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Original Article

PHYSICAL PROPERTIES OF PROLIPOSOME FOR INDUSTRIAL QUALITY CONTROL AND RECONSTITUTION OF PROLIPOSOME IN PORCINE INTESTINAL MUCOSA

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ABSTRACT

Objective: The aim of this research was to examine the physical properties of proliposome granules for industrial quality control and to develop the proliposome tablet. The reconstitution of proliposome into liposome in porcine intestinal mucosa was also examined.

Methods: Proliposome granules of bovine serum albumin (BSA) were prepared by granulation method with lecithin and cholesterol as coating lipid. The physical properties which were granular size, flow-ability, moisture content and adsorption isotherm of granules were examined and set as quality control (QC) standards. The obtained proliposome granules were further compressed into tablets with addition of filler/binders. Proliposome granules were also studied in porcine intestinal mucosa at specific time of 0, 5, 10 and 20 minutes to observe the reconstitution of proliposome into liposome.

Results: Granular size was decreased regarding the drop of BSA while the amount of lipid had no obvious effect on granular size. Granular size with properties of fair flow-ability and the granular moisture of less than 1.5% was capable for the good tablet compression. These parameters could be set as the standard for quality control of proliposome granules. Proliposome granules displayed type V of BET adsorption isotherm which could be exploited as the fingerprint of proliposome formula and set as QC standard. The reconstitution of proliposome into liposome by mucus on surface of small intestine was clearly observed at 10 minutes onward. The addition of PVP as dry binder along with Avicel® increased the hardness of proliposome tablet, suitable for further experiment of an enteric coating.

Conclusion: Industrial quality control of proliposome granules could be accessed by physical properties of granules. PVP combined with Avicel® were the appropriate binders for proliposome tablet compression. The reconstitution of proliposome into liposome could be displayed on the surface of the small intestine.

Keywords: Proliposome, Industrial quality control, Reconstitution, Porcine intestinal mucosa.

INTRODUCTION

Proliposome, a dry granular product, is a new innovation designed to overcome the limitation of liposomal stabilities. It composes mainly of phospholipid and drug which can be reconstituted into liposomes upon hydration by liquid [1]. Interest has been drawn to proliposome of protein [2]-[3] since proteins are decayed by various factors whereas a range of medicines recently used are proteins. Of the various methods to prepare proliposome, granulation is the simplest method that is relatively easy to scale up in the industrial level [4]. However, production of proliposome in the industrial level requires the quality control standard to enable the quality monitoring of proliposome in every production lot. The quality control standard can be accessed by the physical properties of proliposome granules.

Study on physical properties of proliposome granule for industrial quality control such as granule size, flow-ability, moisture content and adsorption isotherm is a tempting research topic. So far to our knowledge, none of any research group has done the study on adsorption isotherm of proliposome in order to set the standard for the industrial quality control before.

The principle of BET adsorption isotherm is the adsorption and desorption of gas on the solid surface to determine the pattern of surface area and porosity which are the total pore volume and pore diameter, of solid surface [5]- [6]. Physical adsorption-desorption isotherms of different solid are dissimilar even though they have the same physical dimensions. The isotherms are different due to the characteristics of pore volume, pore diameter and surface area in which they could be called the texture of solids. The type of isotherm could be divided into six types in general depending on the gas adsorption-desorption characteristics. Thus, the adsorption isotherm could be considered as the fingerprint of those individual solid [7]. Performance characteristics of solid depend on their physical characteristics capable to be illustrated by the BET adsorption isotherm approach.

The target site of proliposome to be reconstituted into liposome is the mucus of the intestine. Thus, the reconstitution efficiency of proliposome using porcine intestinal mucosa as a model tissue is of interest in order to have the visual of how the proliposomes are reconstituted into liposomes over a period of time in real mucus.

The aim of this research was to examine the physical properties of proliposome, especially the adsorption isotherm, in order to set the standard of industrial quality control. The reconstitution of proliposome into liposome in porcine intestinal mucosa was also studied. The formulation development of proliposome tablet for the industrial level production was further investigated.

MATERIALS AND METHODS

Chemicals and drug

Bovine serum albumin (BSA) was procured from Merck (Darmstadt, Germany). Cholesterol (CHOL) and D-Mannitol (MAN) were purchased from Carlo Erba Reagents (Rodano, Italy).

