SÍNTESIS DE NANOPARTÍCULAS POLIMÉRICAS DE QUITOSANO FUNCIONALIZADAS CON PÉPTIDOS Y EVALUACIÓN DE SU ACTIVIDAD IN VITRO FRENTE *A ESCHERICHIA COLI* Y *STAPHYLOCOCCUS AUREUS*

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Trabajo de grado presentado como requisito para optar al título de Químico

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DEDICATORIA

A mis padres, Ana F. y Cesar A. y a mis hermanos Nadia V. y Cesar G. que con su arduo trabajo y constante apoyo me brindaron la oportunidad de alcanzar esta meta.

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TITULO: SÏNTESIS DE NANOPARTÍCULAS POLIMÉRICAS DE QUITOSANO FUNCIONALIZADAS CON PÉPTIDOS Y EVALUZCIÓN DE SU ACTIVIDAD IN VITRO FRENTE A *ESCHERICHIA COLI* Y *STAPHYLOCOCCUS AUREUS*

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PALABRAS CLAVE: Nanopartículas de quitosano, SARM, *E*. *coli* O157:H7, péptido, actividad antibacterial.

DESCRIPCIÓN:

La actividad biológica y estabilidad de los péptidos puede ser mejorada mediante sistemas de liberación. Uno de estos sistemas son las nanopartículas de quitosano, las cuales son adecuadas para la entrega de péptidos antimicrobianos debido a su biodegradabilidad, no toxicidad y actividad contra hongos y bacterias. En este trabajo, hemos sintetizado nanopartículas de quitosano (NPs), cargadas con los péptidos JC16, JC17 y Vapreotide, a través del proceso de gelificación iónica entre el quitosano y los aniones del tripolifosfato de sodio, obteniendo NPs con cargas superficiales positivas (>20 mV), bajo índice de polidispersión (< 0.3) y tamaños hidrodinámicos entre 280 y 330 nm. La actividad antibacterial In vitro de las nanopartículas de quitosano cargadas con los péptidos JC16, JC17 y Vapreotide fue superior a los péptidos libre contra *Escherichia coli* O157:H7 y *Staphylococcus aureus* resistente a meticilina como se evaluó mediante la determinación de la concentración mínima inhibitoria (CMI). De hecho, CMI_{50} contra E. coli de estas nanopartículas cargadas estuvo entre 1.3 y 3.4 µM, todavía más bajo que el CMI_{50} del péptido libre y más potente contra SARM que *E. coli* O157:H7 mostrando inhibición en todas las concentraciones probadas. Interesantemente, el CMI_{50} contra SARM de las nanopartículas cargadas con JC16, JC17 y Vapreotide fueron 75, 12.5 y 50 veces menores que el CMI_{50} de péptido libre, respectivamente. Por lo tanto, la nano-encapsulación de estos péptidos mejoró su actividad antibacteriana en comparación con péptidos libres.

▪Trabajo de grado

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ABSTRACT

TITLE: SYNTHESIS AND CHARACTERIZATION OF PEPTIDE LOADED CHITOSAN NANOPARTICLES WITH ACTIVITY AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI O157:H7

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KEYWORDS: Chitosan nanoparticles, MRSA, *E. coli* O157:H7, peptide, antibacterial activity.

DESCRIPTION:

Peptide biological activity and stability can be improved by using delivery systems. One of these systems is Chitosan nanoparticles, which is suitable for antimicrobial peptide delivery because its biodegradability, nontoxicity and activity against fungi and bacteria. In this work, we synthesized chitosan nanoparticles (NPs), loaded with peptides JC16, JC17 or Vapreotide, through the process of ionic gelation between chitosan and tripolyphosphate anions, obtaining NPs with positive surface charges (> 20 mV), low polydispersity index (< 0.3) and a hydrodynamic sizes between 280 and 330 nm. *In vitro* antibacterial activity of Chitosan nanoparticles loaded with peptides JC16, JC17 and Vapreotide, was higher than the free peptides against *Escherichia coli* O157:H7 and Methicillin resistant *Staphylococcus aureus* (MRSA) as was evaluated by determining the minimum inhibitory concentration (MIC). Indeed, MIC_{50} against *E. coli* of these peptide nanoparticles was between 1.3 and 3.4 µM, still lower than the MIC_{50} of free peptide and more potent against MRSA than *E. coli* O157:H7 showing inhibition at all concentrations tested. Interestingly, the MIC_{50} against MRSA for nanoparticles loaded with peptides JC16, JC17 and Vapreotide were 76, 12.5 and 50 times lower than the MIC_{50} of free peptide, respectively. Therefore, nano-encapsulation of these peptides improved their antibacterial activity compared with free peptides.

▪ Bachelor Thesis

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INTRODUCTION

Since the penicillin discovery, antimicrobial drugs have caused a great impact in the treatment of infectious diseases and therefore in the fate of mankind. New classes of antimicrobial agents were developed to control pathogens, resulting in global improvements of control infectious diseases and increments in life expectancy (Omulo, Thumbi, Njenga, & Call, 2015). However antimicrobial resistance (AMR) is a global threat (Huttner et al., 2013), which is not only a modern problem but a natural phenomenon that predate the modern selective pressure of clinical antibiotic use (D'Costa et al., 2011).

Staphylococcus aureus, one of the most problematic human pathogens, acquired resistance very quickly to sulphonamides when they were in use; then penicillin was effective against this bacterium, but in 1942 resistant strains producing penicillinase appeared (Purrello et al., 2014). In 1960 Methicillin, an alternative semi-synthetic to penicillin was developed but as early as in 1961 was isolated in the UK the first Methicillin resistant strain of *Staphylococcus aureus* (MRSA) (Saga & Yamaguchi, 2009); which is one of the major worldwide public health problem owing to its frequent resistance to a wide range of antimicrobial agents (Egyir et al., 2015). For example, more than 60% of all *S. aureus isolated* from Spain hospitals were methicillin resistant in 2004. In the US, 46.3 per 1000 of hospitalized patients were colonized or infected by MRSA (Muto et al., 2003). In Spain, the MRSA prevalence increased from 1.5% in 1986 to 31.2% in 2002, similar to the rest of Europe (Siegel, Strausbaugh, Jackson, Rhinehart, & Chiarello, 2004). In some Latin American countries infections caused by MRSA have resulted in 25%–63% mortality. Moreover, *Staphylococcus epidermidis* has replaced to *Escherichia coli* as the first bacteremia agent producer.

