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Cholesterylsuccinyl-*N*-hydroxysuccinimide as a cross-linking agent for the attachment of protein to liposomes

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Immunoglobulin-coated liposomes containing drugs have been examined (i) for their ability to specifically interact with cognate surface antigen on tumour cells and (ii) for targeting to tumours in animals [1]. Antibody coated liposomes have been prepared by Hashimoto *et al.* [1] who incorporated *N*-(*m*-maleimido benzoyl) dipalmitoyl-phosphatidyl ethanolamine into liposomes which were then reacted with immunoglobulin. Covalent attachment occurs via protein sulphydryl-SH groups to the maleimido area of the modified phospholipid. Attachment of immunoglobulins has also been achieved through a disulphide sulphydryl group exchange reaction using liposomes containing dipalmitoyl phosphatidyl-ethanolamine-3-(2-pyridyldithio) propionate [2]. Less-specific cross-linking procedures have made use of the coupling reagents toluene-2,4-diisocyanate and 1-ethyl-3-(dimethylaminopropyl) carbodiimide [3, 4].

In the present communication we describe an alternative procedure for attaching proteins and amino group-containing molecules to liposomes. The method makes use of cholesteryl-succinyl-*N*-hydroxysuccinimide incorporated into membrane structures of liposomes (Fig. 1). The resulting liposomes were found to be capable of interacting with amino group-containing substances to give molecues covalently attached to the surface.

Materials and Methods

Chemicals. Cholesteryl hemisuccinate, *N*-hydroxysuccinimide and *N,N'*-dicyclohexyl carbodiimide were purchased from the Sigma Chemical Co., Pool, U.K.). [^{3}H]Puromycin (5 Ci/mmol) was supplied by Amersham (Bucks, U.K.). All other reagents were of analytical grade.

Preparation of cholesteryl hemisuccinyl-*N*-hydroxysuccinimide (Fig. 1). Cholesteryl hemisuccinate (97.4 mg, 0.2 mmol) and *N*-hydroxysuccinimide (25.3 mg, 0.02 mmol) were dissolved in 1 mL of dioxane. To this solution was added *N,N'*-dicyclohexyl carbodiimide (41.3 mg, 0.2 mmol) dissolved in 0.3 mL dioxane. The reaction mixture was allowed to stand at room temperature overnight. Dicyclohexylurea was removed by filtration and the clear filtrate taken to dryness at 37° under vacuum. The residue was taken up in 2 mL dioxane and allowed to stand at room temperature for 2 hr until no further crystallization of dicyclohexlurea occurred. Following filtration the final solution was concentrated to dryness and the residue recrystallized from isopropanol. m.p. 151–152°. Chromatography on silica gel $60F_{254}$ TLC plates developed in $CHCl_3$:methanol (9:1, v/v) gave a single spot (hydroxylamine/$FeCl_3$ for active ester and 1% $HClO_4$ with

heating for steroids), $R_f = 0.81$. Found C, 70.93; H, 9.87; N, 2.23. Calculated for $C_{35}H_{53}O_6N$. 1 Isopropanol:C, 70.88; H, 9.55; N, 2.18.

Liposome preparations. Liposomes containing dipalmitoyl phosphatidyl choline and cholesterol were prepared by a reverse phase method in the presence of sucrose [5, 6]. For the preparation of liposomes containing active ester, cholesterol and cholesteryl hemisuccinyl *N*-hydroxysuccinimide were used at a mole ratio of 1.0 to 0.54. Liposomes used in protein binding studies were unfractionated whilst puromycin binding studies were performed with trisacryl spun column purified liposomes.

Interaction between liposomes containing cholesteryl hemisuccinyl-N-hydroxysuccinimide and [^{3}H]puromycin. Aliquots of control and activated liposomes (0.7 mg cholesterol/1 mL PBS were separately incubated with 10 μCi [^{3}H]puromycin for 4 hr at 37°. Incubation mixtures were then filtered through columns of Sephadex G-50 (1 × 20 cm) and eluted with PBS at 20°. Fractions (2.0 mL) were collected and radioactivity determined by liquid scintillation with appropriate controls. Optical densities (276 and 620 nm) were also determined on each fraction.

