RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2014, **4**, 58137

Chitosan modified cerasomes incorporating poly (vinyl pyrrolidone) for oral insulin delivery

Yushen Jin,[ab] Hongjie Pan,[b] Yanyan Li[ab] and Zhifei Dai*[a]

The most significant finding of this study is that a hybrid liposomal cerasome with high stability and good biocompatibility was successfully developed for oral insulin delivery by incorporating poly (vinyl pyrrolidone) (PVP) into the cerasome followed by coating with chitosan (CS). These PVP and CS modified cerasomes (PCCs) were positively charged with an average size of about 247.8 ± 3.3 nm. It was found that insulin-loaded PCCs could maintain their size and retain the encapsulated insulin over a period of 90 days, suitable for uptake within the gastrointestinal tract. PCCs had an encapsulation efficiency of 82.03 ± 7.53% and drug loading content of 0.36 ± 0.03 (g g^{-1}), much higher than that of unmodified cerasomes (45.91 ± 1.87% and 0.27 ± 0.01 (g g^{-1})). The *in vitro* experiments showed that less than 65% insulin was released from PCCs after 160 h under physiological conditions. The oral administration of insulin-loaded unmodified cerasomes resulted in almost no change in blood glucose levels in hyperglycemic rats. On the contrary, the insulin-loaded PCCs rapidly decreased the plasma glucose levels which reached their maximum around 2 h (17.15% from the initial value) after oral administration. Especially, the insulin-loaded PCCs displayed a prolonged and stable glucose-lowering profile over a period of over 10 h. Both the PVP and the CS can increase the half life of the cerasomes *in vivo*. In addition, CS as a mucoadhesive polymer can open tight junctions of intestinal epithelial layers, resulting in high oral bioavailability. In other words, the PCCs have great potential as oral formulations for delivering therapeutic protein or peptide drugs.

Received 4th September 2014
Accepted 22nd October 2014

DOI: 10.1039/c4ra09771h

www.rsc.org/advances

Introduction

Over the past three decades, the number of people suffering diabetes mellitus has more than doubled globally, which makes diabetes mellitus one of the most important public health challenges to all nations.[1] Insulin is a very important biologically active molecule used to control glucose levels in the blood.[2] However it is well known that insulin possesses very short biological half-life and extremely low stability due to its low permeability through the intestinal mucosa associated with its hydrophilicity, high molecular weight, and proteolytic inactivation and degradation.[3,4] Therapeutic insulin for diabetes is usually administered *via* subcutaneous injection[5,6] and three or four injections per day are required,[3] which causes many disadvantages such as pain, tenderness, local tissue necrosis, psychologic stress, microbial contamination, nerve damage and hypoglycemic shocks.[7–9] Thus, to enhance patient compliance and convenience, there is a pressing need to investigate the possibility of administering insulin orally to replace the painful subcutaneous route, which is more natural, less invasive, and can be self-administered.[8,10,11] However, the oral delivery of insulin has two main barriers to overcome: (1) the solution is able to remain stable inside the gastrointestinal environment;[12–15] (2) it manages to pass the intestinal epithelium.[11,14,16]

In order to overcome these obstacles, many strategies to enhance the oral delivery of insulin have been or are currently being pursued, such as co-administration with bioadhesive agents, synthesis of their stable derivatives and the development of special dosage forms by utilizing micro- or nanoparticles as a drug carrier.[17–20] The drug carriers can protect insulin from degradation during transport through the acidic environment of the stomach and enhance transport across the intestinal wall. Among numerous drug carrier, liposomes are one of the most potent candidates for such a drug carrier system due to their unique properties compared with other delivery systems, *e.g.* biodegradability, good biocompatibility, and non-immunogenicity, protecting insulin from enzymatic attack, and improving enteral absorption of insulin. However, the utilization of liposome for oral insulin delivery is limited due to a range of problems including: low encapsulation efficiency, poor storage stability and disruption in gastric media and duodenal enzymes.[21] Recently, liposomal nanohybrid cerasome has attract intensive research interests as an ideal drug delivery system because its atomic layer of polyorganosiloxane surface endows higher morphological stability than conventional

[a]*Department of Biomedical Engineering, College of Engineering, Peking University, Beijing, 100871, China. E-mail: zhifei.dai@pku.edu.cn; Web: http://bme.pku.edu.cn/~daizhifei*

[b]*Nanomedicine and Biosensor Laboratory, School of Life Science and Technology, Harbin Institute of Technology, Harbin 150080, China*

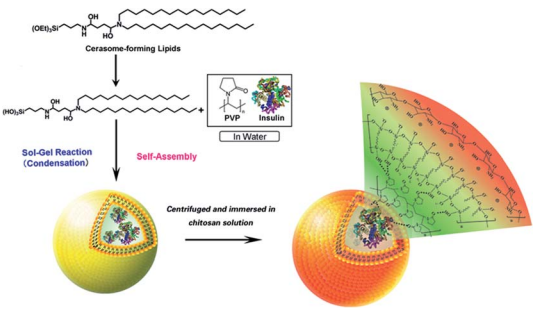

Fig. 1 Schematic of the formation of the insulin-loaded PVP and CS modified cerasomes (PCCs).

liposomes and its liposomal bilayer structure reduces the overall rigidity and density greatly compared to silica nanoparticles.[22] It has been demonstrated that drug loaded cerasomes exhibited sophisticated controlled release behavior and remarkably high stability towards surfactant solubilization, long-term storage, acidic media, and all factors susceptible to destabilize conventional liposomes.[23–26]

