Thiol-norbornene Photoclick Chemistry for Grafting Antimicrobial Peptides onto Chitosan to Create Antibacterial Biomaterials

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ABSTRACT.

Covalent conjugation of antimicrobial peptides (AMP) is considered an effective approach to overcome their potential degradation *in vivo*. As the high cost of AMP-based therapies is one of the major hurdles towards their clinical application, it is urgently needed to explore high yield reactions. Herein, the highly efficient thiol-norbornene photoclick chemistry (TNPC) was explored for AMP grafting onto chitosan. Norbornenes were introduced onto chitosan (NorChit) in an aqueous/organic system, followed by UV-triggered conjugation of *N*- and *C-terminus* cysteine-modified Dhvar5 (NorChit-Dhvar5). Up to 0.38 norbornene groups per chitosan repeating unit and up to 43% conjugation yield in NorChit-*Nt*-Dhvar5 (80 μmol of Dhvar5/g) were achieved, while in NorChit-*Ct*-Dhvar5 conjugation yield was 30% (55 μmol of Dhvar5/g). Finally, NorChit-Dhvar5 ultrathin films showed up to a 35% reduction of total adhered Gram-positive *Staphylococcus epidermidis* and increased the adhesion and killing of Gram-negative *Pseudomonas aeruginosa* compared to unmodified chitosan. Moreover, NorChit-Dhvar5 was non-cytotoxic to human neonatal dermal fibroblasts, according to ISO 10993-1. Overall, our findings indicate TNPC as a high yield strategy for AMP grafting onto norbornene-functionalized biopolymers towards the fabrication of antibacterial biomaterials.

1. Introduction

Chitosan possesses several reactive functional groups (hydroxyls and amines) amenable to participate, directly or indirectly, in a wide range of [bio]functionalization, and/or crosslinking reactions[1,2]. Besides possessing mild antimicrobial activity, chitosan can also act as a sensitizer to potentiate the antimicrobial activity of other molecules, including antimicrobial peptides (AMP)[3,4]. The covalent conjugation of AMP onto chitosan may improve its bactericidal properties, allowing,

for instance, the fabrication of coatings to address medical devices-related infections[5,6] or to fight infection in biofilms established in highly proteolytic environments, such as skin chronic wounds, where covalent conjugation may protect AMP from enzymatic degradation[7].

Cationic peptidopolysaccharides using chitosan have been reported, showing excellent bactericidal and fungicidal activity[4]. Among them, previous approaches in our group studied the covalent conjugation of Dhvar5 (LLLFLLKKRKKRKY) onto chitosan. Dhvar5 is a synthetic peptide, derived from the histatins family, whose mechanism of action is still not fully elucidated, but studies using yeast cells suggested that, in solution, Dhvar5 acts at the membrane level by binding to it and promoting leakage of intracellular content through the formation of transient pores[8–10]. However, short cationic peptides (for instance, a stretched peptide with 12 amino acids and a glycine linker has 5.5 nm, approximately), such as Dhvar5, do not have sufficient length to insert across the bacterial membrane (20-50 nm). Thus, when conjugated onto polysaccharides, their most likely mechanism of action against bacteria may be through the formation of small perturbances on the bacteria surface, leading to electrostatic imbalances and triggering cell death[11]. Our previous approaches provided the opportunity to retrieve important information about the specificities of Dhvar5 conjugation onto chitosan, namely the influence of using spacers with different length and/or flexibility and the effect of altering the orientation of the exposed AMP terminus[10,12,13]. Nonetheless, the labile nature of disulfide bonds, or the use of a prohibitively large excess of peptide, augmenting the overall cost of the peptide grafting methods previously addressed, left room for improvement. Thus, we pursued an alternative chemoselective approach of higher efficiency for Dhvar5 tethering onto chitosan that could be promoted in absence of metal catalysts. In this connection, the thiol-norbornene “photoclick” chemistry (TNPC) was selected, in which thiol-containing moieties react with norbornene groups triggered by UV or visible light

in the presence of a suitable photoinitiator[14,15]. TNPC can achieve high yields with fast reaction rates, while not being hindered by ambient oxygen or water, leading to a single regioselective product. It occurs with the use of any non-sterically hindered terminal ene, but reaction rates are faster when using an electron-rich and/or strained ene, such as norbornene[16]. Thiol-norbornene reactions have been extensively studied for biomaterial functionalization in recent years. Although the most common polymers explored so far have been cellulose, pectin, gelatin and hyaluronic acid, a few recent reports have also used chitosan as starting biomaterial, but not for antimicrobial peptide conjugation, to the best of our knowledge[17–19]. Considering that reaction of chitosan with carbic anhydride (CA) may allow for the introduction of norbornene onto both hydroxyl and amine groups[14,18,20], there is an enhanced probability of obtaining a high degree of substitution (DS). Thus, herein, the hypothesis to be tested is that TNPC is an improvement over former approaches for AMP conjugation onto polysaccharides (chitosan, in this case), while providing enough norbornene groups to remain available for additional reactions.

2. Experimental Section

2.1. Synthesis of norbornene-functionalized chitosan (NorChit)

2.1.1. Chitosan purification and characterization

Commercial squid pen chitosan (France Chitine; Degree of acetylation (DA) = 2% and molecular weight (MW) = 355 ± 22 kDa) was purified by the reprecipitation method[2]. Briefly, a chitosan solution was filtered through a 20 μm filter and precipitated using 1 M NaOH. Then, the regenerated chitosan was washed with type I water (ultrapure water with a resistivity > 18 MΩ.cm, conductivity of < 0.056 μS/cm and < 50 ppb of total organic carbon) until pH = 7, freeze-dried

and grounded to obtain a powder. Chitosan powder was dried in a vacuum oven (Raypa) for 24 h at 60 ºC, before further use.

DA was calculated using ^{1}H NMR, according to section 2.3.2 and using **Equation S1 (Figure S1A)**, as described elsewhere[18,21,22], based on comparing the integral of the 3 protons of the acetyl group, with that of the H-2 proton from $GlcNH_2$ and $GLcNHCOCH_3$ units. Further characterization was performed by Fourier Transform InfraRed (FTIR) spectroscopy **(Figure S1B)**, according to section 2.3.1. Chitosan MW was determined in a Lovis 2000 M/ME viscometer (Anton Paar). Briefly, the intrinsic viscosity of a range of dilute chitosan solutions (0.3 mg mL^{-1} to 0.7 mg mL^{-1}; buffer: 0.3 M acetic acid (AppliChem) / 0.2 M sodium acetate (Merck)) was measured by recording the rolling time of a sphere in a capillary filled with sample solution. Then, the MW was obtained by applying the Mark-Houwink equation (constants: a = 0.76, $K \times 10^5$ (dL g^{-1}) = 82.0)[23].

2.1.2. Establishment of an optimal co-solvent (aqueous/organic) system for chitosan modification

Organic solvents tested included acetonitrile (ACN, Carlo Erba Reagents) and *N*,*N*-dimethylformamide (DMF, Merck). For the chosen 40:60 (aqueous:organic) ratio, chitosan (30 mg) was hydrated in type I water (2957 µL) for 8 h, then solubilized upon addition of glacial acetic acid (17 µL) and magnetic stirring overnight at room temperature (RT). Further glacial acetic acid (26 µL) was added to set the final acetic acid concentration to 0.1 M. After 10 min, the organic solvent (4500 µL) was added dropwise, under strong magnetic stirring. Chitosan was then left to solubilize in the co-solvent system for 30 min.

2.1.3. Reaction of chitosan with carbic anhydride

This reaction was based on a previous report[14]. Firstly, chitosan was solubilized in DMF as explained in 2.1.2. Then, optimizations regarding the molar excess of carbic anhydride (CA; Acros Organics) and reaction time were performed **(Table S1)**. For the selected protocol, a freshly prepared solution of CA in DMF (0.15 M) was added (411 µL) every 30 min, three times. The reaction proceeded under strong magnetic stirring and protected from light, for 3 h at RT. The mixture was then dialyzed (Thermo Scientific SnakeSkin 10 K MWCO Dialysis Tubing) against decreasing concentrations of NaCl (VWR chemicals; 30 g, 25 g, 20 g (twice), 15 g, 10 g (twice) in 4 L of type II water) for 2.5 days, and against type II water (resistivity of >1 MΩ.cm, a conductivity of <1 μS/cm and <50 ppb of total organic carbon) for 1.5 days. Dialysis buffer was changed three times every day and kept in a bath at a constant temperature of 40 ºC (using a thermocouple, VWR) during the first 48 hours, to improve carbic anhydride solubility. The final solution was subsequently frozen, lyophilized and stored at -20 ºC, under inert atmosphere (nitrogen). Degree of substitution (DS) of NorChit (0.38 norbornene groups per chitosan repeating unit) was determined as described in **Figure 1** and **Equation (1)**, by comparing the integral of the peaks of norbornene vinyl protons (2-H, i) to that of the peak from the H-2 proton (f) of chitosan repeating units.

