

Project title: Design, characterisation and understanding of target sensitive liposomes for signal enhancement in ELISAs, ELONAs and biosensors

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Project Objective: The project objective is to design and characterise liposomes for maximal encapsulation of reporter molecules (fluorophores, magnetic nanoparticles, enzymes) to facilitate the development of ultrasensitive biosensors. The overall aim should be to try and understand the underlying mechanism of target sensitive liposomes and to develop tools to probe this mechanism.

Sub-objectives of the project are: i) optimise liposome preparation to realise homogenous, stable liposome populations of optimal size (in terms of compromise between maximal encapsulation and steric hindrance); ii) obtain liposomes with rapid lysing kinetics; iii) application of system to alternate biomolecules (aptamer, antibody fragments, DNA); iv) realisation of washless, ultrasensitive system

Introduction: Liposomes, phospholipid vesicles, are spherical lipid bilayers capable of entrapping water soluble solutes within an aqueous domain or alternatively lipid molecules within the lipid bilayers. They are biodegradable, biocompatible and non-immunogenic in nature. Phospholipids have a variety of shape behaviour in aqueous media, e.g. conical, inverted cone, truncated cone, cylindrical and others; this is due to the different charges and interactions between the molecule and the surrounding media. Generally, a liposome is made with different lipids, a main component and one or two stabilising lipids. Biological membranes are, in fact, a fluid mixture of lipids and other molecules; the richness of their chemical and mechanical properties is often dictated by an asymmetric distribution of these molecules. Depending on the selected phospholipids and its ratio in the pre-mixture to construct the vesicle, the final liposome will be more or less stable because of the tendency or not to spontaneously form micelles. This means that a diverse range of different applications can be developed regarding the enormous possibilities given by the distribution of those molecules in the bilipidic layer; moreover, chemical or biochemical conjugations can be added.

Types of Liposomes

pH Sensitive The pH can be calculated at which the lamellar phase becomes unstable, a value that depends continuously on the system composition. The understanding serves for preparing tunable, pH-sensitive vesicles.

Temperature Sensitive These liposomes are formed by a mixture of synthetic lipids that exhibits a gel to sol transition at temperature few degrees above the physiological temperature, a range easily obtainable by local hyperthermia.

Target Sensitive Target sensitive liposomes are liposomes that are stabilised by certain molecules (e.g. antibody). These liposomes can be exploited to avoid the need for a washing step in ELISAs, ELONAs and biosensors. Antibody linked target sensitive liposomes are very stable, as they allow the acylated antibody comprising part of the bilayer to freely move within the lipid bilayer, the antibody adding stability to the bilipidic structure. Even if these antibody liposomes bind to antigen in solution, they remain stable – however, when the liposome linked antibody-antigen complex links to surface (electrode) immobilised antibody, the acylated antibody is now anchored and no longer free to move, and the integrity of the liposome stability is

disturbed and lyses, releasing the encapsulated enzyme to interact with the surface immobilised substrate and provide signal.

Antibody linked liposomes that have not interacted with the immobilised capture antibody (i.e. antibodies not linked to antigen, the target molecule), remain stable and the encapsulated enzymes remain encapsulated and unavailable for detection, and thus a wash step to remove unbound liposome labelled antibody is not required, fulfilling the requirements of a washless immunosensor. This is depicted schematically in Figure 1:

