ORIGINAL ARTICLE
Published 18 July 2025
DOI: https://doi.org/10.17533/udea.vitae.v32n2a358908

DEVELOPMENT AND CHARACTERIZATION OF LIPOPOLYMERIC PARTICLES FOR THE ENCAPSULATION OF A SYNTHETIC HYDROPHILIC NATURE PEPTIDE

DESARROLLO Y CARACTERIZACIÓN DE PARTÍCULAS LIPOPOLIMÉRICAS PARA LA ENCAPSULACIÓN DE UN PÉPTIDO SINTÉTICO DE NATURALEZA HIDROFÍLICA

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ABSTRACT

Background: The development of micro- and nanoparticulate systems for the controlled delivery of synthetic peptides represents a significant advancement in creating new pharmaceutical products with diverse applications. **Objectives:** This study aimed to develop a lipopolymeric hybrid system (LPS) designed to encapsulate a hydrophilic synthetic peptide, using a traditional polymeric system (PS), a lipidic system (LS) as references. The systems were prepared using the double emulsion formation method (W1/O/W2), followed by the extraction and evaporation of the organic solvent. **Results:** All systems exhibited a spherical shape, a negative Z-potential, and encapsulation efficiencies ranging from 40.4% to 57.42%, with micrometric sizes for the polymeric (3,080-4,920 nm) and lipopolymeric systems (3,030-3,930 nm). The lipidic system showed nanometric sizes (136-163 nm). The encapsulated synthetic peptide was fully released in about 25 days in the LPS, compared to 45 days for the PS and 8 days in the LS. Although PS provides benefits in encapsulation efficiency and release time, incorporating a lipid component can significantly extend its residency time in the body. This is particularly crucial when utilizing a peptide for targeted therapies, as it helps the body avoid recognizing it as a foreign substance, leading to more effective treatment outcomes. **Conclusion:** this innovative experimental approach successfully establishes a highly effective hybrid lipopolymeric system for encapsulating an hydrophilic synthetic peptide, merging the beneficial features of both polymeric microparticles and solid lipid nanoparticles, and positioning itself as a leading option for their encapsulation and delivery in pharmaceutical applications.

KEYWORDS: particles, lipopolymeric, polymeric, lipidic, synthetic peptide, drug delivery system.

JOURNAL VITAE
School of Pharmaceutical and Food Sciences
ISSN 0121-4004 | ISSNe 2145-2660
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Medellin, Colombia

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Received: 15 November 2024
Accepted: 14 July 2025
Published: 18 July 2025

RESUMEN

Antecedentes: El desarrollo de sistemas micro y nano particulados para la liberación controlada de péptidos sintéticos, representan un avance significativo en la creación de nuevos productos farmacéuticos con diversas aplicaciones. **Objetivos:** Este trabajo tuvo como objetivo crear un sistema híbrido lipopolimérico (LPS) diseñado para la encapsulación de un péptido sintético de naturaleza hidrofílica, usando un sistema polimérico (PS) tradicional y un sistema lipídico (LS) como referencia. Los sistemas fueron obtenidos por el método de formación de doble emulsión ($W_1/O/W_2$) con posterior extracción/evaporación del solvente orgánico. **Resultados:** Todos los sistemas presentaron forma esférica, potencial Z negativo, eficiencia de encapsulación entre el 40.4% y el 57.42% y tamaños micrométricos para el sistema polimérico (3,080-4,920 nm) y para el sistema lipopolimérico (3,030-3,930 nm). El sistema lipídico mostró tamaños nanométricos (136-163 nm). El péptido sintético encapsulado fue liberado en su totalidad en aproximadamente 25 días para el LPS, mientras que el PS requirió 45 días y el LS 8 días. A pesar de que el SP muestra beneficios en la eficiencia de encapsulación y el tiempo de liberación, al incorporar un componente lipídico en el sistema se podría extender el tiempo de residencia en el organismo. Esto es particularmente importante al utilizar un péptido para terapias dirigidas, ya que este ayuda a que el cuerpo no lo reconozca como una sustancia externa, permitiendo tener resultados más efectivos en el tratamiento de enfermedades. **Conclusión:** esta innovadora aproximación experimental permitió crear exitosamente un sistema híbrido lipopolimérico altamente efectivo para la encapsulación de un péptido sintético de naturaleza hidrofílica, asociando las características benéficas de ambos, las micropartículas poliméricas y las nanopartículas sólidas lipídicas, posicionándolo como una opción importante para su encapsulación y la administración en aplicaciones farmacéuticas.

PALABRAS CLAVE: partículas, lipopolimérico, polimérico, lipídico, péptido sintético, sistema de entrega de fármacos

INTRODUCTION

Peptides are molecules that correspond to short chains of amino acids, linked by peptide bonds. Many of these molecules occur naturally in the human body and play a role in numerous biological processes (1,2). Currently, it is possible to obtain synthetic peptides with biological activity, such as antibacterials, antifungals, antivirals, and antitumor agents. They may also have potential applications in the development of vaccines and diagnostic methods. In general, they are considered an excellent alternative for creating cutting-edge pharmaceutical products (3). Organic synthesis in solution and solid phase are the most suitable methodologies for obtaining these molecules with fewer than 40 residues (4). In contrast, synthetic peptides may face some limitations, such as low oral bioavailability, restricted biodistribution, and poor chemical and metabolic stability (5). Still, they also offer significant advantages, including high specificity and effectiveness. These molecules are relatively safe and well tolerated, unlike conventional active pharmaceutical ingredients (APIs) (3,6). Consequently, synthetic peptides are viewed as a promising alternative in the search for APIs in developing novel treatments for various pathologies (7). Currently, approximately 85 peptides are used in the development of FDA-approved drugs, more than 170 are in clinical trials, and over 500 are in preclinical studies. Furthermore, between 2016 and 2024, more than 11% of FDA-approved drugs were synthetic peptides. (8,9).

In the pharmaceutical field, recent years have seen work on developing novel and innovative strategies to overcome the limitations of synthetic peptides as APIs (10). One of these approaches is micro- and nanoparticulate systems, which have been proposed for the safe and controlled administration of these molecules, because they protect them from rapid enzymatic degradation, prolong their lifetime, improve their activity, and ultimately allow for the simplification of administration schemes (11).

The systems that have shown the best results as nano/micro carriers of API (Active pharmaceutical ingredient) are liposomes, biodegradable polymeric particles, and solid lipid particles (12). Research and development have been conducted to integrate two distinct drug delivery systems into a single entity, leveraging synergistic advantages. (liposomes/polymeric particles) or (solid lipid particles/polymeric particles). This new and innovative system has been named lipid-polymer hybrid particles, polymer-lipid hybrid carriers, lipoparticles, lipid-coated particles, lipomers (13,14), and lipopolymeric particles. The term hybrid is used since it possesses the characteristics of both systems (15–17). This system has demonstrated advantages over the individual use of lipidic or polymeric nano/microparticles (18). Specifically, the polymeric component primarily serves to protect the encapsulated molecule against the enzymatic attack and to control its release, while the lipid component enhances the system's interaction with biological membranes (19).