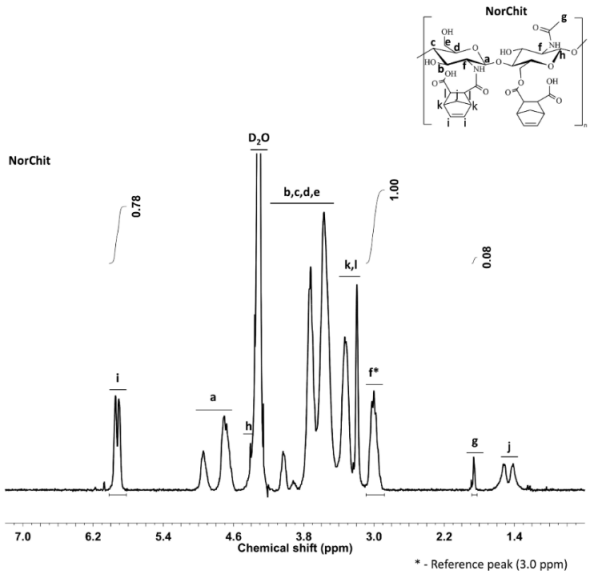


Figure 1. Determination of NorChit degree of substitution (DS) by ^{1}H NMR (400 MHz, 0.5 M DCl in D2O, 70 ºC), applying Equation 1 (for NorChit 1-fold, 3h). For integration, peak (f) from the H-2 proton of chitosan was used as reference.

(Equation 1) $$\text{Substitution degree} = \frac{\int \text{HC} = \text{CH from CA (i)}}{\int \text{H} - 2 \text{ from Chit (f)}} \times \frac{\mathbf{1}}{\mathbf{2}}$$

2.2. Peptide conjugation to NorChit via TNPC

2.2.1. Peptide synthesis

Two modified derivatives of the AMP Dhvar5 (LLLFLLKKRKKRKY, *C*-terminal amide) were produced, with a cysteine (Cys) residue at either the *N*- or *C*- *terminus*, through a flexible 6-aminohexanoic acid (Ahx) spacer to the original AMP sequence (Cys-Ahx-Dhvar5 and Dhvar5-Ahx-Cys, respectively; MW = 2062 Da)[10]. Peptides were synthesized, analyzed and purified as previously reported[12]. Final peptides used had a purity of, at least, 95%, as determined by high-performance liquid chromatography (HPLC), and their expected MW was confirmed by electrospray ionization/ion trap mass spectrometry (ESI-IT MS) **(Figure S2 and S3)**. Lyophilized peptides were solubilized in sterile filtered 0.1 M acetic acid, aliquoted, freeze-dried and stored under nitrogen at -20 ºC until further use. The quantity of peptide per aliquot was accurately determined by measuring the absorbance of 2 μL of peptide 0.1 M acetic acid solution (n=3) in NanoDrop One (Thermo Scientific) at 205 nm wavelength using an extinction coefficient of 31 for 1 mg mL^{-1} peptide solutions

2.2.2. Phototriggered conjugation

Peptide conjugation reaction parameters (namely, UV exposure time) were firstly optimized using *N*-acetyl-L-cysteine (Merck) as a model. NorChit (10 mg) was hydrated in type I water (1985 µL) for 8 h, then solubilized upon addition of 15 µL of glacial acetic acid and magnetic stirring overnight at RT. Then, Cys-Ahx-Dhvar5 or Dhvar5-Ahx-Cys (3.8 mg) was added (final concentration: 1.2 mg mL^{-1}). Reaction was performed in the presence of 0.4 wt% VA-086, under UV light (365 nm, RoHS, Hamamatsu) at 10 mW cm^{-2} for 5 min, under magnetic stirring. The mixture was immediately dialyzed (Thermo Scientific SnakeSkin 10 K MWCO Dialysis Tubing) against HCl 0.1% v/v (VWR chemicals) for 2 days and against type II water for 1 day. The final solution was subsequently frozen, lyophilized and stored at -20 °C, saturated under nitrogen. For the adsorbed Dhvar5, the same protocol was performed, but in the absence of the photoinitiator and without UV exposure.

2.3. Polymer characterization

Chitosan DA and purification were confirmed by proton Nuclear Magnetic Resonance (^{1}H NMR) and Fourier Transform Infrared spectroscopy (FTIR). The potential impact of the co-solvent system on chitosan structure and the extent of norbornene conjugation onto chitosan (NorChit) were assessed by FTIR, ^{1}H NMR and X-ray Photoelectron Spectroscopy (XPS). Peptide conjugation via TNPC between the AMP and NorChit (NorChitAMP) was confirmed by Attenuated Total Reflectance Fourier Transform InfraRed spectroscopy (ATR-FTIR), ^{1}H NMR, XPS and Amino Acid Analysis (AAA).

2.3.1. Fourier Transform InfraRed (FTIR) spectroscopy

FTIR spectra were acquired using transmission mode (Slide Holder coupled to a Perkin Elmer Frontier). Spectra resulted of the average of 32 consecutive interferograms at 4 cm^{-1} resolution, between 4000 and 400 cm^{-1}. ATR-FTIR spectra were acquired (Universal ATR module couple to a Perkin Elmer Frontier) with an average of 100 consecutive interferograms at 4 cm^{-1} resolution, between 4000 and 400 cm^{-1}. Spectra were fitted using the Spectrum (version 10.5.2.636) software.

2.3.2. Nuclear Magnetic Resonance (NMR)

Samples (chitosan and chitosan derivatives: 10 mg mL^{-1}; AMP: 2 mg mL^{-1}) were dissolved in 0.5 M deuterium chloride solution (DCl, Sigma-Aldrich) in deuterium oxide (D_2O, Sigma-Aldrich). Chemical shifts are reported in ppm (δ units) and are referenced to the residual HOD signal from D_2O (δ = 4.31 ppm, 70 ºC)[24]. ^1H NMR spectra were recorded on a 400 MHz Bruker AVANCE III spectrometer (Centro de Materiais da Universidade do Porto - CEMUP). ^1H NMR spectra were recorded at 70 ºC, to increase the solubility of samples and minimize solvent interference[21,25].

2.3.3. X-ray Photoelectron Spectroscopy (XPS)

Samples were prepared as thin pellets in a hydraulic press. Measurements were carried out on a Kratos Axis Ultra HSA spectrometer at CEMUP, as previously described[12]. Spectra were fitted using the CasaXPS (version 2.3.17PR 1.1) software and peak intensities of high-resolution spectra (*C1s*, *N1s*, *O1s* and *S2p*) were normalized using *C1s* peaks as reference.

2.3.4. Amino Acid Analysis (AAA)

Dhvar5 content in the final NorChitAMP conjugates was determined by AAA, as previously reported[12]. Phenol was added to the hydrolysis reaction mixture to avoid degradation of the tyrosine side chain, which would lead to an incorrect quantification of this amino acid[26].

2.4. Evaluation of antibacterial effect and cytotoxicity

2.4.1. UV light exposure effect on Dhvar5 antimicrobial activity

Antimicrobial activity of Dhvar5, Dhvar5-Ahx-Cys and Cys-Ahx-Dhvar5 before and after UV treatment (10 mW cm^{-2} for 5 min), was assessed in solution, against *Staphylococcus epidermidis* (*S. epidermidis*, ATCC 35984), by an adaptation of the microtiter broth dilution method[27]. Briefly, peptide dilutions were prepared in acetic acid (0.01% v/v) with bovine serum albumin (BSA) solution (0.2 wt%) in a range concentration from 0.5 μg mL^{-1} to 256 μg mL^{-1}. Bacteria inoculum (100 μL, 2×10^5 CFU mL^{-1}) in Mueller-Hinton Broth (MHB) (Sigma-Aldrich) was added to a rounded-end shape microtiter 96-well plate (Costar). Dhvar5 and related derivatives at each concentration were added (11 μL) to the respective well and incubated for 18-24 h at 37 ºC. Uninoculated MHB was added to the last column as a control of sterility. The Minimum Inhibitory Concentration (MIC) corresponded to the first well where no visible bacteria growth was observed. These wells were serially diluted and plated in Tryptic Soy Agar (TSA, Merck) and incubated overnight, to determine the Minimum Bactericidal Concentration (MBC). Two replicates for each concentration were used. Two independent assays were performed.

2.4.2. Preparation of chitosan, NorChit and Dhvar5-NorChit thin films

Gold (Au) square substrates (1 cm^2) and chitosan ultrathin films were prepared as previously described[13]. Briefly, a chitosan solution (0.4 wt% in 0.1 M acetic acid) was spin-coated at 9000 rpm for 1 min (Laurell Technologies Corporation) onto the Au substrate. This step was then repeated to create double-layered chitosan ultrathin films. NorChit and NorChit-Dhvar5 (0.4 wt% in 0.1 M acetic acid) solutions were deposited on Au substrates previously coated with an ultrathin film of unmodified chitosan. After spin-coating, the double-layered ultrathin films were neutralized with 0.1 M NaOH for 5 min and then rinsed twice with type I water. Samples were dried individually with a gentle stream of argon and stored in a desiccator, saturated under argon. For cytotoxicity evaluation, this process was performed using tissue culture (TCPE) coverslips as substrates.

2.4.3. Surface antimicrobial activity evaluation

Samples were disinfected twice in ethanol 70% (30 min) and washed twice in sterile-filtered type II water, and then dried with a gentle stream of Argon in sterile conditions. A suspension of *S. epidermidis* or *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853) in phosphate buffered saline (PBS 1x; 5 µL, 10^8 CFU mL^{-1}) was deposited onto each sample and then covered with a sterile glass coverslip (13 mm diameter) to promote contact between bacteria and surface. After incubation at 37 ºC for 5 h, coverslips were gently removed and samples were rinsed twice with sterile-filtered aq. NaCl (0.9 wt%, 500 μL). Then, samples were prepared either for Live/Dead assay or CFU counting after sonication, as described below.

