M. Reza Mozafari
Editor

Nanomaterials and Nanosystems for Biomedical Applications

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NANOMATERIALS AND NANOSYSTEMS
FOR BIOMEDICAL APPLICATIONS

Nanomaterials and Nanosystems for Biomedical Applications

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This book is dedicated to Dr I. Joseph Okpala whose help, support and encouragements made it possible

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FOREWORD

It is not so far from now, although it is just the end of the XX century, the time when we discussed outlooks of the use of biotechnologies in medicine and pharmacy. These hopes were connected mainly with new microbiological products and new materials (polymers) for pharmaceutics, biomedicine and organ transplantation. Now in the XXI century, we are much more enthusiastic about outlooks of nanotechnologies for our life and environment. Nanotechnology, when fused with biotechnology, creates nanobiotechnology and nanobiomedical technology; the products of which hardly resemble the parent biotechnology products. These new scientific disciplines, by overall opinion, can even change the face of our civilization in this century. The important point is that dealing with nanotechnologies, we faced new phenomenon: the transition of compounds to nanostate dramatically changes their characteristics such as electrical, magnetic, optical, mechanical, biological and so on. This phenomenon permits creation of novel functional materials with unique custom-made properties.

Development of completely new technologies and innovative nanomaterials and nanosystems with exceptional desirable functional properties lead to a new generation of products that will improve the quality of life and environment in the years to come. There are numerous new generation nanomaterial products of high quality including biocompatible biomaterials, antimicrobial biodevices, surgical tools, implants, decorative and optical devices, and, finally, nanocarriers and nanosystems.

One of the most important applications of the so called nanomedicine/nanotherapy appeared to be the targeting of medicines or additives to the desired organs and tissues using special nanoparticles and nanocapsules of various nature to cure human diseases. Because of their unique characteristics, nanosystems enhance the performance of medicines by improving their solubility and bioavailability, increasing their in vivo stability, creation of high local concentrations of bioactives in target cells and cellular compartments in order to gain therapeutic efficiency.

Nanocarrier systems used for medicine targeting are mainly consisting of lipid molecules, surfactants, and certain polymers, such as dendrimers, which are specially designed to be drug carriers. Hybrid organic/inorganic materials have also become popular now. Carbon-based nanostructures (nanotubes, etc.) are used for implant construction and as nanosystems for drug targeting. In our view, however, detailed toxicological studies are needed because of high chemical reactivity of carbon nanostructures as a result of their small size and high surface area.

Research efforts in such a complex area require interdisciplinary approach covering physics, chemistry, biology, material science and technology. This approach is realized in this volume at the highest degree. This book is the second one devoted to nanotherapy/nanomedicine and issued by Springer. It continues, and it is beneficially complemented to the previous Springer volume "Nanocarrier Technologies: Frontiers of Nanotherapy". Both of these volumes are edited by an internationally recognized scientist, Dr. M. Reza Mozafari. He succeeded to collect in each volume quality chapters authored by highly creative scientists from variety of countries throughout the World. The present volume starts with Dr. Nesrin Hasirci (Ankara, Turkey), an expert in biomaterial science and tissue bioengineering; Dr. Valentin Vlassov (Novosibirsk, Russian Federation), a famous specialist in antisense DNA-based medicines; Dr. Ali Azghani (Texas, USA) a world renowned biomedical scientist and Dr. Abdelwahab Omri (Ontario, Canada) expert in antibacterial and antioxidant delivery using archaeosomes. These follow by manuscripts from other world-class laboratories leaded by Dr. Ozlen Sahin, Dr. Jaspreet Singh, and Dr. M. Reza Mozafari. The book ends with chapters by Dr. Costas Demetzos (Athens, Greece), a famous specialist in dendrimers and liposomal anticancer delivery; and Dr. Yekta Ozer (Ankara, Turkey), an expert in radiopharmacy and nanocarrier targeting.

If the first volume, published last year, was devoted almost totally to the delivery systems of "nano-" scale, e.g., archaeosomes for medicine and vaccine delivery; solid lipid nanoparticles; hydrotropic nanocarriers; biomimetic approach to medicines' delivery; drug delivery using nanoemulsions; the use of new class of gemini surfactants and non-viral vectors for gene delivery; and dendrimers, the second one is of more general interest. It covers also new types of nanomaterials, which have outlooks as artificial implants and for variety of biomedical implications along with a description of traditional micro- and new nanocarrier systems and their release characteristics.

The role of nanomaterials and nanosystems for current pharmaceutical and biomedical research/technologies, and for our life is very hard to overestimate. We are sure that this volume, its outstanding contributions, creativity of the authors, and excellent editing as well will beneficially contribute to the field of biomedical nanotechnologies and nanotherapy.

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PREFACE

Nanotechnology has been defined as the scientific area, which deals with sizes and tolerances of 0.1 to 100 nm (Albert Franks). This is a working definition that refers to the properties of materials, in the above size range. More specifically, nanotechnology can produce, characterize and study devises and systems by controlling shape and size at nanometer scale. At that scale level, the chemical, physical and biological properties of the materials have fundamental differences in comparison to the material at the conventional scale level, because of the quantum mechanic interactions at atomic level.

During the last decade, research on nanoparticles properties has tremendously increased. In the European Union and in the USA a huge number of research projects on nano-devices are ongoing. Europe has already responded to challenges in the emerging field of Nanotechnology, participating with scientific experts from academia, research institutes and industry to the vision regarding future research and applications in Nanoscience.

Even though nanotechnology has become synonymous to innovation, there are challenges, which comprise issues of toxicity, long term stability and degradation pathways of nanoparticles, which may affect the environmental integrity and balance. The harmonization as well as the protection of the intellectual properties of the industries, which produce nanoparticles, is a concern of the regulatory authorities and experts. They have to identify issues incorporated into the existing regulatory framework or to evaluate new regulatory developments.

The economical landscape of nanobiotechnological products based on the definition that nanoscience includes system, devises and products for healthcare, aimed at prevention, diagnosis and therapy the total market segment for medical devices and drug / pharmaceuticals, represented in 2003 a value of 535 billion euros. The drugs segment values 390 billion euros. European Biotech companies have made great efforts mainly in drug development and medical devices, but commercialization effectiveness is relatively weak compared to the USA, with only half as many companies as in the United States.

These facts described above, concerning the scientific area of nanotechnology urge the need for studies and publications in order to characterize the impact of nanomaterials, nanotools and nanodevices in healthcare.

This volume edited by Dr. M. Reza Mozafari, presents important chapters, which refer to micro and nano systems, lipid vesicles and polypeptides as well as

applications of niosomes in the encapsulation and delivery of bioactive molecules by using different routes of administration.

It is well known that the design of new drug delivery systems which are able to transport toxic or poorly soluble bioactive molecules in aqueous media is driven by the need to improve drug effectiveness and to minimize side effects. Therefore, chapters concerning drug carriers are of great importance and useful for the readers of this volume.

Nasal and pulmonary routes for drug delivery depend on the type of nanoparticle such as liposomes, microspheres etc and the relevant chapter describes effectively the nasal and pulmonary drug delivery mechanism. It is worth noticing that inhalation, dermal and oral administration routes for preparing appropriate nanoparticles are of great importance.

The field of active implants has grown in recent years. Liposomal antibiotics, as coating for implants, are the subject of one of the chapters.

Cancer is known to be one of the main causes of death in the developed world. Nanotechnology through the use of drug delivery systems participates in the struggle against cancer. Liposomes are widely accepted as drug delivery systems. Particularly, nanoliposomes are considered as promising carriers especially in the case of bioactive agents, cosmetics and nutraceuticals. They can be studied by several techniques one of which is the Microscopy. This volume incorporates a chapter which deals with the study of liposomes by applying light and electron microscopy while in another chapter liposomes incorporated cytotoxic molecules have been tested against cancer cell lines and their uptake by the cancer cells was investigated.

Based on the aforementioned brief description of the contents of this volume, I conclude that the chapters are extremely important and the volume obviously covers a great range in the field of nanotechnology, gaining a great impact in the international literature. The Editor Dr. M. Reza Mozafari completed this effort successfully and the results should encourage him for relevant publishing efforts in the future. The excellent chapters that he gathered from high quality scientists contribute positively to the bibliography in the field of nanotechnology.

It is my honor to foreword this volume and I firmly believe that the prefix nano – derived from the Greek word '*νάνος*' which means something very small – will be the word of the 21st century.

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CHAPTER 1

MICRO AND NANO SYSTEMS IN BIOMEDICINE AND DRUG DELIVERY

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Abstract: Micro and nano sytems sysnthesized from organic and inorganic materials are gaining great attention in biomedical applications such as design of biosensors, construction of imaging systems, synthesis of drug carrying and drug targeting devices, etc. Emulsions, suspensions, micelles, liposomes, dendrimers, polymeric and responsive systems are some examples for drug carrier devices. They have lots of advantages over conventional systems since they enhance the delivery, extend the bioactivity of the drug by protecting them from environmental effects in biological media, show minimal side effects, demonstrate high performance characteristics, and are more economical since minimum amount of expensive drugs are used. This chapter provides brief information about micro and nano systems used in biomedicine, nanobiotechnology and drug delivery

Keywords: micelles, liposomes, dendrimers, drug carriers, responsive polymers

1. INTRODUCTION

Development of metal, ceramic, polymer or materials of biological origin for use in medicine is a very important research area of the last decades. Scientists made great innovations in the production of artificial organs and tissues such as dental and orthopedic prostheses, artificial veins and heart valves, contact lenses, tissue engineering scaffolds, diagnostic systems, etc. As the knowledge on materials and biological systems improved, new areas such as interaction between the material and cells, effect of therapeutic agents at molecular level, the relation between the molecular structure and macroscopic properties became important research lines. Scientists are increasingly interested in mimicking the biological systems, understanding cell-cell communications and modeling the structures that already exist

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in nature. This curiosity makes them search individual molecules, study interactions between the functional groups, signaling between the cells at micro and nano levels to be able to control the properties of the artificial and biological systems. Technologies based on micro and nano levels involve synthesis and utilization of materials, devices and systems in which at least one dimension is less than 1 mm or in the submicron range, respectively.

