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Hydrophobic solid lipid-based microparticles for the protection of gastric-sensitive hydrophilic active biomolecules for oral administration in the treatment of EPI

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Abstract

Exocrine Pancreatic Insufficiency (EPI), induced by conditions such as cystic fibrosis, chronic pancreatitis, and Crohn's disease, is a frequently overlooked and underdiagnosed gastrointestinal disorder. It leads to inadequate intestinal digestion due to insufficient secretion of pancreatic juice, resulting in discomfort, pain, and ultimately severe malnutrition. Despite numerous treatments proving ineffective over the past three decades, a strictly hydrophobic solid lipid formulation, administered orally, is proposed in this study to restore digestive function. This technology relies on the hydrophobic nature of the matrix to physically protect the hydrophilic active principle from the gastric environment while enabling its immediate release in the duodenum by targeting the amphiphilic nature of bile salts. Results demonstrate that this formulation effectively protects an acid-sensitive active ingredient during gastric passage (Simulated Gastric Fluid or SGF), facilitating its rapid release upon entering an artificial duodenal environment (Simulated Intestinal Fluid or SIF). Furthermore, it has been demonstrated that the preservation of a protein-based active ingredient extends beyond its primary protein structure to include its functional aspects, such as enzymatic activity. This drug delivery technology could enable the protection of hydrophilic active biomolecules, such as pancreatin, which are sensitive to gastric acidity, while promoting their immediate release upon contact with bile salts in the proximal duodenum, with the ultimate goal of correcting the digestive defect induced by EPI.

Keywords: solid lipid-based formulation, biomolecules, drug delivery, therapeutic efficacy, exocrine pancreatic insufficiency, controlled release.

1. Introduction

Exocrine Pancreatic Insufficiency (EPI) is a pathological condition characterized by a dysfunction of the exocrine function of the pancreas, resulting in insufficient or absent production of pancreatic enzymes, bicarbonate ions, or an obstruction of the Wirsung duct[1].

Symptoms associated with EPI encompass digestive disorders such as abdominal pain, steatorrhea, diarrhea, flatulence, and bloating, attributed to the malabsorption of dietary fats and the fermentation of undigested food in the colon. Significantly, EPI leads to malnutrition, anemia (especially vitamin B-12 or iron deficiency), bone pathology (vitamin D deficiency)[2], coagulation dysfunction, and internal bleeding (vitamin K deficiency). Its prevalence in the general population remains undetermined, although a German survey in 2005 suggested that approximately 11.5% of individuals over the age of 50 observed in general medical practice exhibited an asymptomatic form of the disease[3]. It is worth noting that EPI is typically an underlying complication of various pathologies that affect the structure

EPI: Exocrine Pancreatic Insufficiency; **SGF:** Simulated Gastric Fluid;
SIF: Simulated Intestinal Fluid; **PE:** Pancreatin Extract; **PEG:** PolyEthylene Glycol;
SMEDDS: Self-MicroEmulsifying Drug Delivery System; **HLB:** Hydrophilic-Lipophilic Balance;
PEP: Pancreatic Enzyme Preparations; **LMP:** Lipid MicroParticles; **SEM:** Scanning Electron Microscopy;
USP: United States Pharmacopeia; **(B)SA:** (Bovine) Serum Albumin; **O/W:** Oil in Water

or function of the pancreas, such as chronic pancreatitis, pancreatic cancer, cystic fibrosis, or Crohn's disease[4]. Fundamentally, EPI results from a deficiency in the breakdown of macronutrients from food[5] (Figure 1).

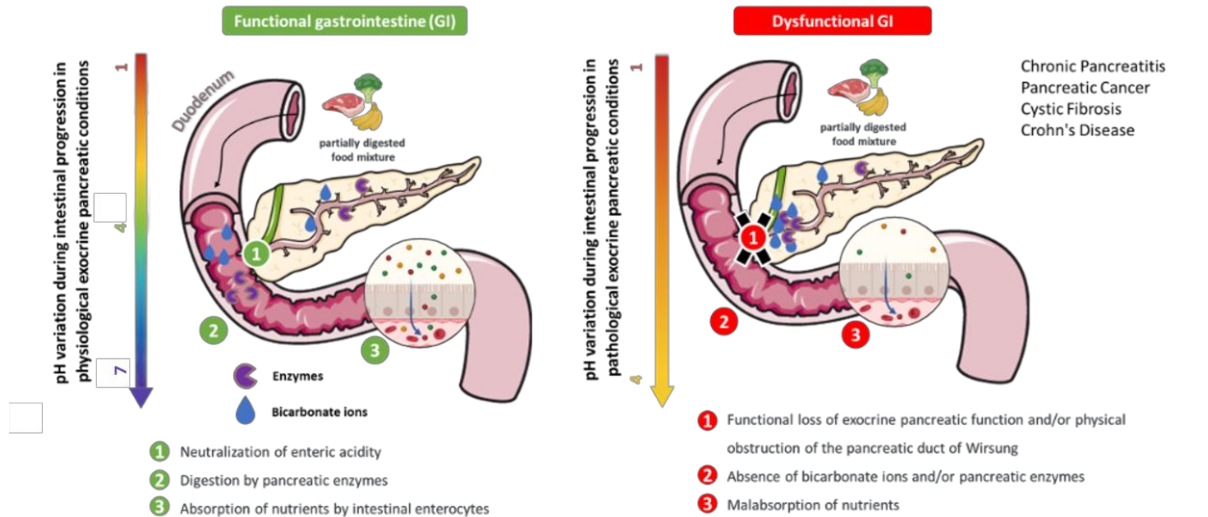


Figure 1: Physiological and pathological condition of Exocrine Pancreatic Insufficiency (EPI) in the gastrointestinal sphere during food intake. Created with BioRender software.

Unfortunately, EPI remains underdiagnosed and undertreated, despite the availability of screening tests[6]. This situation is primarily due to the prolonged asymptomatic phase of the disease, the scarcity of initial symptoms, and the late manifestation of complications. Diagnosis relies on a correlation between clinical elements, biological data, and the results of stool tests. The treatment of EPI is based on the use of Pancreatic Extracts (PE), although guidelines may vary among pharmaceutical companies. Porcine pancreatic extracts used as substitutes are composed of acidic-sensitive pancreatic enzymes such as lipase, alpha-amylase, and proteases (trypsin, chymotrypsin, carboxypeptidases...), enabling the replacement of the nutrient degradation mechanisms normally mediated by absent endogenous pancreatic enzymes. Additionally, adequate nutritional support is crucial to counter malnutrition and its consequences. Commercial PE products are presented as gastro-resistant microgranules containing porcine-derived pancreatin, available in free form or as capsules. These microgranules include various digestive enzymes, with lipase being the primary dosage unit expressed[7].

Gastro-resistant microspheres are equipped with a polymeric coating designed to withstand the acidic environment of the stomach and dissolve in the alkaline conditions of the duodenum[8], [9]. The coating of the microgranules is pH-dependent, protecting the enzymes from acidic degradation in the stomach and allowing their release when the pH reaches higher levels in the duodenum (above 5)[10], [11], [12] (Figure 2).

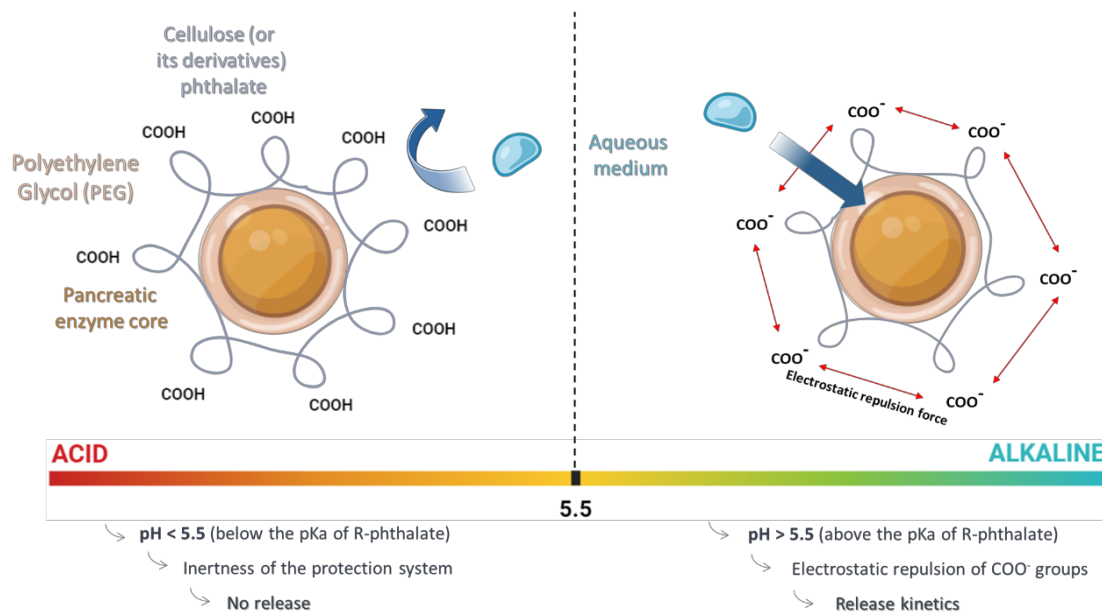


Figure 2: The drug delivery technology of current commercial Pancreatic Enzyme Replacement Therapies (PERTs). Created with BioRender software.

