ENCAPSULATING LIPID STRUCTURES: PREPARATION AND APPLICATION IN BIOSENSORS, NANOPARTICLES SYNTHESIS AND CONTROLLED RELEASE

Rukan Genç

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Supervised by: Dr. Ciara K. O'Sullivan
Dr. Mayreli Ortiz

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UNIVERSITAT ROVIRA I VIRGILI

Tarragona
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Departament d'Enginyeria Química
Universitat Rovira i Virgili
Campus Sescelades,
Avda. Paisos Catalans, 26
43007 Tarragona
Tel: 977 55 96 58
Fax: 977 55 96 67

Dr. Ciara K. O'Sullivan and Dr. Mayreli Ortiz,

CERTIFY:

That the present study, entitled "Encapsulating lipid structures: Preparation and application in biosensors, nanoparticles synthesis and controlled release" presented by Rükan Genç for the award of the degree of Doctor, has been carried out under my supervision at the department of Chemical Engineering Department of University of Rovira i Virgili, and that it fulfils the requirements to obtain the Doctor European Mention.

Tarragona, 24 Enero 2011

Dr. Ciara K. O'Sullivan Dr. Mayreli Ortiz

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Dr. Ciara K. O'Sullivan and Dr. Mayreli Ortiz,

CERTIFICO:

Que el present treball, titulat "Encapsulating lipid structures: Preparation and application in biosensors, nanoparticles synthesis and controlled release" que presenta Rükan Genç per a l` obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament d'Enginyeria Química d`aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

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To my beloved family from whom I learned to love,

To my friends from whom I learned the forgiveness

To my sweetheart from whom I learned to be loved

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RESUMEN

El objetivo central de la tesis es el desarrollo de un método eficiente para la preparación de liposomas en ausencia de disolventes orgánicos, así como su empleo para aplicaciones analíticas en biosensores, como nanoreactores en la síntesis de nanopartículas de oro, y en la liberación controlada de fármacos.

En el **Capítulo I** se introducen los conceptos fundamentales y se describe el estado del arte.

El **Capítulo II** describe la optimización de un nuevo método que involucra una sola etapa de preparación de poblaciones homogéneas de nanoliposomes unilaminares muy estables.
Este método ha sido desarrollado teniendo en cuenta el fenómeno de formación espontánea de vesículas a partir de los lípidos y la teoría de curvatura. Se basa en la combinación de un cambio rápido de pH 7.4 a 11 y finalmente a 7.4, seguido por un período definido de equilibrio. El método fue aplicado a un amplio rango de formulaciones y se evaluaron varios parámetros experimentales en términos de sus efectos sobre el tamaño y la forma de los liposomas, siendo el intervalo de tiempo de la variación de pH, la temperatura de operación, el tiempo de equilibrio, y el tipo de lípidos los factores que determinan el tamaño, forma y monodispersión de los liposomas.

Una vez establecido el método de preparación de liposomas, en el **Capítulo III** se describe la utilización de liposomas termosensibles como elementos amplificadores de la señal en el desarrollo de un inmunosensor amperométrico para la detección del antígeno carcinoembrionario (CEA), que es considerado un marcador tumoral para el diagnóstico de varios tipos de cáncer como el de mama y colon. Se estudiaron cinco métodos diferentes de bio-conjugación de un anticuerpo anti-CEA a liposomas encapsulando la peroxidasa de rábano (HRP). Se realizaron medidas de -potencial ζ antes y después de cada método de modificación, así como después de la incubación

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con el antígeno CEA como una herramienta para monitorear el éxito de la modificación y la afinidad por el analito de los anticuerpos enlazados al liposoma. Los mejores resultados fueron obtenidos para las modificaciones en las que se emplearon los sistemas SATA/sulfo-SMCC y biotina-estreptavidina como vías de bioconjugación. Durante la detección amperométrica, se observó una amplificación de la señal de aproximadamente 9 y 11 veces respecto al conjugado de anti-CEA-HRP. Se obtuvieron límites de detección de 2 órdenes de magnitud menores que los obtenidos con un conjugado anti-CEA-HRP, tomado como referencia. Además se evaluaron las condiciones de ruptura de liposoma (temperatura v.s. adición de detergente) observándose que la ruptura por aumento de temperatura es un método extremadamente efectivo que evita el uso de detergentes que pueden afectar sistemaza actividad de la enzima. Los resultados obtenidos demuestran claramente el gran potencial de los liposomas con enzimas encapsuladas como herramientas para la amplificación de la señal analítica en biosensores (Genç et al, Anal. Chem. 2010).

En el **Capítulo IV**, se describen diferentes métodos para la síntesis de nanopartículas cuya forma y tamaño son controladas por nano-liposomes y otras estructuras de lípidos empleando los liposomas como nanoreactores o nanoplantillas.

En primera sección de este capítulo, el ácido tetracloroáurico y el mercaptohexanol (MCH) fueron encapsulados en los nanoliposomas preparados con *1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)* **(**DOPG**).** Estos nanoliposomas fueron estudiados como nanoreactores para la síntesis de nanopartículas de oro en presencia de borohidruro de sodio, empleado como agente reductor ($NaBH_4$). Las nanopartículas preparadas empleando diferentes cantidades de $NaBH_4$ fueron comparadas en términos de tamaño de partícula y estabilidad a la agregación. La membrana de la vesícula actúa como barrera para el control de la velocidad de reacción y la protección de las nanopartículas de oro de la agregación rápida a altas concentraciones de reductor.

El glicerol es un conocido poliol empleado para estabilizar los liposomas. También ha sido reportado como agente reductor en la síntesis de liposomas pero empleando altas temperaturas (190 ^{o}C) para completar la reducción del Au(III) to Au(0) en la formación de nanopartículas. En esta sección del **Capítulo IV**, nosotros diseñamos un método ecológico para la síntesis de nanopartículas. El glicerol es incorporado en las

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membranas internas y externas de los liposomas y éstos son empleados como nanoreactores en la síntesis de nanopartículas de oro a temperaturas moderadas. El glicerol, que se encuentra semi-móvil en la membrana de los liposomas, produce sitios de nucleación para la formación de nanopartículas de entre 2 y 8 nm. El tamaño de partículas resulto dependiente de la temperatura de trabajo, la presencia del agente estabilizador, y la concentración de glicerol.

En la sección final del **Capítulo IV** se describe la formación de diferentes superestructuras a partir de lípidos que poseen un centro con carga positiva y otro con carga negativa: *1,2-dioleoyl-sn-glycero-3-phosphocholine* **(**DOPC) y *1,2-dipalmitoyl-sn-glycero-3-phosphocholine* (DPPC) o cargados negativamente y con una cadena carbonada corta como *1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)* (DMPG). Los resultados obtenidos se discuten en función de la geometría del lípido y efectos de temperatura.
Se describen además las aplicaciones de estas superestructuras de lípidos con formas únicas como plantillas en la síntesis de interesantes estructuras metálicas. Se obtuvieron estructuras hexagonales, rectangulares, cintas y trenzas con potenciales aplicaciones en catálisis y electrónica.

El **Capítulo V** describe el estudio de liposomas sensibles a la temperatura en términos de la cinética de liberación del compuesto encapsulado a diferentes concentraciones de calcio y suero, con el objetivo de evaluar sus potenciales aplicaciones en la liberación controlada de fármacos. Se estudió la integridad de la membrana de lípidos a diferentes cantidades molares de *1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine* (DPPE). Este lípido incluido en la membrana de liposomas que contienen fármacos, puede ser eventualmente utilizado para enlazar anticuerpos, mediante sus grupos aminos, facilitar la liberación de la molécula encapsulada. Los liposomas fueron caracterizados empleando diferentes métodos como espectroscopia de correlación de fotones, calorimetría diferencial de barrido y espectroscopia de fluorescencia. Se estudio la estabilidad térmica de los liposomas preparados con la composición de lípidos más estable (DPPE 3 %) en términos la liberación de calceina (molécula fluorescente encapsulada) a temperatures entre 35 y 60 ºC. Se observó un incremento de la velocidad de liberación con el aumento de la temperatura.

En **Capítulo VI** se exponen las conclusiones generales y un plan de trabajo para el futuro derivado de los resultados obtenidos y las potenciales aplicaciones.

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En resumen, en la presente tesis doctoral se ha desarrollo un método de preparación de liposomas, extremadamente rápido, eficiente y que no emplea disolventes orgánicos. Las poblaciones de liposomas obtenidas resultan altamente homogéneas y estable. Se prepararon además diferentes superestructuras de lípidos de una manera controlada donde la composición de lípidos y las condiciones del proceso son determinantes en la estructura final del agregado de lípidos. Tanto los liposomas como las estructuras de lípidos obtenidas fueron exitosamente empleadas en diferentes áreas de aplicación: como agentes amplificadores de la señal en determinaciones electroquímicas de un marcador de cáncer, como nanoreactores/ nanoplantillas en la preparación de diversas estructuras de oro y como vesículas para la liberación controlada de fármacos.

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SUMMARY

The focus of the present thesis is to develop an organic solvent-free method for the preparation of encapsulating liposomes and their use in analytical applications, nanoreactors and template directed synthesis of gold nanoparticles with controlled shape and size as well as their use for controlled release of drugs.

Chapter I is the introduction which covers the general information on lipids and lipid structures and the state of the art.

The core of the thesis, **Chapter II**, is concerned with the optimization of a one-step, time efficient method for the preparation of small unilamellar liposomes, which obviates the need for organic solvents and the lengthy thin film preparation step. Taking into account the phenomena of spontaneous vesiculation and curvature theory together together with critical discussions regarding existing methods and their underlying phenomenon, a new method, coined the "Curvature tuned preparation method" of liposome preparation is described. The method exploits a rapid pH jump from pH 7.4 to pH 11 and instant pH drop to pH 7.4 followed by a defined equilibration time at a controlled temperature. The method was applied to a wide range of lipid formulations, chosen due to their characteristics (e.g type of the lipid polar group, tail length, melting temperature), resulting in diverse nanosized liposomes.

Once the method was established; in **Chapter III**, the application of enzyme encapsulating thermo-sensitive liposomes for their use as signal enhancers in biosensor development is described. A horse radish peroxidase (HRP) encapsulating liposome base amperometric immunoassay was developed for the detection of carcinoembriyonic antigen (CEA), which is one of the most commonly detected cancer marker related to several cancer types such as breast, colon and liver cancer. Liposomes , with their huge capacity to encapsulate hydrophilic molecules in the internal aqueous core coupled with the ease of which they can be conjugated to receptor molecules, highlights their potential as versatile tools for signal enhancement. Enzyme encapsulating liposomes were bioconjugated to antibodies and the encapsulating capacity of the liposome labels, results in an enhanced signal proportional to the amount of encapsulated reporter molecules. The success of a liposomal label is highly dependent on the bioconjugation efficiency to the recognition element with the liposomes, and to this end, five different bioconjugation methods based on affinity and covalent interactions were discussed in terms of their recognition capacity, liposome morphology following bioconjugation as well as the signal

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enhancement obtained with each conjugation method studied. The best results were obtained with biotin-streptavidin and SATA/sulfo-SMCC conjugation approaches, with 11 and 9 time higher signals obtained, respectively, as compared to a Ab-HRP conjugate. The liposome-antibody conjugates were compared in terms of their stability, background signal and limit of detection. Lysing conditions (temperature vs. detergent) were evaluated, with the application of temperature providing an extremely effective means of liposome lysis, thus obviating the need for surfactants, again highlighting the environmental friendliness of the system. The detection limits were two orders of magnitude lower than that obtained with the HRP-antibody reporter conjugate, clearly demonstrating the potential of the enzyme encapsulating liposomes as signal enhancement tools.

Chapter IV is dedicated to several approaches for the synthesis of gold nanoparticles where their shape and size is controlled by the nanosized liposomes and other lipidic superstructures designed either as nanoreactors or templates.

In the first section of this chapter, chloroauric acid ($HAuCl_4$) and capping agent, mercaptohexanol (MCH), encapsulating nanosized liposomes of 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) **(**DOPG) were studied as nanocompartments for nanoparticle synthesis in the presence of increased concentration of a strong reducer, sodium borohydrate ($NaBH_4$), and compared to solution based synthesis in terms of the size and aggregation of the resulting nanoparticles. The barrier effect of the liposomal membrane protecting the gold atoms being formed from rapid aggregation at high reducing agent concentrations is discussed and compared to bulk synthesis.

Glycerol is a known polyol, routinely used to stabilize liposomes, and has also been reported as a reducing agent for gold to form nanoparticles requiring high temperatures of around 190 ^{o}C or more for a complete transformation of the Au(III) to Au(0) and nanoparticle formation. In this section of the **Chapter IV**, we designed a greener method of synthesis by incorporating the glycerol in the external and internal membrane of the liposomes and using them as nanoreactors for nanoparticle synthesis, and compared the nanoparticles obtained with those produced via solution synthesis under the same conditions. Whilst, semi-mobile glycerol molecules localized on the membrane provided nucleation sides for nanoparticle formation and ultrasmall nanoparticles of 2 to 8 nm were obtained, where the particle size was found to be strongly dependent on temperature, presence of capping agent and glycerol concentration.

In the final section of **Chapter IV**, the formation of different lipidic superstructures from either zwitterionic lipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine **(**DOPC) and 1,2-dipalmitoyl-*sn*-

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glycero-3-phosphocholine (DPPC) or negatively charged short tailed lipid 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DMPG) is discussed in terms of the lipid geometry and temperature effect. The potential to exploit the diverse lipid superstructures with unique shapes as templates for the synthesis of interesting metallic nanostructures was explored.. Twisted ribbons, hexagonal and square shaped lipid disks were studied as templating platforms where sodium citrate was used as a reducing agent. In the case of hexagonal and square shaped lipidic disks, a fusion based synthesis strategy was used for exchange of reactants encapsulated in separate populations of lipidic templates. For the ribbon lipid structures, four different methodologies were studied including diffusion based synthesis strategies by immersing the chloroauric acid encapsulating template either in PBS or citrate solution in PBS or citrate encapsulating template in chloroauric acid in PBS as well as the fusion based strategy where two populations of encapsulating templates were mixed. The lipidic superstructures were clearly demonstrated to function as effective templates for the synthesis of metallic nanostructures such as hexagonal disks, planar disks, twisted ribbons, as well as chain-like structures, highlighting the applicability of the approach to synthesize a huge range of diverse nanostructures, which would have tremendous interest in the fields of plasmonics and catalysis.

Finally, in **Chapter V**, thermo-sensitive liposomes with three lipid components were examined in terms of their release kinetics at different serum and calcium ion concentrations in order to establish their potential as encapsulating liposomes for controlled release in drug delivery. The membrane integrity at different molar ratios of *1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine* (DPPE) from 0 to 6 % of the total lipid concentration was also evaluated, where the DPPE would eventually be used to link drug encapsulating liposomes to antibodies so as to facilitate targeted drug delivery. Several methods, such as photon correlation spectroscopy, differential scanning calorimetry as well as fluorescence spectroscopy were used to characterize the liposomes. Following characterization of the liposomes, the thermal sensitivity of the most stable formulation, composed of 3 % of DPPE, was further studied in terms of the calcein release at temperatures from 35 ^{o}C -60 ^{o}C, where an increased release rate from hours to minutes was achieved at increased temperatures.

Overall, the presented Ph.D thesis has contributed to development of a solvent-free, extremely rapid method for the preparation of highly homogenous populations of encapsulating liposomes. Nanoliposomes and several other lipid structures were prepared in a controlled manner where the lipid type and process conditions were observed to strongly influence the final structure of the lipid aggregate. These liposomes and lipid structures were successfully implemented in three different application areas including enzyme encapsulating liposome based signal enhancement in biosensors for the detection of tumor markers, liposomes and lipid structures as nanoreactors

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and/or templates for metal nanoparticle synthesis where the shape and size of the resulting nanoparticles was controlled by template structure and finally characterization of thermo-sensitive liposomes prepared with the developed preparation method for their use as controlled release system.

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CHAPTER

1 INTRODUCTION

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1.1.2 Lipids

Lipids make up a large and diverse group of naturally occurring organic compounds which have an essential role in the cell membrane structure. Although there is great structural variety among the lipids, including fats, phospholipids and steroids, they are related by their solubility in non-polar organic solvents such as ether, chloroform and benzene, and also their general insolubility in water (Roberts and Gabriel 1991), (Segota and Tezak 2006).

Phospholipids are one of the major groups of the four membrane lipids (glycolipids, cholesterol and gliyceride derivatives). They are basically derived from the three-carbon alcohol, glycerol, or from sphingosine. The common alcohol components of phospholipids derived from glycerol are called phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI). They occur via the esterification of one of the primary hydroxyl groups of glycerol to phosphoric acid through its hydroxyl group (polar head). The two remaining hydroxyl groups of the glycerol backbone are esterified to fatty acids (saturated or unsaturated) and form the non-polar tails of the lipid (Segota and Tezak 2006). Figure 1, shows a schematic representation of phosphatidylcholine as an example.

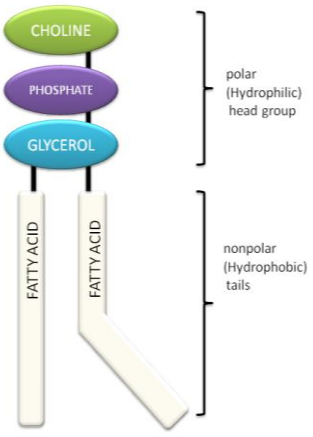

Figure 1. A model of phosphatidylcholine showing one saturated (left) and one monounsaturated (right) fatty acid ester (Alberts, et al. 2002).

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1.1.3 Liposomes and lipid meso-structures

Lipids are a particular kind of naturally occurring surfactant group that form non-covalent aggregates in the presence of an aqueous environment like water (Tomohiro, Shoko and Masahiko 2006). Liposomes (Figure 2) are the most known structures composed of lipids curled into spherical shells in an aqueous environment (Vamvakaki, Fournier and Chaniotakis, 2005). In principle they are identical to the lipid bilayers that form the cell membrane, and share many dimensional, structural and functional properties (A. T. Bangham 1989). The lipids that make up the cell membrane fall into four basic categories; phospholipids, sphingolipids, glycolipids and sterols all of which possess characteristic head (polar) and tail (non polar) structures. Phospholipids are the most commonly used in the preparation of lipid vesicles, as they are cheap, readily available in bulk quantities and have very well established and characterized preparation techniques.

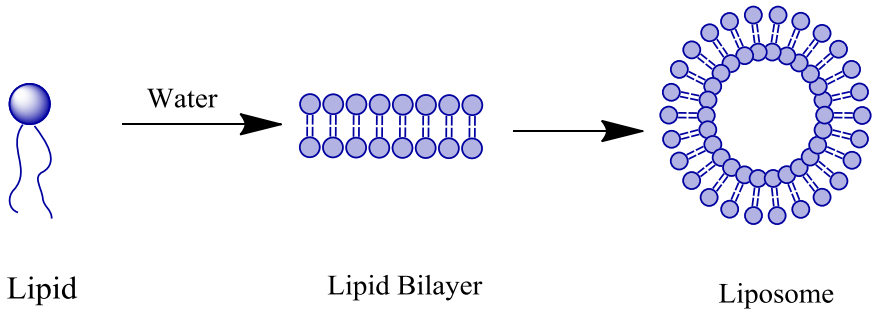

Figure 2: Phospholipids to liposomes

Both liposomes and cell membranes can be described by the "fluid mosaic model", which was proposed in 1972 by Singer and Nicholson (Yeagle 1989). According to this model the head groups of these lipids are hydrophilic and can therefore be left exposed to the aqueous environment while the lipid tails are hydrophobic and therefore orientate towards each other. As a result these lipids can spontaneously arrange in concentric spheres and sheets of bilayers when dispersed in water (Figure 2). However, they may assemble into other types of structures, so called meso structures (tubes, ribbons, and cubosomes) rather than vesicles under specific conditions (Boyd, et al. 2007, Mouritsen 2005). The shape, size and other characteristics of the resulting aggregates mostly depend on lipid geometry and chemical properties, as well as physical conditions (e.g. temperature, pH and salinity). In addition to these properties, attractive-repulsive forces (Derjaguin, Landau, Verwey and Overbeek (DLVO) Theory) and thermodynamic interactions, due to the amphiphilic and ionic properties of the lipids have a role in the vesiculation process (Mouritsen 2005). The self-assembly of lipids to vesicles relies on two main phenomena, the theory of spontaneous vesiculation and the curvature theory,

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dominated by the physiochemical properties of the phospholipids such as lipid head group, tail length and saturation level (Crommelin and Schreier 1994).

1.1.3.1 Phenomenon of Spontaneous Formation and Curvature Theory

Spontaneous vesiculation is a process that occurs without energy supply to the lipid dispersion (Hauser 1989). This phenomenon can simply be explained by the study of the intermolecular forces and also thermodynamics. Attractive forces are one of the important intermolecular forces in vesicle formation. Hydrogen bonds between the head group and water are one of the other important types of attractive forces contributing to the stabilization of the head groups.

Moreover, London forces, occurring via ion_dipole interactions between the charged head groups and water, also have an effect on the stabilization of the polar headgroups (Segota and Tezak 2006), (Tomohiro, Shoko and Masahiko 2006). In addition to the attractive forces, repulsive forces occurring via ion_ion repulsion formed between head groups with the same type of charge are important in vesicle formation. However, it is important to point out that the lipid aggregation needed to form the vesicles or micelles requires attractive forces to be greater than the repulsive forces (Ostrowsky and Sornette 1983).

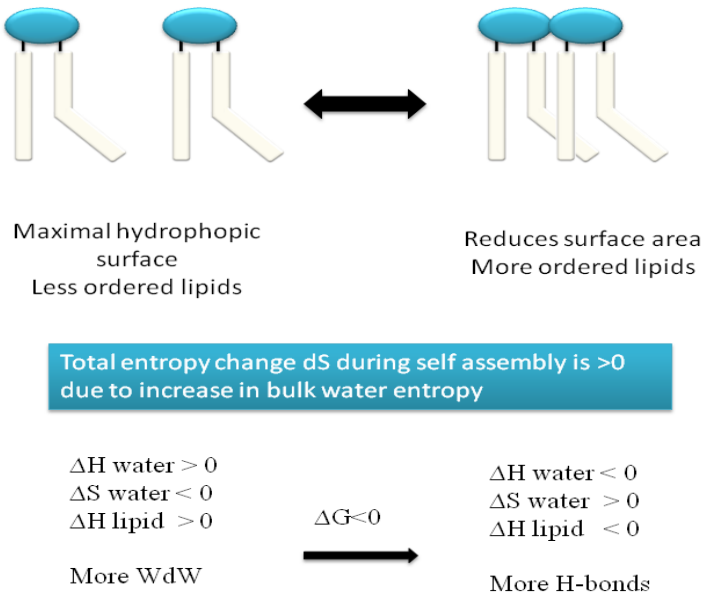

Figure 3. Entropy changes due to lipid organization (http://biology.ucsd.edu/classes/bibc110.SU2.07/webreader/Thermodynamics.htm)

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From the thermodynamic point of the view, the driving force of the lipid self-assembly into various supramolecular structures is the hydrophobic effect. (Figure 3) The stability of the phospholipids within the aggregate is due to both the hydration of the polar head groups and the immersion of the apolar residues in hydrophobic solvent (Alberts, et al. 2002, Segota and Tezak 2006). The first is an enthalpic gain in solvation due to hydrogen bond formation (potential energy minimization) and the second is a gain in the entropy of bulk water. The hydrophobic effect is only valid at low temperatures (e.g. room temperature) and the enthalpic component of solubilization increases with increasing temperature. Since biochemical reactions occur mostly around room temperature, it is assumed that the hydrophobic effect is also entropically driven (Ostrowsky and Sornette 1983).

Under the right conditions (e.g temperature, lipid concentration), a decrease in the overall Gibbs free energy of the system through the clustering, leads to the lipid self-assembling. Nonetheless, these generalized self-assembly phenomena do not specify the exact relation between the resulting structure of the aggregate and the size and shape of the cluster, and only govern the system choice to form lipid particles in order to maximize the degrees of freedom and the number of polar interactions of its water molecules (Nieh, et al. 2004), (Wen-Jun Chen 2003). The resulting structure must have a water-free hydrophobic core and minimized water-soluble surface to favor the rules of thermodynamics.

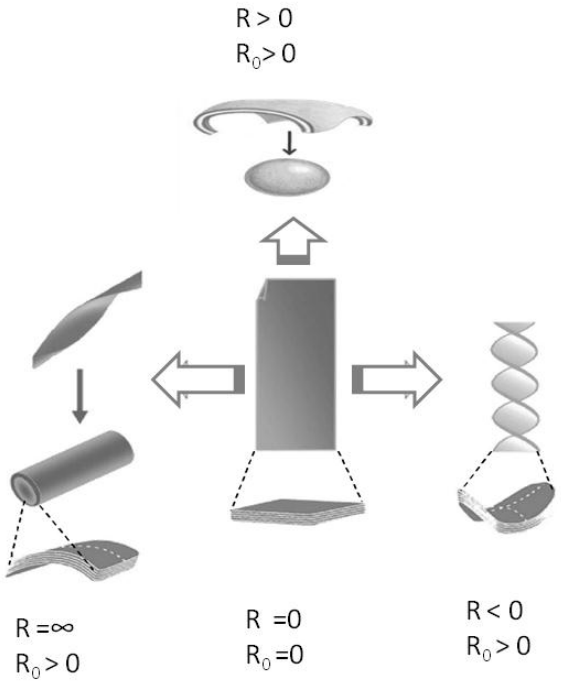

Figure 4: Lipid bilayer from the point of thermodynamics (Oda, et al. 1999).

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The so called P value is a fundamental term explaining the *packing geometry* of the lipid molecule. It can be defined as the ratio between the effective volume occupied by a single lipid molecule and the length of the lipid tail (l) and head group size (*a*), equation 1. In spontaneous vesiculation processes, the conformational change of the amphiphile (hydrophobic effect) raises the P value, thus lowering the curvature.

$$P = V/l.a \qquad \text{(Eq.1)}$$

where, P is the packing factor, V is effective volume occupied by a single lipid molecule; (*l*) length of the lipid tail and (*a*) head group size.

Another term *Instrict Radius of Curvature* defined in equation 2 is the radius of the curvature that minimizes the elastic energy and does not vary significantly with the lipid geometry, but rather with the factors affecting the tails, such as temperature and degree of unsaturation, as well as with properties that lead to an an increase in the effective headgroup area (e.g., charge of the headgroup) (Figure 4) (Gruner 1985).

$$\mu_e = k\,(1/R - 1/Ro)^2 \qquad \text{(Eq.2)}$$

where, μ_e is elastic energy, k is elastic constant, R is the radius of curvature/water interface, Ro is instrict radius of the curvature.

If no other energies compete with μ_e, the lipid monolayers will curl so that R = Ro to lower the elastic energy. The tendency for lipid monolayers to bend in order to lower μ_e results in packed vesicles which are energetically favored (Gruner 1985, Lasic, et al. 2001).

1.1.3.2 Parameters affecting the aggregate size and shape

Although, lipid vesicles are the most known structures of the lipid aggregates, they can also assemble into several other meso-structures, such as tubes, cubosomes and sponge-like structures (Boyd, et al. 2007, Mouritsen 2005). The most important factors on the shape being formed are the chemical structure of the lipids and lipid mixtures, water content (lipid concentration), temperature, pH and ionic strength (Ostrowsky and Sornette 1983).

The chemical structure of the amphiphile is a fundamental property affecting the final structure of the aggregate. The term "chemical structure" includes several properties of the lipid molecule such as shape of the molecule, the relative size and proportion of its polar and non-polar parts, if it is flexible or rigid, charged or uncharged, short or long, branched or linear. This

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effect becomes more important in lipid mixtures since the behavior of the mixture is different to that of the individual lipid components (Segota and Tezak 2006, Engberts and Kevelam 1996).

Water content The supramolecular organization of the lipids also depends on the water content or in other words, lipid concentration in aqueous solution. This effect can be explained bythe term "critical micelle concentration (CMC)" (Gurr, Harwood and Frayn 2005). Below the critical micelle concentration the lipids form a single layer on the liquid surface and are dispersed in solution. At the first critical micelle concentration (CMC-I), the lipids organize in spherical micelles, at the second critical micelle concentration (CMC-II) into elongated pipes, and at the lamellar point (LM or CMC-III) into stacked lamellae of pipes. The CMC depends on the chemical composition and mainly on the ratio of the head area and the tail length (Figure 5) (Segota and Tezak 2006, Zhu, et al. 2005, Maurer, O. and Hofer, 1991).

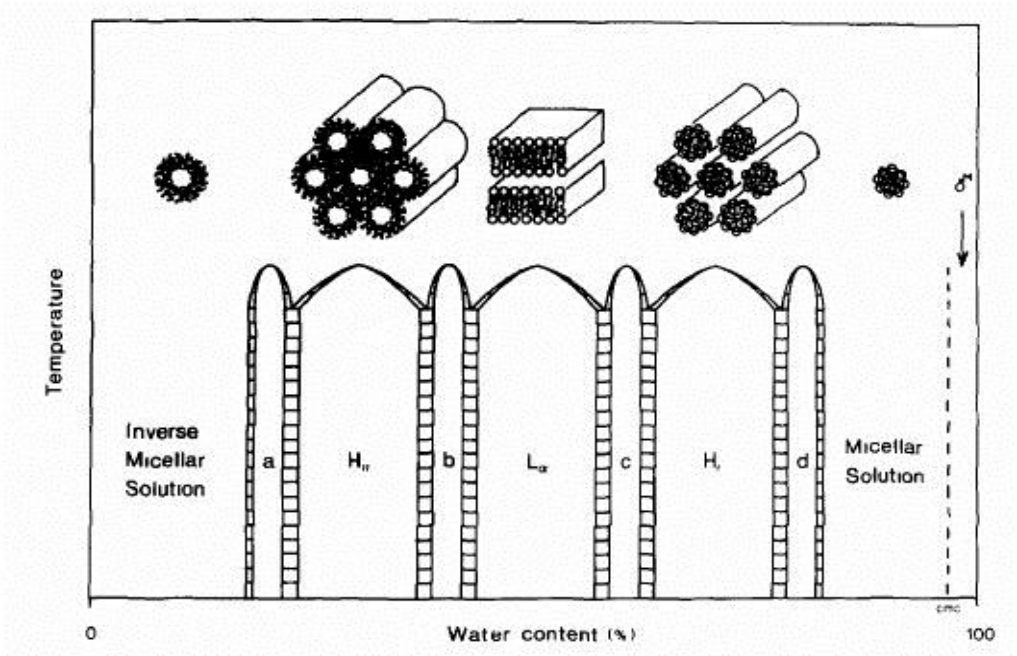

Figure 5. Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids (Seddon 1990).

Effect of Temperature Each phospholipid has a specific phase transition temperature or in other words, critical melting temperature (T_M), which determines the phase behavior of the lipids (Seddon 1990, Mozafari and Mortazavi 2005, Mozafari, Reed, et al. 2002). Hence the temperature has a great effect on the thermal phase behavior of phospholipid monolayers. Above this temperature, the lipid is in the liquid crystalline phase and is fluid. Below this temperature, the lipid is in a gel phase and is rigid (Engberts and Kevelam 1996). The lower the temperature, the smaller the limiting area per molecule due to the increased rigidity. This means

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that low temperature induces solid compressed or crystalline phases. As the temperature rises, the lipid bilayer changes state from a solid state, where the fatty acid tails of the lipids are packed together in a regular arrangement, to a liquid state where the fatty acid tails are more free to move (Figure 6) (Engberts and Kevelam 1996, Seddon 1990).

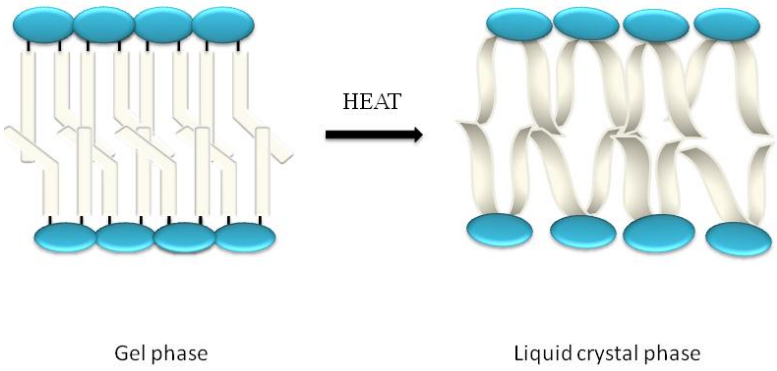

Figure 6: Phase transition of the lipid layers

Effect of pH and ionic strength pH and ionic strength affect the phase behavior of lipid monolayers due to the effect of mobile ions interacting with the fixed charge on the head group of the surfactant. (M. Mozafari, 2005, Hauser and Gains, 1982). The surface potential of a monolayer is influenced by the nature of the polar head groups in the interface. Essentially three components of the phospholipid head group effect the surface potential: (1) the molecular dipole of the head group, (2) the molecular dipole of the ester bond carbonyls, and (3) the charge-charge interaction of the zwitterionic structure (Hauser, et al. 1990) To increase the surface potential of the individual clusters is a known procedure in order to maintain the stability of the lipid clusters (since they are thermodynamically unstable), since higher surface potential result in higher repulsive forces and less aggregation (Lasic, et al. 2001).

Buffer solution In chemistry and biology, a buffer is a substance that minimizes changes due to pH adjustment. In liposomology, they are used to suspend liposomes (Seddon 1990). It is well known that biological processes depend on the salt concentration and the properties of the buffer. The maintenance of the cell membrane and the mass transfer through the membrane are some of the many examples of the use of buffer in nature. Dispersion forces are the forces that serve in the electrodynamic fluctuation of the biological systems and depend on the polarisability of each ion and are thus, very ion-specific. It is known that in high salt buffer systems, the electrostatic forces dominate owing to the high dispersion forces (Seddon 1990). In

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addition to this, the salinity of the environment and the presence of the additional counter-ions induce the formation of lamellae through the low head group hydration and cross-sectional headgroup area due to the increased counter-ion binding. (Engberts and Kevelam 1996, Lasic, et al. 2001).

Lipid asymmetry Lipid asymmetry is one of the important parameters that drives the lipid layer to bend in order to form vesicles by increasing the P value of the system to 0.5 - 1.0 (Lasic, et al. 2001). The use of a mixture of short and long tail lipids, detergents and single tail lipids in an appropriate ratio as well as the use of oppositely charged lipid mixtures moderates the fluidity and transition temperature of the bilayers (Segota and Tezak 2006). Liposomes or other supramolecular structures formed by lipids are thermodynamically unstable due to the transmembrane gradient. An asymmetric distribution of the lipids through the lipid bilayer leads to a compensation effect on this gradient and results in a high homogeneity of the liposome population (Lasic, et al. 2001).

1.1.4 Liposomes: Classification, Preparation and Applications

1.1.4.1 Classification of liposomes

Liposomes can be classified into a number of different categories based on their structural properties and composition. In research, various vesicle structures and lipid compositions have been studied to identify the optimal type of liposome for a particular application (Crommelin and Schreier 1994, Gupta 2006). Liposomes can differ from each other in their size and physical morphology depending on their lipid composition and preparation method, where prepared liposomes can be composed of one or several lipid bilayers. Unilamaller liposomes are classified according to their size, for example, small unilamellar vesicles (SUV) have a diameter of twenty to one hundred nm, while the larger unilamellar vesicles (LUV) are over one hundred nm in diameter and intermediate sized unilamellar vesicles have a size distribution between one and two hundred nm. The largest of the unilamellar vesicles are the giant unilamellar vesicles (GUVs) with a diameter of one to one hundred μm, and are mostly used as model systems to study properties of living cell membrane. All of the LUVs have had pharmaceutical applications however it is the smallest SUVs that are the most commonly used. This is due partly to their small size which facilitates their size-selective cellular uptake into cells as well as their size dependant accumulation in areas such as the brain and spleen (Harasym, Bally and Tardi 1998, Nagayasu, Uchiyama and Kiwada 1999, Poznansky and Juliano 1984, Patil, Rhodes and Burgess 2004, Mamot, et al. 2003, Maestrelli, et al. 2006, Banerjee 2001). In addition to these unilamellar liposomes, other larger liposome structures are also possible. These structures include multilamellar, oligolamellar and multivesicular vesicles. In contrast to the unilamellar

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vesicles, which are composed of a single bilayer, these larger vesicles are composed of many layers.

Liposomes can capsulate diverse type of hydrophilic molecules inside the aqueous core and hydrophobic ones through the lipid membrane due to their amphiphilic character. Those encapsulating liposomes have potential use in several applications requiring a versatile carrier system, such as drug delivery applications, gene therapy, cosmetics and so on. These liposomes with large encapsulating capacity can also be classified by their response sensitivity to their environmental changes such as pH and temperature gradients (e.g. pH-responsive, temperature sensitive liposomes), as well as a spontaneous response when the mobility of the bilayer is altered (e.g. target sensitive liposomes), and they become unstable and spontaneously release their contents when they faced to the necessary conditions such as pH changes (Leroux 2004), or temperature changes (Needham and Dewhirst 2001). These types of liposomes are often used for the delivery of macromolecules to the site of interest in drug delivery (Leroux 2004, Huang 1994).

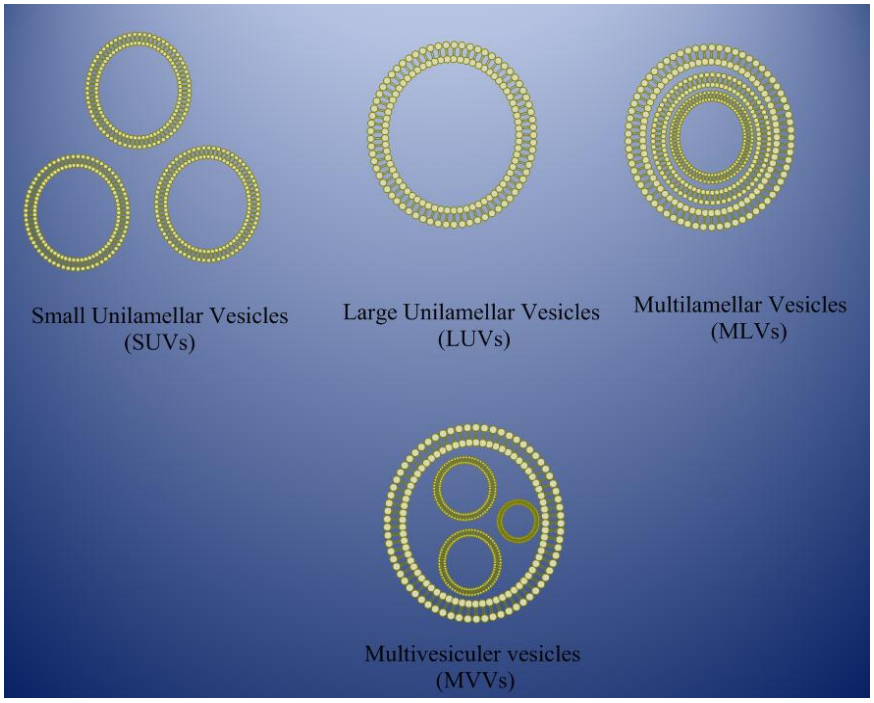

Figure 7: Classification of liposomes

1.1.4.2 Preparation Methods

Several methods of liposome synthesis have been described in the literature. These methods include dry lipid films or emulsions as well as those involving the use of micelle-forming

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detergents or the principles of solvent injection (Figure 8). Despite the vast amount of literature on the subject a fully defined system that results in liposomes of high homogeneity and high stability is still required.

