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In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin

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Abstract

In this study, niosomes of polyoxyethylene alkyl ethers (Brij^{TM}) were prepared for encapsulation of insulin by film hydration method. Without cholesterol, brij 35 and brij 58 did not form niosomes, apparently because of relatively large polar head groups in comparison with their alkyl chains. The size of vesicles depended on the cholesterol content, charge incorporation or hydrophilicity of surfactants. Entrapment of insulin in bilayer structure of niosomes protected it against proteolytic activity of α-chymotrypsin, trypsin and pepsin in vitro. The maximum protection activity was seen in brij 92/cholesterol (7:3 molar ratios) in which only 26.3 ± 3.98% of entrapped insulin was released during 24 h in simulated intestinal fluid (SIF). The kinetic of drug release for most formulations could be best described by Baker and Lonsdale equation indicating diffusion based delivery mechanism. These results indicate that niosomes could be developed as sustained release oral dosage forms for delivery of peptides and proteins such as insulin.

Keywords: Insulin; Niosomes; Oral delivery; Polyoxyethylene alkyl ethers; Proteolytic enzymes

1. Introduction

The delivery of protein pharmaceuticals to the systemic circulation through oral administration is hindered by numerous barriers, including proteolytic enzymes, sharp pH gradients and low epithelial permeability (Lee and Yamamoto, 1990). Recently, different systems and technologies such as enteric-coated capsules (Hosny et al., 2002), gel beads (Sriamornsak, 1998), intestinal patches (Whitehead et al., 2004), liposomes (Takeuchi et al., 1996; Kim et al., 1999; Iwanaga et al., 1999; Kisel et al., 2001; Wu et al., 2004), microparticles (Agarwal et al., 2001; Morcol et al., 2004; Morishita et al., 2004), microspheres (Morishita et al., 1992), mucoadhesive tablets (Caliceti et al., 2004; Krauland et al., 2004), nanocubicles (Chung et al., 2002), nanospheres (Damge et al., 1997; Carino et al., 2000; Foss et al., 2004), and non-ionic surfactant vesicles (Yoshida et al., 1992) have been used to overcome these barriers and improve protein absorption following oral delivery. Among these, vesicular systems such as liposomes have been investigated more than the other systems. Unfortunately, a number of serious limitations exist with the use of liposomes such as in vitro (Ausborn et al., 1992) and in vivo (Poste, 1983) instability.

One alternative of phospholipids, the main constituents of liposomes, is the hydrated mixture of cholesterol and non-ionic surfactants such as alkyl ethers, alkyl esters or alkyl amides non-ionic surfactants (Manosroi et al., 2003). This type of vesicle formed from the above mixtures has been known as niosomes or non-ionic surfactant vesicles (NSVs). The low cost, greater stability, ease of storage and also large numbers of available vesicle forming non-ionic surfactants make these vesicles more attractive than liposomes for industrial production both in pharmaceutical and cosmetic applications (Uchegbu and Vyas, 1998).

Both hydrophilic and hydrophobic substances can be embedded in niosomal vesicles. Some proteins and peptides such as alpha-interferon (Niemiec et al., 1995), bovine serum albumin (Brewer and Alexander, 1992; Murdan et al., 1999),

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cyclosporine A (Niemiec et al., 1995; Waranuch et al., 1998), 9-desglycinamide-8-arginine vasopressin [DGAVP] (Yoshida et al., 1992), GnRH-based anti-fertility immunogen (Ferro et al., 2004), haemagglutinin (Murdan et al., 1999), influenza viral antigens (Chattaraj and Das, 2003), insulin (Khaksa et al., 2000; Varshosaz et al., 2003), luteinizing hormone releasing hormone [LHRH] (Arunothayanun et al., 1999), ovalbumin (Brewer et al., 1998; Rentel et al., 1999) and recombinant human granulocyte-macrophage colony stimulating factor [rhGM-CSF] (Memisoglu et al., 1997) have been successfully encapsulated in niosomes. The encapsulation of pharmaceutical materials in niosomes can decrease drug toxicity, increase drug absorption, stability or activity and retard removal of drug from the circulation due to slow drug release.

In previous article we reported the encapsulation of insulin in sorbitan ester vesicles which led to protection of protein against proteolytic enzymes and sustained release of insulin (Varshosaz et al., 2003). In this study, the ability of polyoxyethylene alkyl ethers (C_nEO_m, Brij^{TM}) mixed with cholesterol to form bilayer vesicles and encapsulate recombinant human insulin was studied. Furthermore, encapsulation efficiency of insulin, protection against enzymes, thermal analysis and size distribution study of vesicles and characterization of niosomes with optical microscope were carried out.

2. Materials and methods

Recombinant human insulin (27.5 IU/mg, Eli Lilly, France) was a kind gift from Exir Pharmaceutical Co., Iran. The non-ionic surfactants used as vesicle-forming materials were Brij 52 (polyoxyethylene 2 cetylether, $C_{16}EO_2$), Brij 72 (polyoxyethylene 2 stearylether, $C_{18}EO_2$), Brij 92 (polyoxyethylene 2 oleylether, $C_{9=9}EO_2$), Brij 76 (polyoxyethylene 10 stearylether, $C_{18}EO_{10}$), Brij 97 (polyoxyethylene 10 oleylether, $C_{9=9}EO_{10}$), Brij 58 (polyoxyethylene 20 cetylether, $C_{16}EO_{20}$) and Brij 35 (polyoxyethylene 23 laurylether, $C_{12}EO_{23}$) and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin (from porcine stomach mucosa, 3200–4500 U/mg protein), α-chymotrypsin (from bovine pancreas, 40–60 U/mg protein), trypsin (from bovine pancreas, 10,000 BAEE U/mg protein) and dicetylphosphate (DCP) were also obtained from Sigma. Cholesterol (Chol) was bought from Fluka, Switzerland. Immunoradiometric assay kit was purchased from Biosource, Belgium. All organic solvents and the other chemicals were of analytical grade and were obtained from Merck, Germany.

2.1. Preparation of non-ionic surfactant vesicles

Multilamellar vesicles (MLV) were prepared by modification of hand-shaking method (Baillie et al., 1985) in which 300 μmol of surfactant or surfactant/Chol or surfactant/Chol/DCP mixtures were dissolved in 10 ml chloroform in a 100-ml round-bottomed flask. The organic solvent was then removed at 50 °C, under reduced pressure, in a rotary evaporator (Buchi, Switzerland). The flask was dried overnight in a desiccator to remove any residual solvent. The dried lipid film was hydrated with 5 ml phosphate buffered saline (PBS) (pH 7.4) containing insulin (20 IU/ml) with a gentle rotation in water bath at 55 °C for 10 min. The resulting multilamellar non-ionic surfactant vesicle dispersions were then left to cool slowly. To study the effect of composition of the vesicles, a series of formulations with different molar ratios (m.r.) were designed (Table 1).

Table 1
Vesicle forming ability of studied polyoxyethylene alkyl ethers (Brij^{TM}) non-ionic surfactants; non-ionic surfactant vesicles (NSVs, niosomes) were prepared by classic film method

Surfactant	HLB	Surfactant/cholesterol molar ratio				
		10/0	9/1	7/3	6/4	5/5
Brij 35	16.9	−	−	+[a,b]	+[a,b]	+[a]
Brij 52	5.3	+[c]	+[c]	+[c]	+[c]	+[c]
Brij 58	15.7	−	−	+	+	+[a]
Brij 72	4.9	+	+	+	+	+
Brij 76	12.4	+	−	+	+	+
Brij 92	4.9	+[c]	+[c]	+[c]	+[c]	+[c]
Brij 97	12.4	+	−	+	+	+

[a] Some cholesterol crystals were observed by optical microscopy.
[b] The number of formed niosomes was relatively low.
[c] Creaming was seen after standing of formulation.

