A study on the interaction of nanoparticles with lipid membranes and their influence on membrane fluidity

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Abstract. In recent years, liposomes encapsulated with nanoparticles have found enormous scopes in various biomedical fields such as drug design, transport, imaging, targeted delivery and therapy. These applications require a clear understanding about the interaction of nanoparticles with cell membranes. The present work aims to investigate the effect of encapsulation of uncharged and positively charged nanoparticles in three different types of lipids such as 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (SOPC-POPS) mixture and archaeal lipids. Through the temperature dependent fluorescence anisotropy measurements, we have found that the entrapment of nanoparticles in the bilayer has decreased the lipid transition temperature and increased the membrane fluidity of all three types of lipid vesicles. The results were more predominant in SOPC-POPS mixture because of high density encapsulation of nanoparticles in the vesicles due to electrostatic interaction between negatively charged membrane and positively charged iron oxide nanoparticles.

1. Introduction

Liposomes, the self-assembled lipid structures have received extensive attention due to their potential application in various fields. Because of versatile organization, well-defined physicochemical properties and ability to mimic membrane scaffolding, they are widely studied as model membranes. The encapsulation of nanoparticles in liposomes provides a biologically inspired route in designing therapeutic agents and as a means of reducing nanoparticle toxicity. The hybrid lipid/nanoparticle conjugates have diverse biomedical applications including imaging of cancer cells, drug/gene delivery, targeted therapy, immunoassay, cell/protein separation, biosensing etc. Currently, little is known about the influence of nanoparticles on physicochemical properties of lipid vesicles such as stability, elasticity, membrane fluidity and bilayer phase behavior [1].
Interest in the synthesis of metal nanoparticles (NPs) is steadily growing due to their unique properties and potentialities. Nanoparticles are highly effective to penetrate the plasma membrane and to alter the natural processes within the cell. They serve as excellent carriers of therapeutic cargos through the membrane, likely due to internalization mechanisms like physical rupturing, membrane mediated transport, pore formation, etc. Recent studies on the effects of different metal NPs on membrane stability and/or deformation have revealed that the incorporation of metal NPs within membrane have altered the phase behavior of the lipids by decreasing the phase transition temperature and increasing fluidity of the bilayer. Since the polymorphic phase behavior of lipids influence different membrane related processes, it has become very important to study the effect of nanoparticle interaction with different lipid membranes [2].

Enormous research has been carried out with homogeneous bilayers consisting of zwitterionic phospholipids, but very less work has been done to understand the electrostatic attraction between the negatively charged lipid bilayers and positively charged nanoparticles. Therefore we intend to study this property in detail and prepared negatively charged lipid vesicles by mixing SOPC and POPS lipids in the ratio of 4:1, respectively. Due to opposite charges, cationic iron oxide NPs are electrostatically attracted towards the negatively charged phosphate group of phospholipids and gets adsorbed. The adsorption process of NPs onto lipid molecules was thermodynamically favorable, and enhanced due to the ultrafine size of the nanoparticles [3].

We have also analyzed the influence of nanoparticles on archaeal lipids. The domain Archaea represents a third evolutionary form of life and their ability to survive in extreme environmental conditions is attributed to their unique lipid composition. The presence of ether linkages and highly branched isoprenoid side chains offers more stability archaeal membranes [4, 5]. We have grown the Aeropyrum pernix K1 archaeal cells in our lab [6] and extracted the lipid from them to study their membrane properties. We encapsulated uncharged Cobalt Ferrite (CoFe$_2$O$_4$) NPs and positively charged Iron Oxide (Fe$_2$O$_3$) NPs in archaeosome and studied their influence on membrane fluidity. The polar lipids of A. pernix K1 consist solely of C$_{25, 25}$-archaeol (2, 3-di-sterpanyl-sn-glycerol), with C$_{25, 25}$-archetidyl (glucosyl) inositol (AGI) accounting for 91mol%, and the remaining 9 mol% by C$_{25,25}$-archetidylinositol (AI) [4, 5].

