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A novel strategy affords high-yield coupling of antibody Fab′ fragments to liposomes

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Abstract

A new assay for the production of reactive sulfhydryl-bearing antibody Fab′ fragments has been utilized to develop conditions affording high efficiencies of coupling of mouse and rabbit IgG-derived Fab′ fragments to lipid vesicles containing maleimidyl-functionalized phospholipids. Cysteine and mercaptoethylamine, but not dithiothreitol, reduce antibody $F(ab')_2$ to Fab′ fragments in very good yields under conditions where overreduction to heavy and light chains is minimized. Surprisingly, however, a large fraction of the Fab′ fragments generated under these conditions can lack maleimide-reactive sulfhydryl groups, as demonstrated using a maleimidyl-poly(ethylene glycol) conjugate to shift selectively the electrophoretic mobility of the reactive sulfhydryl-bearing Fab′ fragments. After modification of $F(ab')_2$ reduction conditions specifically to maximize the yield of the latter fraction, it is possible to achieve high and very reproducible coupling of functional Fab′ fragments to liposomes (equivalent to coupling of ca. 70% of total input protein and almost 100% of the reactive sulfhydryl-bearing Fab′ fraction). A novel phospholipid-poly(ethylene glycol)-maleimide 'anchor' allows particularly efficient coupling of Fab′ fragments to liposomes, even using relatively low liposome concentrations and molar percentages of the liposome-incorporated 'anchor' species. These results demonstrate that with appropriate optimization of the conditions for Fab′ production and liposome coupling, Fab′ fragments can be coupled to liposomes with efficiencies comparable to or exceeding those reported for coupling of intact antibodies. These results should facilitate the wider use of Fab′ fragments as a potentially advantageous alternative to intact antibodies for liposomal targeting in various applications.

Keywords: Liposome; Liposomal targeting; Antibody; Liposome-antibody conjugation; Poly(ethylene glycol)-modified lipid

1. Introduction

'Targeting' of liposomes via surface-coupled antibodies has proven an effective method to modify the biodistribution and/or the pharmacokinetic behaviour of liposomes in various applications [1–8]. A variety of techniques has been proposed and exploited for the covalent coupling of antibodies to liposomes (for reviews see [9,10]). With few exceptions [10–12], an inherent feature of these approaches is the largely nonselective modification of several amino acid residues per antibody molecule, an aspect of the methodology that, in at least some cases [13], is in fact required for optimal efficiency of antibody-liposome coupling. Such multiple and largely random modifications of the antibody molecules increase significantly the potential both for alteration of biological activity and, using some modifying agents, for the creation of neoepitopes [14] in a significant fraction of the antibody population.

In principle, antibody Fab′ fragments represent an attractive alternative to whole antibodies as liposomal targeting agents for many applications. Fab′ fragments can be coupled to liposomes at a well defined site (the thiol(s) of the 'hinge' region) that is remote from the antigen-recognition site, thus avoiding perturbation of the latter and the

Abbreviations: BMP-, *N*-(β-maleimidopropionyl)-; BMPS, β-maleimidopropionic acid *N*-hydroxysuccinimide ester; BSA, bovine serum albumin; CF, 5-(and-6)-carboxyfluorescein; DCCD, *N,N′*-dicyclohexylcarbodiimide; DMF, dimethylformamide; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EMC-, *N*-(ε-maleimidocaproyl)-; EMCS, ε-maleimidocaproic acid *N*-hydroxysuccinimide ester; ePC, egg phosphatidylcholine; ePG, egg phosphatidylglycerol; HBSS/Hepes, Hank's balanced salt solution with 10 mM *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (pH 7.2); HOBT, *N*-hydroxybenzotriazole; LUV, large unilamellar vesicle(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol); PDP-, *N*-(3-(2-pyridyldithio)propionyl)-; PE, phosphatidylethanolamine; Rho-PE, *N*-lissamine rhodaminesulfonyl-PE; SUV, small unilamellar vesicle(s); TEA, triethylamine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TLC, thin-layer chromatography; tPE, transphosphatidylated egg phosphatidylethanolamine.

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introduction of random amino acid modifications into the protein. Moreover, the absence of the antibody Fc domain in Fab′ fragments eliminates a range of Fc-mediated biological activities, some of which (e.g., complement activation via the classical pathway and humoral responses or clearance via antibody-Fc receptor interactions [15–17]) may be undesirable in various applications. These advantages, coupled with the potential to produce humanized and otherwise modified antibody fragments by biotechnological methods [18,19], may aid in the exploitation of antibody fragments as targeting factors while minimizing unwanted biological side effects (e.g., immunogenicity) of such molecules.

Various methodologies have been proposed for coupling Fab′ fragments to liposomes, via either thioether [8,20–25] or disulfide [1,26] linkages to suitable 'anchor' lipids. However, in applying such methodologies to couple a murine monoclonal Fab′ fragment to liposomes, we encountered low and frequently irreproducible coupling yields, as has also been encountered in previous studies using similar approaches. Such complications pose a potentially significant limitation to the application of this promising targeting strategy, as the surface density of coupled antibodies has been shown to be an important determinant of the selectivity and avidity of liposome-cell binding [27–29].

In an effort to overcome the generally low efficiency of Fab′-liposome coupling, we have identified the factors that compromise the efficiency of this process, and we have developed procedures (and new liposome-coupling reagents) to control better and to optimize the extent of liposome-Fab′ coupling. As a result we have identified conditions permitting both rabbit and mouse Fab′ fragments to be coupled to liposomes with efficiencies (ca. 65–70%) that considerably exceed those previously observed for liposomal coupling either of Fab′ fragments or, in most previous reports, of intact immunoglobulin molecules. The availability of such methodologies should facilitate the employment of Fab′ fragments as potential alternatives to whole (derivatized) antibodies as liposome-'targeting' agents in a variety of applications.

2. Materials and methods

Egg phosphatidylcholine (ePC) and transphosphatidylated (egg) phosphatidylethanolamine (tPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from NuChek Prep (Elysian, MN). Egg phosphatidylglycerol (ePG) was prepared by enzymatic transphosphatidylation of ePC with phospholipase D as described previously [30]. Rhodamine-PE (Rho-PE) was prepared from tPE as described previously [31].

