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A simple method for preparation of immuno-magnetic liposomes

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Abstract

A simple and readily manoeuverable method for preparing immuno-magnetic liposomes that indigenously contain binding sites for attaching other molecules like antibodies on their exterior surface is described. In this method magnetic unilamellar vesicles are prepared from a mixture of phosphatidylcholine, cholesterol, small amounts of a linear chain aldehyde and colloidal particles of magnetic iron oxide, using a reverse phase evaporation technique. The aldehyde (dedecanal) molecules align themselves among the lipid molecules in the bilayer with their aldehyde groups exposed to the aqueous phase, allowing straight attachment of antibody molecules (human-antimouse IgG-FITC in this case) in one single step. The success of this approach is confirmed by fluorescence microscopy as well as binding of the resulting immuno-magnetic liposomes to their corresponding target cells.

Keywords: Liposomes; Immuno-liposomes; Magnetic; Antibody; Attachment; Aldehyde; Immuno-magnetic

1. Introduction

Ever since their discovery, liposomes (lipid vesicles) have been a subject of great interest in a wide variety of applications, namely as simplified biomembrane models or as potential biocompatible carriers of genetic and pharmaceutical agents (Bangham and Horn, 1964; Gregoriadis and Allison, 1980; Knight, 1981; Gregoriadis and Florence, 1992; Ostro, 1987; Peeters et al., 1987; Pidgeon and Hunt, 1987; Lopezs-Berestein and Fidler, 1989; Gray and Morgan, 1991; Jones and Chappman, 1995). Despite the extensive efforts and achievements, there still are many obstacles to their in-vivo use as drug carriers in the body, e.g. their recognition and capture by the defence system and their accumulation in the liver and spleen (Gregoriadis and Ryman, 1972). Many approaches have been improvised to avoid such problems and to increase their lifetime in blood (Patel and Baldeschwieller, 1983; Proffit, 1986; Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen and Hasan, 1989; Klibanov et al., 1991; Gabizon, 1992; Woodle and Lasic, 1992), e.g. the use of immunoliposomes (Huang et al., 1980; Stonsons and Mallett, 1981; Schwendener et al., 1990), pH sensitive (Yatvin and

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Krutz, 1980; Connor and Huang, 1986; Holmberg and Reuer, 1994), and thermosensitive liposomes (Yatvin and Weinstein, 1990; Viroonchatapan and Sato, 1996). Another alternative has been the introduction of magneto-sensitive liposomes in order to help localize the drug loaded particles at therapeutic site of interest, (Margolis and Namit, 1983; Kato and Kiwada, 1986; De Cuypur and Joniau, 1988; Scheffold and Milteni, 1995; Viroonchatapan and Sato, 1996).

Several techniques have been developed and applied for attachment of antibodies to the exterior surface of liposomes, (Huang and Kennel, 1979; Magee and Miller, 1979; Sollivan and Huang, 1986; Wessing and Lasch, 1986; Wang and Huang, 1989; Allen, 1990; Furukawa and Sahasrabuddhe, 1990). For example, glutaraldehyde, carbodiimde, and *N*,succinimidyl-3-(2-pyridyldithio) proprionate has been used as a bridge for the coupling of antibodies onto liposomes, (Torchilin and Khaw, 1979; Allen et al., 1995; Hansen et al., 1995; Aslam and Dent, 1998). However, glutaraldehyde is highly reactive and may inadvertently interact with other proteins, thereby affecting their performance. Besides, the number of functional groups that appear on liposome surfaces cannot be known quantitatively. The second compound suffers from the disadvantage that it only attaches to phosphatidylethanolamine thus it is of limited use and cannot be used for liposomes lacking this component. Therefore, one of the goals of this research was to develop a simpler and more dependable alternative. To this end, the use of a linear chain aldehyde (e.g. dedecanal) was investigated as a replacement for the linkers currently in use, like carbodiimide or glutaraldehyde.