Lecithin (LEC) was kindly provided as a gift from Shreenidhi Enterprises (India). Dichloromethane (DCM) was obtained from Labscan (Stillorgan, Co Dublin, Ireland). All bradford protein assay reagents were procured from Bio-Rad (Hercules, California, USA). Avicel® and Emcompress® were purchased from Asahikasei Chemical Corporation (Tokyo, Japan) and Labscan (Stillorgan, Co Dublin, Ireland). Explotab® was obtained from Penwest (Lestrem, France). Aerosil® and Magnesium stearate were procured from Merck (Darmstadt, Germany) and Peter Greven (Netherland). Other chemicals were of analytical grade and used as received.

Proliposome granules

Preparation of proliposome granules

BSA-entrapped proliposome granules were prepared by the method modified from Charernruttanakul [8]. In brief, mannitol powder was granulated with BSA solution at mannitol to BSA solution ratio of 10:0.125, 10:0.25 and 10:0.5. Damp mass was passed through sieve no.18 and dried in hot air oven at 45°c for 1 hour to obtain the dry granules. The lipid mixture composed of LEC to CHOL at the molar ratio of 7:3 was dissolved in DCM. The dry granules were subsequently granulated again with the lipid mixture at ratio of total lipid: Granules of 0.5:10, 1.0:10, 1.5:10 and 2.0:10. Granules were then dried again in hot air oven at 45°c for 45 minutes.

Physical properties of proliposome granules for industrial quality control

Size of proliposome granules by sieve analysis

Particle size of proliposome granules was evaluated by standard sieve analysis using sieve no.16, 18, 20, 30 and 40, respectively. The process was accomplished by placing the proliposome granules onto the uppermost sieve and vibrating. The residue weights on any individual sieve were recorded and calculated. The graphs of cumulative percentage oversize of proliposome granule were subsequently plotted.

Flow-ability by bulk density and tapped density

Flow-ability of selected proliposome granule was obtained from the percentage of powder compressibility which could be calculated from the tapped density and bulk density. Tapped density was investigated by adding proliposome granule into 100 ml cylinder and tapping for 100 times by tapped density tester (Vankel, USA). The obtained values of bulk volume and tapped volume were then calculated for the percentage of compressibility.

Moisture content

Moisture content of selected proliposome granule was determined by moisture content analyzer (Scaltec SMO 01, Germany). The 1 gram of proliposome granule was heated at 130^{o}C until the weight was constant. The moisture was subsequently calculated and reported.

BET adsorption isotherms

The selected proliposome granule was examined for its surface and porosity characteristics by nitrogen adsorption-desorption process. The Y-shape chemisorptions cell (P/N 74067) was used. The proliposome granule was outgassed in a chemisorptions cell using automated sequence at 100^{o}C outgas temperature. The surface was pretreated to remove the surface moisture and the granule was subsequently dried to remove all the moisture before examination.

Reconstitution of proliposome granules on surface of porcine intestinal mucosa

Porcine intestinal mucosa preparation

Porcine intestinal mucosa was prepared regarding the method of mucosal tissue preparation of Pietzonkaa [9] with modification. Briefly, small intestine of porcine was separated from gastrointestinal tract of dead porcine at certified abattoir. Intestinal tissues were used within 25 minutes after slaughter in order to maintain the tissue viability [10]. The duodenum segment was cut open along the mesenteric path and the segment was cut into 1x1inch per piece. Then, pieces of tissue were immersed and swung in 250 ml, 8^{o}C PBS, pH 6.8 for 3 seconds to clean the intestinal surface. Finally, each piece was casted in small volume of 8^{o}C PBS, pH 6.8 and placed on the ice in order to transport to the lab.

Evaluation of proliposome granules reconstituted in small intestine by SEM

The 50 mg of selected proliposome granules were sprinkled onto the surface of porcine intestinal tissues. Subsequently, the pieces of tissues, casted in small volume of 8^{o}C PBS pH 6.8, were placed on the vibrator plate. At any point of time of 0, 5, 10, 15 and 20 minutes, tissues were picked and prepared for the photograph taken by scanning electron microscope (SEM, JSM-5410LV, Jeol, Japan) to examine the reconstitution of proliposome granules on surface of intestinal mucosa. For tissue preparation, tissues were fixed with 1% osmium tetroxide for 30 minutes and rinsed for 5 minutes with PBS, following by distilled water and then, dehydrated three times by a gradient series of 30%, 50%, 70%, 90% and 100% alcohol, respectively. The critical point dryer was used to dry tissues. The tissues were subsequently coated by gold and observed under SEM. The SEM photograph of pure proliposome granules was also taken as a reference.

