ANTIMICROBIAL ACTIVITY OF A NEW SYNTHETIC ENCAPSULATED ANTIMICROBIAL PEPTIDE AGAINST *Pseudomonas aeruginosa, Escherichia coli* O157:H7 AND METHICILLIN RESISTANT *Staphylococcus aureus*

JAIR ALEXANDER FLÓREZ PLATA

UNIVERSIDAD INDUSTRIAL DE SANTANDER

FACULTAD DE CIENCIAS

ESCUELA DE QUÍMICA

BUCARAMANGA

2016

ANTIMICROBIAL ACTIVITY OF A NEW SYNTHETIC ENCAPSULATED ANTIMICROBIAL PEPTIDE AGAINST *Pseudomonas aeruginosa, Escherichia coli* O157:H7 AND METHICILLIN RESISTANT *Staphylococcus aureus*

JAIR ALEXANDER FLÓREZ PLATA

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

Director:
CLAUDIA CRISTINA ORTIZ LÓPEZ
Microbióloga, *PhD*

Co-Director:
JENNIFFER CRUZ LAITÓN
Química, *Msc*

UNIVERSIDAD INDUSTRIAL DE SANTANDER
FACULTAD DE CIENCIAS BÁSICAS
ESCUELA DE QUÍMICA
BUCARAMANGA
2016

DEDICATORIA

Al Rey de Reyes y Señor de Señores, Mi Padre Amado Dios.

Al ser más maravilloso y el más importante de mi vida, Mi Hermosa Madre María Teresa Plata.

A mi abuelo Santiago Plata, mi mayor motivación y ejemplo de vida.

AGRADECIMIENTOS

A Dios por darme la vida, por tantas bendiciones y por la sabiduría necesaria para alcanzar este gran logro.

A mis padres por su infinito amor, comprensión, dedicación esfuerzo y sacrificio; no sólo en este proceso sino durante mis años de vida y sobre todo por los valores inculcados para ser de mi la persona que soy ahora.

Al resto de mi familia: mis hermanos, mis abuelos, mis tíos, mis primos por toda la colaboración y el cariño brindado. Especialmente a mi tío Juan, quien desde la distancia siempre me colaboró en todos los aspectos, pero sobre todo y lo más importante con los consejos y la motivación que a diario me daba.

Al profesor Rodrigo Torres y la profesora Claudia Ortiz quienes me brindaron la oportunidad de pertenecer al grupo de investigación GIBIM, por toda la colaboración y todas las enseñanzas tanto académicas como personales.

A Jenniffer Cruz que más que mi codirectora fue como una segunda madre y como una mentora para mí. Por toda su gran paciencia, colaboración, cariño, confianza y enseñanzas durante este tiempo de trabajo y amistad.

A los doctores Mauricio Urquiza y Jorge Gutiérrez porque a pesar del poco tiempo de haber llegado al grupo, fueron de gran ayuda para la terminación de este proceso de formación y porque además de eso logré establecer con ellos una gran amistad. A los demás integrantes del GIBIM quienes fueron fundamentales en el desarrollo de mi investigación, quienes me colaboraron de la mejor manera, con quienes compartí y viví diferentes momentos, en especial a mis dos buenos amigos César Ariza y Johan Gallo con quienes también compartí experiencias en el ámbito deportivo.

A mi segunda Familia conocida como LOS BONITOS FÚTBOL CLUB: Yesenia, Alberto, Jorge, Oscar, Johanny, Jonathan, Fabián y Carlos; por ser parte de este gran triunfo, por ser esas personas maravillosas con las que compartí toda clase de momentos, porque cada uno aportó algo para mi vida, por la amistad incondicional, por el cariño brindado, por ser únicos, gracias mil gracias. Siempre los llevaré en mi corazón.

A la Familia Pérez Santamaría por el especial acogimiento, ayuda y el cariño brindado.

A todos mis amigos. Los de la carrera: Alba López, Monica Vega, Susana, Judith, Lyda, Terry, Isidro; y los del barrio: Karen, Tati, Silvia Vargas, Silvia Joya, Adri, Lalis, Jhosman, Shecho, Jhoao, Ferney, Chucho, Yeiner, Jota, Marlon y William por creer y estar siempre conmigo en las buenas y en las malas. Los quiero mucho.

A la Universidad Industrial de Santander y a la escuela de Química por su formación.

CONTENTS

Pág.

INTRODUCTION 14
1. MATERIAL AND METHODS 17
1.1 MATERIALS 17
1.2 PEPTIDE SYNTHESIS AND CHARACTERIZATION 17
1.3 CIRCULAR DICHROISM 18
1.4 PREPARATION OF POLYMERIC NANOPARTICLES 18
1.5 PHYSICOCHEMICAL PROPERTIES OF NPs 19
1.6 DETERMINATION OF PEPTIDE LOADING EFFICIENCY OF NANOPARTICLES 19
1.7 IN VITRO RELEASE 20
1.8 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) 20
1.9 SERUM STABILITY ASSAY 21
1.10 PEPTIDE LOADED NP HEMOLYTIC ACTIVITY ON HUMAN ERYTHROCYTES 21
1.11 STATISTICAL ANALYSIS 22
2. RESULTS AND DISCUSSION 23
2.1 SYNTHESIS AND CHARACTERIZATION OF GIBIM-P5S9K LOADED POLYMERIC NANOPARTICLES 23
2.2 ANTIBACTERIAL ACTIVITY OF GIBIM-P5S9K PEPTIDE FREE AND LOADED IN POLYMERIC NANOPARTICLES 28
2.3 IN VITRO PEPTIDE RELEASE FROM PLA- OR PLGA-NPS 31
2.4 PLA- OR PLGA-NP STABILITY IN HUMAN SERA 32
2.5 HEMOLITIC ACTIVITY OF PEPTIDE LOADED PLA- OR PLGA-NPS 33
3. CONCLUSION 35
ACKNOWLEDGMENTS 36
BIBLIOGRAPHY 37
ANNEXES 44

LIST OF FIGURES

Pág.

Figure 1. SEM micrograph and Zeta potential of GIBIM-P5S9K loaded NPs. a) and b) PLANPs, c) and d) PLGANPs.....26

Figure 2. Antimicrobial activity against *E. coli* O157:H7. a) GIBIM-P5S9K, b) GIBIM-P5S9K- loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (●) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◄) 25µM, (►) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.....29

Figure 3. Antimicrobial activity against MRSA. a) GIBIM-P5S9K, b) GIBIM-P5S9K-loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (●) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◄) 25µM, (►) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.....29

Figure 4. Antimicrobial activity against *P. aeruginosa*. a) GIBIM-P5S9K, b) GIBIM-P5S9K- loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (⬬) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◄) 25µM, (►) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.....30

Figure 5. In vitro release of NPs. Release profile of polymeric nanoparticles under the same incubation conditions, pH and stirring speed.....31

Figure 6. Hemolytic activity GIBIM-P5S9K encapsulated in PLGA and PLA. The final concentrations used PGLA and PLA were to C1 and C2 were 5 and 0.5 mg/mL, respectively. *p<0.05 versus viability cells without compound (positive control).....34

LIST OF TABLES

Pág.

Table 1. Physicochemical properties of peptide GIBIM-P5S9K. 24
Table 2. Physicochemical properties of PLA and PLGA nanoparticles.....27
Table 3. Antimicrobial activity of free and encapsulated GIBIM-P5S9K. 30
Table 4. Serum stability of GIBIM-P5S9K-loaded PGLA and GIBIM-P5S9K-loaded PLA NPs. 33

LIST OF ANNEXES

Pág.

Annex A. RP-HPLC of GIBIM-P5S9K peptide.....44
Annex B. MALDI-TOF spectrum of GIBIM-P5S9K. 45
Annex C. CD spectrum of GIBIM-P5S9K peptide. 46

RESUMEN

TÍTULO: ACTIVIDAD ANTIMICROBIANA DE UN NUEVO PÉPTIDO ANTIMICROBIANO SINTÉTICO ENCAPSULADO, FRENTE A *Pseudomona aeruginosa*, *Escherichia coli* O157:H7 Y *Staphylococcus aureus* RESISTENTE A METICILINA*

AUTOR: JAIR ALEXANDER FLÓREZ PLATA**

PALABRAS CLAVE: Nanopartículas de PLA y PGLA, SARM, *E. coli* O157:H7*,* péptido, actividad antibacteriana.

DESCRIPCIÓN: Los sistemas de nanotransporte están siendo desarrollados para la entrega de péptidos, proteínas y genes debido a que los protegen en la circulación sanguínea y en el tracto gastrointestinal. En esta investigación, se obtuvieron nanoparticulas (NPs) de Ácido Poliláctico (PLA) y Ácido Poliláctico-co-Glicólico (PLGA) cargados con un nuevo péptido antimicrobiano por el método de doble de emulsión/evaporación de solvente. Las NPs de PLA y PLGA, caracterizadas por dispersión de luz dinámica, potencia Zeta, microscopía electrónica de barrido (SEM), presentaron tamaños esféricos entre 300 a 400 nm para PLA y 200-300 nm para PLGA con carga superficial > 20mV e índice de polidispersidad < 0,3. La eficiencia de carga del péptido fue de 75% y 55% para NPs de PLA y PLGA, respectivamente. Alrededor del 50% de péptido fue liberado de las NPs de PLA y PLGA durante las primeras 8 horas. Las NPs de PLA y PLGA cargadas con el péptido, incubadas en suero humano al 10%, aumentaron los tamaños de estás entre 25,2% y 39,3%, con un rango de PDI de 3.2 a 5.1 y una carga superficial entre -7,15 y -14,6 mV. Tanto el péptido cargado en NPs de PLA y PLGA inhibieron el crecimiento de *Escherichia coli* (*E. coli)* O157:H7, *Staphylococcus aureus* resistente a meticilina SARM y *Pseudomona aeruginosa* a una concentración de péptido al 0.5 µM. Por el contrario, el péptido libre no inhibió a 0.5 y 1 µM, pero inhibió a 10 µM. Estas NPs fueron hemocompatibles porque presentaron hemólisis <10% y son promisorias para la entrega y la protección del sistema del péptido GIBIM-P5S9K.

