

Research Article

Theme: Formulation and Delivery of Macromolecules
Guest Editors: Jason McConville and Javier Morales

Formulation and Characterization of Bovine Serum Albumin-Loaded Niosome

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Abstract. Niosomal vesicle, as a unique novel drug delivery system, is synthesized by non-ionic surfactants. Both hydrophilic and lipophilic drugs and also biomacromolecular agents, such as peptides and proteins can be encapsulated in this vesicular particle. Regarding polypeptide-based component loading, and delivery potential of the niosome, some valuable studies have been conducted in recent years. However, exploring the full potential of this approach requires fine tuned optimization and characterization approaches. Therefore, this study was conducted to achieve the following two goals. First, formulation and optimization of bovine serum albumin (BSA) load and release behavior as a function of cholesterol (CH) to sorbitan monostearate (Span 60) molar ratio. Second, investigating a cost- and time-effective polypeptide detecting method via methyl orange (MO) dye. To this aim, BSA-loaded niosomes were prepared by reversed-phase evaporation technique. The effect of CH to Sorbitan monostearate (Span 60) molar ratio on niosome entrapment efficiency (EE%) and release profile of BSA was studied using a ultraviolet (UV) spectrophotometer technique (NanoDrop 2000/2000c). Niosome with a 60% CH content showed the highest BSA EE% and release behavior. Then, BSA was dyed using MO in an acidic solution and used in BSA-niosome formulation. The MO-colored protein, loaded into the vesicles, was successfully assessed by an inverted light microscope, in order to observe the protein location in the vesicle. The results obtained in this study can be useful for various applications in different fields, including pharmaceutical, cosmetics, and drug delivery in biomedical and tissue engineering.

KEY WORDS: bovine serum albumin; controlled release; drug delivery; dyeing; methyl orange; niosome; vesicle.

INTRODUCTION

Niosomes are one type of vesicles with numerous advantages, such as high biocompatibility, low toxicity and immune system activation, and capability of being synthesized by suitable components for targeted drug delivery (1–3). Both hydrophilic and lipophilic drugs and also biomacromolecular agents, such as peptides and proteins can be encapsulated in this vesicular particle. Non-ionic surfactant such as Span® and cholesterol (CH), as a stabilizer additive, are the main components of these nano/micro-carriers. CH content in niosomal formulation is one of the most important parameter that affect the vesicular properties such as entrapment efficiency (EE%), storage time, and release (4,5). Encapsulating polypeptide-based biomacromolecules, including proteins and growth factors are of great interests in many pharmaceutical and

biomedical applications, for their safe and efficient delivery and release.

Bovine serum albumin (BSA; ~66 kDa) is a physiological protein that is widely used in tissue engineering and drug delivery applications, as a suitable carrier because of its biodegradability, non-toxicity, non-antigenicity, and simple synthesis (6,7). The hydrophobicity of BSA has been obtained by determining the number of equilibrium binding of the apolar fluorescent dye 1-anilinonaphthalene-8-sulphonate to BSA which has been 10 (8). BSA is used as combination with other controlled release systems mainly for increasing the stability of the system and prolonging the release profile (6). There are some reported studies using BSA for carrying drugs, such as aspirin, ganciclovir, doxorubicin, etc. and administrated by various routes, such as ocular, brain, intravenous, etc. (9–13).

In the entrapment characterization, detection and observation of the loaded molecules into the vesicles is quite helpful and exciting. As shown in Table I, usually fluorescent assay is used. For which, there are various methods for detecting the fluorophore attached to the loaded biomolecules, luminescence spectrometer, epifluorescence, and confocal microscopy are often used for this purpose (17–19,21). Despite having advantages such as clarity of results and ability to use for numerous applications, fluorescence labeling method has significant disadvantages such