Proliposome Tablet

Formulation development

Proliposome tablets were prepared by direct compression method with independent variables which were; the percentage of granule, percentage and type of filler/binder and percentage of disintegrant as shown in Table 1. For the preparation, proliposome granules with other chemicals were mixed together in a dry mixer for 10 minutes, after which. Magnesium stearate was added as a lubricant and further mixed with other powders for 3 minutes. The mixtures were subsequently compressed into tablets with the tablet weight of 350 mg approximately using a single punch tablet machine (FETTE, Hanseaten Exacta I) with a 3/8" punch and die set.

Characterization of Tablet

Thickness, diameter, hardness, weight variation and friability

Ten proliposome tablets were randomly sampled and their thickness, diameter and hardness were individually measured by a multipurpose measuring device. Their means and standard deviations of these parameters were reported. The friability of proliposome tablets as well as the weight variation were also determined based on the method described in USP 36.

Disintegration time

Disintegration time of proliposome tablets was determined by USP tablet disintegration testing apparatus (Model QC21, Hanson Research) under the method of uncoated tablets described in USP 36. Six tablets of each formulation were evaluated individually. The average disintegration time and standard deviation was subsequently calculated.

RESULTS AND DISCUSSION

Physical properties of proliposome granules for industrial quality control

Size of proliposome granules by sieve analysis

Sieve analysis was a standard method industrially employed to measure the size of granules. It was observed that an increase of lipid concentrations coated onto proliposome granules of BSA had small effect on granular size as illustrated in Figure 1(A-C).

Table 1: Formulation of proliposome Tablets

Composition (%w/w)	Formulation code								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Proliposome granules	30	50	70	30	50	70	30	50	70
Avicel® pH102	38.5	28.5	18.5	-	-	-	38.5	28.5	18.5
Emcompress® (DCP)	-	-	-	38.5	28.5	18.5	-	-	-
Explotab®	30	20	10	30	20	10	25	15	5
Polyvinyl alcohol	-	-	-	-	-	-	5	5	5
Aerosil®	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Magnesium stearate	1	1	1	1	1	1	1	1	1

On the contrary, the size of proliposome granules was decreased regarding the drop of BSA at the similar lipid concentration as shown in Figure 1(D-G). The high amount of BSA solution was directly related to the agglomeration of powder. Thus, proliposome granules prepared at high concentration of BSA tended to be larger than those at the lower concentrations. In the industrial scale, the physical properties of proliposomes are supposedly changed, especially the size of granules, when the dose of active protein is adjusted. The difference in proliposomal sizes generates effect on the compressibility of tablet. Thus, granular size need to be measured in every production lot after protein dose adjustment. Obtained granules at any size after the dose adjustment should contain the satisfied flow-ability in order to be compressed into tablet with good characteristics.

Flow-ability and Moisture content of proliposome granules

The selected proliposome formulation was at the ratio of mannitol: BSA 10:0.5 and total lipid: BSA granules 0.5:10. This formulation was selected for further experiment regarding its physical property of high protein loading efficacy. In the industrial level, the flow-ability and moisture content of granule require to be monitored and controlled, since these properties influence the compressibility of tablet. The percentage of compressibility in relation to the flow-ability could be calculated from tapped density and bulk density as shown in Table 2. The flow-ability of selected proliposome granules was fair at the percentage of compressibility 16.36% and the moisture content 1.33% w/w. These limits of flow-ability and moisture content could be set as a standard for quality control of proliposome granules when the compressed tablets are with the satisfied characteristics.