Live/dead assay

Live/dead assay was performed as before[13]. A minimum of nine fields on each of triplicate replicates were acquired in an Inverted Fluorescence Microscope with a 630× magnification (oil objective), which is equivalent to a net area of 0.3429 mm^2 per sample. Three independent assays were performed, unless stated otherwise. The acquired images were segmented and bacteria were outlined using a machine-learning software, Ilastik. Then, bacteria were automatically counted on an imaging processing software, CellProfiler. Green bacteria were considered viable, whereas red bacteria were considered dead. Double-stained bacteria were classified as yellow, unless the signal intensity from propidium iodide (PI) or from Syto9 was too dim in comparison to the other, which was attributed to signal cross-over (criteria: Syto9 intensity above average and PI intensity below average – live; Syto9 intensity above average and PI intensity below average – dead).

CFU counting after sonication

Bacteria were recovered from the surface of the samples and plated in agar for CFU counting, as reported by Monteiro et al.[28]. Briefly, samples were transferred to 5 mL tubes (Sarstedt) with 1 mL of 0.5% Tween 80 in PBS, placed on ice, and then sonicated for 15 min at 160 W (BactoSonic®, Bandelin), placed on ice for 5 min, sonicated again for 15 min and put on ice. Then, serial dilutions were plated on agar for CFU counting. Three independent assays were performed.

Statistical analysis was performed in GraphPad Prism (version 9.1.2). When gaussian distribution was confirmed, statistical analysis was performed using one-way ANOVA with Holm-

Sidak's multiple comparison test. When data sets did not follow a gaussian distribution, Kruskal-Wallis non-parametric test with Dunn's multiple comparison test was performed. Data is presented as mean ± standard deviation and p values below 0.05 were considered statistically significant.

2.4.4. Evaluation of cytotoxicity

Human neonatal dermal fibroblasts (hNDFs) isolated from human neonatal foreskin samples (Coriell Institute for Medical Research, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Sigma-Aldrich) and amphotericin B (2.5 mg/L, Sigma-Aldrich) and kept at 37 ºC, 5% CO_2. Cells (passage 10) were trypsinized before reaching confluence using 0.05 wt% trypsin/ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) and were plated on top of chitosan, NorChit and NorChit-Dhvar5 films on TCPE coverslips, at a density of 1.0 x 10^5 cells, in 24-well suspension plates (Greiner Bio-one). Bare TCPE coverslips were used as positive control. After 24h of incubation, metabolic activity of hNDFs was assessed by the resazurin assay. Samples were incubated in 20 v/v% resazurin (Sigma-Aldrich) solution in DMEM medium for 2 h at 37 ºC. After that, three 100 μl/well of metabolized resazurin solution were transferred to a 96-well black plate and fluorescence was measured using a microplate reader (Synergy MX, Biotek) at wavelength 530 nm/590 nm (excitation/emission). Finally, cells on each sample were trypsinized and counted in a Neubauer chamber to normalize metabolic activity by the number of adherent cells.

3. Results and discussion

A two-step approach was designed to produce AMP-functionalized chitosan: (A) reaction of chitosan with carbic anhydride in a co-solvent (aqueous/organic) system, to produce norbornene-chitosan (NorChit); (B) phototriggered thiol-ene conjugation of the cysteine-bearing peptide (Cys-Ahx-Dhvar5 or Dhvar5-Ahx-Cys) with NorChit (NorChit-Dhvar5). Schematic representation of both needed reactions to obtain AMP-functionalized chitosan (NorChit-Dhvar5) is presented in **Figure 2**.

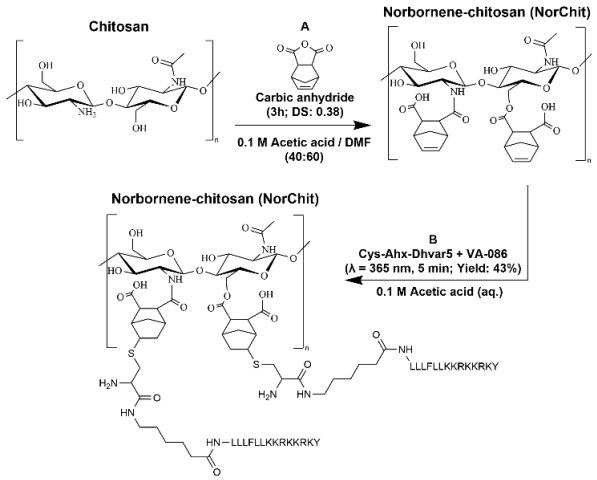


Figure 2. Schematic representation of the two-step approach to produce the AMP-functionalized chitosan (NorChit-Dhvar5). The first reaction (A) involves conjugation of norbornene groups into chitosan upon reaction with carbic anhydride, while in the second step (B) UV light triggers the chemoselective peptide (Cys-Ahx-Dhvar5)-chitosan conjugation in the presence of the photoinitiator VA-086, thus yielding the target polymer (NorChit-Dhvar5).

3.1. Synthesis of norbornene-functionalized chitosan (NorChit)

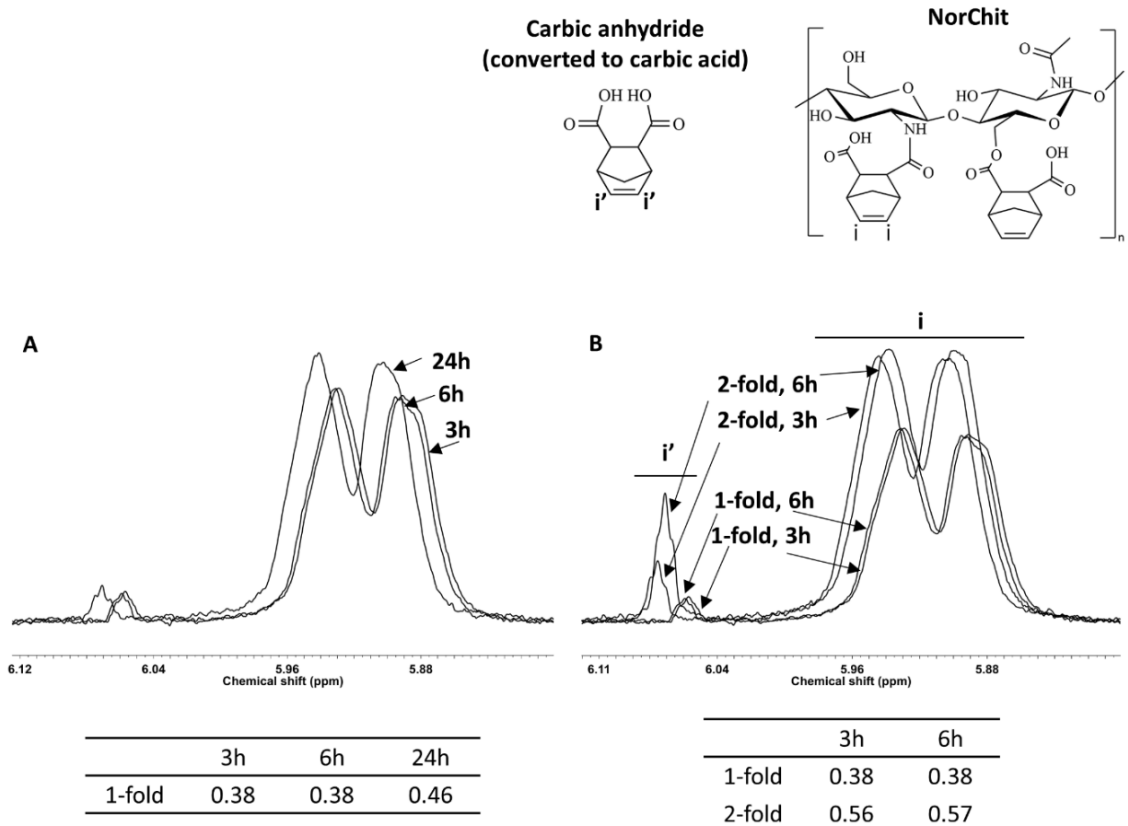
3.1.1. Establishment of a co-solvent system

The norbornene moiety was introduced into chitosan via reaction with CA. At the concentration used, CA exhibits limited solubility in the acidic aqueous solvents required to solubilize chitosan[18,20]. In other previous reports, some strategies to address solubility issues included; i) using heterogenous systems, where the polysaccharide is suspended on an organic solvent, and/or reacted with the norbornene-introducing reagent at high temperatures (at least 50 ºC)[18,29] or by ii) the modification of the polysaccharide (hyaluronic acid, pectin) to increase its solubility in organic solvents prior to modification with norbornene[14,30]. Here, to overcome this solubility problem, and considering that chitosan has poor solubility in organic media or alkaline aqueous solvents, a co-solvent system was created. Chitosan solubilization was tested by creating co-solutions of 0.1 M aq. acetic acid and a water-miscible organic solvent (ACN or DMF), without the extra need of chitosan modification, neither heating. The selection of the best co-solvent system was based on having a solution with low viscosity, hence the volume ratio of 40% 0.1 M aq. acetic acid to 60% DMF was chosen. No significant impact of the co-solvent system on chitosan structure and composition was observed **(Figure S4 and Table S2)**.

