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Can the interaction between the antimicrobial peptide LL-37 and alginate be exploited for the formulation of new biomaterials with antimicrobial properties?

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ABSTRACT

In this study, we took advantage of the strong interaction between the antimicrobial peptide LL-37 and anionic polysaccharides, such as alginate, to design and evaluate a new biomaterial with putative antibacterial properties.

To begin with, we have investigated the effect of different biocompatible polysaccharides on both the cytotoxicity and the antimicrobial activity of LL-37, a powerful endogenous antimicrobial peptide of human origin, whose use in therapy has been hampered by its toxicity to host cells. Interactions of the peptide with polysaccharides were evaluated by circular dichroism analyses, which revealed a different capacity of the polymers to induce the active helical conformation in the peptide. Among the polysaccharides tested, sodium alginate was the only one that significantly reduced the toxicity of LL-37 toward mammalian cells. A sodium alginate/LL-37 preparation was then tested on four bacterial strains. The Gram-negative bacteria resulted susceptible to the mixture, while the growth of the Gram-positive ones was poorly affected and only at the highest peptide concentration tested. Following the positive results with Gram-negative species, the alginate/LL-37 binary system was used for the preparation of calcium alginate beads, which were tested for peptide release.

1. Introduction

In the last few years, the number of clinical isolates resistant to the antibiotics in use has dramatically increased, producing, as a consequence, a need for antibacterial compounds with novel mechanisms of action (Livermore, 2004; Payne, 2008; Spellberg et al., 2008; Theuretzbacher, 2009; Vicente et al., 2006). In this perspective, a promising class of molecules is that of AntiMicrobial Peptides (AMPs) (Brogden, 2005). These are ancient components of the innate immune system and represent a first line of defense against bacterial infections in plants, invertebrates and vertebrates, including humans (Boman, 1995; Bowdish, Davidson, Speert, & Hancock, 2004; Giangaspero, Sandri, & Tossi, 2001; Nicolas & Mor, 1995). AMPs have been shown to be effective against drug-resistant pathogens of both bacterial (Hancock & Chapple, 1999) and fungal (Tanida, Okamoto, Ueta, Yamamoto, & Osaki, 2006) origin. Another advantage is that they are in general not antigenic (Hancock & Lehrer, 1998) and, in addition, they may modulate various aspects of the immune response (Tjabringa, Rabe, & Hiemstra, 2005) at variance with conventional antibiotics.

Among antimicrobial peptides, LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (Dürr, Sudheendra, & Ramamoorthy, 2006) is of particular interest for its biological properties. It is the only human peptide belonging to the cathelicidin family, a heterogeneous group of antimicrobial peptides characterized by a common pro-region in their precursors (Zanetti, Gennaro, & Romeo, 1995) and is endowed with a powerful antibacterial activity (Zanetti, 2004). In common with many other AMPs, LL-37 can assume an amphipathic α-helical conformation, with a moderate hydrophilicity (0.56) according to the Hopp and Woods scale (Hopp & Woods, 1981). The presence of both positively and negatively charged residues confers to the peptide a tendency to assume a helical structure even in water (Johansson, Gudmundsson, Rottenberg, Berndt, & Agerberth 1998). In such an ordered conformation, hydrophobic and hydrophilic residues are localized on opposite sides of the helix surface and the net positive charge (+6 at physiological pH) allows the interaction of the peptide with the negatively charged surfaces of bacteria and fungi.

The wide spectrum of antibacterial activity and the LPS-neutralizing effect of LL-37 would make it a molecule of choice for

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treatment of bacterial infections and, particularly so, in the presence of antibiotic-resistant strains (Hiemstra, 2007; Shai, 2002). Unfortunately, the significant toxicity of LL-37 to mammalian cells has so far hampered its use in clinical contexts. In fact, LL-37, at variance with many other antimicrobial peptides, is not very selective, with Minimal Inhibitory Concentration values (MIC) ranging from 1 to 10 μM for a variety of Gram-positive and Gram-negative bacteria, and eukaryotic cytotoxicity occurring at 13–25 μM *in vitro* (Johansson et al., 1998), as indicated by its significant haemolytic activity and its toxicity for human leukocytes and T lymphocytes (Ciornei, Sigurdardòttir, Schmidtchen, & Bodelsson, 2005).

AMPs are of interest for biomaterials science, since they yield promising results in the preparation of biomaterials endowed with antimicrobial properties, to contrast, for instance, infections associated with implant surgery (Shukla et al., 2010; Statz, Park, Chongsiriwatana, Barron, & Messersmith, 2008). Some attempts have been made to exploit specifically LL-37 in the preparation of antimicrobial surfaces, either by covalently linking it to the material (Gabriel, Nazmi, Veerman, Amerongen, & Zentner, 2006) or by entrapping it during the scaffold preparation, thus allowing its release after implantation (Izquierdo-Barba et al., 2009; Steinstraesser, Ring, Bals, Steinau, & Langer, 2006). Results reported in these papers are preliminary but encouraging.

A common mechanism shared by bacteria to try to limit the effect of cationic AMPs consists in the release of polyanions, namely exopolysaccharides like alginate, which act as an "auxiliary bacterial membrane" (Chan, Burrows, & Deber, 2005), limiting the accessibility of the peptides to the bacterial surface and thus decreasing their antibiotic efficacy (Llobet, Tomás, & Bengoechea 2008).

