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Research Article

Surface Modification of Liposomal Vaccines by Peptide Conjugation

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Abstract

The aim of the present work was to prepare liposomal vaccine formulation by incorporating naked plasmid DNA that can trigger humoral and cell mediated protective immunity against infection. For these cationic lipids like dimyristoyl phosphatidylcholine (DMPC), dioleyl phosphatidyl ethanolamine (DOPE), [1, 2 – dioleyloxy -3-(trimethyl ammonium propane)] (DOTAP), were taken in the ratio of 4:2:1 respectively. The liposomal formulations thus prepared were surface modified by peptide conjugation with the help of EDC and NHS. Physical characterization of liposomal formulations was done by estimating the average size distribution, which gives an average liposomal size of 53.0nm. Concentration of peptide bound liposomes was estimated by Lowry method which entails that bound protein concentration was 30.5μg/ml.

Keywords: Liposomes, plasmid DNA, vaccine delivery, surface modification

Introduction

The use of nanotechnology in medicine and more specifically drug delivery is set to spread rapidly. Recent years have witnessed unprecedented growth of research and applications in the area of nanoscience and nanotechnology. There is increasing optimism that nanotech-nology, as applied to medicine, will bring significant advances in the diagnosis and treatment of disease. [1] Anticipated application in medicine include drug delivery, both in vitro and in vivo diagnostics, nutraceuticals and production of improved biocompatible materials.1The science of drug delivery may be described as the application of chemical and biological principles to control the in vivo temporal and spatial location of drug molecules for clinical benefit. Spatial control allows for high anatomic specificity, lower dosage, and decreased side effects.[2]Temporal control of drug release allows for sustained dosing and minimized fluctuations from the therapeutic window.[2] Most mini aturized drug delivery systems are based on either organic materials such as polymers, gels, vesicles, lipoproteins and liposomes or inorganic metallic and semiconducting nanoparticles suggested to minimize drug degradation and loss, prevent harmful side effects, and increase drug bioavailability. Each of these carriers offers its own advantages and has its own shortcomings, therefore the choice of a certain carrier for each given case can be made only by taking into account all of relevant considerations. To this extent, this project, more precisely delineates the possibilities and scope for usage of liposome as suitable drug delivery system. The common feature of classical liposomes i.e., made preferentially of phospholipids, and of vesicles made of amphiphilic molecules, was their ability to form dynamic lamellar structures with barrier properties separating the interior of the vesicles from the outside medium. Liposomes are generally considered non-toxic, biodegradable and non-immunogenic.

Use of vaccines for the prevention of microbial infections is a preferred alternative to treatment. It has been applied successfully, for instance, in the eradication of smallpox as well as against tetanus, diphtheria, whooping cough, polio and measles, thus preventing millions of deaths each year. A novel and exciting concept developed, namely de novo production of the required vaccine antigen by the host's cells in vivo, revolutionize vaccination especially where vaccines are either ineffective or unavailable. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way into the nucleus where it transfect the cells episomally.3Produced antigen, recognized as foreign by the host, is then subjected to pathways similar to those undergone by the antigens of internalized viruses (but without their disadvantages) leading to protective humoral and cell mediated immunity. Vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf

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the plasmid. Some of the DNA may also be endocytosed by APCs infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics. The extent of DNA degradation by extracellular deoxy-ribonucleases is unknown but, depending on the time of its residence interstitially, degradation could be considerable. In the gut milieu, naked DNA has failed to elicit an immune response, probably because of its anticipated complete degradation.[4]It follows that approaches to protect DNA from the extracellular biological milieu, introduce it into cells more efficiently or target it to immunologically relevant cells should contribute to optimal DNA vaccine design. The use of liposomes as carriers of peptide, protein, and DNA vaccines requires simple, easy-to-scale-up technology capable of high-yield vaccine entrapment. It has been proposed that,[5] as APCs are a preferred alternative to muscle cells as targets for DNA vaccine uptake and expression; liposomes would be a suitable means of delivery of entrapped DNA to such cells. Locally injected liposomes are known to be taken up avidly by APCs infiltrating the site of injection or in the lymphatics, an event that has been implicated in their immune-adjuvant activity.[3]Liposomes would also protect their DNA content from deoxyribonuclease attack. Because of the structural versatility of the system, its transfection efficiency could be further improved by the judicial choice of vesicle surface charge,6size and lipid composition or by the co-entrapment of cytokine genes and other adjuvant (e.g. immunostimulatory sequences), together with the plasmid vaccine. Moreover, as a number of injectable liposome-based drug formulations including vaccines against hepatitis A and influenza have been already licensed in the USA and Europe for clinical use,[7] Acceptance of the system clinically would be less problematic than with other systems that are still at an experimental stage. In the current project work plasmid DNAs were quantitatively entrapped into the aqueous phase of multi-lamellar cationic liposomes by a mild dehydration–rehydration procedure.[6,8,9] This consists of mixing preformed small uni-lamellar vesicles (SUVs) with a solution of the DNA destined for entrapment, freeze–drying of the mixture and controlled rehydration of the formed powder followed by centrifugation to remove non-entrapped material.

The preparation of modified liposomes with controlled properties requires the chemical conjugation of proteins, peptides, polymers and other molecules to the liposome surface. In general, the conjugation methodology is based on three main reactions, which are quite efficient and selective: reaction between activated carboxyl groups and amino groups, which yields an amide bond; reaction between pyridyldithiols and thiols, which yields disulphide bonds; and reaction between maleimide derivatives and thiols, which yields thioether bonds. Many lipid derivatives used in these techniques are commercially available.[12] Other app- roaches also exist, such as those that yield the carbamate bond via the reaction of p-nitrophenylcarbonyl- and amino-group.[11,13,14]Numerous techniques were developed during the 1980s, which are based on the nucleophilic reactivity of free amino groups of the protein or peptide. In terms of selectivity, high-yield coupling, and preservation of biological activity of the protein or peptide, a two stage coupling procedure involving carboxy-acyl derivatives of phophatidyl ethanolamine proved to be superior. The lipid free carboxylic group is first activated with the water soluble carbodimide [1-ethyl-3(3-dimethyl amin -opropyl) carbodimide, EDC)] at pH = pKa–1, where Ka is the ionization constant of the given carboxylic group. In the second stage the protein or peptide solutions are added with a simultaneous change to pH 8.

Chemically EDC/EDAC is 1-ethyl-3-[3-dimethylamino propyl] carbo- dimide hydrochloride and is a zero-length cross-linking agent generally used to couple carboxyl groups to primary amines. This cross-linker has been used in diverse applications such as forming amide bonds in peptide synthesis, attaching haptens to carrier proteins to form immunogens, labeling nucleic acids through 5-phosphate groups and creating amine-reactive NHS-esters of bio molecules. EDC reacts with a carboxyl to form an amine-reactive O-acylisourea intermediate. This intermediate does not encounter an amine; it will hydrolyze and regenerate the carboxyl group.11 In the presence of N-hydroxysulfosuccinimide (Sulfo-NHS), EDC can be used to convert carboxyl groups to amine-reactive Sulfo-NHS esters. This is accomplished by mixing the EDC with a carboxyl containing molecule and adding Sulfo-NHS shown in Fig.1.

Materials and Methods

Preparation of liposomal vaccine

Lipids like DMPC, DOPE, and DOTAP in the ratio of 4: 2: 1 were accurately weighed for each formulation (Table 1) and kept in separate round bottom flask. The weight ratio of drug to lipid was 1:200 for each formulation. Unsaturated phospholipids are extremely hygroscopic as powders and will quickly absorb moisture and become gummy upon opening the storage container. So they were dissolved in a suitable solvent chloroform (here 25ml). The mouth of the round bottom flask was made tightly closed to avoid solvent evaporation. The round bottom flask was placed in the rotary evaporator to get a homogeneous mixture of all lipids by evaporating the chloroform in a reduced pressure of (100mbar) at 45°C.The mixture was kept in rotary evaporator for approximately 2hrs to