Table 2: Flow-ability and Percentage of compressibility of proliposome granules

Parameters	Value
Tapped density	0.55 g/ml
Bulk density	0.46 g/ml
Percentage of compressibility	16.36%
Flow-ability	Fair

BET adsorption isotherms

Adsorption isotherm of selected proliposome granules prepared at the ratio of mannitol: BSA 10:0.5 and total lipid: BSA granules 0.5:10, corresponded to type V of BET adsorption isotherms as illustrated in Figure 2. Characteristics of type V isotherm could be implied that the adsorption of nitrogen gas, the inert gas used in the adsorption experiment, had no monolayer completion on the surface of solid which was the proliposome granules in this study. The no monolayer completion was due to the stronger gas to gas interaction compared to gas to surface interaction which would be occurred when the surface of solid was hydrophobically high [7]. Proliposome surface was hydrophobically high since its surface was covered by lipid. It could be concluded that the proliposome granules possessed the hydrophobic surface regarding BET adsorption isotherm corresponded to the preparation composition and process. Therefore, the result of preparation composition and process met the assumption of the obtained adsorption isotherms.

BET adsorption isotherm also showed the heteresis of the graph due to the uneven rate of gas adsorption and desorption. This was happened as a result of the incomplete nitrogen adsorption on the proliposome surface as a consequence of porous characteristics of solid granules. The heteresis shown in Figure 2 was the H2 type. The solid characteristic of H2 heteresis type was often disordered assuming that the pore diameters as well as the pore volume on the surface of proliposome and inside the proliposome were dissimilar. Therefore, the distributions of pore size and shape of solid with H2 heteresis were not well defined [7], [11]-[12].

The surface area of proliposome granules was 114.722m^2/g, average pore volume and pore diameter, were 0.167cc/g and 4.308 nm, respectively. From the result, the pore diameter was within the mesophore size. In general, the pore characteristic of substances categorized in BET isotherm type V was within the mesophore limit of 2 nm – 50 nm.

Therefore, the character of the proliposome granular pore in this study could be determined as non-geometric. The average diameter of pore was 4.308 nm, within the range of mesophore. BET adsorption isotherm is able to be used for quality control of Proliposme granules as it shows the unique pattern of granules, supposedly similar in different lot of industrial production.

Reconstitution of proliposome granules in porcine intestinal mucosa by SEM

Porcine intestinal tissues were incubated with proliposome granules and the visuals of tissue surfaces at 5, 10, 15 and 20 minutes were shown in Figure 3A-3E, respectively. The photograph of proliposome granules was taken as reference as shown in Figure 3F. It was observed that at 0 minute (Figure 3A), the surface of intestinal tissue was covered with mucus resembled to mesh layer. The visual of intestinal tissue was taken from the part that was covered by either mucus or mucus with the dissociated granules, not from the segment that was shielded by the associated granules. At 5 min (Figure 3B), the spherical particles were begun to form and at 10 min (Figure 3C), spherical particles were more structured. At 15 and 20 min (Figure 3D and 3E), the spherical particles could be clearly observed. It was seen that the observed amount of particles at 20 min was relatively less than at 15 min, supposedly as a result of particle uptake by intestinal tissue. Foreign particles such as the lipid based drug delivery particles, were efficiently trapped in the mucus layer by steric obstruction and/or adhesion [13] and supposedly taken up in maximum of 30 minutes [14].

Physical properties of proliposome tablets prepared from proliposome granules

Physical properties and hardness of proliposome tablet are shown in Table 3 and Figure 4, respectively. Proliposome granule selected for further experiment of tablet compression was at the ratio of mannitol: BSA 10:0.5 and total lipid: BSA granules 0.5:10.

Physical properties of tablets prepared from proliposome granules were different among nine formulations of F1-F9. The control variables in this study were the thickness, diameter and average weight of tablets. It was illustrated in Table 3 that these three variables were satisfactory controlled in all nine formulations. The independent variables were the percentage of granule, percentage of disintegrant, percentage and type of filler/binder as displayed in Table 1 above. The dependent variables were the hardness as shown in Figure 4, friability and disintegration time as displayed in Table 3. The hardness of tablets tended to drop when the percentage of granules was increased. The tablet formulation suitable to be further studied was F7 since the hardness was quite high and appropriate for tablet coating experiment. It was also observed that an increase of percentage of granules resulted into the raise of friability percentage as shown in Table 3. This was obviously observed in formulations of 70% granules prepared either with Avicel® or Emcompress® as filler/binder as shown in formulation F3 and F6, respectively. Formulation prepared with Avicel® and PVP of 70% granules (F9) showed the good result of small percentage of friability eventhough the percentage of granule addition was high (70%). This was happened as a result of PVP added into the formulation, consequences into the good hardness with small friability tablets. PVP could act as dry binder [15].