ε-Maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS), β-maleimidopropionic acid *N*-hydroxysuccinimide ester (BMPS), rabbit non-specific IgG, pepsin and L-cysteine were obtained from Sigma (St. Louis, MO). Bio-Gel P-6DG and all electrophoresis reagents were obtained from Bio-Rad (Mississauga, Ontario). $Na^{125}I$ was obtained from ICN (Mississauga, Ontario). All cell culture reagents except for apotransferrin were obtained from Gibco Laboratories (Grand Island, NY). All other common chemicals and synthetic reagents were reagent grade or better. Calcein and carboxyfluorescein (Sigma) were purified by chromatography on Sephadex LH-20 as described previously [32]. Hybridoma cells producing the murine TFR1 (anti-human transferrin receptor) mAb [33] were obtained from the laboratory of Dr. R.M. Johnstone (McGill University) and were cultured in RPMI medium supplemented with insulin, holotransferrin (prepared from human apotransferrin (Sigma) as described previously [34]), ethanolamine and sodium selenite [35]. The antibody was purified from culture supernatants by chromatography on protein A-Sepharose CL-4B (Pharmacia) as described previously [36].

BMP-PE was prepared by reacting tPE (100 mM in 99:1 chloroform/triethylamine (TEA)) with 2 molar equivalents of BMPS (Sigma) under argon for 16 h at 25°C, then purified by preparative thin-layer chromatography (TLC) on silica gel 60 plates in 75:20:5:4 chloroform/methanol/acetonitrile/water [37]. EMC-PE was prepared similarly from EMCS (Sigma) and tPE and was purified by preparative TLC in 56:12.5:1:1 chloroform/methanol/water/ammonium hydroxide. To prepare BMP-PEG5K, α-amino-ω-methoxyPEG5K, synthesized as described previously [38], was reacted with 2 molar equivalents of BMPS (100 mM, in 99:1 chloroform/TEA) under argon for 16 h at 25°C, and the product was purified by preparative TLC in 90:10:1 chloroform/methanol/water.

α,ω-Diamino-PEG1K was prepared as follows: 4 g of PEG1K (Sigma, further purified by the method of Honda [39]) was dissolved at −10°C in 25 ml methylene chloride containing 6% TEA. A 4-fold molar excess of methanesulfonyl chloride was then added slowly at −10°C with stirring. The sample was stirred at −10°C for 1 h, then allowed to warm slowly to 10°C and concentrated under a stream of nitrogen at this temperature. 8 ml concentrated ammonium hydroxide and 6 ml ethanol were then added, and the sample was sealed and incubated at 65°C for 40 h. The mixture was dried under nitrogen, with warming and repeated addition of chloroform/methanol (2:1) to aid water removal, and the waxy residue was redissolved in chloroform and purified by flash chromatography [40] on silica gel 60 (Merck), using 15% methanol in chloroform as the eluant.

BMP-PEG1K-PE (Fig. 1) was prepared by the following protocol. *N*-Succinyl-PE was first prepared from tPE as described previously [41]. The product (25 mg/ml in dry DMF) was reacted with 4, 3, and 3 molar equivalents of α,ω-diamino-PEG1K, HOBT, and DCCD, respectively, under argon for 90 min at 25°C. The product,

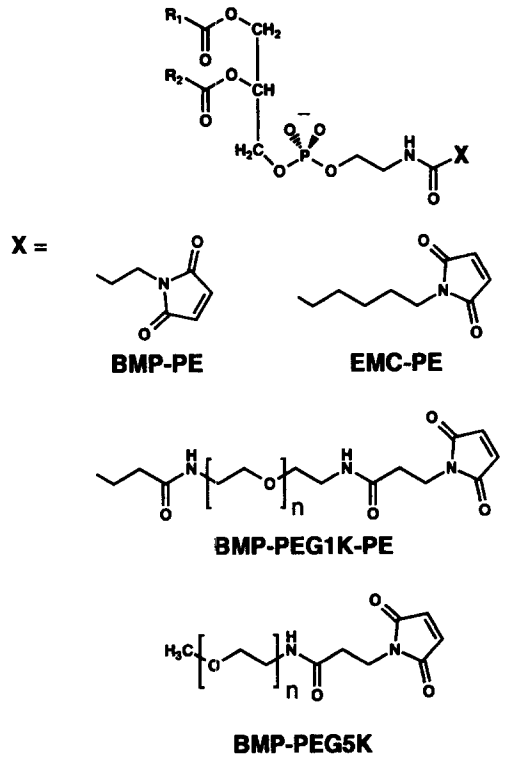

Fig. 1. Structures of maleimidyl-PE conjugates used in this study and of BMP-PEG5K (n = ca. 23 and ca. 114 for BMP-PEG1K-PE and BMP-PEG5K, respectively).

NH_2PEG1K-PE, was purified by preparative TLC in 50:20:10:10:5 chloroform/acetone/methanol/acetic acid/water and reacted (at 100 mM in 99:1 chloroform/TEA) with 2 molar equivalents of BMPS under argon for 2.5 h at 25°C. The phosphate-positive, ninhydrin-negative product was purified by preparative TLC in 85:15:1.5 chloroform/methanol/water (R_f = 0.35). Concentrations of all phospholipid and phospholipid-conjugate stocks were assayed by the method of Lowry and Tinsley [42].

2.1. Preparation of liposomes

Liposomes were prepared from ePC/ePG/cholesterol (3:1:2 molar proportions) plus small proportions of Rho-PE and/or PE-maleimide conjugates (specified as molar percentages with respect to total lipids) as indicated in the text. After drying for several h under high vacuum, the lipids were initially vortexed in an appropriate aqueous solution as specified for each type of experiment. The resulting dispersions were converted either to small unilamellar vesicles (SUV), by bath sonication for 10 min under argon, or to large unilamellar vesicles (LUV) by repeated freeze-thawing followed by hand extrusion through 0.1 μm pore-size polycarbonate filters [43]. SUV and LUV samples prepared in this manner gave average diameters of ca. 30 nm and ca. 110 nm, respectively, by calcein-encapsulation measurements carried out similarly to the carboxyfluorescein-encapsulation assay of Wilschut et al. [45]. The predominantly unilamellar character of the LUV preparations was demonstrated using comparable vesicles in which 10% of the PC component was replaced by PE; the trinitrobenzenesulfonic acid labeling procedure of Nordlund et al. [46] showed 42–45% of the PE component (whose distribution in such vesicles parallels that of the total lipids [46]) to be exposed at the vesicles' outer surface.

