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A non-covalent method of attaching antibodies to liposomes

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A novel non-covalent method of attaching antibodies to liposomes which exploits the high affinity of streptavidin for biotin, is described. The two-step coupling protocol involves the initial attachment of streptavidin to liposomes containing biotin PE, followed by the coupling of biotinated antibodies to streptavidin-liposomes. The association of streptavidin with liposomes containing biotinated PE is rapid (< 5 min), resulting in a maximum association of 40 molecules of streptavidin per 100 nm vesicle. In the presence of equimolar cholesterol, the amount of streptavidin bound is twice that observed when biotin PE/egg PC liposomes are used. Irrespective of the mole ratio of biotin to antibody (e.g. for 1–6 biotins per antibody), or the molar ratio of antibody to streptavidin in the second incubation step, equimolar amounts of antibody bind to streptavidin. It is shown that anti-rat-erythrocyte IgG or $F(ab')_2$ complexed to liposomes via the streptavidin linker bind specifically to rat erythrocytes but not to human erythrocytes. This coupling protocol can be readily extended to other biotinated antibodies.

The ability to conjugate antibodies and other targeting molecules to liposomal vesicle systems has many potential applications for drug delivery to cells in vitro and in vivo as well as for diagnostic protocols [1]. Most such procedures are based on covalent coupling of the protein to vesicles via a suitably modified version of phosphatidylethanolamine (PE) [2,3]. These coupling procedures are time consuming and involve the exposure of the proteins and vesicles to harsh, toxic reagents. Alternative, non-covalent approaches involve the non-specific adsorption of proteins to vesicles [4] or the binding of proteins to specific surface components. Non-specific adsorption, however, only occurs for particular proteins, and can be of questionable stability [5]. More reliable, specific non-covalent conjugation can be achieved employing protein A [2] (for binding of antibodies) or avidin [6] (for binding to biotinated proteins or lipids). These latter approaches have been employed in targeting protocols where target cells coated with antibody or biotinated antibody, will subsequently sequester protein A or avidin coated liposomes.

Complete targeted liposomes containing biotinated PE coupled to biotinated antibodies via streptavidin (a biotin-binding protein produced by streptomycetes [7]) have not been characterized, however. In this work we employ streptavidin (to avoid the non-specific binding properties of native avidin [8]) and demonstrate a rapid two-step method of attaching biotinated antibodies to biotinated liposomes. Such antibody-liposome complexes can be produced rapidly by a simple, gentle incubation procedure. It is further demonstrated

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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that these antibody-liposome complexes bind specifically to target cells as directed by the coupled antibody.

The first series of experiments were aimed at characterizing the binding of streptavidin to liposomes containing biotinated PE. This binding is a crucial step, as the four biotin binding sites on streptavidin can give rise to inter-vesicle coupling, with associated aggregation and precipitation effects. The major aims of these experiments were therefore first to show that streptavidin couples to liposomes containing biotinated PE and second to determine the maximum liposomal concentration of biotinated PE for which streptavidin induced aggregation can be avoided. Biotinated PE was therefore incorporated into egg phosphatidylcholine (egg PC) at a molar ratio of 0.1% (with respect to egg PC) and LUVs produced by an extrusion procedure [9] through 100-nm pore size filters. This results in vesicle systems which exhibit a mean diameter of approx. 100 nm. These vesicles were then incubated in the presence of streptavidin (10-fold molar excess with respect to biotin PE) and, as shown in Fig. 1 binding proceeded rapidly to achieve streptavidin-biotinated PE ratios of 1 : 12 (mol/mol). This association was found to be independent of pH over the pH range 5–9, and all subsequent streptavidin-liposome coupling was performed for 30 min at pH 8.0.

In the preceding experiment, a maximum of 5.8 μg of streptavidin was bound per μmol lipid, which corresponds to about 7–8 copies of streptavidin per vesicle for a vesicle diameter of 0.1 μm. In order to increase the amount of streptavidin bound per liposome (and thus increase the number of sites available for biotinated antibody to couple to liposomes), the amount of biotinated PE in the vesicles was increased over the range 0–0.5% of total lipid, maintaining a constant ratio of streptavidin to total lipid. As shown in Fig. 2 when the amount of biotin PE is increased, a linear increase in the amount of streptavidin bound per μmol lipid is observed, both in the presence and absence of cholesterol. In the presence of 50 mol% cholesterol, a 50% increase in the streptavidin bound per μmol total lipid is observed compared to egg PC liposomes. If the amount of biotin PE is increased further than 0.35% (in the presence of cholesterol) and 0.5% (in the absence

