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Lectin-mediated targeting of liposomes to a model surface

An ELISA method

F.J. Hutchinson and M.N. Jones

Department of Biochemistry & Molecular Biology, Biomolecular Organisation and Membrane Technology Group, University of Manchester, Manchester M13 9PT, England

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Wheat germ agglutinin has been conjugated to the surfaces of sonicated phospholipid liposomes by reacting the protein derivatised with *N*-succinimidyl-*S*-acetylthioacetate (SATA) with the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) derivative of dipalmitoylphosphatidylethanolamine (DPPE) incorporated into the liposomal bilayers. The liposomes as characterised by photon correlation spectroscopy had a weight-average radius of 44 ± 10 nm and the number of WGA molecules per liposome was in the range up to approx. 120. An ELISA method has been developed to assess the efficiency of targeting the conjugated liposomes to the antigenic determinants on a surface coated with glycophorin A (blood group B). For liposomes in which the degree of conjugation was controlled by varying the mol% DPPE-MBS from 3 to 27% targeting efficiency as assessed from the extent of inhibition of the ELISA increased by a factor of 10.

Lectin; Liposome; Targeting; ELISA

1. INTRODUCTION

The widespread interest in the use of liposomes as carriers of drugs, vaccines and other therapeutic agents [1-3] has led to studies directed to methods of incorporating glycoproteins [4-7] into liposome bilayers or covalently coupling proteins, particularly antibodies, to liposomal surfaces [8-10]. The objective of many of these studies was to improve the affinity of the liposomes for specific target sites on biosurfaces by exploiting biochemical specificity.

The binding specificities of lectins for sugars [11] make them an interesting alternative for targeting liposomes to the glycocalyx of membrane surfaces and other biosurfaces and lectins have been used as mediators in the targeting of liposomes to erythrocytes by either physically coating the erythrocytes with lectin [12] or by physically coating the liposomes [13]. Although there is no substitute for the in vivo assessment of targeting efficiency it is nevertheless desirable to have an in vitro system of assaying the effectiveness of targeting of liposomes bearing site-directed surface groups. When assessing parameters which affect targeting it is important to target to a reproducible model surface.

Here, sonicated liposomes incorporating phosphatidylethanolamine activated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide have been coupled with the *N*-succinimidyl-*S*-acetylthioacetate (SATA) derivative of wheat germ agglutinin (WGA). The efficiency of targeting of the resulting proteoliposomes to a glycophorin A surface has been investigated using a newly developed competitive enzyme-linked immunosorbent assay (ELISA).

Correspondence address: M.N. Jones, Department of Biochemistry & Molecular Biology, Biomolecular Organisation and Membrane Technology Group, University of Manchester, Manchester M13 9PT, England

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2. EXPERIMENTAL

2.1. *Materials*

L-α-Dipalmitoylphosphatidylethanolamine (DPPE, product no. P-0890), L-α-dipalmitoylphosphatidylcholine (DPPC, product no. P-0763), WGA (from *Triticum vulgaris*, product no. L-9640), glycophorin (from human blood type B, product no. G9511) and Sigma 104 phosphatase substrate tablets were obtained from Sigma (Poole, Dorset, England). SATA was from Calbiochem (Cambridge, England). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce Warriner (Chester, England). Phosphatidylinositol (PI) grade I was from Lipid Products (South Nutfield, Surrey, England). Anti-B (mouse hybridoma) was from Chembiomed (Edmonton, Canada) and alkaline phosphatase conjugate goat anti-mouse IgM antibody (μ chain specific) was from Tago Immunodiagnostics (Burlingame, CA). [^{3}H]DPPC was from Amersham International.

2.2. *Methods*

2.2.1. Derivatisation of WGA with SATA [14]

The SATA derivative of WGA was prepared by addition of SATA (0.69–6.9 μmol in 50 μl dimethylformamide) to WGA [0.28 μmol in 2.5 ml phosphate (50 mM)-EDTA (1 mM) buffer, pH 7.5] at room temperature. The solution always contained less than 10 μl dimethylformamide/ml. The reaction was complete in 15 min after which derivatized WGA was separated from unreacted SATA by gel filtration on a Sephadex G-50 column (15 × 2 cm). Fractions (2 ml) were collected and the WGA fractions (detected at $A_{280\ nm}$) were pooled and stored at 4°C. The extent of derivatisation of the WGA was determined after deacetylation of the sulphydryl group of the SATA by reaction with a solution of hydroxylamine (0.5 M plus 25 mM EDTA and solid Na_2HPO_4 to pH 7.5). The sulphydryl content of the deprotected WGA derivative was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Ellman [15]. Protein was assayed according to Lowry et al. [16] using a WGA (0–200 μg/100 μl) standard.

