylation reaction time. Values represent the average  $\pm$  SD ( $n=10$ ).

In what concerns surface free energy, the polar component of surface free energy ( $\gamma_{\text{sv}}^{\text{p}}$ ) increased with reaction time, leading to the increase of the total surface free energy from  $35.42$  to  $51.58\ \text{mJ}\cdot\text{m}^{-2}$ , as the membranes were reacted from 0 to 48 h, respectively (Table 3). These results are in agreement with the presence of polar

groups provided by phosphate functionalities. The plot of  $\gamma_{sv}^p$  versus phosphorus surface concentration was fitted to a positive exponential-like regression function (Fig. 3). The non-linear increase of  $\gamma_{sv}^p$  is probably due to the reorientation of phosphate groups at the solid-air interface. During the vacuum drying process, the hydrophilic phosphate groups are probably reoriented towards the inner part of the membranes, in order to minimize the total surface free energy. As the P surface concentration increases, this reorientation may be partially hindered, leaving the phosphate groups more exposed, and thus to the exponential increase of  $\gamma_{sv}^p$ . As compared to XPS, contact angle measurements are likely to be more sensitive to surface reorientation phenomena. While the contact angle depends on the outermost surface molecular layers,<sup>39</sup> XPS data resulting from emitted photoelectrons analyzed at a 90° take-off angle regards a thicker surface layer, typically ranged in about 10 nm.<sup>50</sup>

In this study, the conventional technique of sessile drop in air was used, which requires preliminary drying of the samples. An alternative would have been the use of immersion techniques for wettability studies, which allow surface characterization of samples in the hydrated state. However, the measurement of surface energy of water-containing polymers is problematic. The use of the captive air bubble or the inverted

**Table 3.**

Surface free energy ( $\gamma_{sv}$ ) and correspondent dispersive ( $\gamma_{sv}^d$ ) and polar ( $\gamma_{sv}^p$ ) components (%) of unmodified and P-chitosan, as a function of phosphorylation reaction time.

| Reaction time<br>(h) | Phosphorus<br>(at. %) <sup>23</sup> | $\gamma_{sv}^d$ (mJ·m <sup>-2</sup> ) | $\gamma_{sv}^p$ (mJ·m <sup>-2</sup> ) | $\gamma_{sv}$ (mJ·m <sup>-2</sup> ) |
|----------------------|-------------------------------------|---------------------------------------|---------------------------------------|-------------------------------------|
| 0                    | 0.00                                | 93.3                                  | 6.7                                   | 35.4                                |
| 1                    | 1.06                                | 90.9                                  | 9.1                                   | 37.2                                |
| 4                    | 2.31                                | 88.4                                  | 11.6                                  | 38.3                                |
| 8                    | 2.87                                | 84.1                                  | 15.9                                  | 39.0                                |
| 12                   | 2.96                                | 82.4                                  | 17.6                                  | 40.1                                |
| 24                   | 3.42                                | 74.3                                  | 25.7                                  | 44.6                                |
| 48                   | 4.71                                | 64.3                                  | 35.7                                  | 51.6                                |

octane droplet techniques maintains the surface in a hydrated state but makes it difficult for the droplet to displace the adsorbed water layers. According to the work of Barnes et al.,<sup>51</sup> the values of the surface free energy components determined in such a system are a function of both adsorbed and surface layers.

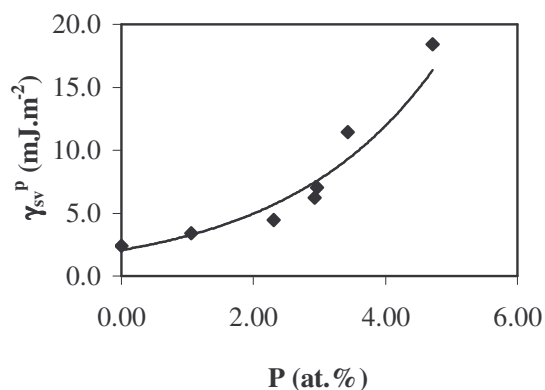


**Figure 3.** Polar component of surface free energy ( $\gamma_{sv}^p$ ) of unmodified and P-chitosan, as a function of phosphorus surface atomic percentage.