In this study we investigated the possibility to take advantage of this bacterial defense mechanism and exploited the electrostatic interaction of LL-37 with differently charged polysaccharides, with the aim of modulating the peptide availability and reducing its cytotoxicity by avoiding a too rapid and massive interaction with eukaryotic membranes. In particular, we focussed our attention onto three polysaccharides (PS), whose repeating units are reported in Table 1, namely alginate (Herasimenka et al., 2005), hyaluronic acid and ChitLac, a derivative of chitosan prepared by reductive *N*-alkylation with lactose (Donati et al., 2005; Yalpani & Hall, 1984). All of these have excellent biocompatibility properties, and would be suitable for further biomedical applications.

Alginate and hyaluronic acid are polyanions, while ChitLac is a low charge density polycation. At neutral pH, the average distance between the projection of charges on the polymer axis *b*, is 4.66 Å for alginate, 9.5 Å for hyaluronic acid and 5.85 Å for chitosan, which correspond to a (dimensionless) linear charge density (ξ) of 1.53, 0.75 and 1.22, respectively. The introduction of the lactitol group on chitosan to give ChitLac causes a decrease of the charge density, which becomes approximately 0.32 at neutral pH. As LL-37 is a cation with a net charge of +6 at neutral pH, an attraction is expected to build up with alginate and hyaluronic acid, and a repulsion with ChitLac, although the presence of several negatively charged residues in the peptide might in some way mediate the interaction with this latter carbohydrate polymer.

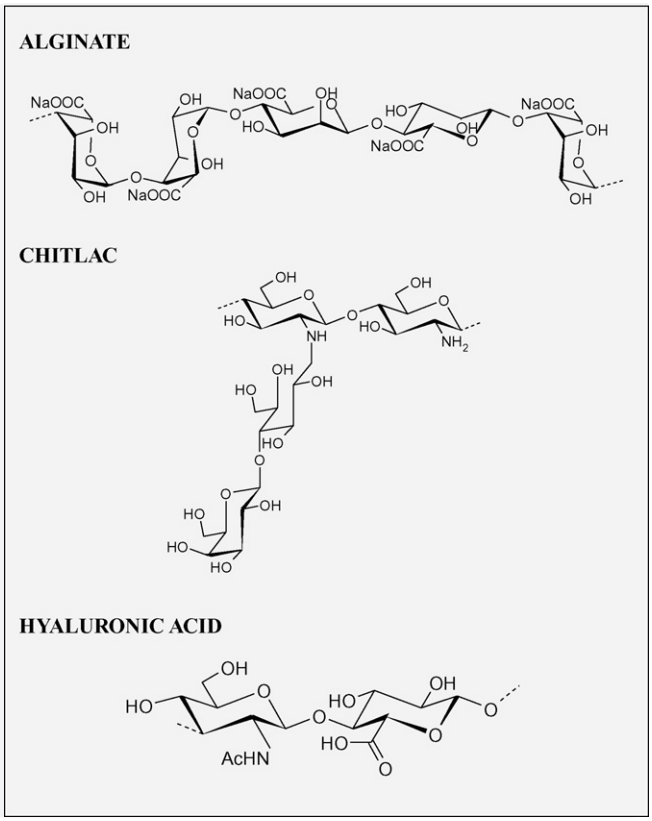
The study of the mechanism of interaction of AMPs with bacterial capsules (Campos et al., 2004; Domenico, Salo, Cross, & Cunha, 1994; Llobet et al., 2008) and exopolysaccharides (Benincasa et al., 2009; Foschiatti, Cescutti, Tossi, & Rizzo, 2009; Herasimenka et al., 2005) is a key point for the modulation of their activity.

It has been demonstrated that the interaction of LL-37 with sodium alginate induces conformational changes in the peptide structure by promoting α-helix formation and self-association (Chan, Burrows, & Deber 2004; Foschiatti et al., 2009; Herasimenka et al., 2005; Yeaman & Yount, 2003). The proposed mechanism involves the neutralization of the positive charge by the anionic polysaccharide, which in turn causes a more dominant role of the peptide hydrophobicity (Chan et al., 2004, 2005). In fact, peptide structuring exposes the hydrophobic sector on the helix surface that may cause self-association of peptide molecules via hydrophobic interactions. Therefore, the interaction can be considered a consequence of both a hydrophilic and a hydrophobic effect. At variance with moderately hydrophilic peptides, such as LL-37, for highly hydrophobic peptides such as the model peptide KKAAAAAAAAAAAAWAAAAAAKKKK-NH_2, helix induction is essentially caused by hydrophobic interactions with hydrophobic microdomains of sodium alginate (Chan et al., 2004).

Binding to glycosaminoglycans like heparin and dermatan sulphate also has an effect similar to that of sodium alginate (Andersson et al., 2004). This interaction causes, as a general consequence, a decreased antimicrobial efficacy of the AMPs, and it is supposed to be the cause of failure of their antimicrobial activity in microenvironments such as biofilms, of which exopolysaccharides are major components.

Starting from the above considerations, we tested the variation of cytotoxicity of LL-37 when sodium alginate, hyaluronic acid or ChitLac are added to the peptide solution. The most promising system was evaluated in terms of its antimicrobial effect on four different bacterial species and exploited for the preparation of beads.

Table 1
Structure of the polysaccharides used in this study.