The polymeric coating-based protection technique, in the context of active ingredient release, has a major drawback, namely a very slow release of the active ingredients. The slowness of release is largely attributed to an underlying pathological mechanism. The deficiency in pancreatic juice and bicarbonate secretion hinders proper chyme buffering in the duodenum. As a result, pH alkalization above 5.5 occurs late in the intestine, significantly reducing the pH-dependent release rate of the active ingredient and thus impeding nutrient absorption by the body[13].

This slow-release renders 70% of current treatments unsatisfactory, resulting in a high daily dose of up to 24 capsules per day for patients due to the low availability of enzymes. In addition to its impact on patient comfort, it is imperative to avoid overdosing as much as possible to reduce the risk of fibrosing colonopathy[12]. This severe complication can lead to intestinal obstructions resistant to medical

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treatment and massive bleeding[14], [15]. Furthermore, the absence of a suitable formulation for children raises other concerns regarding administration, both in terms of quantity and dosage form.

Thus, the polymeric formulation technology appears to be reaching its limits regarding the challenges of rapid release, thereby impacting the efficacy of treating the digestion impairment induced by EPI.

In order to enhance the protection and delivery mechanisms of pancreatic extract sensitive to gastric acidity, lipid-based formulation approaches seem to be an interesting alternative. Over the past decade, oral formulations based on lipids have experienced significant growth[16] (Figure 3).

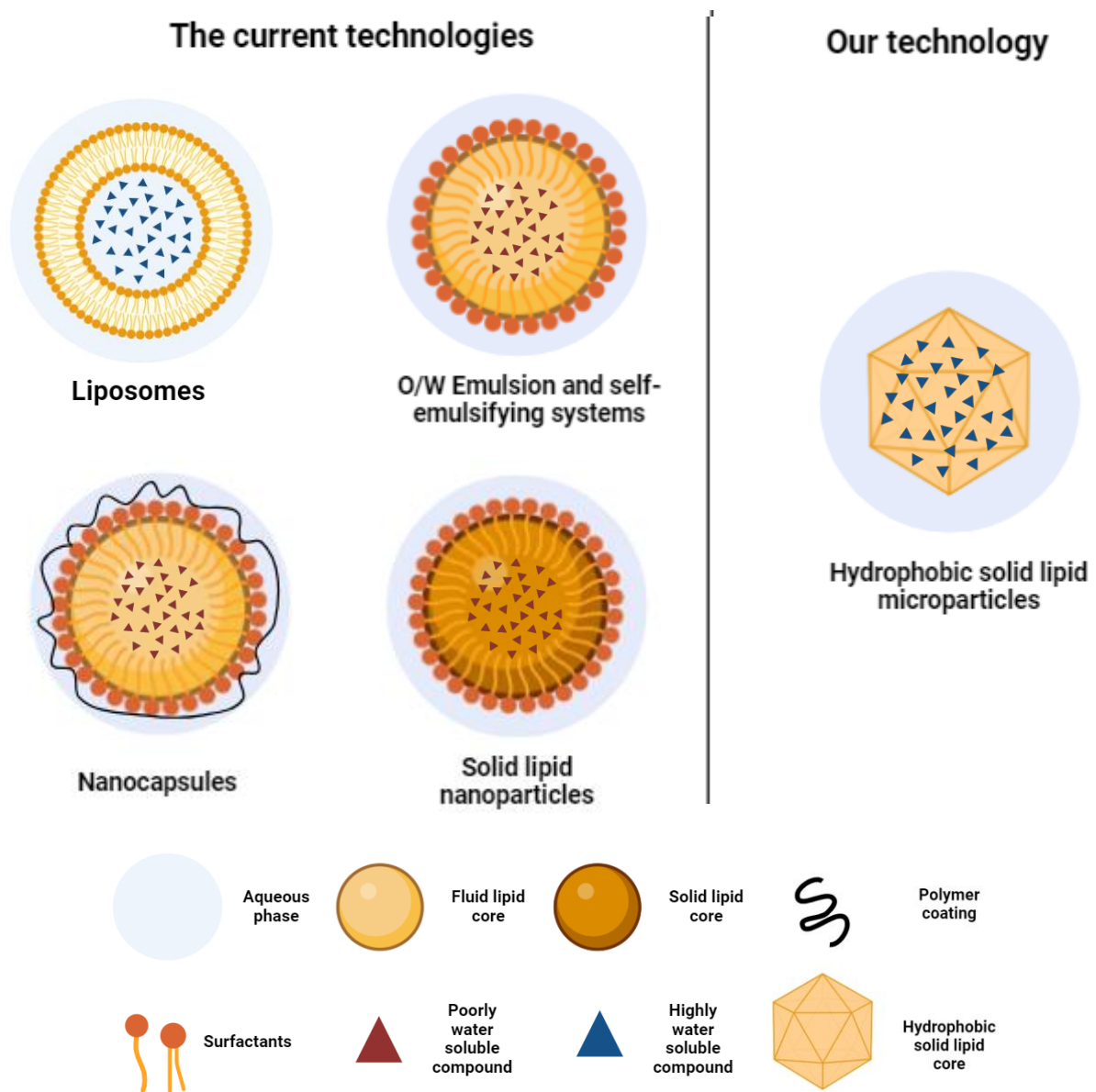


Figure 3: Current lipid-based technologies for transporting hydrophobic or hydrophilic compounds and innovative technology for transporting fragile hydrophilic compounds in oral drug delivery (Inspired by Plaza-Oliver & al, 2021). Powered by BioRender software.

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3 Technologies such as self-microemulsifying drug delivery systems (SMEDDS) notably improve the
4 dispersion and/or absorption of water-insoluble compounds[17]. SMEDDS rely on solubilizing the
5 lipophilic active ingredient in a liquid mixture of triglyceride oil, surfactant, partial glycerides, or co-
6 surfactant within a gelatin capsule[18]. This mixture, upon contact with the gastrointestinal
7 environment, forms a fine emulsion allowing for facilitated absorption of the compound due to a drastic
8 increase in specific surface area. These formulations have particularly demonstrated their strength in
9 enhancing the bioavailability of previously poorly assimilated compounds[19]. A solid alternative to
10 this formulation is known as solid SMEDDS, which has addressed issues such as bitterness, capsule
11 softening, leaks, and precipitation of the active compound induced by the liquid state of SMEDDS[20].
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13 More recently, a solid lipid formulation with sustained release has been described. It enables controlled
14 release of a poorly soluble active ingredient over several hours through the tandem action of lipase
15 inhibitor incorporation and a triglyceride-based matrix[21].
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31 Thus, the latest innovations in lipid drug delivery have significantly advanced the administration of
32 poorly soluble compounds. However, these formulations may not be suitable for incorporating acid-
33 sensitive hydrophilic compounds[22]. The presence of surfactants[23], glyceride derivatives (typically
34 with an HLB greater than 5)[24], or liquid forms[25] would lead to phenomena of wetting and
35 dispersion of the formulation in the stomach. For the challenge of oral administration of highly soluble
36 compounds, liposome technologies are at the forefront. However, the instability of liposomes,
37 combined with poor bioavailability under the extreme conditions of the gastrointestinal tract, poses a
38 significant barrier to their use in oral delivery[26]. Moreover, in the specific case of incorporating
39 pancreatin, a mixture of hydrophilic enzymes, into the aqueous compartment of the liposome, the
40 enzymatic activity of proteases could potentially degrade the structure of the other co-incorporated
41 enzymes, while lipase could disrupt the lipid organization of the liposome. This phenomenon ultimately
42 tends to lead to structural and functional loss of the liposome, as well as instability and poor targeting
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of the hydrophilic active principle. Ultimately, these technologies would not allow for the gastric resistance of the formulation and would ultimately lead to gastric acid degradation of the sensitive hydrophilic compound before reaching the duodenum. To overcome this, the LMP (Lipid MicroParticles) formulation consists of various strictly hydrophobic triglycerides ($HLB \leq 2$) and an acid-sensitive hydrophilic active ingredient uniformly dispersed in the matrix. This strictly hydrophobic configuration prevents access to the active compound sensitive to degradation in the stomach but allows for its immediate release upon contact with endogenous bile salts in the duodenum (Figure 4).