### Cytotoxicity evaluation

#### 24 h assay

No statistical significant differences were observed in the cell metabolic activity of MG-63 osteoblast-like cells cultured for 24 h in the presence of unmodified or P-chitosan extracts, when compared to the control, independently of the dilution used (Fig. 4). In the case where a 10% diluted P-chitosan extract was used, an exception occurred, resulting in an average relative metabolic activity of 87%. In addition, no correlation was found between the dilution of the extracts and the metabolic activity of the cells, showing the absence of cytotoxic products released from chitosan membranes subjected to this phosphorylation method.

#### 120 h assay

Figure 5 shows the cell metabolic activity assessed after a 120 h period of cell incubation with the extracts. A statistically significant decrease of cell metabolic activity in the presence of unmodified chitosan extract was observed, particularly noticeable in the case of the less diluted extracts. In fact, a Pearson correlation

coefficient of -0.76, significant at the 0.001 level, was determined between the dilution of the extracts and the cell metabolic activity, indicating a linear dependence between these two variables. The present results suggest a potential inhibitory effect of chitosan extract on the number of viable cells at the end of the 120 h period, and thus on cell proliferation. The inhibition of cell proliferation in the presence of chitosan extracts was possibly related to the depletion of nutrients from the culture medium ( $\alpha$ -MEM), promoted by the presence of positively charged amine functionalities from chitosan, which have the ability to ionically bind to negatively charged functional groups.<sup>52,53</sup> When cells were cultured in the presence of extracts from P-chitosan, the above mentioned inhibitory effect on cell proliferation was not observed. Additionally, no correlation was found neither between cell metabolic activity and the dilution of the extracts. It is possible that the presence of the negatively charged phosphate functionalities contributed to counterbalance the positively charged amines from chitosan, leading to a less specific binding of elements from the culture medium.

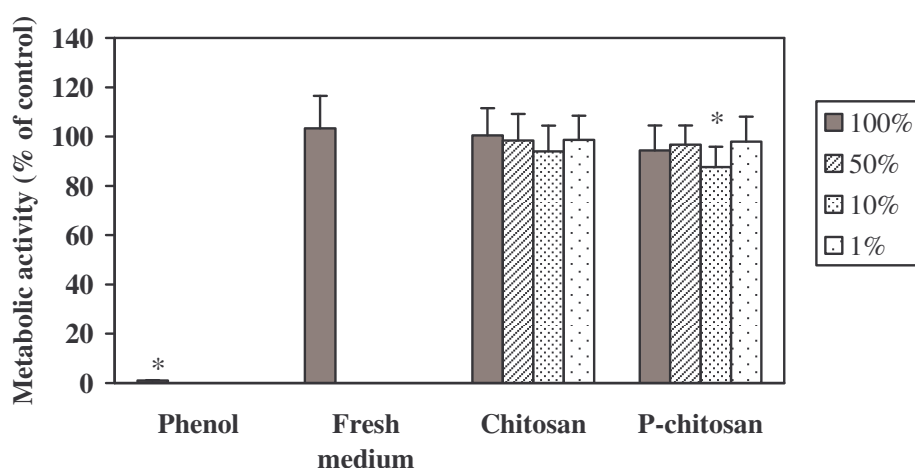


**Figure 4.** MG-63 osteoblasts-like cell viability assessed by the MTT assay, after 24 h of cell incubation with serially diluted extracts of chitosan membranes and 8-h phosphorylated chitosan membranes. Phenol (64g/L) in fresh culture medium and fresh culture medium were used as positive and negative controls, respectively. Values are the average  $\pm$  SD of 8 cultures. Data result from one of three replicate experiments. Asterisks indicate a statistical significant difference from the control. ( $p \leq 0.01$ ).