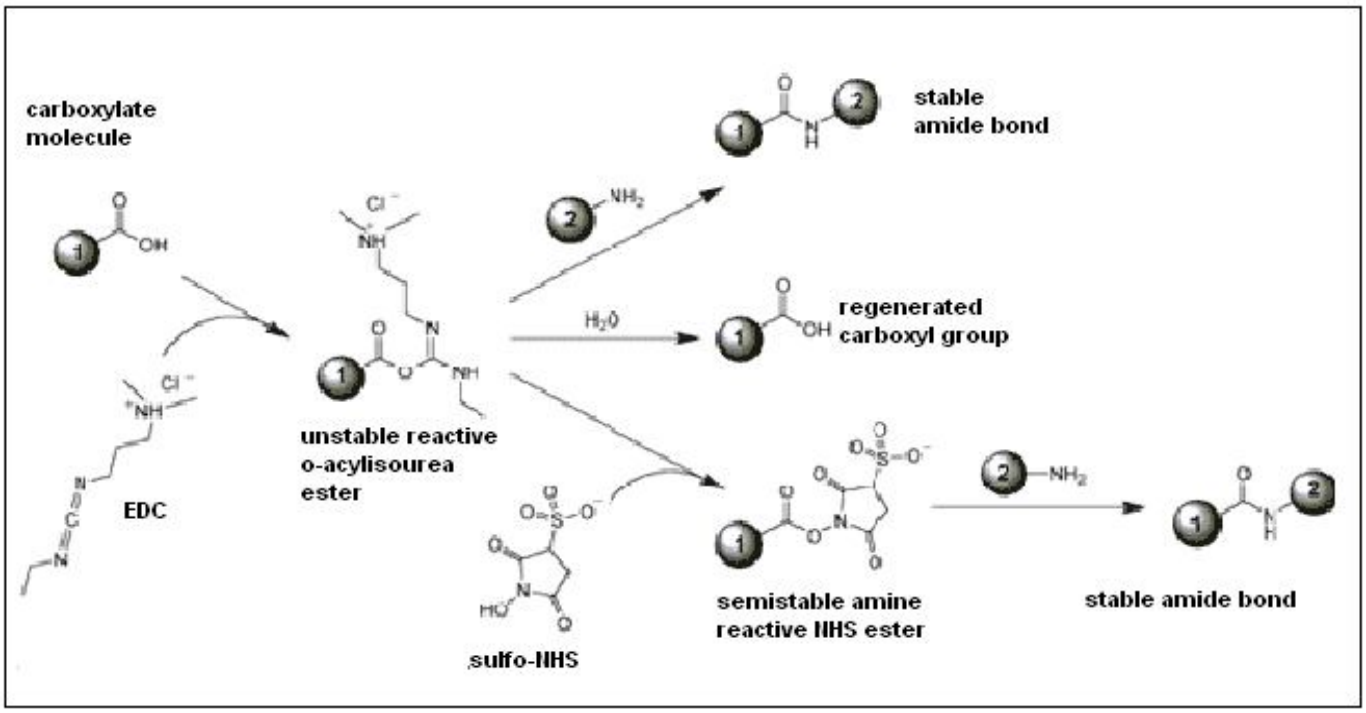


Fig. 1: EDC reacts with a carboxyl group on molecule #1, forming an amine-reactive O-acylisourea intermediate. This intermediate may react with an amine on molecule #2, yielding a conjugate of the two molecules joined by a stable amide bond. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The addition of Sulfo-NHS (5 mM) stabilizes the amine-reactive intermediate by converting it to an amine-reactive Sulfo-NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions. The amine-reactive Sulfo-NHS ester intermediate has sufficient stability to permit two-step cross-linking procedures, which allows the carboxyl groups on one protein to remain unaltered.

get a dried film of phospholipid around the round bottom flask. Flask was flushed with Nitrogen gas (N2) to evaporate traces of chloroform from the flask. The thin film of lipid of each formulation was hydrated with 25ml of glutamate-trehalose buffer pH 5.5 (Table 2) to get multi-lamellar vesicles (MLVs).

