Volume 231, number 2, 381–384 FEB 05789 April 1988

Protein immobilization on the surface of liposomes via carbodiimide activation in the presence of *N*-hydroxysulfosuccinimide

A.A. Bogdanov, jr, A.L. Klibanov and V.P. Torchilin

Institute of Experimental Cardiology, USSR Cardiology Research Center, Academy of Medical Sciences, 3-d Cherepkovsky 15 A, 121552 Moscow, USSR

Received 16 February 1988

A method of the covalent immobilization of proteins on the surface of liposomes, containing 10% (by mol) of *N*-glutaryl phosphatidylethanolamine, is described. Carboxylic groups of liposomal *N*-glutaryl phosphatidylethanolamine were activated in the presence of water-soluble carbodiimide and *N*-hydroxysulfosuccinimide and reacted subsequently with protein amino groups. The liposome-protein conjugates formed contained up to 5×10^{-4} mol protein/mol lipid. Lectins (RCA_I and WGA) upon immobilization on liposomes retained saccharide specificity and the ability to agglutinate red blood cells. The immobilization of mouse monoclonal IgG in a ratio of 3.5×10^{-4} mol IgG/mol lipid was achieved. The liposome activation in the absence of *N*-hydroxysulfosuccinimide resulted in a 2-fold decrease of protein coupling yields.

Liposome; *N*-Hydroxysulfosuccinimide; Carbodiimide; Lectin; Monoclonal antibody; Immobilization

1. INTRODUCTION

Recently a number of methods of protein coupling to preformed liposomes was described (reviewed in [1,2]). The immobilization of thiolated proteins on liposomes bearing *N*-[4-(*p*-maleimidophenyl)butyryl] or *N*-[(3-pyridyl-2-dithio)propionyl] groups seems to be one of the most effective procedures [3,4]. Proteins lacking free SH groups are usually subjected to pretreatment with SPDP [5,6] or *N*-hydroxysuccinimidyl-*S*-acetylthioacetate [7]. The evident drawback of the method is that this modification may lead to partial loss of the binding specificity of protein or even to its complete denaturation.

Recently we applied the Con A-liposome conjugates for binding studies in cell-liposome experimental system [8,9]. The covalent attachment of thiolated Con A to liposomes did not affect noticeably the D-mannose-binding properties of Con A. Nevertheless, with other lectins (RCA_I and WGA) the SPDP-coupling method was found to be unacceptible due to the loss of the lectin specificity towards saccharides.

Here we describe a simple method of protein conjugation with liposomes directly via protein amino groups by carbodiimide activation in the presence of *N*-hydroxysulfosuccinimide. High binding yields were obtained in 2.5—3 h of incubation of the native protein with preactivated lipo-

Correspondence address: V.P. Torchilin, Institute of Experimental Cardiology, USSR Cardiology Research Center, Academy of Medical Sciences, 3-d Cherepkovsky 15 A, 121552 Moscow, USSR

Abbreviations: WGA, wheat germ agglutinin; RCA_I, agglutinin from *Ricinus communis*; Con A, concanavalin A; $HoSu(SO_3^-)Na^+$, *N*-hydroxysulfosuccinimide, sodium salt; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DPPC, L-α-phosphatidylcholine dipalmitoyl; PE, phosphatidylethanolamine; DPPE, L-α-phosphatidylethanolamine dipalmitoyl; SPDP, *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate; BBS, 50 mM sodium tetraborate, 0.1 M NaCl, pH 7.5; MesBS, 5 mM 2-(*N*-morpholinoethane) sulfonic acid, 0.15 M NaCl, pH 5.5; SUV, small unilamellar vesicle; REV, reverse-phase evaporation vesicle

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

somes at pH 7.5. The immobilized proteins retain their specific activity.

2. MATERIALS AND METHODS

WGA was purchased from Calbiochem. Affinity purified RCA_I was a generous gift of Dr A.G. Tonevitsky; mouse monoclonal antibody 9B9 (IgG_1 subclass) was kindly provided by Dr S.M. Danilov (both at the Institute of Experimental Cardiology, USSR Cardiology Res. Center). Lipids and other chemicals were obtained from Sigma unless specified.

$HoSu(SO_3^-)Na^+$ was prepared as described in [10]. *N*-Glutaryl PE was obtained according to the recommendations of Weissig et al. [11]. The product was purified by preparative TLC performed on Kieselgel 60 (Merck) plates developed with chloroform/methanol/water (65:25:4).

