Norbornene-chitosan spray-dried microspheres for peptide conjugation using thiol-ene "photoclick" chemistry

Premio Áccesit Congreso SIBB 2022

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Abstract

The action of bioactive peptides, such as antimicrobial peptides (AMP), in the human body is often compromised by limited residence time and stability in the target site. Bioconjugation of peptides to biomaterial surfaces is one of the strategies that may overcome these limitations. Herein, norbornene-chitosan (NorChit) microspheres were engineered to react with thiolated peptides by thiol-ene "photoclick" chemistry. NorChit microspheres were produced by spray drying and crosslinked with dithiothreitol (DTT) to prevent their solubilization. Microspheres with a diameter of 5 ± 2 µm showed round and smooth morphology with pockets over the surface that could be related with hydrophobic interactions between internal norbornene groups. Thiol-ene bioconjugation carried out using a fluorescent model peptide, showed a yield of 45%, whereas using the peptide but without UV exposure indicated a maximum of peptide adsorption of 30%. Altogether, NorChit microspheres show the potential for carrying bioactive peptides, which may open avenues for AMP activity onto harsh environments in the body.

Keywords*: norbornene-chitosan, covalent conjugation, peptide, spray-drying, scanning electron microscopy, thiol-ene*

Resumo

A ação de péptidos bioativos, tais como péptidos antimicrobianos (AMP), no corpo humano é frequentemente comprometida pelo seu limitado tempo de permanência e estabilidade no local alvo. A bioconjugação de péptidos em superfícies de biomateriais é uma das estratégias que pode ajudar a ultrapassar estas limitações. Neste trabalho, microesferas de quitosano-norborneno (NorChit) foram produzidas e modificadas para reagir com péptidos contendo grupos tiol, através da química tiol-eno promovida por exposição a luz UV. As microesferas de NorChit foram produzidas por spray-drying *e reticuladas com ditiotreitol (DTT) para evitar a sua solubilização. As microesferas apresentaram um diâmetro de 5 ± 2 µm e uma morfologia redonda e lisa, com poços ao longo da superfície que podem estar relacionados com interações hidrofóbicas internas entre os grupos norborneno. A bioconjugação tiol-eno, efetuada com um péptido modelo fluorescente, teve um rendimento de 45%, enquanto que usando o péptido sem exposição ao UV levou a um máximo de adsorção de 30%. No geral, as microesferas de NorChit têm potencial para transportarem péptidos bioativos podendo vir a ser usadas para proteger e manter os péptidos ativos em ambientes mais adversos no corpo humano.*

Palavras-chave: *quitosano-norborneno, conjugação covalente, péptido, spray-drying, microscopia eletrónica de varrimento, tiol-eno*

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Introduction

Antimicrobial peptides (AMP) are mostly peptides of cationic, hydrophobic and/or amphiphilic nature that can have up to 100 amino acids. They exhibit a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, viruses, fungi and protozoa.[1–3] However, free AMP have short-term activity due to their in vivo inhibition by hydrolysis, oxidation, proteolysis, and other conditions found in certain body environments such as the alkaline pH in skin wounds.[4]

To circumvent peptide degradation, their sequences may be changed from L-amino acids to D-amino acids, which are more resistant to protease degradation, as they are less common in nature. However, the cost increase associated with using D-amino acids and the possibility of increasing toxicity are causes for concern.[5] Alternatively, the ends of the peptide sequence may be capped (C-amidation and/or N-acetylation) to difficult the access to enzymes via steric hinderance.[6]Although this is a simple method, which does not alter the native peptide sequence, it is considered a process with limited effectiveness in protecting peptides against degradation, as they may remain vulnerable to certain endopeptidases.[5] A more successful approach has been their bioconjugation in biomaterials in the form of polymeric scaffolds, nanoparticles, self-assembled peptides and peptide-polymer conjugates.[4,7] These formulations may not only protect AMP from degradation, but also enhance their bioactive properties.

