Synthetic Communications

Facile Regioselective Synthesis of a Novel Chitosan-Pexiganan Conjugate with Potential Interest for the Treatment of Infected Skin Lesions

Mary K. S. Batista [ab]; Marçal Gallemí [a]; Alberto Adeva [c]; Carlos A. R. Gomes [b]; Paula Gomes [a]
[a] CIQUP, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal [b] LAQUIPAI, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal [c] Unitat de Síntesi de Pèptids, Serveis Científicotècnics de la Universitat de Barcelona, Parc Científic de Barcelona, Barcelona, Spain

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis
Taylor & Francis Group

Facile Regioselective Synthesis of a Novel Chitosan–Pexiganan Conjugate with Potential Interest for the Treatment of Infected Skin Lesions

Mary K. S. Batista,[1,2] Marçal Gallemí,[1] Alberto Adeva,[3] Carlos A. R. Gomes,[2] and Paula Gomes[1]

[1]CIQUP, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal
[2]LAQUIPAI, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal
[3]Unitat de Síntesi de Pèptids, Serveis Científicotècnics de la Universitat de Barcelona, Parc Científic de Barcelona, Barcelona, Spain

Abstract: A pexiganan–chitosan conjugate was designed to combine the exceptional bioadhesion and tissue-regenerating abilities of chitosan with the excellent antibiotic properties of pexiganan. We herein report our first results on the successful synthesis, including Fourier transform infrared (FT-IR) and amino acid analysis of such conjugate, which was prepared by regioselective covalent attachment of a Cys-containing pexiganan analog to the chitosan's amino groups. Further results from ongoing research will be reported.

Keywords: Antimicrobials, biopolymers, chitosan, diabetes, drug delivery, peptides, pexiganan

INTRODUCTION

Pexiganan (or MSI-78) is a 22-amino acid linear analog of magainin-2, a natural antimicrobial peptide isolated from the skin of the African clawed

frog, *Xenopus laevis*.[1–3] Pexiganan is remarkably effective against more than 3,000 species of gram-positive and gram-negative bacteria that cause infections on skin and soft tissues, including methicillin-resistant *Staphylococcus aureus* (MRSA), and has no propensity to induce bacterial resistance. In two phase III clinical trials involving 835 patients with infected diabetic foot ulcers, both topical pexiganan acetate 1% and oral ofloxacin (800 mg/day) achieved clinical cure or improvement in 90% of patients, so it rapidly underwent commercial development for topical application as a cream (pexiganan acetate 1%).[4] However, in 1990, the U.S. Food and Drug Administration denied its approval on the basis that its effectiveness in completely eradicating the pathogen (66% of treated patients) was not higher than that achieved by ofloxacin taken orally (82% of treated patients),[5] which led the industrial developer (Genaera Corporation, formerly Magainin Pharmaceutical Inc.) to abandon the production of pexiganan acetate. Even so, the very large and growing incidence of diabetes in northwestern countries, together with the lack of valuable topical antimicrobials to treat infected diabetic foot ulcers and the well-known problems associated with oral drug delivery, makes further exploration of pexiganan-based systems worthwhile, explaining the recent (October 2007) acquisition by the U.S. pharmaceutical corporation MacroChem of the exclusive option to license pexiganan worldwide.[6] This was followed by the latest (as of 13 March 2008) press release on new phase III clinical trials promoted by this company that confirm the efficacy of pexiganan to be statistically equivalent to that of oral antibiotic therapy.[7]

Taking advantage of our prior experience in the synthesis and study of antimicrobial peptides,[8–10] as well as in the development of novel chitosan-based biopolymers for biomedical applications,[11–13] we decided to break through to the preparation of a novel chitosan–pexiganan conjugate with potential application in the topical treatment of infected diabetic foot ulcers. We hope such a conjugate will combine the excellent antimicrobial properties of pexiganan with the well-known biocompatibility and skin-injury-healing ability of chitosan. Chitosan-based materials have been used for several biomedical purposes, including drug delivery, vascular grafts, cartilage regeneration, sutures, and wound-healing materials, because among chitosan's most appealing characteristics are its bioadhesive and self-aggregation properties, as well as its ability to enhance cell proliferation, thus promoting tissue regeneration.[14–25] In 2004, Gopinath et al. reported pexiganan-incorporated collagen matrices that led to improvement of tissue reconstruction in infected wounds in mice as compared to collagen films alone.[26] However, so far nothing has been attempted concerning the development of

pexiganan–biopolymer conjugates where this peptide is covalently attached to the polymer matrix. This communication reports our primary results concerning the synthesis and characterization by Fourier transform infrared (FT-IR) spectroscopy and amino acid analysis (AAA) of a covalent pexiganan–chitosan conjugate. Further results from ongoing physicochemical, morphological, and biological studies on this conjugate will be eventually published as a full paper.

