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SHORT COMMUNICATION

The Interaction of Serum Albumin with Cholesterol Containing Lipid Vesicles

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Abstract In this paper, the interaction of both human blood serum (the primary fraction of which is serum albumin) and pure human serum albumin (HSA) with surface immobilised lipid vesicles was measured by combined Surface Plasmon Resonance (SPR) and Surface Plasmon enhanced Fluorescence (SPEFS), and fluorescence microscopy. It was found that both blood serum and HSA showed specific binding to vesicles which contained cholesterol, resulting in increased membrane permeability and release of encapsulated fluorescent dye. This effect was not seen with heat inactivated blood serum, heat inactivated HSA or in vesicles not containing cholesterol. These results suggest that HSA may have a physiological role over and beyond that of fatty acid carrier, possibly acting to regulate vascular endothelial cell cholesterol concentration.

Keywords Human-serum-albumin · Surface-plasmon-enhanced-fluorescence · Vesicles · Liposomes · Cholesterol

Introduction

Human Serum Albumin (HSA) is a globular protein with a molecular weight of 66 KDa. It is synthesised in the liver, and degraded within the kidneys. It is believed to have various physiological roles including the transport of hydrophobic molecules such as fatty acids, bilirubin and some vitamins through the vascular (circulatory) system [1]. HSA is the major component of human blood serum, at around 60 wt %. There is clinical evidence that lowered HSA levels in blood correlate with increased risk of vascular diseases and mortality [2]. However, there is some dispute, with later papers not finding clear correlative clinical data between lowered HSA levels and the formation of carotid plaques [3].

There is considerable interest in how cholesterol concentrations in blood may correlate with risk of atherosclerosis. Consequently, drugs such as statins have been developed to lower blood cholesterol and low density lipoprotein concentration, with apparent success in reducing atherosclerosis and other vascular problems [4]. The interaction of HSA with cholesterol has been studied by various researchers. A study by Ji-Sook Ha et al showed that HSA can mediate the efflux of cholesterol from cultured endothelial cells [5]. Other studies have shown that HSA binds non-esterified cholesterol in blood and transports it in the circulatory system, with around 24% of cholesterol bound to albumin in blood serum [6]. These findings taken together may suggest a causal link between the observation that persons with lowered HSA levels have greater vascular problems since HSA plays a critical role in both cholesterol transport and cell cholesterol levels.

Studies of the interaction of lipid vesicles with serum albumins date back to the 1980s, where increased interest in utilising lipid vesicles as drug delivery vehicles led to studies of albumin induced vesicle leakage [7]. Mui et al. in addition to reviewing much of the earlier work in this area, measured the osmotic sensitivity of lipid vesicles exposed to blood serum and found that the presence of blood serum significantly decreases the osmotic gradient required to cause vesicle rupture [8].

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In this paper we have made a simple model membrane system consisting of surface tethered lipid vesicles, and have studied how both serum and HSA bind to such membranes and the effect these systems have on vesicle stability.

Experimental

Formation of supported vesicles on surface

Thin gold films (ca. 50 nm thick) were made by thermal evaporation of gold in an Emitech K975 thermal evaporator onto high refractive index (n=1.80) $LaSFN_9$ glass slides (Berliner glass) at a pressure of 4×10^{-6}mbar. The coated glass was subsequently annealed at 450°C for 2 min and then cleaned under a high intensity UV lamp / ozone for 10 min before rinsing in ethanol and water for 20 min. A binary SAM consisting of a biotin thiol and mercaptounadecanol lateral spacer was formed by immersion of the clean gold / glass slides in an ethanolic solution of the two thiol moieties at 1 mol % 11-mercaptododecanoic-(-8-biotinoyl-amido-,3,6-dioxaoctyly) amide (thiol – biotin) and 99% mercaptoundecanol (Sigma) for 16 hours before rinsing in ethanol and water. The biotin thiol consists of a C11 mercapto-alkyl chain, three ethoxy spacer units and a biotin head group [9]. The biotin thiol was synthesised in our laboratory following the procedure described by Booth et al. [10]. The biotin surface was coupled to streptavidin (500 nmol dm^{-3} in 10 mmol dm^{-3} HEPES buffer containing 2 mmol dm^{-3} $CaCl_2$ and 150 mmol dm^{-3} NaCl, to create the 'capture' surface for the lipid vesicles. Experiments were run at ambient temperature of 22 +/− 1°C.