Peptide bioconjugation onto biomaterials can be achieved in a controlled chemoselective way through several click chemistry reactions, such as UV-triggered thiol-ene conjugation, classic Michael-type additions, copper catalysed azide-alkyne 1,3-dipolar cycloadditions (CuAAC) or strain-promoted azide-alkyne cycloadditions (SPAAC).[8]The thiol-ene conjugation can be triggered by UV, visible light or heat, and typically occurs between an alkene-modified polymer and a thiol-containing moiety, in the presence of a photoinitiator.[8] Thiol-ene reactions can occur with the use of any non-sterically hindered terminal ene, but reaction rates are faster when using electron-rich and/or strained enes, such as norbornenes. The thiol-ene reaction has optimal efficiency between pH 4 and 7.[9] Contrarily, it is highly hindered at high pH, due to the formation of thiolates (RS-) that impair the generation of thiyl radicals (RS•), which are necessary to target the alkene double bonds towards facilitating the thiol-ene reaction.

Recently, we have reported the production of norbornene-chitosan (NorChit) in a high yield, simple manner.[10] Chitosan was first modified with norbornene groups in a 3-h reaction with carbic anhydride, using a dilute acetic acid/dimethylformamide co-solvent system. This allowed the introduction of an average of 0.38 norbornene groups per chitosan monomer, which enabled, in turn, to conjugate the antimicrobial peptide Dhvar5 with up to 43% yield. Building on this work, herein we explore the possibility of producing spray-dried microspheres using the NorChit polymer previously developed and of coupling a model peptide onto the microspheres by thiol-norbornene photoclick chemistry (TNPC). We hypothesize that the use of microspheres may increase the peptide conjugation efficiency and peptide exposure, due to the higher surface area.

Materials and methods

Spray-dried norbornene-chitosan microspheres

NorChit was synthesized as previously described by us.[10] Then, NorChit (0.9% w/v) was first hydrated for 8 h in Type I water (ultrapure water with a resistivity >18 MΩ.cm, conductivity of <0.056 $\mu S.cm^{-1}$ and <50 ppb of total organic carbon), at 4 °C, under mild magnetic stirring, followed by dropwise addition of glacial acetic acid to obtain a final acetic acid concentration of 0.1 M. Unless stated otherwise, dithiothreitol (DTT) was added at 1.7 mmol per g of NorChit (0.5:1 DTT to norbornene ratio) following overnight solubilization. Microspheres were produced by spray drying (Mini Spray dryer BÜCHI B-290 advanced with a standard 0.5 mm nozzle spray dryer (Flawil) at Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE, University of Porto)). Spray-drying settings were the following: air and solution flow rates – 40 $m3.h^{-1}$ and 4 $mL.min.^{-1}$, respectively; air pressure – 6.0 bar; inlet temperature - 120 °C; outlet temperature – 63 ± 3 °C. Microspheres were collected and stored at room temperature in a desiccator, under nitrogen atmosphere and protected from light until further use.

To study the effect of UV crosslinking, NorChit microspheres (1 $mg.mL^{-1}$) were resuspended in Type I water, in the presence of the photocrosslinker VA-086 (0.4% w/v; Wako Chemicals). The suspension of NorChit microspheres was then exposed to UV light (λ = 365 nm; 10 $mW.cm^{-2}$) for 5 minutes. NorChit microspheres were then washed by centrifugation at 16 000 g for 5 minutes, and the supernatant discarded.

Scanning Electron Microscopy (SEM)

Spray-dried microspheres were adhered onto carbon tape and coated with a gold/palladium thin film by sputtering (SPI Module Sputter Coater equipment) for 80 s with a 15 mA current. SEM analyses were performed at "Centro de Materiais da Universidade do Porto" (CEMUP), using a high resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis (FEI Quanta 400 FEG ESEM/ EDAX Genesis X4M). Images were taken at 5000× and 10000× magnification.

Stability testing

Microspheres (3.33 $mg.mL^{-1}$) were re-suspended in Type II water (resistivity of >1 MΩ.cm, a conductivity of <1 $\mu S.cm^{-1}$ and <50 ppb of total organic carbon), sonicated in short pulses (1× 3 sec pulse per mL), centrifuged at 16 000 g for 5 min and re-suspended in either phosphate buffer saline (PBS; pH 7.4) or simulated gastric fluid (SGF; pH 1.2). Suspensions were kept at 4 ºC, protected from light, and aliquots were taken at days 0 and 8, for quantification.