2.2. Differential scanning calorimetry analysis (DSC)

A small amount (typically 5 mg) of freeze-dried NSVs (9:1 and 7:3 surfactant/Chol m.r.) or pure semisolid surfactant (Brij 52, Brij 72, and Brij 76) was sealed in a 40-μl-aluminium crucible. For preparation of freeze-dried samples, 1 ml of niosomal suspension was frozen in liquid nitrogen and freeze dried all over the night in a lyophilizer (FD-81, Eyela, Japan). A second crucible containing the equivalent amount of PBS (pH 7.4) was sealed as the reference cell. The temperature of the pans was raised from 20 to 80 °C, at a rate of 5 °C/min and a sensitivity of 1 mW/s using a differential scanning calorimeter (TA 4000, Mettler, Germany). The heat flow calibration was performed with indium. The reproducibility of the thermograms was determined by repeating the temperature cycle three times for each sample.

2.3. Measurements

The particle size and particle size distribution of niosomes were determined by laser-light scattering (Mastersizer X, Malvern Instruments, UK). Some micrographs were prepared by a camera attached to the optical microscope (HFX-DX, Nikon, Japan) in 10 × 40 and 10 × 100 magnifications. Immunoreactive insulin in samples was measured using An INS-IRMA^{TM} radioimmunoassay kit using a gamma counter device (Minigamma Gamma counter, LKB Wallac, Turku, Finland).

2.4. Insulin encapsulation efficiency

To separate non-entrapped insulin, the vesicle suspensions were centrifuged (90 XL Ultracentrifuge, Beckman, USA) at 40,000 × g for 30 min at 4 °C and washed with PBS (pH 7.4). The amount of insulin in the supernatant and also in the

pellets was analyzed radioimmunometrically, after disrupting the niosomes by isopropyl alcohol.

2.5. In vitro release of insulin from the vesicles

One ml of each niosomal suspension was centrifuged (40,000 × *g* for 30 min at 4 °C), washed twice and the obtained pellet was resuspended to 1 ml in PBS. The pellets obtained from centrifugation were washed twice and resuspended in PBS. The niosome suspensions were diluted to 10-fold in SIF (pH 6.8) with or without pancreatin and in SGF (pH 1.2) with or without pepsin. A 1-ml sample of the diluted suspension was used for each time point; samples in screw cap Eppendorf tube were incubated under gentle shaking conditions (10 cycles/min) at 37 °C. The release study was carried out for 4 h in SGF and for 24 h in SIF. Triplicate samples were taken for analysis according to planned schedule. Vesicle pellets were then separated by centrifugation in a microcentrifuge at 13,000 × *g* for 10 min. The supernatant removed and the pellets were resuspended in PBS. After disruption by isopropyl alcohol, the amount of remained insulin in the pellets was determined radioimmunometrically.

2.6. Physical stability of vesicles at different temperatures

Aggregation or fusion of the vesicles as a function of temperature was determined as the changes in vesicle diameter by a laser light scattering method. The vesicles were stored in glass vials at room temperature or kept in refrigerator (4 °C) for 3 months. The changes in morphology of MLVs and also the constituent separation were assessed by an optical microscope. The retention of entrapped insulin was also measured 72 h after preparation and after 1, 2 or 3 months in some formulations. No special precautions were taken in order to improve the stability of vesicles.

2.7. Protection of encapsulated insulin

Three enzyme solutions were used: the pepsin solution (5 IU/ml) in glycine buffer adjusted to pH 1.2, the trypsin solution (704 IU/ml), and the α-chymotrypsin solution (4.16 IU/ml) in phosphate buffer at pH 7.8. Pepsin solution (0.5 ml) was incubated for 1 h at 37 °C with 10-fold diluted niosome suspension (0.5 ml) or free insulin solution in PBS, pH 7.4 (0.5 ml, 2 IU/ml). For the other enzymatic solutions the same conditions were applied, except that the incubation time was 3 h (Morishita et al., 1992). The studied niosomes composed of 7:3 m.r. of surfactant/Chol. After appropriate incubation, samples (200 μl) were taken, centrifuged at 13,000 × *g* for 5 min and the supernatant was removed. Then 200 μl of 0.05% trifluoroacetic acid (TFA) was added to the pellets to inactivate traces of enzyme present at the niosomes surfaces (Shao and Mitra, 1993). Subsequently, 0.5 ml of isopropyl alcohol was added to dissolve the pellets and the concentration of remained active insulin was determined.

2.8. Statisitcs

One-way ANOVA tests were conducted for the obtained results, followed by Scheffe post hoc tests in SPSS 11 for windows. $P < 0.05$ was denoted for statistical significance.

3. Results and discussion

3.1. Vesicle-forming ability of surfactants

The ability of the studied surfactants to form vesicles by lipid hydration method (classic film method or hand shaking) is summarized in Table 1. Among the C_nEO_m surfactants, the $C_{16}EO_2$ (Brij 52), $C_{18}EO_2$ (Brij 72), C_{18} EO_{10} (Brij 76), $C_{9=9}EO_2$ (Brij 92) and $C_{9=9}EO_{10}$ (Brij 97) were able to form stable NSV suspensions in the absence of cholesterol. The micrographs in Fig. 1 confirm the formation of vesicular structures from C_nEO_m by classic film hydration method. The other surfactants needed different amounts of cholesterol to form vesicles.

The ability of surfactants to form vesicles is depending on the structure of the surfactant. The packing properties of surfactants depend on the balance between hydrophilic surface area a_0, the volume V of the hydrocarbon chain, and the maximum length L_c that the chains can assume (Israelachvili, 1985). The dimensionless packing parameter (V/a_0L_c), CPP, indicates the surfactant ability to form spherical micelles (CPP < 1/3), non-spherical micelles (1/3 < CPP < 1/2) or bilayers (1/2 < CPP < 1) (Bouwstra and Hofland, 1996).

It has been reported that without cholesterol, vesicle formation was observed for only surfactant with stearyl chain (C_{18}) such as Brij 72 with a proper HLB and CPP (Manosroi et al., 2003). As the HLB of the surfactant increases above 10, the minimum amount of cholesterol necessary to form vesicles increases (Uchegbu and Vyas, 1998). More cholesterol is necessary to compensate for the larger head group. This is demonstrated by the results in Table 1. In the present study $C_{12}EO_{23}$ (Brij 35) has the highest HLB (16.9) indicating low hydrocarbon chain volume in comparison with hydrophilic surface area. This amphiphile could not form niosomes in the absence of bilayer inducting agent, cholesterol. It was not possible to prepare $C_{12}EO_{23}$ containing niosomes by lipid hydration method even in the presence of equal weight amount of cholesterol (Parthasarathi et al., 1994). In this study, $C_{16}EO_{20}$ (Brij 58, HLB 15.7) also did not form niosomes in spontaneous vesicle formation method.

It appeared that C_nEO_2 surfactants were able to form vesicles. Since this group of surfactants possesses a rather small hydrophilic head group in comparison with their alkyl chain, it seems that vesicle formation is confirmed by the Israelachvili's CPP theory.