3.1.2. Optimization of reaction conditions on norbornene-functionalized chitosan (NorChit)

Functionalization of chitosan with norbornene (NorChit) was optimized regarding reaction time (3, 6 and 24 h) and molar excess of CA (1- or 2-fold) **(Table S1)**. Firstly, the aim was to minimize reaction time, while obtaining NorChit with a DS near 0.38, which was reported elsewhere as an appropriate DS for efficient thiol-ene reaction[18]. Indeed, reaction times of 3h and 6h yielded NorChit with a DS of 0.38, whereas a reaction time of 24 h yielded a DS of 0.46 **(Figure 3)**.

Nonetheless, the latter was discarded as the greater length of the protocol translated to only a slight increase in DS. Optimization proceeded with short reaction times (3h and 6h), and the effect of increasing CA molar excess was evaluated. Using 2-fold molar excess of CA led to higher DS (0.56 - 0.57), but there was also a steep increase in unreacted carbic anhydride (converted to carbic acid; peak i' (vinyl protons from carbic acid) in the NMR spectrum, Figure 3B). Using 1-fold molar excess, DS was lower (0.38), but it still provided many functional sites for peptide conjugation, with minimal quantity of unreacted CA. Therefore, as no differences were found between 3 h and 6 h of reaction time, the optimized protocol for functionalization of chitosan with norbornene (NorChit) includes the reaction of chitosan with CA at a 1:1 molar ratio during 3 h. Importantly, reaction was performed at RT and without prior chemical modifications on chitosan, making the protocol straightforward and simple to implement. DS was 0.38 norbornene groups per chitosan repeating unit, corresponding to an estimated 1725 μmol of norbornene per g of NorChit. DS was higher than the described by other authors (below 0.20)[14,29,30]. Moreover, similar DS (0.38) was reported using chitosan with a lower MW (50-190 kDa, instead of 355 ± 22 kDa used here) and higher DA (24%, instead of 2% used here)[18]. However, to achieve this DS, authors used considerably longer reaction time (48 h vs 3 h used herein) and higher temperature (50 ºC vs RT).



	3h	6h	24h
1-fold	0.38	0.38	0.46

	3h	6h
1-fold	0.38	0.38
2-fold	0.56	0.57

Figure 3. Influence of reaction time in the degree of substitution of NorChit (A) and of increasing CA:Chitosan molar ratio from 1-fold to 2-fold (B). ^{1}H NMR spectra recorded at 400 MHz, 0.5 M DCl in D_2O, 70 ºC. Peaks labelled with i and i' refer to those from vinyl protons of norbornene in NorChit and of unbound carbic anhydride, respectively.

The resulting polymer was evaluated by FTIR as depicted in **Figure 4A**. Chitosan characteristic bands were observed at: 1620 cm^{-1} (amide I, *C=O* stretching from secondary amides), 1521 cm^{-1} (amide II, *N-H* deformation combined with C-N stretching from secondary amides and also *N-H* bending from primary amines), 1386 cm^{-1} (*CH_3* deformation), 1075 cm^{-1} (*C-O-C* stretching vibration)[2]. New bands appeared in FTIR spectra at 1765 and 1693 cm^{-1}, which can be assigned to

new carbonyl (C=O) (from CA) and amide (N-C=O) (from CA grafted through the free amines of chitosan) vibrational modes, respectively[29], suggesting the successful introduction of norbornene groups into chitosan. In ^{1}H NMR spectra **(Figure 4B)**, new peaks at 1.3-1.6 (j), 3.1-3.2 (k,l) and 5.8-6.0 (i) ppm are observed, the latter assigned to the vinyl protons of norbornene (HC=CH)[14,15,18].

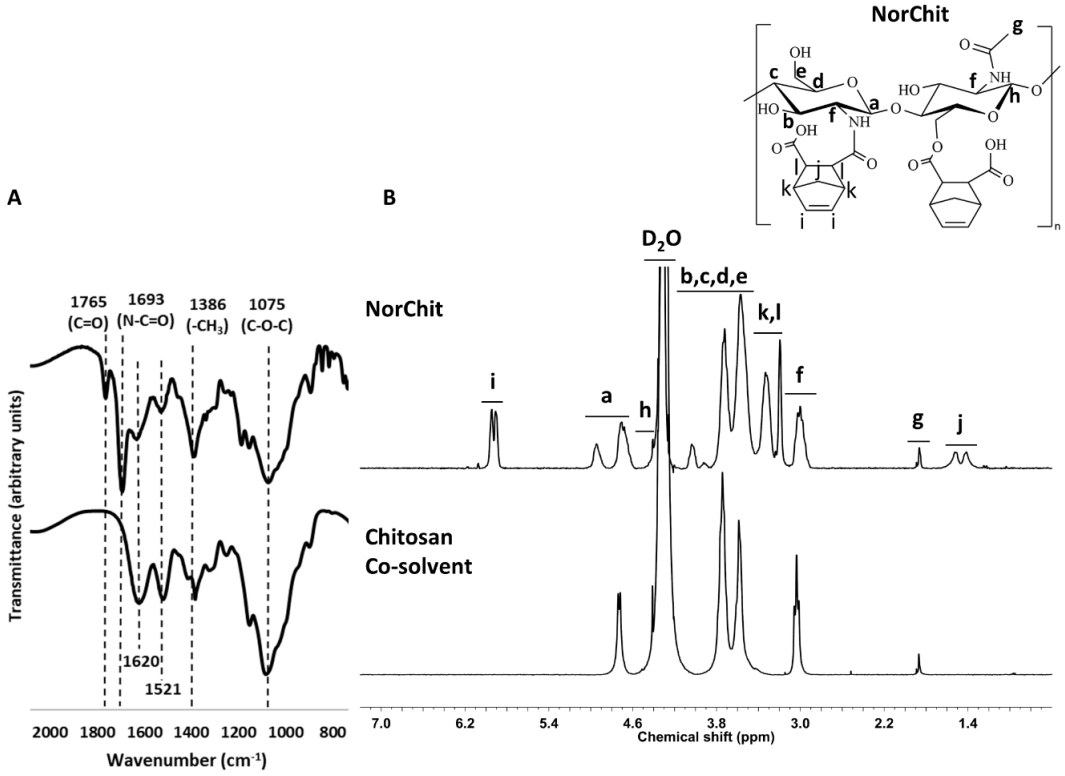


Figure 4. NorChit obtained with chosen reaction conditions (1-fold, 3h) characterized by FTIR (A) and by ^{1}H NMR (B) (400 MHz, 0.5 M DCl in D_2O, 70 ºC).

3.2. Chemoselective grafting of Dhvar5 onto NorChit via TNPC

3.2.1. Grafting of Cys-modified Dhvar5 onto chitosan via TNPC (synthesis of NorChit-Dhvar5)

Grafting of Cys-modified Dhvar5 (3.8 mg) onto NorChit (10 mg), which corresponds to approximately 1:10 molar ratio of Dhvar5 to norbornene groups, was pursued using the conditions firstly optimized with *N*-acetyl-*L*-cysteine (NAC) as model for the AMP of interest: VA-086 at 0.4 wt% and UV exposure for 5 min at 10 mW cm^{-2} intensity **(Figure S5)**. The 1:10 Dhvar5 to norbornene molar ratio was chosen to maintain free norbornene groups for subsequent reactions and also to minimize the quantity of AMP used relatively to our previous approaches for Dhvar5 conjugation onto chitosan[12]. ^{1}H NMR spectra of the resulting NorChit-Dhvar5 conjugates **(Figure 5)** showed the appearance of new peaks at δH 0.6 ppm, attributed to methyl protons ($-CH_3$) from the side chain of leucine residues, and 6.5-7.3 ppm, assigned to the aromatic protons (Ar-H) of phenylalanine and tyrosine side chains[31]. These peaks could also be observed in the spectrum of the peptide alone (Cys-Ahx-Dhvar5 and Dhva5-Ahx-Cys), confirming their assignment.

Of note, when the reaction was performed without promoting phototriggered AMP conjugation (in the absence of photoinitiator and UV exposure; Adsorbed Dhvar5), peptide characteristic peaks were barely observed, showing that unbound peptide is efficiently removed by dialysis against acidified water, while covalently conjugated peptide remains present in NorChit-Dhvar5 (Figure 5). Importantly, acidification of the dialysis buffer was required to ensure the removal of unreacted peptide, as the solubility of Cys-Ahx-Dhvar5 and chitosan (due to its high MW) are increased under acidic conditions. These data strongly suggest that TNPC efficiently promotes covalent grafting, and not mere adsorption of the peptide onto NorChit.