Chitosan (CS) is a natural cationic polysaccharide[27] derived by deacetylation of chitin, which is found in exoskeletons of crustaceans. It is a hydrophilic, biocompatible, and biodegradable polymer with low toxicity.[28] It has also received substantial attention in novel bioadhesive drug delivery systems due to its bioadhesive properties which can improve the bioavailability of drugs by prolonging the residence time at the site of absorption and enhancing the transport of drugs across epithelia surface.[10,29–31]

With the aim to develop mucoadhesive cerasomal dosage forms to facilitate the enteral absorption of insulin and further enhance the hypoglycemic effect after oral administration, a cerasomal insulin formulation was successfully fabricated from *N*-[*N*-(3-triethoxysilyl)propylsuccinamoyl]-dihexadecylamine by incorporating PVP into cerasome followed by coating with chitosan according to the Bangham method in combination of sol–gel reaction and self-assembly process[32–34] (Fig. 1). The physicochemical properties of the PVP and chitosan modified cerasomes (PCCs) were characterized and the stability of vesicles was investigated in terms of particle size. In addition, the *in vitro* release experiments and *in vivo* hypoglycemic effect of the insulin-loaded vesicles were also further examined.

Materials and methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Nanjing Kangsente Chemical Engineering Company and cholesterol (Chol) was obtained from Shanghai Advanced Vehicle Technology Pharmaceutical Ltd. Streptozotocin (STZ; MW: 265.2, Sigma), polyvinyl pyrrolidone (PVP; MW: 40 000, Sigma), chitosan (CS; MW: 200k, Sigma), and insulin (INS, 28.8 U mg^{-1}; MW 5807.69, Xuzhou Wanbang Biopharmacy Company) are commercially available products. Cerasome forming lipid (CFL), *N*-[*N*-(3-triethoxysilyl)propylsuccinamoyl]-dihexadecylamine, was synthesized according to the reported method.[22] Dialysis membrane was obtained from Spectrum Laboratories Inc. (MWCO: 1.2–1.4 kDa, California, USA). Deionized water (DI water) (resistivity 18.2 MΩ cm^{-2}) was obtained by purification of house-distilled water with a Milli-Q Gradient System. The other chemicals were of analytical grade.

Fabrication of the insulin-loaded unmodified cerasomes (UCs) and insulin-loaded liposomes

Insulin was encapsulated into UCs according to the Bangham method[35] in combination of sol–gel reaction with self-assembly process (Fig. 1). Briefly, 6 mg of CFL was accurately weighed into a 25 mL round bottom flask and dissolved with 4 mL chloroform. The solvent was removed by rotary evaporation (Laborota model 4010, Heidolph, Germany). The obtained CFL thin film was then dried overnight in vacuum and rehydrated in the presence of 3 mL of insulin solution at 40 °C for about 30 min. The warm solution was sonicated in water bath for 5 min and then ultrasonicated with a probe-type sonicator for 3 min in ice bath. Then, the insulin-loaded UCs were centrifuged at 30 000 rpm for 45 min to remove the free insulin and re-dispersed into DI water. The obtained insulin-loaded UCs suspension was stored in tight containers at 4 °C for further experiments. Conventional liposomes and insulin-loaded liposomes were prepared for a control study using the same process.

Fabrication of the insulin-loaded modified cerasomes

The CFL thin film prepared as described above was hydrated with phosphate buffered saline (PBS, pH 7.4) containing insulin (molar ratio of insulin and CFL was 1 : 10) and PVP (1%, 1 mL per 3 mg CFL) and sonicated in water bath for 5 min. Then, the solution was allowed for 3 min ultrasonication with a probe-type sonicator. After incubation for 24 h at room temperature, the samples were centrifuged at 30 000 rpm for 45 min to remove the free insulin and PVP molecules, and re-suspended with CS solution for 15 min. Then these obtained modified cerasomes were centrifuged at 30 000 rpm for 45 min and washed with PBS for 3 times. The obtained insulin-loaded PCCs were re-suspended with DI water, and stored at 4 °C for further experiments. For comparative studies, UCs, insulin-loaded UCs, insulin-loaded CS coated cerasomes (CCs) and PVP-incorporated cerasomes (PCs) were also prepared.

Characterization of the vesicles

Vesicle size and surface potential measurements. The mean geometric diameter, size distribution and surface potential of the vesicles were analyzed by a 90Plus/BI-MAS dynamic light scattering analyzer (Brookhaven Instruments Co., USA). Vesicle suspensions were diluted with DI water before the measurement. A helium laser was used as the light source and the incidence of light was 90°. The obtained data were analyzed in automatic mode. Vesicle size is expressed as intensity-weighted mean diameter ± SD of values collected from six different batches.

Shape and surface morphology analysis. The shape and surface morphology of the vesicles were analyzed by transmission electron microscopy (TEM) (Hitachi H-7650, Tokyo,

Japan). For TEM analysis, samples (10 μL) were placed on 400-mesh copper grids covered by carbon-stabilized Formvar film. After 1 min, excess fluid was removed and the grids were negatively stained with 2% uranyl acetate in water for 30 s. Samples were viewed in a Hitachi H-7650 electron microscope operating at 100 kV.

Fourier transform infrared spectroscopy. Fourier transform infrared (FTIR) spectra of lyophilized samples of all kinds of vesicles and free insulin were recorded on using a Varian Resolution FTIR spectrophotometer (Varian FTS 3100, USA). The samples for recording FTIR spectra were prepared by blending with KBr powder with the vesicles (99 : 1, w/w) followed by compacting using an IR hydraulic press at a pressure of 8 tons for 1 min. For each spectrum a 512-scan interferogram was collected with a 4 cm^{-1} resolution from the 4000 cm^{-1} to 400 cm^{-1} region at 2 mm s^{-1} at room temperature with nitrogen gas. The resultant spectra were smoothed with a seven-point Savitsky Golay smooth function to remove the noise.[36–38]

In vitro stability of the polymerized vesicles

Vesicles' stability is defined as their capability to prevent leakage of their aqueous contents and keep the structural integrity of the bilayer.[39] The stability of the PCCs was investigated in comparison with that of the CCs, PCs, UCs and liposomes.

