



Lipid and PLGA hybrid microparticles as carriers for protein delivery



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ABSTRACT

The present study aimed at investigating the influence of lipid excipients on protein carriers when proteins are encapsulated in lipid and PLGA hybrid particles. PLGA and lipid hybrid microparticles (MP) were prepared by a double emulsion method, and lysozyme was used as the model protein. The encapsulation efficiency (EE) of lysozyme in hybrid MP, particle surface morphology, as well as the release profile of lysozyme were investigated. The results showed that higher content of PLGA in the hybrid MP resulted in better EE of protein and smoother surface of the MP. Burst release of lysozyme from the MP was positively correlated to the chain length of acyl groups in lipids as well as the content of lipids in the hybrid MP. The polymorphic form of lipids in the hybrid MP affected both the EE of protein in the MP and the protein release from the MP, suggesting that EE of protein in the hybrid MP and the protein release profile could be regulated by changing lipid excipients as well as the level of lipids in hybrid MP. The present study provides a good basis for further investigation of the application potentials of lipid and PLGA hybrid MP in drug delivery.

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1. Introduction

Therapeutic peptide and protein may provide special treatment on numerous diseases and disorders. Most of them have a short half-life and need to be injected frequently, therefore, formulations for long-term release of peptide and protein have gained great attentions [1,2]. There are a few PLGA microparticles (MP) products on the market for sustained delivery of peptides, such as leuprolide (Lupron Depot[®], TAP Pharmaceutical Products Inc.), octreotide (Sandostatin LAR[®], Novartis) and triptorelin (Trelstar[®], Debiopharm). However, PLGA MP for sustained delivery of proteins is challenging and is still under development. One of the problems is the degradation of proteins during the preparation and storage of PLGA MP [3,4], especially the decreased pH during PLGA degradation affected the protein stability [5]. Additionally, non-specific adsorption to PLGA polymer may lead to incomplete protein release [6].

Lipids, one of the major human nutrients, are biocompatible and biodegradable. The safety and diversity of lipids have attracted more interests to their applications in drug delivery. It has been reported that triglyceride implant did not result in significant inflammatory response and cytotoxic reaction in mice over 2 months

[7]. Additionally, the slow digestion rate of selected lipids might be applied to sustained delivery of peptide and protein drugs [8,9], for instance lipid MP have been used for sustained delivery of GnRH antagonist (antide) for 30 days *in vivo* [10].

Lipid and polymer hybrid system draw more and more attention in drug delivery system to merge benefit of lipid and polymer [11]. Our hypothesis was that adding lipids into PLGA MP could maintain the ability of MP for sustained drug release and avoid some of the disadvantages of PLGA MP. Limited studies have been conducted on the combination of lipids and PLGA in drug delivery in general, especially for sustained delivery of proteins. It has been reported that combining phospholipids with polymer affected drug release from the nanoparticles, which could result in delayed release or burst release of drug depending on the properties of phospholipids [12–14]. The phospholipids were mainly detected on the surface of polymer particles because of the amphiphilic property of phospholipid [15]. It has also been reported that addition of medium-chain triglyceride, tricaprins, in PLGA MP could accelerate drug release from the MP [16]. The present study aimed at achieving a better understanding of the effects of lipid structures and compositions on the release of proteins from PLGA MP. Therefore, lipid and PLGA hybrid MP were prepared with different contents of lipids in hybrid MP, as well as different lipid excipients, i.e. triglycerides containing medium-chain (TG8, TG12), long-chain (TG16) and very-long-chain fatty acids (TG22), and monoglycerides such as glycerol monostearate (GMS).

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2. Materials and methods

2.1. Materials

The mono-acid triglycerides trilaurin (Dynasan D112, TG12), tripalmitin (Dynasan D116, TG16), and Tribehenate (Dynasan D122, TG22) were kindly donated by IOI Oleo GmbH (Hamburg, Germany). Captex 300 (medium chain triglycerides (MCT), caprylic ($\approx 70\%$) and capric acids) was bought from ABITEC Corporation (Columbus, USA) and Geleol mono- and diglycerides NF (Glycerol monostearate 40–55 type 1, GMS) were obtained from Gattefossé (Lyon, France). PLGA (75/25, RG 753 S, inherent viscosity: 0.32–0.44 dl/g, around 50 KDa) was bought from EVONIK (Darmstadt, Germany). Lysozyme from chicken egg (70,000 U/mg), sodium azide, polyvinyl alcohol (PVA, average MW 85,000–124,000, 87–89% hydrolyzed) were bought from Sigma Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Microparticles preparation

