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Silica nanoparticle coated liposomes: A new type of hybrid nanocapsule for proteins

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ABSTRACT

A hybrid silica–liposome nanocapsule system containing insulin has been developed and the encapsulation, protection and release properties are evaluated. The formulation strategy is based on using insulin-loaded 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and cholesterol liposomes as a template for the deposition of inert silica nanoparticles. The influence of formulation and process variables on particle size, zeta potential and liposome entrapment of insulin is reported. The ability to protect against lipolytic degradation and sustain insulin release *in vitro* in simulated GI conditions is also reported. Depending on the concentration and charge ratio of liposomes and silica nanoparticles, nanoparticle coated liposomes with varied size and zeta potential were obtained with an insulin entrapment efficiency of 70%. The silica nanoparticle coating protected liposomes against degradation by digestive enzymes *in vitro*; the release rate of insulin from silica coated liposomes was reduced in comparison to uncoated liposomes. Thus the liposomal release kinetics and stability can be controlled by including a specifically engineered nanoparticle layer. Silica nanoparticle–liposomes hybrid nanocapsules show promise as a delivery vehicle for proteins and peptides.

1. Introduction

Liposomes have been extensively studied as potential drug delivery systems, because of their unique properties such as the ability to protect drugs from degradation, specific targeting, minimized toxicity or side effects, reasonable ease in manufacturability, biocompatibility and versatility in carrying drugs such as nucleic acids, proteins, and chemotherapeutic agents such as doxorubicin and antibiotics with narrow therapeutic windows (Smyth Templeton, 2002; Li et al., 2009; Hashida et al., 2005; Gupta and Moulik, 2008; Iwanaga et al., 1997; Takeuchi et al., 1998). For the successful design and development of liposome-based formulations, their colloidal stability and the chemical stability of lipids are critical factors that have to be considered and optimized.

Liposomes suffer physical instability during manufacturing and storage. Vesicle aggregation, fusion and creaming are the most problematic destabilization processes that lead to liposomal size increase, thereby limiting liposomes as effective drug delivery vehicles. It is therefore desirable for a liposome formulation that the size distribution of the vesicles remain relatively constant over time. Liposome use in oral delivery is limited due to a range of problems including; low encapsulation efficiency, poor storage stability and degradation in gastric media and duodenal enzymes (Barratt, 2000; Takeuchi et al., 2001). In addition, liposome phospholipids are sensitive to acidic or basic hydrolysis and unsaturated phospholipids are susceptible to oxidative degradation. Two types of chemical degradation reactions can affect the performance of phospholipid bilayers; hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and peroxidation of unsaturated acyl chains. The oxidation and hydration of lipids may lead to a decrease in the quality of liposomes along with drug degradation in the intestine during this process resulting in reduced absorption, poor bioavailability and inefficient delivery (Brayden, 2001; Kisel et al., 2001; Takeuchi et al., 2001).

A number of systems have been developed to improve the stability of liposomes, e.g. PEGylated liposomes (Rosen and Abribat, 2005), polymerosomes (Discher and Eisenberg, 2002), polymer caged liposomes (Lee et al., 2007), lyophilosomes (Daamen et al., 2007), polyelectrolyte layer-by-layer assembly (Peyratout and Dahne, 2004) and colloidosomes (Dinsmore et al., 2002; Noble et al., 2004). Liposil are hollow silica nano-shell systems obtained by chemically assembling silica layers onto the external surface of liposomes and have attracted considerable research interest due to their ability to retain the fundamental properties of free liposomes (Zhang and Granick, 2006; Begu et al., 2007). Zhang and Granick used hydrophilic negatively charged carboxyl-modified polystyrene latex nanoparticles to stabilize liposomes against fusion and to stop liposomes from leaking their contents (Zhang

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and Granick, 2006). These particle-stabilized liposomes encapsulated the fluorescent dye (Rhodamine B) without any leakage for 4 days and facilitated suspension stability, i.e. negligible fusion for up to 50 days. However, there is a limited work presented in the literature where well defined liposomes are used as templates for the deposition of silica nanoparticles from aqueous solution to obtain silica nanoparticles coated liposomes (SNCL).

Silica nanoparticles have been extensively used as stabilizers for emulsion droplets (Simovic and Prestidge, 2003, 2004, 2008; Prestidge and Simovic, 2006; Binks and Whitby, 2005; Binks and Lumsdon, 1999) and their ability to control or facilitate enhanced drug dissolution has been previously reported (Simovic and Prestidge, 2007). Silica nanoparticle layers successfully prevented coalescence of emulsion droplets by forming stable networks of emulsion droplets and creating a rigid protective barrier at the liquid–liquid interface (Ghouchi Eskandar et al., 2007; Prestidge and Simovic, 2006). Enhanced *in vitro* dissolution and *in vivo* absorption of the poorly soluble drugs, celecoxib (Tan et al., 2009) and indomethacin (Simovic et al., 2009) from silica–lipid hybrid microcapsules has been reported; this was considered due to the stabilizing effect and nanostructure introduced by silica nanoparticles. Surface functionalized mesoporous silicon microparticles have been used to improve the dissolution and oral absorption of the poorly soluble drug, indomethacin (Wang et al., 2010).

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. It is used in the management of diabetes mellitus and has been applied therapeutically, via parenteral routes. An oral dosage form is the preferred mode of delivery because of ease of administration, patient compliance and cost. In this study we aim to generate novel hybrid liposome carriers for insulin. Specifically, nanoparticulate layers are employed to improve the pharmaceutical performance of liposomes, i.e. by developing a hybrid nanocapsule using liposome as a template for the deposition of silica nanoparticles. The major focus is optimization of liposome stability, demonstration of structural and dynamic property retention, effective encapsulation and protection of insulin under simulated gastro-intestinal delivery conditions, and enhanced storage stability.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Avanti Polar Lipids, USA), cholesterol with a purity of >95% were supplied from Lab Chem, Australia. Chloroform and sodium chloride as used for liposome preparation were of analytical grade. Colloidal silica (Ludox) is supplied by Sigma–Aldrich (USA) have a surface area of ~135 m^2/g and a particle size of 20 ± 4 nm. Insulin from bovine pancreas, pancreatin from porcine pancreas, egg lecithin, sodium taurocholate and Bradford protein assay kit were obtained from Sigma–Aldrich (Sydney, Australia). All other solvents and materials were laboratory grade and of the highest purity available. Milli-Q water was used throughout this study.