Despite the recognized advantages of lipid-polymer hybrid systems, a significant gap remains in understanding their behavior and performance compared to their individual polymer and lipid counterparts. This is particularly evident in the controlled release of hydrophilic peptides (20). Most studies focus on developing and meticulously characterizing a new hybrid lipopolymeric microparticulate system (LPS) composed of biodegradable materials for the controlled release of synthetic hydrophilic peptides. Our study innovatively establishes a direct comparison by evaluating the LPS alongside a reference polymeric microparticulate system (PS) and a lipidic nanoparticulate system (LS) (17). Our findings indicate that while the polymeric system (PS) demonstrated superior encapsulation efficiency and extended-release time, incorporating a lipidic component in the lipopolymeric system (LPS) is crucial. This addition is anticipated to improve the *in vivo* residence time and overall biocompatibility of the system, ultimately resulting in a more optimized and versatile drug delivery platform (21). Thus, the developed LPS shows intermediate behavior regarding release time and physicochemical properties, representing a significant advancement that merges the strengths of both individual systems for peptide delivery.

MATERIALS AND METHODS

Materials:

Poly-D, L-lactide-co-glycolide (PLGA 50:50) polymer with inherent viscosity 0.8 dl/g (LACTEL, Birmingham Polymers Inc., Alabama, USA), Dichloromethane (DCM) (Honeywell, Muskegon USA), polyvinylalcohol (PVA) (Mw 31,000-50,000) (Sigma Aldrich, St. Louis, MO, USA), Isopropyl alcohol (IPA) (Mallinckrodt Chemicals, New Jersey, USA), TG C12-C18 triglycerides (Novata BC PH) (BASF, Ludwigshafen, Germany, Poloxamer 188 (Plx) supplied by (HANDLER, Bethel, CT, USA), Micro BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Phosphate-buffered saline (PBS) was prepared from individual salts (sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate), all analytical grade and sourced from Sigma-Aldrich (St. Louis, MO, USA). Sodium azide, used as an antimicrobial agent, was also obtained from Sigma-Aldrich (St. Louis, MO, USA) All other reagents used in the study were of analytical grade.

In this study, the peptide was produced and physicochemically characterized at the Fundación Instituto de Inmunología, Bogotá, Colombia. The molecule was synthesized using the solid-phase synthesis described by Merrifield (22) and modified by Houghten (23), according to the *t*-Boc technique. The peptide's amino-acid sequence is: CGDELEAE-TQNVYAAPNANPYSLFQKEKMVLPNANPPANKK-NAGC, which has been explored extensively in the past (24–27). Additionally, this molecule has been extensively characterized by other research groups employing different analytical techniques. This information can be found in earlier publications (28–31). The peptide was received as a lyophilized material, stored at room temperature, and used for the encapsulation experiments.

METHODS:

Obtaining the systems

The PS, LS, and LPS containing the encapsulated synthetic peptide (loaded) or not (empty), were obtained by the double emulsion formation methodology, followed by the extraction/evaporation of the organic solvent (27,32–36). Briefly, the organic phase (O) consisted of a solution in DCM of the material responsible for forming the matrix structure of each of the systems. The aqueous inner phase (W_1) was an aqueous solution of the synthetic peptide for the loaded systems, or deionized water in the case of the empty systems. In contrast, the aqueous outer phase (W_2) contained a solution of an emulsion stabilizing agent. The first emulsion was formed by adding the aqueous phase (W_1) to the organic phase (O) and subsequently homogenizing with ultrasound (Qsonica sonicators, Q125, Newtown, CT, USA); at 100% amplitude for 60 seconds. The resulting emulsion (W_1/O) was dispersed in the aqueous phase (W_2), and a turbine homogenizer (Miccra, Miccra D-1 010049, Müllheim, Germany) was then used at 20,000 rpm for 5 minutes. The double emulsion formed (W_1/O/W_2) was kept in agitation, and a 2% aqueous solution of IPA was added to promote the extraction/evaporation of the organic solvent at room temperature, thus inducing the insolubilization of the formed particles (32–37).

The system underwent a 5-minute centrifugation process in a laboratory centrifuge (Maschinenfabrik Berthold Hermle AG, Z 206 A; Gosheim, Germany) at 6,000 rpm, and the collected particles were washed with deionized water. Finally, each system was subjected to a freeze-drying (LABCONCO, Lyph-lock 6, Kansas City, MO, USA) process for subsequent characterization. The phase composition for each system obtained is reported in Table 1.

Table 1. Composition of the phases to obtain each system (PS, LS, and LPS).

System	Organic Phase DCM (FO) (1mL 100mg/mL)	Internal aqueous phase (FW1) (0.2 mL peptide 50mg/mL or water)	External aqueous phase (FW2) (10 mL)
PS	PLGA 50:50	Peptide/Water	PVA 5%
LS	TG C12-C18	Peptide/Water	Plx 188 0.5%
LPS	PLGA 50:50-TG C12-C18 (9:1)	Peptide/Water	PVA 5%

System characterization:

Morphology: The scanning electron microscopy (SEM) technique was employed to ascertain the shape and surface of the developed systems. Briefly, particle dispersion was applied to a stub sample holder and allowed to dry at room temperature. The dried sample was then sputter-coated with gold. Images were taken on a JEOL JXA 8230 electron microscope (Jeol Ltd, Tokyo, Japan) at a voltage of 25 kV. Images were taken at 1,000X and 5,000X magnification and then processed directly on the microscope using Gatan Microscopy Suite Digital Micrograph software version 2.32.888.0. (37,38). Samples obtained from independent batches for each system type were morphologically characterized by SEM.

Particle size: For this analysis, an aqueous dispersion of particles from each of the systems was employed. The ZetaSizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) was utilized to determine the size of LS, using a dispersion angle of 173° and a temperature of 25°C. The Master Sizer 3000E (Malvern Instruments Ltd, Malvern, UK) was used to determine the size of PS and LPS (38,39).

Z potential: The ZetaSizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) was used to determine the zeta potential of all the systems obtained, and a DTS1070 cell was utilized (27,38).

Encapsulation efficiency: The amount of encapsulated synthetic peptide was determined directly in each specific lyophilized system. Specifically for SP, a precisely weighed amount of lyophilized particles was taken in an analytical balance (OHAUS, PX224, Parsippany, NJ, USA) and dispersed in a 0.2 M NaOH aqueous solution, with rotational agitation at room temperature, until complete dissolution was achieved. For LS and LPS, a precisely weighed amount of lyophilized particles was taken and dispersed in DCM, and the extraction of the synthetic peptide was then performed using deionized water. In all cases, the concentration of the synthetic peptide was determined using a microplate reader with the Micro BCA kit (Thermo Scientific, Waltham, MA, USA) and incubated at 37°C for 2 hours. The reading was conducted at 590 nm using an Expert Plus plate reader from Biochrom Ltd. (Cambridge, United Kingdom). A calibration curve was created with a control solution of the synthetic peptide, from which the encapsulated peptide was quantified in each of the systems (32,33). Finally, the encapsulation efficiency was determined using the following relationship (Equation 1):

$$\text{Eq.1.} \quad EE = \frac{\text{Peptide amount in particles}}{\text{Initial peptide amount}} \times 100$$