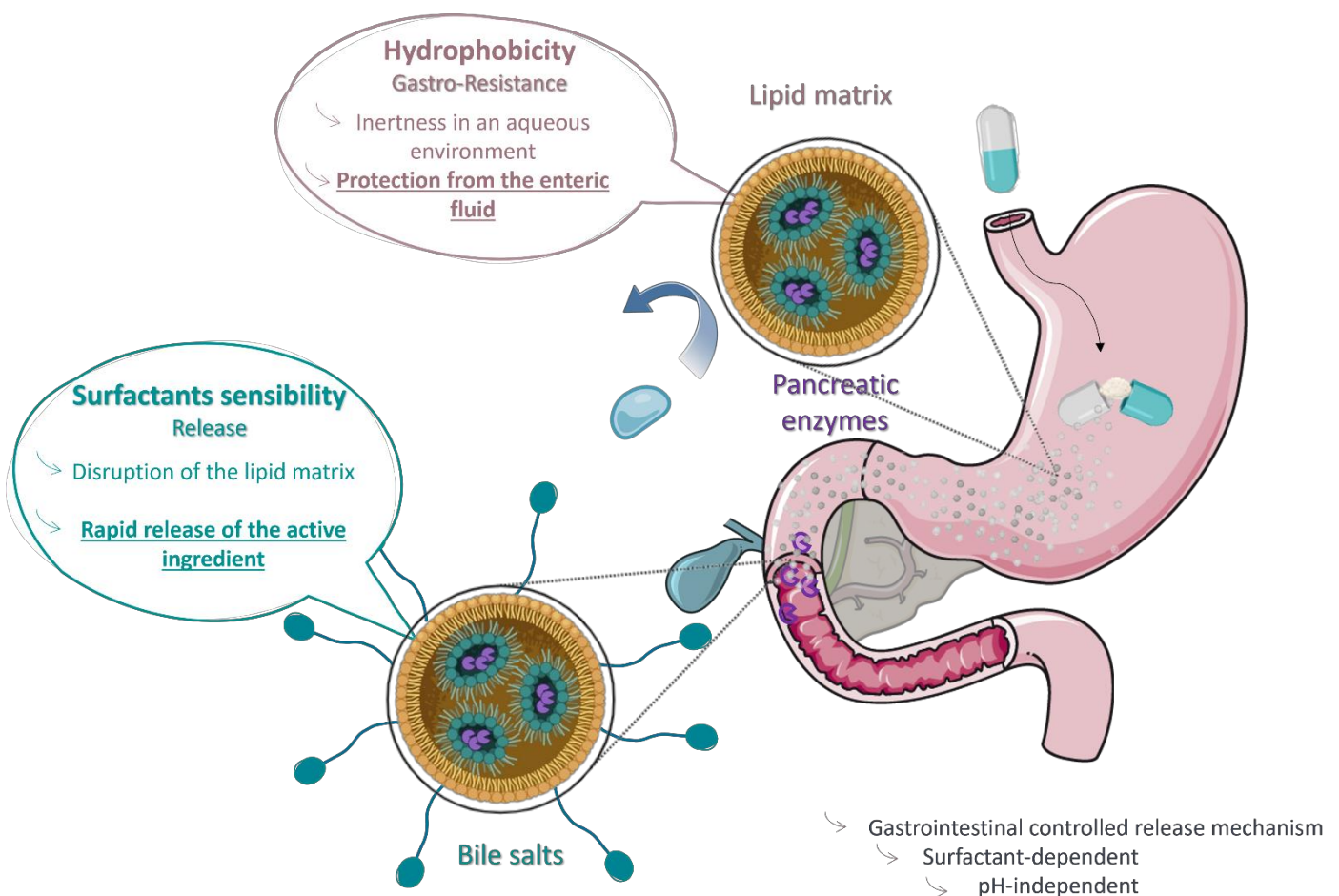


Figure 3: The drug delivery strategy of the Lipid MicroParticles (LMP) formulation technology in a simulated gastrointestinal tract. Created with BioRender software.

The LMP formulation approach would allow for the treatment of exocrine pancreatic insufficiency (EPI), aiming to address the shortcomings of traditional pancreatic enzyme preparations (PEP) that have remained unchanged for three decades. Furthermore, it's entirely composed of naturally digestible products, thereby avoiding issues of irritation and stenosis in the digestive tract associated with

1 polymeric coatings of PEP. The overarching goal of innovative approach is to revolutionize the
2 treatment of EPI by ensuring optimal efficacy while enhancing the quality of life for patients, without
3 causing harmful side effects[27].
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7 **2. Materials and methods**

8 **1. Materials**

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10 The lipid matrix of interest consists of a blend of three distinct hydrophobic excipients, Gelucire™
11 43/01, Gelucire™ 39/01, and Compritol ATO 888™, all characterized by a low HLB ≤ 2 . Gelucire™ 43/01
12 (Gattefosse, Saint Priest, France) is composed of triglycerides of fatty acids (C8 to C18), with a melting
13 point of 43°C and Gelucire™ 39/01 (Gattefosse, Saint Priest, France) is mainly composed of triglycerides
14 of fatty acids (C8 to C18) with a melting point of 39°C. Only the ratio of these different glycerides is
15 altered between the two excipients. Compritol ATO 888™, on the other hand, is composed of mono-,
16 di-, and triesters of behenic acid (C22).
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30 Pancreatin (Merck KGaA, Darmstadt, Germany), composed of a mixture of pancreatic enzymes
31 (amylase, lipase, proteases) of porcine origin, is akin to pancreatic extracts used in the treatment of
32 EPI.
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38 The LMP lipid matrix corresponds to an equivalent ratio of 70% Gelucire™ 43/01, 29.5% Gelucire™
39 39/01, and 0.5% Compritol ATO 888™. The encapsulated active ingredient is dosed at 0.2 g/g of final
40 product, resulting in a loading rate of 20%.
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46 All products and reagents used in this study were supplied by Merck KGaA (Darmstadt, Germany),
47 except for the starch, which was provided by Roquette Frères (Lestrem, France) and all lipid excipients,
48 which were provided by Gattefossé (Saint-Priest, France).
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53 **2. Methods**

54 **2.1. LMP Preparation**

1 The protocol for preparing divided hydrophobic matrices in the form of particles (LMP: Lipid
2 MicroParticles) containing the active ingredient involves the dry incorporation of the hydrophilic active
3 ingredient into the hydrophobic lipid matrix through thermal crystallization and cryo-milling.
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7 For this purpose, the various lipid excipients are melted in a double-jacketed reactor at a temperature
8 of 80°C under stirring at 250 rpm until complete liquefaction phase transition. The addition of the active
9 ingredient follows, with the stirring rate increased to 500 rpm to achieve homogeneous dispersion of
10 the product in the formulation. To maintain homogeneous dispersion of the hydrophobic
11 matrix/hydrophilic active ingredient, agitation is sustained under supercooling until the temperature is
12 lowered to 38.5°C. The pasty formulation is poured by pressure upon valve opening onto a plate
13 previously cooled to 4°C. After complete solidification of the formulation, manual cryo-pre-milling is
14 performed to obtain particle sizes ranging from 1 mm to 1 cm. The obtained pre-milled material is
15 transferred to a cryo-mill sieve (AS22 – 1200µm, Atelier de Lasserre, Cadarcet, France) cooled with
16 liquid nitrogen and milled at 3000 rpm to obtain a particle size ranging from 100 to 1200 µm. Finally,
17 the resultant particles are subsequently stored at 4°C in a dry place away from light.
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34 **2.2. Macromorphology Observation**

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37 Microscopic examinations were carried out using a scanning electron microscope (JSM 6400, Jeol,
38 Akishima, Japan). The specimens were meticulously prepared by fixation, dehydration, and a thin
39 carbon coating to enhance conductivity and mitigate charge-related effects. Prior to examination, the
40 specimens were affixed to SEM-compatible sample holders and subsequently placed inside the
41 electron microscope chamber. Observations were conducted under controlled conditions,
42 encompassing an appropriate acceleration voltage, working distance, and field of view. High-resolution
43 images of the specimens were captured using the SEM.
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54 **2.3. Morphology Distribution**

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57 Morphological distribution analyses were executed using a Mastersizer 3000 morphogranulometer
58 (Malvern Instruments, Malvern, Worcestershire, United Kingdom). The morphology distribution
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1 measurements involved the injection of 20 mg of prepared samples into the Mastersizer 3000 system.
2 A brief pressure pulse (4 bar) was applied to the sample, followed by projection onto a microscopic
3 analysis plate, and observations were performed using x2 and x20 objectives. Particle size distribution
4 data were analyzed utilizing the software provided by the manufacturer (Malvern Mastersizer
5 Software, Malvern, Worcestershire, United Kingdom). The results were presented in the form of
6 particle size distribution curves and particle size parameters, including convexity, circularity, and aspect
7 ratio. CE Diameter is a measure that represents the size of a particle as a circle having the same area
8 as the actual particle. Convexity is the perimeter of the convex hull perimeter of the object divided by
9 its Perimeter **(1)**. The Aspect Ratio is the ratio of the Width to the Length of the particle **(2)**.

$$Convexity = \frac{Perimeter_{A+B}}{Perimeter_A}, \quad (1)$$

$$Aspect\ Ratio = \frac{Width}{Length}, \quad (2)$$

2.4. Release Test

31 Release testing of the formulations was conducted employing a calibrated dissolution apparatus (Smart
32 A7, Sotax, Aesch, Switzerland) equipped with paddles rotating (USP2) at 100 revolutions per minute,
33 while maintaining the bath temperature at 37.0 ± 0.5 °C. The formulations underwent initial exposure
34 to a simulated gastric medium (pH 1.2) for one hour, followed by a subsequent one-hour immersion in
35 a simulated intestinal medium (pH 6.8), following established protocols. In cases of single release
36 measurements, the formulation was exclusively exposed to the intestinal medium (pH 6.8) for one
37 hour. A simulated intestinal fluid (pH 6.8) containing 16 mM of hydrated sodium cholate was used as
38 the release medium, exceeding the critical micellar concentration set at 9-15 mM (20-25°C). The
39 simulated gastric fluid was prepared by dissolving 1 g of sodium chloride (NaCl) in approximately 450
40 mL of deionized water and adjusting the pH to 1.2 ± 0.1 with diluted hydrochloric acid. The preparation
41 of the simulated intestinal fluid followed a similar procedure, involving the dissolution of 3.55 g of
42 monobasic potassium phosphate and 3.44 g of hydrated sodium cholate in 450 mL of deionized water,
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with pH adjustment to 6.8 ± 0.1 using 1 N sodium hydroxide (NaOH). Finally, the volume of each fluid was adjusted to 500 mL with deionized water. Samples were extracted from the dissolution vessel at 1, 2.5, 5, 10, 15, 30, 45, and 60 minutes during dissolution in the simulated intestinal fluid, with an equivalent volume of release medium promptly replaced. Subsequently, the sample solutions were filtered through a 0.45 μm nylon filter (25 mm, Branchia, Labbox France, Rungis, France). The methodology adhered to the United States Pharmacopeia standards.