MLVs of each formulation were passed through high-pressure homogenizer at 40KPSI for 5 times to get single uni-lamellar vesicles (SUVs). SUVs within the range of 50-120nm size were obtained which were checked by zeta-sizer. SUVs of each formulation were filtered through 0.45μm syringe filter under laminar air flow. The SUVs were then mixed with the S DNA, NE DNA, NE protein, HBsAg protein (Table 3 for the list of antigens used) separately in respective vials. Each formulation was transferred into 4.0 ml vial under laminar air flow. The content of each vial was freeze dried to form the cake in the vial.

Surface modification of liposomes by peptide conjugation

Modification of the liposome surface is a powerful approach to control liposome properties and biological behavior. Attachment of certain specific ligands (such as antibodies, peptides, hormones, sugars) makes liposome targeted. Attachments of

Table 2: Composition of glutamate buffer, pH 5.5 (500ml)

Components	Quantity
Glutamic acid powder	1.47 gm
NaCl	0.87 gm
Trehalose	42.78 gm
WFI	500 ml
PH	5.5

contrast agents convert liposomes into efficient diagnostic tools. Coating liposome with polymers such as polyethylene glycol imparts to them an ability to circulate long without being opsonized and recognized by the cell of RES. Protein conjugated liposomes have attracted a great deal of interest, principally because of their potential use as targeted drug delivery systems and in diagnostic application.[11] Since the first description of conjugating proteins to liposomal surfaces via free amino groups. Below is the description of procedure of peptide conjugation to the liposomal surface.

Covalent coupling of water-soluble peptides to dehydration-rehydration vesicle-Peptide conjugation to the surface of liposome

Table 1: Composition of phospholipid in formulations of plasmid DNA vaccine

Formulations	DMPC(μg)	DOPE (μg)	DOTAP(μg)
Formulation 1	110.5	60.55	28.93
Formulation 2	110.5	60.55	28.93
Formulation 3	110.5	60.55	28.93
Formulation 4	221	121.1	57.86
Formulation 5	110.5	60.55	28.93
Formulation 6	221	121.1	57.86
Formulation 7	221	121.1	57.86
Formulation 8	442	242.2	115.72
Formulation 9	110.5	60.55	28.93
Formulation 10	1105	605.5	289.3
Formulation 11	110.5	60.55	28.93
Formulation 12	1105	605.5	289.3
Formulation 13	221	121.1	57.86
Formulation 14	2210	121.1	57.86

2.6 ml of SUVs were taken in a glass bottle. The pH of the suspension was adjusted to pH3.5 using 0.01(N) HCl. 15 mg of EDC was added to get cross-linked with liposomal surface.9 mg of NHS was added. The suspension was kept in rocker for 15 min, for gentle mixing.

Dialysis of the solution against borate buffer to remove the NHS and EDC

The peptide conjugated SUV suspension is then poured in dialysis bag having 10 kD pore size and the bag was kept in borate buffer at pH8.5.The NHS and EDC came out of the bag to the external borate buffer solution. The solution was taken out of the dialysis bag; 200 g of peptide was added in the presence of borate buffer into the SUV solution. Then it was incubated with gentle stirring for 4 h.

Dialysis of the peptide bound SUV solution against PBS buffer to remove the free peptide

Dialysis of peptide conjugated SUV solution was done against PBS buffer (Table 4) overnight. Free peptide will come out of the dialysis bag to the external buffer solution. The dialysis bag will only retain peptide conjugated liposome. Peptide conjugated liposome were recovered from the dialysis bag.