Microsphere size and concentration

Microspheres size and concentration were evaluated using high content screening microscopy (IN Cell Analyzer 2000; GE Healthcare) at i3S BioSciences Screening scientific platform. Microspheres were first stained with carmoisine food-dye (1:500) for 30 minutes, centrifuged at 16 000 g for 5 minutes, and then washed twice in Type II water. Samples (100 µL) were then sonicated in three short pulses, at 70% amplitude. Afterwards, 15 µL of the suspension, in triplicates, were transferred to 96 half-well plates (#675090, Greiner Bio-One). The whole well was imaged with a Nikon 20×/0.45 NA Plan Fluor objective (binning of 2 × 2, 2.5 D acquisition mode with a Z section of 2.5 µm), using a TexasRed filter. Microspheres were identified using ilastik, a machine learning segmentation software, and processed with CellProfiler, as previously described.[11] Microspheres with a diameter inferior to 1 µm were not considered, in order to discard small residues.

Peptide conjugation

Microspheres (4 $mg.mL^{-1}$) were washed once in Type I water, centrifuged at 16 000 g and re-suspended again, to remove excess DTT. Then, the microspheres suspension was sonicated at 70% amplitude (1x 3-secs pulse per mL) and the VA-086 photoinitiator (4% w/v) as well as a model peptide with a fluorescein (FITC) tag (0.8 $mg.mL^{-1}$; CGGGGRGDSP-FITC) were added. The solution was perfused at 210 $\mu L.min^{-1}$ through a PVC tubing (MasterflexTM 30526-14) placed between two UV LEDs (365 nm; power source: 3.3 V, 0.26 A; ~30 s of UV exposure) on top and below (distance ~1.5 cm). Suspension was then centrifuged at 16 000 g for 5 minutes, supernatant collected and re-suspended in Type I water. This process was repeated twice. Concentration of peptide-FITC in collected supernatants (washes 0, 1 and 2) was then determined by fluorescence spectroscopy ($\lambda_{ex/em}$ = 495/520; Perkin Elmer), relatively to initial peptide solution.

To evaluate the maximum amount of peptide adsorbed, we performed the same assay, but without exposure to UV light, thus preventing the TNPC from occurring.

Results and discussion

Norbornene-chitosan (NorChit) microspheres were produced by spray-drying with and without addition of dithiothreitol (DTT), to evaluate the need for the crosslinker to improve microsphere stability in solution. DTT has one thiol group on each end that can react with the C=C bond on norbornenes via TNPC, i.e., in the presence of a photoinitiator and upon UV irradiation. This enables UV-triggered generation of thiol radicals at each end of the DTT molecule which then react with C=C bonds in norbornenes, yielding the covalent crosslink. In other words, covalent crosslinking by TNPC will expectedly occur only when NorChit microspheres prepared in the presence of DTT are exposed to UV radiation in the presence of a photoinitiator.

The resulting microspheres from the spray-drying process were imaged by scanning electron microscopy (SEM), as presented in Figure 1.

Spray-dried NorChit microspheres (5 ± 2 µm) exhibited a round morphology with a smooth surface, showing pockets (0.3 to 2 µm) over the surface. These pockets have previously been observed in spray dried microspheres prepared with arabic gum or with water insoluble chitosan.[12] In opposite, microspheres produced with water soluble chitosan and with the chitosan used in this work [11], presented a very smooth surface. The observed pockets on NorChit microspheres may be related with hydrophobic interactions between the norbornene groups, which may face preferably towards the core of the microspheres.[13] This mor-

phology was not affected by the presence of DTT. França et al.[13] had previously reported chitosan microspheres with an increasingly collapsed appearance when the sample solution fed to the spray-drier increased in complexity (i.e., chitosan was modified with other chemical groups). Nonetheless, in that work, raw chitosan (DA 15%) microspheres also presented a shape with pockets appearing along its surface. Indeed, the higher DA used in that work (15%) compared to the chitosan used herein (6%) confers higher hydrophobicity to chitosan. In another work, spray-dried microspheres of carboxymethyl chitosan also presented an irregular shape, showing collapsed structures in their morphology. Interestingly, despite the irregular appearance, their structure was found to be very stable.[15]

In this work, NorChit microspheres prepared in the absence of DTT were not visible in transmitted light optical microscopy immediately after suspension in phosphate buffer saline (PBS; pH 7.4). Contrarily, microspheres produced in the presence of DTT were clearly visible under identical conditions (Figure 2). This suggested that the presence of DTT in the core of the microspheres, even without triggering the TNPC by exposure to the photoinitiator and UV light, contributes to stabilizing the microspheres in suspension. Therefore, only NorChit-DTT microspheres continued to be used.