2.2.2. Synthesis of the MBS derivative of DPPE

DPPE (40 mg) was dissolved in a mixture of dry chloroform (16 ml), dry methanol (2 ml) and dry triethylamine (20 mg). MBS (20 mg) was added and the reaction mixture was stirred under nitrogen at room temperature for 24 h, after which the organic phase was washed three times with phosphate-buffered saline (PBS, pH 7.3) to remove unreacted MBS. The DPPE-MBS derivative was recovered from the organic phase by rotary evaporation and analysed by TLC using a silica plate and a solvent mixture containing chloroform, methanol and glacial acetic acid (65:25:13, by vol.). TLC confirmed that there was negligible contamination of DPPE-MBS (R_f 0.78) with DPPE (R_f 0.56). The DPPE-MBS was stored in a chloroform-methanol (9:1, v/v) mixture at 4°C.

2.2.3. Vesicle preparation and conjugation

DPPC (9 mg), PI (1 mg) and variable amounts of DPPE-MBS (0.4–4.5 mg) plus 100 μl [^{3}H]DPPC (4 μCi/ml) were dissolved in chloroform (20 ml) and methanol (5 ml) in a 1 l round-bottomed flask. The mixture was rotary evaporated at 60°C to yield a thin lipid film. The lipid film was dispersed in 5 ml nitrogen-saturated PBS at 60°C, vigorously shaken and transferred to a glass test-tube, purged with nitrogen, sealed with a suba-seal and sonicated in a bath sonicator (Decon FS100) at 60°C until visibly clear (1 h). The initial diameters of the vesicles were measured by photon correlation spectroscopy (PCS) using a Malvern autosizer. The liposome suspension was then applied to a Sephadex G-200 column (30 × 2 cm) previously equilibrated with PBS (pH 7.3) at a flow rate of 0.2 ml/min. Fractions (2 ml) were collected and aliquots (100 μl) of the fractions taken for scintillation counting. The size of the liposomes in the peak fraction was also measured by PCS.

The liposome fractions containing the highest [^{3}H]DPPC counts were mixed with derivatized WGA in appropriate proportions and reacted overnight at 4°C after which vesicle size was again monitored. The reaction mixture was applied to the Sephadex G-200 column and fractions (2 ml) collected. The fractions were assayed for protein content [14] and lipid from the [^{3}H]DPPC count.

2.2.5. Targeting assay

The targeting of the WGA-conjugated liposomes to a model biosurface (glycophorin A from blood group B) was assessed by ELISA. The microtitre plate (Dynatech M129B) wells were incubated with 100 μl glycophorin solution (0.02 g/l in PBS) for 12 h at 4°C or 2 h at 37°C. After washing (3 × 300 μl PBS), blocking of non-specific sites [300 μl of 1% bovine serum albumin (BSA) in PBS for 30 min at 37°C followed by 0.1% BSA/PBS] various amounts of liposomes were added in 100 μl of 0.1% BSA/PBS for 2 h at 37°C, after which the plates were washed with 0.1% BSA/PBS. Antibody-antibody conjugate (100 μl) mixture (100 μl anti-B mouse hybridoma plus 10 μl alkaline phosphatase goat anti-mouse IgM conjugate in 4.89 ml of 0.1% BSA/PBS premixed for 30 min) was added and incubated for 2 h at 37°C. After incubation the plate was washed with PBS (2 × 300 μl) and then water (4 ×) and 100 μl substrate [5 mg *p*-nitrophenylphosphate disodium hexahydrate in 5 ml glycine buffer (0.1 M glycine plus 2 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10.4)] added. The plate was left in the dark for 30 min and the absorbances measured at 405 nm. The washing steps were performed using a Dynatech 96-channel automatic washer (AM56) and the plates were read using a Dynatech MR610 reader coupled to an Apple IIe microcomputer.

3. RESULTS AND DISCUSSION

The results of derivatisation of WGA by SATA are shown in fig.1 which illustrates that the number of SATA residues which can be incorporated into WGA by the method described reaches a limiting value of approx. 3 per molecule. The number of lysine plus N-terminal residues in the WGA dimer is 14–16 [17,18]. The extent of derivatisation of WGA is comparable to that found for other proteins using SATA [14]. In this work the M_r of dimeric WGA was taken to be 36 000 [17,19]. Routinely, a reaction mole ratio of 10:1 SATA to WGA was used, giving approx. 2 reactive sulphydryl groups per dimeric WGA.