Large Unilamella Vesicles (LUVs) were made by mixing 60 mol % dimyristolyl-phophatidylcholine (DMPC), 35 mol % cholesterol and 5 mol % dipalmitoyl-phosphoethanolamine-N-biotin (DPPE-biotin) (Sigma U.K.) in chloroform, and drying under vacuum for 12 hours and re-suspending in the HEPES / $CaCl_2$ / NaCl buffer described above, with 1 µmol dm^{-3} water soluble bodipy dye ($ex._{max}$=650 nm, $em._{max}$=665 nm) added to give a final lipid concentration of 0.4 mg mL^{-1} [11]. The dye was purchased as an NHS ester but hydrolysed to the carboxylic acid in acidified water (pH 5) water prior to use, removing the active NHS group. The lipid suspension was extruded through 100 nm diameter pores in a polycarbonate membrane in a lipid extruder (Avestin). The biotin tagged vesicles were added to the streptavidin catcher surface and binding followed using Surface Plasmon Resonance. Vesicles were allowed to bind for 30 min before rinsing in buffer to remove non-encapsulated fluorophore and non-bound vesicles from the system. Further details of the construction of the sensor surface can be obtained in reference [12].

Surface Plasmon Resonance(SPR) and Surface Plasmon enhanced Fluorescence (SPEFS) measurements

SPR was used to follow the construction of the modified surface. Full details of the SPR / SPEFS system are provided in references [13] and [14]. SPR measures the angle of resonance when light is coupled into a thin metal film, normally via a prism. The angle at which surface plasmon resonance takes place, observed as a reflection minimum, is highly sensitive to changes in the dielectric constant. The adsorption or loss of a surface film within the evanescent field of a metal surface can thus be measured *in-situ* and in real time. The home-built instrument used in these experiments followed the changes in reflection intensity at an angle around 1.5° lower than the resonance angle. Hence, changes in reflected light intensity at the fixed angle allowed real-time measurement of HSA binding. SPEFS utilizes the

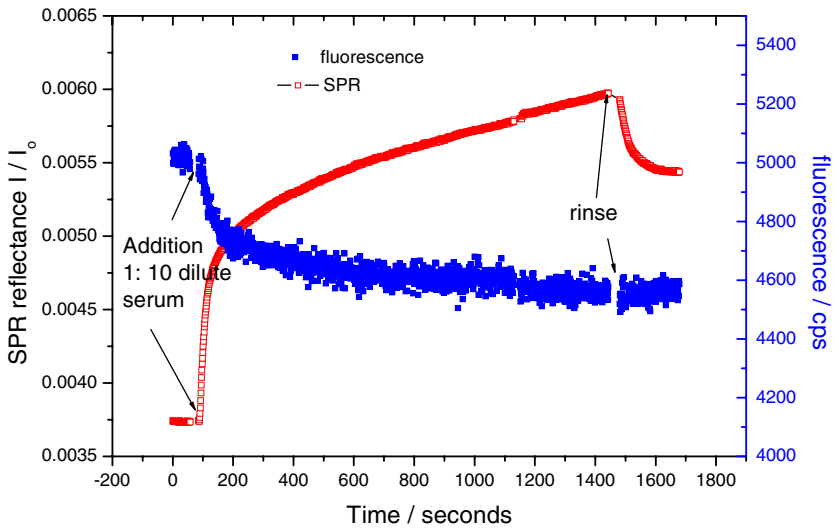

Fig. 1 Combined SPR and SPEFS response from the addition of human blood serum (1:10 diluted in HEPES-buffer) to vesicles containing cholesterol. The almost simultaneous binding of serum components and release of dye is clearly seen. The SPR reflectance is plotted on the left (open squares) with fluorescence from the encapsulated dye in vesicles being plotted on the right hand side