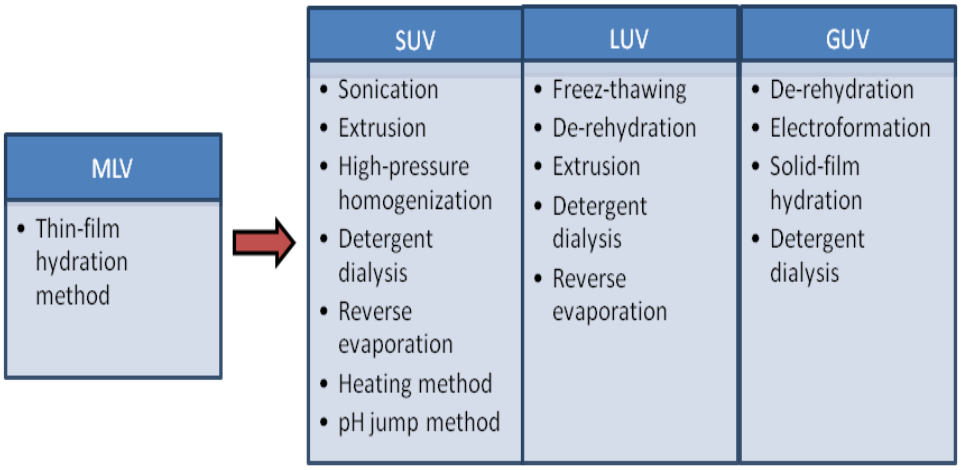

Figure 8: Common preparation techniques for different types of liposomes, categorized by lamellarity and size range (Jesorka and Orwar 2008).

Bangham thin film hydration The first of these methods was described by A. Bangham and colleagues in 1965 (Bangham, et al. 1965). This method, which is known as the thin film preparation/hydration method, is based on the preparation of a thin film of lipids on a glass surface, typically the surface of a round bottom flask connected to a rotary evaporator (Figure 9). In this method lipids are firstly dissolved in an organic solvent, such as chloroform or methanol, and are then dried by the evaporator. Alternatively, this step can be carried out at low pressure for many hours in the presence of a neutral desiccant (Jesorka and Orwar 2008). Following drying the lipid film is rehydrated in excess of aqueous media such as buffer, which in the case of encapsulating liposomes, usually contains the molecules to be encapsulated. As a result of this method, a highly heterogeneous population of MLVs, SUVs are obtained and it is necessary to include an additional, and often, lengthy step, to provide homogenously dispersed liposomes. Some of the most commonly used techniques, particularly for encapsulating liposomes, are sonication, freeze-thaw and extrusion.

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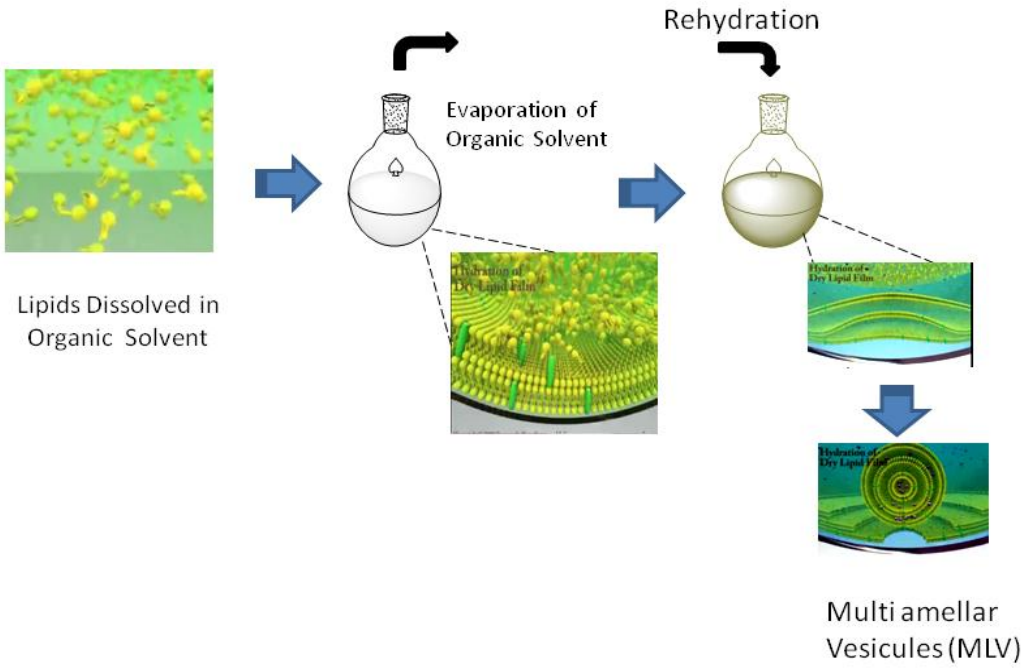

Figure 9: Bangham Thin Film Method. (http://www.encapsula.com/)

Size Reducing and Homogenization Methods

Prepared MLV vesicles via thin-film preparation method requires further sizing and homogenisation methods in order to obtain liposome populations with certain lamellar properties and size (Figure 8): Some of those methods are:

Sonication is a simple method of preparing SUVs. The most common sonication method involves treating hydrated vesicles with titanium tipped sonicator probe for several minutes. The resulting vesicles are then passed through a membrane filter to remove any residual titanium particles. However, this method often results in liposomes of irreproducible homogeneity which is partly due to the incontrollable process conditions. Additionally, the high energy input required in this method often results in damage of the encapsulated material (Richardson, Pitt and Woodbury 2007).

Extrusion The extrusion method for homogenisation of re-hydrated liposomes results in the generation of unilamellar liposomes of uniform size (Berger, et al. 2001). However this is not an easy method to scale up. In this method previously prepared MLVs are subsequently forced

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through polycarbonate membranes of decreasing pore size at elevated temperatures higher than lipid melting temperature (T_M). This procedure is commonly repeated five to fifteen times until vesicles of the desired size are obtained (Jesorka and Orwar 2008). Typical sizes of liposomes obtained for the extrusion through 100 nm pore size are changes in the range of 120-140 nm depending on the lipid composition (Lasic, 1997).

Hydration-dehydration This is a rapid method for the preparation of liposomes, typically requiring minutes to produce unilamellar vesicles in high yields. However, this method results in mixtures of multilamellar as well as giant unilamellar liposomes. The first step in this method is to obtain a dry lipid film, which is subsequently exposed to an aqueous medium. Due to the presence of the buffer salts within the lipid film, an osmotic pressure gradient is created, which forces water between the individual bilayers. This causes the lamellae of the lipid film to separate and form liposomes (Kirby and Gregoriadis 1984). The degree of lamellar separation is a critical factor that affects the formation of liposomes. This degree of separation is influenced by a number of factors such as lipid composition and it has been observed that the inclusion of negatively charged lipid molecules can enhance the separation of the lipid film. (Jesorka and Orwar 2008).

"pH jump" method The so called "pH jump" method, first described in 1982 by Hauser et al, is a relatively rapid method for the preparation liposomes, particularly small unilamellar vesicles, SUV (200-600 Å) (Hauser and Gains, 1982). This method involves a rapid change in buffer pH which effectively breaks MLVs previously prepared using the thin film method, into the more desirable SUVs (Figure 10).

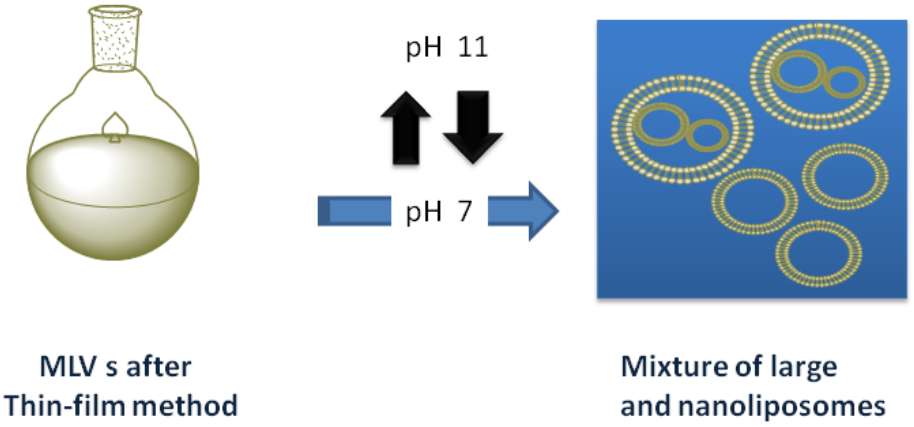

Figure 10: Hauser's pH-jump Method

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However, this procedure must then be followed by separation of the SUVs from any remaining MLVs and LUVs. This separation generally requires size exclusion chromatography (Lundahl, et al. 1999). However, there are again disadvantages associated with this method, such as heterogeneous population of SUVs (around % 40 of the total population) with larger liposomes requiring post homogenisation steps (e.g extrusion), use of organic solvents, and much research has gone into improving this particular method of liposome formation; however, these improved methods require thin film formation which is a lengthy process (Hauser, Henry and Hector, 1990), and little effort has gone into improving the homogeneity of the liposomes produced. As a result there is still a need for a well-defined procedure which results in liposomes of high homogeneity and stability.

One-step SUV Preparation by Heating Method Mozaffari's heating method is a solvent free method where glycerol is used as dispersant agent, which facilitates the homogenous solubilization of lipids and encapsulated materials as well as stabilise the liposome membrane by interacting with the lipid polar head group, and the use of heat as the driving force for the formation of SUVs (Mozafari and Mortazavi 2005). The system is modified depending on the lipids used in the formulation, for example; when cholesterol or any other sterol is integrated in the system liposome preparation takes places at temperatures ≥120 ^{o}C (Figure 11) and in the absence of cholesterol or related sterols, the lipids and the material to be encapsulated would be heated to a temperature around 60-70 ^{o}C depending on the melting temperature of the lipids used (Mortazavi, et al. 2007).

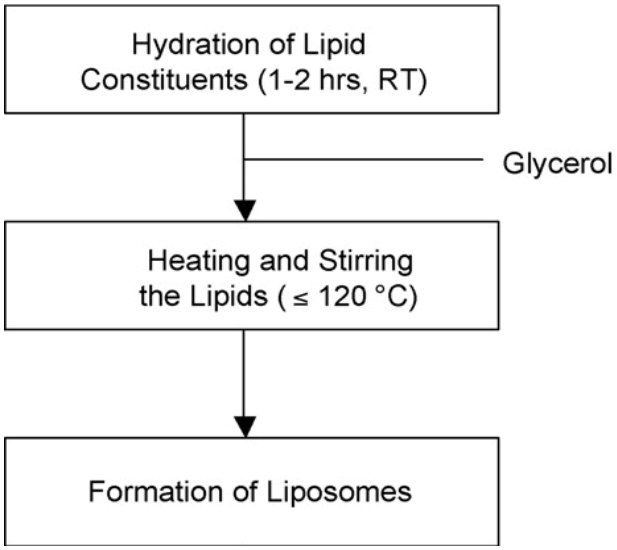

Figure 11: Lipoplex (DNA carrying liposomes) preparation using heating method (Mortazavi, et al. 2007).

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In this method, encapsulated material can be incorporated into the nanoliposomes using three approaches: i) direct addition of the material to be encapsulated to the lipids in glycerol and buffer mixture and heating; ii) addition of the material to be encapsulated to a lipid solution in a glycerol-buffer mixture that has already been heated to a temperature above the melting temperature of the lipids; and iii) primarily preparing the nanoliposomes, and subsequently incorporating the material to be encapsulated e.g. plasmid DNA with Ca^{2+} ions to obtain liposome- Ca^{2+}-plasmid complex, where Ca^{2+} is used as mediator (Mouritsen 2005, M. Mozafari, 2005) (Mortazavi et al, 2007). Whilst this is a very attractive, direct and solvent free method, resulting in highly homogenous liposomes, the temperatures used are high and could denature or affect the activity of protein molecules to be encapsulated e.g. enzymes.

1.1.4.3 Applications

1.1.4.3.1 Analytical applications: Signal amplifiers in biosensors

A **biosensor** is "*a chemical sensing device in which a biologically derived recognition entity is coupled to a transducer, to allow the quantitative development of some complex biochemical parameter*".

(Ahujaa et al. 2007)

An **electrochemical biosensor** is "*a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element.*" (IUPAC)

(Thévenot, et al. 1999)

Biosensors can be further classified as: (**i**) conductimetric, (**ii**) amperometric, and (**iii**) potentiometric based on the parameter measured and as (**i**) catalytic or (**ii**) affinity sensors based on the recognition mechanism.

A widely reported class of biosensorusing biocomplexing receptors are based on immunochemical reactions where the affinity betweenan antigen (Ag) and a specific antibody (Ab) is exploited. Enzymes are linked to antigen (competition) or secondary antibody (sandwich) and the amount of antigen is quantified by measuring the enzymatic activity via measuring electrochemically, optically or fluorometrically (Thévenot, et al. 1999). In the case of immunoliposomal biosensors, reporter molecules are encapsulated within a liposome microenviroment attached to the antibody or antigen. (Edwards and Baeumner 2006).

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Liposomes can be linked to antibodies using several methods including covalent and non-covalent bioconjugation techniques, which can be chosen depending on the chemistry of the ligand and the phospholipid used in the formulation (Martinkova, et al. 2009). Thousands of enzymes can easily be encapsulated into the aquatic core of a signal vesicle or immobilized on its external surface,.

The first liposome based immunosensor was reported by Haga et al. in 1980 for the detection of theophylline, where the detection is based on the use of target sensitive liposomes where their lysis is induced by the binding of anti- theophylline antibody conjugated on the liposome surface with theophylline, and resulting in the release of encapsulated enzyme. Oxygen depletion, which is proportional to the theophylline concentration, was recorded amperometrically on oxygen permeable teflon membrane which is immersed in the analyte solution. With this separation-free detection method, an incremented sensitivity over existing enzyme immunosensor and spin membrane immunoassay was reported (Haga, et al. 1980).

Ho et al., used calcein encapsulating liposomes as biolabels the detection of biotin using an immunoaffinity chromatographic biosensor based on the competition between the carboxyfluorescein (CF) encapsulating, biotin-tagged liposomes and biotin for a known number of binding sites of antibodies, which were previously immobilised on sepharose beads packed into a tubular column. Measurement of the CF amount released from the lysed liposomes via methanol was used for the indirect quantification of biotin with a detection limit as low as 5.0 pg/mL(Ho and Hung 2008).

Vamvakaki and Chaniotakis reported a fluorescent-based biosensor for pesticide detection (with a LOD down to 10^{-10} M) using a pH-sensitive fluorescent agent and enzyme encapsulating nanoliposomes where, mimicking the living cell membrane, the liposome membrane was engineered via the integration of porins on the membrane. The porins were used to facilitate the diffusion of Acetylcholine substrate into the liposomal core. Biocatalytical hydrolisis of the Acetylcholine produces choline and acetic acid which leads to a pH decrease and the consequent enzyme inhibition was measured by measuring the pH change which is directly related to the enzymatic activity so the analyte concentration with a detection limit of 10^{-10} M of pesticide (Vamvakaki and Chaniotakis 2007).

Alfonta et. al. implemented biotin-capped, horseradish peroxidase (HRP) encapsulating liposomes as a reporter probe for antigen-antibody immunorecognition or sensing of

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oligonucleotide-DNA hybridisation using a two-step detection method by the use of impedance spectroscopy and chronopotentiometry. In the first step, binding of the liposomes to the interface alters the interfacial properties of electrode due to the microenviroment of the liposomes, and subsequent precipitation of insoluble product by HRP enhances the primary detection of the analyte, achieving a detection limit of 6.5×10^{-13} M of DNA with current design of biosensor (Alfonta et al. 2001).

Another type of liposome based immunoassay was described by Durst et al., 1993 for the herbicide alachlor detection in short periods of time at low concentrations. The detection system was based on competition between an alachlor-tagged, dye-containing liposome and alachlor in the sample, subsequent to their migration through an anti-Alachlor antibody zone. Quantification of the analyte concentration of alachlor was measured via the quantification of unbound liposomes that migrated to a liposome capture zone either visually or by densitometry, where the alachlor concentration in the sample is proportional to the measured concentration of liposome-entrapped-dye (Durst, 1993), (Siebert, 1993).

Those are some examples of the potential of using encapsulating liposomes for an enhanced signal and sensitivity and a subsequent decreased limit of detection due to their capacity to encapsulate a wide range of reporter molecules into the large aquatic core or external surface, and their flexibility providing facile conjugation with antigens or antibodies.

Nanoreactors/Templates for Synthesis of Metal Nanoparticles

Recent developments in the field of metal nanoparticles with diverse shapes (cubes, disks, tubes, and stars) and size have led to enormous interest in their use for electronic, catalytic, optical and sensor technologies (Eustis and El-Sayed 2006). Today, there are variety of chemical and physical preparation methods, such as colloidal crystallization, monolayer deposition, multilayer casting, molecular crosslinking, the use of complementary interactions and the synthesis of nanoparticles in patterned etch pits, offering nanoparticles with several shapes and unique properties; however, there are very few techniques that allow the control of the shape and the size of the resulting nanoparticles (Shenton et al. 1997). An example of a technique that addresses the requirement for shape controlled synthesis of nanoparticles is template directed synthesis, a simple and cost effective method, where the template material is used as a pattern for the generation of nanostructures assembled via the template geometry where increasing information is becoming available on the template directed synthesis of nanoparticles of controlled shape (Simmons, et al., 2002). Despite the fact that, nanoparticle shapes and properties to be achieved by template directed synthesis is limited only by

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imagination, it is still a challenging topic, since the question of the relation between the template shape and the geometry of the resulting particles is not yet clear (Simmons, et al., 2002).

Shenton et al. reported the preparation of organic-inorganic nanotubes patterned by the tobacco mosaic virus in a hollow tube shape with charged amino acid residues on the internal and external surfaces, providing various nucleation sides for the deposition of nanoparticles being synthesised (Shenton 1999). Sun et al performed a series of studies using solid metals, which can later be converted into soluble species, as templates for the production of hollow nanostructures with several shapes (triangular, cube, tubes and so on) by a replacement reaction (Sun, Mayers and Xia 2002). In another study, the wire-like self-assembly of cadmium sulfide (CdS) was generated on the core of the amphiphilic polymer brushes (Zhang, et al. 2008).

Apart from these solid inorganic and biological templates (e.g. DNA, viruses, polymers and diatoms) as well as reverse micelles, liposomes are suitable candidates for the design of submicron sized reactors for their use as templates for the 2D and 3D organization of inorganic materials. There are several recent studies on liposomes as reactors. Spherical nanoparticles were synthesized in gigantic or small unilamellar liposomes where the particle size is controlled by the reactor volume, however, to date; there have been very few reports on the use of other types of lipid based structures as patterns for metallic nanoparticle synthesis, with atoms being assembled in a shape directed by the template geometry.

Yang et al, reported highly efficient method resulting in nanoparticles in the size range of 4 nm or 50 nm depending on the strategies used for the exchange of the liposomal content. Two strategies were discussed for the exchange of the encapsulated reactant: (i) electrofusion driven vesicle fusion, or (ii) use of nanotubes as channels to connect two liposome populations encapsulating either reducing agent or metal salt (Yang et al. 2009).

In an earlier study by Chow et al., lipidic nanoreactors, in which palladium is incorporated as catalyst, were used for the synthesis of nanosized Au particles where Au(III) reduction by membrane bound Pd catalysts which facilitated the reduction of Au ions by hypophosphite, and resulted in heterogeneously distributed population of mostly multiply twinned particles (Chow, et al. 1996).

In another study reported by Korgel and Monbouquette, phosphatidylcholine vesicles were demonstrated as nanoreactors for the synthesis of various type nanoparticles from CdS, $Zn_yCd_{1-y}S$, and $Hg_yCd_{1-y}S$ as well as core−shell alloys of CdS−HgS and CdS−ZnS nanocrystals within the vesicle core by addition of cations and sulfide to build layered particles. Those particles with tunable size and compositions showing absorbance and luminescence features are providing promising income for the state of the art (Korgel and Monbouquette 2000).

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In addition to use of liposomes as nanoreactors, there are few other reports on the use of other lipid structures. For example, phospholipid tubes, which are used as templates for the formationof patterned arrays of gold nanoparticles (Burkett and Mann 1996). In addition to the tubes, Meister et al reported a study on 1D alignment of AuNPs (5 nm diameter) onto the helically structured nanofibres of self-assembled bipolar phospholipids (Meister, et al. 2008).

Despite the fact that lipid structures providing quite versatile tools for the size and shape controlled synthesis of nanoparticles with their capacity to encapsulate various hydrophilic materials in the internal core or absorbed on the external lipid bilayer, methods resulting in different shapes of lipid structures in a controlled manner are required for revealing their full potential as templates.

Liposomes in controlled release of drugs

Drugs used in the treatment of diseases such as cancer usually have a narrow therapeutic index (Hacker, Messer and Bachmann 2009). Although, there are many approved and developmental drugs which are well-tolerated, there are still numerous drugs that need advanced delivery technologies to improve pharmacokinetics, decrease toxicity, and also increase tolerability and ultimately enhance the therapeutic index.

The toxicity of these drugs may be minimised by the application of drug carrier systems (dendrimers, liposomes, polymers, micelles) (Zhang, et al. 2008). Moreover, surface modification of these carriers by the addition of a targeted ligand (antibodies, peptides, proteins, vitamins) can enhance the selective uptake of drugs to target tissues or cells, thereby minimising non-specific binding to non-target tissues and cells whilst significantly altering the bio-distribution of the drug away from drug sensitive normal tissues and cells.

The concept of site-specific drug targeting was first proposed by Paul Ehrlich in 1906 (Ehrlich 1906). Since then, there have been an enormous number of studies to develop systems to meet the objective of delivery of the right drug at the right time to the right cell. In other words, what is needed is a vehicle capable of carrying the cytotoxic agent, at highly concentrated levels, to the targeted tissue or site in order to provoke maximum damage to the tumour cells whilst sparing the normal cells. Monoclonal antibodies being naturally target-specific are the most general targeting ligands used to modify the carrier system for drugs. Several different routes of administration are available in the design of drug delivery systems, such as oral, intravenous, intraperitoneal, nasal, direct injection, and transdermal. However, in most cases, the best way is to utilize the natural distribution method of the body itself, which is the vascular system. Each

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route has a large number of physiological barriers and is a very important parameter in the design of the carrier system. To develop an efficient drug delivery agent, one should first give attention to: 1) the desired site of action of the drug, 2) the route of the drug administration, and 3) the barriers that the drug must pass before reaching the right place at the right time, 4) the sites that the drug should avoid (e.g. tissue/cells sensitive to drug, or physiological environments that may damage the carrier, such as the high pH in the stomach or the low pH of the intestinal organs) (Karsa and Stephens 1996).

After the consideration of the route and the barriers for drug delivery, the critical parameter is the carrier system. Liposomes are one of the most attractive carrier materials due to the amphiphilic nature of the lipids, and liposomes have the capacity to encapsulate a huge number of water-soluble substances in their aquatic core, as well as oil-soluble substances among the lipid bilayer. Besides this, being a natural product and being easily modified (e.g. via targeting agents such as antibodies for targeted drug delivery), liposomes have seen enormous, increasing interest as carrier and encapsulation material since their discovery by Bangham in 1965 (Bangham, et al. 1965)(Chonn and Cullis 1995), (Medina et al. 2004), (Oku and Namba 1994) and there are growing numbers of proven and commercially available lipid based drug carrying systems such as AmBisome ® , Visudyne ®, DOXIL ® and DaunoXome with highly improved entrapment of soluble compounds as well as liposomal vaccine adjuvants (e.g Inflexal ®) (Li 2006).

A lowered cytotoxicity, higher encapsulation capacity and maintenance of the active component in their aqueous microenvironment, and possibility of formulating sensitive liposomes with environmentally triggered release kinetics, liposomal carriers are attracting great interest of the market with growing numbers of products under clinical development.

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1.2 Concluding Remarks

Lipids can form diverse types of structures and liposomes are closed structures formed by the self-assembly of lipids into bilayers, which further curl into vesicles due to several physical and chemical phenomena including spontaneous vesiculation phenomenon and curvature. Liposomes are currently being successfully used in several areas of chemistry, medicine, and biotechnology due to their capacity to encapsulate molecules in their cavity or through the lipid bilayer. Analytical applications of liposomes are mainly in the areas of sensors, and immunoassays exploiting the signal enhancement due to the capacity of liposomes to encapsulate huge amounts of signal elements as well as their facile modification using non-covalent and covalent bioconjugation methods taking advantage of the chemistry of polar head groups of the phospholipids. Furthermore, liposomes are often used as drug carriers for the targeted delivery of encapsulated active compounds to the site decreasing drug uptake from normal cells and tissues minimising non-specific binding to non-target. Liposomes and other lipid base nanostructures such as tubes, cubosomes and planar bilayers are of increasing interest for their use in micro- and nanoreactors, as well as for the template directed synthesis of metallic nanoparticles of controlled morphology.

Whilst liposomes have tremendous potential in a wide range of applications, to date the laborious techniques required for the preparation of homogenous encapsulating/non-encapsulating liposomes, as well as their subsequent stability, has limited the wide-spread use of liposomes. In this direction, the present thesis is a contribution to the development of a rapid and efficient method of liposome preparation, and explores different applications of the liposomes demonstrating the versatility of the method.

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1.3 Objectives

The overall objective of the present doctoral thesis is the development and optimization of a one-step environmentally-friendly method for the preparation of encapsulating liposomes and their application as signal enhancers in biosensor development, as nanocompartments for metal nanoparticle synthesis as well as their application in controlled release systems for drug delivery.

The thesis has the following specific objectives:

I. Evaluation of different optimization parameters for the development of an organic solvent-free method of liposome preparation taking into account physical mechanisms and the phenomenon of spontaneous vesiculation and its implementation to various lipid types in order to demonstrate the versatility of the optimized method.

II. Design of a surfactant free liposome based amperometric immunosensor system with enhanced signal and sensitivity and improved limit of detection for the quantification of carcinoembryonic antigen (CEA) using horseradish peroxidase (HRP) encapsulating bioconjugated thermo-sensitive liposomes.

III. Evaluation of different reducing agents (such as sodium borohydrate and glycerol) and operation conditions for the preparation of gold nanoparticles using liposomes as nanoreactors.

IV. Study of the application of lipid superstructures with different shapes (hexagonal and rectangular lipid disks and twisted ribbons) for the controlled synthesis of gold nanoparticles of controlled morphology.

V. Evaluation of the basic properties of thermo-sensitive liposomes with changing lipid composition, such as membrane integrity, morphological properties, size, stability in serum proteins and temperature-triggered release mechanisms for use as a controlled release system in drug delivery applications.

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1.4 Articles resulting from PhD studies

I. **Curvature tuned preparation of nanoliposomes.**

Rukan Genç, Mayreli Ortiz, Ciara K. O'Sullivan. *Langmuir*, **2009**, 25(21),12604-12613.

II. **Signal-enhancing thermosensitive liposomes for highly sensitive immunosensor development**

Rukan Genç, Deirdre Murphy, Alex Fragoso, Mayreli Ortiz, Ciara O'Sullivan, *Anal. Chem.*, **2011**, 83 (2), 563–570.

III. **Using Biotechnology in the laboratory: Using an immobilized-laccase reactor-system to learn about waste water treatment**

Rukan Genç, Susana Rodriquez, *Biochemistry and Molecular Biology Education*, **2009**, 37, 182-185.

IV. **Diffusion driven size controlled synthesis of gold nanoparticles: nano-sized liposomes as mass transfer barrier.**

Rukan Genç, Mayreli Ortiz, Ciara K. O'Sullivan, *Article in Progress (will be submitted to Chem. Comm.)*

V. **Green synthesis of gold nanoparticles using glycerol incorporated nanoliposomes.**

Rukan Genç, Gael Clergeaud, Mayreli Ortiz, Ciara K. O'Sullivan, *Article in Progress (will be submitted to Langmuir)*

VI. **Template directed synthesis of gold nanostructures using lipid nano-structures.**

Rukan Genç, Gael Clergeaud, Mayreli Ortiz, Ciara K. O'Sullivan, *Article in Progress (will be submitted to ACS Nano)*

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VII. **Three-component thermo-sensitive liposomes: size, membrane integration and stability.**

Rukan Genç, Mayreli Ortiz, Dimitris Fotouros, Ciara K. O'Sullivan, *Article in Progress (will be submitted to Journal* of *Liposome* Research*)*

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CHAPTER

2

PREPARATION OF LIPOSOMES AND LIPID MESO-STRUCTURES

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Curvature-Tuned Preparation of Nanoliposomes

Rükan Genç,[†] Mayreli Ortiz,*[,†] and Ciara K. O'Sullivan*[,†,‡]

[†]*Nanobiotechnology and Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Av. Paisos Catalans, 26, 43007 Tarragona, Spain, and* [‡]*Institució Catalana de Recerca i Estudis Avançats, Passeig Lluis Companys 23, 08010 Barcelona, Spain*

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Numerous methods have been reported for the preparation of liposomes, many of which, in addition to requiring time-consuming preparative steps and the use of organic solvents, result in heterogeneous liposome populations of incontrollable size. Taking into consideration the phenomenon of spontaneous vesiculation and the theory of curvature, here we present an extremely rapid and simple, solvent-free method for the preparation of monodisperse solutions of highly stable small unilamellar vesicles using both charged and zwitterionic lipids mixed with lyso-palmitoylphosphatidylcholine, exploiting a combination of a rapid pH change followed by a defined period of equilibration. Various experimental parameters and their interactions were evaluated in terms of their effect on resulting liposome size and shape, as well as on liposome stability and size distribution, with transmission electron microscope imaging being used to visualize the formed liposomes, and photon correlation spectroscopy to obtain statistical data on mean diameter and monodispersity of the liposome population. ζ potential measurements also provided information about the interpretation of vesiculation kinetics and liposome stability. The time interval of pH jump, operation temperature, equilibration time, and lipid type were shown to be the determining factors controlling the size, shape, and monodispersity of the liposomes. Buffer type was also found to be important for the long-term storage of the liposomes. Ongoing work is looking at the application of the developed method for encapsulation of bioactive molecules, such as drugs, genetic materials, and enzymes.

1. Introduction

The self-assembly of building blocks into nano- or microstructures is an area of intense interest, with lipids being particularly attractive in the formation of closed spherical lipid bilayers known as liposomes.[1,2] Because of the amphiphilic nature of the lipids, liposomes have the capacity to encapsulate a huge number of water-soluble substances in their aquatic core, as well as oil-soluble substances in the lipid bilayer.[3,4] Furthermore, being a natural product, liposomes are of enormous interest as a carrier and encapsulation material in several research areas such as pharmaceuticals,[5] cosmetics,[6] and biosensor technologies,[7,8] and as a model system in cell biology (cell membrane physiology, function, and cell trafficking).[4]

Liposomes can be classified according to their lamellarity, multilamellar (MLV) or unilamellar (UV), and size, being defined as small unilamellar vesicles (SUVs, < 100 nm), large unilamellar vesicles (LUVs, from 100 nm to 1 μm), or giant vesicles (> 1 μm).[1] There is a wealth of literature detailing the specific applications of liposomes taking into consideration their size and structure, and the reader is directed to very recent comprehensive reviews that summarize several advantages of SUVs over larger liposomes with regard to their implementation.[3−5,9−13] Examples of these advantages include size-selective cellular uptake through tumor cells (cancer diagnosis and treatment)[10,13] and from narrow skin capillaries (transdermal drug delivery and cosmetics)[2,14] as well as their size- and charge-dependent accumulation in brain, spleen, bone marrow, and lung cells (topically or targeted delivery of drugs)[5,15] and higher transfection capacity after cellular uptake (gene therapy).[9,12] Moreover, they are less recognizable to macrophages, increasing their half-life in the bloodstream.[4]

Numerous methods have been described for liposome synthesis.[2] The so-called thin-film preparation method, first described by Bangham in 1965,[16] is the method most used for the preparation of MLVs and is based on the preparation of a thin film of lipids, dissolved in organic solvent (e.g., chloroform/methanol), on the surface of a round-bottom flask via rotary evaporation under vacuum, followed by rehydration of the film in an aqueous medium containing the substance to be encapsulated.[2,5] The monodispersity of the resulting liposomes depends on the quality of the thin film, hydration time, and agitation conditions. Once MLVs are obtained, additional steps, such as detergent depletion, reverse phase evaporation, sonication, and high-pressure extrusion, are required to prepare LUVs and SUVs.[2,6] Although sonication is an easy way to prepare SUVs, irreproducibility

*To whom correspondence should be addressed. M.O.: telephone, +34-977-558555/8722; fax, +34-977-559621/8205; e-mail, mayreli.ortiz@fundacio.urv.cat. C.K.O.: telephone, +34-977-558740/8722; fax, +34-977-559621/8205; e-mail, ciara.osullivan@urv.cat.

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due to uncontrollable process conditions as well as low encapsulation efficiency and stability of the vesicles formed and potential damage of encapsulated material (DNA and enzyme) caused by the high energy input are major limitations of the method.[6,17] On the other hand, the method of high-pressure extrusion has the advantage of producing homogeneously sized SUVs of controllable dimension but is not an easy method to scale up.[6] The "pH jumping method", first reported by Hauser et al. in 1982, is a relatively fast method for the preparation of nanoliposomes.[22] The method is based on a rapid change in pH which breaks down MLVs into SUVs (LUVs and MLVs require subsequent separation using gel chromatography or centrifugation).[20−24] Recently, other research groups have adopted and reported improved versions of the method;[24,25] there is, however, still a requirement for the step of thin-film formation, thus lengthening the preparation time and necessitating the use of organic solvents. Moreover, little attention has been paid to improving the homogeneity in size of the produced liposomes. Mozaffari et al.[26,28] and Otake et al.[29] reported on the possibility of liposome formation from a nonhomogeneous mixture of phospholipids swollen in aqueous solution, demonstrating the possibility to force the lipids to curve into a liposome, without any pretreatment with organic solvent or use of any additional size homogenization steps, but simply by tuning the curvature of the lipid bilayer under the optimal conditions. There is a growing amount of literature[5,30−36] detailing the curvature theory and the phenomenon of spontaneous vesiculation, which highlights the essential parameters for forcing lipid bilayers to curl into liposomes (i.e, operation temperature, pH, and physicochemical properties of the phospholipids).

Although there are many methods for liposome preparation, fully defined systems that can provide monodisperse liposome populations of high stability are still required. Taking into account the dynamics of curvature theory, in this study we report on a rapid one-step, solvent-free, method of liposome preparation using a rapid change in pH ("pH jump") as one of the driving forces. Several operation parameters and lipid types were studied to probe the effect of each possible factor on the aggregate shape and size. Extremely stable nanoliposomes highly homogeneous in size were successfully obtained using both charged (negative/positive) and zwitterionic phospholipids with different melting temperatures (T_M). The size distribution and shape of the resulting liposomes were evaluated using both transmission electron microscope imaging and photon correlation spectroscopy measurements. Liposome stability was reported as a function of the change in mean diameter and distribution of it and was confirmed by ζ potential value, which is an indication of the strength of the repulsive interactions between liposomes, which prevents particle flocculation or aggregation.[37] Hence, each variable and its relationship with other factors are discussed in relation to the curvature theory to provide a clear understanding of the underlying physical phenomena of the reported method.

2. Materials and Methods

2.1. Materials. 1,2-Dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG), 1,2-dioleoyl-3-trimethylammonium propane (chloride salt) (DOTAP), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DPPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and lyso-palmitoylphosphatidylcholine (lyso-PPC) were supplied as a powder by Avanti Polar Lipids, Inc., and used without further purification. Sodium hydroxide, hydrochloric acid, disodium hydrogen phosphate (anhydrous, reagent grade) (Na_2HPO_4), sodium dihydrogen phosphate (anhydrous, extra pure) (NaH_2PO_4), and glycerol (99.5%, reagent grade) were purchased from Scharlau Chemie SA. Sodium chloride was provided by Riedel-de Haen, and MES and HEPES were obtained from Sigma. Milli-Q water (18.2 MΩ cm) used to prepare buffers and liposomes was obtained using a Simplicity 185 Millipore-Water System.

2.2. Liposome Preparation. As an alternative to the thin-film preparation step, a lipid mixture (50 mg) was directly hydrated in 4 mL of buffer (Milli-Q water or 0.01, 0.05, and 0.1 M MES, HEPES, and PBS solutions) which had previously been heated to a predetermined temperature, T_o. The temperature was kept constant by lacing a glass flask (15 mL) in a water jacket connected to an UltraTerm 200 Model (P-Selecta) thermocycler. The mixture was vortexed in a 10 mL falcon tube (with glass beads) for 1−3 min and added to 6 mL of the buffer solution (pH 7.4) [3% (v/v) glycerol, total]. Glycerol was added because of its antioxidative properties, via interaction with the polar headgroup of the lipid, which is useful for long-term storage of the liposomes.[38] The mixture was left to stir for ∼15 min while the temperature was kept constant at T_o. The pH was then subsequently increased to a maximum (pH ∼11) using NaOH and adjusted to pH 7.4 using HCl for a fixed time period, which we termed the "pH jumping time", Δjt. The resulting mixture was left to mix for a fixed time period of equilibration, Δeq, under the same conditions. Finally, the stirring and heating was stopped, and the solution was left to cool to room temperature for 25 min; subsequently, the liposome solution was centrifuged at 1500 rpm for 15 min to eliminate possible aggregations and larger sized liposomes and stored at 4 °C. All steps were conducted under argon. Until otherwise described, all liposome formulations consisted of phospholipid and lyso-PPC (88:12 molar ratio) which is a typical formulation for the well-known temperature sensitive liposomes, where lyso-PPC is used because of its temperature-dependent inducer role in the flexibility and permeability of the liposome membrane.[39] The final lipid mass concentration was kept constant for all lipid formulations at 0.5% (w/v).

2.3. Visualization of Liposome Size and Shape Using Transmission Electron Microscopy (TEM). The prepared liposome dispersion was diluted at least three times with buffer.

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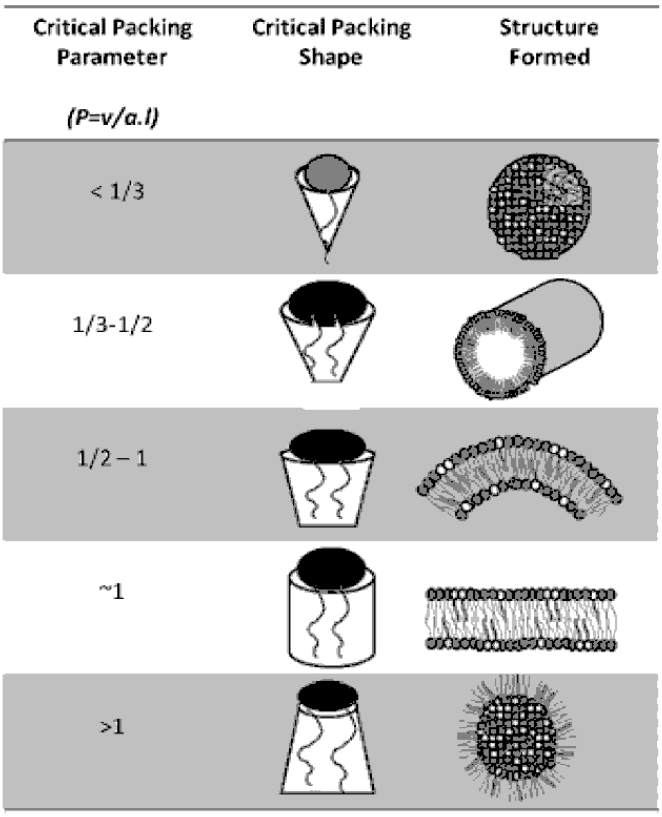

Figure 1. Shematic representation of the packing parameter and corresponding structure of the lipid aggregate (modified from ref 1).