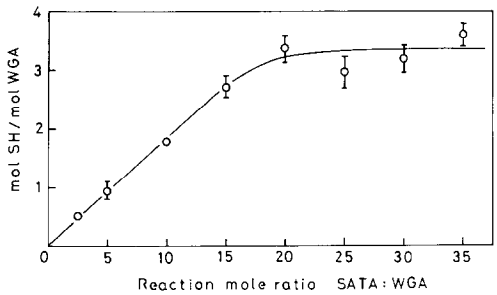


Fig.1. The derivatisation of wheat germ agglutinin (WGA) by SATA.

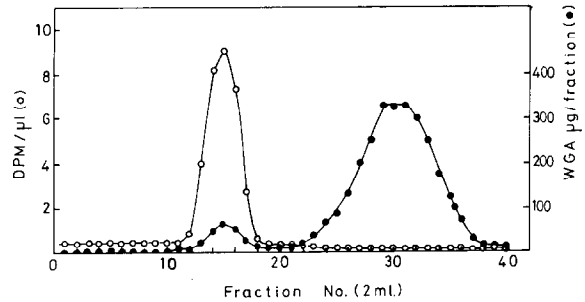


Fig.2. The elution profile of WGA-conjugated liposomes and unreacted WGA on a Sephadex G-200 column. Left-hand ordinate, activity of [^{3}H]DPPC (○); right-hand ordinate, WGA concentration (●). The conjugated liposomes elute in the void volume of the column. The fraction volume was 2 ml.

Fig.2 shows the gel filtration profile after conjugation in a typical liposome preparation. After sonication the unconjugated liposomes eluted in the void volume on a G-200 column; after reaction with derivatised WGA the unreacted WGA gave a peak clearly separated from the WGA-conjugated liposomes with a K_d of 0.64.

Fig.3 shows the results of an ELISA assay in which liposomes conjugated with WGA were targeted to glycophorin with blood group B antigenic determinants and their effectiveness in blocking access to B-antibodies, as a result of liposome-bound WGA binding to sugar (preferentially *N*-acetylglucosamine) residues was assayed. It is seen that in contrast to 'naked' liposomes, liposomes conjugated with WGA effectively inhibit the assay for B group activity. In this example, inhibition was ~83% when the glycophorin layer had been exposed to 0.4 mM liposomal lipid. The extent of conjugation was approx. 40 µg WGA/µmol lipid so that the concentration of conjugated WGA was approx. 1 µM. It is significant that free WGA at this and higher (up to 4 µM) concentrations did not inhibit the assay. Some inhibition by free WGA might be expected, however it is possible that in contrast to liposomal WGA, free WGA is more easily removed in the wash steps which proceed antibody-antibody-conjugate addition either as free WGA or possibly as partially complexed glycophorin, alternatively it is possible that inhibition requires multivalent binding which may occur more readily with liposomal WGA.

The relationship between targeting efficiency (ability to inhibit the ELISA assay) and the degree of coupling of WGA to the liposomes was investigated in a series of experiments in which the amount of DPPE-MBS in the liposomes was varied. Fig.4 shows targeting efficiency for liposomes in which the mole percentage of DPPE-MBS was varied from 3 to 27%. We found that when the amount of DPPE-MBS exceeded 27% the liposomes readily agglutinated and uncoupled WGA could not be satisfactorily separated by gel filtration. In the range up to 27 mol% DPPE-MBS the extent of WGA coupling increased monotonically up to 50 µg WGA/µmol lipid (i.e. 1.4 mmol WGA/mol lipid). The efficiency of targeting increases with the degree of coupling. As follows

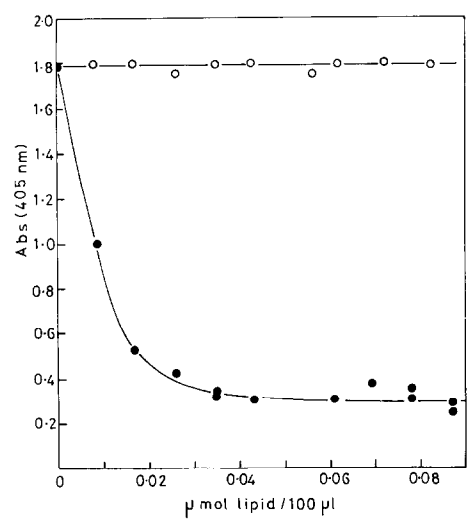


Fig.3. Competitive inhibition of the ELISA of glycophorin A (blood group B) antigenic determinants by WGA-conjugated liposomes (●). Control, unconjugated liposomes (○). WGA was conjugated to liposomes incorporating 10 mol% DPPE-MBS to give approx. 40 µg WGA/µmol lipid.