---

*Trabajo de grado

**Facultad de Ciencias, Escuela de Química. Director: Claudia Cristina Ortiz López, *PhD.* Codirector: Jenniffer Cruz Laitón, *Msc.*

ABSTRACT

TITLE: ANTIMICROBIAL ACTIVITY OF A NEW SYNTHETIC ENCAPSULATED ANTIMICROBIAL PEPTIDE AGAINST *Pseudomonas aeruginosa, Escherichia coli* O157:H7 AND METHICILLIN RESISTANT *Staphylococcus aureus**

AUTHOR: JAIR ALEXANDER FLÓREZ PLATA**

KEYWORDS: PLA and PLGA nanoparticles, MRSA, *E. coli* O157:H7, *Pseudomona aeruginosa*, peptide, antibacterial activity.

DESCRIPTION: Nanocarrier systems are currently being developed for peptide, protein and gene delivery because protect them in the blood circulation and in the gastrointestinal track. Herein, we obtained Polylactic Acid (PLA) and Poly (Lactic-co-Glycolic) Acid (PLGA) nanoparticles (NPs) loaded with a new antimicrobial peptide by the double emulsion solvent extraction/evaporation method. PLA- and PLGA-NPs, characterized by dynamic light scattering, Zeta potential, scanning electron microscopy (SEM), were spherical with sizes between 300-400 nm for PLA and 200-300 nm for PLGA with >20mV surface charge and <0.3 polydispersity index. The peptide-loading efficiency was 75% and 55% for PLA-NPs and PLGA-NPs, respectively. Around 50% of peptide was released from PLA- or PLGA-NPs over 8 hours. Peptide loaded PLA- and PLGA-NPs in 10% human sera increased the nanoparticle size between 25.2% and 39.3%, the PDI from 3.2 to 5.1 and the surface charge from -7.15 to -14.6 mV. Both, peptide loaded PLA- and PLGA-NPs inhibited *Escherichia coli* (*E. coli*) O157:H7*,* Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* growth at 0.5µM peptide concentration. In contrast, free peptide did not inhibit at 0.5 and 1 µM but inhibited at 10µM. These NPs were hemocompatible because presented <10% hemolysis. These PLA- and PLGA-NPs are a promising for delivery and protection system of GIBIM-P5S9K peptide.

---

*Bachelor Thesis

**Faculty of Sciences, School of Chemistry. Director: Claudia Cristina Ortiz López, *PhD.* Codirector: Jenniffer Cruz Laitón, *Msc.*

INTRODUCTION

The worldwide infectious diseases caused by resistant microorganisms are turning in pathologies of difficult therapeutic treatment. Particularly, a serious public health problem is the increasing prevalence of antibiotic resistant bacterial infections (Rai, Yadav, & Gade, 2009),(Kim et al., 2007). The emergence of antibiotic resistant bacteria is associated to the inappropriate antibiotic use; which in these microorganisms generates a selective pressure leading to the appearance of antibiotic resistance genes (Kola & Urba, 2001), (Davies & Davies, 2010). In consequence, the development of novel antimicrobial compounds, acting on a broad variety of targets is required (Singh, Smitha, & Singh, 2014). Antimicrobial peptides (AMPs) have recently emerged as one of these compounds due to its multiple mechanisms of action, broad activity spectrum, relatively low bacterial resistance and great potential for clinical use (Marr, Gooderham, & Hancock, 2006), (Seo, Won, Kim, Mishig-ochir, & Lee, 2012).

The AMPs are effector molecules of the innate immune system often isolated from various natural sources such as plants, animals and bacteria (Hancock & Sahl, 2006; Haney, Nazmi, Lau, Bolscher, & Vogel, 2009). AMPs are cationic peptides, because the lysine and arginine content with a net charge between +2 to +9 at physiological pH; having around 30 amino acid (a size less than 10 kDa), amphipathic sequences and characteristic secondary structures. These properties confer them antimicrobial properties because the better interaction with the cell membrane of pathogenic bacteria (Park, Park, & Hahm, 2011; Povey, Howard, Williamson, & Smales, 2008; Rozek, Powers, Friedrich, & Hancock, 2003). However, the AMP therapeutic use is restricted because their susceptibility to degradation in human fluids before reaching the bacterium cell wall by the acidic pH of the stomach, the enzyme proteolytic action, high ionic strength media (Sohani & Gaikwad, 2013). Fortunately, the AMP bioavailability in the patient and stability against extreme conditions faced in the human body can be improved by

using different techniques as for example loading the AMPs in nanoparticles. In fact the nanoparticles are alternatives for peptide and protein delivery, because they exhibit versatility for oral administration, site-specific release, biocompatibility with tissues and cells and improved bioavailability of the active molecules by minimizing degradation and toxicity of the active ingredient (Atyabi, Talaie, & Dinarvand, 2009; Fievez, Garinot, Schneider, & Préat, 2006; Jung & Jik, 2011).

Polymeric nanoparticles (NPs) are defined as solid colloidal particles formed by polymeric assemblies at nanoscale in which the drug or active principle is dispersed within a polymer matrix, acting as carriers and nanocapsules of drugs or active principles (Hans & Lowman, 2002). They can be classified as nanospheres, in which the drug is dispersed evenly throughout the polymer matrix (typically as a solid solution), or nanocapsules in which the drug is present in the core either as aqueous or oil solution (Kumari, Yadav, & Yadav, 2010).

Different techniques have been developed to prepare polymeric nanoparticles (NPs) loaded with peptides and proteins such as: emulsion /solvent evaporation, double emulsion, solvent spread, coacervation, nanoprecipitation, ionotropic gelation, precipitation by salts (Forier et al., 2014). In general, the NPs preparation begins with entrapment or immersion of the drug in the same or different phase with the polymer matrix. These obtained nanoparticles are purified by successive washings, centrifugation or filtration, among other methods (Alvarez-fuentes & Fern, 2008; Danhier et al., 2012; Reis & Neufeld, 2006).

NPs have been prepared from synthetic and natural polymers (Feng, 2004). However, the search for natural polymers for biomedical applications has decreased in the last three decades because their purity variations and the frequent required crosslinking to increase system stability, affecting drug encapsulation (Hans & Lowman, 2002). Polylactic acid (PLA) and the copolymer formed by monomer units of lactic and glycolic acid called poly-lactic-co-glycolic

(PLGA),–have been FDA (Food and Drug Administration) approved for polymeric NP preparation for drug delivering by parenteral route and they are broadly used in the controlled release of encapsulated drugs, due to their biodegradability and biocompatibility (Kumari et al., 2010; Prior, Gamazo, Irache, Merkle, & Gander, 2000; Vauthier & Bouchemal, 2009). The polymer and concentration used for drug encapsulation affect properties, particle structure, possible applications and the administration route (Reis & Neufeld, 2006). Indeed, PLGA can be modified to control the encapsulation degree, the drug release and degradation rate (Wu et al., 2013).

In this work, the GIBIM-P5S9K peptide was loaded into PLA and PLGA nanoparticles by double emulsion-solvent evaporation technique. Subsequently, the peptide-loaded NPs were characterized by DLS, Z potential, and SEM. The antimicrobial activity *in vitro* of free peptide and peptide-loaded NPs by the broth microdilution method on pathogenic strains of methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* O157: H7 (*E. coli* O157: H7) and *Pseudomonas aeruginosa* (*P. aeruginosa*) was evaluated and compared. Finally, peptide hemolytic activity and NP stability was evaluated. The results showed that NPs are no toxic and were stable (size and surface charge) in the presence of human serum.

1. MATERIAL AND METHODS

1.1 MATERIALS

L-amino acids protected at N-terminal with Fmoc group was purchased from IRIS Biotech GmbH; TBTU, HBTU, HoAT, DCC and DIPEA was obtained from Alfa Aesar, and Rink amide 4MBHA resin from Merck Millipore.

Poly-lactic acid (PLA, MW 85-160 KDa) and Poly Lactide-co-Glycolide acid (PLGA, 50:50, MW 38-54 KDa), Poloxamer 407 (POLO, MW 12.5 KDa), Ethyl acetate and Polyethylenimine (PEI, 2 KDa) were obtained from Sigma-Aldrich. Acetonitrile (ACN) and Methanol (MetOH) HPLC grade (J.T BAKER), Dichloromethane (DCM) and growth media Mueller Hinton (MH) from MERCK, the Brain Heart Infusion (BHI) from OXOID. The three bacterial strains were donated by School of microbiology at Pontificia Universidad Javeriana, Bogotá, Colombia, and were kept in BHI until analysis. The three bacterial strains were donated by School of microbiology at Pontificia Universidad Javeriana, Bogotá, Colombia, and were kept in BHI until analysis.