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Table I. Some Methods for Detecting Loaded Biomacromolecule

| Loaded agent | The method of detecting (agent-observation tool) | Aim | Ref. |
|--|---|--|------|
| Glycosaminoglycans (GAG) | Dimethylmethylene blue dye—spectrophotometry | Evaluating growth factor delivery via microsphere in biopolymer scaffolds for osteochondral tissue engineering | (14) |
| Transforming growth factor-b3 (TGF-b3) and dexamethasone (Dex) | Fluorescein isothiocyanate (FITC), confocal laser microscope | Determination of dual delivery for stem cell differentiation using dexamethasone and TGF-b3 in/on polymeric microspheres | (15) |
| Nerve growth factor (NGF) | Coomassie Brilliant Blue-γ counterstain | Evaluating the transport of nerve growth factor encapsulated into liposomes across the blood-brain barrier: <i>in vitro</i> and <i>in vivo</i> studies | (16) |
| Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) | Fluorescence-dequenching assay-LS55 spectrofluorometer | Evaluating HPTS delivery via polysorbate 20 vesicles as oral delivery system: <i>in vitro</i> characterization | (17) |
| BSA | Fluorescence labeling confocal invert microscope | Evaluating microsphere-integrated collagen scaffolds for tissue engineering: effect of microsphere formulation and scaffold properties on protein release kinetics | (18) |
| Dextran | Fluorescence labeling confocal invert microscope | Evaluating the influence of polysaccharide coating on the interactions of nanoparticles with biological systems | (19) |
| Diallyl disulfide (DADS) | Fluorescent dye (calcein)—phase contrast microscopic techniques | Evaluating the efficacy of niosomal diallyl disulfide in the treatment of disseminated murine candidiasis | (20) |

as difficulty of handling, requiring dark room, and requiring treatment with detergents to permeate into cell membrane (22–24). Therefore, using simple chemical dyes such as methyl orange (MO) for coloring the test compound and then visualization of the colored compound by a light microscope are cost- and time-effective methods. In this method, the protein and dye molecules, MO, form a complex. Color change of the MO-protein complex is caused by a proton-exchange reaction, and its intensity depends on the pH of the reaction solution (25). Easy handling is another advantage of this method. Up to now, methyl orange dye has been used for dyeing some other agents such as poly-L-lysine hydrobromide (26), cetyltrimethylammonium bromide (27), CdCl₂ (28), lysozyme (29), glutaraldehyde (30), etc.

In this study, BSA-loaded niosomes were prepared by reversed-phase evaporation technique.

The effect of CH to Sorbitan monostearate (Span 60) molar ratio on noisome EE% and release profile of BSA was studied using an ultraviolet (UV) spectrophotometer technique (NanoDrop 2000/2000c). In addition, BSA was dyed using MO in an acidic solution and used in BSA-niosome formulation. The MO-colored protein, loaded into the vesicles, was assessed by an inverted light microscope, in order to observe the protein location in the vesicle. It is worth to mention that, although there are some studies in which BSA loading into the vesicles has been briefly studied, there is still a lack of detailed analysis of BSA loading into the niosome in the previously published literature. Moreover, the colored biomacromolecule loading into the niosome and its microscopic observation in such a simple way has not been reported before.

MATERIALS AND METHODS

Chemicals and Instruments

Span 60, CH, and BSA were purchased from Sigma Chemical Co. (Germany); diethyl ether was from Merck

Chemical Co. (Germany). MO and standard acidic solution (pH = 4) were purchased from GhataranShimi Co. (Iran). All other chemicals were of the highest grade commercially available.

Obtaining of BSA Optical Density—Concentration Standard Curve

Considering that NanoDrop 2000/2000c Spectrophotometer reports the level of the solution concentrations according to the level of UV absorption, the standard curve was drawn from the resulted optical density (OD) of solutions with standard concentration. For preparation of solutions with standard concentration, BSA was dissolved in deionized water with different BSA concentration (0, 250, 500, 750, and 1000 μg/ml). The OD of 2 μl of these solutions was determined by measuring their absorbance at 280 nm with NanoDrop 2000/2000c Spectrophotometer and OD concentration standard curve has been drawn.

Preparation of BSA-Loaded Niosomes

Niosomes were prepared by reversed-phase evaporation method (Fig. 1) (31). Briefly, set amounts of Span 60 (Table I) with different percentages of CH (0%, 20%, 40%, 60%, and 80%) (Table II) were dissolved in 5 ml of diethyl ether. Subsequently, BSA was dissolved in deionized water (1 mg/ml); 2 ml of this solution was added to the flask and mixed by vortexing for 60 s. The mixture was then emulsified by 5 min sonication in a water bath at 10°C. Afterwards, the emulsion was rotary evaporated at 40°C with a rotating speed of 60 rpm to remove the organic solvent. Material types and concentration of BSA-loaded niosomes preparation were reported in Table II.