2. Materials and methods

ChitLac (CAS registry number 85941-43-1) was synthesized starting from commercial chitosan from Aldrich, USA (degree of acetylation 11%, $[\eta] = 6.43$ dL/g, viscosity-average relative molar mass approximately 6.9×10^5) (Donati et al., 2004). The used degree of substitution was 70%, which leaves 19% of free amines. The estimated relative molar mass of ChitLac was 1.5×10^6. Algi-

nate (sodium salt) from *Laminaria hyperborea* LF 10/60 (F_G = 0.69; F_{GG} = 0.56; $[\eta]$ = 5.46 dL/g, viscosity-average relative molar mass approximately 1.3×10^5) (Donati et al., 2004) was from Protanal (Norway). Hyaluronic acid ($[\eta]$ = 15 dL/g, viscosity-average relative molar mass approximately 7.75×10^5) was from FMC Biopolymer AS/NovaMatrix (Japan). Dialysis membranes with cut-off of 12,000 were purchased from Sigma–Aldrich Co. (St. Louis, MO).

2.1. Synthesis of LL-37

Solid phase peptide synthesis was performed using the Fmoc-chemistry on a Liberty Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC, USA). The synthesis was carried out at a 0.05 mmol scale (208 mg of PEG-PS resin with a substitution of 0.24 mequiv./g). Double coupling with 2-(6-chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) as an acylating agent was carried out at all positions. The peptide was cleaved from the resin and deprotected using a mixture consisting of trifluoroacetic acid, water, and triisopropylsilane (95:2.5:2.5, v/v/v). The crude peptide was obtained in high yield and was precipitated and washed several times with chilled *tert*-butylmethyl ether. Its mass was confirmed by ESI-MS using a Bruker Esquire 4000 instrument (Bruker Daltonics, Billerica, MA, USA). No further purification was necessary since homogeneity of the peptide was >95% by analytical RP-HPLC.

2.2. Circular dichroism

Circular dichroism measurements were carried out with a Jasco-700A spectro-polarimeter in the wavelength range between 205 and 370 nm, using a quartz cuvette with 1 cm optical path. Spectra were recorded in PBS buffer, pH 7.4, with a value of the ionic strength corresponding to 150 mM (Phosphate solution at Ionic strength of 150 mM, PI-150) and in 5 mM sodium phosphate buffer, pH 7.4, with a value of ionic strength corresponding to 20 mM (PI-20). The former ionic strength was selected as it corresponds to standard physiological conditions, the latter one for comparison with the conditions used by others (Chan et al., 2004, 2005).

Polysaccharides were solubilised both in PI-150 and in PI-20 at a concentration of 12.8 mM of the repeating unit. Solutions of LL-37 were also prepared in the two buffers. The PSs were stepwise added to each of the LL-37 solutions so as to have a constant peptide concentration of 10 μM and the following polysaccharide concentrations: 50.4, 100.8, 151.8, 201.6, 302.4, 403.2, and 504 μM.

2.3. MTT assay

Cell viability was evaluated in terms of mitochondrial oxidative capacity, by the MTT (3,4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide) assay. The experiments were performed by using two osteoblast-like cell lines, MG-63 and Saos-2, treated with LL-37 alone and in mixture with alginate, ChitLac or hyaluronic acid.

After 24 h from seeding, the cells were treated with solutions of increasing concentration of LL-37 in Dulbecco's Modified Eagle Medium (DMEM), in the absence or presence of alginate and hyaluronan.

The cells (5000 cells/well) were plated in a 96-well microtiter plate and incubated for 24 h at 37 °C. After incubation, the MTT solution was added at a final concentration of 0.5 mg/mL and the plates were further incubated for 4 h at 37 °C in 5% CO_2 atmosphere. The MTT-containing medium was then removed and 100 μL of dimethyl sulfoxide (DMSO) per well were added to solubilise violet formazan crystals. Absorbance was measured in a microplate reader (Tecan Trading AG, Switzerland) at 620 nm, and cell viability was expressed as percent relative to untreated control.

2.4. Antibacterial activity

The antibacterial activity of LL-37 alone and mixed with polysaccharides was tested on four bacterial strains: two Gram-negative, namely *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and two Gram-positive, i.e. *Staphylococcus aureus* ATCC 25923 and a clinical isolate of *Staphylococcus epidermidis*. All bacterial strains were grown in Mueller-Hinton (MH) broth at 37 °C.

The inhibition of the bacterial growth was evaluated with a Tecan microplate reader (Tecan). Mid-log-phase bacterial suspensions were dispensed in triplicate in microtiter plates at approximately 1×10^6 cells/mL in 20% MH broth in the presence or absence of LL-37 and polysaccharides (final volume of 200 μL). Microtiter plates were then incubated for 4 h at 37 °C in the plate reader with 5 s shaking every 5 min and recording of the OD_{620} every 10 min.

2.5. LL-37 release from beads

Beads were prepared by blending the mixture of alginate and hydroxyapatite with an aqueous solution of LL-37 peptide. This solution (2.5 mL), containing alginate 2% (w/v), hydroxyapatite 3% (w/v) and LL-37 0.5 mg/mL (110 μM, MW 4493.3 g/mol), was dripped manually with a syringe into 400 mL of 50 mM $CaCl_2$ under stirring for 30 min. Obtained beads presented an average diameter of about 2 mm. At the end, the beads were removed and washed with water.

To achieve the release profile, the beads were divided in several fractions containing 15 beads each. These fractions were incubated with 1 mL of PBS buffer and the beads were removed and hydrolysed under reflux with HCl at regular time intervals. Quantitative analysis of the released arginine was performed by capillary electrophoresis (CE-UV conditions: 100 mM borate buffer; pH 8.95; 15 kV; fused silica capillary of a total length of 64 cm; effective length of 56 cm; internal diameter 50 μm; detection wavelength 195 nm). The percentage of released peptide was calculated by subtracting the peptide amount recovered in the beads at different time intervals from the total peptide initially encapsulated in the beads.