2.2. F(ab')$_2$ preparation

F(ab')$_2$ fragments were prepared from rabbit nonspecific IgG (Sigma) as described previously [26] or from the murine IgG_{2a}-subclass mAb TFR1 using a modification of previous protocols [44], as follows. Briefly, in the latter protocol intact antibody (2 mg/ml) was incubated with pepsin (60 μg/ml) in 0.1 M sodium acetate (pH 4.2) for 4 h at 37°C. Digestion was terminated by adding 2 M Tris (pH 8.8) to a final concentration of 80 mM, then centrifuging (10000 × g, 30 min, 4°C). The supernatant was dialyzed at 4°C against 150 mM NaCl, 20 mM phosphate, pH 7.0, then chromatographed on a column of Protein A-Sepharose CL-4B to remove any undigested IgG. A portion of F(ab')$_2$ was radioiodinated using Iodobeads (Pierce) as per the manufacturer's protocol. Protein concentrations were determined by a modification of the Lowry method [47].

2.3. Fab' preparation

All solutions used for Fab' production and liposome coupling were extensively degassed by nitrogen purging before use. Rabbit or mouse F(ab')$_2$ (5 mg/ml) was reduced under argon in 30 mM cysteine, 100 mM Tris, pH 7.6, for 15 min at 37°C except where otherwise indicated. The solution was then diluted 2-fold with an equal volume of the appropriate experimental buffer (as indicated) and spun through a 0.4 ml spin-column of Bio-Gel P-6DG, packed in a punctured 0.5 ml microfuge tube over a glass wool plug, that had been pretreated with BSA (40 μg in 20 μl) and washed three times with buffer.

2.4. Fab'-liposome coupling

Freshly reduced Fab' (1 mg/ml final), including a trace amount of ^{125}I-labelled protein, was centrifuged through a Bio-Gel spin-column as just described into an argon-blanketed sample of LUV (0.2–10 mM total lipid), normally in 150 mM NaCl, 10 mM Mops, 0.1 mM EDTA, 50 μM DTPA, pH 6.5 (final antibody concentration = 1 mg/ml). The mixture was then incubated under argon in a sealed tube for 16 h at 4°C with gentle stirring. For control samples, similar liposomes were preincubated with 50 mM cysteine (1 h, 25°C) before addition of Fab' fragments. The liposome-Fab' coupling reaction was quenched by the

addition of cysteine (0.5 mM, 15 min at 25°C), then the mixture was chromatographed on a column of Sepharose CL-4B (Pharmacia, Baie D'Urfe, Québec) for LUV or Sepharose CL-6B for SUV. Fab′ was quantitated in the eluted fractions by gamma counting, using an LKB 1270 Rackgamma II counter, and liposomal lipid was monitored by fluorescence of liposome-incorporated Rho-PE (1 mol%), using a hand-held blacklight for qualitative observation and a Perkin Elmer LS-5 spectrophotometer for quantitation.

2.5. Fab′-PEG5K shift assay

Freshly reduced Fab′ (20 μl at 1 mg/ml, with or without a trace amount of radiolabelled protein) was centrifuged as above through a Bio-Gel spin-column into an equal volume of BMP-PEG5K (500 μM, in pH 6.5 buffer as above) and allowed to react under argon for 1 h at 25°C. The reaction was quenched by the addition of NEM (25 mM, 25°C, 30 min). The samples were then analyzed by non-reducing SDS-PAGE [48], using a 12% resolving gel, and were visualized by coomassie blue staining and/or autoradiography. Quantitation of protein bands was performed by densitometry using a Fujix BAS 2000 imaging system.

2.6. Leakage assay

LUV, prepared as described above in isotonic (100 mM) calcein, pH 7.4 were gel-filtered on Sepharose CL-4B (buffer = 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA, pH 7.4) at 4°C to remove unencapsulated dye. After incubation under various conditions as indicated in the text, aliquots of the calcein-loaded LUV were withdrawn and analyzed for fluorescence (excitation and emission wavelengths 490 and 520 nm, respectively) on a Perkin Elmer LS-5 spectrophotometer before and after the addition of Triton X-100 to 1% (w/v). The percentage of calcein retained in the liposomes was calculated from the extent of fluorescence dequenching upon Triton addition [44,49,50], assuming 100% encapsulation of the dye in liposomes freshly eluted from the Sepharose column.

2.7. Fluorescence microscopy

LUV, prepared in 45 mM carboxyfluorescein, 10 mM Mops, 0.1 mM EDTA, pH 6.5 that was adjusted to isotonicity (270 mosM) with NaCl, were coupled to Fab′ fragments (10 mM lipid, 1 mg/ml Fab′) as described above but using the carboxyfluorescein-containing buffer just noted. The sample was then adjusted with NaOH to pH 7.4, and the mixture was allowed to equilibrate for 1 h at 25°C, then gel-filtered on Sepharose CL-4B to remove unencapsulated dye. CV-1 cells, grown on glass coverslips to approx. 75% confluency, were washed once and then incubated for 1 h at 37°C in serum-free medium, then washed twice with Hanks' buffered saline solution including 10 mM Hepes (HBSS/Hepes) and cooled to 4°C. To reduce nonspecific binding of liposomes, cells were normally preincubated with PC/PG/cholesterol SUV (50 μM in HBSS/Hepes) for 15 min at 4°C. The cells were washed twice with cold HBSS/Hepes, then incubated for 30 min at 4°C with Fab′- or cysteine-coupled LUV (40 μm lipid, 1 ml per coverslip) in HBSS/Hepes. After the liposome-cell incubation, the cells were washed twice with serum-free medium and incubated at 37° for 1 h, then washed with HBSS/Hepes and observed using a Zeiss epifluorescence microscope. Kodak TMY400 film was used for photography.

3. Results

In initial experiments, the murine mAb TFR1 (IgG2a subclass) was converted to Fab′ fragments, using cysteine as the reducing agent [51], and coupled to large unilamellar liposomes containing 2.5 mol% of the maleimide-derivatized lipid EMC-PE [13,52]. To obtain an optimal yield of Fab′ fragments as assessed by nonreducing SDS-PAGE, we initially reduced $F(ab')_2$ fragments using 10 mM cysteine, pH 7.6 for 45 min at 37°C. Considerably lower yields of Fab′ fragments (typically < 35%) were obtained, under separately optimized conditions, using DTT or β-mercaptoethanol as alternative reducing agents (results not shown). However, while the yield of Fab′ fragments obtained using the 'optimal' cysteine-reduction conditions described above typically exceeded 75%, only a low and variable fraction of the Fab′ fragments (2% to 13% in 21 experiments) could be coupled to EMC-PE-containing LUV. LUV containing different thiol-reactive lipid 'anchors' (BMP-PE, BMP-PEG1K-PE (see Fig. 1) or PDP-PE) showed modest differences but in no case any pronounced improvement in the efficiency of Fab′-liposome coupling (maximum yield < 20%), which moreover remained highly variable. Similar results were obtained in experiments using SUV in place of LUV or increasing to 5 mol% the proportion of the thiol-reactive lipid 'anchor' in the liposomes. Similarly low efficiencies of coupling of Fab′ fragments to liposomes (typically < 30%) have been reported by other workers [1,8,20–24,26].

