Hollow Microparticles as a Superior Delivery System over Solid Microparticles for the Encapsulation of Peptides

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ABBREVIATIONS

GLP-1	Glucagon Like Peptide-1
h-MPs	Hollow microparticles
s-MPs	Solid microparticles
PLGA	Poly(D, L-lactide-co-glycolide)
HTRF	Homogeneous time resolved fluorescence
HPLC	High performance liquid chromatography
pI	Isoelectric point

ABSTRACT:

Purpose: Peptides are gaining significant interests as therapeutic agents due to their high targeting specificity and potency. However, their low bioavailability and short half-lives limit their massive potential as therapeutics. The use of dense, solid particles of biodegradable polymer as a universal carrier for peptides also has its challenges, such as inefficient peptide release and low bioactivity. In this paper, it was established that h-MPs instead of s-MPs, as peptide carriers, could improve the release efficiency, while better preserving their bioactivity.

Methods: Glucagon like Peptide-1 (GLP-1) was encapsulated as a model peptide. Mass loss, average molecular weight changes, intraparticle pH, polymer-peptide interaction and release studies, together with bioactivity assessment of the peptides for solid (s-MPs) and hollow (h-MPs) were systematically analyzed and evaluated for efficacy.

Results: The intraparticle pH of s-MPs was as low as 2.64 whereas the pH of h-MPs was 4.99 by day 7. Consequently, 93% of the peptide extracted from h-MPs was still bioactive while only 58% of the peptide extracted from s-MPs was bioactive. Likewise, the cumulative release of GLP-1 by day 14 from h-MPs showed a cumulative amount of 88 ± 8% as compared to 33 ± 6% for s-MPs.

Conclusions: The cumulative release of peptide can be significantly improved, and the bioactivity can be better preserved by simply using h-MPs instead of s-MPs as carriers.

INTRODUCTION

Peptides are extremely popular as therapeutics, because of their exquisite specificity for molecular targets that make them extremely potent.(1, 2) Unlike small molecular drugs, peptides, because of their high selectivity, do not accumulate in specific organs such as the kidneys or liver, and any toxic side effects are thus minimized.(3) Over the decades, several therapeutic peptide-based drugs such as Lupron, Zoladex, Copaxone, Sandostatin have made their way into the commercial market, each generating more than $1 billion in annual sales.(3) There are currently over 500 peptide drugs in pre-clinical trials, 140 in clinical trials and 60 Food and Drug Administration (FDA)-approved peptide drugs in the market.(4) With the approval rate of peptide being twice as high as small molecules, the peptide drug market is therefore growing twice as fast.(5) In 2015, the global peptide therapeutics market was valued at $21.3 billion and is estimated to reach $46.6 billion in 2024.(6)

Still, the commercial potential of peptides as therapeutics is significantly hampered from two major drawbacks: 1. low bioavailability and 2. short half-lives, due to their rapid degradation by proteolytic enzymes in the blood plasma and digestive system(7, 8). Currently, marketed peptide and protein drugs are mostly administered by parenteral routes (subcutaneous, intravenous, intramuscular injection) as this can avoid biological barriers, in contrast to pulmonary or oral delivery.(9) Nonetheless, their therapeutic potential is significantly hampered by their rapid elimination from the circulation through renal filtration and enzymatic degradation.(10) To overcome this, peptides are therefore encapsulated into particles composed of biodegradable polymers (e.g. polyesters, polyanhydrides, etc.). At the same time, encapsulation also provides the means to control their release.(11, 12) Poly(D, L-lactide-co-glycolide) (PLGA), in particular, has been a popular encapsulation choice of material owing to its excellent biocompatibility, tunable

degradation rate and the relative ease in designing them for controlled and sustained delivery.(13, 14) In addition, it is approved by the FDA for parenteral human use. (14, 15) Upon administration, these polymer particles become hydrated and undergo mainly bulk degradation,(14) through the hydrolysis of their ester bonds. Subsequently, the polymer matrix becomes more porous, aiding in a faster rate of diffusion and release of the encapsulated peptide.(16)

However, it is worth noting that the release of peptides from PLGA particles is reported to be rather inefficient.(17, 18) Peptide release is often characterized by an initial burst release, followed by a sluggish and often incomplete release.(18, 19) This undesirable, incomplete peptide release, as reported in the literature,(20, 21) can be attributed to the interactions between the peptide and the encapsulating polymer. When the peptide-loaded particle is delivered to a physiological environment, hydrolysis of the polymer occurs and shorter polymer chains or oligomers, with carboxylic acid-terminated end-groups, are generated. The microenvironment within the particles therefore becomes acidic over time that results in an auto-catalytic degradation effect.(22, 23) Auto-catalytic degradation occurs when the acidic degradation products themselves act as a catalyst for the same degradation reaction.(24) In addition, as many peptides contain positive charges, or transition from negative to positive charges at low pH(25) (shown in **Supplementary Information**), electrostatic interaction between a highly acidified (i.e. negatively-charged) polymer matrix and the encapsulated peptides(26, 27) will ensue. Also, under a low pH environment, acylation of the peptides with lactic and glycolic acids and their oligomers is catalyzed and polymer-peptide adducts are formed.(28, 29) All these may influence release kinetics and possibly compromise the bioactivity of the peptide. Understanding such a phenomenon during release and developing a delivery system that circumvents these issues is therefore paramount and the key aim of this paper.