2.5. Tolerance Test

Tolerance tests were conducted in a simulated gastric medium of 500 mL (pH 1.2) with agitation at 100 rpm, and the bath temperature was maintained at 37°C. Each sample remained in this simulated gastric medium for one hour, after which it was transferred to a thermal extraction medium at 46°C containing 1.775 g of monobasic potassium phosphate and 1.72 g of hydrated sodium cholate, with agitation at 750 rpm for three minutes. In cases of extraction alone, the formulation was exclusively exposed to the extraction medium. A 1 mL sample was ultimately withdrawn using a syringe, then filtered through a 0.45 μm nylon filter (25 mm, Branchia, Labbox France, Rungis, France) into labeled glass tubes.

2.6. Protein Quantification

Internal standards of the protein of interest were prepared by mixing 1 mL of Bradford reagent with a solution of the protein of interest (1 mg/mL) to achieve final concentrations of 0, 4, 8, 12, 16, or 20 $\mu\text{g/mL}$. Deionized water was added as needed to maintain a total sample volume of 2 mL. Initially, 20 μL of the protein of interest solution and 80 μL of deionized water were combined with 1 mL of Bradford reagent. Each sample was briefly agitated for a few seconds immediately after adding the protein. The samples were then incubated at room temperature for 15 minutes, and the absorbance of each sample was measured at 595 nm using a UV/Vis spectrophotometer Jasco 730 (Jasco, Jena, Germany). Release percentages (%) were calculated using the following equation (3):

$$\text{Release (\%)} = 100 \cdot \frac{\text{Release amount}}{\text{Labeled amount}} \quad (3)$$

2.7. Activity quantification

The procedure initiates with the preparation of a 10 g/L solution of soluble starch in deionized water, heated to 95°C for 45 minutes with continuous stirring. For each set of experiments, blank control samples and certified amylase controls are meticulously prepared. In glass bottles, a mixture is created by combining 25 mL of the starch solution, 1 mL of the tested amylase at a concentration of 0.25 mg/mL, and 80 mL of phosphate buffer. This blend is then incubated at 37°C for 10 minutes to facilitate the enzymatic reaction.

To halt the enzymatic reaction, 2 mL of 1M hydrochloric acid is introduced. In the same vials, a simultaneous addition of 10 mL of a potassium iodide solution and 45 mL of sodium hydroxide takes place. The solution is then allowed to incubate in the dark for 15 minutes. Finally, to stop the reaction between potassium iodide and the reducing ends, 4 mL of 4N sulfuric acid is added. Any excess iodine in the vials is subsequently titrated with a 0.1N solution of sodium thiosulfate. The volume of thiosulfate required to reach the point of complete colorimetric extinction in the reaction is recorded.

The determination of the enzymatic activity of amylase (in USP/mg) in accordance with the specifications of the United States Pharmacopeia (USP) is determined by the following definition: one USP unit of amylase activity is contained in the amount of amylase that decomposes starch at an initial rate such that 0.16 μ Eq of glycosidic linkage is hydrolyzed per minute under the conditions of the USP method. This step allows for the quantification of the quantity of reducing ends liberated by the amylase. Amylase activity (USP/mg) is then determined using the formula **(4)**. The variables used correspond to volumes for n (in mL), masses for m (in mg), and amylase activities for A (in USP/mg of enzyme), as follows. n is the volume of thiosulfate added during titration for the test sample, while n_1 corresponds to the same volume for the reference sample. The variables n' and n'_1 refer respectively to the volume of thiosulfate added for determining the blank of the sample (n') and the reference (n'_1). Additionally, m and m_1 are the variables for the mass added to the enzymatic reaction of the sample

(m) and the reference (m_1). Finally, A corresponds to the enzymatic activity of the standardized amylase.

$$\text{activity of amylase} = A \cdot \frac{(n' - n) \cdot m_1}{(n'_1 - n_1) \cdot m} \quad (4)$$

3. Results and discussion

3.1. Granulometric properties of the LMP particle

As there is limited existing information about this formulation, a particle size analysis was conducted to provide a better understanding of its physical properties. In addition to its release technology following a chemical mechanism, the intrinsic physical properties are equally involved in this phenomenon.

During macroscopic observation, the particles appear to have a somewhat irregular parallelepiped shape. This organization is likely induced by the manufacturing process, which involved low-temperature grinding, resulting in distinct structural fractures, as illustrated in Figure 5.

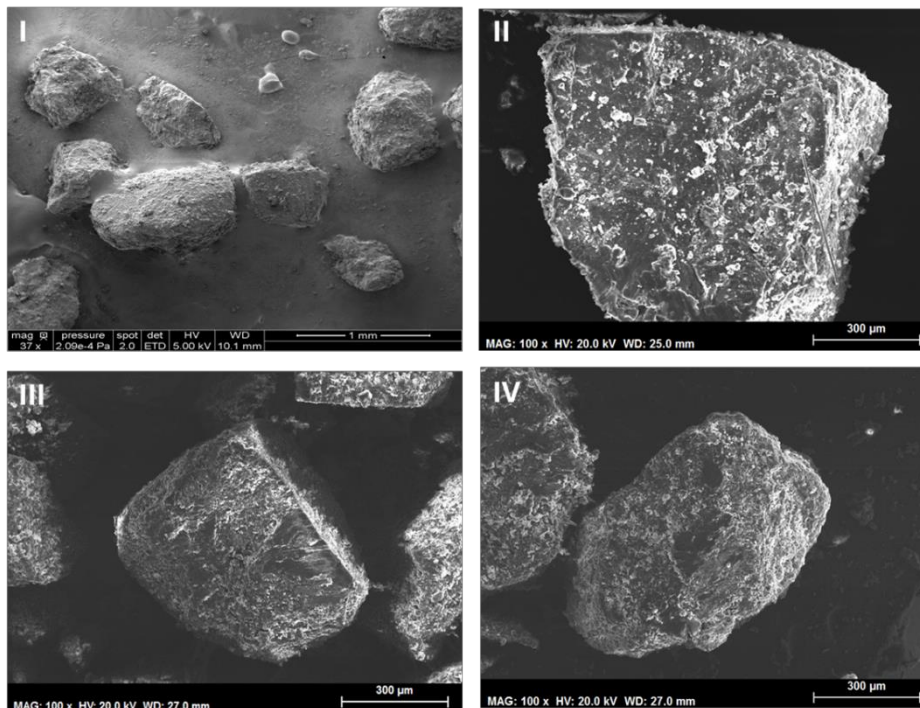


Figure 4: SEM micrographs showing the macrostructure of the LMP formulation and its surface state, performed using a Cryo-SEM (I) or a conventional SEM (II, III and IV).

Parameters such as size, convexity, or aspect ratio have a definite impact on the release kinetics and gastric resistance of the LMP formulation. In fact, these parameters are intimately linked to the specific surface area of a particle, exerting influence on both release and tolerance kinetics.

	Replicate 1	Replicate 2	Replicate 3	Mean	Standard deviation
Particles count	302650	491658	221329		
CE diameter D [4.3] (µm)	660.90	520.10	653.20	611.40	79.16
CE diameter D [v.0.1] (µm)	144.50	104.30	152.60	133.80	25.87
CE diameter D [v.0.5] (µm)	690.90	492.70	691.90	625.17	114.72
CE diameter D [0.9.v] (µm)	1081.00	983.90	1071.00	1045.30	53.41
Aspect Ratio Mean	0.69	0.69	0.69	0.69	0.00
Convexity D Mean	0.982	0.98	0.98	0.98	0.00

Table 1: Morphological distribution (CE diameter, aspect ratio, and convexity) of the LMP formulation.

As seen in Table 1, 80% of the particles have sizes ranging between [133.8 < n < 1045.3] µm with an average value of 611.4 µm. Moreover, we observe a standard deviation ranging between 5% and 20% for all size classes, indicating a good uniformity in the size distribution of this powder. Furthermore, the aspect ratio of the formulation, which indicates the width-to-length ratio, reveals that the particles have a predominantly elongated shape. With an average value of 0.69, the particles are 31% longer than they are wide. Finally, convexity, as an indicator of overall shape regularity, exhibits a value close to 1, indicating a macroscopically regular character devoid of indentations, despite a visually rough surface texture.

3.2. Properties safeguarding the primary structure of proteins and controlled release

The objective of this study is to determine whether the LMP formulation, as such, would protect a protein of interest, serving as a model for a generic hydrophilic active principle, from gastric degradation while maintaining rapid release in an intestinal environment.