2.2. Preparation of silica nanoparticles coated liposomes (SNCL)

SNCL were prepared in a two-step process. Firstly, multilamellar vesicles were prepared using the conventional thin film hydration method (Bangham, 1980) and then the liposomes were coated with silica nanoparticles. A lipid mixture, composed of DPPC and cholesterol in a 7:3 molar ratio, was suspended in solution containing chloroform and evaporated to remove chloroform. A 1 mM NaCl solution in 1% acetic acid (with or without insulin) was added to hydrate the phospholipid film at above the transition temperature of the lipid components to obtain a suspension. The liposome suspension was then subjected to ten passes through polycarbonate membranes of 200 nm pore diameter (Lipex™ extruder, Canada) to obtain highly monodisperse (pdi < 0.25) and unilamellar liposomes. The liposome suspension was then centrifuged at 30,000 rpm for 45 min at 4 °C to remove supernatant. To the sedimented liposomes, 1.33 mM NaCl solution was added (i.e. to maintain the same ionic strength) followed by various concentrations of hydrophilic silica nanoparticles (20 ± 4 nm) dispersed in Milli-Q water. These were tumbled overnight to facilitate adsorption equilibrium of the silica onto the surface of liposomes. Throughout our study the lipid concentration (3.67 mg/mL) was kept constant and mixed with the following concentrations of silica (0, 0.01, 0.1, 1, 10, 20, 50, and 100 mg/mL); the resultant silica:lipid mass ratios are given in Table 1.

2.3. Particle size and zeta potential

The interaction between silica nanoparticles and liposomes was monitored by measuring the zeta potential and particle size of liposomes as a function of the silica:lipid mass ratio. The size and size distribution of both uncoated liposomes and SNCL were determined by photon correlation spectroscopy using a Zetasizer Nano instrument (Malvern Instruments, Malvern, UK). The zeta potential of particles was determined by laser Doppler anemometry using a

Table 1
Composition of uncoated liposomes and SNCL with/without insulin and the influence of silica on particle size and zeta potential.

Sample	Lipid content of liposome suspension (mg/mL)	Silica:lipid mass ratio	Type (empty/insulin loaded)	Mean particle size (nm)[a]	PDI	Zeta potential (mV)
Uncoated	3.67	0	Empty	118	0.61	21 ± 7
			Loaded	235	0.25	25 ± 7
SNCL 1	3.67	0.0068	Empty	146	0.17	20 ± 7
			Loaded	360	0.24	27 ± 6
SNCL 2	3.67	0.068	Empty	396	0.54	18 ± 3
			Loaded	441	0.29	24 ± 4
SNCL 3	3.67	0.68	Empty	3060	1.00	−7 ± 5
			Loaded	2128	0.93	3 ± 5
SNCL 4	3.67	1.36	Empty	295	0.21	−16 ± 4
			Loaded	787	0.54	−13 ± 6
SNCL 5	3.67	3.40	Empty	255	0.32	−14 ± 3
			Loaded	297	0.38	−15 ± 4
SNCL 6	3.67	6.81	Empty	190	0.37	−15 ± 5
			Loaded	255	0.35	−16 ± 6

[a] Size measurement data are the average value of three measurements +SD ($n = 3$).

Zetasizer Nano. For each sample, the mean ± SD for 3 repeat measurements was established.

2.4. Morphology and surface composition

The morphology and elemental analysis of SNCL were characterized using freeze-fracture scanning electron microscopy (FESEM) (Philips XL 30 FEG scanning electron microscope with Oxford CT 1500 cryotransfer system) coupled with X-ray microanalysis (EDAX Genesis v.5.21). In general, samples (50 μL) were deposited on a flat copper substrate holder and cryofixed by rapid cooling with liquid nitrogen (−196 °C) to reach the vitreous state. The frozen sample was fractured using freeze fracture equipment and the surface ice was removed by sublimation, i.e. by increasing the sample temperature to −92 °C for 2 min. The fractured sample was then coated with platinum, prior to SEM imaging.

2.5. Encapsulation efficiency of insulin

Insulin-loaded liposomes were separated by ultracentrifugation at 30,000 rpm for 45 min at 4 °C and liposomes entrapping insulin and free insulin were collected. The amount of insulin entrapped in the liposomes was calculated by the difference between the total amount of protein in the initial formulation medium and the free protein, using the Bradford protein assay (Smith et al., 1985). The principle of the assay is based on the proportional binding of the dye Coomassie blue to proteins. This method is rapid, sensitive and well established for determination of total concentrations of amino acids in biological samples. The protein concentration of a test sample was determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile with correlation co-efficient value (R^2) greater than 0.99. The insulin encapsulation efficiency (EE) was then calculated from Eq. (1).

$$\text{EE}(\%) = \frac{\text{Total amount of insulin} - \text{Free insulin}}{\text{Total amount of insulin}} \times 100 \quad (1)$$

The method, which produced the highest EE, was used for preparation of SNCL.

2.6. Lipolysis study

In vitro lipid digestion studies were conducted in digestion media designed to simulate fasted intestinal conditions (1.25 mM phosphatidylcholine (PC) and 5 mM sodium taurodeoxycholate (BS) in a digestion buffer comprising 50 mM trizma maleate, 150 mM NaCl, and 5 mM $CaCl_2 \cdot 2H_2O$ at pH 7.4) and were conducted in a similar manner to those described by Sek et al. (2002). The liposome suspension (1.5 mL) was then added to 18 mL of simulated fasted intestinal medium, and the pH adjusted to 7.400 ± 0.005, prior to addition of 20,000 TBU equivalents of porcine pancreatin enzyme in 2 mL of digestion buffer. Addition of enzyme stimulate lipid digestion, and the progress of digestion was followed using a pH stat (Radiometer Pacific, Australia) by monitoring the volume of titrant (0.2 M NaOH) required to maintain the pH at 7.400. Titration profiles for the blank, lipid free medium (i.e., 18 mL of simulated fasted intestinal medium alone) were subtracted from the uncoated and SNCL sample profiles to obtain digestion profiles of the lipids alone. The lipid concentration hydrolyzed in the digestive media at set time points was then plotted as a function of time.

2.7. In vitro protein release study

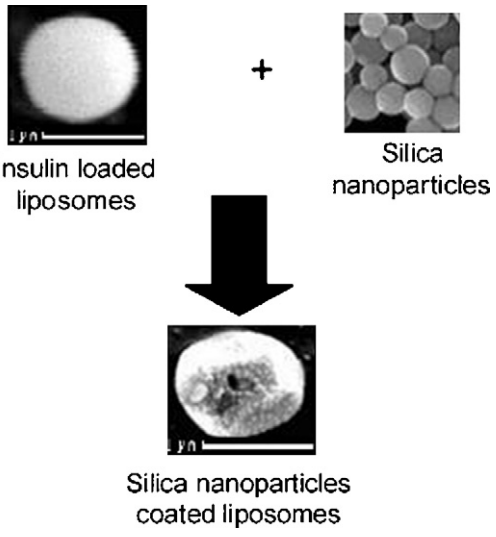
A known quantity (1 mL) of insulin-loaded uncoated liposomes and SNCL were suspended and incubated in either 4 mL of phosphate buffer pH 7.4, or simulated gastric fluid at pH 2.0, each with controlled agitation at 37 °C. An equivalent method of release study was used previously for release of insulin from polysaccharide nanoparticles (Sarmento et al., 2007). The concentration of insulin used by Sarmento et al. was 4 mg in 20 mL (200 μg/mL), whereas in our case 100 μg in 5 mL was used. At designated time intervals, samples were centrifuged (21,000 rpm for 20 min at 4 °C) and 500 μL of the supernatant removed and replaced by an equal volume of fresh medium. The amounts of insulin released at various time intervals (5, 15, 30, 45, 60, 120, 240, 360, and 480 min) were determined. All measurements were performed in triplicate. The empty uncoated liposomes along with empty SNCL were incubated at 37 °C and analyzed by the same method to act as controls.