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Here, we hypothesized that encapsulating peptides into hollow microparticles instead of solid ones could help in improving the release efficiency of the encapsulated peptides and better preserve their bioactivity upon release. We speculate that the accumulation of acidic degradation products can be significantly reduced through the use of hollow microparticles so as to minimize polymer-peptide interactions. In this study, we have encapsulated Glucagon like Peptide-1(GLP-1), a 30-amino acid peptide that stimulates post-prandial insulin secretion, into hollow PLGA microparticles fabricated through a unique one-step, osmogen-mediated technique.(30) Comparisons were made against GLP-1 loaded solid microparticles, as control.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-co-glycolide) polymer (Purasorb PDLG 5002A; intrinsic viscosity (IV): 0.2 dl/g) was purchased from Purac Biochem (Netherlands). Poly(vinyl alcohol) (PVA) (MW: 30–70 kDa), trifluoroacetic acid (TFA) and sodium chloride (NaCl) were purchased from Sigma Aldrich. Human GLP-1, was purchased from Prospec, and used without any further purification. HTRF active GLP-1 assay kit was obtained from Cisbio Bioassays. High performance liquid chromatography (HPLC)-grade deionized water (Millipore, 0.22 μm), dichloromethane (DCM) and acetonitrile (Tedia Co., Inc.) were used as solvents.

Methods

GLP-1 loaded microparticle (MP) synthesis

GLP-1 loaded hollow microparticles (h-MPs) were fabricated using a modified (oil/water) emulsion solvent evaporation technique. Briefly, 0.2 g of PLGA, 2 mg GLP-1 and 5mg of NaCl

(2.5% (w/w)) were added to 3 ml of dichloromethane (DCM). The content was put under magnetic stirring for 3.5 hours and vortexed for 20 minutes to ensure that the polymer was completely dissolved, and the finer salt and peptide crystals were uniformly distributed within the polymer solution. Subsequently, the polymer/salt solution was emulsified with 50 ml of polyvinyl alcohol (PVA) aqueous solution (3% (w/v)) for 3.5 hours at 350 rpm using an overhead stirrer. The hardened MPs were then centrifuged (3000 rpm, 5 mins) and washed three times to get rid of the PVA. These MPs were dried overnight in a freeze-dryer and stored in -20 °C until further use. For comparison, GLP-1 loaded solid microparticles (s-MPs) were also prepared by similar technique, but without any salt introduced into the emulsion.

Characterization of MPs

Morphological analysis: The surface and internal morphologies of the fabricated MPs of each formulation were analyzed using a scanning electron microscope operated at 5 KeV (SEM JEOL 6360A). Samples were prepared by uniformly dispersing freeze-dried MPs onto metal stubs and cross-sectioning approximately at the center line with a surgical grade razor blade. Cross-sectioned MPs were finely coated with gold using sputter coater model SPI-Module and inspected using the SEM. ImageJ software was used to analyze the SEM images for particle size distribution. For each batch, an average of 100 random MPs were measured.

Encapsulation efficiency and release study: GLP-1 loaded MPs (10mg, n=3) of each formulation were dissolved in 0.2 ml acetonitrile and 1 ml of DI water was added to the solution. The solution was vortexed, allowed to settle and centrifuged (5000 rpm, 5 minutes) to separate any undissolved impurities. 500 µl of upper supernatant was then withdrawn and analyzed for GLP-1 amount by using a reversed-phase HPLC Agilent separations module equipped with a UV-visible detector.

Likewise, release study was carried out by incubating 20 mg of MPs in 1.5 ml PBS with 0.01% BSA in Eppendorf tube and kept on a rotor-shaker at 37 °C. At regular time interval, the tubes were centrifuged (3000 rpm, 3 minutes) and 1 ml of the supernatant was withdrawn for analysis by HPLC and replenished with 1 ml of fresh medium.

HPLC measurement was carried out using a BC-Zorbax SB-C18 Analytical (4.6 x 250 mm, 5 µm) chromatographic column. The mobile phase comprised of Eluent A (20 % ACN; 80% water, 0.05% TFA) and eluent B (50% ACN and 50% water, 0.05% TFA) eluted at a flow rate of 2 ml/min in gradient mode. The gradient used for separation was 50% A from 0 to 4.5 min, followed by a linear gradient that increased the concentration of B from 50% to 60% from 4.5 to 7.5 min, and 60% to 100% for additional 0.5 min and then returned to starting condition for 2 minutes. Sample injection volume was 100 µl and effluent was monitored at 200 nm. Each time, the mobile phase was freshly prepared before use. Freshly prepared GLP-1 solutions in PBS of known concentrations (0.001-0.1 mg/ml, n=3) were measured and plotted against the total peak area of the peptide to prepare the calibration curve of GLP-1.