The drug leakage experiments were carried out by incubating the vesicles at 4 °C. The total amount of insulin entrapped in vesicles was calculated from the drug loading content (DLC) (eqn (1)) and/or the encapsulation efficiency (EE) (eqn (2)). At indicated time points, 100 μL of the suspension was centrifuged at 30 000 rpm for 45 min, then the supernatant was diluted 10 times with DI water and the amount of the insulin was determined by UV-vis spectrophotometer (Varian 4000, USA). The results were reported as mean ± SD ($n = 4$).

$$\mathrm{DLC}(\%) = \frac{W_{\text{drug in vesicles}}}{W_{\text{vesicles}}} \times 100\% \quad (1)$$

$$\mathrm{EE}(\%) = \frac{W_{\text{drug in vesicles}}}{W_{\text{feeding drug}}} \times 100\% \quad (2)$$

The structural integrity of the bilayer was calculated through detecting the diameter of all the vesicles at different storage time and different storage temperature. Five kinds of samples at a lipid concentration of 1.25 mg mL^{-1}, UCs, liposomes, CCs, PCs and PCCs, were stored at 37 °C or 4 °C for 90 days. The diameter of the vesicles was evaluated at the predetermined time.

In vitro release properties of the vesicles

Insulin release behaviour was investigated using the dialysis method in PBS (pH 7.4, 10 mM) under agitation (320 rpm) in a thermostatic bath at 37 °C. Briefly, 1 mL of vesicles solution containing 2 mg vesicles was placed into a dialysis bag, which was immersed into a vial containing 25 mL PBS (pH 7.4) with slowly stirring the release medium using a magnetic stirrer. At scheduled time intervals, 3 mL of release medium was withdrawn and replaced using the same amount of fresh buffer. The amount of released insulin was estimated spectrophotometrically by reading the absorbance of the solution at 276 nm (UV-vis spectrophotometer, Varian 4000, USA) without further treatment. Experiments were run in quintuplicate for each time point of release kinetics.

Cell culture and antiproliferative activity of various vesicles

Human umbilical vein endothelial cells (HUVECs) were cultured in RPMI-1640 medium supplemented with 2 mmol L^{-1} L-glutamine, 500 U mL^{-1} penicillin, 10% fetal bovine serum (FBS), and 50 μg mL^{-1} streptomycin under a 5% CO_2 atmosphere at a density of 8×10^3 cells per well in a 96-well plate at 37 °C for 24 h. Various concentrations of blank vesicles were added and incubated for another 24 h. Then, 20 μL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) solutions at a concentration of 5 mg mL^{-1} were added to each specimen. The cell culture medium was removed from the specimens after 4 h of incubation, and 150 μL DMSO was added to each specimen. The optical density of each specimen at a wavelength of 490 nm, proportional to the number of live cells, was measured by a microplate reader (Thermo Multiskan MK3).

In vivo studies of the insulin-loaded vesicles in diabetics rats

Animals. For *in vivo* studies, we used adult male rats of SD strain (body weight, 200 ± 20 g) fed in a standard laboratory diet. Rats were housed in an air-conditioned animal room at 25 °C and allowed free access to food and water for two weeks before initiation of the experiments. All animal procedures were in agreement with institutional animal use and care committee and carried out ethically and humanely.

Diabetes induction and monitoring. Diabetes was induced according to our previously-reported protocol.[3] Briefly, streptozotocin (STZ, 70 mg kg^{-1}) dissolved in cold 50 mM citrate buffer (pH 4.5) was administrated intraperitoneally. Every animal was weighted before and after STZ injection daily. The fasting blood glucose levels were determined by withdrawing blood from the tail vein and measuring with a Free Style Blood Glucose Meter (Yi Cheng, China) and the corresponding reagent paper. This experimental procedure causes a type I diabetes. Rats were considered diabetic only when their fasting blood glucose levels were higher than 16.5 mM.

Therapeutic responses after oral administration. At the beginning of each experiment run ($t = 0$), blood samples were collected from tail vein from each rat, and assayed for glucose levels. Rats, whose fasting blood glucose levels were higher than 16.5 mM, were considered diabetic, and taken to the experiment. All the diabetics rats were divided into six groups ($n = 6$). 1 mL of different insulin formulations (or saline) was administrated orally using a rodent disposable gavage needle at a dose of 100 IU kg^{-1} of body weight, and blood samples were drawn from the tail vein of rats without anesthesia after being treated for 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h and 24 h, respectively. Blood glucose levels were measured with a Free Style Blood Glucose Meter (Yi Cheng, China) and the

corresponding reagent paper. Furthermore, in order to estimate these hypoglycemic effects quantitatively, real time levels of the plasma glucose (RL%) were calculated according to the following equation:

$$\mathrm{RL}(\%) = \frac{G_t}{G_0} \times 100\% \quad (3)$$

where G_t refers to the plasma glucose levels of the rats at t h after oral administration of the samples and G_0 refers to the fasting blood glucose level at zero-time.

Statistics

In order to test the reliability of the experimental data, all experiments were done at least thrice and the curves drawn were shown along with the respective error bars. The one-tailed Student's *t*-test (SPSS, Chicago, USA) was performed to analyze the data of the two groups. p values of 0.05 or less were considered significant, while p values of 0.01 or less were considered very significant.