To achieve this, assays for total cumulative protein quantification and total cumulative enzymatic activity were conducted relative to the theoretical total incorporated reference value for gastric degradation tests and active ingredient encapsulation (All the figures labeled "A" in the manuscript), which amounted to 200 mg of total proteins, equivalent to 0.4 g/L, and 105.78 USP/mg of enzyme

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corresponding to the total amylase activity. Meanwhile, formulation release tests were compared to the measured total quantity/activity of encapsulated protein/enzyme. (All the figures labeled "B" in the manuscript).

In this case, bovine serum albumin (BSA) is the best candidate for this study as it is considered a well-known reference hydrophilic protein for this purpose[28]. However, BSA is only minimally sensitive to acidic conditions[29]. Therefore, a simulated gastric fluid (SGF) medium with gastric enzymes, pepsin (3.2g/L), had to be used[30]. It has been demonstrated that the residence of BSA alone in an SGF gastric medium (with enzymes) significantly degraded its protein primary structure by more than 95%. Furthermore, the BSA formulation process had no significant impact on this same structure. Finally, the gastric protection of the formulation is significantly more effective at over 84% compared to gastric residence alone (Figure 6.A).

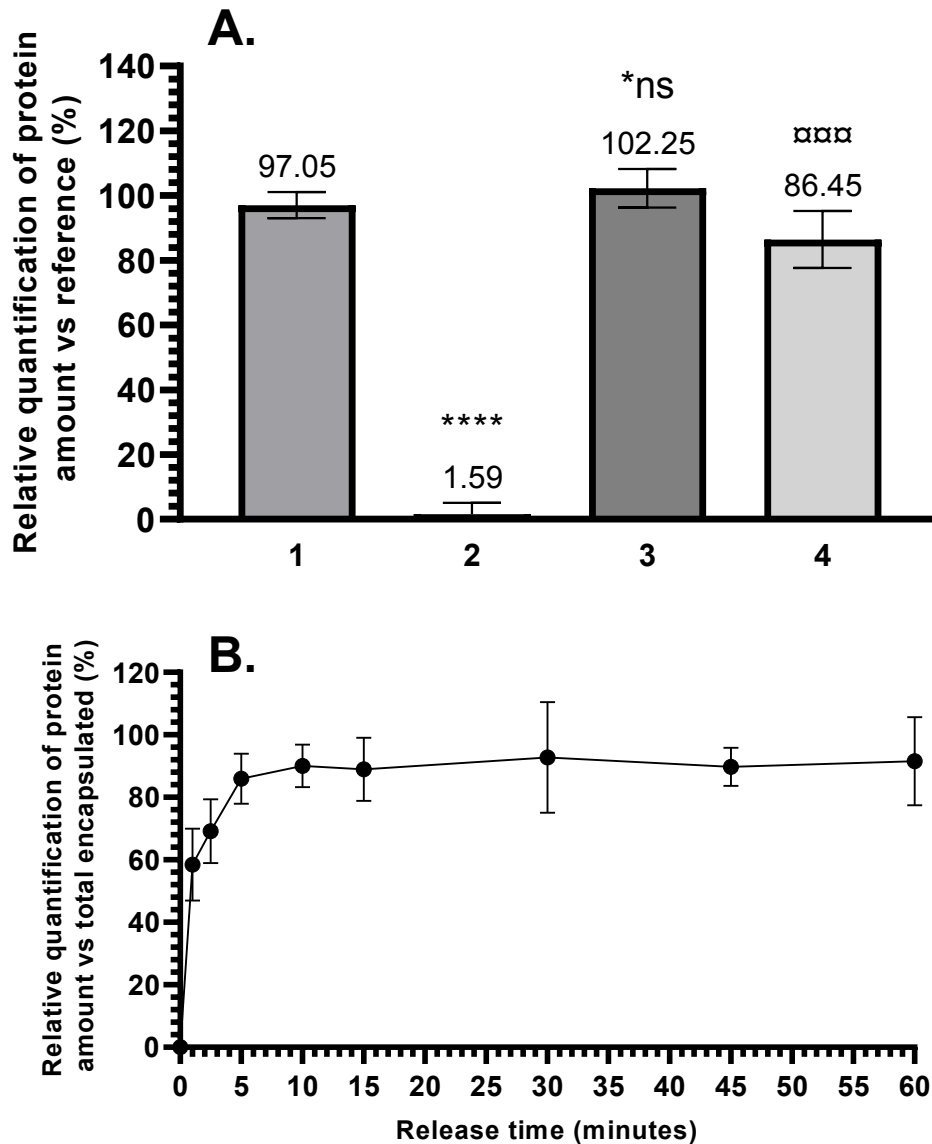


Figure 5: The impact of formulation on post-production protein primary structure and on the enteric resistance of a model protein, BSA (6.A), as well as its release rate in a simulated intestinal fluid (SIF) medium (6.B). The reference value corresponds to the theoretical total amount of incorporated proteins (200 mg/g of formulation), while the total value of encapsulated proteins corresponds to the measured post-production protein quantification. The caption for figure 6.A is described as follows: 1. Raw BSA; 2. Raw BSA in SGF; 3. Formulated BSA; 4. Formulated BSA in SGF. *ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs Raw BSA; xxx $p < 0.001$ vs Raw BSA in SGF. The described p -values obtained using t-student test.

This result supports the fact that the manufacturing process of the formulated particle does not degrade the primary structure of a model protein while significantly improving its resistance to a gastric environment.

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In terms of the release of formulated BSA in an *in vitro* intestinal environment, an equivalent amount of protein was found to be 58.41% (\pm 11.5) at 1 minute, 85.9% (\pm 8.1) at 5 minutes, with a maximum value of 92.7% (\pm 17.7) at 30 minutes. This notably demonstrates a very rapid release kinetics during the first five minutes (Figure 6.B).

3.3. Properties safeguarding functional protein activity and controlled release.

With the established protection of a model protein, it remains that, in the context of a hydrophilic active ingredient, its functionality is often the central element of therapeutic technology. Thus, preserving this function is of paramount importance to ensure the efficiency of enzymatic treatment. Therefore, the choice was made for a well-described intestinal enzyme in the literature[31], relevant to the targeted hydrophilic active ingredient, pancreatic α -amylase.

For this study, quantification of α -amylase protein was coupled with enzymatic activity measurement. The determination of the protein quantity after simulated gastric medium exhibited a significant decrease of over 28% compared to the reference value. Furthermore, no significant difference was detected either between the quantity of the formulated protein and the reference, or between the quantification of the formulated enzyme and the enzyme alone, following the simulated gastric medium (Figure 7.A).

