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Multifuntional role of liposome-mimicking vesicles – Potential *nanoreactors* and effective *storehouses* for hemoglobin

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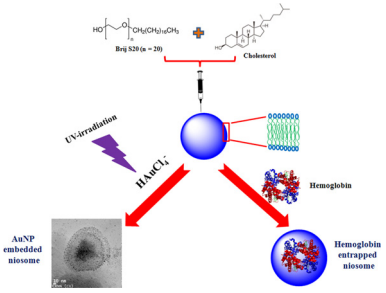
GRAPHICAL ABSTRACT



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ABSTRACT

Liposome-mimicking niosomal vesicles were synthesized using a surfactant having high *hydrophile : lipophile* (HLB) value i.e. Brij S-20. The effect of Brij concentration, preparation method and Brij : Cholesterol ratio on the niosome structure was thoroughly investigated. Due to high HLB of the surfactant, a low Brij : Cholesterol ratio leads to a more compact niosome structure. Fluorescence studies with diphenyl hexatriene (DPH) and a coumarin dye (C-153) indicate disruption of bilayer integrity of niosomes upon increase of temperature. More significantly, the niosomes show two important functions: (i) they can function as very efficient *nanoreactors* for synthesis of gold nanoparticles (GNPs); (ii) they also serve as "*effective storehouses*" for the physiologically important protein hemoglobin (Hb). In niosomes with 1:1 Brij : Cholesterol ratio, small highly crystalline GNPs are formed in the bilayer and larger ones in the aqueous core, thus leaving the surface free for further functionalization. The protein-surfactant interaction has also been studied in detail with an aim to understand the role of the niosomal bilayers in maintaining protein stability. It has been inferred from fluorescence and circular dichroism studies that Hb remains well encapsulated within the niosomal bilayers and is thus not exposed to external perturbations. Moreover, the Soret absorption of Hb is unaffected for several days upon encapsulation in niosomes. More importantly, Hb encapsulated in niosomes does not show significant denaturation even in presence of the well-known denaturant urea. One practical application of this may be in cases of acute renal failure where high urea concentration leads to severe anemia.

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1. Introduction

Vesicular systems are foundabundantly in living systems. Bio-mimicking vesicular systems are formed from phospholipids. They are popularly known as liposomes [1]. Niosomes are vesicles formed by highly ordered curved bilayer systems composed of nonionic surfactants. They were first introduced by Handjani-Vila et al. [2]. Niosomes offer several advantages over convensional liposomes such as higher stability, enhanced skin-penetration properties and lower cost of production [3,4]. Different classes of nonionic surfactants, e.g. polyglycerol alkylethers, polyoxyethylene alkyl ethers, etc have been employed for the preparation of niosomes [5,6]. The role of cholesterol is also important. It imparts rigidity and orientational order to the niosomal bilayer [7]. Liposomal vesicles are good models for cells. The applications of niosomes in the various fields have been studied extensively [8–11]. They are mostly used in the field of controlled delivery of hydrophobic and hydrophilic drugs [12] as their permeability is greatly enhanced above the membrane melting temperature [13].

Vesicular systems are good candidates for controlled reactions and they serve as excellent microreactors for synthesis of nanoparticles [14–18]. The flexible bilayer can tune the growth of the nanomaterials [19]. Though there are some reports of successful synthesis of metal and metal oxide nanoparticles in the liposomal systems, reports of nanoparticle synthesis in niosomal systems are very few [20,21]. Hybrids of vesicles and inorganic nanoparticles are very promising in the field of "theranostics" [21,22]. Particularly gold nanoparticle (GNP) containing niosomal systems can be employed in the field of controlled delivery, nanotoxicology, selective imaging of biological tissues etc. They can be used also as probes to follow different biological processes inside the cells or blood vessels. Iron oxide loaded liposomes exhibit "triggered release of NPs" upon applying alternating magnetic fields [23]. Vesicle-nanoparticle hybrids too have interesting applications viz. medical imaging and targeted drug delivery [22]. GNPs have been synthesized earlier in liposomes [19] and ethosomes [16]. GNPs are good as therapeutic agents. Their strong surface Plasmon resonance (SPR) enables diverse biological applications. Thus GNPs encapsulated in vesicular systems are promising for biological and biomedical applications.

In this work niosomal systems have been prepared from a nonionic surfactant with a relatively high *Hydrophile : Lipophile Balance* (HLB) value. The effect of temperature on bilayer rigidity has been studied using different polarity probes. Having a structure consisting of both hydrophilic and hydrophobic regions, niosomes accommodate a wide variety of solutes (i.e. precursors and reductants for GNP synthesis) and act as excellent microreactors for nanoparticle synthesis. The effect of nanoparticles on bilayer rigidity has also been studied.