The observations noted above suggested a clear need to better define (and thereby to optimize) the factors governing Fab′-liposome conjugation. A key question was whether the extent of Fab′-liposome coupling was limited by some property of the Fab′ preparation itself or rather by an inherent feature of the liposome-Fab′ conjugation process (e.g., kinetic competition between liposomal coupling and reoxidation of maleimide-reactive sulfhydryl groups). To address this question, we devised an assay, using a maleimide-bearing derivative of PEG-5000 (BMP-PEG5K (see Fig. 1)), to determine quantitatively the proportion of Fab′ fragments in a given preparation that bears reactive

sulfhydryl residues. As shown in Fig. 2, coupling of BMP-PEG5K to mouse Fab′ sulfhydryl groups substantially altered the mobility of the Fab′ fragments upon (nonreducing) SDS-PAGE analysis, allowing ready quantitation of the proportions of maleimide-reactive Fab′ fragments (upper arrow) and nonreactive Fab′ fragments (lower arrow) by gel-scanning densitometry. A lower molecular weight maleimidyl-PEG conjugate, BMP-PEG2K, shifted essentially the identical proportion of Fab′ fragments in parallel reactions, although as expected by a smaller distance (not shown). These shifts were in all cases completely blocked by prior treatment of the Fab′ fragments with NEM (Fig. 2, compare lanes 1 and 2). Reducing SDS-PAGE analysis of BMP-PEG5K-reacted Fab′ showed that only the heavy chain was shifted by the maleimidyl-PEG (not shown), consistent with the expected selective reaction of this reagent with the reduced 'hinge'-region sulfhydryl(s) of the Fab′ fragments [51]. In preliminary experiments we found that even a modest molar excess (5-fold) of BMP-PEG5K over Fab′ was sufficient to shift the maximal fraction of Fab′ fragments in a given preparation. However, a 25-fold molar excess of BMP-PEG5K was routinely used to ensure maximal derivatization of Fab′ sulfhydryl groups in all cases.

Using the assay just described, we found that Fab′ preparations obtained using different reduction conditions varied widely in their contents of maleimide-reactive material, even when all preparations contained high proportions of 55 kDa (apparent molecular mass) Fab′ fragments as assessed by conventional (non-reducing) SDS-PAGE analysis. As illustrated in Fig. 2 (lanes 2–4), during extended incubation of $F(ab')_2$ fragments with either cysteine or (not shown) mercaptoethylamine there is a gradual and pronounced decline in the proportion of Fab′ fragments that is maleimide-reactive (band denoted by upper arrow), although the proportion of total Fab′ fragments decreases only modestly with increasing time of incubation. As a result, the conditions initially developed to optimize the production of Fab′ fragments as assessed by conventional SDS-PAGE analysis (10 mM cysteine, pH 7.6, 37°C, 45 min) prove to yield only a low proportion of maleimide-reactive Fab′ (Fig. 2, lane 3). An analogous, though somewhat slower time-dependent loss of maleimide-reactive Fab′ fragments was also detected in the reduction of rabbit IgG-derived $F(ab')_2$ under similar conditions (not shown). For both $F(ab')_2$ species, it was possible to identify modified incubation conditions (employing shorter incubations and higher cysteine concentrations) that afford high yields of reactive sulfhydryl-bearing Fab′ fragments (60–80% based on total input protein) while increasing only minimally the extent of overreduction of Fab′ to isolated heavy and light chains.

The results just presented suggested that using conventionally 'optimized' protocols for Fab′ production, the efficiency of subsequent liposome-Fab′ coupling can be limited most fundamentally by inefficient production of maleimide-reactive Fab′ fragments. This suggestion was supported by a comparison of the efficiency of coupling of antibody TFR1 Fab′ fragments, prepared using a re-optimized reduction protocol as just described (30 mM cysteine, pH 7.6, 15 min at 37°C), to either BMP-PEG5K or SUV containing 2.5 mol% BMP-PEG1K-PE. In a representative experiment, a preparation of Fab′ fragments containing 78% of reactive sulfhydryl-bearing Fab′ (assessed

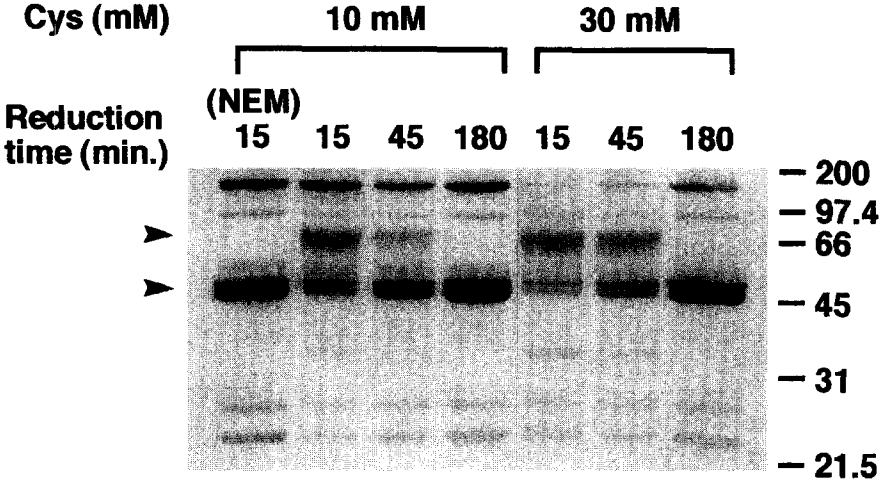

Fig. 2. Representative non-reducing SDS-PAGE results using the BMP-PEG5K 'shift assay' for various preparations of TFR1-Fab′ fragments. Lane 1: mAb TFR1-derived $F(ab')_2$ was incubated with 10 mM cysteine (37°C, pH 7.6) for 15 min, then rapidly gel-filtered and reacted with NEM (50 mM, 30 min at 25°C). Lanes 2–4: $F(ab')_2$ was incubated with 10 mM cysteine for 15 min, 45 min or 180 min, respectively, then gel-filtered and reacted successively with BMP-PEG5K (0.5 mM, 60 min at 25°C) and with 50 mM NEM. Lanes 5–7: as for lanes 2–4, but using 30 mM cysteine in the initial incubation. Other experimental conditions were as described in Section 2. The upper and lower arrows denote the positions of migration of BMP-PEG5K-reacted Fab′ and of unreacted Fab′ (observed as a doublet due to alternate sites of pepsin cleavage), respectively. For reference, the uppermost (ca. 121 kDa) band in lane 1 represents intact $F(ab')_2$ fragments, and the lowest two bands represent heavy chain fragments (ca. 26 kDa, seen as a doublet in some lanes) and light chains (ca. 23 kDa) released by Fab′ overreduction.