2. MICRO AND NANO TECHNOLOGY IN MEDICINE

Micro and nanotechnology have significant applications in the biomedical area, such as drug delivery, gene therapy, novel drug synthesis, imaging, etc. In diagnostics and treatment of many disorders, micro-electro-mechanical systems (MEMS) and biocompatible electronic devices have great potentials. MEMS are formed by integration of mechanical elements, sensors, actuators and electronics on a common silicon wafer with microelectronics and micromachining technologies. Sensors collect information from the environment by measuring mechanical, thermal, biological, chemical, optical or magnetic parameters; electronics process these information and actuators respond by moving, positioning, regulating, pumping or filtering. Therefore a desired response occurs against the stresses and environment is controlled by the system.

Use of nano devices in imaging is another important area especially in the detection of tumor cells. In principle, nanoparticles injected into the body detect cancer cells and bind to them. They behave as contrast agents making the malignant area visible so that the anatomical contours of the cancer lesion can be defined. For this purpose iron-oxide nanoparticles whose surfaces were modified by amines were prepared by Shieh et al (2005) and a fast and prolonged inverse contrast effect was shown in the liver in vivo that lasted for more than 1 week. Medical applications of metallic nanoparticles were studied by different groups. For example Dua et al (2005) constructed a non-toxic, biomimetic interface for immobilization of living cells by mixing colloidal gold nanoparticles in carbon paste and studied its electrochemical exogenous effect on cell viability. Pal et al (2005) prepared gold nanoparticles in the presence of a biopolymer, sodium alginate by UV photoactivation. Carrara et al (2005) prepared nanocomposite materials of poly(*o*-anisidine) containing titanium dioxide nanoparticles, carbon black and multi-walled carbon nanotubes for biosensor applications. The synthesized materials were deposited in thin films in order to investigate their impedance characteristics. Lee et al (2005) prepared ultrafine poly(acrylonitrile) (PAN) fibers containing silver nanoparticles. Silver ions in a PAN solution were reduced to produce Ag nanoparticles and the resulting solution was electrospun into ultrafine PAN fibers.

Morishita et al (2005) associated HVJ-E (hemagglutinating virus of Japan-envelope) with magnetic nanoparticles so that they can potentially enhance its transfection efficiency in the presence of a magnetic force. It was reported that, heparin coated maghemite nano particles enhanced the transfection efficiency in the analysis of direct injection into the mouse liver. They proposed that the system could potentially help overcome fundamental limitations to gene therapy in vivo.

3. MICRO AND NANO DRUG DELIVERY SYSTEMS

One of the most attractive areas of micro and nano research is drug delivery. This includes the design of micro and nano carriers, synthesis of nanomedicines and production of nanosystems that are able to deliver therapeutic drugs to the specific organs or tissues in the body for appropriate periods. For drug delivery vehicles it is very important that these systems have good blood and biocompatibility properties. They themselves or the degradation products should not have any toxic, allergic or inflammatory effects. The systems should also protect the activity of the drugs and improve their transport through the biological barriers. If some specific functionality is added on the system, it would also be possible to deliver the drug to the target site where the system is stimulated by an appropriate signal.

In the design and formulation of delivery systems, the key parameters are the size of the device, entrapment method, stability of drug, degradation parameters of the matrix and release kinetics of drugs. Nanosystems have many advantages over the micro systems such as circulation in blood stream for longer periods without being recognized by macrophages, ease of penetration into tissues through capillaries and biological membranes, ability to be taken up by cells easily, demonstrating high therapeutic activity at the target site, and sustaining the effect at the desired area over a period of days or even weeks. In the last decades, numerous publications came up to describe the design of delivery systems with novel preparation methods, physicochemical properties, and bioactivities.

Drug delivery is an interdisciplinary area of research that aims to make the administration of complex drugs feasible. Over the recent years there has been an increasing interest in developing new delivery systems by collaborative research of basic scientists, engineers, pharmacologists, physicians and other health related scientists. The main purpose is to deliver the drug to the desired tissue in the biological system so that it would achieve higher activity for prolonged period at the site without risk of side effects. Micro and nano drug delivery systems are developed for these purposes especially to target the drugs to a specific area or organ in a more stable and reproducible controlled way.

Entrapment or conjugation of a drug to a polymeric system may protect the drug from inactivation and help to store its activity for prolonged durations, decrease its toxicity, as well as may achieve administration flexibility. Various delivery systems, such as emulsions, liposomes, micro and nanoparticles, are of major interest in the field of biomedicine and pharmaceutics. Generally biodegradable and bioabsorbable matrices are preferred so that they would degrade inside the body by hydrolysis or by enzymatic reactions and does not require a surgical operation for removal.

Targeted delivery can be achieved by either active or passive targeting. Active targeting of a therapeutic agent is achieved by conjugating the therapeutic agent or the carrier system to a tissue or cell-specific ligand. Passive targeting is achieved by coupling the therapeutic agent to a macromolecule that passively reaches the target organ. Muvaffak et al (2002, 2004a, 2004b, 2005) prepared anticancer drug-containing gelatin microspheres and conjugated antibodies on the surfaces of these biodegradable microspheres. It was reported that the systems prepared in this

way demonstrated specific activity towards its antigen. Monsigny et al (1994) reviewed the main properties of neoglycoproteins and glycosylated polymers which have been developed to study the properties of endogenous lectins and to carry drugs which can form specific ligands with cell surface receptors. The glycoconjugates have been successfully used to carry biological response modifiers such as *N*-acetylmuramyldipeptide which is hundreds of times more efficient in rendering macrophages tumoricidal when it is bound to this type of carriers. Complexes of polycationic glycosylated polymers with plasmid DNA molecules are also very efficient in transfecting cells in a sugar-dependent manner.

Bioactive agents can be incorporated in micro and nano systems or in systems which have microporous structures. Local delivery of drugs or growth factors which are embedded in microporous gelatin structures was reported by Ulubayram and coworkers (2001, 2002). They examined release kinetics of bovine serum albumin proteins from gelatin matrices (Ulubayram et al 2002) and also reported fast and proper healing of full skin defects on rabbits with application of gelatin sponges loaded with epidermal growth factor (EGF) (Ulubayram et al 2001). EGF was added in gelatin microspheres which were crosslinked with various amounts of crosslinkers (Ulubayram et al 2001, 2002). Similar systems were studied by Sakallioglu and colleagues (2002, 2004) and positive effects of low-dose EGF loaded gelatin microspheres in colonic anastomosis were reported. Uguralp et al (2004) also reported positive effects of sustained and local administration of EGF incorporated to biodegradable membranes on the healing of bilateral testicular tissue after torsion. Guler et al (2004) examined the effects of locally applied fibroblast containing microporous gelatin sponges on the testicular morphology and blood flow in rats.

There are a large number of studies investigating the drug releasing responses to various stimuli such as pH, temperature, electric field, ultrasound, light, or other stresses. Kim et al (2000) prepared nanospheres with core-shell structure from amphiphilic block copolymers by using PEO-PPO-PEO block copolymer (Pluronic) and poly(ε-caprolactone). Release behaviors of indomethacin from Pluronic/PCL block copolymeric nanospheres showed temperature dependence and a sustained release pattern. Chilkoti et al (2002) described recursive directional ligation approach to synthesis of recombinant polypeptide carriers for the targeted delivery of radionuclides, chemotherapeutics and biomolecular therapeutics to tumors by using a thermally responsive, elastin-like polypeptide as the drug carrier. Determan et al (2005) synthesized a family of amphiphilic ABCBA pentablock copolymers based on the commercially available Pluronic® F127 block copolymers and various amine containing methacrylate monomers. The systems exhibited both temperature and pH responsiveness. They suggested that the copolymers have high potential for applications in controlled drug delivery and non-viral gene therapy due to their tunable phase behavior and biocompatibility. Micro and nano systems for drug delivery applications can be studied in the classes of micelles, liposomes, dendrimers, and particles of polymeric and ceramic materials as explained in the following sections.

3.1. Micelles

Micelles are ideal bioactive nanocarriers, especially for water insoluble agents. Many amphiphilic block copolymers can be used for this purpose. Polymers can self-associate to form spherical micelles in aqueous solution by keeping hydrophilic ends as the outer shell and the hydrophobic ends as the core. Hydrophobic drugs can be entrapped in the core during micelle formation process. Polymeric micelles have good thermodynamic stability in physiological solutions, as indicated by their low critical micellar concentration, which makes them stable and prevents their rapid dissociation in vivo. The sizes of micelles are generally less than 100 nm in diameter. This provides them with long-term circulation in blood stream and enhanced endothelial cell permeability in the vicinity of solid tumors by passive diffusion. If site-specific ligands or antibodies are conjugated to the surface of the micelles, the drug targeted delivery potential of polymeric micelles can be enhanced.