SUVs were prepared by sonication of DPPC and *N*-glutaryl PE mixture (9:1, molar ratio, 5 mM lipids) in MesBS. The sonication was performed with Ultratip instrument (Lab-Line Inc., USA) for 20 min at 45°C. Liposomes were centrifuged at 40000 × *g* for 1 h and supernatant was used in further experiments. Large liposomes (REVs) were prepared from DPPC, cholesterol and *N*-glutaryl PE (6:3:1 by mol, 50 mM lipid) in MesBS by the method described in [12]. REVs were extruded through stacked 0.4 and 0.2 μm Nucleopore membranes [13]. Trace amounts of cholesteryl-[1-^{14}C]oleate (56 Ci/mol, Amersham) were used as the radioactive lipid marker.

Protein solutions were centrifuged before use at 105000 × *g* for 1 h to remove aggregates. ^{125}I-labeled proteins were obtained using $Na^{125}I$ and Iodogen (Pierce) [14]. All the proteins were transferred to BBS by gel filtration on Sephadex G-25 (Pharmacia) or Bio-gel P-4 (Bio-Rad) columns.

The immobilization was run as follows: (i) to 200 μl of liposomes (1 μmol lipids) in MesBS, 20 μl EDC (0.25 M in water) and 20 μl of 0.1 M $HoSu(SO_3^-)Na^+$ were added, and then incubated for 10 min; (ii) 200 μl of protein solution in BBS (1 mg of protein/ml, 4000 cpm/μg of protein) were added and the resultant mixture (pH 7.4) was incubated for 2.5–3 h at room temperature with mixing. Liposomes were separated from the unbound protein on Sepharose CL-6B of CL-4B (in the case of REVs) presaturated with lipid and BSA [15]. The ^{125}I and ^{14}C radioactivities were counted in the eluate. The ^{14}C activity values were corrected after the determination of ^{125}I counting rate efficiency in a ^{14}C channel.

In the agglutination studies glutaraldehyde-fixed canine RBCs were used. 50 μl of 2% RBC suspension were mixed with 50 μl liposomes (or lectin solution) in round-bottom Microtest plates. To study the inhibitory effect on hemagglutination, sugar solution (0.1 M final concentration) was added to the RBC suspension. The endpoint agglutination titer was estimated after 30 min of incubation using the Salk pattern method.

3. RESULTS AND DISCUSSION

As has been shown recently by Papisov et al. [16], a two-stage immobilization procedure may be useful for high-yield coupling of proteins to the carboxylic carrier by water-soluble carbodiimide. In this case, first the short-time activation of carboxylic groups by water-soluble carbodiimide is carried out at pH = $pK_a - 1$, where K_a is the ionisation constant of the given carboxylic group. In the second stage, the protein solution is added to the activated carrier with simultaneous change of pH to 8. This procedure was already performed for the immobilization of IgG on liposomes, which contained carboxylic derivatives of PE [17]. Certain drawbacks of the method exist due to the side reactions of the EDC-activated carboxylic derivative (*O*-acylisourea) (fig.1). *O*-Acylisourea is susceptible to fast hydrolysis resulting in the formation of the starting components (pathway 2) and isomerisation to non-active *N*-acylurea (pathway 3, fig.1). The introduction of *N*-hydroxysulfosuccinimide [10] to the reaction mixture during EDC-activation leads to the formation of the more stable to hydrolysis and non-isomerizable hydrophilic activated derivatives of carboxylic groups (*N*-hydroxysulfosuccinimide esters) (pathway 4). The hydrolysis of $HoSu(SO_3^-)$ esters is much slower than the reaction with primary amino groups [18]. Thus, a higher coupling yield can be obtained compared to the traditional carbodiimide coupling procedure.

In a study presented here we coupled lectins and mouse monoclonal antibody to SUVs and REVs, which were activated with EDC in the presence of $HoSu(SO_3^-)Na^+$. A starting ratio of 0.01 mol protein/mol *N*-glutaryl DPPE was used. The maximal coupling efficiency was achieved after a 2.5 h incubation of proteins with activated liposomes (table 1). By using the immobilization procedure the protein-liposome conjugates were obtained containing up to 5×10^{-4} mol protein/mol liposomal lipid. If the activation of liposomes was carried out in the absence of $HoSu(SO_3^-)Na^+$, the coupling yields were diminished by 2- or 3.5-fold for RCA_I and WGA, respectively. The highest percent of nonspecific binding of lectins to nonactivated liposomes was observed in the case of Con A: 15% of the total protein added to liposomes. With RCA_I and WGA the nonspecific binding was not higher than 3%. In some control experiments the level of intramolecular crosslinking of proteins was estimated when the procedure was performed in the absence of liposomes. The formation of protein aggregates was detected only for Con A (1–3% of protein used). These ag-