Protein-loaded MP were prepared by double emulsion and solvent evaporation method based on the method described previously [17]. Briefly, 400 mg lipids and PLGA at different percentage were dissolved in 1 ml of chloroform (Table 1), mixed with 0.1 ml of lysozyme (50 mg/ml) and followed by probe sonication (Sonics & Materials, CT, USA). The probe sonication (130 W, 20 KHz) was operated at a pulse mode with 3 s work, and 3 s off, 60% of intensity, 10 times. Then, 3 ml of 2.5% PVA solution (room temperature) was added into the primary emulsion and probe sonicated again. The working condition of probe sonication was: 3 s work, 3 s off, 40% of intensity, 10 times. The obtained w/o/w emulsion was transferred into 200 ml of PVA solution (0.1%) at room temperature, which was stirred (200 rpm) for 4 h to evaporate the organic solvent. MP were obtained after centrifugation (6200 g, 10 min), and washed with purified water, followed by freeze-drying (0.08 Mbar, $-50\text{ }^{\circ}\text{C}$, overnight). Table 1 lists the used lipid excipients (MCT, TG12, TG16, TG22, GMS), and the weight percentage of lipids in the formulations (5%, 10%, 20%, 33.3%, 50%, 66.7%).

2.3. Encapsulation efficiency determination

Lysozyme was extracted from the formulations using the method described previously with minor modification [18]. 10 mg of MP, accurately weighted, was dissolved in 10 ml of ethyl acetate

at $40\text{ }^{\circ}\text{C}$, and lysozyme was pelleted by centrifugation at 1500 g for 10 min. After removing the supernatant, the residual ethyl acetate was evaporated under nitrogen at room temperature. 2 ml of purified water was added to dissolve lysozyme, and the concentration of lysozyme was quantified by the HPLC method described below. The EE of lysozyme in the MP was calculated by:

$$\text{EE\%} = \text{Actual drug content in MP/Theoretical drug content} \times 100$$

The HPLC method was slightly modified from the one reported by Christophersen et al. [19]. A Dionex HPLC system (Agilent Technologies, Waldbronn, Germany) was used with a C18 column ($4.60 \times 100\text{ mm}$, $5\text{ }\mu\text{m}$, 300 \AA , Waters, USA). A binary solvent system was used at room temperature at a flow rate of 1 ml/min. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in purified water and solvent B was 0.1% (v/v) TFA in acetonitrile. A gradient method was used: 0–2 min: 23% B, 2–12 min: 23–50% B, 12–13 min: 50–100% B, 13–15 min: 100% B, 15–16 min: 100–23% B, 16–18 min: 23%. Lysozyme was detected at 220 nm by UV detection.

2.4. Particle size analysis

The size and size distribution of MP were analyzed based on laser diffraction principle using Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK) equipped with Hydro 2000S (Malvern Instruments Ltd., UK) for wet dispersions. The volume-mean diameter of MP was measured. Wet dispersions for particle size analysis were prepared as follows: MP suspension was transferred to the optical measurement cell containing the dispersing medium, i.e. 0.1% PVA solution. Measurements were performed while the sample was in the cell under stirring (1200 rpm) and ultrasound (50%). The obscuration was set between 8% and 12%.

2.5. Scanning electron microscopy (SEM)

Surface morphology of MP was observed by scanning electron microscopy (SEM) (Hitachi, TM3030, Japan) after particle preparation. The samples were coated with gold under an argon atmosphere for 20 s and examined under an accelerating voltage of 5 kV.

2.6. Differential scanning calorimetry (DSC)

DSC (Perkin Elmer, Shelton, USA) measurements were carried out to determine the polymorphic changes of lipids and glass transition points of PLGA. Approximately 5 mg of samples were weighed into DSC pans and crimp sealed. They were heated from $10\text{ }^{\circ}\text{C}$ to $100\text{ }^{\circ}\text{C}$ at a heating rate of $10\text{ }^{\circ}\text{C}$ per min under a 20 ml/min of nitrogen. DSC system was controlled by the software Trios v3.3.1.

2.7. X-ray powder diffraction (XRPD)

X-ray diffraction measurements were performed to elucidate the solid state of TG16 and PLGA in hybrid MP both after particle preparation and *in vitro* release experiments by using a PANalytical X'Pert Pro diffractometer equipped with a PIXcel detector (PANalytical B.V., Almelo, Netherlands). Measurements were conducted at ambient conditions by using Cu K α radiation at 40 mA and 45 kV, with an angular increment of $0.04^{\circ}/\text{s}$ and count time of 2 s. Data were collected at 0.05° (2 theta) within a 5° – 35° range. Data were analyzed by X'Pert high score plus version 2.2.4 (PANalytical B.V.).

2.8. In vitro release profiles

MP (approximately 50 mg, accurately weighed, $n = 3$) were suspended in 1.0 ml of the release medium (PBS-buffer pH 2.5,

Table 1
Characterization of the various lipid PLGA hybrid MP.