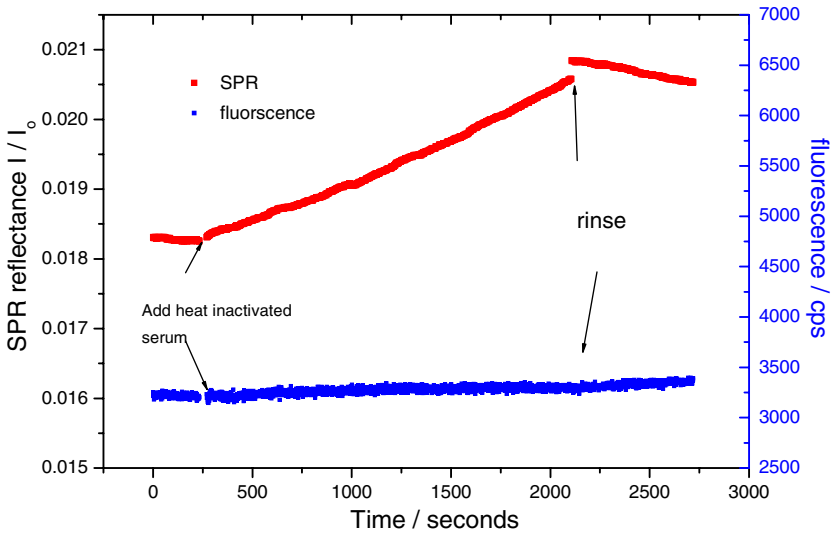

Fig. 2 Results from a control experiment, showing the combined SPR and SPEFS response from the addition of heat inactivated human blood serum to vesicles containing cholesterol. Weak non-specific binding of deactivated blood serum is observed but no dye release

intense electric field created by the resonating surface plasmons to excite fluorophores situated within the evanescent field. This approach gives three advantages over conventional fluorescent excitation: 1. Only fluorophores located close to the surface i.e. in this case in surface attached vesicles are excited; 2. There is a significant enhancement of the fluorescence excitation and emission; [14] The SPEFS and SPR responses are monitored simultaneously and in real-time.

Preparation of blood serum, HSA and heath inactivation

Blood serum was obtained from a research student in the laboratory. Whole blood was taken and centrifuged to separate the serum component. This was diluted at a 1: 10 in HEPES in all experiments which measured whole serum. HSA was obtained from Sigma (UK) and used at 40 mg mL^{-1}, approximately equivalent to typical blood serum concentrations. Where appropriate, HSA and serum were heat deactivated by heating at 60°C for 15 min.

Results and discussion

The interaction of blood serum with surface immobilized lipid vesicles

Blood serum was applied to the surface immobilized vesicles. Figure 1 shows the effect of the serum on the vesicles, with specific binding of serum components being observed by SPR and the release of the encapsulated fluorescent dye measured by SPEFS. A control experiment with heat inactivated serum (60°C, 15 min) was also performed (Fig. 2). It can be seen that no specific binding