MATERIALS AND METHODS

Chemicals

4-Methylbenzhydrylamine (MBHA) LL resin (100–200 mesh), p-{(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl}-phenoxyacetic acid (Fmoc-Rink) linker, Fmoc-protected amino acids (Fmoc-AA-OH), and N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) were all from NovaBiochem (VWR International, Lisbon, Portugal). (N-ε-Maleimidocaproyloxy)-sulfosuccinimide ester (Sulfo-EMCS) was from Pierce (Sopachem, Ap Ochten, the Netherlands). Low-molecular-weight chitosan from coarse ground crab (~150 kDa, degree of acetylation 0.10, 4.86 mmol –NH$_2$ groups/g; ref. 44.886-9), N-ethyl-N,N-diisopropylamine (DIEA), EDTA, piperidine, triisopropyl-silane (TIS), trifluoroacetic acid (TFA), KBr (p.a., for IR spectroscopy grade), and all remaining common chemicals and solvents (p.a. quality) were from Sigma-Aldrich (Lisbon, Portugal). The dialysis membrane (MW cutoff ~12–14 kDa) was from Medicel International (London, UK).

Instrumentation

FT-IR spectra were recorded from KBr pellets of pexiganan and pexiganan–chitosan on a Jasco FT/IR-460 instrument (each spectrum, run in duplicate, was obtained from 32 scans between 4000 and 400 cm^{-1} at a resolution of 4 cm^{-1}). AAA of peptide and peptide–chitosan hydrolysates was carried out on a Beckman 6300 amino acid analyzer. Mass spectra (MS) were obtained by the matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) technique on a MALDI-TOF Voyager DE Perspective spectrometer from Applied Biosystems. High-performance liquid chromatographic (HPLC) analyses were run on an analytical Beckman System Gold, and peptide purification was carried out on a preparative HPLC Waters DeltaPrep

4000 system, using C-18 reverse-phase columns. Peptide and peptide–chitosan conjugate were freeze-dried on a Christ alpha l-4 lyophilizer.

Peptide Synthesis

Pexiganan-Ala-Ala-Cys amide (Gly-Ile-Gly-Lys-Phe-Leu-Lys-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Leu-Lys-Lys-Ala-Ala-Cys-NH$_2$; molecular weight, 2721 Da) was synthesized manually on a Fmoc-Rink-MBHA resin by standard Fmoc/tBu solid-phase peptide synthesis (SPPS) strategies.[8,9] The target peptide was isolated from the crude mixture by preparative HPLC and characterized by analytical HPLC, AAA, and MALDI-TOF MS (data not shown).

Modification of Chitosan with the Sulfo-EMCS Cross-Linker

Chitosan ($\sim$10 μmol –NH$_2$ groups) was dissolved in acetic acid/sodium acetate buffer at pH 6.0 (1 ml), and phosphate-buffered saline (PBS) buffer (pH 7.4, 5 ml) was added to the solution together with EDTA (5 mmol). The mixture was put under vigorous stirring at 4 °C, and Sulfo-EMCS ($\sim$70 μmol) was then slowly added to the solution. The reaction was allowed to proceed at 4 °C for 30 min and then at room temperature for 90 min. The solution was then dialyzed against PBS over 24 hours (three buffer renewals) and then immediately used in the subsequent reaction.

Attachment of Pexiganan-Ala-Ala-Cys to the Modified Chitosan

The modified chitosan solution, obtained as described in the previous section, was put under vigorous stirring at 4 °C. The peptide (3.2 μmol) was then slowly added to the solution, and the reaction was allowed to evolve at 4 °C for 30 min and then at room temperature for a further 90 min. The reaction was quenched by addition of free cysteine 100 mM in PBS (1 ml) and further reaction for 15 min at room temperature. The final mixture was dialyzed against PBS (2 days, four buffer renewals) and then against deionized water (3 days, six solvent renewals), after which it was freeze-dried and characterized by FT-IR.