Using a glass pipet, a drop of sample was added to a 200 mesh copper grid with a thin film of Formvar polymer and left at room temperature until a dried film was obtained. TEM analyses were performed using a JEOL 1011 transmission electron microscope operated at 80 keV with an ultra-high-resolution pole piece providing a point resolution of 2 Å. Micrographs (1024 pixels × 1024 pixels) were acquired using a Megaview III multiscan-CCD camera. Images were analyzed with an iTEM image analysis platform by measuring the diameter of more than 100 particles from the photos captured from different parts of the grid and calculating the mean diameter from the data of experiments ($n \geq 3$) conducted using the same parameters obtained from the program.

2.4. Size and Size Distribution Studies Using Photon Correlation Spectroscopy (PCS). The mean diameter of the liposome emulsions and the size distribution, presented as a function of polydispersity index (PI), were measured using Zeta Sizer 3000H [He−Ne laser (633 nm), detector angle of 90°] equipment from Malvern Instruments, Inc., which measures the rate of fluctuation of the light scattered from the particles using photon correlation spectroscopy (PCS). Standard deviations were calculated from the mean of the data of a series of experiments ($n \geq 3$) conducted using the same parameters.

2.5. ζ Potential Measurements. ζ potential measurements were used to characterize changes in the surface charge of lipid aggregates to evaluate the relationship between surface charge and stability. ζ potential values were measured using a Zeta Sizer 3000H instrument (Malvern Instruments, Inc.). Presented standard deviations were calculated from the mean of ζ potential values of a series of experiments ($n \geq 3$) conducted using the same parameters.

3. Results and Discussion

3.1. Elucidation of Optimum Parameters. Lipids spontaneously form aggregates of different shapes depending on their geometry and chemical properties, as well as physical conditions (e.g., temperature, pH, and salinity). In addition to these properties, attractive−repulsive forces [Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory] and thermodynamic interactions, due to the amphiphilic and ionic properties of the lipids, have an influence on spontaneous vesiculation.

Two important terms related to the formation of vesicles from lipids are the critical packing parameter, P, and the radius of intrinsic curvature, R_o.

The value of the critical packing parameter, P, is a fundamental term used to explain the packing geometry of the lipid molecule and can be defined by

$$P - V/la \qquad (1)$$

where P is the critical packing parameter, V is the molecular volume of the surfactant chain, a is the area per surfactant head, and l is the length of the surfactant chain. P should have a value of 0.5−1 to yield a truncated cone-shaped lipid, which is the most suitable shape for the lipid to curl into vesicles (Figure 1).[5] During

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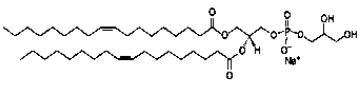
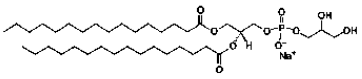
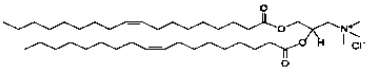
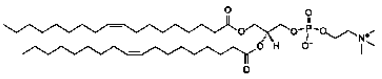
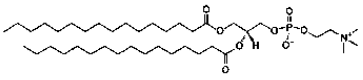
Table 1. Properties of the Phospholipids Evaluated for the Preparation of Liposomes Using the Developed Method

Lipid name	Chemical Structure	Degree of Saturation	Surface charge	T_M(°C)
1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (DOPG)		18:1	Negative	**-18**
1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (Sodium Salt) (DPPG)		16:0	Negative	**41**
1,2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt) (DOTAP)		18:1	Positive	**0**
1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC)		18:1	Zwitterion	**-20**
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC)		16:0	Zwitterion	**41**

the spontaneous vesiculation process, changes in amphiphile geometry increase the value of P, lowering the curvature (see eq 1). The effective shape of the molecule can be tuned by changing lipid properties, such as the effective size of the headgroup and the charge of the ionic headgroup, and by changing the degree of hydration or, alternatively, can be temperature tuned.[30]

Another important factor, the radius of intrinsic curvature, R_o, defined in eq 2, is the radius of the curvature (see also ref 35) that minimizes the elastic energy. It does not vary significantly with lipid geometry but is influenced by factors affecting the lipid tail, such as the temperature and degree of unsaturation. It also increases with properties that lead to an increase in the effective headgroup area (e.g., charge of the headgroup).

$$\mu e - k(1/R - 1/R_o)^2 \qquad (2)$$

where μe is the elastic energy, k is the elastic constant, R is the radius of curvature at the water interface, and R_o is the intrinsic radius of the curvature.[30,35]

Finally, the phase behavior of the lipid has a significant effect on the bilayer curvature. Each phospholipid has a specific main phase transition temperature (T_M) that determines the phase behavior of the lipids. Above this temperature, the lipid is in a liquid crystalline phase (L_α) and is fluid; below its T_M, it is in a gel phase and is rigid. A low temperature induces the solid compressed or crystalline phases where the fatty acid tails of the lipids are packed together in a regular arrangement, and thus, the limiting area per molecule is decreased. As the temperature increases, the lipid bilayer changes state from a solid state to a liquid state where the fatty acid tails are more flexible.[2,16,30] In L_α, R is ∞, so if the intrinsic curvature of the radius is high, then eq 2 will be $\mu e - k(1/R_o^2)$.[35] In this case, $R - R_o$, lowering the elastic energy of the lipid monolayer, facilitating it to curl into energetically favored vesicles. This situation is only valid if no other energies compete with μe. If R_o is small, then the packing stress will increase, disturbing the formation of bilayers. On the other hand, if R_o is too large, it will engender a large amount of packing energy, and the bilayer is then difficult to destruct, thus inhibiting release of encapsulated contents. In the case of an intermediate value of R_o, the bilayer would be stable and could be affected only by an external factor.[35]

Extrapolating from eqs 1 and 2, we can predict the parameters determinative of liposome shape and stability. To elucidate optimum conditions for liposome formation, in the work reported here, the roles of (i) the length of the equilibration time after pH jump (Δeq), (ii) pH jumping time (Δjt) which corresponds to the time interval between pH values, (iii) operation temperature (T_o), and (iv) buffer type were evaluated in terms of their effect on the critical packing shape of lipids and their spontaneous vesiculation into liposomes.

Initial method developmental work was conducted with the negatively charged phospholipid DOPG which has a very low melting temperature (T_M − −18 °C). Once the optimal equilibrium time, pH jumping time, and buffer type were evaluated, the method was demonstrated with various phospholipids specifically selected according to their surface charge and T_M values, as outlined in Table 1, and as discussed in section 3.5.

3.2. Temperature Control and Equilibrium State of the Vesiculation. There are several reports of the use of a change in pH as the driving force for the formation of liposomes; however, the resulting liposomes in these reports were highly heterogeneous in size with a mixture of GUVS, LUVs, and SUVs, thus requiring further treatment to yield liposome populations homogeneous in size.[20−26] Our studies have demonstrated that this heterogeneity in size is principally due to a lack of temperature control and monodisperse liposome populations can be obtained under tight

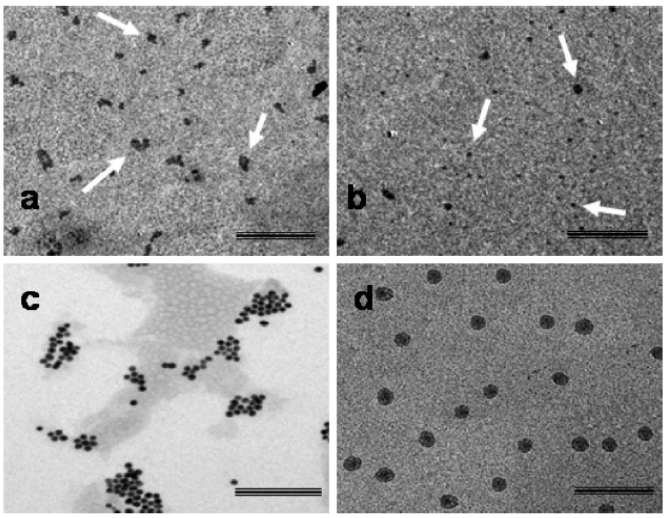


Figure 2. TEM images of lipid organization of a DOPG/lyso-PPC (88:12 molar ratio) lipid mixture with changing equilibrium time, Δeq, (a) just after a pH jump or after (b) 15, (c) 25, or (d) 45 min. Δjt = immediate (~1 s), and T_o = 45 °C, in PBS buffer (pH 7.4). The scale bar is 250 nm.

Table 2. List of the Operation Parameters Studied and Corresponding ζ Potential Values, Mean Diameters, and Polydispersity Index (PI) Values of DOPG/Lyso-PPC (88:12 molar ratio) Liposomes[a]

operation temperature (T_o) (°C)	Δjt (min)	Δeq (min)	buffer type	mean diameter (nm) ± SDV (n − 3)	PI value[b]	ζ potential (mV) ± SDV (n − 3)
45	immediate (~1 s)	0	PBS	–	1	−34.6 ± 4.9
45	immediate (~1 s)	15	PBS	–	1	−35.4 ± 6.8
45	immediate (~1 s)	25	PBS	24.5 ± 1.1	0.181	−50.1 ± 3.3
45	immediate (~1 s)	45	PBS	56.7 ± 4.7	0.249	−24.9 ± 4.9
45	0.5	25	PBS	–	–	−47.1 ± 3.6
45	1	25	PBS	–	–	−45.2 ± 4.1
45	5	25	PBS	–	–	−36.4 ± 3.9
45	25	25	PBS	–	–	−37.2 ± 13.8
45	immediate (~1 s)	25	water	–	1	−56.2 ± 106.0[c]
45	immediate (~1 s)	25	HEPES	28.6 ± 2.3	0.240	−41.1 ± 4.6
45	immediate (~1 s)	25	MES	30.9 ± 2.6	0.222	−40.8 ± 4.9
25	immediate (~1 s)	25	PBS	22.4 ± 1.0	0.200	−47.5 ± 3.8
65	immediate (~1 s)	25	PBS	52.6 ± 3.2	0.242	−43.6 ± 4.9

[a] Δjt is the interval of the pH jump, and Δeq is the duration of the equilibrium step at a certain temperature. [b] A particle population is accepted as monodisperse when the polydispersity index, PI, is in the range of 0-0.5. A value higher than this shows a heterogeneous size distribution. [c] Unequal ζ potential values of three repetitions.

temperature control. In the work detailed here, temperature is used in a controlled way to adjust the curvature of the lipids in a synergistic manner with the quick jump in pH.

Moreover, not only the control of the temperature through the system but also the duration of the application of heat has an effect on the formation of vesicles. As mentioned before, to obtain closed packed bilayers or vesicles, the lipid packing shape should be switched from a cylindrical to a truncated cone to obtain a P value of 0.5−1, and the intrinsic curvature of the radius, R_o, should be equal to R to lower the elastic energy. Increasing the applied temperature to some extent affects the fluidity of the hydrophobic tails, thus increasing the curvature, facilitating the lipid layer to curl into a liposome (see section 3.1). The first change in the lipid shape, enforced by the change in pH, is followed by a temperature-provoked secondary change as the lipid aggregates curl into vesicles. Using TEM imaging, the monitoring of structural changes in the lipid aggregates over time provided powerful insight into the process of vesiculation (Figure 2). Four different equilibration times, 0, 15, 25, and 45 min, were studied with mixing at 45 °C (T_o), and the Δjt was fixed at immediate (~1 s). ζ potential studies were also used to characterize the stability of lipid aggregates. As one can see in Figure 2, at Δeq − 0 min, at the time just after the pH jump, lipid clusters relatively homogeneous in size were formed. After 15 min, the clusters show a decrease in size with an increase in surface potential (from −32 to −35 mV), while at Δeq − 25 min, monodisperse liposomes of 24.5 ± 1.1 nm (PI − 0.181) with a high surface charge of −50 mV, indicating high colloidal stability, were observed (see Figure 2 and Table 2).[8,15] As one can see in Figure 2d, when the lipid solution was left for 45 min, larger liposomes [56.7 ± 4.7 nm (PI − 0.270)] with a sharp decrease in surface potential (−25 mV) were observed (Table 2). Their low ζ potential value indicates a relatively lower stability of these liposomes as compared to those formed at Δeq − 25 min. These results are in agreement with the data obtained from PCS measurements showing the increased mean diameter (78.5 ± 7.7 nm) and polydispersity index (0.432) of the liposomes prepared at Δeq − 45 min with respect to the slight increase (26.2 ± 2.3 nm and 0.211, respectively) in the ones

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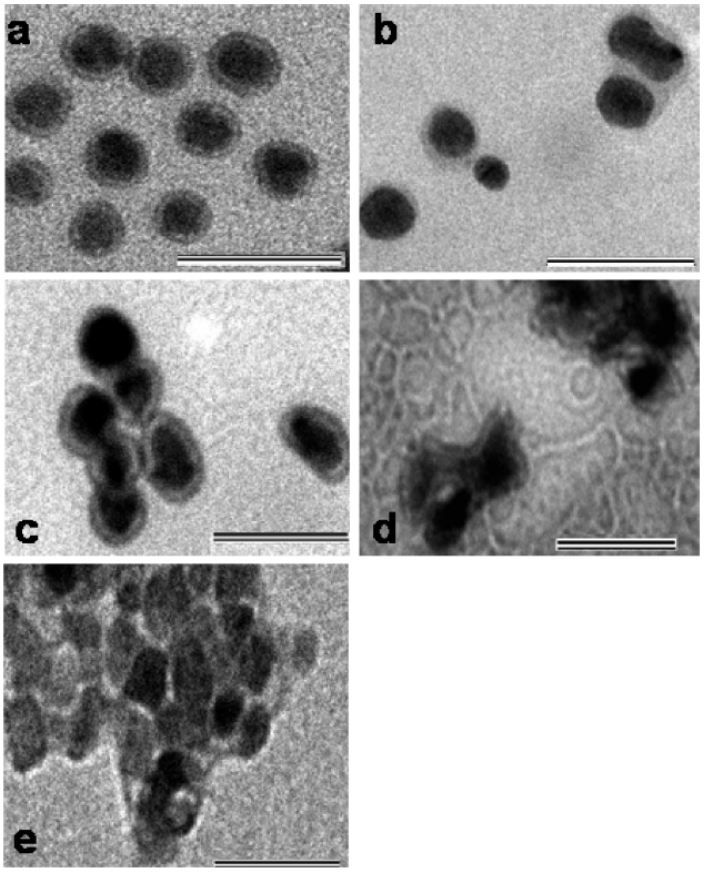

Figure 3. Structural representation of the lipid aggregates formed from a DOPG/lyso-PPC mixture (88:12 molar ratio) with jumping times (Δjt) of (a) immediate (~1 s) and (b) 0.5, (c) 1, (d) 5, and (e) 25 min. Δeq = 25 min, and T_o = 45 °C, in PBS buffer (pH 7.4). The scale bar is 50 nm.

prepared at Δeq − 25 min after storage for 7 months at 4 °C . Although there are reports describing the increase in liposome size, with an increase in the duration of the hydration step for specific methods, to the best of our knowledge, there is not much work explaining the change in size over time at a constant temperature. In their study, Carnerio and Santana explain the change in size by time saying that "The mean diameter increases with time, indicating the aggregation of lipids and a vesiculation phenomenon".[40] However, this argument is not in agreement with our results as no sign of aggregation or fusion was observed in TEM analysis of the samples after preparation. Another study by Garcia-Manyes et al.[41] offered a deeper understanding of the changes in the bilayer from the perspective of molecular interactions. Basically, they stated that the temperature provokes structural changes, by softening the membrane due to several intermolecular changes, such as an increased headgroup area per molecule, *A*, decreased headgroup order, and increased disorder in the hydrocarbon chains across the membrane, in a temperature range of 10−60 °C above T_M.[41] It is possible to hypothesize that the lipid bilayer finds the optimum chain fluidity and headgroup size and forms stable liposomes at Δeq − 25 min. After that time period, an increase in time increases the fluidity of the tails, increasing the distance between the lipid molecules resulting in less stable liposomes larger in size compared to the ones formed at Δeq − 25 min having a less fluid membrane (Figure 2d).

3.3. Optimization of pH Jumping Time. The effect of the length of the jumping time, Δjt, was evaluated. Liposomes prepared using different Δjt values, immediate (~1 s) or 0.5, 1, 15, and 25 min, while T_o (45 °C) and Δeq (25 min) were kept constant were studied. The results were in agreement with the previous reports of Hauser,[22] which also looked at the effect of the time interval of the pH jump. An instantaneous decrease from pH 10−11 to neutral pH was observed to result in more stable liposomes. The longer the duration of the pH jump, the greater the degree of liposome aggregation, possibly due to a lower surface potential (reduced from −50 to −37 mV) that facilitates aggregation (see Table 2). Although the degree of aggregation of the liposomes is acceptable for the samples prepared at 1 min of Δjt, at a Δjt of 5 min it is widespread and at a Δjt of 25 min there are no longer vesicles but rather lipid precipitations (Figure 3).

These results can be explained by two phenomena: (1) increasing pH gradient across the membrane layer which influences the attractive−repulsive forces, affecting the stability of the bilayer,[23,42] and (2) the curvature induced by an increase in the headgroup

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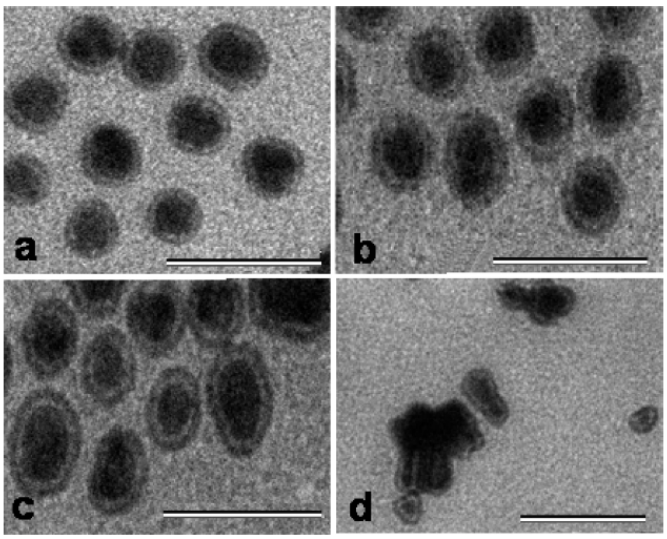

Figure 4. TEM images of DOPG/lyso-PPC (88:12 molar ratio) liposomes prepared using different aqueous solutions: (a) PBS, (b) HEPES, (c) MES, and (d) water. Δjt = immediate (~1 s). Δeq = 25 min. T_o = 45 °C. The scale bar is 50 nm.

area:lipid molecule ratio of the lipids.[30,35] In the first phenomenon, the longer the lipids are subjected to high pH, the greater the extent of deprotonation of the headgroup. Thus, when the pH is reversed back to neutral, the headgroup charge of the lipids located in the core of the vesicles (more alkaline core) would have a higher value than the ones located on the outer layer (neutral), and this difference increases depending on the duration of the time that the lipids are left at high pH. This difference through the liposome layer may disturb the bilayer dynamics by disturbing the attractive forces keeping the lipids together in liposome form and by reducing the total surface potential of the vesicles which decreases the repulsive forces between individual liposomes which prevent them from aggregating. Some studies claim that in the case of a lipid bilayer with an alkaline inner/neutral outer core, there is lipid migration from the outer layer to the inner layer depending on time and temperature.[43,44] The decreasing surface charge of the liposomes over increasing Δjt is in agreement with the findings of Hope et al. who also reported a decrease in the surface potential (less negative membrane) of the liposomes prepared from different phospholipids, including DOPG, due to the migration of lipid across the lipid bilayer (with neutral outer layer/more basic inner core).[43]

From the curvature point of view, the aggregation mechanism is similar. The higher the level of deprotonation of the lipid, the larger the headgroup area of the lipid and the larger the intrinsic curvature of the radius, R_o, of the resulting bilayer. In fact, this is the underlying mechanism for the spontaneous formation of the liposomes due to a rapid change in pH (pH jump); however, an increased curvature difference between the inner and outer layer enlarges the packing energy which competes with the decreased elastic free energy. As Δjt increases, it promotes an increase in the packing energy of the bilayer; thus, liposomes aggregate, decreasing their overall free energy.[35]

3.4. Type of Buffer Solution. Liposomes are frequently employed as vesicles for the encapsulation of biological materials, such as enzymes, DNA, and drugs. Buffer type is an important factor in the encapsulation of biological molecules which require electrolyte solutions to maintain their activity. Prior to the preparation of encapsulating liposomes, one should choose the best buffer according to the needs of the encapsulated material. Here, we studied the formation of the DOPG/lyso-PPC liposomes using water and organic (HEPES and MES) and inorganic (PBS) buffer solutions of varying ionic strength (0.01, 0.05, and 0.1 M, respectively), which are frequently used in encapsulating liposome preparations. ζ potential measurements, combined with TEM imaging and PCS studies, were conducted to determine the relation between the stability and surface potential of the resulting forms taking into account the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory.

According to the DLVO theory, colloidal stability is achieved only when the repulsive forces between two particles are sufficiently high to compete with the attractive forces (London–van der Vaals interactions):

$$V_T - V_A + V_R \quad (3)$$

where V_T is the interaction potential between particles, V_A is the potential for attractive interactions, and V_R is the potential for repulsive interactions.[37] Repulsive interactions are strengthened as a function of the ζ potential value of two particles, which has a maximum corresponding to the highest colloidal stability with an increase in ionic strength.[45]

As one can clear see in particle size distribution histograms in Figure 5 and TEM images in Figure 4, highly monodisperse spherical liposomes of decreasing size, 30.9 ± 2.6 nm (PI = 0.222), 28.6 ± 2.3 nm (PI = 0.242), and 24.5 ± 1.1 nm (PI = 0.181), were obtained using MES, HEPES, and PBS buffers, respectively. The stability of the liposomes formed using HEPES and MES was very good, with no change in liposome size or aggregation observed over 2.5 months and a slight increase in the mean diameter and PI value of the vesicle population after storage for

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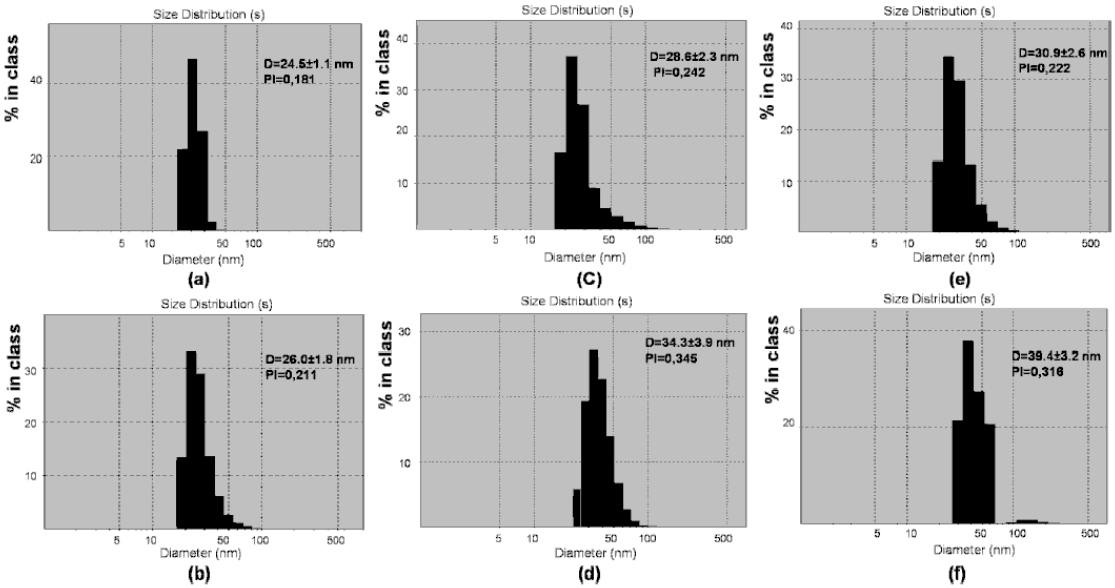

Figure 5. Particle size distribution histograms of DOPG/lyso-PPC (88:12 molar ratio) liposomes, prepared using different aqueous solutions as a function of storage time: (a) PBS, first day; (b) PBS, after storage for 7 months at 4 °C; (c) HEPES, first day; (d) HEPES, after storage for 5 months at 4 °C; (e) MES, first day; and (f) MES, after storage for 5 months at 4 °C. Δjt = immediate (~1 s). Δeq = 25 min. T_o = 45 °C. D is the mean diameter of the liposomes ± the standard deviation (SDV) calculated from the mean of the data of a series of experiments ($n \geq 3$) conducted using the same parameters.

5 months at 4 °C (Figure 5d,f). However, the liposomes formed using PBS were notably more stable, with a smaller increase in the population size and polydispersity after at least 7 months (Figure 5b). ζ potential studies also supported the results that showed that the surface potential values of liposomes prepared in HEPES and MES buffers were almost the same, with a high value of approximately −42 mV, while the liposomes prepared using PBS have the highest ζ potential value of −50 mV (Table 2), indicative of the high colloidal stability. Liposomes prepared from different lipid formulations using either MES or PBS at the same concentrations were also studied, and again, the liposomes prepared using PBS were more stable than those formed using MES (results not shown). This improved stability of liposomes may be attributable to the higher ionic strength of PBS compared to those of HEPES and MES even at the same concentration.[46] These findings are consistent with those of Claessens et al.[46] who demonstrated that at a low concentration of salt in buffer, vesicle radius decreases with an increased ionic strength and the highest stability of the DOPG liposomes studied was achieved at a high salt concentration. In our work, in the case of the liposomes prepared using water, the ζ potential value was very broad, resulting in a precipitated liposome population (Figure 4d) having a PI value of 1 indicating a broad size distribution through the sample. As expected from the DLVO theory, the prepared liposomal population of unequal ζ potential tended to aggregate due to an insufficient amount of repulsive force over attractive force in the absence of salt. In conclusion, the size and stability of liposomes prepared using the new method reported here are mainly related to the ionic strength of the buffer, and the method works effectively in both organic and inorganic buffers.

3.5. Lipid Type: T_M Value and Headgroup Charge. As previously stated, it is not only the lipid packing shape but also the properties of the lipids, such as melting temperature, overall headgroup charge, and degree of saturation, that significantly affect the shape and mechanical properties of the formed liposomes.[30] Moreover, these physicochemical properties of the lipids are also important for the encapsulated material−liposome and liposome−target interactions which are essential properties of encapsulating liposomes for specific applications, such as drug delivery or gene therapy.[47−49] Therefore, we studied several phospholipids to demonstrate the implementation of the method to a wide range of lipid types and also to provide a deeper understanding of the underlying mechanisms of the developed method. Lipids with different properties [negatively charged, 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPPG), positively charged, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and zwitterionic, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)], having differently charged headgroups and representing a wide range of T_M values (from −20 to 41 °C), were studied. Detailed properties of the phospholipids are outlined in Table 1.

As previously mentioned, the T_M determines the phase behavior of lipids, and thus, to probe the influence of lipid T_M, we prepared liposomes from lipids with different T_M values, at three different operating temperatures (25, 45, and 65 °C).

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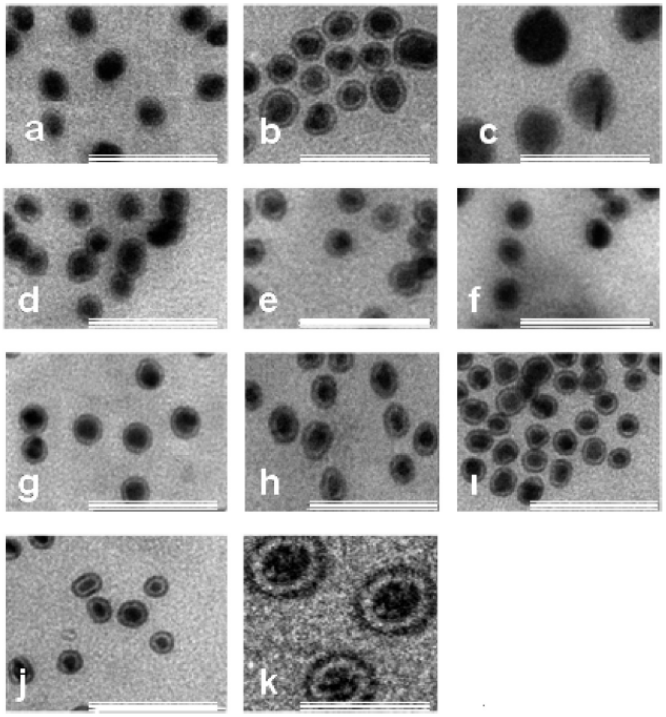

Figure 6. Liposomes formed from different lipids at changing operation temperatures, T_o, using the vesiculation method: (a−c) DOPG/lyso-PPC (88:12 molar ratio) liposomes prepared at 25, 45, and 65 °C, respectively; (d−f) DPPG/lyso-PPC (88:12 molar ratio) liposomes prepared at 25, 45, and 65 °C, respectively; (g−i) DOTAP/lyso-PPC (88:12 molar ratio) liposomes prepared at 25, 45, and 65 °C, respectively; (j) DOPC/lyso-PPC (88:12 molar ratio) liposomes prepared at 25 °C; and (k) DPPC/lyso-PPC (88:12 molar ratio) liposomes prepared at 65 °C. Δjt = immediate (~1 s). Δeq = 25 min, in PBS buffer (pH 7.4). The scale bar is 100 nm.

Photon correlation spectroscopy studies showed that, as one can clearly see in TEM images presented in Figure 6 b,c, the size of the liposomes prepared using negatively charged DOPG (T_M − −18 °C) increased from 24.5 ± 1.1 nm (PI − 0.181) to 52.6 ± 3.2 nm (PI − 0.242), with an increase in the operation temperature (from 45 to 65 °C). However, in the case of another negatively charged phospholipid, DPPG (T_M − 41 °C), as well as the positively charged DOTAP (T_M − 0 °C), increasing temperature only led to a very small increase in liposome size (Figure 6). This sharp increase in the size of the DOPG liposomes at temperatures highly above its T_M is possibly due to the lipid phase transition to a liquid crystalline meso-phase where the lipids are more liquid[30] and the bilayer curvature is less competent against increased hydrocarbon packing energy. In their study of different types of egg yolk lecithins with a changing degree of saturation, Nii et al. also reported increasing liposome size at increasing operation temperatures,[50] stating that in the case of lipids with lower degrees of saturation, corresponding to lower main transition temperatures, T_M,[51] the increase in size becomes more significant than that of lipids with higher T_M values.[50]

However, in the case of DPPG, liposomes are observed to be formed even at a temperature (25 °C) below its T_M (Figure 6d), which may be due to the removal of hydrocarbon packing energy, resulting in the formation of curved structures at relatively low temperatures, by virtue of the fact that there is no other energy to compete with the curvature.[35] In a previous study, Träuble and Eibl[52] suggested that the phenomena could be due to the increased charge concentration of the lipid's headgroup, which increases the T_M value of the lipid due to an expected change in the electrostatic free energy of the charged lipid bilayers. Hence, thanks to the headgroup properties of DPPG, its curvature appears to be so high following the pH jump that it can easily overcome the packing stress caused by the hydrocarbon tails and can form liposomes below its particular T_M value.

With regard to the behavior of zwitterionic lipids, DPPC and DOPC, the results were very different in comparison to those for the charged lipids. As one can see from Figure 6j, DOPC with a T_M of −20 °C formed 20.2 ± 1.4 nm (PI − 0.263) liposomes at just 25 °C and nonlamellar spongelike structures at 45 and 65 °C (results not shown). Barauskas et al., who also obtained similar structures using a particular lipid combination of diglycerol monoolate (DGMO), glycerol diolate (GDO), P80, and water, reported that under particular conditions, a decreased curvature of the lipids (e.g., heat treatment) can force the lipids to form nonlamellar mesophase structures [the sponge phase (L_3) is one of those internal mesophases].[53,54] Moreover, in the case of DPPC (T_M − 41 °C) and DPPG (T_M − 41 °C), we observed the same counter-relationship as the one between DOPG and DOPC. Having the same T_M value as the negatively charged DPPG,

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the zwitterionic DPPC formed 69 ± 6.7 nm (PI = 0.281) liposomes only at 65 °C (see Figure 6k). At temperatures below this, it forms cubic (25 °C) and hexagonal (45 °C) lipid structures (results not shown) as it undergoes an insufficient curvature for the bilayer to curl into liposomes. Considering the phase theory outlined above, it is not surprising that DOPC and DPPC behave in an extremely different manner compared to their negative counterparts, DOPG and DPPG, despite the fact that they have almost the same T_M value.

In summary, there is strong evidence of multicollaborative synergistic interactions of pH change, lipid type, and temperature in the phase behavior of the lipids, and one can carefully tune and control the size and shape of the liposomes by taking into consideration the operation temperature and physicochemical properties of the lipids used. Although the effect of Δjt was found to be same for all the types of lipids studied, one may need to reoptimize the equilibrium times for different liposome formulations of other lipid types, especially zwitterionic lipids. Furthermore, the results indicate that as a new method, the curvature-tuned liposome preparation method could provide flexibility in the design of liposome-based carrier systems of different phospholipid types which is important for the immobilized and/or encapsulated material−lipid interaction and important for the control of the delivery route of the liposomes used for drug delivery systems.

4. Conclusions

A new ultrarapid method for the preparation of liposomes and an explanation of the physical phenomena involved are reported. The developed method does not require the use of organic solvents and is completed in less than 1 h. The method produces monodisperse liposomes of controllable size, using both charged and neutral lipids without any pre- or postpreparative steps. The synergistic effect of several factors, such as the pH change, operation temperature, time interval of the change in the factors or duration of them, buffer type, and lipid properties, on the bilayer curvature was investigated, and formed liposomes were analyzed using transmission electron microscope imaging, photon correlation spectroscopy, and ζ potential measurements. The findings using different lipid types enhanced our understanding of the underlying mechanism of the spontaneous vesiculation phenomenon and allow us to predict the optimal conditions for curvature tuning of the lipid bilayer to form stable liposomes. We also demonstrated the possibility of using the method for preparation of liposomes encapsulating bioactive molecules since these liposomes were observed to be completely stable, with no evidence of leaching or aggregation, upon being stored for at least 7 months depending on preparation conditions. Further work will focus on the encapsulation capacities of these liposomes for the implementation of the method in applications such as drug or gene delivery, cosmetics, and biosensor technologies.

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CHAPTER

3 APPLICATION TO BIOSENSOR TECHNOLOGY

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Signal-Enhancing Thermosensitive Liposomes for Highly Sensitive Immunosensor Development

Rükan Genç,[†] Deirdre Murphy,[†] Alex Fragoso,[†] Mayreli Ortiz,*[,†] and Ciara K. O'Sullivan*[,†,‡]

Nanobiotechnology and Bioanalysis Group, Departament d'Enginyería Química, Universitat Rovira i Virgili, Avinguda Països Catalans, 26, 43007 Tarragona, Spain, Institució Catalana de Recerca i Estudis Avançats, Passeig Lluis Companys 23, 08010, Barcelona, Spain

Liposomes are potential candidates as nanovesicles for the development of detection systems with improved sensitivity and detection limits, due to their capacity to encapsulate diverse types of signal enhancing molecules. An amperometric immunosensor exploiting enzyme encapsulating thermosensitive liposomes for the ultrasensitive detection of carcinoembryonic antigen (CEA) is reported. Five different bioconjugation methods to link an anti-CEA antibody to horseradish peroxidase (HRP) encapsulating liposomes were studied and compared to HRP–Ab conjugate. ζ-Potential measurements of liposomes before and after each modification method as well as following incubation with CEA were used as a tool to monitor the success of modification and probe the affinity of the liposome linked antibodies. The use of different lysing conditions (temperature vs detergent) was evaluated, with the application of temperature providing an extremely effective means of liposome lysis. Finally, thermosensitive liposomes modified using biotin–streptavidin and *N*-succinimidyl-*S*-acetylthioacetate (SATA)/sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-1-carboxylate (Sulfo-SMCC) chemistries were used to detect CEA and compared in terms of their stability, background signal, and limit of detection. Detection limits of 2 orders of magnitude lower than that obtained with the HRP–antibody reporter conjugate were obtained (0.080 ng CEA/mL and 0.0113 ng CEA/mL), with 11-fold and 9-fold amplification of signal, for the biotin–streptavidin and SATA/Sulfo-SMCC modified liposomes respectively, clearly demonstrating the powerful potential of enzyme encapsulating liposomes as signal enhancement tools.

Serum tumor markers are of increasing interest for the early diagnosis and/or follow up of cancer patients,[1,2] and the carcinoembryonic antigen (CEA) is one of the most studied biomarkers, being related to several cancer types including liver, colon, breast, and colorectal cancer.[3,4] CEA is also present in healthy tissue in very low concentrations, with the CEA serum concentration for healthy male and female nonsmokers being 3.4 and 2.5 ng·mL^{-1}, respectively, while for healthy smokers the serum concentration doubles and it is markedly higher in individuals with the specific cancer types listed previously.[4] In a recent review, CEA was shown to be highly sensitive for the monitoring of reappearance of the disease after therapy or surgery and considerably less expensive than radiology and endoscopy, which are typically used for patient follow-up.[4] A highly sensitive, cost-effective, rapid, and easy-to-use analytical tool for the monitoring of CEA levels would thus be very useful for the early diagnosis and follow-up monitoring. Immunosensors address these requirements and also facilitate analysis at the point of care, showing significant potential advantage over the laboratory based immunoassays currently used for analysis of CEA.[5,6] As depicted in Figure 1, electrochemical immunosensors exploiting enzyme labels for signal generation are analogous to their enzyme linked immunosorbent assay, with the exception that the capture antibody is immobilized directly on the surface of the transducer and signal generation is due to the oxidation/reduction of an electrochemical substrate/product of the enzyme label.[5] While there are many reports of the application of electrochemical immunosensors in clinical diagnostics, the sensitivity and achievable limit of detection is restricted by the number of enzyme labels present on the reporting antibody, which is normally one enzyme per antibody. There are an increasing number of reports demonstrating the possibility of signal amplification strategies using various approaches, including the use of inorganic nanoparticles,[7,8] liposomes,[9,10] and/or hybrid materials.[11]

Liposomes are promising candidates as molecular signal enhancement tools, coupling their capacity to encapsulate biomolecules in the aquatic core or within the lipid membrane, with

* To whom correspondence should be addressed. E-mail: ciara.osullivan@urv.cat (C.K.O'S.); mayreli.ortiz@urv.cat (M.O.). Fax: +34 977 55 96 21 (C.K.O'S. and M.O.). Tel. +34 977 55 8740 (C.K.O'S.); +34 977 55 8576 (M.O.).
† Universitat Rovira i Virgili.
‡ Institució Catalana de Recerca i Estudis Avançats.