1.2 PEPTIDE SYNTHESIS AND CHARACTERIZATION

Peptide GIBIM-P5S9K was synthesized via solid phase peptide synthesis (SPPS) (Matsuzaki, 1999), using the tea-bag procedure reported by Houghten for multiple peptide synthesis(Houghten, 1985), in accordance with standard Fmoc chemistry and using 0.63 substituted rink amide 4MBHA resin (100-200 mesh; Loading: 0.63 mmol/g) and Fmoc amino acids(Fields & Noble, 1990). These peptides were cleaved by treatment with trifluoroacetic acid (TFA)/Triisopropylsilan (TIS)/Ethanedithiol/H_2O (92.5/2.5/2.5:2.5) for 2 hours and then precipitated with cold diethyl ether(Jofré, Guzmán, Cárdenas, Albericio, & Marshall, 2011). The peptides were desalted by gel exclusion chromatography using G-10 columns (Amersham, USA).

Peptides were purified by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (Jasco Corporation, Tokyo, Japan) with the Vydac C-18 preparative column, mobile phase of buffer A: H_2O with TFA 0.1% (v/v) and buffer B: Acetonitrile (ACN) containing TFA 0.1% (v/v); using the gradient 5-70% of B during 30 minutes at 1 mL/min and detection at 220 nm. Molar mass of purified peptides was determined by MALDI-TOF mass spectrometry (MS)(Granados, Pinto, Chaves, Calvo, & Rodrõ, 2002; Oliva, Guzman, Patarroyo, & Santovena, 2002).

1.3 CIRCULAR DICHROISM

CD spectra between 190-260 nm of designed and analogues peptides were carried out at 25 °C in a 1 mm path length cuvette in a CD Spectrometer (J-815 Jasco Corporation, Japan) using a 0.2 mM peptide solution dissolved in 50 mM sodium phosphate buffer, (pH 7.4) / 30% (v/v) 2,2,2-trifluoroetanol (TFE) (Houghten, 1985). Each spectrum was obtained as an average of three scans taken at 20 nm/min with a spectral band of 1 nm (Carvajal-rondanelli, Marshall, & Guzman, 2011).

1.4 PREPARATION OF POLYMERIC NANOPARTICLES

NPs were prepared using double emulsion solvent evaporation method as reported by Cohen-Sela *et al*. with some modifications(Cohen-Sela, Chorny, Koroukhov, Danenberg, & Golomb, 2009); 1 mL of (1 mg/ml) GIBIM-P5S9K peptide solution (W_1 phase) was dispersed in 4 mL of DCM containing 10 mg PLA or 4 mL of Ethyl acetate containing 10 mg PLGA (O phase). The W_1 and O phases were vigorously mixed for 30 s to form a simple W_1/O emulsion using a homogenizer-disperser (IKA Ultra-turrax T-18) at maximum speed (15500 rpm).

This first emulsion was then dispersed in 10 mL 1% Poloxamer 407 surfactant solution (W_2 phase). The suspension was then homogenized at 15500 rpm during

30 s to obtain $W_1/O/W_2$ double emulsion. Finally, DCM and Ethyl acetate was removed in a conventional rotary evaporator. This suspension was spinned at 22000 g to obtain the pellet that was washed and lyophilized. The dried product was then evaluated for re-dispersibility in aqueous medium.

1.5 PHYSICOCHEMICAL PROPERTIES OF NPs

The size and charge of nanoparticles was measured by dynamic light scattering (DLS) and z potential in Zetasizer Nano Series Nano-zs90 equipped with 633 nm laser at 25°C; all measurement were performed by triplicate and the polydispersity index was lower than 0.3. SEM microscopy in STEM and conventional mode was used to determine morphology and confirm sizes of gold coated nanoparticles in FEI Quanta 650 microscope at Microscopy Laboratory, Guatiguará Technology Park, Universidad Industrial de Santander, Colombia (Schneider, Rasband, & Eliceiri, 2012).

1.6 DETERMINATION OF PEPTIDE LOADING EFFICIENCY OF NANOPARTICLES

Peptide loading efficiency of nanoparticles (EE) was determined by an indirect quantification method, measuring the peptide concentration that remained in solution after separation from nanoparticles by centrifugation, using UV-Vis Spectroscopy at 220 nm and peptide standard calibration curve. Loading peptide concentration was calculated indirectly by calculating the differences between the initial and free peptide concentrations.

$$EE= (A-B/ A) *100$$

Where A is the total peptide amount, B is the free peptide amount (Taha, Singh, & Dennis, 2012).

1.7 IN VITRO RELEASE

GIBIM-P5S9K NPs (2 ml suspension) were diluted in PBS (20 ml) 50mM, pH=7.4 and incubated at 37 °C under magnetic stirring (180 rpm). At predetermined time points, a sample of the NP suspension (0.2 ml) was withdrawn, centrifuged at 22000 *g* and the amount of GIBIM-P5S9K in the supernatant was quantified at 220 nm in a Nanodrop Thermo Scientific (Cohen-Sela et al., 2009). The volume of the assay was kept constant by adding 0.2 ml of PBS pH 7.4, after every sample removing. The release assays were carried out in triplicate.

1.8 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC)

Bactericidal activity was evaluated by broth microdilution method (Martinez-gutierrez, Olive, & Banuelos, 2010; Ortiz & Torres, 2014). Initially, the preinoculum of *E. coli* O157:H7 and MRSA in Luria-Bertani (LB) and Muller-Hilton (MH) broth, respectively were prepared at 37°C, shaking at 200 rpm during 12-14 h; then the inoculum for each strain was set at 0.5 in McFarland scale (10^8 CFU/mL); 100 µL aliquots of these cell suspensions were mixed with 100 µL NPs solution at different concentrations in a 96 well microplate and incubated at 37°C in an orbital shaker (200 rpm for 8h). The growth kinetics of each microorganism was determined measuring absorbance at 595 nm in Elisa lector (Biorad, imarck). All assays were done by triplicate and the controls were 1) just growth media, 2) growth media plus bacteria,3) growth media, bacteria and Ofloxacina and 4) growth media, bacteria and non-loaded nanoparticles.

Minimum inhibitory concentration at 50% (MIC_{50}) was defined as the lowest concentration of nanocomposite capable to reduce the bacterial concentration to 50% in comparison with the growth of non-treated bacteria. After 8 hour incubation 100 µL aliquots of the previously treated bacterial culture were poured in 900 µL of BHI to determine MBC; the cultures were incubated at 37°C for 24 h. Then 10 µL

of this culture were seeded over BHI-agar petri dishes, incubated for 1 day at 37°C and the appearance of colonies were recorded. MBC was the lowest nanoparticle concentration producing >99,9% reduction of Colony-Forming Unit (CFU) comparing with the non-treated bacteria (Yoon et al., 2014).

1.9 SERUM STABILITY ASSAY

To evaluate the NP stability near to physiological conditions, 3.0 mg freeze-dried NPs were resuspended in 1.5 mL of 10 % v/v Human Serum (HS) in PBS. The suspensions were incubated at 37 °C, samples of this suspension were analyzed by NPs size and ζ-potential at fixed time intervals (0, 60, 120, and 240 min) to determine aggregations, changes in physical properties (particularly the ζ- pot), and the dimensional growth produced by the serum protein adsorption on the particles surface (Ruozi et al., 2015).

1.10 PEPTIDE LOADED NP HEMOLYTIC ACTIVITY ON HUMAN ERYTHROCYTES

Peptide hemolytic activity was evaluated on human erythrocytes; 1 mL of human blood obtained by venipuncture on citrate tubes was spun at 1000 × g per 10 min at 4 °C, the plasma and the white blood cells were discarded and the erythrocytes were washed four times with isotonic PBS and $2x10^7$erythrocytes/mL in PBS was prepared; aliquots of 100 µl of this suspension were transferred to 1.5 ml tubes and mix with free peptide or peptide loaded NP at the desired concentrations at 37 °C for 4 h. Then the erythrocyte suspension was spun at 13,000 xg for 5 min and 80 µL supernatant was transferred to a 96 well plate to measure hemoglobin released at 550 nm in a plate reader. 100% hemolysis was obtained by using 0.1% Triton X-100. The comparison was performed with the hemolytic concentration 50 (HC_{50}) (Andreu & Cativiela, 2009).

1.11 STATISTICAL ANALYSIS

Normalized data were expressed as the mean ±SEM and analyzed using the Statistica software (StatSoft Inc., Tulsa, OK, USA). The analysis of variance (one-way ANOVA) and t-student comparisons were used to assess statistical significance at $p<0.05$.

2. RESULTS AND DISCUSSION

2.1 SYNTHESIS AND CHARACTERIZATION OF GIBIM-P5S9K LOADED POLYMERIC NANOPARTICLES

The 17 amino acid peptide GIBIM-P5S9K designed by Cruz et al; taking into account the structural properties, such as hydrophobic moment, net charge and amphipathic helical structure (table 1), was synthesized in our laboratory by Fmoc (9-fluorenylmethoxycarbonyl) technique; analyzed by liquid chromatography high efficiency reverse phase (RP-HPLC) to establish its purity, by MALDI-TOF to measure its molecular weight and by Circular Dichroism (CD) to determine secondary structure (See annex A, B and C). According to the CAMP database (http://www.camp.bicnirrh.res.in/) and Protparam in Expasy (http://web.expasy.org/cgi-bin/compute_pi/pi_tool), this peptide displayed >95 % probability to be antimicrobial, a low instability index and positive charge.

