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Binding of biotinated-liposomes to streptavidin is influenced by liposome composition

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Abstract

We describe an 'in vitro' assay which allows rapid quantification of the binding of biotinated-vesicles to streptavidin immobilised on microtitre plates by estimating levels of a liposome encapsulated fluorescent molecule, rhodamine 123. It is shown that optimal vesicle binding to streptavidin occurs when a six carbon biotin spacer arm derivative of distearoylphosphatidylethanolamine (biotin-X-DSPE) is incorporated in liposomes. This alleviates steric hindrance arising due to the inclusion of small amounts of large bulky amphiphiles such as monosialoganglioside (G_{M1}, 5 mol%) in vesicles. In contrast the ability of liposomes containing poly(ethylene glycol) derivatives of DSPE (PEG_{2000}-DSPE, 5 mol%) to bind streptavidin was only marginally better when biotin-X-DSPE was substituted for biotin-DSPE in vesicles. It is further shown that amounts of biotinated-vesicles bound to streptavidin were minimally influenced by the fluidity of the liposome preparation when assayed at 4°C. However, at elevated temperatures (37°C) lipid estimates as determined by vesicle entrapped rhodamine 123 were low due to leakage of this marker from vesicles. This was shown by comparing amounts of biotinated-liposomes bound to streptavidin coated plates using the lipid marker [^{3}H]cholesteryl hexadecyl ether to estimates determined by vesicle entrapped rhodamine 123. The 'in vitro' assay protocol described here is a general method applicable in the optimisation of other targeting protocols. In conclusion our work suggests that liposomes containing G_{M1} and the spacer arm derivative biotin-X-DSPE bind optimally to immobilised streptavidin which should aid in the use of biotinated-liposomes in 'in vivo' targeted delivery applications.

Keywords: Liposome; Streptavidin; Biotin; Ganglioside; Poly(ethylene glycol); Targeted liposome

1. Introduction

The ability to conjugate antibodies and other targeting proteins to liposomes has many potential applications such as drug delivery to cells 'in vitro' and 'in vivo' as well as in diagnostic protocols [1,2]. To this end a variety of methods have been developed for coupling proteins to liposomes. These include the use of detergent dialysis to incorporate fatty acyl derivatised antibody into vesicles [3], direct coupling of proteins to the liposome surface by covalent chemistry [4] or approaches based on a liposome bound 'sandwich' protein which recognises a targeting antibody such as protein A (binds certain IgG isotypes by the Fc portion [5]), anti-mouse IgG (species specific recognition [6]), or streptavidin (high-affinity binding of biotin derivatised proteins [7,8]). In addition, the use of liposomes containing small haptens has been explored [9,10]. The merits of each of these approaches with regard to ease of preparation and flexibility related to the types of targeting ligands capable of associating with vesicles, has been discussed [11].

Levels of liposomes associated with target surfaces or cells will depend on the ability of protein or hapten derivatised lipids in vesicles to bind their target ligand. Few studies have addressed how parameters such as lipid composition will affect this critical step. In particular, the adaptability of liposome formulations optimised for 'in vivo' applications in targeted liposome

Abbreviations: G_{M1}, monosialoganglioside; PEG, poly(ethylene glycol); biotin-DSPE, *N*-biotinoyl distearoylphosphatidylethanolamine; biotin-X-DSPE, *N*-((6-biotinoyl)amino)hexanoyl) distearoylphosphatidylethanolamine; Hepps, *N*-2-hydroxyethylpiperazine-*N*'-3-pro panesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid; HBS, 25 mM Hepes/150 mM NaCl (pH 7.5); PBS, phosphate-buffered saline.

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protocols has not been fully addressed. The aim of this work was first to develop a sensitive assay for screening parameters such as lipid composition which could significantly influence this step. The assay was based on liposome encapsulated rhodamine 123, a highly fluorescent laser dye which localises to mitochondria in cells [12]. This intra-cellular distribution in conjunction with the maintenance of a substantial membrane potential across the membrane of the organelle suggested to us that rhodamine 123 would be a good candidate for active liposome loading strategies based on pH gradients [13]. In this report we first describe optimised conditions for the preparation of highly loaded rhodamine-123 vesicles containing biotinated lipids. We then describe a microtitre based protocol which was used to examine the influence of lipid composition on the binding of biotinated-liposomes to streptavidin. Our results show that liposome composition plays a critical role in the ability of streptavidin to bind biotinated-liposomes. This information will aid in optimising targeting strategies based on the streptavidin biotinated-liposome system prior to their use 'in vivo'.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylcholine (EPC) were obtained from Avanti Polar Lipids (Alabaster, USA). *N*-Biotinoyl distearoylphos phatidylethanolamine (biotin-DSPE), *N*-((6-biotinoyl)amino) hexanoyl) distearoylphosphatidylethanolamine (biotin-X-DSPE) and *N*-((monomethoxy-poly(ethylene glycol)$_{2000}$) succinoyl)-2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (MePEG$_{2000}$-S-POPE) were obtained from Northern Lipids (Vancouver, B.C., Canada). Rhodamine 123 was purchased from Molecular Probes (Eugene, OR, USA). [^{3}H]Cholesteryl hexadecyl ether was obtained from NEN (Hertfordshire, UK). Monosialioganglioside (G$_{M1}$), cholesterol, Hepes and citric acid were purchased from Sigma. All other chemicals were of standard grade.