Table 1
Summary of Fab'-liposome coupling using maleimide-PE linkers

Maleimide-PE linker	Type of liposome	mol% linker	Number of samples tested	% Fab' bound	mol Fab'/ mol linker
EMC-PE	LUV	0.5	2	28.4 ± 0.4	0.074 ± 0.002
BMP-PE	LUV	0.5	6	30.6 ± 2.0	0.081 ± 0.007
		2.5	4	51.4 ± 9.4	0.029 ± 0.007
	SUV	0.5	2	25.8 ± 0.9	0.078 ± 0.003
		2.5	4	56.1 ± 1.2	0.039 ± 0.002
BMP-PEG1K-PE	LUV	0.5	6	56.1 ± 2.5	0.166 ± 0.009
		2.5	4	63.9 ± 7.2	0.037 ± 0.005
	SUV	0.5	2	55.3 ± 1.3	0.166 ± 0.009
		2.5	8	70.6 ± 1.9	0.046 ± 0.002
BMP-PEG1K-PE + rabbit Fab'	SUV	2.5	2	64.3 ± 0.2	0.049 ± 0.002

Freshly reduced Fab' (1 mg/ml final concentration) was coupled to ePC/ePG/cholesterol (3:1:2 molar proportions) liposomes incorporating the indicated molar percentages of different maleimidyl lipids (10 mM total lipid). Conditions of the coupling reactions were as specified in Section 2. Tabulated values represent means ± 1 S.D.

by BMP-PEG5K conjugation [1]) showed 76% coupling to the vesicles at lipid and Fab' concentrations of 10 mM and 1 mg/ml, respectively. The similarity of these two values indicates that a very high proportion of the maleimide-reactive Fab' fragments in the preparation can readily be coupled to liposomes under these conditions. There thus appears to be no intrinsic limitation to the efficiency of coupling of free sulfhydryl-bearing Fab' fragments to appropriately derivatized liposomes under coupling conditions like those just described.

The results described above do not rule out the possibility that factors other than the availability of free sulfhydryl-bearing Fab' fragments may affect the efficiency of Fab'-liposome coupling under certain conditions. Different applications of Fab'-targeted liposomes may dictate the use of liposomes of different sizes, compositions and/or degrees of surface modification. Accordingly, we examined the efficiency of coupling of Fab' fragments to both LUV and SUV containing either the conventional maleimidyl lipids BMP-PE and EMC-PE or a novel lipid 'anchor', BMP-PEG1K-PE, which incorporates a PEG spacer between the lipid and maleimide groups. Spacer-containing lipid 'anchors' like BMP-PEG-PEs will likely be required to achieve efficient coupling of Fab' fragments to liposomes that bear surface-anchored polymers as steric masking agents to reduce the rate of liposomal clearance in vivo [10,53–58].

[1] The weight fraction of maleimide-reactive Fab' in a given sample (as a fraction of total protein mass) was estimated from the Coomassie staining intensity of the BMP-PEG-shifted band, expressed as a fraction of the total staining intensity measured for all protein bands in the same sample. This calculation rests on two assumptions: first, that reduction of a given mass of $F(ab')_2$ to Fab' (or to light chains and heavy-chain fragments) does not substantially alter the total Coomassie-staining intensity summed over all of the product bands, and second, that the addition of PEG chains to Fab' fragments does not significantly alter their Coomassie-staining intensity. Both assumptions were supported by the results of control experiments designed to test these points.

In Fig. 3 are shown representative results illustrating the dependence of the Fab' coupling efficiency on the lipid concentration for LUV differing in their content and type of maleimidyl-lipid 'anchor.' It is clear that higher concentrations of liposomes promote greater efficiency of Fab' coupling, even though in these experiments the liposome-bound maleimidyl groups are in all cases far from saturated by coupled Fab' fragments (see Table 1). Liposomes incorporating a lower molar percentage of maleimidyl-lipid

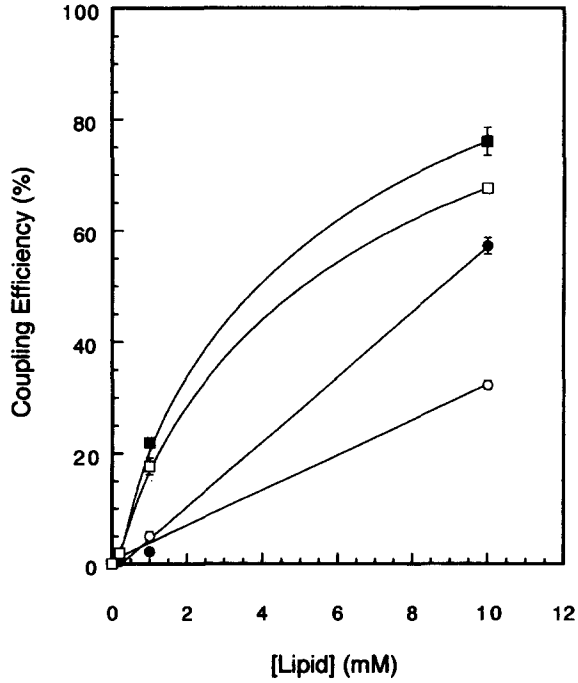

Fig. 3. Lipid concentration dependence of the coupling of TFR1 Fab' fragments (1 mg/ml) to PC/PG/cholesterol/Rho-PE (3:1:2:0.06 molar proportions) LUV containing 0.5 mol% BMP-PE (○), 0.5 mol% BMP-PEG1K-PE (●), 2.5 mol% BMP-PE (□) or 2.5 mol% BMP-PEG1K-PE (■). Coupling reactions were carried out as described in Section 2. Error bars represent the range of values measured for duplicate samples incubated at each lipid concentration.

(0.5 mol% vs. 2.5 mol%) must be present at higher concentrations to maximize the fraction of Fab′ that becomes liposome-bound. Interestingly, however, replacement of BMP-PE by the spacer-containing BMP-PEG1K-PE significantly enhances the efficiency of liposome-Fab′ coupling at lower lipid concentrations, particularly when using a low proportion (0.5 mol%) of the maleimidyl-lipid 'anchor'.