Although the vesicle formation of $C_{12}EO_7$ (HLB 13.2), $C_{14}EO_7$ (HLB 12.5), $C_{16}EO_7$ (HLB 11.8) and $C_{18}EO_7$ (HLB 11.2) in the absence of cholesterol has not been observed (Van Hal, 1994), but we prepared NSVs of $C_{18}EO_{10}$ (HLB 12.4) and $C_{9=9}EO_{10}$ (HLB 12.4) without any added cholesterol. The ability of $C_{18}EO_{10}$ or $C_{9=9}EO_{10}$ to form vesicles compared to a non-vesicle forming surfactant, $C_{14}EO_7$, with a similar HLB may be related to their longer hydrocarbon chain and an optimum CPP. Surprisingly in the presence of 10 molar ratio of cholesterol neither Brij 76 ($C_{18}EO_{10}$) nor Brij 97 ($C_{9=9}EO_{10}$) did not form niosomes (Table 1). This may be related to the solubilizing property of these two surfactants, their high HLB values and

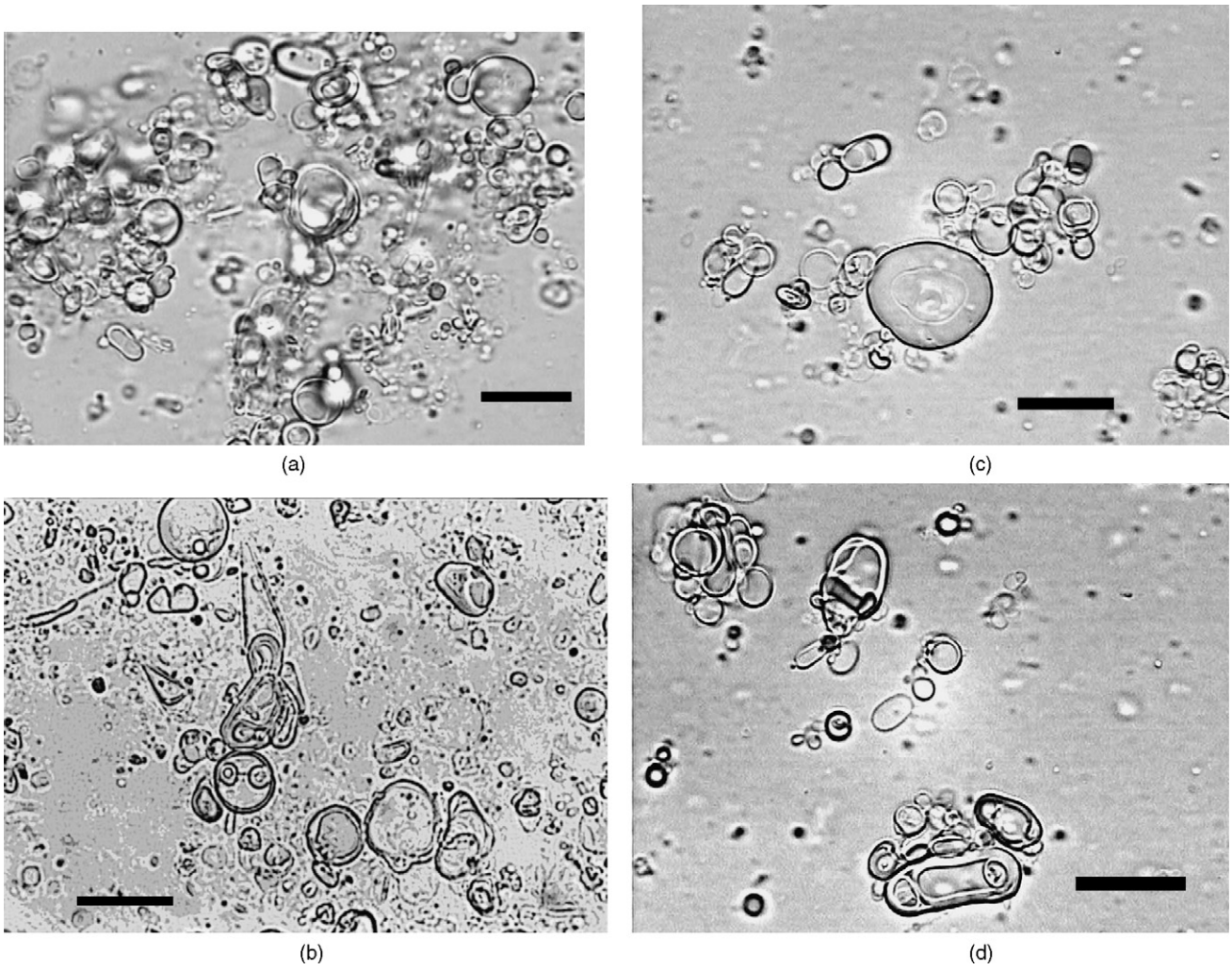

Fig. 1. Micrographs (1000×) of insulin-loaded vesicles composed of polyoxyethylene alkyl ethers (Brij^{TM}) prepared by classic film method: (a) Brij 52/Chol (7:3 m.r.); (b) Brij 52/Chol/DCP (6:3:1 m.r.); (c) Brij 92/Chol (7:3 m.r.); Brij 92/Chol/DCP (6:3:1 m.r.). Bar = 10 μm.

therefore their micelle formation ability that dissolve the small amounts of cholesterol.

Because of poor ability of Brij 35 and Brij 58 to form vesicles, further studies on these two surfactants were not carried out.

3.2. Differential scanning calorimetry

In present study the phase transition temperature of freeze-dried vesicles or pure surfactants were determined from thermograms as the midpoint (main phase transition) temperature of endothermic peaks (Lawrence et al., 1996; Tabbakhian, 1998). For $C_{16}EO_2$ (Brij 52) and $C_{18}EO_2$ (Brij 72), T_c were determined as 32.5 and 44 °C, respectively. Therefore increasing the acyl chain from C_{16} to C_{18} led to raising of T_c. Similar conditions are valid in NSVs: increasing the fatty acid chain length in polyoxyethylene alkyl ether surfactants (C_mEO_3) from C_{16} to C_{18} increased T_c from 30 to 42 °C (Van Hal, 1994). Following the temperature raising, the fatty acid side chains go from a closely packed ordered gel-phase to a more loosely packed, less ordered liquid-crystalline state where the chains are capable of rotational motion. The liquid state surfactants, $C_{9=9}EO_2$ (Brij 92) and $C_{9=9}EO_{10}$ (Brij 97), showed no transition endotherm in the used temperature range (20–80 °C) both in pure form and freeze-dried NSVs (data not shown).

The effect of gradually increasing the molar content of cholesterol in NSVs is similar to that seen using phospholipids. Cholesterol is known to abolish the gel-liquid phase transition of liposomal (New, 1990) and niosomal systems (Cable, 1989) resulting in niosomes that are less leaky (Rogerson et al., 1987). In present investigation, cholesterol increase from 1 to 3 m.r. abolished the T_c in all formulations (Fig. 2). However the study of hydrated formulations with high sensitivity DSC (HSDSC) will reveal more useful data.

The DSC results for non-hydrated samples, revealed single transition peaks for $C_{16}EO_2$ and $C_{18}EO_2$, but two distinct peaks were observed for $C_{18}EO_{10}$ (Fig. 2). This could imply that the EO_2 surfactants are comparatively pure, and that the EO_{10} surfactant contains more than one molecular species in sizeable quantities.