Interaction between liposomes containing cholesteryl hemisuccinyl-N-hydroxysuccinimide and peroxidase. To aliquots (0.9 mL) of either control or activated liposomes (0.4 mg cholesterol) was added peroxidase (1.2 mg in 0.4 mL PBS) and imidazole (6 mg). The suspensions were incubated at 21° for 12 hr and passed through a Biogel A1.5 column (1 × 55 cm) at 11 mL/hr. To each fraction collected (2.3 mL) was added 100 μL of colour reagent containing hydrogen peroxide (0.006%) and *O*-phenylenediamine (0.04%) in 100 mM sodium phosphate, pH 5.0. Colour development was arrested after 5 min by the addition of 6 N H_2SO_4 (100 μL) and intensities determined at 491 nm (λ_{max}). Liposome concentration was obtained from cholesterol assays using the Lieberman Burchard method.

Results and Discussion

Liposomes containing the activated ester cholesterylsuccinyl-*N*-hydroxysuccinimide in their membranes (Fig. 1) react with and covalently bind amino group-containing substances. Results concerning Sephadex G-50 column chromatography of reaction mixtures containing either activated or control liposomes previously incubated with [^{3}H]puromycin are shown in Fig. 2. It is seen that the liposomes containing activated cholesteryl ester (Fig. 2B) bind considerably larger amounts of puromycin than the control liposomes (Fig. 2A).

Further experiments were carried out with a protein to ascertain if activated liposomes were capable of interacting with larger molecules. Peroxidase was chosen for this purpose as its association with liposomes could be determined by a colour reaction following separation of conjugates from unbound peroxidase. Control and activated liposomes were incubated with peroxidase overnight and products fractionated by gel filtration (Fig. 3). Peroxidase containing fractions were detected with *O*-phenylenediamine/hydrogen peroxide. The results indicate that activated liposomes bind peroxidase whilst control liposomes do not bind the protein. Moreover, it seems reasonable to conclude that the peroxidase is covalently attached to the liposomes and not merely entrapped as no apparent association takes place with control liposomes. The rapid colour development observed in detecting the presence of peroxidase does not permit a reliable estimation of the stoichiometry of complexes.

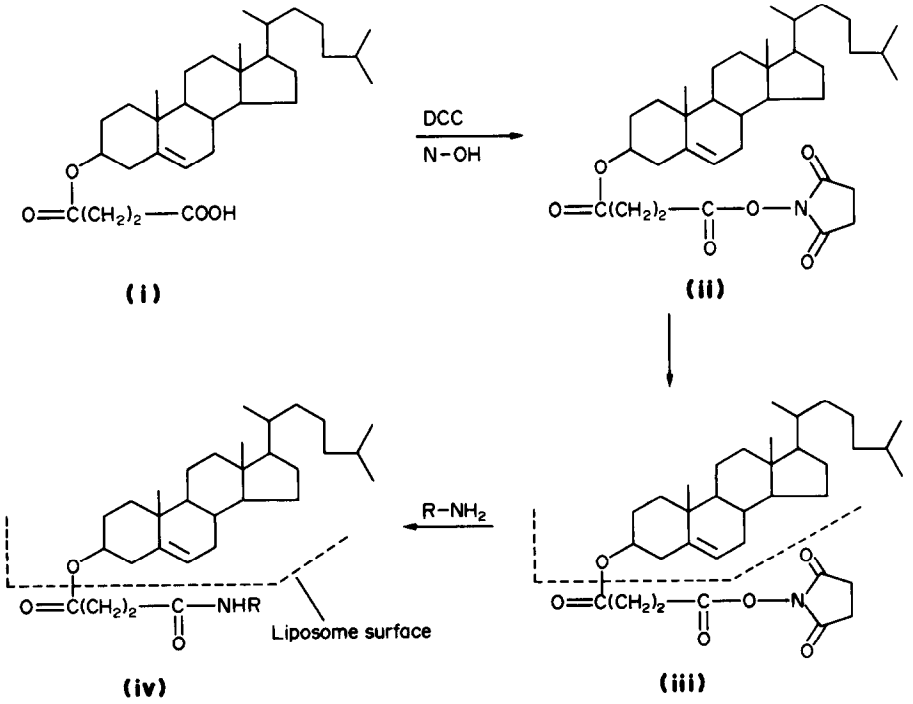

Fig. 1. Synthesis of cholesteryl hemisuccinyl-*N*-hydroxysuccinimide (ii) from cholesteryl hemisuccinate (i) and *N*-hydroxysuccinimide (N-OH) using *N*,*N*'-dicyclohexyl carbodiimide (DCC). Incorporation of (ii) into liposomes to give activated liposomes (iii), followed by reaction of the activated liposomes with either puromycin or horseradish peroxidase (RNH_2) to give (iv).