Kataoka et al (2000) studied the effective targeting of cytotoxic agents to solid tumors by polymeric micelles. They conjugated doxorubicin to poly(ethylene glycol)-poly(β,α-aspartic acid) block copolymers and showed that these micelles achieved prolonged circulation in the blood compartment and accumulated more in the solid tumor, leading to complete tumor regression against mouse C26 tumor. Rapoport (1999) studied stabilization and activation of Pluronic micelles for tumor-targeted drug delivery. Aliabadi et al (2005a) examined the potential of polymeric micelles to modify the pharmacokinetics and tissue distribution of cyclosporine A (CsA). Their results demonstrated that PEO-b-PCL micelles can effectively solubilize CsA confining CsA to the blood circulation and restricting its access to tissues such as kidney, perhaps limiting the onset of toxicity. They also investigated micelles of methoxy poly (ethylene oxide)–b–poly (ε–caprolactone) (PEO–b–PCL) as alternative vehicles for the solubilization and delivery of Cyclosporine A (Aliabadi et al 2005b). They concluded that these nanoscopic PEO–b–PCL micelles have high potential as drug carriers for efficient solubilization and controlled delivery of CsA. Prompruk et al (2005) synthesized a functionalized copolymer with three polymeric components, poly (ethylene glycol)–block–poly (aspartic acid–stat-phenylalanine) and investigated its potential to form micelles via ionic interactions with diminazene aceturate as a model water-soluble drug.

Wasylewska et al (2004) entrapped human prostatic acid phosphatase (PAP) entrapped in AOT–isooctane–water reverse micelles and studied the kinetics of 1–naphthyl phosphate and phenyl phosphate hydrolysis, catalyzed by PAP. Wang et al (2004) prepared polymeric micelles from poly (ethylene glycol)–distearoyl phosphoethanolamine conjugates (PEG–DSPE) loaded with Vitamin K3 (VK3) and with 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU). These micelles were stable for 6 months during storage at 4°C and no change in their size or release of the incorporated drugs were observed. They showed that these loaded micelles resulted in synergistic anticancer effects against both murine and human cancer cells in vitro. Kang et al (2004) prepared A-B-A triblock and star-block amphiphilic copolymers such as poly (*N*–(2–hydroxypropyl) methacrylamide)–*block*–poly

(D,L–lactide)–*block*–poly (*N*–(2–hydroxy propyl) methacrylamide), poly (*N*–vinyl-2–pyrrolidone)–*block*–poly (D,L–lactide)–*block*–poly (*N*–vinyl–2–pyrrolidone), star–poly (D,L–lactide)–*block*–poly (*N*–(2–hydroxypropyl) methacryl amide) and star–poly (D,L–lactide)–*block*–poly (*N*–vinylpyrrolidone). They reported that all copolymers self-assembled in aqueous solution to form supramolecular aggregates of 20–180 nm in size. The prepared triblock copolymer micelles were examined as carriers for two drugs, indomethacin and paclitaxel, which are poorly water-soluble. Carrillo and Kane (2004) studied the formation and characterization of self–assembled nanoparticles of controlled sizes based on amphiphilic block copolymers synthesized by ring-opening metathesis polymerization. They showed that the monomer undergoes living polymerization and forms assembled nanoparticles of controlled size. The obtained micelles were fairly monodisperse with dimensions of 30–80 nm depending on the composition of the block polymer.

Synthetic copolymers containing phosphorylcholine structure can also be used in the formation of micelles. Phosphorylcholine-based polymers mimic the surface of natural phospholipid membrane bilayers and therefore demonstrate good biocompatability. Salvage et al (2005) copolymerised 2-methacryloyloxyethyl phosphorylcholine (MPC) with two pH responsive comonomers, 2–(diethylamino) ethyl methacrylate (DEA) and 2–(diisopropyl amino) ethyl methacrylate (DPA), in order to develop pH responsive biocompatible drug delivery vehicles. Koo et al (2005) studied sterically stabilized micelles (SSM) and evaluated camptothecin-containing SSM (CPT–SSM) as a new nanomedicine for parenteral administration where camptothecin is a well-established topoisomerase I inhibitor against a broad spectrum of cancers. Konno et al (2001) have shown that 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer immobilized on poly (l–lactic acid) nanoparticles effectively suppressed any unfavourable interactions with biocomponents and improved the blood compatibility of the nanoparticles. It has been suggested that the nanoparticles immobilized with the MPC polymer have the potential use as long–circulating micelles and are good candidates for carrying drugs and diagnostic reagents which can come in contact with blood components. Nishiyama et al (2005) published a review article about construction and characteristic behaviors of intracellular environment-sensitive micelles that selectively exert drug activity and gene expression in live cells. Xiong et al (2005) grafted poly (lactic acid) to both ends of Pluronic F87 block copolymer (PEO–PPO–PEO) to obtain amphiphilic P(LA-*b*-EO-*b*-PO-*b*-EO-*b*-LA) block copolymers. Various types of particles consisting of small micelles were obtained due to the complex structure of the copolymers and a constant initial release rates were observed for procain hydrochloride. Sot and coworkers (2005) investigated the behaviour of *N*–hexadecanoyl sphingosine (Cer16), *N*–hexanoylsphingosine (Cer6) and *N*–acetyl sphingosine (Cer2) ceramides in aqueous media and in lipid-water systems. Cer16 behaved as an insoluble non-swelling amphiphile while both Cer6 and Cer2 behaved as soluble amphiphiles in aqueous solutions. They observed micelle formations for Cer6 and Cer2 at high concentrations as well as phospholipid monolayer formation when the air-water interface is occupied by a phospholipid.

Responsivity can be added to micelles by combining pH or temperature sensitive functional groups into the structures. Cammas et al (1997) prepared thermo-responsive polymeric micelles from amphiphilic block copolymers composed of N–isopropylacrylamide as a thermo-responsive outer shell and styrene as hydrophobic inner core. Leroux et al (2001) studied N–isopropylacrylamide bearing pH-responsive polymeric micelles and liposomes as a delivery system for the photosensitizer aluminum chloride phthalocyanine (AlClPc), which was evaluated in photodynamic therapy. pH-responsive polymeric micelles loaded with AlClPc were found to exhibit increased cytotoxicity against EMT-6 mouse mammary cells in vitro. Liu et al (2003) synthesized cholesteryl end-capped thermally responsive amphiphilic polymers with two different hydrophobic/hydrophilic chain-length ratios from the hydroxyl-terminated random poly (N–isopropylacrylamide–co–N, N–dimethylacrylamide) and cholesteryl chloroformate. The micellar nanoparticles prepared from the amphiphilic polymers demonstrated temperature sensitivity. It was suggested that these nanoparticles would make an interesting drug delivery system. Nostrum (2004) reviewed the results of photosensitizers for photodynamic therapy including drug loading, biodistribution studies, and therapeutic efficiency and concluded that pH-sensitive micelles appeared to be promising candidates for photosensitizer delivery.

3.2. Liposomes

Liposomes are small spherical vesicles in which one or more aqeous compartments are completely enclosed by molecules that have hydrophilic and hydrophobic functionality such as phospholipids and cholesterol. Properties of liposomes vary substantially with composition, size, surface charge and method of preparation. They can be formed as single lipid bilayer or in multiple bilayers. Liposomes containing one bilayer membrane are termed small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) based on their size ranges (Mozafari and Sahin 2005). If more than one bilayer is present then they are called multilamellar vesicles (MLV). Liposomes are commonly used as model cells or carriers for various bioactive agents including drugs, vaccines, cosmetics and nutraceuticals.

The introduction of positively or negatively charged lipids provides the liposomes a surface charge. Drugs associated with liposomes have markedly altered pharmacokinetic properties compared to free drugs in solution. Liposomes are also effective in reducing systemic toxicity and preventing early degradation of the encapsulated drug after introduction to the body. They can be covered with polymers such as polyethylene glycol (PEG) – in which case they are called pegylated or stealth liposomes – and exhibit prolonged half-life in blood circulation (Mozafari et al 2005). Furthermore, liposomes can be conjugated to antibodies or ligands to enhance target-specific drug therapy. Visser et al (2005) studied targeting of pegylated liposomes loaded with horse radish peroxidase (HRP) and tagged with transferrin to the blood-brain barrier in vitro. They have shown effective targetting of liposomes loaded with protein or peptide drugs to the brain capillary endothelial

cells and suggested that the system is an attractive approach for drug delivery to brain. Lopez-Pinto and coworkers (2005) examined the dermal delivery of a lipophilic drug, minoxidil, from ethosomes versus classic liposomes by appliying the vesicles non-occlusively on rat skin. They studied the permeation pattern, depth into the skin and the main permeation pathway of different liposomal systems. Ozden and Hasirci (1991) prepared small unilamellar vesicles composed of phosphatidylcholine, dicetyl phosphate and cholesterol and entrapped glucose oxidase in them. They obtained loading efficiency as one protein per liposomal vesicle.

Liposomes containing the expression vector pRSVneo coding for neomycin phosphotransferase–II were studied by Leibiger et al (1991) for a gene transfer into rat liver cells in vivo. After intravenous application of liposomes to male Wistar-rats, nonintegrated vector DNA was detected by blot-hybridisation in isolated nuclei of hepatocytes. Cirli and Hasirci (2004) prepared calcein encapsulated reverse phase evaporation vesicles carrying photoactive destabilization agent suprofen in the lipid bilayer. They investigated the effect of UV photoactivation of liposomal membrane-incorporated suprofen on the destabilization of the liposome bilayer and the release of encapsulated calcein as a model active agent.

Liposomes are also studied as carriers for cells, genes or DNA fragments. Ito et al (2004) studied the effect of magnetite cationic liposomes which have positive surface charge to enrich and proliferate Mesenchymal stem cells (MSCs) in vitro. Kunisawa et al (2005) established a protocol for the encapsulation of nanoparticles in liposomes, which were further fused with ultra violet-inactivated Sendai virus to compose fusogenic liposomes and observed that fusogenic liposome demonstrated a high ability to deliver nanoparticles containing DNA into cytoplasm. Ito et al (2005) investigated whether coating the culture surface with RGD (Arg–Gly–Asp) conjugated magnetite cationic liposomes (RGD-MCLs) was able to facilitate cell growth, cell sheet construction and cell sheet harvest using magnetic force without enzymatic treatment. They reported that cells adhered to the RGD-MCLs coated bottom of the culture surface, spreaded and proliferated to confluency. Detachment and harvesting of the cells did not need enzymatic process. Fuentes et al (2003) studied the adjuvanticity of two gamma inulin/liposomes/Vitamin E combinations in the mouse, in contraceptive vaccines by using sperm protein extracts or a synthetic HE2 peptide (Human Epididymis gene product; residues 15–28) as antigen. They showed that the gamma inulin/liposomes/Vitamin E combination, with sperm protein extracts, was better than Freund's adjuvant. When the synthetic HE2 peptide was used as antigen, the gamma inulin/liposomes/Vitamin E combination was less effective than Freund's adjuvant.