On the other side, gram-negatives bacteria are now resistant to almost every available antibiotic, including the most recent generation (Carlet & Pittet, 2013). For example *Escherichia coli,* naturally found in human and animal intestinal tracts and spread by faecal contamination to food and water, present one particular strain named Shiga toxin-producing *E. coli* (STEC), O157, which is a major cause of food-borne illness producing haemolytic-uraemic syndrome in humans. This strain displays antibiotic resistance to ampicillin and carbenicillin among others. *E. coli* O157:H7 cause an estimated morbidity in children younger than 5 years between 0.6%–2.4% and specifically 15%–36% of all diarrhoea with blood (Koyange et al., 2004). The prevalence in Colombia is 4.7% for this strain according to one study carried out in Spain, Argentina, Chile, Canada, and Belgium (Mattar, Visbal S, & Arrieta, 2001). Some researchers do not recommended antimicrobial treatment of STEC owing to bacterial lysis and toxin release (Folster, Pecic, Stroika, Rickert, & Whichard, 2014).

New antibiotics development has been gradually reduced over the last several years, pharmaceutical companies have abandoned this work given their high cost and low yield (Huttner et al., 2013). There is a urgent need to develop antimicrobial compounds with new mechanisms of action; in this way antimicrobial peptides (AMPs) seen to be a promising solution (Oren & Shai, 1998). These compounds are bactericidal, fungicidal, virucidal and/or tumouricidal turning them promising candidates for therapeutic treatment (Reddy, Yedery, & Aranha, 2004).
AMPs are very interesting molecules due to their broad-spectrum effectiveness, probably because in many cases the action mechanism targets the cell membrane and not a specific receptor or membrane proteins. It is thought that bacteria can hardly alter its membrane as alter other target to develop resistance (Soblosky, Ramamoorthy, & Chen, 2015). There are four major AMP classes based on their structures: α-helical, β-sheet, loop and extended peptides (Powers & Hancock, 2003). Peptide structure is associated to its antimicrobial activity and one of challenges is to understand how the peptide secondary structure influences the mechanism of action (Marr, Gooderham, & Hancock, 2006). For example the amphipathic α-helical peptides are predominantly membrane disruptive but clearly not all α-helical peptides target the microbial membrane (Powers & Hancock, 2003). In this context peptide charge distribution appears to play an important role to interact with the cell membrane; indeed cationic antimicrobial peptides (CAMPs) interact with the phospholipid head group charge on cell membranes (L. Zhang, Rozek, & Hancock, 2001). Moreover, CAPMs target cell surface negative charge of bacteria through lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria and teichoic and teichuronic acids in their cell wall of Gram-positive bacteria (Guilhelmelli et al., 2013).

There are also peptides having a receptor-mediated mechanism of killing bacteria; these are mainly produced by some bacteria and are highly active on a specific bacterial target (Shai, 2002). Despite the high antimicrobial activity of AMPs their use as therapeutic agents present some drawbacks; for example their short half-life and susceptibility to proteases in biological fluids. To improve the stability and extend half-life of peptides, they can be protected in nanoparticles.

Biodegradable nanoparticles have been explored for antimicrobial molecule delivery, displaying a good bactericidal activity *in vitro*. The active substance can be loaded through of physical encapsulation, adsorption or chemical conjugation and its release can occur through contact, adsorption or endocytosis to the target cell (Xie et al., 2014). Different methods to prepare delivery systems for peptides has been developed using polymers such poly-(lactic-co-glycolic acid) (PLGA) (Takeuchi, Yamamoto, & Kawashima, 2001), poly –(lactic acid) (PLA), poly-(caprolactone) (PCL), dextran, chitosan and others (Herrero, Alonso, & Csaba, 2012; Prego et al., 2006). Chitosan exhibits antibacterial activity influenced for the type of chitosan, polymerization degree and some of its physicochemical properties (Qi, Xu, Jiang, Hu, & Zou, 2004). Its biodegradability, nontoxicity, and broad spectrum of activity against fungi, and bacteria make it suitable to build drug delivery vehicles (Ing, Zin,

Sarwar, & Katas, 2012). Amongst the methods of the chitosan nanoparticle preparation, ionotropic gelation provides synthesis conditions friendly to peptide stability. This method based on the property of polyelectrolytes to cross link in the presence of counter ion to form gelispheres, allows the biomolecules to retain their three dimensional structure into these hydrogel beads (Patil, Chavanke, & Wagh, 2012).

In this work, two cationic antimicrobial peptides synthesized by solid phase chemistry using Fmoc strategy and a control peptide (Vapreotide) were encapsulated by ionotropic gelation method in chitosan/sodium-trypoliphosphate (TPP) nanoparticles. This nanoparticles were characterized by Scanning Electron Microscopy (SEM) for morphology, Dynamic Ligth Scattering (DLS) technique for particle size and polydispersity index (PDI) and Zeta potential to charge. The antibacterial activities of three nanosystem were tested against *Escherichia coli* O157:H7 and Methicillin resistant *Staphylococcus aureus* (MRSA) determining the minimum inhibitory concentration (MIC) using the broth microdilution methodology.