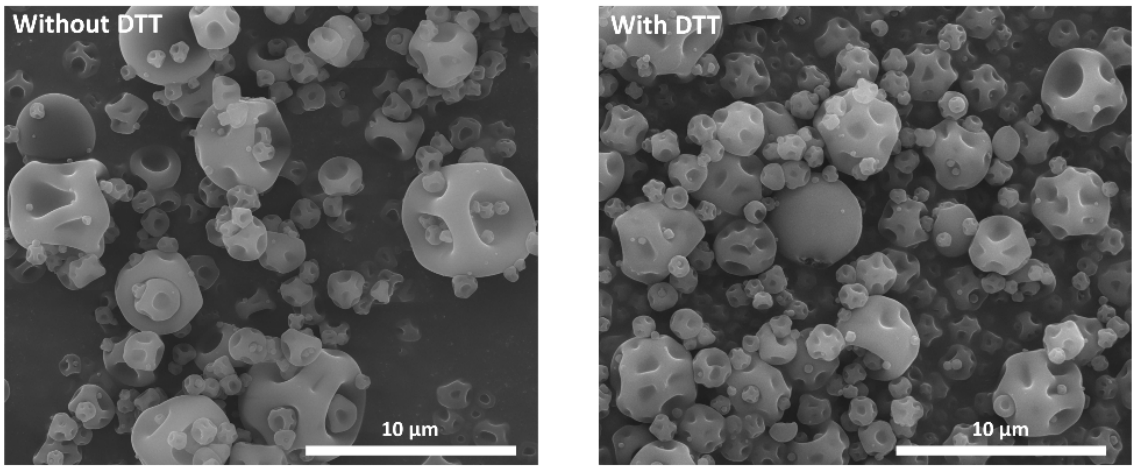

Figure 1. Scanning electron microscopy (SEM; 10 000x magnification) images of norbornene-chitosan (NorChit) microspheres prepared without (left) and with DTT (right) after spray-drying process

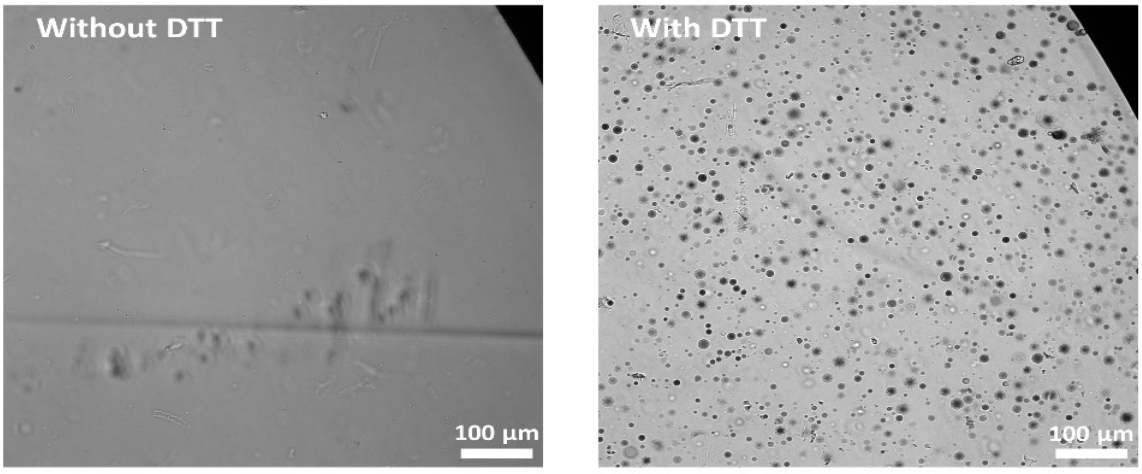

Figure 2. Transmission optical microscopy images of norbornene-chitosan (NorChit) microspheres prepared without (left) and with DTT (right) immediately after suspension in PBS (pH 7.4)