Loading capacity: Based in the information obtained from the encapsulation efficiency, the loading capacity was determined using the following relationship (Equation 2):

$$\text{Eq.2.} \quad LC = \frac{\text{Peptide amount}}{\text{Particles amount}} \times 100$$

Release profile: For this determination, an accurately weighed amount of particles in an analytical balance (OHAUS, PX224, Parsippany, NJ, USA) was taken and suspended in 1 mL of PBS buffer (pH: 7.4) preserved with 0.1% sodium azide. The system was maintained under constant rotary shaking at 37°C. After 24 hours under these conditions, all systems were centrifuged at 6,000 rpm for 10 min, and 500 µL of the supernatant was collected to determine the amount of surface-associated peptide (SAP) or fast-releasing peptide. The volume removed from each system was replaced with an equivalent volume of fresh buffer solution to continue the release assay. Subsequently, new samples were taken at pre-established time intervals until the study concluded. In all samples, the peptide was quantified by the micro-BCA technique (26,37). The peptide release profile obtained for each system was reported as the cumulative percentage of peptide released as a function of time. The release assay was performed in triplicate for each system.

All physicochemical characterization data presented in Table 2, including particle size, polydispersity index (PDI)/SPAN, Z-potential, encapsulation efficiency (EE), surface-adsorbed peptide (SAP), and loading capacity (LC), were

obtained from independent experimental preparations. Specifically, empty systems (PS1, LS1, LPS1) were prepared and characterized in one independent batch. Loaded systems (PS2/PS3, LS2/LS3, LPS2/LPS3) represent two independent batches for each formulation.

Statistical Comparative Analysis: All statistical analyses were performed using RStudio software (version 2025.05.1 Build 513). To comprehensively assess the overall differences in the physicochemical characteristics (particle size, zeta potential, encapsulation efficiency, and adsorbed peptide) among the various encapsulation systems (polymeric, lipidic, and lipid-polymer hybrid particles), a Multivariate Analysis of Variance (MANOVA) was conducted. This approach was employed to determine whether significant differences existed across the multivariate response space, accounting for the inherent intercorrelations among these dependent variables. MANOVA allowed for a robust initial evaluation of the global impact of the formulation type on the combined physicochemical profile.

Following the overall MANOVA, and to pinpoint specific differences within each physicochemical characteristic, univariate Analyses of Variance (ANOVA) were performed for particle size, zeta potential, encapsulation efficiency, and the amount of adsorbed peptide. Recognizing the increased risk of Type I errors associated with multiple comparisons, a Bonferroni correction was rigorously applied to the p-values obtained from each ANOVA. This adjustment ensured a more conservative and statistically sound interpretation of the individual comparisons. A two-tailed statistical significance level of $p<0.05$ was predetermined for all analyses.

RESULTS:

Morphology: The morphology of all developed systems was characterized by Scanning Electron Microscopy (SEM). Representative micrographs are presented in Figure 1.

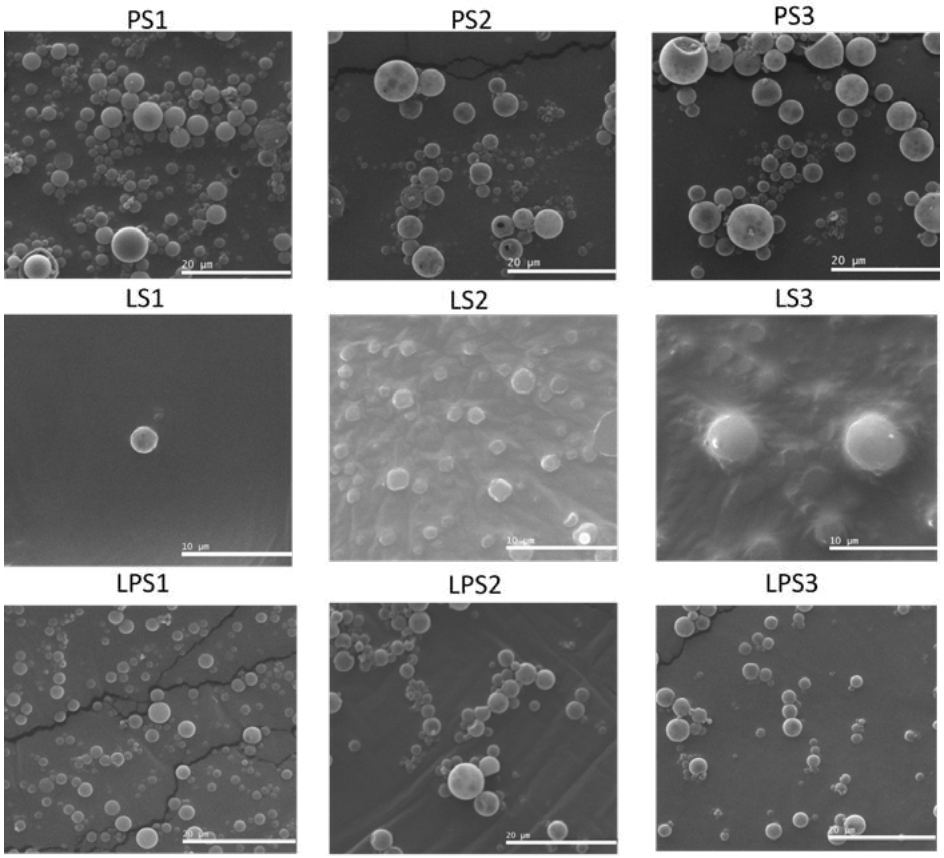

Figure 1. Morphological characterization by SEM of the developed systems. PS1 represents empty polymeric microparticles, while PS2 and PS3 are polymeric microparticles loaded with peptide. LS1 denotes empty lipid particles, and LS2 and LS3 are lipid particles loaded with peptide. LPS1 indicates empty lipopolymeric particles, with LPS2 and LPS3 lipopolymeric particles loaded with peptide. Cavities were observed.

Physicochemical Characterization: The developed particulate systems were further characterized regarding size, polydispersity index (PDI) or SPAN, zeta potential, peptide encapsulation efficiency (EE), surface-adsorbed peptide (SAP), and loading capacity (LC). The results are summarized in Table 2.

Table 2. Physicochemical characterization of each of the specific systems developed.

System	Size (nm)	PDI/ SPAN*	Zeta Potential (mV)	EE (%)	SAP (%)	LC (%)
PS1	3,080	1.224*	-24.5	N.A.	N.A.	N.A.
PS2	4,590	1.324*	-19.3	57.32	48.90	5.21
PS3	4,920	1.242*	-18.5	57.53	37.40	5.23
LS1	136.0	0.167	-29.9	N.A.	N.A.	N.A.
LS2	163.0	0.174	-15.5	39.21	76.40	2.40
LS3	155.0	0.190	-18.4	41.60	78.00	2.60
LPS1	3,030	1.115*	-26.1	N.A.	N.A.	N.A.
LPS2	3,930	1.132*	-20.8	42.68	36.44	3.88
LPS3	3,840	1.123*	-20.0	43.70	34.10	3.97

PDI: Polydispersity index. EE: Peptide encapsulation efficiency. SAP: Particle Surface associated peptide. LC: Peptide loading capacity in the system. N.A.: Not applicable

In Vitro Peptide Release: The *in vitro* release profile of the encapsulated peptide was determined for each of the developed systems. The obtained release profiles are presented in Figure 2.