In Table 1 are summarized the results of a number of experiments to measure the effects of liposome size (ca. 30 nm. vs. 110 nm. average diameter), and of the content and type of maleimidyl-lipid 'anchor', on the efficiency of Fab′-liposome coupling at a fixed lipid concentration (10 mM). These experiments were carried out using preparations of Fab′ fragments containing 60% to 78% (weight fraction of total protein) of reactive sulfhydryl-bearing Fab′ fragments as assessed by the gel-shift assay described above. Several points are evident. First, using 2.5 mol% maleimidyl-lipid in either SUV or LUV, it is possible reproducibly to achieve coupling of a very high proportion of the reactive sulfhydryl-bearing Fab′ fragments to the liposomes, particularly using liposomes incorporating BMP-PEG1K-PE. Second, only minor reductions in coupling yield are observed using as little as 0.5 mol% of BMP-PEG1K-PE in the liposomes. By contrast, for liposomes containing the more conventional maleimidyl-lipids BMP-PE and EMC-PE, reduction in the content of maleimidyl lipid from 2.5 mol% to 0.5 mol% considerably reduces the efficiency of Fab′ coupling. Third, as the standard deviations in Table 1 demonstrate, procedures optimized specifically for the production of free sulfhydryl-bearing Fab′ fragments afford very good reproducibility as well as efficiency of liposome-Fab′ coupling. Finally, as shown by the final entry in Table 1, high efficiencies of liposomal coupling of rabbit polyclonal as well as mouse monoclonal Fab′ fragments can be achieved using the coupling protocols and lipid 'anchors' described here.

To monitor the effects of liposome-incorporated maleimidyl-lipids and of bound Fab′ fragments on retention of liposomal contents, two types of experiments were carried out. First, liposomes were prepared containing calcein at a high, self-quenching concentration (100 mM), and retention of calcein at 37°C was measured by a fluorescence-dequenching assay [45,49,50] as a function of the liposomal content of different maleimidyl-lipids and of the presence of bound Fab′ fragments. Representative data from such experiments are shown in Fig. 4. As shown in Fig. 4A, control liposomes prepared without any maleimidyl-lipid component lose roughly 12% of entrapped calcein after 24 h of incubation at 37°C. Under the same conditions liposomes incorporating 2.5 mol% of EMC-PE or BMP-PE show similar losses of encapsulated contents after 24 h incubation while liposomes incorporating 2.5 mol% of BMP-PEG1K-PE release a slightly greater fraction of their contents, mainly within the first few hours

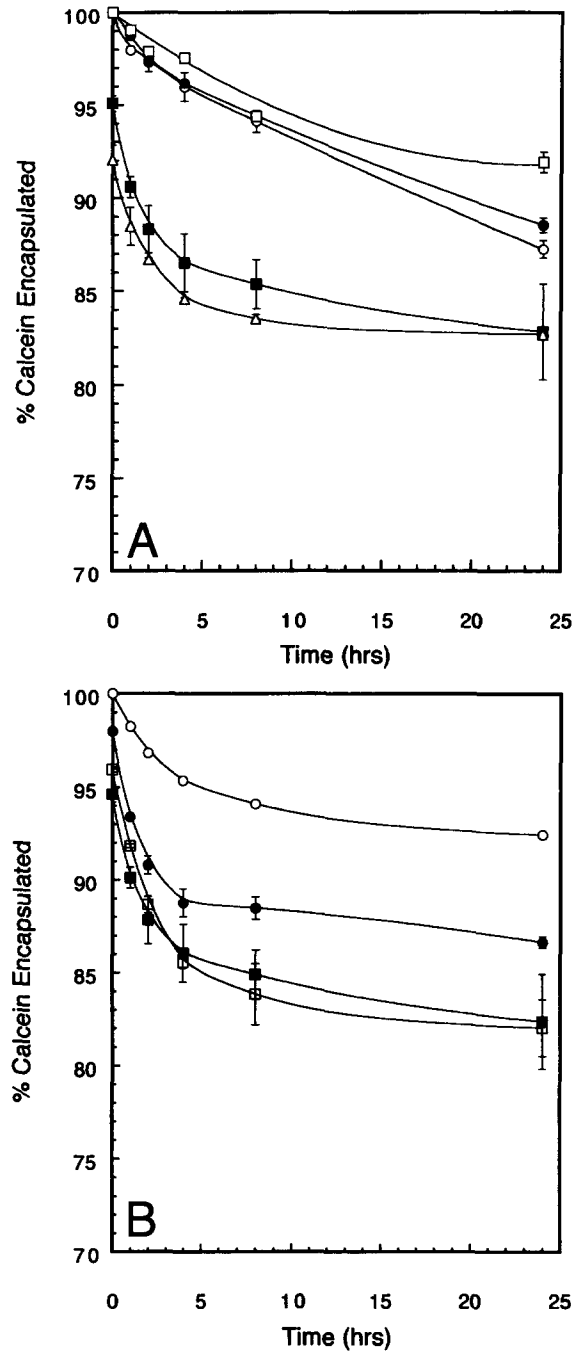

Fig. 4. Effect of maleimidyl-functionalized PEs on liposomal stability as determined by retention of encapsulated calcein. (A) PC/PG/cholesterol (3:1:2) LUVs, containing no maleimidyl-lipid (○), 2.5 mol% of either EMC-PE (●), BMP-PE (□) or BMP-PEG1K-PE (■), or 2.5 mol% BMP-PEG1K-PE coupled to TFR1 Fab′ fragments (△), were incubated at 37°C. Calcein retention at the indicated time points was determined and liposome-Fab′ coupling carried out as described in Section 2. (B) PC/PG/cholesterol LUVs containing 0 mol% (○), 0.5 mol% (●), 1.0 mol% (□) or 2.5 mol% (■) BMP-PEG1K-PE were incubated for varying times before determining the percentage encapsulation of calcein as above. Error bars represent the range of values measured for duplicate samples independently incubated for the indicated times.

of incubation, after which further leakage slows markedly. As illustrated in Fig. 4B, less leakage of entrapped calcein is seen for liposomes incorporating lower mol fractions of maleimidyl-lipid (in this figure, BMP-PEG1K-PE). The presence of coupled Fab′ fragments did not significantly affect the rate of calcein leakage from liposomes containing 2.5 mol% BMP-PEG1K-PE (Fig. 4A, open triangles).