Formulation	Lipids content (%)	Size (μm)	EE (%)
PLGA MP	0	14.69 ± 1.49	60.48 ± 1.84
TG16 PLGA MP	1.0	16.46 ± 2.37	55.48 ± 3.26
MCT PLGA MP	5.0	15.32 ± 2.00	60.29 ± 1.51
TG12 PLGA MP	5.0	$24.16 \pm 2.78^*$	62.67 ± 8.09
TG16 PLGA MP	5.0	13.04 ± 2.74	57.86 ± 4.45
TG22 PLGA MP	5.0	16.71 ± 1.03	$47.38 \pm 4.55^*$
TG16 PLGA MP	10.0	17.05 ± 1.89	50.19 ± 4.50
TG16 PLGA MP	20.0	16.76 ± 1.40	52.85 ± 13.64
TG16 PLGA MP	33.3	16.84 ± 1.16	$42.61 \pm 8.15^*$
TG16 PLGA MP	50.0	$20.54 \pm 2.60^*$	$24.58 \pm 6.10^*$
TG16 PLGA MP	66.7	17.31 ± 2.40	$12.61 \pm 6.57^*$
GMS PLGA MP	1.0	16.21 ± 4.16	54.70 ± 10.15
GMS PLGA MP	5.0	12.14 ± 4.17	$17.33 \pm 2.55^*$
GMS PLGA MP	10.0	12.17 ± 4.07	$12.92 \pm 5.44^*$

*: $P < 0.05$ vs PLGA MP, PLGA: poly (D, L-lactide-co-glycolide), EE: entrapment efficiency; TG12: triglycerides trilaurin; TG16: tripalmitin; TG22: Tribehenate; GMS: Glycerol monostearate; MCT: medium chain triglycerides, dominated by TG8:0. Results are given as average values \pm S.D., $n = 3$.

0.02% (w/v) Tween 20) in 1.5 ml Eppendorf tube and incubated at 37 °C under constant agitation (30 rpm). At pre-defined time, samples were centrifuged (2400 g, 5 min) and the release medium was collected for analysis of lysozyme by HPLC. MP was re-suspended in 1 ml of fresh release medium to continue the *in vitro* release study.

2.9. Statistics

The results were expressed as mean \pm standard deviation (SD). Statistical analysis was carried out using the Student's *t* tests. The difference between two groups was considered to be statistically significant when the *p* value was less than 0.05.

3. Results and discussion

The effects of lipid excipients and the composition of the hybrid MP on particle characters and protein release behavior were investigated. The differences between the hybrid MP and pure PLGA MP as well as pure lipids MP were evaluated.

3.1. Effects of lipid excipients on MP characters

The particle size of different lipid and PLGA hybrid MP was between 10 and 25 μm (Table 1). The size of MP showed Gaus distribution and 90% of the particles (*D*(0.9)) was less than 50 μm , suggesting that the MP were suitable for subcutaneous and intramuscular injection [20]. Fig. 1 shows the surface morphology of the hybrid MP observed by using SEM. All the hybrid MP were

spherical. The surface of lipids and PLGA hybrid MP was gradually changed from smooth to coarse when the content of TG16 increased from 5% to 66.7%. Lipid crystals were observed on the particle surface when the hybrid MP contained 50% or 66.7% of lipids.

There was no clear trend in the change of particle size when the lipid content was altered, neither the lipid excipients. However, the physicochemical properties of lipids and the lipid content in hybrid MP affected the EE of lysozyme in MP significantly. The EE of lysozyme was around 60% for PLGA MP, which was similar to that for the hybrid MP when the content of triglyceride (MCT, TG12, TG16) in the MP was 20% or less ($p > 0.05$). However, 5% of TG22 in the hybrid MP resulted in a significant lower EE of lysozyme ($p < 0.05$), as well as MP contained more than 33.3% of lipids ($p < 0.05$). Addition of monoglycerides in the hybrid MP had more profound effects on reducing the EE of lysozyme; only 15% of lysozyme was encapsulated in the hybrid MP when it contained 5% of GMS or more (Table 1). Pure triglyceride MP (TG16 MP) had around 5% of lysozyme EE, therefore it was not included in further investigation in the present study.

In order to understand how the different lipids and lipid composition affected the EE of protein in MP, both XRPD and DSC were used to evaluate the MP. Fig. 2A and Fig. 3A illustrated the XRPD diffractograms and DSC thermograms for lipid and PLGA hybrid MP. All the triglycerides (MCT, TG12, TG16, TG22) were found in amorphous form in the hybrid MP when the MP contained 20% of lipids or less (Supplement A). The results suggest that lipids might be dispersed in the polymers unordered after the solidification process. 20% or less lipids in the hybrid MP may have similar