In a second set of experiments, to some fractions, the supernatant was removed and replaced with fresh buffer once a day. The quantification of the peptide released was then performed as described above.

3. Discussion

3.1. Circular dichroism

Since electrostatic interactions are considered to be the principal interactions occurring between LL-37 and the ionic PS, CD spectra of polysaccharide/peptide mixtures were recorded in phosphate buffer, pH 7.4, at two different values of ionic strength, a parameter that is known to strongly modulate charge attraction.

The first set of analyses was carried out in 5 mM phosphate buffer, pH 7.4, ionic strength 20 mM, to reproduce the experimental conditions reported in the literature for previous analyses of PS/AMP mixtures (Herasimenka et al., 2005). PBS, pH 7.4, ionic strength 150 mM, was instead used to mimic the physiological conditions. In previous studies, it was shown that the α-helical conformation of LL-37 is anion-, pH- and concentration-dependent (Chan et al., 2005; Johansson et al., 1998). It is generally assumed that structuring to an α-helical conformation is principally driven by the hydrophobic effect, and the promotion of helix formation by some anions (HCO_3^-, SO_4^{2-}, CF_3COO^-, and to a significantly

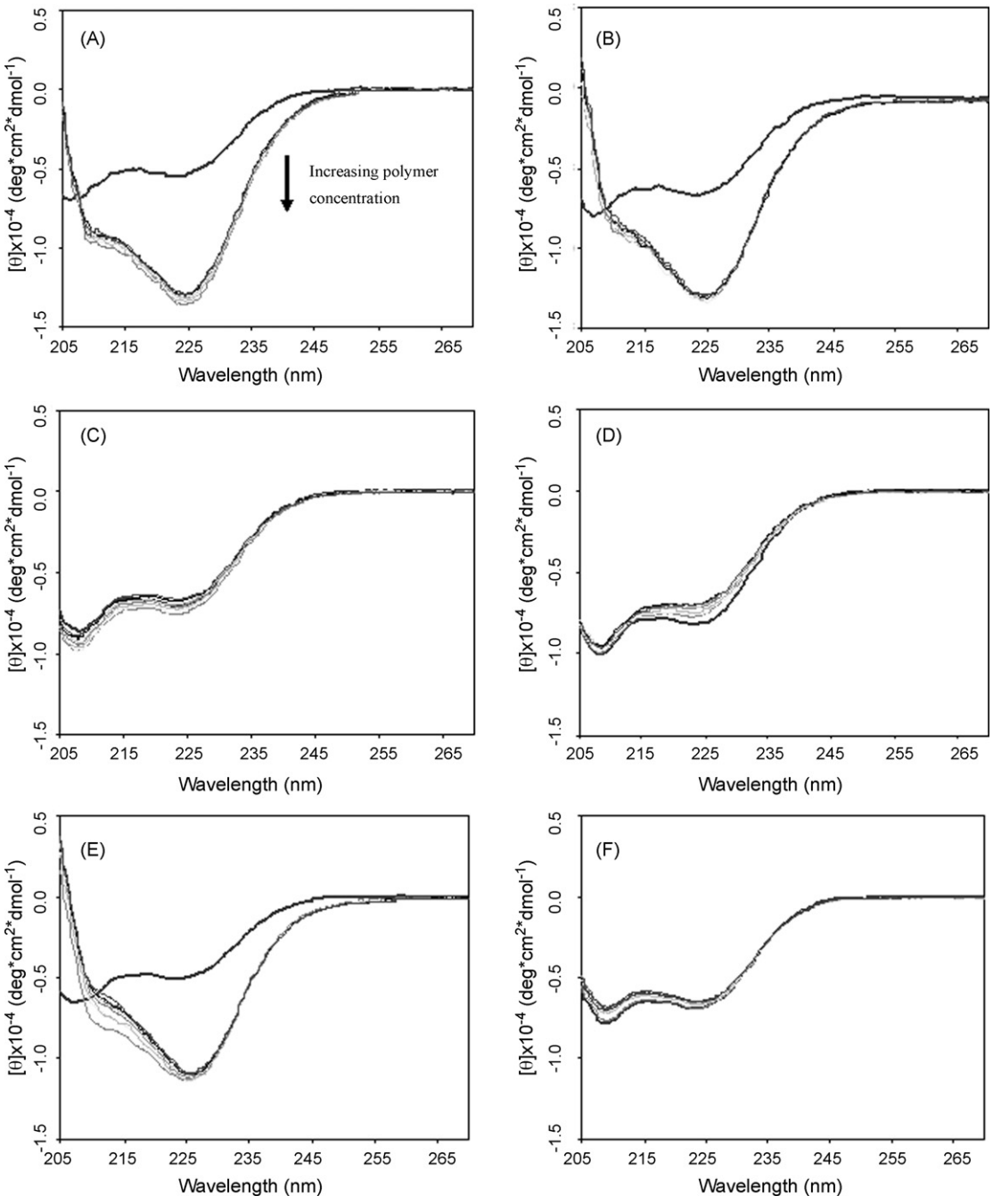


Fig. 1. Circular dichroism spectra of: LL-37/alginate; LL-37/ChitLac; LL-37/hyaluronan mixtures in PI-20 buffer (A, C, E) and in PI-150 buffer (B, D, F). Measurements were performed on solutions with a constant LL-37 concentration (10 μM) and increasing polysaccharides concentrations (from 50.4 to 504 μM). The spectrum of each PS alone, measured under the same conditions, was subtracted to that of the respective mixture.

lesser extent Cl^-) follows the Hofmeister series (Baldwin, 1996; Johansson et al., 1998).