Vierling et al (2001) published a review on fluorinated liposomes made from highly fluorinated double-chain phospho- or glyco-lipids as well as fluorinated lipoplexes, e.g. complexes made from highly fluorinated polycationic lipospermines and a gene. The properties of the fluorinated lipoplexes including stability and in vitro cell transfection in the presence of serum or bile were reported. El Maghraby et al (2004) showed that incorporation of activators (surfactants) into liposomes improved estradiol vesicular skin delivery. They examined the

interactions of additives with dipalmitoylphosphatidylcholine (DPPC) membranes by using high sensitivity differential scanning calorimetry. Lopes and colleagues (2004) investigated the encapsulation of acid (AD) and sodium diclofenac (SD) in small unilamellar liposomes (SUV) prepared by sonication from multilamellar liposomes containing soya phosphatidylcholine and diclofenac at various proportions. The interactions of the drug with the bilayers were examined. They proposed a schematic model for interaction of SD with phosphatidylcholine of the liposomes in which the diclofenac anion interacts with the ammonium group of the phospholipid and the dichlorophenyl ring occupies a more internal site of bilayer near phosphate group. Simard et al (2005) prepared multilamellar vesicles by shearing a lamellar phase of lipids and surfactants. They reported formation of vesicles with mean diameter of less than 300 nm in which hydrophilic drugs can be loaded with high yield. They coated the vesicles with PEG and loaded them with 1-β-D-arabinofuranosylcytosine. Following injection of the vesicles intravenously to rats they observed that the surface-modified liposomes exhibited longer circulation times compared to uncoated liposomes.

Koynova and MacDonald (2005) examined the lipid exchange between model lipid systems, including vesicles of the cationic lipoids ethyl dimyristoyl phosphatidylcholine, ethyl dipalmitoyl phosphatidylcholine or their complexes with DNA, and the zwitterionic lipids by using differential scanning calorimetry. They observed that, exchange via lipid monomers was considerably more facile for the cationic ethylphosphatidylcholines than for zwitterionic phosphatidylcholines and for the cationic liposomes. The presence of serum in the dispersing medium strongly promoted lipid transfer between cationic vesicles while almost no effect was reported for zwitterionic liposomes. This phenomenon was proposed as an important point for the application of cationic liposomes as nonviral gene delivery. Foco et al (2005) studied the delivery of sodium ascorbyl phosphate (SAP), an effective oxygen species scavenger to prevent the degenerative effects of UV radiation on skin. SAP was encapsulated into liposomes to improve its penetration through the stratum corneum into the deeper layers of the skin. They prepared two types of multilamellar vesicles, one from non-hydrogenated and the other from hydrogenated soybean lecithin, together with cholesterol. Sinico et al (2005) studied transdermal delivery of tretinoin and examined the influence of liposome composition, size, lamellarity and charge on transdermal delivery. They studied positively or negatively charged liposomes of different types, i.e. multilamellar vesicles (MLV) or unilamellar vesicles (ULV), prepared from hydrogenated soy phosphatidylcholine (Phospholipon® 90H) or non-hydrogenated soy phosphatidylcholine (Phospholipon® 90) and cholesterol, in combination with stearylamine or dicetylphosphate. It was reported that negatively charged liposomes strongly improved newborn pig skin hydration and tretinoin retention.

Arcon et al (2004) encapsulated an anticancer agent, cisplatin, in sterically stabilized liposomes and studied the systems with extended X-ray absorption fine structure (EXAFS) method, and concluded that the liposome-encapsulated drug is chemically stable and does not hydrolyze. Sapra and Allen (2003) published

a review article about the ligand-targeted liposomes (LTLs) for the delivery of anticancer drugs. In this article, new approaches used in the design and optimization of LTLs was discussed and the advantages and potential problems associated with their therapeutic applications were described.

3.3. Ceramic Nanoparticles

Use of ceramics in medicine is especially significant in dental and orthopedic applications as strengthening materials for the hard tissue implants. Hydroxyapatite (HA) is a ceramic naturally existing in the bone structure and therefore its use in the hip or knee prosthesis can reduce the risk of rejection and stimulate the production of osteoblasts which are the cells responsible for the growth of the bone matrix.

Ceramic particles effectively protect the doped molecules (enzymes, drugs, etc) against denaturation induced by external pH and temperature. In addition, their surfaces can be easily modified with different functional groups. They can be conjugated to a variety of monoclonal antibodies or ligands for targeting purposes in vivo. Ceramic particles with entrapped biomolecules have a great potential in delivery of drugs. Such particles, including silica, alumina, titania, etc, are known for their compatibility with biological systems. They have several advantages such as the ease of preparation with the desired size, shape and porosity under ambient conditions, high stability such as no swelling or change in shape in environmental conditions.

McQuire et al (2005) synthesized hydroxyapatite sponges by using aminoacid coated HA nanoparticles dispersed within a viscous polysaccharide (dextran sulfate) matrix and examined the use of these materials for the viability and proliferation of human bone marrow stromal cells in order to search possibility for cartilage or soft tissue engineering. Rusu et al (2005) studied size-controlled hydroxyapatite nanoparticles prepared in aqueous media in a chitosan matrix from soluble precursors salts bone for the purpose of tissue engineering applications. Serbetci et al (2000, 2002, 2004) prepared acrylic bone cements with addition of HA microparticles. They examined the effect of HA addition on the properties of the cement. They reported enhancement of mechanical, thermal and biological properties depending on the added amount of HA.

Christel and co-workers (1984) implanted calcium phosphate bioglass ceramics in the tibiae of rabbits to study the interface of bioceramics. It was reported that hydroxyapatite surface give rise to a closer contact with new bone than calcium phosphate glass ceramics. Lin and colleagues (1996) implanted bioglass discs into the condyle area of rabbits. The failure load, when an implant detached from the bone or when the bone itself broke, was measured by a push-out test and compared with sintered hydroxyapatite bioceramic. Vogel and coworkers (2001) implanted bioglass particles in the distal femoral epiphysis of rabbits and examined bone formation at the implant site. They discussed the parameters (implantation model, particle size and surface-area-to-volume ratio) as possible parameters determining bone regeneration. Recently Amaral and colleagues (2002) studied wettability and

surface charge properties of Si_3N_4–bioglass biocomposites. They determined that the examined bioglass had comparatively higher hydrophilic character and surface tension value than the most common bioceramics. The presence of very high negative zeta potential at neutral pH influenced albumin adsorption. They also studied mechanisms in terms of entropy and enthalpy gains from conformational unfolding and cation coadsorption (Amaral et al 2002).

Zeng and co-workers (2002) prepared Al_2O_3–A/W bioglass coating through tape casting process by selecting low melting point A/W bioglass to decrease the Al_2O_3 sintering temperature and modify the bioactivity of implant. On the other hand, Xin and colleagues (2005) investigated the formation of calcium phosphate (Ca-P) on various bioceramic surfaces in simulated body fluid (SBF) and in rabbit muscle. The bioceramics were sintered porous solids, including bioglass, glass-ceramics, hydroxyapatite, α-tricalcium phosphate and β-tricalcium phosphate. They compared the ability of inducing Ca-P formation and obtained similar results in SBF but observed considerable variations in vivo.

3.4. Dendrimers

Dendrimers are small molecules which have a core and a series of branches symmetrically formed around the core resulting in a monodisperse, symmetrical macromolecule. They can be synthesized either starting from the core molecules and going out to the periphery by connecting the branch groups or by forming the branches first and then collecting all around the core. Functionality of the branching units is generally 2 or 3, which makes the layer of branching units doubles or triples. The interior cavity is very suitable for the entrapment of the drugs and their unique properties such as high degree of branching, multivalency, globular architecture and well-defined molecular weight, make dendrimers promising new carriers for drug delivery. Their nanometer size, ease of preparation and functionalization, and their ability to display multiple copies of surface groups for biological reorganization processes increase their attraction in biomedical applications.

Interaction of dendrimer macromolecules with the molecular environment is predominantly controlled by their terminal groups. By modifying their termini, the interior of a dendrimer may be made hydrophilic while its exterior surface is hydrophobic, or vice versa. Drug molecules can be loaded both in the interior of the dendrimers as well as attached to the surface groups. Water-soluble dendrimers are capable of binding and solubilizing small molecules and can be used as coating agents to protect or deliver drugs to specific sites in the body or as time-release vehicles for transporting biologically active agents. In the last decades, research has increased on the design and synthesis of biocompatible dendrimers and their application to many areas of bioscience including drug delivery, immunology and the development of vaccines, antimicrobials and antivirals gained great attantion.

A series of lipidic peptide dendrimers based on lysine with 16 surface alkyl (C_{12}) chains has been synthesised by Florence et al (2000). A fourth generation dendrimer with a diameter of 2.5 nm was studied for its absorption at different organs after

oral administration to female Sprague–Dawley rats. The results showed that the total percentage of the dose absorbed through Peyer's patches depend on the loaded dose as well as the size of the nanoparticules. Wang et al (2000) investigated the fifth generation of ethylenediamine core dendrimer for its ability to enhance gene transfer and expression in a clinically relevant murine vascularized heart transplantation model. They formed complexes of the plasmids with dendrimers which were perfused via the coronary arteries during donor graft harvesting, and reporter gene expression was determined by quantitative evaluation. Yoo and Juliano (2000) studied the behavior of dendrimer-nucleic acid complexes at the cell interior. They prepared dendrimers conjugated with the fluorescent dye Oregon green 488 and used these in conjunction with oligonucleotides labeled with a red (TAMRA) fluorophore in order to visualize the sub-cellular distribution of the dendrimer-oligonucleotide complex and of its components by two-color digital fluorescence microscopy. They observed that oregon green 488-conjugated dendrimer was a better delivery agent for antisense compounds than unmodified dendrimers.