## Analysis of the Peptide and the Peptide–Chitosan Conjugate by AAA

The amount of peptide and its amino acid composition, for both the pure peptide and its chitosan conjugate, were determined by standard AAA

procedures.[8,9] Briefly, a known amount of the sample was submitted to acid hydrolysis in aqueous HCl 6 M containing 1 mM of aminobutyric acid as internal reference. The hydrolysis was carried out in a sealed glass tube at 100 °C and over 24 h, for full cleavage of peptide bonds and concomitant quantitative release of the amino acids. The hydrolysate was then evaporated to dryness, and the residue was retaken with a known volume of citric acid/sodium citrate buffer (60 mM, pH 2), of which, after adequate dilutions, a known aliquot was filtered through a nylon filter (Ø = 0.45 μm) and injected into the analyzer.

RESULTS AND DISCUSSION

The preparation of covalent peptide and protein conjugates is often accomplished by means of maleimide-based crosslinkers.[27,28] Other elegant approaches include enzyme-catalyzed conjugations, such as the preparation of a gelatin–chitosan conjugate through tyrosinase-mediated conversion of Tyr residues into their reactive o-quinone moieties.[29] Recently, Masuko et al. used N-ε-maleimidocaproyloxy-sulfosuccinimide ester (Sulfo-EMCS) to promote the attachment of bovine serum albumin (BSA) to thiolated chitosan.[30] Thus, we have investigated whether it was possible to prepare a pexiganan–chitosan conjugate by a similar procedure, but in our case using unmodified chitosan and a pexiganan analog having an additional C-terminal Ala-Ala-Cys segment to provide the peptide the Cys thiol necessary to accomplish the desired reaction (Scheme 1). The target peptide was successfully synthesized and isolated (not shown), and its attachment to unmodified chitosan was then promoted by Sulfo-EMCS, yielding a product that was characterized by FT-IR and AAA.

The FT-IR spectrum of chitosan (Fig. 1a) is characterized by a large and intense band at 3450–3200 cm^{-1} (hydrogen-bonded O-H stretching and N–H stretching bands, with maximal absorption at 3376 cm^{-1}) partially overlapped with C–H stretching bands at ∼2800 cm^{-1}; bands due to the N-acetyl-D-glucosamine (GlcNHAc) units, with amide I (C=O stretching) at 1645 cm^{-1}, amide II (N–H bending) at 1557 cm^{-1}, amide III (C−N stretching coupled with N–H plane deformation) at 1412 cm^{-1} and symmetrical angular deformation of –CH$_3$ at 1384 cm^{-1}; C–N stretching of the amino groups at 1325 cm^{-1}; O–H plane deformation at 1259 cm^{-1}; C–O stretching band at 1078 cm^{-1}; C–O–C stretching vibration in the glucopyranose ring at 1030 cm^{-1}; and the specific bands of the $\beta(1 \rightarrow 4)$ glycoside bridge at 1153 cm^{-1} and 899 cm^{-1}.[12,13] In comparison, the FT-IR spectrum of the peptide conjugate (Fig. 1b), while still displaying the characteristic bands due to the polysaccharide chains (O–H bending at
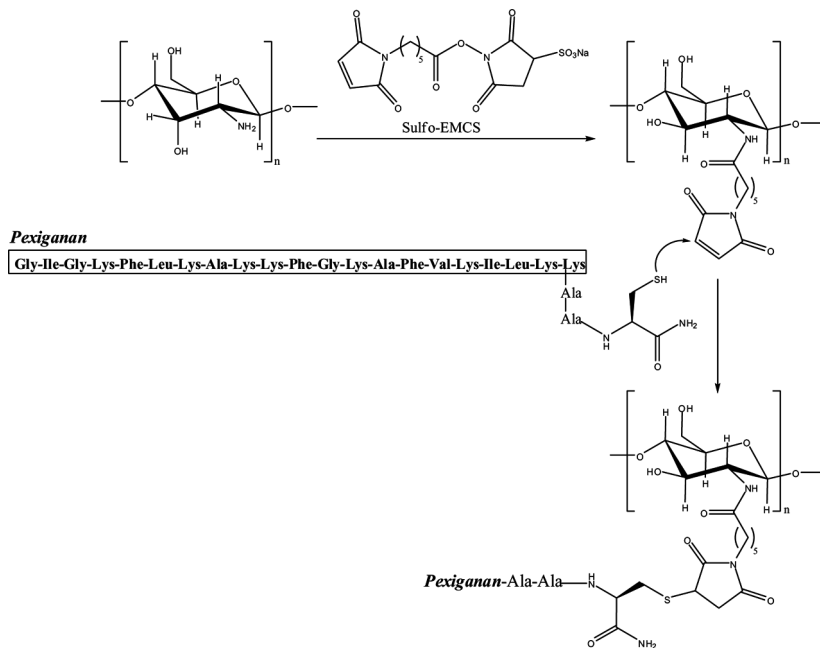
Scheme 1. Modification of chitosan D-glucosamine units by sulfo-EMCS and subsequent attachment of pexiganan-Ala-Ala-Cys through its *C*-terminal Cys thiol.