Results and discussion

Fabrication of insulin-loaded and unloaded vesicles

The insulin loaded and unloaded vesicles were readily formed using the thin-film hydrated method followed by sonication with probe-type sonicator. CS was deposited successfully onto the surface of the UCs or PCs *via* the strong electrostatic interaction between the negatively charged UCs and the positively charged CS. The mean diameter of the vesicles in suspension was shown in Table 1. The diameter of the UCs (195.8 ± 2.4 nm) is larger than liposomes (143.4 ± 3.1 nm) and the CS absorption and/or PVP incorporation didn't show significant influence on the size distribution of the vesicles. TEM (Fig. 2) clearly confirmed the formation of spherical vesicles with a diameter range from 100 to 300 nm, well consistent with the hydrodynamic diameter evaluated by DLS measurement (Table 1).

After surface modification with CS, the zeta potential of the UCs and PCs increased significantly from −33.54 ± 0.95 mV and −32.93 ± 1.23 mV to +19.12 ± 0.42 mV and +21.49 ± 1.94 mV, respectively. This inversion of zeta potential indicates the successful adsorption of CS. As seen from Table 1, the insulin

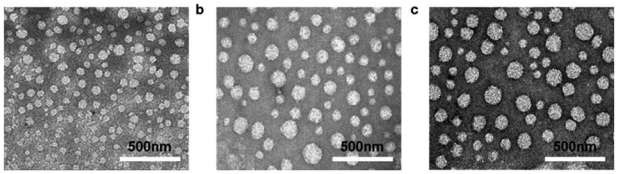

Fig. 2 TEM images of the insulin-loaded liposomes (a), UCs (b) and PCCs(c).

encapsulation caused an apparent decrease in zeta potentials for all vesicles including UCs, PCs, CCs and PCCs due to the negative charge of the insulin molecules at neutral pH. A positively charged surface was considered to be an advantage from biopharmaceutical viewpoint, which would help the insulin transport across the epithelia surface[40,41] and enhance the bioavailability of the insulin. However, the zeta potential of the DPPC/Chol (4 : 1) liposomes was −0.93 ± 1.73 mV. Such a neutral charge may cause the destabilization of the liposomes and the formation of large aggregates in long-term storage due to low interparticle repulsion.

FTIR spectroscopy

FTIR spectroscopy was used to further confirm that the polysiloxane networks were successfully formed on the surface of the UCs (Fig. 3). Compared with the conventional liposomes, a band at about 1100 cm^{-1}, attributed to Si–O–Si asymmetric stretching mode, was seen in the FTIR spectra of cerasomes, PCCs, insulin-loaded PCs and insulin-loaded PCCs. It indicated the formation of polysiloxane networks on the surface of the cerasomes.

FTIR spectroscopy was also used to confirm the encapsulation of insulin and the incorporation of the PVP and CS into UCs (Fig. 3). In the insulin-loaded vesicles, peaks at 1261 cm^{-1} attributed to the C–O stretching vibrations are slightly blue-shifted compared to that observed for insulin at 1242 cm^{-1}. The amide group of free insulin displayed a characteristic bending vibration band at the ~1540 cm^{-1} region. However, this band shifted to ~1560 cm^{-1} in the vesicles because of ionic cross-linking of the amide group of insulin.[42] Nevertheless, the FTIR spectrum of PCCs showed stronger amide region bands

Table 1 Characterization of insulin-loaded vesicles

Type of vesicles	Diameter (nm)	PI	Zeta potential in DI water (mV)	DLC (g g^{-1})	EE (%)
UCs	195.8 ± 2.4	0.20	−33.54 ± 0.95	—	—
Liposomes	143.4 ± 3.1	0.27	−0.93 ± 1.73	—	—
CCs	201.3 ± 4.8	0.19	+19.12 ± 0.42	—	—
PCs	193.4 ± 8.1	0.34	−32.93 ± 1.23	—	—
PCCs	217.3 ± 5.2	0.24	+21.49 ± 1.94	—	—
Insulin-loaded UCs	221.4 ± 2.5	0.19	−36.41 ± 1.05	0.25 ± 0.01	45.91 ± 1.87
Insulin-loaded liposomes	162.9 ± 4.6	0.24	−3.06 ± 0.81	0.27 ± 0.01	49.27 ± 2.58
Insulin-loaded CCs	205.7 ± 5.2	0.26	+17.21 ± 1.07	0.24 ± 0.01	48.31 ± 2.51
Insulin-loaded PCs	199.0 ± 1.6	0.12	−33.81 ± 2.71	0.33 ± 0.02	72.29 ± 4.32
Insulin-loaded PCCs	247.8 ± 3.3	0.21	+18.58 ± 0.56	0.36 ± 0.03	82.03 ± 7.53

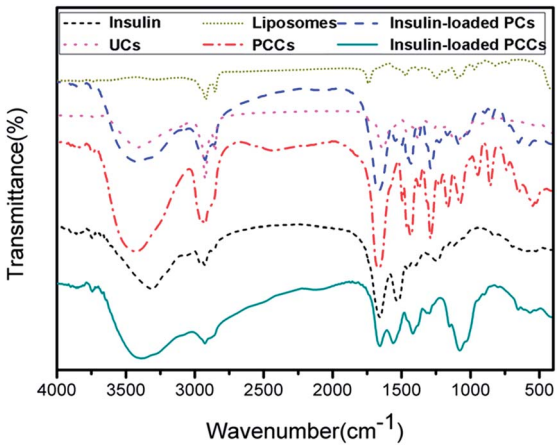

Fig. 3 FTIR spectroscopic analysis of different types of vesicles: liposomes, UCs, PCCs, insulin-loaded PCs and insulin-loaded PCCs.

compared with PCs, indicating that CS was successfully adsorbed onto the surface of the PCs *via* electrostatic interaction.

