**D-Polyarginine Lipopeptides as Intestinal Permeation Enhancers**

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An estimated 285 million people were living with diabetes in 2010, and this number is expected to reach 440 million by 2030. Current treatment of this disease involves the intradermal injection of insulin analogues. Many alternative administration routes have been proposed, the oral route being the most widely studied. One of the most interesting approaches for insulin delivery is the use of permeation enhancers to increase its transport across the gastrointestinal tract (GIT). Cell-penetrating peptides (CPPs) are a remarkable example of this family of compounds. Another alternative is the use of medium-chain fatty acids (MCFAs) to temporally disrupt the tight junctions of the GIT, thereby allowing greater drug transport. A combination of both strategies can provide a synergistic way to increase drug transport through the GIT. In this study we evaluated the complexation of insulin glulisine, an insulin analogue administered subcutaneously or intravenously in clinical practice, with a well-known CPP modified with the MCFA lauric acid. We prepared several formulations, examined their stability, and tested the best candidates in an intestinal cell-based model. In particular, two compounds (C₁₂-r₄ and C₁₂-r₆) were found to significantly increase the transport of insulin, and therefore show promise as a new delivery system worthy of further evaluation.

**Introduction**

Recent research in the fields of biomedicine and pharmacology have led to promising strategies to treat and cure several diseases. However, despite these huge breakthroughs, effective treatment for many others is still elusive. Although therapeutic agents achieve the desired purpose, they can cause long-term side effects. A clear example is the administration of insulin and its analogues, which provide the most convenient and effective treatments for diabetes mellitus but require multiple subcutaneous injections per day. This administration has multiple side effects, including pain, swelling and redness at the site of injection.[3] The development of other ways to administer insulin, such oral delivery, would provide a painless and friendlier delivery route for this protein.

Peptides are now essential tools in pharmaceutical research owing to high specificity toward their targets, low immunogenic response, and relatively affordable price.[2] Among these molecules, peptide enhancers are widely used in biomedicine to improve the transport of therapeutic agents across biological barriers.[3] Regarding their mechanism of action, peptide enhancers are classified into three main groups. The first comprises transcellular enhancers, mostly CPPs (Figure 1(1)) short peptides that interact with the plasma membrane, thus causing their cell uptake. Given their properties, transcellular enhancers can serve as a drug delivery system, increasing the absorption of the cargo into the desired tissue or cell type. The second group is formed by paracellular enhancers (Figure 1(2)), which interact with tight junctions (TJs). These structures are closely associated areas between two cells responsible for cell–cell adhesion and provide high impermeability toward substances. TJ modulators derive mainly from TJ proteins or toxins and in-
clude peptides with the ability to transiently open TJs. The third and final group comprises targeting peptides derived predominantly from phage display. These specific peptide enhancers direct the transport of a macromolecular agent into a specific tissue or cell type (Figure 1(3)).

A wide range of CPPs have been described, including those derived from venoms and viruses, and synthetically designed peptides such as oligoarginines. Many research groups have addressed their use to transport drugs across biological barriers. Examples include arginine-rich peptides like HIV-1 TAT peptide, non-natural oligoarginines such as d-octaarginine (r8), and amphipathic peptides such as the Drosophila antennapedia homeodomain (penetratin), among others.

CPPs can be used in several ways to promote the absorption of therapeutic agents. They can be covalently conjugated or electrostatically bound to the biotherapeutic, or in combination with nanoformulations. Many attempts with several CPPs have been made to deliver insulin into the bloodstream by crossing the gastrointestinal barrier as an alternative to the common subcutaneous administration method. One of the first studies in the field involved the use of a chemically synthesized TAT covalently bound to fluorescently labelled insulin (insulin-FITC). The use of TAT/insulin-FITC caused a 6- to 8-fold increase in insulin transport across a Caco-2 cell monolayer (the gold-standard cellular model to simulate the epithelial cell layer) relative to insulin-FITC alone. A few years later, Morishita and colleagues tested the capacity of 10 distinct CPPs electrostatically bound to insulin to enhance cell membrane permeation. In this regard, l-penetratin and l-pVec, followed by d-octaarginine (r8), showed the best performance with regard to increasing insulin bioavailability and low toxic effects. In another study, r8 was co-administered as a physical mixture (electrostatically bound) with various peptide drugs. They found that the peptide drugs with higher transport through in situ ileum loop were those with negative charges. Peptide drugs with a neutral charge or positive charged were not able to cross the ileum membrane. These results demonstrated that the binding affinity (electrostatic) between drugs and CPPs is crucial for drug absorption in the intestine. Moreover, the binding ratio between insulin and r8 was a key factor as an increase in the ratio of CPP bound led to enhanced intestinal absorption of insulin.