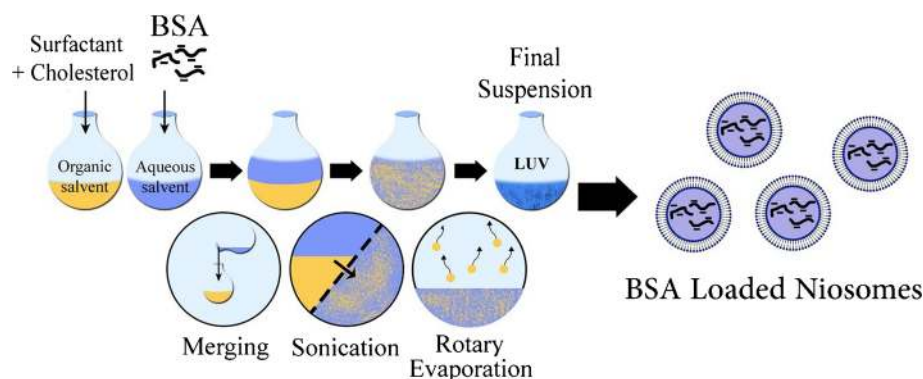


Fig. 1. Schematic illustration of preparation method of large unilamellar vesicle (LUV) (1)

BSA EE%

Final niosomal suspension contained loaded and free BSA. Free BSA was separated by two-step ultra-centrifuge (16,000×g, 4°C, and 25 min), and loaded BSA was washed with deionized water. BSA concentration was determined by NanoDrop 2000/2000c Spectrophotometer, and EE% was determined based on the proportion between the concentration of loaded BSA found in the supernatants and the total concentration of BSA in niosomal suspensions. EE% was obtained using the following equation:

$$EE\% = (WT - WF) / WT \times 100; \quad (1)$$

Where WT is the concentration of BSA in niosomal suspensions and WF is the concentration of free BSA found in the supernatants.

The Study of Protein Release from Niosome Systems

BSA-loaded niosomes with different percentages of CH were separated from non-encapsulated BSA prior to the release studies (ultra-centrifuged 16,000×g, 25 min), and the pellets were redistributed in phosphate buffer saline (PBS; pH 7.4). The samples were incubated at 37°C under a gentle shaking condition. Released BSA was measured at 1, 2, 24, 48, and 72 h. Every time, the samples were centrifuged (16,000×g, 25 min) and the amount of protein in the supernatant was measured with NanoDrop 2000/2000c Spectrophotometer. Considering the EE% obtained in the previous section and the volume of the suspension used in this measurement (VA),

the following formula was used to calculate the total amount of BSA in the suspension:

$$WA = (EE\%) \times VA / 100; \quad (2)$$

Where WA is the total amount of protein in suspension in micrograms and VA is the volume of suspension in milliliters used for the measurement.

Then the released protein (R) in micrograms was placed in the equation below, and the release (%) was obtained from Eq. 3.

$$\text{Release} (\%) = WN / WA \times 100; \quad (3)$$

Where WN is the amount of released protein.

BSA Dying with MO

MO in reaction with BSA molecules caused a color change from colorless to orange in solution. The MO-reacted dried BSA powder has a rich orange color in comparison with the un-MO-reacted dried BSA powder which was white in color. For the preparation of colored BSA, first a mixture of MO (5%, W/V) and the standard acidic buffer was prepared (1:1, V/V, pH=4, adjusted by HCL) and then the uncolored BSA was added to the MO mixture (2 mg/ml). In fact, we add 25 mg/ml MO and 2 mg/ml BSA into the mixture. Regarding the molecular weight of Mo (327.33 g/Mol) and that of BSA (66,000 g/Mol), the number of molecules of each compound and as a result the molar ratio can be calculated, which is 75 mM of Mo for 3 mM of BSA in this experiment. After