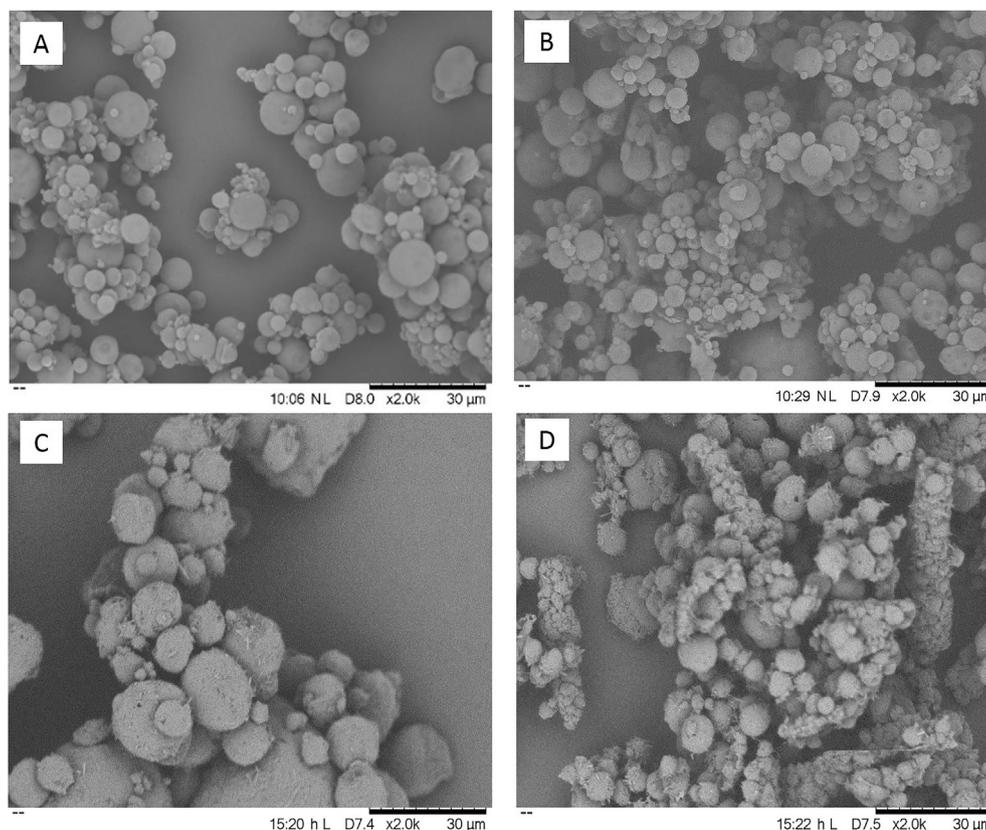


Fig. 1. SEM images of lipids PLGA hybrid MPs containing different amount of the TG16 lipid. A: 5% TG16 PLGA MP. B: 20% TG16 PLGA MP. C: 50% TG16 PLGA MP. D: 66.7% TG16 PLGA MP.

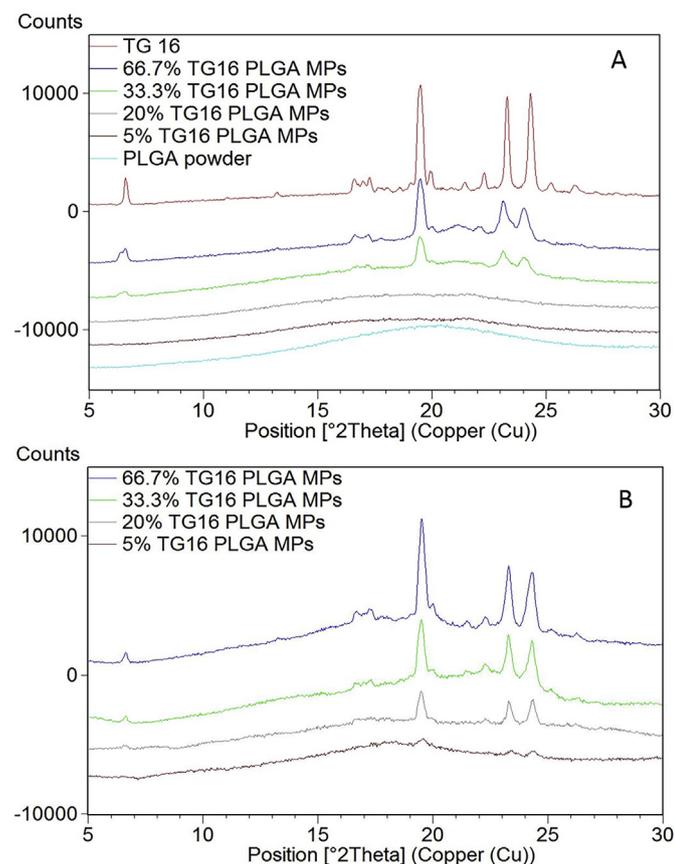


Fig. 2. Diffractograms of PLGA hybrid MPs containing different amounts of the TG16 lipid. A: after MP preparation. B: after *in vitro* protein release study.

solidification process with pure PLGA MP, which results in the similar EE with PLGA MP. When the hybrid MP contained more than 33.3% of lipids, polymorphic forms of triglycerides were observed both by XRPD and DSC. The XRPD result of the hybrid MP was similar to that of bulk TG16 and demonstrated the crystalline structures of lipids (Fig. 2A). Those hybrid MP showed endothermic peaks at 45 °C, 52.5 °C, and 65.5 °C, corresponding to α form, β' form, and β form of TG16, respectively (Fig. 3A). Previous research has shown that the crystallinity of poly l-lactides had an unfavourable effect on drug encapsulation during MP solidification process [21]. Therefore the crystallinity of lipids in hybrid MP during the solidification process may also reduce the EE of lysozyme. Additionally, it has also been reported that using lower concentration of PLGA in preparation of MP led to lower EE [22]. So, another reason for the lower EE of protein in the hybrid MP with high content of lipids could be decreased content of PLGA in the hybrid MP.