Table 1. Physicochemical properties of peptide GIBIM-P5S9K.

Peptide	Sequence	Charge	MW (Da)	m/z	PAP(%)[a]	pI	II[b]	GRAVY[c]
GIBIM-P5S9K	ATKKCGLFKIL KGVGKI	5	1804.31	1804.19	96.0	10.2	-18.06	0.38

[a] Probably Antimicrobial Peptide

[b] Instability Index

[c] Grand Average of Hydropathicity

NPs loaded with peptide GIBIM-P5S9K were prepared taking into account that drug formulations at nanoscale allow a more effective drug interaction with bacterial membranes and enhance the drug bioavailability at infection sites (Rogueda & Traini, 2007; Suk et al., 2009). Considering the properties of GIBIM-P5S9K, PLA and PLGA, the double emulsion-solvent evaporation method was used for both PGLA and PLA GIBIM-peptide P5S9K nanoparticles preparation. This technique is considered the most suitable technique for the encapsulation of water-soluble molecules such as peptides and proteins (Hachicha, Kodjikian, & Fessi, 2006).

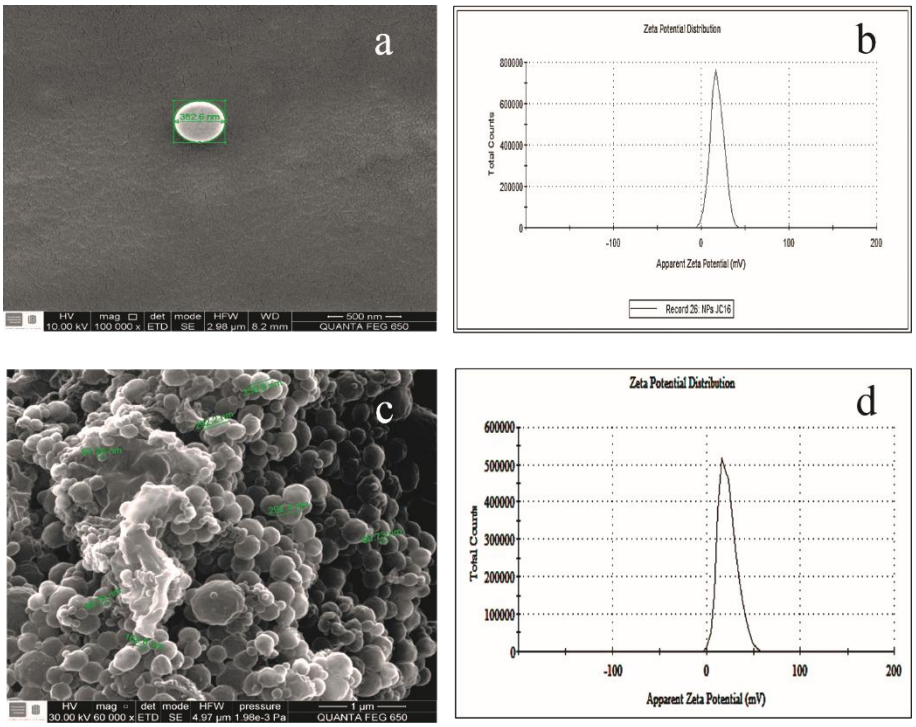
The PLA- and PLGA-NPs obtained in this work were between 350 nm and 250 nm in diameter respectively with polydispersity index < 0.3 as determined by DLS and SEM (Figure 1), indicating a homogeneous particle population or monomodal size distribution (figures 1(a) and 2(a)). These results are similar to other studies of hydrophilic drug encapsulation to obtain nanoparticles using this technique. The particle size is one of the most important characteristics because is related to stability, cellular uptake, release profile, degradation rate and biodistribution (Derman, Mustafaeva, Abamor, Bagirova, & Allahverdiyev, 2015; Kumari et al., 2011; Patel, Kulkarni, Nandekar, & Vavia, 2008; Roussaki et al., 2014).

The morphology of thin gold covered NPs determined by SEM, showed all of them spherical in shape and homogeneous dispersion sizes between 300-400 nm for PLA and 200-300 nm for PLGA. Both NPs were stabilized using the non-ionic surfactant Poloxamer 407 conformed by a Polypropylene (PP) and Polyoxyethylene, where the hydrophobic PPs interact with NP surface and hydrophilic “heads” with the aqueous media. This kind of capping modifies the NP z potential, which is related to electric repulsion and attraction between particles in a colloidal solution (Santander-Ortega, Jódar-Reyes, Csaba, Bastos-González, & Ortega-Vinuesa, 2006). In this sense, Polyethylenimine (PEI) was used to increase the positive surface charge of PLA and PLGA NPs because PEI is branched

molecule with high density of primary amino groups; in fact the z potential was +22 and +30 mV for PLA and PLGA, respectively (figures 1(b) and (d)). In our case, positively surface charged NPs were necessary to enhance the interaction with negatively charged bacterial membrane (Khayata, Abdelwahed, Chehna, Charcosset, & Fessi, 2012).

It is known that when second emulsion is formed, the amount of NP loaded peptide, NP size and morphology can be modified. In this case was not possible to determine that nanocomposites were either nanocapsule or nanosphere because the NP core was not analyzed.

Figure 1. SEM micrograph and Zeta potential of GIBIM-P5S9K loaded NPs. a) and b) PLANPs, c) and d) PLGANPs.



It is clear that the NP peptide loading efficiency (NP-PLE) strongly dependent on peptide properties such as solubility in the polymer matrix, molecular weight and functional groups. NP-PLE was higher in PLA (75.1%) than in PLGA (55.3%), probably because the use of ethyl acetate to dissolve PLGA and dichloromethane for PLA. The water solubility of ethyl acetate is higher than dichloromethane, suggesting faster peptide diffusion into the aqueous phase when using ethyl acetate. It is probably that ethyl acetate allows peptide interaction with the NP surface, facilitating the peptide removal during the washings. On the other side, the peptide adsorption is prevented when using dichloromethane to dissolve PLA because dichloromethane is immiscible in water (Table 2) (Cheow & Hadinoto, 2010). These NP-PLEs were similar to other drugs encapsulated with this methodology (Patel et al., 2008; Ruozi et al., 2015).

Table 2. Physicochemical properties of PLA and PLGA nanoparticles.

Nanoparticles	**Size-Average (d.nm)**	**PdI**	**Zeta Potential (mV)**	**NP-PLE (%)**
GIBIM-P5S9K-Loaded PLA	352.6 ± 3.2	0.19 ± 0.02	+29.4 ± 1.5	75.1% ± 1.5
PLA	311.2 ± 5.2	0.33 ± 0.05	+14.4 ± 0.1	ND
GIBIM-P5S9K-Loaded PLGA	258.3 ± 3.5	0.25 ± 0.1	+22.7 ± 0.3	55.3% ± 2.0
PLGA	280.5 ± 9.5	0.32 ± 0.1	+15.6 ± 0.3	ND

PdI: Polydispersity index, NP-PLE: Nanoparticle-Peptide loading encapsulated

ND: Not determinated

2.2 ANTIBACTERIAL ACTIVITY OF GIBIM-P5S9K PEPTIDE FREE AND LOADED IN POLYMERIC NANOPARTICLES

The antimicrobial activity of free peptide or peptide loaded PLA- or PLGA-NPs was determined using the broth microdilution method to increase the probability of interaction between bacteria and nanoparticles and also because its high sensitivity. Peptide loaded PLA- or PLGA-NPs presented a significant growth inhibition of *E. coli O157:H7,* SARM and *P. aeruginosa,* in a dose dependent manner, even at 0.5 µM peptide concentration. There was no significant differences in growth inhibition of bacteria treated with peptide loaded PLA- or PLGA-NPs, except that the activity of PLA-NPs against *E. coli O157:H7* (MIC_{50} <0.5 µM) was higher than the activity of PLGA-NPs (MIC_{50} between 1 and 10 µM). In contrast, free peptide did not inhibit at 0.5 and 1 µM, but at 10 µM showed almost complete bacterial growth inhibition (Figure 2, 3 and 4). This higher antibacterial activity of peptide loaded PLA- or PLGA-NPs than free peptide could involve an electrostatic interaction of the positive surface charge of these NPs (+22 and +30 mV) with the negative surface charge of both Gram-negative and Gram-positive bacterial membrane (Panyam, William, Dash, Leslie-Pelecky, & Labhasetwar, 2004; Xie et al., 2014). This interaction increases the probability of having a high local peptide concentration on bacterial surface, resulting in higher peptide specificity towards the target cell (Menon et al., 2012). This interaction could be mediated by teichoic acid, lipoteichoic or lipopolysaccharides and phospholipids on the bacterial surface. Non-loaded PLA- and PLGA-NPs, showed no significant growth inhibition of the three bacterial strains used in the study (Chan, Prenner, & Vogel, 2006; Dautin & Bernstein, 2007; Haney et al., 2009; Radovic-Moreno et al., 2012).

Figure 2. Antimicrobial activity against E. coli O157:H7. a) GIBIM-P5S9K, b) GIBIM-P5S9K- loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (●) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◄) 25µM, (►) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.