In our studies, we found that the main effect is due to phosphate ions, which are particularly efficient in promoting the α-helical conformation.

The CD spectra obtained for the three PS/peptide mixtures recorded in the two considered buffers are reported in Fig. 1.

The concentration of LL-37 was kept constant at 10 μM during all the experiments, while the PS concentration was stepwise increased as reported in Section 2. For clarity, results are reported as spectra from which the CD of each PS alone at the same concentration and in the same buffer was subtracted from that of the mixture, so that the contribution of the peptide is highlighted.

Fig. 1A and B shows that even the lowest concentration of sodium alginate tested (50.4 μM) induces LL-37 to assume an α-helical conformation, as indicated by the appearance of a negative minimum at 222 nm (Chen, Yang, & Chan, 1974). These results are qualitatively and quantitatively in agreement with those previously reported for sodium alginate/AMP (Andersson et al., 2004; Chan et al., 2004; Kuo, Chan, Burrows, & Deber, 2007) and, in particular, for sodium alginate/LL-37 mixtures (Benincasa et al., 2009; Foschiatti et al., 2009; Herasimenka et al., 2005). As for the role of ionic strength, there is no apparent difference between the spectra recorded at low and high salt concentrations.

Fig. 1C and D shows the spectra obtained with ChitLac/LL-37 mixtures. It is evident that the presence of the polysaccharide

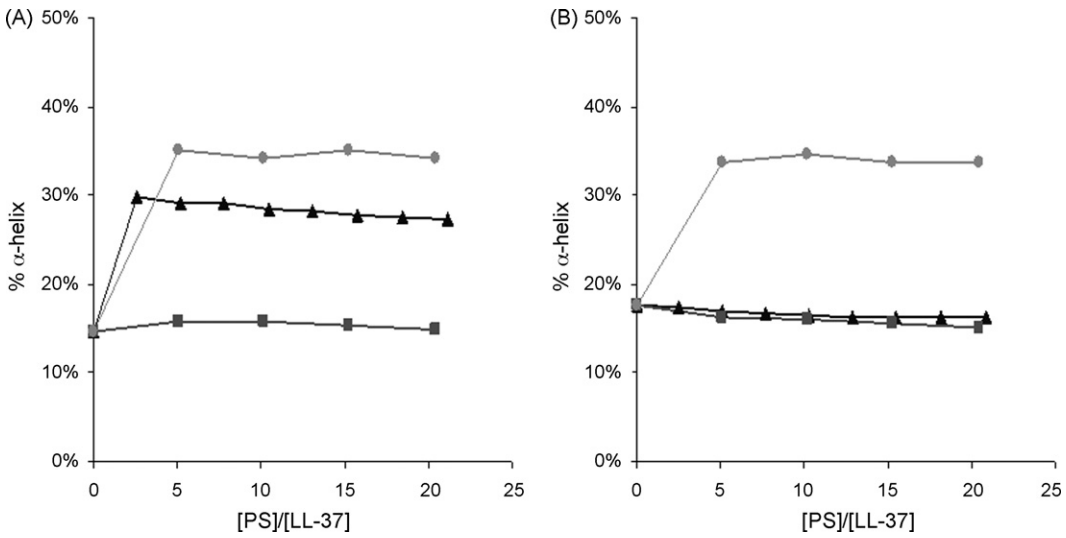


Fig. 2. Percentage of α-helical content in LL-37 in the presence of alginate, hyaluronic acid and ChitLac. Panel A: PI-20; panel B; PI-150. Each value was corrected for the dilution factor. Graphs were obtained by calculating the α-helix percentage determined by using the CD spectra reported in Fig. 1.

does not influence the peptide conformation. Although a possible LL-37/PS interaction devoid of conformational effects cannot be completely ruled out, this is unlikely due to the cationic nature of the macromolecules. Also in this case there is no significant difference between the spectra recorded at low and high ionic strength.

In contrast, the behaviour of the peptide in the presence of hyaluronic acid is peculiar. In the high ionic strength buffer, hyaluronic acid, at all the concentrations tested, does not influence the conformation of LL-37 (Fig. 1F), while, at low ionic strength, a transition to a more helical and aggregated form takes place (Fig. 1E), although less evident than that observed for sodium alginate (Fig. 1A).

The α-helical content of LL-37 was determined as the ratio $[\theta]/[\theta]_\alpha$, where $[\theta]$ is the observed molar ellipticity at 222 nm and $[\theta]_\alpha$ is the molar ellipticity of a fully structured peptide calculated using the equation $[\theta]_\alpha = -40{,}000(1 - 2.5/n)$, where n is the number of amino acid residues in the peptide (Juban, Javadpour, & Barkley, 1997).

Fig. 2 summarizes all the CD analyses by reporting the percentage of α-helix in LL-37 as a function of the [PS]/[peptide] ratio, in the presence of each of the three polysaccharides and in the two buffers used.

As mentioned above, the buffer itself plays a role in inducing the conformational transition of the peptide chain independently of the PS used, as shown by the LL-37 spectra in the absence of PS. By increasing the ionic strength from 20 to 150 mM, the α-helix percentage in LL-37 alone rises from about 14 to 19%, which corresponds to a mean of approximately 5 and 7 residues per chain taking part into the helix, respectively. The transition to an α-helical conformation is driven essentially by electrostatic interactions, since at high ionic strength charges of the same sign are allowed to get closer, giving rise to the helix, while at a lower salt concentration the electrostatic repulsions are predominant and destabilize the helix.