1. MATERIALS AND METHODS

1.1. Materials

Fmoc-L-amino acids were purchased from IRIS Biotech GmbH. Rink amide resin 4MBHA was obtained from Merck Novabiochem. All reagents and organic solvents for peptide synthesis were of HPLC grade. Sodium tripolyphosphate was purchased from Sigma and chitosan used has 129 kDa average molecular weight with about 79.5 % degree of deacetylation.
Pathogenic strain Methicillin-resistant *Staphylococcus aureus* was donated by Hospital Universitario de Santander, Bucaramanga, Colombia, and *Escherichia coli* O157:H7 was obtained from Laboratorio de Biotecnología, Pontificia Universidad Javeriana, Bogotá, Colombia. Both strains *E. coli* O157:H7 and SARM were kept on brain heart infusion (BHI) agar slants (Oxoid, Basingstoke, UK).

1.2. Methods

1.2.1. Peptide synthesis and characterization

Peptides were synthesized by solid phase peptide synthesis (SPPS) (Amblard, Fehrentz, Martinez, & Subra, 2006; Merrifield, 1963; Stawikowski & Fields, 2002), on 0.35 substituted rink amide 4MBHA resin (100-200 mesh; Loading: 0.35 mmol/gt); using Fmoc strategy (Carpino & Han, 1972; Fields & Noble, 1990) and T-bag simultaneous synthesis mode as reported by Houghten (Houghten, 1985). Peptides were cleaved from the resin by treatment with trifluoroacetic acid (TFA)/triisopropylsilan (TIS)/ethanedithiol/H_2O (37/40:1/40:1/40:1/40) for 2 hours and finally precipited with cold diethyl ether (King, Fields, & Fields, 1990). The salts were removed from peptides by gel exclusion chromatography using G-10 columns (Amersham, USA). Peptides were purified on Sep-Pak C18 and molecular weight was determined by ESI-MS.

1.2.2. Preparation and physicochemical characterization of chitosan nanoparticles

Chitosan nanoparticles were prepared according to a modified Calvo protocol (P. CALVO, C. REMUÑÁN-LÓPEZ, J. L. VILA-JATO, 1997). Preliminar experiments were performed to determine a range of conditions enabling the formation of nanoparticles. These conditions were varied to find the optimal conditions for nanoparticle formation and the amount of encapsulated peptide, keeping the size and surface charge of nanoparticles. Temperature, pH of the chitosan solution and

peptide-polymer mass ratio were the variables used in the factorial design 2^3. The best conditions found for formation of nanoparticles containing peptides were 1 mg/ml chitosan dissolved in an aqueous solution of acetic acid (0.1% w/v), stirred overnight at room temperature, pH adjusted to 6 in CS solution using aqueous sodium hydroxide solution and 1 mg/ml TPP solution pH 9.5. The nanoparticle were spontaneously formed upon slow incorporation of 2 ml of cold TPP solution in 10 ml of the CS at 60±1 °C. Cationic peptides and Vapreotide were dissolved in CS before adding the TPP. All solutions were passed through 0.45 µm filter before use.

The peptide-loaded CS/TPP nanoparticles were characterised by dynamic light scattering (90 degrees) using a Zetasizer Nano ZS90 (Malvern instrument Ltd). It was determined the particle size, particle size distribution and polydispersity index (PDI). Measurements were performed utilizing a standard 4 mW He-Ne gas laser and a detector of avalanche photodiode, Q.E. >50% at 633nm. Zeta potential of nanoparticles was measured utilising Zetasizer Nano ZS90. The sample volume used for analysis was in all cases 1 mL. Morphology of the nanoparticles and their size were confirmed by Scanning Electron Microscopy (SEM)

1.2.3. Determination of encapsulation efficiency and release profile

Following formation of nanoparticles, non-encapsulated peptide was separated utilizing Amicon ultra centrifugal filter of 10 kDa (Merck Millipore). Then the nanoparticles were broken with HCl [0.05M] for two 2h for releasing the encapsulated peptide. Peptide non-encapsulated and encapsulated was quantified by Revesed-Phase High-Performance Liquid Chromatography (RP-HPLC) using Agilent 1100 Series Diode Array detector. The peptide encapsulation efficiency (EE) of the CS nanoparticles was calculated according to the equation 1:

$$EE_D(\%) = \frac{W_2}{W_0} \times 100 \quad \text{(Equation 1)}$$

Where EE_D represent the encapsulation efficiency calculated by direct method, W_0 is the total amount of peptide used for the preparation of nanoparticles and W_2 is the weight of peptide enveloped in the chitosan nanoparticles.

Rate of peptide release from nanoparticles was studied at physiological conditions (pH 7.4, 100 rpm and 37°C). 250 µL of PBS and 250 µL nanoparticles were poured into a dialysis tubing cellulose membrane (flat width 10 mm, 6 mm when full) and the tubing was immersed in 5 ml of PBS at pH 7.4 and placed in an orbital shaker at 100 rpm and 37 °C. Aliquots of 500 µL of the external medium was taken at different times and PBS was added to keep the constant volume. RP-HPLC using Agilent 1100 Series fluorescence detector quantified the amount of released peptide.

1.2.4. Determination of minimum inhibitory concentration (MIC) of CSNPs

MIC, defined as the lowest concentration of antimicrobial substance that inhibit the in vitro growth of bacteria under standard conditions, was determined in multi-well culture plates of 96 wells according to standard protocols previously published. According to this, MIC_{50} was the antimicrobial concentration that inhibited 50% of growth bacterial (Cruz, Ortiz, Guzmán, Cárdenas, & Fernandez-Lafuente, 2013; Paredes, Ortiz, & Torres, 2014).

Briefly, *E. coli* O157:H7 and MRSA were grown in liquid Luria-Bertani (LB) and Müeller Hinton medium, respectively, at 37 °C and 200 rpm over 12-16 h. Samples of approximately $4.6x10^8$ Colony Forming Units (CFU) per ml of each bacterium were incubated with different concentrations of peptide loaded chitosan nanoparticles (NP) to determine their antibacterial activity at 37°C with constant stirring (Holowachuk, Bal'a, & Buddington, 2003). Bacterial cell growth was determined using the optical density (OD) at 595 nm measured in a microplate reader at intervals of 1 h, over 9 h, (Biorad, Hercules, CA, USA)

2. RESULTS

2.1. Peptide synthesis and characterization

The peptides JC16 and JC17 designed by Cruz et al (Cruz et al; under revision) were synthesized and characterized; both peptides having 17 amino acids in its sequence (primary structure not shown) are cationic, hydrophobic and displayed an alpha-helix structure suggesting a characteristic amphipatic alpha helix peptide (Table 1).

