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PRELIMINARY STUDIES OF A STEARYLAMINE-BASED CATIONIC LIPOSOME

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ABSTRACT

Cationic liposomes are the leading drug delivery systems and are gaining increasing importance in gene therapy as an alternative to recombinant viruses. This article is a preliminary work on the characterization and in vitro study of a stearylamine-based cationic liposome. Multilamellar vesicles were prepared using the lipid film hydration technique. Vesicle morphology was evaluated by digital light microscopy. Particle size, zeta potential and polydispersity were determined using photon correlation spectroscopy. Susceptibility of Staphylococcus aureus to amoxicillin encapsulated in stearylamine-based cationic liposome was compared to 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)–based cationic liposome and the marketed drug. The vesicles of the stearylamine based cationic liposome were spherical and concentric with a core. Particle size distribution had a peak at 94 nm, zeta potential was 34 mV and polydispersity was greater than 0.3. The clinical isolate of Staphylococcus aureus was most susceptible to stearylamine-cationic liposome with an inhibitory zone diameter (IZD) of 25.00 mm + 0.58 > DOTAP-based cationic liposome with IZD of 24.67mm + 0.67 > tablet amoxicillin with IZD of 23.00 mm + 0.58.

Keywords: *stearylamine; cationic liposome; vesicles; amoxicillin; Staphylococcus aureus.*

INTRODUCTION

Fifty years have passed since liposomes were first discovered by A.D. Bangham.[1] He demonstrated that a wide variety of molecules could be encapsulated with the aqueous spaces of liposomes or inserted into their membranes. Liposomes are artificial vesicles composed of concentric lipid bilayers which are separated by aqueous compartments. The typical characteristic of bilayer forming lipids is their amphiphilic nature: a polar head group covalently attached to one or two hydrophobic hydrocarbon tails.[2] When these lipids are exposed to an aqueous environment, interactions between themselves and with water lead to spontaneous formation of closed bilayers.[3] In this time, liposomes have evolved from a model for biomembranes to drug carriers with clinical utility. The range of medical applications of liposomes extends from chemotherapy of cancer and fungal infections to vaccines and most recently to gene therapy. [4] Gene therapy is still in its infancy and liposomal gene carriers have only progressed to the clinical trial stage[2]. Gene therapy offers a fundamental approach to the treatment of diseases of inherited or acquired origin. However, transfer to the nucleus of target cells of either a functional gene, or a structure capable of interfering with a cellular gene, is a daunting task when considering the number of barriers the vector/DNA complexes must overcome. Numerous roles are required of the vector which include protection of the DNA from the extracellular environments, facilitated uptake into the target cells, and escape from intracellular compartments and finally release of the plasmid, which must subsequently pass across the nuclear membrane before transgenic expression can occur.[5-7] Problems of immunogenicity and toxicity associated with gene delivery using viruses added to the practical issues of large scale production and quality control, have led to the emergence of cationic gene delivery vectors as non-viral alternatives. [8] Advances in the field of non-viral vectors are now made in two distinct structural categories: cationic polymers and cationic lipids, both of which must face the barriers to gene delivery. [9-11] Cationic liposomes are structures that are made of positively charged lipids and are increasingly being researched for use in drug and gene delivery due to their favourable interactions with negatively charged cell membranes.[12,13] Cationic lipids are amphiphilic molecules having a lipophilic region, commonly comprising one or more hydrocarbon or alkyl groups, and a hydrophilic region comprising at least one positively charged polar head group. They are internalised by cells by a classical receptor-mediated endocytosis using cell surface receptors which contain specific binding sites for, and are able to internalise cationic molecules.[14-18] Most eukaryotic cells are negatively charged and as a result positively charged liposomes will bind to antigen presenting cells and other immune cells.[19-21] This leads to better uptake and in vivo cytotoxic T-lymphocyte induction and humoral

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responses. Cationic liposomes are also potential carriers for oral drug delivery due to their protective effects on encapsulated antigens and their ability to be taken up by Peyer's patches in the intestine. They provide enhanced antigen processing through their ability to target phagocytosis by professional antigen presenting cells. The large majority of cationic liposome formulations consist of a cationic lipid mixed with a neutral phospholipid and cholesterol. The preparation procedure is very simple. The cationic liposomes, usually vesicles with diameters < 100 nm are mixed with the drug in dilute solution. The liposomes form spontaneously due to electrostatic charge interactions. The major parameters determining the final product are the charge ratio, the ionic strength of the solution and the overall concentration of the reactants. Cationic liposomes are always prepared with a slightly positive surface charge to allow for interaction with negatively charged cell surfaces, thus increasing cellular uptake. [22]