2.2. Methods

2.2.1. Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared as described by Hope et al. [14]. Briefly, appropriate amounts of lipid mixtures dissolved in chloroform were deposited in a tube and dried to a lipid film under a stream of nitrogen followed by high vacuum for 2 h. Lipid samples (EPC, DPPC or DSPC, 59.9–59 mol%, cholesterol 40 mol% and biotin-DSPE or biotin-X-DSPE, 0.1–1 mol%) were hydrated in 300 mM citric acid buffer (pH 4.0), frozen and thawed five times and extruded ten times through two stacked 100 nm polycarbonate filters. In some cases, [^{3}H]cholesteryl hexadecyl ether, a non-exchangeable lipid marker [15] was included in preparations (1 μCi/μmol total lipid). For stealth liposomes, 5 mol% of G$_{M1}$ or DSPE-PEG$_{2000}$ were added to the lipid sample. Liposome size as estimated by quasi-elastic light scattering (Brookhaven laser light scattering apparatus, BI-90) for all liposome preparations studied was between 105 and 130 nm.

2.2.2. Preparation of rhodamine-123-loaded liposomes

Rhodamine-123-loaded liposomes containing biotinated DSPE derivatives (0.1–1.0 mol%) were prepared by a modification of the procedure of Mayer et al. [13]. Normally liposomes prepared in 300 mM citric acid buffer (pH 4.0) were exchanged on PD-10 columns equilibrated with 25 mM Hepps, 150 mM NaCl (pH 8.5). Rhodamine 123 (400 nmol) was incubated with chromatographed liposomes (1 μmol, 5 mM total lipid) for 1 h at 60°C. Unencapsulated rhodamine 123 was removed from liposomes by gel filtration on PD-10 columns equilibrated with 25 mM Hepes, 150 mM NaCl (pH 7.5) (HBS). Rhodamine 123 to lipid ratios were estimated by monitoring fluorescence for rhodamine at 536 nm using a Perkin-Elmer Luminescence Spectrophotometer LS50B with an excitation wavelength of 512 nm and phospholipid by the colorimetric method of Fiske and SubbaRow [16].

2.2.3. Microtitre based liposome binding assay

Binding of liposome preparations containing biotin derivatives of DSPE to streptavidin was studied on microtitre plates. In the majority of experiments the fluorescent molecule rhodamine 123 which was actively loaded into liposomes was used as a liposome reporter molecule. Microtitre plates (96 well, Nunc) were coated with streptavidin by incubating protein (0.1–1.0 μg/well in 50 mM carbonate buffer (pH 9.6)) with plates at 37°C for 1 h. Streptavidin binding to microtitre plates was by non-specifc absorption. Plates were then washed with Tween wash solution (0.05% Tween 20 in 150 mM NaCl) three times and then once with HBS to remove unbound streptavidin. Biotinated-liposomes (0–50 nmol in 200 μl of HBS) containing rhodamine 123 (250–390 nmol/μmol total lipid) were added to wells and plates were incubated for a further hour at 37°C. After four washes of plates with HBS, levels of plate associated liposomes were determined after addition of ethanol (80%, 300 μl/well) by estimating rhodamine-123 fluorescence as described above. Non-specific binding of liposomes to plates was determined by estimating levels of biotinated-liposomes bound when incubated in the presence of excess free biotin (1000 molar excess to the biotinated lipid

derivative in liposomes). In a number of experiments, the non-exchangeable lipid marker [^{3}H]cholesteryl hexadecyl ether [15] was employed to estimate levels of biotinated-liposomes bound to streptavidin coated microtitre plates. Binding studies were carried out essentially as described for rhodamine-123-loaded biotinated-vesicles. For radioactive lipid estimates, liposomes were first solubilised in phosphate-buffered saline containing 0.5% Triton X-100 (300 μl) by heating plates for 5 min at 60°C and lipid was quantified by counting aliquots (200 μl) in a Beckman LS 1801 scintillation counter in Beckman Ready Safe scintillation fluid. Each micotitre experiment was performed in triplicate and presented results are representative of an experiment that was repeated a minimum of three times.