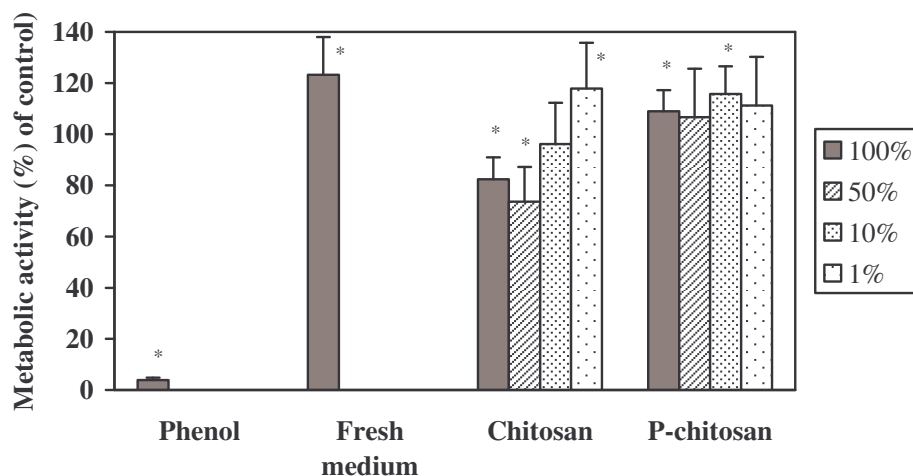


**Figure 5.** MG-63 osteoblasts-like cell viability assessed by the MTT assay, after 120 h of cell incubation with serially diluted extracts of chitosan membranes and 8-hrs phosphorylated chitosan membranes. Phenol (64g/L) in fresh culture medium and fresh culture medium were used as positive and negative controls, respectively. Values are the average  $\pm$  SD of 8 cultures. Data result from one of three replicate experiments. Asterisks indicate a statistical significant difference from the control. ( $p \leq 0.01$ ).

According to other reports, both phosphorylated chitin and phosphorylated chitosan have shown *in vivo* biocompatibility, when used as additives of calcium phosphate cements.<sup>54,55</sup> Moreover, polyelectrolyte complexes containing phosphorylated chitin and chitosan revealed no apparent cytotoxicity towards rat calvarial osteoblasts.<sup>56</sup> The present results revealed no cytotoxic effect of the phosphorylated chitosan, in agreement with the previous studies.

## CONCLUSIONS

Phosphorylation of chitosan membranes by the  $\text{H}_3\text{PO}_4/\text{Et}_3\text{PO}_4/\text{P}_2\text{O}_5/\text{butanol}$  reaction system led to a decrease of the surface roughness with reaction time at the nanometric scale, as shown by AFM studies, although the surface morphology of the membranes was not significantly altered. Wettability experiments clearly showed an increase of the polar component of surface free energy with reaction time and, as a consequence, of the total surface free energy. Cytotoxicity studies revealed no cytotoxic effect of

phosphorylated chitosan membranes towards cultured bone cells, enabling their use in further biological assays, such as the study of osteogenic differentiation of osteoprecursor cells on both phosphorylated and mineralized membranes.

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### CONCLUDING REMARKS AND FUTURE WORK

#### 1. Concluding remarks

##### 1.1. Chapters II, III and IV

The research purpose of the first part of this thesis was to investigate the effect of the degree of acetylation (DA) on the ability of chitosan matrices to promote cell anchorage, proliferation, and osteogenic differentiation of osteoprecursor cells, *in vitro*, envisaging its application in cell-matrix constructs for bone tissue engineering therapies. For that purpose, chitosan samples with DAs in the range of 4 to 49% were initially prepared and characterized, to produce chitosan films and sponges.