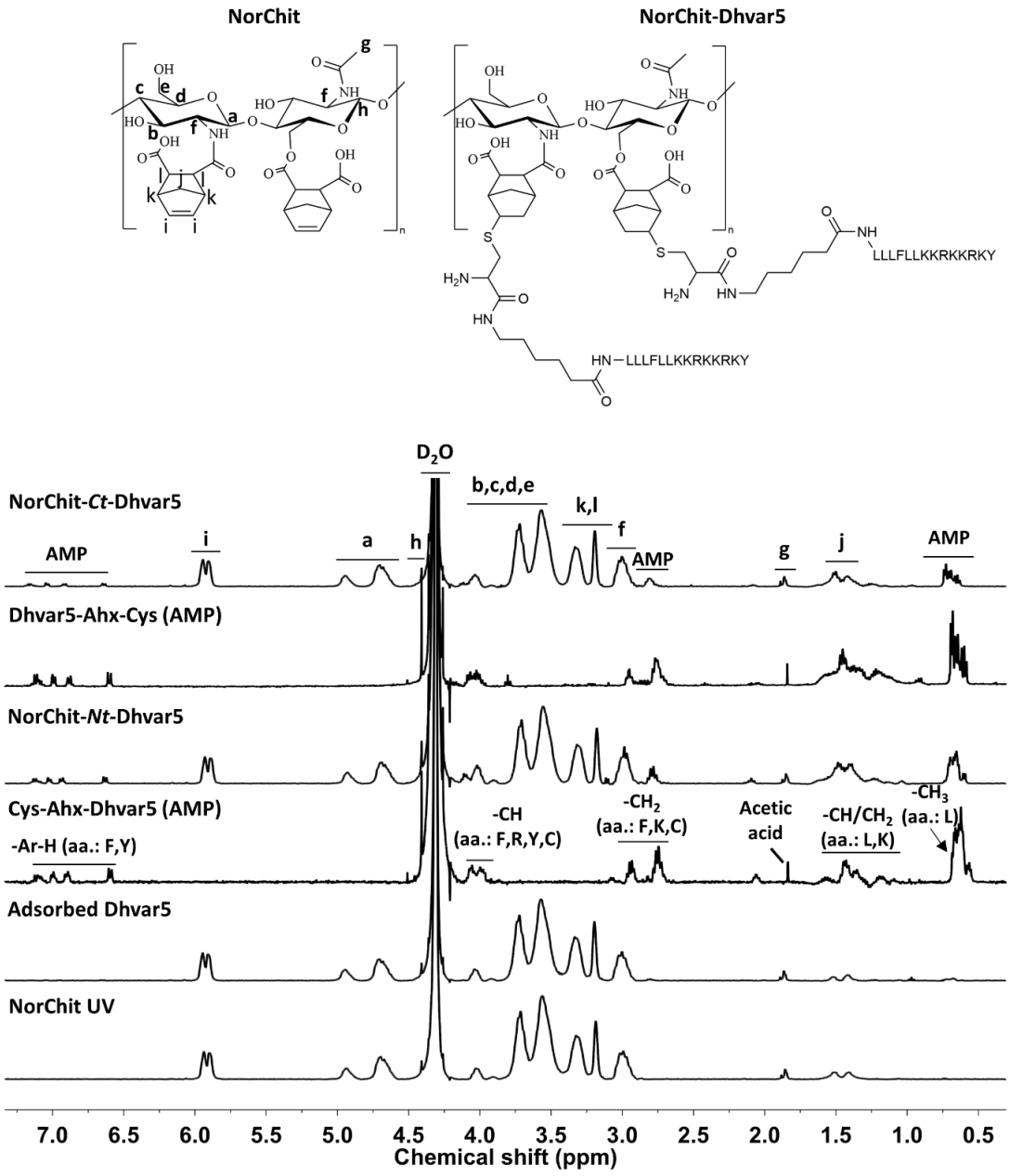


Figure 5. Conjugation of Dhvar5 onto NorChit by the *C*- or *N*- termini monitored by ^{1}H NMR (400 MHz, 0.5 M DCl in D_2O, 70 ºC) shows the appearance of characteristic Dhvar5 peaks. When

conditions for covalent conjugation were not present (absence of photoinitiator and UV exposure) adsorbed Dhvar5 peaks present are negligible after acidic dialysis.

As the ratio of AMP:norbornene was 1:10, only up to 10% of norbornene groups would react (in ideal conditions), therefore, it could be difficult to confirm successful Dhvar5 conjugation through the reduction in the norbornene vinyl protons peak in the ^{1}H NMR spectrum. Thus, Dhvar5 tethering onto NorChit was confirmed by XPS **(Table 1)**. NorChit atomic percentages reveal, as expected, an increase in the relative amount of carbon and a decrease in the relative amount of nitrogen and oxygen, compared to chitosan. The high increase of *N1s* relative percentage in NorChit-Dhvar5, as compared to the parent polymer and the controls (including Adsorbed Dhvar5), agrees with insertion of peptide bonds and nitrogen-bearing side chains from lysine and arginine residues. Also, the *O1s* content decreased in the conjugate relative to the parent polymer, which is in accordance with the expected theoretical composition for the target NorChit-Dhvar5 polymer. In addition, the increase in *S2p* in NorChit-Dhvar5, assigned to peptide cysteine thiol, clearly demonstrated the presence of Dhvar5. Moreover, the relative percentage of sulfur on NorChit-Dhvar5 was close to the theoretical value (0.2%).

Figure 6 shows that, in most samples, a *S2p* peak appears at 168 eV, which can be assigned to sulfur in an oxidized state[32,33] that can arise from surface contaminants[12] and/or from thiol oxidation in adsorbed Dhvar5, which may occur during the light-triggered TNPC performed under ambient conditions. The success of peptide tethering via TNPC was suggested by the major *S2p* peak at 164 eV assigned to the thioether (C-S-C) bond, in NorChit-Dhvar5 conjugates, obtained after reaction between the peptide cysteine thiol and the double bond in the NorChit norbornene moiety. Comparing the *S2p* peak intensity at 164 eV and also the relative quantities of *N1s* and *S2p*, in Table 1, it is clear that the quantity of Dhvar5 in NorChit-*Nt*-Dhvar5 is higher than in NorChit-*Ct*-

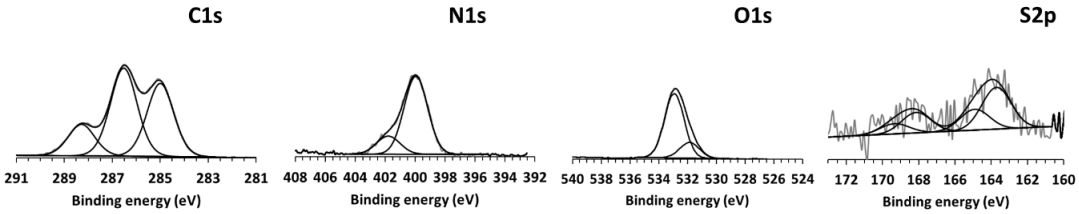
Dhvar5. Additionally, when comparing NorChit-Dhvar5 to NorChit and Adsorbed Dhvar5, the increase of *C1s* at 285.0 eV (C-C/C-H bonds, most likely from the peptide structure)[2] relatively to *C1s* at 286.7 and 288.5, suggests the presence of higher amounts of Dhvar5.

Table 1. Atomic percentages obtained by XPS of chitosan co-solvent, NorChit, NorChit UV, Adsorbed Dhvar5 and NorChit-Dhvar5. Data are presented as experimental [theoretical] values.

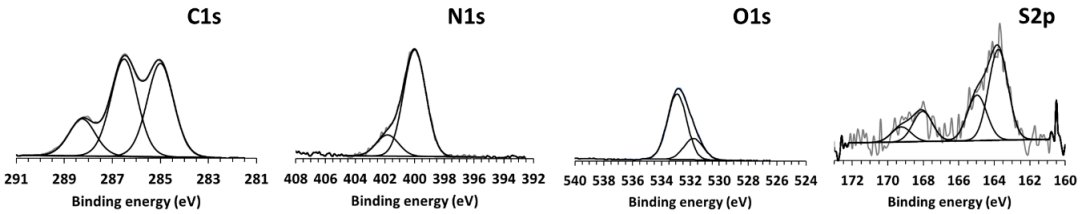
	C1s	N1s	O1s	S2p		
				Total	164 eV	168 eV
Chitosan co-solvent	62.5 [54.5]	7.6 [9.1]	30.2 [36.4]	N.D.		
NorChit	68.2 [60.7]	6.1 [6.3]	25.6 [33.0]	0.12	N.D.	0.12
NorChitUV	67.8	6.0	26.1	0.07	N.D.	0.07
Adsorbed Dhvar5	69.0	5.8	25.1	0.06	N.D.	0.06
NorChit-*Nt*-Dhvar5	69.6 [62.8]	9.1 [9.8]	21.1 [27.2]	0.17 [0.20]	0.13	0.04
NorChit-*Ct*-Dhvar5	68.5 [62.8]	8.1 [9.8]	23.3 [27.2]	0.14 [0.20]	0.09	0.05