In lipid vesicles, nanoparticle encapsulation can be achieved by trapping the particles within the aqueous core or in the hydrophobic bilayer. To be embedded in the lipid bilayers, the nanoparticles must possess two important features. They should be smaller in size to fit within a lipid bilayer and should have a hydrophobic surface (by coating with appropriate agents such as sterylamine). When the nanoparticles are entrapped within bilayers, it can lead to changes in lipid packing and may disrupt lipid-lipid interactions amongst the head groups and/or acyl tails. Disruption of such interlipid interactions can result in changes in lipid bilayer phase behavior, which is related to the degree of lipid ordering and bilayer viscosity [8]. When some charged proteins or nanoparticles are adsorbed onto cell surface, the membrane undergoes deformation and lipids in the constituent bilayers will be reorganized due to electrostatic interaction between the lipids and nanoparticles/proteins. Since the membrane is negatively charged, positively charged nanoparticles are attracted more towards the surface of cell-membrane and show higher levels of internalization when compared to uncharged and negatively charged particles. Hence, depending on their size and surface chemistry, embedded nanoparticles may influence the stability and function of hybrid vesicles, domain formation, phase separation etc [9].

### 1.1 Modes of nanoparticle interaction in lipid bilayer

Depending upon the size, electrostatic charge and hydrophobicity, the nanoparticle may be partly or fully trapped in the bilayer. Three different possibilities of nanoparticle entrapment are shown in the figure 1. The first possibility indicates a structure in which a nanoparticle is partially embedded in the bilayer or resting on the vesicle surface. The second possibility shows that the nanoparticle spans the hydrophobic region making equivalent contact with the two quencher populations. The third mode
depicts the entrapment of nanoparticle perfectly in middle of the bilayer due to hydrophobic interactions [10].

Figure 1. Sketches of possible nanoparticle/bilayer interactions

2. Materials and methods

2.1. Synthesis of nanoparticles

The superparamagnetic maghemite nanoparticles (γ-Fe₂O₃) are synthesized through a controlled chemical coprecipitation method. An aqueous mixture of ferric, ferrous salts and sodium hydroxide were prepared as alkali stock solutions. The corresponding metal hydroxides were precipitated during the reaction between the alkaline precipitating reagent and the mixture of metal salts and subsequently oxidised in air to form γ-Fe₂O₃. To achieve purification and uniform size distribution, the iron oxide nanoparticles were precipitated out from the solution using ethanol and separated from the supernatant by differential centrifugation method. The purified sample was dried under argon and redispersed in double distilled water. Their surfaces were stabilised electrostatically with positive charge by adsorption of citric acid on their surface. Citric acid provides strong negative surface charge and creates repulsive forces which prevents their aggregation and ensures the stability. The nanoparticles were characterised using X-ray diffractometry and Transmission Electron Microscopy (TEM). The size of the synthesized γ-Fe₂O₃ nanoparticles was found to be 10±2 nm by TEM analysis [7].

The cobalt ferrite nanoparticles were purchased from Sigma Aldrich. They were synthesized by coprecipitating the stoichiometric mixtures of Fe(NO₃)₆.9H₂O and Co(NO₃)₂.6H₂O in aqueous solutions. The pH was maintained between 9.5-11 using 10% NaOH solution and the temperature was set between 70-95°C for 4-5 hours under vigorous magnetic agitation. The resulting mixture was then centrifuged for fifteen minutes at 3000 rpm. The supernatant was then decanted and centrifuged rapidly until a thick black precipitate was obtained. The precipitate was then washed thoroughly with water and acetone for purification and dried overnight at 100 °C in hot air oven. The dried samples were then dispersed in double distilled water. In order to avoid the nanoparticle agglomeration in aqueous solutions, strong surface charges are applied by varying the pH of the solution. This results in high zeta potential value and increased nanoparticle stability. The size of CoFe₂O₄ nanoparticles were found to be in the range of 5-15 nm by TEM and the zeta potential value was estimated to be ± 34 using DLS.