Table 3. Properties of JC16, JC17 and Vapreotide.

Peptide	Sequence	MW (Da)	m/z	#AA	Charge[a]	<μ>[b]	volume (A^3)	<H>[c]
JC16 GIBIM-P5S9K	Data not shown	1804.03	1804.03	17	+5	0.279	2183	0.422
JC17 GIBIM-P5F8W		1802.24	1802.20	17	+4	0.221	2180	0.505
Vapreotide	Phe-Cys-Tyr-Trp-Lys-Val-Cys-Trp-NH_2	1134.37	1134.29	8	+1	----	1372	----

[a]Net positive charge at neutral pH. [b]Mean hydrophobic moment of the peptide in a-helix conformation**.** [c]Mean hydrophobicity calculated from the sum of hydrophobicity values

2.2. Preparation and characterization of CSNPs

The peptide-loaded CS/TPP nanoparticles were prepared based on ionotropic gelation of chitosan upon contact with the triphosphate pentaanion (Amidi, Mastrobattista, Jiskoot, & Hennink, 2010). A 2^3 factorial experimental design allowed the identification of the most relevant factors in the formation of CS nanoparticles resulting in nanoparticles with CS/TPP ratio of 5:1 functionalized with JC16, JC17 and Vapreotide. Peptide functionalized Chitosan nanoparticles were very similar; most of them were regularly spherical in shape (Fig1A), with a very homogenous radius around 300 nm and PdI around 0.28 (Table2 and Fig 1B). There were no

significant differences in size and PdI (Table2). On the contrary, surface charge was higher on Vapreotide functionalized nanoparticles and lower on JC16 nanoparticles according to the results of Zeta potential; this surface charge was very homogenous as can be observed in the dispersion of Zeta potential (Fig 2C).

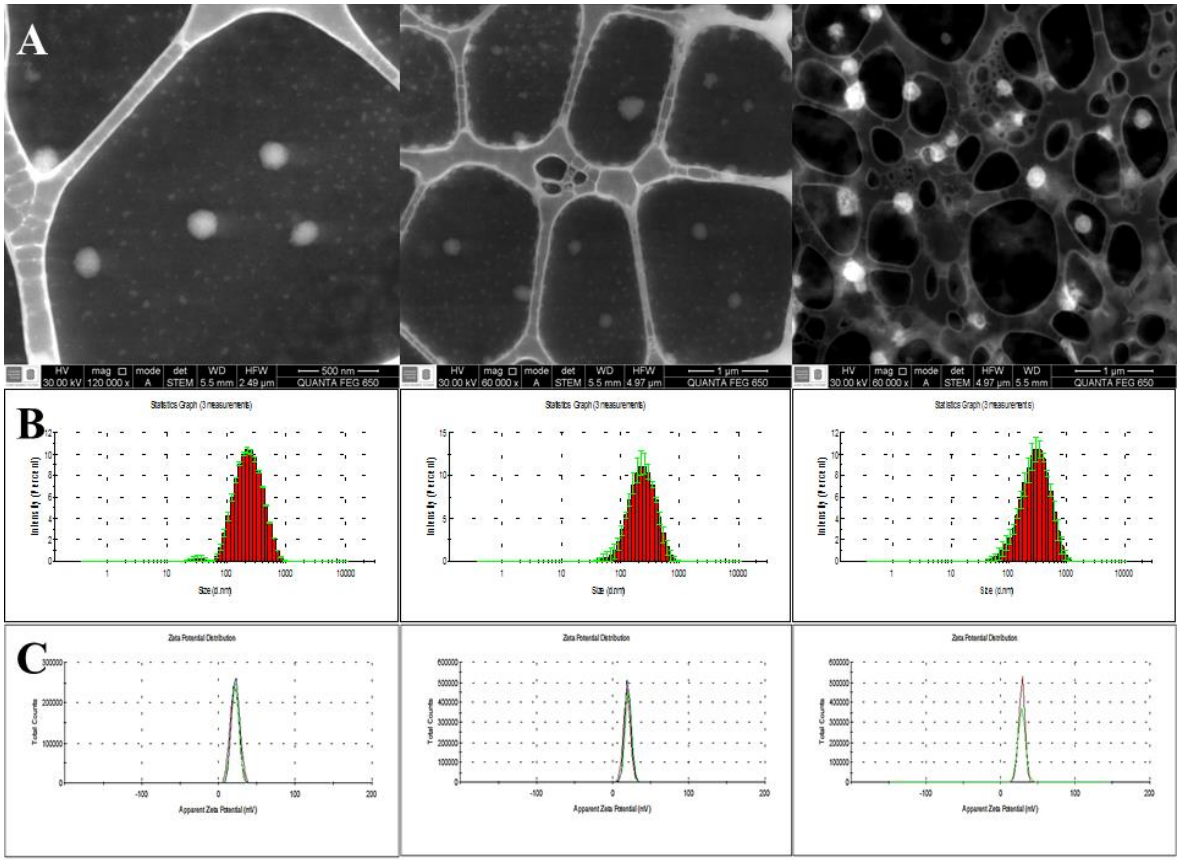

Figure 6. Characterization of chitosan nanoparticles functionalized with the three different peptides. **(A)** Morphology of CS-NPs visualized by S-TEM images; **(B)** Particle size distribution measured by DLS; **(C)** Zeta potential distribution measured by LDE. From left to right the images correspond to nanoparticles functionalized with peptides JC16, JC17 and Vapreotide respectively.

Table 4. Physicochemical properties of CS nanoparticles. Particle size, zeta potential and polydispersity index of empty or peptide functionalized chitosan nanoparticles.