In addition, the niosomal systems play the role of a perfect carrier of the heme protein hemoglobin (Hb). It is an iron-containing metalloprotein in mammalian red blood cells and has four oxygen binding sites [24]. Hb was selected for our study as it is a physiologically important protein involved in many diseases like leukaemia, anaemia etc [25]. Moreover, major insight is required into the interactions of vesicles and proteins to understand actual behavior in living systems and to explore the viability of using these systems as biological models Hb is suitable in this respect as it has distinct spectral signature i.e. UV–vis absorption, fluorescence and circular dichroism. Hence the Brij S-20 niosomes open up new possibilities for their multivaried applications in the field of bionanotechnology.

2. Materials and methods

2.1. Materials

Cholesterol and the non-ionic surfactant Brij S-20 were purchased from Himedia and Sigma Aldrich, respectively. Diethyl ether, Chloroform and Methanol were purchased from Merck (India). Chloroauric acid ($HAuCl_4$) was purchased from SRL, India. Bovine Hemoglobin was from Fluka. 1,6-diphenyl-1,3,5- hexatriene (DPH) and Coumarin 153 were from Sigma Aldrich and Rhodamine B (RhB) was purchased from Merck., Germany. All chemicals were used without further purification. Doubled distilled water was used for preparation of all solutions.

2.2. Methods

2.2.1. Preparation of Brij S-20 niosomes

Niosomes were prepared by ether injection technique. The mixture of non-ionic surfactant Brij S-20 and cholesterol were dissolved in 10 mL diethyl ether. Then the solution was injected at a fixed rate in a round bottom flask containing 5 mL of water maintained at 60 °C. The suspension was then sonicated for 15 min to produce the final niosomal suspension. To find out the optimum concentration, niosomes were prepared at different molar ratios of surfactant/cholesterol.

To compare various niosome preparation methods, niosomes were also prepared using lipid film hydration method. The mixture of Brij S-20 and cholesterol was dissolved in chloroform/methanol mixture (2:1) in a round bottomed flask. The solvent was evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. During drying, the flask was rotated at 100 rpm speed until a smooth dry lipid film layer was obtained. It was kept overnight in a vacuum desiccator. The film was hydrated using double distilled water. A milky suspension was formed. The suspension was then sonicated for a few minutes to produce the final niosomal suspensions. The niosomes were prepared at various molar ratios of surfactant/cholesterol i.e. 1:1/4, 1:1 and 1:2.

2.2.2. Preparation of GNPs in Brij S-20 micelle

GNPs were prepared using UV irradiation. Accurately weighed amounts of Brij S-20 were used for preparation of surfactant solutions of different concentration. To these solutions different amounts of Au precursor ($HAuCl_4$) stock solution was added to find out the optimum concentration. The optimum concentration of $HAuCl_4$ and Brij S-20 were found to be 9×10^{-4} M and 5×10^{-3} M respectively. Absorption spectra were recorded at regular time intervals. The appearance of wine red color and the SPR peak at 520 nm confirms the formation of GNPs.

2.2.3. Preparation of GNPs in Brij S-20 niosomes

After obtaining the final niosomal suspension, gold nanoparticles were prepared in this medium using sodium citrate as reducing agent under UV irradiation. Final concentration of Au precursor was 9×10^{-4} M. Absorption spectra were recorded at regular time intervals.

2.2.4. Membrane rigidity study

Membrane rigidity of the niosomes was studied using two well known polarity probes DPH and Coumarin 153. The final concentration of DPH was maintained at 10 μM. For Coumarin 153 the final concentration was 2 μM.

2.2.5. Stability of hemoglobin in niosome

For all studies with Hb, the protein stock solution was prepared in Tris buffer and the final concentration was maintained at 3 μM to avoid quenching of Trp fluorescence [11,25]. The final working pH was ~7. For denaturation studies, the urea concentration was varied from 0 to 7 M.

2.3. Instrumentation

A Philips 16 W lamp was used for UV irradiation. The absorption spectra were recorded in a Shimadzu UV-2401PC spectrophotometer. High Resolution Transmission Electron Microscopy (HRTEM) studies of the nanoparticles were carried out at a resolution of 1.9 Å with a JEOL JEM-2100 electron microscope. TEM specimens were prepared by placing micro-drops of solution on a carbon film supported by a copper