GMS was found in amorphous form in the GMS PLGA hybrid MP when the content of GMS was 10% or less (Supplement A). Emulsifiers with low HLB values had a destabilizing effect on the w/o/w emulsion, and could induce the transfer of part of the internal phase to the external phase [23]. The lower EE of protein in the hybrid MP could be explained by that GMS is a lipophilic emulsifier with a HLB value around 3.8, which could disrupt the stability of double emulsion system during the MP preparation process and lead to protein leakage into outer water phase.

3.2. Effect of fatty acyl groups in triglyceride on protein release profiles

To investigate the impact of fatty acyl groups in triglycerides on the characters of the hybrid MP, 5% of triglycerides with medium-chain (MCT and TG12), long-chain (TG16), and very-long-chain fatty acids (TG22) were used in preparing the hybrid MP. The release profile of lysozyme from different MP was investigated in a medium at pH 2.5. The selection of pH value for the release medium was based on the lysozyme stability study and research from literature. Only 40% of lysozyme could be detected by HPLC after one day incubation in the release medium at pH 7.4 and 37 °C under agitation. Significant aggregation of lysozyme at pH 7.4 has also been reported previously [24,25]. While almost 100% of lysozyme was detected after five days at pH 2.5 and 37 °C under agitation (data not shown). Additionally, a good *in vitro-in vivo* relationship has been reported when the *in vitro* release of lysozyme was performed in pH 2.5 [26].

The results of *in vitro* release of lysozyme showed that the chain length of fatty acyl groups in triglyceride had significant impact on the release profile of lysozyme from the hybrid MP. The release of lysozyme from all the hybrid MP initiated with a burst release in the first 24 h, followed by slow release in 20 days (Fig. 4A). The level of burst release of lysozyme was positively correlated to the chain length of fatty acyl groups in the triglycerides in the following order MCT < TG12 < TG16 < TG22 (Fig. 4A). This phenomenon may be related to the compatibility of triglycerides with the polymer PLGA. The calculated logP value of the PLGA is around 10, while the logP value of MCT, TG12, TG16 and TG22 is 12.2, 15.5, 17.3, and 26.9, respectively. The hydrophobicity of triglyceride is increased when the chain length of fatty acyl groups become longer, resulting in less compatibility between lipids and PLGA in the hybrid MP. It has been reported that the release profile of drug from PLGA nanoparticles could be modulated by manipulating the hydrophobicity of pro-drug conjugates [27]. Therefore the increased hydrophobicity of long-chain lipids may be responsible for the increased burst release of lysozyme from the hybrid MP.

3.3. Effects of triglyceride content in the hybrid MP on protein release profiles

To further clarify the effect of lipids on the release of proteins, hybrid MP were prepared with different contents of TG16 in hybrid MP. The lipid content in the hybrid MP was varied from 5% to 66.7% (Table 1). The general release profile of lysozyme from the hybrid MP was similar to that from the other hybrid MP, i.e. it initiated with a burst release of lysozyme within 24 h and followed with sustained release in 20 days (Fig. 4B). The burst release of lysozyme increased when the content of TG16 in the hybrid MP changed from 33.3% to 66.7% (Fig. 5). The EE of hybrid MP also decreased at the same time, suggesting that more lysozyme stay on the surface of MP and lead to increased burst release. However, the burst release of lysozyme from hybrid MP with 20% lipids was higher than that of hybrid MP with 33.3% lipids (Fig. 5). The differences of burst release may be caused by the variation of the solid state of lipids. Fig. 6 illustrates the solid state of lipids and how TG16 molecules might interact with PLGA molecules during hybrid MP preparation and lysozyme release processes. The lipids were in amorphous form when it is less than 20% in the hybrid MP (Fig. 6, left), while it is in crystalline form when it is more than 33.3% in hybrid MP (Fig. 6, right). Lipids in amorphous form could lead to faster drug release from solid lipid particles than lipids in crystalline form [28,29].