Chitosan films were prepared by solvent casting and characterized using different surface analysis techniques, namely, atomic force microscopy imaging and roughness analysis, as well as contact angle and streaming potential measurements (**Chapters II and III**). In addition, the films were evaluated regarding their ability to adsorb fibronectin (Fn), chosen as a model of cell adhesive molecule, both from single protein solution and from diluted serum, using radiolabeled Fn ( $^{125}\text{I}$ -Fn) (Chapter III). Briefly, AFM studies revealed a tendency to an increase of surface roughness on increasing the DA, for films with DAs  $\leq 30\%$ , indicating the presence of an increased number of polymeric fibrillar aggregates. The sample with the highest DA (49%) showed a significantly higher surface roughness, when compared to the others, indicating the presence of a higher amount of insoluble polymer. In which concerns wettability studies, a decrease of the water contact angle with the decrease of DA was observed, for DAs  $\leq 30\%$ , which was mainly attributed to the increase of amine functionalities at the surface, provided by glucosamine residues. Streaming potential measurements showed the highest  $\zeta$ -potential for the sample with the lowest DA (4%), pointing out a higher surface concentration of amine groups on these films, as compared to the others. Films with higher DAs revealed significantly lower values of  $\zeta$ -potential, and a tendency to lower values of  $\zeta$ -potential on increasing the DA.

However, a linear dependence of the  $\zeta$ -potential with the DA was not observed, as films with DAs comprised between 13 and 30% revealed minimal differences in terms of  $\zeta$ -potential. Molecular reorientations might have occurred at the chitosan/air interface during the preparation of the chitosan films, leading to the reorientation of polar groups like amines toward the inner part of the film and, consequently, to a lower surface concentration of amine groups, even after hydration. Protein adsorption studies revealed poor Fn adsorption for samples with DAs in the range of 13 to 49%, and no significant differences among samples, regardless the DA. In contrast, for the lowest DA (4%), a significantly higher amount of adsorbed Fn was found when compared to any of the other chitosan samples, both from single Fn and serum solutions. In this study, the higher levels of adsorbed Fn found for a DA of 4% were attributed to a higher surface concentration of amine functionalities, in agreement to the highest  $\zeta$ -potential found for this sample. Furthermore, the levels of Fn adsorbed from serum found for this DA were 3-fold higher than for tissue-culture polystyrene (TCPS) used as control in the biological assays, indicating that in the presence of competitive proteins chitosan is more specific towards Fn adsorption than TCPS.

In a first study (**Chapter II**), human osteoblastic cells (MG-63 osteoblast-like cells) were used to study the DA effect on cell adhesion, spreading, and short-term proliferation of osteogenic cells on chitosan films. The results showed that for a short-term incubation period (3 h) cell attachment was similar among films with DAs in the range of 4 to 30%. This initial cell attachment was mainly attributed to non-specific interactions including electrostatic interactions occurring between protonated chitosan amine groups and negatively charged carboxylate and sulphate groups found in cell surface proteoglycans, as suggested by Chatelet et al.<sup>1</sup> The lower cell attachment observed on films with the lowest  $\zeta$ -potential (DA 49%), is in agreement with this assumption. After 24 hours of incubation, the percentage of cells remaining attached to the films clearly depended on the DA, decreasing from 60 to 5% of the control, as the DA increased from 4 to 49%, respectively. The examination of cell morphology at 24 h also suggests a higher cell attachment strength onto films with lower DAs, as cell spreading was only observed on samples with DAs  $\leq 13\%$ . With time, cell spreading and cytoskeleton filamentous organization were also only attained on films with DAs  $\leq 13\%$ , while on films with higher DAs, cells were found mainly clustered into spheroid-like cellular aggregates, displaying faint F-actin staining. Cell proliferation