Although the insulin molecule has an amphiphilic nature and can interact with the membrane of vesicles and even could sometimes result in an alteration of the physical characteristics of

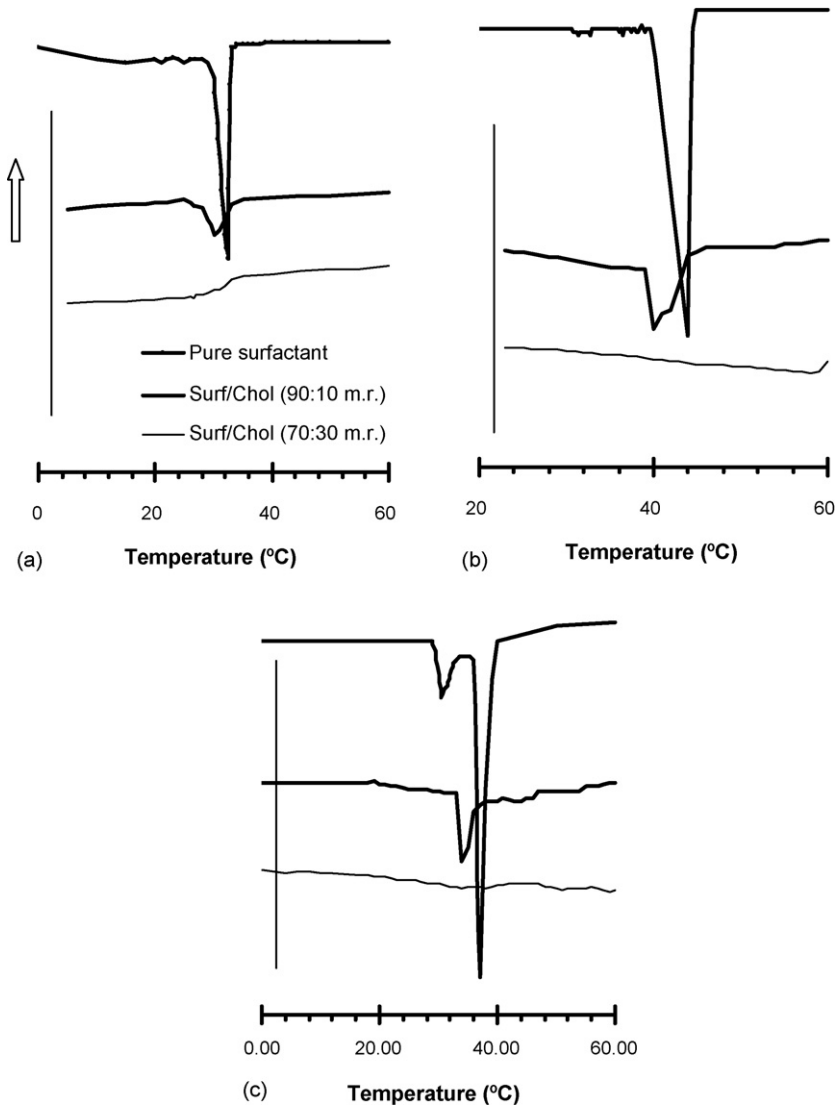

Fig. 2. DSC thermograms of pure semisolid surfactants and freeze dried niosomes composed of different molar ratios of surfactant/cholesterol: (a) Brij 52; (b) Brij 72; (c) Brij 76.

bilayer membranes (Lai et al., 1988), in our experiments, it had a negligible effect on thermograms or T_c of the studied vesicles.

3.3. Vesicle size

The mean volume diameters (d_v) of prepared NSVs are presented in Table 2. The main factors affecting the size and size distribution of vesicles are cholesterol and charge inclusion and also HLB of surfactants.

3.3.1. Cholesterol content effect

Increasing the amount of cholesterol content from 1 to 5 m.r. reduced the volume diameter $C_{16}EO_2$ (Brij 52) significantly ($P<0.05$) (Fig. 3). This effect was also observed in $C_{18}EO_2$ (Brij 72) vesicles when the molar ratio of cholesterol was increased from 0 to 30 ($P<0.05$) (Fig. 4). A similar effect has been reported earlier for $C_{12}EO_3$, $C_{18}EO_3$, $C_{9=9}EO_{10}$ (Brij 97) (Hofland, 1992), $C_{12}EO_7$, $C_{16}EO_5$, and $C_{18}EO_7$ (Van Hal, 1994). As cholesterol increases the chain order, stabilizes the bilayers of vesicles, especially the smaller ones. It is expected that vesicles with relatively high cholesterol content be smaller than vesicles with low amounts of cholesterol (Van Hal, 1994).

The cholesterol-free vesicles consist of highly flexible bilayers which allow the vesicles to attain a variety of shapes, especially at elevated temperatures; the bilayers will take an energetically favored form by stretching and decreasing the curvature. This will result in larger vesicles and also can be observed in the case of $C_{16}EO_2$, $C_{18}EO_2$, $C_{18}EO_{10}$ and $C_{9=9}EO_{10}$ in the absence of cholesterol (Fig. 4).

3.3.2. Charge inclusion

The incorporation of negatively charged dicetylphosphate (DCP) led to a decrease of vesicle size in $C_{16}EO_2$/Chol or $C_{18}EO_2$/Chol NSVs (Fig. 5) ($P>0.05$). This is similar to *Z*-average diameter decrease of polyoxyethylene alkyl ether and the sugar ester vesicles, following the insertion of DCP (Van Hal, 1994). An equation has been proposed (Israelachvili, 1985), whereby the minimum vesicular radius (R) could be calculated

Table 2
Mean volume diameter of niosomes and insulin encapsulation efficiency at different time intervals after preparation and storage at 4 °C

Surfactant	Surfactant/Chol[a]/DCP[b] molar ratio	Mean volume diameter (μm) ± S.D.				Insulin encapsulation efficiency (EE%)		
		72 h	1 month	2 months	3 months	72 h	1 month	3 months
Brij 52	10/0	24.00 ± 0.54	–	–	–	–	–	–
	9/1	26.71 ± 2.09	–	–	–	–	–	–
	7/3	13.39 ± 0.25	8.24 ± 0.36	7.16 ± 1.02	6.28 ± 0.99	40.20 ± 4.12	31.42 ± 3.68	20.16 ± 8.10
	6/4	9.38 ± 0.20	7.21 ± 0.71	5.92 ± 0.55	5.74 ± 0.75	29.80 ± 7.25	–	–
	5/5	8.10 ± 0.27	–	–	–	35.95 ± 2.96	–	–
	6/3/1	9.55 ± 0.14	8.74 ± 1.03	8.04 ± 0.94	7.58 ± 1.33	25.14 ± 6.00	–	–
	5/3/2	7.98 ± 0.09	–	–	–	19.76 ± 6.35	–	–
Brij 72	10/0	41.84 ± 0.91	–	–	–	–	–	–
	9/1	33.89 ± 2.58	–	–	–	–	–	–
	7/3	12.13 ± 0.57	17.25 ± 1.22	21.56 ± 1.52	23.25 ± 1.65	40.06 ± 5.09	27.69 ± 3.64	15.28 ± 4.19
	6/4	15.94 ± 0.29	18.50 ± 0.57	19.10 ± 1.23	20.20 ± 1.44	27.93 ± 2.13	–	–
	5/5	15.14 ± 0.63	–	–	–	39.30 ± 2.43	–	–
	6/3/1	12.68 ± .76	15.62 ± 0.98	17.21 ± 1.12	18.14 ± 2.11	22.85 ± 4.95	–	–
	5/3/2	7.68 ± 0.17	–	–	–	20.97 ± 4.18	–	–
Brij 76	10/0	12.65 ± 0.65	–	–	–	–	–	–
	9/1	VNF[c]	–	–	–	–	–	–
	7/3	4.55 ± 0.13	7.23 ± 0.42	8.92 ± 0.57	10.26 ± 0.85	28.69 ± 3.97	21.05 ± 5.11	9.25 ± 3.37
	6/4	5.69 ± 0.35	7.12 ± 0.58	8.20 ± 0.89	8.87 ± 0.93	37.55 ± 4.05	–	–
	5/5	4.94 ± 0.10	–	–	–	32.58 ± 2.76	–	–
	6/3/1	5.39 ± 0.05	6.50 ± 0.76	7.92 ± 0.63	8.69 ± 0.77	25.23 ± 5.72	–	–
	5/3/2	4.71 ± 0.17	–	–	–	17.44 ± 5.37	–	–
Brij 92	10/0	5.23 ± 0.34	–	–	–	–	–	–
	9/1	18.22 ± 2.98	–	–	–	–	–	–
	7/3	6.41 ± 0.09	6.52 ± 0.25	6.32 ± 0.52	6.17 ± 0.84	41.50 ± 1.86	35.20 ± 8.10	30.28 ± 3.65
	6/4	21.98 ± 0.67	12.32 ± 0.75	8.01 ± 1.25	6.57 ± 0.65	39.39 ± 3.25	–	–
	5/5	11.22 ± 1.32	–	–	–	39.43 ± 2.39	–	–
	6/3/1	6.31 ± 0.10	6.50 ± 0.66	6.02 ± 0.72	5.56 ± 0.98	30.19 ± 6.00	–	–
	5/3/2	6.26 ± 0.01	–	–	–	21.76 ± 4.26	–	–
Brij 97	10/0	21.50 ± 4.00	–	–	–	–	–	–
	9/1	VNF[c]	–	–	–	–	–	–
	7/3	4.16 ± 0.03	5.52 ± 0.41	6.00 ± 0.71	6.04 ± 0.41	39.22 ± 2.92	19.79 ± 7.45	8.12 ± 2.34
	6/4	4.63 ± 0.09	5.12 ± 0.12	5.60 ± 0.89	5.55 ± 0.85	34.37 ± 6.33	–	–
	5/5	4.95 ± 0.08	–	–	–	30.00 ± 4.52	–	–
	6/3/1	5.70 ± 0.07	6.50 ± 0.63	7.12 ± 0.35	7.65 ± 0.56	27.14 ± 5.81	–	–
	5/3/2	4.81 ± 0.06	–	–	–	19.29 ± 8.06	–	–