To evaluate the stability of the NorChit-DTT microspheres at different pH values, the particles were immersed in either PBS (pH 7.4) or simulated gastric fluid (SGF, pH 1.2) for 8 days (Figure 3). NorChit-DTT microspheres not subjected to TNPC conditions (photoinitiator and UV light exposure) degraded faster in SGF (nearly 70% degradation) than in PBS (about 40% degradation), likely due to the acidic pH that protonates primary amines of chitosan leading to its solubilization.[16] The microspheres exposed to UV light in the presence of the photoinitiator did not show any relevant degradation in PBS (the apparent increase in microspheres percentage after 8 days is related to particles aggregation/disaggregation in PBS). However, about half of these UV-irradiated microspheres were degraded when incubated in SGF for 8 days (53% UV+ microspheres remained after this incubation period). Therefore, exposure of NorChit-DTT microspheres to UV/photoinitiator did not seem to significantly increase their stability in SGF. Of note, the DTT:norbornene ratio in the precursor NorChit solution was 50%, to ensure the presence of free norbornenes after spray-drying for the subsequent peptide conjugation. Further increasing the DTT:norbornene ratio could lead to higher stabilization of the microspheres after TNPC. Nonetheless, in the current conditions, the increased stability of NorChit-DTT particles, as compared to NorChit ones, is not due to TNPC-mediated crosslinking, as discussed. One possible explanation for this observation is that thiol-ene reactions can also be triggered by heat, usually in the presence of a thermal initiator, such as 2,2'-azoisobutyronitrile, at 80 ºC.[17] Although the reaction rate for heat-triggered thiol-ene conjugation in the absence of a thermal initiator is very low, we hypothesize that the brief exposure of the NorChit-DTT suspension to the significantly higher temperature (120 ºC) at which the spray-dryer operates can promote covalent crosslinking by reaction of DTT with norbornene groups to an extent that is enough to stabilize the microspheres. This hypothesis agrees with the lack of significant additional stabilization after UV irradiation, which can be due to a low number of surface-exposed norbornene groups that remain free after the thermally-induced crosslinking.

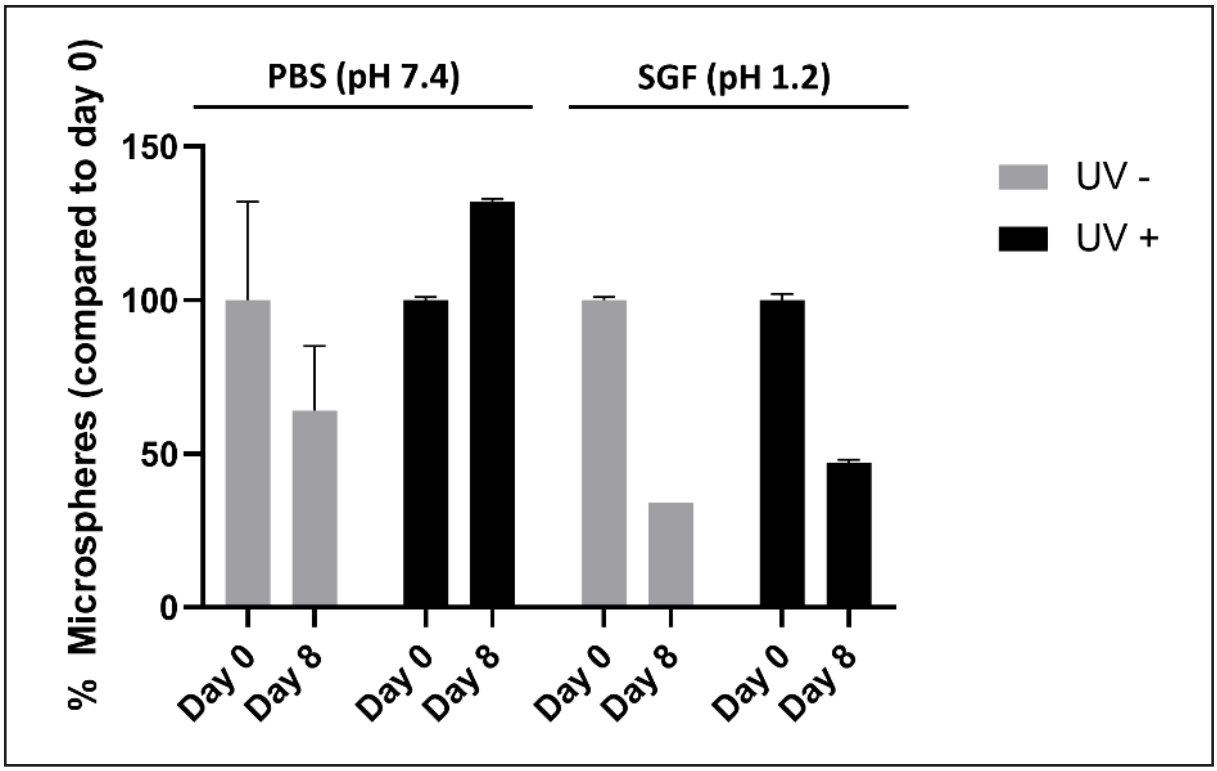

Figure 3. Stability of NorChit-DTT microspheres without (UV-) and with (UV+) exposure to UV light after immersion in PBS (pH 7.4) or SGF (pH 1.2) for 8 days. Results are expressed in microspheres percentage compared to day 0