Bound protein estimation by Lowry procedure

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen(s) with the copper (II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdicphospho tungstic acid to heteropolymolybdenum blue by the copper catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10-10.5. The Lowry method is sensitive to low concentration protein. Dunn (1992) suggests concentrations ranging from

0.10-2 mg of protein per ml. The major disadvantage of Lowry method is the narrow pH range within which it is accurate.

Table 3: Different type of antigens entrapped in liposomal vaccine formulations

No of formulations	Liposome formulations
1.	Empty liposome
2.	1µg pVAX1 vector
3.	1 µg NE protein
4.	1 µg NE DNA + 1 µg NE protein
5.	1 µg X1 Ag protein
6.	1 µg S DNA +1 µg X1Ag protein
7.	1 µg NE protein+1 µg X1Ag protein
8.	1 µg NE DNA + 1 µg NE protein + 1 µg S DNA + 1 µg X1Ag protein
9.	1 µg S DNA
10.	10 µg S DNA
11.	1 µg NE DNA
12.	10 µg NE DNA
13.	1 µg S DNA + 1 µg NE DNA
14.	10 µg S DNA + 10 µg NE DNA

Table 4: Phosphate buffer saline (PBS) pH7.4 (10mM)

Components	Quantity
$Na_2HPO_4.7\ H_2O$	2.07gm
$NaH_2PO_4.\ H_2O$	0.311gm
NaCl	8.7 gm
WFI	1000 ml
PH	7.4
Filter	0.22 micron

Preparation of solutions

Solution A: 0.2 % Na_2CO_3 in 0.1N NaOH

Solution B: 1% NaK tartarate in H_2O Solution C: 0.5% $CuSO_4.5H_2O$ in H_2O

Solution D: 48 ml of A, 1ml of B, and C

Solution E: Phenol reagent- 1 part Folin -phenol (2N): 1 part water.

Preparation of standard BSA solution

Six sets of test tubes were kept in a rack. 10, 20, 40, 80, 100 l of BSA solution (1mg/ml) were added to these tubes (Table 5). To each of the standard solution test tube 2 ml of solution D was added. 0.2 ml of Folin reagent was then added. The test tubes were then kept in incubation for 30 min. The absorbance of each sample was measured at 600nm. A standard curve was obtained by plotting absorbance vs. mg protein.

Table 5: Preparation of standard BSA solution

Test tube marketing	BSA(µl)	Water(µl)
1	0	1000
2	10	990
3	20	980
4	40	960
5	80	920
6	100	900

Preparation of sample

Sample A: 1000 l of SUV solution + 2 ml solution D + 0.2 ml of Folin reagent.

Sample B: 10 l of peptide bound SUV solution + 990 l of water + 2 ml solution D + 0.2 ml Folin reagent.

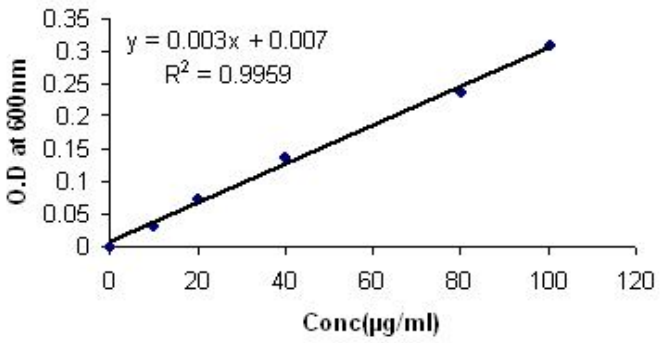


Fig. 2: Standard curve of BSA solution

Results

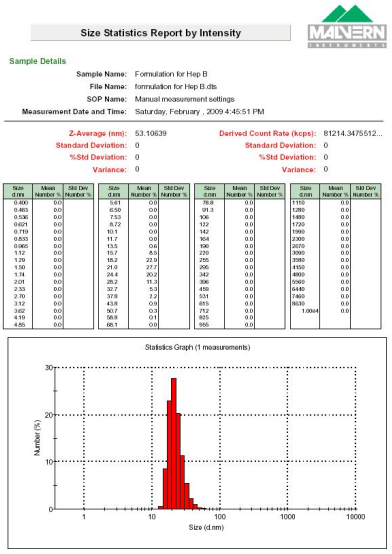
Average Particle Size distribution of liposomal formulations containing plasmid DNA