EXPERIMENTAL

Materials

Cholesterol (Sigma Grade, minimum 99 % Sigma Aldrich Chemie, GmbH, St. Louis, USA), stearylamine (Sigma-Aldrich, USA), Phospholipon 90H (GmbH Nattermannallee 1.D50829 Köln, Germany), methanol (extra pure ph Eur, NF,Scharlau Chemie S.A. EU.), chloroform (Sigma-Aldrich GmbH Germany), amoxicillin (donation from JUHEL Pharmaceutical Company, Nigeria), dioleoyltrimethylammonium propane (DOTAP, Sigma Aldrich, USA), clinical isolates of Staphylococcus aureus were obtained from Bishop Shanahan Hospital, Nsukka.

Methods

Preparation of the cationic liposome

Multilamellar vesicles were prepared using a technique based on established dry lipid film hydration method.[23] Chosen molecular ratios of cholesterol: phosphatidylcholine and stearylamine were formulated. The concentrations of stearylamine were varied slightly to obtain maximum encapsulation efficiency. This is presented in Table 1. The combining ratios were dissolved in 10 ml of chloroform. Thereafter, the chloroform was completely evaporated to dryness in a water bath maintained at 60 °C to form films on the wall of the round bottom flasks used. Each of the resulting films was then hydrated with 2 mg/ml aqueous solution of the amoxicillin. The resulting dispersion was agitated for 10 min to ensure thorough mixing and finally kept for 15 min to anneal.

Table 1: *Combining molecular ratios for optimum encapsulation*

Cholesterol (mg)	Phosphatidylcholine (mg)	Stearylamine (mg)	DOTAP (mg)
12.37	25.12	1.08	
12.37	25.12	1.61	
12.37	25.12	2.15	
12.37	25.12		0.46

Photomicrography of the vesicles: Morphological analyses of the stearylamine based-cationic liposome was carried out by digital light microscopy using Moticam 1000 1.3MP live resolution, Macintosh OSX. Simply, a small drop of sample (about 0.05 ml) was smeared on slide to form a thin film which was then viewed with x40 objective lens. Photographs of the vesicles were captured with the digital camera.

Vesicle size and zeta potential: The z-average vesicle diameter of the stearylamine based-cationic liposomes in distilled water was measured by photon correlation spectroscopy using nanosizer 3000 HS, Malvern Instruments, UK. Zeta potential was calculated from the mean of three runs. The polydispersity index was determined as a measure of homogenity.

Sensitivity studies of the clinical isolates

Molten nutrient agar (15 ml) was inoculated with 0.1ml of *Staphylococcus aureus* broth culture. It was mixed thoroughly, poured into Petri dishes and rotated to ensure even distribution of the organism. The agar plates were allowed to set and a sterile cork borer (5 mm diameter) was used to cut three cups in the agar medium plate. Liposomal samples containing stearylamine, DOTAP and then conventional amoxicillin respectively were prepared and used as follows: 3 drops each of the samples were added respectively into the different cups in each of the plates using sterile Pasteur pipettes. The plates were allowed to stand at room temperature for 15 min to enable the samples diffuse into the medium before incubating at 37 °C for 24 h. The inhibition zone diameters were carefully measured and recorded.

Statistical analysis

For analysis sensitivity study, data were tested by analysis of variance (more than two groups) which was performed to compare the mean values of different groups. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Photomicrographs of the vesicles

Photomicrographs of the vesicles of stearylamine based cationic liposome after formulation and 28 days after formulation as shown if Figures 1 and 2 reveal structures that are spherical, look like water bubbles and of varying sizes. Some of the vesicles look like concentric circles which show that they may be multilamellar vesicles while others that are not concentric may be large unilamellar vesicles. Some reveal the hollow core in which a solute which is hydrophilic can be entrapped or otherwise embedded in the lipid bilayer if it is lipophilic or amphiphilic. The lipid composition and method of preparation also contribute to its structural organization. Bilayer elasticity such as tensile strength, compressibility and bending

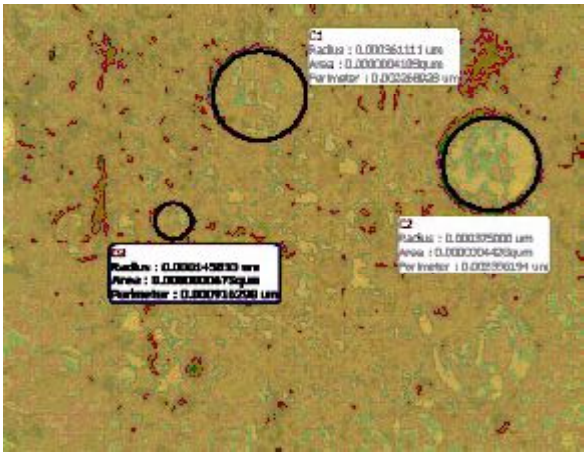


Fig. 1: *Photomicrographs of the stearylamine-based cationic liposome after formulation.*