3. Results and discussion

3.1. Preparation of silica nanoparticles coated liposomes (SNCL)

DPPC/cholesterol liposomes of average diameter in the range of 100–200 nm were readily formed at 5 mM concentration using the thin film hydration method followed by extrusion. On inclusion of charged silica nanoparticles, adsorption to the phospholipid bilayer membrane leads to the formation of a nanoparticle–liposome hybrid structure as shown in Scheme 1. The preparation described is primarily based on electrostatic interaction between liposomes and silica nanoparticles resulting in the formation of regular structures with diameters in the range 150–500 nm. The strong electrostatic interaction between the positively charged lipid head groups and the negatively charged silica particles is the main driving force for the templating (Begu et al., 2004, 2007). It is noted that the formation of SNCL is strongly influenced by the lipid and silica ratio. In general, the formation of small SNCL (<500 nm) was confined to specific silica:lipid mass ratios of 0.0068, 0.068, 3.40 and 6.81. The mean diameter of the liposomes in suspension (Table 1 and Fig. 1) increased from around 120 nm in the absence of silica nanoparticles to >3000 nm at a silica:lipid mass ratio of 0.68, indicating the formation of large aggregated structures. These aggregates eventually phase separated and formed a precipitate.

In line with the electrostatic mechanism for nanoparticle coating, the zeta potential of the liposomes decreased significantly from +24.8 to −15.8 mV on addition of increasing levels of silica nanoparticles (Table 1 and Fig. 1). The reduction in zeta potential indicates the strong adsorption of hydrophilic silica nanoparticles due to electrostatic interaction with the phospholipid head group



Scheme 1. Scheme of the experimental strategy used to produce insulin-loaded SNCL.

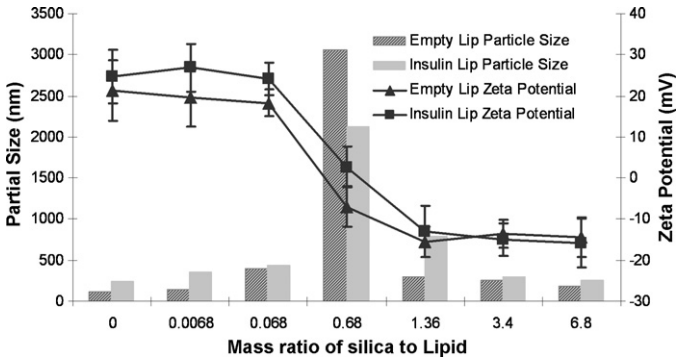


Fig. 1. Influence of silica nanoparticles concentration on the particle size and zeta potential of empty and insulin-loaded 5 mM DPPC liposomes.

(Prestidge and Simovic, 2006; Zhang and Granick, 2006). From our zeta potential results, we could identify three distinct interaction regions of silica nanoparticles and liposomes: (i) up to a silica to lipid mass ratio of 0.068 the liposomes are positively charged (>+18 mV) and show no significant changes in size. This positive charge proves that at low silica:lipid ratio, the levels of silica nanoparticles are less than those necessary for monolayer coverage, resulting in partial coverage of liposomes by nanoparticles; (ii) at a silica:lipid mass ratio of 0.68 the net charge on the liposomes was close to zero (±6 mV). Such charge neutralization causes destabilization of the liposomes due to an increase in density and a decrease in interparticle repulsion, resulting in formation of large aggregates; (iii) at silica:lipid mass ratios >1.36 liposome charge reversal occurs ($\zeta = -13$ mV), suggesting complete coating, and resulting in electrostatic repulsive forces between the negatively charged silica coated liposomes that overcome the attractive forces and redisperses the liposomes. In addition, at high silica:lipid ratios (>3) weak aggregation of thickly coated, charge-reversed liposomes followed by phase coacervation occurs. It suggest at higher silica:lipid ratios (3.40 and 6.81), silica nanoparticles adsorbed to the liposome surface until the liposomal membrane was fully covered thereby preventing further adsorption and the excess silica act as a colloidal protectant via steric hindrance, preventing SNCL from coagulation. This is the reason for decreased particle diameter (297 and 255 nm) for SNCL of higher silica:lipid mass ratios (3.40 and 6.81), compared to SNCL of intermediate silica:lipid ratios (0.68 and 1.36).

Equivalent variation of size and zeta potential was observed for insulin-loaded liposomes. The incorporation of insulin into liposomes caused an increase in both size and zeta potential of uncoated and SNCL, compared to the unloaded liposomes (Fig. 1). This significant increase in particle size and zeta potential give a good indication of the incorporation of insulin and suggest that the protein formed a close association with the liposome matrix materials.

3.2. Incorporation of insulin into liposomes

Insulin is practically insoluble in water at pH 7 and dissolves only in dilute mineral acids. Hence, in our study we used pH 3.0 as the formulation medium. At this pH, insulin is positively charged (isoelectric point of 5.3) and electrostatic repulsion would occur with DPPC and the encapsulation efficiency (EE) is anticipated to be low or moderate. We obtained an EE ~70%, which compares to values reported by Zhang et al. (30.3%) and Bi Ru et al. (43.6%) for insulin-loaded liposomes (Zhang et al., 2006; Bi et al., 2008). Zhang et al. also reported a significant increase in insulin EE to 69.3 and 82.5% when the liposomes are modified with wheat germ agglutinin (WGA) and tomato lectin, respectively (Zhang et al., 2006). At neutral pH insulin is negatively charged while both tomato lectin and WGA are positively charge, and increased liposome's EE due to electrostatic attraction.

Generally, protein encapsulation is optimized at pH values greater than the protein's isoelectric point. In addition to protein-liposome association by electrostatic interaction, other forces including hydrogen bonding, hydrophobic interactions and the reduction of protein solubility near its isoelectric point play a role in encapsulation. E.g. the liposomal encapsulation efficiency of an active substance can be significantly increased either by using a long carbon chain lipids (Kim and Han, 1995) or cholesterol in the formulation (Kim et al., 1999). In our case, both these approaches are used to improve insulin incorporation, i.e. the long carbon chains of DPPC and presence of cholesterol enhance the interaction of insulin with the liposomes and also increase the liposomal membrane rigidity.

3.3. Morphology of empty and insulin-loaded SNCL

Typical freeze-fracture SEM images of insulin-loaded uncoated liposomes and SNCL (Fig. 2) show that they have a spherical structure. The uncoated liposomes showed a non-smooth spherical structure, while the silica coated liposomes exhibited a dense and spherical structure with rough surfaces (i.e. structured nanoparticle surface layers are visible), indicating the presence of silica nanoparticles distributed across the liposome surface. The particle size increase from uncoated liposomes loaded with insulin to the SNCL occurred as a consequence of the formation of silica nanoparticle (Ludox®) coatings.