Table II. Material Types and Concentration of BSA Loaded Niosomes Preparation

| CH percentage (%) | CH: span 60 ratio (mM) | Diethyl ether (5 ml) | | Deionized water (2 ml) |
|-------------------|------------------------|----------------------|---------|------------------------|
| | | Span 60 (mg) | CH (mg) | BSA (mg) |
| 0 | 0:20 | 13.780 | 0.000 | 4.000 |
| 20 | 4:16 | 11.024 | 2.472 | 4.000 |
| 40 | 8:12 | 8.268 | 4.948 | 4.000 |
| 60 | 12:8 | 5.512 | 7.424 | 4.000 |
| 80 | 16:4 | 2.756 | 9.896 | 4.000 |

1 h mixing, colored BSA was separated by centrifuge (3000×g, 10 min) and the plate was air dried. The spectrophotometry of BSA, colored BSA, BSA-loaded niosome, and colored BSA-loaded niosome were performed by NanoDrop 2000/2000c Spectrophotometer.

Statistics

Statistical analysis of the data from at least three experiments ($N=3$) was carried out using one-way analysis of variance (ANOVA) for one independent factor and two-way ANOVA for two independent factors using the SPSS statistical program (version 19, SPSS Inc) by running a post hoc analysis to the ANOVA. The p value of <0.05 was considered significant. Each value was reported as mean \pm SD.

RESULTS

BSA OD—concentration standard curve

Considering that NanoDrop 2000/2000c Spectrophotometer reports the level of the solution concentrations according to the level of UV absorption, the standard curve was drawn from the resulted OD of solutions with standard concentration. Five samples with defined BSA concentrations were made, and their absorbance was measured to obtain the conversion formula (Eq. 3) from BSA OD concentration standard curve (as seen in Fig. 2).

$$\text{Con} = 544.740 (\text{OD}) + 26.624; (R^2 = 0.993); \quad (4)$$

Effect of CH on BSA EE%

CH is an additive agent for niosome preparation which improve stability and mobility of final vesicles (1,32). BSA EE% in niosomes with different CH percentages were obtained. The data were plotted in a bar graph (Fig. 3). According to the statistical analysis, there were significant differences between all the groups when compared pairwise, for a $p < 0.05$.

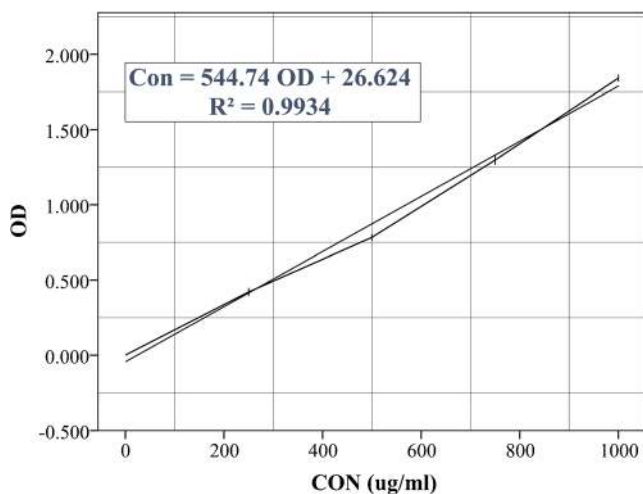


Fig. 2. BSA OD-concentration standard curve

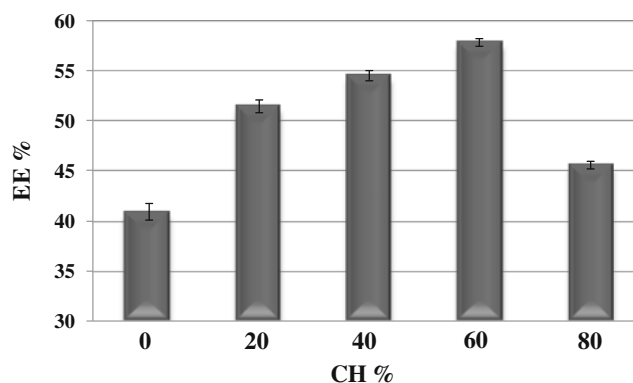


Fig. 3. Effect of CH percentage on the EE% (mean \pm SD, $N=3$; N number of experiments)

In Vitro BSA Release Studies

The release test of BSA in PBS (pH 7.4, 1×) was performed at 37°C and the amount of released BSA was calculated. Having the total amount of BSA in the systems, calculated from Eq. 2, the percentage of released BSA was obtained. This was done by calculating the ratio between the amounts of released BSA in PBS and the amount of total BSA existing in the sample. The release diagram of BSA-loaded niosomes with different CH percentages is shown in Fig. 4.