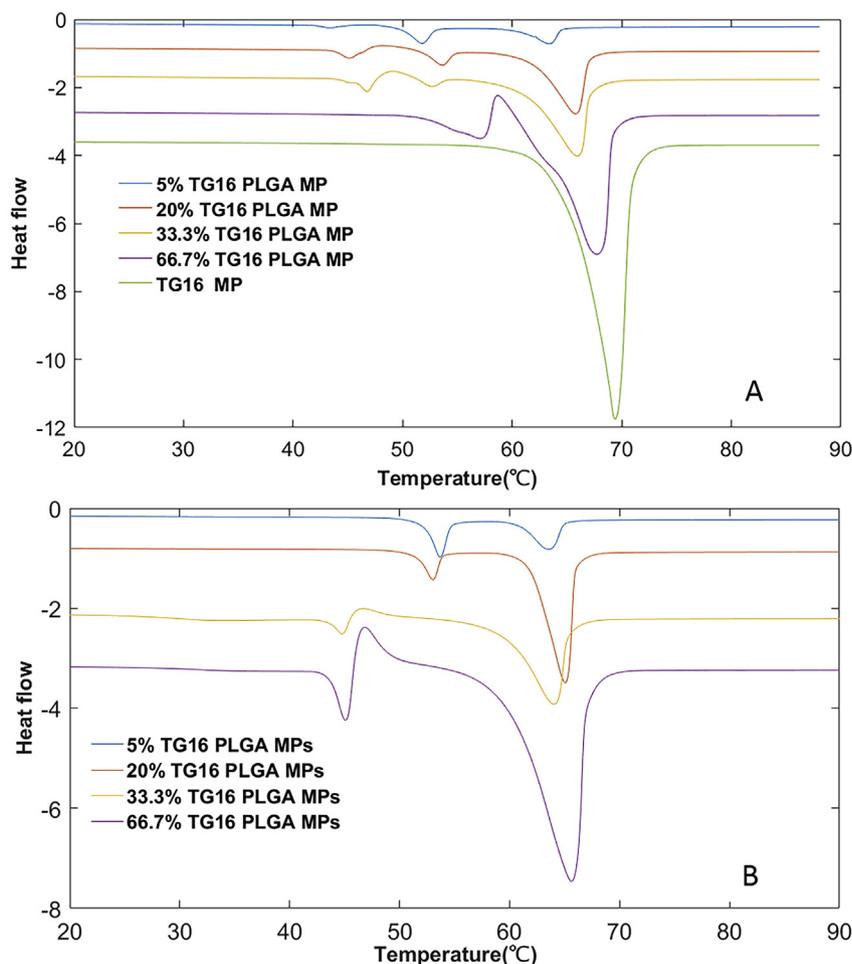


Fig. 3. Thermograms of PLGA hybrid MPs containing different amounts of the TG16 lipid. A: after MP preparation. B: after *in vitro* protein release study (α form: 45 °C, β' form: 52.5 °C, β form: 65.5 °C).

Additionally, amorphous form of compounds had higher solubility than compounds in crystalline form [30]. Therefore, TG16 in amorphous form may diffuse faster to the surface of the hybrid MP than the crystalline form of TG16. The diffusion of amorphous TG16 in hybrid MP formed pores on the hybrid MP, which accelerated lysozyme release and led to high burst release. In hybrid MP contained less than 20% of lipids, lipid transformed from amorphous form to crystalline form after the *in vitro* release study (Figs. 2B and 3B). It may be that lipid diffuse to the surface of hybrid MP and then re-crystallized.

3.4. Effects of GMS content in the hybrid MP on protein release

In our previous studies we found that lipid MP with different lipid structures, i.e. GMS, GDS and TG, had different effect on protein release, and the mechanism of protein release from lipid MP depended on lipid structures [31]. In the present study, we also investigated the influence of different lipid structures on the release of protein from the hybrid MP. GMS PLGA hybrid MP were prepared with different contents of GMS (1%, 5%, and 10%) (Fig. 4C). The *in vitro* protein release study showed that GMS PLGA hybrid MP had similar protein release behavior as TG16 PLGA hybrid MP (Fig. 4A and C). Similar to TG PLGA hybrid MP, higher content of GMS in the hybrid MP also induced higher burst release of protein from the hybrid MP. XRPD and DSC studies showed that all GMS

were in amorphous form both before and after the protein release study (Supplement A).

3.5. Effects of lipids in the hybrid MP on protein incomplete release

PLGA matrix had non-specific adsorption to protein which could lead to incomplete protein release [6]. In the present study, hybrid MP which had higher content of lipids achieved higher protein release, and PLGA MP contains 5% TG22 or high ratio of TG16 realized almost complete protein release (Fig. 4). This could be due to that the non-specific adsorption of protein to PLGA matrix was decreased by the addition of lipids in the hybrid MP.

4. Conclusion

In the present study, lipid and PLGA hybrid MP were prepared successfully by the double emulsion method. Both the chemical structure of lipids and the chain-length of fatty acyl groups of triglycerides affected the EE of lysozyme in the hybrid MP as well as the release profile of lysozyme. Additionally, the content of lipids in the formulation had significant influence on the EE of protein in the hybrid MP and protein release behavior from the hybrid MP. Increased content of lipids in the hybrid MP resulted in reduction of the EE of lysozyme and enhancement of the burst release of lysozyme from the hybrid MP. The variation of protein EE in the hybrid