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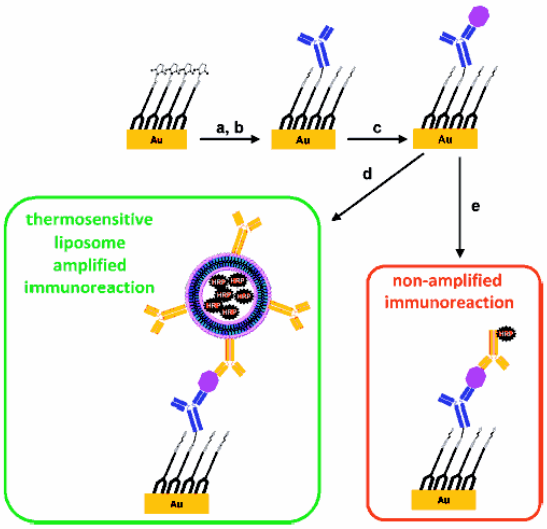

Figure 1. Liposome based sandwich immunosensor for CEA detection: (a, b) Modification of the bare gold electrode by 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester (DT2) and further activation of the carboxyl groups of the chemisorbed dithiol using EDC/NHS; (c) immobilization of primer antibody on electrode surface and addition of the target protein; (d) amplified immunosensor using enzyme encapsulating liposomes, (e) traditional nonamplified enzyme immunosensor.

the straightforward biofunctionalization of the phospholipid polar head groups with, for example, antibodies, facilitating the realization of multifunctional vesicles.[12] Although liposomes were first reported as signal amplifiers in immunosensors in 1980, there have been a limited number of reports detailing their exploitation for increased sensitivity and decreased detection limit.[13,14] Viswanathan et al. used potassium ferrocyanide-encapsulated and ganglioside (GM1)-functionalized liposomes for the detection of cholera toxin (CT), where the CT is detected by a "sandwich-type" assay on the electronic transducers, where the toxin is first bound to the anti-CT antibody and then to the GM1-functionalized liposome. The potassium ferrocyanide molecules are released from the bound liposomes on the electrode by lyses with methanolic solution of Triton X-100 and were able to detect as low as 10^{-16} g of cholera toxin (equivalent to 100 μL of 10^{-15} g mL^{-1}). The authors used the same concept for the detection of CEA, achieving a detection limit of 1×10^{-12} g mL^{-1}.[15,16] Ou et al. described a novel rolling circle amplification (RCA) immunoassay based on DNA-encapsulating liposomes, liposome-RCA immunoassay, exploiting antibody-modified liposomes with DNA primer probes encapsulated, which following lysis were used to initiate a linear RCA reaction, generating a long tandem repeated sequence that was detected using fluorescence. This combination of amplification via the use of liposomes followed by a secondary RCA amplification, facilitated the detection of prostate specific antigen (PSA) over a 6-decade concentration range from 0.1 fg mL^{-1} to 0.1 ng mL^{-1} with a limit of detection as low as 0.08 fg mL^{-1}.[17] In another strategy reported by the same authors, alkaline phosphatase (ALP)-encapsulated and detection antibody-functionalized liposomes were used as the reporter antibody. The model analyte PSA was first captured by anti-PSA capture antibody immobilized on the electrode and then sandwiched with the functionalized liposomes. The bound liposomes were then lysed with surfactant to release the encapsulated ALP, which served as secondary signal amplification means, and a detection limit as low as 0.007 ng mL^{-1} was obtained.[9]

In all reports, the encapsulating liposomes are lysed using lysing agents such as Triton-X; however, these surfactants can have a detrimental effect on encapsulated biological material, such as enzymes,[18,19] and potential alternatives are the use of liposomes with leakage triggered by environmental factors such as temperature or pH.[20] Thermosensitive liposomes have been widely reported for drug delivery;[21] there are, however, no reports to date of their use as tools for signal amplification, either for immunoassays or for immunosensors.

The objective of the work reported here is the development of an amperometric immunosensor exploiting enzyme encapsulating thermosensitive liposomes for the ultrasensitive detection of carcinoembryonic antigen (CEA). A method developed by our group, based on pH jump, was used to prepare the liposomes, and different bioconjugation methods including covalent and bioaffinity based interactions for the binding of these horse radish peroxidase (HRP)-encapsulating liposomes with an anti-CEA monoclonal antibody were evaluated. The different methods were applied to the detection of low levels of CEA and compared in terms of stability, background signal, and limit of detection, and the approach was demonstrated to be very effective in facilitating an enhancement in signal, with a concomitant decrease in detection limit.

EXPERIMENTAL SECTION

Reagents and Materials. Sodium dihydrogen phosphate (NaH_2PO_4; anhydrous, reagent grade, ACS), disodium hydrogen phosphate (Na_2HPO_4; anhydrous, reagent grade, ACS), sodium hydroxide, hydrochloric acid, glycerol (99.5%, reagent grade, ACS), and hydroxylamine hydrochloride were purchased from Scharlau Chemie SA. PBS-Tween (10 mM) prepreparedlyophilized buffer (NaCl 0.138 M, KCl 0.0027 M) Tween 20 0.05%, L-cysteine, (97%), sodium acetate, Triton X-100, horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), glutaraldehyde, chloroform, ferric chloride ($FeCl_3 \cdot 6H_2O$), ammonium thiocyanate (NH_4SCN), sulfuric acid, hydroquinone (98%), hydrogen peroxide, dimethylsulfoxide (DMSO), maleimide activated streptavidin, maleimide activated HRP, and Sephadex G-100 were purchased from Sigma-Aldrich.

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Phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoylphosphoethanolamine (DPPE), biotin-capped-1,2-dipalmitoylphosphoethanolamine (Biot-DPPE), and lyso-palmitoylphosphatidylcholine (lyso-PPC) were supplied by Avanti Polar Lipids Inc. as powders and were used without further purification. *N*-Hydroxysuccinimide (NHS) (98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC), and 2-mercaptoethanol were purchased from Acros Organics. Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-1carboxylate (Sulfo-SMCC), *N*-succinimidyl-*S*-acetylthioacetate (SATA), Microcon nominal weight cut off centrifugal filters (YM-10, YM-30, YM-50, YM-200), and affinity column Freezyme Conjugation purification kit were supplied by Pierce Chemicals. Monoclonal antibodies (anti-CEA) and carcinoembriyonic antigen (CEA) used were provided by Fujirebio Diagnostics AB. One millimolar stock solutions of 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide (DT2) (SensoPath Technologies (Bozeman, MT)) were prepared in ethanol and stored at −20 °C.

Liposome Preparation and Purification. *Curvature-Tuned Preparation of HRP-Encapsulated Liposomes.* The liposomes were prepared using a method recently developed in our laboratories.[22] Briefly, 10 mL of 0.1 M sodium phosphate buffer solution (PBS, pH 7.4) was heated in a 15 mL glass round bottomed flask to the predetermined temperature of 45 °C. This flask was placed in a water jacket, which was connected to an UltraTerm 200 Model (P-Selecta) Thermocycler to maintain a constant temperature. From this buffer, 4 mL was taken and used to directly hydrate a 50 mg lipid mixture composed of DPPC, DPPE, and lyso-PPC with a constant molar ratio (86.1:1.9:12.0). The mixture was vortexed for ∼2 min with glass beads and then placed in a water bath to maintain the temperature. To the PBS remaining in the glass flask, 300 μL of glycerol and 20 mg of the horseradish peroxidase (HRP) enzyme were added, followed by the hydrated lipids, and the mixture was left to stir for 15 min at a constant temperature under an argon environment. The pH of the buffer was then increased to pH 11 using NaOH and immediately returned to pH 7.4 using HCl. All of the above steps were carried out under argon. The mixture was then left in the same conditions for a fixed period of time (∼25 min) before being allowed to cool to room temperature and finally was stored in the fridge at 4 °C until used.

Liposome Purification. Size exclusion chromatography (SEC) was used to physically separate the liposomes from unencapsulated HRP. A size exclusion chromatography column was prepared using Sephadex G-100. The column was left to equilibrate overnight before being washed with 100 mL of 0.1 M PBS buffer solution. The liposome solution was then added to the column, and eluate aliquots were collected for further analysis.

Liposome Characterization. *Size Measurements by Photon Correlation Spectroscopy.* The mean diameter of the liposome emulsions was measured using Zeta Sizer 3000H (laser He−Ne (633 nm), detector angle 90°) equipment from Malvern Instruments, Inc. The standard deviations were calculated from the mean of the data of a series of experiments ($n \geq 3$).

Visualization of Liposome Size and Shape Using Transmission Electron Microscopy (TEM). A drop of sample was added to a 200-mesh copper grid with a thin film of Formvar polymer and left at room temperature until a dried film was obtained. TEM analyses were performed using a Transmission Electron Microscope JEOL 1011 operated at 80 keV with an ultrahigh-resolution pole piece providing a point resolution of 2 Å. Micrographs 1024 pixels × 1024 pixels in size were acquired using a Megaview III multiscan-CCD camera. Images were analyzed by iTEM image analysis platform.

ξ-Potential Measurements. ξ-Potential measurements were carried out to characterize the change in the surface charge of the liposomes before and after modification, as well as following addition of CEA as a means of monitoring the affinity of the anti-CEA−liposome linked antibody for CEA after modification. Liposome samples were diluted in 2 mL of Milli-Q water, subsequently incubated for 30 min at room temperature, and finally analyzed after purification from nonattached proteins.

Evaluation of Lipid Content Using Stewart's Method.[23] In order to measure the lipid content, a small amount of each sample (50 μL) was taken and mixed with 500 μL of chloroform (to break up the liposomes into individual lipids) and 1 mL of ammonium ferrothiocyanate, which binds to the phosphoryl group of the lipids, leading to the formation of a pink colored complex. The samples were then vortexed and centrifuged at 1000 rpm for 10 min, and the absorbance of each aliquot was measured at 496 nm using a Cary UV−vis spectrophotometer, and using a previously prepared calibration curve, the lipid contents were estimated.

Liposome Modification. *Conjugation of Biotin-Capped-DPPE Liposomes with Streptavidin Coupled Anti-CEA (Method I).* First, liposomes were prepared using biotin-capped-DPPE in the place of DPPE. Anti-CEA monoclonal antibody (1 mg) was dissolved in 0.1 M PBS (pH 7.4) to a final volume of 1500 μL. A 10 μL aliquot of a solution containing 1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution, and the mixture was gently stirred for 30 min at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl (pH 7.2) was added to the SATA−antibody mixture and allowed to react for 2 h at room temperature, again protected from light. Subsequently, 1 mg of maleimide activated streptavidin was added to the SATA modified antibody in a 1 to 5 molar ratio of Ab to lipid, and the solution was incubated for 2 h at 37 °C. To deactivate unconjugated maleimide groups, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and stirring continued for 15 min. Finally, the conjugate was purified and concentrated by passing through a YM-50 nominal weight cutoff filter, and the conjugate washed three times with 0.1 M PBS (pH 7.4). The protein concentration was calculated by measuring the absorbance at 280 nm and using the relationship of [Protein] mg/mL = $A_{280} \times 1.38$. The mixture was left to incubate for 2 h at 4 °C under continuous stirring, and the resulting conjugate was passed through a YM-100 nominal weight cutoff filter and stored at 4 °C.

SATA/Sulfo-SMCC (Method II). First, maleimide activated liposomes were prepared by adding 40 μL of Sulfo-SMCC (4.8 mg/mL in PBS pH 7.4) to a 1 mg/mL liposome solution and incubating for 2 h at 4 °C. Meanwhile, anti-CEA monoclonal antibody (1 mg) was dissolved in 0.1 M PBS (pH 7.4) to obtain a final volume of 1500 μL. A 10 μL aliquot of a solution containing

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1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution, and the mixture was gently stirred for 30 min at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl (pH 7.2) was added to the SATA–antibody mixture and allowed to react for 2 h at room temperature, again protected from light. The resulting solution was purified using YM-50 nominal weight cutoff filter, and the protein concentration was again measured by absorbance. Subsequently, thiol-activated anti-CEA monoclonal antibody and maleimide activated liposomes were again mixed with a molar ratio of 1:5, and the solution incubated for 2 h at 4 °C. To deactivate unconjugated maleimide groups, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and gentle stirring continued for 15 min. Finally, the conjugate was passed through a YM-100 nominal weight cutoff filter and was washed three times with 0.1 M PBS (pH 7.4).

Modification Using NHS and EDC (Method III). In this method, 1 mg of anti-CEA monoclonal antibody was diluted in 1 mL of acetate buffer (pH 5), and an excess of NHS and EDC dissolved in PBS was added. The mixture was kept at 4 °C, under stirring conditions for 30 min, and excess unbound NHS/EDC was then removed using a YM-50 nominal weight cutoff filter; the antibody concentration was measured using absorbance. The liposome buffer was exchanged from PBS (pH 7.4) by centrifuging for 10 min at 10 000 rpm and resuspending the pellet (liposomes) in 1 mL of acetate buffer (pH 5). The lipid content was measured as previously described, and the liposomes were suspended in acetate buffer, mixed with activated antibody again in a 1:5 mol/mol ratio, and left to incubate overnight at 4 °C. Modified liposomes were then purified using a YM-100 nominal weight cutoff filter, and the lipid content was again estimated.

Sodium Periodate Method (Method IV). In this method, in the first step, monosaccharide residues in the antibody are oxidized with periodate to produce aldehyde groups, and in the second step, the aldehyde groups are allowed to react with amino groups in the liposomes; finally, the Schiff bases formed are reduced. In more detail, the anti-CEA monoclonal antibody (1 mg) was dissolved in 250 μL of water, and 50 μL of freshly prepared sodium periodate (100 mM) was added and the solution stirred for 20 min at room temperature, protected from light. The activated antibody was then dialyzed overnight against 1 mM acetate buffer, pH 4.4. Meanwhile, the liposome buffer solution was exchanged by centrifugation as previously described, and the liposome pellet was reconstituted in 10 mM sodium carbonate buffer. The pH of the dialyzed antibody solution was adjusted to pH 9.0 by addition of 0.2 M sodium carbonate buffer (pH 9.5), and the liposome solution was immediately added, again at a 1:5 (mol/mol) lipid to antibody ratio, and the mixture stirred for 2 h at room temperature. Freshly prepared sodium borohydride solution (25 μL of a 4 mg/mL solution) was then added, and the mixture again stirred for 2 h, this time at 4 °C. The modified liposomes were then purified using a YM-100 nominal weight cutoff filter.

Glutaraldehyde Method (Method V). In this method, liposomes are first activated using excess glutaraldehyde. The liposome–glutaraldehyde mixture was then left at room temperature (25 °C) for 10 min before being passed through a YM-100 nominal weight cutoff filter to remove excess glutaraldehyde. The liposomes activated with glutaraldehyde were then mixed with the anti-CEA monoclonal antibody again at a 1:5 (mol/mol) lipid to antibody ratio. Antibody stock (2.5 mg/mL) was diluted in 0.1 M PBS to give a final concentration, which was dependent on liposome lipid content. Liposome–antibody mixtures were incubated overnight at 4 °C.

Preparation of Horseradish Peroxidase (HRP)–Anti-CEA Conjugate.[24] Horse radish peroxidase (HRP) was conjugated to the reporter anti-CEA monoclonal antibody using the maleimide activation method described in Method I above, replacing the streptavidin with the HRP. Purification of the resulting conjugate was carried out with the metal chelate affinity chromatography Freezyme Conjugation purification kit. Finally, the pure conjugate was concentrated by passing through a YM-50 nominal weight cutoff, and the conjugate was washed three times with 0.1 M PBS (pH 7.4).

Electrochemical Instrumentation. All the electrochemical measurements were performed using a PGSTAT12 potentiostat (Autolab) controlled with the General Purpose Electrochemical System software, with built-in frequency response analyzer FRA2 module. A three-electrode configuration of Ag/AgCl-3 M NaCl as a reference (CH Instruments., model CHI111), Pt wire as a counter (BAS model MW-1032), and bare or modified Au (BAS model MF-2014, 1.6 mm diameter) as working electrode was used.

Electrode Preparation. Bare gold electrodes were thoroughly cleaned prior to surface modification. The gold surface was polished with alumina powder of 0.3 μm and sonicated in ethanol and Milli-Q water twice for 5 min each. The electrodes were then placed in cold Piranha's solution (1:3 v/v H_2O_2 to H_2SO_4) for 5 min and once again sonicated twice in ethanol and Milli-Q water for 5 min each. (*Caution: Piranha's solution is highly corrosive and violently reactive with organic materials; this solution is potentially explosive and must be used with extreme caution).* The electrodes were dried using nitrogen and electrochemically cleaned in 0.5 M H_2SO_4 solution via cyclic voltametry for 40 cycles at 10 mV/s and characterized in 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl (pH 7.2). Clean bare electrodes were incubated with 1 mM of 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester for 3 h at room temperature. The carboxyl groups of the chemisorbed dithiol were activated by with a mixture of 200 mM EDC and 50 mM NHS solution for 40 min. Three micrograms per milliliter of the anti-CEA capture antibody solution in 10 mM acetate buffer (pH 5.0) was then added to the washed electrode and left for 2 h at room temperature. Active ester sites were blocked by incubating the modified electrodes in 10 mM ethanolamine solution in PBS (pH 8.0) at room temperature for 30 min. CEA analyte solutions were prepared in 0.1 M PBS (pH 7.4), and electrodes were incubated in these solutions for 30 min before their incubation with secondary anti-CEA-liposome or anti-CEA-HRP solutions for 40 min at room temperature. Electrode regeneration was carried out treating the electrodes in 10 mM glycine solution (pH 2.5) for 5 min.

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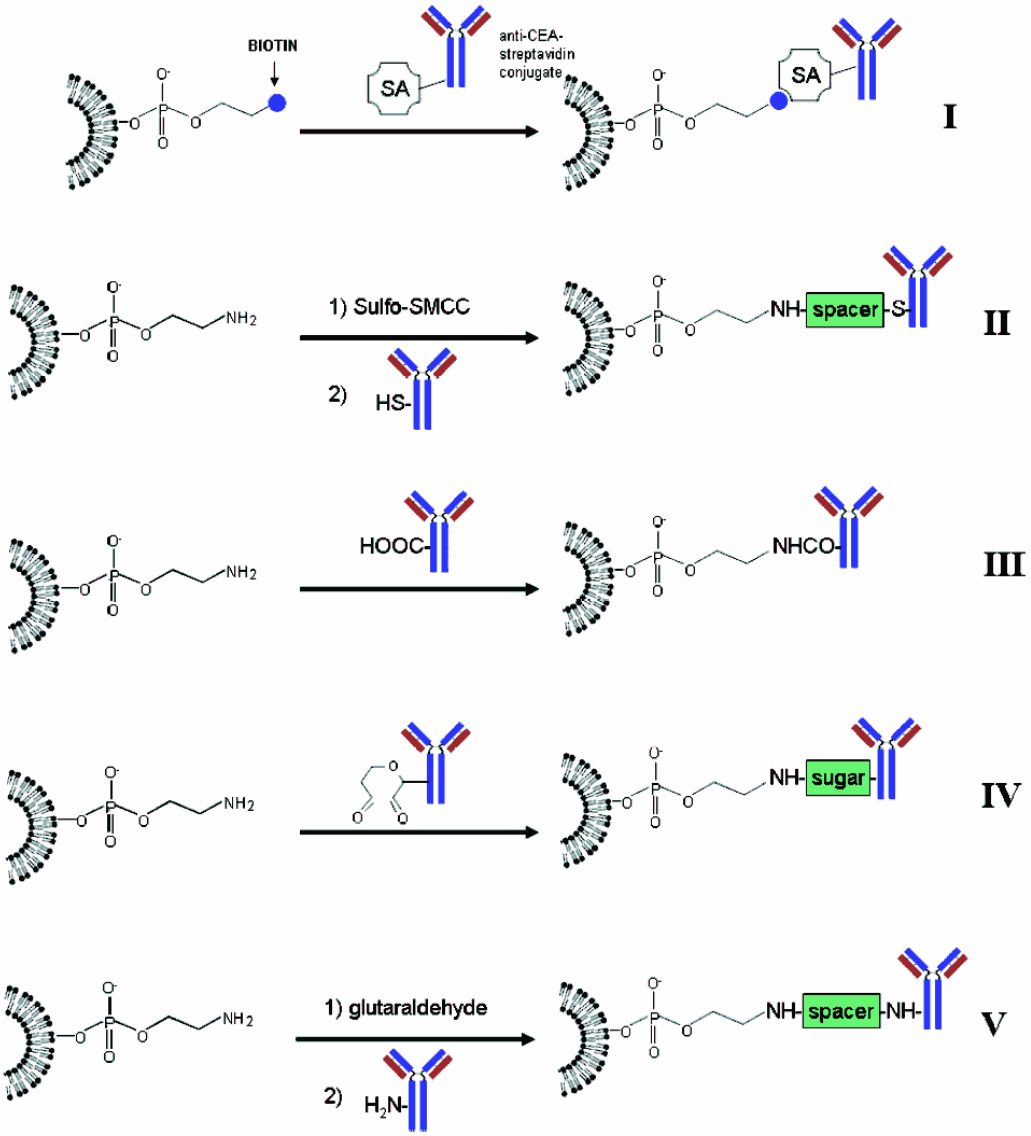
Scheme 1. Flow Scheme of Modification Methods Studied



RESULTS AND DISCUSSION

Characterization of Antibody Modified Liposomes. A known lipid formulation was used to prepare thermosensitive liposomes. Depending on the concentration of DPPE/biotin-capped DPPE used in the formulation, the liposome size was found to be tunable (results not shown). In this study, an optimized formulation resulting in a monodisperse population of liposomes with diameter of 200 ± 13 nm was used (Figure 1). The prepared liposomes were modified with anti-CEA using five different modification methods: the first method exploiting the use of biotin-capped DPPE in the formulation and subsequent attachment to the streptavidin linked anti-CEA antibody and the other four being based on the covalent binding of the anti-CEA antibody to the amine group of the DPPE lipid using different functionalities and activation methods (Scheme 1). The modification methods were compared in terms of the size, shape, and stability of the resulting liposome−anti-CEA complexes, as well as for their affinity for the CEA antigen.

As a means of characterization, transmission electron microscopy (TEM) was carried out on the liposomes before and after modification to probe the shapes formed (e.g., aggregates of liposomes). The liposomes modified with glutaraldehyde were observed to be shapeless and bulky with a significantly increased size of the vesicles (see Figure 2e) while all other methods resulted in evenly shaped liposomes with an increased bilayer thickness from 4 to 12 nm and an increase in the diameter in the order of 16−20 nm, as exemplified in Figure 2 where the modified liposomes resulting from Methods I−V are shown. Glutaraldehyde links amine groups, and it is highly probable that a polymer of

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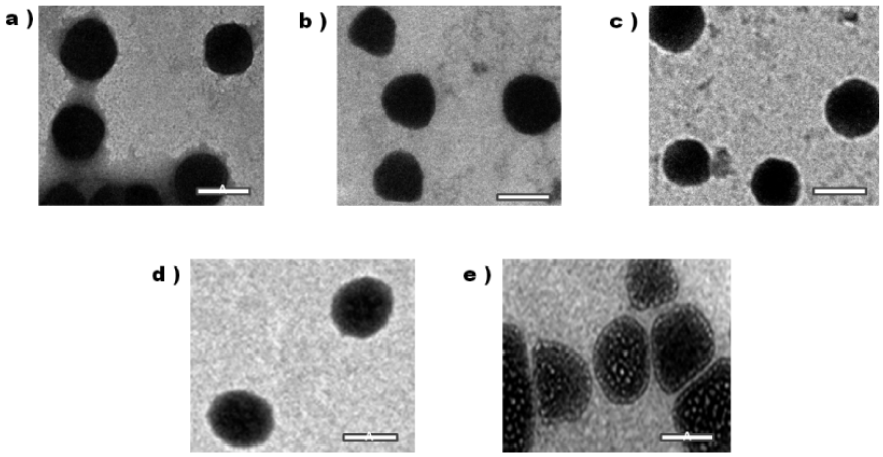

Figure 2. TEM images of liposomes modified using Methods (a) I, (b) II, (c) III, (d) IV, and (e) V. Scale bar 200 nm.

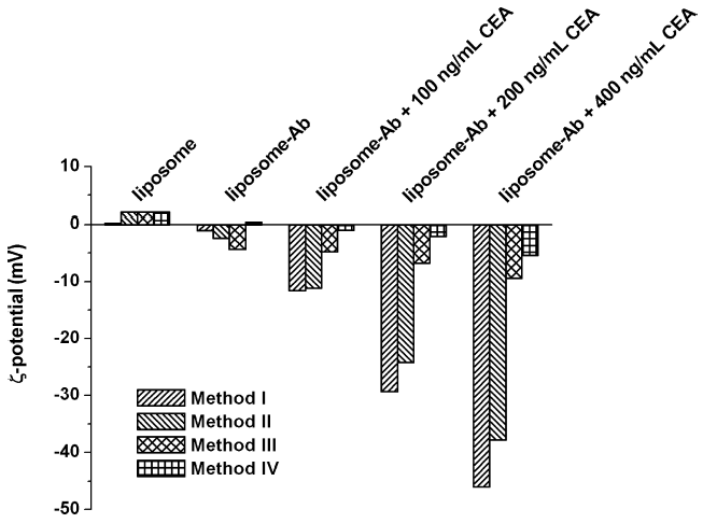

Figure 3. ζ-Potential values of liposomes biofunctionalized using Methods I, II, III, and IV. Measurements were obtained before and after modification with anti-CEA and following incubation with increasing concentrations of CEA.

liposomes were formed with liposomes being conjugated to each other and not to the antibody. In the other methods, differential functional groups are linked, e.g., maleimide with thiol and amine with carboxyl, avoiding this problem.

For each method, the modification was monitored with ζ-potential measurements in order to see the effect of each layer on the total charge of the particle surface. In Figure 3, results obtained with the antibody functionalized liposomes prepared according to Methods I, II, III, and IV are shown, and as can be seen, following functionalization with the anti-CEA monoclonal antibody, a decrease in the ζ-potential was obtained. Following incubation with the target antigen CEA, with increasing concentrations of the CEA, there was a shift in ζ-potential to more negative values. This is an expected result since proteins are negatively charged at a pH higher than their p*I* value. In this case, the highly acidic nature of CEA leads to a negative charge at pH 7.4, resulting in a change on the surface of the liposomes which increases depending on the concentration of CEA localized on the surface. These results demonstrated the great potential of ζ-potential measurements to be used as a versatile technique, not only confirming the functionalization of the liposomes with antibody and subsequent binding of the target antigen but also providing comparative data for the evaluation of the different methods. From the ζ-potential results, Methods I and II are the preferred methods for biofunctionalization of the liposomes, despite the fact that lower levels of antibodies appear to be linked to the surface, presumably due to a preferred orientation of the antibody for binding to the target antigen.

Temperature vs Detergent Triggered Enzyme Release. Triton-X100 is one of the most commonly used detergents for breaking the vesicle membrane and promoting the leakage of the encapsulated material from the liposomes. However, Triton X-100 is known to have a denaturing effect on enzymes, having a pronounced effect on their activity.[18]

As an alternative to the use of detergents to lyse liposomes, the use of thermosensitive liposomes (DPPC/DPPE/lyso-PPC =

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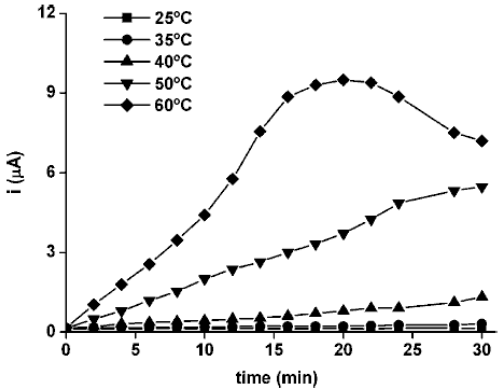

Figure 4. Analysis of thermo-sensitivity and release kinetics of biofunctionalized liposomes using amperometry prior to their modification.

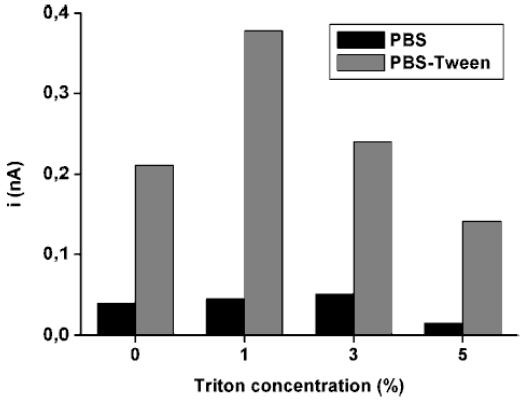

Figure 5. Time dependence of the amperometric signal for the HRP release from the liposomes prepared by Method I at increasing concentrations of Triton X-100 in PBS (10 mM) and PBS-Tween (10 mM).

86.1:1.9:12.0 mol/mol) was evaluated, and the release capacities of the prepared liposomes after biofunctionalization with Method I were analyzed at different temperatures. Amperometric measurements were carried out with modified electrodes after their incubation with liposomes at room temperature before and after the addition of a mixture of H_2O_2 and hydroquinone (HQ), followed by incubation at different temperatures, measuring the changes in current over 30 min. As can be seen in Figure 4, the highest signal was achieved at about 20 min at 60 °C, and a decrease in signal was obtained at longer time periods due to decreased activity of enzyme. These results are as expected as the T_M of the thermosensitive liposomes is 41 °C, and they are expected to be stable below this temperature, which is clearly reflected in Figure 4 where no or negligible release of HRP is observed until the temperature is elevated to 50 °C.

Control studies monitoring HRP activity colorimetrically were carried out with free HRP to see if the HRP is still active at 60 °C over time, and the results showed that the HRP had conserved 98% of its activity compared to that at 25 °C for 20 min while a decreasing activity was measured at increasing incubation times, in agreement with amperometric studies.

In order to compare to lysis using Triton X-100, amperometric measurements were carried out with increasing concentrations of Triton X-100 (0, 1, 3, and 5% v/v) in PBS (10 mM, pH 7.4) or PBS-Tween (10 mM, pH 7.4) and the signal was recorded over time following addition of TMB and hydrogen peroxide. It is expected that the presence of Tween should assist the lysis of the liposomes and as expected can be seen in Figure 5; the signal obtained is consistently higher in PBS-Tween. However, the signal obtained in all cases is markedly lower than that obtained using elevated temperature, and furthermore, increasing the concentration of the Triton X-100 results in decreasing signals. Control experiments monitoring HRP activity colorimetrically with increasing concentrations of Triton X-100 were in agreement with the electrochemical studies, demonstrating a possible denaturation of the released HRP, further highlighting the usefulness of thermosensitive liposomes for the encapsulation of enzymes for signal enhancement.

Evaluation of Signal Enhancement Using Biofuntionalized Thermosensitive Liposomes. HRP encapsulating liposomes modified with anti-CEA monoclonal antibody were analyzed in terms of their capacity to recognize the CEA target and amplify the signal. The various modification methods studied were compared to each other, as well as to a covalent HRP–anti-CEA conjugate. Recently, we have reported on the preparation of highly stable self-assembled monolayers of dithiols enabling rapid chemisorption of proteins on gold surfaces.[25,26] In this work, the same methodology was used to prepare monolayers of the anti-CEA capture antibody on dithiol-modified electrodes (see Figure 1). Gold electrodes modified with DT2 covalently linked to a catching anti-CEA primary antibody were incubated with 18.75 ng/mL CEA for 30 min and subsequently incubated with 20 μg/mL of the biofunctionalized liposomes, followed by elevating the temperature to 60 °C for 20 min. The electrochemical signal was measured before and after heat treatment. Following heat treatment, the solution was allowed to cool down to room temperature before the electrochemical measurement was carried out. A control experiment was also carried out to test the effect of the temperature on CEA, where a range of concentrations of CEA alone was heated to 60 °C for 20 min and was then measured using sandwich enzyme linked immunosorbent assay (ELISA), and the response was obtained compared with that of CEA that had not being heated; no difference in signal was observed.

As can be seen in Figure 6, Methods I and II yielded the highest amplification factors with liposomes modified according to Method I demonstrating an 11-fold signal enhancement as compared to the HRP–anti-CEA conjugate and those modified according to Method II providing a 9-fold signal enhancement. This is in excellent agreement with the ζ-potential measurements shown in Figure 3. The reason for the superior performance of the biofunctionalized liposomes obtained using Methods I and II can be attributed to the conjugation methods used. As can be seen from Scheme 1, EDC/NHS (Method III) and periodate (Method IV) based methods are two-step methods, with the first step involving the activation of antibody and the second step involving the mixing of the activated antibody with liposomes. For the

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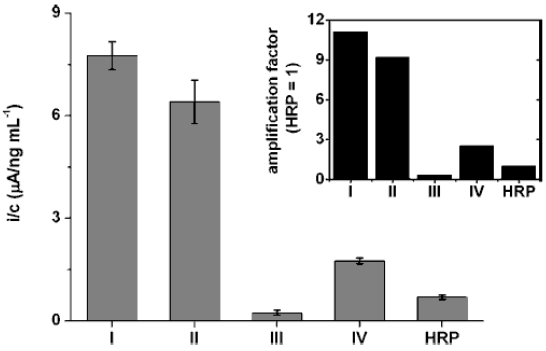

Figure 6. Effect of modification method: Signal enhancement capacities of anti-CEA modified liposomes prepared by Methods I–IV in the amperometric detection of 18.75 ng/mL CEA. The amplification factors are shown in the inset.

Table 1. Analytical Parameters Obtained for the Detection of CEA Using Anti-CEA Functionalized Liposomes and HRP–Anti-CEA Conjugate

	sensitivity (μA·mL/ng·cm^2)	EC_{50} (ng/mL)	LOD (ng/mL)
method I	7.765	17.96	0.080
method II	6.412	18.25	0.113
HRP–anti-CEA	0.697	4.416	2.159

antibody–liposome conjugates based on the bioaffinity of biotin–streptavidin and the covalent interaction via SATA/sulfo-SMCC, the bioconjugation process was carried out in three steps, where the liposomes were activated with one reagent while the antibody was separately activated with another reagent, followed by mixing of the activated moieties to form the conjugating bridge between liposomes and antibodies. The advantage of this approach is that the linkage between either SATA/sulfo-SMCC or biotin–streptavidin cannot be formed if one component is not present, and this protects against undesired cross-linking reactions, such as liposome–liposome and antibody–antibody self-conjugation.

Table 1 reports the analytical parameters, and Figure 7 shows the calibration curves obtained for the detection of CEA using liposomes prepared by Methods I and II and HRP–anti-CEA conjugate. As can be seen in Figure 7, linear dynamic ranges of CEA concentrations in the ranges of 0.59–37.5 and 1.17–37.5 ng·mL^{-1} were achieved using Methods I and II with R^2 values of 0.989 and 0.992, respectively. The limit of detection (LOD) values calculated from a linear regression equation using three times the standard deviation of blanks were 0.080 and 0.113 ng·mL^{-1}, for Methods I and II, respectively, which are significantly lower than that obtained with the HRP–anti-CEA conjugate. In addition, the electrochemical assay sensitivities obtained with the liposomes are improved in more than 1 order of magnitude with respect to the covalent HRP conjugate (Table 1).

Stability of Biofunctionalized Liposomes. The stability of the anti-CEA–liposome conjugates modified by Methods I and II were monitored over 30 days of storage in PBS (0.1 M, pH 7.4) at 4 °C in terms of change in electrochemical signal at CEA concentration of 18.75 ng·ml^{-1} and their size. Although there was slight increase in the liposome diameter (~4–5 nm), there was no significant change in the electrochemical signal (1.35% for Method I and 1.65% for Method II).

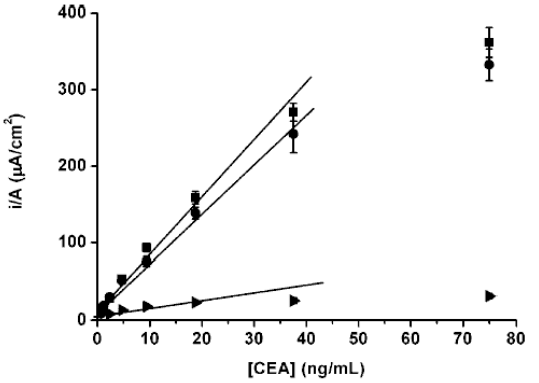

Figure 7. Amperometric calibration curves for the detection of CEA using liposomes modified via SATA/sulfo-SMCC (●) and biotin–streptavidin based methods (■). The calibration curve obtained with HRP–anti-CEA conjugate (arrowhead) is also shown for comparison.

CONCLUSIONS

An amperometric immunosensor using enzyme encapsulating thermosensitive liposomes for signal enhancement for the ultrasensitive detection of carcinoembryonic antigen (CEA) is reported. The biotin–streptavidin (Method I) and SATA-Sulfo-SMCC (Method II) approaches were demonstrated to be the most suitable biofunctionalization methods of the five methods studied for the modification of liposomes with anti-CEA Ab in terms of their stability, shape, and signal enhancement capacity. The ξ-potential of the prepared liposomes was measured before and after modification, and the change in the total surface potential was demonstrated to be useful for monitoring of the modification success or antigen capturing capacity of the liposome-linked antibodies. Heating thermosensitive liposomes to an optimized temperature rather than lysing with Triton X-100 facilitated a higher level of enhancement, maintaining the activity of the encapsulated enzymes. The use of thermosensitive liposomes modified using Methods I and II for amperometric detection of carcinoembryonic antigen provided 11- and 9-fold signal amplification, with significantly decreased detection limits as compared to HRP–anti-CEA-Ab, clearly demonstrating the potential of these nanotools for signal amplification.

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CHAPTER

4

APPLICATION TO INORGANIC SYNTHESIS OF METALLIC NANOPARTICLES

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Diffusion driven size controlled synthesis of gold nanoparticles: nano-liposomes as mass transfer barrier

Rukan Genç, Mayreli Ortiz [*], Ciara O'Sullivan [*]

[1]Nanobiotechnology and Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Av. Països Catalans, 26, 43007, Tarragona, Spain

[2]Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23,

08010 Barcelona, Spain.

Abstract

Reverse micelles, lipid vesicles and other types of amphiphilic materials are of great interest in the size-controlled synthesis of metal nanoparticles. In this study, we present a facile synthesis method of small (2-6 nm) gold nanoparticles in the cavity of nano-sized liposomes (18 nm) under mild conditions at variable concentrations of $NaBH_4$. The barrier role of the vesicle membrane in controlling the reaction rate and protecting the gold nanoparticles from rapid aggregation at high reducing agent concentrations is discussed and compared with the traditional method of nanoparticle synthesis.