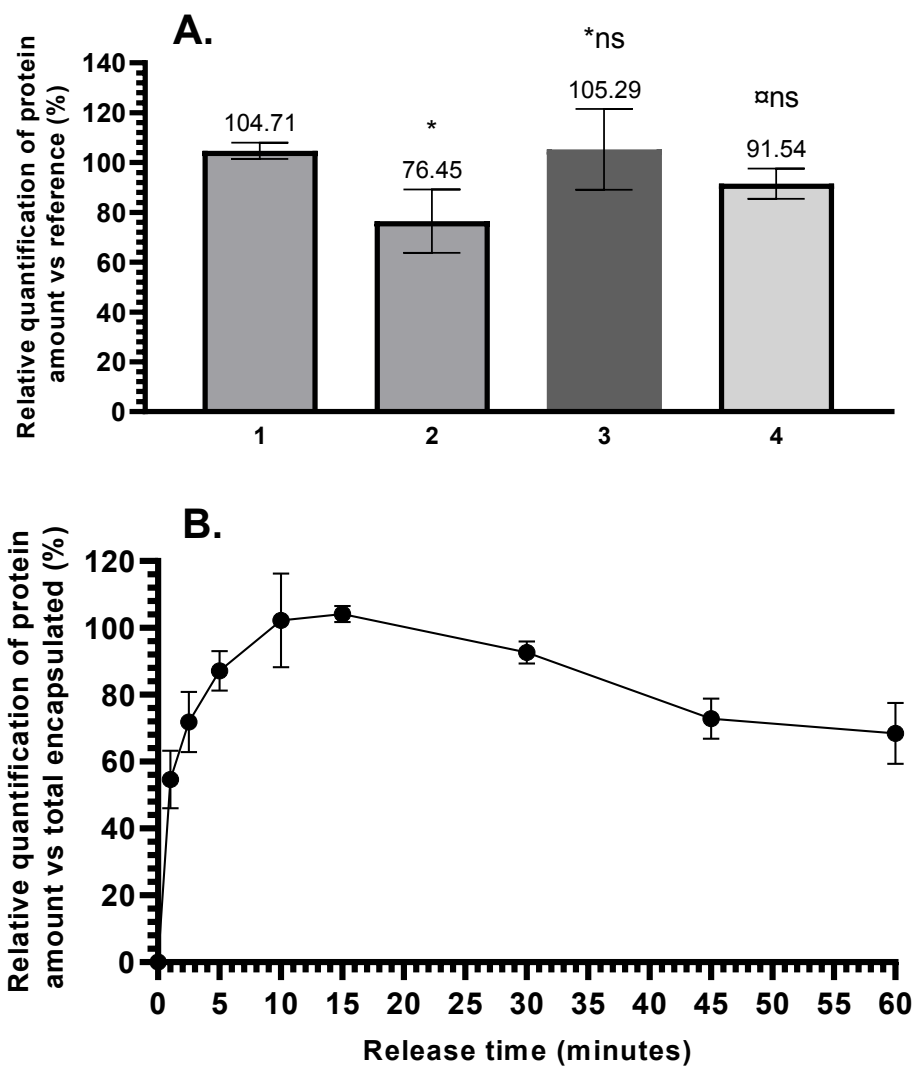
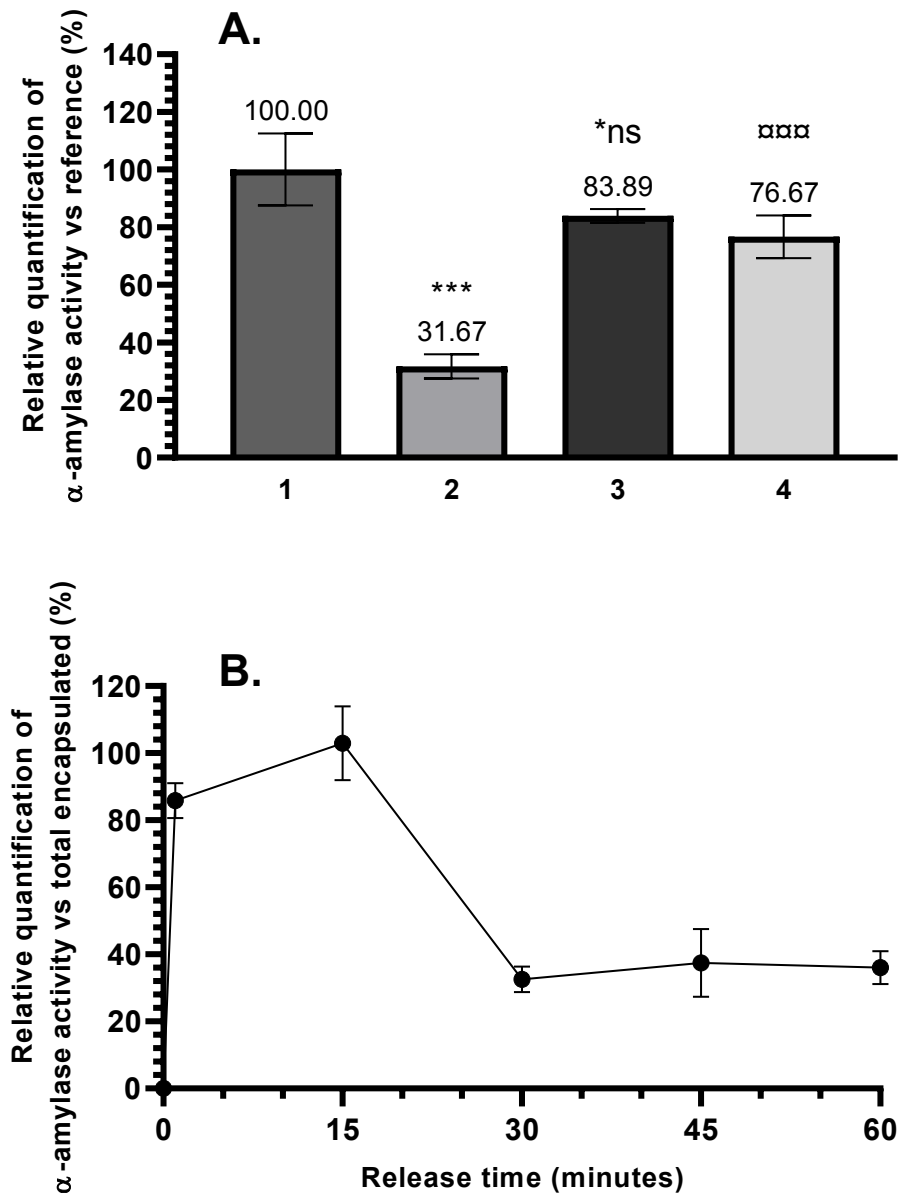


Figure 6: The impact of formulation on post-production protein primary structure and on the enteric resistance of a model enzyme, α -amylase (7.A), as well as its release rate in a simulated intestinal fluid (SIF) medium (7.B). The reference value corresponds to the theoretical total amount of incorporated alpha-amylase (200 mg/g of formulation), while the total value of encapsulated alpha-amylase corresponds to the measured post-production protein quantification. The caption for figure 7.A is described as follows: 1. Raw α -amylase; 2. Raw α -amylase in SGF; 3. Formulated α -amylase; 4. Formulated α -amylase in SGF. *ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs Raw α -amylase; ns $p < 0.001$ vs Raw α -amylase in SGF. The described p -values obtained using t-student test.

Regarding the release of the formulated α -amylase in an *in vitro* intestinal setting, the protein content was measured at 54.6% (± 8.6) at 1 minute, 87.2% (± 5.9) at 5 minutes, reaching a peak value of 104.1% (± 2.5) at the 15-minute mark. This notably illustrates a swift release trend within the initial five minutes (Figure 7.B).

Thus, the primary structure of pancreatic α -amylase is minimally affected by the acidity of the gastric environment simulated by SGF medium.

1 But the challenge in studying pancreatic α -amylase (α -amylase 2) as a model enzyme is its function
 2 thermolability[32]. However, it was the only pancreatic enzymatic candidate, as lipase and proteases
 3 introduced too much autocatalytic bias and/or variability in the assays. Nevertheless, the loss of
 4 function due to thermal denaturation of its tertiary structure can be delayed by the presence of Ca^{2+}
 5 cations as well as proteins derived from SA (serum albumin)[33].
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53 **Figure 7:** The impact of the formulation on the post-production and post-enteric enzymatic
 54 functional activity of a model enzyme, α -amylase (8.A), as well as on its rate of release of
 55 enzymatic activity in a simulated intestinal fluid (SIF) medium (8.B). The reference value
 56 corresponds to the theoretical total amylase activity of the incorporated alpha-amylase (200 mg/g
 57 of formulation), while the total value of encapsulated alpha-amylase corresponds to the measured
 58 post-production amylase activity quantification. The caption for figure 8.A is described as follows:
 59 1. Raw α -amylase; 2. Raw α -amylase in SGF; 3. Formulated α -amylase; 4. Formulated α -amylase
 60 in SGF. *ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs Raw α -amylase; **** $p < 0.001$
 61 vs Raw α -amylase in SGF. The described p -values obtained using t -student test.
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In the case of pancreatic α -amylase activity, a significant decrease of over 68% is observed when the enzyme has a one-hour gastric residence time prior to enzymatic measurement. Furthermore, amylase is not significantly denatured during the formulation process, although a declining trend is evident, likely due to its thermolability. Finally, the activity of the formulated α -amylase is significantly better preserved, with an increase of over 44%, during an **gastric** passage compared to its isolated activity. Despite the presence of a loss in enzymatic activity during the formulation, the protection of this function by the formulation during an **gastric** stay is far superior to the enzyme alone (Figure 8.A).

As for the release kinetics of the functional formulated pancreatic α -amylase, characterized by its enzymatic activity, it reaches a value of 85.8% (± 5.2) in the first minute and 103% (± 11.1) in the fifteenth minute. However, starting from the thirtieth minute, enzymatic activity experiences a sharp decline down to 32.6% (± 3.8) of the reference activity, which is indicative of the thermolability of α -amylase even in a 37°C environment. Nevertheless, this experiment effectively demonstrates the rapid release of the enzyme of interest as well as the preservation of its enzymatic activity in a simulated intestinal fluid (Figure 8.B).

3.4. Properties preserving the bioactivity of therapeutic molecules.

Having demonstrated the effectiveness of the lipodic microparticle (LMP) formulation in **preserving protein structure** and maintaining its function in an unfavorable gastrointestinal environment, as well as its rapid release at the intended target, it was then possible to move to a more advanced stage targeting a complex hydrophilic gastro-sensitive therapeutic molecule. Therefore, pancreatin, a biological mixture containing porcine pancreatic enzymes, was an ideal candidate to test the limits of this formulation method. Indeed, current formulations are suboptimal in delivering this active substance rapidly to the targeted site. The aim of this study was to determine whether the LMP formulation could be a serious contender to these existing formulations. However, incorporating an enzyme such as lipase poses a real challenge for a formulation composed mainly of lipids since this