Hydrolytic degradation of the MPs: 10 mg of GLP loaded MPs (n=3) of each formulation were suspended in 1 ml PBS in Eppendorf tubes and incubated at 37 ºC in a shaking incubator. At predetermined time points, MPs were centrifuged (5000 rpm, 5 minutes), washed with DI water and lyophilized overnight. The samples were analyzed for the change in molecular weight by Gel permeation chromatography (GPC) using Agilent 1100 Series LC System. Briefly, the MPs from each formulation were dissolved in 1 ml of THF. This polymer solution was then analyzed at 30 °C with THF as solvent, using a refractive index detector on Agilent 1100 Series LC System. The flow rate was maintained at 1 ml/min. Calibration of the average molecular weight was determined using

polystyrene standards with molecular weights ranging from 162 to 47,190 g/mol (Polystyrene Easycal Vial, Agilent).

Measurement of change in intraparticle pH upon degradation

The average pH of intraparticle microenvironment in both h-MPs and s-MPs was studied using a previously described technique (31, 32) that is based on the total aqueous volume of hydrated MPs. Calibration curve was prepared by obtaining a correlation between the known pH of lactic acid monomers in PBS and lactic acid monomer in the mixture of polymer, PBS and DI water. Briefly, 10 mg of MPs (n=3) for each formulation were incubated in 1 ml of PBS in 1.5 ml Eppendorf tubes on a rotor-shaker at 37 °C. At predetermined time points, the tubes were centrifuged, and the supernatant was removed. 800 µl of acetonitrile was then added to the centrifuged polymer pellet to dissolve it. To this, 200 µl of DI water was added and vortexed vigorously and measured for pH with a pH small probe (Mettler Toledo, Columbus, OH). The pH probe is reported to behave satisfactorily in organic solvent-water mixtures up to 90 wt% or organic solvent.(32) Hence the readings obtained are considered reliable. Also, the pH of the supernatant was measured.

Measurement of peptide adsorption to polymer by bulk concentration measurement

A simple method was used wherein blank polymer MPs are dispersed in appropriate media and mixed with peptide solution allowing enough interaction time.(33) MPs are then removed by centrifugation and the supernatant is measured for the peptide concentration. The adsorption of peptide to the polymer was measured in two conditions 1. varying particle concentration and 2. varying pH of the medium. For the first experiment, 25 mg of MPs was suspended in 0.5 ml PBS adjusted to different pH values (7, 4 and 2). Then GLP-1 solution was added to the MPs suspension

to achieve the final peptide concentration of 2.5 µg/ml. This suspension was then kept at roto-shaker at 37 °C for 30 mins. The suspension was then centrifuged (5000 rpm, 5 mins) and 200 µl of clear supernatant was withdrawn and analyzed by HPLC for peptide concentration. Likewise, study was also carried out with varying MPs concentration i.e. 0, 1, 10, 50 and 100 mg/ml in PBS adjusted to pH 2. All the samples were in triplicate and controls in the absence of polymer MPs were included.

Bioactivity assessment of the encapsulated peptide

The microparticles of both formulations from release study were collected at day 7 and the peptide that was still entrapped into the particles was extracted by similar method discussed earlier and analyzed for degradation and measured for concentration by HPLC. The extracted GLP-1 from the two formulations, h-MPs and s-MPs, were then diluted with DI water with 0.05% Triton X100 and 0.04% of Tween 20 to normalize the concentration to 1000 pg/ml. These solutions were analyzed for bioactivity using HTRF active GLP-1 assay kit from Cisbio following the protocol provided with the kit.

RESULTS AND DISCUSSION

Fabrication of hollow microparticles through osmogen-mediated one-step technique

Microparticle batches having two different morphologies were fabricated for this study viz: solid (s-MPs) and hollow (h-MPs), with GLP-1 loaded in solid crystal form as provided by the supplier. Both samples were fabricated using the emulsion-solvent evaporation technique, with a slight modification in the protocol to produce MPs with a hollow-core structure. The latter were fabricated

through the addition of an osmogen in the oil phase (polymer solution in organic solvent) before emulsifying with the water phase (water with surfactant).(34)