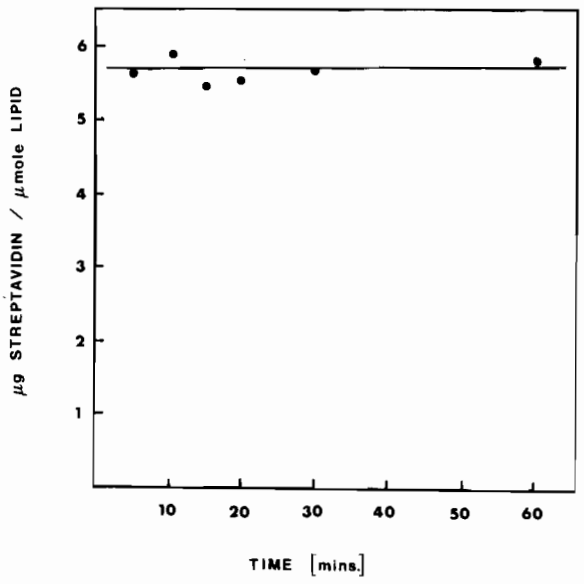

Fig. 1. Coupling of streptavidin to LUVs containing biotin phosphatidylethanolamine. Egg phosphatidylcholine was purified from egg yolks using established procedures and was greater than 99% pure as determined by thin-layer chromatography. Cholesterol, streptavidin, Hepes and all salts were purchased from Sigma Chemical Co. Biotin-phosphatidylethanolamine was obtained from Molecular Probes, $Na^{125}I$ from Amersham, iodobeads from Pierce, and Sepharose CL-4B from Pharmacia. Liposomes (99.9% egg PC, 0.1% biotin PE) were prepared according to the procedure described by Hope et al. [9] employing a freeze-thaw protocol [14] and 100 nm pore size polycarbonate filters. ^{125}I-streptavidin was prepared using iodobeads [15]. Streptavidin (1 μCi ^{125}I/mg, 4 mg/ml) was incubated at room temperature with vesicles at a 10-fold excess to biotin-PE in 20 mM Hepes-buffered saline (pH 8). At various time points, aliquots were fractionated on Sepharose CL-4B columns (5 ml) to separate liposomally bound streptavidin from free streptavidin. The ratio of streptavidin bound per μmol total lipid was determined by counting ^{125}I (Packard, Auto Gamma 5650) and a standard phosphate assay for phospholipid.

of cholesterol) with respect to total lipid, complete aggregation and precipitation of liposomes is observed on addition of streptavidin. Thus the maximum number of copies of streptavidin per 100 nm vesicle that can be achieved without aggregation is approx. 40, both in the presence and absence of cholesterol.

The next step in the coupling procedure involves the attachment of biotinated antibody to the streptavidin-coated liposomes. A preliminary study was performed to determine whether an

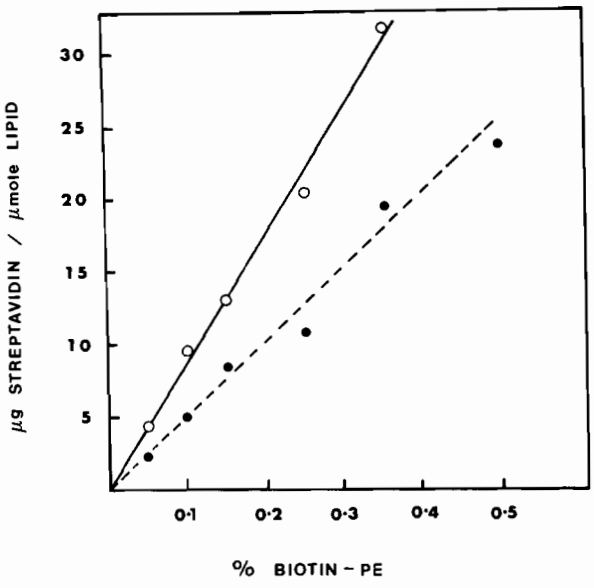

Fig. 2. Influence of cholesterol on streptavidin coupling to LUVs containing variable amounts of biotin-phosphatidylethanolamine. Vesicles (25 μmol egg PC, 25–125 nmol biotin PE (●— — —●), or 12.5 μmol cholesterol, 12.5 μmol egg PC, 25–125 nmol biotin PE (○——○)) were prepared as in Fig. 1. Streptavidin (1 μCi ^{125}I/mg, 0.68 mg) was incubated with liposomes (1 μmol total lipid) for 30 min at room temperature in 20 mM Hepes-buffered saline (pH 8). The ratio of streptavidin bound per μmol total lipid was determined as in Fig. 1.