Elemental analysis (EDAX) data (Fig. 2) from the liposome surface clearly showed an increasing Si peak intensity upon increasing silica coating levels, i.e. no Si signal is observed for uncoated liposomes and the Si peak intensity increases with the silica:lipid mass ratio of SNCL. These findings confirm our understanding of silica nanoparticles adsorption on liposome surfaces and the formation of an interfacial layer with increased surface coverage at high silica:lipid ratio compared to the low.

3.4. Lipolysis study

Lipid degradation is a critical process in determining the *in vivo* fate of liposomes. In the body, the sn-2 fatty acyl bonds of liposomal phospholipids are hydrolyzed by enzymes to liberate free fatty acids and lysophospholipids. We have quantified the enzymatic degradation (hydrolysis) of phospholipids in uncoated liposomes and SNCL under simulated gastro-intestinal conditions. The extent of lipid hydrolysis and corresponding pseudo-first-order kinetic plots of the formulations in digestive media with porcine pancreatin enzyme are presented in Fig. 3. The digestion profile of both uncoated and SNCL 2 exhibited rapid digestion, i.e. ~80% over the initial 15 min, followed by relatively slow digestion of remaining 20% over the following 30 min. In general, at all the time points, the extent of lipid digestion for SNCL 2 was lower than for the uncoated liposomes, though this is not highly significant. This marginal resistance of SNCL 2 to enzymatic degradation is considered due to the partial silica nanoparticle coverage blocking enzyme action. For more complete nanoparticle layers a more pronounced reduction in lipid digestion was observed, e.g. the extent of lipid digestion was significantly lower for SNCL 5 than SNCL 2 and uncoated liposomes, i.e. 10% digestion in the initial 15 min followed by another 10% in the next 15 min after which virtually no digestion occurred. An equivalent enzyme effect has been observed for triglyceride droplets (Tan et al., 2010).

Muller et al. and Garcia et al. reported that anchoring of lipase to the surface of solid lipid nanoparticles is a prerequisite for enzymatic degradation (Müller et al., 1996; García-Fuentes et al., 2003).

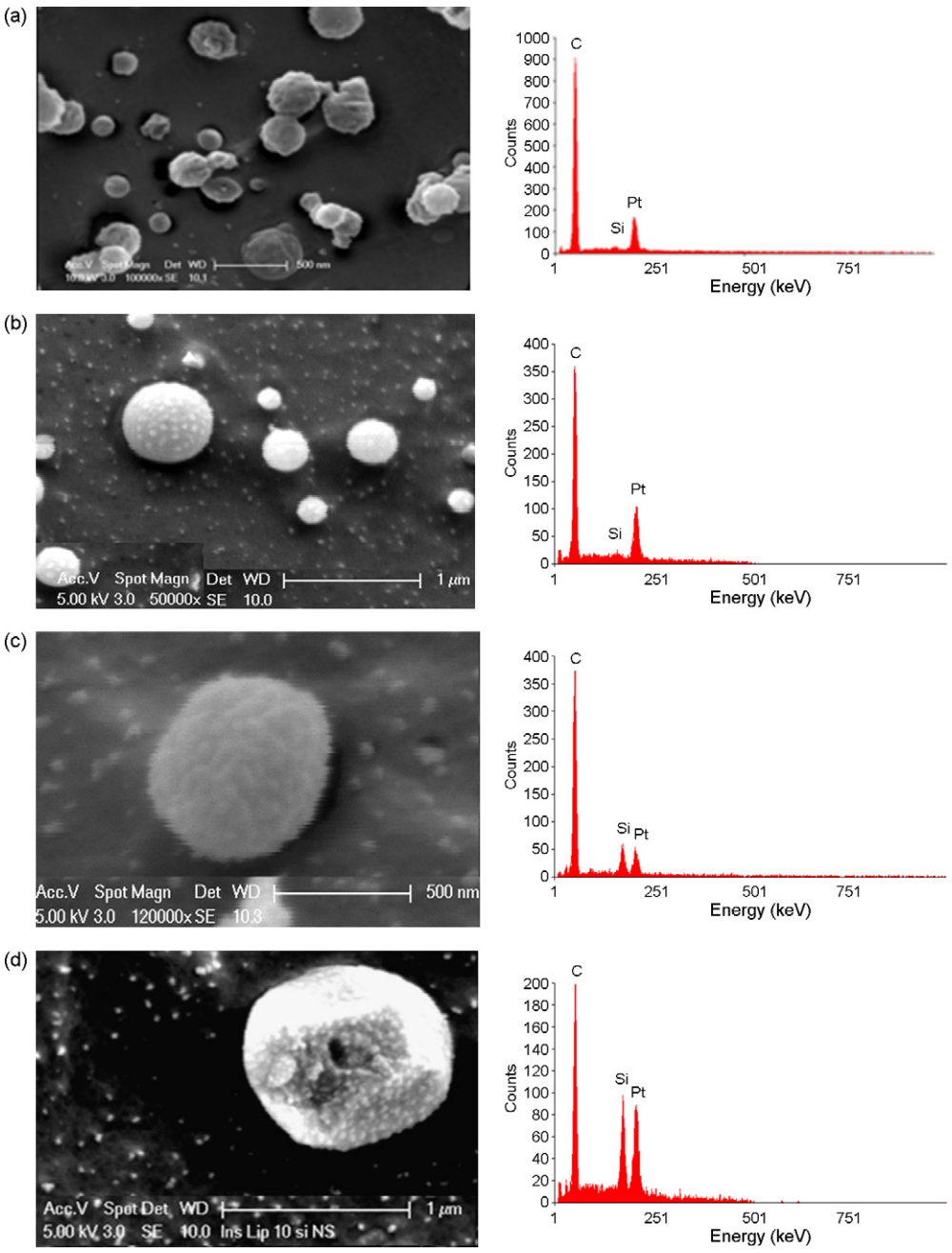


Fig. 2. Freeze-fracture SEM images of uncoated and SNCL coupled with corresponding EDAX spectrum obtained from selected area on liposome surface: (A) Uncoated; (B) SNCL 2; (C) SNCL 5; (D) SNCL 6.

It was reported that the structural irregularities present in strongly curved bilayers of uncoated DPPC liposomes increased the binding affinity of enzyme and hence increase lipid hydrolysis (Kisel et al., 2001). One proposed mechanism for the action of silica nanoparticle coatings on the digestion of liposomes is reduced enzyme attachment and hence resulting in reduced degradation compared to uncoated liposomes. The low lipid degradation of coated liposomes suggests a physical shielding effect of silica nanoparticles at the liposome interface, providing a protective effect to the system.