Statistical Comparative Analysis: Multivariate Analysis of Variance (MANOVA): The overall effect of formulation type (polymeric, lipidic, and lipid-polymer hybrid) on the combined dependent variables: particle size, zeta potential, encapsulation efficiency, and adsorbed peptide. This initial analysis aimed to identify whether the different nanoparticle systems exhibited a significant global difference in their physicochemical profiles. MANOVA statistics obtained were Wilks' Λ:1.9307e-05, F statistic: 75.528, degrees of freedom: 2, and the associated p value: 0.01312. The MANOVA revealed a significant overall effect of formulation type on the combined physicochemical characteristics.

Univariate Analyses of Variance (ANOVA) with Bonferroni Correction: This subsection presents the findings for each physicochemical characteristic, as shown in Table 3.

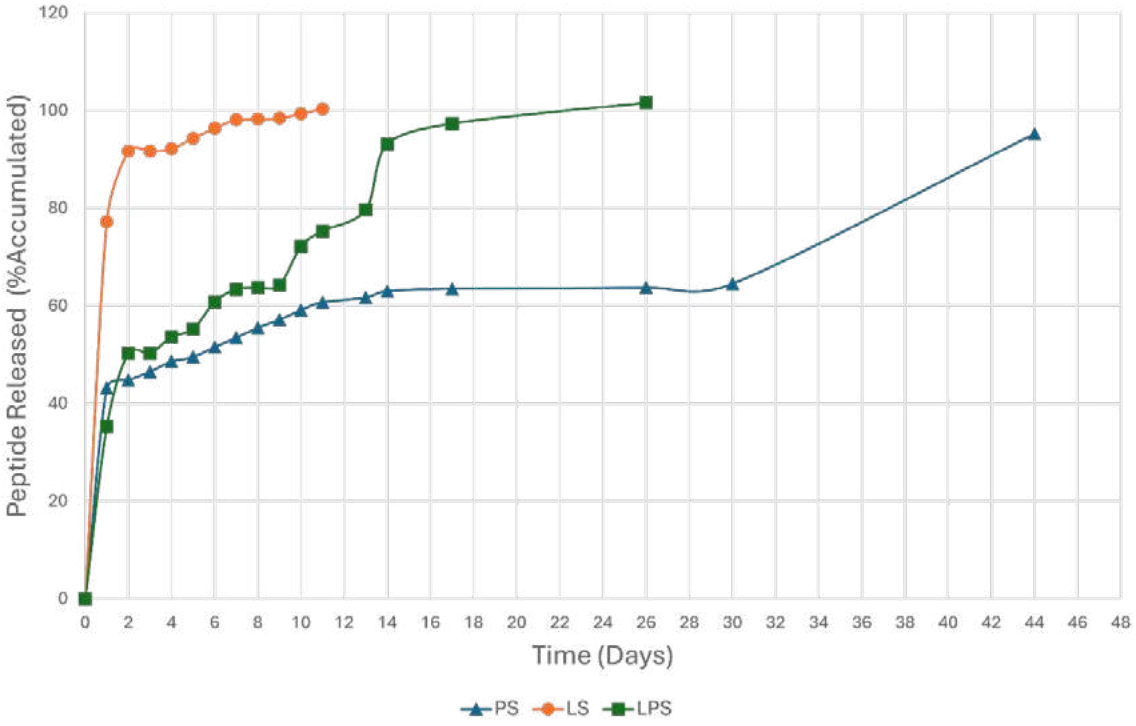

Figure 2. Release profiles from the encapsulated peptide in the obtained systems. Symbols represents: Δ PS, •LS and □LPS.

Table 3. Summarized ANOVA results conducted to compare the physicochemical characteristics

Physicochemical Characteristic	F statistic	Degrees of Freedom	p value	Bonferroni Corrected p value
Particle Size	611	2	0,000121	LS-PS: 0.00019*
				LPS-PS: 0.02503*
				LPS-LS: 0.00035*
Zeta Potential	3.706	2	0.155	LS-PS: 0.67
				LPS-PS: 0.97
				LPS-LS: 0.155
Encapsulation Efficiency	148.4	2	0.001	LS-PS: 0.0016*
				LPS-PS: 0.0027*
				LPS-LS: 0.2330
Surface Adsorbed Peptide	42.48	2	0,0063	LS-PS: 0.0177*
				LPS-PS: 0.6050
				LPS-LS: 0.0097

Post-hoc comparisons for Particle Size, adjusted with Bonferroni, revealed size differences across all systems studied. In contrast, Zeta Potential comparisons with the same adjustment indicated no differences in Zeta Potential characterization among the studied systems. For Encapsulation Efficiency, Bonferroni-adjusted post-hoc comparisons showed significant differences only between LS and PS, as well as between LPS and PS, with no differences found between LPS and LS. Similarly, Adsorbed Peptide post-hoc comparisons revealed differences only between LS and PS and between LPS and LS, with no differences between LPS and PS. The corresponding graph is presented in Figure 3.