was also found to be dependent on the DA, cells proliferating at a higher rate on films with the lowest DA (4%). However, cells on films with DAs in the range of 13 to 30% showed similar specific growth rates among samples, despite the different cell attachment rates revealed after 24 hours of incubation. On films with a DA of 49% cell proliferation was impaired, concomitantly to the lowest cell attachment rate found for these films, after 24 hours of cell culture. Cell adhesion, spreading, and proliferation on films with the DA of 4% were attributed to the ability of amine groups in chitosan glucosamine units to adsorb Fn from serum, and not merely to the higher cationicity and hydrophilicity of the films. MG-63 cells express several Fn receptors, among them the  $\alpha_5\beta_1$  integrin, a high affinity receptor for Fn.<sup>2</sup> According to the literature, Fn presents a more favorable conformation for  $\alpha_5\beta_1$  integrin binding and osteoblast adhesion upon adsorption on self-assembled monolayers (SAMs) exposing amine groups as compared to SAMs exposing methyl groups (which are present in chitosan acetylated units).<sup>3</sup> The cell spreading observed for a DA of 13% suggests that in spite of the lower  $\zeta$ -potential, cell attachment strength was enough to allow the synthesis of endogenous ECM molecules and consequently cell spreading. The ability of cells to proliferate within multi-cellular spheroids on films with DAs of 22 and 30%, despite the poor cell attachment, was related to the nature of the cells, since cells from osteosarcoma may show, as other transformed cells, an anchorage-independent growth.<sup>4</sup> Finally, the hamper of cell adhesion observed on films with the highest DA (49%) was attributed to the significantly higher surface roughness together with the higher hydrophobicity and lower  $\zeta$ -potential observed for this substrate. Taken together, the results showed not only a marked effect of the DA on cell adhesion at 24 h, lower DAs favoring cell adhesion, as reported for other anchorage dependent cells, but also an effect on cell morphology and cytoskeleton organization, as well as on cell growth kinetics. While cell spreading was only possible on films with DAs  $\leq 13\%$ , cell proliferation was favored on films with a lower DA.

In view of the previous findings, the DA effect on cell adhesion and osteogenic differentiation of osteoprecursor cells on chitosan films was investigated, using rat bone marrow stromal cells (rBMSCs) (**Chapter III**). Cell attachment, cytoskeleton organization, proliferation, and osteogenic differentiation over a 21 day period were assessed. At 24 h, the percentage of rBMSCs remaining attached was less than 19% of

the control, independently of the DA, although a tendency to higher cell attachment levels on decreasing the DA was found, following the trend observed with MG-63 cells. In time, rBMSCs cultured on chitosan films with a DA of 4% were able to spread, proliferate and differentiate, reaching a higher level of osteogenic differentiation than on the control, as shown by the increased levels of ALP activity and osteocalcin synthesis at days 14 and 21 of culture. For DAs  $\geq 13\%$  cell attachment strength was low, as shown by the spherical morphology displayed by the cells, and cell proliferation was impaired. As among chitosan samples, a DA of 4% showed a significantly higher Fn adsorption from serum, when compared to the others, it is suggested that cell adhesion, spreading and osteogenic differentiation of rBMSCs on chitosan may be mediated by the adsorbed layer of fibronectin.

These two studies revealed a similar tendency to increased cell adhesion on decreasing the DA. However, it is clear that cell response of bone-derived cells to chitosan films with different DAs is cell-type dependent, as shown by the different rates of cell adhesion found, as well by the cell morphology displayed on films with a DA of 13%. The poorer cell attachment on chitosan observed for stromal cells when compared to MG-63 cells, can be attributed to differences in terms of integrins profile. Although this osteosarcoma cell line is considered a model for the study of cell adhesion of osteoblasts, it is known that on this, the  $\alpha_4$  subunit of the  $\beta_1$  Fn receptor is overexpressed when compared to bone derived cells, while on BMSCs, the same is absent.<sup>2,5</sup>