1. Introduction

Metal nanoparticles of diverse sizes and shapes have garnered great interest in the last decade due to their exceptional unique optical, electronic and chemical properties, which are not displayed in the bulk state of the metal.[1, 2] Among those metals, gold (Au) based nanoparticles (NPs) have been of particular interest due to their widespread application in catalysis, plasmonics, sensors, and biomedical technologies (e.g. drug delivery) as well as electronics.[3,4]

As an alternative to wet synthesis methods (e.g Turkevich citrate method), the use of porous or hollow materials with aqueous cores, such as reverse micelles, lipid vesicles and polymer cages are of great interest as they provide a controlled environment in submicron volumes for the particle synthesis. [5,6]

Liposomes are potential candidates as reaction compartments with their capability to encapsulate metal salt with hydrophilic nature in their aqueous core. For the specific application of nanoparticle synthesis and specifically using liposomes (rather than reverse micelles), to date, the most used liposome types have been giant liposomes, due to their ease of preparation. Onion like multivesicular liposomes (MVL) have been used for the spontaneous synthesis of gold nanoparticles where the particle size (around 100 nm) was controlled by the distance between the two neighbouring bilayers.[7] In another approach, CdS particles of just 2.5-5.9 nm with just one nanoparticle per liposome were obtained.[8] Although these studies were only reported as a "proof-of-concept", they have drawbacks such as poor yield, as well as the relatively large size of the resulting particle sizes compared to wet-synthesis. Yang et al., succeeded in the

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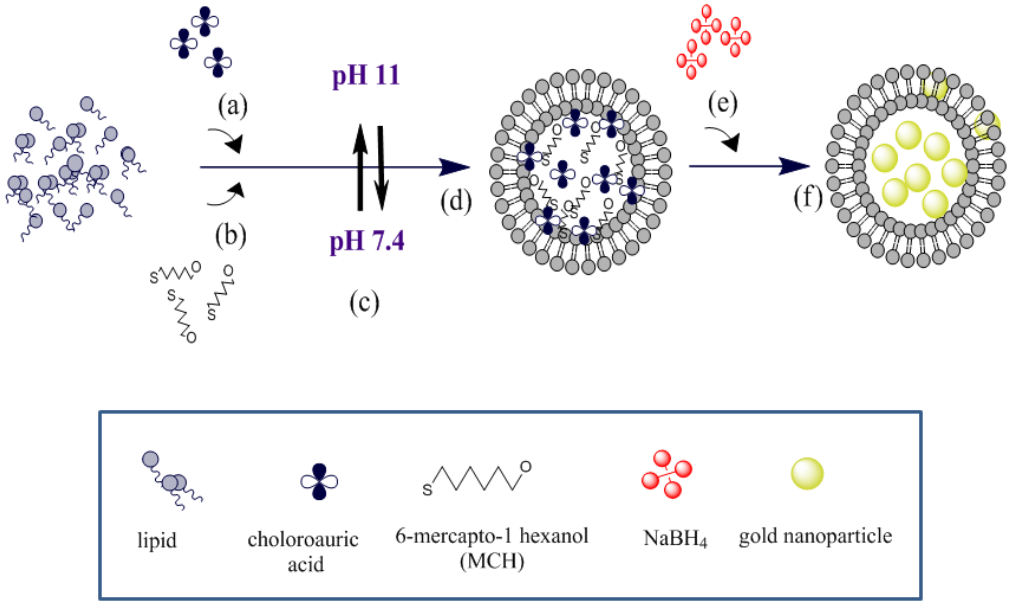
preparation of a higher yield of of 4 or 50 nm CdS nanoparticles in electrofused giant liposomes where particle size was dependent on the protocol used.[9]

In this report, gold nanoparticles (Au-NP) were synthesized within liposomal nanoreactors. These nanoreactos were prepared using a solvent free, one-step preparation method (curvature tuned preparation method)[10] based on a rapid change of pH to a maximum around 11, followed by an instant decrease to a pH around 7.4 and equilibration period at a certain temperature chosen depending on the lipid formulation (scheme 1).



Scheme 1: Schematic representation of liposome mediated gold nanoparticle synthesis: a) Mixing lipids with $HAuCl_4$ and b) and capping agent 6-mercapto-1-hexanol (MCH) in buffer, c) further instant pH jump from pH 7.4 to pH 11 and turn back to pH 7.4 at constant temperature, d) formation of liposomal nanoreactors, and e) titration of the reducing agent ($NaBH_4$) which leads f) the particle formation in the liposomecore.

2. Experimental section

2.1 Preparation of encapsulating nano-liposomes: $HAuCl_4$ encapsulating liposomes were prepared via the curvature tuned preparation method (Figure 1)as reported previously. Briefly, 50 mg of phospholipid formulation of 1,2-dioleoyl-snglycero-3-[phosphor-rac-(1-glycerol)] (DOPG) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC) was directly rehydrated in $HAuCl_4$ and 6-mercapto-1-hexanol (capping agent, 1:50 $HAuCl_4$/MCH molar ratio) mixture in PBS buffer (0.1 M, pH 7.4) at room temperature under argon supply while the mixture was continuously stirred. The solution was then subjected to a rapid pH jump (pH 7.4→pH 11 → pH 7.4) followed by an equilibration period of 25 min, where lipid clusters curl into encapsulating liposomes of 20 nm in diameter. The resulting liposomes were purified using G25 Sephadex column and stored at 4 °C.

2.2 Synthesis of gold nanoparticles in the presence and absence of liposomes: Encapsulated and non-encapsulated solutions of $HAuCl_4$ and capping agent, 6-mercapto-1-hexanol (MCH)

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were mixed in 1:50 molar ratio in phosphate buffer saline (PBS, 10 mM, pH 7.4) and were titrated with $NaBH_4$ solutions at room temperature. Solutions were continuously stirred under argon. The morphology and size distribution of the resulted nanoparticles were characterized using transmission electron microscopy (TEM) after their purification from lipids and excess reactants by centrifugation using methanol:ethanol mixture (1:4 v/v) and their dispersion in toluene.

3. Results and Discussion

Earlier studies on the effect of membrane charge of the vesicles on particle size had shown that particles produced using membranes of negatively charged lipids are smaller than that of mixtures of negatively charged and zwitterionic lipids.[11] Thus, a negatively charged phospholipid, 1,2-dioleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (DOPG), which previously formed 22±4 nm sized liposomes when mixed with a small amount of one tail lipid (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, lyso-PPC)- with a 88:12 molar ratio was used to produce the nanoreactors.[10]

Particle synthesis using both the classic solution based method and liposomal nanoreactors were carried out in parallel under the same conditions to evaluate the importance of liposomes as a mass transfer barrier. Thiols are often used as stabilizing or capping agent to prevent nanoparticle aggregation.[5] In this work a short chain alkanethiol, 6-mercapto-1-hexanol (MCH) (1:50 Au:MCH molar ratio) was used as a capping agent and two different routes of its administration to the system were studied. Firstly, two different solutions were prepared: a) $HAuCl_4$ and MCH encapsulated in liposomes and b) $HAuCl_4$ and MCH in 10 mM phosphate buffer saline (PBS, pH 7.4). Sodium borohydrate, $NaBH_4$, was then added drop-wise to both solutions. In the second route, two other solutions were prepared c) $HAuCl_4$ encapsulated in liposomes and d) $HAuCl_4$ in 10 mM PBS (pH 7.4). A mixture of MCH and $NaBH_4$ was slowly titrated to both solutions. The final solution was purified by centrifugation with methanol:ethanol solution (1:4 v/v) and kept in toluene for further characterization of them by transmission microscope. TEM studies showed that the second route resulted in large gold nanoparticles in both cases. The nanoparticles prepared in the absence of liposomes were a heterogeneous population of particles with a wide size distribution from 10 nm to 500 nm (Figure 1-a, 1-c, 1-d) whilst in the presence of liposomes, nanoparticles of 10-20 nm were produced, which represented the 80 % of the total particle number (Figure 1-b, 1-d), indicative of a high degree of homogeneity.

However, using the first synthetic route where MCH is present in the system together with $HAuCl_4$ smaller nanoparticles (17±7 nm (in solution) and 4.7± 0.2 nm (within nanoreactors)) were obtained at 1:40 $HAuCl_4/NaBH_4$ molar ratio. A pale red color solution was observed. In the second route, the particle size increased by several nanometers, as when reducing agent and capping agents are titrated together, the kinetics of aggregation are more rapid than the diffusion of the capping agents to the nanoparticles being formed. Thus, further experiments were carried out mixing the MCH capping agent together with $HAuCl_4$.

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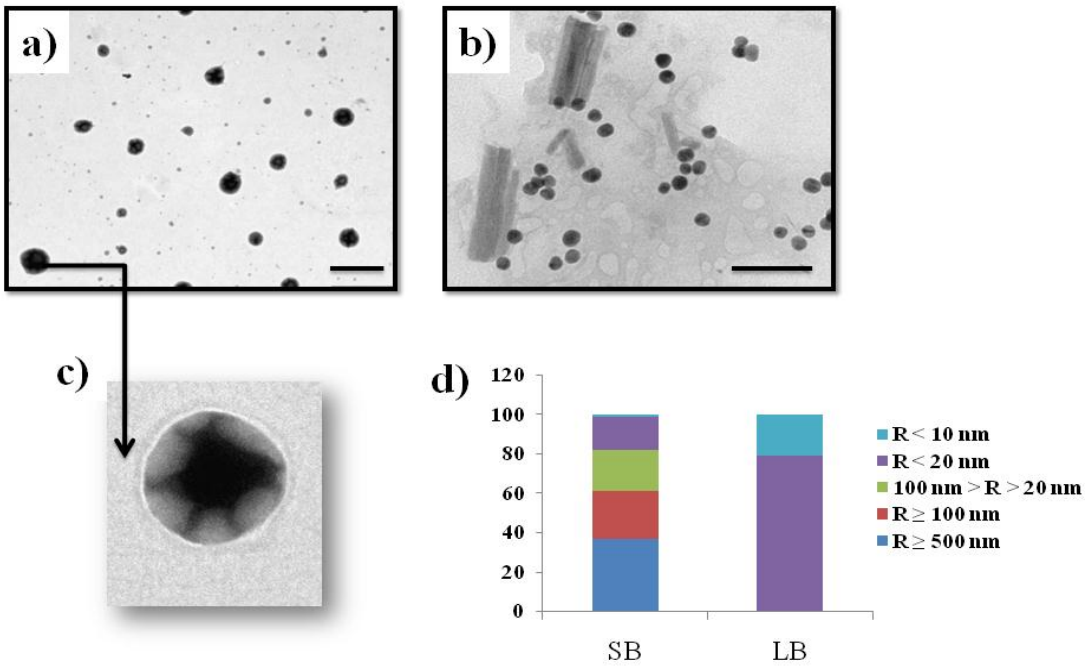

Figure 1 Effect of the route of the capping agent addition to the synthesis process. TEM images of large aggregates of Au where capping agent integrated to the synthesis together with the reducing agent (1:20 $HAuCl_4$ /$NaBH_4$ molar ratio) a) direct synthesis in solution and b) liposome mediated synthesis. c) Magnified TEM image of a single gold particle produced via solution based synthesis and d) graphical representation of the approximate size distribution of the particles formed calculated using TEM images, n≥3, particle number ≥100. Scale bars = 2 μm.

To develop a nanoreactor, it is crucial to understand in which part of the liposome the synthesis reaction occurs in order to elucidate the mechanism and role of the liposome in particle formation. The liposome membrane is semi-permeable to small molecules and water akin to living-cell membranes and while uncharged and small molecules can pass through the bilayer easily, charged and macromolecules are retarded by the lipid barrier.[12] Due to its small size, the BH_4^- anion can diffuse through the membrane from outside to the liposome core. As can be seen from the TEM image of a single liposomal nanoreactor captured before (Figure 2-a) and during the synthesis (Figure 2-b), there are numbers of Au-NPs inside the liposomes where some others were formed at sites close to the membrane as depicted in the cartoon in Figure 2-c.

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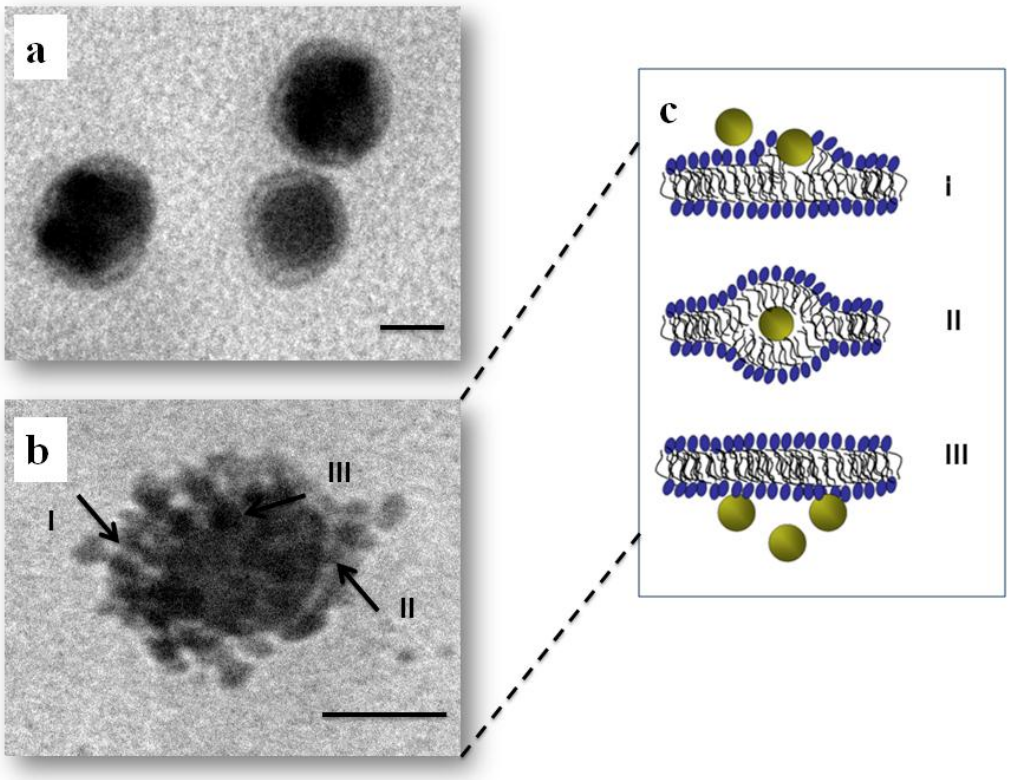

Figure 2: Captured transmission electron microscope image of single liposomal nanoreactor a) before the synthesis and c) during the synthesis. c) Proposed ways of the particle synthesis; I) on the membrane surface, II) among the lipid bilayer, and III) in membrane cavity.

There are several reports in the literature regarding the effect of reducing agent concentration on particle size.[13-14] In agreement with the literature, studies on the effect of increased concentration of $NaBH_4$ either in solution based or liposome mediated Au-NP synthesis resulted in the increased size of NPs with increasing concentration of reducing agent (Figure 3) at constant concentrations of the capping agent MCH (1:50 molar ratio $HAuCl_4$/MCH).[15] The $HAuCl_4/NaBH_4$ molar ratio, was varied in the range of 1:20 – 1:1000. In the case of non-encapsulated $HAuCl_4$ solutions, 5 ± 1 nm gold-nanoparticles with a low polydispersity was achieved at a molar ratio of 1:20 whilst at 1:40, a broad size distribution of 17±7 nm and aggregates of several nm (Figure 3-1st line) appeared with a color change to bluish purple, and at a ratio of 1:100 a blue color (Figure 4-a) emulsion of amorphous aggregates with varying sizes in the submicron range is obtained. Thus, in solution, the size of the nanoparticles formed, is, as expected, highly influenced by the ratio of reducing and capping agents, and in the presence of increasing concentrations of reducing agent at a constant concentration of capping agent, the aggregation of smaller nanoparticles into larger particles is observed both due to an inadequate amount of capping agent, as well as the kinetics of nanoparticle formation being far more rapid than the diffusion of the capping agent to the formed nanoparticles.

On the other hand, nanoparticles synthesized in the liposomal reactor showed a great degree of protection from aggregation and was in fact only observed after the addition of $NaBH_4$ in a 1:1000 molar ratio. The particle size ranged from 2.9 ± 0.7 to 4.7± 1.1 at increasing $NaBH_4$:MCH molar ratios of 1:20 and 1:40, respectively, indicating the decelerating effect of

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the liposomal membrane on the reaction rate, by effectively placing a mass transfer barrier between the two reaction species (Figure 3-2nd line). The small size of the liposome is also effective in the production of smaller nanoparticles by providing a reaction volume in nano-ranges for controlling the chloroauric acid concentration so that most of the metal salt is consumed in the nuclei synthesis and less metal salt is accessible for further growth of the larger nanoparticles.[6]

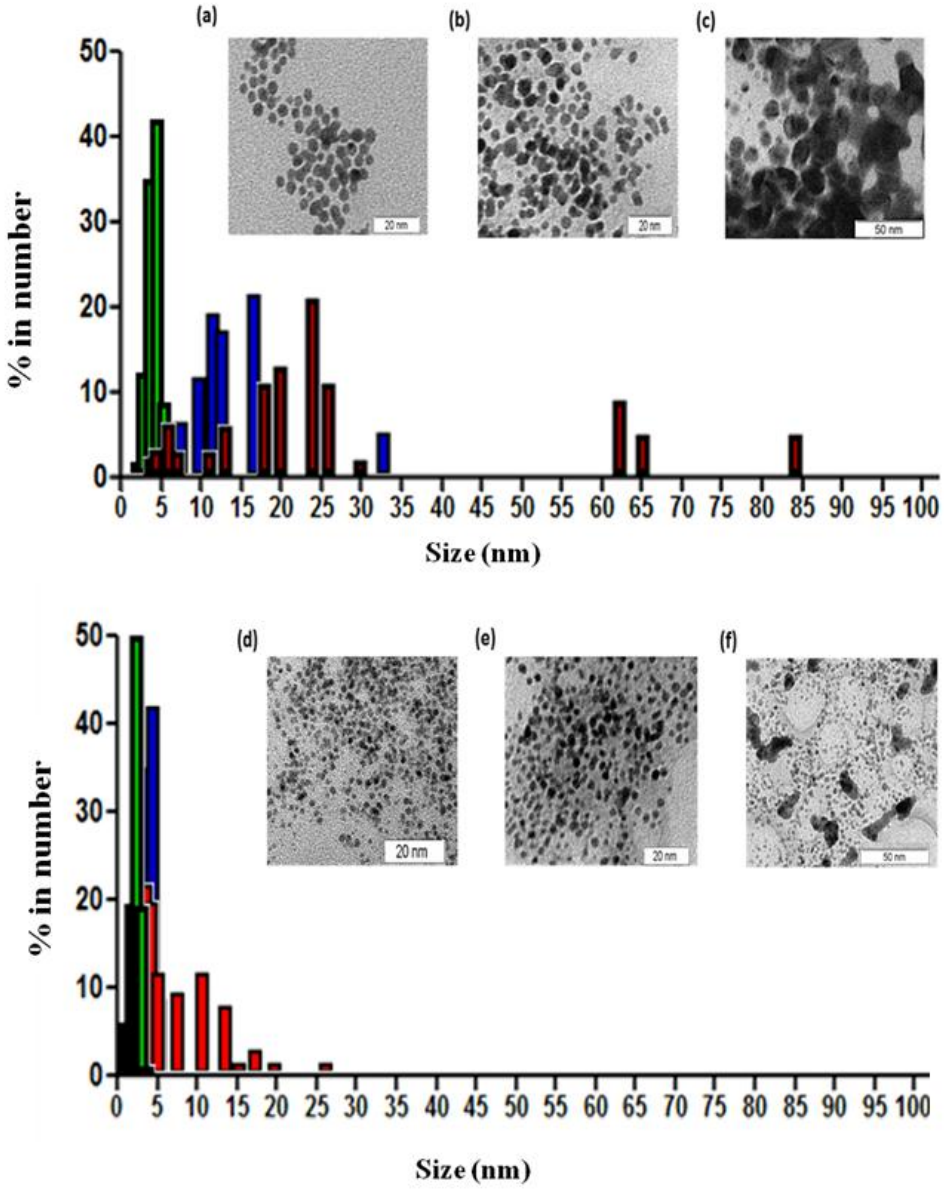

Figure 3 Size distribution of the gold nanoparticles in toluene prepared at changing molar ratios of $HAuCl_4/NaBH_4$: 1st line using solution based method (■) 1:20, (■) 1:40 and (■) 1:100 and their corresponding TEM images (a), (b) and (c), respectively, and graph on the 2nd line using liposomal nanoreactors (■) 1:20, (■) 1:40 and (■) 1:1000 and their corresponding TEM images (a), (b) and (c), respectively.

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AuNPs having size around 3 nm were a very pale pink, whereas particles formed at increased $NaBH_4$ concentrations was of a deepening and more intense pink color with increased reducing agent concentrations, indicative of the increased size of the gold particle formed Figure 4 b.

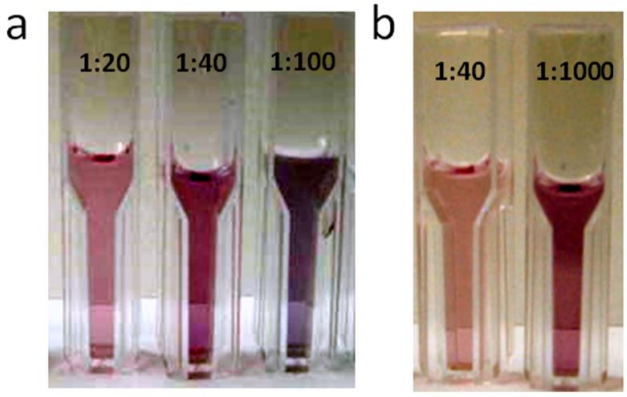

Figure 4: Final colors of the gold nanoparticles synthesized using a) classic in-solution synthesis and b) in liposomal nanoreactors.

Regarding the stability of the AuNPs produced, nanoparticles prepared at a molar ratio of 1:20 either in solution or within the liposomal nanoreactors were monitored by TEM microscopy and spectrophotometry over a month at 4 ^{o}C. Results showed that particles prepared within the liposome nanoreactors exhibited a 3.2% increase in size, whilst the increase in the size reached to 12.5 % for the nanoparticle population which prepared via traditional method. These results showed that presence of liposomes in the nanoparticle preparation process renders gold nanoparticles of increased stability, due to the controlled synthesis process resulting in a better interaction between the capping agent and gold nanoparticles, this avoiding aggregation, even over long storage periods.

In summary, the liposome membrane effectively acts as a barrier membrane, controlling the mass transfer through the membrane, and thus controlling the reaction rate of nanoparticle synthesis and capping, protecting against the rapid aggregation of Au-NPs at high concentrations of $NaBH_4$, whilst nanoparticles of 2-5 nm were obtained using nanoliposomal reactors and the particle aggregation was started only after 1000 times higher moles of reducing agent respect to the initial metal salt concentration.

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Green synthesis of gold nanoparticles using glycerol incorporated nanosized liposomes

Rukan Genç, Gael Clergeaud, Mayreli Ortiz [*], Ciara O'Sullivan

[1]Nanobiotechnology and Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Av. Països Catalans, 26, 43007, Tarragona, Spain

[2]Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23,

08010 Barcelona, Spain.

*

Abstract

There has been enormous interest in method development for the inorganic synthesis of metallic nanoparticles of desired size and shape in the last decade, due to their unique properties and extensive application in catalysis, electronics, plasmonics and sensing, to name a few. This article reports on an environmentally-friendly, one-pot synthesis of metallic nanoparticles, which avoids the use of organic solvents, and only requires mild experimental conditions. The developed method uses liposomes as nanoreactors, where the liposomes were prepared encapsulating chloroauric acid and exploited the use of glycerol, incorporated within the lipid bilayer as well as in its hydrophilic core, as a reducing agent for the controlled preparation of highly homogenous populations of gold nanoparticles. The effect of temperature, the presence of capping agent and the concentration of glycerol on the size and homogeneity of the nanoparticles formed was investigated and compared with solution based glycerol mediated nanoparticle synthesis. Well-distributed gold nanoparticle populations in the range of 2-8 nm were prepared in the designed liposomal nanoreactor with a clear dependence of the size on the concentration of glycerol, temperature and presence of capping agent whilst large and heterogeneous populations of nanoparticles with amorphous shapes were obtained in the absence of liposomes. Particle morphology and sizes were analyzed using transmission electron microscopy imaging and liposome size was measured using Photon Correlation Spectroscopy.

1 Introduction

Metal nanoparticles of diverse sizes and shapes have garnered great interest in the last decade due to their exceptional unique optical, electronic and chemical properties, which are not displayed in the bulk

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state of the metal.[1, 2] Among those metals, gold (Au) based nanoparticles (NPs) have been of particular interest due to their widespread application in catalysis, plasmonics, sensors, and biomedical technologies (e.g. drug delivery) as well as electronics.[3,4] The development of synthesis methods to obtain homogenous and tunable sized and shaped nanoparticles for specific application requirements is a priority in nanoparticle based technology development.[1] In the majority of the reported chemical synthesis methods of gold NPs, the basic principle is the reduction of Au(III) to Au(0), often exploiting sodium citrate and sodium borohydride as reducing agents in an aqueous solution. Subsequently, reduced gold atoms assemble in small clusters and finally these clusters provide nucleation sides for other molecules to adhere to and grow forming nanoparticles.[5] To avoid uncontrolled aggregation into larger particles, stabilizing or capping agents are usually added to the mixture. [6] Addressing environmentally friendly methods of producing nanoparticles, renewable reagent sources such as bacteria,[7] plant extracts[5], alcohols[6] and polyols[8] have been demonstrated as successful reducing agents and/or capping agents.

Glycerol is a known polyol used as a moistening and preservative agent to extend shelf life, as well as a sweetener in food technology and in the manufacture of many drugs. Oxidation products of glycerol are also of great interest and the development of new cost-effective methodologies for their production, using gold or palladium nanoparticles as catalysts have been reported.[9-12] However; there are very few attempts exploiting the reverse reaction, where the metals are reduced by glycerol, thus forming nanoparticles. To date, the synthesis of nanoparticles with polyols (most often ethylene glycol) is based on heating a polyol-inorganic salt mixture, typically to high temperatures over 100^{o}C depending on the melting temperature of the polyol, under continuous stirring conditions.[13] In their recent study, Grace and Pandian reported on the synthesis of gold nanoparticles and nanoprisms using glycerol as a reducing agent, both under reflux and microwave conditions, where the glycerol- $HAuCl_4$ mixture was heated to boiling point, resulting in the synthesis of spherical or prism-shaped nanoparticles depending on the reaction time.[14] Nisaratanaporn and Wongsuwan prepared silver powders of larger than 63 nm from silver alkoxide using glycerol as a reducing agent, again heating the metal and undiluted glycerol solution to high temperatures (150 - 180 ^{o}C),[15] whilst, Sarkar et al achieved the glycerol-mediated reduction of silver to form nanoparticles of 25 nm at room temperature but reduction required the addition of NaOH.[16] To the best of our knowledge, there is no report to date demonstrating the formation of extremely small nanoparticles using glycerol as a reducing agent at low temperatures and not requiring any additional reactants.

A widely reported method for the preparation of nanoparticles is the reverse micelle method, which exploits water-in-oil droplets stabilized by a surfactant (most often AOT (Aerosol OT, sodium bis(2-ethylhexyl) sulfosuccinate)).[17,18] They have been used as nanoreactors for the synthesis of structures

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having the same shapes as the micelle nuclei, such as metal nanoparticles [19] or metal hybrids,[20] ceramic materials, and [21] quantum dots[22] as well as polymer composites. [23] Reverse micelles tend to fuse and disperse randomly due to Brownian motion and the content exchange between two fused reverse micelles results in the formation of nanosized particles with their size being defined by the micelle volume.[24]

As an alternative to reverse micelles as nanoreactors, liposomes are promising candidates for the synthesis of metal nanoparticles as they provide a controllable environment, not only in the core, but also within the lipid bilayer.[25] However, the preparation of nano-sized liposomes is labor intensive. In a previous study carried out in our group, nanosized liposomes have been prepared using a one-step preparation method based on a pH jump,[26] which was not only environmentally friendly as it avoids the use of organic solvents, but is also extremely rapid as it requires no homogenisation step such as extrusion and sonication, with the preparation of a highly uniform population of nano-sized liposomes being achieved in less than an hour.

In this work, we report a new environmentally friendly, low-temperature method to obtain a homogenous population of ultrasmall gold nanoparticles using liposomes incorporating glycerol. The glycerol, which is incorporated on both the external and internal polar surface of liposomes encapsulating chloroauric acid, $HAuCl_4$, facilitates the reduction of Au(III) to form Au(0) atoms and subsequent nanoparticles. The effect of parameters such as temperature, use of capping agent and glycerol concentration was investigated in terms of particle size and monodispersity. The resulting nanoparticles were characterized by transmission electron microscope (TEM). Highly monodisperse Au-NPs in a size range of 2-7 nm were obtained after 1 day of incubation at room temperature depending on the conditions used.

2 Experimental Sections

2.1 Materials

PBS buffer (10 mM, pH 7.4) supplied as a sachet of prepared lyophilised buffer, glycerol, $HAuCl_4$ and 6-mercapto-1-hexanol (MCH) were purchased from SIGMA. Liposomes were prepared using phospholipids supplied by Avantin® Polar Lipids Inc. All lipids were supplied as powders and were used without further purification. Sodium hydroxide and hydrochloric acid, reagent grade, ACS, were also purchased from Scharlau Chemie SA.

2.2 Preparation of encapsulating nano-liposomes Twenty mg $HAuCl_4$ or MCH/ $HAuCl_4$ encapsulating liposomes were prepared via the curvature tuned preparation method as reported previously.[26] Briefly, 50 mg of a phospholipid formulation of 1,2-dioleoyl-snglycero-3-[phosphor-rac-(1-

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glycerol)] (DOPG) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC) were dissolved in $HAuCl_4$ and/or 6-mercapto-1-hexanol (MCH, 1:50 mol/mol) mixture in 10 mL of PBS buffer (10 mM, pH 7.4) at various concentration of glycerol (% v/v) at room temperature under argon and stirring conditions. The mixture was then treated with a rapid pH jump (pH 7.4→pH 11 → pH 7.4) followed by an equilibration period of 25 min, where lipid clusters curl into encapsulating liposomes of 20 nm in diameter. The resulting liposomes were purified using G25 Sephadex column and used freshly prepared.

2.3 Synthesis of gold nanoparticles in the presence of liposomes Solutions of nanoliposomes prepared as explained in section 4.2 were incubated at predetermined temperatures for 24 hours. Solutions were continuously shaken in a temperature controlled shaker. Following incubation, nanoparticles were purified by centrifuging at 1000 rpm for 5 min, 3 times with methanol/ethanol mixture (1:4 v/v), and the collected pellet was re-suspended in toluene and kept at 4 °C for further characterization studies.

2.4 Synthesis of gold nanoparticles in the absence of liposomes I) 20 mg of $HAuCl_4$ was re-suspended in constant concentration of glycerol solution (x % v/v to y% v/v) in PBS (10 mM, pH 7.4) and incubated at predetermined temperatures for 24 hours. II) 20 mg of $HAuCl_4$ was re-suspended in constant concentration of glycerol solution in PBS (10 mM, pH 7.4) and the mixture exposed to a rapid pH jump from pH 7.4 to pH 11 and subsequent decrease to pH 7.4. Solution was again incubated at predetermined temperatures for 24 hours. III) $HAuCl_4$ and/or capping agent and 6-mercapto-1-hexanol (MCH) were mixed with a constant concentration of glycerol solution (x % v/v to y% v/v) in PBS (10 mM, pH 7.4) and the mixture exposed to a rapid pH jump from pH 7.4 to pH 11 and subsequent decrease to pH 7.4 and further incubated at predetermined temperatures for 24 hours. Solutions were continuously shaken in a temperature controlled shaker, and following nanoparticles were purified by centrifuging at 1000 rpm for 5 min, 3 times with methanol/ethanol mixture (1:4 v/v). The collected pellet was re-suspended in toluene and kept at 4 °C for further characterization studies.

2.5. Photon Correlation Spectroscopy (PCS) The mean diameter of nanoreactor liposomes was measured using Zeta Sizer 3000H equipment from Malvern Instruments, Inc., [He-Ne laser (633 nm), detector angle of 90 ^{o}] which measures the rate of fluctuation of the light scattered from the particles using photon spectroscopy (PCS). Standard deviations were calculated from the mean of the data of a series of experiments ($n \geq 3$).

2.6 Transmission electron microscopy (TEM) imaging using a glass pipette, a drop of sample was added to a 200 mesh copper grid with a thin film of Formvar polymer and carefully dried using a filter

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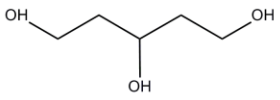
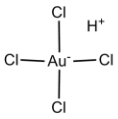
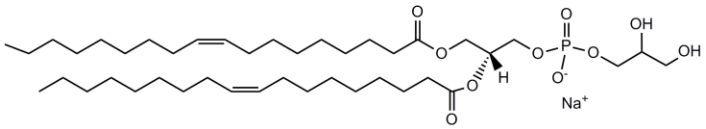
paper. The sample was left at room temperature until a dried film was obtained. Transmission Electron Microscopy (TEM) analyses were performed using a JEOL 1011 transmission electron microscope operated at 80 keV with an ultra-high-resolution pole piece providing a point resolution of 2 A°. Micrographs (1024 pixels x 1024 pixels) were acquired using a Megaview III multiscan-CCD camera. Images were analyzed with an iTEM image analysis platform and the mean diameter was calculated at least measuring 100 particles from the series of experiments (n±3).



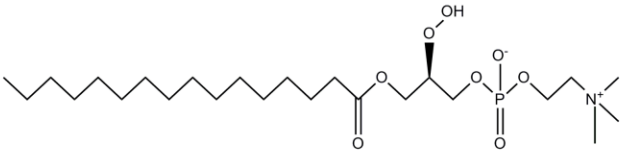
glycerol



Chloroauric acid



1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG)



1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC)

Scheme 1: Structures of molecules used for the nanoreactor design.

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3 Results and Discussion

3.1 Solution based synthesis of gold nanoparticles in glycerol

We have previously reported on an environmentally friendly method for the rapid (<1h) preparation of highly homogenous spherical liposome populations, the size of which can be carefully controlled via a combination of lipid composition and temperature applied during an equilibration stage following a rapid pH jump from 7.4 to pH 11 and back to pH 7.4, which was coined as curvature tuned liposome preparation.[26] To demonstrate the concept of producing gold nanoparticles via the glycerol mediated reduction of chloroauric acid ($HAuCl_4$) solution based synthesis of nanoparticles was primarily demonstrated, using this method. Briefly, 20 mg chloroauric acid was mixed with glycerol in solution (15 % v/v) at 25 ^{o}C in PBS (pH 7.4, 10 mM), in the absence of liposomes. As illustrated in figure 5, after 1 day of incubation at room temperature, relatively large and heterogeneous particles (10-50 nm or more) with amorphous shape were obtained.

Polyol based reduction-oxidation reactions mainly depend on the reaction pH, thus one of the important parameters of the curvature-tuned liposome preparation method, which exploits a pH jump, will have an impact on reaction kinetics.[27, 28] Thus, solution based synthesis was also carried out at a constant concentration of glycerol (15 % v/v) after an instant pH jump to pH 11 and subsequent drop back to pH 7.4, followed incubation at room temperature for 24 hours. TEM images of the nanoparticle population following elimination of the glycerol excess by centrifugation with methanol/ethanol solution (1:4 v/v) (Figure 5-b) demonstrated that there was a decrease in the particle size and an increase in the number of particles (see Figure 5-b). However, the particles were still amorphous and mainly aligned as chains of several particles (inset of Figure 5-b) with a length of around 20 nm.

Further studies on the effect of capping agent at a glycerol concentration of 15 % v/v in the absence of liposomes resulted in smaller particles (5-10 nm) compared to the ones obtained without MCH (around 20 nm). These results clearly show that the reduction of the Au(III) to Au(0) at room temperature using glycerol produces relatively small nanoparticles when they are exposed to a instant pH change compared to those formed directly in PBS solution (10 mM, pH 7.4), in the absence of capping agent.

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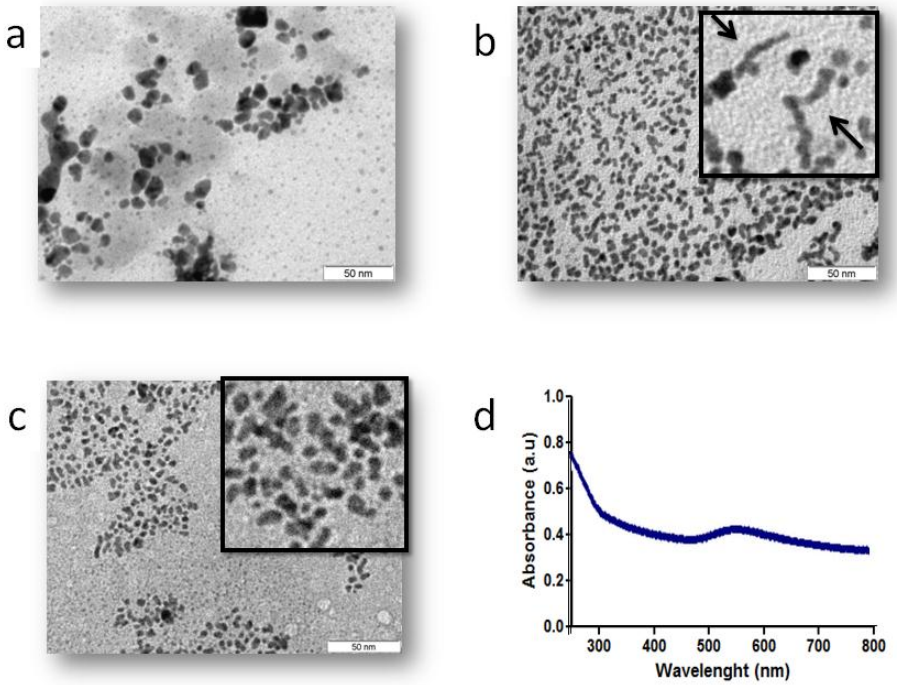

Figure 5: TEM images of the particles synthesized using direct synthesis of gold nanoparticles using glycerol in PBS after 24 hours of incubation: a) Glycerol-$HAuCl_4$ mixture in PBS (10 mM, pH 7.4) incubated at 25 ^oC without capping agent, b) Glycerol-$HAuCl_4$ mixture in PBS (10 mM, pH 7.4) incubated at 25 ^oC without capping agent after an instant pH jump (arrows are showing the particle chains), and c) Glycerol-$HAuCl_4$ mixture in PBS (10 mM, pH 7.4) incubated at 25 ^oC at constant concentration of capping agent after an instant pH jump. Insets are magnified images of corresponding particles. d) UV-Vis spectra of the sample (c), band observed at 546 nm.

3.2 Liposomal nanoreactor design

As an alternative to the well-established technique of the reverse micelle method for the preparation of nanoparticles, we proposed the use of liposomes as a nanoreactor for nanoparticle synthesis. It was expected that in the presence of liposomes, the nano-environment of the interior core, would provide a semi-solid reaction environment by keeping glycerol semi-mobile, thus facilitating the formation of nanoparticles in a more controlled manner than their synthesis in solution.

In the work reported here, we incorporated glycerol into the nanoliposome formulation. Glycerol is commonly used in liposomal formulations as it increases the solubility of lipids and encapsulated materials in water, as well as enhancing the stability of formed liposomes via interaction with the polar

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head groups of the phospholipids. [29] Furthermore, glycerol can be used to reduce chloroauric acid to form gold nanoparticles. There are many reports on polyol-mediated synthesis of metal nanoparticles, but not much information is available on the underlying mechanism. Leiva et al. postulated that although the exact mechanism is not fully understood, that the gold reduction reaction in the presence of alcohols most likely occurs due to the –OH groups of the reducing agent. [30] Like all other redox reactions, the reduction of the metal is driven by the difference between the redox potentials (ΔE) of the oxidation capacity of the metal salt and the reductivity of the polyol.

We thus proposed the preparation of nanoliposomes encapsulating chloroauric acid, whilst also incorporating glycerol in the lipid bilayer as a reducing agent, thus producing gold nanoparticles both in the lipid bilayer and in the liposome core, subsequently released by centrifugation in alcohol, to form uniformly sized, non-aggregating gold nanoparticles.

In the designed nanoreactor, hydrophilic Au (III) would expect to be encapsulated in the aquatic core of the membrane whilst glycerol would be located on the internal and external surface of the liposomes as well as within the aquatic core providing nucleation sites for the gold nanoparticle formation (Figure 1).