3. Results

The aim of this study was first to develop a rapid and sensitive method to detect binding of liposomes containing biotinated lipid derivatives to streptavidin. The fluorescent molecule rhodamine 123 was chosen as a liposome marker. In Fig. 1 it is shown that this molecule is efficiently encapsulated in liposomes by pH remote loading strategies [13]. The optimum external pH for uptake was found to be pH 8.5. Encapsulation efficiency was routinely between 85–95% for all liposome compositions examined when a rhodamine to lipid ratio of between 250 ando 400 nmol/μmol total lipid was employed. Liposomes retained rhodamine 123 and were stable with respect to vesicle size for several months when stored at 4°C.

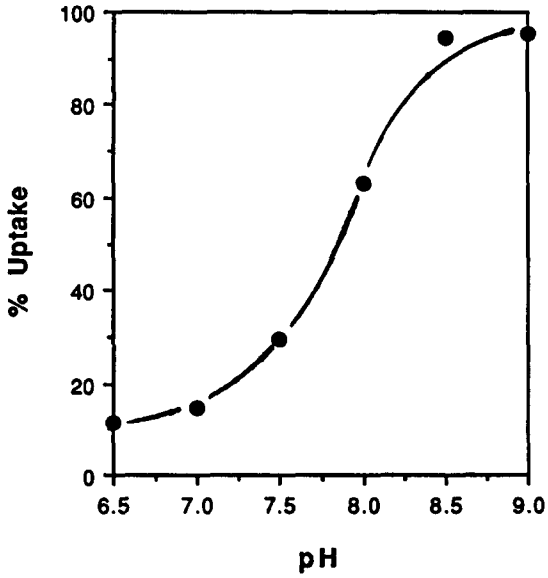

Fig. 1. Effect of pH on accumulation levels of rhodamine 123 in biotinated-liposomes. Liposomes (DSPC, 59.75 mol%; cholesterol, 40 mol%; biotin-DSPE 0.25 mol%, 5 mM) prepared in citric acid buffer (300 mM, pH 4) were incubated for 1 h at 60°C with rhodamine 123 (400 nmol/μmol total lipid) at various pH values (pH 6.5 to pH 9.0). Samples were chromatographed on PD 10 columns to remove unencapsulated rhodamine 123. Rhodamine 123 to lipid ratios were determined as described in Materials and methods.

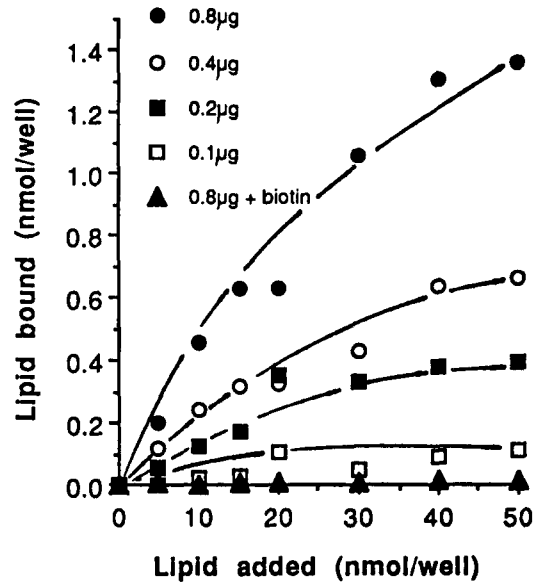

Fig. 2. Optimisation of the binding of biotinated-liposomes to streptavidin coated microtitre plates. Microtitre plates were coated with various amounts of streptavidin (0.1 to 0.8 μg per well) as described in Materials and methods. Liposomes (DSPC, 59.75 mol%; cholesterol, 40 mol%; biotin-DSPE 0.25 mol%.) containing rhodamine 123 (rhodamine 123 to lipid ratio: 372.4 nmol/μmol total lipid) were added to plates (0 to 50 nmol lipid in 200 μl per well) and incubated 37°C for 1 h. Levels of liposomes bound were quantified by measuring vesicle associated fluorescence due to rhodamine 123 after lyses of liposomes by ethanol (see materials and methods). For non-specific lipid binding estimates, biotin-DSPE liposomes were incubated in plates in the presence of excess free biotin (1000 molar excess to the biotinated lipid component in liposomes). Data is presented as an average of triplicate measurements.