$1252\,cm^{-1}$, C–O stretching at $1079\,cm^{-1}$, C–O–C stretching vibration in the glucopyranose ring at $1036\,cm^{-1}$ and the specific bands of the $\beta(1 \to 4)$ glycoside bridge at $1153\,cm^{-1}$ and $899\,cm^{-1}$), it also exhibits significant differences as compared to the spectrum of the parent polymer. For instance, the large and intense O–H + N–H stretching band has its maximal absorption shifted down to $3289\,cm^{-1}$ ($\Delta = -87\,cm^{-1}$), which certainly reflects the significant contribution from N–H stretching vibrations due to the primary amino groups from the *N*-terminal amino acid and Lys side-chains (10 groups per peptide chain) and to the amide bonds (25 secondary plus 1 primary amide groups per peptide chain); this interpretation is reinforced by the changes registered in the amide I and amide II bands, whose relative intensities are dramatically changed from one spectrum to the other, as these are the two most intense bands in the spectrum of the peptide–chitosan conjugate, but not in the spectrum of unmodified chitosan. The wavenumbers for the amide I and amide II bands are also different in the conjugate, where they appear at 1653 ($\Delta = +8\,cm^{-1}$) and $1541\,cm^{-1}$ ($\Delta = -16\,cm^{-1}$), respectively. If we go further and take a look at the hyperfine structure of the conjugate's amide I band (inset in
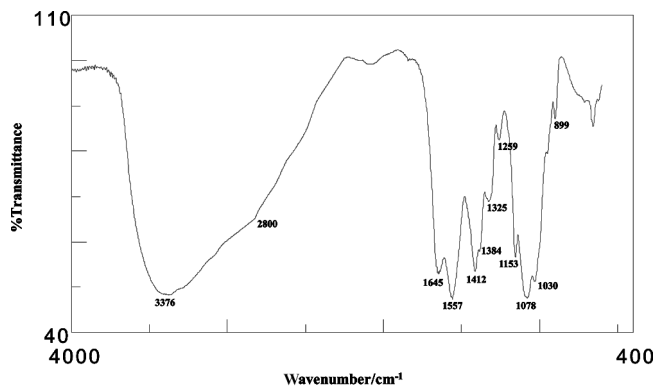
(a)
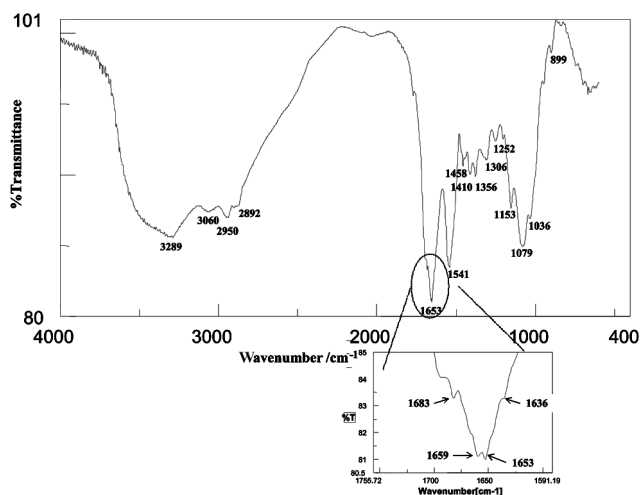
(b)

Figure 1. FT-IR spectra acquired from KBr pellets of (a) chitosan and (b) pexiganan–chitosan conjugate.