The EE of liposomes, UCs and CCs was evaluated to be 49.27 ± 2.58%, 45.91 ± 1.87% and 48.31 ± 2.51%, and their DLC was about 0.25 ± 0.01, 0.27 ± 0.01 and 0.24 ± 0.01 (g g^{-1}), respectively. Interestingly, the EE of insulin in PCs and PCCs increased to 72.29 ± 4.32% and 82.03 ± 7.53%, and their DLC increased to 0.33 ± 0.02 and 0.36 ± 0.03 (g g^{-1}), respectively, suggesting that the presence of PVP was favored for insulin association and hence leads to an obvious increase of the EE due to the hydrogen-bonding between the carbonyl groups of the PVP and the amino groups of insulin (as shown in Fig. 1).

In vitro stability of the vesicles

The size of the vesicles affects the half-life of the vesicles in the circulation system because the particles over a certain size may be more easily removed from the circulation by reticulo-endothelial system (RES). The stability of vesicles in different formulations was evaluated over three months of storage at 4 °C or 37 °C by measuring the diameter of the vesicles using DLS. As shown in Fig. 4a and b, the conventional liposomes remained stable in the first few days, but their average size increased from about 164.5 nm to about 1308.4 nm and 608.1 nm due to aggregation or fusion after 90 days storage at 37 °C or 4 °C, respectively. On the contrary, the average size of the UCs, CCs, PCs and PCCs had almost no change, which demonstrated that the UCs, CCs, PCs and PCCs are much more stable than the conventional liposomes. In agreement with the DLS measurements, the TEM image showed that the insulin-loaded PCCs had almost no change after 3 months storage (Fig. 4c). The results may be due to the highly stable polysiloxane networks and/or the significantly negative or positive charges on the surface of vesicles. In addition, we found that the liposomes stored at 4 °C were more stable than that stored at 37 °C during the period of 90 days. However, there was no obvious difference in the stability of UCs, CCs, PCs and PCCs between two storage temperatures of 4 °C and 37 °C. It indicated that the temperature had no significant effect on the storage stability of the UCs, CCs, PCs and PCCs. And all the results suggested that the UCs, CCs, PCs and PCCs were all suitable for long-term storage.

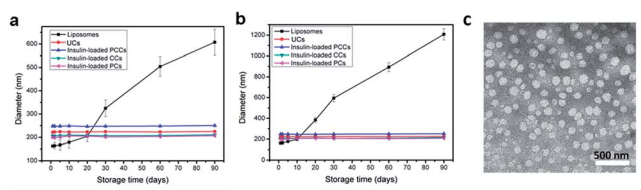

Fig. 4 The hydrodynamic diameter (D_{hy}) of the insulin-loaded UCs, liposomes, CCs, PCs and PCCs after storage 3 months (a) stored at 4 °C, (b) stored at 37 °C; the TEM image of insulin-loaded PCCs after storage 3 months at 37 °C (c).

The drug leakage during the storage period is a critical parameter that affects the efficacy of the drug. By using a sampling separation method we examined the drug leakage property of the insulin-loaded vesicles at 4 °C. As shown in Fig. 5, 57.51 ± 3.13% of the initially encapsulated insulin released from liposomes after 90 days storage. In stark contrast, only 9.56 ± 2.11%, 9.51 ± 3.13%, 8.43 ± 1.83% and 8.03 ± 1.45% of the drug payload released from UCs, CCs, PCs and PCCs over the same period of time, respectively. These results were likely due to the formation of polysiloxane networks on the surface of the UCs, CCs, PCs and PCCs, which inhibited the diffusion-driven release of insulin. Additionally, the hydrogen-bonds between insulin and PVP may give a little contribution to further enhance the retention of insulin in PCCs.

In vitro drug release behavior of the insulin-loaded vesicles

The release studies of insulin from vesicles in different formulations were performed by a dialysis method at physiological pH and temperature (pH 7.4 and 37 °C) (Fig. 6). The cumulative release was calculated with respected to the drug content presented in Table 1. As a control study, insulin was released about 17% from the dialysis bag in 1 h, and the release almost completed within 20 h, indicating that the dialysis bag would not affect the insulin release from vesicles during a long release

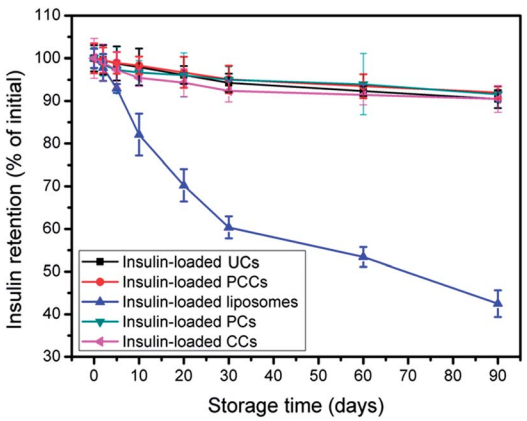

Fig. 5 Retention of encapsulated insulin in the liposomes (▲), UCs (■), PCCs (●), PCs (▼) and CCs (◄) as a function of time. Data shown as means ± SD (n = 4).