Here we examined peptides, in particular CPPs, as penetration enhancers. The selected CPPs were modified with a certain MCFA, reported as a penetration enhancer. The unmodified CPP were not included in this work because they have already been widely studied. Fatty acid based compounds are a great source of absorption enhancers. Despite the potential of these compounds, some have been raised regarding their toxicity. Sodium caprate (C12) in particular has been extensively studied for in vitro and in vivo studies. This MCFA, as well as its homologues (C9, C10, C14, C16, etc.), cause cytotoxicity in a concentration- and time-dependent manner. In spite of this, several reports have studied the safe concentration range in which these compounds can be used as permeation enhancers in vitro. More importantly, fine control of the concentration used has allowed the use of C10 in clinical trials. Therefore, modulation of the dose of MCFA can enhance the intestinal absorption of biotherapeutics without causing remarkable cytotoxicity. In addition, the combined effect of TJ modulator peptides and MCFA had a greater effect on paracellular transport, where C14 covalently bound to the TJ modulator peptide protected it from degradation and aggregation. In 2015, Zhang et al. reported a synergistic effect when using amphiphilic lipopeptide-insulin complexes relative to r8 alone. In that case, the use of stearic acid and incorporation of glutamic acid and tryptophan increased the stability of the complex and, therefore, the transport of insulin.

Although lipopeptides have the potential to increase insulin bioavailability through the intestinal tract, they have several drawbacks. One of the main problems is the low peptide stability in the gastrointestinal environment, which makes them susceptible to enzymatic degradation. Furthermore, it has been observed that the complexes formed between CPPs and insulin show instability across the intestinal tract, possibly because of the high ionic strength in the intestinal media. In addition, at a certain molar ratio, insoluble aggregates are observed. In this regard, many strategies, such as the use of polymer coatings, have been tested to preserve the stability of these complexes during intestinal absorption.

In this study, four distinct lipopeptides (Figure 2) were used to form complexes with insulin glulisine (commercially known as Apidra®). Glulisine is a new generation insulin analogue characterized by its rapid onset of action. We hypothesized that self-aggregation occurs as a result of the amphipathic nature of lipopeptides, which would lead to micelle formation. Lipopeptide micellization may affect the structure of CPPs and insulin and thus cause precipitation of the complex.

![Figure 2. Structures of the four lipopeptides used: C12-r8 (n = 1), C12-r5 (n = 2), C12-r6 (n = 3), C12-r12 (n = 5).](image-url)
Results and Discussion

Determination of critical micelle concentration

Lipopeptide aggregation behavior in solution is a crucial factor to study with respect to stability of the lipopeptide-glulisine complex. Weak complex stability caused by lipopeptide self-aggregation could lead to decreased glulisine transport across the intestinal barrier. We therefore explored whether the formation of micelles triggers complex aggregation.

ITC is a calorimetric high-precision technique that can be used to determine thermodynamic parameters associated to micelle formation.23 In our case titration experiments of each lipopeptide into HBSS were performed to obtain the CMC (Supporting Information, Figure S1). As an example, various interactions were observed in the lipopeptide C12-T8 (Figure S1B): peaks 1 to 14 showed thermal effects produced by exothermic interactions, while peaks 15 from 24 showed the process derived from endothermic interactions. The concentration obtained from the transition of peak 14 to 15 corresponds to the CMC. Corrected heat, in kJ mol−1, correspond to each injection of lipopeptides into HBSS, and the heat rate is represented inversely (Figure S2). CMC and other thermodynamic parameters were calculated for all the lipopeptides.24 The results for each lipopeptide are shown in Table 1.

| CPP | CMC [mM] | ΔHmic [kJ mol−1] |
|-----|----------|------------------|
| C12-T12 | 4.56 ± 0.46 | 2.16 ± 0.73 |
| C12-T6 | 2.04 ± 0.78 | 7.66 ± 4.19 |
| C12-T8 | 4.07 ± 0.86 | 1.24 ± 2.69 |
| C12-T6 | 0.67 ± 0.25 | 0.50 ± 0.72 |

[a] Data are expressed as the mean ± SD, n = 2.