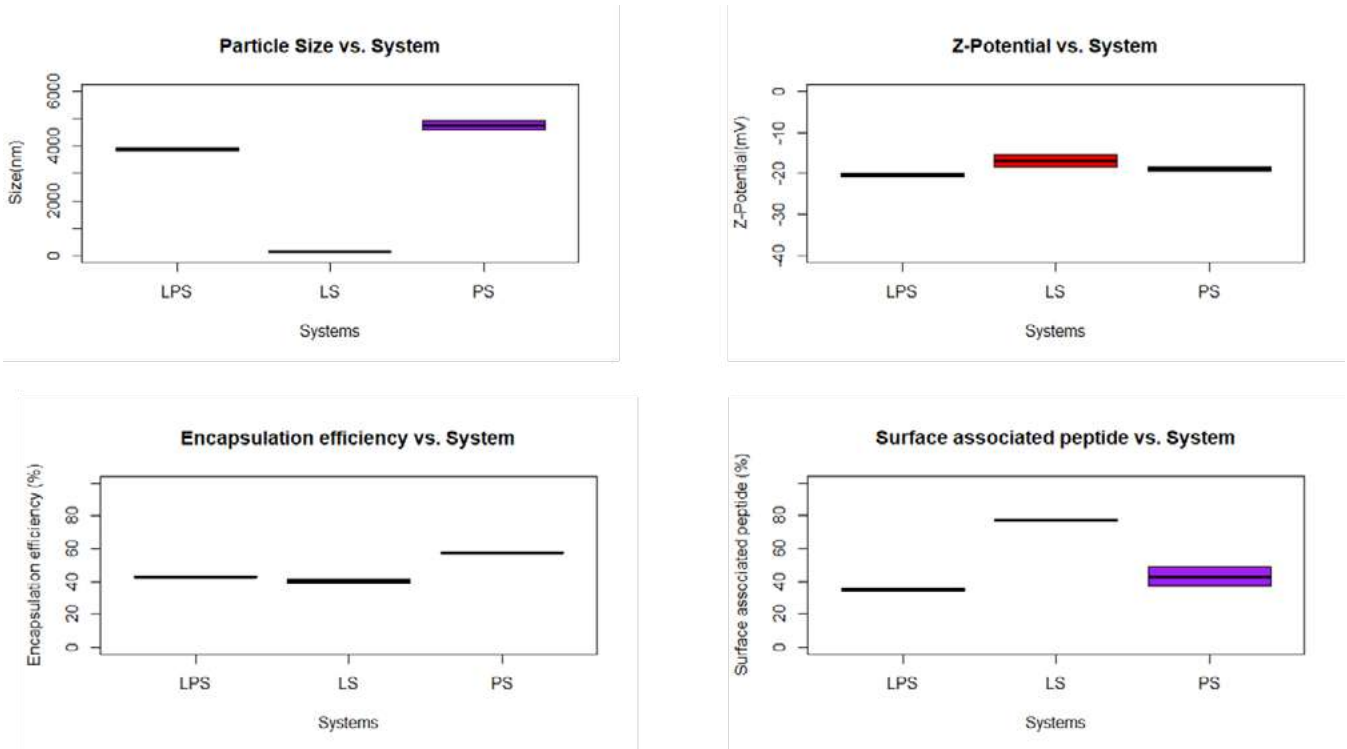

Figure 3. Comparative analysis of the physicochemical characteristics studied as a function of the systems obtained. This graph summarizes the results from the system characterization.

Following the strategy used for physicochemical characteristics, a Multivariate Analysis of Variance (MANOVA) was likely employed, treating the percentage release at each time point as a dependent variable. The MANOVA statistics obtained were Wilks' Λ: 0.00034344, F statistic: 17.654, degrees of freedom: 2, and the associated p-value: 0.05457. The MANOVA didn't reveal a significant overall effect of formulation type on the *in vitro* release profile.

To study the effect at specific time points, subsequent univariate Analyses of Variance (ANOVA) were conducted for each time point, as shown in Table 4.

Table 4. Summarized ANOVA results conducted to compare Release Profile

Day	F statistic	Degrees of Freedom	p value	Bonferroni Corrected p value
1	7.357	2	0.0697	LS-PS: 0.097
				LPS-PS: 0.808*
				LPS-LS: 0.279*
6	44.26	2	0.00594	LS-PS: 0.0079
				LPS-PS: 0.1375*
				LPS-LS: 0.0281
11	36.49	2	0.00785	LS-PS: 0.010
				LPS-PS: 0.099*
				LPS-LS: 0.053*

Post-hoc comparisons with Bonferroni adjustment indicated that, at the studied time point beginning from the release profile, there were clear differences between LS and PS, but not between LPS and PS. On day 6, there were significant differences between LS and LPS-PS, while LPS and PS exhibited similar behavior. Finally, on day 11, differences were found only between LS and PS. The resulting graph is shown in Figure 4.

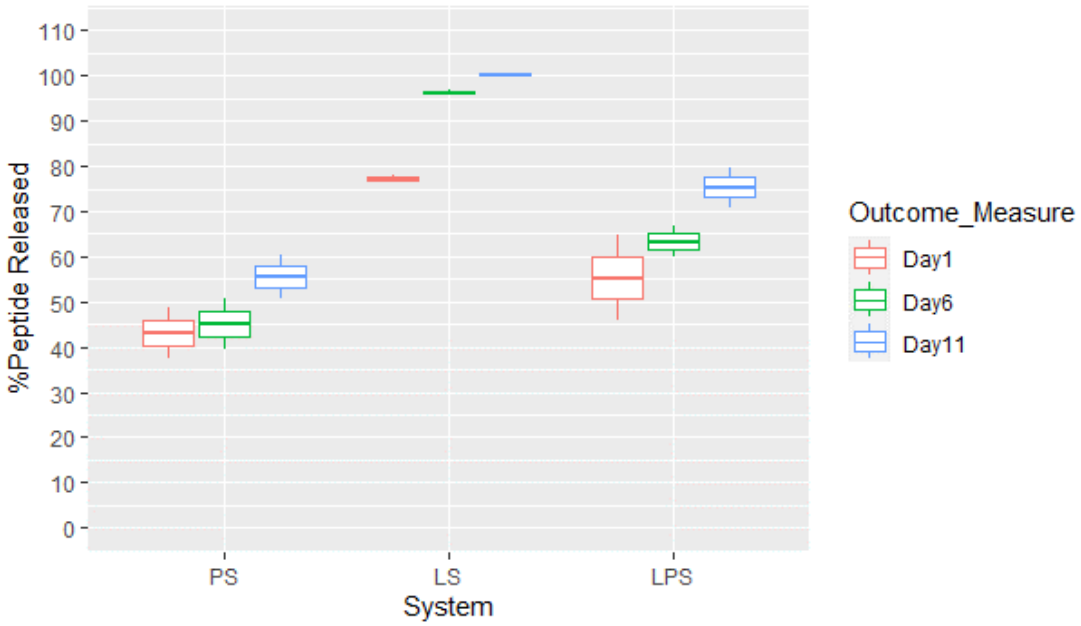

Figure 4. Comparative boxplot graph of the release profiles obtained for each system at different comparable time points.

DISCUSSION:

Obtaining the systems:

The selection of the double emulsion (W/O/W) technique, followed by solvent extraction/evaporation for fabricating the polymeric, lipid, and hybrid particulate systems, was based on a rigorous evaluation of the delivery system requirements and the encapsulated active's intrinsic nature. This method was initially chosen due to our research group's established expertise and successful track record in encapsulating sensitive hydrophilic compounds, as documented in previous works (24,26,37). Crucially, the hydrophilic nature of the peptide to be encapsulated rendered simpler methods like single emulsion or coacervation unsuitable, as they would not provide adequate protection or the required encapsulation efficiency for a water-soluble molecule within a continuous oil phase. Furthermore, the peptide's known thermal sensitivity required excluding high-temperature processing methodologies such as spray drying or fluidized bed techniques, which could compromise its stability and integrity (37,39).

The W/O/W double emulsion remains an exceptionally robust and cutting-edge strategy for encapsulating thermosensitive hydrophilic molecules, including peptides, owing to its inherent capacity to confine the active within an inner aqueous phase and its remarkable versatility. Recent studies continue to validate and optimize this technique, underscoring its enduring relevance. For instance, authors like Romero–Carmona et al. have demonstrated the efficacy of double emulsions in enhancing the stability and bioavailability of bioactive peptides such as insulin (40). Similarly, this methodology's capability to generate hybrid systems, combining the advantages of both polymers and lipids, represents a field of constant innovation, allowing for designs that optimize controlled release and stability against enzymatic degradation, as evidenced by recent investigations by Shah, S, et al. in peptide drug delivery systems (12). These advancements collectively reinforce the suitability of the double emulsion as a primary technique for the elaborate fabrication of peptide-loaded particulate systems. Our results, which demonstrate a high encapsulation efficiency (approx. 40-5% for polymeric and lipopolymeric systems) while preserving peptide integrity, further corroborate the appropriateness of this methodological approach in our study.