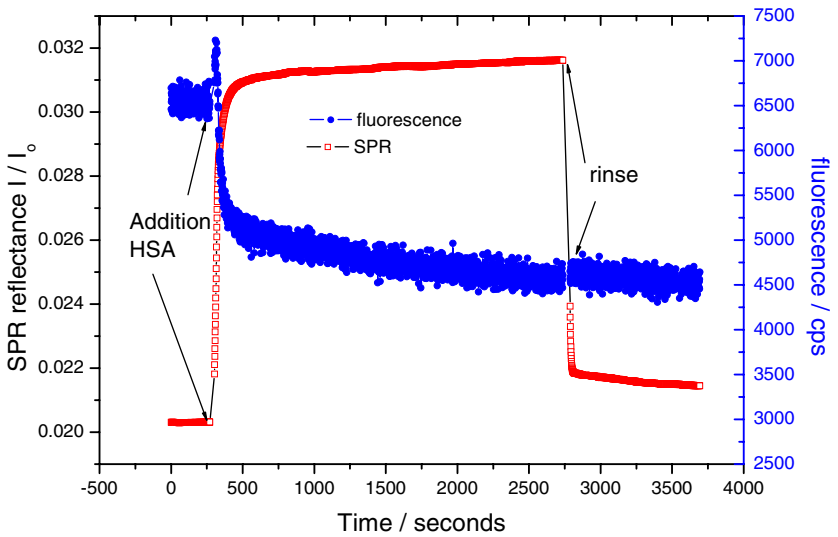

Fig. 3 Combined SPR and SPEFS response from the addition HSA at 40 mg mL^{-1} to vesicles containing cholesterol. A response similar to that from blood serum is observed, although with a faster protein binding and a greater proportionate decrease in fluorescence, expected since HSA is more concentrated than in the diluted blood serum

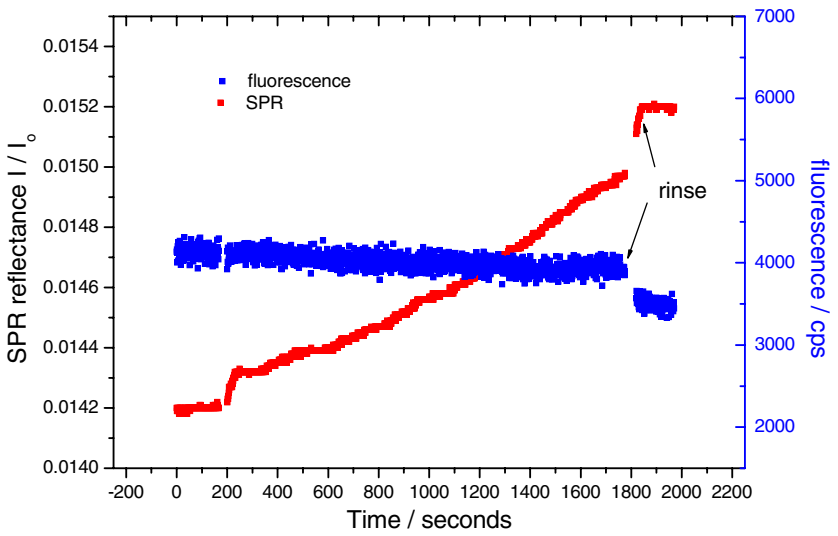

Fig. 4 Addition of heat-denatured HSA at 40 mg mL^{-1}. No dye release is observed and only slow non-specific binding seen

of serum components are observed, although a small amount of non-specific binding of the serum to the surface was seen. The important point is that no destruction of the membrane resulting in release of fluorescent dye was observed.

The initial interpretation of these measurements was that complement factors present in blood serum were interacting with the membrane, via the formation of a Membrane Attack Complex (MAC), causing permeation and dye release. However, experiments with various concentrations of known alternative pathway complement inhibitor Sbi-E, failed to suppress the binding of serum components and concomitant dye release [15]. This was an unexpected result, which led us to consider whether the effect was due to interaction of the primary component of human serum: HSA, might be causing the observed behaviour.

The interaction of Human Serum Albumin (HSA) with surface immobilized lipid vesicles

The physiological concentration of HSA in human blood is around 40 mg mL^{-1}. This concentration of HSA was used to attempt to replicate the conditions found within the human vascular system.