In a second series of experiments, we examined the retention of calcein in liposomes during the process of Fab′ coupling at 4°C; Bredehorst et al. [21] have reported very substantial losses of liposomal contents during coupling of Fab′ fragments to liposomes containing a different maleimidyl-lipid 'anchor'. Calcein-loaded LUV (1 mM) containing different proportions of either BMP-PE or BMP-PEG1K-PE were incubated at 4°C with Fab′ fragments (1 mg/ml), then treated with cysteine to quench excess liposome-associated maleimidyl groups, using the same conditions as in our standard liposome-Fab′ coupling protocol. In a representative experiment, after coupling to Fab′ fragments (1 mg/ml) for 16 h at 4°C, liposomes containing 0.5 mol% or 2.5 mol% BMP-PE retained 91% and 84% of their originally entrapped calcein, respectively, while similar liposomes containing BMP-PEG1K-PE retained 89% and 85%, respectively. After subsequent quenching with 0.5 mM cysteine (15 min at 4°C), liposomes containing 0.5 mol% or 2.5 mol% BMP-PE retained

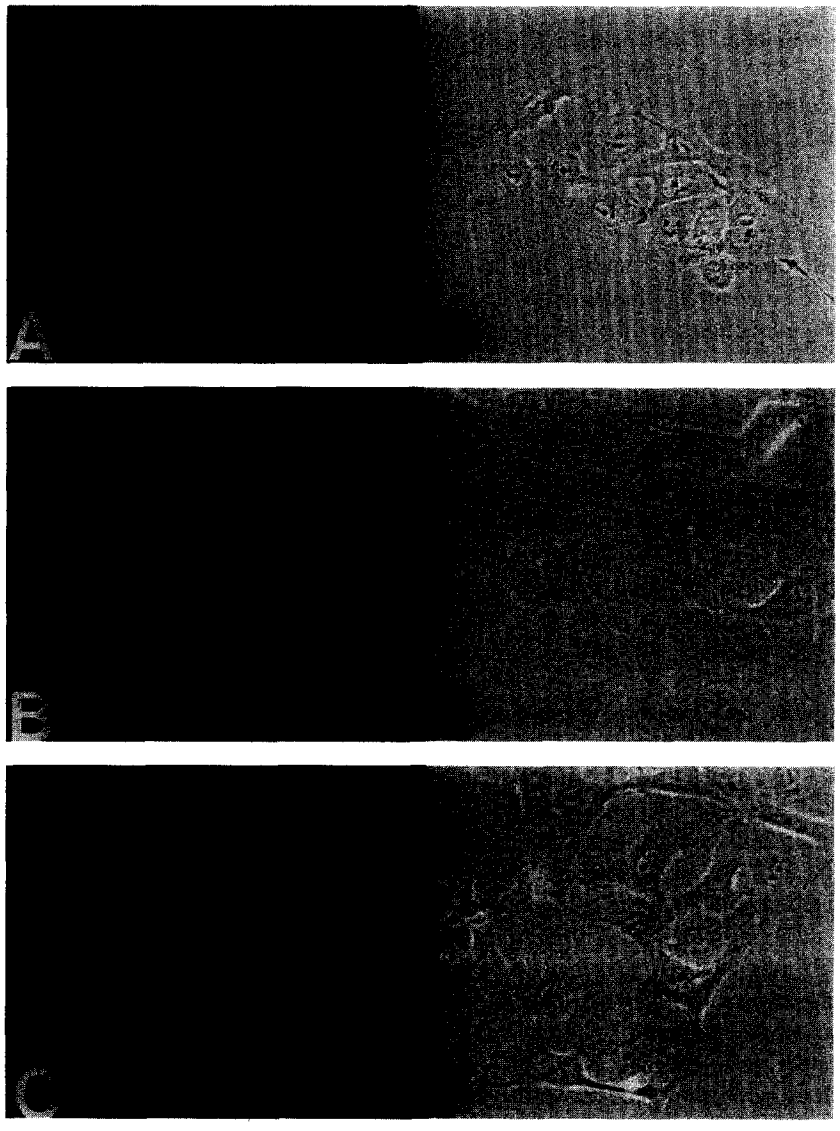

Fig. 5. Fluorescence microscopy of CV-1 cells incubated at 37°C with TFR1 Fab′- or cysteine-conjugated PC/PG/cholesterol/BMP-PEG1K-PE (3:1:2:0.015) LUV loaded with carboxyfluorescein (45 mM). (A) Fab′-conjugated liposomes (40 μM lipid) in the absence of free (TFR1) mAb. (B) Fab′-conjugated liposomes in the presence of 1 mg/ml free mAb. (C) Cysteine-conjugated ('untargeted') liposomes in the absence of free mAb. (D–F) Phase-contrast images of the fields shown in panels A–C. Details of sample preparation, liposome-cell incubations and fluorescence microscopy are given in Section 2.

91% and 74% of their original (pre-Fab'-incubation) calcein content, respectively, while similar liposomes containing 0.5 mol% or 2.5 mol% BMP-PEG1K-PE retained 77% and 66%, respectively. Control liposomes lacking maleimidyl-lipid retained 96% of initially encapsulated calcein after the initial 4°C incubation with Fab' fragments and showed negligible further calcein release upon subsequent incubation with cysteine. As noted above, once coupled to Fab' fragments liposomes showed very slow rates of leakage ($< 0.4\%/h$) upon subsequent incubation at 37°C.

To confirm that Fab'-coupled liposomes prepared in the manner described here are functionally competent to recognize the 'targeted' cell-surface determinant(s), we examined the uptake by CV-1 cells of carboxyfluorescein (CF)-loaded vesicles conjugated to TFR1 Fab' fragments, which recognize the extracellular domain of the transferrin receptor [33]. CF-loaded LUV containing 2.5 mol% BMP-PEG1K-PE were conjugated either to TFR1 Fab' fragments or to cysteine, and the liposomes thus derivatized were incubated for 1 h at 37°C with CV-1 cells, which were subsequently washed and examined by fluorescence microscopy. As shown in Fig. 5 (panels A/D), cells incubated with the Fab'-conjugated liposomes show a diffuse intracellular fluorescence which is due to endocytic uptake of the liposomes and subsequent diffusion of the protonated dye from the acidic endosomal lumen to the cytoplasm [49]. By contrast, cells incubated with TFR1 antibody (1 mg/ml) but not with an irrelevant antibody (not shown) before and during incubation with the Fab'-conjugated liposomes (panels B/E) show greatly reduced endocytic uptake of carboxyfluorescein. Similarly, non-Fab'-coupled ('untargeted') liposomes, treated with cysteine to block reactive maleimidyl groups [24] (panels C/F), show very low levels of endocytic uptake under the above incubation conditions. The results shown in Fig. 5 were obtained using cells preincubated with ePC/ePG/cholesterol SUV (50 μM), to reduce nonspecific binding of liposomes. A qualitatively similar pattern of results was observed using cells that were not pretreated with SUV (not shown), but in this case higher nonspecific uptake of carboxyfluorescein was observed using untargeted liposomes or targeted liposomes in the presence of blocking antibody. Interestingly, in the absence of SUV pretreatment the nonspecific binding of liposomes incorporating BMP-PEG1K-PE (with or without bound Fab') was much less than that of similar liposomes lacking this lipid 'anchor.' This result is likely attributable to steric interference by liposome-bound PEG groups with nonspecific interactions between the liposome and cell surfaces.