In Fig. 2 it is shown that liposomes containing biotinated lipid derivatives bound in a specific manner to streptavidin on streptavidin-coated microtitre plates. Lipid was estimated from measurements of fluorescence due to vesicle-associated rhodamine 123. Increases in the coating concentration of streptavidin resulted in a corresponding rise in levels of biotinated-liposomes (59.75 mol% DSPC, 40 mol% cholesterol, 0.25 mol% biotin-DSPE) bound to plates. For example, increasing the amount of streptavidin from 0.1 μg to 0.8 μg resulted in a 14-fold increase in lipid levels bound to plates when 50 nmol of lipid were added to wells. Lipid binding was dependent on the incorporation of biotin-DSPE in vesicles as minimum amounts of biotinated-liposomes bound to plates in the presence of excess free biotin (1000 molar excess to the biotinated lipid derivative in Liposomes; 0.8 μg streptavidin per well). A coating concentration of 0.4 μg streptavidin per well was chosen for all subsequent experi-

ments as this gave a high signal to background within the lipid range 5–50 nmol/well. As emphasised here differences in amounts of streptavidin used in the microtitre plate coating step can significantly influence estimates of lipid bound to plate. To discount the possibility that this contributed to variations in estimates of lipid levels bound to immobilised streptavidin for the various liposome systems under study, a standard liposome preparation (DSPC/cholesterol/biotin-DSPE, 59.75 mol%, 40 mol%, 0.25 mol%) was routinely run. In all further figures data are corrected for non-specific binding and are presented as averages of triplicate measurements.

In Fig. 3, binding of liposomes (59.9–59 mol% DSPC, 40 mol% cholesterol,) containing either biotin-DSPE (0.1–1 mol%; Fig. 3A) or a six carbon spacer arm biotin derivative of DSPE (biotin-X-DSPE, 0.1–1 mol%; Fig. 3B) to streptavidin coated plates was examined as a function of lipid concentration. Estimates of biotin-DSPE vesicle levels bound to immobilised streptavidin correlated with the mol% of the biotinated lipid derivative included in the preparation. For example, on addition of 50 nmol of lipid to wells, 0.75, 1.25 and 2 nmol of lipid bound to streptavidin on microtitre plates when vesicles contained 0.1, 0.25 and 1 mol% biotin-DSPE, respectively. For biotin-X-DSPE-liposome, amounts of lipid bound to streptavidin coated plates were significantly higher and shown to be independent of the mol% of biotinated lipid derivative included in vesicles within the range of 0.1–1 mol%. For example on average 2.2 nmol of lipid were detected bound to immobilised streptavidin when 50 nmol of lipid were added to wells.

Results shown in Fig. 4 suggest that the binding of biotinated-liposomes to streptavidin was influenced by the degree of saturation of the fatty acid and the fatty acyl chain length of the phosphatidylcholine lipid component. For example when EPC, DPPC or DSPC cholesterol liposomes containing biotin-DSPE were added at 50 nmol per well, 0.4, 0.7 and 0.8 nmol of lipid bound to microtitre plates respectively as estimated by vesicle associated rhodamine 123 (Fig. 4A). Similarly when compared to EPC and DPPC vesicles, DSPC/Chol vesicles containing biotin-X-DSPE bound to immobilised streptavidin best (0.8, 1.2 and 1.6 nmol of lipid bound for EPC/Chol, DPPC/Chol and DSPC-/Chol liposomes, respectively, when 50 nmol lipid was added per well, Fig. 4B). To address whether low estimates of vesicles bound to plates was due to rhodamine-123 leakage from fluid liposome preparations, liposome binding and subsequent washing steps were carried out at 4°C. In Fig. 4C it is shown that under these conditions binding of EPC/Chol liposomes containing biotin-X-DSPE to streptavidin on plates was significantly higher than estimates obtained at 37°C over a range of lipid concentrations. In addition