As there was no significant gain in stability by exposing microspheres to TNPC conditions, studies proceeded with NorChit-DTT microspheres not subjected to UVs, i.e., NorChit-DTT(UV-) microspheres. After the 8-day incubation period in either PBS or SGF, NorChit-DTT(UV-) microspheres presented a slight shift in size distribution towards higher diameters, probably due to particle aggregation or preferential degradation of smaller microspheres (Figure 4). Overall, microspheres presented a diameter of 5 ± 2 μm, which is in line with previous reports for chitosan microspheres.[11,12] Ultimately, the desired size for microspheres will depend on their intended application and/or route of administration. For instance, size may be less important for skin and gastrointestinal applications than in pulmonary therapies, where microspheres larger than 5 μm are retained in the nose and throat, whereas rapid clearance by macrophages occurs when particle size is between 1 and 5 μm.[18,19]

The potential of the NorChit-DTT(UV-) microspheres for bioconjugation was evaluated using a fluorescent model peptide. Fluorescence spectroscopy of the washed out peptide indirectly show that around 45% of initial peptide was grafted onto the microspheres, which corresponds to nearly 84 μg of peptide per mg of microspheres (Figure 5). To evaluate the amount of peptide adsorbed onto the microspheres surface, we performed the same assay, but without exposure to UV light, thus removing the trigger for bioconjugation. A high percentage (30%) of the peptide was still retained by the microspheres in these conditions, highlighting the occurrence of significant unspecific interactions between the peptide chain and the surface of the microspheres. Additionally, the fact that there is not a dramatic difference in the percentage of peptide retained by adsorption *versus* covalent grafting may be a further indication that, as previously hypothesized, there are only a few surface-exposed norbornene groups left for peptide grafting via TNPC after the thermal thiol-ene crosslinking that possibly occurs during the spray drying of NorChit-DTT(UV-) microspheres.

The percentage of peptide grafting onto the microspheres is approximately half of that previously reported by us when using classical thiol-maleimi-