In order to investigate the effect of the DA on the ability of chitosan porous scaffolds to support cell anchorage, cytoskeleton organization, activity, and proliferation of osteogenic cells, human osteoblastic cells (MG-63 osteoblasts-like cells) were used (**Chapter IV**). Porous chitosan matrices were initially prepared from 2% (w/v) chitosan acidic solutions via thermally induced phase separation and subsequent sublimation of the ice crystals, and further characterized in terms of microstructure, porosity, and pore size. Collagen sponges were equally prepared and used as 3-D positive control. SEM micrographs of the seeding surfaces revealed for DAs  $\leq 30\%$  a similar highly porous microstructure consisting of perpendicularly oriented and interconnected macropores, regardless the DA. For a DA of 49%, a more heterogeneous pore distribution together with the presence of fibers was found, which was attributed to the presence of an insoluble chitosan fraction. Observation of the

hydrated samples by widefield microscopy revealed average pore diameter values in the range of 61 to 87  $\mu\text{m}$ , with few pores outside the 47 to 102  $\mu\text{m}$  range, and no significant correlation was found between average pore diameter and the DA.

Results showed that in terms of cell proliferation, alkaline phosphatase activity and cell viability, cells cultured in chitosan sponges performed as well as in the 3-D control regardless the DA. Still, on sponges prepared from highly acetylated chitosan (49%) cell proliferation rate was poorer, as shown by the significantly lower cell number found from 72 h on, as compared to collagen sponges. In addition, a marked effect of the DA on cell morphology and cytoskeleton organization was found, the latter being progressively higher with the decrease of the DA, following the tendency previously observed on films. While on sponges with DAs  $\leq 13\%$  cells attached and spread similarly to collagen sponges, displaying long cell filopodia and numerous cell-to-cell contacts, infiltrating into the pores of the scaffolds, on sponges with higher DAs cells tended to remain spherical with no discernable actin filament formation, and grow into spheroid-like cellular aggregates, suggesting for these a lower cell attachment strength. The hamper of cell spreading and actin cytoskeleton organization observed for DAs higher than 13% was associated to a reduced adsorption of cell adhesion peptides on these substrates, since integrin binding induces the rearrangement of F-actin fibers leading to cell anchorage and spreading.<sup>6</sup> In this study, the results obtained were not correlated simplistically with the ability of the correspondent chitosan films to adsorb Fn from serum, since the surfaces involved are different. In the case of chitosan sponges, polar groups like amines are expected to remain at the surface after sublimation of the ice crystals, resulting in surfaces with an amine group concentration basically dependent on the DA used. Therefore, the increase of cell spreading on decreasing the DA was attributed to the presence of a higher amount of adsorbed adhesive proteins, such as Fn, at the surface, promoted by amine groups from chitosan glucosamine units. Finally, the poorer cell proliferation found for sponges with the highest DA (49%), can also be due to an increase of surface roughness, as previously observed for chitosan films, resultant from chain aggregation in chitosan acidic solutions. Taken together, the results highlight the importance of the DA in determining the affinity of osteoblasts-like cells towards porous chitosan scaffolds, possibly by influencing the nature of the initial adsorbed protein layer. Furthermore, these results emphasize the importance of the careful

selection of the scaffold in terms of DA, envisaging the culture of bone cells, *in vitro*, since differences in the DA as small as 9% were shown to be critical in terms of osteoblastic cells response to porous chitosan scaffolds. Moreover, it is suggested that chitosan sponges with a  $DA \leq 13\%$  may be useful as temporary supports for application in cell-matrix constructs for bone tissue engineering. However, a  $DA \leq 13\%$  may not be suitable for the culture of BMSCs. As was previously shown using films as substrates, the DA effect is cell-type dependent, and, according to the behavior of rBMSCs on films, lower DAs may be required. Therefore, this aspect should be further investigated.