Fig. 1b), we find that it reflects the contributions of the different amide bonds present and of peptide secondary structure determinants. In fact, the bands at 1682, 1659, and $1636 \, cm^{-1}$ observed in the present case are close to those reported by Shanmugam et al. for the FT-IR absorption spectrum of pexiganan in methanol, respectively, at 1680, 1659, and $1630 \, cm^{-1}$,[31] with slight differences probably due to the additional *C*-terminal Ala-Ala-Cys stretch in our peptide. These authors have analyzed the structure of pexiganan in aqueous and different organic media using FT-IR absorption and other spectroscopic techniques and found

that this peptide exhibits conformational polymorphic behavior in different solvents, which is reflected on the wavenumbers associated to peaks in the amide I region. Thus, a comparison of our data with those from these authors would suggest the peptide adopt a dominant β-sheet structure with β-turn and minor α-helix content, when inserted into the chitosan matrix.[31] However, this hypothesis must be regarded with some caution, as our spectra were acquired from KBr pellets, a procedure that has been previously found to change protein structure from α-helix to β-sheet.[32] Additional information on peptide secondary structure within the chitosan matrix can be withdrawn from the amide III region. In fact, among the spectral regions arising out of vibrational modes of amide bonds, amide I and amide III bands are the most sensitive to variations in secondary structure folding. The amide I region from spectra of proteins and peptides suffers from several limitations, including interference with bands from water or hydroxylated solvents, and a relatively unstructured contour due to overlap of orbital bands that arise from various secondary structures; yet, it is still the most commonly used for secondary structure analysis, given its high intensity. In contrast, the corresponding amide III region ($\sim$1350–1200 cm^{-1}), although fairly weak in intensity, is not affected by these constraints, so it should be preferred to gain reliable information on peptide secondary structure.[32,33] This region is quite different between both spectra displayed in Figs. 1a and b, attesting the significant alterations brought by peptide insertion. Thus, amide III bands arise at 1356–1306 cm^{-1} in the peptide conjugate, which, according to Cai and Singh, reflects a predominant peptide α-helical structure.[33] This interpretation follows the opposite direction of that given by the analysis of the amide I region, reinforcing the need to analyze these data with caution, at least concerning peptide secondary structure predictions.

Finally, differences in the C–H stretching region are also noteworthy: in contrast to what is observed in the spectrum of chitosan, the FT-IR spectrum for the peptide conjugate exhibits three perfectly defined bands at 3060, 2950, and 2892 cm^{-1}, attributed to C–H stretching vibrations in, respectively, aromatic, methylene, and methyl groups, most probably from the side chains of the aromatic amino acid Phe (three per peptide chain) and of aliphatic amino acids such as Leu, Ile, Val, Ala and Lys. C–C (aliphatic and aromatic) stretching vibrations are hindered by amide I and II bands, but a C–C in-ring stretching, typical of aromatics, can still be distinguished at 1458 cm^{-1}.

In view of this, FT-IR data alone definitely support the successful insertion of the peptide into the chitosan matrix. Still, characterization of the peptide-chitosan conjugate by AAA provided the ultimate proof of a profitable attachment of the antimicrobial peptide to the polymer.

Thus, as shown in Table 1, acid hydrolysis of the product allowed determining the amino acids whose identities and relative proportions fully agree with what was expected from the peptide sequence.