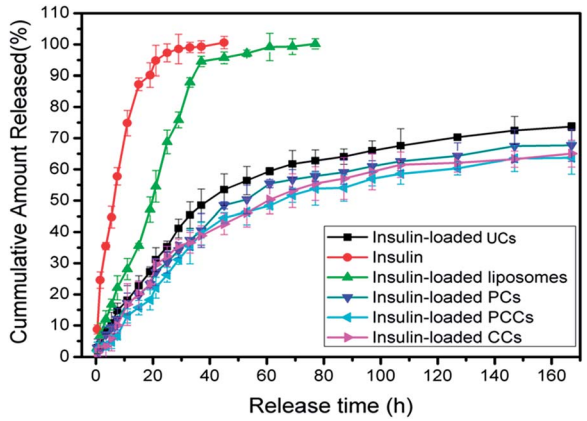

Fig. 6 *In vitro* release of insulin from free insulin (●), insulin-loaded liposomes (▲), insulin-loaded UCs (■), insulin-loaded CCs (►), insulin-loaded PCs (▼) and insulin-loaded PCCs (◄) into the release medium at 37 °C. All the *p* values were lower than 0.01, which of the UCs, CCs, PCs and PCCs compared with liposomes or free insulin. ($n = 4$).

period. It was obvious that insulin release from all insulin-loaded vesicles with different formulations *in vitro* underwent a burst and rapid release of the insulin in the first 32 h followed by slow dissolution. The initial burst and rapid release of insulin may be due to the drugs dissociating from the surface of the carriers,[43] the large concentration gradient and the states of the encapsulated drugs in carriers (molecularly dispersed drugs and bound drugs).[44,45] We thought the ratio of the two different state drugs dictates the amount of the initial burst release.

In addition, a significant difference in the insulin release was observed between the insulin-loaded liposomes and insulin-loaded UCs. The drug release from insulin-loaded liposomes was much faster than insulin-loaded UCs and almost completed within 60 h, while only 73.7% insulin was released from UCs within 167 h at 37 °C due to the siloxane networks of a high degree of polymerization on the surface of UCs, which would block the drug release channels. Nevertheless, the siloxane network is not so highly developed on the surface of the UCs as the length of the Si–O–Si bond is much shorter than the diameter of the cross-section of the dialkyl tail of the amphiphiles constituting the molecular packing of the stable liposomal bilayer structure. This effect perturbs the bilayer membrane and may induce the formation of pores that are sufficiently large to allow for the leakage of the insulin contents. These results demonstrate that the UCs can be used as an effective carrier for controlled release of insulin.

As shown in Fig. 6, the release rate of insulin-loaded CCs (65.1%, 167 h), PCs (67.7%, 167 h) and PCCs (63.7%, 167 h) was slight lower than that of insulin-loaded UCs (73.7% 167 h), suggesting that the introduction of PVP and chitosan may reduce the insulin release rate from vesicles. The hydrogen-bonding between PVP and insulin not only improved the encapsulation efficiency (Table 1) but also had a little contribution to lower the drug release rate. In addition, the CS adsorbed on the surface of the vesicles can further lower the membrane permeability, which is partially responsible for the sustained insulin release from insulin-loaded PCCs.

Inhibitory effects of all vesicles on cell proliferation

It is well recognized that all materials can't be considered as biomaterials until and unless they meet certain criterion and regulatory requirements. For instance, the materials must be non-toxic. In this study, the biocompatibility of the prepared vesicles has been evaluated by incubating with HUVECs. The MTT assay with HUVECs in the presence of liposomes, UCs, PCs, CCs or PCCs concentrations up to 1.0 mg mL^{-1} resulted in more than 80% viability after incubation with all vesicles for 24 h (Fig. 7). These results demonstrate the biocompatibility of our vesicles up to high concentrations on HUVECs. The lack of toxicity together with the specific release profiles supports the potential medical use of the vesicles used in our study for protein delivery.

Hypoglycemic effects of insulin-loaded PCCs after oral administration

To evaluate the pharmacological efficacy of administered insulin, blood glucose levels in STZ-induced diabetic rats were measured after oral administration of insulin solutions, insulin-loaded vesicles and the mixture of insulin and blank vesicles (Fig. 8). The experimental conditions used to STZ-induced type I diabetes caused a severe hyperglycemia characterized by fasting blood glucose levels up to 16.5 mM in all treated animals. The oral administration of insulin solutions, insulin-loaded liposomes or insulin-loaded UCs did not result in a significant change of blood glucose levels in hyperglycemic rats (data were not shown). As shown in Fig. 8, a mild reduction in glycaemia was observed after insulin-loaded PCs orally administrated in hyperglycemic rats. On the contrary, treatment with insulin-loaded CCs or PCCs caused a significant blood glucose level reduction in time-dependent fashion compared to the control group. This implied that the insulin was absorbed into the systemic circulation and kept its activity. The maximum hypoglycemic effect was observed 2 h after administration of insulin-loaded PCCs (17.15% from the initial value) and PCs (76.96% from the initial value), whereas insulin-loaded CCs

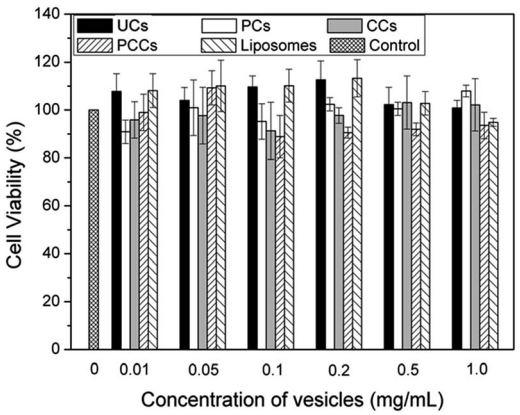

Fig. 7 Anti-proliferative effect of blank liposomes, UCs, CCs, PCs and PCCs on HUVECs at different concentrations ($n = 6$) after 24 h incubation.