[a] Cholesterol.
[b] Dicetylphosphate.
[c] Vesicle was not formed.

for molecule if L_c, V and a_0 were known:

$$R = \frac{L_c}{(1 - V)/a_0 L_c}$$

A disproportionate distribution of DCP in the bilayers may increase the curvature of the bilayer through the effect on electrostatic repulsion between the ionized head group, thus increasing the hydrophilic surface area (a_0). This effect will result in smaller vesicles by using the mentioned equation. The same results in mono and dialkyl glycerol ether niosomes prepared by sonication method were obtained (Cable, 1989). It must be kept in mind that the size of MLVs depends on the properties of the molecules in the bilayer as well as interactions between bilayers, with the ultimate size of the vesicle determined by the distance between the bilayers and the number of bilayers present. The inclusion of charged molecules in the composition increased the separation between bilayers of MLVs. This effect was observed in formulation of MLVs of alkyl glycerol ethers prepared by hand shaking method (Cable, 1989).

DCP inclusion in the other C_nEO_m/Chol/DCP NSVs to maximum 5/3/2 m.r. had no statistically significant effect on the size distribution of them (Fig. 5) ($P > 0.05$).

3.3.3. Effect of HLB on vesicle size

It has been reported that the mean size of niosomes increased with progressive increase in the HLB value in different sorbitan ester surfactants (Ruckmani et al., 2000). The same results were obtained from sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85) NSVs (Yoshioka et al., 1994).

Dissimilar results were obtained when $C_{18}EO_2$ (HLB 4.9), $C_{16}EO_2$ (HLB 5.3) and $C_{18}EO_{10}$ (HLB 12.4)/Chol m.r. was changed from 9:1 to 6:4 (Figs. 3 and 4). On the basis of the vesicles' *Z*-average diameter measurement, composed of $C_{12}EO_3$ (HLB 9.4), $C_{14}EO_3$ (HLB 8.6), $C_{16}EO_3$ (HLB 8) or $C_{18}EO_3$

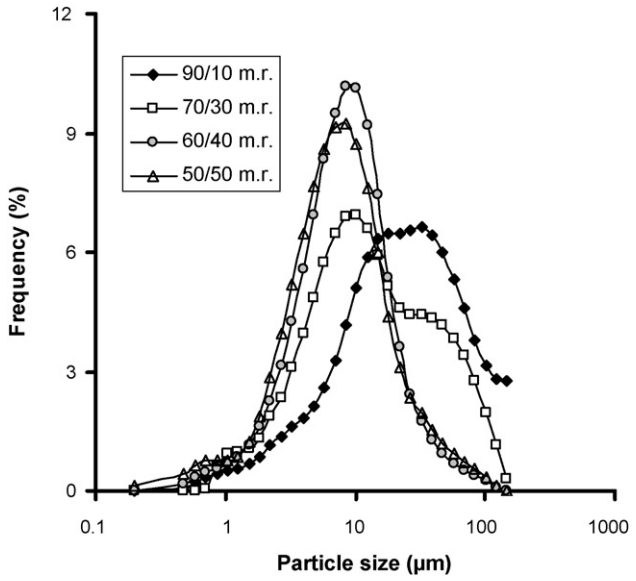

Fig. 3. The effect of cholesterol content on the size distribution of niosomes composed of Brij52/Chol.

(HLB 7.4) prepared by sonication method, it has been concluded that the Z diameters increased as the length of the alkyl chain of the surfactant increased (Van Hal, 1994). This is in accordance with our results when comparing the d_v of $C_{16}EO_2$ and $C_{18}EO_2$ in the free cholesterol niosomes or in the presence of 1, 4 or 5 m.r. of cholesterol (Table 2). The size differences among all surfactant/Chol m.r. were significant ($P < 0.05$). It is obvious that when the polyoxyethylene head group is constant and the alkyl chain length is increased both micellar volume and vesicular size prepared by sonication method (Hofland et al., 1993) or film hydration method (present study) will be increased. Similar results were obtained in vesicles composed of $C_{16}EO_5$/Chol or $C_{18}EO_5$/Chol prepared by dehydration-rehydration (DRV) technique (Stafford et al., 1988).

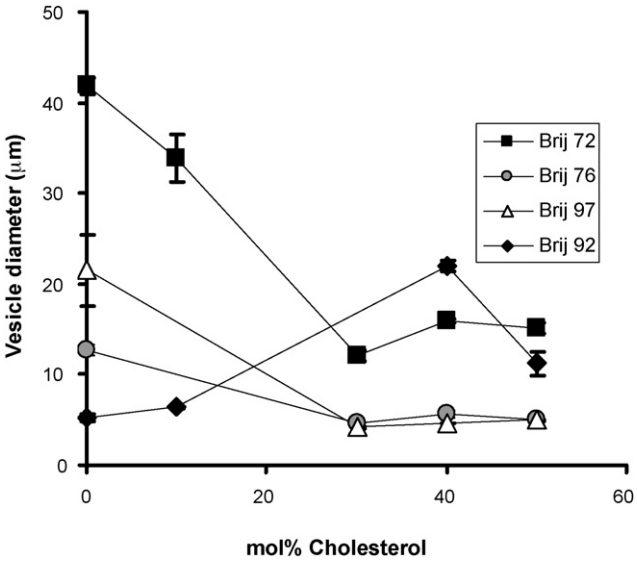

Fig. 4. The effect of cholesterol content on the mean volume diameter (mean ± S.D.) of niosomes composed of polyoxyethylene alkyl ethers/Chol. Diameters were determined by static laser light scattering ($n = 3$).