Overall, we established an easy, fast, and profitable method for the covalent attachment of pexiganan to chitosan. An alternative approach would be the synthesis of the peptide as a C-terminal carboxyl and then its attachment to the chitosan's amino groups by means of condensation agents such as a water-soluble carbodiimide,[34] which has been used in polymer chemistry as a cross-linking agent[35–37] and also to attach other peptides to the chitosan matrix.[38] However, both the peptide N-terminal α-amine and Lys-side chain ε-amino groups would be reactive under those conditions, and extensive cross-linking between peptide chains would most probably occur. To overcome this problem, a fully protected peptide analog, assembled on an acid hyperlabile resin such as 2-chloro-trityl chloride (2-CTC) resin,[39] could be used; but then peptide's solubility in aqueous media would be minimal, so the condensation to chitosan should be carried out in a suitable organic solvent such as DCM. This, in turn, would require the conversion of chitosan (insoluble in current organic media) into a suitable organic-soluble derivative, such as O-trityl-chitosan.[40–43] Taken together, these considerations show that our route for peptide insertion into the polymer matrix is incontestably advantageous, as it is regioselective, where only the sulfo-EMCS-activated amino groups of chitosan will be able to react exclusively with the peptide Cys-side chain thiol groups. This offers an efficient and controlled attachment of the peptide with minimal, if any, perturbation of its bioactive sequence.

Table 1. Amino acid composition of the peptide–chitosan conjugate

Amino acidresidue[a]	Concentration[b] (mM)	Relative proportion	
		Experimental	Expected[c]
Gly	0.4240	3.25	3
Ala	0.5230	4.00	4
Val	0.1271	0.97	1
Ile	0.2461	1.88	2
Leu	0.2596	1.99	2
Phe	0.3899	2.98	3
Lys	1.661	8.93	9

[a]Cys was not considered, as its quantitation by AAA is not reliable.
[b]In injected sample, after known dilutions of the original hydrolysate.
[c]From the pexiganan-Ala-Ala-Cys amino acid sequence.

CONCLUSION

We have successfully achieved the synthesis (FT-IR and AAA) of a pexiganan–chitosan conjugate prepared by regioselective attachment of a Cys-containing pexiganan analog to the chitosan's amino groups. The conjugate will most probably find clinical application for topical treatment of infected diabetic foot ulcers and other skin injuries, as it combines the well-known antimicrobial potency of pexiganan with the remarkable properties of chitosan for bioadhesion and tissue regeneration. Results from ongoing biological studies toward this goal will be reported in due course.

ACKNOWLEDGMENTS

REFERENCES

1. Ge, Y.; McDonald, D. L.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M. In vitro antibacterial properties of Pexiganan, an analog of Magainin. *Antimicrob. Agents Chemother.* **1999**, *43*, 782–788.
2. Jenssen, H.; Hamill, P.; Hancock, R. E. W. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **2006**, *19*, 491–511.
3. Giuliani, A.; Pirri, G.; Nicoletto, S. F. Antimicrobial peptides: An overview of a promising class of therapeutics. *Central Eur. J. Biol.* **2007**, *2*, 1–33.
4. Lamb, H. M.; Wiseman, L. R. Pexiganan acetate. *Drugs* **1998**, *56*, 1047–1052.
5. Moore, A. The big and small of drug discovery: Biotech versus pharma: Advantages and drawbacks in drug development. *EMBO Rep.* **2003**, *4*, 114–117.
6. BIOTECH Patent News. Accessed 27 February 2008. http://www.entrepreneur.com/tradejournals/article/168795681.html
7. MacroChem Corp. Accessed 17 March 2008. http://www.bio-medicine.org/medicine-technology-1/MacroChem-Announces-Presentation-of-Pexiganan-Phase-3-Study-Results-for-Topical-Treatment-of-Diabetic-Foot-Infection-1515-1/
8. Abrunhosa, F.; Faria, S.; Gomes, P.; Thomaz, I.; Pessoa, J. C.; Andreu, D.; Bastos, M. Interaction and lipid-induced conformation of two cecropin–melittin hybrid peptides depends on peptide and membrane composition. *J. Phys. Chem. B* **2005**, *109*, 17311–17319.

9. Bastos, M.; Bai, G.; Gomes, P.; Andreu, D.; Goormaghtigh, E.; Prieto, M. Energetics and partition of two cecropin-melittin hybrid peptides to model membranes of different composition. *Biophys. J.* **2008**, *94*, 2128–2141.

10. Adão, R.; Seixas, R.; Gomes, P.; Pessoa, J. C.; Bastos, M. Membrane structure and interactions of a short Lycotoxin I analogue. *J. Pept. Sci.* **2008**, *14*, 528–534.