Sashiwa and Aiba (2004) investigated the role of individual functional groups in applications of chitosan. They modified chitosan by attaching sugars, dendrimers, cyclodextrins, crown ethers, and glass beads to chitosan and concluded that among these derivatives, sugar-modified chitosans were excellent candidates as drug delivery systems or for cell culture while chitosan–dendrimer hybrids were interesting multifunctional macromolecules in biomedicinal applications.

The most commonly synthesized and studied dendrimers are the ones prepared from polyamidoamine (PAMAM). Wiwattanapatapee et al (2000) investigated the effects of size, charge, and concentration of PAMAM dendrimers on uptake and transport across the adult rat intestine in vitro using the everted rat intestinal sac system. They used cationic PAMAM dendrimers (generations 3 and 4) and anionic PAMAM dendrimers (generations 2.5, 3.5, and 5.5) and labelled the dendrimers with I-125. They concluded that, the anionic PAMAM dendrimers displayed serosal transfer rates faster than that of other synthetic and natural macromolecules (including tomato lectin). PAMAM dendrimers were also prepared by Tripathi et al (2002) by linking methyl methacrylate and ethylenediamine successively on an amine core and the surfaces were modified with fatty acids. They studied the release rates of chemotherapeutic drug, 5-fluorouracil (5-FU), which was entrapped in dendrimer grafts. In vitro studies, release rate was examined across cellulose tubing in PBS, and in vivo studies release rates were performed in albino rats by determining the amount of 5-FU in plasma. Jevprasesphant et al (2004) investigated the mechanism of transport of G3 PAMAM dendrimer nanocarriers and surface-modified (with lauroyl chains) dendrimers across Caco-2 cell monolayers. Optical sectioning of cells incubated with fluorescein isothiocyanate (FITC)-conjugated dendrimer and lauroyl–dendrimer using confocal laser scanning microscopy revealed colocalisation of a marker for cell nuclei (4',6-diamidino-2-phenylindole) and FITC fluorescence, also suggesting cellular internalisation of dendrimers. Effect of various concentrations PAMAM dendrimers (generations 2, 3, and 4) on human red blood cell morphology, and membrane integrity was studied by

Domanski et al (2004). They observed a change in erythrocyte shape from biconcave to echinocytic in dendrimers as well as cell aggregation and haemolysis depending on concentration and generation of dendrimers. Sagidullin et al (2004) studied the self-diffusion coefficients and nuclear magnetic relaxation of poly (amidoamine) dendrimers with hydroxyl surface groups (PAMAM-OH) by dissolving dendrimers in methanol over a wide range of concentrations. The generalized concentration dependence of PAMAM-OH self-diffusion coefficients were found to be coincide with analogous curve obtained for poly (allylcarbosilane) dendrimers of high generations.

To establish an effective nonviral gene transfer vector to hepatocytes, various oligo-carrier complexes were developed by Mamede et al (2004) by employing dendrimer (G4) and avidin–biotin systems (Av–bt). It was reported that for In-111-labeled-oligo, without any carriers, low uptake in normal organs other than the kidney were observed. In contrast, In-111-labeled-oligo coupled with avidin through biotin had very high accumulation in the liver. If G4 complexed forms are used, high uptake in the kidney and spleen were observed with relatively low hepatic uptake. They concluded that avidin–biotin systems have high potential as a carrier of oligo-DNA to the liver. ^{111}In-oligo-bt-Av, which exhibited the highest hepatic uptake in vivo, showed high and rapid internalization into hepatocytes. Okuda et al (2004) also studied non-viral gene delivery systems and showed that dendritic poly (L-lysine) of the 6th generation (KG6) had high transfection efficiency into several cultivated cells with low cytotoxicity. They synthesized KGR6 and KGH6, in which terminal amino acids were replaced by arginines and histidines, respectively. DNA-binding analysis showed that KGR6 could bind to the plasmid DNA as strongly as KG6, whereas KGH6 showed decreased binding ability. Wada et al (2005) studied in vitro and in vivo gene delivery efficiency of polyamidoamine starburst dendrimer (generation 2) conjugate with α-cyclodextrin bearing mannose with various degrees of substitution of the mannose moiety as a novel non-viral vector in a variety of cells. Sampathkumar et al (2005) described bifunctional PAMAM-based dendrimers that selectively target cancer cells. The targeting moiety for the folate receptor was complexed to an imaging or therapeutic agent by a DNA zipper. Choi et al (2005) produced amine-terminated, generation 5 polyamidoamine dendrimers conjugated to different biofunctional moieties (fluorescein and folic acid), and then linked them together using complementary DNA oligonucleotides to produce clustered molecules that target cancer cells that over express the high-affinity folate receptor. Kolhe et al (2003) studied the interaction between the drug and polyamidoamine dendrimers (generations 3 and 4 with $-NH_2$ functionality) and Perstrop Polyol (generation 5, hyperbranched polyester with –OH functionality) by using ibuprofen as a model drug. They found that hyperbranched Polyol (with 128 –OH end groups) appears to encapsulate approximately 24 drug molecules.

Singh and Florence (2005) synthesized lipidic polylysine dendrimers. They examined the effect of concentration on the diameter and stability of nanoparticles formed from two short homologous series of dendrimers. Raju et al (2005) described the synthesis of a new scaffold derived from iminodipropionic acid for

the preparation of peptide dimers and tetramers. Pan et al (2005) synthesized polyamidoamine (PAMAM) dendrimer on the surface of magnetite nanoparticles to allow enhanced immobilization of bovine serum albumin (BSA). They concluded that there were two major factors that improved the BSA binding capacity of dendrimer-modified magnetite nanoparticles: either the increased surface amine can be conjugated to BSA by a chemical bond; or the available area has increased due to the repulsion of surface positive charge.

Schatzlein and colleagues (2005) studied the transfection activity of polypropylenimine dendrimers and the effect of the strength of the electrostatic interaction between carrier and DNA on gene transfer. They evaluated the in vivo gene transfer activity of low molecular weight, non-amphiphilic plain and quaternary ammonium gene carriers and concluded that the polypropylenimine dendrimers were promising systems, which may be used in gene targeting. Recently Namazi and Adeli (2005) applied citric acid–polyethylene glycol–citric acid triblock dendrimers as biocompatible compounds for drug-delivery. They investigated the controlled release of molecules and drugs in vitro conditions and reported that the drug/dendrimer complexes were stable while the drugs were not released after storage at room temperature for about 10 months. Marano and co-workers (2004) described the synthesis of lipid–lysine dendrimers and their ability to deliver sense oligonucleotide ODN-1 to its target. It is important to mediate the reduction in VEGF concentration both in vitro and in vivo during ocular neovascularisation. They demonstrated that lipophilic, charged dendrimer mediated delivery of ODN-1 resulted in the down-regulation of in vitro VEGF expression. Time course studies showed that the dendrimer/ODN-1 complexes remained active for up to two months indicating the dendrimer compounds provided protection against the nucleases. Ooya and colleagues (2003) developed systems to increase the aqueous solubility of paclitaxel (PTX), a poorly water-soluble drug. They reported that graft and star-shaped graft polymers consisting of poly (ethylene glycol) (PEG 400) graft chains increased the PTX solubility in water by three orders of magnitude. Polyglycerol dendrimers dissolved in water at high concentrations without significantly increasing the viscosity and by increasing the solubility of PTX while the release rate was found as a function of the star shape and the dendrimer generation. Rittner and co-workers (2002) studied the design of basic amphiphilic peptides, ppTG1 and ppTG20 (20 amino acids), and evaluated their efficiencies *in vitro* and *in vivo* as single-component gene transfer vectors. Based on the structure–function studies, and sequence variants, they suggested that the high gene transfer activity of these peptides was correlated with their propensity to exist in α-helical conformation, which seems to be strongly influenced by the nature of the hydrophobic amino acids.

Dendrimers were also studied in the production of biosensors. For example, Alonso et al (2004) used ferrocene–cobaltocenium dendrimers in the preparation of glucose electrodes. For this purpose, enzyme glucose oxidase (GOx) was immobilized electrostatically onto carbon and platinum electrodes which were modified with dendrimers and the effects of the substrate concentration, the dendrimer

generation, and the thickness of the dendrimer layer, interferences, and storage on the response of the sensors were investigated. Devarakonda et al (2004) investigated the effect of low generation (G0–G3) ethylenediamine (EDA) core poly (amidoamine) dendrimers on the aqueous solubility of nifedipine in different pH values. It was reported that generation size, surface functional group and the pH of the aqueous media determined the aqueous solubility and solubility profiles of nifedipine. For amine and ester terminated dendrimers the highest nifedipine solubility was observed at pH 7.0.

Smith et al (2005) published a review about the properties of dendritic molecules and focused on examples in which individual dendritic molecules are assembled into more complex arrays via non-covalent interactions. This review emphasises how the structural information programmed into the dendritic architecture controls the assembly process, and as a consequence, the properties of the supramolecular structures which are generated, and how the use of non-covalent (supramolecular) interactions provide the assembly process with reversibility, with a high degree of control. The review also illustrates how self-assembly offers an ideal approach for amplifying the branching of small, synthetically accessible, relatively inexpensive dendritic systems (e.g. dendrons), into highly branched complex nanoscale assemblies and how assembled structures encapsulate a templating unit.