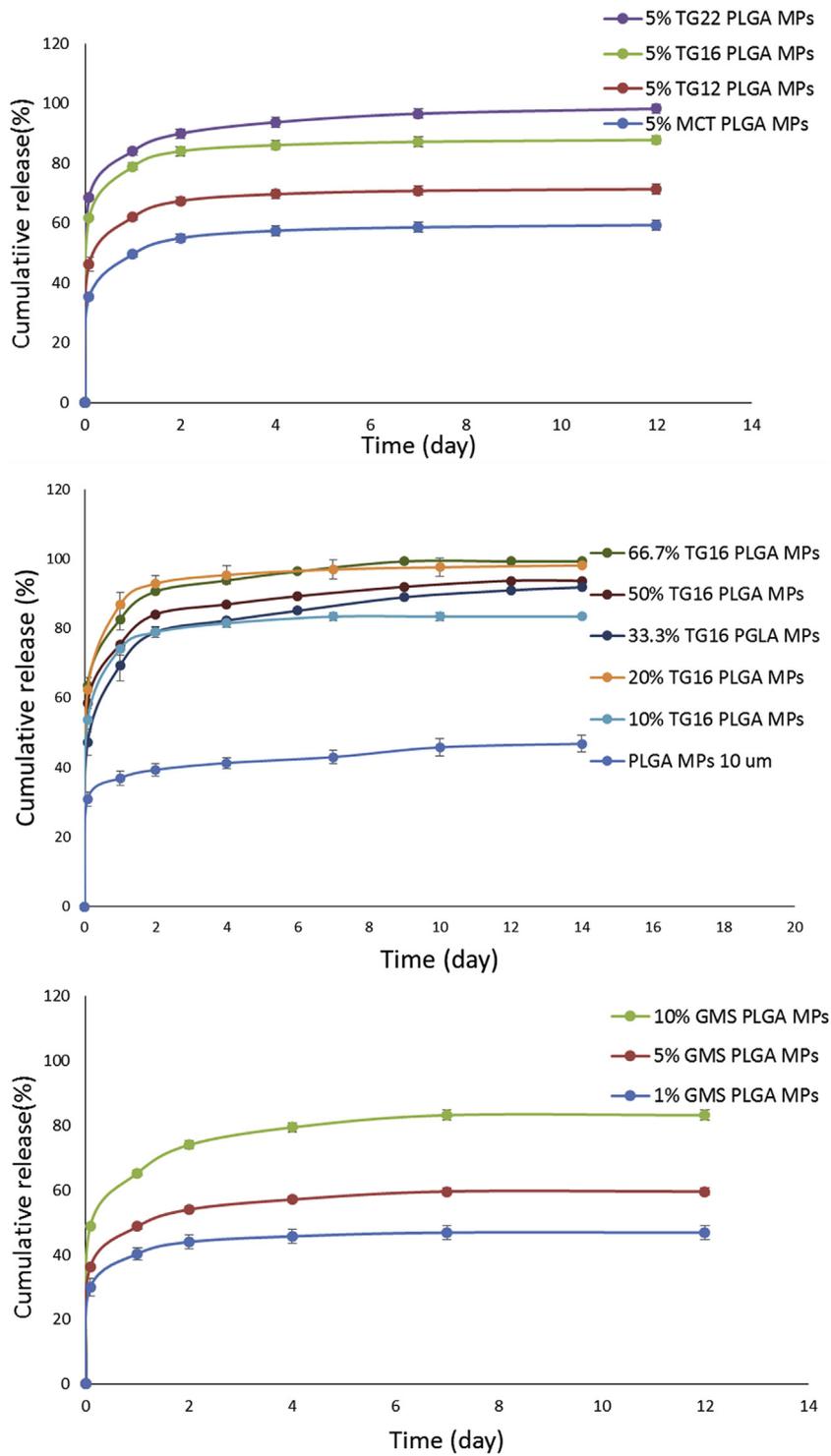
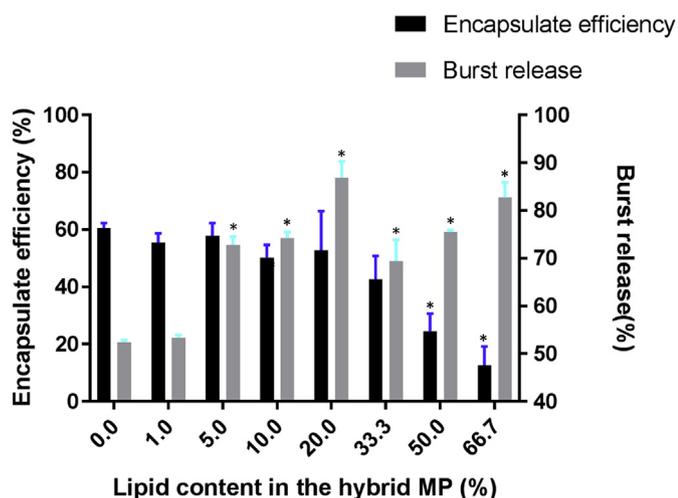


Fig. 4. *In vitro* release profile of lysozyme from lipids PLGA hybrid MPs (A: 5% lipids PLGA MPs; B: TG16 PLGA MPs; C: GMS PLGA MPs. $n = 3$. In some cases, the error bars are within the size of data points.).



MP and the release profile of protein was closely related to the physical state of lipids in the hybrid MP. The results obtained in the present study provide a good basis for further investigation of the application potentials of lipids and PLGA hybrid MP for drug delivery.