11. Kogan, G.; Skorik, Y. A.; Žitňanova, I.; Križková, L.; Ďuračková, Z.; Gomes, C. A. R.; Yatluk, Y. G.; Krajčovič, J. Antioxidant and antimutagenic activity of N-(2-carboxyethyl)chitosan. *Toxicol. Appl. Pharm.* **2004**, *201*, 303–310.

12. Batista, M. K. S.; Pinto, L. F.; Gomes, C. A. R.; Gomes, P. Novel highly-soluble peptide–chitosan polymers: Synthesis and spectral characterization. *Carbohydr. Polym.* **2006**, *64*, 699–305.

13. Gomes, P.; Gomes, C. A. R.; Batista, M. K. S.; Pinto, L. F.; Silva, P. Synthesis, structural characterization, and properties of water-soluble *N*-(γ-propanoyl-amino acid)-chitosans. *Carbohydr. Polym.* **2008**, *71*, 54–65.

14. Ping, H.; Daves, S. S.; Illum, S. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *Int. J. Pharm.* **1998**, *166*, 75–88.

15. Gupta, K. C.; Ravikumar, M. N. V. Drug release behavior of beads and microgranules of chitosan. *Biomaterials* **2000**, *21*, 1115–1119.

16. Bernkop-Schnürch, A. Chitosan and its derivatives: Potential excipients for peroral peptide delivery systems. *Int. J. Pharm.* **2000**, *194*, 1–13.

17. Bernkop-Schnürch, A.; Kast, C. E. Chemically modified chitosans as enzyme inhibitors. *Adv. Drug Deliv. Rev.* **2001**, *52*, 127–137.

18. Berger, J.; Reist, M.; Mayer, J. M.; Felt, O.; Gurny, R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 35–52.

19. Berger, J.; Reist, M.; Mayer, J. M.; Felt, O.; Peppas, N. A.; Gurny, R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 19–34.

20. Iwasaki, N.; Yamane, S.-T.; Majima, T.; Kasahara, Y.; Minami, A.; Harada, K.; Nonaka, S.; Maekawa, N.; Tamura, H.; Tokura, S.; Shiono, M.; Monde, K.; Nishimura, S.-I. Feasibility of polysaccharide hybrid materials for scaffolds in cartilage tissue engineering: Evaluation of chondrocyte adhesion to polyion complex fibres prepared from alginate and chitosan. *Biomacromolecules* **2004**, *5*, 828–833.

21. Amaral, I. F.; Lamghari, M.; Sousa, S. R.; Sampaio, P.; Barbosa, M. A. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. *J. Biomed. Mat. Res.* **2005**, *75A*, 387–397.

22. Amaral, I. F.; Sampaio, P.; Barbosa, M. A. Three-dimensional culture of human osteoblastic cells in chitosan sponges: The effect of the degree of acetylation. *J. Biomed. Mat. Res.* **2005**, *76A*, 335–346.

23. Krauland, H.; Guggi, D.; Bernkop-Schnürch, A. Thiolated chitosan microparticles: A vehicle for nasal peptide drug delivery. *Int. J. Pharm.* **2006**, *307*, 270–277.

24. Martien, R.; Loretz, B.; Bernkop-Schnürch, A. Oral gene delivery: Design of polymeric carrier systems shielding toward intestinal enzymatic attack. *Biopolymers* **2006**, *83*, 327–336.

25. Kotzé, A. F.; Lueβen, H. L.; Thanou, M. M.; Verhoef, J. C.; de Boer, A. G.; Junginger, H. E.; Lehr, C.-M. Chitosan and chitosan derivatives as absorption enhancers for peptide drugs across mucosal epithelia. In: *Bioadhesive Drug Delivery Systems: Fundamentals, Novel Approaches, and Development*; E. Mathiowitz, D. E. Chickering, C.-M. Lehr (Eds.); Marcel Dekker: New York, 1999, pp. 341–387.

26. Gopinath, D.; Kumar, M. S.; Selvaraj, D.; Jayakumar, R. Pexiganan-incorporated collagen matrices for infected wound-healing processes in rat. *J. Biomed. Mat. Res.* **2005**, *73A*, 320–331.

27. Xiao, S.-J.; Wieland, M.; Brunner, S. Reactions of surface amines with heterobifunctional cross-linkers bearing both succinimidyl ester. *J. Coll. Interface Sci.* **2005**, *290*, 172–183.