All the CMCs determined were above the maximum concentration used to form the complexes (0.48 mM) as the maximum concentration used for C12-T12. With these results, the hypothesis that concentrations above the CMC would trigger complex precipitation was discarded. Thus, lipopeptides were in their non-micellar form and electrostatic interactions with glulisine were not altered by micellization processes.

Bile salts act as a surfactant, emulsifying dietary fats into micelles and thus facilitating their digestion. As the lipopeptide structure contains a fatty acid chain, the bile salts may cause early micellization. To study variations in the lipopeptide CMCs caused by pH variation or the presence of bile salts, we prepared a fasted simulated intestinal medium (FaSSIF). Again, ITC was performed to study C12-T8 in FaSSIF medium at the same concentration studied previously in HBSS (Figure S3). Our results showed that all the titrations were exothermic and, in contrast to what was expected, the CMC was not reached in this case. Therefore, we assumed that an increase in CMC for all the lipopeptides would be observed in FaSSIF. The interaction of the lipopeptides with the bile salts of the media could explain this phenomenon.

Evaluation of the formulation of physicochemical properties

We studied the effect of factors such as the preparation of glulisine stock solution, molar ratio between the lipopeptide and glulisine and ionic strength of the buffer on complex size, charge and tendency to aggregate.

Procedure to dissolve glulisine

A well reported procedure to solubilize insulin for the formation of complexes with positively charged peptides is the addition of HCl (0.1 M), followed by the addition of the desired buffer, and finally pH adjustment with NaOH (0.1 M).20,22 Insulin and peptides are then mixed to form the complexes at a specific molar ratio.

In our hands, this methodology resulted in highly polydisperse samples regarding particle size (measured by DLS), thereby indicating that glulisine was not well dissolved and aggregation was taking place. The same results were obtained when insulin was first dissolved with NaOH (0.1 M), followed by pH adjustment with HCl (0.1 M).

We found that the most efficient approach to solubilize glulisine was with a NaOH solution (0.01 M). The complexes with the lipopeptide were then formed. Next, the desired buffer was added and the pH was adjusted. In addition, the pH achieved when dissolving glulisine in NaOH 0.01 M conferred the complexes extra negative charges, thus increasing electrostatic interaction efficiency with the positively charged lipopeptides and resulting in samples with greater monodispersity.

Lipopeptide/glulisine molar ratio

Particle size and ζ-potential were measured after preparing the formulations. The effect of a range of molar ratios between lipopeptides and glulisine was tested on these two parameters. Lipopeptide concentration ranged from 15 μM to 480 μM while glulisine concentration remained constant at 15 μM. The results are given in the Supporting Information (Table S1). On the one hand, for both C12-T8 and C12-T6, a molar ratio higher than 4:1 resulted in nanometric particles with an average size of 200 nm (Figure 3A, B, respectively). However, molar ratio of 1:1 resulted in aggregation and, consequently, particle size exceeded 2 μm in both cases. On the other hand, C12-T6 and C12-T8 showed a different tendency. For complexes formed by C12-T6/glulisine, molar ratios ranging from 8:1 to 1:1 resulted in particle aggregation (Figure S3). Nevertheless, molar ratios of 16:1 and 12:1 resulted in nanometric particles. In the case of C12-T8, completely different behavior was observed, as molar ratios of 1:1 and 32:1 resulted in particles with a diameter slightly over 200 nm (Figure 3D). However, molar ratios ranging from 4:1 to 16:1 yielded particle aggregation.