The highest percentage of α-helix is found for the LL-37/sodium alginate mixture both in low (PI-20, Fig. 2A) and high (PI-150, Fig. 2B) ionic strength buffers. Under these conditions, a helical content of about 35% is observed, corresponding to a mean number of 13 residues per molecule of peptide taking part into the helix. This result is in agreement with similar studies reported in the literature (Kuo et al., 2007), and it suggests that the electrostatic interaction between LL-37 and sodium alginate is very strong and that 35% is likely the upper limit of helix percentage attainable in this environment (for comparison, this limit is higher than 60% in trifluoroethanol). Of course, it cannot be excluded that, by pure chance, an equivalent compensation of the two opposite effects takes place, or, more likely, that this derives from a compensating contribution by hydrophobic interactions (Chan et al., 2004, 2005).

In the case of ChitLac, this weakly charged polycation does not have any effect on the peptide conformation. This is likely due to the fact that the two molecules are both positively charged and thus repel each other. As a consequence, the presence of the PS does not bring in any additional effect on the helical conformation in addition to that induced by the ionic strength of the medium.

Hyaluronic acid has a density of the negative charges which is approximately half that of sodium alginate. As expected, in this case the extent of electrostatic interactions is lower and thus the peptide/PS interaction is more susceptible to the surrounding environment. At low ionic strength (buffer PI-20, Fig. 2A), the charge density on the polymer, albeit low, is sufficient to cause the interaction of the peptide molecules with the sugar chain. This allows the transition to the α-helical conformation, although to a lower extent compared to sodium alginate. When the ionic strength is increased (PI-150), the interaction between opposite charges is shielded and the structuring of LL-37 cannot take place, at variance with what observed in the presence of sodium alginate. Under these conditions, hyaluronic acid behaves similarly to ChitLac. The strong, negative dependence of the PS/peptide interaction from ionic strength suggests that the hydrophobic interactions between LL-37 and the "hydrophobic patch" in hyaluronan (Scott, 1989) are comparatively small, if present at all.

From the results reported above, it can be concluded that sodium alginate has always a strongly helicogenic effect, while hyaluronic acid has the same effect only at low ionic strength.

3.2. Binary alginate-ChitLac system

Since alginate, as a delivery system for LL-37, could be in contact with cationic macromolecules competing for its interaction with the AMP (Etrych, Leclercq, Boustta, & Vert 2005), the influence on the amount of α-helix in LL-37 in the presence of sodium alginate was investigated after addition of ChitLac as a model polycation.

In particular, the reversibility of the interaction between LL-37 and sodium alginate was tested by gradually increasing the positively charged ChitLac in the mixture, under conditions corresponding to those used to obtain the first curve of Fig. 1B (alginate

50.4 μM, LL-37 10 μM, buffer PI-150). For each addition, the ratio of the sum of the positive charges on LL-37 and ChitLac, calculated taking into account their effective charge at the given pH, over the negative ones of sodium alginate was calculated. The investigated ratio range varied from 1.15 (initial value in the absence of ChitLac) to 13.15, thus reaching conditions of a large excess of potentially competing positive charges. CD spectra recorded in the above ratio range are totally superimposable (data not shown), indicating that the percentage of α-helix remains constant and that the ordered conformation stabilized by the interaction of LL-37 with sodium alginate is very strong with no competition by ChitLac.

3.3. Cytotoxicity of LL-37/polysaccharide mixtures

The aim of these experiments was to verify whether the relevant cytotoxicity of LL-37 towards mammalian cells could be mitigated or not upon binding to charged macromolecules that alter the peptide conformation in solution.

With this in mind, we carried out a series of experiments aimed at evaluating the toxic effect of LL-37 on eukaryotic cells as a function of the presence/absence of PS. The CD data reported above showed that only polyanions effectively bind LL-37 and influence its conformation. The cytotoxicity tests were therefore carried out in the presence of sodium alginate and hyaluronic acid at the concentration of 500 μg/mL. For this purpose, the MTT assay was used on two osteoblast cell lines: MG-63 and Saos-2. The choice of these cell lines was based on a potential future application of the peptide/PS mixture in the field of biomaterials, and in particular for orthopaedic implants. Osteoblasts were plated at a density of 5000 cells/well. The ionic strength of DMEM medium is 166 mM, very close to the PI-150 buffer used in the CD experiments.

The results obtained with the MG-63 cells are reported in Fig. 3 and show that sodium alginate is able to abolish the cytotoxic effect of LL-37 up to a peptide concentration of 50 μM and to significantly reduce it at higher concentration. In contrast, hyaluronan was unable to protect the cells at all the peptide concentrations tested.

The results with sodium alginate are in good agreement with what predicted from CD data: the higher the fraction of LL-37 bound and aggregated onto sodium alginate and, consequently, the percentage of α-helix, the lower its cytotoxic effect. Highly similar results were also obtained with the Saos-2 cell line (data not shown).

The *in vitro* experiments with osteoblasts showed that the mixture sodium alginate/LL-37 is the only system here tested that effectively reduces the cytotoxic activity of LL-37 and it was therefore selected to evaluate its antimicrobial activity on both Gram-negative and Gram-positive bacterial species.