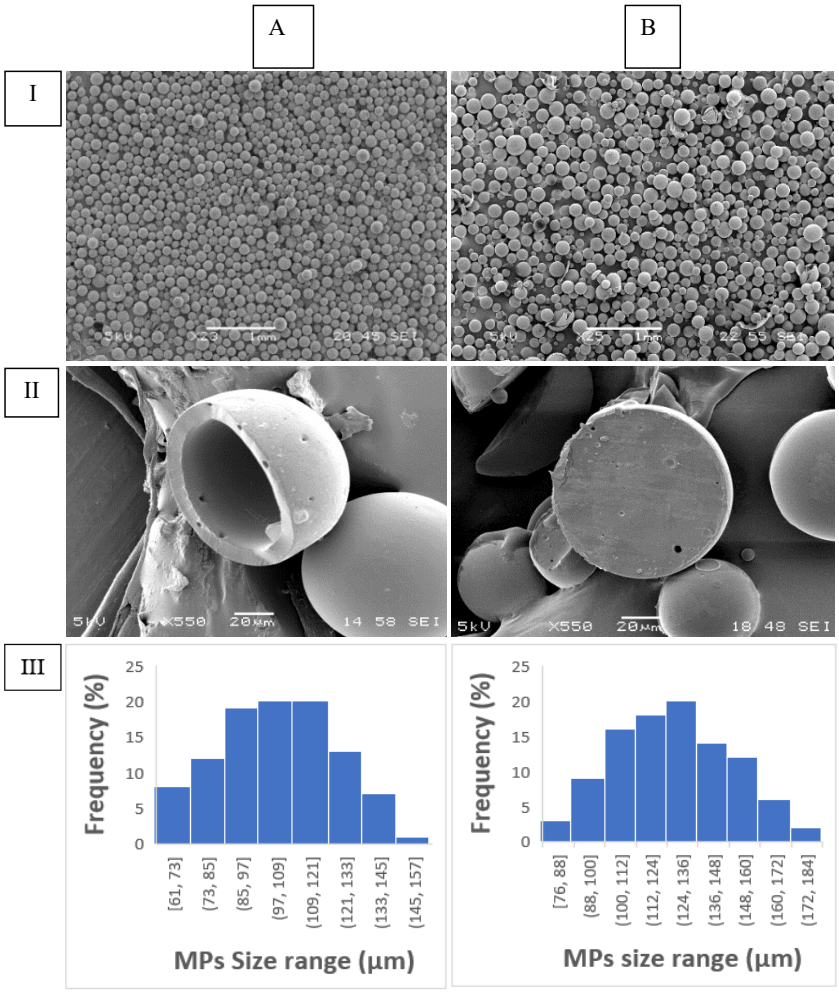

Figure 1. Scanning electron micrograph (SEM) of (A) h-MPs and (B) s-MPs, showing (I) overall size distribution (II) cross section of a particle and (III) particle size distribution histogram

For h-MPs, the presence of the NaCl (salt) in the polymer solution droplet creates an osmotic pressure that drives water from the external continuous phase into the polymer droplets. With careful adjustments of the amount of NaCl as the osmogen, the amount of water that penetrates into the polymer solution droplet is controlled so as to facilitate the coalescing of the penetrating water droplets to be at the core of the emulsion. By removing the water core through freeze drying, h-MPs are formed. In contrast, s-MPs were obtained, without the addition of salt during the fabrication process. **Figure 1** shows the SEM micrographs of the MPs fabricated. For both formulations of h-MPs and s-MPs, spherical and smooth exterior was observed. Cross-sectioning the MPs revealed a solid core for s-MPs and a hollow core for h-MPs. The average particle sizes measured were 127 ± 23 µm and 103 ± 20 µm for h-MPs and s-MPs respectively.

Degradation of the MPs and intraparticle pH varies with the morphology

During peptide release, the hydrolysis of the PLGA backbone produces oligomers that are carboxylic acid-terminated.(35) The progressive degradation of the polymer leads to the accumulation of these acidic oligomers that builds up towards an acidic microenvironment within the particle.(36) **Figure 2** plots the mass loss and average molecular weight of the MPs against time. **Figure 2A** shows the loss of polymer mass of the MP samples, whereby no significant mass loss was observed for both formulations during the initial 7 days of hydrolysis. With time, even though both particles were composed of the same PLGA polymer, s-MPs experienced an earlier and more significant mass loss. For instance, mass loss for s-MPs was 36 ± 3% at day 11 that increased to 54 ± 5% at day 14, whereas h-MPs experienced only 6.7 ± 8% and 33 ± 6% mass loss at the same time points respectively.

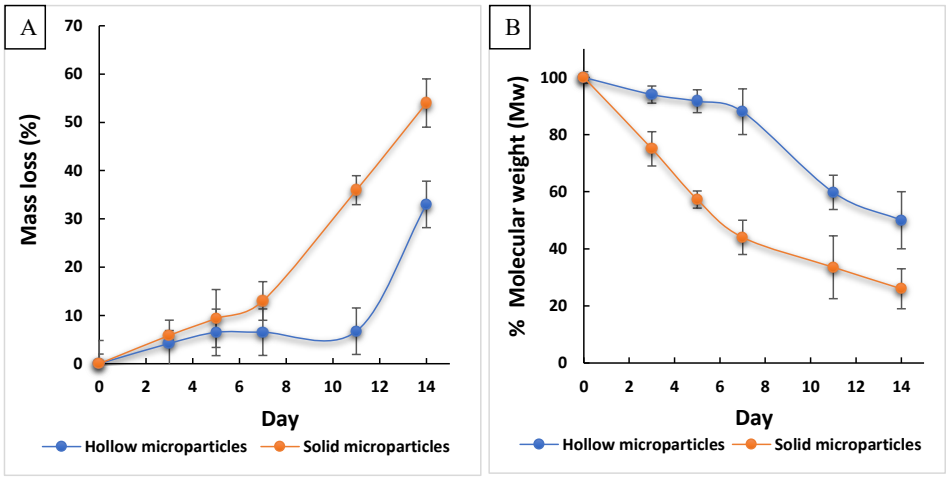

Figure 2. (A) Mass loss of h-MPs and s-MPs of PLGA at different degradation time points (B) % change in average molecular weight of polymer particles of h-MPs and s-MPs at different degradation time points