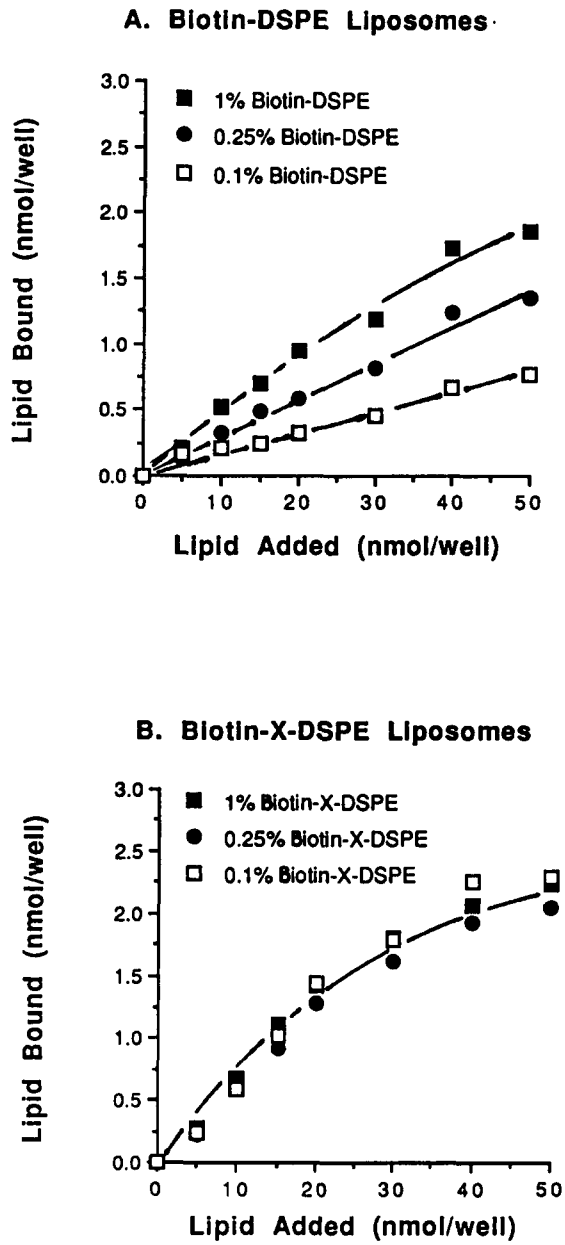

Fig. 3. Effect of inclusion of a biotin spacer arm derivative of DSPE in liposomes on levels of vesicle binding to streptavidin coated microtitre plates. Liposomes (DSPC, 59.75–59 mol%; cholesterol, 40 mol%; biotin-DSPE (Fig. 3A) or biotin-X-DSPE (Fig. 3B); 0.1 to 1 mol%) containing rhodamine 123 (250–350 nmol/μmol lipid) were added to streptavidin coated plates at various concentrations (0 to 50 nmol total lipid/well in 200 μl). Plates were incubated for 1 h at 37°C, washed and lipid remaining bound was estimated as described in Fig. 2. Presented data is an average of triplicate measurements and corrected for non-specific binding of biotinated-liposomes to plates.

amounts of DPPC/Chol/biotin-X-DSPE liposomes bound to streptavidin on plates approached that of DSPC/Chol/biotin-X-DSPE liposomes when assayed at 4°C.

To verify that the fluidity of liposome samples did not physically influence the binding of biotinated-lipo-

somes to streptavidin, liposomes with incorporated biotin-X-DSPE were prepared containing both the lipid marker [^{3}H]cholesteryl hexadecyl ether and aqueous entrapped rhodamine 123. Amounts of biotinated-vesicles bound to streptavidin-plates were estimated independently using these two markers in the same microtitre plate at equivalent streptavidin coating concentrations. High concentrations of streptavidin (1 μg per well) were employed in the coating step in this experiment in order to obtain reliable estimates for radioactive lipid samples. In Table 1 it is shown that levels of plate associated biotinated-liposomes, as determined by vesicle entrapped rhodamine 123, were dependent on both the temperature at which the experiment was performed as well as the type of phosphatidylcholine component included in the vesicle preparation. These findings confirm results presented in Fig. 4. For example, at elevated temperatures (37°C) amounts of DPPC/Chol/biotin-X-DSPE-vesicles associated with streptavidin on plates was significantly reduced when compared to levels detected at 4°C (3.814 and 4.495 nmol/well, respectively). In contrast, when [^{3}H]cholesteryl hexadecyl ether was employed to estimate lipid concentrations in binding experiments, levels of DPPC-biotinated-liposomes associated with streptavidin coated plates was found to be independent of temperature. In the case of saturated DSPC-vesicles