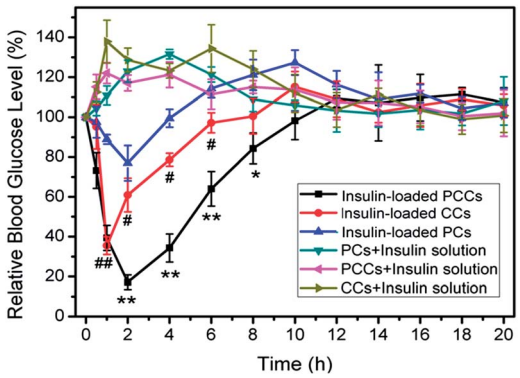

Fig. 8 Plasma glucose levels after oral administration of different insulin formulation: insulin-loaded PCCs 100 IU kg^{-1} (■), insulin-loaded CCs 100 IU kg^{-1} (●), insulin-loaded PCs 100 IU kg^{-1} (▲), blank PCCs mixed with insulin solution 100 IU kg^{-1} (◀), blank CCs mixed with insulin solution 100 IU kg^{-1} (▶), and blank PCs mixed with insulin solution 100 IU kg^{-1} (▼). Data represent the mean ± SD, $n = 6$ per group. $^{**}p < 0.01$ (compared with insulin-loaded CCs and PCs), $^{*}p < 0.05$ (compared with insulin-loaded CCs) and $^{\#\#}p < 0.01$ (compared with insulin-loaded PCs), #p < 0.05 (compared with insulin-loaded PCs).

rapidly decreased the plasma glucose levels which reached the maximum around 1 h (35.48% from the initial value). Especially, the level increased gradually and the insulin effect vanished until 4 h, 7 h and 10 h after oral administration of insulin-loaded PCs, CCs or PCCs, respectively, revealing that the insulin-loaded PCCs exerted a very significant and linger hypoglycemic effect. It suggested that both the PVP and the CS could increase the half life of the cerasomes *in vivo* and the high oral bioavailability may be attributed to the ability of CS to act as a mucoadhesive polymer and to open tight junctions of intestinal epithelial layers.[20,46] Otherwise, the oral administration of the mixture of insulin and the blank vesicles showed no hypoglycemic effect. During these experiments, a slight increase in blood glucose levels above the baseline was observed in Fig. 8. We believe that this increase may be attributed to the change of metabolisms and the increase of endogenous secretion of glucagon in the diabetic rats due to stress in the animals during blood sampling.

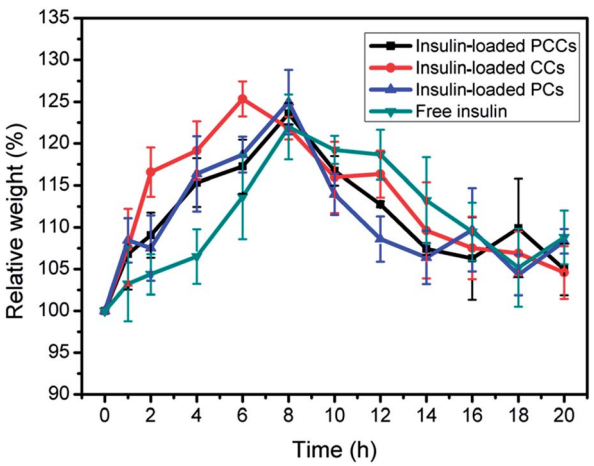

Fig. 9 Body weight of rats at different time points after oral administration of different insulin formulations ($n = 6$).

As seen in Fig. 9, a significant increase in the body weight was observed in the initial time during the experiments due to the free access for food and water after fasting for 12 h, which could induce the increase of blood glucose levels in the control groups after oral administration of the mixture of insulin and the blank vesicles. In a word, the PCCs have great potential as oral formulations for delivering therapeutic protein or peptide drugs.

Conclusions

A cerasomal insulin formulation was successfully fabricated for oral insulin delivery by incorporating PVP into cerasome followed by coating with CS according to the Bangham method in combination of sol–gel reaction and self-assembly process. The *in vitro* experiments showed that both modified and unmodified cerasomes exhibited higher stability, lower drug leakage and more sustained release compared with conventional liposomes. Especially, the incorporation of PVP led to a substantial increase in encapsulation efficiency and drug loading content. More importantly, insulin-loaded PCCs significantly enhanced intestinal absorption of insulin after oral administration, conducing a remarkable and prolonged hypoglycaemic effect, which lowered plasma glucose levels of STZ-induced diabetic rats at insulin doses of 100 IU kg^{-1} up to 81% of their basal glucose level and kept for more than 10 h. Therefore, cerasomal technique can be an efficient strategy to achieve oral delivery of peptide drugs by using appropriate materials for doping and coating cerasomes.

Acknowledgements

This work was financially supported by the State Key Program of National Natural Science of China (no. 81230036), National High Technology Research and Development Program of China (no. 2013AA032201), National Natural Science Foundation of China (no. 21273014 and 81371580) and National Natural Science Foundation for Distinguished Young Scholars (no. 81225011).

References

1 O. S. Y. Nazmi and B. Turan, *Diabetic Cardiomyopathy*, 2014.
2 H. M. Joshi, D. R. Bhumkar, K. Joshi, V. Pokharkar and M. Sastry, *Langmuir*, 2006, **22**, 300.
3 J. Zheng, X. L. Yue, Z. F. Dai, Y. Wang, S. Liu and X. F. Yan, *Acta Biomater.*, 2009, **5**, 1499.
4 C. Damge, P. Maincent and N. Ubrich, *J. Controlled Release*, 2007, **117**, 163.
5 R. N. Brogden and R. C. Heel, *Drugs*, 1987, **34**, 350.
6 J. A. Galloway and R. E. Chance, *Horm. Metab. Res.*, 1994, **26**, 591.
7 R. L. Chin, R. Martinez and G. Garmel, *Am J. Emerg. Med.*, 1993, **11**, 622.