2.2. Isolation and purification of Archaeal lipids

“Aeropyrum pernix” K1 was purchased from Japan Collection of Microorganisms (number 9820; Wako-shi, Japan) and the archaeal cells are cultivated in our lab. They were grown in 800 mL growth medium in 1000 mL heavy-walled flasks, with a magnetic stirring hot plate and forced aeration (0.5 L·min⁻¹) at 92°C. After 40 h, the suspensions were cooled and centrifuged at 11,000×g for 10 min at 10°C. The cell pellets were washed twice with the corresponding buffer (20mM Hydroxyethylpiperazineethanesulfonic acid (HEPES), pH 7.0, containing 3% NaCl). Later the archaeal cells were lyophilized to extract polar-lipid methanol fraction (PLMF) containing approximately 91% C₂₅₂₅-archetidyl (glucosyl) inositol (AGI) and 9% C₂₅₂₅-archetidylinositol (AI). The lipids were fractionated using adsorption chromatography and analysed by Thin Layer Chromatography (TLC) with chloroform/methanol/acetic acid/water (85/30/15/5) solvent. The methanol fraction containing the
polar lipids (PLMF) was used for further analysis. This lipid solution was dried by slow evaporation under a constant flow of dry nitrogen, followed by vacuum evaporation of solvent residues [3, 4].

2.3. Preparation of liposome – nanoparticle conjugates
Adequate volumes of SOPC, SOPC-POPS (both purchased from Avanti Polar Lipids) and Archaeal lipids were dissolved in chloroform and transferred into round-bottomed glass flasks. The solvent from the lipid samples were evaporated using a Rotavapor under reduced pressure (17 mbar). The dried lipid films were then hydrated with the aqueous nanoparticle solutions so that the final concentration of the lipids was made to 1 mg·mL\(^{-1}\). Multilamellar vesicles (MLVs) were prepared by vortexing the lipid suspensions vigorously with glass beads for 10 minutes. The MLVs were further transformed into small unilamellar vesicles (SUVs) by sonication for 30 minutes with 10 s on-off cycles at 50% amplitude with a Vibrcell Ultrasonic Disintegrator VCX 750 (Sonic & Materials, Newtown, USA). To separate the debris from SUVs after sonication, the sample was centrifuged for 10 min at 14,000 rpm (Eppendorf Centrifuge 5415C). The control lipid vesicles without nanoparticles were prepared in a similar way but diluted with 1 ml of 20 mM HEPES buffer instead of nanoparticle solution.

2.4. Bilayer melting and fluidity: Fluorescence anisotropy
Bilayer melting temperatures and fluidity were examined by fluorescence anisotropy measurements using 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethyl-ammonium-6-phenyl-1,3,5-hexatriene (TMA-DPH) in control liposomes and nanoparticle encapsulated liposomes in a 10 mm-path-length cuvette using a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Australia), in the temperature range from 20° C to 90° C. Varian autopolarizers with slit widths of 5 nm for both excitation and emission were used. Here, 10 μL DPH or TMA-DPH (Sigma- Aldrich Chemie GmbH, Steinheim, Germany) in dimethyl sulphoxide (Merck KGaA, Darmstadt, Germany) was added to 2.5 mL of 100 μM SUV solution in the relevant buffer, to reach a final concentration of 0.5 μM DPH and 1.0 μM TMA-DPH. DPH and TMA-DPH fluorescence anisotropy was measured at the excitation wavelength of 358 nm, with the excitation polarizer oriented in the vertical position, while the vertical and horizontal components of the polarized emission light were recorded through a monochromator at 410 nm for both probes. The anisotropy \(<r>\) was calculated using built-in software of the instrument using below formula:

\[
r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}
\]

where \(I_\parallel\) and \(I_\perp\) are the parallel and perpendicular emission intensities, respectively.

2.5. Characterization of nanoparticle encapsulated liposomes
Liposomes were further analyzed by X-ray photoelectron spectroscopy (XPS) to determine their chemical composition [3].