The change in average molecular weight over time (**Figure 2B**) further corroborated with the above observations, whereby s-MPs had an earlier onset and a relatively more drastic reduction of its average molecular weight. At day 7, the average molecular weights of s-MPs and h-MPs were 44 ± 6% and 88 ± 8% of the original molecular weights respectively. A decrease in average molecular weight is evidence of hydrolysis,(37) and this leads to the formation of acidic degradation products, i.e. oligomers. As a result of water uptake by the polymer, ester bonds in the polymer backbone is hydrolyzed by the water and increasingly shorter polymer chains with carboxylic acid end groups are produced as acidic by-products as shown in **Figure 3**.(38) These acidic oligomers dissolve in the aqueous environment, and dissociate into carboxylate anions.(25) As the acidic degradation products accumulate within the particles, these anions increase the negative charge of the polymer matrix. At the same time, the accumulation of hydrophilic carboxylate anions further

increases water diffusion into the microparticles, leading to a faster hydrolysis of PLGA, i.e. an auto-catalytic effect.

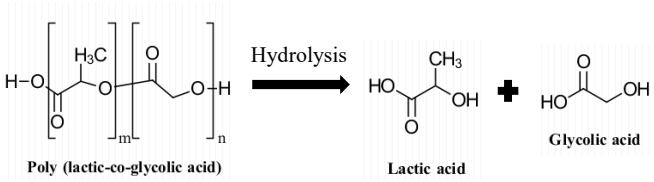

Figure 3. Hydrolysis of PLGA forming the degradation products

To validate the above observations, the intraparticle pH of both formulations were measured. **Figure 4** shows how intraparticle pH changes with hydrolysis time. For both microparticles, intraparticle pH was observed to decrease with hydrolysis, but with s-MPs having a significantly lower intraparticle pH (an average difference of 1.63 ± 0.65 as compared to h-MPs). The accumulation of the oligomeric by-products within the microspheres as a result of their relative hydrophobicity, results in an acidic microenvironment, leading to a correspondingly lower intraparticle pH (**Figure 4A**). On the other hand, although the average molecular weight of h-MPs decreased significantly after day 7 (**Figure 2B**), its intraparticle pH did not seem to be influenced by this.

Next, the pH values of the release medium for both degrading formulations were measured. Measuring the pH of the release medium would provide information on how much of the acidic oligomers has diffused out into the physiologically-relevant environment. **Figure 4B** shows pH of the release medium for h-MPs decreased steadily over time. This is unlike s-MPs where its release medium only showed a significant decrease in pH after day 7. This sudden reduction in pH for s-MPs after day 7, coincides with its drastic mass loss at this time point (**Figure 2A**). Interestingly,

the intraparticle pH values for both samples increased after hitting their lowest values at Day 5 and Day 7 respectively (**Figure 4A**), although the general trend is still a decreasing one. Essentially, both samples are degrading over time, but at different rates (Figure 2A). After Day 7, as the solid microparticles degrade rapidly, acidic by-products are quickly released into the release medium, resulting in a rapid decrease in pH of the supernatant (**Figure 4B**). This quick release of acidic by-products into the supernatant may cause this temporal rise in particle microenvironment, though its microenvironment is general still acidic.

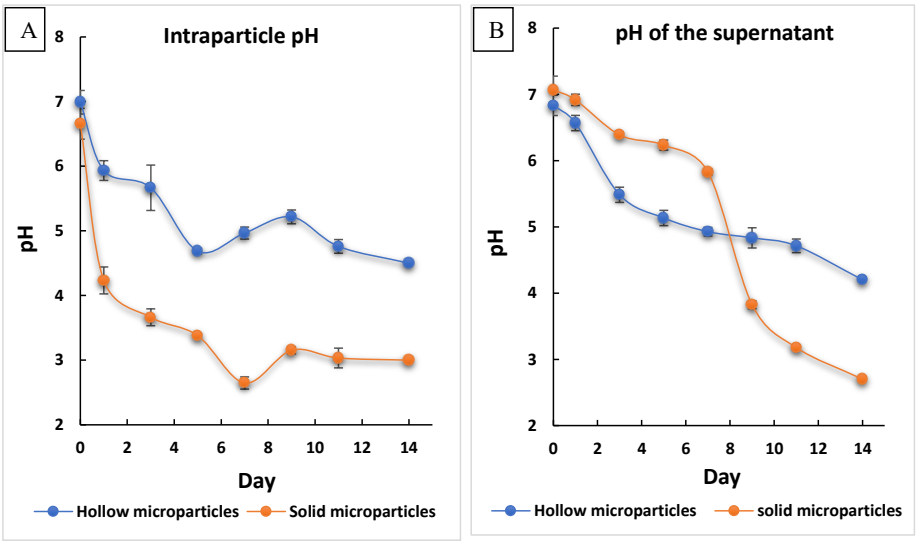

Figure 4. (A) Intraparticle pH measurements for h-MPs and s-MPs, (B) Corresponding supernatant pH measurements for the microparticle formulations