Peptide	CS/peptide ratio	Size (d.nm)	Zeta potential (mV)	PdI
JC16	5:1	300 ± 15	26.1 ± 1.6	0.262 ± 0.005
JC17	5:1	283 ± 23	21.2 ± 0.8	0.277 ± 0.011
Vapreotide	5:1	328 ± 15	28.7 ± 0.4	0.279 ± 0.009
Blank	---	331 ± 18	21.7 ± 0.9	0.295 ± 0.010

Size corresponds to hydrodynamic diameter of nanocompounds.

2.3. Peptide loading efficacy and rate of peptide release

The ability of the CS-NPs to entrap and release the peptides JC16, JC17 and Vapreotide was determined. Peptides JC16 and JC17 showed similar and Vapreotide showed lower encapsulation efficiency (%) (Fig 2). The results shown that peptide loading was moderately high and comparable to those obtained with different drugs by the same methodology (Gan & Wang, 2007). Over the first 20 minutes peptides JC16, JC17 and Vapreotide were release from chitosan nanoparticles to high rate reaching 50% for JC16, 40% for JC17 and 10% for Vapreotide; then these rates decreased specially for JC16 which is almost zero at 6 h despite there is 40% of peptide present in the nanoparticles. The total amount of release peptide during the first six hours of measurement was 60% for JC16, 50% for JC17 and 30% for Vapreotide (Figure 3).

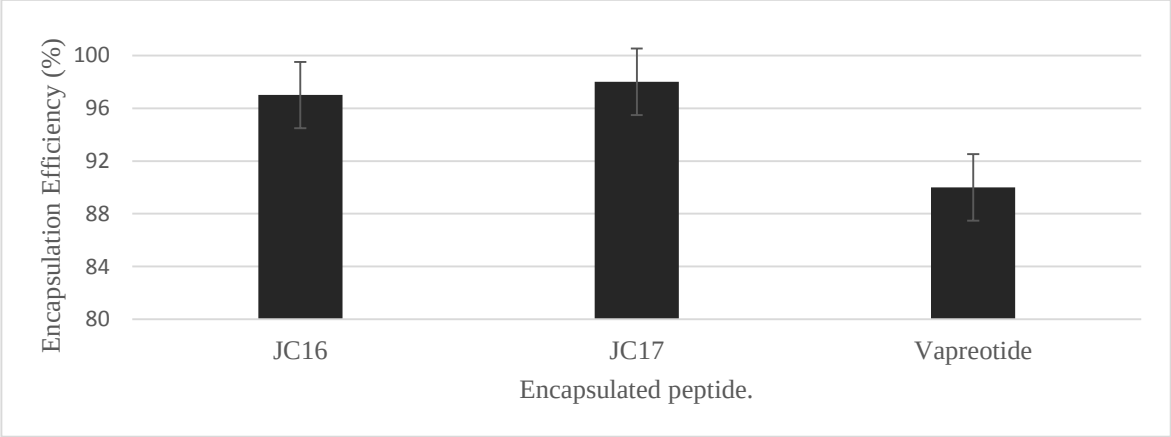

Figure 7. JC16, JC17 and Vapreotide encapsulation efficiency of CS NP.

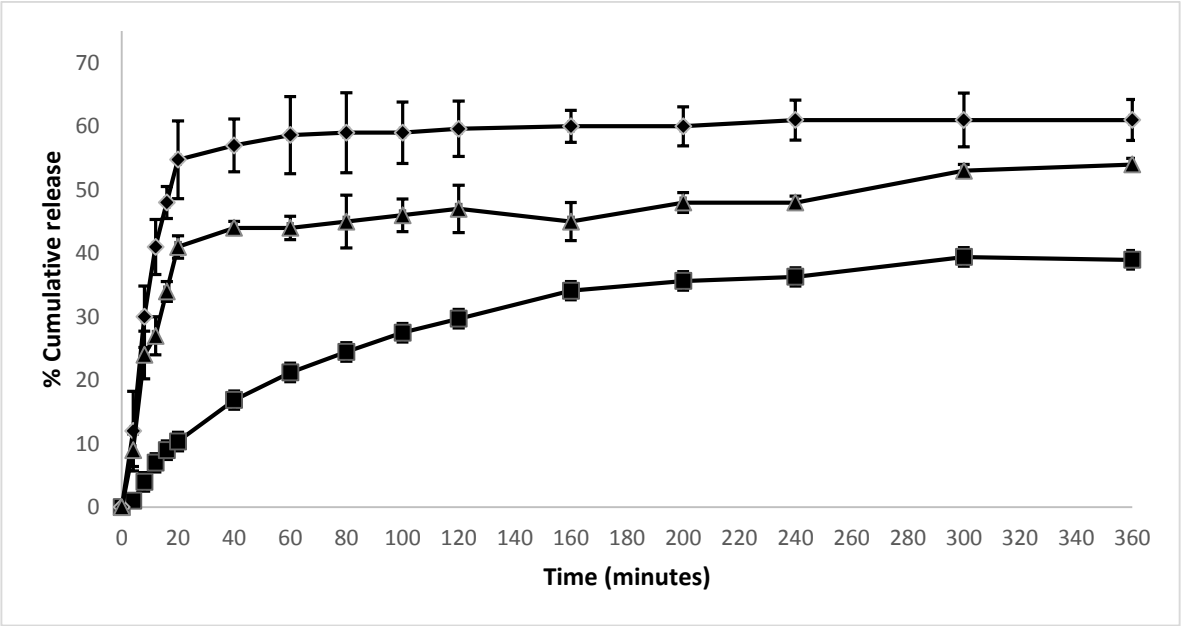

Figure 8. In vitro release of JC16 (◆), JC17 (▲) and Vapreotide (■) from CS NPs at pH 7.4, 100 rpm and 37°C