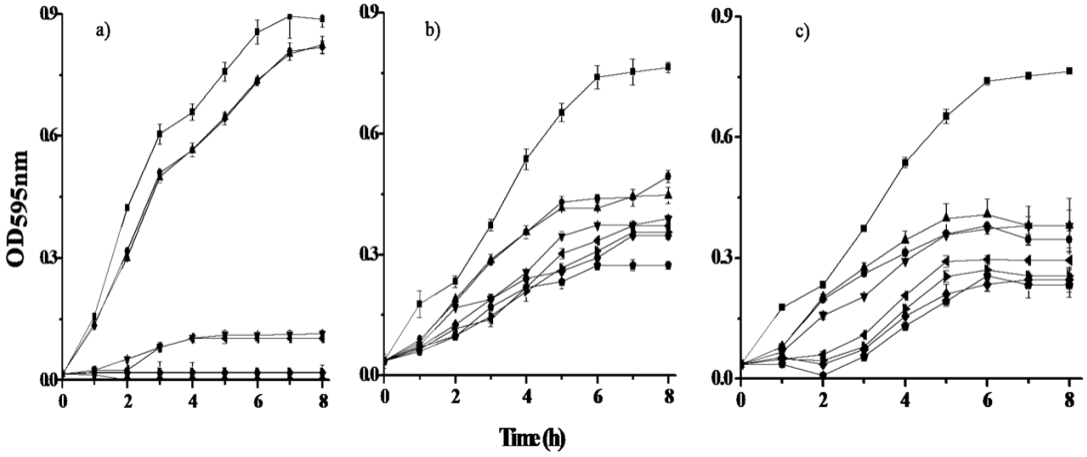


Figure 3. Antimicrobial activity against MRSA. a) GIBIM-P5S9K, b) GIBIM-P5S9K-loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (●) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◄) 25µM, (►) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.

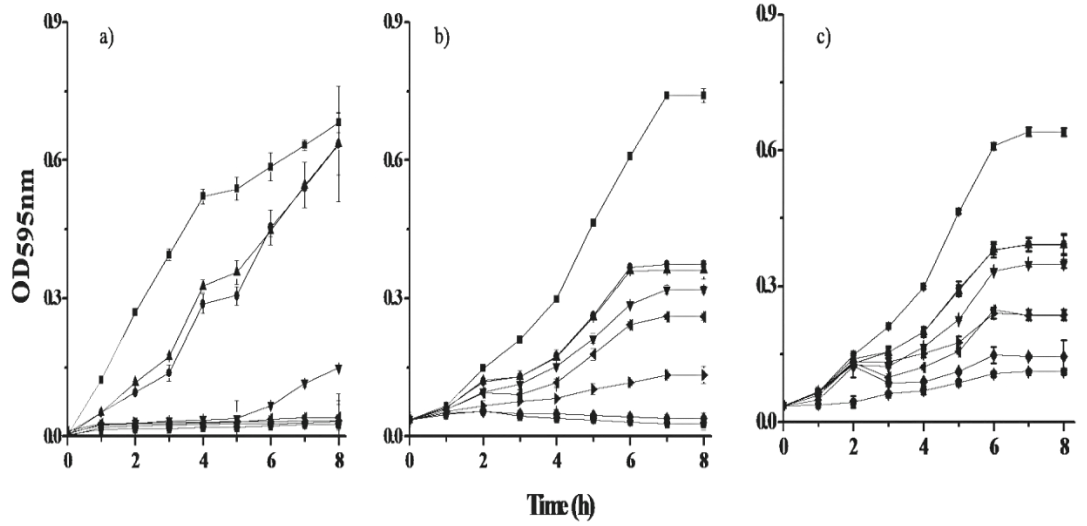


Figure 4. Antimicrobial activity against P. aeruginosa. a) GIBIM-P5S9K, b) GIBIM-P5S9K- loaded PLGA NPs c) GIBIM-P5S9K-loaded PLA NPs. (■) Control, (●) 0.5µM, (▲) 1.0µM, (▼) 10.0 µM, (◀) 25µM, (▶) 50µM, (◆) 75µM and (⬟) 100µM. Error bars indicate SD.

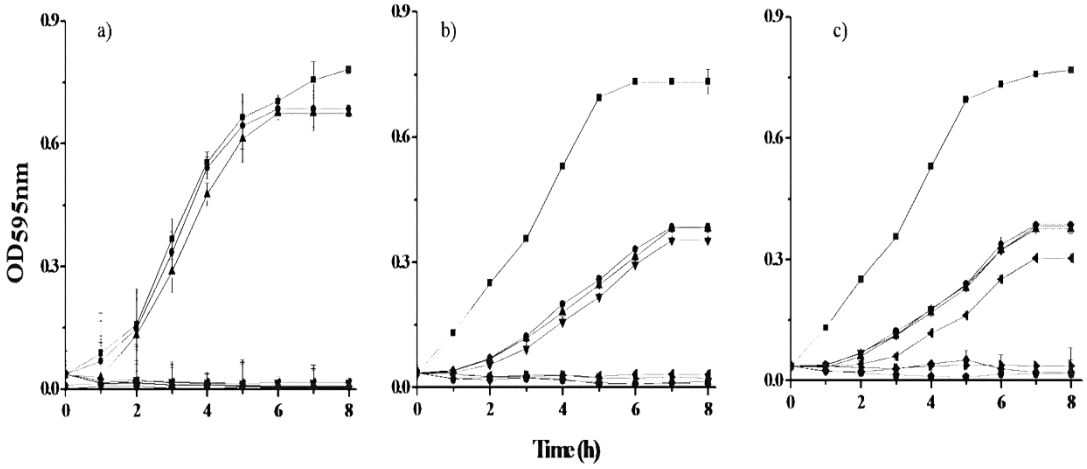


Table 3. Antimicrobial activity of free and encapsulated GIBIM-P5S9K.

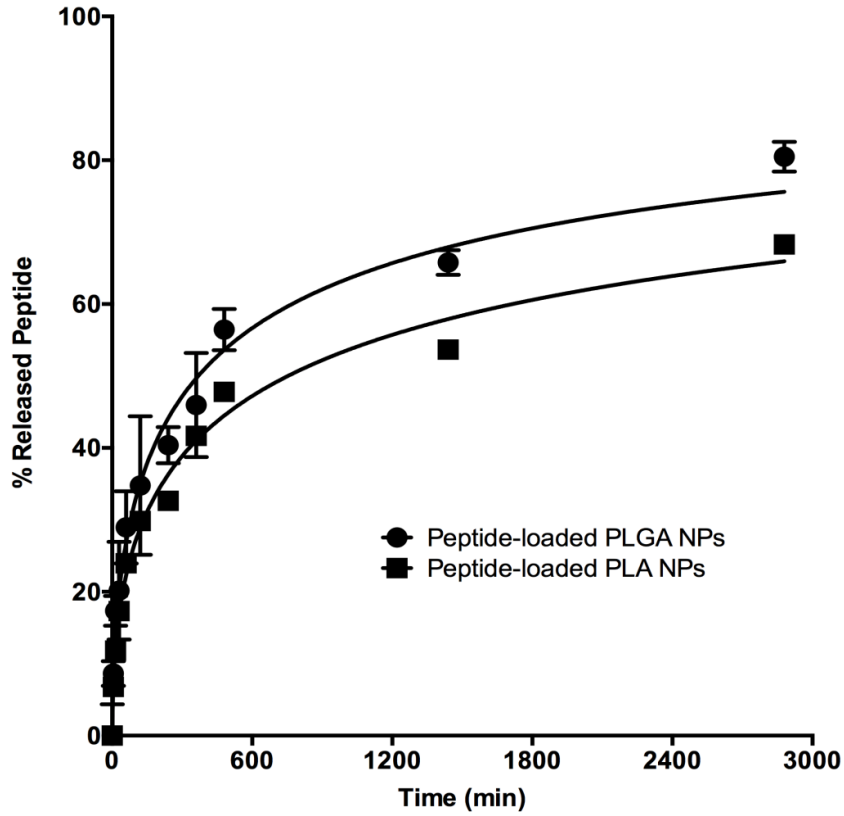
Compound	***E. coli* O157:H7**		**MRSA**		***P. aeruginosa***	
	MIC_{50} (µM)	**MBC (µM)**	**MIC_{50} (µM**	**MBC (µM)**	**MIC_{50} (µM)**	**MBC (µM)**
GIBIM-P5S9K	<10	50	>1.0	75	<10	>100
GIBIM-P5S9K-Loaded PLGA NP	<10	>100	<0.5	>100	<0.5	>100
GIBIM-P5S9K-Loaded PLA NP	<0.5	>100	<1.0	>100	<0.5	>100

*PLA and PLGA NPs did not inhibit bacterial growth at the concentration tested.

2.3 IN VITRO PEPTIDE RELEASE FROM PLA- OR PLGA-NPS

The drug release mechanism from nanoparticles generally involve three stages. In the first stage the drug bound to the surface or trapped in the nanoparticle is released. In the second stage the released drug diffuses through the polymer matrix and pores generated by matrix degradation. Finally, in the third stage drug is released to the media during the polymer matrix disintegration (Derman et al., 2015; Hu et al., 2008; Ortiz & Torres, 2014).Kinetics of peptide release from PLA- or PLGA-NPs showed two phase process; the initial one in the first 8 hours being the peptide release faster than final one for both NPs. In the initial phase around 50% of peptide was released from both PLA- or PLGA-NPs but a slightly faster for PLGA-NPs (Figure 5).