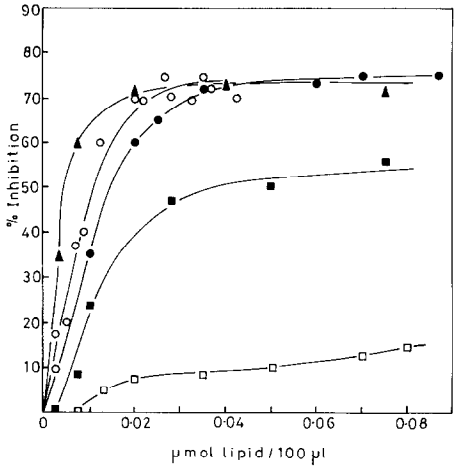


Fig.4. The effect of the degree of conjugation of WGA in WGA-conjugated liposomes on the competitive inhibition of the ELISA of glycophorin A (blood group B) antigenic determinants. The degree of conjugation was controlled by the mol% of DPPE-MBS in the liposomes which were as follows; □, 3%; ■, 5%; ●, 10%; ○, 15%; ▲, 27%.

from fig.4 for WGA-conjugated liposomes at a lipid concentration of 0.2 mM (0.2 μmol/100 μl in the microtitre well) inhibition increases by a factor of approx. 10 between liposomes conjugated with 3 and 27% DPPE-MBS.

The liposomes were characterised by photon correlation spectroscopy during each stage of their preparation. In the series of experiments shown in fig.4 the weight-average radii (r_w) of the initial sonicated liposomes was 36 ± 4 nm; after gel filtration on Sephadex G-200 the peak fractions taken for conjugation had an r_w of 35 ± 5 nm. After conjugation the size increased to 40 ± 6 nm and the peak fractions after separation of unreacted WGA by gel filtration on G-200 had an r_w of 44 ± 10 nm. Taking an area per lipid molecule of 0.5 nm^2 and a bilayer thickness of 7.5 nm [20] the number of WGA molecules per liposome based on the weight-average radii range from 17 (3% DPPE-MBS) to 117 (27% DPPE-MBS).

These experiments demonstrate that liposome targeting can be very rapidly and conveniently assessed using competitive inhibition of an ELISA system and that targeting with lectins is convenient and effective for carbohydrate antigenic determinants. The method described should be capable of adaptation and development to assess the efficiency of liposome targeting to many cellular antigens which can be immobilised.

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REFERENCES

[1] Knight, C.G. (1981) Liposomes: from Physical Structure to Therapeutic Applications, Elsevier, Amsterdam, New York.
[2] Ostro, M.J. (1987) Liposomes from Biophysics to Therapeutics, Marcel Dekker, New York.
[3] Weinstein, J.N. and Leserman, L.D. (1984) Pharmac. Ther. 24, 207–233.
[4] Juliano, R.L. and Stamp, D. (1976) Nature 261, 235–237.
[5] Redwood, W.R. and Polefka, T.G. (1976) Biochim. Biophys. Acta 455, 631–643.
[6] Goodwin, G.C., Hammond, K., Lyle, I.G. and Jones, M.N. (1982) Biochim. Biophys. Acta 689, 80–88.
[7] Schmitz, B. and Klein, R.A. (1986) Biochem. Biophys. Res. Commun. 141, 1274–1278.
[8] Hashimoto, Y., Sugawara, M. and Endoh, H. (1983) J. Immunol. Methods 62, 155–162.
[9] Hashimoti, Y., Sugawara, M., Kamiya, T. and Suzuki, S. (1986) Methods Enzymol. 121, 817–828.
[10] Connor, J., Sullivan, S. and Huang, L. (1985) Pharmac. Ther. 28, 341–365.
[11] Goldstein, I.J. and Poretz, R.D. (1986) in: The Lectins, Functions and Applications in Biology and Medicine (Liener, I.E. et al. eds) Chap. 2, pp. 103–115, Academic Press, New York.
[12] Eriksson, H., Mattiasson, B. and Sjögren, H.-O. (1984) J. Immunol. Methods 75, 167–179.
[13] Salame, M.Y. and Patel, H.M. (1986) Biochem. Soc. Trans. 14, 301–302.
[14] Duncan, R.J.S., Weston, P.D. and Wrigglesworth, R. (1983) Anal. Biochem. 132, 68–73.
[15] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Nagata, Y. and Burger, M.M. (1974) J. Biol. Chem. 249, 3116–3122.
[18] Wright, C.S., Gavilanes, F. and Peterson, D.L. (1984) Biochemistry 23, 280–287.
[19] Allen, A.K., Neuberger, A. and Sharon, N. (1973) Biochem. J. 131, 155–162.
[20] Janiak, M.J., Small, D.M. and Shipley, G.G. (1979) J. Biol. Chem. 254, 6068–6078.