In totality, the results obtained cohesively imply that both MP formulations undergo hydrolysis when exposed to the physiologically-relevant release medium. Although both formulations were composed of the same polymer, i.e. PLGA, they experienced different hydrolysis rates due to their difference in particle morphology. Because s-MPs possess a higher polymer mass per particle, due

to its dense solid morphology, more degradation by-products are expected to be formed within s-MPs. These acidic oligomers in s-MPs tend to be entrapped within these particles, leading to a lower intraparticle pH (**Figure 4A**). Because of the solid morphology of s-MPs, these oligomers could not diffuse efficiently out into the release medium,(39) due to a slower diffusion rate through a dense polymer matrix and a longer diffusion distance. On the other hand, although acidic oligomers are also formed in h-MPs (**Figures 2** and **4**), this did not seem to affect its intraparticle pH as significantly. As the shell of h-MPs degrades, the acidic products have a shorter diffusion distance to the release environment. In addition, the water-filled hollow cavity also helps to dilute the concentration of the acidic oligomers, and further promotes diffusion. As such, the less acidic oligomers in h-MPs were better able to leach or diffuse into the release medium. These results therefore consistently explain why a huge variation exists between the intraparticle pH of s-MPs and the pH of its release medium, and this is attributed to the dense particle morphology of s-MPs.

Intraparticle pH affects the release kinetics of GLP-1

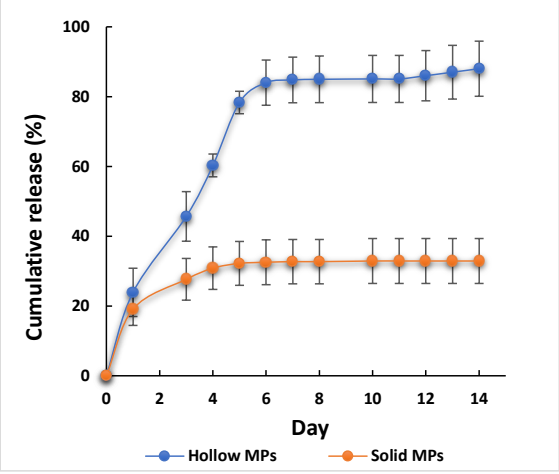

Figure 5. Comparative in vitro release profiles of GLP-1 peptide encapsulated in hollow and solid microparticles

Next, release study of GLP-1 from both formulations were investigated to understand how particle morphology can influence release kinetics of the encapsulated peptide. **Figure 5** shows the cumulative release profiles of hollow and solid MPs over the 14 days release period. While the cumulative release of GLP-1 from h-MPs showed a cumulative amount of 88 ± 8%, the cumulative release from s-MPs plateaued at 33 ± 6%, without any further release. In other words, a more complete and efficient release of GLP-1 was achieved from h-MPs.

This stark difference in the release profiles can be attributed to two main factors. First, because of particle morphology, the rate of peptide release is different in each formulation. While a hollow morphology promotes peptide release through diffusion, a solid morphology tends to restrict diffusion because of a greater diffusion distance and a denser polymer matrix. This was further verified by calculating the release kinetics of both the batches using various mathematical models as shown in **Table S2**. The calculations reflect that the hollow microparticles best fit to the Higuchi model (rate constant = 5.56; $r^2 = 0.86$) – a diffusion controlled release model from a matrix system.

Table S2. Release kinetics of peptide GLP-1 from hollow and solid microparticles.

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	Coefficient (r^2)			Rate constant K ($h^{-1/2}$)
Batches	**Zero order**	**First order**	**Higuchi**	**Higuchi**
Solid microparticles	0.46	0.49	0.55	2.23
Hollow microparticles	0.689	0.8	0.86	5.56

Second, the intraparticle pH of the degrading MPs can also influence the release kinetics of the peptide.(25) When the intraparticle pH drops below the isoelectric point of the encapsulated

peptide, the peptide attains a positive charge and will be electrostatically bound to the negatively-charged degrading PLGA matrix. This electrostatic interaction between positively-charged peptide and a negatively-charged polymer can significantly impede peptide release. Nevertheless, hydrophobic interaction between peptide and polymer has also been reported to promote peptide adsorption and poor release, though its effect would be of a lesser extent here.(40, 41) To validate if peptide-polymer interaction is a significant factor impeding the release, we calculated the net charge of the peptide as a function of pH. For this, the amino acid sequence of GLP-1 was analyzed using the Bachem's peptide calculator with this formula:

$$Z = \sum_{i} N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_{j} N_j \frac{10^{pKa_j}}{10^{pH} + 10^{pKa_j}}$$

Z	Net charge of the peptide sequence
N_i	Number of arginine, lysine, and histidine residues and the N-terminus
pKa_i	pKa values of the N-terminus and the arginine, lysine, and histidine residues(42)
N_j	Number of aspartic acid, glutamic acid, cysteine, and tyrosine residues and the C-terminus
pKa_j	pKa values of the C-terminus and the aspartic acid, glutamic acid, cysteine, and tyrosine residues(42)
pH	pH value