The selection of the appropriate stabilizing agent was equally crucial for the particulate systems' successful formation and morphological stability. For the predominantly polymeric systems (PS and LPS), polyvinyl alcohol (PVA) was chosen as the standard stabilizer in the external aqueous phase. This decision was based on PVA's well-established efficacy in forming stable emulsions, preventing droplet coalescence, and controlling particle size during the preparation of polymeric micro- and nanoparticles via double emulsion methods (41). However, during the development of the solid lipid particles (LS), the incorporation of PVA led to the rapid insolubilization of the lipid, significantly compromising the integrity and homogeneous dispersion. This incompatibility can likely be attributed to unfavorable interactions between PVA and the lipid matrix, or perhaps its lower affinity for the more hydrophobic or rigid surfaces characteristic of solid lipids, which contrasts with its compatibility with more flexible polymers (42).

To circumvent this challenge in lipid systems (LS), Poloxamer 188 was strategically incorporated. This non-ionic triblock copolymer (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)) is widely recognized for its amphiphilic structure, which enables effective interaction with both hydrophilic and hydrophobic interfaces (43). The central hydrophobic segment of Poloxamer 188 (PPO) optimally adheres to the lipid matrix surface, while its hydrophilic PEO chains extend into the aqueous phase. This configuration provides superior steric stabilization that effectively prevents the aggregation and rapid insolubilization observed with PVA in this class of systems. Poloxamer 188's ability to adaptively interact with the lipid matrix and its proven efficacy in stabilizing lipid nanoparticles make it the ideal choice for obtaining LS systems (44).

Morphology:

Morphological characterization was performed using scanning electron microscopy (SEM), and presented in Figure 1, provided critical insights into the physical attributes of the developed particulate systems. All formulations consistently exhibited spherical morphology, a desired characteristic for drug delivery systems. A notable distinction in size was observed: polymeric (PS) and lipo-polymeric (LPS) systems formed micro-sized particles, whereas the lipid-based (LS) particles were within the nanometer range. This inherent size difference between the two leading platforms is consistent with the distinct fabrication mechanisms often associated

with the chosen materials and methods; for instance, the double emulsion method typically yields larger particles when using high molecular weight polymers like PLGA, while lipid nanoparticle formation often results in smaller, sub 200nm structures (45,46). Beyond size, alterations in surface integrity were observed explicitly in the peptide-loaded PS and LPS systems. These images revealed the presence of surface-located pores and cavities (Fig.1, PS2, PS3, LPS2, LPS3). This phenomenon can be attributed to the peptide's hydrophilic nature and tendency to diffuse from the internal aqueous phase to the external aqueous phase through the polymeric matrix during the organic solvent evaporation/ extraction process. Such outward diffusion of the active substance during particle formation is a well-documented challenge in W/O/W emulsion systems, leading to the formation of surface irregularities and potential loss of encapsulated material (47). This suggests that while the double emulsion successfully protected the peptide, the kinetics of solvent removal and peptide partitioning might have influenced the final particle morphology.

In contrast, the lipid-based systems (LS) consistently displayed a smoother and more integrated surface (Fig.1; LS1; LS2, LS3), with no evidence of cavities observed in their polymeric counterparts. This difference strongly suggests that the lipid matrix, due to its distinct physicochemical properties and potentially lower permeability to the peptide than the polymeric network, significantly hinders the outward diffusion of the hydrophilic peptide. The lack of robust encapsulating nature of lipid matrices for hydrophilic compounds generates burst release and surface accumulation, which have been frequently highlighted in recent literature (48). Notably, the magnification employed for the LS systems in Figure 1 differs from that of the PS and LPS systems. This choice was deliberate, driven by the lipid particles' inherently smaller, nanoscale dimensions and our interest in closely examining their surface features to correlate with release profiles. While an image at the same magnification for all systems would offer a broad comparative overview, focusing on the higher resolution for LS particles was deemed essential to capture the nuanced surface details critical for their characterization. The observed differences in background and occasional shape alterations in the LS micrographs, particularly for LS2 and LS3, were primarily related to the susceptibility of these lipid structures to the electron beam under high vacuum conditions of the SEM analysis. The relatively low melting point of the lipid used (33.0 - 34.5°C) (49) makes these systems particularly susceptible to electron beam-induced thermal effects, which can cause partial melting or structural changes (50). These effects can influence the background appearance and subtle deformities, rather than issues with sample preparation steps such as moisture elimination or gold coating, which were rigorously controlled for all samples. The observed micrograph charge, which contributes to the background variation, is also a direct consequence of the electron beam interaction with these sensitive lipid materials (51).

Particle Size Distribution (PDI/Span):

Particle size and distribution are critical parameters influencing drug delivery systems' biodistribution, cellular uptake, and release characteristics. As detailed in Table 2, the determination of particle size for polymeric and lipo-polymeric systems (PS and LPS) was performed using laser diffraction, while dynamic light scattering (DLS) was employed for the lipid systems (LS) due to their nanoscale dimensions. A clear distinction in size was observed across the systems: PS (empty and loaded) ranged from 3,080 to 4,920 nm, LS from 136 to 163 nm, and LPS from 3,030 to 3,930 nm.

Regarding particle size distribution, assessed by PDI for LS and SPAN for PS/LPS, the lipid systems (LS) exhibited the highest uniformity (PDI <0.2), indicative of a narrow and monodisperse population. The lipo-polymeric systems (LPS) showed moderate uniformity, particularly those encapsulating the peptide, followed by the polymeric systems (PS), which displayed broader distributions (higher SPAN values). Despite incorporating a lipid component, the LPS systems maintained a similar size range to the polymeric microparticles (PS). This observation strongly suggests that the overall architectural assembly of the hybrid (LPS) particles is predominantly dictated by the polymeric component, which constitutes approximately 90% of the matrix composition. This phenomenon is consistent with findings in hybrid particle formation, where the more abundant or rigid material dictates the system size and shape (52).

The inherent size differences between the lipid (nano-sized) and polymeric/lipo-polymeric (micro-sized) systems warrant further discussion. Literature consistently reports that solid lipid nanoparticles typically range from 50 to 1,000 nm, a significantly smaller scale than polymeric microparticles. This can be primarily attributed to lipid materials'

fundamental nature: smaller molecules than polymers, they possess a greater propensity for rapid self-assembly into more compact, smaller structures. Moreover, the type of lipid, its proportion, and the selected stabilizer critically influence the final system size (53). In the context of the LS; the strategic use of Poloxamer 188 instead of PVA likely, played a significant role in achieving their nanoscale dimensions. Poloxamer 188's superior steric stabilization, particularly for lipid interfaces, promotes the formation of smaller, more stable nanoparticles (54,55).

Additionally, the highly organized and compact structure of lipid materials inherently contrasts with the more voluminous and less densely packed polymeric matrices (55). This fundamental structural difference offers a compelling explanation for why PS and LPS systems result in significantly larger particles than LS systems, even when similar material quantities are employed.