A pseudo-first order kinetic model (Eq. (2)) was used to describe lipid degradation.

$$[L]_t = [L]_o e\left(\frac{-k}{RT}\right) \quad (2)$$

where L_o is the initial amount of lipid, L_t is the amount of lipid hydrolyzed in time t, k is the degradation rate constant for pseudo-first order model, R is the universal gas constant and T is the absolute temperature. Fits are shown in Fig. 3b, with the corresponding rate constants for lipolysis (k) given in Table 2. The linearity ($R^2 > 0.99$) of the plots in Fig. 3b confirms that lipid digestion kinetics are pseudo-first-order in nature. Uncoated liposomes and SNCL 2 fitted

Table 2
Pseudo-first order lipid digestion rate constant (k) of uncoated liposomes and SNCL.

Liposomes sample	Lipid digestion rate constant k (min^{-1}) ± SD
Uncoated	0.179 ± 0.105
SNCL 2	0.176 ± 0.112
SNCL 5	0.063 ± 0.006

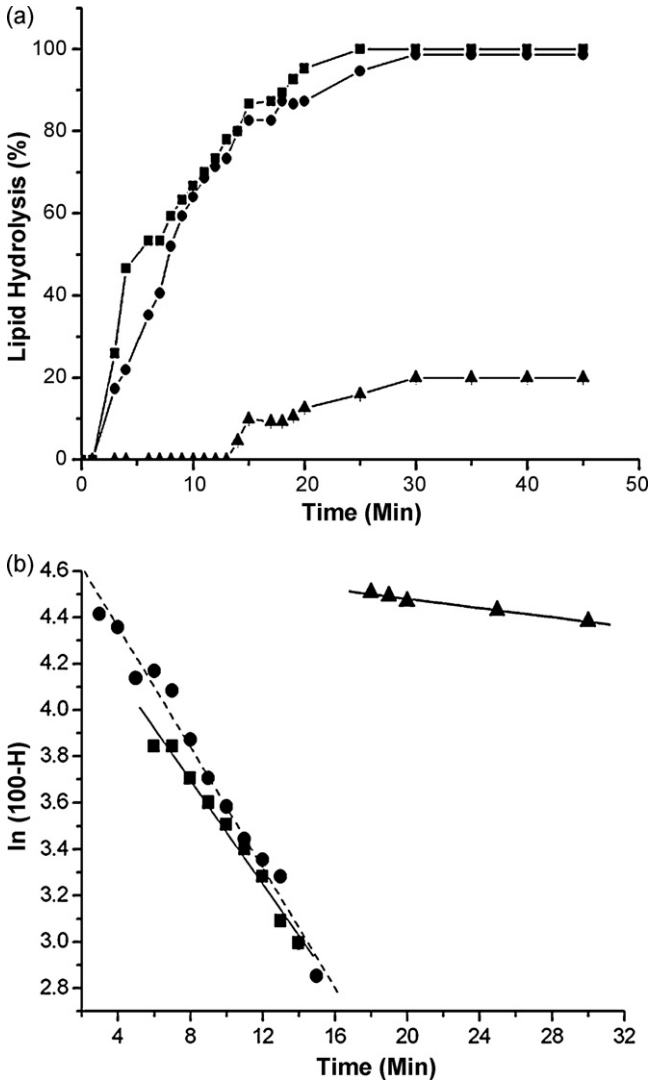


Fig. 3. (A) Percentage of lipid digested against time after incubation of liposomes (■ Uncoated, ● SNCL 2 and ▲ SNCL 5) in simulated lipid digestive media. (B) Pseudo-first order kinetic plots for uncoated and SNCL. The lines represent best fits to the data.

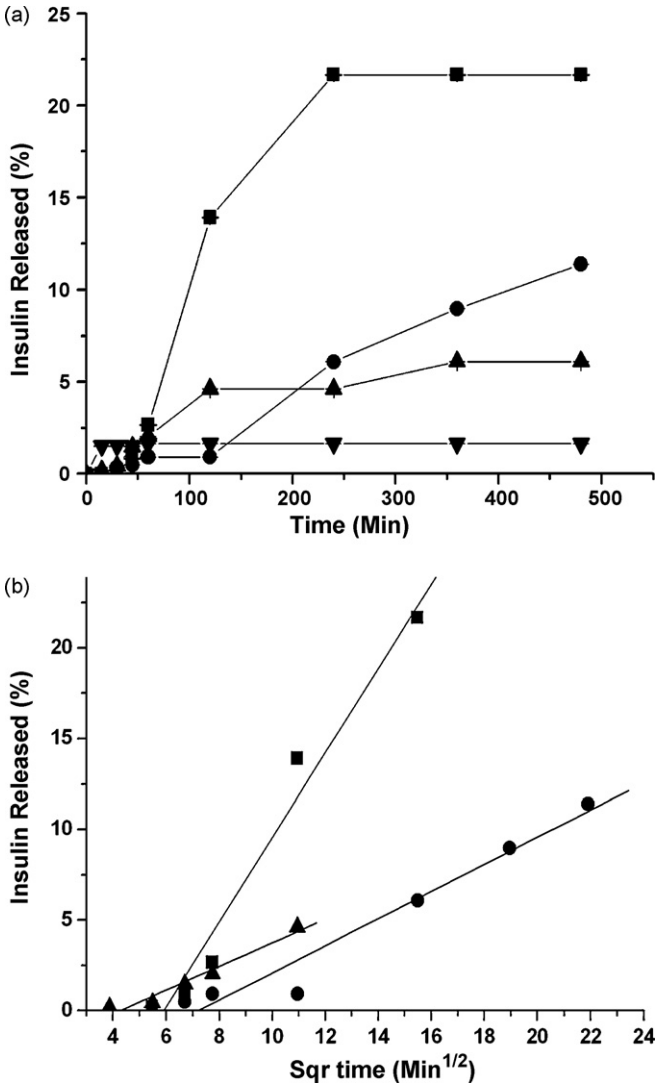


Fig. 4. (A) Release profile of insulin from liposomes (■ Uncoated, ● SNCL 2, ▲ SNCL 5 and ▼ SNCL 6) in SGF pH 2.0. Each point on the curve is the mean of at least three experiments (bars represent SD values). (B) The linear fits of the release data against square root of time.

the model between time points 5 and 15 min and exhibited equivalent lipid digestion rate constants. In contrast, SNCL 5 followed pseudo-first-order behaviour between 18 and 32 min and with a significant reduction in lipid digestion rate constant. The relatively high silica:lipid mass ratio of SNCL 5 and hence the more completely covered interfacial silica layer on the liposome facilitates low levels of lipid digestion and a high level of stabilization.

The resistance of SNCL 5 against enzymatic degradation confirms that a critical silica coverage is required to significantly reduce lipolysis, in agreement with the previous observations that a high coverage of nanoparticles results in enhanced stabilization and protection compared to a low surface coverage (Simovic and Prestidge, 2003; Zhang and Granick, 2006; Tan et al., 2009). For oral delivery, silica is a good tool due to its resistance to acidic pH and enzymatic degradation (Barbé et al., 2004). Since silica is stable in the presence of enzymes, the SNCL show improved chemical stability in this destabilizing environment. In addition, it has been reported for solid lipid nanoparticles that particle size has an influence on enzymatic degradation (i.e. larger sized particles degrade faster). Here we show that the larger sized SNCL exhibit lower lipid degradation than uncoated liposomes, which supports Olbrich's claim that the stabilizer is the determining factor for degradation and particle size differences have little effect or no effect (Schwab et al., 2009; Olbrich et al., 2002).