The ζ-potential of the nanocomplexes is another parameter indicating particle aggregation. Lipopeptides containing a greater number of arginine residues in their sequence (C12-T12, C12-T6, and C12-T8) showed a more negative ζ-potential than the complexes with C12-T8. The lower ζ-potential of the nanocomplexes indicated the presence of aggregated particles.
and C₁₂-r₆ yielded higher ζ-potential values at lower molar ratios compared with C₁₂-r₄ and C₁₂-r₆. Thus, at molar ratios of 4:1 and 8:1, C₁₂-r₁₂ and C₁₂-r₄ formed complexes with enough positive surface charge to be stable (Figure 3A,B). Nevertheless, in both cases, a molar ratio of 1:1 gave a lower ζ-potential and, as a result, larger particle size.

The ζ-potential is a key indicator of particle stability. In many cases, values close to zero indicate poor stability as a result of weak repulsion between charges. This concept is reflected in Figure 3D, where an almost zero ζ-potential (between +10 and −10 mV) correlates with particle aggregation. Otherwise, values higher than ±10 mV, such those corresponding to molar ratios of 32:1 and 1:1 for C₁₂-r₁₂ resulted in nanometric complexes. Only one exception was observed for C₁₂-r₁₂ at molar ratio of 1:1. In this case, aggregation takes place at high ζ-potential. Several studies outline the importance of the CPP concentration for the insulin association efficiency. Thus at low concentrations, such as the one corresponding to 1:1, C₁₂-r₁₂ would not bind properly to glulisine, thus causing instability and aggregation.

### Ionic strength

Given that ionic strength plays a key role in the colloidal stability of electrostatic formulations, we studied the physicochemical properties of complexes in a range of buffer solutions, analysing both particle size and ζ-potential. First, HBBS was selected because it is the most commonly used buffer for in vitro intestinal models. Results showed that the high amount of salts have a strong effect on the size of the complexes (Figure 4A). For the complexes formed with C₁₂-r₁₂ and C₁₂-r₈, the increase in the ionic strength led to aggregation, thereby modifying particle size from around 200 nm to more than 1000 nm at molar ratios (CPP/glulisine) of 8:1 (Figure 4A) and 4:1 (Table S1).

![Figure 3. Measurement of particle size and ζ-potential: A) C₁₂-r₁₂, B) C₁₂-r₆, C) C₁₂-r₆, and D) C₁₂-r₄. Data are expressed as the mean ± SD for particle size, and mean ± SD for ζ-potential, n=6–18.](image)

![Figure 4. Effect of ionic strength on: A) particle size and B) ζ-potential. Data are expressed as the mean ± SD, n=6–18; ns (p > 0.05), * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001), **** (p ≤ 0.00001).](image)
In the case of C₁₂₋₆ and C₁₂₋₈, there was also an increase in the particle size but it was less than that observed for the previous complexes (Figure 4A). In all cases, the particle size increased when HBSS buffer was used, compared with complexes formed in aqueous solution.

Regarding the ζ-potential, lower values were registered in all the complexes assayed (Figure 4B and Table S1), except for the molar ratio 1:1 of C₁₂₋₆, which led to an increase in this parameter (Table S1).

The high ionic strength of the buffer has been reported to affect electrostatic and hydrophobic interactions. Therefore, we hypothesized that HBSS decreases the repulsive electrostatic lipopeptide-glulisine interactions. There is a strong correlation between protein solubility and protein-protein interactions. In this regard, a decrease in electrostatic repulsion results in a decrease in protein solubility. Complexes present positive charges (conferred by arginine residues) and negative charges (glulisine), while HBSS contains both cations and anions in solution. Consequently, these ions interact with both lipopeptides and glulisine, thus hindering the formation of the complex, as reflected by an increase in their size.

As previously mentioned, ζ-potential is used as a parameter to measure particle stability in solution. The decreases in ζ-potential brought about by the salts are the main cause of complex aggregation owing to weak positive-positive repulsion between surface charges. However, even with this decrease in ζ-potential and the evident aggregation, no visible precipitation of the complexes was observed after 2 h in HBSS. In summary, the selection of an optimal ionic strength is a difficult issue that must be addressed by achieving an equilibrium between the deleterious effect of high ionic strength on particle size and ζ-potential and the minimal salt content required to ensure cell survival during transport assays. Although the use of polymers to protect the complexes could be useful, this approach would introduce other drawbacks, such as potential polymer toxicity or long degradation times.