The graph plot of the charge of peptide as a function of pH revealed that the isoelectric point of GLP-1 is at pH 7.7. With a decrease in the environmental pH, the positive net charge of this peptide increases to be as high as +5 net charge per kDa, when the pH is at or below 3 (see **Supplementary Information**). Considering the results from our intraparticle pH measurements, it is evident that the encapsulated GLP-1 in s-MPs attains a more positive net charge per mass than in h-MPs. With an intraparticle pH of 2.64 (for s-MPs – **Figure 4A**), this would imply that the peptide would

achieve a +5 net charge per kDa compared to +1.5 per kDa for h-MPs. A stronger electrostatic interaction therefore exists between a more positively-charged peptide and a more negatively-charged (i.e. acidic) polymer matrix (**Figure 4A**). It should also be noted that presence of NaCl could have some degree of influence on this electrostatic interaction due to the salt induced-electrostatic screening. For example, Mehta SB. et al. showed that interfacial gels of protein observed in formulations containing 50 mM NaCl exhibited slightly increased elastic moduli, with no further increases at higher salt concentrations.(43)

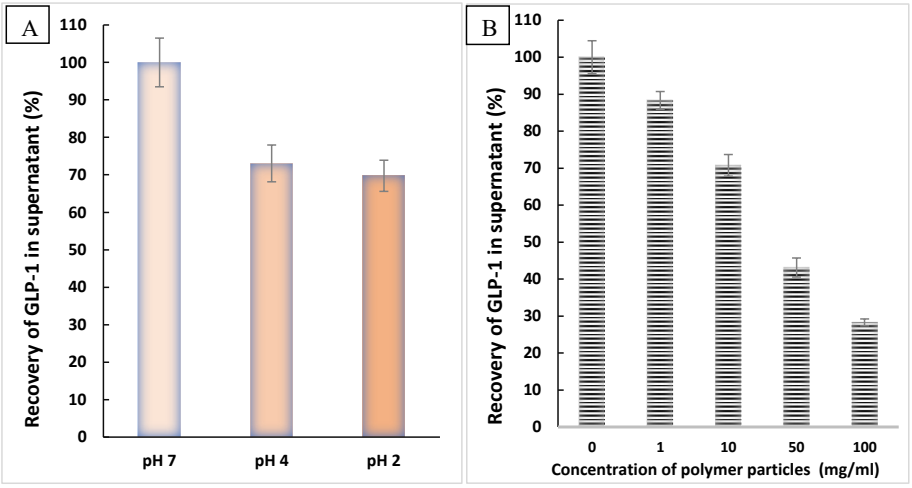

Figure 6. (A) the influence of pH on the adsorption of peptide GLP-1 to PLGA polymer (B) the influence of increased amount of polymer on the adsorption of the peptide GLP-1

In a recent study by Balmert et al, it was also shown that there is an inverse correlation between the net positive charge of the peptide and its release rate.(25) Hence, we carried out a simple, bulk concentration measurement experiment(44) to confirm if peptide-polymer interaction, as a result of a considerably stronger acidic environment, is the main reason for the incomplete release observed

for s-MPs. First, we investigated the amount of GLP-1 lost from the GLP-1 solution when exposed to: (a) the same amount of blank polymer MPs in medium at different pH, and (b) different amounts of the polymer at a constant pH. **Figure 6A** shows that there was a significant decrease in the recovery of the GLP-1 amount in the solution when interacted with an equal amount of MPs at acidic pH 2 compared to neutral pH 7.

While the amount of GLP-1 in the solution remained almost unaffected at pH 7, 30 ± 4% of the initial amount of the GLP-1 couldn't be detected in the solution at pH 2. Evidently, when the peptide was exposed to polymeric MPs suspended in the solution adjusted to pH 2, there was significant adsorption of the peptide to the polymer. Likewise, when the peptide solution was mixed with 0, 1, 10, 50, and 100 mg of MPs in release medium adjusted at pH 2, it was observed that with an increasing amount of MPs, the recovery of peptide in the solution also decreased (**Figure 6B**). The peptide solution alone, however, was confirmed to be stable at pH 2 by comparing the HPLC chromatograms with that of peptide at pH 7 (see **Supplementary Information**). While the control without polymer MPs in the solution showed no loss (100 ± 5) in the recovery of GLP1, the recovery of peptide was 88 ± 2%, 71 ± 3%, 43 ± 3% and 28 ± 1% of the initial amount for 1mg, 10 mg, 50 mg and 100 mg of MPs in the solution respectively. This strongly suggests that the disappearance of GLP-1 from the solution is likely due to peptide-polymer interaction. In summary, the results do conclude that particle morphology influences particle hydrolysis and intraparticle pH, which subsequently would affect the release profile and kinetics of the encapsulated peptide. Here, the morphology of h-MPs seems to promote a more efficient release of GLP-1 that would otherwise not have been possible if it was encapsulated within s-MPs. From this, we hypothesize that this could therefore similarly translate to improved bioactivity and release of peptides *in vivo* through hollow microparticles.