3.5. Polymeric Micro and Nano Particles

In the delivery of bioactive agents, generally the agent is dissolved, entrapped, adsorbed, attached or encapsulated in a polymeric matrix that has a micro or nano dimension. Depending on the method of preparation, micro or nano particles, spheres or capsules can be obtained with different properties and different release characteristics. Capsules are vesicular systems in which the drug is trapped in the central cavity which is surrounded by a polymeric membrane, whereas spheres are systems in which the drug is physically and uniformly dispersed in the matrix. Scientists have carried out numerous studies describing the effect of preparation parameters on the properties of micro and nano particles. Boguslavsky et al (2005) prepared polyacrylonitrile nanoparticles in sizes ranging from approximately 35 to 270 nm by dispersion/emulsion polymerization of acrylonitrile. They investigated the influence of various polymerization parameters (e.g. concentration of monomer and initiator, type and concentration of surfactant, temperature and time of polymerization, ionic strength, pH and co-solvent concentration) on the properties (e.g. size and size distribution, yield, stability, etc.) of the particles. Recently He and colleagues (2005) prepared polyaniline nanofibers and polyaniline/CeO_2 composite microspheres by stabilizing the emulsion by CeO_2 nanoparticles. They also synthesized sub-micrometer fibers of polyaniline/nano-ZnO composites in a toluene/water emulsion stabilized by ZnO nanoparticles and examined effects of volume ratio of toluene to water on properties of the composites. Akin and co-workers (1990) designed and synthesized polymeric hydrophobic membranes which have micro hydrogel channels and examined permeabilities towards various chemicals. They

found that, permeability depends on the crosslinking of hydrogel part, as well as the chemical structure and the charge of the permeant.

Nanoparticles of poly (DL-lactic acid) (PDLLA), poly (DL-lactic-co-glycolic acid) (PLGA) and poly (ethylene oxide)–PLGA diblock copolymer (PEO–PLGA) were prepared by the salting-out method by Zweers et al (2004). They examined the in vitro degradation of the prepared nanoparticles in PBS (pH 7.4) at 37°C. The effects of particle size, molecular weight of the polymers and the amount of lactic and glycolic acids on the degradation were examined. It was reported that, PDLLA nanoparticles gradually degraded over a period of 2 years while faster degradation was observed for PLGA nanoparticles such as complete degradation in 10 weeks.

Natural polymers such as gelatin, chitosan, proteins and starch are all interesting materials for medical applications since they are biodegradable and bioabsorbable where the degradation products do not have any toxic effect. Akin and Hasirci examined the properties of gelatin microspheres prepared under different conditions (1995) and also examined release of 2,4-D from these systems (1994). Burke et al (2000, 2002) examined iron ion adsorption capacity of chitosan microspheres to remove iron from the blood for the treatment purpose of thalasemmia. Yilmaz et al (2002) also examined chelating capacity of chitosan flakes and microspheres for complexed iron (III) for the removal of iron ions. Ulubayram et al (2001, 2002) examined cytotoxicity of microporous gelatin sponges prepared with different crosslinkers. In a series of studies Muvaffak et al (2002, 2004a, 2004b, 2005) prepared gelatin microspheres and conjugated antibodies to their surfaces. They studied targeting and release of chemotherapeutic drugs such as 5-fluoroucil and colchicines and showed that the system had a high affinity towards its antigens and the release rate of drugs depended on the preparation parameters of microspheres. They suggested the systems are promising and have high potential as anticancer drug targeting systems to specific tumor locations.

One advantage of delivery systems is that they allow the delivery of drugs that are highly water-insoluble or unstable in the biological environment. Zhang and Zhuo (2005) prepared a BAB type amphiphilic triblock copolymers consisting of poly (ethylene glycol) (PEG) (B) as hydrophilic segment and poly (ε-caprolactone) (PCL) (A) as hydrophobic block. A poorly water-soluble anticancer drug 4'-dimethyl-epipodophyllotoxin (DMEP) was encapsulated into the polymeric nanoparticles for controlled drug release. In vitro results showed that the drug release rate can be modulated by the variation of the copolymer composition. Long-term sustained delivery is a desired property and is affected by the diffusion kinetics of the drug and degradation of the matrix which controls the rate of drug release. It is possible to extend this period from hours to months. A review was published by Sinha et al (2004) about long-term delivery from poly-ε-caprolactone (PCL) microspheres and nanospheres. They reported that biodegradation of PCL is very slow in comparison to other polymers, which makes it suitable for long-term delivery, extending the release duration to more than one year.

Alonso and colleagues (2004) studied nanosystem drug carriers for mucosal administration. In vitro cell culture studies and in vivo experiments have proved the

potential of nanocarriers in overcoming mucosal barriers such as intestinal nasal and ocular barriers. Recently Dinauer et al (2005) prepared gelatin nanoparticles and antibodies specific for the CD3 antigen of lymphocytic cells were conjugated to the nanoparticle surface. Cellular uptake and effective internalization of antibody-conjugated nanoparticles into CD3 expressing cells were examined. Dinauer et al (2004) also developed a carrier system for antisense oligonucleotides (AS-ODN) and antisense phosphorothioate analogs (AS-PTO). They prepared nanoparticles by using protamine to complex AS-ODN and AS-PTO and concluded that cellular uptake of these nanoparticles significantly enhanced the uptake in comparison to naked oligonucleotides. Dong and Feng (2005) prepared poly (d,l-lactide-co-glycolide)/montmorillonite (PLGA/MMT) nanoparticles by emulsion/solvent evaporation method as bioadhesive drug delivery system for oral delivery of paclitaxel. It was reported that the system extended residence time in the gastrointestinal (GI) tract and promoted the effect of the drug.

Ciardelli et al (2004) studied formation of poly (methyl methacrylate-*co*-methacrylic acid) nanospheres which were imprinted with theophylline through template radical polymerization. Effect of the nature of the functional monomer in the recognition and in the release of template was studied. These systems can be considered as promising systems for the recognition and isolation of the biologically important template molecules. Chen and Subirade (2005) prepared chitosan/β-lactoglobulin core–shell nanoparticles with the aim of developing a biocompatible carrier for the oral administration of nutraceuticals. Uniform size nanoparticles were prepared by ionic gelation with sodium tripolyphosphate and were highly sensitive to medium pH. When transferred to simulated intestinal conditions, the β-lactoglobulin shells of the nanoparticles were degraded by pancreatin.

Responsive hydrogels gained great importance in 1990‘s and lots of research is going on since then. Yoshida et al (1989) synthesized some thermo-responsive hydrogels containing α-amino acid groups as side chains from copolymerizing 2-hydroxypropyl methacrylate and polyethylene glycol dimethacrylate, using gamma irradiation. They investigated swelling-deswelling as well as thermo-responsive kinetics of drug release. Dong and Hoffman (1990) investigated progesterone release from thermally reversible hydrogels of N-isopropylacrylamide (NIPAAm) and bis-vinyl-terminated polydimethylsiloxane (VTPDMS) synthesized by gamma irradiation. They proposed existance of microdomain structure in the gels based on differential scanning calorimetry results and observed zero-order release of progesterone. Kabra et al (1992) synthesized poly (vinyl methyl ether) thermally responsive gels by gamma irradiation and examined the shrinking rates of the gels. They observed that enhancement in rate was related to the development of a microporous structure which allows the convective expulsion of solvent from the network which occurs more quickly than the diffusive motion of the network. Low et al (2000) designed microactuator valves made of metal or polymeric substances for responsive delivery of drugs. The reversible polymeric valve systems acted as artificial muscle and were prepared from a blend of redox polymer and hydrogel (polyaniline and poly (2–hydroxyethylmethacrylate)–poly (N–vinylpyrrolidinone).

They concluded that responsive controlled drug delivery by these microactuator valves is possible. Shantha and Harding (2000) examined biocompatible and biodegradable pH-responsive hydrogels based on N-vinyl pyrrolidone (NVP), polyethylene glycol diacrylate (PAC) and chitosan. In-vitro release profiles of theophylline and 5-fluorouracil were examined in enzyme-free simulated gastric and intestinal fluids, observing that more than 50% of the entrapped drugs were released in the first 2 h in gastric pH. Goldraich and Kost (1993) prepared hydrogel matrices for immobilization of glucose oxidase and release of insulin responsive to glucose concentration. They did the synthesis by chemical polymerization of 2-hydroxyethyl methacrylate, N,N-dimethyl-aminoethyl methacrylate, tetraethylene glycol dimethacrylate, ethylene glycol in the presence of water solutions of glucose oxidase, bacitracin or insulin. They observed faster and higher swelling and release rates at lower pH or at higher glucose concentrations. Chen et al (2000) prepared colloidal platinum nanoparticles in the size range of 10–30 Å in the presence of poly (*N*-vinylisobutyramide) (PNVIBA). The formed colloidal PNVIBA–Pt nanoparticles exhibited inverse temperature solubility and a cloud-point temperature of 38.9°C in water.