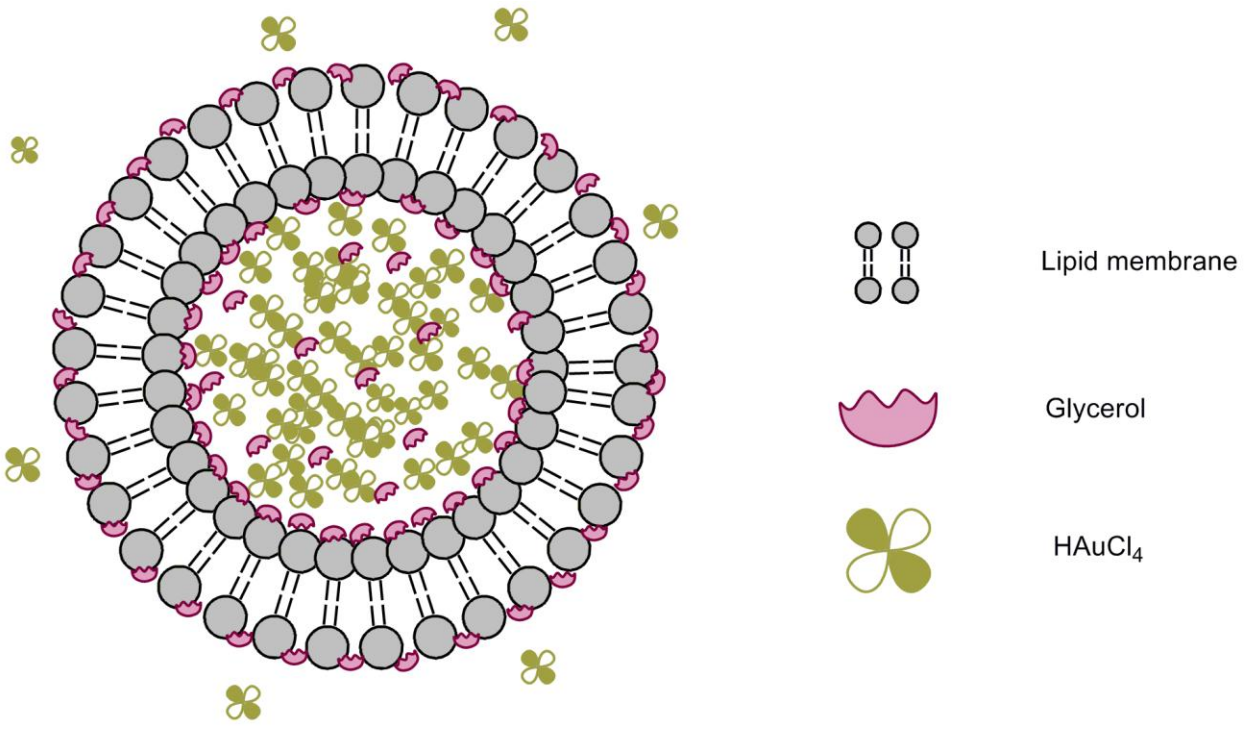

Figure 1: Representation of designed liposomal nanoreactor system.

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Liposomal nanoreactors of 24 ± 1 nm radius, as measured by photon correlation spectroscopy (PCS) were prepared using a formulation of 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC) in 88:12 molar ratio via our previously reported curvature tuned preparation (CTP) method,[18] at 25 ^{o}C in the presence of a constant concentration of $HAuCl_4$ and at varying concentrations of glycerol (0-15 % (v/v)). Figure 2 depicts the TEM images showing the stages of the reduction reaction in the presence of glycerol incorporated liposomal nanoreactors before reduction and during the reaction with a clear appearance of nanoparticles within the nanoreactor as well as the homogenous nanoparticle population in toluene observed following the elimination of lipidic membrane by centrifugation with methanol/ethanol mixture (1:4 v/v. The particle formation occurs mostly throughout the liposome membrane where the glycerol molecules are less immobile, as well as inside the liposome core, as was expected. No particle formation was observed in control experiments carried out with $HAuCl_4$ encapsulating liposomes in the absence of glycerol under the same conditions studied.

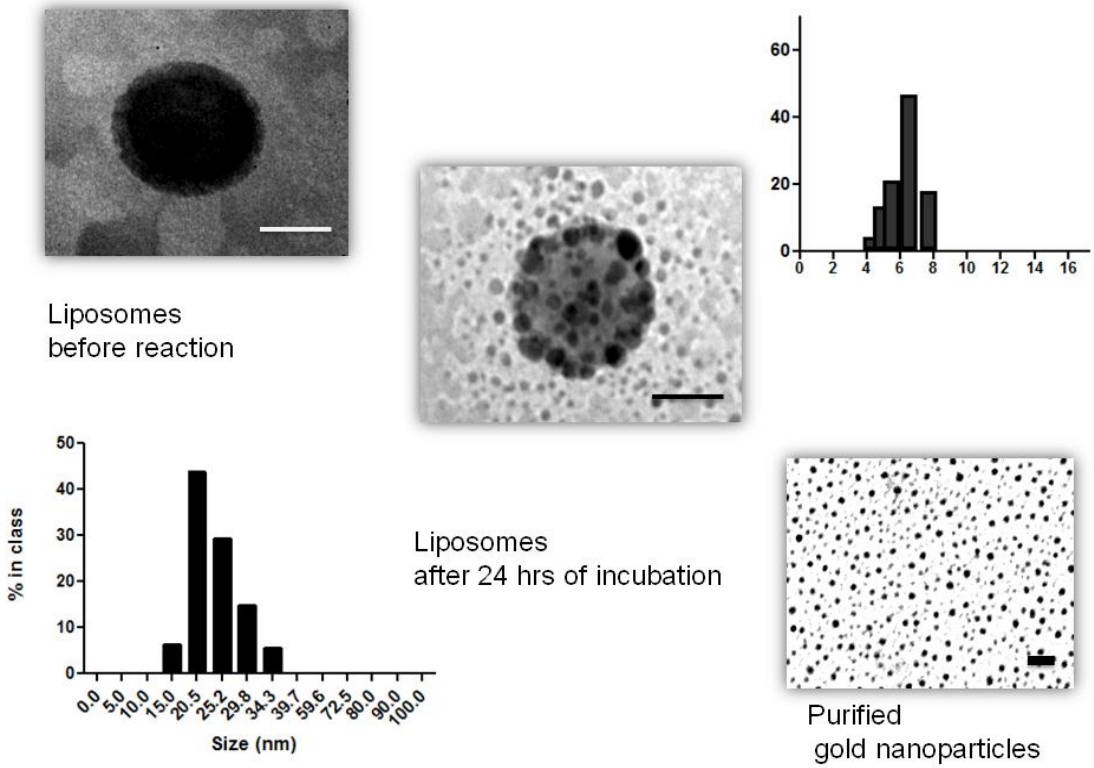

Figure 2: Formation stages of gold nanoparticles inside the glycerol incorporated (15% v/v) liposomes: Liposomes before reaction and liposomes during the reaction (scale bars = 10 nm) and purified gold nanoparticle synthesized in the nanoreactor (scale bar 20 nm). Photo correlation spectroscopy graph of

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liposome size distribution before the reaction, PI= 0.232 (bottom left corner) and graph of calculated nanoparticle size distribution using iTEM (top right corner).

3.3 Evaluation of the effect of reducing agent concentration and capping agent.

In previous reports, 3% v/v of glycerol was found to be optimum for the long-term storage of liposomes.[26,29] However, the reducing agent concentration is an important parameter for a well-defined method of metal nanoparticle synthesis and to this end the effect of glycerol concentration in a range from 3-15 % v/v was studied. At higher concentrations of glycerol no liposomes were formed. In addition to glycerol concentration, the influence of the presence of a capping agent was also evaluated using a short chain alkanethiol, 6-mercapto-1-hexanol (MCH) as a model, which was encapsulated in liposomes with $HAuCl_4$ in an excess of 1:50 $HAuCl_4$/MCH molar ratio. The formed liposomes were incubated in sealed glass bottles at room temperature for 24 hours under stirring conditions. In the absence of MCH, the solution colour changed from pale yellow to green-brown after the pH jump, with the intensity proportional to the glycerol concentration, which over time deepened to a very dark brown (Figure 2-1st line) indicative of oxidation by-products of glycerol formed due to the reaction between Au (III) and glycerol. As can be seen in Table 1, no significant changes in size were observed (from 7.7 ± 1.7 nm to 6.4 ± 1.3 nm) with increasing glycerol concentrations of 3% and 15 % v/V, respectively.

Tabel 1: Particle size after 24 hours of incubation at different glycerol concentration in the presence and absence of capping agent at 25 ºC Standard deviations were calculated from the mean of the data of a series of experiments (n≥3)

Glycerol concentration v/v	Absence of capping agent (nm)	Presence of capping agent (nm)
15 %	6.4±1.3	2.9±0.2
10 %	7.3±1.5	3.5±0.3
3 %	7.7±1.7	4.9±1.4

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As summarized in Table 1, the presence of the MCH capping agent encapsulated in the liposomes together with the $HAuCl_4$ lead to a sharp decrease in particle size, with particles of 2.9 nm to 4.9 nm obtained using MCH with glycerol concentrations of 15 % v/v and 3 % v/v, respectively. Similar correlations between the particle size and the stabilizer and/or reducing agent have been reported elsewhere.[33] In a recent study on the use of poly(e-caprolactone)/poly(N-vinyl-2-pyrrolydone) triblock copolymer as stabiliser and reducing agent for the AuNPs, a decrease in the particle size with increased ratio of copolymer to gold salt was reported.[31] In another study on the effect of Au/thiol ratio by Frenkel et al. (2005) where they used x-ray absorption fine-structure EXAFS spectroscopy technique, concluding that the mean cluster size strongly depends on the Au/thiol ratio, with the lower the Au/thiol ratio, the smaller the nanoparticles formed. [33]

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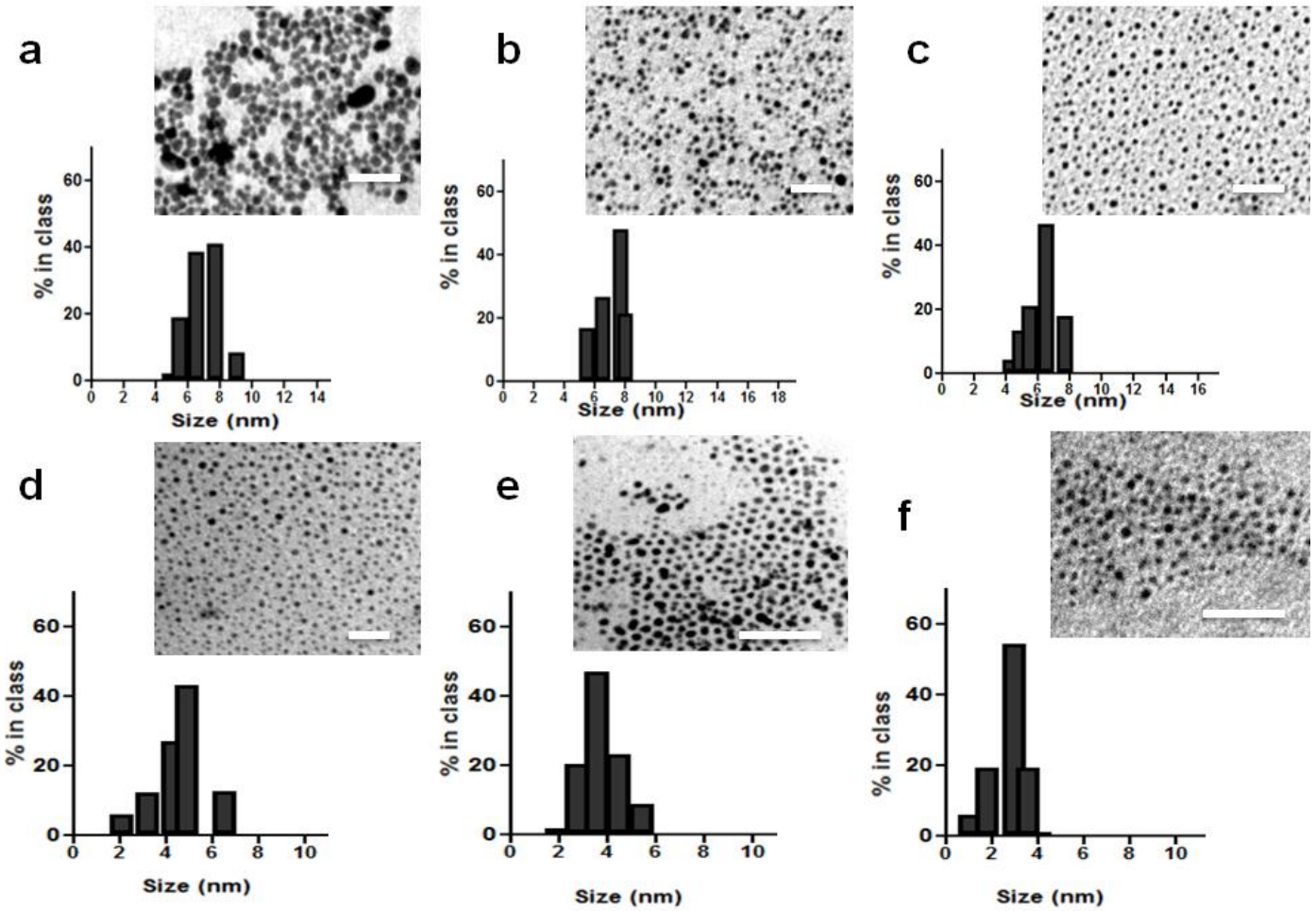

Figure 3: Effect of capping agent and glycerol concentration: Calculated particle size distributioan and corresponding TEM images of particles prepared in the liposomes in the absence of capping agent (MCH) (a-c) and in the presence of MCH (d-f) using changing concentrations of glycerol : a and d) 3 %, b and e) 10 %, c and f) 15 % after 24 hours of incubation at 25 ^{o}C. Scale bars are 20 nm.

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3.4 Influence of Temperature

In addition to the reactant concentrations, reaction kinetics are also governed by temperature and pH.[30] Thus, polyol driven synthesis reactions are more efficient at high temperatures, although at optimized concentrations of metal salt, slow reduction might occur at low temperatures.[33] Therefore, it is crucial to study the effect of temperature on the particle properties since the parameters influencing the reaction will control the shape and the size of the particles formed. Here, we investigated the effect of temperature (in the range of 4-50 ^{o}C), on the particles formed. MCH/$HAuCl_4$ encapsulating liposome solutions (glycerol, 15 % v/v) were incubated for 24 hours under constant stirring conditions at a defined constant temperature. As shown in Figure 4, decreasing particle sizes were obtained with increasing temperature over the range of 4 - 50 ^{o}C, with a significantly larger nanoparticle size observed at 4 ^{o}C compared to the particles obtained at 50 ^{o}C (6.3 nm, 1.9 nm, respectively). As the temperature increases, the reaction rate increases as a result of rapid nucleation, and thus the amount of metal consumed for the nucleation increases, resulting in a decrease in the number of molecules available for the further growth of nanoparticles, resulting in smaller nanoparticles (Figure 4-e).[33,34]

Even in the presence of capping agent, solution based direct synthesis of the nanoparticles without liposomal nanoreactors did not provide the same quality of homogeneous and small sized spherical nanoparticle populations synthesized using designed liposomal nanoreactors under the same operation conditions, highlighting the potential of the method for the high-yield preparation of ultra small Au nanoparticles at mild temperatures with their size being tightly controlled by application of specific temperatures, and glycerol concentrations and the presence of capping agent.

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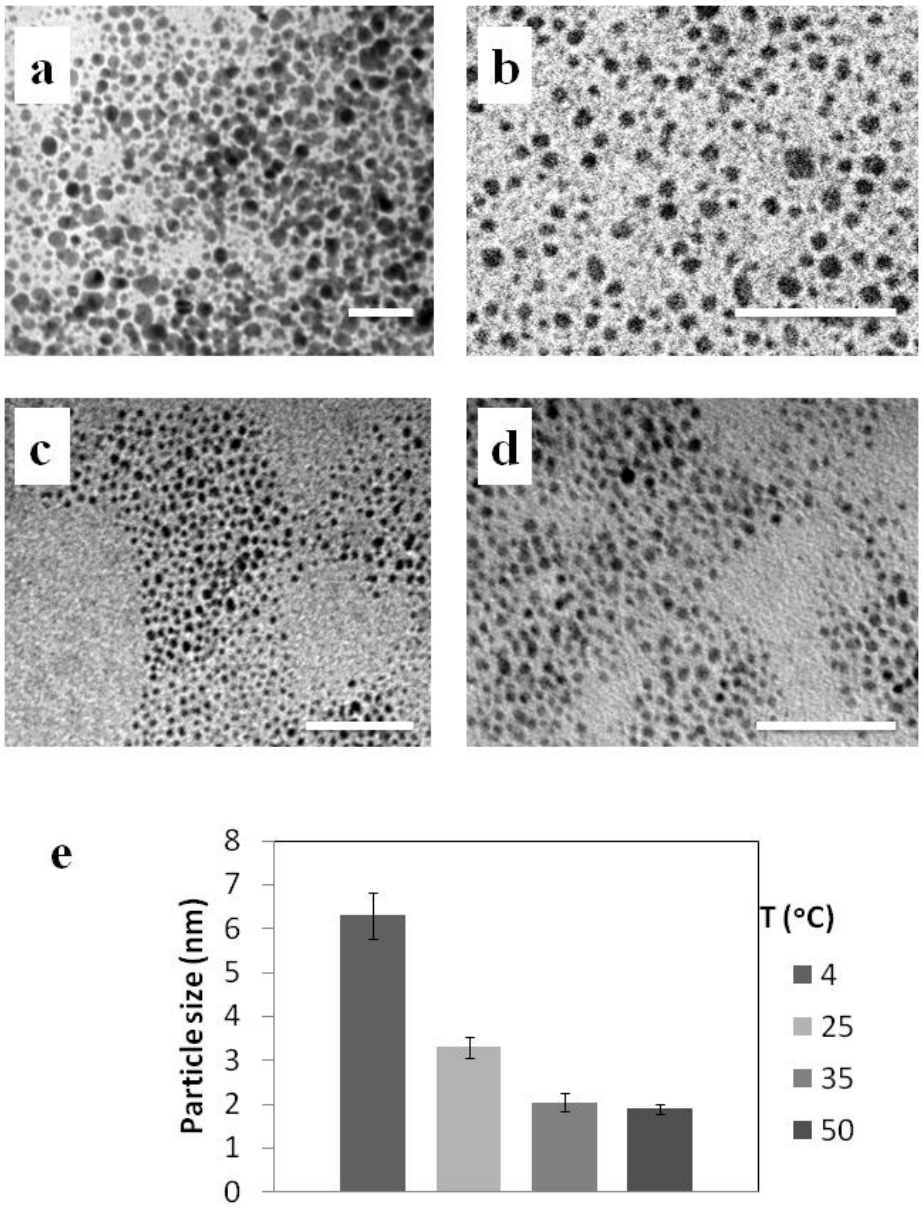

Figure 4: Effect of temperature on the particle size and shape synthesized in glycerol (15 % v/v) incorporated liposomes in the presence of capping agent MCH at changing temperatures: a) 4 ^{o}C, b) 25 ^{o}C, c) 35 ^{o}C, d) 50 ^{o}C after 24 hours of incubation, and e) graph presenting the calculated mean diameter of the particles, n≥3, scale bars = 20 nm.

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4 Conclusions

In this report, a known polyol, glycerol, was studied as a green catalyst for the reduction of gold to assemble in nanoparticles without the use of any harsh chemicals. Nanosized liposomes were used as functional nanoreactors exploiting glycerol incorporated in the external and internal surface of the lipid bilayer, maintaining the reducing agent semi-mobile in their nanoenviroment. Reaction parameters such as temperature, glycerol concentration, and the effect of capping agent were studied in terms of their effect on size and the homogeneity of nanoparticles formed. Increased concentrations of glycerol resulted in a decreased size of the nanoparticles and, furthermore, nanoparticles synthesized in the presence of capping agent showed almost a 2 fold decrease in the particle size leading to ultrasmall gold nanoparticles of around 2 nm. Moreover, a decrease in the nanoparticle size at constant concentrations of capping agent and glycerol was observed when the temperature was increased in the range of 4 ^{o}C to 50^{o}C. Comparison studies of gold nanoparticle synthesis in solution under the same conditions without the use of the nanoliposome reactors, resulted in highly heterogeneous nanoparticles with amorphous shape. These results indicate that with the designed liposomal nanoreactors, with glycerol integrated in the membrane as a reducing agent, a one-pot synthesis of highly homogenous nanoparticles was successfully achieved as a result of semi-solid reaction environment provided by the liposome.

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Template directed synthesis of gold nanoparticles from lipid superstructures

Rukan Genç, Gael Clergeaul, Mayreli Ortiz [*], Ciara O'Sullivan [*]

[1]Nanobiotechnology and Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Av. Països Catalans, 26, 43007, Tarragona, Spain

[2]Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23,

08010 Barcelona, Spain.

Abstract

Different shaped lipidic superstructures were demonstrated as templates for the inorganic synthesis of gold nanoparticles where their shape was directly related to the template geometry. Zwitterionic, 2 -dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) together with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC) lipid mixtures resulted in square and hexagonal shaped lipid disks, which were used as templates encapsulating chloroauric acid and sodium citrate to synthesize gold nanoparticles with the same shape as the template used. In the case of twisted ribbon lipid structures formed from the negatively charged lipid, DMPG, the lipid template encapsulating chloroauric acid served for the synthesis of a ribbon shaped one-dimensional organization of gold nanoparticles in the absence of a reducing agent, while further studies on different administration routes of reducing agent, citrate, and chloroauric acid resulted in different organizations of gold nanoparticles aligned and guided by the template structure. The obtained gold nano-structures of different shapes and sizes have great potential in catalysis and plasmonics

1. Introduction

As one of the building blocks of living-cell membrane, lipids and their interaction with neighboring lipids and other molecules, as well as their ability to form different kinds of structures have garnered immense interest.[1] There are several structural factors and environmental conditions that affect the self-assembly of lipids into nano-sized structures, influencing the phase behavior and the curvature of the lipid. Lipid superstructures have been studied intensively over the last 30 years[2] and the interest in nanostructured materials formed by self-organisation has continuously increased. [3] Vesicles, or liposomes, are the most known lipid based structures, however, lipids are also known to be able to self-assemble into several other structures, such as lipid tubes and rods, [4] lipid ribbons, [5] hexasomes [6] as well as cubosomes [7]

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and non-lamellar mesophase lipid aggregations.[8] As well as being used as model systems to understand cell membrane nature,[9] these nano- and micro-structures are attractive as substrates for protein crystallization,[10] and as templates for the synthesis of one-dimensional inorganic materials,[11] as well as vectors for drug delivery.[12]

In parallel, there is enormous interest in the development of methods for the preparation of metallic nanoparticles of diverse sizes and shapes, particularly for application in catalysis, where structures such as cubes, [13] disks, [14] tubes, [15] stars, [16] and nanocages [17] which provide crystal plane architectures that can be tailor-designed according to the specific catalytic application.[1] Spherical nanoparticles are mainly synthesized by wet-state preparation methods such as the Turkewitch method (1951)[18] or the Schmid method (1981) [19] which is based on reduction/oxidation reactions. However, further control of the one and two-dimensional shapes of nanoparticles is still largely unaddressed. The seed mediated synthesis of rod-like structures exploiting the use of a growth-directing agent,[20] as well as vapor-phase synthesis, [21] vapor-solid-liquid synthesis methods, [22] patterning on a solid surface by etching or lithography methods [23] are some of the synthetic methods reported to achieve particle growth in tailor-designed directions.[24] However, those synthetic methods are limited due to the inherent complexity of the reactions and requirement for high-tech laboratory conditions. To this end, template directed synthesis using self-assembled structures have introduced new possibilities, not only due to the relative inexpensiveness of the technique and its simplicity and inherent applicability to scale-up, as well as the unlimited combinations lipids to form diverse template structures that could be used as pattern for the growth of a plethora of different nanoparticle morphologies.

A highly reported method that has been used for the preparation of nanoparticles has been the so-called reverse micelle method. In this method, the inner core of the reverse micelles is considered as a nanoreactor,[25] within which controlled reactions leading to the formation of nanosized metallic and metal halide particles are carried out, [26] where the size of the micelle core is controlled by the molar ratio of water to surfactant/lipid molecules in solution. Typically, individual reverse micelle populations are prepared containing metallic salts e.g. $HAuCl_4$ and reducing agent (e.g. sodium citrate), respectively. When these reverse micelles are mixed in solution, they exchange the contents of their cores via fusion and re-dispersion processes.[27] As a result the reduction of a metal salt within the cores of the reverse micelle results in the growth of metallic nanoparticles within the core of the micelle. The exchange process occurs when the micelles collide due to Brownian motion as well as the attractive forces between the micelles, resulting in a fusion of the reverse micelles, an exchange of the contents within the cores, and a re-dispersion of the micelles. [28] This method has found widespread application and has been used, for example, for the synthesis of semi-conductor materials,[29] metallic nanoparticles[30] and

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nanoalloys.[31,32] However, to date this method has been used for the preparation of spherical nanoparticles and to the best of our knowledge has not been exploited to produce nanomaterials of tailor-designed shapes.

Recently, we reported on an ultra-rapid and environmentally friendly method for the preparation of highly stable liposomes using both charged and zwitterionic lipids with different critical melting temperature (T_M), exploiting a combination of a rapid pH change followed by a defined period of equilibration, called curvature-tuned liposome preparation method, resulting in monodisperse and stable liposome populations.[33] In the work reported here, we exploit this method for the formation of several lipidic superstructures using different formulations of phospholipids of diverse tail length, head groups, surface charges, and T_M. These parameters affect the effective shape and thermal-phase behavior of the lipid, which, in turn, influence the curvature of the lipidic bilayer and subsequently leads to self-assembly of the lipids into structures different from liposomes, such as twisted ribbons, planar bilayers as well as square and hexagonal shaped lipidic disks. We proposed to use these diverse lipid shapes as templating nanoreactors, modeled on the reverse micelle method, for the production of metallic nanoparticles of controlled size and shape. The prepared lipid superstructures encapsulating metallic salt and reducing agent, respectively, were mixed, with the size and shape of the resulting nanoparticle dictated by the lipid templates. Analysis of the lipid superstructures and the metallic nanostructures formed was carried out using transmission electron microscope (TEM), cryo-TEM and confocal microscopy, which clearly demonstrated the feasibility of the approach for the preparation of ribbon, hexagonal and cubic metallic nanostructures.

2. Experimental section

2.1 Materials

Phospholipids were supplied as a powder by Avanti Polar Lipids, Inc. and used without further purification. Sodium hydroxide, hydrochloric acid, di-sodium hydrogen phosphate (anhydrous, reagent grade), Na_2HPO_4), sodium dihydrogen phosphate (anhydrous), extra pure, (NaH_2PO_4) and glycerol 99.5%, reagent grade, were purchased from Scharlau Chemie SA. Sodium chloride was provided by Riedel-de Haën. Milli-Q water (1.82 MΩ.cm-1) used to prepare buffers and liposomes were obtained using a Simplicity 185 Millipore-Water System.

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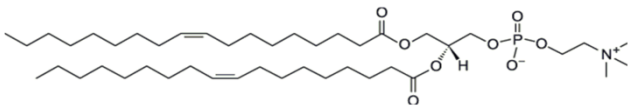
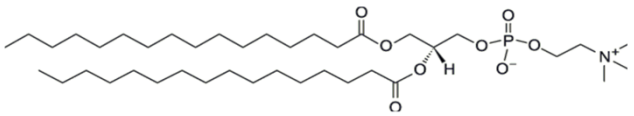
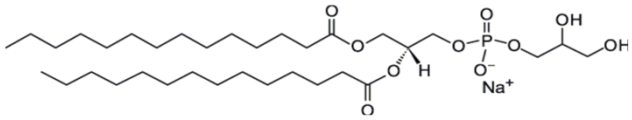


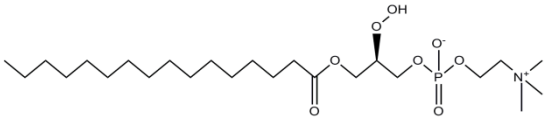
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)



1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)



1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG)



1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC)

Scheme 1: Structures of the phospholipids used in this work.

2.2 Curvature tuned preparation method [33]

Different lipid mixtures (50 mg) were directly hydrated in 4 mL of buffer (0.1 M PBS, pH 7.4), which had previously been heated to a pre-determined temperature, T_O. The temperature was kept constant by placing a glass flask (15 mL) in a water jacket connected to an UltraTerm 200 Model (P-Selecta) thermocycler. The mixture was vortexed in a 10 mL falcon tube (with glass beads) for 1-3 min and added to 6 mL of the buffer solution (pH 7.4) including either $HAuCl_4$ (20 mg) or sodium citrate (115 mg) and was left to stir for 15 min while the temperature was kept constant at T_O. The pH was then subsequently increased to a maximum (pH 11) using NaOH and immediately re-adjusted to pH 7.4 using HCl. The resulting mixture was left to mix for a 25 min equilibration period under the same conditions. Finally, stirring and heating was stopped, and the solution was left to cool to room temperature for 25 min, and, subsequently, samples were stored at 4 ºC before use. All steps were conducted under Argon. Unless otherwise described, all lipid formulations consisted of phospholipid and lyso-PPC (88:12 molar ratio). The final lipid mass concentration was kept constant for all lipid formulations at 0.5% (w/v).

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2.3 Gold nanostructure synthesis patterned by lipid structures

In the case of hexagonal and square shaped lipid structures as template, two populations of the template either encapsulating citrate or choroauric acid were mixed, or, as a control, choroauric acid encapsulating lipid structures were immersed in PBS. In the case of twisted ribbon shaped lipid templates, four features were studied. Chloroauric acid encapsulating lipid were immersed in PBS or citrate solution in PBS, or, alternatively were mixed with citrate encapsulating ribbons. In addition, citrate encapsulating lipid ribbons were immersed in chloroauric acid solution in PBS. Mixtures were incubated in room temperature and monitored by TEM over 72 hours. Daily, samples were taken and purified using centrifugation with methanol/ethanol solution (1:4 v/v) and kept in toluene at 4 ^{o}C until analyzed.

4.4 Characterization of structural changes and determination of size

Photon correlation Spectroscopy (PCS): The mean diameter of the liposomal structures were measured using the Zeta Sizer 3000H (laser He−Ne (633 nm), detector angle 90°) equipment from Malvern Instruments, Inc.. The presented standard deviations were calculated from the mean of the data of a series of experiments 56 ($n \geq 3$) carried out using the same parameters. A prepared dispersion of lipid structures was diluted at least three times with buffer.

***Negative- stain Transmission electron Microscopy (TEM) imaging via phosphotungstic acid hydrate*:** Using a glass pipette, a drop of sample was added to a 200 mesh copper grid with a thin film of Formvar polymer and kept at room temperature for 1 min, followed by addition of a drop of 2 % v/v? phosphotungstic acid hydrate (PA) (Panreac) solution (pH 7.2) in distillate water was dropped on grid and left in touch with the sample for 2 min. Subsequently, the PA was carefully dried using filter paper and the sample was left at room temperature until a dried film was obtained. Transmission Electron Microscopy (TEM) analyses were performed using a JEOL 1011 transmission electron microscope operated at 80 keV with an ultra-high-resolution pole piece providing a point resolution of 2 A°. Micrographs (1024 pixels x 1024 pixels) were acquired using a Megaview III multiscan-CCD camera. Images were analyzed with an iTEM image analysis platform by measuring the dimensions of particles from the photos captured from different parts of the grid and calculating the mean diameter from the series of experiments (n±3) conducted using the same parameters.

Scanning Electron Microscope (SEM): Scanning electron microscope studies were carried out using Scanning electron microscope, SEM (Jeol JSM 6400, 40 kV). Spectrometric measurements were performed by Spectrophotometer UV-Vis-NIR, Cary 500- Varian.

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3 Results and Discussion

3.1 Formation of lipid superstructures

There are several structural factors that determine the final structure of lipid self-assembly. The packing factor, P, is fundamental in determining the effective shape of the lipids and their organization.[34] The value of P (Equation 1) is directly proportional to the effective volume occupied by a single lipid molecule (*V*) and inversely proportional to the length of the lipid tail (*l*) and head group size (*a*).

$$P = V / l\, a \qquad \text{(Eq.1)}$$

It has been found that the three-dimensional organization of a lipid is influenced by the value of P. Hence, lipids form vesicles at a value of P between 0.5-1, while other structures are formed at P values below and above that interval (Figure 1).[34]

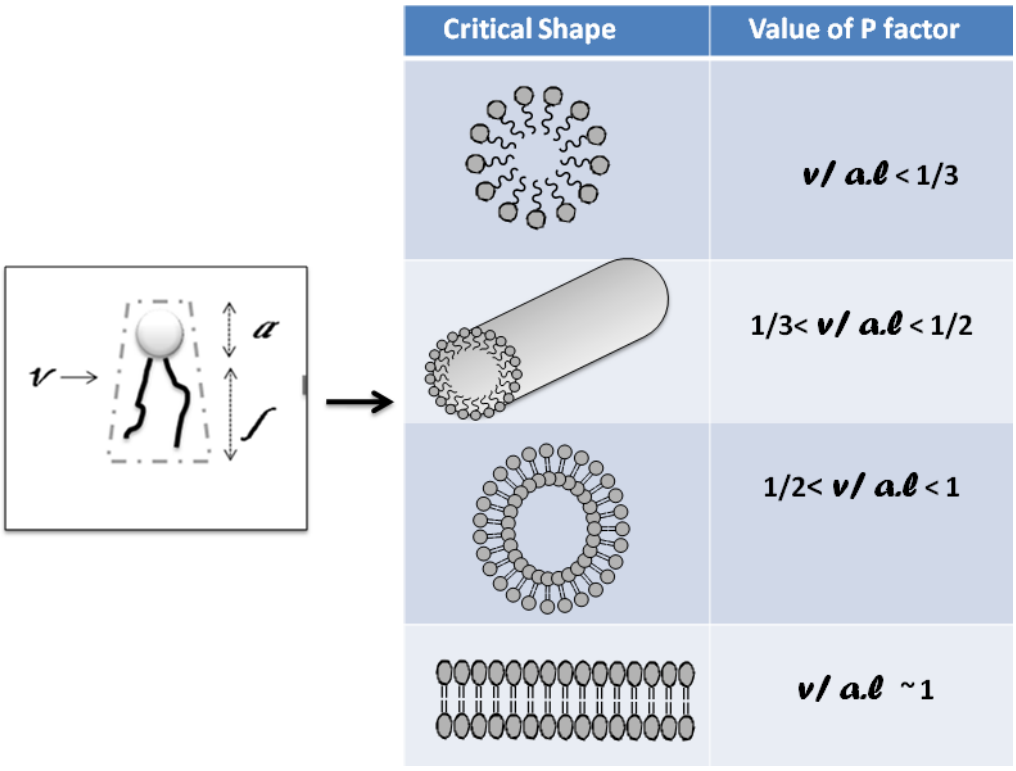

Figure 1: The value of critical packing factor, P, and corresponding basic lipid aggregate shapes.

Another important lipid property is the critical melting temperature (T_M), which is the temperature at which lipids change phase from gel to liquid crystalline. The T_M is indicative of the thermal behavior of the lipids as well as their physical characteristics, such as lipid tail length, head group size and the effective volume occupied by a single lipid, at changing thermal

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conditions.[34] To this end, the effect of temperature on the formation of diverse lipid structures was studied.

3.1.2 Evaluation of the effect of head group type

Lipids with the same tail length but different head groups (DPPC vs. DPPG and DOPG, DOTAP *vs*. DOPC) were used to evaluate the effect of head group size (***a)*** on the lipid phase behavior and organization at different temperatures ranging from 25 - 65 ^{o}C. The head group had a definitive effect on the shape of the lipid aggregates formed, due to the differences in the electrical charge density, which affected the effective size of the lipid head (***a***) of charged lipids, as well as influencing the attractive-repulsive forces between two lipid polar heads. When ***a*** or head group orientation is changed by external forces, a change in the lipid organization is expected.[34] This was clearly demonstrated via the formation of spherical lipid vesicles from long tailed charged lipids (DPPG, DOPG, DOTAP) in all the conditions studied[33] and the final structure obtained with the zwitterionic lipids (DOPC and DPPC) where a strong temperature dependence on structure formation was observed (Figure 2).

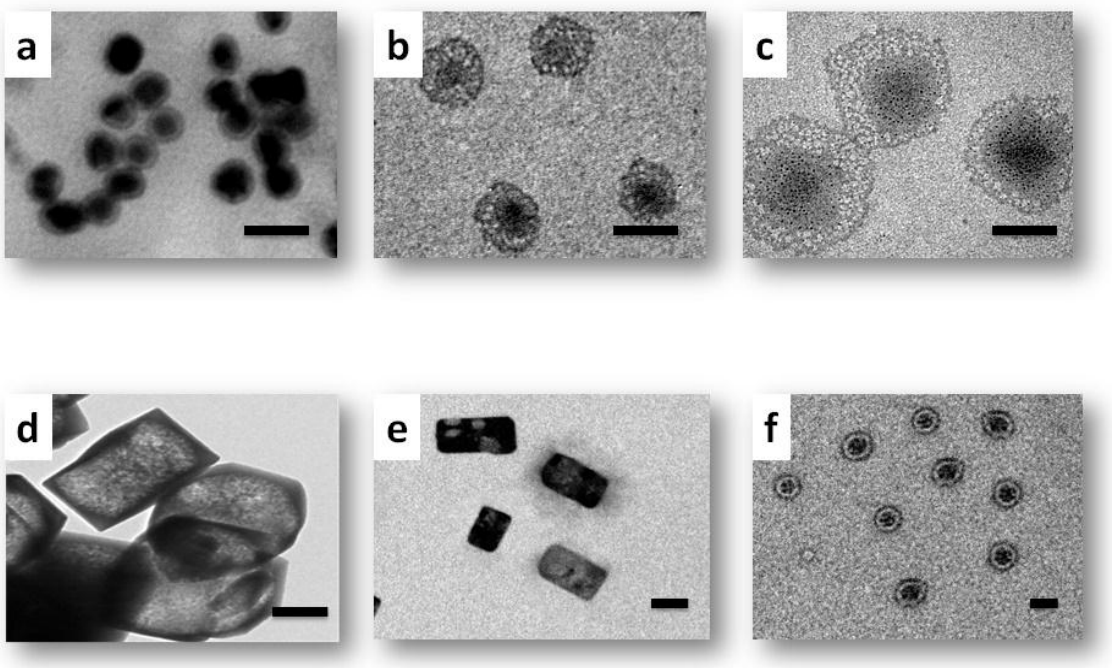

Figure 2. TEM images of lipid organization of zwitterionic lipids DOPC (first line) and DPPC (second line) prepared at various temperatures: 25 ^{o}C (a, d), 45 ^{o}C (b, e) and 65 ^{o}C (c, f), respectively. Scale bars are 50 nm.