The disintegration time of tablets was increased regarding the decrease of disintegrant, Explotab®, obviously observed in formulation prepared with Emcompress®(F4-F6). The decrease of filler/binder amount resulted into the decline of hardness and increase of friability in all filler/binder types. The formulation suitable for further experiment of tablet coating was F7. The physical properties of the obtained tablets were satisfied. Thus, the proliposome granules with only fair flow-ability and the moisture content of 1.5% approximately was good enough to be compressed into tablet. These properties could be exploited as quality control standard in the industrial scale of protein proliposome tablet preparation following this study.

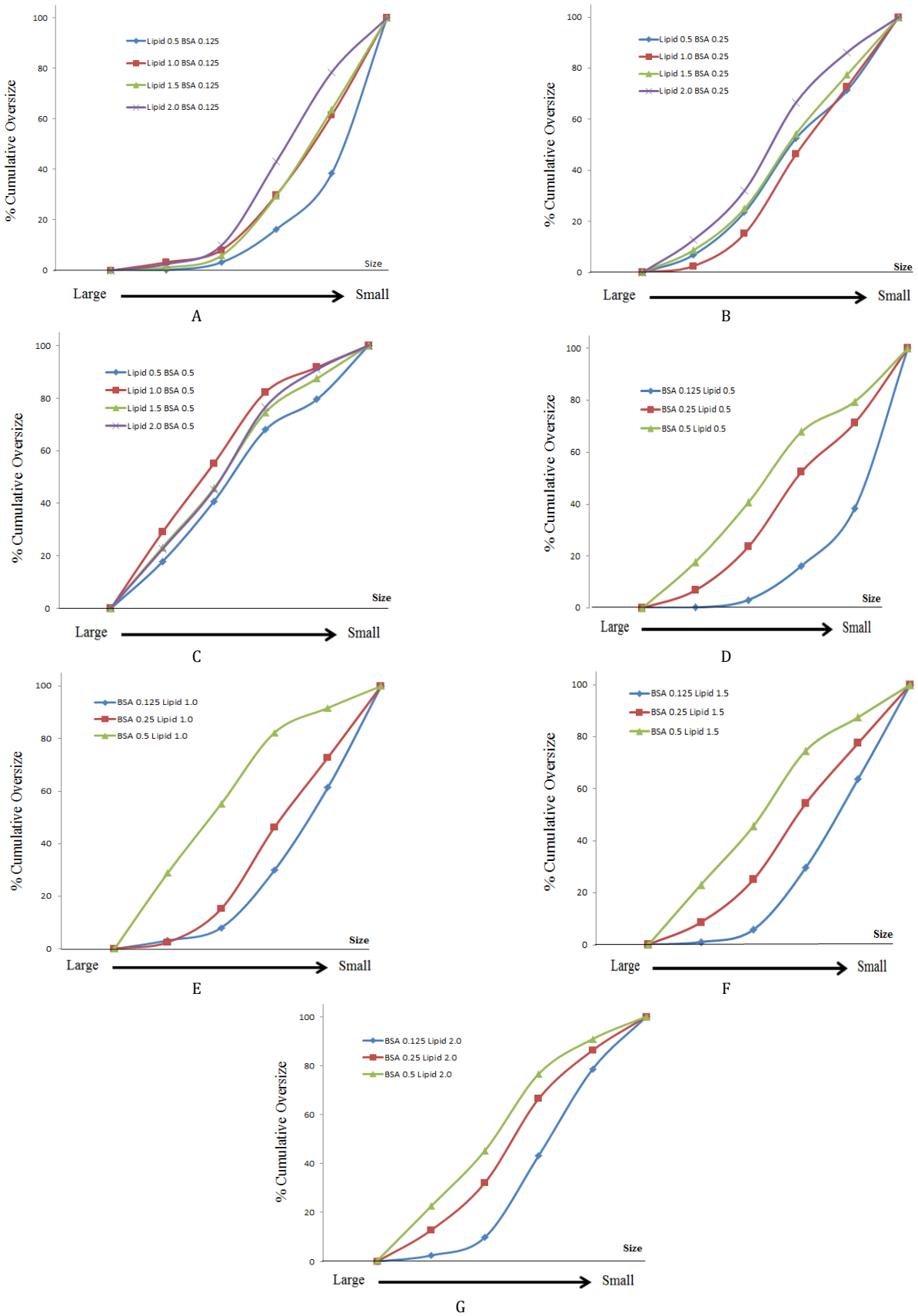

Fig. 1: Granular sizes of proliposome granules by sieve analysis prepared at different ratio of lipid to BSA; A: Different concentrations of lipid at BSA ratio of 0.125, B: Different concentrations of lipid at BSA ratio of 0.25, C: Different concentrations of lipid at BSA ratio of 0.5, D: Different concentrations of BSA at lipid ratio of 0.5, E: Different concentrations of BSA at lipid ratio of 1, F: Different concentrations of BSA at lipid ratio of 1.5 and G: Different concentrations of BSA at lipid ratio of 2. The graphs are plotted as cumulative percentage oversize.