2. Materials and methods

2.1. Materials

Acetone, petroleum ether, chloroform, methanol, diethylether, hydrochloric acid, acetic acid, sulfuric acid, ammonia, aluminum oxide, iodine, sodium chloride, potassium dihydrogen phosphate, sodium bisulfate, disodium hydrogen phosphate, fuchsine, sodium tetraborate, sodium chromate, dedecanol, sucrose, silica gel G, and cholesterol, were all reagent grade from Merck, AG, (Darmstadt, Germany), and were used without further purification. Hydrated ferric chloride was from Hopkin & Williams, Ltd. (UK) and Analar Hipak and Ficol from Sigma, Inc. (St Louis, Missouri, USA). CD8, and antimouse IgG-FITC antibodies were gifts from the Blood Transfusion Organization (Tehran) and from the immunology laboratory at the College of Health Education, Tehran Medical Sciences University.

2.2. Instruments

Instruments used were a UV-visible spectrometer (model TCC-240A, Shimadzu, Japan), infrared spectrometer (model IR-4300, Shimadzu, Japan), particle size analyzer (gravito–centrifugal type-model SA-P3, Shimadzu, Japan), probe type ultrasonic generator (model MK2-3.75, MSE, France), variable autoinjector (model AG, Melsongen, Germany), rotary evaporator (model NAJ, EYELA, Japan), pH meter (model pH 196, WTW, Weilheim, Germany), fluorescent microscope (model Z3-UV 50 HP Zeiss, Germany), and a refrigerated ultracentrifuge (model 6-6,Imaco, Co, Iran) and ordinary equipment like oven, magnetic stirrer and blender.

2.3. Methods

2.3.1. Colloidal magnetic particles

These were prepared by reacting a 25% ammonium hydroxide solution with ferro/ferric chloride according to standard procedures (Hamada and Matuevic, 1982) and were washed with phosphate buffered saline solution PBS (NaCl 137 mM; KCl 2.67 mM; Na_2HPO_4 6.4 mM; KH_2PO_4 1.4 mM, pH 7.2). Size distributions and their median were determined via the particle size analyzer as described below.

2.3.2. Synthesis of dedecanal

This compound was prepared from the oxidation of dedecanol (Furniss et al., 1989) and the product was ascertained by the Schiff reagent (0.1 g fuchsine, 1.8 g $NaHSO_4$, water 100 ml, 2 mM HCl) and IR spectroscopy.

2.3.3. Preparation of magnetic liposomes

Purified phosphatidylcholine (PC) was prepared from fresh egg yolk according to Singleton (Singleton and Gray, 1965), and its purity ascertained via thin layer chromatography on silica gel plates using a mixture of 65/5/1 vol/vol of chloroform/methanol/water as eluent. Two lipid compositions were used for the making of liposomes: (1) a mixture of 10/5 (w/w) of PC/cholesterol (blank); (2) a mixture of 10/5/1 (w/w/w) PC/dedecanal/cholesterol (for preparation of immunoliposomes) via a modification of reverse phase evaporation technique (Szoka and Papahadjopoulous, 1978). In this method 46 mg of the solid lipid mixture was dissolved in 4 ml of diethylether and 1 ml of PBS containing 0.01 g colloidal magnetic iron oxide added before sonication.

The resultant homogenate was placed in a rotary evaporator at 42°C and the organic solvent evaporated. Magnetic liposomes thus obtained were washed with borate buffer (6.84 g H_3BO_3, 9.53 g $Na_2B_4O_7$, 4.364 g NaCl in 1l water, pH 8.5), using a variable autoinjector connected to 0.2 ml bubble pipette whose bulb was placed in a strong hand held magnet (to retain the magnetic liposomes during continuous flow of the washing liquid; Fig. 1). Washing was continued until the emerging liquid was free of aldehyde (no color change against the Schiff indicator).