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3.1.2.1. Zwitterionic lipids

Although lipid vesicles (20 ± 1nm) were obtained with a DOPC (T_M= -20°C):lyso-PPC (88:12 mol/mol) based lipid formulation at T_0 =25 °C (Figure 2a), a change in the equilibration time, T_0, from 25 °C to 45 °C resulted in melted lipid particles with an increased density in their core (Figure 2b). According to Barauskas et al.[8] , the formation of these non-lamellar meso-structures occurs under specific thermally enforced conditions where lipids are in an internal phase, termed the *sponge phase (L_3).* The phase transfer of DOPC, from L_α (liquid crystalline phase), where lipids can form bilayers, to L_3, occurs at temperatures between 25 °C and 45 °C, and the resulting melted lipids organize into sponge-like structures (Figure 2 b). At 65 °C, an increase in size was observed (Figure 2 c), due to an enhanced fluidity of the melted lipid tails, leading to an increasing distance between lipid molecules and a consequent increase of the effective lipid volume (***V***) at a temperature markedly above its T_M. [34]

In the case of the zwitterionic DPPC (T_M = 41 °C) with lyso-PPC (88:12 mol/mol), a strong temperature-dependent formation of different aggregate structures was observed. At 25 °C, sub-micron sized hexagonal disks and rectangular shaped lipid structures were obtained (Figure 2-d), whilst at 45 °C nano-sized lipidic rectangles were observed (Figure 2-e). At a temperature of 65 °C, which is higher than the T_M of the DPPC , monodisperse liposomal structures were obtained with a diameter of 69 ± 7 nm (Figure 1f). It is evident that at this temperature the lipid bilayer is in L_α and has enough curvature to form vesicles, whilst at temperatures below and around its T_M, it is in its gel crystalline phase and forms disk shaped structures. In agreement with our observations, Wang et al., postulated that this intermediate-phase-driven disordered lipid organization may transform the lipids into closed vesicles at higher temperatures, which in our case are obtained at an increased T_0 of 65 °C.[36]

From the point of view of the packing parameter and attractive-repulsive forces between the lipid polar head groups, studies reported by Mbamala and Fahr[35]on the electrostatic model of mixed cationic and zwitterionic lipid membranes demonstrated that in membranes formed of zwitterionic lipids, lipids orient themselves parallel to the membrane interface (neutral pH) and a single dipole of the lipid with a moving positive charge and fixed negative charge (Figure 3-a) tilts at an angle (θ) which is dependent on the changes in attractive-repulsive forces, thus defining the area per lipid molecule **(a)** (Figure 3-b).[35]

In the case of CTP method which involves an instant deprotonation/protonation of lipids moities (pH jump) for charged lipids, phase changes due to temperature changes from gel to liquid crystal, and the orientation of the head groups based on the repulsion effect (because their head has the same charge) contribute in the same direction for the formation of spherical

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structures. However, for zwitterionic lipids (which contain both positive and negative centers) the system is more complicated. They assemble in different lipid structures rather than liposomes, because, in addition to those effects, the orientation of the lipid dipole head can change deppending on their interaction with neighboring lipids, in a different way to the charged lipids, due to the pH jump (Figure 3).[37]

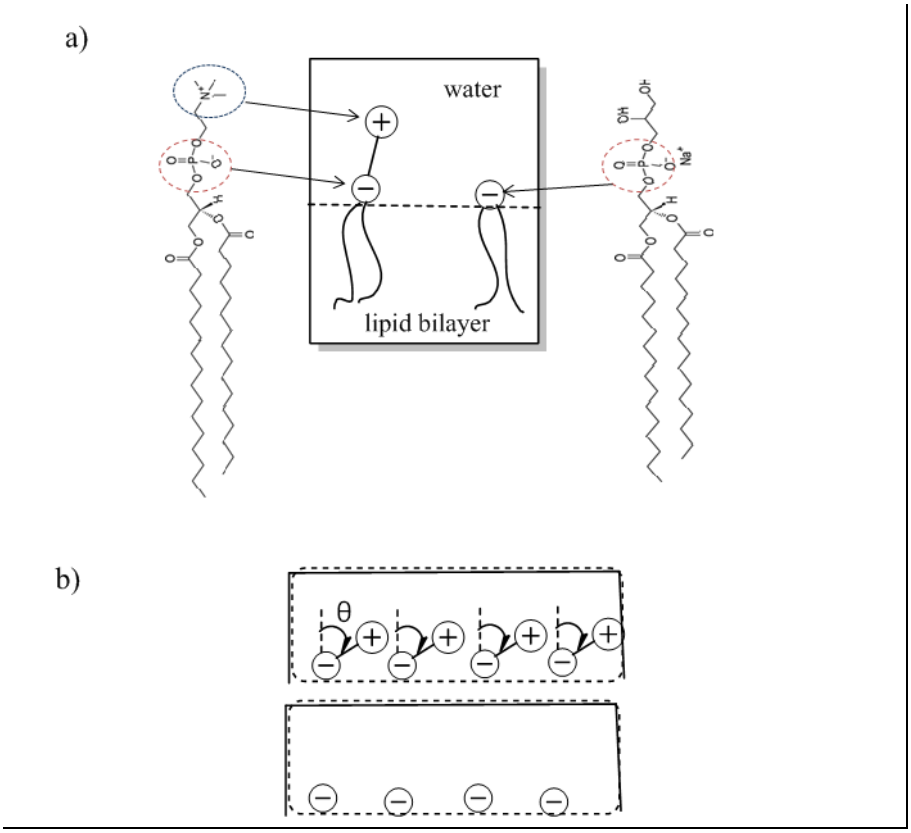

Figure 3: Scheme showing a) the zwitterionic and charged lipids in lipid membrane where zwitterionic lipid (in this case DPPC) has a single dipole with a fixed negative charge and mobile positive charge where it orientates with an angle (θ) and cannot penetrate into the bilayer. On the other hand charged lipids (DPPG) have a single charge which is immobile, b) parallelly oriented zwitterionic lipids to the membrane interface at neutral pH due to attractive-repulsive forces (top) and orientation of charged lipids (bottom). [modified from reference [35]].

3.1.3 Lipid tail length

The effect of lipid tail length (***l***), and thus of the lipid packing factor, on aggregate shape and the lipid superstructure obtained, was also evaluated. For this purpose, phospholipids with a phosphatidylglycerol (*-PG) head group of different tail lengths (14C, 16C, and 18C) were examined. Lipid formulations of DOPG:lyso-PPC (88:12 mol/mol) and DPPG:lyso-PPC (88:12 mol/mol) formed nano-sized liposomes at all temperatures studied, with a temperature-dependent size increase in liposomes prepared from DOPG.[33]

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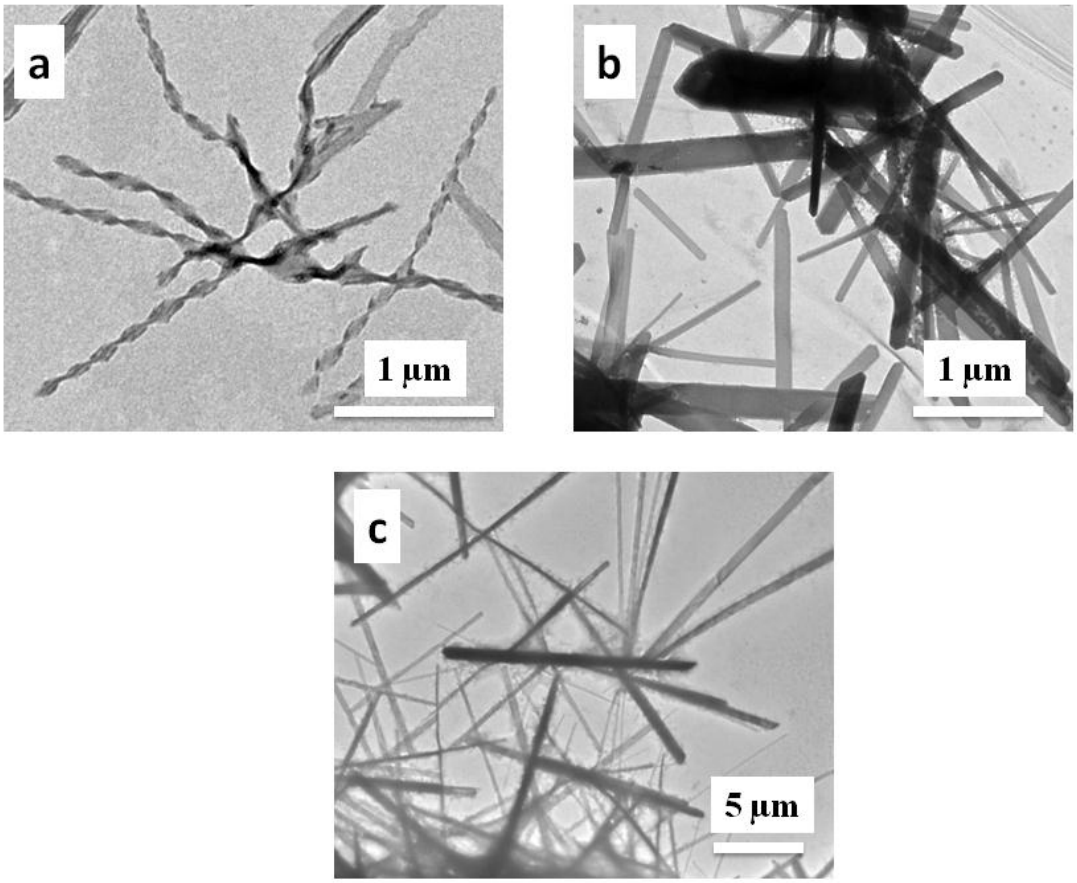

Figure 4: Negative stained TEM images of twisted ribbons prepared at 25 ^{o}C, nanostrips at 45 ^{o}C and nanotubes prepared at 65 ^{o}C from negatively charged short tail phospholipid, DMPG via CTP method.

Interesting superstructures were obtained with the lipid formulation of the short tailed, negatively charged DMPG (14C, T_M = 23 ºC):lyso-PPC (88:12 mol/mol), which formed twisted ribbons of ~100-150 nm width, ~200 nm pitch and several microns in length, at T_0=25 ºC (Figure 4-a). When the T_0 was increased to 45 ºC, nano-sized planar strips of 200 nm in width and of several microns in length (Figure 4-b) were observed, and at 65 ºC, (Figure 4-c) tubes with a width of approximately 1 µm were obtained. Oda et al also reported that short tailed amphiphiles have a higher tendency to form twisted ribbons than long tailed ones. In a more recent study of Spector et al., they reported that the formation of these kinds of lipid assemblies is related to the molecular packing of the two lipids and their chirality, which force the lipid molecules to pack into membranes tilted at a small angle where planar bilayers form twisted ribbons and helices, [9] which close into tubes.[38]

Comparing the lipids of the same type of head group, taking Eq.1 into account and assuming that the P value of the lipid bilayers of DOPG and DPPG is in the range of 0.5-1, DMPG with shorter lipid tail (*l*) can be assumed to have a value of P closer to 1, the value where lipid bilayers curl into planar bilayers with zero curvature rather than liposomes (positive curvature).

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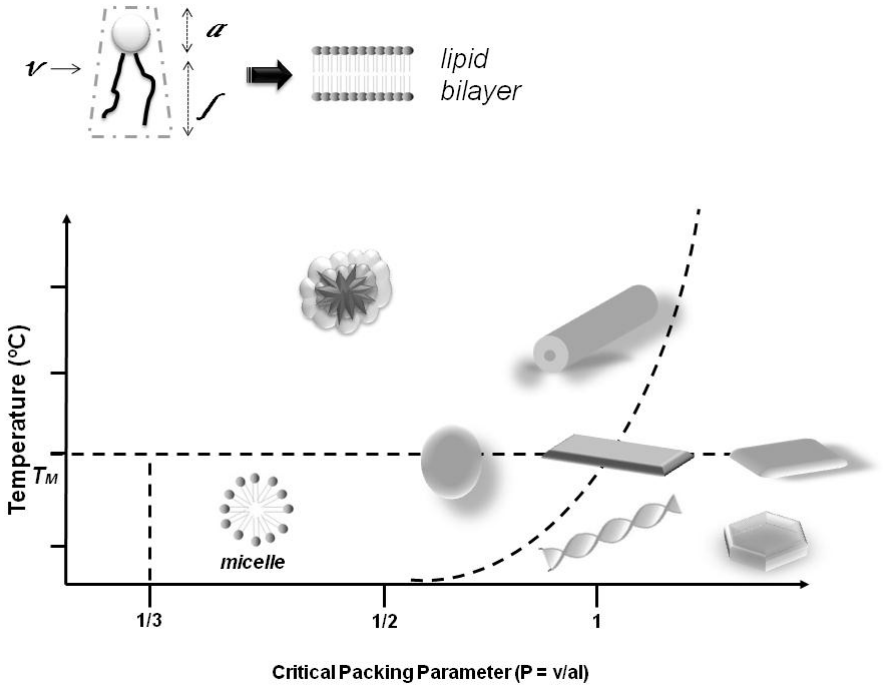
Furthermore, due to the melting temperature dependent phase transmission of the lipid bilayer, the planar structures evolve into twisted ribbons at lower temperatures or tubes at increased temperatures high enough to obtain cylindrical curvature (Figure 4 and 5).



Scheme 2: Cartooned relation between the packing factor, lipid melting temperature and corresponding lipid structures from DPPC, DOPC and DOPG as a result of the circumstances of the CTP method.

The dependence of the vesicle shape on the packing factor at different temperatures is depicted in scheme 2. In conclusion, exploiting our curvature-tuned method of liposome preparation, we can control diverse lipid superstructure organizations by tuning the temperature to the appropriate value determined by nature of the lipids used, resulting in a wide range of diverse lipidic shapes and sizes.

3.2 Lipid Structures: Templates for the shape controlled gold nanoparticle synthesis

The lipidic nanostructures, hexagonal and square shaped lipid disks prepared from DPPC and lyso-PPC at different temperatures (25 ^{o}C and 45 ^{o}C, respectively), as well as DMPG based twisted ribbons prepared at 25 ^{o}C, are not only interesting as self-assembled lipid superstructures, but also for the possibility for their use as templating nanoreactors for the preparation of metallic nanostructures of controlled size and shape. To this end, lipid structures were prepared as described above, to form ribbons, cubes, hexagonal architectures as well as

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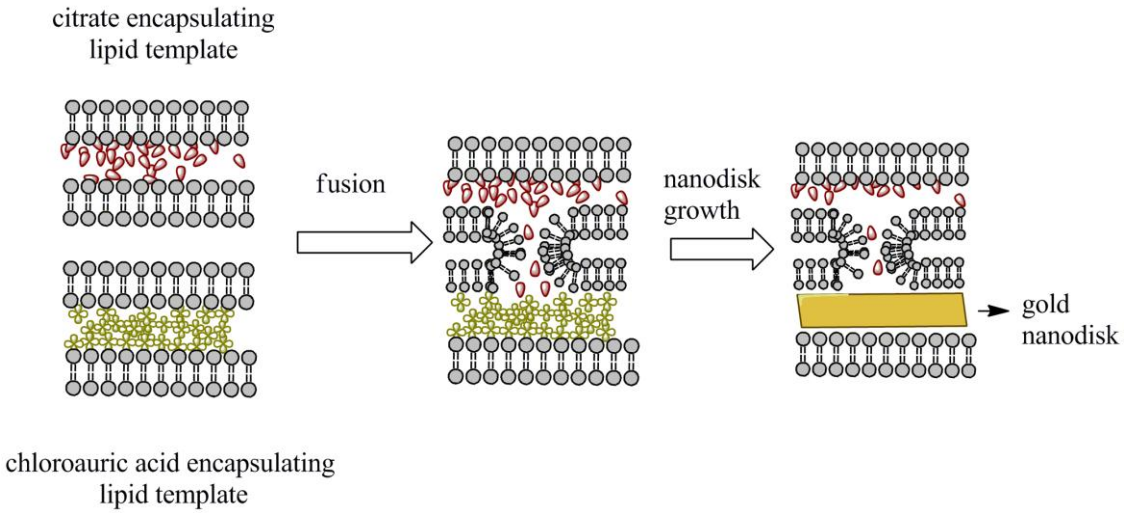
rods. In the case of the hexagonal, cubic and twisted ribbon-like structures, two lipid populations were prepared, encapsulating chloroauric acid and sodium citrate, respectively. Upon mixture of these two lipid populations, the structures fused, resulting in an exchange of contents and metallic nanostructure synthesis due to the reduction of the metallic salt by the citrate reducing agent. The fused lipid was then re-dispersed and the resulting encapsulated nanostructure was formed in the shape and size of the lipid interior core template, resulting in cubic, hexagonal and ribbon-like metallic nanostructures. In the case of the ribbon lipids, an alternate approach was also investigated, and the citrate reducing agent was added to a population of chloroauric acid encapsulating ribbon lipids, and diffused across the lipid membrane to the interior core. This core effectively acted as a nanoreactor, and reduction of the encapsulated chloroauric acid took place, resulting in a gold ribbon-shaped nanoparticles.

3.2.1 Mechanism of 2D gold nanodisk assembly by lipid bilayer fusion

There have been a plethora of reports detailing the reverse micelle method,[25-32] where a clear correlation between template size due to the ratio of water:surfactant/lipid ratio, and the resulting nanoparticles formed, has been established. Reverse micelles are dynamic lipid clusters where they continuously fuse and redisperse due to Brownian motion, exchanging their contents.[40] However, in the case of lipid bilayers, fusion is a more complex process where the membrane fluidity and hydrophobic interactions have been reported to be important factors for liposome membrane fusion, and is mostly an irreversible process. [41] Zwitterionic lipids fuse at temperatures below their critical melting temperature while fusion of the charged lipids occurs at higher temperatures. [42]



Scheme 3: 2D growth of nanodisks as a result of the membrane fusion of reactant encapsulating lipid structures.

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The proposed mechanism of the 2D growth of rigid particles with a shape patterned by single template due to fusion of the two lipid structures encapsulating $HAuCl_4$ and citrate, respectively, is depicted in scheme 3. With electrostatic interaction between $AuCl_4^-$ with a net negative charge and the zwitterionic –PC head with a fixed negative charge and mobile positive-charge provide nucleation sites for the synthesis. Subsequent to the fusion of two lipid structure, citrate molecules slowly diffuse into the bilayer and reduce the Au(III) to Au(0) resulting in the formation of solid gold hexagonal and rectangular disks. Hexagonals nanodisks mixed with rectangular ones with a size of around 250 nm and homogenous distributuion of rectangular nanosdisk around 200 nm length and 80 nm width were obtained using hexagonal and rectangular shaped lipid templates, respectively. Ribbon shaped template was lead to either solid ribbons or aligned spherical structures with several nm length depending on the method used

3.2.2 Rectangular shaped lipid disks as template

Rectangular-shaped lipid disks were prepared at 45 ^oC and used as templates. The prepared encapsulating lipids were purified from excess reagents using a Sephadex G-200 gel purification column. Nanoparticle synthesis was carried out by mixing $HAuCl_4$ encapsulating lipid disk templates with citrate encapsulating ones in a 1:1 ratio, and monitored over 72 hours. For control experiments, citrate was replaced by PBS. As can be seen in Figure 5-c, very homogenous population of shaped rectangular disk gold nanoparticles with a large area in the {111} crystal plane dimension were obtained using rectangular shaped lipid disks as a template (Figure 5-a).

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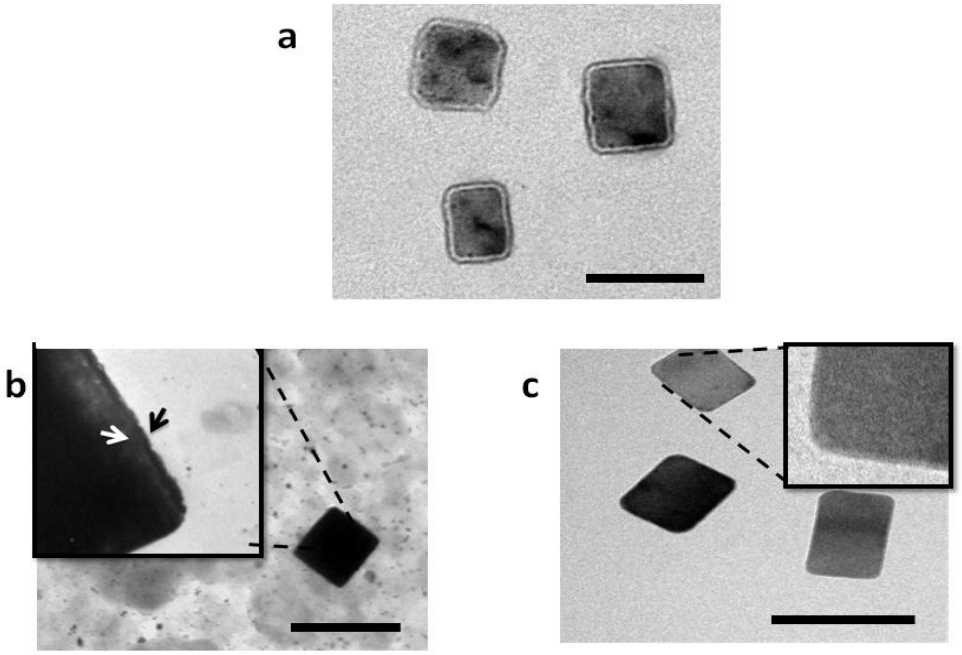

Figure 5: TEM images representing the square shaped lipid templates formed from DPPC: lyso PPC prepared at 45 ^{o}C (a), TEM image of a single gold nanodisk before purification from lipids (Inset is magnified TEM image which the lipid layer on the particle was highlighted by arrows (b), purified gold nanodisks in toluene which prepared after 72 hours of incubation at room temperature and inset magnified image highlighting the smooth edges of the gold nanodisk and their {111} plane face (c). Scale bars=200 nm.

In Figure 5-b, nanodisks surrounded by the lipid bilayer is presented. The formed nanodisks were purified from this lipid bilayer and excess citrate by centrifugation using methanol/ethanol mixture (1:4 v/v) and stored in toluene. TEM studies showed that the resulting nanodisks were around 10-20 nm smaller than the average template size with 200±11 nm length and 80±7 nm width, where the aspect ratio varies between 1-1.5 (Figure 5-c) and no aggregation was observed following purification of nanoparticles from lipids.

3.2.3 Hexagonal shaped lipid disks as template

As with the disk shaped lipids, lipidic hexagonal structures were prepared encapsulating chloroauric acid and sodium citrate, respectively. Again, a 1:1 molar ratio of each was mixed and monitored over 72 hours using UV-Vis, transmission electron microscope (TEM) and scanning electron microscope (SEM) before synthesis, during synthesis and following separation of the lipids from gold nanoparticles by centrifugation using a methanol/ethanol solution. Even though the yield was not very high, a heterogeneous population of rectangular and hexagonal shaped gold nanoparticles was obtained after 24 hours where the number of

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particles increased after 72 hours (Figure 6-c). In the absence sodium citrate encapsulating liposomes, no formation of nanoparticles structures was observed.

The template size was around 250 nm (Figure 6-a), and the resulting particles were around 200-250 nm (Figure 6-c and 6-d). As can be seen from the inset of figure 6-b a lipid bilayer surrounding the produced nanoparticle can be observed prior to purification. The particles produced had a tendency to grow in the {111} crystal plane (see 3D SEM image of single crystal in Figure 6-d). However, the heterogeneity of the template itself, obviously limits the quality of the obtained nanodisk population, which is inherently also heterogenous. However, the results clearly indicate the ability of the lipidic superstructure to be used as a template for metallic nanoparticlate synthesis.

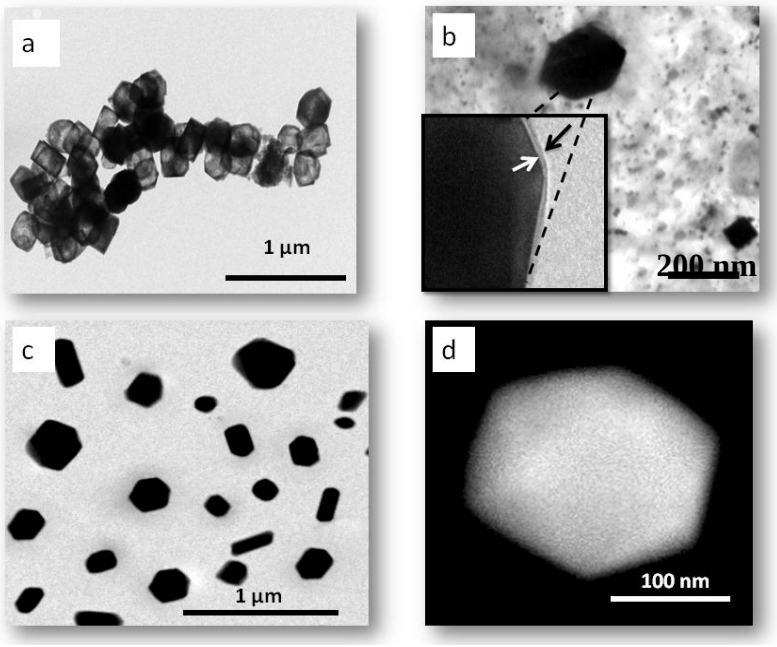

Figure 6: (a) TEM images representing the hexagonal shaped lipid templates formed from DPPC: lyso PPC prepared at 25 ^{o}C , (b)TEM image of a single hexagonal shaped gold nanodisk before purification from lipids (inset is a magnified TEM image which the lipid layer on the particle, highlighted by the arrows) , (c) TEM image of purified gold nanodisks after 72 hours of incubation at room temperature and SEM image demonstrating the {111} face of a single hexagonal gold nanodisk synthesized.

3.3.3 Twisted ribbons as templates

Lipidic twisted ribbon-like superstructures were prepared encapsulating chloroauric acid and citrate, respectively. However, due to the multiple fusion sites available with the ribbons, which could lead to aggregation and collapse of the template, an alternative route for preparation of the twisted ribbon metallic nanostructures was also pursued, where chloroauric acid encapsulated structures were immersed in a solution containing sodium citrate, or sodium citrate encapsulated

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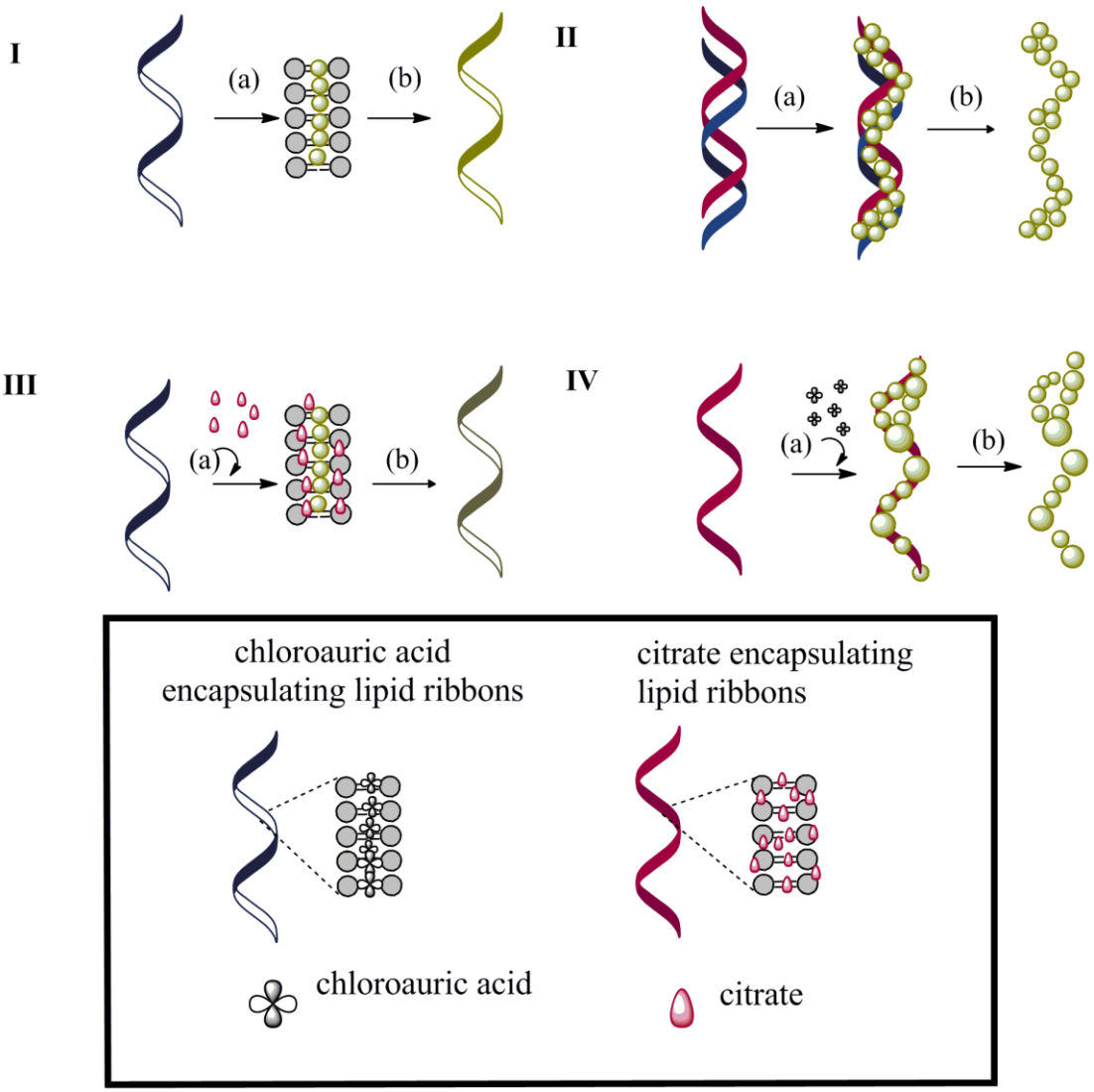
structures were immersed in a solution containing chloroauric acid. In this case, fusion did not occur for exchange of the lipidic contents, but rather diffusion of the respective reagents across the lipid bilayer into the interior core of the ribbon-like structure. As a control, the chloroauric acid encapsulated structres were immersed in PBS alone.

Using different templating methods (Scheme 4), the lipid structures were mixed or immersed and monitored for over 72 hours of incubation at 25 ^{o}C. Incubations over 72 hours resulted in presipitations and aggregations most probably due to oxidation of the phospholipid.



Scheme 4: Four different features of gold nanostructure formation which their shape was templated by twisted lipid ribbons((a) particle growth, (b) purification of the gold structures from lipid residues).

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As expected, the four different approaches investigated resulted in four different final structures (see Figure 7 and Figure 8). As can be seen, after 72 hours of incubation at 25 ^{o}C, ribbon like thin rods with slight appearance were spontanously formed even when no additional reducing agent was used. This is attributed to the ability of the DMPG to act as a capping agent on gold nanoparticles, as has been reported previously, [43] and additionally, there are examples of the spontaneous formation of gold nanoparticles in lipid bilayers without the use of reducing agents.[44]

In the second method chloroauric acid encapsulated structures were immersed in a solution containing sodium citrate, twisted ribbon shaped nanoparticles (scheme 4-3) with a more solid appearance were obtained (Figure 7-b) as a result of the citrate reduction enhanced by the capping property of the lipid.

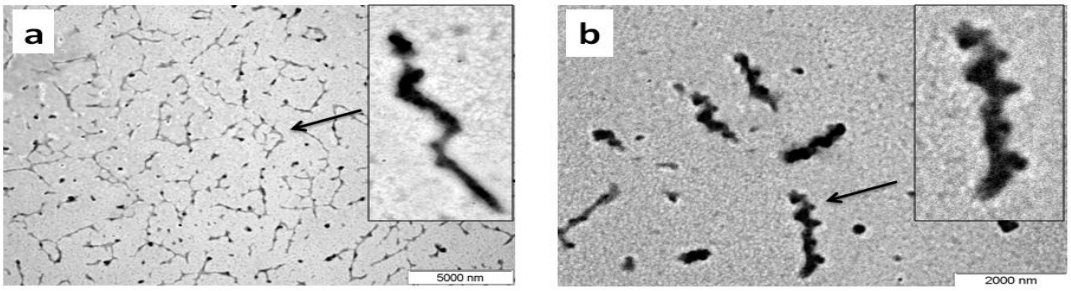

Figure 7: TEM images of ribbon-like gold nanoparticles prepared in chloroauric acid encapsulating ribbon-shaped template (a) spontanously after 72 hours and (b) via addition of citrate solution in PBS (pH 7.4, 10 mM) which were purfied after 24 hour of incubation at 24 ^{o}C.

However, when citrate and $HAuCl_4$ encapsulating twisted lipid ribbons were mixed, tiny nanoparticles arranged in a way that they built 1D lace-like structures (Figure 8-a) after 24 hour of incubation at 25 ^{o}C. As depicted in Scheme 4-II, when two twisted encapsulating lipid ribbons are mixed, the chloroauric acid and citrate interact at the contact points and particles form at these points due to the reduction of the Au(III) to Au(0), resulting in a one-dimensional nanoparticle chain type structure guided by the template.

The same type of one-dimensional particle alignment was observed when the citrate encapsulating lipid template was immersed in chloroauric acid solution in PBS (pH 7.4, 10 mM, 25 ^{o}C). The particles were slightly larger than the previous method, but again arranged in nanopartcle chain type structure guided by the dimensions of the lipid template. As shown in Scheme 4-IV, these structures were formed due to the interaction between the chloroauric acid and the citrate diffusing from the bilayer core which provided nucleation cites for the particles which later aggregates in a manner that they form chains aligned one after the other (Figure 8-b inset).

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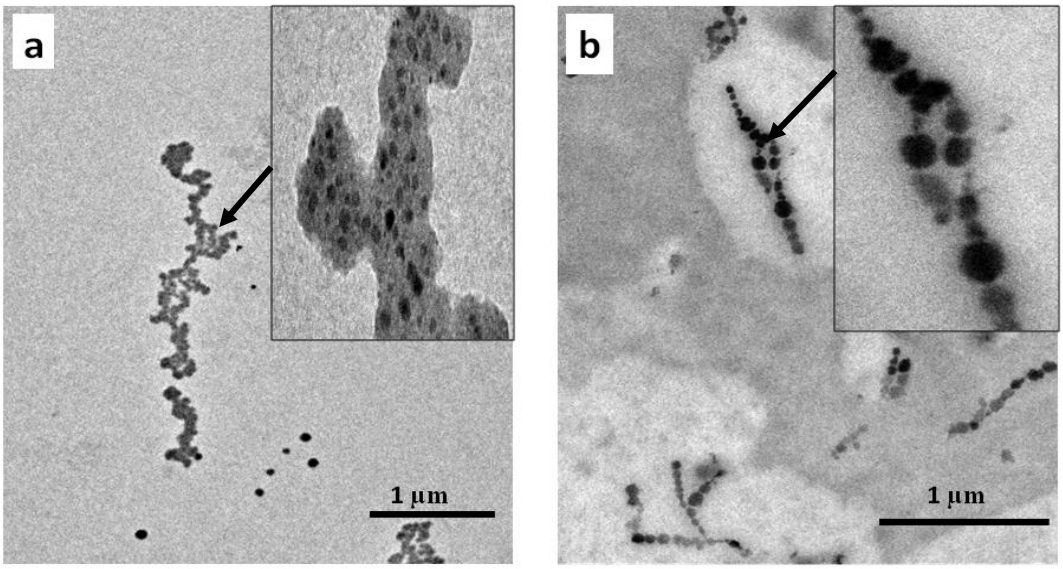

Figure 8: (a) TEM images of gold nanoparticle chain-like alignment prepared using chloroauric acid encapsulating ribbon-shaped template mixed with citrate encapsulating template (inset magnified section of the particle structure), whereas, (b) is the TEM image of a gold-nanoparticle chain-like arrangment prepared via citrate encapsulating lipid template immersed in chloroauric acid solution in PBS (pH 7.4, 10 mM) (inset is magnified section of a single nanostructure) in toluen which were purfied after 24 hours of incubation at 25 ^{o}C.

Whilst there are many studies on the lipid tubes as templates for inorganic particle synthesis, there are only couples of studies on lipid ribbons as templates. [24] In a review by Zhou and Shimizu, different approaches of lipid tubes resulting in different nanoparticles arrangement were discussed.[4] Jung et al, in a recent study, reported self-assembled helical lipid ribbons as templates for the synthesis of palladium nanoparticles using ascorbic acid as a reducing agent, where they observed either tiny nanoparticles embedded on the template surface or solid nanostructures depending on the patterning method used. [45] Jin et al, in another study, reported silica nanostructures where their shape was patterned using lipid structure including ribbons, hollow sphere and other chiral materials prepared at different temperatures together with the use of co-structure-directing agents.[5] Although there is not much information on twisted ribbon shaped templates, it could be concluded that the addition of reducing agent in PBS to the metal salt encapsulating template leads solid nanostructures with the same morphology as the template itself since the reducing agent is more mobile to diffuse into the template bilayer to interact with the metal salt. However, when two reactors were mixed or citrate encapsulating template were immersed in the metal salt solution spherical nanoparticles aligned in nanoparticle chain-like structure was observed, due to the limited diffusion of the reaction species.

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4 Conclusions

The work reported here examines the effect of lipid characteristics (such as lipid tail length, head group type, lipid phase transition) on final lipid organization when exposed to conditions exploiting the curvature of the lipids as pH jump and heat treatment so called curvature preparation method. These results demonstrate that with this method, it is possible to obtain lipid aggregates with diverse shape, which could be tuned by changing the lipid formulations with different thermal behaviors or operation temperature. Long tail (16C - 18C), charged lipids formed liposomes at all temperatures studied, whilst the lipid organization of zwitterionic lipids and short tail charged lipids were strongly dependent on lipid phase transition and they can form diverse type of aggregate shapes such as hollow hexagonals and tubes with encapsulating capacity, and planar strips as well as rectangular shaped lipidic structures and ribbons, when right conditions were given. Furthermore, hexagonal, rectangular shaped disks and twisted ribbon lipidic superstructures were studied as templates for the synthesis of nanoparticles of controlled morphology. Different strategies for the synthesis of gold nanoparticule structures were explored. Hexagonal and rectangular gold nanodisks were produced with their shape patterned by the template; with the homogeneity of the particles formed being directly dependent on the homogeneity of the template population. Ribbon shaped lipidic superstructure templates resulted in diverse types of alignment of the gold atoms synthesized, resulting in structures again patterned by the template (e.g ribbon and chain-like alignments). In conclusion, lipidic nanotemplates were demonstrated to be promising tools for the synthesis of metal nanoparticles of controlled morphology and size. The possibility to prepare nanoparticles in mild conditions with defined size opens the door for a plethora of potential applications in catalysis, plasmonics and electronics.

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CHAPTER

5 APPLICATION TO CONTROLLED RELEASE

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Three-component thermo-sensitive liposomes: size, membrane integration and stability

Rukan Genç[1], Mayreli Ortiz[1], Dimitris Fatouros,[2] Ciara O'Sullivan [1,3]

[1]Nanobiotechnology and Bioanalysis Group, Department of Chemical Engineering, Universitat Rovira I Virgili, Av. Països Catalans, 26, 43007, Tarragona, Spain.

[2] Department of Pharmaceutical Technology, School of Pharmacy, Aristotle University of Thessaloniki, GR-54124, Thessaloniki, Greece.

[3]Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23,

08010 Barcelona, Spain.

Abstract

Liposomes are naturally suitable vehicles for targeted drug delivery approaches. However, time-consuming preparation methods and possible toxicity issues as a result of the organic solvents used for their preparation limit their potential for in vivo use. Avoiding the use of solvents, we exploited our previously reported curvature tuned preparation method for the preparation of thermosensitive calcein-encapsulating liposomes exploiting a known temperature-sensitive liposome formulation composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE). A range of molar ratios of DPPE to DPPC at constant concentrations of lyso-PPC was studied in terms of its effect on membrane integration, release kinetics and stability, as well as on liposome size. Differential scanning calorimetry (DSC, transmission electron microscope (TEM), photon correlation spectroscopy as well as fluorimetry were used to characterize the liposomes formed. A decrease in liposome size from 320 nm to 80 nm was achieved at increased DPPE concentrations whilst liposomes of 3 % w/v DPPE were found to be more stable both at elevated concentrations of serum or $CaCl_2$ when they were incubated at 37° C for 24 hours. Liposomes encapsulating calcein were analysed in terms of calcein release over a range of temperatures with liposomes composed of 3% w/v DPPE demonstrated calcein release from vesicles at

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temperatures higher than 38 ^{o}C, with the release rate reaching a maximum at 60 ^{o}C, highlighting the potential of the liposomes as thermo-sensitive liposomes for localised drug delivery.