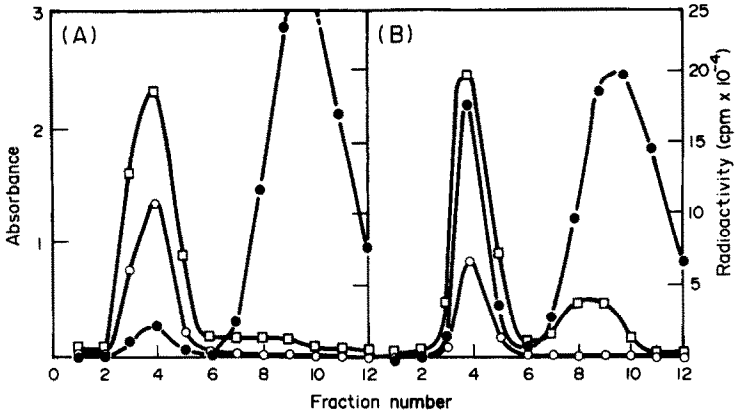

Fig. 2. Chromatography of liposomes on Sephadex G-50 following interaction with [^{3}H]puromycin. Reaction mixtures were subjected to gel filtration through columns (1 × 20 cm) as described in Materials and Methods. Fractions were assayed for radioactivity of [^{3}H]puromycin (●) and optical densities at 276 nm (□) and 620 nm (○), respectively, were determined. (A) Control liposomes. (B) Activated liposomes containing cholesteryl hemisuccinyl-*N*-hydroxysuccinimide.

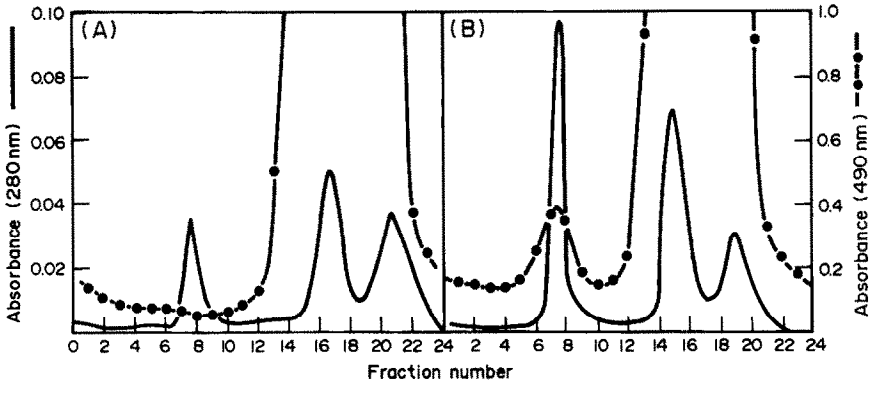

Fig. 3. Gel filtration of liposomes following interaction with horseradish peroxidase. Reaction mixtures were applied to a Biogel A1.5 column (1 × 55 cm) and eluted with phosphate buffered saline. Eluates were monitored at 280 nm (—) and individual fractions were assayed for peroxidase (●—●). (A) Control liposomes. (B) Activated liposomes containing cholesteryl hemisuccinyl-*N*-hydroxysuccinimide.

In summary, it has been shown that it is possible to prepare liposomes containing the activated ester, cholesteryl hemisuccinyl-*N*-hydroxysuccinimide for the purpose of covalently attaching amino group-containing molecules and proteins to the surface of such particles.

Department of Biochemistry
University of Durban-Westville
Durban, 4000
South Africa

GERALD M. RADFORD
MARIO ARIATTI*
ARTHUR O. HAWTREY†

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* To whom correspondence should be addressed.
† Present address: Department of Pharmacology, Faculty of Medicine, University of Stellenbosch, PO Box 63, Tygerberg, 7505, South Africa.