Hollow MPs preserve the bioactivity of the peptide better than solid MPs

The bioactivity of GLP-1 was assessed in vitro using HTRF active GLP-1 assay kit. This kit uses sandwich immunoassay involving two monoclonal antibodies, one labelled with Lumi4Tb-Cryptate (Donor) and the second with d2 (Acceptor).(45) The principle of detection is based on HTRF® technology. The two antibodies bind to the active GLP-1 and upon the excitation of the donor with a light source, Fluorescence Resonance Energy Transfer (FRET) towards the acceptor is triggered which in turn fluoresces at 665 nm wavelength. This fluorescence signal intensity is proportional to the antigen-antibody complexes formed and therefore to the active GLP-1. This kit was used to test the level of bioactivity of the peptide extracted at day 7 from all the formulations suspended in PBS.

The results in **Figure 7A** showed that 93% of the extracted peptide was still bioactive for the h-MPs. In contrast, a significant drop in the bioactivity of peptide was observed for s-MPs and only 58% of the peptide was observed to be bioactive. These results are in good correlation with the intraparticle pH study whereby the pH of s-MPs was extremely low (pH 2.64) compared to h-MPs (pH 4.99) by day 7. This high level of acidity has been widely reported to be detrimental to the bioactivity of the encapsulated bioactive molecules such as peptides and proteins.(46, 47) Also, it has been reported that at such acidic microenvironment, cationic peptides form new covalent bonds with the PLGA oligomers by acylation reactions resulting in PLGA-peptide adjunct.(25, 48) Acylation of peptides significantly decreases the bioactivity of peptides. HPLC chromatograph analysis of the extracted peptides at day 7 confirmed on the acylation of GLP-1. HPLC chromatograph of peptide extracted from h-MPs showed a very small peak for intact peptide showing presence of very little amount of non-released intact peptide (see **Figure 7B**). However, for s-MPs, not only a significantly large peak was observed for non-released intact peptide, a second

peak was also observed which well indicates an acylation product of the peptide. Because of this peptide degradation, the bioactivity of the encapsulated peptide will be compromised in s-MPs. This limitation can thus be ameliorated through the use of h-MPs as a delivery vehicle considering the results above. Hence, h-MPs that can be easily fabricated through this one-step, osmogen-mediated technique for a more efficient and effective delivery of peptides.

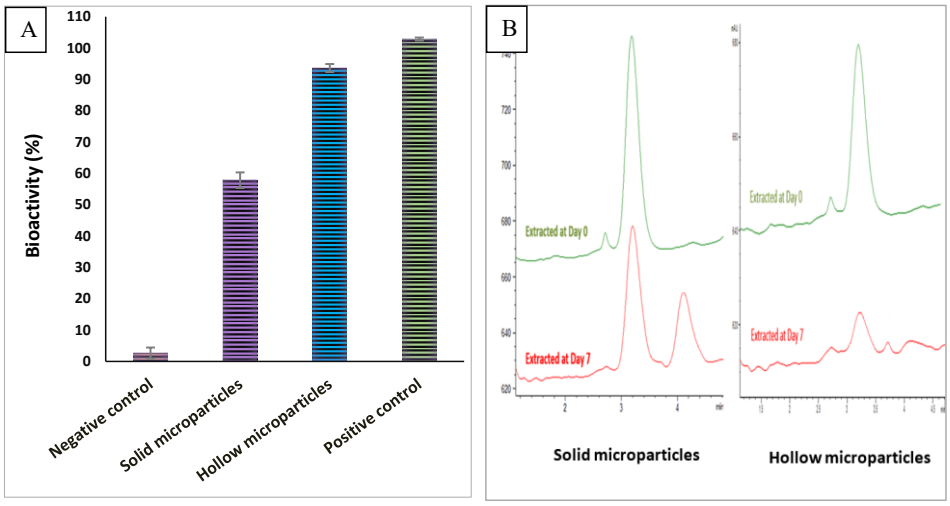

Figure 7. (A) Results showing the bioactivity of the peptide extracted from hollow and solid microparticles at Day 7, normalized to 1000 pg/ml. (B) HPLC chromatogram of the peptide extracted from microparticles at Day 7 showing intact and degraded peaks of GLP-1

CONCLUSION

Peptide GLP-1 was successfully encapsulated into PLGA h-MPs using a one-step fabrication technique. Mass loss, changes to average molecular weight and intraparticle pH were measured and compared between h-MPs and s-MPs. The results in its entirety indicate that there is significantly less accumulation of polymer degradation products, or acidic oligomers, in the h-MPs. In contrast,

s-MPs suffered from a highly acidified microenvironment that drastically decreased the intraparticle pH. The acidic polymer matrix was found to impede peptide release through an electrostatic interaction between the negatively-charged polymer and a positively-charged peptide. In addition, the peptide from h-MPs maintained its bioactivity. Hollow microparticles in h-MPs was therefore shown to be a promising solution to the inherent problems of s-MPs as a delivery vehicle.

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