Figure 2. XPS survey depicting the difference in chemical composition (at.%) between 2 spectrums. Upper spectra: Control magnetosomes without Fe\(_2\)O\(_3\) NPs, Lower spectra: magnetosomes encapsulated with Fe\(_2\)O\(_3\) NPs.
3. Results and discussion

Figure 2 shows the chemical composition of a set of liposomes containing magnetic nanoparticles and control liposomes without nanoparticles. In the first case, we can see oxygen and carbon originating from phospholipids as well as high peaks due to Si and Fe which are constituents of magnetic nanoparticles, while in the second case, we can observe only oxygen and carbon originating from phospholipids.

Given that the nanoparticles had diameters exceeding the thickness of a bilayer, this work suggests that lipid bilayers can distort to accommodate such particles and this distortion reduces lipid ordering. This result is consistent with the ability for a cell membrane to accommodate large transmembrane proteins. Since the colloidal particles in bilayer are in dynamic state, the particles are moving and vibrating continuously above absolute zero temperature. Also, the motion of colloidal particles increases with temperature due to bilayer melting. Thus, the movement of colloidal particles would disturb the crystalline structure of bilayer, resulting in decrease of phase transition temperature and increase of the membrane fluidity.

![Figure 3A. Temperature dependent fluorescence anisotropy measurement of Archaeal lipid; control; CoFe₂O₄ NP; Fe₂O₃ NP. DPH with head groups.](image)

![Figure 3B. Temperature dependent fluorescence anisotropy measurement of SOPC-POPS mixture. control; Fe₂O₃ NP.](image)

![Figure 3C. Temperature dependent fluorescence anisotropy measurement of SOPC control; Fe₂O₃ NP.](image)

The upper graph depicts the interaction of DPH and lower graph TMA DPH

DPH and TMA-DPH are the widely used fluorescent probes to study the membrane properties. The results of anisotropy measurements of DPH and TMA DPH in three different types of lipids are shown.
in the figure 3. Anisotropy is a measure of lipid ordering and the bilayer microviscosity. It is inversely related to the membrane fluidity, that is, lower anisotropy values indicate an increase in the membrane fluidity. In order to study the influence of size of nanoparticle in bilayer uptake, we also encapsulated CoFe$_2$O$_4$ NP (20-30 nm) in archaeal lipids. These NPs are almost three times bigger in size compared to Fe$_3$O$_4$ NPs. The initial values of the order parameter of DPH at 20°C were: 0.23±0.01 for control archaeosome; 0.22±0.01 for CoFe$_2$O$_4$ encapsulated archaeosome; and 0.20±0.01 for Fe$_3$O$_4$ incorporated archaeal lipids 0.08±0.01, 0.07±0.01, and 0.06±0.01 respectively. In case of SOPC, the DPH anisotropy values at 20°C were: 0.20±0.01 for control and 0.18±0.01 for Fe$_3$O$_4$ loaded liposome. For SOPC-POPS mixture the initial values were 0.24±0.01 for control and 0.19±0.01 for Fe$_3$O$_4$ loaded liposome. The results have shown that the NPs show lower anisotropy values in all the three types of lipids when compared with the control liposomes without nanoparticles. The differences were significant in DPH rather than TMA-DPH. Due to electrostatic attraction, the cationic iron oxide NPs are attracted more towards the negatively charged SOPC-POPS lipid membrane. The observed encapsulation ratio is increased comparing the uncharged archaeal and SOPC lipids.

4. Conclusions
Fluorescence anisotropy of DPH and TMA-DPH gradually decrease with increasing temperature in all three types of liposome-nanoparticles conjugates. Though there was a gradual decrease in the anisotropy values of all these samples, the difference was predominant in the case of negatively changed lipid mixture (SOPC-POPS) containing positively charged iron oxide NPs. This result is in good agreement with the understanding that electrostatic interactions promote the encapsulation process. It is well known that the fluorescent probe DPH locates primarily in the core of the hydrophobic tails whereas TMA-DPH is anchored within the head group region close to the lipid-water interface. The fact that stronger quenching and significant decrease in the anisotropy values in case of DPH when compared to TMA-DPH confirms that the nanoparticles are trapped predominantly in the middle of the bilayer rather than partially embedding on the membrane surface or equally spanning the bilayer. The results from XPS spectra also confirm that the nanoparticles are successfully encapsulated in the liposomes.

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