Figure 5. In vitro release of NPs. Release profile of polymeric nanoparticles under the same incubation conditions, pH and stirring speed.



2.4 PLA- OR PLGA-NP STABILITY IN HUMAN SERA

The NP stability in serum was evaluated to determine whether or not there are changes in the physical properties of NPs. This is very critical when the nanocompound is intravenously delivered. The sizes of peptide loaded PLA- or PLGA-NPs in 10% human serum were 65% of the size in aqueous solution and started to increase along incubation time, reaching 82% and 92% of the size in aqueous solution at 240 min for PLGA-NPs and PLA-NPs, respectively (Table 4).
The PDI was between 3.2 and 5.1, indicating a homogenous NP population during the incubation time in serum. The surface charge of the PLGA- and PLA-NPs in 10% human serum was negative, increasing along the incubation time, especially the negative charge of PLA-NPs that increased 50% during 240 min of incubation in serum. This suggests interactions between NPs and serum molecules forming hybrid structures. One of these serum molecules could be human serum albumin (HSA), which is the most abundant serum protein and is negatively charged at pH 7.2. HSA binding to NPs could explain the increase in both size and in negative surface charge of NPs during the incubation in serum. These changes in NP physical properties during serum incubation could be due to an osmotic effect exerted by the salts present in the buffer and human serum as has been reported (Menon et al., 2012; Ruozi et al., 2015).

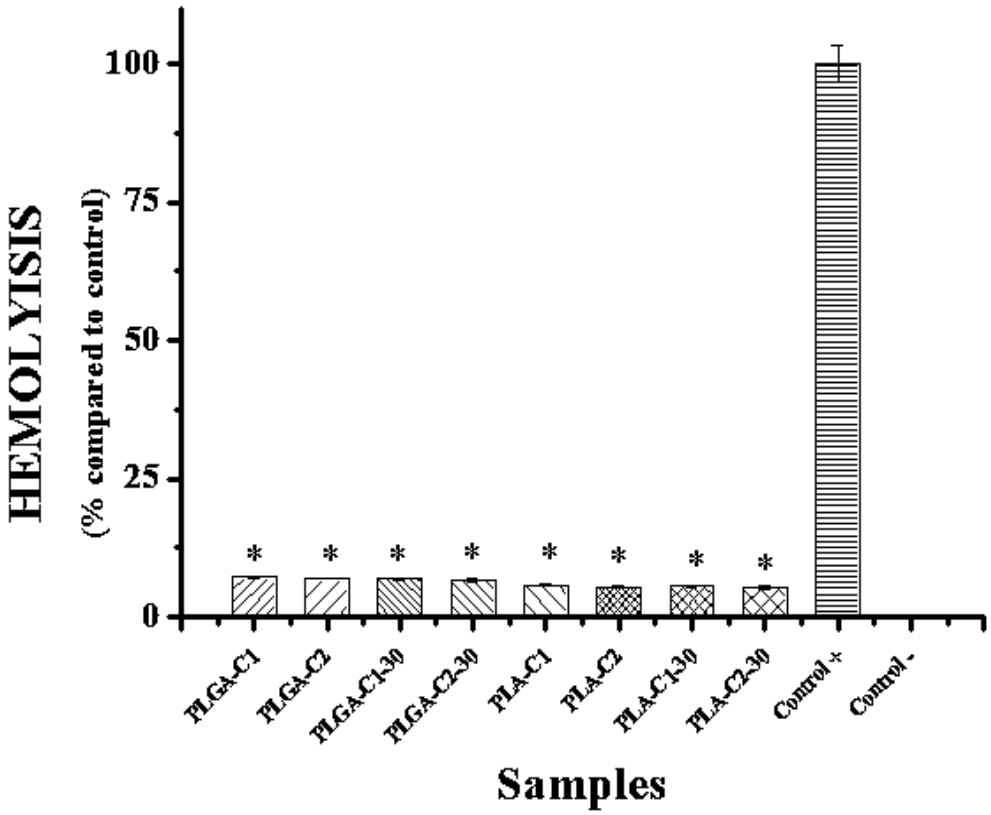
Table 4. Serum stability of GIBIM-P5S9K-loaded PGLA and GIBIM-P5S9K-loaded PLA NPs.

Time (min)	GIBIM-P5S9K-loaded PGLA NPs		GIBIM-P5S9K-loaded PLA NPs	
	Size average (nm)	Z potential (mV)	Size average (nm)	Z potential (mV)
0	169.3±4.5	-7.15±0.6	139.5±3.2	-9.44±0.5
60	182.2±3.2	-7.17±0.2	146.5±2.1	-12.7±0.3
120	184.8±4.8	-7.65±0.4	163.6±3.2	-14.6±0.3
240	211.9±5.1	-7.67±0.7	194.4±4.0	-14.3±0.4
NPs without Serum	258.3±3.5	+22.7±0.3	352.6±5.2	+29.4±1.53

2.5 HEMOLITIC ACTIVITY OF PEPTIDE LOADED PLA- OR PLGA-NPS

Human erythrocytes were incubated with peptide loaded or non-loaded PLA- and PLGA-NPs at two concentrations (0.5 to 1% w/v); the hemolysis percentages of these NPs were below 10%. Having into account that a compound causing >25% hemolysis is classified as hemolytic and causing <10% hemolysis is considered non-hemolytic or hemocompatible (Figure 6), these NPs are considered hemocompatible substances which could potentially be administered parenterally. Moreover, this result suggests a specific effect on bacterial membrane considering that the peptide target is the membrane structure (Nidhi, Indrajeet, Khushboo, Gauri, & Sen, 2011; Patel et al., 2008).

Figure 6. Hemolytic activity GIBIM-P5S9K encapsulated in PLGA and PLA. The final concentrations used PGLA and PLA were to C1 and C2 were 5 and 0.5 mg/mL, respectively. *p<0.05 versus viability cells without compound (positive control).



3. CONCLUSION

GIBIM-P5S9K peptide has been successfully loaded in PLA and PLGA nanoparticles via double-emulsion solvent evaporation. These peptide loaded NPs presented higher antibacterial activity than the free peptide; around 50% of peptide is released in vitro during the first 8 hours. Moreover, they were stable in human sera. The results obtained indicate that synthesized nanoparticles are a promising alternative method for GIBIM-P5S9K peptide delivery and even more important as a protection system of enzymatic degradation.

ACKNOWLEDGMENTS

Authors are grateful with Vicerrectoría de Investigación y Extension (VIE) of Universidad Industrial de Santander and with Colombian government through COLCIENCIAS from for financial support to JC and MU. This project was supported by COLCIENCIAS (Project Number: 110265740828).

BIBLIOGRAPHY

ALVAREZ-Fuentes, J., & Fern, M.***Synthesis of lidocaine-loaded PLGA microparticles by flow focusing Effects on drug loading and release properties*** 2008. 358: 27–35.

ANDREU, D., & Cativiela, C.Therapeutic Index of Gramicidin S is Strongly Modulated by D -Phenylalanine Analogues at the -Turn. ***Journal of Medicinal Chemistry***. 2009. 52: 664–674.

ATYABI, F., Talaie, F., & Dinarvand, R.Thiolated Chitosan Nanoparticles as an Oral Delivery System for Amikacin : In Vitro and Ex Vivo Evaluations. ***Journal of Nanoscience and Nanotechnology***. 2009. 9(8): 4593–4603.

CARVAJAL-Rondanelli, P. A., Marshall, S. H., & Guzman, F.***Antifreeze glycoprotein agents : structural requirements for activity Patricio A Carvajal-Rondanelli , a , b ∗ Sergio H Marshall b , c , d*** 2011. (July): 2507–2510.

CHAN, D. I., Prenner, E. J., & Vogel, H. J.Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. ***Biochimica et Biophysica Acta - Biomembranes***. 2006. 1758(9): 1184–1202.

CHEOW, W. S., & Hadinoto, K.Enhancing encapsulation efficiency of highly water-soluble antibiotic in poly(lactic-co-glycolic acid) nanoparticles: Modifications of standard nanoparticle preparation methods. ***Colloids and Surfaces A: Physicochemical and Engineering Aspects***. 2010. 370(1-3): 79–86.

COHEN-Sela, E., Chorny, M., Koroukhov, N., Danenberg, H. D., & Golomb, G.A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. ***Journal of Controlled Release***. 2009. 133(2): 90–95.

DANHIER, F., Ansorena, E., Silva, J. M., Coco, R., Le Breton, A., et al.PLGA-based nanoparticles: An overview of biomedical applications. ***Journal of Controlled Release***. 2012. 161(2): 505–522.

DAUTIN, N., & Bernstein, H. D.Protein secretion in gram-negative bacteria via the autotransporter pathway. ***Annual Review of Microbiology***. 2007. 61: 89–112.

DAVIES, J., & Davies, D.Origins and Evolution of Antibiotic Resistance. ***American Society for Microbiology***. 2010. 74(3): 417–433.

DERMAN, S., Mustafaeva, Z. A., Abamor, E. S., Bagirova, M., & Allahverdiyev, A.Preparation, characterization and immunological evaluation: canine parvovirus synthetic peptide loaded PLGA nanoparticles. ***Journal of Biomedical Science***. 2015. 22: 89.