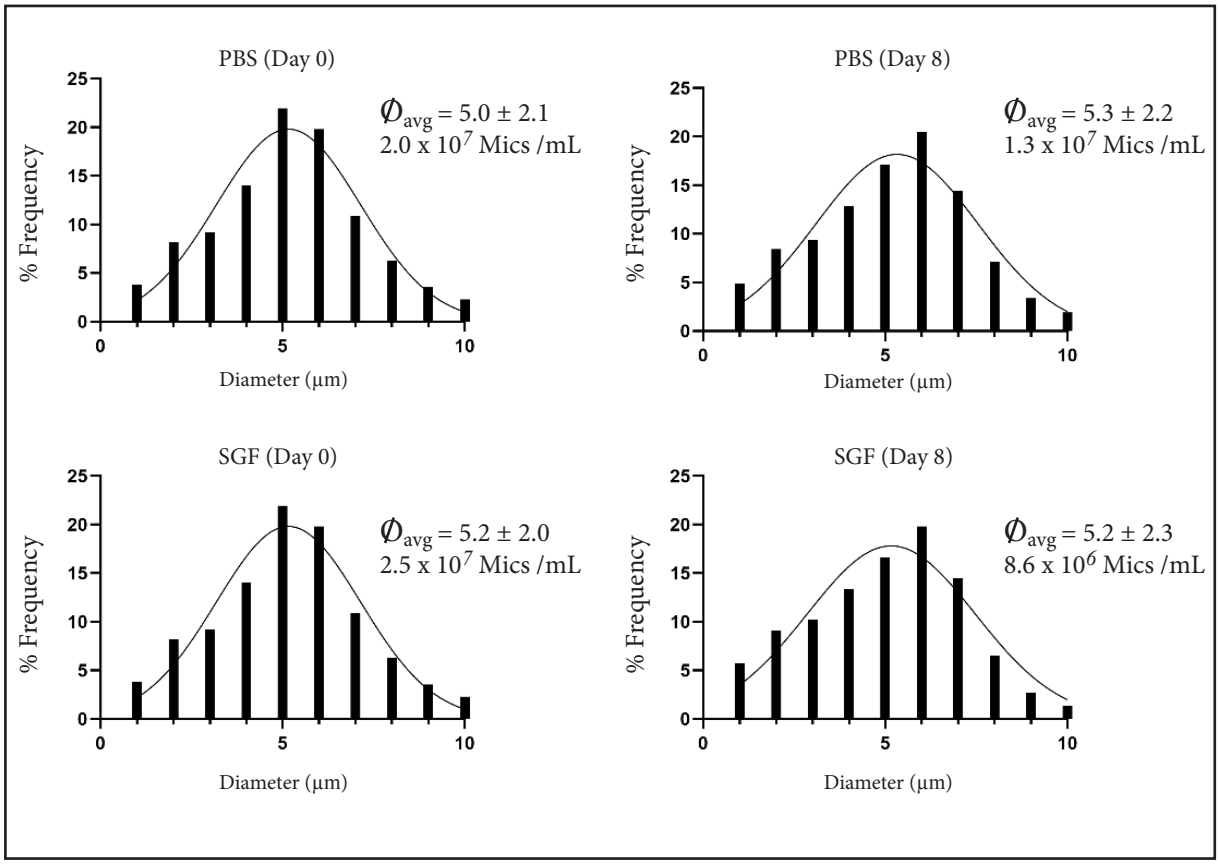

Figure 4. Size distribution and concentration of NorChit-DTT(UV-) microspheres at day 0 (left) and 8 (right) in PBS (pH 7.4, upper panel) and SGF (pH 1.2, bottom panel)

de Michael addition and the antimicrobial peptide MSI-78A (GIGKFLKKAKKFAKAFVKILKK-ahx-SH) bearing a PEG_{113} spacer (91%).[11] In turn, it is comparable to that of grafting the same MSI-78A peptide when bearing a PEG_2 spacer (51%), meaning that spacer length greatly impacts the conjugation efficiency. Of note, only a 16% of adsorbed peptide was observed in this previous study using thiol-maleimide Michael addition; yet, peptide MSI-78A was conjugated to chitosan-genipin microspheres functionalized with a PEG spacer that, by being non-fouling, decreases the likelihood of peptide adsorption to the surface.

In another work, poly(ε-caprolactone) (PCL): gelatin:(gelatin-methacryloyl) nanofiber microspheres were produced to conjugate bone morphogenic protein-2 (BMP-2)- and vascular endothelial growth factor (VEGF)-mimicking peptides through methacrylate phototriggered crosslinking.[20] Peptide grafting yield was between 46-48%, with around 32% adsorbed peptide, which is comparable to this work. Interestingly, the morphology of PCL-gelatin-methacryloyl microspheres was similar to that of NorChit-DTT ones, i.e., a smooth spherical surface with visible pockets, which may also explain the high retention of adsorbed peptide.

Altogether, this work demonstrates that, as compared to adsorption, peptide grafting onto NorChit-DTT(UV-) microspheres via TNPC enables to increase not only the amount of peptide retained by the microspheres, but also the stability of the peptide-particle assembly, by establishment of a covalent thioether link. This is of wide interest in biomedical engineering, as several applications of microparticles or microparticulate dressings have been described in the literature[21–27], although most of them involve the delivery of bioactive drugs or peptides that are embedded in the core of the particles, not grafted onto their surfaces. Therefore, this work opens the door for the straightforward production of peptide-conjugated chitosan microparticles that offer stability and, potentially, protection, of peptides for delivery even onto harsh environments in the body. Of note, chitosan microspheres are especially interesting for targeting bacterial biofilms, given their mucoadhesive properties that enable their binding to mucins present in the biofilm.[18] Thus, peptide-grafted microspheres as those herein reported are ideal candidates for the local treatment of complex skin and soft tissue infections.