Here in this project work cationic vesicles containing DOPE had been used to facilitate endosomal release of plasmid DNA as a 'helper' lipid attracting much attention as a potential DNA vaccine delivery vehicle. In this project work we prepared cationic liposomes for vaccine delivery and physically characterize it by checking the size distribution. These formulations were seen to be in the range of 53.0nm and hence can be categorized under Single Unilamelar vesicle (Table 3).

Estimation of bound protein (in surface modified liposome) by Lowry procedure

Surface modification of liposomal surface was done by peptide conjugation. After dialysis the bound protein was estimated by Lowry Method. Standard plot for BSA solution was prepared

Table 6: Particle size distribution of liposomal vaccine formulation



(Fig. 2) and then and then by measuring the optical density peptide bound liposome concentration was measured. Results show (Table 6) the concentration of protein bound liposome was 30.5μg/ml.

Discussion

During the past 30 years liposomes have received increased attention from the scientific community, as well as from the Pharmaceutical Industry, due to the possibility of being a pharmaceutical carrier for numerous problematic drugs. It was recognized at an early stage that the effective delivery or targeting of drugs using liposomes is dependent on one or more of the following factors: quantitative retention of drugs by the carrier en route to its destination; control over the rate of clearance of vesicles from the circulatory system (or other compartments of the body when the drug is locally administered); and access to, and preferential (or at least sufficient) uptake by, the target. Within this project work we had concentrated in two aspects of the utilization of liposomes: as carriers of plasmid DNA of an antigen X1 as vaccine formulation and surface modification of the prepared liposomes by peptide conjugation. These delivery vehicles facilitate the transfer of DNA across membranes and release their DNA contents following fusion with endosomes. Cationic liposomes have been successfully used for gene delivery in human clinical trials without limiting toxicity. There is evidence that cationic and anionic liposome formulations can enhance both the humoral and cell-mediated immune responses induced in mice by plasmid DNA immunogens. Here in this project work cationic vesicles containing DOPE had been used to facilitate endosomal release of plasmid DNA as a 'helper' lipid attracting much attention as a potential DNA vaccine delivery vehicle. In this project work we prepared cationic liposomes for vaccine delivery and physically characterize it by checking the size distribution. These formulations were seen to be in the range of 53.0nm and hence can be categorized under single uni-lamellar vesicle. Surface modification of liposomal surface was done by peptide conjugation. After dialysis the bound protein was estimated by Lowry method, which shows the concentration of protein bound liposome was 30.5μg/ml.

Conclusion

DNA immunization is a promising approach to the design of vaccines for situations where antigens are either ineffective or unavailable. However, plasmid DNA vaccines used as such are vulnerable to attack by deoxy- ribonuclease following their administration and do not normally target antigen presenting cells.[15,16] Such problems can be circumvented by entrapping the DNA within cationic liposomes. The technique of entrapment is simple (one step), easy to use and generates a freeze–dried preparation that can be used on rehydration. Entrapped plasmid is not accessible to nuclease nor can it be replaced by other competing anionic molecules. Peptide coated liposomes may be able to remain homogeneously dispersed in the physiological system and will be uptaken by the target cell receptors for DNA vaccine delivery. This will be aided by cationic phospholipids which are usually composed of cationic lipid derivatives and a neutral phospholipid such as dioleoylphosphatidyl ethanol-amine (DOPE). The negatively charged genetic material, for example, plasmid, is not encapsulated in liposomes but complexed with cationic lipids

they can be commercialized. Determining how to change or modify preparation procedures from laboratory scale to mass production is also important. Resolving these realistic difficulties is the challenge and mission for future development of new products and formulations related to liposomes.

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