FENG, S.Nanoparticles of biodegradable polymers for new-concept chemotherapy. ***Expert Rev. Medical Devices***. 2004. 1: 115–125.

FIELDS, G. B., & Noble, R. L.***Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids*** 1990. 161–214.

FIEVEZ, V., Garinot, M., Schneider, Y., & Préat, V.Nanoparticles as potential oral delivery systems of proteins and vaccines : A mechanistic approach. ***Journal of Controlled Release***. 2006. 116: 1–27.

FORIER, K., Raemdonck, K., Smedt, S. C. De, Demeester, J., Coenye, T., et al.Lipid and polymer nanoparticles for drug delivery to bacterial biofilms. ***Journal of Controlled Release***. 2014. . https://doi.org/10.1016/j.jconrel.2014.03.055.

GRANADOS, G., Pinto, M., Chaves, F., Calvo, J., & Rodrõ, R.***Double dimer peptide constructs are immunogenic and protective against Plasmodium falciparum in the experimental Aotus monkey model*** 2002. 62–70.

HACHICHA, W., Kodjikian, L., & Fessi, H.Preparation of vancomycin microparticles : Importance of preparation parameters. ***International Journal of Pharmaceutics***. 2006. 324: 176–184.

HANCOCK, R. E. W., & Sahl, H.***Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies*** 2006. 24(12): 1551–1557.

HANEY, E. F., Nazmi, K., Lau, F., Bolscher, J. G. M., & Vogel, H. J.Novel lactoferrampin antimicrobial peptides derived from human lactoferrin. ***Biochimie***. 2009. 91(1): 141–154.

HANS, M. L., & Lowman, A. M.B iodegradable nanoparticles for drug delivery and targeting. ***Current Opinion in Solid State and Materials Science***. 2002. 6(September): 319–327.

HOUGHTEN, R. A.***General method for the rapid solid-phase synthesis of large numbers of peptides : Specificity of antigen-antibody interaction at the level of individual amino acids Immunology : Houghten*** 1985. 82(August): 5131–5135.

HU, B., Pan, C., Sun, Y., Hou, Z., Ye, H., et al.Optimization of fabrication parameters to produce chitosan-tripolyphosphate nanoparticles for delivery of tea catechins. ***Journal of Agricultural and Food Chemistry***. 2008. 56(16): 7451–7458.

JOFRÉ, C., Guzmán, F., Cárdenas, C., Albericio, F., & Marshall, S. H.***Peptides A natural peptide and its variants derived from the processing of infectious pancreatic necrosis virus (IPNV) displaying enhanced antimicrobial activity : A novel alternative for the control of bacterial diseases*** 2011. 32: 852–858.

JUNG, A., & Jik, Y.“ Nanoantibiotics ” : A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. ***Journal of Controlled Release***. 2011. 156(2): 128–145.

KHAYATA, N., Abdelwahed, W., Chehna, M. F., Charcosset, C., & Fessi, H.Preparation of vitamin e loaded nanocapsules by the nanoprecipitation method: From laboratory scale to large scale using a membrane contactor. ***International Journal of Pharmaceutics***. 2012. 423(2): 419–427.

KIM, J. S., Kuk, E., Yu, N., Kim, J., Park, S. J., et al.Antimicrobial effects of silver nanoparticles. ***Nanomedicine: Nanotechnology, Biology, and Medicine***. 2007. 3: 95–101.

KOLA, M., & Urba, K.Antibiotic selective pressure and development of bacterial resistance. ***International Journal of Antimicrobial Agents***. 2001. 17: 357–363.

KUMARI, A., Yadav, S. K., Pakade, Y. B., Kumar, V., Singh, B., et

al.Nanoencapsulation and characterization of Albizia chinensis isolated antioxidant quercitrin on PLA nanoparticles. ***Colloids and Surfaces B: Biointerfaces***. 2011. 82(1): 224–232.

KUMARI, A., Yadav, S. K., & Yadav, S. C.Colloids and Surfaces B : Biointerfaces Biodegradable polymeric nanoparticles based drug delivery systems. ***Colloids and Surfaces B: Biointerfaces***. 2010. 75: 1–18.

MARR, A. K., Gooderham, W. J., & Hancock, R. E. W.Antibacterial peptides for therapeutic use : obstacles and realistic outlook. ***Current Opinion in Pharmacology***. 2006. 6: 468–472.

MARTINEZ-gutierrez, F., Olive, P. L., & Banuelos, A.Synthesis , characterization , and evaluation of antimicrobial and cytotoxic effect of silver and titanium nanoparticles. ***Nanomedicine: Nanotechnology, Biology, and Medicine***. 2010. 6(5): 681–688.

MATSUZAKI, K.***Why and how are peptide ^ lipid interactions utilized for self-defense ? Magainins and tachyplesins as archetypes*** 1999. 1462: 1–10.

MENON, J. U., Kona, S., Wadajkar, A. S., Desai, F., Vadla, A., et al.Effects of surfactants on the properties of PLGA nanoparticles. ***Journal of Biomedical Materials Research - Part A***. 2012. 100 A(8): 1998–2005.

NIDHI, K., Indrajeet, S., Khushboo, M., Gauri, K., & Sen, D. J.Hydrotropy: A promising tool for solubility enhancement: A review. ***International Journal of Drug Development and Research***. 2011. 3(2): 26–33.

OLIVA, A., Guzman, F., Patarroyo, M. E., & Santovena, A.***Chromatographic characterization of synthetic peptides : SPf66 malaria vaccine*** 2002. 766(2001): 3–12.

ORTIZ, C., & Torres, R.***Synthesis , characterization , and evaluation of antibacterial effect of Ag nanoparticles against Escherichia coli O157 : H7 and methicillin- resistant Staphylococcus aureus (MRSA)*** 2014. 1717–1729.

PANYAM, J., William, D., Dash, A., Leslie-Pelecky, D., & Labhasetwar, V.Solid-state solubility influences encapsulation and release of hydrophobic drugs

from PLGA/PLA nanoparticles. ***Journal of Pharmaceutical Sciences***. 2004. 93(7): 1804–1814.

PARK, S., Park, Y., & Hahm, K.***The Role of Antimicrobial Peptides in Preventing Multidrug-Resistant Bacterial Infections and Biofilm Formation*** 2011. 5971–5992.

PATEL, A. R., Kulkarni, S., Nandekar, T. D., & Vavia, P. R.Evaluation of alkyl polyglucoside as an alternative surfactant in the preparation of peptide-loaded nanoparticles. ***Journal of Microencapsulation***. 2008. 25(8): 531–540.

POVEY, J. F., Howard, M. J., Williamson, R. A., & Smales, C. M.***The effect of peptide glycation on local secondary structure*** 2008. 161: 151–161.

PRIOR, S., Gamazo, C., Irache, J. M., Merkle, H. P., & Gander, B.Gentamicin encapsulation in PLA / PLGA microspheres in view of treating Brucella infections. ***International Journal of Pharmaceutics***. 2000. 196: 115–125.

RADOVIC-Moreno, A. F., Lu, T. K., Puscasu, V. A., Yoon, C. J., Langer, R., et al.Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics. ***ACS Nano***. 2012. 6(5): 4279–4287.

RAI, M., Yadav, A., & Gade, A.Silver nanoparticles as a new generation of antimicrobials. ***Biotechnology Advances***. 2009. 27(1): 76–83.

REIS, C. P., & Neufeld, R. J.***Nanoencapsulation I . Methods for preparation of drug-loaded polymeric nanoparticles*** 2006. 2: 8–21.

ROGUEDA, P. G., & Traini, D.The nanoscale in pulmonary delivery. Part 1: deposition, fate, toxicology and effects. ***Expert Opinion on Drug Delivery***. 2007. 4(6): 595–606.

ROUSSAKI, M., Gaitanarou, A., Diamanti, P. C., Vouyiouka, S., Papaspyrides, C., et al.Encapsulation of the natural antioxidant aureusidin in biodegradable PLA nanoparticles. ***Polymer Degradation and Stability***. 2014. 108: 182–187.

ROZEK, A., Powers, J. S., Friedrich, C. L., & Hancock, R. E. W.Structure-Based Design of an Indolicidin Peptide Analogue with Increased Protease Stability. ***Biochemistry***. 2003. 42(48): 14130–14138.

RUOZI, B., Belletti, D., Sharma, H. S., Sharma, A., Muresanu, D. F., et al.PLGA

Nanoparticles Loaded Cerebrolysin : Studies on Their Preparation and Investigation of the Effect of Storage and Serum Stability with Reference to Traumatic Brain Injury. ***Mol Neurobiol***. 2015. 52: 899–912.

SANTANDER-Ortega, M. J., Jódar-Reyes, A. B., Csaba, N., Bastos-González, D., & Ortega-Vinuesa, J. L.Colloidal stability of Pluronic F68-coated PLGA nanoparticles: A variety of stabilisation mechanisms. ***Journal of Colloid and Interface Science***. 2006. 302(2): 522–529.

SCHNEIDER, C. A., Rasband, W. S., & Eliceiri, K. W.HISTORICAL commentary NIH Image to ImageJ : 25 years of image analysis. ***Nature Methods***. 2012. 9(7): 671–675.

SEO, M., Won, H., Kim, J., Mishig-ochir, T., & Lee, B.Antimicrobial Peptides for Therapeutic Applications: A Review. ***Molecules***. 2012. 17: 12276–12286.