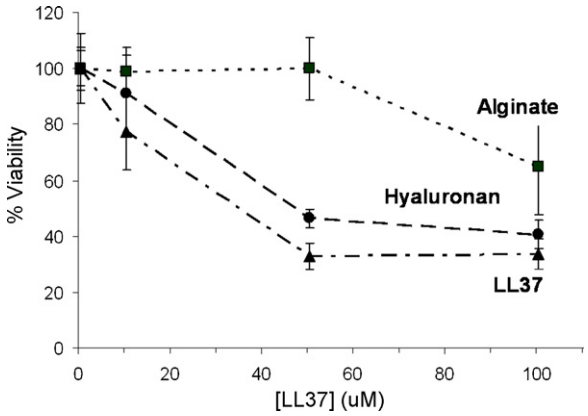


Fig. 3. Cytotoxic effect of increasing concentrations of LL-37 alone or in the presence of 500 μg/mL alginate or hyaluronan on MG-63 cells. The cells were treated with solutions of increasing concentration of LL-37 in DMEM, in the absence or presence of alginate and hyaluronan.

3.4. Antimicrobial activity of alginate/LL-37 mixtures

The antimicrobial activity of the sodium alginate/LL-37 system was tested on *E. coli* and *S. aureus*. It should be noticed that sodium alginate alone already slows down bacterial growth. Therefore, to highlight the effect of the mixture, analyses were performed using a concentration of sodium alginate (500 μg/mL), which has only a modest effect on bacterial growth. Under these conditions, the kinetics of bacterial growth was followed for 4 h in the presence of two peptide concentrations (2 and 10 μM). As reported in Fig. 4A, *E. coli* cells in 20% MH grow rapidly, as indicated by the fast increase of absorbance at 620 nm. The presence of sodium alginate alone causes a modest reduction in the growth kinetics with respect to untreated cells (18%). LL-37 alone, at both concentrations tested, effectively blocks the growth of *E. coli* cells and this effect is also maintained in the presence of 500 μg/mL of sodium alginate (Fig. 4A). These results indicate that under the selected conditions there is enough peptide available to affect bacterial cells, either free in solution and/or released from the complex with sodium alginate. On *S. aureus*, LL-37 is active only at the concentration of 10 μM while all other mixtures cause only a moderate decrease of the bacterial growth rate (Fig. 4B).

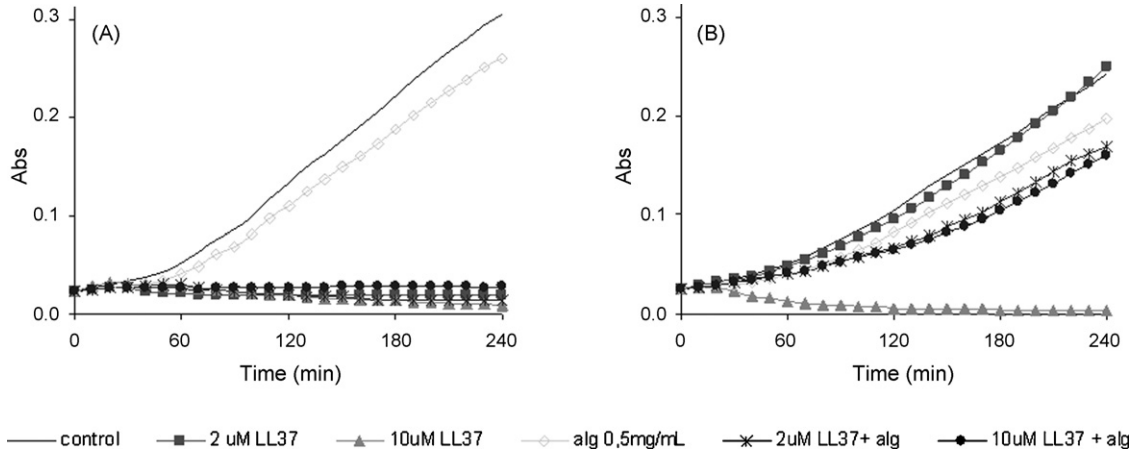


Fig. 4. Growth kinetics of: *E. coli* (panel A); *S. aureus* (panel B), in 20% MH broth in the presence of alginate (500 μg/mL), LL-37 (2 or 10 μM) or a mixture of both.

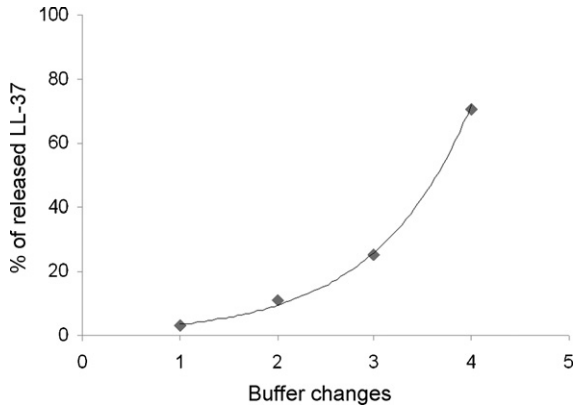


Fig. 5. Release from calcium alginate beads loaded with LL-37 by replacing the supernatant once a day. The release of LL-37 parallels the bead structure degradation.

To confirm the results reported above, the experiment was performed with two other bacterial strains, i.e. *P. aeruginosa* and *S. epidermidis*, that showed the same behaviour of respectively *E. coli* and *S. aureus* (data not shown), depending on the Gram-type.

This result suggests that the charge density at the surface of the Gram-negative cells is high enough and/or the binding sites have enough affinity to displace the peptide from sodium alginate. In contrast, this seems not the case for the Gram-positive species tested.