Gomez-Lopera et al (2001) prepared colloidal particles responsive to magnetic field. They did the synthesis of biodegradable poly (dl-lactide) polymer around a magnetite nucleus by using biodegradable poly (dl-lactide) with a double-emulsion technique. The main purpose was to develop responsive drug delivery systems. Vihola et al (2002) investigated behaviours and release kinetics of model drugs (β-blocking agents nadolol and propranolol and a choline-esterase inhibitor tacrine) from thermally responsive polymeric nanoparticles composed of poly (N-vinylcaprolactam) (PVCL). They observed that the more hydrophobic drug substances, propranolol and tacrine, considerably swell the PVCL-microgels. The β-blocking agents were tightly bound to the microgels especially at higher temperatures and on the contrary, the release of tacrine across the cellulose membrane was increased when PVCL particles were present. Taniguchi et al (2003) investigated temperature, pH, and salinity effects for adsorption and desorption of anti-α-feto protein (anti–AFP) onto polystyrene-core-poly (*N*-isopropylacrylamide)-shell particles. They observed that adsorption was mainly governed by electrostatic interactions. Twaites et al (2004) prepared poly (*N*-isopropyl acrylamide) (PNIPAm) co-polymers responsive to temperature and pH. They examined the binding of plasmid DNA to these materials and to control polymers of poly (ethyleneimine) (PEI) and poly (ethyleneimine)-octanamide. They observed the complexes of plasmid DNA with thermoresponsive cationic polymers displayed variations in gel retardation behaviour above and below polymer phase transition temperatures such as, lesser affinity for high molecular weight linear cationic PNIPAm co-polymer complexes, and higher affinity for branched PEI-PNIPAm co-polymers above LCST. Zhang et al (2004) prepared composite membranes from nanoparticles of poly (N-isopropylacrylamide-co-methacrylic acid) of various NIPAAm:MAA ratios dispersed in a matrix of a hydrophobic polymer. Permeation of N-Benzoyl-L-tyrosine ethyl ester HCl, momany peptide, Leuprolide, vitamin B12, insulin,

and lysozyme were examined as a function of temperature. Kovacs et al (2005) demonstrated that anionic microspheres coated with an ornithine/histadine-based cationic peptide (O10H6) were effective carriers of short oligonucleotides. They reported that microspheres stabilize the DNA and O10H6 through complexation. They proposed that, this self-assembly system can be an effective delivery vehicle for DNA-based formulations. Venkatesan et al (2005) studied the feasibility of nanoparticulate adsorbents in the presence of an absorption enhancer for the administration of erythropoietin (EPO) to the small intestine. Liquid filled nano and micro particles were prepared using solid adsorbents such as porous silicon dioxide, carbon nanotubes, carbon nanohorns, fullerene, charcoal and bamboo charcoal. The serum EPO levels were compared for the prepared systems. Among the adsorbents studied, carbon nanotubes showed the highest capacity. Recently Jo and coworkers (2004) carried out mathematical modeling of release of encapsulated indomethacin from poly (lactic acid-co-ethylene oxide) nanospheres and investigated in vitro release behavior based on the proposed mathematical models. Effects of several key parameters were examined according to two different types of mathematical models.

4. CONCLUSION

Use of micro and nano particles in biomedicine and especially in drug delivery has a great deal of advantages over conventional systems such as: the enhanced delivery, high performance characteristics of the product, use of lesser amounts of expensive drugs in the delivery systems, extension of the bioactivity of the drug by protecting it from environmental effects in biological media, more effective treatment with minimal side effects. In addition, research for the design of more effective delivery systems is more economical for the discovery of a new bioactive molecule. Micro and nano colloidal drug delivery systems such as emulsions, suspensions and liposomes have been used for decades for this purpose and recently, nanosized systems with dimension of less than 100 nm gained significant attention. Nanotechnology promises to generate a library of sophisticated drug delivery systems that integrate molecular recognition, diagnostic and feedback. Nanotechnology is expected to create lots of innovations and play a critical role in various biomedical applications including the design of drug and gene delivery systems, molecular imaging, biomarkers and biosensors. By understanding the signalling and interaction between the molecules at nano levels, it would be possible to mimic biological systems.

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CHAPTER 2

NEW LIPID- AND GLYCOLIPID-BASED NANOSYSTEMS FOR TARGETED GENE DELIVERY: CHOLENIMS, GLYCOCLIPS, GLYCOLIPIDS AND CHITOSAN

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Abstract: Cationic lipid vesicles and polypeptides represent common non-viral gene delivery systems for in vitro and in vivo applications. New non-viral vectors for targeted gene delivery, namely, mono-, di- and tricholesterol derivatives of oligoethyleneimine, glycolipids and chitosan derivatives are reported in this chapter. Testing of genotoxicity, cytotoxicity and gene transfer activity against transformed monolayer and suspension cell cultures is carried out for all of these mediators of gene transfer. Experimental results show that GLYCOLIPID VI containing a lactose residue, which was used to form liposomes for gene delivery into tissues (using ^{14}C-adenosine-labeled or plasmid DNA), expressed the affinity of corresponding lipoplexes for kidney, liver, and spleen tissues. GLYCOLIPID VI is a prospective tool for designing new generation of nonviral vectors for targeted gene delivery to tissues. In addition, mCHIT preparation demonstrated high gene transfer activity (β-Gal and CSEAP plasmids) for both monolayer and suspension cell lines

Keywords: cholesteroyl derivatives of oligoethylenpropylenimine; cationic lipid; cationic glycolipid; lactosolipid, modified chitosan; cytotoxicity; genotoxicity; gene transfer; gene delivery

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Abbreviations: CLIP: cationic lipid; GLYCOCLIP: cationic glycolipid; DOPE: dioleyl phosphatidyl ethanolamine; PC: phosphatidyl choline; CHOLENIM: monocholesteroyl ester of ethylen/propylene/imine co-oligomer; TsO: p-toluenesulfonate; RGGN: rat Gasserian ganglion neurinoma; RLU: relative luminescence unit

1. INTRODUCTION

Cationic lipid based vesicles and polypeptides represent common non-viral delivery systems for *in vitro* and *in vivo* functional gene transfer for gene therapy purposes [1–5]. There exist a great variety of types of non-viral vectors [1, 6, 7]. They possess a number of advantages comparing to the viral vectors: they are not immunogenic like adenoviruses, not randomly integrated into genome like retro viruses, not infectious, not patogenic (oncogenic) and cheap. Neutralizing DNA negative charge they facilitate adsorbic endocytosis of self-assembled complexes between plasmid DNA and polycation and/or cationic lipid particle – lipoplexes. Another possiblity for genosomes to be internalized is receptor-mediated endocytosis [8–10]. The most promissing approach to the latter mechanism of targeted gene transfer/delivery is to employ specific oligosaccharide-conjugated vector systems [11, 12]. Systems for targeted delivery and receptor-mediated gene transfer could be also designed on the basis of polycations, but mainly using coupling with carbohydrates [10]. Polycations conjugated with carbohydrate residues were introduced into gene transfer field, and appeared to be one of the most effective group of transfection agents due to the moieties employed responsible for the receptor-mediated gene transfer [12, 13]. A number of chitosan preparations were recently reported as gene transfer and delivery systems [14–16]. Galactose derivative of cholesterol was introduced to provide gene targeting to hepatocytes [17]. In our study we emploied the incapsulation of reporter plasmid DNA into new delivery systems based on glycolipids, which are combining the advantages of both gene transfer mechanisms: non-specific (adsorbic endocytosis) and receptor-mediated ones, along with DNA incapsulation into hydrophobic oligocations.

Here we report new systems for nanotherapy comprising encapsulation of reporter genes into lipoplexes based on the use of cholesterol derivatives of oligoethylenpropylenimine I-III (CHOLENIMs) [18, 19]; cationic glycolipid containing glucose moiety V (GLYCOCLIP) [20], liposomal preparations based on lactosylated lipid (GLYCOLIPID) VI [21]; as the cytofectins and helper phospholipids, for gene transfer and delivery. Evaluation of the cyto- and geno-toxicity and gene delivery activity of these lipoplex and glycolipoplex systems were carried out in cell culture. To this end we also used modified natural polycationic polysaccharide, chitosan–modified chitosan derivative (mCHIT) VII, which can be prepared by deacetylation of chitin – linear poly–(N-acetyl-glucosamine) followed by methylation of deacetylated chitosan.

2. MATERIALS AND METHODS

All reagents used in this study were of reagent grade. Organic solvents were distilled before use. All lipid preparations (Sigma; Avanti) were stored at –80°C.

2.1. Cholenims

2.1.1. Synthesis and properties

Cholenims were synthesized as described earlier [18]. Cholenim I is tris- [2- *N*- (3- aminopropyl) aminoethyl] amine monocholesteroyl formiate; cholenim II, tris- [2- *N*- (3- aminopropyl) aminoethyl] amine dicholesteroyl formiate; and cholenim III, tris- [2- *N*- (3- aminopropyl) aminoethyl] amine tricholesteroyl formiate. Salmon sperm genomic DNA (1.7×10^4 kDa) was fragmented by mild sonication to duplexes with an average size of 4 kb. After dialyzing aqueous DNA solution (1.5 mg/ml) against 10 mM NaCl and 1 mM Tris-HCl (pH 7.2), its concentration was determined spectrophotometrically ($\lambda = 260$ nm) using the molar extinction coefficient $\varepsilon = 6600\ M^{-1}cm^{-1}$ [22]. Melting temperature of DNA duplexes in buffer solution was 72°C at a hyperchromic effect of 40%, indicating that the two-helix native structure of the duplexes was retained during sonication of genomic DNA. Melting curves of the complexes between genomic DNA fragments and cholenims were recorded on aVS4-2P spectrophotometer at 260 nm; the accuracy of measurements of temperature was $+/-0.5$ °C. Pyrene fluorescence spectra [23] were recorded on an MPF-44B Perkin-Elmer spectrofluorometer. Circular dichroism spectra of the lipoplexes containing the pCMV-SPORT-β-Gal plasmid (BioLifeTech, catalogue no. 10586-04) and cholenims were recorded on a Jasco J-600 spectropolarimeter. Electron micrographs of lipoplexes were obtained on a JEM 100B electron microscope at accelerating voltage of 80 kV. Briefly, an aliquot of the cholenim/DNA lipoplex was placed on a copper grid covered with a collodion film and dried. Excess complex was removed, and the remainder was negatively stained with 4% aqueous uranyl acetate. After removal of the dye, the film was dried. Micrographs were obtained on Kodak photographic plates.