The three studies performed revealed that lower DAs are preferable for the culture of bone cells *in vitro*, and that for osteogenic differentiation of rBMSCs on chitosan films, DAs lower than 13% are required. However, as both enzymatic degradation and chitosan biological aptitude to enhance tissue regeneration *in vivo*, are dependent on the DA,<sup>7,8</sup> the use of such low DAs may not always be desirable. As a consequence, an alternative strategy to promote bone cells adhesion and differentiation on chitosan was sought.

## **1.2. Chapters V, VI, and VII**

The research purpose of the second part of this thesis was to enhance the osteoconductive properties of chitosan matrices by inducing the deposition of an apatite-like layer. As previously mentioned, grafting of negatively charged groups such as phosphates is a well-known strategy to induce the deposition of apatite-like layers under simulated physiological conditions. In this study, the phosphorylation of chitosan membranes was attempted using the  $H_3PO_4/Et_3PO_4/P_2O_5$ /hexanol reaction route,<sup>9</sup> substituting hexanol by 2-butanol. Clear and transparent chitosan membranes ( $DA \cong 30\%$ ) prepared by solvent casting of ca. 50  $\mu m$  thick were phosphorylated for different time periods, up to 48 hours. Fully-acetylated chitosan membranes, in which amines are absent, were also phosphorylated for a period of 8 h. Alternatively, acetylated and original chitosan membranes which were phosphorylated for 8 h were both subjected to neutralization, in order to convert chitosan ammonium groups into uncharged amine groups, with consequent release of ionically bound phosphates. Surface characterization was performed using different surface analysis techniques

namely XPS, ATR-FTIR, SEM, AFM, and static contact angle measurements (**Chapters V and VII**). In addition, cross-sections of the modified membranes were also observed by scanning electron microscopy, fitted with an energy dispersive X-ray analysis system, in order to evaluate the extent of phosphorylation along the membrane thickness (**Chapter V**).

Briefly, the results showed an increase of the phosphate content with the reaction time, as shown by XPS and ATR-FTIR, a maximum of 4.7 at.% P being reached after 48 h of treatment. If expressed as a function of nitrogen, an atomic ratio of 0.73 P/N was obtained for this reaction period which can be correlated to the degree of substitution at the surface, given that one nitrogen atom is present in each chitosan monomeric unit. The NaOH neutralization step led to a decrease of surface phosphorus amount in the range of 50%, for the 8-h phosphorylated chitosan membranes, which was attributed to the release of phosphate ions, ionically bound to protonated amines. The remaining phosphorus probably corresponds to the covalently bound phosphates (chitosan phosphate esters), as phosphate monoesters of polysaccharides were shown to be remarkably stable to base-catalyzed hydrolysis. For the same phosphorylation period, acetylated membranes revealed a lower phosphorus amount, which was attributed to the decrease of hydroxyl groups available for phosphorylation, as a result of the increase of hydrogen bonding between adjacent chains. After the NaOH treatment, an almost complete retention of P amount was observed, as expected. SEM examination of phosphorylated membranes revealed a smooth and homogeneous film surface. AFM studies revealed a decrease of the surface roughness with reaction time at the nanometric scale, although the surface morphology of the membranes was not significantly altered. The smoothing of chitosan membranes during chemical modification was related to the acidic nature of the phosphorylation reaction medium. Wettability experiments clearly showed an increase of the polar component of surface free energy with reaction time and, as a consequence, of the total surface free energy, which was attributed to the increase in phosphate surface concentration. EDS analysis of cross-sections revealed a gradual P reduction up to 50% towards the inner part of the membrane, indicating that phosphorylation also occurred in the inner of the membrane, although to a lower extent.