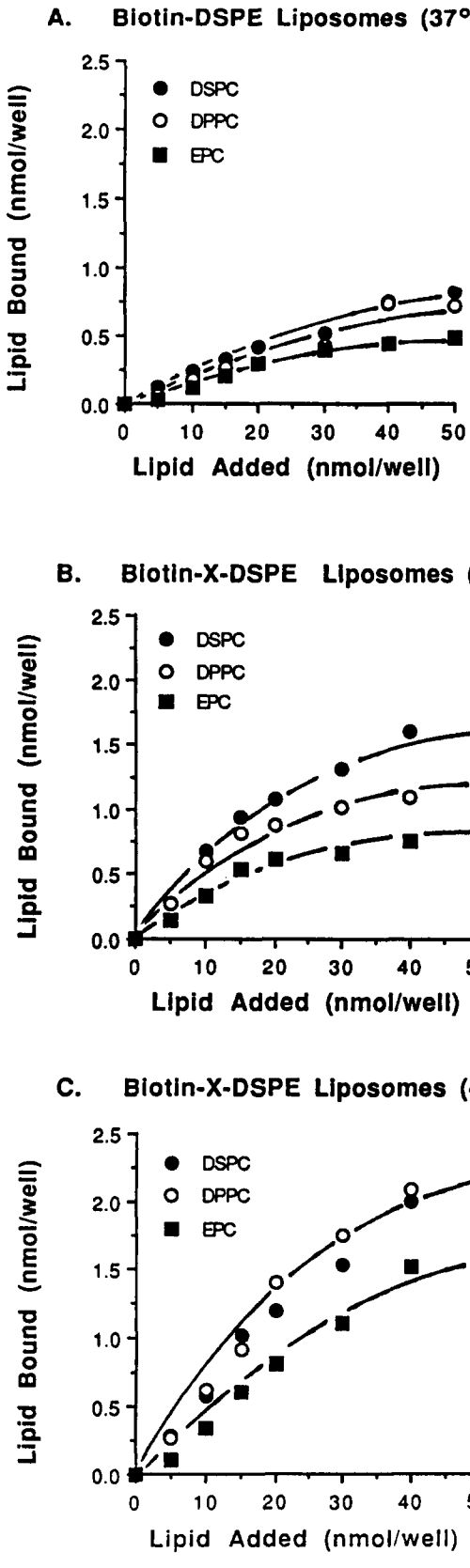

Table 1
Estimation of amounts of biotin-X-DSPE liposomes bound to streptavidin-coated microtitre plates by lipid and aqueous entrapped liposome markers

Biotinated-liposome sample	Temperature of assay (°C)	Lipid bound to streptavidin coated plates (nmol per well)		
		Rhodamine 123 lipid estimate	[^{3}H]Chol. hex. ether lipid estimate	Ratio [a]
DPPC vesicles	4°	4.495 ± 0.09	3.244 ± 0.04	1.386
	37°	3.814 ± 0.09	3.491 ± 0.28	1.076
DSPC vesicles	4°	4.590 ± 0.11	3.069 ± 0.14	1.496
	37°	4.475 ± 0.11	3.046 ± 0.23	1.469

Biotin-X-DSPE-liposomes (DPPC or DSPC 59.75 mol%; cholesterol, 40 mol%; biotin-X-DPSE, 0.25 mol%) with incorporated [^{3}H] cholesteryl hexadecyl ether (1 μCi/μmol total lipid) and containing rhodamine 123 (DPPC liposomes, 269 nmol/μmol total lipid; DSPC-liposomes, 288 nmol/μmol total lipid) were prepared. Amounts of biotinated-liposomes bound to streptavidin-coated plates (1 μg per well) at 4°C and 37°C were estimated as described in Materials and methods.

[a] Ratio of amounts of biotinated-liposomes bound to streptavidin-coated plates determined using vesicle encapsulated rhodamine 123 to levels estimated using the lipid marker [^{3}H] cholesteryl hexadecyl ether.

Fig. 4. Influence of lipid fluidity on biotinated liposome binding to streptavidin coated microtitre plates. Binding of rhodamine-123-loaded liposomes (EPC, DPPC or DSPC 59.75 mol%; cholesterol, 40 mol%; rhodamine 123 to lipid ratio: 290 to 372.4 nmol/μmol total lipid) containing biotin-DSPE (0.25 mol%; 4A) or biotin-X-DSPE (0.25 mol%, 4B and 4C) to streptavidin on microtitre plates was assayed at 37°C (4A and 4B) or 4°C (4C) as described in Fig. 2.

containing biotin-X-DSPE lipid, levels of lipid bound to plates as estimated by both lipid and aqueous markers did not differ when binding studies were carried out at either 4°C or 37°C. We therefore conclude that slow leakage of aqueous entrapped rhodamine 123 from vesicles at elevated temperatures can give rise to under estimates for levels of biotinated-vesicles bound to streptavidin coated microtitre plates, particularly when fluid phospholipids such as DPPC are included in the liposome preparation.