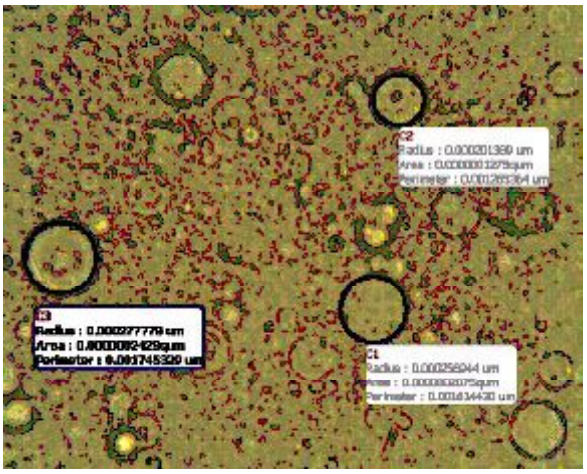


Fig. 2: *Photomicrographs of the stearylamine–based cationic liposome after 28 days*

also come from the lipid composition and contributes to the liposome withstanding mechanical stress[3]. The fluidity of the liposomal bilayer, when it is made from a single lipid depends on the lipid phase transition temperature (Tc) and its relative position compared to ambient temperature. When ambient temperature is increased and reaches Tc, the membrane passes from a solid gel phase, where the lipid hydrocarbon chains are in an ordered state to a fluid liquid crystal phase, which is a disordered state where molecules have more freedom of movement.[24] Membrane fluidity can be controlled by supplementing the lipid bilayer with cholesterol, a mechanism that enhances membrane rigidity and stability.[25] The properties of liposomes and their subsequent applicability depend on the physical and physicochemical characteristics of the liposomal membrane. Usually, a zwitter ionic or non-ionic lipid is used as the basic lipid for the preparation of liposome but this is easily destroyed by the lipases. The net surface charge can be modified by the incorporation of positively charged lipids which resist degradation in the gastric environment. Presence of charge tends to increase the interlamellar distance between successive bilayers in multilamellar vesicle structure and leads to greater overall entrapped volume.

Determination of particle size and distribution

A nanosizer (Malvern Instruments) employing dynamic light scattering was used in determining the particle size. Figure 3 shows the results of the particle size where the particles are heterogenous ranging from about 80 nm-120 nm. The size of the vesicles and encapsulation efficiency is greatly influenced by the method of preparation. The reverse phase evaporation technique, the first to use water-in-oil emulsion encapsulates up to 50 % solute.[26] Another method that gives a high yield of solute entrapment is dehydration-rehydration technique which is both simple and easy to scale up.[27] The third method requires the use of detergent. [28] This method produces relatively small vesicles (mean diameter in the range of 0.08 to 0.2 ¼m) with a narrow size distribution. The main properties governing circulation lifetimes of liposomes are size, lipid composition and dose.[29] The longest half-life has been obtained when liposomes are relatively small (diameter < 0.05 ¼m). The maximum percent encapsulation efficiency of the stearylamine based cationic liposomes was 66.5 % using the film hydration method. This is shown in Table 2. Encapsulation of drugs in liposomes has several advantages. Stable encapsulation of drugs in liposomes changes the drug elimination characteristics and biodistribution. Encaspulation of drugs in liposomes can reduce the volume of distribution and decrease toxic side effects for healthy tissues as compared to free drugs which usually have a large volume of distribution when injected and as a consequence exhibit significant toxicity for healthy tissues.[2] Amoxicillin is soluble and slightly acidic in aqueous solution, therefore an electrostatic association will be formed between it and the positively charged liposome thus facilitating adsorption and entrapment in the cationic liposome.