4. Discussion

While the strategy of coupling Fab' fragments to liposomes via their exposed 'hinge' thiols was first introduced in the early 1980s [20,26], this approach has achieved relatively limited use in practice, due in part to the low and variable coupling yields that may be achieved. Dithiothreitol reduction typically gives substantial over-reduction under conditions in which a majority of $F(ab')_2$ is cleaved, severely complicating efforts to obtain high and reproducible yields of Fab' fragments by this approach ([59]; and present results (not shown)). The use of monovalent thiol compounds as reducing agents greatly diminishes this problem but, as we have shown here, can still entail a more insidious one, namely the formation of appreciable amounts of 'non-couplable' Fab' fragments under conditions where Fab' production appears optimal by conventional SDS-PAGE analysis. Using the maleimidyl-PEG 'shift' assay to detect and to minimize this latter problem, conditions can be determined that routinely afford high efficiencies of liposome-Fab' coupling, equivalent to up to ca. 70% of input $F(ab')_2$ protein and to virtually 100% of the reactive sulfhydryl-containing Fab' fraction in a given preparation.

The phenomenon of gradual and substantial formation of 'non-couplable' Fab' fragments during reduction of $F(ab')_2$ with thiols such as cysteine or cysteamine has to our knowledge not hitherto been described, perhaps due to the previous absence of a direct assay for the formation of such species. In our view the most likely explanation for this phenomenon is a gradual reoxidation of the reduced hinge thiol(s) of freshly generated Fab' fragments, to form either Fab'-cyste(am)ine mixed disulfides or, potentially, an intrachain disulfide bond in Fab' fragments containing two reduced sulfhydryl groups. Such disulfides may form much more readily than do $F(ab')_2$ fragments, explaining the fact that the gradual appearance of 'non-couplable' Fab' during prolonged treatment with monovalent thiols is accompanied by only modest reformation of $F(ab')_2$.

The maleimidyl-PEG-based 'shift assay' described here provides a convenient means to optimize conditions for production of Fab' fragments bearing reactive sulfhydryl groups, as is required to prepare Fab'-containing conjugates with liposomes, toxins and other agents. The maleimidyl-PEG reagent, which can readily be prepared from commercially available α-amino-ω-methoxy-PEG5K and BMPS, efficiently and rapidly derivatizes Fab' thiol groups when present at relatively low concentrations and in only a modest molar excess over the Fab' fragments. A sample of an Fab' preparation can be derivatized and electrophoretically analyzed within as little as two hours (during which time Fab' fragments can be stabilized by cold and reduced pH [51]), in principle allowing the proportion of maleimide-reactive Fab' in a given preparation to be determined before liposomal coupling in applications where very fine control of coupled-Fab' density is critical. For most purposes, however, as the results shown in Table 1 indicate, very good reproducibility of Fab'-liposome coupling can be achieved simply by using a standard optimized protocol for Fab' production and well-controlled conditions for the coupling reaction.

The maleimide-based coupling of Fab′ fragments to liposomes containing an already-encapsulated solute has been reported to induce severe leakage of liposome-encapsulated molecules [21]. Coupling of Fab′ fragments to liposomes incorporating the maleimidyl-PE 'anchors' examined here induced comparatively modest, albeit significant, losses of encapsulated contents specifically during the coupling and cysteine-quenching steps. As observed previously, the presence of liposome-coupled Fab′ fragments has very little effect on retention of liposomal contents after the initial coupling is complete. For optimal encapsulation of solutes using the protocols described here, liposome-Fab′ coupling should thus ideally be performed with the solute present inside and outside the vesicles; unbound antibody and (where necessary) unencapsulated solute could then be removed together upon subsequent gel-filtration. Where this approach is not feasible, however, using appropriate levels of the maleimidyl-lipids described here, Fab′ fragments can still be coupled to liposomes in good yields and with acceptable (> 75%) retention of contents throughout the entire coupling procedure.

The novel PE-PEG-maleimide 'anchor' lipid employed here, BMP-PEG1K-PE, is analogous to a number of other functionalized PE-PEG's that have been described recently [11,12,58,60,61] and offers at least two potential advantages over previously described maleimidyl-lipids with shorter spacers between the lipid and maleimidyl moieties. First, proteins coupled to functionalized lipids containing PEG spacers typically retain good reactivity with cell-surface recognition determinants when incorporated into liposomes containing other PEG-modified lipids as steric masking agents [11,12,58,61]. This is not true for proteins coupled to lipid 'anchors' containing shorter spacer groups when incorporated in similar sterically 'masked' liposomes [10,53,61]. Second, as we have shown here, the extended spacer in BMP-PEG1K-PE permits more efficient coupling of Fab′ groups to liposomes, particularly at suboptimal lipid concentrations and at even low (0.5 mol%) mole fractions of the incorporated maleimidyl-lipid 'anchor'. This finding likely reflects an enhanced steric accessibility of maleimidyl groups to reactive Fab′ fragments when the maleimidyl groups are tethered to the liposome surface via a long, flexible PEG 'spacer'.

The efficiencies of liposome-Fab′ coupling routinely observed using the approach described here (ca. 70% of input protein for liposomes containing BMP-PEG1K-PE) are considerably better than those reported previously for Fab′ fragments (typically < 30% [1,8,20–23,26]) and are in fact comparable to the best liposomal coupling yields reported, using any coupling methodology, for whole antibodies. Since virtually 100% of free sulfhydryl-bearing Fab′ fragments can be coupled to liposomes using the conditions described here, it is possible that further improvements in the efficiency of production of such fragments could improve overall Fab′-liposome coupling efficiencies still further. Fab′ fragments offer potentially significant advantages over intact antibodies for certain applications, e.g., to avoid a variety of biological responses mediated by the antibody Fc region [16,17,62]. The results reported here should facilitate the wider use of Fab′ fragments for liposomal targeting in such cases.

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