BSA Dying with MO

MO, also called Orange III, is a colored compound used in dyeing and printing textile materials. MO is also used as an indicator in the titration of weak bases with strong acids by

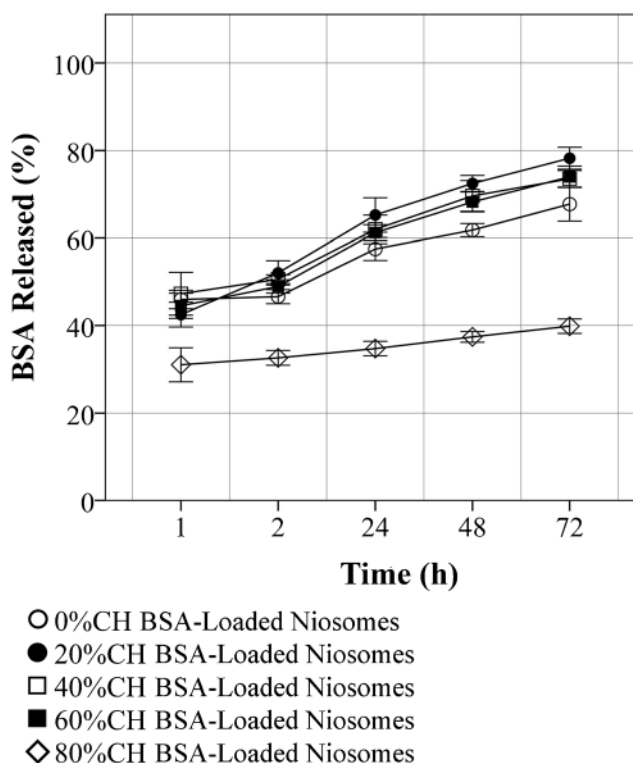


Fig. 4. BSA release diagram from BSA-loaded niosomes with different CH percentages (mean \pm SD, $N=3$; N number of experiments)