N.D. – not detected

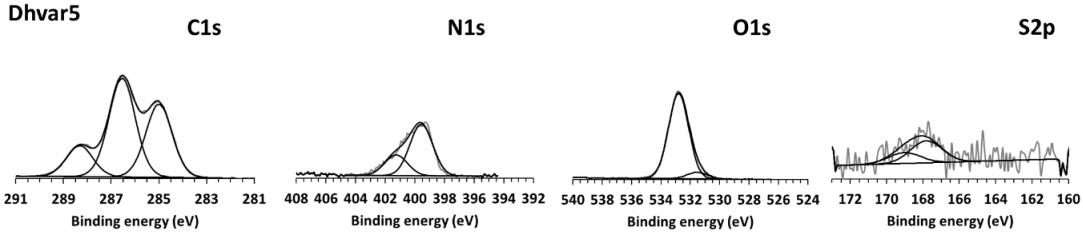
v) NorChit-*Ct*-Dhvar5



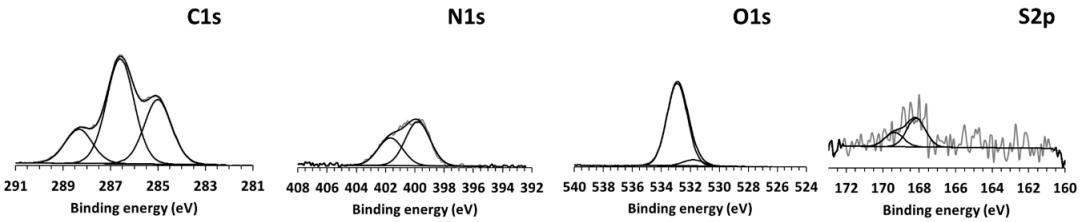
iv) NorChit-*Nt*-Dhvar5



iii) Adsorbed Dhvar5



ii) NorChit UV



i) NorChit

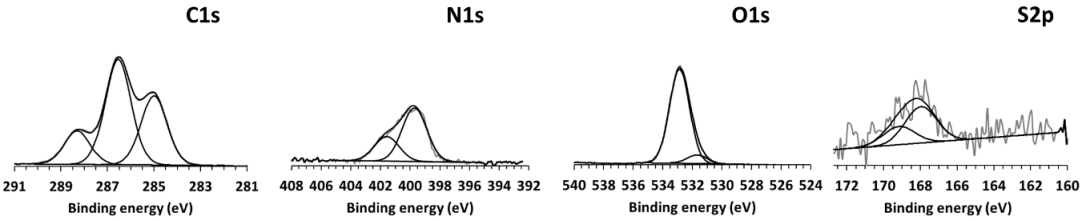


Figure 6. High resolution XPS spectra of i) NorChit; ii) NorChit exposed to UV light in the absence of Cys-modified Dhvar5, but in the presence of the photoinitiator (NorChit UV); iii) NorChit in the absence of the photoinitiator and UV exposure, but in the presence of Cys-modified Dhvar5 (Adsorbed Dhvar5); iv) NorChit in the presence of both photoinitiator and *N*-terminus Cys-modified Dhvar5 (NorChit-*Nt*-Dhvar5); and v) *C*-terminus Cys-modified Dhvar5 (NorChit-*Ct*-Dhvar5).

The presence of peptide in the final conjugates was additionally confirmed by Attenuated Total Reflectance Fourier Transform InfraRed (ATR-FTIR) **(Figure 7)**. A novel peak appears in NorChit-Dhvar5 conjugates at 1630 cm^{-1} (amide II, *N-H* bending, which is an indication of the presence of amide bonds from the peptide's backbone[34].

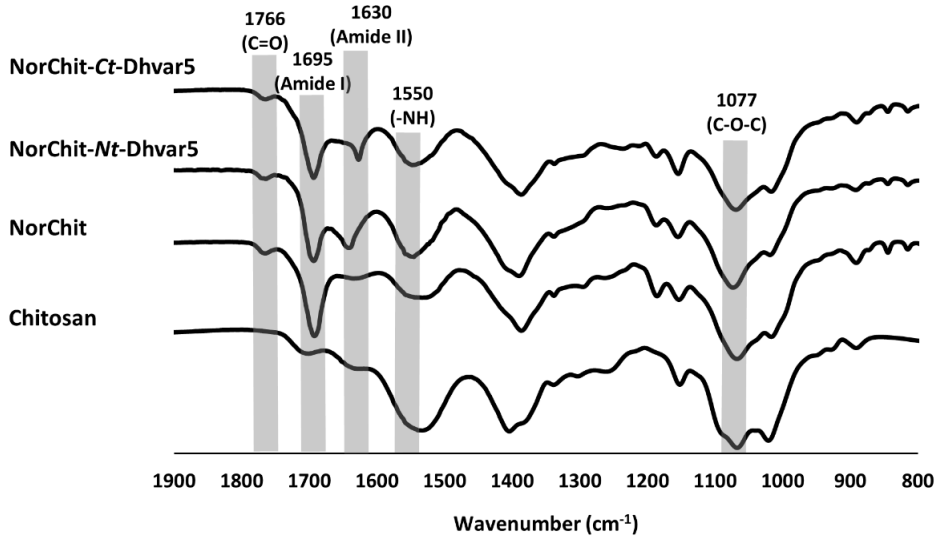


Figure 7. Attenuated Total Reflectance Fourier Transform InfraRed (ATR-FTIR) spectra of chitosan, norbornene-chitosan (NorChit) and peptide-NorChit conjugates (NorChit-*Nt*-Dhvar5 and NorChit-*Ct*-Dhvar5).

Finally, total peptide content in the NorChit-Dhvar5 conjugate was evaluated by Amino Acid Analysis (AAA) **(Table 2)**. Experimental and theoretical ratios of the amino acids in the Dhvar5 sequence were similar for the peptide alone and for the conjugate, suggesting that peptide amino acid sequence remained unchanged during TNPC. Moreover, AAA results indicate that approximately 80 µmol of Cys-Ahx-Dhvar5 were conjugated per gram of NorChit-Dhvar5, corresponding to a 43% conjugation yield. Therefore, based on AAA results, approximately 5% of the norbornene groups available in NorChit (79.6 µmol of the 1725 µmol norbornene groups per gram of NorChit) reacted with the AMP.

Table 2. AAA of adsorbed Dhvar5 and NorChit-Dhvar5 obtained upon AMP grafting through its *N*-terminus or *C-terminus*.

Amino acid	nmol (from hydrolyzed conjugate)			Theoretical ratio	Experimental ratio		
	Adsorbed	*N-terminus*	*C-terminus*		Adsorbed	*N-terminus*	*C-terminus*
Arginine	9.7	71.8	59.1	2	2	2	2
Tyrosine	3.6	29.6	18.6	1	1	1	1
Lysine	19.9	206.9	133.8	5	5	5	5
Leucine	22.8	199.5	139.1	5	5	5	5
Phenylalanine	2.7	38.9	26.7	1	1	1	1
AMP content*	10.6	79.6	55.2				

*estimated (μmol of AMP per g of NorChit-Dhvar5)

Concerning *C-terminus* conjugation, 55 µmol of peptide were present per gram of NorChit-Dhvar5 (30%, conjugation yield), (Table 2). This represents a reduction of 30% in the amount of

conjugated peptide compared to Cys-Ahx-Dhvar5 (*N-terminus* conjugation). The higher amount of peptide after *N-terminus* conjugation can be justified by hydrophobic interactions between the hydrophobic *N-terminus* of Dhvar5 (LLLFLLKKRKKRKY; head-to-tail amphipathicity) and the hydrophobic norbornene groups. Indeed, hydrophobic interactions are one of the driving forces involved in the self-assembly of amphiphilic peptides and the degree of hydrophobic attraction influences the spatial arrangement of such peptides[35,36]. Therefore, we hypothesize that non-covalent interactions between the hydrophobic region of Dhvar5 and norbornene groups of NorChit are favored in solution, bringing the cysteine moieties closer to norbornenes when this amino acid is linked to the peptide *N-terminus* (Cys-Ahx-Dhvar5). Conversely, when the cysteine is present at the *C-terminus* (Dhvar5-Ahx-Cys), it may be placed further away from norbornenes, reducing the likelihood of covalent conjugation.

Nevertheless, 43% and 30% conjugation yield for NorChit-*Nt*-Dhvar5 and NorChit-*Ct*-Dhvar5, respectively, is similar or higher than that described in other works using thiol-ene reaction. For instance, Guindani et al.[37] used thiol-ene chemistry to functionalize the surface of poly(Globalide-*Co*-*ε*-Caprolactone) nanoparticles, first with NAC, as a proof of concept, and then with bovine serum albumin (BSA). Despite the high surface area of nanoparticles, the availability of alkene bonds at the surface of nanoparticles and the fact that BSA was modified to have 5-fold more cysteine groups per molecule, the grafting yield decreased from 58% when using NAC (MW = 163 g/mol), to 36% when using BSA (MW ≈ 66 kDa). Thus, the concept of "high efficiency" should be tuned to the specificities of the reaction. For a peptide (HHC10, MW ≈ 1548 Da) with a MW in the range of Dhvar5 (MW = 2062 Da), only around 0.10 mg of peptide were conjugated in a reaction of 75 seconds (5 × 15 seconds), when using an initial peptide amount of 40 mg (approximate yield: 0.25%)[38]. Moreover, Ding et al.[39] conjugated the peptide CGGGREDV (MW

= 792 Da) to polyurethane in an 1 h thiol-ene reaction, achieving a yield of 32%. The conjugation yield reported herein using a thiol-ene reaction (43% and 30%) was a significant improvement over our previous method for Dhvar5 conjugation onto chitosan. The previous method of copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, "click" reaction) allowed to conjugate 50 μmol of peptide per g of chitosan, but only with a 4% conjugation yield[12], since it uses a ratio of Dhvar5/chitosan that is, approximately, 7 times higher than the used in this work. Herein, the quantity of peptide used in the conjugation reaction by TNPC (3.8 mg of Dhvar5 per 10 mg of modified chitosan) was much lower compared to that needed using CuAAC (25 mg of Dhvar5 per 10 mg of modified chitosan)[12] or other chemistries[40,41], while the efficiency of the reaction was considerably improved.