Caco-2/HT-29 transport assay

Caco-2 cells are the gold-standard to simulate the intestinal barrier as they recapitulate the morphological and functional characteristics of mature enterocytes. Their ability to form a monolayer and express TJJs are crucial properties that make them ideal as a model to study glulisine transport across the intestinal epithelium. In addition, these cells express microvilli, enzymes and transporters that are unique to enterocytes. However, they present some limitations, such as the lack of mucus secretion. Therefore, to work with a more realistic intestinal environment, we used co-cultures with mucus-secreting HT-29 cells.

The optimized formulations of the amphiphilic lipopeptides and glulisine were assayed in this cellular model as drug delivery system across the Caco-2/HT-29 cell monolayer. Transepithelial electrical resistance (TEER) is a quality indicator of cell monolayer integrity as it measures the electrical resistance that this monolayer offers against electrical current. In transport studies, TEER values were measured at 0, 2, and 24 h in order to monitor membrane disruption.

Various profiles were observed when the complexes were assayed in the Caco-2/HT-29 model (Figure S4). Glulisine did not affect TEER as it remained stable throughout the 24-h experiment. However, the complexes formed with the four lipopeptides had different effects on the Caco-2/HT-29 cell monolayer. After 2 h, complexes formed with C₁₂₋₆, decreased TEER from 100% to 53% and 42% for molar ratios of 4:1 and 8:1, respectively (Figure S4A). At 24 h, TEER was reduced to 32% and 30% for molar ratios of 4:1 and 8:1. The lipopeptide C₁₂₋₆ showed similar results for the two molar ratios assayed: TEER was reduced from 100% to 97% in 2 h and decreased to 67% in 24 h at a molar ratio of 4:1. When the molar ratio was 8:1, TEER also decreased, from 100% to 74% in 2 h and to 49% in 24 h.

A completely different scenario was observed for C₁₂₋₈. In this case, TEER decreased after 2 h at the higher molar ratios tested (Figure S4B); however, none of the values fell below 75%. At 24 h, only the molar ratio of 4:1 maintained TEER around 100%, the rest of the formulations maintained the decreased TEER constant.

Two distinct profiles were observed when the complexes were formed with C₁₂₋₆ (Figure S4C): molar ratios corresponding to 32:1 and 1:1 registered a notable reduction in TEER after 2 h, followed by a recovery of this parameter at 24 h, while molar ratios of 8:1 and 4:1 showed a gradual increase of TEER up to 110% and 120%, respectively, over the 24-h experiment.

Analysis of the TEER measurements in the Caco-2/HT-29 transport assay revealed that the complexes formed with longer oligoarginine (C₁₂₋₆ and C₁₂₋₈) had a more pronounced effect on the cell monolayer resistance compared with the others. This finding was reflected in a decrease in TEER, with no apparent recovery at 24 h, thus indicating cell monolayer disruption. In contrast, the effect of C₁₂₋₆ and C₁₂₋₈ on the cell monolayer was not as harmful as that of the richer arginine peptides. This observation was reflected in a reduction in TEER, which was not below 75% in any case and was recovered or maintained in most of the cases.

Comparing the same lipopeptides but different molar ratios, in all cases TEER decreased when the molar ratio increased. This effect can be attributed to the higher number of MCFAs present in the complex.

To quantify glulisine transport, the samples from the acceptor compartment were collected and analyzed by UPLC–MS. The molecular weight of glulisine was detected in C₁₂₋₆/glulisine (4:1) and C₁₂₋₆/glulisine (1:1) but no quantitative results were obtained.