1 matrix constitutes a substrate hydrolysable by this enzyme. The advantage of this hydrophobic solid
2 reformulation method is to significantly limit the incorporation of water, preventing the hydrolytic
3 mechanisms of lipases. Thus, the following experiments will demonstrate whether this reformulation
4 would be a viable strategy for the controlled release of pancreatin during gastrointestinal passage.
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9 Firstly, it was essential to ensure the reproducibility of the result concerning this therapeutic target of
10 interest. Thus, a significant decrease of over 44 % in the quantity of proteins derived from the biological
11 mixture was observed after a one-hour gastric residence time. Furthermore, no significant difference
12 was noted regarding the effect of the formulation process on protein degradation. However, a
13 significant increase of more than 33% was found in the total protein quantity of the formulated active
14 ingredient compared to the active ingredient alone, after an acid treatment. This result confirms the
15 ability of this formulation in gastro-protective mechanisms for the raw protein structure without loss
16 during manufacturing, even in the case of potential therapeutic biomolecules (Figure 9.A).
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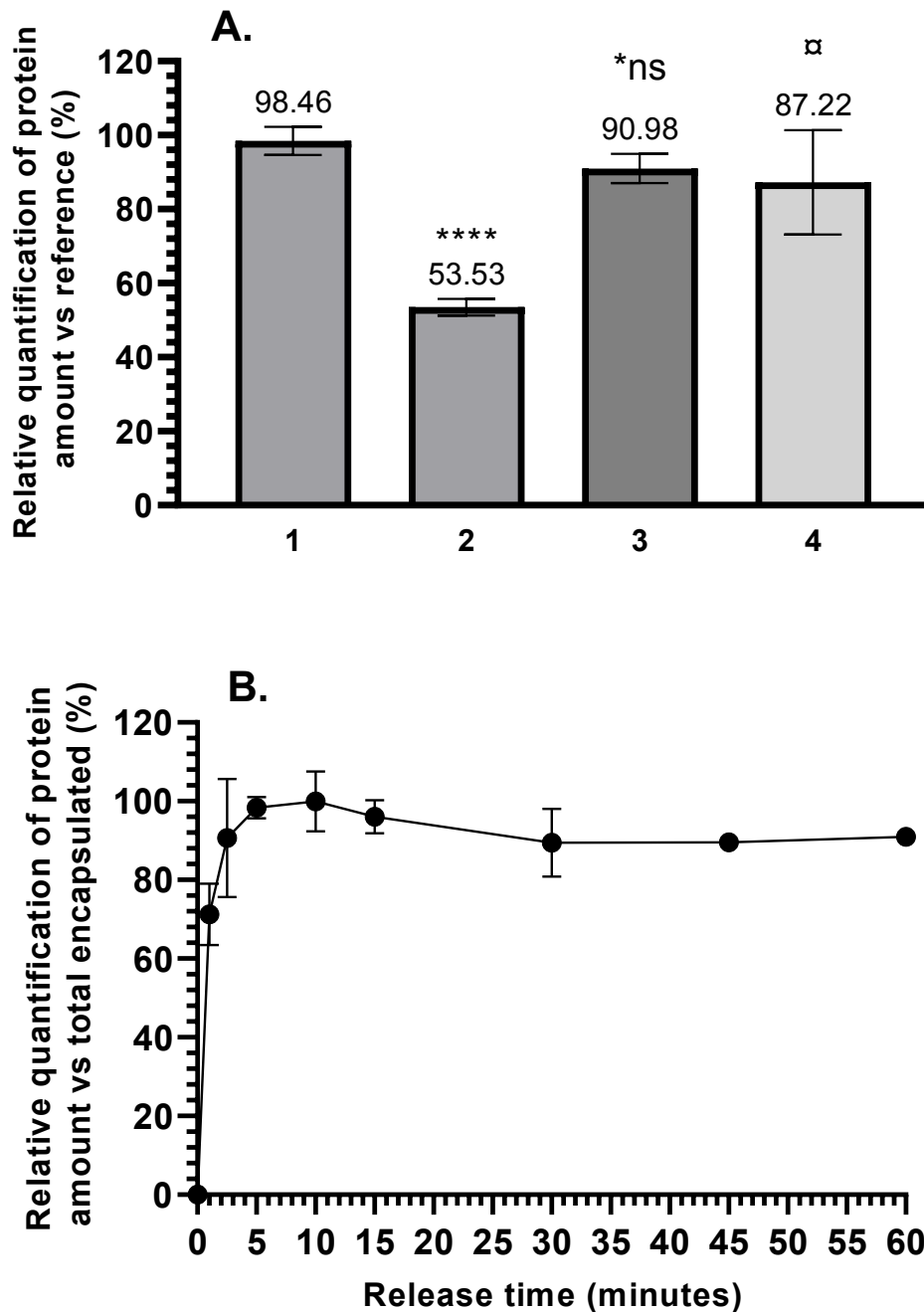


Figure 8: The impact of formulation on post-production protein primary structure and on the enteric resistance of a model active biomolecule, pancreatin (9.A), as well as its release rate in a simulated intestinal fluid (SIF) medium (9.B). The reference value corresponds to the theoretical total amount of incorporated pancreatic enzyme preparations (200 mg/g of formulation), while the total value of encapsulated pancreatic enzyme preparations corresponds to the measured post-production protein quantification. The caption for figure 9.A is described as follows: 1. Raw pancreatin; 2. Raw pancreatin in SGF; 3. Formulated pancreatin; 4. Formulated pancreatin in SGF. *ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs Raw pancreatin; α $p < 0.001$ vs Raw pancreatin in SGF. The described p-values obtained using t-student test.

In the study of the release kinetics of this active compound over time, a total release of 71.2% (± 7.8) was observed at the end of the first minute, reaching a maximum value after ten minutes of 99.7% (\pm

8.1). The result from this figure once again suggests a rapid release of our hydrophilic active principle of interest in a simulated intestinal environment (Figure 9.B).

Having established the protection of the primary structure of pancreatin through gastric resistance and rapid intestinal release, the critical determinant for ensuring the potential therapeutic efficacy of this hydrophilic active biomolecule is the assessment of its enzymatic function.

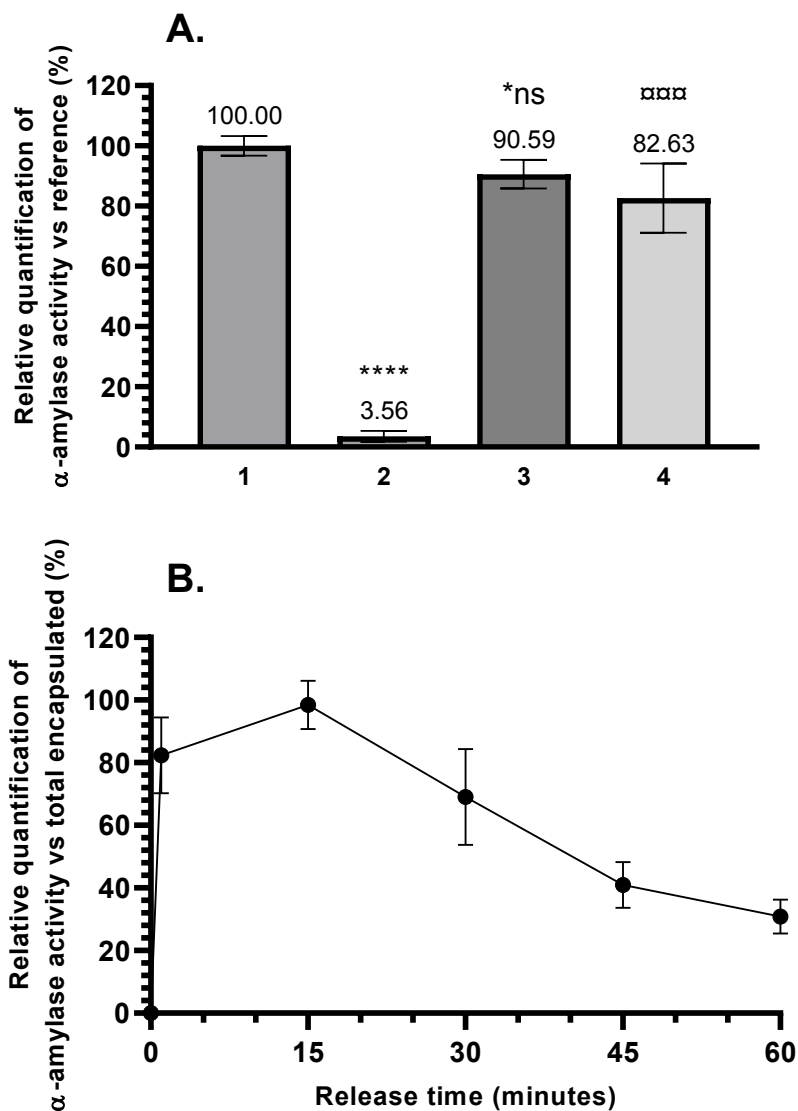


Figure 9: The impact of the formulation on the post-production and post-enteric enzymatic functional activity of a model active biomolecule, pancreatin (10.A), as well as on its rate of release of enzymatic activity in a simulated intestinal fluid (SIF) medium (10.B). The reference value corresponds to the theoretical total amylase activity of incorporated pancreatic enzyme preparations (200 mg/g of formulation), while the total value of encapsulated pancreatic enzyme preparations corresponds to the measured post-production amylase activity quantification. The caption for figure 10.A is described as follows: 1. Raw pancreatin; 2. Raw pancreatin in SGF; 3. Formulated pancreatin; 4. Formulated pancreatin in SGF. *ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs Raw pancreatin ; *** $p < 0.001$ vs Raw pancreatin in SGF. The described p -values obtained using t-student test.