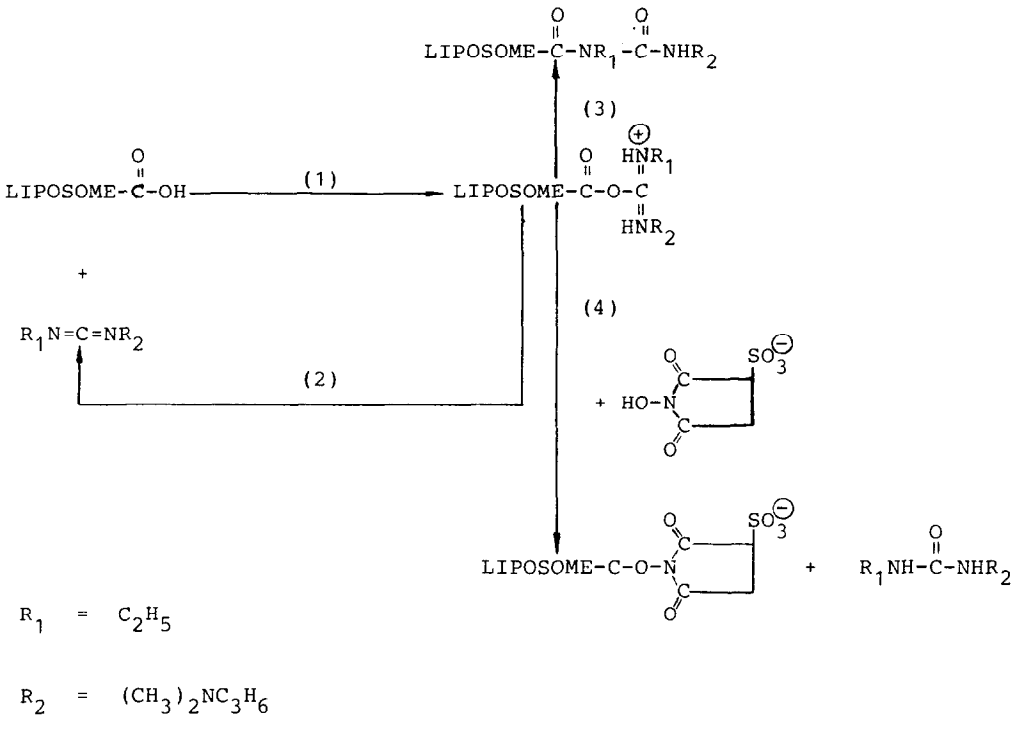

Fig.1. The carbodiimide activation of liposomes carrying carboxylic groups: (1) the modification of carboxylic group with the formation of *O*-acylisourea; (2) the hydrolysis of *O*-acylisourea; (3) the isomerisation of *O*-acylisourea with the formation of *N*-acylurea; (4) the formation of *N*-hydroxysulfosuccinimide ester in the presence of *N*-hydroxysulfosuccinimide.

Table 1

The immobilization of proteins on liposomes prepared from *N*-glutaryl PE/DPPC (1:9, molar ratio) by EDC

Liposomes were incubated with			Protein/ lipid molar ratio in conjugates	Coupling yield (%)[a]
EDC	$HOSu(SO_3^-)Na^+$	Protein		
+	+	RCA_I	5×10^{-4}	43
+	–	RCA_I	2.5×10^{-4}	21
–	+	RCA_I	7×10^{-6}	1.5
+	+	WGA	3.3×10^{-4}	40
+	+	Con A	4.7×10^{-4}	42
+	+	IgG	3.5×10^{-4}	40[b]

[a] The coupling yield was calculated dividing the protein/lipid ratio in the purified conjugates by the protein/lipid ratio in the incubation mixture

[b] For the immobilization REVs [12] were used. Liposomes were separated from unbound protein by gel filtration on Sepharose CL-4B

Reagents were used in the following concentrations: 0.45 mg/ml protein, 10 mM EDC, 4.5 mM $HoSu(SO_3^-)Na^+$, 2 mM lipid

gregates can be removed effectively by flotation of liposomes in discontinuous gradients of metrizamide [17] or Ficoll [19].

The method used in our study proved to be suitable for the immobilization of monoclonal IgG on large liposomes. The resultant conjugates (immunoliposomes) were proposed earlier as an effective tool for drug targeting [2]. The short-time incubation of IgG with activated REVs in the presence of $HoSu(SO_3^-)Na^+$ afforded 40% of the protein to be coupled with liposomes. The conjugates contained 3.5×10^{-4} mol IgG/mol liposomal lipid (table 1).