We then lyophilized the samples and concentrate them 10 fold. After resuspension, samples were analysed by UPLC and transported glulisine was calculated by the following equation [Eq. (1)]:

\[
\text{Transport (%) = } \frac{\text{Amount of Glulisine}_{\text{acceptor}}}{\text{Amount of Glulisine}_{\text{total}}} \times 100
\]
This cellular model allowed us to identify two formulations that can improve the transport of glulisine through the intestinal barrier. C\textsubscript{12}-t\textsubscript{2} at a molar ratio of 4:1 and C\textsubscript{12}-t\textsubscript{3} at a molar ratio of 1:1 significantly increased glulisine transport across the Caco-2/HT-29 monolayer. In the case of the C\textsubscript{12}-t\textsubscript{3} and C\textsubscript{12}-t\textsubscript{8} formulations (Figure 5A), the amount of glulisine detected was similar to the control. In contrast, for C\textsubscript{12}-t\textsubscript{6} formulations (Figure 5B), a slight increase in glulisine transport was observed in all the conditions assayed except for the molar ratio of 12:1. Remarkably, the molar ratio of 4:1 increased the transport of glulisine by 30%. Similar results were observed for C\textsubscript{12}-t\textsubscript{6} formulations (Figure 5C). A slight increase in glulisine transport was detected for molar ratios of 32:1 and 4:1. However, the 1:1 molar ratio significantly increased glulisine transport up to 40%. These results highlight the potential of these formulations. Although the use of Caco-2/HT-29 monolayers to evaluate intestinal permeability is widely used, direct correlation with in vivo transport cannot be done. The information extracted from this model should be considered only as qualitative indication of transport.

These results reveal that the transport of glulisine through the Caco-2/HT-29 monolayer is inversely proportional to the number of arginine residues in the lipopeptides used in the formulations. Furthermore, in our hands, as the molar ratio increased, glulisine transport decreased.

A recent study highlighted the importance of negative and neutral surface charge particles for diffusivity through porcine intestinal mucus. On the basis of finding on negative mucus glycoproteins such as the mucin secreted by HT-29 cells, electrostatic binding between mucin and complexes could be the plausible cause of the poor transport of the more polar positively charged formulations (those containing C\textsubscript{12}-t\textsubscript{3} and C\textsubscript{12}-t\textsubscript{6}). In contrast, neutral and negative formulations such those corresponding to C\textsubscript{12}-t\textsubscript{4} and C\textsubscript{12}-t\textsubscript{2} would be electrostatically trapped to a lesser extent in the mucus and could promote the transport of glulisine across the cell monolayer more efficiently. If we consider the particle size of these complexes, C\textsubscript{12}-t\textsubscript{4}: glulisine 3715 nm, C\textsubscript{12}-t\textsubscript{2}: glulisine 992 nm, a disaggregation process that results in size reduction upon interaction with mucus can be proposed. These phenomena have to be further evaluated.

Conclusions

Here we studied complexes formed by amphiphilic lipopeptides and glulisine as potential permeation enhancers. Several parameters were examined and optimized. As general trend, the size and homogeneity of the complexes were strongly affected by changes in pH, molar ratio and ionic strength. These changes can be caused by the self-assembly of the lipopeptides which can occur at concentrations below the CMC due to their amphipathic character. Two of the formulations tested, namely positively charged C\textsubscript{12}-t\textsubscript{2} at a molar ratio of 4:1 and negatively charged C\textsubscript{12}-t\textsubscript{6} at a molar ratio of 1:1, enhanced the passage of glulisine through the Caco-2/HT-29 model approximately a 30 and 40%, respectively. These promising formulations need to be further evaluated.

Experimental Section

Lipopeptide synthesis: Lipopeptides were synthesized by Solid-phase Peptide Synthesis (SPPS) following the Fmoc/Bu strategy. H-Rink Amide-ChemMatrix\textsuperscript{®} resin was used to obtain C-terminal amidation. Peptide elongation was performed manually using PyAOP and DIEA as coupling reagents. After the introduction of each amino acid, the Kaiser test\textsuperscript{[31]} was used to ensure high coupling efficiency. Fmoc deprotection was performed by the addition of 20% piperidine in DMF. Lauric acid was coupled to the N-terminus using the previous strategy. The peptides were deprotected and cleaved from the resin using the following mixture: TFA/TIS/H\textsubscript{2}O (95:2.5:2.5) for 4–5 h. Peptides were purified by RP-HPLC at semi-preparative scale (Sunfire C\textsubscript{18} column (150 x 10 mm x 5 μm, 100 Å, Waters), flow rate 6.6 mL/min using acetonitrile (0.045 %T FA)) and characterized by UPLC and UPLC–MS (Acquity UPLC\textsuperscript{®} BEH C\textsubscript{18} column (50 x 2.1 mm x 1.7 μm, Waters) coupled to a PDA Acquity detector and SQ detector 2, flow rate 0.6 mL/min using acetonitrile (0.036 %TFA) and H\textsubscript{2}O (0.045 % TFA)). All peptides were obtained with high purity (>95%) and stored lyophilized at −20 °C.