2.3.4. Determination of encapsulation efficiency

Non-magnetic or magnetic liposomes (lacking aldehyde) were separately prepared in sodium chromate (0.1 M) and dialyzed against buffer to remove the unencapsulated sodium chromate. Subsequently, liposomes were lysed by treatment with Triton-X100. The concentration of the chromate ion encapsulated in the liposomal samples was determined by UV spectrophotometry at 380 nm (using a standard curve).

2.3.5. Size determination

Size and distribution, both of the colloidal magnetic particles as well as the magnetic liposomes, were determined via the particle size analyzer. Since such determinations require a prior knowledge of the particle densities and since the densities of liposomes were unknown and unavailable, their approximate values were determined using a sucrose density gradient column.

2.3.6. Antibody attachment

At first blank and aldehyde liposomes were incubated with the antibody (CD8, 5 μg/mg lipid) at 4°C for 48 h and the antibodies were then conjugated with fluorescein isothiocyanate (FITC) at 4°C for 48 h. Unreacted antibodies and FITC were removed by chromatography on a G-25 Sephadex column (1 × 15 cm). Magnetic liposomes were contacted with human anti-mouse IgG–FITC conjugate, (5 μg/mg lipid, pH 8.5), and incubated at 4°C for 48 h. Unreacted antibodies were washed with the aid of a magnetic field in the same fashion as unreacted dedecanal described above. After thorough washing the fluorescent immuno- and immuno-magnetic lipo-

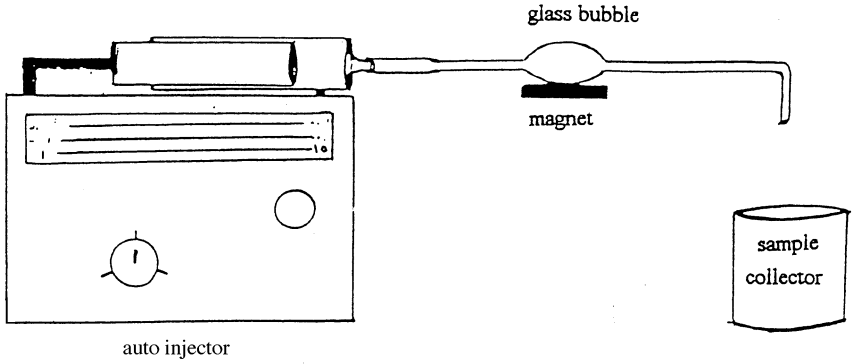

Fig. 1. The washing system.

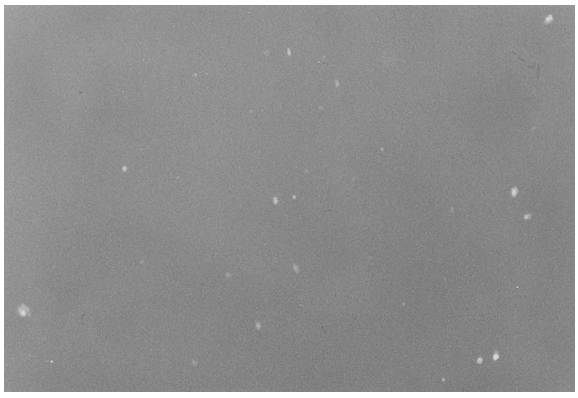

Fig. 2. The liposomes conjugated with antibody and then FITC (fluorescence microscopy magnified 40 ×).

somes were observed and photographed under a fluorescence microscope (Fig. 2).

2.3.7. Attachment of lymphocytes to immunomagnetic liposomes

Lymphocytes were separated using a Ficol solution (Duby et al., 1986) and subsequently attached to the CD8 antibody. The CD8 lymphocytes were mixed with the immunomagnetic liposomes prepared above and incubated at 4°C for 1 h. After separating the unattached liposomes (by centrifugation), the liposome-bonded cells were taken for fluorescence microscopy (Figs. 3 and 4).