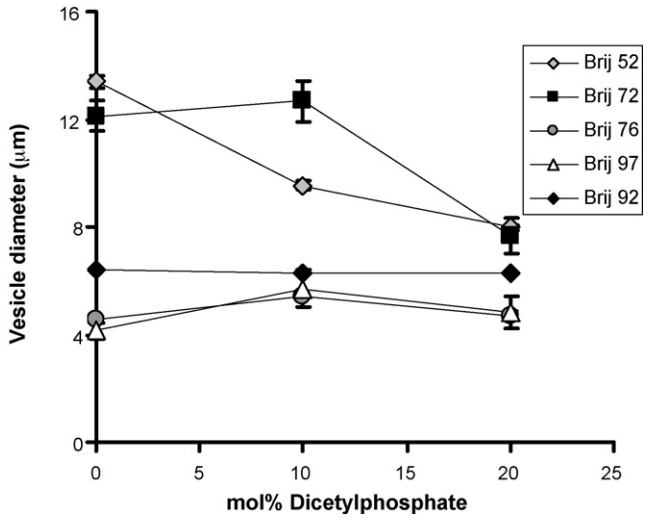

Fig. 5. The effect of dicetylphosphate (DCP) content on the mean volume diameter (mean ± S.D.) of niosomes composed of polyoxyethylene alkyl ethers/Chol/DCP. Diameters were determined by static laser light scattering ($n = 3$).

3.4. Insulin encapsulation efficiency

Insulin encapsulation efficiencies (EE) of all studied formulations are shown in Table 2. Increasing the amount of cholesterol in vesicle's bilayer structure had no significantly effect on insulin EE in the studied formulations ($P > 0.05$). This is in contrast with 5(6)-carboxyfluorescein (CF) incorporation in Span™ family niosomes prepared by hand-shaking method, which increased with increasing cholesterol content (Yoshioka et al., 1994). The surface activity property of large water soluble protein molecule, insulin, in comparison with small water soluble molecule, CF, must be kept in mind in addition to protein flexibility, lipid compressibility, electrostatic interactions, and water structuring about the lipid polar groups when studying the interaction between insulin and lipid bilayers (Schwinke et al., 1983).

Inclusion of negatively charged molecule, DCP, in all formulations led to decrease of insulin encapsulation efficiency (Table 2). This is due to negatively charged nature of insulin molecule in PBS (pH 7.4) that has less affinity to negatively charged niosomes because of electrostatic repulsion.

Encapsulation of insulin in $C_{16}EO_2$, $C_{18}EO_2$, $C_{18}EO_{10}$, $C_{9=9}EO_2$ and $C_{9=9}EO_{10}$ were almost identical (Table 2) ($P > 0.05$). This was similar to encapsulation of 5(6)-carboxyfluorescein in $C_{18}EO_3$, $C_{18}EO_7$ and $C_{12}EO_7$ vesicles (Van Hal, 1994).

3.5. Stability at different temperatures

Stable niosome dispersion must exhibit a constant particle size and a constant level of entrapped drug. There must be no precipitation of the membrane components, which are highly insoluble in aqueous media (Uchegbu and Vyas, 1998). In present study, among the investigated niosomes the rank order for insulin encapsulation efficiency maintenance under storage at 4 °C for 3 months was Brij 92 > Brij 52 > Brij 72 > Brij

76 > Brij 97 (Table 2). However, the difference among the insulin EE maintenance in different formulations was not significant ($P > 0.05$).

Except for Brij 92, other formulations that showed high EE contained gel-state niosome forming surfactants (Brij 52 and Brij 72). However, ideally these systems should be stored dry for more stability by freeze-drying or proniosome formulations (Uchegbu and Vyas, 1998).

In $C_{18}EO_2$ niosomes, increasing the molar ratio of cholesterol from 30 to 40 or incorporation of charged molecules (DCP) in the bilayer composition led to increased stability in the form of less size variation during storage (Table 2). Upon agitation the size increase was not reversible in any formulation. In a similar manner of Lawrence et al. (1996) conclusions about the 1,2-dialkyl glycerol polyoxyethylene ether niosomes, we suggested that size increment was due to growth rather than vesicle aggregation.

A unique and different behavior in size change was seen in $C_{16}EO_2$ (Brij 52) vesicles; after 3 months storage under 4 °C, the mean volume diameter decreased in Brij 52/Chol (7:3 m.r.) vesicles from 13.39 to 6.28 μm, in Brij 52/Chol (6:4 m.r.) niosomes from 9.38 to 5.74 μm and in Brij 52/Chol/DCP (60:30:10 m.r.) NSVs from 9.55 to 7.58 μm (Table 2). This situation was also observed in $C_{9=9}$EO2/Chol (6:4 m.r.) niosomes. Incomplete or slow hydration process was possibly attributed to this kind of size change in vesicles.

Incorporation of charge, led to prevention of creaming or sedimentation of niosomes due to electrostatic stability. In some systems, incorporation of polyoxyethylene carrying addition such as Solulan-C24 causes steric stabilization (Uchegbu et al., 1996, 1997). However because of polyoxyethylene chains presence in C_nEO_m NSVs, the physical stability of them was much more than sorbitan ester vesicles, reported in previous article (Varshosaz et al., 2003), even in the absence of negatively charged DCP.

3.6. Protection of insulin

In the presence of trypsin, there was not any significant difference among protein protection ability of different Brij niosomes (Fig. 6a) ($P > 0.05$), but all formulations protected insulin significantly in comparison with free protein solution ($P < 0.05$). In the presence of α-chymotrypsin, the immunoreactive insulin in Brij 92 and Brij 76 were 87.5 ± 6.52 and 80 ± 8.65%, respectively, which significantly were superior in comparison to the other Brij NSV formulations or free insulin solution ($P < 0.05$). Exposure of encapsulated insulin in Brij niosomes to pepsinic degradation showed a significant difference with non-encapsulated insulin in all studied formulations as showed in Fig. 6b ($P < 0.05$). In pepsin solution the highest protection among Brij NSV dispersions was related to $C_{9=9}EO_2$ (Brij 92), which was not significantly different from the other ones ($P > 0.05$). Slow release of insulin from Brij 92 niosomes may be the reason for high protectibility of this formulation. However, insulin encapsulation in all Brij formulations effectively protected this protein against the proteolytic effects of enzymes efficiently as discussed above.

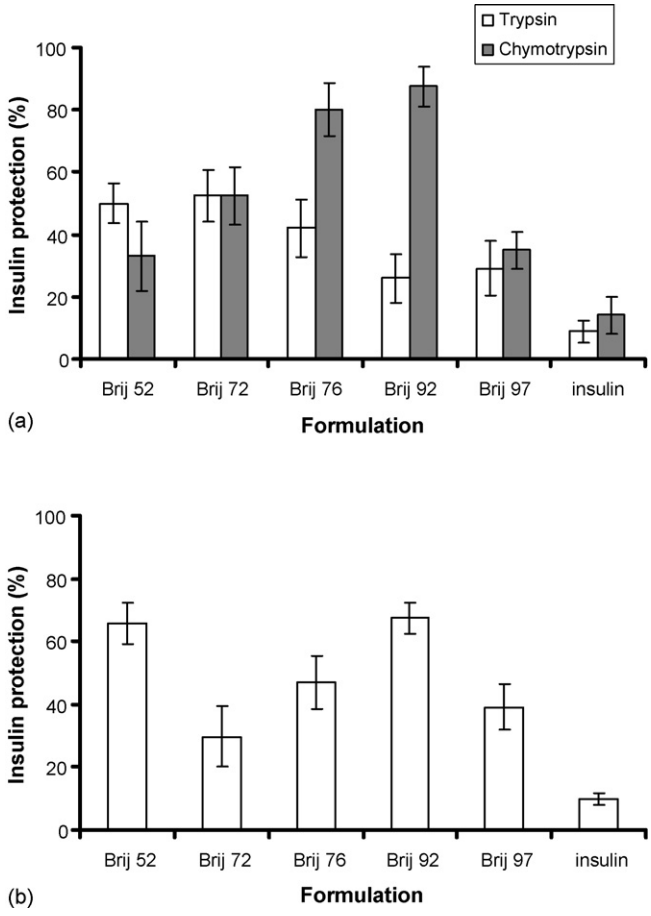

Fig. 6. The insulin protection property (mean ± S.D.) of different Brij/Chol (7:3 m.r.) niosomes against (a) α-chymotrypsin and trypsin solution incubated for 3 h and (b) pepsin solution incubated for 1 h at 37 °C (proteolytic effect of pepsin at 37 °C ($n = 3$).