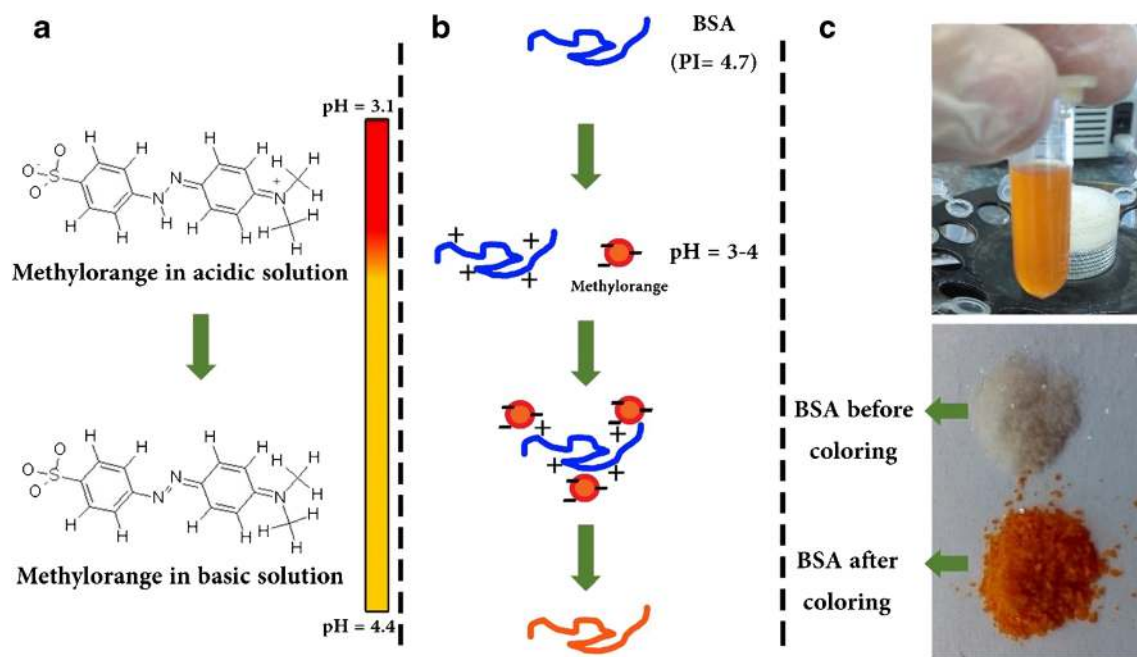


Fig. 5. **a** MO changes the color from red (at pH 3.1) to orange yellow (at pH 4.4) (<http://antoine.frostburg.edu/chem/sense/101acidbase/faq/methyl-orange.shtml>); **b** schematic showing the mechanism of dyeing of BSA by MO; **c** final colored BSA with MO

chemists. It changes the color from red (at pH 3.1) to orange yellow (at pH 4.4) (Fig. 5).

As a polypeptide, BSA is an amphoteric compound due to the presence of amino acids, functional side groups, and of the terminal amino and carboxyl groups. In acidic media (i.e., in the presence of high concentrations of H^+ ions), BSA is positively charged. In an alkaline medium (i.e., in the presence of OH^- ions), BSA is negatively charged. At the isoelectric point (IEP), positive charges from NH_3^+ groups are equal to negative charges from COO^- groups.

Also, the results of spectrophotometry of BSA, colored BSA, BSA-loaded niosome, and colored BSA-loaded niosome are reported in Table III. Running a post hoc analysis to the ANOVA indicated significant differences between all the groups when compared pairwise, for a $p < 0.05$.

DISCUSSIONS

Effect of CH on BSA EE%

As it can be seen, niosomes with 60% CH has shown the highest EE%, at 57.904 ± 0.388 . Compared with other articles with protein-loaded particles, this entrapment percentage is considered an appropriate EE% (1,33–35). Also, as shown in Fig. 3, the amount of loading has been raised by increasing the CH percentage to 60%, but for higher CH ratios than 60%, EE% has experienced a decrease. Similar results have also

been observed in other reported studies (36–39). As mentioned earlier, running a post hoc analysis to the ANOVA indicated significant differences between all the groups when compared pairwise, for a $p < 0.05$.

Essentially, the value of non-ionic surfactant hydrophilic-lipophilic balance (HLB) must be in the range of 3–8 for obtaining niosomal vesicle. For surfactant with higher HLB, cholesterol must be added in order to form a bilayered vesicle. Therefore, surfactant/cholesterol molar ratio is an important parameter and directly affect the EE% (1,32). This has been already shown in a few studies, for example, insulin as a protein has been loaded (EE = 40%) in niosomal vesicle which was prepared from Span 60 and cholesterol (33). The EE% of BSA in liposomal system involving cholesterol has been reported to be 23.55% (40). Comparing with niosomal system, EE% of BSA in liposomes is located in a lower position. Finally, the results suggested that for obtaining a higher loading percentage of BSA in niosomal vesicles, the CH must be used in a optimum CH/Span ratio.

In Vitro BSA Release Studies

In Fig. 4, 30–50% of the loaded BSA was released in the first hour, because of the fast diffusion of BSA molecules located on the surface. As can be seen in Fig. 4, niosomes with 0% and 80% CH showed slower release;

Table III. Spectrophotometry of BSA, Colored BSA, BSA-Loaded Niosome, and Colored BSA-Loaded Niosome

| Samples | BSA | Colored BSA | BSA-loaded niosome | Colored BSA-loaded niosome |
|------------|-------------------|-------------------|--------------------|----------------------------|
| OD in A280 | 0.998 ± 0.012 | 1.502 ± 0.055 | 2.330 ± 0.024 | 2.869 ± 0.956 |