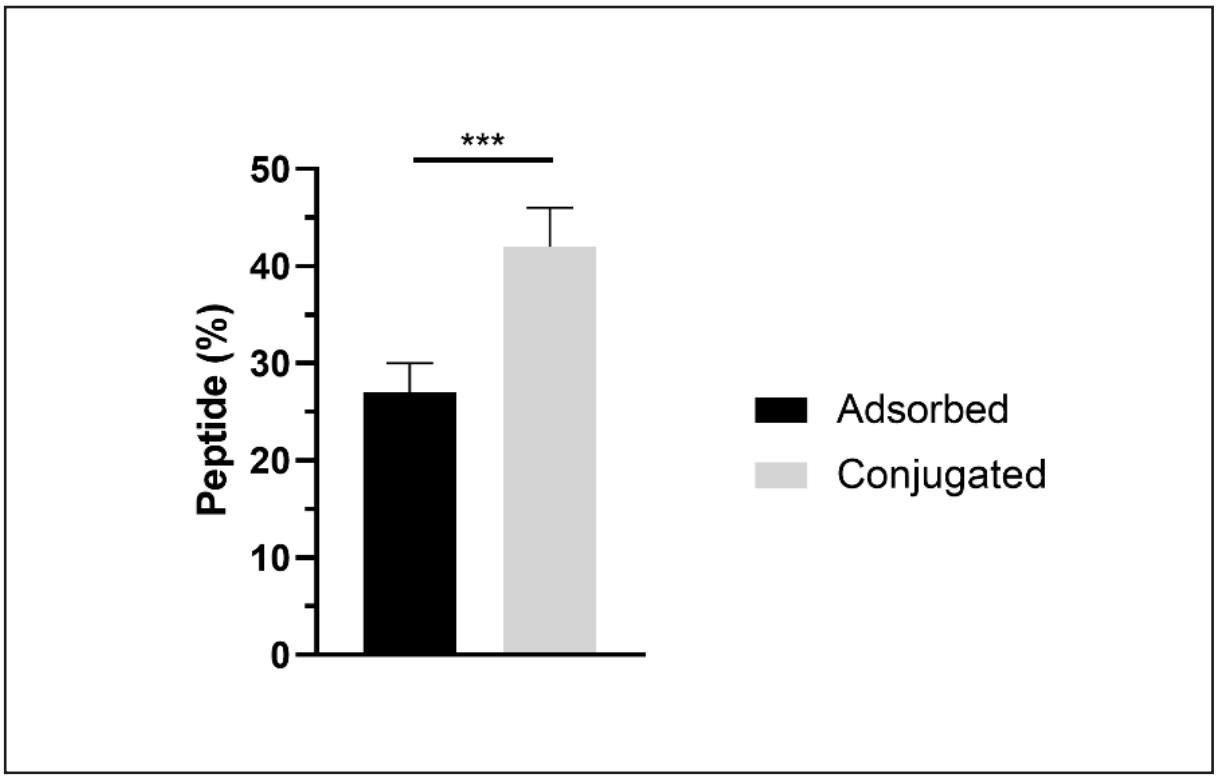

Figure 5. Relative amount of adsorbed and conjugated peptide-FITC in NorChit-DTT(UV-) microspheres, expressed as percentage of the initial peptide amount. ***$p<0.001$ (unpaired t-test, assuming Gaussian distribution)

Conclusion

Herein, the spray-drying of norbornene-chitosan microspheres was described. Smooth shape microspheres with pockets on the surface were obtained, which were stable in solution at neutral pH upon the inclusion of dithiothreitol (DTT), even without UV crosslinking. Thiol-ene conjugation of a model peptide was successful, increasing the amount of peptide retained by the microspheres, which have shown propensity to also adsorb the peptide. Overall, the norbornene-chitosan microspheres developed have the capacity of carrying bioactive peptides, which is of great interest for the therapeutic effect of bioactive peptides onto the target site with simultaneous protection from enzymatic degradation.

Funding

This work was financed by Portuguese funds through FCT/MCTES (Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Inovação) in the framework of the projects 2022.06048.PTDC (i3S), UIDB/50006/2020 (LAQV-REQUIMTE), LA/P/0045/2020 (ALiCE) and UIDB/00511/2020 (LEPABE). P.A. (SFRH/BD/145471/2019) and D.F. (SFRH/BD/146890/2019) doctoral grants, were financially supported by national (FCT/Norte 2020 Framework) and European Union (ESF – European Social Fund) funds. B.E. acknowledges FCT for the contract based on the "Lei do Emprego Científico" (DL 57/2016).

Maria Cristina L. Martins also acknowledges FCT (LA/P/0070/2020), project Bio2Skin Advanced (2021-24):NORTE-01-0247-FEDER-047225; and MOBILIsE Project, which has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement no. 951723.

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