antibody (anti-rat erythrocyte IgG), which had four biotin molecules covalently bound, would associate with pre-formed streptavidin liposomes. Approximately equimolar amounts of antibody bound per liposomal streptavidin irrespective of the amount of antibody available (1–8 antibody molecules per streptavidin). Further, equimolar amounts of antibody bind to streptavidin liposomes irrespective of the amount of antibody biotination (1–6 biotins per antibody). In the absence of liposomally bound streptavidin, no detectable levels of antibody bind (data not shown). Thus, since streptavidin liposomes can be prepared with 40 copies of streptavidin bound per vesicle, the maximum number of copies of antibody bound per vesicle is also 40.

In order to demonstrate that these targeted liposomes will bind specifically to target cells, biotinated anti-rat IgG or derived (biotinated) $F(ab')_2$ fragments were coupled to vesicles containing ^{125}I-labelled tyramine insulin (^{125}I-TI, see Ref. 10). The high specific activity of this trapped marker allowed the in vitro distribution of the vesicle-antibody complexes to be determined. Approximately 7 copies of IgG or $F(ab')_2$ were coupled per 100 nm vesicle. As shown in Table I, little non-specific binding of biotinated liposomes to rat or human erythrocytes was observed. Anti-rat erythrocyte IgG or $F(ab')_2$ liposomes complexes bind specifically to rat erythrocytes but not to human erythrocytes. Such specific binding is effectively blocked by the presence of a 1000 molar excess of biotin to streptavidin. Pre-incubation of

TABLE I

BINDING OF TARGETED LIPOSOMES TO RAT ERYTHROCYTES

^{125}I-Inulin liposomes (25 μmol egg PC, 25 nmol biotin PE, 0.027 μCi ^{125}I-inulin/μmol lipid) were prepared as described by Sommerman et al. [10]. Streptavidin-liposomes were prepared as in Fig. 1. Biotinated anti-rat erythrocyte IgG (cappel) was prepared with four biotins covalently bound per mol of antibody by the method of Bayer er al. [16]. For the preparation of biotinated $F(ab')_2$ fragments, biotinated anti-rat erythrocyte IgG (4 biotins/IgG) was digested with pepsin in 0.1 M sodium acetate (pH 4.5) at 37 °C overnight [17]. The products were fractionated on a Sephadex G-150 column and fractions containing $F(ab')_2$ fragments, as determined by 10% SDS polyacrylamide gel [18], were pooled. Antibody (or $F(ab')_2$) streptavidin liposomes were prepared by incubating biotin antibody (or biotinated $F(ab')_2$; 1 mg/ml) with streptavidin liposomes (1 μmol/ml) for 30 min at room temperature, at a 4-fold mole ratio of antibody (or $F(ab')_2$) to streptavidin. For erythrocyte cell binding studies, rat or human erythrocytes were washed with 20 mM Hepes-buffered saline (pH 8), three times. Lipid (0.62 μmol/ml) was incubated with 10^9 erythrocytes in each experiment for 1 h at 4 °C, with the exception of (a), where the lipid concentration was 1.76 μmol/ml. Cells were washed three times with 20 mM Hepes-buffered saline (pH 8) and were counted to determine levels of erythrocyte-associated liposomes. n.d., not done.

Sample	No. of liposomes bound per erythrocyte	
	rat	human
Liposomes	18	n.d.
Streptavidin-liposomes	20	n.d.
(a) Pre-incubation with IgG + streptavidin-liposomes	542	n.d.
IgG streptavidin-liposomes	416	11
IgG streptavidin-liposomes + biotin	73	n.d.
$F(ab)_2$ streptavidin liposomes	302	11
$F(ab)_2$ streptavidin liposomes + biotin	50	n.d.

rat erythrocytes with biotinated antibody, followed by addition of streptavidin liposomes also leads to binding of liposomes to rat erythrocytes. For these targeted vesicle systems, a maximum of 20% of liposomes conjugated to anti-rat erythrocyte IgG were bound to rat erythrocytes, which is comparable to binding efficiencies of other targeted vesicle systems [11–13].

In summary, this study was initiated in order to determine whether biotinated antibodies could be coupled via streptavidin to liposomes containing biotin PE resulting in targeted vesicle systems. We demonstrate that such antibody liposome complexes can be prepared by a more rapid and gentle procedure than conventional covalent methods allow. The antibody liposome complexes obtained are observed to bind specifically to their target cells as directed by the antibody with very little non-specific binding. A particular advantage of this technique is that it should be readily extended to couple any biotinated antibody or protein to liposomal systems.

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