The effect of adding HSA to the cholesterol containing surface immobilised vesicles gave results that were not dissimilar to that observed for blood serum, with the exception that on rinsing, a far greater proportion of the initially adsorbed protein appeared to be washed away. Figures 3 and 4 shows the fast binding of HSA to the surface (by SPR) and the rapid release of encapsulated dye from the vesicles, as measured by SPEFS. These results immediately suggested that the results from the experiments

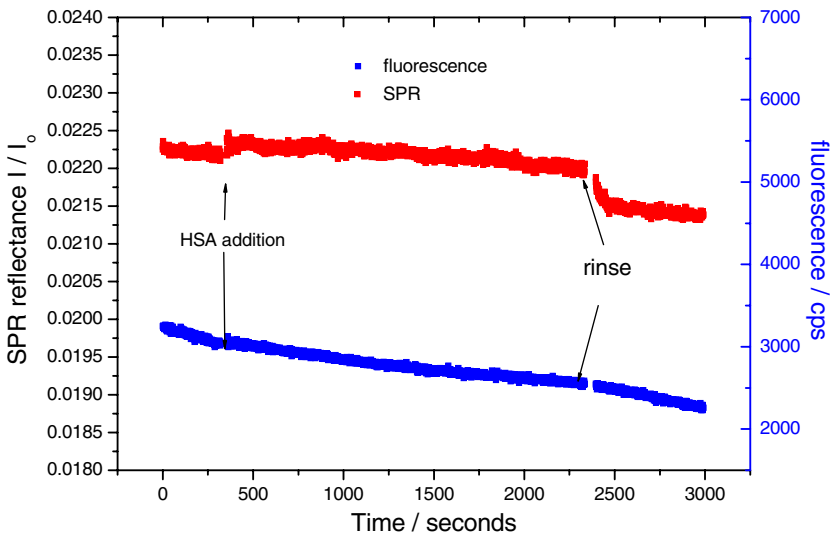

Fig. 5 Addition of 40 mg mL^{-1} HSA to vesicles without cholesterol. No binding or release is observed, confirming the essential role of membrane cholesterol as a substrate to HSA

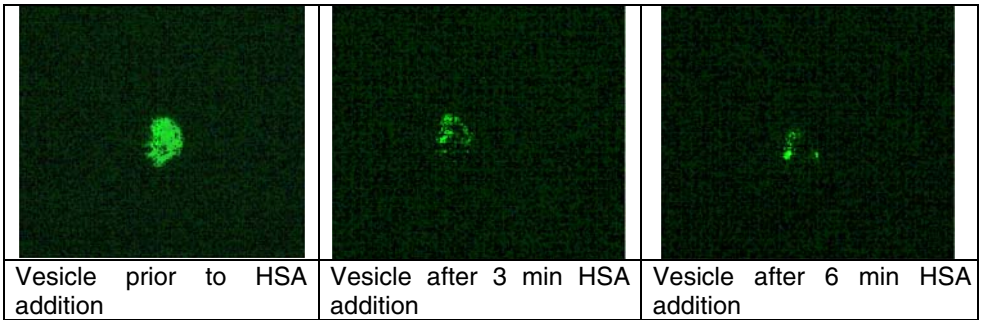

Fig. 6 Fluorescence microscopy of surface immobilised giant unilamella vesicle with identical lipid composition as LUVs used in SPR / SPEFS study. Leakage of dye mediated by HSA is clearly seen, visually supporting the results from SPR / SPEFS

with serum were probably due to HSA interaction with the membrane. A control experiment was also carried out, with HSA being heated at 60°C for 15 min before it was added to the vesicles.

Following these experiments, it was important to ascertain whether cholesterol in the vesicle membrane was critical to HSA binding and subsequent vesicle destabilisation. Lipid vesicles without cholesterol were less stable than vesicles with cholesterol, with a greater degree of passive dye leakage being observed (Fig. 5). Addition of HSA (at 40 mg mL^{-1}) to cholesterol free vesicles showed no significant binding of HSA or increased rate of dye permeation through the membrane.

The final measurements utilised Giant Unilammelar Vesicles (GUVs) immobilised on collagen coated Petri dishes. The vesicles were obtained following the procedure described by Moscho et al. [16]. Water soluble fluorescein dye was used in these measurements, with images being obtained on a Nikon Eclipse TE-2000-S fluorescence microscope.

Figure 6 shows the rapid loss of fluorescence from an attached vesicle following HSA addition. A control measurement, with a vesicle being imaged over the same time scale in the absence of HSA showed no measureable fluorescence loss.