2.4. Antibacterial activity of CS NP against E. coli O157:H7 and MRSA

The antibacterial activity of chitosan nanoparticles, functionalized with JC16, JC17 and Vapreotide, carried out by determining minimum inhibitory concentration using broth microdilution method for both strains was determined. The growth of *E. coli* O157:H7 and MRSA showed typical kinetics as determined OD at 595 nm (Holowachuk et al., 2003); bacterial growth decreased in presence of different peptide nanoparticle concentrations; especially for peptide JC16 that inhibited at all tested concentration of the growth of E. *coli* O157:H7; on the other hand peptide JC17 presented the lowest potency to control the growth of *E. coli* O157:H7 (Fig 4); in deed these peptide nanoparticles presented MIC_{50} against *E. coli* O157:H7 between <1.3 and 3.4 uM that were always lower than the MIC_{50} of free peptide (Table 3). These peptide nanoparticles also inhibited the growth of MRSA, being more potent against MRSA than *E. coli* O157:H7 showing inhibition at all concentrations tested (Fig 5). There were not significant differences in the potency to arrest the MRSA growth of these peptide nanoparticles as determined comparing the MIC_{50} of these three peptides (Table 3). The MIC_{50} against MRSA for JC16, JC17 and Vapreotide peptide nanoparticles were 76, 12.5 and 50 times lower than the MIC_{50} of free peptide, respectively (Table 3).

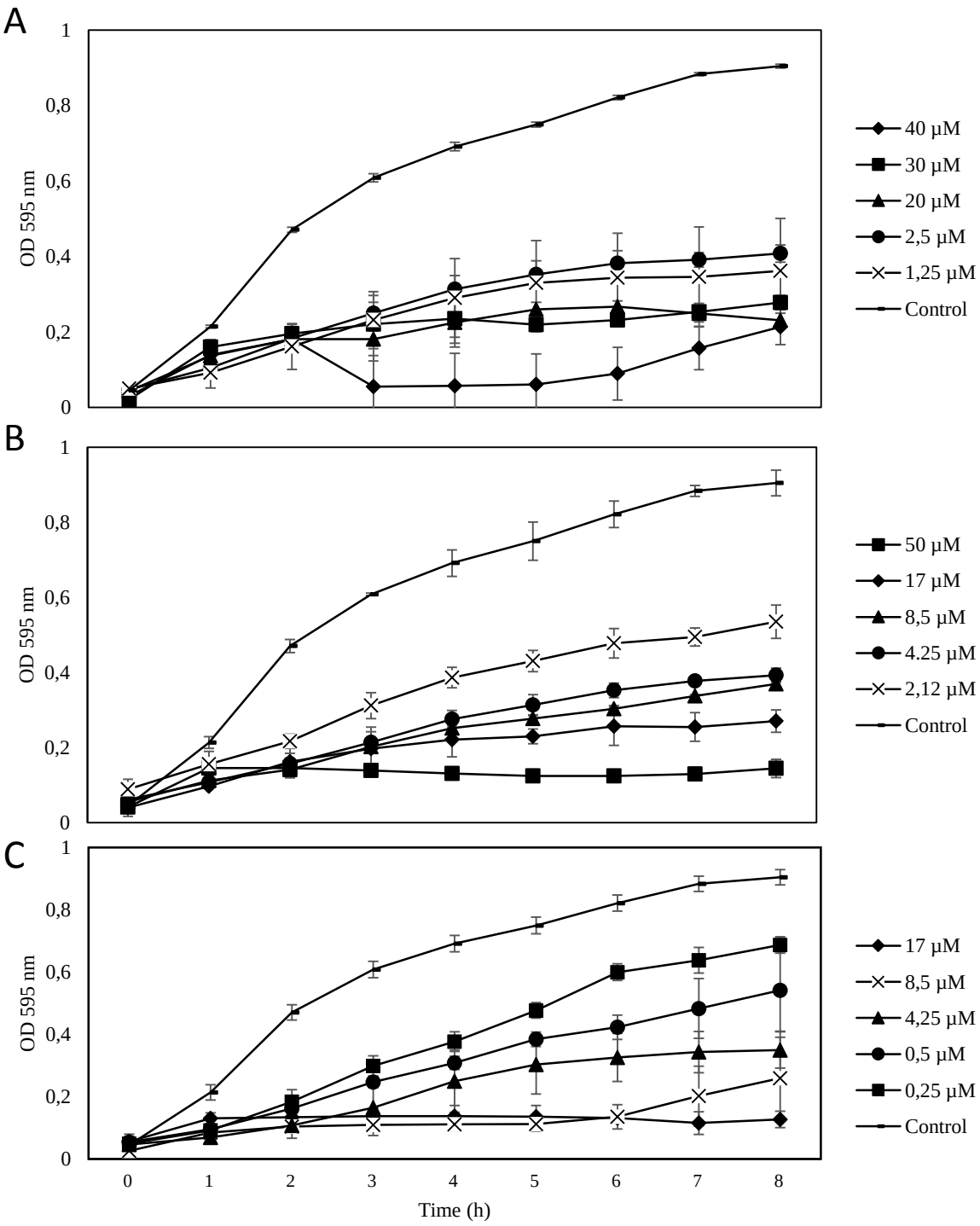

Figure 9. Microbial growth kinetics of *Escherichia coli* O157:H7 in the presence of CSNPs functionalized with: **(A)** JC16, **(B)** JC17 and **(C)** Vapreotide