Notably, reaction efficiency could be further improved by optimizing the operating parameters involved in the thiol-ene reaction, such as the UV exposure time, light intensity or the photoinitiator concentration and/or type. However, a balance between the overall UV dose and the formation of free radicals should the considered in order to preserve the antimicrobial activity of the peptide. Therefore, a good balance was achieved herein between having a reaction with a good efficiency and preserving the antimicrobial activity of the peptide, while using a simple and timesaving protocol.

In the present report, thiol-ene reaction was performed with lower UV light intensity and exposure time (10 mW cm^{-2}, 5 min), than the described in the literature, which can be due to the high absorbance of the photoinitiator VA-086 at 365 nm[42]. Moreover, reaction kinetics of thiol addition onto norbornenes is not only driven by electron density, but also by mitigating the ring tension in norbornene versus its saturated counterpart. In fact, as mentioned in the introduction, norbornene is one of the most reactive enes within the thiol-ene reactions, and has been found to

undergo quantitative reaction even at very low concentrations as polymer end groups[43]. Previous reports describe peptide conjugation (EPL-chitosan and AMP-dextran) in a 12 h reaction under UV light (365 nm, 20 mW cm^{-2}) at RT[40,41]. Elsewhere, immobilization of cysteine-modified AMP (Inverso-CysHHC10, 1 mg mL^{-1}) directly onto hydroxyapatite (HA) discs modified with an alkene-terminating organic acid (16-heptadecenoic acid) film was performed over 3 h under UV light and in the presence of Irgacure 2959, in inert conditions; this resulted in an AMP content of 8850 μg (around 5.8 μmol) per g of modified HA[44]. More recently, when using norbornene as an alkene donor, HHC10-Cys was linked to oxy-norbornene modified alginate hydrogels using 5 min of UV-triggered reaction on each side of the hydrogel, after a lengthy 24 h soaking step of the hydrogel in the AMP solution (1 to 4 mg mL^{-1}), to allow the AMP to infiltrate within the hydrogel mesh[45]. The amount of initial AMP retained within the hydrogels varied between 91% and 37.5% depending on AMP concentration in solution. However, quantification of AMP content in the hydrogel was based on the total amount of sulfur present and it is not clear if the washing step performed (washing the hydrogels in PBS) ensured complete removal of unreacted peptide.

Additionally, ^{1}H NMR results (and, indirectly, AAA results, by quantifying the amount of AMP present in NorChit-Dhvar5) confirmed the presence of norbornene groups available after Dhvar5 conjugation (~95% and 97% for NorChit-*Nt*-Dhvar5 and NorChit-*Ct*-Dhvar5, respectively) that can be used for subsequent reactions, such as conjugation of additional bioactive peptides and/or crosslinking towards the development of materials with tunable mechanical properties. The importance of having free norbornenes for subsequent reactions has been recently highlighted[46]. For instance, this versatility of TNPC was shown in a report where norbornene-functionalized starch (DS = 0.25) was crosslinked with PEG dithiol and the remaining free norbornene groups were used to immobilize an AMP (Cys-KR12)[47].

3.3. Assessment of antibacterial properties and cytotoxicity

3.3.1. Influence of UV exposure on soluble Dhvar5 antimicrobial activity

The effect of UV exposure on Dhvar5 and related derivatives (Dhvar5-Ahx-Cys and Cys-Ahx-Dhvar5) antimicrobial activity was assessed by determining MIC (the lowest concentration of antimicrobial agent that inhibits visible bacteria growth) and MBC (the lowest concentration of antimicrobial agent that kills 99.9% of bacteria) against *S. epidermidis*. As shown in **Table 3**, the introduction of the Cys residue contributed for a slight increase of both MIC and MBC values. These were not further modified by the peptide exposure to UV light, under the same TNPC conditions (10 mW cm^{-2} for 5 min).

Table 3. MIC and MBC values of Dhvar5, Dhvar5-Ahx-Cys and Cys-Ahx-Dhvar5 against *S. epidermidis* before and after exposure to UV light.

Sample	MIC (μg/mL)	MBC (μg/mL)
Dhvar5	0.5	0.5
Dhvar5*	0.5	0.5
Dhvar5-Ahx-Cys	2	2
Cys-Ahx-Dhvar5	1	2
Cys-Ahx-Dhvar5*	1	2

*after UV exposure

3.3.2. Evaluation of NorChit-Dhvar5 antimicrobial activity

The effect of the Dhvar5 conjugation chemistry (TNPC) on its antimicrobial activity was evaluated by bacterial adhesion (and viability) to thin coatings prepared by the spin coating of solutions of chitosan, NorChit, NorChit-*Ct*-Dhvar5 and NorChit-*Nt*-Dhvar5 on gold substrates.

The amount and viability of the Gram-positive *S. epidermidis* and the Gram-negative *P. aeruginosa* after adhesion onto these thin films is shown in **Figure 8A** and **8B**, respectively.

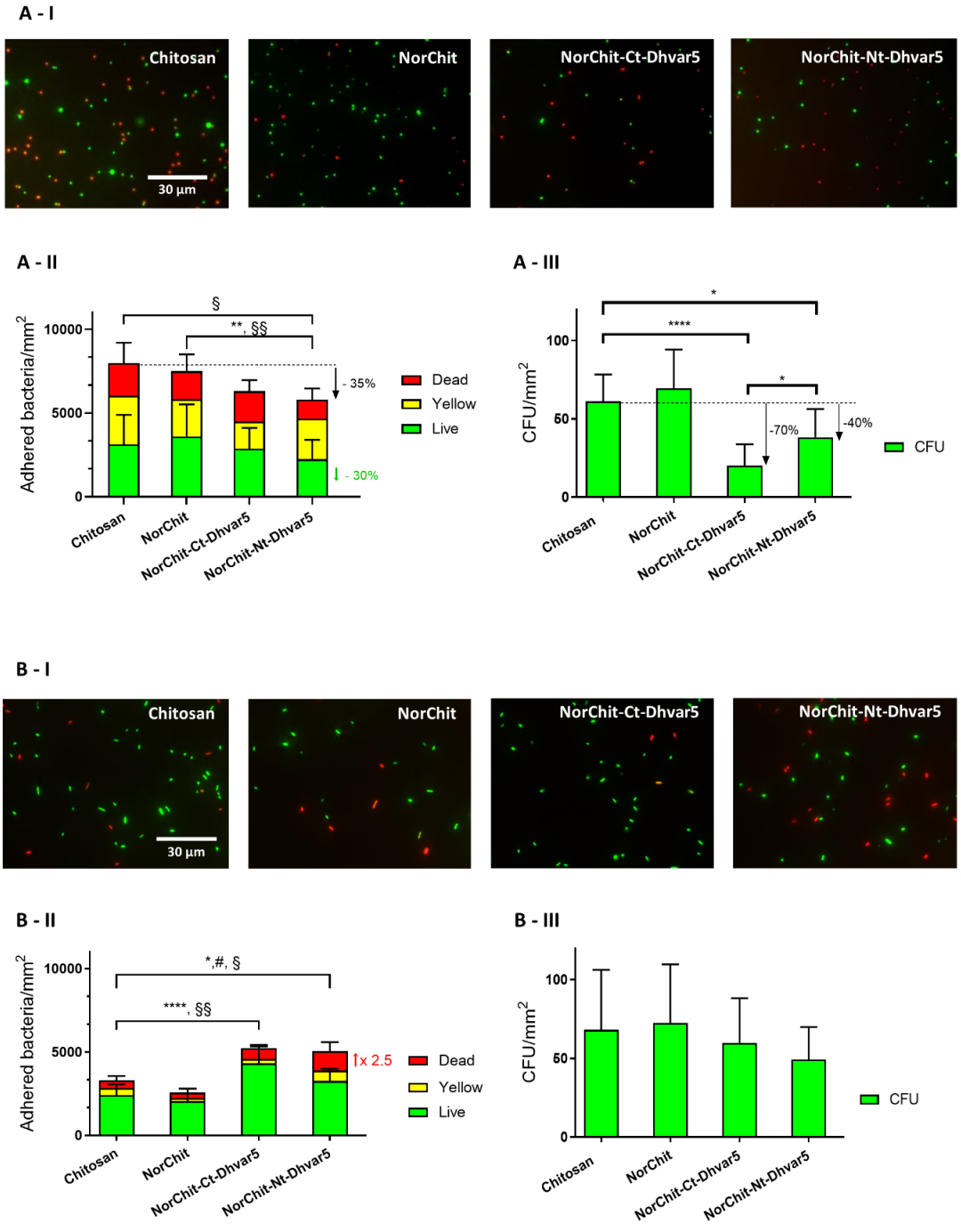


Figure 8. A (*S. epidermidis*) and B (*P. aeruginosa*)– I: Representative micrographs of bacterial adhesion assay against chitosan, NorChit, NorChit-*Ct*-Dhvar5 and NorChit-*Nt*-Dhvar5 thin films using LIVE/DEAD® Bacterial Viability staining (BacLight™) with a magnification of 630×. II: Graph shows the viability of adhered bacteria incubated at 37 ºC for 5 h (Live: ** $p<0.01$, *** $p<0.001$; Dead: # $p<0.05$; Total: § $p<0.05$, §§ $p<0.01$). Data are presented as mean ± standard deviation (A*:* 3 replicates × 12 independent assays for chitosan and NorChit; 3 replicates × 9 independent assays for NorChit-*Ct*-Dhvar5 and NorChit-*Nt*-Dhvar5*;* B: 3 replicates × 3 independent assays). x% (red bars) = % of dead bacteria; III – Viability of CFU recovered from the samples after sonication and vortexing (*$p<0.05$; ***$p<0.001$; 3 replicates x 3 independent assays.