3.7. Release of insulin

From the release profiles, it appears that insulin efflux from niosomes is a biphasic process containing of an initial relatively fast release and an equilibrium state or a slower release phase achieved within approximately 1 h in SGF and 2–4 h in SIF (Fig. 7). The rapid initial phase may be originated from desorption of insulin from the surface of niosomes and the slower phase related primarily to the diffusion of insulin through the bilayers (Manosori and Bauer, 1989). This kind of release profiles has been observed for insulin delivery from coated and uncoated liposomes (Tabbakhian, 1998) and also for ^{125}I-labeled luteinizating hormone releasing hormone (^{125}I-LHRH) delivery in plasma and 5% muscle homogenate from hexadecyl diglycerol ether/Chol vesicles (Arunothayanun et al., 1999).

Statistically there were not any significant differences between the overall release percent of insulin in SIF with or without enzyme from different C_nEO_m (Brij) niosomal formulations (Table 3, $P > 0.05$). In these formulations, the drug release mechanism in SIF (with and without pancreatin) can be best described by two diffusion based models, i.e. Fick's second law and Baker and Lonsdale equation (Baker and Lonsdale, 1974) (Table 4). According to Baker and Lonsdale model, for a drug

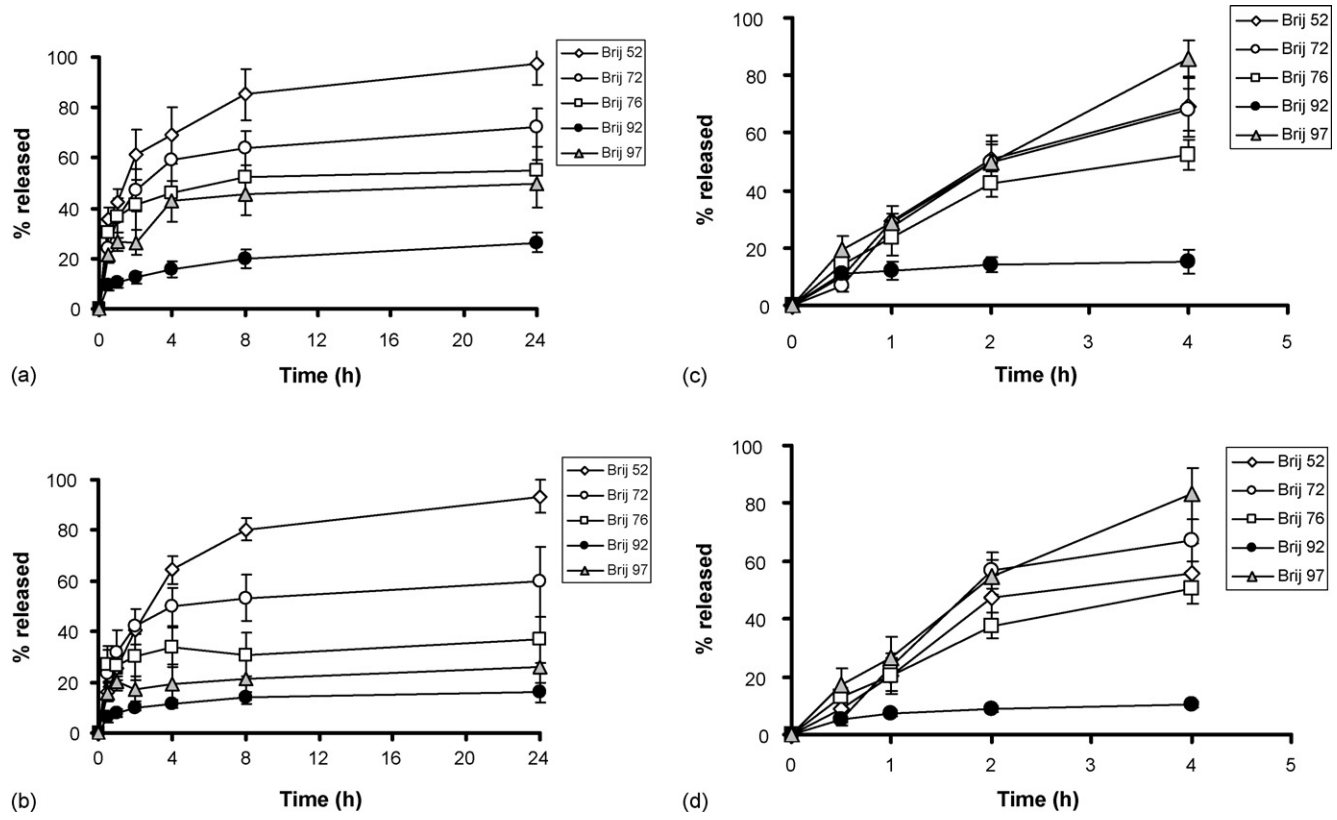

Fig. 7. Release of insulin (mean ± S.D.) from vesicles composed of polyoxyethylene alkyl ether/Chol (7:3 m.r.) prepared by film hydration method in different media at 37 °C. (a) in SIF; (b) SIF without pancreatin; (c) SGF; (d) SGF without pepsin ($n = 3$).

incorporated in a spherical matrix, a straight line is expected for the $(3/2)[1-(1-Q)^{2/3}]-Q=kt$, where Q is release percent of incorporated drug at time t and k is release constant. These results showed that insulin release from the vesicles might be attributed to the diffusion mechanism. On the basis of Baker and Lonsdale's equation, release kinetic constants of different formulations at pH 6.8 (SIF) were from 0.0178 to 0.0204 (Table 4).

When SGF was the insulin delivery medium, in Brij 52 or Brij 76 containing niosomes, drug release profiles were better fitted with Hixson–Crowell model and it might be concluded that drug was released by erosion of niosomal bilayers (Table 4).

The retention profiles or delivery rates are rather variable and can be regulated by several factors including: (a) particle size, (b) lamellarity, and (c) membrane fluidity, as a function of either acyl chain length and saturation or cholesterol content (Weiner et al., 1989). The introduction of double bonds into the hydrocarbon chains and decreasing chain length causes a marked enhancement of vesicle permeability (De Gier et al., 1968). In the case of Brij/Chol niosomes, the short hydrocarbon chain surfactant ($C_{16}EO_2$, Brij 52) released approximately all of entrapped insulin after 24 h in SIF (Fig. 7b). In SGF the presence of double bound in hydrocarbon chain of $C_{9=9}EO_{10}$ (Brij 97) led to rapid release of 85.63 ± 6.5% of encapsulated insulin, during 4 h (Fig. 7d). Similar results have been reported about insulin release from liquid state Span 20 and Span 80 niosomes in comparison with gel state Span 40 and Span 60 NSVs (Varshosaz et al., 2003). In spite of the presence of a double bound in acyl chain of $C_{9=9}EO_2$ (Brij 92), the final released amount of insulin from this NSV dispersion was significantly different from the other Brij formulations ($P < 0.05$). This formulation released only 15.32 ± 4.12 and 10.36 ± 0.89% in SGF and SGF without pepsin, respectively (Fig. 7a and b).