SINGH, R., Smitha, M. S., & Singh, S. P.The Role of Nanotechnology in Combating Multi-Drug Resistant Bacteria. ***Journal of Nanoscience and Nanotechnology***. 2014. 14(7): 4745–4756.

SOHANI, O. R., & Gaikwad, U. B.Nanoencapsulation system for delivery of protein and peptide-A review. ***Journal of Biomedical and Pharmaceutical Research***. 2013. 2(2): 58–64.

SUK, J. S., Lai, S. K., Wang, Y. Y., Ensign, L. M., Zeitlin, P. L., et al.The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles. ***Biomaterials***. 2009. 30(13): 2591–2597.

TAHA, M. A., Singh, S. R., & Dennis, V. A.***Biodegradable PLGA85 / 15 nanoparticles as a delivery vehicle for Chlamydia trachomatis recombinant MOMP-187 peptide*** 2012. 325101. https://doi.org/10.1088/0957-4484/23/32/325101.

VAUTHIER, C., & Bouchemal, K.Expert Review Methods for the Preparation and Manufacture of Polymeric Nanoparticles. ***Pharmaceutical Research***. 2009. 26(5): 1025–1058.

Wu, J., Kong, T., Wai, K., Yeung, K., Cheung, H., et al.Acta Biomaterialia

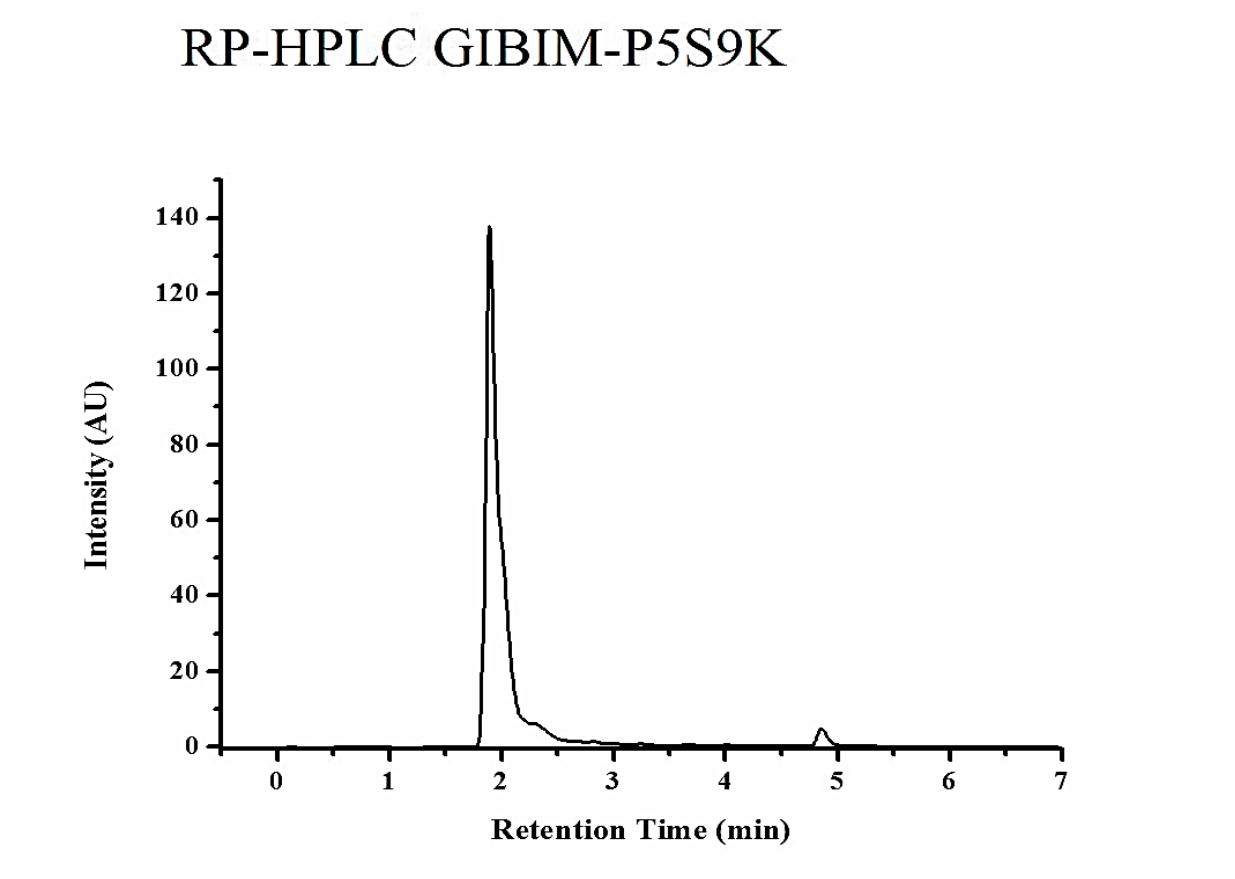
Fabrication and characterization of monodisperse PLGA – alginate core – shell microspheres with monodisperse size and homogeneous shells for controlled drug release. ***Acta Biomaterialia***. 2013. 9(7): 7410–7419.

XIE, S., Tao, Y., Pan, Y., Qu, W., Cheng, G., et al.Biodegradable nanoparticles for intracellular delivery of antimicrobial agents. ***Journal of Controlled Release***. 2014. 187: 101–117.

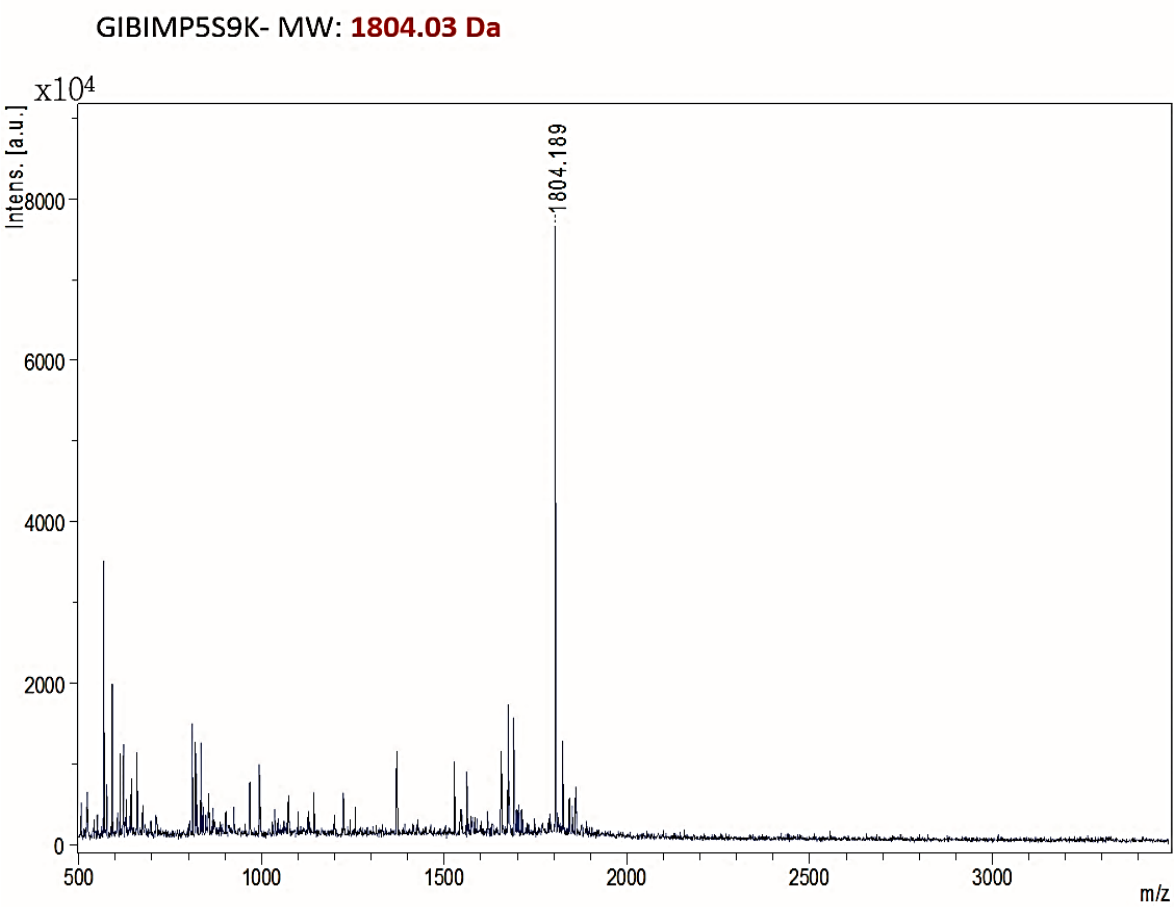
YOON, J. H., Ingale, S. L., Kim, J. S., Kim, K. H., Lee, S. H., et al.Effects of dietary supplementation of synthetic antimicrobial peptide-A3 and P5 on growth performance , apparent total tract digestibility of nutrients , fecal and intestinal microflora and intestinal morphology in weanling pigs. ***Livestock Science***. 2014. 159: 53–60.

ANNEXES

Annex A. RP-HPLC of GIBIM-P5S9K peptide.



Annex B. MALDI-TOF spectrum of GIBIM-P5S9K



.

Annex C. CD spectrum of GIBIM-P5S9K peptide.