8 X. W. Zhang, J. P. Qi, Y. Lu, W. He, X. Y. Li and W. Wu, *Nanomedicine*, 2014, **10**, 167.
9 G. P. Carino and E. Mathiowitz, *Adv. Drug Delivery Rev.*, 1999, **35**, 249.
10 M. Thanou, J. C. Verhoef and H. E. Junginger, *Adv. Drug Delivery Rev.*, 2001, **52**, 117.
11 A. Sood and R. Panchagnula, *Chem. Rev.*, 2001, **101**, 3275.
12 L. L. Chang and J. P. Bai, *Pharm. Res.*, 1996, **13**, 801.
13 R. J. Schilling and A. K. Mitra, *Pharm. Res.*, 1991, **8**, 721.
14 M. Goldberg and I. Gomez-Orellana, *Nat. Rev. Drug Discovery*, 2003, **2**, 289.
15 J. M. Gamboa and K. W. Leong, *Adv. Drug Delivery Rev.*, 2013, **65**, 800.
16 C. W. Andrews, L. Bennett and L. X. Yu, *Pharm. Res.*, 2000, **17**, 639.
17 E. Lee, J. Lee and S. Jon, *Bioconjugate Chem.*, 2010, **21**, 1720.
18 Y. Zhang, W. Wei, P. Lv, L. Wang and G. Ma, *Eur. J. Pharm. Biopharm.*, 2011, **77**, 11.
19 M. C. Chen, K. Sonaje, K. J. Chen and H. W. Sung, *Biomaterials*, 2011, **32**, 9826.
20 Z. H. Wu, Q. N. Ping, Y. Wei and J. M. Lai, *Acta Pharmacol. Sin.*, 2004, **25**, 966.
21 V. J. Mohanraj, T. J. Barnes and C. A. Prestidge, *Int. J. Pharm.*, 2010, **392**, 285.
22 K. Katagiri, M. Hashizume, K. Ariga, T. Terashima and J. Kikuchi, *Chemistry*, 2007, **13**, 5272.
23 Y. S. Jin, X. L. Yue, Q. Y. Zhang, X. Y. Wu, Z. Cao and Z. F. Dai, *Acta Biomater.*, 2012, **8**, 3372.
24 Z. Cao, X. L. Yue, Y. S. Jin, X. Y. Wu and Z. F. Dai, *Colloids Surf., B*, 2012, **98**, 97.
25 Z. Cao, Y. Ma, X. L. Yue, S. Z. Li, Z. F. Dai and J. Kikuchi, *Chem. Commun.*, 2010, **46**, 5265.
26 X. L. Yue and Z. F. Dai, *Adv. Colloid Interface Sci.*, 2014, **207**, 32.
27 K. Sonaje, K. J. Lin, M. T. Tseng, S. P. Wey, F. Y. Su, E. Y. Chuang, C. W. Hsu, C. T. Chen and H. W. Sung, *Biomaterials*, 2011, **32**, 8712.
28 O. Felt, P. Buri and R. Gurny, *Drug Dev. Ind. Pharm.*, 1998, **24**, 979.
29 S. Surini, H. Akiyama, M. Morishita, T. Nagai and K. Takayama, *J. Controlled Release*, 2003, **90**, 291.
30 W. Wei, G. H. Ma, L. Y. Wang, J. Wu and Z. G. Su, *Acta Biomater.*, 2010, **6**, 205.
31 L. Yin, J. Ding, C. He, L. Cui, C. Tang and C. Yin, *Biomaterials*, 2009, **30**, 5691.
32 M. L. Liu, M. S. Li, S. L. Sun, B. X. Li, D. Du, J. L. Sun, F. Y. Cao, H. C. Li, F. Jia, T. F. Wang, N. D. Chang, H. Yu, Q. Wang and H. S. Peng, *Biomaterials*, 2014, **35**, 3697.
33 L. Li, J. Hou, X. Liu, Y. Guo, Y. Wu, L. Zhang and Z. Yang, *Biomaterials*, 2014, **35**, 3840.
34 Q. Yao, Z. T. Luo, X. Yuan, Y. Yu, C. Zhang, J. P. Xie and J. Y. Lee, *Sci. Rep.*, 2014, **4**, 3848.
35 A. D. Bangham, M. M. Standish and J. C. Watkins, *J. Mol. Biol.*, 1965, **13**, 238.
36 L. Jorgensen, C. Vermehren, S. Bjerregaard and S. Froekjaer, *Int. J. Pharm.*, 2003, **254**, 7.
37 W. G. Dai and L. C. Dong, *Int. J. Pharm.*, 2007, **336**, 58.
38 B. Sarmento, D. C. Ferreira, L. Jorgensen and M. van de Weert, *Eur. J. Pharm. Biopharm.*, 2007, **65**, 10.
39 P. I. Lelkes and H. B. Tandeter, *Biochim. Biophys. Acta*, 1982, **716**, 410.
40 A. F. Kotze, H. L. Luessen, B. J. de Leeuw, A. G. de Boer, J. C. Verhoef and H. E. Junginger, *J. Controlled Release*, 1998, **51**, 35.
41 J. Smith, E. Wood and M. Dornish, *Pharm. Res.*, 2004, **21**, 43.
42 X. Z. Shu and K. J. Zhu, *Int. J. Pharm.*, 2002, **233**, 217.
43 J. Wang, B. M. Wang and S. P. Schwendeman, *J. Controlled Release*, 2002, **82**, 289.
44 W. Teng, J. Cappello and X. Wu, *J. Controlled Release*, 2011, **156**, 186.
45 L. Zeng, L. An and X. Wu, *J. Drug Delivery*, 2011, **2011**, 370308.
46 N. Shrestha, M. A. Shahbazi, F. Araujo, H. Zhang, E. M. Makila, J. Kauppila, B. Sarmento, J. J. Salonen, J. T. Hirvonen and H. A. Santos, *Biomaterials*, 2014, **35**, 7172.