Table 3
The overall release percent of insulin from niosomal formulations (70:30 surfactant/cholesterol molar ratio) different media (mean ± S.D., $n = 3$)

Surfactant	Medium			
	SIF	SIF without enzyme	SGF	SGF without enzyme
Brij 52	97.56 ± 8.47	93.46 ± 6.54	69.01 ± 10.35	55.91 ± 7.29
Brij 72	72 ± 7.6	59.8 ± 13.72	68.23 ± 7.32	67.09 ± 5.14
Brij 76	55.13 ± 9.11	36.8 ± 4.45	52.16 ± 5.3	50.38 ± 3.12
Brij 92	26.31 ± 3.98	15.97 ± 3.75	15.32 ± 4.12	10.36 ± 0.89
Brij 97	49.87 ± 9.32	26.24 ± 3.91	85.63 ± 6.5	83.42 ± 8.77

In SGF (with or without enzyme) and SIF (with or without enzyme), the release data were obtained during 4 or 24 h, respectively.

Table 4
Regression coefficient (r^2) of insulin release data from niosomes formulations in different media according to different kinetic models

Release kinetic model	Brij 52/Chol (7:3 m.r.)				Brij 72/Chol (7:3 m.r.)				Brij 76/Chol (7:3 m.r.)			
	SIF	SIF-WE	SGF	SGF-WE	SIF	SIF-WE	SGF	SGF-WE	SIF	SIF-WE	SGF	SGF-WE
Baker–Lonsdale $((3/2)[1-(1-Q)^{2/3}]-Q=kt)$	0.971 (0.0202)	0.975 (0.0211)	0.951 (0.1299)	0.968 (0.1341)	0.929 (0.0198)	0.916 (0.0194)	0.945 (0.1297)	0.965 (0.1346)	0.859 (0.0186)	0.791 (0.016)	0.974 (0.1308)	0.952 (0.1294)
Fick's second law $(Q_0/(Q_0-Q)=e^{-kt})$	0.974 (−0.2451)	0.999 (−0.2106)	0.881 (−1.2199)	0.961 (−1.2103)	0.982 (−0.1967)	0.986 (−0.1919)	0.887 (−1.046)	0.925 (−1.234)	0.992 (−0.1885)	0.931 −(0.1726)	0.982 (−1.188)	0.895 (−1.190)
First-order $(Q=Q_0\,e^{-kt})$	0.956 (−0.0001)	0.918 (−0.0002)	0.956 (−0.0011)	0.918 (−0.0011)	0.657 (−0.0001)	0.574 (−0.0001)	0.982 (−0.001)	0.924 (−0.001)	0.474 (−0.0001)	0.306 (−0.0001)	0.936 (−0.001)	0.967 (−0.001)
Higuchi $(Q=kt^{1/2})$	0.818 (0.1889)	0.884 (0.2149)	0.963 (0.5354)	0.929 (0.5533)	0.761 (0.1892)	0.725 (0.1826)	0.947 (0.540)	0.902 (0.564)	0.627 (0.1667)	0.468 (0.1423)	0.977 (0.530)	0.985 (0.521)
Hixson–Crowell $(1-(1-Q)^{1/3}=kt)$	0.938 (0.0371)	0.838 (0.0391)	0.985 (0.2481)	0.908 (0.2538)	0.916 (0.0359)	0.901 (0.035)	0.979 (0.246)	0.986 (0.257)	0.845 (0.0334)	0.737 (0.0295)	0.996 (0.245)	0.983 (0.244)
Zero-order $(Q=Q_0-kt)$	0.569 (−0.0298)	0.662 (−0.0353)	0.925 (−0.251)	0.886 (−0.2583)	0.499 (−0.029)	0.462 (−0.256)	0.931 (−0.256)	0.881 (−0.243)	0.372 (−0.0241)	0.261 (−0.0198)	0.889 (−0.241)	0.928 (0.242)
Release kinetic model	Brij 92/Chol (7:3 m.r.)				Brij 97/Chol (7:3 m.r.)							
	SIF	SIF-WE	SGF	SGF-WE	SIF	SIF-WE	SGF	SGF-WE				
Baker–Lonsdale $((3/2)[1-(1-Q)^{2/3}]-Q=kt)$	0.998 (0.0204)	0.975 (0.0199)	0.965 (0.1164)	0.998 (0.1226)	0.889 (0.0199)	0.926 (0.0178)	0.883 (0.1268)	0.912 (1281)				
Fick's second law $(Q_0/(Q_0-Q)=e^{-kt})$	0.961 (−0.222)	0.986 (−0.1921)	0.956 (−1.0128)	0.928 (−0.9135)	0.986 (−0.1903)	0.946 (−0.2136)	0.856 (−1.021)	0.856 (−1.107)				
First-order $(Q=Q_0\,e^{-kt})$	0.767 (−0.0001)	0.581 (−0.0001)	0.544 (−0.0008)	0.712 (−0.0009)	0.571 (−0.0001)	0.431 (−0.0001)	0.974 (−0.0001)	0.992 (−0.0001)				
Higuchi $(Q=kt^{1/2})$	0.912 (0.1878)	0.802 (0.1846)	0.786 (0.481)	0.916 (0.499)	0.747 (0.1861)	0.591 (0.1513)	0.971 (0.497)	0.969 (0.513)				
Hixson–Crowell $(1-(1-Q)^{1/3}=kt)$	0.982 (0.0375)	0.933 (0.0359)	0.917 (0.213)	0.979 (0.221)	0.841 (0.0358)	0.861 (0.0326)	0.945 (0.248)	0.961 (0.2427)				
Zero-order $(Q=Q_0-kt)$	0.716 (−0.0315)	0.556 (−0.029)	0.528 (−0.184)	0.698 (−0.205)	0.493 (−0.0285)	0.386 (−0.0229)	0.985 (−0.239)	0.972 (−0.246)				

Calculated release rate (K) constants are presented in parenthesis. Chol: cholesterol; m.r.: molar ratio; SIF: simulated intestinal fluid; SIF-WE: simulated intestinal fluid without enzyme (pancreatin); SGF: simulated gastric fluid; SGF-WE: simulated gastric fluid without enzyme (pepsin).

Fig. 8. Correlation between decrease of the entrapped insulin retention during 3 months storage of vesicles at 4 °C and the insulin release percent at 37 °C in SGF without enzyme; vesicles were composed of surfactant/cholesterol (7:3 m.r.) ($n=3$).

Unfortunately, on the basis of DSC thermograms we could not explain the strange behavior of liquid state bilayers of Brij 92 niosomes. On the other hand this formulation showed minimal decrease in encapsulation efficiency of insulin during storage at refrigerator temperature (Table 2). There was a good correlation ($r^2=0.9534$) between retention of the entrapped insulin during storage (4 °C) and release of insulin in SGF without enzyme (37 °C); the more leaky formulation during storage, the more percent of insulin release (Fig. 8). This conclusion was not valid for the other studied media.

In all formulations the amount of released insulin in the presence of enzyme is more than the medium without that (Fig. 7) ($P>0.05$). Higher rate and extent of insulin release in the presence of enzymes was driven by the higher concentration gradient due to destruction of protein in the presence of enzymes (Tabbakhian, 1998).

4. Conclusions

The results of this study show that polyoxyethylene alkyl ether type of non-ionic surfactants can be used for preparation of insulin entrapping niosomes. The type of surfactant, the cholesterol content and charge inclusion altered the entrapment efficiency, size distribution range and drug release rate from niosomes. Niosomes composed of Brij 92 and cholesterol was found to most effectively prolong the release of insulin in both SGF and SIF. This formula retained 30.28% of insulin in niosomes after storage at refrigerated temperature for a period of 3 months.

Our study shows that niosomes are able to stabilize insulin against enzymatic degradation and they may be promising candidates as oral carriers of this protein.

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