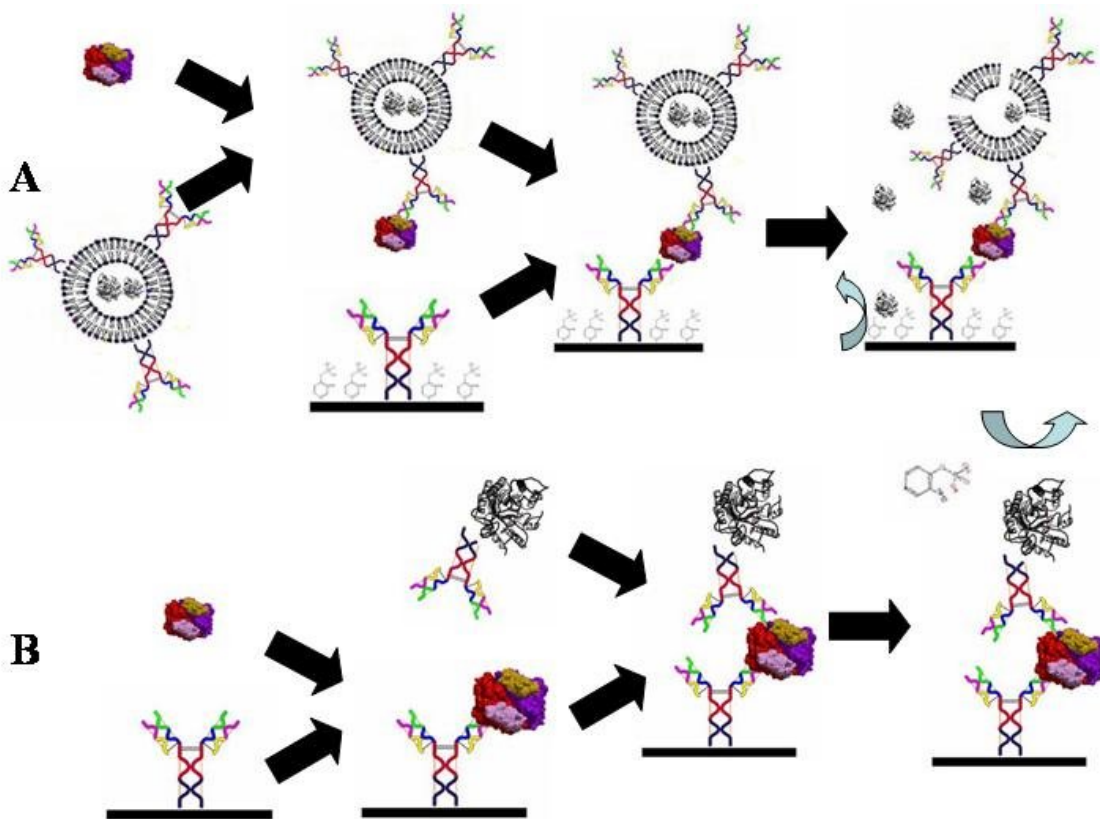


Figure 1 Methodology proposed in this work. The sample is pre-incubated with the functionalised liposome which has encapsulated enzyme. This is added to the pre-functionalised surface with the first bio-recognition molecule and the enzyme substrate. Due to mechanical properties of these liposomes once they become anchored via formation of the immunocomplex with the immobilised capture antibody, they break and release the encapsulated enzymes. Immediately, the enzymes will react with the immobilised substrate and a washing step to eliminate unbound liposome linked antibody is avoided (B). Standard immunoassay. A first incubation has to be made to immobilise the first bio-recognition molecule, then it has to be incubated with the sample and washed. The surface must be blocked and then incubated with the second labelled antibody. Finally, after washing to eliminate unbound labelled antibody, the substrate is added to have detection. In the first approach, much more enzyme molecules will react per unit of antigen.

Project Outline: The project can be divided into four separate inter-dependent sub-projects; i) Proof of concept; ii) Elucidation of mechanism and liposome formulations; iii) Liposome preparation; iv) Application to real systems

1. Proof of concept

Using liposome formulations developed by Huang (1980) for drug delivery, (i.e. using NHS-plamitic acid for antibody incorporation into liposome or linking antibody to amine

functionalised lipid e.g. DPPE, DOPE, DMPE etc.), but with modern liposome preparation techniques such as rehydration in the presence of molecule to be encapsulated and sequential extrusion through 800, 400 and 200nm membranes. ELISA will be carried out to demonstrate target sensitivity. The molecules to be encapsulated as 'model' molecules will be horse radish peroxidase (HRP) or the self quenching fluorescein, calcein.

2.a. Elucidation of mechanism

This element of the work will be quite challenging and may take place in latter stages of the DEA research work, thus allowing the researcher to have some experience but also to leave time for incorporation of the results into the PhD thesis.

A technique to look at the real-time transition from bilayer to hexagonal phase (the transition that causes rupture of the target sensitive liposomes) will be investigated. Preliminary investigations suggest the use of X-ray scattering (SAXS and WAXS). Using lipids of different structures and properties, a series of experiments to probe liposome rupture will be executed. POPE and DEPE are lipids with a lamellar structure, but as the temperature is increased, it is known that they transform to the hexagonal phase. DOPE, the lipid routinely used in target sensitive liposomes, is hexagonal. Thus, preparing liposomes using DOPE, POPE and DEPE (for example) and carrying out ELISA type experiments at room temperature, only the DOPE lipids are expected to be target sensitive if a lamellar to hexagonal phase change is responsible. However, if we increase the temperature when preparing the liposomes, and maintaining a high assay temperature, the POPE and DEPE should also be hexagonal, and if lamellar to hexagonal change is the key factor in the underlying lysis mechanism, the resulting liposomes should also be target sensitive.

This 'quick and dirty' type of approach would be confirmed using SAXS and WAXS. The effect of chain length by comparing DPPE, DMPE and DOPE (all hexagonal) would also be investigated.

2.b. Liposome formulations

The liposome formulation will be optimised in terms of (a) lipids used (b) ratio of linked Ab to lipids. Various formulations using DOPE, DMPE, DPPE, DEPE, DOPC, DMPC, DPPC, DEPC will be looked at and evaluated in terms of target sensitivity (can be evaluated with biotinylated lipids and streptavidin plates or antibody linked lipids and immobilised antigen). The lysis kinetics of each formulation will be studied. The ratio of lipid to antibody linked lipid will be optimised. Different techniques will be used to quantify the actual lipid ratio, rather than the starting lipid mixture which may, or may not correlate to the final liposome formulation. For example Ab and the lipid could each be labelled with different lanthanides, allowing their simultaneous detection and quantitation.