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3 In this case, amylase activity remains the best indicator of the bioactivity of our extract.
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5 Based on this premise, the study of amylase activity demonstrated a significantly weak gastric
6 resistance of over 96% compared to the reference alone. Once again, no significant difference in
7 enzymatic activity quantification was observed between pancreatin alone and formulated. Lastly, a
8 significant increase of over 79% in the hydrolytic activity of the formulated enzyme compared to the
9 enzyme alone was noted after the gastric residence (Figure 10.A).
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15 The quantification of the release of the hydrophilic active ingredient while maintaining its function was
16 subsequently examined. The results revealed a release of 85.2% (± 5.2) within the first minute and an
17 optimal release of 103% (± 11.1) at the fifteenth minute, indicating an effective release of the full
18 enzymatic function (Figure 10.B). These results suggest the protection of hydrophilic sensitive
19 compounds such as pancreatin from gastrointestinal environmental stresses while facilitating its
20 release kinetics under more favorable intestinal conditions. In addition to the preservation of the
21 primary structure, the formulation enables the protection of enzymatic function without significant
22 loss.
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34 35 **3.5. Simulated *in vivo* conditions** 36

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38 In order to closely replicate *in vivo* conditions in humans, a combination of a one-hour residence step
39 in a simulated gastric fluid (SGF) followed directly by a residence step in a simulated intestinal fluid (SIF)
40 was implemented. This study model has the advantage of mimicking the inherent parameters of the
41 gastrointestinal tract during a standard cycle.
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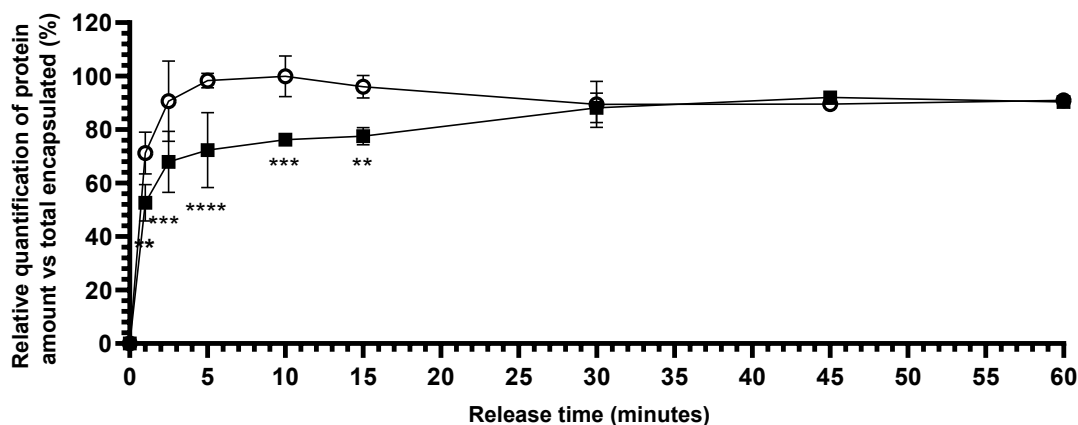


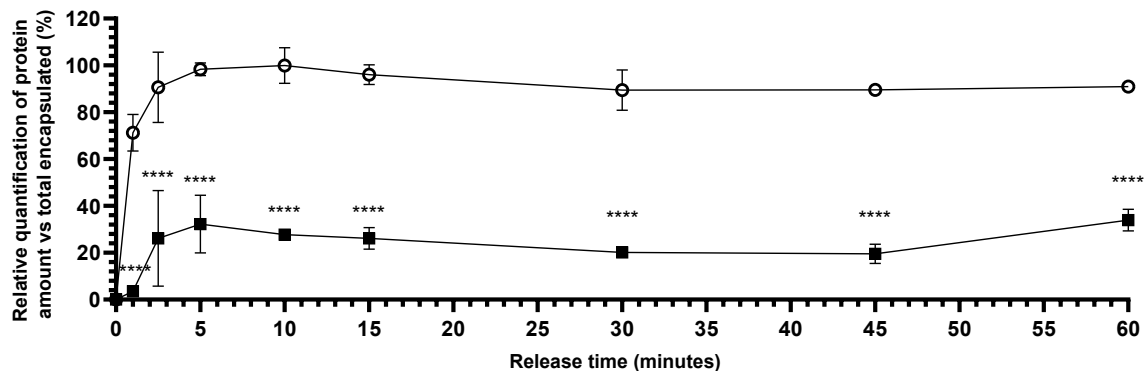
Figure 10: The impact on the quantity of proteins from residence in a simulated gastric fluid (SGF) followed by dissolution in a simulated intestinal fluid (SIF) of the formulated pancreatin. The total value of encapsulated pancreatic enzyme preparations corresponds to the measured post-production protein quantification. The caption for this figure is described as follows: ○: Dissolution kinetics ; ■: Gastro-resistance followed by dissolution kinetics. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs dissolution kinetics. The described p -values obtained using t -student test.

The gastro-dissolution shows that the formulated pancreatin exits at 52.6% (± 6.9) at 1 minute, which remains significantly lower than the dissolution output alone. At 5 minutes, the reduction remains significant for the gastro-dissolution with an average at 72.3% (± 14). However, at 30 minutes, no significant difference stands out between gastro-resistance followed by dissolution and dissolution alone, with a maximum value at 45 minutes reaching 92% (± 2) (Figure 11). This result suggests a loss of approximately 8% due to residence in a simulated gastric environment with significant release kinetics in the first 30 minutes. However, this release delay could hypothetically be coupled with re-aggregation phenomena due to the hydrophobic nature of the formulation immersed in an aqueous environment. Indeed, the hypothesis of a decrease in specific surface area could lead to this release delay.

Despite this phenomenon, the formulation fulfills its role in protecting the hydrophilic biomolecule sensitive to acidic environment while releasing it relatively quickly during the intestinal passage.

3.6. Proof of concept of the controlled release technological mechanism

1 This controlled release technology is not directly dependent on conditions such as pH, as in current
2 commercial formulations, but rather on the presence of naturally occurring surfactant compounds in
3 the body. Indeed, the lipid composition of the reformulation grants it rapid disintegration properties
4 upon contact, particularly with cholate and its derivatives, excreted in the proximal segment of the
5 duodenum.
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Figure 11: The impact of the presence of a surfactant on the release rate of the formulated active ingredient in a simulated intestinal fluid (SIF). The total value of encapsulated pancreatic enzyme preparations corresponds to the measured post-production protein quantification. The caption for this figure is described as follows: ○: Dissolution kinetics ; ■: Dissolution kinetics without surfactant. **** $p < 0.0001$ vs dissolution kinetics. The described p -values obtained using t -student test.

31 In the absence of a surfactant, the release kinetics are significantly reduced across all time points, with
32 an output of 32.3% (± 12.3) at 5 minutes and a maximum value at 60 minutes of 34% (± 4.6). Therefore,
33 the presence of a surfactant is essential for a rapid and complete release of the active ingredient. This
34 release mechanism takes advantage of the bile vesicle and its place of excretion to induce the
35 disintegration of the formulation "at the right place, at the right time".
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43 4. Conclusion

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46 This research marks a pioneering stride toward the formulation of a process aimed at delivering fragile
47 hydrophilic active biomolecules. This approach effectively mitigates challenges associated with gastric
48 degradation and delayed release, showcasing promising potential for advancements in oral drug
49 delivery. This formulation presented in this paper enables the protection of a fragile hydrophilic active
50 principle, preventing the degradation of its primary structure and neutralizing potential functional
51 denaturation effects induced by the environment. Hence, its matrix organization, composed of
52 triglycerides, confers insensitivity to the gastric tract due to its strictly hydrophobic nature.
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It has been shown that the manufacturing process developed has no impact on the active principle, either **in terms of structure or functional folding**. The lipid matrix liberates rapidly in an intestinal environment since its composition allows facilitated disintegration in the presence of a surfactant. This mechanism enables complete release within a few minutes, facilitating the onset of enzymatic activity in the intestine. This phenomenon is crucial as the absorption of various nutrients from the diet is inversely proportional to the progress of chyme.

5. Perspectives

In a simulated *in vivo* context, the formulation allows for passage through the **gastric** barrier for hydrophilic active biomolecules such as pancreatin, providing rapid release in the proximal duodenum to initiate its function at the earliest. This drug delivery mechanism paves the way for the oral administration of hydrophilic active principles sensitive to the gastric environment, currently administered via more invasive routes such as intravenous or intramuscular injections and nasogastric methods.

However, the screening of this new formulation technology is still partial. First, it would be necessary to deepen the study of protein structure under these various conditions, particularly through circular dichroism analysis, in order to confirm any potential phenomena of degradation, denaturation, and protection of proteins inherent to the gastrointestinal environment and the formulation of interest.

Furthermore, it is undeniable that surfactant activity is not the only physical parameter at play. Other parameters such as temperature, re-aggregation phenomena, buoyancy, or even self-hydrolysis due to the incorporation of lipolytic enzymes in the formulation are equally involved in the protective and release mechanisms of this drug delivery technology. **Thus, the study of these parameters to better understand the mechanisms inherent to the protection and release of the biomolecule of interest, coupled with the determination of the formulation's stability profile under controlled storage conditions, constitutes the final steps prior to preclinical efficacy and non-inferiority trials in animals.**

Finally, the ultimate goal would be to bring this formulation to clinical trials in humans, with the main

1
2 focus on inducing increased treatment efficacy without side effects, thereby improving patient comfort
3 and compliance.
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5 **6. Acknowledgments**

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11
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