3.5. Application of LL-37 to solid supports: release from alginate/hydroxyapatite beads

Since the final application of an alginate/LL-37 system might be in the preparation of biomaterials, in particular for orthopaedic implants, the action of the peptide was tested after encapsulation in calcium alginate/hydroxyapatite microbeads. This system is suitable for use as filler for bone microfractures.

The peptide charge has an important role in defining encapsulation efficiency; in the case of LL-37, thanks to its high positive charge, this was a very efficient process. The encapsulation of LL-37 was performed by blending alginate and hydroxyapatite with a peptide aqueous solution, and then the mixture was dripped into a gelling solution containing calcium ions. The gel formation was instantaneous and both the peptide and hydroxyapatite remained entrapped in the calcium alginate gel bead. LL-37 demonstrated to undergo a substantially complete encapsulation without loss of peptide during the process. The amount of encapsulated peptide was calculated after acidic hydrolysis of loaded beads by quantification of arginine in the hydrolysate with capillary electrophoresis. The percentage of LL-37 released from the beads was calculated in the same manner. The procedure comprised the incubation of wet beads in PBS under agitation on a rotary stirrer. At determined time intervals, some beads were collected, hydrolysed and the released peptide was quantified as described above. In this procedure, the supernatant was never replaced. Data of collected fractions (not reported) showed that the release was very slow: after 1 month, only 30% of the peptide was released from the beads. This behaviour indicates that LL-37 is liberated during the degradation of the polymer-based 3D structure of calcium alginate beads and not by diffusion of the peptide through the pores. To confirm this mechanism, a second type of experiment was carried out and the results obtained are presented in Fig. 5. In this case the percentage of peptide released is reported as a function of the number of buffer replacements that were performed at the frequency of once a day.

The results indicated that the release of LL-37 from calcium alginate beads is a consequence of the bead structure degradation, and is not dependent on diffusion through the gel pores. This behaviour has an important advantage over conventional diffusion, since in this way the cytotoxic effect of LL-37 associated to burst release of this molecule can be avoided when the peptide is in complex with alginate.

4. Conclusions

The sodium alginate/LL-37 mixture analyzed in this work appears to be a potential antimicrobial system capable of reducing the toxic effect of the peptide towards mammalian cells in defined ratios of peptide/polysaccharide, while maintaining the ability to arrests the growth of Gram-negative bacteria. In contrast, the peptide/PS mixture is poorly or not at all effective on Gram-positive bacteria. The reason for this dissimilar behaviour is likely to be searched for in the different composition of the bacterial wall of Gram-negative and Gram-positive bacteria. In fact, the surface of the former group of microorganisms is rich in LPS, which confers a high density of negative charge. This density is likely high enough to shift the peptide/PS equilibrium so that at least part of the peptide is transferred to the bacterial wall, where it performs its antimicrobial activity. Under these conditions, the bacterial growth is inhibited at 10 μM peptide, a concentration that results to be completely safe in terms of cytotoxicity (Fig. 3).

In the case of Gram-positive bacteria, the charge on the bacterial wall and/or the affinity of the binding sites are likely not sufficient to displace LL-37 from the sodium alginate/peptide complex, and the presence of the peptide causes only a slight decrease of the growth kinetics. It can be supposed that in this case the mixture behaves as a "peptide trap" inasmuch as the presence of the peptide is not "experienced" by the microorganism.

We tested the alginate/peptide complex for the preparation of microbeads, to verify if this equilibrium can be shifted when applied to localized (semi) solid systems. These experiments revealed that the only possible efficient release mechanism that an LL-37/calcium alginate construct is able to perform is related to the degradation of the construct itself. This result is not completely unexpected, since other papers (Mumper, Huffman, Puolakkainen, Bouchard, & Gombotz, 1994) report that highly charged peptides bind irreversibly to alginate. A way to promote peptide release would be the preparation of mixed beads containing hydrophobic synthetic polymers.

As suggested by the results here reported, a sodium alginate/LL-37 complex used in implants would only be active against Gram-negative strains. This is not ideal, as a large proportion of all implant-related infections are caused by Gram-positive microorganisms, namely *Staphylococci*. However, an increasing number of Gram-negative infections, particularly those caused by opportunistic microorganisms, are becoming effectively untreatable as they are resistant to most of the antibiotics in use. There is thus an urgent medical need for new agents that are active against hospital infections, caused by Gram-negative opportunists, against which only a few new agents are in advanced development (Livermore, 2004). The mixtures LL-37/PS could then be an additional opportunity that could find application in topical contexts.

Although further investigations are needed to fully understand the behaviour of the peptide/PS mixtures in the presence of cells and to improve the release profiles from microbeads, this work shows that it is possible to effectively modulate the action of LL-37 by means of its interaction with a suitable, negatively charged polyelectrolyte. The important goal reached with the LL-37/sodium alginate mixture is the decreased cytotoxicity towards eukaryotic cells. These are fully vital up to 50 μM LL-37 in the presence of sodium alginate, a peptide concentration at which

only less than 50% of the cells are viable in the absence of the polysaccharide.

Application of LL-37/PS mixtures as a systemic drug-delivery system is not immediately at hand, since much more light must be shed onto the variety of possible interactions of the alginate/LL-37 complex with biological polyelectrolytes in the body fluids (Chen et al., 1974). However, the use of the peptide/PS complexes in localized (semi) solid systems, such as in preparation of beads or in coating of biomaterials for orthopaedic applications (Turco et al., 2009), is an interesting perspective in a shorter term.

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