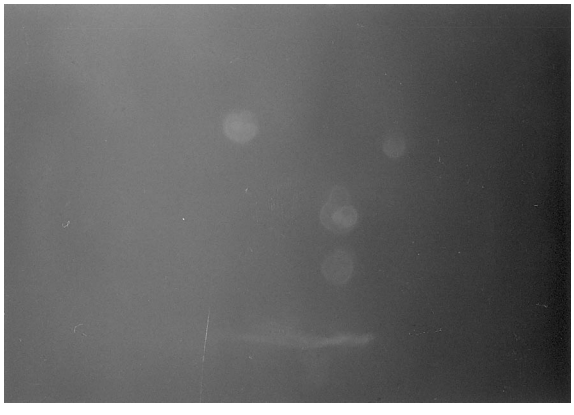

Fig. 3. The magnetic liposomes conjugated with human-anti-mouse FITC antibody (fluorescence microscopy magnified 450 × 1.25).

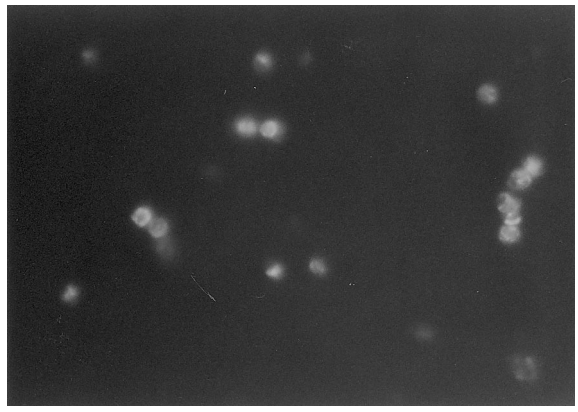

Fig. 4. The immunomagnetic liposomes attached to lymphosite cell (fluorescence microscopy magnified 40 ×).

3. Results and discussion

Encapsulation efficiencies of ordinary and magnetic lioposomes were found to be 29.5 and 19.6%, respectively, which clearly reflect the effect of magnetic particles present within the liposomes. The values are in satisfactory agreement with 20–60% reported by others (Szoka and Papahadjopoulous, 1978). The average size of the magnetic particles was ~ 0.3 μm and that of the magnetic liposomes 1.63 μm. The latter is large compared to 0.2–1 μm usually reported for ordinary liposomes. This difference might be due to: (a) errors involved in the density values used; (b) formation of large unilamellar vesicles encapsulating the magnetic particles and their selective separation during the washing step in the magnetic field.

Blank liposomes (made of PC/cholesterol lacking dedecanal) did not bind the antibodies. Binding of the liposomes containing dedecanal in their bilayer clearly demonstrate that it offers a uniquely simple and easy procedure that is advantageous to the existing methods, like those based on glutaraldehyde, etc., because the binding sites are indigenously present on the liposome surfaces for direct attachment right after their formation, and toxicity of glutaraldehyde or carbodiimmide and their associated multistep processes are avoided.

Availability of such easily prepared immunomagnetic liposomes is an aid in expanding the therapeutic application of liposomes. Their magnetic properties allow rapid and focussed delivery of drug-carrying immunoliposomes, before they are deleteriously affected by the blood system or otherwise. Besides, due to their unique specificity, use can be made of external vibrating magnetic fields in their deliberate, on site, rupture and immediate release of their contents.

4. Conclusions

1. A simple and highly effective method was developed for preparing liposomes that readily bind protein molecules, like antibodies, to their exterior surfaces.
2. The extent of surface coverage can easily be controlled and varied by simply changing the amount of aldehyde initially used in the lipid mixture for preparing the liposomes.
3. Incorporation of colloidal magnetic particles yields liposomes that seem larger than ordinary liposomes.
4. Magnetic immunoliposomes prepared by this method exhibit satisfactory binding to cells carrying their corresponding antigenic determinants.
5. Immunomagnetic liposomes allow quick and focussed delivery of drugs to the sites of interest. Once localized these liposomes may readily and specifically bind to their target cells, where they could be ruptured by external stimuli to release their deadly potions.

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