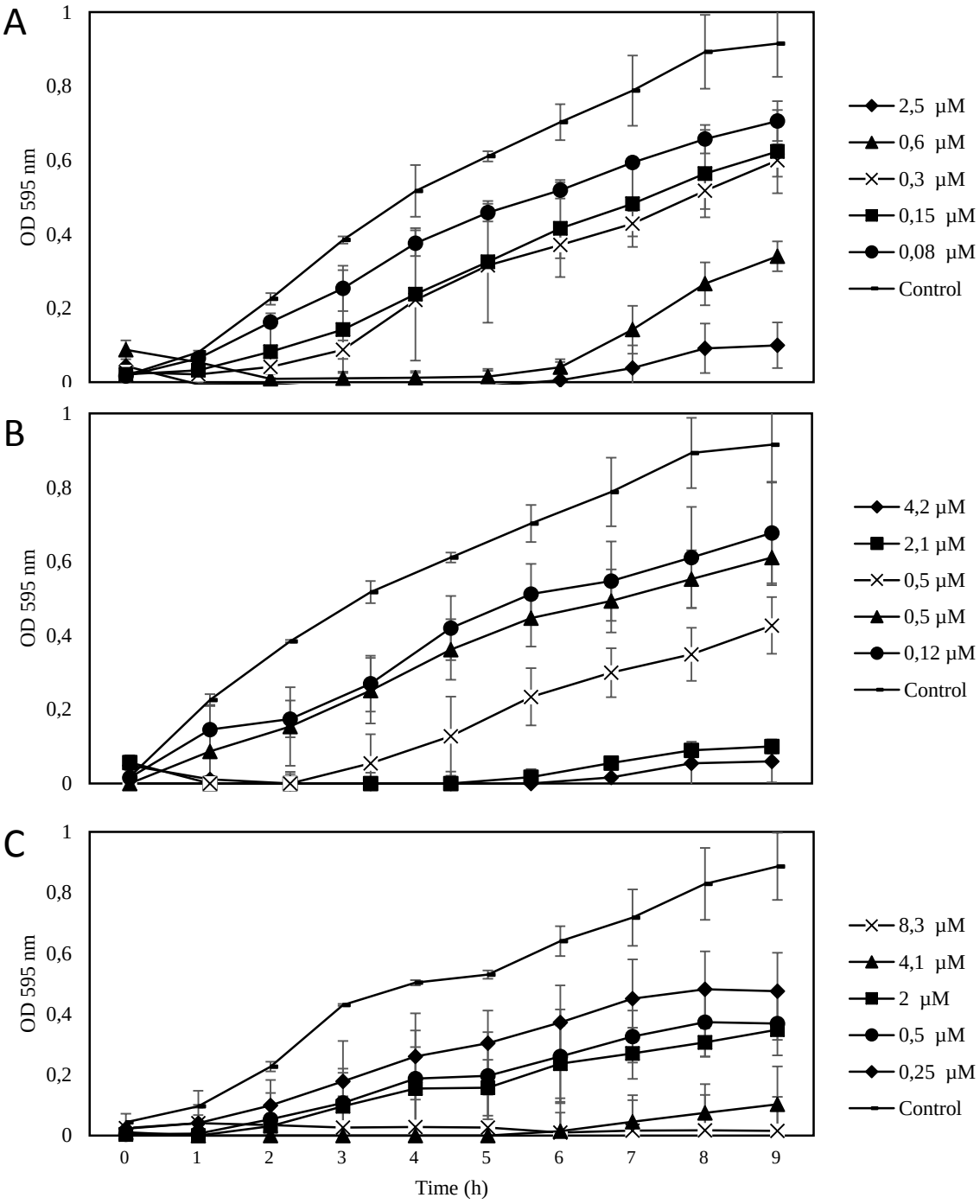

Figure 10. Microbial growth kinetics of Methicillin-resistant *Staphylococcus aureus* in the presence of CSNPs functionalized with: **(A)** JC16, **(B)** JC17 and **(C)** Vapreotide

Table 3. MIC of peptides nanoencapsulated in chitosan nanoparticles against *Escherichia coli* O157:H7 and MRSA determined by the microdilution method.

Microorganism	Peptide nanoencapsulated	MIC_{50} µM	MIC_{90} µM	MIC_{99} µM
***Escherichia coli* O157:H7**	JC16	< 1,3	> 40	-
		>5	**-**	**-**
	JC17	3,4	50	-
		10	**-**	**-**
	Vapreotide	2,4	17	-
		>10	**-**	**-**
Methicillin resistant *S. aureus* (MRSA)	JC16	0,42	2,5	10
		32	**65**	**75**
	JC17	0,5	2,2	8,5
		6,25	**40**	**>50**
	Vapreotide	0,5	4,2	8,5
		25	**45**	**50**

Bold values correspond to MIC of free peptide. MIC of empty nanoparticles (Determined by chitosan concentration) was 20.2 **µM** and 2.6 **µM** against *E coli* and MRSA respectively.

3. DISCUSSION

Peptides JC16 and JC17 designed by Cruz et al. present potent antimicrobial activity that increased by encapsulating them in chitosan nanoparticles. These peptides were designed having into account structural peptide properties, such as hydrophobicity, hydrophobic moment and net charge (Cruz et al; under revision); resulting in cationic peptides with net charge of +5 for JC16 and +4 for JC17, α-helical structure, amphipathic and a total of 17 amino acid residues in each case, as outlined in Table 1. In fact, amphipathic structure play a key role in the antimicrobial mechanism of action, facilitating the electrostatic interaction of antimicrobial peptides with the negatively charged bacterial surface; the hydrophilic (positive charged) could interact with the negatively charged headgroups of bilayer phospholipids, in this way increasing the peptide positive charge correlates with the increase in antimicrobial potency, as it has been seen in other studies (Hancock & Diamond, 2000; Oren & Shai, 1998; Wu, Maier, Benz, & Hancock, 1999). JC16 and JC17 have not structure in aqueous solution; but there is evidence that helix formation can be induced by lipids through direct interaction with the lipid bilayer, which might also assist membrane penetration when these peptides interact with the lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria such as *E. coli* O157:H7 (Epand, Hui, Argan, Gillespie, & Shore, 1986; Robinson, Blakeney, & Mattice, 1982). In gram-positive bacteria such as MRSA, the initial interaction occurs with teichoic and teichuronic acids and with carboxyl groups of peptidoglycan, which give the negative charge to the bacterial surface (Tossi, Sandri, & Giangaspero, 2000). We also studied Vapreotide, a model peptide having a positive net charge given by Lysine residues lower than JC16 and JC17, which is an octapeptide analogue of somatostatin; with molecular weight of 1131.37 Da; potentially candidate to have antimicrobial activity (Calès, 2008; Deghenghi, Papotti, Ghigo, Muccioli, & Locatelli, 2001; Fortune, Jackson, Leonard, & Trotter, 2009; O'Byrne et al., 1999). It has growth inhibitory activity in experimental tumours, it has been tested in treatment of esophageal variceal bleeding and it has demonstrated efficacy in the early management of acute variceal haemorrhage.