Stability studies will also be carried out with each formulation, in the presence and absence of stabilizer (e.g. DOPA).

Storage conditions and liposome preparations for storage will also be investigated (e.g. freeze dried vs freezer vs fridge), and the susceptibility of the liposome formulations to storage conditions (e.g. temperature, humidity, light, oxygen etc.).

3. Liposome preparation

a. Lipid Purity

The purity of lipids received from chemical companies (e.g. Avanti Lipids, Sigma) will be evaluated for their purity using thin layer chromatography.

b. Lipid Formulation

Based on the lipid formulation extrapolated above (Section 2), Ab-lipids will be prepared by SATA and maleimide activated lipid; SMCC/SDDP and lipid; Periodate oxidation) and the final Ab-lipids determined in terms of homogeneity, reproducibility of protocol, stability and efficiency.

c. Rehydration of lipid film

As can be seen in Figure 2a and 2b, the first step in any liposome preparation is the preparation of a lipid film, removal of solvent and rehydration of film in aqueous medium containing the molecule desired to be encapsulated.

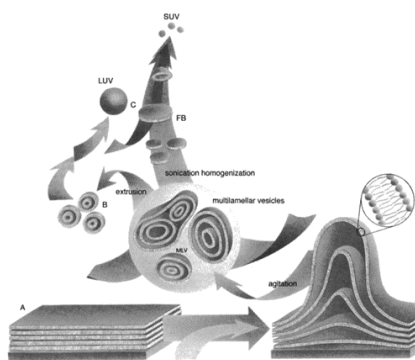


Figure 2a: Schematic of liposome preparation

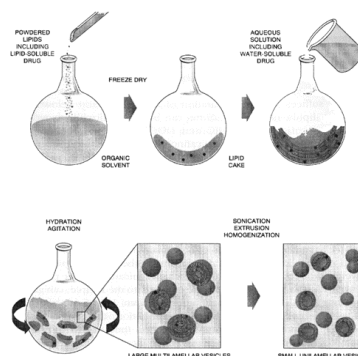


Figure 2b: Schematic of lipid film rehydration

The importance of the temperature of rehydration will be investigated and if deemed important a study carried out to look at the effect of the elevated temperatures on enzymes/fluorophores to be encapsulated. If enzyme activities are badly affected, the use of thermophilic enzymes will be exploited. The length of rehydration time and bulk concentration of the enzyme in the rehydration buffer will be optimised. The effect of buffer, buffer pH, buffer ionic strength will also be optimised. An estimation of the size and heterogeneity of the liposome population at this point will be carried out.

d. Dispersion

Following lipid film rehydration, a heterogeneous population of SUVs, MUVs, MLVs, and LUVs will exist and dispersion techniques are required to realise a homogeneous population of optimal size. Dispersion techniques to be studied include sonication, centrifugation, extrusion, freeze-thaw, ether vaporisation and reverse phase vaporisation. Diverse purification techniques will also be used - such as the use of a column or dialysis - or eliminating the purification step completely.

e. Characterisation

The liposomes will be characterised using a zetasizer for population homogeneity, enzyme activity assay for estimation of number of molecules encapsulated, phosphate assay to calculate enzyme molecule per lipid.

The compromise of liposome size versus steric hindrance will be evaluated. With a range of liposome sizes ELISA with a model system will be carried out and it is anticipated that with increasing liposome size (and thus encapsulation), that the response will increase until such a point where the liposome is too large and starts to cause steric hindrance problems and the response starts to decrease. The optimal liposome size for biosensor/ELISA application can thus be elucidated.

The lysis kinetics will be evaluated, as will the loss of enzyme activity due to liposome encapsulation. Finally, the effect of applying temperature/electric potential on lysis efficiency will be probed.

f. Liposome storage and stability

For preparation of liposomes for storage the following preps will be attempted: (i) Freeze-drying; (ii) Storage at 4°C in liquid form; (iii) Aliquot and freeze at -20°C; (iv) evaporation; (v) storage under nitrogen/argon. Additionally the use of mPEG linked lipids will be looked at. The liposomes will be prepared and over a period of six months their stability looked at by reconstituting them and carry out (a) establishment of population homogeneity by size measurement using zetasizer; (b) establishment of target sensitivity using ELISA; (c) establishment of enzyme activity post-lysis.

4. Application to real systems

Finally the system will be applied to the quantitative detection of specific analytes of interest - for example, coeliac disease toxic gliadin in foodstuffs, stroke markers in serum. The attainable detection limits, matrix effects, response time, sensor stability and cost will be established. The system will also be demonstrated for alternate biocomponents - antibody fragments, aptamers, DNA/mRNA.