3.5. *In vitro release studies*

Insulin release profiles from uncoated liposomes and SNCL in simulated gastric fluid (SGF) and phosphate buffer at 37 °C, are presented in Figs. 4a and 5a. Significant differences in the release profiles of coated and uncoated liposomes were observed. In SGF (pH 2.0), during the initial 45 min there is negligible insulin release, i.e. all formulations are stable, and after 1 h insulin release occurred slowly and continuously for up to 8 h. The uncoated liposomes released 13.9% of insulin in 2 h and further 7.0% over the next 6 h. SNCL 2, SNCL 5 and SNCL 6 released significantly less insulin, i.e. ~11.0, 6.0 and 1.6%, over 8 h, respectively. It is clear that hydrophilic silica nanoparticles create an interfacial layer that significantly retards insulin release from liposomes and that this is considerably more pronounced at increased surface coverage, i.e. at higher silica:lipid ratios. Thus, depending on the silica concentration during preparation, hydrophilic silica coatings can be engineered to

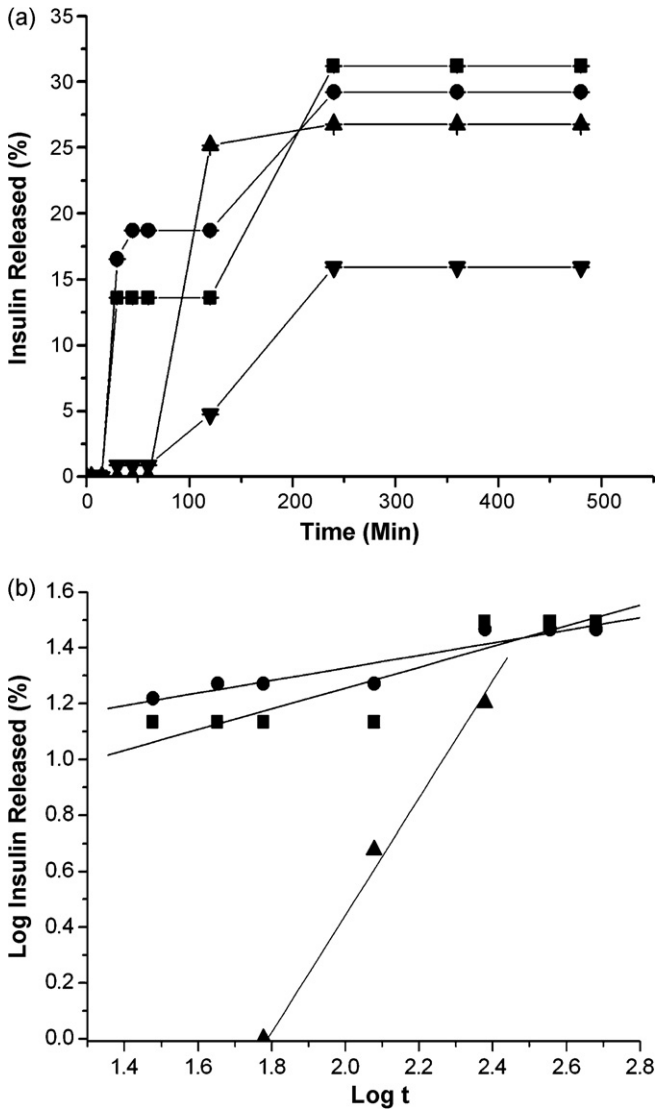


Fig. 5. (A) Release profile of insulin from liposomes (■ Uncoated, ● SNCL 2, ▲ SNCL 5 and ▼ SNCL 6) in phosphate buffer pH 7.4. Each point on the curve is the mean of at least three experiments (bars represent SD values). (B) The linear fits of the log of release data against log time.

control liposome permeability. Possible mechanisms for this phenomenon include: (i) formation of a physical barrier, (ii) the silica nanoparticles form electrostatic bridges between the lipid head groups and make the bilayer less permeable and (iii) the insulin is transported through the lipid bilayer and then restricted from release into solution due to interaction with the silica nanoparticles. Ongoing studies are directed at de-convoluting these mechanisms.

Insulin release kinetics for uncoated and SNCL in phosphate buffer at pH 7.4 (Fig. 5) exhibit a two-step release pattern, i.e. an initial rapid release during the first 2 h followed by a delayed or slow release for up to 8 h. These findings are consistent with those obtained by Sarmento et al., who reported insulin-loaded chitosan/dextran sulfate nanoparticles retained insulin for 2 h at gastric pH followed by a two-step release pattern in pH 7.4, i.e. an initial burst release during the first hour with delayed release over the next 6 h (Sarmento et al., 2007). Insulin release from uncoated liposomes and SNCL in phosphate buffer was significantly more pronounced than in SGF, confirming that DPPC liposomes provide a higher insulin retention capacity in SGF.

It is known that DPPC membranes become protonated at pH ≤ 2.0 and hence the phosphotidyl choline membrane has a positive charge (Jänchenová et al., 2007). SNCL have uniform and dense coatings of silica nanoparticles in simulated gastric conditions; this is considered to reduce the insulin release in comparison to phosphate buffer (simulated intestinal) conditions. Slow release of insulin in SGF compared to phosphate buffer (SIF) is desirable for oral delivery, since it results in reduced insulin gastric degradation and an increase in the active molecule available for absorption in the intestine. Many researchers have used this strategy to developing particulate delivery systems for insulin, e.g. muco-adhesive multivesicular liposomes (Sarmento et al., 2007), polymethacrylic acid alginate microparticles (Sajeesh and Sharma, 2004) and hydroxypropyl methylcellulose phthalate enteric coated microspheres (Qi and Ping, 2004). With this in mind, the insulin release performance of formulations SNCL 5 and 6 protect insulin from gastric degradation and facilitate controllable release in the upper intestine; hence further investigations as oral delivery vehicles are justified.

Finally to gain further insight into the insulin release mechanism from uncoated liposomes and SNCL in SGF and phosphate buffer, we have employed both the Higuchi (Eq. (3)) and Peppas (Eq. (4)) drug release kinetics models:

$$m = kt^{1/2} \tag{3}$$

$$\frac{M_t}{M_\infty} = at^n \tag{4}$$

where m is the amount of drug (%) released in time t, M_t/M_∞ is the fractional drug release of, a is a constant incorporating structural and geometrical characteristics of the dosage form, k is the Higuchi release rate constant and n is the release exponent, used to characterize the release mechanism for insulin in the current study.