28. Mochizuki, M.; Yamagata, N.; Philp, D.; Hozumi, K.; Watanabe, T.; Kikkawa, Y.; Kadoya, Y.; Kleinman, H. K.; Nomizu, M. Integrin-dependent cell behaviour on ecm peptide-conjugated chitosan membranes. *Biopolymers* **2007**, *88*, 122–130.

29. Chen, T. H.; Embree, H. D.; Payne, G. F. In vitro protein–polysaccharide conjugation: tyrosinase-catalyzed conjugation of gelatin and chitosan. *Biopolymers* **2002**, *64*, 292–302.

30. Masuko, T.; Minami, A.; Iwasaki, N.; Majima, T.; Nishimura, S.-I.; Lee, Y. C. Thiolation of chitosan: Attachment of proteins via thioether formation. *Biomacromolecules* **2005**, *6*, 880–884.

31. Shanmugam, G.; Polavarapu, P. L.; Gopinath, D.; Jayakumar, R. The structure of antimicrobial pexiganan peptide in solution probed by FT-IR absorption, vibrational circular dichroism, and electronic circular dichroism spectroscopy. *Biopolymers* **2005**, *80*, 636–642.

32. Kaiden, K.; Matsui, T.; Tanaka, S. A study of the amide III band by FTIR spectrometry of albumin, myoglobin, and γ-globulin. *Appl. Spectrosc.* **1987**, *41*, 180–184.

33. Cai, S.; Singh, B. R. Identification of β-turn and random coil amide III infrared bands for secondary structure estimation of proteins. *Biophys. Chem.* **1999**, *80*, 7–20.

34. Sheehan, C.; Preston, J.; Cruishank, P. A. A rapid synthesis of oligopeptide derivatives without isolation of intermediates. *J. Am. Chem. Soc.* **1965**, *87*, 2492–2493.

35. Bodnar, M. J.; Hartman, F.; Borbely, J. Synthesis and study of cross-linked chitosan-N-poly(ethyleneglycol) nanoparticles. *Biomacromolecules* **2006**, *7*, 3030–3036.

36. Lee, Y.; Jo, W. H. Physicochemical characteristics of self-aggregates of hydrophobically modified chitosans. *Langmuir* **1998**, *14*, 2329–2332.

37. Damink, H. H. O.; Dijkstra, P. J.; van Luyn, M. J. A.; van Wachem, P. B.; Nieuwenhuis, P.; Feijen, J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **1996**, *17*, 765–773.

38. Hojo, K.; Maeda, M.; Mu, Y.; Kamada, H.; Tsutsumi, Y.; Nishiyama, Y.; Yoshikawa, T.; Kurita, K.; Block, L. H.; Mayumi, T.; Kawasaki, K. Preparation of a chitosan hybrid of an antimetastatic laminin-related peptide. *Pharm. Pharmacol. Commun.* **1999**, *5*, 277–280.

39. Bray, L. Large-scale manufacture of peptide therapeutics by chemical synthesis. *Nature Rev. Drug Discov.* **2003**, *2*, 587–593.

40. Kurita, K.; Ikeda, H.; Yoshida, Y.; Shimojoh, M.; Harata, M. Chemoselective protection of the amino groups of chitosan by controlled phthaloylation: Facile preparation of a precursor useful for chemical modifications. *Biomacromolecules* **2002**, *3*, 1–4.

41. Kurita, K.; Hayakawa, M.; Nishiyama, Y.; Harata, M. Polymeric asymmetric reducing agents: Preparation and reducing performance of chitosan/dihydronicotinamide conjugates having D- and L-phenylalanine spacer arms. *Carbohydr. Polym.* **2002**, *47*, 7–14.

42. Holappa, J.; Nevalainen, T.; Soininen, P.; Elomaa, M.; Safin, R.; Másson, M.; Järvinen, T. N-chloroacyl-6-O-triphenylmethylchitosans: Useful intermediates for synthetic modifications of chitosan. *Biomacromolecules* **2005**, *6*, 858–863.

43. Holappa, J.; Nevalainen, T.; Soininen, P.; Másson, M.; Järvinen, T. Synthesis of novel quaternary chitosan derivatives via N-chloroacyl-6-O-triphenyl-methylchitosans. *Biomacromolecules* **2006**, *7*, 407–410.