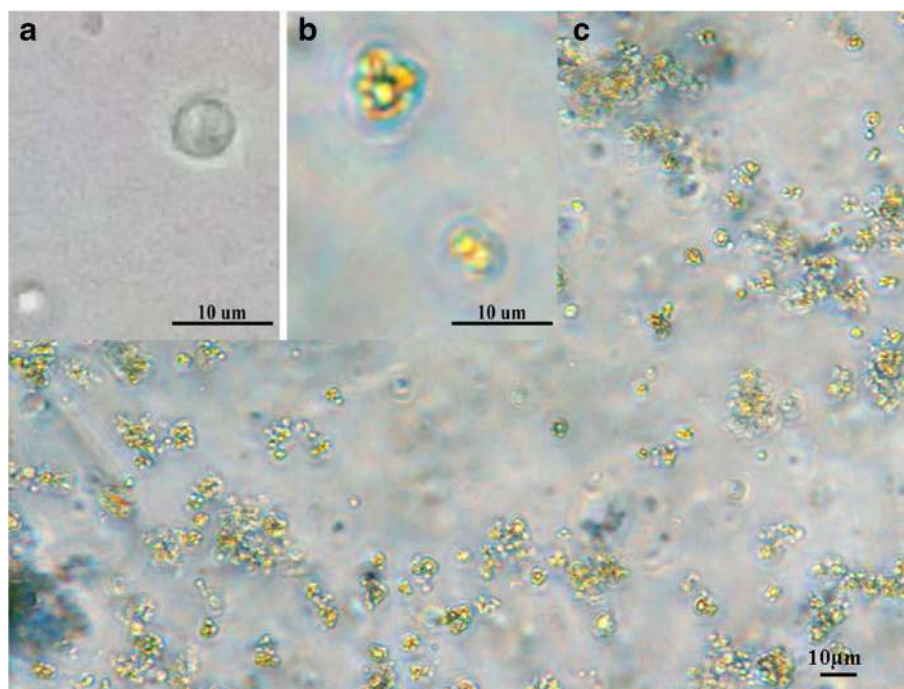


Fig. 6. Light micrographs of uncolored BSA-loaded niosomes (a) and colored BSA-loaded niosome (b, c)

this could be due to the fact that lower EE% caused lower concentration difference between two sides of the niosome membrane in these samples, compared with others. This kind of BSA release profile has also been observed for BSA-loaded niosomal vesicles prepared from Span 80 and Span 85 reported elsewhere (31) in which, 30% of BSA has been released after 3 days. Moreover, other proteins such as insulin, encapsulated in niosomal vesicle, has shown a similar sustained release (38,41). Also, for non-peptide drugs in niosomal systems such as colchicine which is commonly used to treat gout, 10% release after 24 h was reported (42). Regarding the study of protein release from the niosome, there is a statistically significant difference between all different time points ($p < 0.05$). For samples with different CH%, there is a statistically significant difference between all different samples ($p < 0.05$), except 40% to 20% and 60% cholesterol.

BSA Dying with MO

In this study, BSA was treated with Mo in a mixture of acidic buffer solution (pH=4). MO in reaction with BSA molecules caused a color change from white to orange, as can be seen in Fig. 5c. The colored BSA was then used in niosome formulation to indicate the encapsulated BSA, by optical microscopy observation. Therefore, the successful BSA loading was simply confirmed by visualization and imaging via a CCD camera-equipped optical microscope quickly and cost effectively (Fig. 6). Very recently, Ito and Yamamoto have investigated absorbance of the MO-human serum albumin (HSA) complex at 560 nm in the solutions with various pH values (pH 2.4–6.6) (25). Also, they have used surface plasmon resonance (SPR) analysis for confirming MO-HSA binding and reported that one or two MO molecules bind to a HSA molecule at pH 3.6–7.2 (25).

CONCLUSIONS

In this study, a system, based on BSA-loaded niosomes, has been developed which is capable of slowly releasing BSA molecules in PBS. The release profile can be controlled by the CH percentage as one of the formulation parameters. High EE% and sustained release of BSA in niosomes make this system extremely valuable for drug delivery and tissue engineering applications. Another achievement of this article is protein dying by MO as a cost- and time-effective method to ensure the protein loading into the maximal vesicles. This method can be used widely for protein detection in drug delivery applications. Since BSA is very similar to many polypeptide-based biomolecules, in structure, including growth factors, the result obtained in this study could be extended for various applications in different fields, including cosmetics and drug delivery in tissue engineering and biomedicine.

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