The Peppas model is generally used if the release mechanism is not known or when more than one type of release mechanism is in action. The release exponent (n) has previously been used to interpret the diffusional release mechanism for polymeric thin films, i.e. if the release exponent $n = 0.5$, the drug transport mechanism is by Fickian diffusion and reverts to the Higuchi model, and for higher values of n between 0.5 and 1.0 ($0.5 < n < 1.0$), or $n = 1.0$ or $n > 1.0$, is considered as non-Fickian model and the drug transport mechanism is by anomalous or Case-II (purely relaxation controlled delivery) or Super Case-II transport, respectively (Costa and Sousa Lobo, 2001). This approach has also been used for liposomes with PEG layers (Er et al., 2009).

Kinetic model parameters for the release of insulin from uncoated liposomes and SNCL are presented in Table 3. In SGF, all the formulations (except SNCL 6) followed the Higuchi model, whereas in phosphate buffer all formulations (except SNCL 5) showed a good fit to the Peppas model. SNCL 6 in SGF and SNCL 5 in phosphate buffer were poorly fitted by either Higuchi or Peppas models ($R^2 < 0.90$); this is due to insufficient data points on the release profiles and hence their plots not shown.

Table 3
Values of linear regression co-efficient (R^2), k and n values from the release study data to characterize different release mechanism of insulin from uncoated liposomes and SNCL based on Higuchi and Peppas model analysis.

Liposome sample	SGF pH 2.0 (Higuchi model)		Phosphate buffer pH 7.4 (Peppas model)	
	k	R^2	n	R^2
Uncoated	2.32	0.985	0.31	0.904
SNCL 2	0.07	0.978	0.22	0.930
SNCL 5	0.65	0.981	0.04	0.776
SNCL 6	0.20	0.813	2.09	0.991

The linearized fits for insulin release in SGF and PBS ($R^2 > 0.95$) are presented in Figs. 4b and 5b, respectively. In SGF, insulin release is well described by the Higuchi model between 30 and 180 min for uncoated liposomes, 45 and 480 min for SNCL 2 and 15 and 120 min for SNCL 5, whereas, in phosphate buffer, good fits were exhibited between 30 and 480 min for both uncoated liposomes and SNCL 2, and between 60 and 240 min for SNCL 6. These findings confirm that insulin release in SGF is largely governed by diffusion while release from phosphate buffer is a combined effect of diffusion, an interfacial barrier and erosion. In phosphate buffer, the insulin release mechanism from uncoated and SNCL 2 is by diffusion controlled Fickian release, whereas release from SNCL 6 is considered to be Super Case-II transport ($n > 1$). Sriamornsak et al. have reported this type of transport for theophylline release from alginate gel formed in hard capsules and to occur via an erosion mechanism that results in an increased n value (Pornsak and Srisagul, 2007). These results suggest that the diffusion controlled release mechanism is significant for both uncoated and silica nanoparticle coated liposome system under investigation here.

4. Conclusion

Novel hybrid insulin-loaded SNCL capsules have been prepared under low temperature conditions, by templating DPPC/cholesterol liposomes with silica nanoparticles via electrostatic interaction to facilitate encapsulation, protection and controlled release of insulin. The SNCL obtained have excellent physicochemical characteristics and exhibited increased insulin entrapment efficiency (70%) compared to insulin-loaded liposomes reported in literature. The particle size, surface charge, stability and insulin release of SNCL are dependent on the silica:lipid ratio. At silica:lipid ratios >3, charge-reversed SNCL form with a high coverage nanoparticle coating that provided a significant protection against *in vitro* enzymatic degradation under simulated GI environment conditions. Insulin release kinetics and stability of liposomes can be controlled by engineering hydrophilic silica nanoparticle layers at the liposome interface. Thus, SNCL are a promising candidate for the storage and delivery of proteins and peptides.

References

Bangham, A.D., 1980. Liposomes in Biological Systems. John Wiley & Sons Ltd., Norwich.

Barratt, G.M., 2000. Therapeutic applications of colloidal drug carriers. Pharm. Sci. Technol. Today 3, 163–171.

Begu, S., Girod, S., Lerner, D.A., Jardiller, N., Tourne-Peteilh, C., Devoisselle, J.-M., 2004. Characterization of a phospholipid bilayer entrapped into non-porous silica nanospheres. J. Mater. Chem. 14, 1316–1320.

Begu, S., Pouessel, A.A., Lerner, D.A., Tourne-Peteilh, C., Devoisselle, J.M., 2007. Liposil, a promising composite material for drug storage and release. J. Control. Release 118, 1–6.

Bi, R., Shao, W., Wang, Q., Zhang, N., 2008. Spray-freeze-dried dry powder inhalation of insulin-loaded liposomes for enhanced pulmonary delivery. J. Drug Target., 16.

Binks, B.P., Lumsdon, S.O., 1999. Stability of oil-in-water emulsions stabilised by silica particles. PCCP 1, 3007–3016.

Binks, B.P., Whitby, C.P., 2005. Nanoparticle silica-stabilised oil-in-water emulsions: improving emulsion stability. Colloids Surf. A, 253.

Brayden, D.J., 2001. Oral vaccination in man using antigens in particles: current status. Eur. J. Pharm. Sci. 14, 183–189.

Barbé, C., Bartlett, J., Kong, L., Finnie, K., Lin, H.Q., Larkin, M., Calleja, S., Bush, A., Calleja, G., 2004. Silica particles: a novel drug-delivery system. Adv. Mater. 16, 1959–1966.

Costa, P., Sousa Lobo, J.M., 2001. Modeling and comparison of dissolution profiles. Eur. J. Pharm. Sci. 13, 123–133.

Daamen, W.F., Geutjes, P.J., van Moerkerk, H.T.B., Nillesen, S.T.M., Wismans, R.G., Hafmans, T., van den Heuvel, L.P.W.J., Pistorius, A.M.A., Veerkamp, J.H., van Hest, J.C.M., van Kuppevelt, T.H., 2007. Lyophilisomes: a new type of (bio)capsule. Adv. Mater. 19, 673–677.

Dinsmore, A.D., Hsu, M.F., Nikolaides, M.G., Marquez, M., Bausch, A.R., Weitz, D.A., 2002. Colloidosomes: selectively permeable capsules composed of colloidal particles. Science 298, 1006–1009.

Discher, D.E., Eisenberg, A., 2002. Polymer vesicles. Science 297, 967–973.

Er, Y., Barnes, T.J., Fornasiero, D., Prestidge, C.A., 2009. The encapsulation and release of guanosine from PEGylated liposomes. J. Liposome Res. 19, 29–36.

García-Fuentes, M., Torres, D., Alonso, M.J., 2003. Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. Colloids Surf. B 27, 159–168.

Ghouchi Eskandar, N., Simovic, S., Prestidge, C., 2007. Synergistic effect of silica nanoparticles and charged surfactants in the formation and stability of submicron oil-in-water emulsions. PCCP 9, 6426–6434.

Gupta, S., Moulik, S.P., 2008. Biocompatible microemulsions and their prospective uses in drug delivery. J. Pharm. Sci. 97, 22–45.

Hashida, M., Kawakami, S., Yamashita, F., 2005. Lipid carrier systems for targeted drug and gene delivery. Chem. Pharm. Bull. 53, 871.