1 Introduction

The toxicity of drugs used in the treatment of diseases, such as cancer, may be minimized via the application of drug carrier systems (dendrimers, liposomes, polymers, micelles).[1]Liposomes are artificially formed vesicles consisting of lipid bilayers which enclose an aqueous compartment.[2]Liposomes have ben widely used as a model system of a cell membrane, and more recently have been developed for the controlled release of pharmaceutics and macromolecules. Liposomes can increase the tolerance of active compounds by lowering the active toxicity of its encapsulated contents,[3] whilst also opening up the possibility of transporting pharmaceuticals into diseased tissue in a controlled manner.[4]

After administration to the blood stream, liposomes are mainly taken up in by the liver and spleen,[5] thus, it is essential to increase the circulation time of the liposomes in the blood so they can reach the tissues or cells of interest.[6] Improved circulation time can be achieved by designing intelligent liposomes which releases its contents only after they are subjected to triggering conditions, such as instant pH change, hyperthermia, or a magnetic field. Structural properties and characteristics of liposomes (size and lamellarity) can be easily modified by altering their lipid composition and method of preparation.[7] For example, multilamellar vesicles (MLV) can be formed spontaneously when phospholipids are hydrated into aqueous solutions, and large unilamellar vesicles (LUVs) can be prepared from MLVs for example by extrusion or freeze-thawing. On the other hand, preparation of smaller sized, homogenous populations of so called small unilamellar vesicles (SUV) requires -in addition to the thin film preparation method – laborsome size-reducing and homogenization methods (e.g. freeze-thawing, sonication, extrusion, ultracentrifugation),[8] and these time-consuming methods of preparation limit the widespread use of liposomes. Methods for the rapid preparation of homogenous populations of encapsulating trigger-sensitive liposomes are widely sought and would find tremendous application for controlled release. We recently reported on a one-step, ultra-rapid (1 h) method coined the curvature tuned preparation (CTP), method for the preparation of homogenous encapsulating liposomes, based on a rapid pH jump followed by an equilibration period at a specified temperature, which has been successfully developed using diverse types of phospholipids (Scheme 1).[9]

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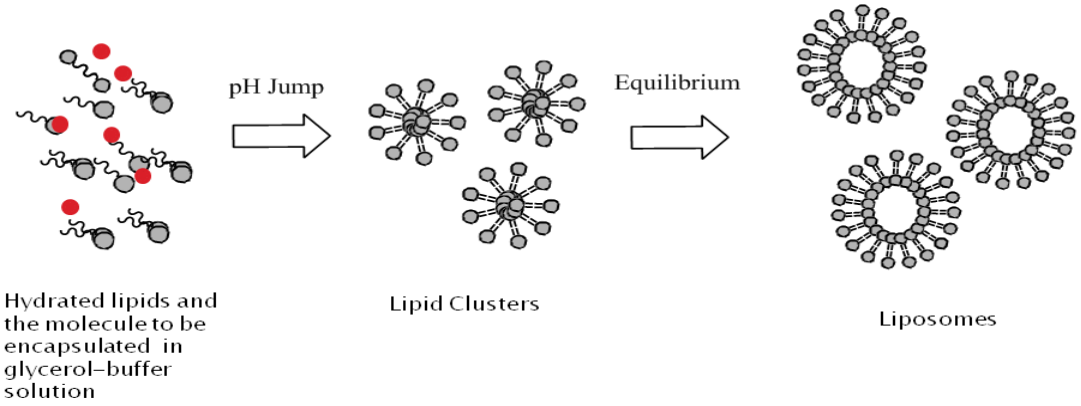

Scheme 1: Curvature tuned preparation of liposomes

Lipid assembly to closed vesicles or other types of structures, such as micelles, tubes and planar bilayers is strongly dependent on the lipid geometry, which is defined by the value of critical packing factor, P, which is the ratio of volume occupied by a single lipid molecule to the area occupied by the lipid polar head group and length of the lipid tail. Another important property of the lipids is the critical melting temperature (T_M) where the lipid phase transition from rigid gel to liquid crystalline phase takes place. External parameters affecting those characteristics (e.g pH, temperature and ionic strength) determine the interaction between the lipids in a synergetic manner with the lipid geometry and the thermal phase behavior of the lipid.[11]

The aim of this study is to implement the CTP method for the preparation of thermo-sensitive liposome for the temperature-triggered drug release using hyperthermia.[10] The prepared liposomes were analyzed in terms of their membrane integration by differential scanning calorimetry (DSC), fluorometric measurement of calcein release in the presence of serum, liposome fusion at increasing calcium ions as well as the triggering effect of temperature for controlled release.

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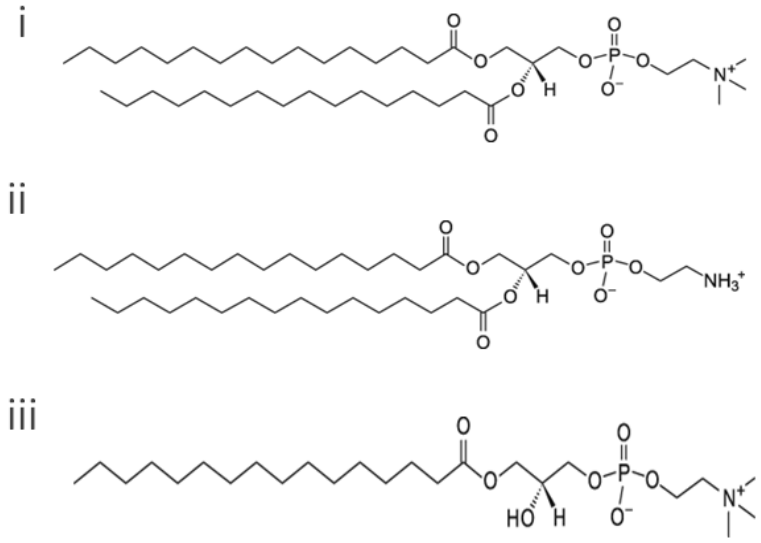

Scheme 2: Structural representation of the phospholipid composition of the thermo-sensitive liposomes: i) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, ii) 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, and iii) 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine.

2 Experimental Section

2.1 Materials

Phospholipids were supplied as a powder by Avanti Polar Lipids, Inc. and used without further purification. Sodium hydroxide, hydrochloric acid, di-sodium hydrogen phosphate (anhydrous, reagent grade), Na_2HPO_4), sodium dihydrogen phosphate (anhydrous), extra pure, (NaH_2PO_4), calcein and glycerol 99.5%, reagent grade, were purchased from Scharlau Chemie SA. Sodium chloride was provided by Riedel-de Haën. Milli-Q water (1.82 MΩ.cm-1) was obtained using a Simplicity 185 Millipore-Water System.

Preparation of encapsulating liposomes: Calcein encapsulating liposomes were prepared via curvature tuned preparation method reported previously.[9] Briefly, 50 mg of a phospholipid formulation of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine

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(lyso-PPC) and phospoholipid 1,2-dipalmitoyl-s*n*-glycero-3-phosphoethanolamine (DPPE) in predetermined molar concentrations were directly rehydrated in a pre-heated calcein mixture in PBS buffer (0.1 M, pH 7.4) at 45 ^{o}C under argon supply while the mixture was continuously stirred. The solution was then subjected to a rapid pH jump (pH 7.4→pH 11 → pH 7.4) followed by an equilibration period of 25 min, where lipid clusters curl into encapsulating liposomes. The resulting liposomes were purified using G25 sephadex coloumn and stored at 4 °C.

Transmission electron Microscopy (TEM) imaging: Using a glass pipette, a drop of sample was added to a 200 mesh copper grid with a thin film of Formvar polymer and carefully dried using filter paper. The sample was then left at room temperature until a dried film was obtained. Transmission Electron Microscopy (TEM) analyses were performed using a JEOL 1011 transmission electron microscope operated at 80 keV with an ultra-high-resolution pole piece providing a point resolution of 2 A°. Micrographs (1024 pixels x 1024 pixels) were acquired using a Megaview III multiscan-CCD camera. Images were analyzed with an iTEM image analysis platform and the mean diameter was calculated measuring at least 100 particles from the series of experiments (n±3).

***Differential scanning calorimetry* (*DSC) studies*:** All calorimetric scans were performed on a Perkin-Elmer DSC7 operating at a scan rate of 1 K/min with a sample mass of approximately 5 mg and a reference pan containing an equal mass of buffer. The scan rate was carefully optimised to be the minimum allowable with a sufficient signal-to-noise output. Calibration of the calorimeter was performed prior and subsequent to scanning of the lipid dispersion. Three aliquots from each preparation were scanned and the results averaged. Preparations were repeated and further aliquots scanned to check the reproducibility of the preparation method.

Size and Size Distribution Studies Using Photon Correlation Spectroscopy (PCS): The mean diameter of the liposome emulsions and the size distribution, presented as a function of polydispersity index (PI), were measured using Zeta Sizer 3000H [He-Ne laser (633 nm), detector angle of 90^{o}] Malvern Instruments, Inc., which measures the rate of fluctuation of the light scattered from the particles using photon correlation spectroscopy (PCS). Standard deviations were calculated from the mean of the data of a series of experiments (n > 3) conducted using the same parameters.

Calcein release: Liposomes were incubated at changing concentrations of liposomes over 24 hours at 37 ^{o}C, and intermitent measurements of calcein leakage from liposomes were analyzed. The change in

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fluorescence intensity due to calcein release from the vesicles was monitored with an Cary Eclipse Fluorometer where excitation and emission wavelengths were set at 490 and 520 nm, respectively. The amount of calcein released after time (*t)* was calculated according to:

$$RF\ (\%) = 100\ (I_t - I_0)/(I_{max} - I_0) \qquad \text{Eq. (1)}$$

Where, I_{max} is the measured intensity after the Triton X-100 treatment and I_0 intensity at time zero.

3 Results and Discussions

3.1 Characterization of liposomes

Thermosensitive liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) (scheme 2) were used to prepare liposomes. The liposome properties were studied at different DPPE concentrations (from 0 to 6 mol %) at a constant percentage of lyso-PPC (12 mol %). During preparation, the temperature was kept constant at 45 ^{o}C, which is higher than the melting temperature of DPPC (41 ºC). Transmission electron microscopy (TEM) showed DPPE was required for the formation of liposome vesicles (Figure 1). The liposome size decreased from 310 to 80 nm when the molar % of DPPE was increased from 1.5 to 6% (Figure 1 and 2). At higher contents of DPPE, due to the truncated cone shape of DPPE[11] the lipid bilayer membrane had a higher curvature, which leads to smaller sized liposomes (Table 1).

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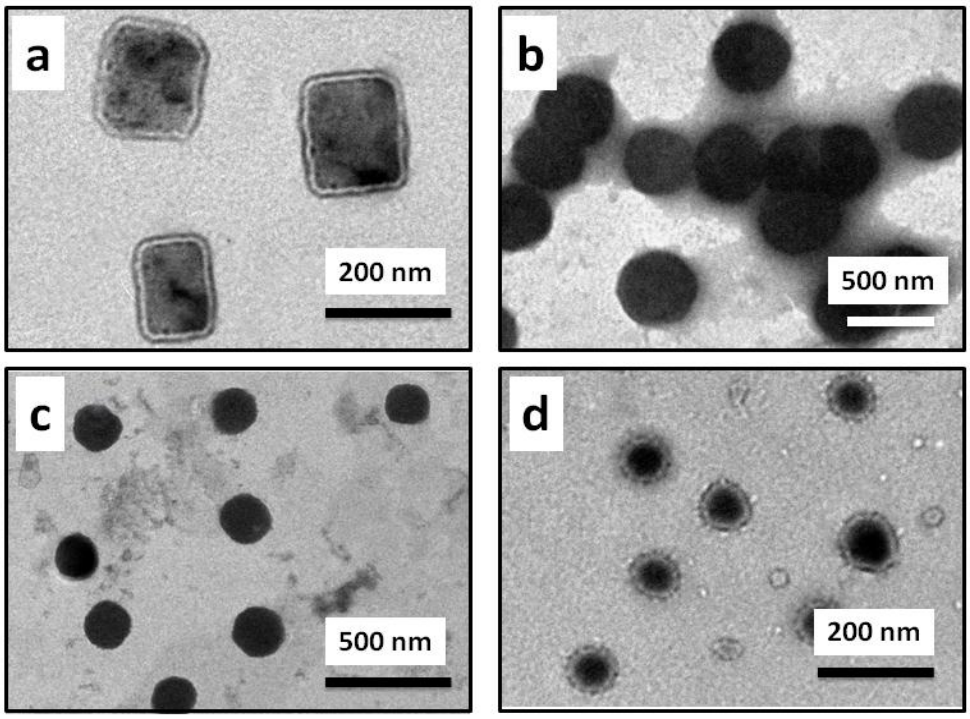

Figure 1: TEM images of liposomes prepared using DPPE molar concentration of a) 0, b) 1.5 %, c) 3%, and d) 6% at 45 ^{o}C in PBS, 10 mM (pH 7.4) using the curvature tuned preparation method.

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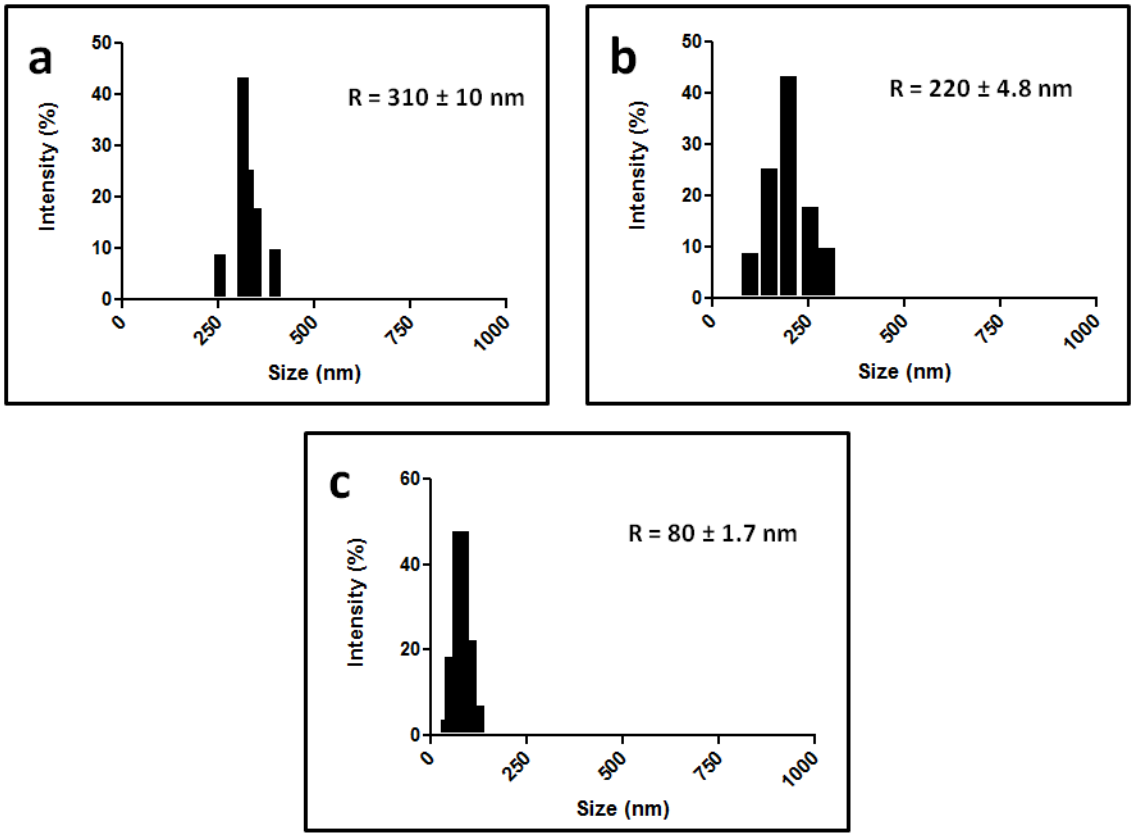

Figure 2: Size distribution of the liposomes prepared at changing DPPE molar ratios a) 1.5 %, b) 3% and c) 6 % measured by Photon Correlation Spectroscopy (PCS).

Further DSC studies, Table 1, showed an increase of melting temperature from 41.41 to 41.72 ºC at increasing DPPE concentrations from 1.5 to 3 mol % and a subsequent decrease to 41ºC for 6 mol % of DPPE composition.. The specific heat capacity, which is the heat capacity per unit mass of a material (Cp), showed the same pattern with a higher value observed for the 3 mol % DPPE formulation, demonstrating the higher stability of this liposome formulation over others. The decreased Cp observed with the 6 % DPPE formulation is attributed to the disorganization of the lipid bilayer caused by a 'phase segregation' due to the increased molar percentage of the DPPE in the formulation.

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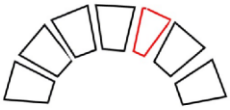
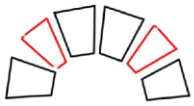
Table 1: Characterization of liposomes composed of different DPPE molar percentage.

DPPE (% in mol)	T_M (°C)	Cp (j/g*K)	Size (nm)	Representation of liposome bilayer
1.5	41.41	2.535	310 ± 10	
3	41.72	3.450	220 ± 4.8	
6	41.00	1.677	80 ± 1.7	

2.2 Stability analysis in the presence of serum

The stability of liposomes at different serum concentrations was evaluated in terms of % of the retention of calcein inside the liposomes at 37 °C after 24 hours exposure to serum, for different mol % of DPPE. Supporting the results obtained from DSC, the lipid formulation of 3 % DPPE was the most stable when incubated in the absence of serum. In the presence of 50 % serum, (approximately equivalent to levels found in blood), the retention is similar slightly higher than with 1.5 mol% of DPPE and clearly higher as compared to that obtained with the 6 mol % DPPE formulation. However, when the serum concentration was increased to 80 %, the retention of the calcein shows a decrease proportional to the DPPE concentration as at high DPPE contents, there is an increased amine group concentration on the liposome surface, and the attack by serum proteins to these amine groups, disturbs the membrane stability. Size analysis with nanosizer showed that at increasing serum concentrations, liposomes start to form an increasing number of micelles of 8-15 nm, with increasing amounts of DPPE (Figure 3), which can be explained by the charge and phase dependent destruction of the membrane by DPPE.

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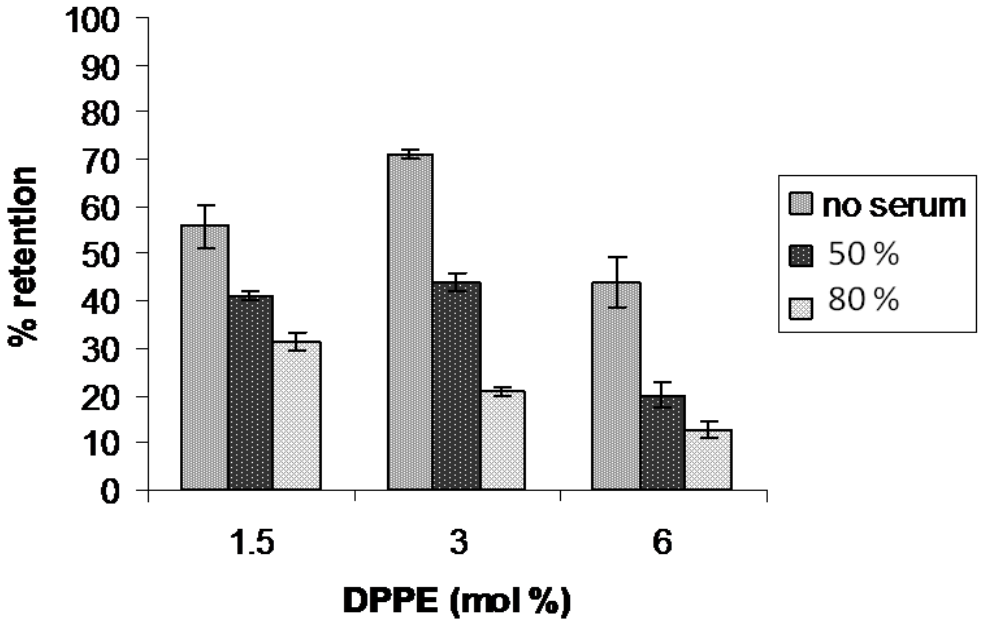

Figure 3: Stability of liposomes evaluated in terms of % retention of calcein inside the liposomes at 37 ^{o}C after 24 hours at changing concentrations of serum.

2.3 Stability of membrane in presence of Ca^{2+} ions. The interaction of cations with phospholipid monolayers is of interest, particularly for calcium as this divalent cation plays a special role in biological membranes. In general, calcium induces more crystalline-like phase behavior due to its ability to chelate negatively charged phospholipid head groups.[12] Phosphatidylserine (PS), Phosphatidic acid (PA), and Phosphatidylinosito (PI) containing monolayers are more sensitive to the presence of calcium than monolayers composed of neutrally charged lipids. As well as lateral lipid self-diffusion, calcium ions, affect the rotational diffusion of the lipid head group. Therefore, calcium, as a fusogenic agent, could be expected to have an influence on a phospholipid membrane.[13]

To probe the influence of Ca^{2+} on membrane integration, DSC studies were performed with the samples after 24 hours of incubation. Transition peak which is the indicator of a transition from gel to gel crystalline was recorded and corresponding melting temperatures (TM) and specific heat capacitance were calculated. As can be seen from Figure 4, no transition peak was observed at calcium concentrations higher than 0.4 mM for 1.5 % DPPE and 1.2 mM for 6 % DPPE . On the other hand, a decrease in Cp starting from 0.4 mM $CaCl_2$ was observed for the liposome sample composed of 6 % DPPE molar concentrations and corresponding T_M values support this observation. At concentrations higher than 0.4 mM $CaCl_2$ concentration, the effect of calcium is more discernable, and at higher calcium concentration, the lipid formulation of 3% DPPE is again observed to be the most stable. Size measurements of the

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encapsulating liposomes before and after incubation in changing concentrations of alcium chloride resulted in an increased liposome size for all the formulations studied. Small micelles of 8-15 nm started to dominate in the case of 6% DPPE at increased concentration of Ca^{2+} due to the dissociation of the DPPE from the lipid membrane, resulting in lower stability and rapid calcein leakage.

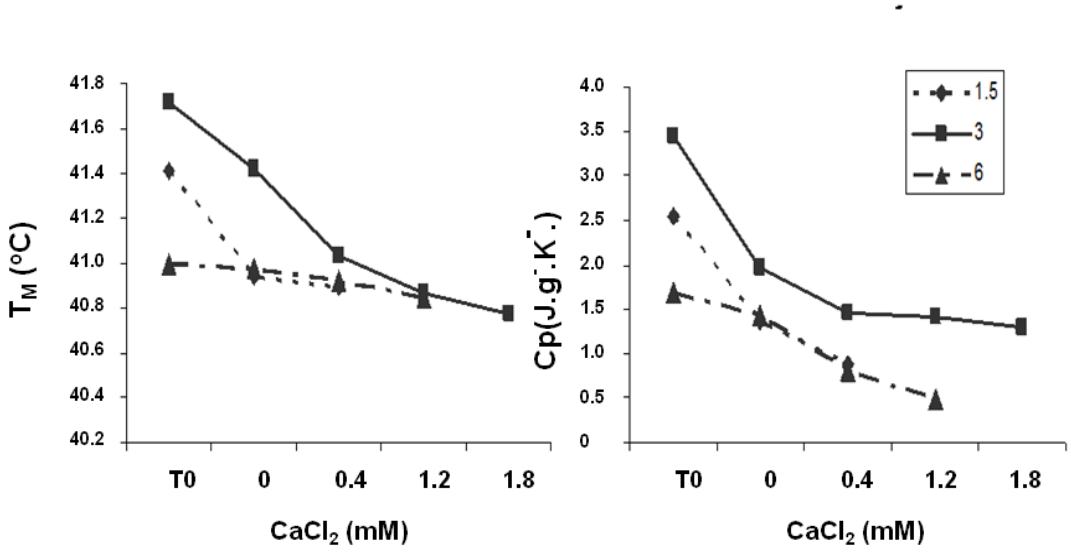

Figure 4: T_M values and calculated Cp values of the liposome formulations treated 24 hours with $CaCl_2$ at 37 ^oC. t_0 = initial time.

Supporting these results, Peschkaa et al. previously demonstrated that liposomes composed of phospholipids with -PE headgroup are more sensitive to serum proteins and calcium than –PC phospholipids due to a phase transfer from liquid crystalline to hexagonal.[14]

Temperature Triggered Calcein Release from DPPC:DPPE: lyso-PPC liposomes (85:3:12 mol/mol)

Both DSC and release studies indicated that liposomes of 3 % were more stable in the presence of increased serum and Ca^{2+} concentrations, than the other liposome formulations studied, . Since the main objective of this report is to demonstrate the curvature tuned preparation method for the preparation of thermo-sensitive liposomes for drug delivery, the efficiency of the thermosensitive liposomes to release the encapsualted calcein at elevated temperatures was evaluated. As illustrated in Figure 5, an increase in temperature from 37 ^oC to 39 ^oC resulted in the release of 20 % of the calcein from the lipids after 2 hours, while further increasing the temperature to 40^oC lead to a more rapid leakage of up to 60% of the

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encapsulated calcein. At even higher temperatures, 50 ^{o}C and 60 ^{o}C a very rapid release of almost all of the encapsulated calcein was achieved in 120 min and 30 min, respectively, whilst almost no release was observed at temperatures below 38 ^{o}C, highlighting the liposome stability and applicability to controlled release and clearly demonstrating that the liposomes prepared in the absence of any solvent using the curvature tuned liposome preparation method, can be triggered to release their contents by increasing the temperature (in a mild range), which is comparable to the temperature-sensitive liposomes reported by Needham.[10]

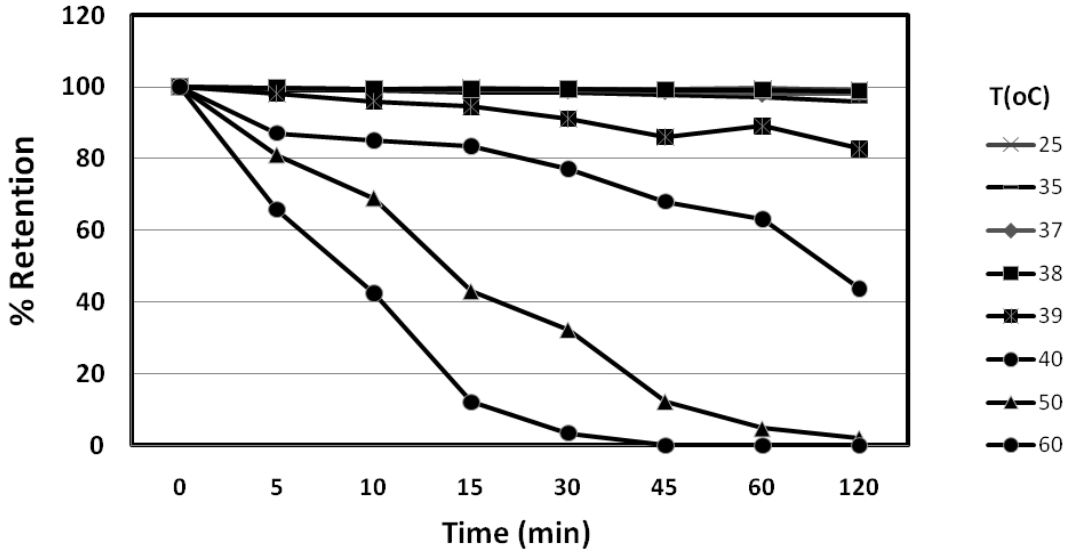

Figure 5: Temperature triggered calcein release from liposomes composed of DPPC:DPPE:lyso-PPC (85:3:12 mol:mol).

4 Conclusions

Thermo-sensitive liposome formulations of DPPC:DPPE: lyso PPC with changing DPPE mol % were studied for their potential use as controlled drug release systems. A decreasing liposome size with increasing concentrations of DPPE was observed as a result of the truncated cone shaped DPPE providing a higher membrane curvature. Furthermore, an increased melting temperature (T_M) and heat capacity (C_p) of liposomes was obtained with liposome formulations prepared with with 3 % of DPPE as compared with 1.5 % indicating the incorporation of DPPE molecules with a melting temperature around 60 ^{o}C to the membrane and increased stability of those membranes compared to that of other formulations. When

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the DPPE concentration was increased to 6 % both the T_M and C_p decreased, which can be attributed to a disorganized membrane due to the incorporation of a higher amount of DPPE. In the presence of $CaCl_2$, an increased calcium concentration leads to decreased membrane stability, and no transition peak which relates was observed after 0.4 mM of $CaCl_2$ for 1.5 % DPPE and 1.2 mM for 6 % DPPE liposomes, whilst the3 % DPPE liposome formulation was shown to be more stable at high concentrations of calcium. A critical factor to be studied when evaluating liposomes for in vivo drug delivery is the effect of serum on liposome stability. Again, the liposomes prepared with 3 % of DPPE were observed to be more stable, when they incubated in the presence of 50 % serum (blood concentration) than the other formulation studied. After 24 hours of incubation the liposomes were observed to have retained 73 and 45 % of the encapsulated calcein, in the absence and presence of serum, respectively. In conclusion, the solvent-free curvature tunable preparation method was used to prepare thermosensitive liposomes for controlled drug release. The concentration of DPPE in the liposome formulation was found to be an important parameter and it effected liposome size, release kinetics as well as the membrane integraty if liposomes of DPPC:DPPE:lyso-PPC. Liposomes of 3 % DPPE were demonstrated to be thermo-sensitive, showing a temperature-dependent calcein release with increased release kinetics at temperatures higher than 38 ^oC, whilst being completely stable at physiological temperatures of 37 ^oC, and lower.

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CHAPTER

6 CONCLUSIONS AND FUTURE WORK

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6. Conclusions and Future Work

6.1 Conclusions

The self-assembly of building blocks into nano- or microstructures is an area of intense interest, with lipids being particularly attractive in the formation of closed spherical lipid bilayers known as liposomes. Numerous methods have been reported for the preparation of liposomes, many of which, in addition to requiring time-consuming preparative steps and the use of organic solvents, result in heterogeneous liposome populations of uncontrolled size.

In the first part of the presented doctoral thesis, taking into consideration the phenomenon of spontaneous vesiculation and the theory of curvature, we presented an extremely rapid and simple, solvent-free method for the preparation of monodisperse solutions of highly stable small unilamellar vesicles using both charged and zwitterionic lipids mixed with lyso-palmitoylphosphatidylcholine, exploiting a combination of a rapid pH change followed by a defined period of equilibration at a fixed temperature. Various experimental parameters and their interactions were evaluated in terms of their effect on resulting liposome size and shape, as well as on liposome stability and size distribution, with transmission electron microscope imaging being used to visualize the formed liposomes, and photon correlation spectroscopy to obtain statistical data on mean diameter and monodispersity of the liposome population. ζ potential measurements also provided information about the interpretation of vesiculation kinetics and liposome stability. The time interval of pH jump, operation temperature, equilibration time, and lipid type were shown to be the determining factors controlling the size, shape, and monodispersity of the liposomes. Buffer type was also found to be important for the long-term storage of the liposomes.

The so called, curvature-tuned preparation, method was later applied to a wide-range of lipid formulations chosen according to the lipid characteristics (e.g. head group charge, tail length, and melting temperature) affecting the lipid thermal behavior, and as a result affecting their interaction with the neighboring lipids. Temperature dependent formation of tubes, plane bilayers, twisted ribbons as well as square and hexagonal shaped lipid disks was observed using either zwitter-ionic or negatively charged but short tailed phospholipids, whilst nano-sized liposomes were obtained with negatively charged long tailed phospholipids at any temperature studied which were further used in several applications.

In this thesis, for the first time, use of thermo-sensitive liposomes for surfactant-free detection is reported. Previous reports on the use of liposomes in both biosensors and bioassays have required use of lysing agents such as surfactants, e.g. Triton X-100 or sodium dodecyl sulfate (SDS), which are not particularly environmentally friendly, and in order to avoid their use, temperature sensitive liposomes were used as labels for the ultrasensitive detection of carcinoembryonic antigen (CEA), a tumor marker. Five different bioconjugation methods to link an anti-CEA antibody to horseradish peroxidase (HRP) encapsulating liposomes were studied and compared to HRP-Ab conjugate. ζ -Potential measurements of liposomes

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before and after each modification method as well as following incubation with CEA were used as a tool to monitor the success of modification and probe the affinity of the liposome linked antibodies. The use of different lysing conditions (temperature vs detergent) was evaluated, with the application of temperature providing an extremely effective means of liposome lysis. Detection limits of 2 orders of magnitude lower than that obtained with the HRP-antibody reporter conjugate were obtained (0.080 ng CEA/mL and 0.0113 ng CEA/mL), with 11- fold and 9-fold amplification of signal, for the biotin-streptavidin and SATA/Sulfo-SMCC modified liposomes respectively, clearly demonstrating the powerful potential of enzyme encapsulating thermo-sensitive liposomes as signal enhancement tools.

Reverse micelles, lipid vesicles and other types of amphiphilic materials are of great interest in the size-controlled synthesis of metal nanoparticles. As an alternative to reverse-micelles,on the forth chapter of the thesis, use of nano-sized liposomes and other lipid structures as nanoreactors/templates for the inorganic synthesis of gold nanoparticles (AuNPs) with a controlled shape and size using different synthesis approaches were discussed. We presented a facile synthesis method of ultrasmall (2-6 nm) gold nanoparticles in the cavity of nanosized liposomes under mild conditions (25 ^{o}C) at varying concentrations of $NaBH_4$. The barrier role of the vesicle membrane in controlling the reaction rate and protecting the gold nanoparticles from rapid aggregation at high reducer concentrations was discussed and compared with the traditional bulk method. A rapid aggregation at molar ratios of $HAuCl_4$: $NaBH_4$ higher than 1:40 was observed in solution synthesis in the absence of liposomes, whilst aggregation of the formed gold nanoparticles was only observed at1:1000 molar ratios.

This section of the thesis focused on the design of a liposomal nanoreactor where a known polyol, glycerol, as green catalyst for the reduction of gold to assemble in nanoparticles without the use of any harsh chemicals. Nanosized liposomes were used as functional nanoreactors exploiting glycerol incorporated in the external and internal surface of the lipid bilayer, keeping the reducing agent semi-mobile in their nanoenviroment. Several reaction parameters such as temperature, glycerol concentration, and the effect of capping agent were studied in terms of their effect on the size and the homogeneity of the nanoparticles formed. Increased concentrations of glycerol resulted in a decreased size of the nanoparticles and, furthermore, nanoparticles synthesized in the presence of capping agent showed almost a 2 fold decrease in the particle size leading to ultra small gold nanoparticles of around 2 nm. Moreover, a decrease in the nanoparticle size at constant concentrations of capping agent and glycerol was observed when the temperature was increased in the range of 4 to 50^{o}C. Comparison studies on the gold nanoparticle synthesis in solution under the same conditions without the use of the nanoliposome reactors, resulted in highly heterogeneous nanoparticles with amorphous shape. These results indicate that with the designed liposomal nanoreactors, with glycerol integrated in the membrane as a reducing agent, a one-pot synthesis of highly homogenous nanoparticles was successfully achieved as a result of semi-solid reaction environment provided by the liposome.

Different shaped lipid structures with changing polar head groups were demonstrated as templates for the inorganic synthesis of gold nanoparticles which their shape was directly linked to the template geometry. Square and hexagonal shaped lipid disks composed of zwitterionic phospholipid, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) together with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PPC) were used as templates where particle growth was achieved due to content exchange as a result of the spontaneous fusion of two template population (A= $HAuCl_4$ encapsulating lipid template,

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B=citrate encapsulating lipid template). Particle size, shape and the homogeneity were found to be directly linked to the template properties resulting in homogenous populations of square shaped gold nanodisks and heterogeneous mixtures of hexagonal gold disks with other shapes were obtained using square shaped lipid disks and hexagonal disks, respectively.

In the case of twisted ribbons made up of negatively charged lipid, DMPG, taking account the planar-bilayer nature of the template, 4 different approaches were studies: 1) $HAuCl_4$ encapsulating template was immersed in PBS, 2) citrate in PBS or mixed with 3) citrate encapsulating lipid template and finally, citrate encapsulating template was immersed in $HAuCl_4$ in PBS. Due to capping property of DMPG, just template immersed in PBS gave ribbon-like shaped solid structures where addition of citrate to the template increased the appearance of the ribbon shape as a result of additional reduction. Both mixing two template population encapsulating the reactants and immersion of citrate encapsulating template in $HAuCl_4$ in PBS, resulted in organizations of tiny gold nanoparticles aligned in a way which guided by the template structure. The possible assembly mechanism which is highly dependent to the route of the administration of reactants was discussed in terms of the resultant gold nanostructures. Obtained gold-nanodisks with high area in one dimension and nano-sized ribbons with curves could have great potential in catalysis.

The last chapter of the thesis covers the basic properties of thermo-sensitive liposomes for their used in controlled release of drugs. Liposomes are naturally suitable vehicles for targeted drug delivery approaches. Time consuming preparation methods and possible toxicity of them as a result of the organic solvents used limit their potential to be used for such purposes. In this report, curvature tuned preparation method was implemented to a known temperature-sensitive liposome formulation composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*glycero- 3-phosphocholine (lyso-PPC) and phospoholipid 1,2-dipalmitoyl-*sn*-glycero-3- phosphoethanolamine (DPPE). Changing molar ratios of (0-6 % mol: mol total lipid) DPPE at constant concentrations of lyso-PPC (12 % of total lipid) was studied in terms of its effect on membrane integration, release kinetics and stability, as well as liposome size. A decreased liposome size from 320 nm to 80 nm was achieved at increased DPPE concentrations due to truncated cone shape of DPPE molecule leading higher curvature, so smaller size of liposome. Further studies on the calcein leakage through the liposomes at changing concentrations of serum and $CaCl_2$ demonstrated that liposomes of 3 % DPPE was more stable either at higher concentrations of serum or $CaCl_2$ when they incubated at 37° C for 24 hours, whereas liposomes of 6 % DPPE at increased serum and Ca^{2+} concentrations resulted in micelle formation as a result of DPPE dissociation from the membrane. Further, Differential scanning calorimetry (DSC) studies which used to analyze the membrane integration and thermal behaviors changing parameters supported the stability results obtained. Finally, liposome formulation of DPPC:DPPE:lyso-PPC in 85:3.0:12 molar ratios were studied at changing temperatures from 35-60 provided a clear proof of the thermo-sensitivity of those liposomes which the calcein leakage is augmented only after 38 ^{o}C and the release rate increased from several hours to minutes at increased temperature to 60 ^{o}C.

In summary, with the presented doctorate thesis, a new preparation method of liposomes and lipid structures and their use in biosensor development as signal enhancers, nanoreactos and/or templates for the shape and size controlled synthesis of metal nanoparticles, as well as basic characterization of thermo-sensitive liposomes for their use in controlled release of the drugs are reported.

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6.2 Future Works

I. Integration of thermo-sensitive liposomes to a microfluidic system in order to develop a lab-on-chip system with incremented sensitivity and signal with less demand of time.

II. Application of the method to target sensitive liposomes for their use in washless and reagentless immunosensor system.

III. Extension of the use of nanoliposomes and lipid nanostructures for synthesis of other metal nanoparticles (e.g palladium) or metal nanoalloys (e.g Au-Pd).

IV. To study catalytic activity of the prepared gold nanoparticles and nanostructures.

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