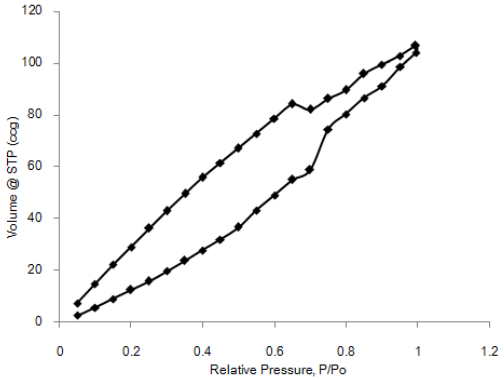

Fig. 2: The BET adsorption isotherm of proliposome granules

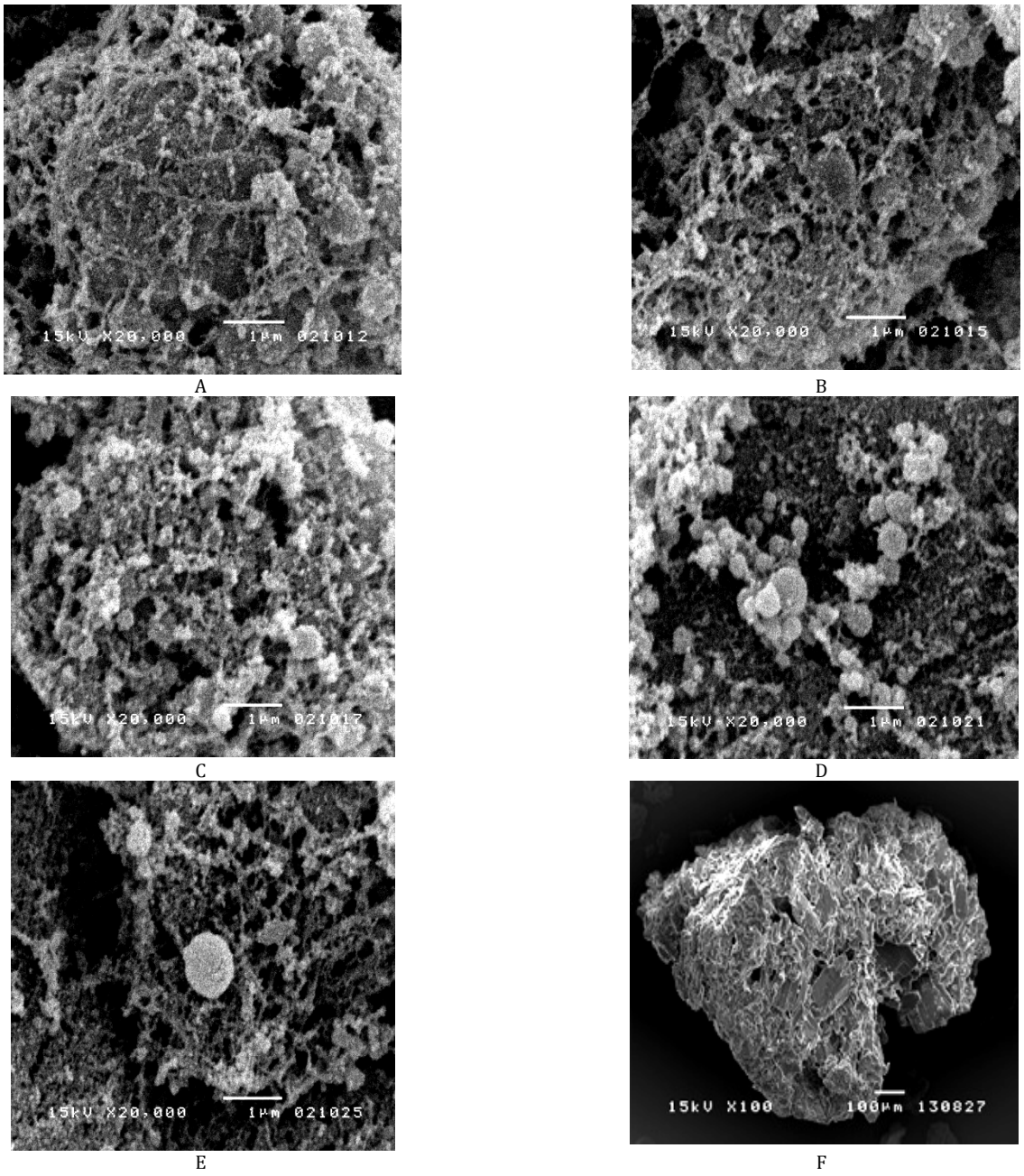

Fig. 3: Porcine intestinal tissues incubated with proliposome granules at 0 (A), 5 (B), 10 (C), 15 (D) and 20 (E) minutes, respectively. Proliposome granules was photographically taken as a reference (F)

Table 3: Physical properties of proliposome tablets

Parameters	Formulation code (Mean±SD)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Thickness (mm)	4.70 (0.049)	4.37 (0.012)	4.62 (0.041)	4.10 (0.045)	4.14 (0.048)	4.44 (0.093)	4.11 (0.01)	4.11 (0.02)	4.08 (0.01)
Diameter (mm)	10.26 (0.025)	10.17 (0.013)	10.22 (0.037)	10.24 (0.032)	10.23 (0.033)	10.25 (0.036)	9.937 (0.01)	9.948 (0.02)	9.947 (0.01)
Weight (mg)	350.56 (0.923)	349.63 (0.871)	349.98 (1.060)	350.52 (0.761)	351.23 (2.037)	350.67 (0.814)	349.05 (0.59)	348.76 (0.67)	348.77 (0.23)
Friability (%)	0.247	0.153	13.69	0.38	0.498	6.298	0.10	0.22	0.19
Disintegration time (min)	1.7 (0.752)	9.10 (1.296)	5.8 (1.427)	3.5 (0.643)	8.1 (2.090)	12.1 (3.411)	7.5 (1.62)	11.2 (1.41)	18.6 (1.68)

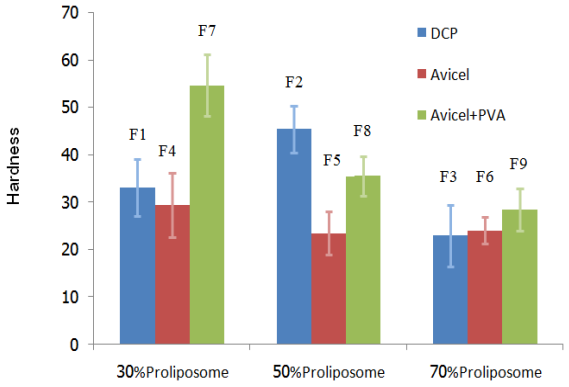

Fig. 4: Hardness of Tablet formulation

CONCLUSION

The granular size after dose adjustment, the flow-ability, the moisture content and the adsorption isotherm of granules should be robustly considered for the proliposome industrial quality control. The effect of protein dose on size of proliposome granules was more pronounced than the effect of lipid. The quality control of granule after protein dose adjustment should be more concerned in the industrial scale. The fair flow-ability with the moisture content of less than 1.5% allowed the satisfied quality control in the industrial scale of proliposome granules preparation. BET adsorption isotherm of type V could be exploited as a fingerprint in the quality control of proliposome batch in the industry. The reconstitution and liposomal formation on small intestine was initially observed at 10 min onward. In the tablet compression of proliposome, the addition of PVP as dry binder along with Avicel® could increase the granules adhesion resulted into the tablet of suitable hardness for further experiment of enteric coating.

CONFLICT OF INTERESTS

Declared None

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