Concerning *S. epidermidis* (**Figure 8A-I & 8A-II**) there is a statistically significant reduction in the total number of adhered bacteria in NorChit-*Nt*-Dhvar5 compared to chitosan (-35%; $p<0.05$) and NorChit (-35%; $p<0.01$). A similar tendency was observed for NorChit-*Ct*-Dhvar5 (-20% relatively to chitosan), but without statistical significance. These results demonstrated an evident antiadhesive effect due to the presence of Dhvar5. Antiadhesive surfaces were previously developed by us when Dhvar5 was grafting to chitosan thin films via disulfide bridge[10], especially when conjugation occurred through the *N-terminus* (exposing its hydrophilic and cationic domain).

Regarding the viability of adherent *S. epidermidis*, using the BacLight™ assay, where live bacteria were labelled as green (Syto9 positive) and dead bacteria as red (PI positive), **Figure 8A-II** shows that NorChit-*Nt*-Dhvar5 caused a reduction in the number of viable bacteria when compared to chitosan (-30%) and NorChit (-40%), although the difference was only statistically significant compared to the latter (**, $p< 0.01$). No difference in the number of viable bacteria was

found for NorChit-*Ct*-Dhvar5 regarding the other films. This fact could be related with the lower amount of Dhvar5 when peptide was grafted by its *C-terminus* (55 μmol Dhvar5 /g NorChit-*Ct*-Dhvar5 versus 80 μmol Dhvar5 /g NorChit-*Nt*-Dhvar5) or with the high number of adherent *S. epidermidis* that were double stained (yellow; Syto9 and PI positive) in all the films and in all the independent assays. This issue is widely described in the literature. In the first report of the use of Baclight® staining, Boulos *et al*.[48] described that "in rare cases, green cells appeared yellowish and red cells appeared orange: yellow cells were considered viable, while orange cells were considered damaged". Conversely, other authors consider that yellow bacteria appear when PI is able to cross damaged bacterial membranes, but does not completely replace Syto9[49]. More recently, PI was found to overestimate death in biofilms of adherent *S. epidermidis* in PBS on glass coverslips, where a large proportion of bacteria were double-stained for Syto9 and PI[50]. This phenomenon was more common in viable biofilms as ethanol treated biofilms consistently showed only PI-stained bacteria. Double-staining was attributed to PI binding to extracellular DNA, which mediates the adhesion of bacteria to surfaces during biofilm establishment. As the interpretation of yellow bacteria, especially in adherent cells, is not straightforward, we reported them separately from green-only and red-only bacteria. Moreover, the Live/Dead assay was validated by CFU counting after bacteria recovery from the sample surface, where a significant reduction in viable bacteria is observed in both NorChit-Dhvar5 conjugates relatively to chitosan (**Figure 8 A-III**), especially for NorChit-*Ct*-Dhvar5 with a decrease of 70% relatively to chitosan (even when Dhvar5 concentration is lower when peptide was immobilized by *C-terminus*). Although these results should be analyzed with caution, since most of bacteria remain on the samples even after sonication and vortexing using a previously optimized protocol[28], no significant differences were found between the bacteria that did not detach from chitosan and from NorChit-Dhvar5 films after

sonication (labelled with 4′ ,6-diamidino-2-phenylindole (DAPI) and quantified by fluorescence microscopy images; data not shown). The difficulty in detaching bacteria from the surfaces may be due to the fact that bacterial adhesion is forced by contact with a glass coverslip. This higher reduction in viable bacteria for NorChit-*Ct*-Dhvar5 (Figure 8 A-III), where the peptide exposed its hydrophobic domain, was previously reported by us using Dhvar5 grafted by CuAAC[13]. Due to the higher number of dead bacteria attached on this surface, it was concluded that the exposure of hydrophobic domain might maximize the insertion into the *Gram-positive* bacterial lipid bilayer[13]. In opposite to the described to *S. epidermidis*, films containing Dhvar5 attract the Gram-negative *P. aeruginosa* (**Figure 8B-I & 8B-II**) independently of their exposition domain. One explanation for this difference could be related with the more negatively charged membrane of Gram-negative bacteria[4]

Concerning the antimicrobial effect, there is a statistical significant increase in dead *P. aeruginosa* (+104%) on NorChit-*Nt*-Dhvar5 relatively to chitosan, which could be related with its higher grafted concentration regarding NorChit-*Ct*-Dhvar5. No significant differences are observed in the number of viable recovered colonies, but, as discussed above, only a small portion of bacteria were recovered from the samples. The killing effect against *P. aeruginosa* is superior in TNPC compared to CuAAC, where no antimicrobial and antiadhesive effect was observed for Dhvar5 *N-terminus* immobilization and an anti-adhesive effect was found for Dhvar5 *C-terminus* immobilization[13].

Altogether, these results further highlight what was previously discussed by us: different peptide conjugation strategies may lead to distinct peptide density, conformation and/or exposure, which altogether influence the antimicrobial performance of the final conjugates[13]. The results here exposed evaluate bacterial adhesion/viability to films prepared with Dhvar5-chitosan conjugates

where the peptide exposition on the surface could not be the ideal. However, the idea is to have a Dhvar5-chitosan conjugated polymer that can be used for the production of antibacterial biomaterial coatings.

3.3.3. Cytotoxicity

The biocompatibility of chitosan, NorChit and NorChit-Dhvar5 conjugates was evaluated against primary fibroblasts in a direct contact assay for 24 h. Metabolic activity was normalized according to the number of adhered cells and is reported relatively to control tissue culture coverslips. The ability of cells to metabolize resazurin to resorufin remained above 70% in all samples (**Figure S6**). Therefore, according to ISO 10993-1, NorChit and NorChit-Dhvar5 conjugates are non-cytotoxic, which is also in accordance with the biocompatibility of our previously reported chitosan-Dhvar5 conjugates[13].

Overall, the employment of the highly efficient TNPC allowed a significant reduction in the amount of necessary Dhvar5 peptide for conjugation, while preserving its antibacterial properties and being non-cytotoxic. Indeed, the higher efficiency in AMP conjugation is of outermost importance for the clinic application of AMP-modified biopolymers, as the high cost of therapies involving AMP is seen as a major hurdle towards their clinical application[51].

4. Conclusion

In the present work, the introduction of 0.38 norbornene groups per modified chitosan repeating unit was achieved using an aqueous/organic co-solvent system. Then, Dhvar5 was successfully grafted onto chitosan using the highly efficient TNPC under mild reaction conditions (RT, low UV light intensity (10 mW cm^{-2}) and exposure time (5 min)). The high amount of Dhvar5 grafted by

its *N-terminus* (~80 μmol per g of modified polymer) demonstrated a superior conjugation yield (43%) when compared to our previously reported conjugation using copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, "click" reaction). In addition, the considerable amount of norbornene groups that remain free provide the opportunity for subsequent modifications (e.g., crosslinking, or combined tethering of other bioactive moieties) or to further increase the peptide quantity, if necessary. Thin films of the resulting polymer, NorChit-*Nt*-Dhvar5 and NorChit-*Ct*-Dhvar5, were able to reduce total *S. epidermidis* and increase the percentage of dead *P. aeruginosa* bacteria adhered onto the surface. Moreover, the number of viable *S. epidermidis* recovered from the surface of NorChit-Dhvar5 conjugate films was reduced compared to chitosan. Finally, the developed biomaterials were non-cytotoxic. Therefore, TNPC is a robust strategy for AMP conjugation onto chitosan to create antibacterial biomaterials, confirming our initial hypothesis.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

Supplementary figures and tables (Word document)

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Declaration of interest

None

Data availability

The raw data and the processed data required to reproduce these findings are available from the corresponding author on reasonable request.

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ABBREVIATIONS

AAA: Amino Acid Analysis; ACN: Acetonitrile; AMP: Antimicrobial Peptides; BSA: Bovine Serum Albumin; CA: Carbic Anhydride; DA: Degree of Acetylation; DMF: *N*,*N*-Dimethylformamide; DS: Degree of Substitution; ESI-IT MS: Electrospray Ionization/ion Trap Mass Spectrometry; FTIR: Fourier Transform InfraRed Spectroscopy; HA: Hydroxyapatite; HPLC: High Performance Liquid Chromatography; MBC: Minimum Bactericidal Concentration; MHB: Mueller-Hinton Broth; MIC: Minimum Inhibitory Concentration; MW: Molecular Weight; MWCO: Molecular Weight Cut-Off; NAC: *N*-Acetyl-*L*-Cysteine; NMR: Nuclear Magnetic Resonance; NorChit: Norbornene-Chitosan; NorChit-Dhvar5: Norbornene-chitosan with conjugated Dhvar5; PBS: Phosphate Buffered Saline; RT: Room Temperature; TNPC: Thiol-Norbornene Photoclick Chemistry; TSA: Tryptic Soy Agar; UV: Ultra-Violet; XPS: X-Ray Photoelectron Spectroscopy

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