Subsequently, the ability of the resulting phosphorylated membranes to nucleate calcium phosphates under simulated physiologic conditions, was investigated (**Chapter VI**). Phosphorylated chitosan (P-chitosan) membranes presenting a surface P at.% of about 2.87 as shown by XPS studies, were used. The membranes were immersed in  $\text{Ca}(\text{OH})_2$  or NaOH solutions, in order to obtain the Na or the Ca salts, respectively. SEM-EDS studies revealed the presence of a calcium phosphate mineral layer all over the surface of P-chitosan membranes, after incubation in  $\text{Ca}(\text{OH})_2$  solution. The release of ionically bound phosphate functionalities under alkaline conditions, possibly contributed to the formation of calcium phosphate precursor sites, due to the chelation of calcium ions from solution. The subsequent immersion in Simulated Body Fluid (SBF) led to the growth of a multilayered porous mineral structure composed of partially carbonated and poorly crystalline apatite, as shown by EDS, ATR-FTIR, and XRD analysis. In addition, Ca was also found in the inner of the membrane, as expected, since the inner of the membrane is also phosphorylated. Unmodified membranes and the sodium salt of P-chitosan membranes did not mineralise under the same conditions, suggesting that Na ions were not exchanged by Ca ions from SBF.

Finally, P-chitosan membranes were evaluated regarding cytotoxicity towards bone cells, by incubating human osteoblastic cells with extracts of 8-hrs phosphorylated chitosan membranes for two different time periods, namely 24 and 120 h (**Chapter VII**). Cytotoxicity studies revealed no cytotoxic effect of P-chitosan membranes towards osteoblastic cells, regardless of the incubation period, enabling their use in further biological assays, such as the study of osteogenic differentiation of osteoprecursor cells on both phosphorylated and mineralized membranes. In addition, the results revealed an inhibitory effect on cell proliferation in the presence of unmodified chitosan extracts. This effect was attributed to the possible depletion of nutrients from the culture medium, promoted by the presence of positively charged amine functionalities from glucosamine chitosan monomeric units.

## 2. Future work

The results reported in the first part of this thesis have put in evidence that differences in the DA as small as 9% may be critical in terms of osteoblastic cells response to

porous chitosan scaffolds. The study of the DA effect on the ability of chitosan matrices to support cell anchorage, proliferation, and osteogenic differentiation of osteoprecursor cells, *in vitro*, (BMSCs or mesenchymal stem cells) should be carried out, using DAs in a narrower range.

The DA effect on the cell response of other anchorage dependent cells currently cultured on chitosan matrices for regenerative approaches, such as endothelial cells and neuronal cells, should be further investigated.

In this thesis protein adsorption studies were limited to a single protein, fibronectin. However, in what concerns the adsorption of other adhesive proteins on chitosan, the influence of this parameter has not been reported yet. Therefore, protein adsorption studies involving other glycoproteins containing cell-binding domains such as laminin, vitronectin and osteopontin, both from pure solutions and from plasma, should be performed, using radiolabeled proteins. Furthermore, it would be interesting to investigate the conformation of these adhesive proteins upon adsorption, by using for instance monoclonal antibodies against their binding domains, together with fluorescent microscopy. As mentioned in the introduction, the conformation of the adsorbed glycoproteins is more important than the amount of the adsorbed protein at the surface, because substrate-induced changes in conformation usually lead to a decrease of protein functional activity and consequently, to lower cell adhesion. As coating with adhesive proteins is a well-known strategy to induce cell adhesion of a number of anchorage dependent cells, the finding of which adhesive proteins adsorb on chitosan films with retention of functionality is important.

In the second part of this thesis, 8-hrs phosphorylated chitosan membranes were shown to be not cytotoxic towards human osteoblastic cells, and to induce the deposition of an apatite-like layer, in SBF. In further studies, the ability of these mineralized P-membranes to support cell adhesion, cytoskeleton organization, proliferation and osteogenic differentiation should be investigated. As mentioned in the introduction, although calcium phosphates were shown to be effective carriers for stem cell transplantation, the effect of CaP mineral substrates on the proliferation and osteogenic differentiation of osteoprogenitor cells varies with both the structure and the composition of the particular CaP mineral.<sup>10-14</sup>

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