Determination of critical micelle concentration: Titration experiments were performed in a Low-Volume Nano ITC (TA Instruments). The sample cell was filled with the buffer. The syringe contained a concentrated solution of the desired lipopeptide in HBSS. Each titration experiment consisted of 24 injections of 2 μL of each lipopeptide into a sample cell (280 sec interval) with a stirring
speed of 207 rpm. A first injection of 0.5 µL was performed to avoid air bubbles. Sample cell and syringe samples were degassed for 15 min and centrifuged for 15 sec at 6000 rpm before each titration experiment. Fasted simulated intestinal fluid (FaSSIF) was used to simulate the effect of bile salts.[16] The experimental data were analyzed by TA Instruments NanoAnalyze® software. CMCs and other thermodynamic parameters were calculated by a Microsoft Excel macro kindly provided by Prof. Dr. Sandro Keller.[24]

Preparation of complexes: 500 µL of glulisine (0.01 M NaOH) solution was placed in a glass vial, and the same volume of lipopeptide aqueous solution was added while the solution was under magnetic stirring. After 10 min, pH was adjusted to 7.4 with HCI 0.1 M. For Caco-2-HT-29 transport assay, lipopeptides and glulisine were mixed as previously explained. Concentrated HBSS was then added in order to obtain the desired lipopeptide/glulisine molar ratio in a standard HBSS solution.

Dynamic light scattering: The particle size and ζ-potential of all the formulations were determined by DLS using a Malvern ZetaSizer (NanoZS, ZEN 3600, Malvern Instruments) upgraded with ζ-potential capability. The equipment was adjusted to 3 measures of 3 runs (10 sec per run) for size measurements and 3 measures of 10 runs for ζ-potential. The temperature was set at 25 °C and the scattering angle to 173°. Data analysis was performed using ZetaSizer software.

Cellular transport model: For the transport model, co-cultures of Caco-2 and HT-29 cells were prepared. Passages from 7 to 11 were used in both lines. The insert (PET, 1 µm pore size, Falcon) was prepared as follows: 300 µL of cold Corning® Matrigel® (10 µLmL−1 in non-supplemented DMEM) was incubated in each insert, in sterile conditions, for 1 h. Matrigel was then removed, and inserts were washed three times with non-supplemented DMEM medium. Caco-2 (90%) and HT-29 (10%) cells were added to each insert (300 000 cells per well) in 500 µL of complete DMEM medium. Next, 1.5 mL of complete DMEM was added to the donor chamber of each well to each well in the donor side, and cells were grown at 37 °C and 5% CO2 for 21 days in order to obtain a cell monolayer. Medium was replaced every other day. TEER was measured as a control of monolayer formation. Medium was changed every two days.

For transport studies, the acceptor (0.5 mL) and a donor (1.5 mL) chambers were equilibrated with HBSS buffer for 30 min at 37°C and 5% CO2. After this time, the lipopeptide complexes were incubated for 2 h in the acceptor chamber at 37°C and 5% CO2. To evaluate the effect of the different formulations on the cell monolayer, TEER was measured before sample addition and 2 h and 24 h after sample addition.

Glulisine quantification: Time0. Donor and Acceptor samples were concentrated 10-fold and analysed by UPLC-MS. The gradient used was from 15% to 65% acetonitrile in 2 min. Glulisine retention time was 1.39 min. A calibration curve of glulisine was used to calculate the concentration transported across the monolayer.

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Conflict of interest

The authors declare no conflict of interest.

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