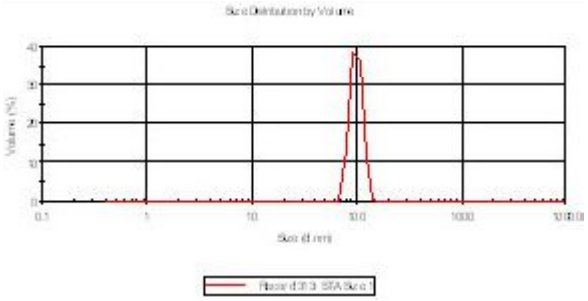


Fig. 3: *Size distribution of the steraylamine cationic vesicles*

Table 2: *Percent Drug Encapsulation Efficiency of Stearylamine*

Stearylamine (mg)	Percent drug entrapment efficiency (%)
1.08	52.0
1.61	66.5
2.50	42.5

Zeta potential distribution and polydispersity of the cationic liposome

The zeta potential distribution ranged from 10 – 60 mV with a peak at 38 mV as shown in Fig 4. It has long been recognised that the zeta potential is a very good index of the magnitude of the interaction between colloidal particles. Dissociation of acidic groups on the surface of a particle will give rise to a negatively charged surface. Conversely, a basic surface will take on a positive charge. In both cases, the magnitude of the surface charge depends on the acidic or basic strengths of the surface groups and on the pH of the solution. The surface charge can be reduced to zero by suppressing the surface ionisation or by decreasing the pH in case of negatively charged particles or by increasing the pH in the case of positively charged particles. The general dividing line between stable and unstable dispersions is generally taken at either +30 or -30 mV. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable. However, if the particles have a density different from the dispersant, they will eventually sediment forming a close packed bed (i.e. a hard cake). [30] The cationic liposomal vesicles have a peak zeta potential greater than +30 mV and so will remain stable for a short period of time. For long stability, it must be stored in a form that confers long stability to the liposomal preparation and its content. Liposomes may be stored as freeze-dried powders. The cryoprotective effect of sugars on liposome integrity has also been studied. [31]

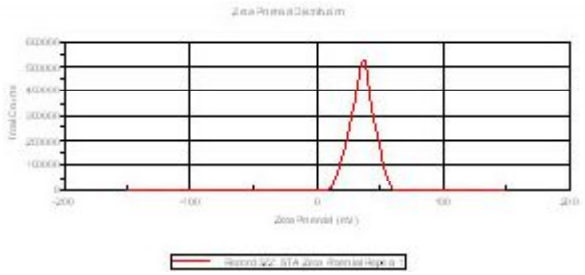
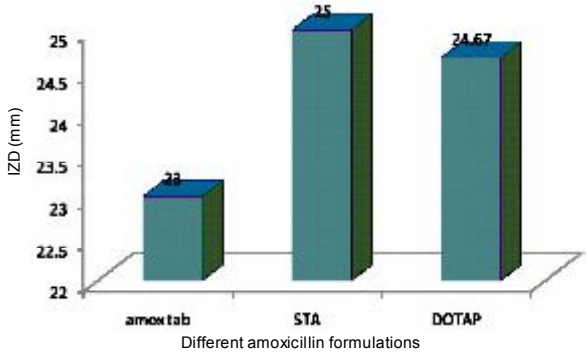


Fig. 4: *Zeta potential distribution of stearylamine cationic liposome*

SENSITIVITY STUDIES

The sensitivity of *Staphylococcus aureus* to stearylamine-based cationic liposome was compared to that of DOTAP-based cationic liposome and amoxicillin tablet is shown in Figure 5. The inhibitory zone diameter showed greatest susceptibility of *Staphylococcus aureus* to stearylamine with an IZD (mm) of 25.00 ± 0.58, followed by DOTAP based liposome with IZD OF 24. 67 ± 0.67 and amoxicillin tablet with IZD of 23.00 ± 0.58. In contrast to conventional liposomes, the large but differential cytotoxicity of cationic liposomes has been advantageously used against pathological microrganisms. This action has been described both *in vitro* against Gram-negative or positive bacterial strains and *in vivo*, against fungus. Given the inexpensive character of certain synthetic cationic lipids and the multiple utility of liposomal models in drug delivery, many pharmaceutical applications are to be anticipated for this convenient joint venture. The cationic agents hypothetically react with phospholipid components in the cytoplasmic membrane, thereby producing membrane distortion and protoplast lysis under osmotic stress.[32] When these cationic vesicles in the rigid gel state do not disintegrate upon adsorption to the bacterial cell surface there is significant evidence for deliverance of drugs inside the vesicle aqueous compartment by endocytosis.



Key : *STA- stearylamine DOTAP- 1, 2-dioleoyl-3-trimethylammonium propane*

Fig. 5: *Susceptibility of Staphylococcus aureus to the different amoxicillin formulations*

CONCLUSION

Stearylamine based cationic liposome encapsulating amoxicllin gave the highest inhibitory zone diameter against *Staphylococcus aureus* showing that it can be used as an antimicrobial agent without loss of antibacterial effects. The technique for formulation of the vesicles could be improved to yield better encapsulation. The choice of the phospholipid can also be modified to improve bilayer arrangement and stability which can withstand serum proteins on parenteral administration.

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