Furthermore, a consistent increase in particle size was observed across all three systems (PS, LS, and LPS) upon peptide encapsulation, accompanied by a noticeable alteration in their size distribution. This finding suggests that incorporating the peptide plays a role in stabilizing the system, potentially by some lateral chains from the residues in its structure. It is plausible that the peptide, acting as an interfacial stabilizer, mitigates droplet coalescence during the double emulsion formation process, thereby influencing the final particle dimensions and distribution (56).

However, it is essential to acknowledge that some systems, particularly the polymeric ones, exhibited relatively wide distribution (high SPAN or PDI values). This observation can be partially attributed to the concomitant decrease in zeta potential upon peptide loading, reducing electrostatic repulsion among particles. Such diminished surface charge can foster particle agglomeration, which would be reflected as broader size distributions in the measurements and is consistent with observations in the micrographs (Figure 1) (57).

Zeta-potential

The zeta potential, a crucial indicator of colloidal stability and surface charge characteristics, was measured for all developed systems, as presented in Table 2. Consistently, all formulations exhibited negative zeta potential values.

For the polymeric (PS) and lipo-polymeric (LPS) systems, this negative charge is primarily attributed to the poly (lactic-co-glycolic acid) (PLGA), a copolymer used in their fabrication. PLGA inherently possesses a carboxylic acid end group that deprotonates in aqueous media at physiological pH, rendering the particle surface negatively charged (58). A noteworthy observation was the slight reduction in the negativity of the zeta potential for peptide-loaded PS and LPS compared to their empty counterparts. This shift, becoming marginally less negative, is most likely due to the adsorption of the peptide on the particle surface. Peptide containing various residues, particularly those with basic side chains (e.g., lysine, arginine), can acquire positive charges at specific pH values, thereby partially neutralizing the inherent negative charge of the PLGA matrix. This phenomenon of charge neutralization or reduction in surface repulsion can, in turn, contribute to an increased tendency for particle agglomeration, a factor previously discussed concerning broader size distributions observed in the DLS/laser diffraction data.

In the case of lipid-based particles (LS), the observed negative zeta potential values are predominantly attributed to the lipid material itself. The triglycerides, which are the main components of the lipid matrix, contain ester bonds that are prone to hydrolysis in aqueous environments, leading to the formation of free fatty acids. These free fatty acids possess carboxyl groups that deprotonate, contributing a negative charge to the lipid particle surface (59). As observed in the polymeric and lipo-polymeric systems, incorporating the peptide into the lipid particles also markedly decreased their zeta potential values (i.e., less negative). This change was notably more pronounced than in the PS and LPS systems. This suggests that within the lipid matrix, the peptide is likely positioned closer to or more extensively distributed across the particle's surface, significantly altering its surface characteristics and electrostatic profile. The greater impact on the zeta potential in LS systems could imply a more direct and extensive interaction between the peptide and the lipid surface, potentially due to the different internal organization and surface chemistry compared to the polymeric particles.

Finally, the overall negative zeta potential values across all systems are favorable for enhancing their physical stability in dispersion. According to established colloidal science principles, systems with zeta potentials outside the range of ± 10 mV and ± 20 mV (typically considered relatively stable) and

particularly those exceeding ±20mV and ± 30 mV (often indicative of high stability) exhibit sufficient electrostatic repulsion to prevent aggregation. The obtained values, particularly for LS systems, are within these ranges, contributing to the colloidal stability of the developed formulations (60).

Encapsulation Efficiency, surface adsorbed peptide, and Loading Capacity

As previously discussed, encapsulation efficiency (EE) and loading capacity (LC) are critical parameters for API dosage. The observed EE values across the different systems highlight crucial insights into their encapsulation capabilities and the underlying mechanisms. The polymeric particles (PS) demonstrated moderate encapsulation efficiencies, consistently around 57%. This is likely influenced by various methodological parameters, such as the solvent evaporation time during particle formation, which can induce porosity within the polymer matrix. Additionally, the hydrophilic nature of our model peptide is known to promote its migration from the polymeric core towards the external aqueous phase during encapsulation. This phenomenon can lead to a partial loss of the peptide or its adherence to the particle surface, with the latter being subsequently removed during washing steps. The substantial percentage of surface adsorbed peptide (SAP) in PS further supports this, indicating that a significant fraction of the peptide is not deeply embedded within the matrix but associated with its periphery.

Conversely, the solid lipid particles (LSs) exhibited lower EE values, averaging approximately 40%. A striking observation for these systems was the exceptionally high percentage of surface adsorbed peptide (SAP), reaching over 76%. We attribute this reduced EE and increased SAP to a limited interaction between the hydrophilic model peptide and the predominantly hydrophobic lipid matrix. This intrinsic low affinity may cause the peptide to be repelled by the lipid during particle formation, promoting its migration toward the system's external aqueous phase. This could lead to an initial burst effect (61) in peptide release due to the molecule's preferential partitioning at the particle–water interface. Consequently, these systems are not deemed optimal for encapsulating hydrophilic molecules unless specific formulation modifications are implemented to enhance peptide–lipid interactions or minimize surface migration (61).

The lipo-polymeric hybrid particles (LPS) presented intermediate EE values (Around 43%) and LC's, strategically positioned between the polymeric and lipid systems. This intermediate behavior is consistent with the hybrid composition of these particles, which combines characteristics from both matrices. Specifically, the incorporation of lipid into the LPS tends to increase the overall hydrophobicity of the matrix compared to polymeric systems (PS). This elevated hydrophobicity reduces the hydrophilic peptide's affinity for the systems compared to the polymeric particles, consequently decreasing the EE value in LPS relative to PS (62,63). However, the SAP percentages for LPS were considerably lower than those for LSs, suggesting that the polymeric component helps to mitigate the strong surface partitioning observed in lipid systems.

Crucially, despite the slightly lower encapsulation efficiency observed for LPS compared to SPs, the presence of the lipid component offers a significant advantage for *in vivo* administration. Lipid-based or lipid-coated systems are generally less prone to recognition by the reticuloendothelial system (RES), an essential physiological barrier to long-circulating nanoparticles. This reduced recognition can lead to a prolonged systemic circulation half-life for LPS compared to purely polymeric systems, enhancing their potential for reaching target sites and improving therapeutic efficacy (64). This highlights the strategic benefit of the hybrid design, where optimized *in vivo* performance outweighs a marginally lower encapsulation efficiency, making LPS a more promising candidate for systemic delivery applications.

In vitro release profile

Understanding the release behavior of the encapsulated peptide from the developed systems is crucial for predicting their therapeutic utility and optimizing their *in vivo* performance. The release profiles were meticulously evaluated, and the results are presented in Figure 2. In all cases, an empty system served as a control to precisely delineate the influence of the matrix materials on the active ingredient's release behavior, minimizing interference.

The distinct release profiles observed across the three particle types reflect fundamental differences in their matrix composition and peptide interaction mechanisms. The initial rapid release, or burst effect, consistently observed across all systems on day 1, is directly attributed to the peptide adsorbed or weakly associated with the particle surface. This aligns with our previous Z-potential

measurements, where a decrease in the negative surface charge upon peptide incorporation for all systems (PS2/3, LS2/3, LPS2/3 a) compared to their empty counterparts) strongly suggests the presence of peptide on the microparticle surfaces. Such rapid initial release is a common phenomenon in various drug delivery systems. It is particularly relevant for achieving a rapid onset of action *in vivo* if a high initial concentration is desired (65).