Author contribution

Chengyu Wu did all the experiments, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis; Huiling Mu and Chengyu Wu analyzed and interpreted the data. Stefania Baldursdottir, Mingshi Yang, Huiling Mu and Chengyu Wu were involved in study concept and design, critical revision of the manuscript. Huiling Mu: obtained the funding.

Fig. 5. The relation between entrapment efficiency (EE) of lysozyme in the MP and the level of burst release of lysozyme from the TG16 PLGA hybrid MP (n = 3). Black: EE of TG16 PLGA hybrid MP; Gray: Burst release of lysozyme from TG16 PLGA hybrid MP in 2 h *: significant difference vs 0.0% lipids in hybrid MP.

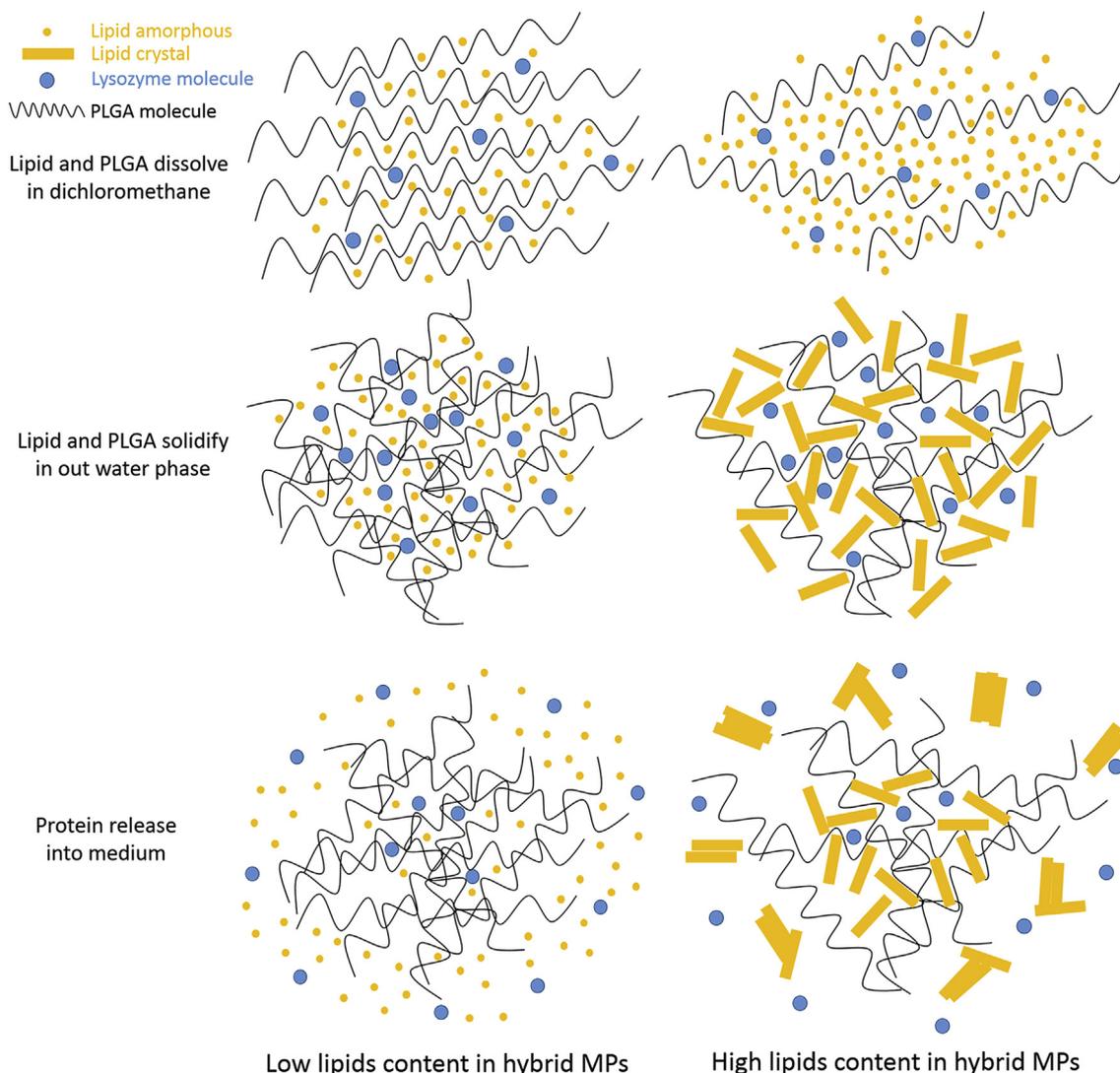


Fig. 6. The schematic illustration of lipids and PLGA MP during dissolve, solidify and release.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jddst.2017.09.006>.

Abbreviations

PLGA	poly (D, L-lactide-co-glycolide)
EE	entrapment efficiency
PVA	polyvinyl alcohol
SEM	scanning electron microscopy
TG12	triglycerides trilaurin
TG16	tripalmitin
TG22	Tribehenate
GMS	Glycerol monostearat
MCT	medium chain triglycerides

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