In Fig. 5 the effect of inclusion of large bulky amphiphiles in biotinated-liposomes on vesicle binding to streptavidin coated plates was investigated. When biotin-DSPE liposomes contain either G_{M1} or PEG-$DSPE_{2000}$ (5 mol%) a dramatic reduction in amounts of lipid bound to streptavidin in plates was detected. For example 0.25 nmol of lipid bound in the case of both G_{M1} or PEG-$DSPE_{2000}$ biotin-DSPE vesicles compared to 0.7 nmol for control biotin-DSPE vesicles on addition of 50 nmol of lipid added to wells. When the six carbon spacer arm biotin-X-DSPE derivative was substituted for biotin-DSPE in G_{M1}-vesicles, steric hindrance due to the large hydrophilic headgroup of G_{M1} was alleviated (Fig. 5B). In this case levels of lipid bound to streptavidin plates were high and comparable to biotin-X-DSPE liposome preparations which contained no G_{M1} (1.4 nmol per well, 50 nmol lipid added to wells). In contrast binding of biotin-X-DSPE vesicles containing PEG_{2000}-DSPE to immobilised streptavidin was significantly lower than levels estimated for control biotin-X-DSPE liposomes (0.5 nmol versus 1.35 nmol per well) and was only marginally higher than amounts detected for biotin-DSPE preparations containing PEG_{2000}-DSPE (0.5 nmol versus 0.25 nmol, respectively, 50 nmol lipid added to wells).

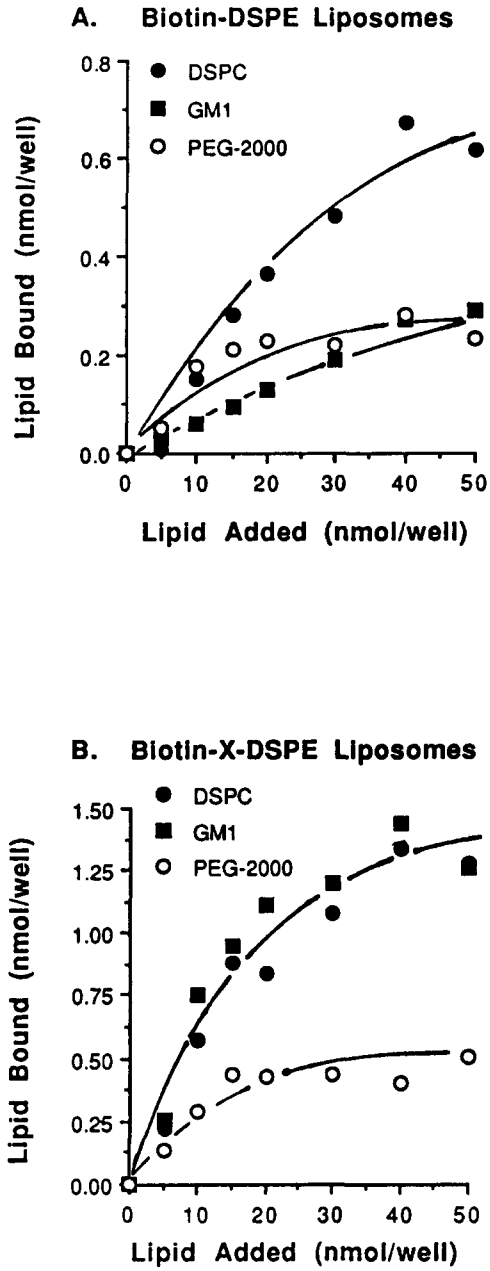

Fig. 5. Effect of incorporation of G_{M1} or PEG2000 in biotinated liposome on vesicle binding to streptavidin. Liposomes (DSPC, 54.75–59.75 mol%; cholesterol, 40 mol%; biotin-DPPE (5A) or biotin-X-DSPE (5B); 0.25 mol%) containing G_{M1} or PEG2000 (5 mol%) were prepared as described in Materials and methods. Binding of biotin-DSPE liposomes (Fig. 5A) or biotin-X-DSPE liposomes (Fig. 5B) to streptavidin plates was determined as described in Fig. 2.

4. Discussion

A major aim of our work is to develop further the use of biotinated-liposomes in pre-targeting 'in vivo' applications such as tumour targeting. In this regard there are two main objectives. The first is that liposomes containing biotinated lipid derivatives should have access to tumours. Secondly, biotinated-liposomes should bind optimally to streptavidin-coated target cells. In relation to the first aim, we recently showed that small amounts of biotin-DPPE in G_{M1} liposomes maintain their ability to circulate for extended periods in the blood and thereby extravasate to tumour sites [17]. To facilitate rapid screening of factors such as liposome composition which may influence the critical streptavidin-biotinated liposome binding step we developed a sensitive fluorescent microtitre based assay. This assay relies on the efficient loading of large quantities of the laser dye rhodamine 123 into vesicles by pH gradients. This results in a liposome preparation which is highly fluorescent. Small amounts of vesicles bound to streptavidin immobilised on microtitre plates were readily quantified by estimating liposome-associated rhodamine 123. Using this approach we show here that vesicles containing a biotin spacer arm derivative of DSPE and the sterically stabilising monosialoganglioside G_{M1} bind optimally to immobilised streptavidin. This property in conjunction with the favourable

'in vivo' circulation behaviour of such vesicle preparations should further the use of biotinated liposome in 'in vivo' targeting applications.