Thus, the method of immobilization presented is efficient enough to obtain conjugates with high protein content. In the case of SUVs (3×10^3 molecules of lipid per liposome [20]) and a protein/lipid molar ratio equal to 5×10^{-4}, each liposome is bearing 1.5 molecules of protein on average. Assuming molecules of lectins are evenly distributed over the liposomal surface, half of the

Table 2

The agglutination of RBC by free lectins and liposome-conjugated lectins

Agglutinin	Endpoint agglutination titer (μg protein/ml)
RCA_I	0.01
RCA_I + Gal[a]	50
WGA	0.04
WGA + NAcGlc	40
RCA_I-liposomes[b]	0.02
RCA_I-liposomes + Gal	78
WGA-liposomes[c]	0.09
WGA-liposomes + NAcGlc	>32[d]

[a] 0.1 M of sugars were used

[b] Lectin-liposome conjugates contained 78 μg RCA_I/mg lipid (5×10^{-4} M protein/M lipid)

[c] Conjugate contained 32 μg WGA/mg lipid (3.3×10^{-4} M protein/M lipid)

[d] WGA-liposomes in the presence of NAcGlc did not agglutinate RBC

liposome population contains at least 2 molecules of lectin per liposome. Such a particle can participate in RBC agglutination mediated by lectins, since there are numerous binding sites for RCA_I and WGA on RBC [21]. The titers of RBC agglutination were compared for free lectins and lectin-liposome conjugates (table 2). From the results presented it is clear that an almost 2-fold higher concentration of immobilized lectins is needed to achieve positive agglutination of RBC compared to free lectin. The result obtained is in agreement with the assumption that at the given protein/lipid ratio half of the liposomes are involved in the agglutination of RBC. The inhibition of agglutination by Gal and NAcGlc was observed for both free and liposome-immobilized lectins. Lectin-free liposomes agglutinated RBC at very high lipid concentrations (2 mg of lipid/ml) only. Thus, immobilized RCA_I and WGA retain the ability to agglutinate RBC as well as saccharide specificity.

Therefore it is reasonable to conclude that the application of *N*-hydroxysulfosuccinimide for protein immobilization on the carbodiimide-activated liposomes enhances the efficiency of the method. Conjugates containing up to 5×10^{-4} mol protein/mol liposomal lipid with high reaction yields were obtained by simple and rapid procedure.

REFERENCES

[1] Torchilin, V.P. (1985) CRC Crit. Rev. Ther. Drug Carrier Systems 2, 65–115.

[2] Connor, J., Sullivan, S. and Huang, L. (1985) Pharmacol. Ther. 28, 341–365.

[3] Martin, F. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.

[4] Martin, F., Hubbell, W. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238.

[5] Carlsson, J., Drevin, H. and Axen, R. (1978) Biochem. J. 173, 723–737.

[6] Lasch, J., Niedermann, G., Bogdanov, A.A. jr and Torchilin, V.P. (1987) FEBS Lett. 214, 13–16.

[7] Derksen, J.T.P. and Scherphof, G.L. (1985) Biochim. Biophys. Acta 814, 151–155.

[8] Bogdanov, A.A. jr, Gordeeva, L.V., Margolis, L.B. and Torchilin, V.P. (1986) Biol. Membr. 3, 674–684.

[9] Margolis, L.B., Bogdanov, A.A. jr, Gordeeva, L.V. and Torchilin, V.P. (1988) in: Liposomes in Drug Targeting: Trends and Progress (Gregoriadis, G. ed.) pp.727–735, Wiley, London.

[10] Staros, J.V. (1982) Biochemistry 21, 3950–3955.

[11] Weissig, V., Lasch, J., Klibanov, A.L. and Torchilin, V.P. (1986) FEBS Lett. 202, 86–90.

[12] Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.

[13] Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 601, 559–571.

[14] Markwell, M.A.K. and Fox, C.F. (1978) Biochemistry 17, 4807–4817.

[15] Torchilin, V.P., Klibanov, A.L. and Smirnov, V.N. (1982) FEBS Lett. 138, 483–487.

[16] Papisov, M.I., Maksimenko, A.V. and Torchilin, V.P. (1985) Enzyme Microb. Technol. 7, 11–16.

[17] Kung, V.T. and Redemann, C.T. (1986) Biochim. Biophys. Acta 862, 435–439.

[18] Staros, J.V., Wright, R.W. and Swingle, D.M. (1986) Anal. Biochem. 156, 220–222.

[19] Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81.

[20] Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. USA 75, 308–310.

[21] Grant, C.W.M. and Peters, M.W. (1984) Biochim. Biophys. Acta 779, 403–422.