For the polymeric particles (SPs), the extended three-phase release profile indicates the peptide's diffusion through and subsequent release from the polymeric matrix. The initial burst is followed by a prolonged, low-release phase, characteristic of diffusion-controlled release from a relatively dense polymeric network. The subsequent resumption of release from day 30 onwards, leading to nearly complete peptide release by day 44, directly correlates with the polymer's physicochemical properties and degradation kinetics. This suggests that the final phase of release is predominantly driven by the erosion and degradation of the polymer matrix, allowing for the complete liberation of the entrapped peptide (66,67).

In contrast, the solid lipid particles (LSs) displayed a comparatively rapid biphasic release, with almost complete peptide liberation by day 6. This accelerated release, particularly after the initial burst, is consistent with our characterization findings, indicating a surface enriched in peptide (high SAP%) and a significant reduction in Z-potential magnitude upon peptide loading. Moreover, a critical factor influencing this rapid release is the melting point of the lipid (approx. 33.0-34.5°C) (49). At the assay temperature (e.g., 37°C), the lipid matrix would be molten or semi-molten, effectively forming an emulsion. This molten state significantly enhances the diffusion of the hydrophilic peptide to the external aqueous medium due to its high affinity for water, leading to a much faster release compared to a rigid polymeric matrix (50). This highlights a potential limitation of lipid systems for prolonged release of highly hydrophilic payloads under physiological temperatures if not adequately stabilized(68).

The lipopolymer hybrid particles (LPS) exhibited a three-phase release profile that represents an intermediate behavior between the polymeric and lipid systems, effectively leveraging the properties of both materials. The initial burst phase in LPS is consistent with the surface-adsorbed peptide, echoing the behavior seen in SPs and further supported by the Z-potential measurements. The subsequent slow and increasing release phase, followed by a more pronounced release, reflects the combined effects of diffusion through the hybrid matrix and the gradual degradation of the polymeric component. The influence of the lipid component is evident in the overall reduction of release times compared to the purely polymeric systems. The incorporation of lipid tends to increase the matrix's hydrophobicity, which, as previously discussed in the EE section, can lead to the peptide migrating towards the particle surface. This increased surface localization, coupled with the potential for lipid melting at physiological temperatures, facilitates faster diffusion of the peptide compared to a purely polymeric matrix. This tunable release profile of LPS underscores their versatility, allowing for a controlled delivery that balances immediate and sustained release, depending on the specific therapeutic need and the desired *in vivo* pharmacokinetic profile (64)

Statistical systems comparison

A comprehensive comparative analysis was conducted to statistically evaluate the distinctions and similarities among the three developed systems: polymeric microparticles (PS), solid lipid particles (LS), and lipopolymeric microparticles (LPS). This statistical approach provided a robust framework for understanding the interplay between the formulation type and the physicochemical and release characteristics.

The statistical analyses performed, beginning with the MANOVA and subsequently validated by individual ANOVAs, provide compelling quantitative evidence for the unique characteristics of each developed system. The Z-potential, lacking significant differences across all three particle types, suggests that the surface charge characteristics, while varying in magnitude upon peptide loading (as discussed previously), are not the primary distinguishing factor between the empty systems or the overall charge trends in the loaded systems at a statistically significant level. This implies that all three matrix types provide a relatively consistent electrostatic environment or that other factors exert a more dominant influence on surface charge in these specific formulations.

Conversely, the statistically significant differences in particle size and encapsulation efficiency (EE) differentiate the systems. As graphically presented in Figure 3, the lipopolymeric microparticles (LPS) consistently exhibit an intermediate behavior for

both size and EE. Specifically, the size of LPS is more akin to that of the larger polymeric microparticles (PS), suggesting that the polymeric component predominantly dictates the overall dimensions of the hybrid structure. Regarding encapsulation efficiency, however, the LPS behave more similarly to the solid lipid particles (LS). This can be primarily attributed to the increased hydrophobicity of the hybrid matrix due to incorporating the lipid component, which, as elaborated in the previous section, reduces the affinity for the hydrophilic peptide and consequently lowers the EE compared to the purely polymeric system. This intermediate behavior underscores the ability of the hybrid LPS platform to offer a tunable approach, combining size characteristics closer to macro- or microparticles with encapsulation efficiencies influenced by both lipid and polymer interactions.

The statistical analysis of the release profiles further reinforces the distinct yet interconnected properties of the systems. The initial lack of significant differences in release kinetics among all systems suggests a common mechanism for the early burst phase, likely driven by surface-associated peptide, irrespective of the matrix composition. However, the divergence in release behavior at later time points (Figure 4) strongly indicates that the specific material properties of each system govern the long-term release kinetics. The fact that the lipopolymeric hybrid system (LPS) presents properties of both the polymeric microparticles and the solid lipid particles in its release profile is a critical finding. It validates the design principle of creating a hybrid system that can modulate release kinetics by combining components with inherently different release characteristics (e.g., slow-degrading polymer vs. faster-releasing lipid at physiological temperatures). This tunable release profile of LPS offers a significant advantage for designing drug delivery systems capable of achieving specific therapeutic objectives, from immediate local effects to prolonged systemic exposure.

CONCLUSIONS

This study successfully developed innovative lipopolymeric microparticles (LPS) by combining PLGA with C_{12}-C_{18} triglycerides, effectively encapsulating a hydrophilic peptide using a double emulsion formation - solvent evaporation method. These LPS exhibited an EE around 43%, sizes between 3,220 and 3,939 nm, a spherical shape, smooth surface, and a stabilizing negative zeta potential.

While LPS didn't surpass polymeric systems (PS) in EE, showing intermediate behavior for size (like PS), and EE/release (closer to lipid particles), this compromise is a strategic advantage. Crucially, the lipid component in LPS significantly benefits *in vivo* administration. Lipid- based systems are less recognized by the reticuloendothelial system (RES), potentially extending their systemic circulation half-life in the body. Despite slightly lower EE or a faster release profile than PS *in vitro*, this enhanced stealth makes LPS a more promising candidate for systemic delivery.

In conclusion, this study provides a solid foundational understanding for the development and characterization of hybrid lipopolymeric systems for peptide encapsulation. It not only contributes to the knowledge base regarding the complex interactions governing the behavior of such sophisticated drug delivery systems but also establishes a strong basis for further research aimed at encapsulating diverse peptide molecules for various therapeutic applications in drug development.

CONFLICT OF INTEREST: The authors declare that have no conflicts of interest related to the work presented.

ACKNOWLEDGEMENTS: The authors thank the Departamento de Farmacia, Universidad Nacional de Colombia, for the use of its facilities to carry out this research. They also thank BASF Chemical for supplying the TG C12-C18 triglycerides (Novata BC PH) used in the development of this research.

AUTHOR CONTRIBUTIONS: Maria Angelica Velandia Paris: Conceptualization, methodology, writing – reviewing, original draft preparation, investigation. **Jaiver Eduardo Rosas Pérez:** Conceptualization, methodology, writing – review & editing, editing, investigation, supervision.

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