JC16, JC17 and Vapreotide can be susceptible to degradation depending on the administration route or be unstable in the biological environment losing their biological activity due to inherent physicochemical and biopharmaceutical features. This is the main reason for using chitosan nanoparticles as carriers because nanoparticles enable the encapsulated molecules to retain their biological activity and allow controlled release. In addition, chitosan is biocompatible, biodegradable, having mucoadhesive properties, low toxicity and permeability enhancement (Abdel-Hafez, Hathout, & Sammour, 2014). The chitosan nanoparticle preparation was based on ionotropic gelation method; which utilizes the ability of polyelectrolytes to cross link in the presence of counterions. We used sodium tripolyphosphate which is a counterion of low molecular weight, nontoxic and multivalent. The formation of chitosan nanoparticles with TPP are friendly for peptides and proteins (Calvo,

Remunan-Lopez, Vila-Jato, & Alonso, 1997; Sinha et al., 2004). The CS nanoparticles prepared in this work exhibited a typical batch of size distribution (figure 1/B) with a mean diameter in the range of approximately 260–350 nm and a narrow size distribution (polydispersity index <0.5) as outlined in Table 2. Nanoparticles functionalized with JC16 and JC17 showed an average diameter lower than empty nanoparticles probably because the positive charge of both peptides induces more compact nanoparticles. The peptide chains could take the place of some CS fragments crisscrossing with TPP anions and other chitosan fragments. When low molecular weight chitosan is used, the nanoparticles tend to decrease their size (H. Zhang, Oh, Allen, & Kumacheva, 2004), thus we assume JC16 and JC17 acted as small polymer chains forcing the nanoparticle to reduce its diameter. This did not happen with Vapreotide because its charge and size is lower than JC16 and JC17.

Chitosan also shows antifungal and antimicrobial activity; the proposed mechanisms involve the positively charged groups amino of chitosan interacting with negatively charged components of fungi and bacteria membranes, resulting in increased membrane permeability, causing the leakage of cellular contents and cell death (Kong, Chen, Xing, & Park, 2010). Other proposed mechanisms are: 1) chitosan binds to trace elements, causing the vital nutrients unavailable for normal growth or 2) chitosan gets inside the cell, binds to its DNA affecting the expression of important proteins (Ing et al., 2012; Kong et al., 2010).

The surface charge of the nanoparticles determined by the zeta potential can influence their stability in suspension through electrostatic repulsion between them. The zeta potential of JC16- and Vapreotide nanoparticles was slightly higher to, and JC17 nanoparticles was similar to the empty nanoparticles. No decrease in the zeta potential of the peptide nanoparticles can be attributed to the positive net charge of peptides, which could increase the nanoparticle stability and increase the repulsion among them avoiding the aggregate formation. The positive net charge of peptides could be responsible of the high encapsulation efficiency as shown in Figure 2, due to interaction with the opposite charges of TPP in the gelling process of the nanoparticle formation. In fact, peptides JC16 and JC17, which have higher positive charges than, Vapreotide, presented slightly higher encapsulation efficiencies than Vapreotide. The rate of peptide release from chitosan nanoparticles seems to be influenced by peptide charge because JC16 having a net charge of +5 achieves rapid release of 60 % in a period of about 20 minutes; in the same period JC17 with lower net charge of +4 reaches a release close to 40 % and Vapreotide with the lowest charge only 15 %. The positive charge of the peptide increased the rate of peptide release from the chitosan nanoparticle. However once these percentages values are reached, the release becomes slower for many hours. Similar peptide release rates and behaviours release have been reported (Hu et al., 2008).

The synergistic effect between the PAMs and chitosan, seen in nanoparticles functioned as carrier for JC16, JC17 and Vapreotide resulted in a lower concentration of antimicrobial peptide necessary to inhibit the growth of the tested

bacteria. For example, MIC_{50} for MRSA of JC16, JC17 and Vapreotide was 75, 12.5 and 50 times lower than the MIC_{50} of the free peptide, respectively. Moreover *E. coli* O157:H7 was less sensitive to the peptide-nanoparticle functionalization than gram positive strain MRSA (Table 3). This differential effect could be due to Gram negative bacteria have an outer membrane composed of proteins, phospholipids and polysaccharides which is not present in gram-positive bacteria and constituting a first line of defence against antimicrobials agents (Chatterjee & Chaudhuri, 2012). The higher activity of peptide nanoparticles compared to the free peptide is due to the combined action of the peptide and chitosan as well as greater availability of active ingredient near the target cell.

4. CONCLUSIONS

The use of peptides is an efficient strategy to combat different types of microorganisms. Peptides are on a par with the best known therapeutic molecules because problems such as peptide stability and cost of large scale synthesis have already potential solutions (Reddy et al., 2004). Peptide stability can be improved as was done in this study, by encapsulating the peptides in biodegradable polymeric nanoparticles that allow controlled release of the active ingredient and protects it from enzymatic degradation and factors such as pH. In this case the nanoparticle not only served as protection for the peptide but also contributed to inhibitory activity due to the antimicrobial properties of chitosan, resulting in improved antibacterial activity for each nanoencapsulated peptide compared to free peptide.

In summary, peptide-loaded chitosan nanoparticles have been synthesized and characterized. These nanoparticles showed positive surface charges (> 20 mV), low polydispersity index (< 0.3) and a hydrodynamic size between 280 and 330 nm. In addition the functionalization of peptides with the nanoparticles was effective against bacterial strains tested in this work and it is emerging as a promising therapeutic strategy.

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