As emphasised in this report the choice of the biotin lipid derivative incorporated into liposomes can dramatically affect the amount of biotinated-liposomes bound to immobilised streptavidin. Lipid derivatives which have a spacer arm between the biotin moiety and the phospholipid head group when incorporated into vesicles bound streptavidin with higher avidity under all experimental conditions examined when compared to vesicles containing DSPE coupled directly to biotin. Incorporation of small amounts of the spacer arm derivative biotin-X-DSPE (0.1 mol%) in liposomes yields vesicles with equivalent binding characteristics to biotin-DSPE samples which contained 10-fold greater amounts of the biotinated lipid derivative. If we assume a vesicle diameter of 100 nm, this corresponds to the presence of 44 versus 4400 molecules in the outer leaflet for the respective preparations. For economic reasons, biotinated-liposomes containing the biotin spacer arm derivative of DSPE would be preferential employed in targeted liposome applications.

The diversity of applications perceived for targeted liposomes may require the use of different lipid compositions. For example, temperature sensitive liposome applications rely on modulation of the fluidity of liposomes by including less saturated phosphatidylcholine lipids [18]. Recently biotinated liposome preparations which contain unsaturated lipids such as dioleoyl phosphatidylethanolamine (DOPE) have been proposed as an approach to preparing target sensitive immunoliposome [19]. Such liposomes become destabilised upon binding to immobilised antibody or cell associated target antigen. Here we show that more fluid biotinated liposome preparations containing EPC or DPPC bind streptavidin with near equal avidity as DSPC biotinated-liposome samples when assayed below the gel to liquid-crystalline phase transition of the preparation (Fig. 4C). However, as suggested by the temperature-dependent leakage of rhodamine 123 from vesicles (Fig. 4 and Table 1), inclusion of fluid phosphatidylcholine lipids in preparations designed for 'in vivo' applications may limit accumulation levels of liposome encapsulated molecules such as small drugs at the tumour site.

Liposomes containing G_{M1} and PEG_{2000} lipid derivatives have been well established as the best system for 'in vivo' applications from the point of view of avoidance of the reticuloendothelial system and localisation to inflammation or tumour sites [20,21]. For 'in vivo' targeting applications, in addition to the ability of vesicles to extravasate to target tissues, it is paramount that targeted-liposomes are capable of recognising their target ligand. Our data indicates that inclusion of the above amphiphiles in liposomes can substantially inhibit the binding of biotin-DSPE liposomes to immobilised streptavidin. This would significantly affect the ability of biotinated vesicles to associate with tumours labelled with biotinated antibodies and coated with streptavidin. In support of this it was recently shown that inclusion of PEG_{1900}-DSPE in liposomes containing biotin-DSPE (0.05 mol%) caused a 20% reduction in lipid levels associated with target cells [22]. As noted here the hindrance on binding of biotinated-liposomes to streptavidin by bulky PEG moieties was not alleviated by introduction of spacer arm between the biotin moiety and the phospholipid head group. However substitution of the sterically stabilising lipid G_{M1} for PEG_{2000} derivatives of DSPE in biotin-X-DSPE vesicles resulted in biotinated vesicle preparations which bound efficiently to immobilised streptavidin. This is consistent with previous work based on streptavidin mediated agglutination of biotin-X-DSPE vesicles (2.5 mol% biotin lipid derivative included in vesicles) which showed that the steric barrier of G_{M1} was weaker than PEG derivatised DSPE in vesicles when the PEG moiety was greater than 750 in length [23]. Therefore, inclusion of G_{M1} in biotin-X-DSPE liposomes has two advantages; vesicles have long circulation half-lives and in addition they maintain their ability to bind optimally to immobilised streptavidin.

In summary, the aim of this study was to optimise the binding step of biotinated liposome preparations to streptavidin with particular emphasis on their use in 'in vivo' targeting applications. To achieve this an 'in vitro' microtitre protocol was developed where the target ligand was absorbed to the plate surface. Target ligand when immobilised on microtitre plates reflects the 'in vivo' scenario of cellular associated target molecules. This approach facilitated rapid screening of factors such as lipid composition which may determine amounts of liposome entrapped molecules delivered to tumour cells 'in vivo'. The described method is a simple and sensitive protocol and is applicable to other targeted-liposome strategies (Loughrey, unpublished observations). This assay should therefore aid in the development and design of vesicles which may contain novel types of hapten or protein derivatised lipids such as those recently described [24], for ultimate usage as targeted-liposomes in 'in vivo' delivery applications.

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