Iwanaga, K., Ono, S., Narioka, K., Morimoto, K., Kakemi, M., Yamashita, S., Nango, M., Oku, N., 1997. Oral delivery of insulin by using surface coating liposomes: improvement of stability of insulin in GI tract. Int. J. Pharm. 157, 73–80.

Jänchenová, H., Lhotský, A., Stulík, K., Marecek, V., 2007. Adsorption and ion-pairing interactions of phospholipids in the system of two immiscible electrolyte solutions. Part I. The behaviour of lecithin at the water/1,2-dichloroethane interface, compared with that of trimethyloctadecylammonium cation. J. Electroanal. Chem. 601, 101–106.

Kim, A., Yun, M.O., Oh, Y.K., Ahn, W.S., Kim, C.K., 1999. Pharmacodynamics of insulin in polyethylene glycol-coated liposomes. Int. J. Pharm. 180, 75–81.

Kim, C.K., Han, J.H., 1995. Lymphatic delivery and pharmacokinetics of methotrexate after intramuscular injection of differently charged liposome-entrapped methotrexate to rats. J. Microencapsulation 12, 437–446.

Kisel, M.A., Kulik, L.N., Tsybovsky, I.S., Vlasov, A.P., Vorob'yov, M.S., Kholodova, E.A., Zabarovskaya, Z.V., 2001. Liposomes with phosphatidylethanol as a carrier for oral delivery of insulin: studies in the rat. Int. J. Pharm. 216, 105–114.

Lee, S.-M., Chen, H., Dettmer, C.M., O'Halloran, T.V., Nguyen, S.T., 2007. Polymer-caged lipsomes: a pH-responsive delivery system with high stability. J. Am. Chem. Soc. 129, 15096–15097.

Li, X., Ding, L., Xu, Y., Wang, Y., Ping, Q., 2009. Targeted delivery of doxorubicin using stealth liposomes modified with transferrin. Int. J. Pharm. 373, 116–123.

Müller, R.H., Rühl, D., Runge, S.A., 1996. Biodegradation of solid lipid nanoparticles as a function of lipase incubation time. Int. J. Pharm. 144, 115–121.

Noble, P.F., Cayre, O.J., Alargova, R.G., Velev, O.D., Paunov, V.N., 2004. Fabrication of "hairy" colloidosomes with shells of polymeric microrods. J. Am. Chem. Soc. 126, 8092–8093.

Olbrich, C., Kayser, O., Müller, R.H., 2002. Lipase degradation of Dynasan 114 and 116 solid lipid nanoparticles (SLN)—effect of surfactants, storage time and crystallinity. Int. J. Pharm. 237, 119–128.

Peyratout, C.S., Dahne, L., 2004. Tailor-made polyelectrolyte microcapsules: from multilayers to smart containers. Angew. Chem. Int. Ed. 43, 3762–3783.

Pornsak, S., Srisagul, S., 2007. Modification of theophylline release with alginate gel formed in hard capsules. AAPS PharmSciTech 8, E1–E8.

Prestidge, C.A., Simovic, S., 2006. Nanoparticle encapsulation of emulsion droplets. Int. J. Pharm. 324, 92–100.

Qi, R., Ping, Q.N., 2004. Gastrointestinal absorption enhancement of insulin by administration of enteric microspheres and SNAC to rats. J. Microencapsulation 21, 37–45.

Rosen, H., Abribat, T., 2005. The rise and rise of drug delivery. Nat. Rev. Drug Discov. 4, 381–385.

Sajeesh, S., Sharma, C.P., 2004. Poly methacrylic acid-alginate semi-IPN microparticles for oral delivery of insulin: a preliminary investigation. J. Biomater. Appl. 19, 35–45.

Sarmento, B., Ribeiro, A., Veiga, F., Ferreira, D., Neufeld, R., 2007. Oral bioavailability of insulin contained in polysaccharide nanoparticles. Biomacromolecules 8, 3054–3060.

Schwab, M., Sax, G., Schulze, S., Winter, G., 2009. Studies on the lipase induced degradation of lipid based drug delivery systems. J. Control. Release 140 (1), 27–33.

Sek, L., Porter, C.J.H., Kaukonen, A.M., Charman, W.N., 2002. Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. J. Pharm. Pharmacol. 54, 29–41.

Simovic, S., Heard, P., Hui, H., Song, Y., Peddie, F., Davey, A.K., Lewis, A., Rades, T., Prestidge, C.A., 2009. Dry hybrid lipid–silica microcapsules engineered from submicron lipid droplets and nanoparticles as a novel delivery system for poorly soluble drugs. Mol. Pharm. 6, 861–872.

Simovic, S., Prestidge, C., 2003. Hydrophilic silica nanoparticles at the PDMS droplet-water interface. Langmuir 19, 3785–3792.

Simovic, S., Prestidge, C.A., 2004. Nanoparticles of varying hydrophobicity at the emulsion droplet-water interface: adsorption and coalescence stability. Langmuir 20, 8357–8365.

Simovic, S., Prestidge, C.A., 2007. Nanoparticle layers controlling drug release from emulsions. Eur. J. Pharm. Biopharm. 67, 39–47.

Simovic, S., Prestidge, C.A., 2008. Colloidosomes from the controlled interaction of submicrometer triglyceride droplets and hydrophilic silica nanoparticles. Langmuir 24, 7132–7137.

Smith, P.K., Krohn, R.I., Hermanson, G.T., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76.

Smyth Templeton, N., 2002. Liposomal delivery of nucleic acids in vivo. DNA Cell Biol. 21, 857–867.

Takeuchi, H., Yamamoto, H., Kawashima, Y., 2001. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Deliv. Rev. 47, 39–54.

Takeuchi, H., Yamamoto, H., Toyoda, T., Toyobuku, H., Hino, T., Kawashima, Y., 1998. Physical stability of size controlled small unilameller liposomes coated with a modified polyvinyl alcohol. Int. J. Pharm. 164, 103–111.

Tan, A., Simovic, S., Davey, A.K., Rades, T., Boyd, B.J., Prestidge, C.A., 2010. Silica nanoparticles to control the lipase-mediated digestion of lipid-based oral delivery systems. Mol. Pharm., doi:10.1021/mp9002442.

Tan, A., Simovic, S., Davey, A.K., Rades, T., Prestidge, C.A., 2009. Silica–lipid hybrid (SLH) microcapsules: a novel oral delivery system for poorly soluble drugs. J. Control. Release 134, 62–70.

Wang, F., Hui, H., Barnes, T.J., Barnett, C., Prestidge, C.A., 2010. Oxidized mesoporous silicon microparticles for improved oral delivery of poorly soluble drugs. Mol. Pharm. 7, 227–236.

Zhang, L., Granick, S., 2006. How to stabilize phospholipid liposomes (using nanoparticles). Nano Lett. 6, 694–698.

Zhang, N., Ping, Q., Huang, G., Xu, W., Cheng, Y., Han, X., 2006. Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin. Int. J. Pharm. 327, 153–159.