The effect of cholesterol on model cell membranes has been long studied. A review from 1972 by Oldfield and Chapman details differential scanning calorimetry measurements of gel — liquid phase transitions in lipid membranes, and how addition of cholesterol removes the sharp transition, probably by preventing lipids crystallizing below the T_c [17]. More recent work by Corvera et al studied the effect of acyl chain length and cholesterol concentration on lipid membranes [18].

Although these results need to be interpreted with some care, it could be hypothesised that HSA is binding to the vesicles and in doing so removing or changing the location of cholesterol within the membrane, in the process destabilising the vesicle causing the observed rupture and dye release. The suggested process is schematically illustrated in Fig. 7. The removal of cholesterol from the vesicles might be expected to cause phase separation of the remaining lipids, especially the longer chain DPPE-biotin. The inherent 'leakiness' of non-cholesterol containing vesicles can be seen in Fig. 5. The results from Figs. 1, 2, 3 and 4 suggest that the inclusion of cholesterol in the membrane not only stabilises the vesicles in the absence of correctly folded HSA or serum, but also provide a specific binding substrate for HSA. This is perhaps unsurprising

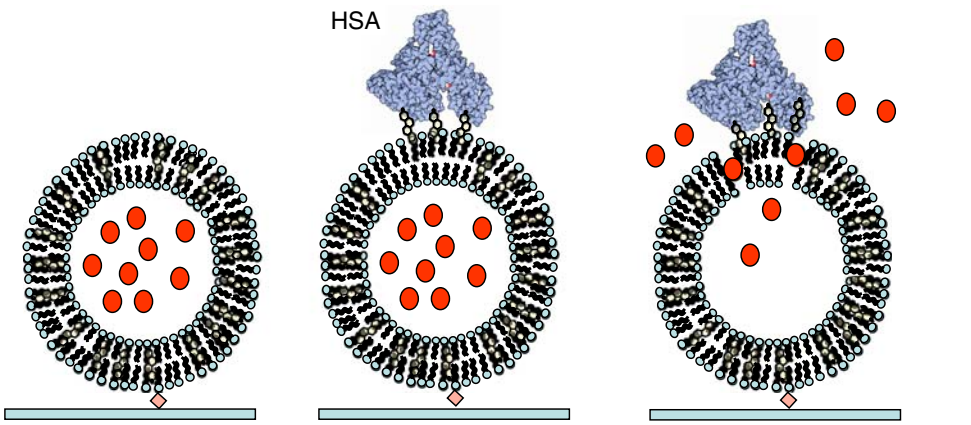

Fig. 7 Illustration of processes that may be involved in HSA binding to cholesterol in vesicle membrane. HSA binds to the cholesterol in the membrane and sequestrates cholesterol from the membrane into protein binding pockets, resulting in a destabilization of the vesicle structure and dye release

given the known physiological role of HSA. The model system described in this paper provides a relatively straight forward platform for further study of the interaction of cellular cholesterol with HSA which may have potential applications in screening for drugs that might control cellular cholesterol concentration.

Conclusions

This work complements many of the early studies of serum induced lysis of lipid vesicles, but critically shows that it is the serum albumin component of blood serum and not other factors that induces lysis. Moreover, the vesicles used in this study were isotonic with the surrounding buffer, so an osmotic gradient was not required to induce lysis. Finally we have shown that cholesterol in the membrane appears to be either the binding substrate for HSA or critically involved in organising membrane lipids such that HSA could bind and lyse the vesicles. These results have some relevance, since cholesterol has been used or proposed for the stabilisation of lipid vesicles containing drugs, for applications in targeted drug delivery systems [19]. It is clear from the results presented in this paper that this strategy, without the addition of further stabilizing components (such as PEGs), will not be successful. Finally, the results support the observations made by Zhao et al. and Ji-Sook Ha et al. that HSA regulates cholesterol efflux from endothelial cells in the vascular system [1, 5].

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