2.1.2. Cytotoxicity, genotoxicty and gene transfer

The effectiveness of gene transfer using the cholenim lipoplexes was studied with eukaryotic cells RGGN-1 (NGUK-1, rat Gasserian gland neurinoma) and PC-12 (rat adrenal gland pheochromocytoma). Cells were cultured in an RPMI-1640 medium (Flow, United Kingdom) supplemented with 10% fetal bovine serum (PANECO) and 50 μg/ml gentamycin at 37°C in 5% CO_2 in a CO_2 incubator (Flow, United Kingdom) [4]. To form transfection complexes, plasmid DNA and cholenims were mixed, shaken on a Vortex, and incubated at room temperature for 30 min. RGGN-1 and PC-12 cells were transfected with the pCMV-SPORT-β-Gal plasmid 24 h after passage of cells in 96-well plates (5×10^4 cells per well). For this purpose, cultural liquid was removed from wells, and the monolayer was washed with a serum-free medium. Then, the DNA/cholenim lipoplex in a serum-free medium was

added to cells, and plates were incubated at 37°C for 5 h in 5% CO_2.Thereafter, an equal volume of culture medium containing 20% serum was added to cells, and incubation continued for another 48 h. After transfection, the medium was thoroughly removed from wells without disrupting the monolayer, and lysing solution containing 0.1% Triton X-100 and 0.25 M Tris-HCl (pH 8.0) was added to cells. Then, cells were frozen at −70°C and thawed at room temperature for 10 min. The activity of the marker β-galactosidase gene was determined as described [4,24], using chlorophenol-red-β-D-galactopyranoside (*N*-Gal; Sigma, United States) as a standard. Incubation was conducted in a phosphate buffer (pH 8.0) containing 1 mg/ml *N*-Gal, 1 mM$MgSO_4$, 10mM KCl, 50 mM mercaptoethanol and 0.5% bovine serum albumin at 37°C until color development (15 min). The enzyme content in samples was determined using dilutions of the standard β-galactosidase sample (Sigma, United States). Liposomes were obtained by evaporation from reverse phase with subsequent sonication at 4°C for 5 min. Liposomes consisting of phosphatidylcholine and dicholenim (1 : 1, w/w) were used to transfer β-galactosidase gene at the lipid-to-DNA ratio of 1.6:1 (w/w) using intravenous injections, as described [4]. To detect expression of the bacterial β-galactosidase gene, mouse organs (kidneys, liver, heart, lungs, intestine, and spleen) were frozen at −80°C. Pieces of tissue were used to prepare sections (25 μm thick) on a cryostat microtome, which were then mounted on slides.

2.2. Glycoclip

Proton magnetic resonance (^{1}H-n.m.r.) spectra were measured with radiospectrometer "Bruker" MSL-200 (200 MHz) in $CDCl_3$ with $Si(CH_3)_4$ as internal standart. Mass-spectra were recorded with MSBKH time-off-flight mass-spectrometer ("Elektron", Sumy-city, Ukraine) with the ionization by nuclear fragments of californium-252; accelerating voltage was +/− 5 kV or +/− 20 kV. Optic rotation angles were measured with Jasco photoelectric spectropolarimeter, model DIP 360 (Japan).

The cationic lipids used are *rac*-N-[2,3-di (octadecyloxy) propyl] pyridinium p-toluenesulfonate (IV, CLIP) that was synthesized by interaction of *rac*-1,2-di-O-octadecyl-3-O-(4-toluenesulfonyl)glycerol with pyridine (90°C, 4 hrs.) with the yield of 85%. Properties: R_f 0.6 (silicagel (Merck), chloroform/methanol, 4:1); mass spectrum: m/z for $[M\text{-}TsO^-]^+$ 658.7; ^{1}H-n.m.r., δ: 0.86 (t, J 7, 6H, $2(CH_2)_{15}CH_3$), 1.24 (br. s, $2(CH_2)_{15}$ CH_3), 1.55 (m, 4H, $2OCH_2$ CH_2), 2.33 (s, 3H, $C_6H_4CH_3$), 3.25 (t, 4H, J 7.1, $2OCH_2CH_2$), 3.3–3.5 (m, 2H, CH_2 $OC_{18}H_{37}$), 3.85 (m, 1H, $CHOC_{18}H_{37}$), 4.61 (d. d, J 8.5, 13; 1H, CH_2N^+), 7.16 (m, 2H) and 7.71 (m, 2H, $C_6H_4CH_3$), 8.04 (m, 2H), 8.52 (m, 1H) and 8.89 (m, 2H, $C_5H_5N^+$).

rac- 1,2-Dioctadecyl-3-O- (2,3,4-tri-O-acetyl-6-deoxy-6-pyridinium-β-D-glucopyranosyl) glycerol p-toluenesulfonate, GLYCOCLIP, V was synthesized by the glycosylation of *rac*-1,2-dioctadecylglycerol [25] with 6-O-(4-toluenesulfonyl)-2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide in the presence of $Hg(CN)_2$ and $HgBr_2$ as previously described [26], followed by interaction of the resulting compound (R_f 0.54, silicagel, petrol.ether/ether, 1:1.5) with pyridine. Properties: $[\alpha]^{20}$D −4.3° (Cl.5, chloroform/methanol, 3:2); R_f 0.45 (silicagel, chloroform/methanol, 4:1); mass

spectrum: m/z for $(M\text{-}TsO^-)^+$ 946.2; ^{1}H-n.m.r., δ: 0.85 (t, J 7, 6H, $2(CH_2)_{15}CH_3$), 1.27 (br. s, $2(CH_2)_{15}CH_3$), 1.52 (m, 4H, $2OCH_2CH_2$), 2.00, 2.15 and 2.35 (s, 9H, 3 ($COCH_3$), 2.61 (s, 3H, $C_6H_4CH_3$), 3.27–3.67 (m, 9H, $2OCH_2CH_2$), $CHOCH_3$, OCH_2CHCH_2O protons of Gro), 4.02–4.22 (m, 1H at C-5 Glc); 3.97–5.37 (m, 6H at C-2, C-3, C-4, C-5 and C-6 Glc); 7.12 (m, 2H) and 7.72 (m, 2H, $C_6H_4CH_3$); 8.10 (m, 2H), 8.57 (m, 1H) and 8.97 (m, 2H, $C_5H_5N^+$).

2.2.1. Liposome preparation

GLYCOCLIP/DOPE (1:1), GLYCOCLIP/DOPE/CHOLENIM (1:1:2), and CLIP/PC (1:1, w/w) liposomes were prepared by the reverse phase evaporation technique normally providing small monobilayer particles [27]. GLYCOCLIP-based liposomes were prepared by slow addition of ether lipid solution to water at 50°C, followed by complete evaporation of organic solvents under reduced pressure and oil pump as described [28]. The value of +/− charge ratio was 1.0 for CLIP/PC vesicles, 1.6 for GLYCOCLIP/DOPE ones, and 3.2 in the case of mixed GLYCOCLIP/DOPE/CHOLENIM liposomes. The size of lipoplex particles formed of liposomes used and plasmid DNA is ca. 100-200 nm (the data are not shown), which is normal for *in vitro* experiments. Oxidation index of liposomal lipids (PC, DOPE), OD^{233}/OD^{215} ratio, was measured after extraction from liposomal preparations, and it didn't exeed 0.1–0.2.

2.2.2. Lipofection procedure

CHO cells were maintained in the RPMI 1640 medium with L-glutamine, and 10% fetal calf serum. The cells were washed, and incubated at 37°C in serum-free OPTIMEM medium (Boeringer-Manheim) before transfection. Genosomes (3 µg of pCMV-Luc/3 µg of liposomes in 100 µL of medium) were added to the CHO cell monolayer (2×10^5 cells) up to 1 ml of total volume, and were incubated for 4 hours (37°C, 4.5% CO_2) (including 15 min period on microshaker 326M) [29]. Medium was then removed, cells were washed twice with HEPES buffer, and incubated with full medium for 24 hrs (postincubation). Then the lysis buffer was added. DNA-liposomes complexes (2 µg of DNA/2 µg of lipid) were prepared by mixing in OPTIMEM medium, added to cells, and incubated in the same way [3]. Luciferase activity was measured after 30 min incubation in the lysis buffer using Promega kit with LUMAT luminometer. The transfection efficiency values were represented as relative lumenescense units (RLU). The data in all cases represent the means of 4 series of independent experiments (four experiments each) with standart deviation ($M+/-\sigma$). The statistical significance was evaluated by Student t-test ($p < 0.05$).

2.3. Lactosylated Lipid, GLYCOLIPID, VI

In this study, we used DMSO, $CaCl_2$ (chemical purity and tissue-culture grades), egg phosphatidylcholine and cholesterol from Fluka, X-Gal (5-bromo-4-chloro-3-indolyl-1,3-*D*-galactopyranoside) from Aldrich, and N-Gal (chlorophenol-red-β-*D*-galactopyranoside). All solutions were sterilized using 0.22-µm nitrocellulose membranes (Millipore). Reagents and media were prepared in autoclaved deionized

water. The modified glycolipid, lactosolipid, was synthesized from lactose thioderivative by the method described [30, 31]. This method allows obtaining neutral and positively charged glycolipids with symmetrical and asymmetrical aglycone structure. The last stage of this synthesis and the removal of protective groups are shown in the scheme. Thiogalactose **1** at the double bond of dihexadecyl ester of maleic acid **2** (scheme) was attached using triethylamine as an activator of reaction. The structure of synthesized compound **3** was confirmed by the results of 1H NMR and IR spectroscopy and mass spectrometry.

2.3.1. DNA, liposomes and lipoplexes

^{14}C-adenosine-labeled DNA was isolated from *E. coli* cells grown on a Luria-Bertani medium with adding ^{14}C-adenine (56 mCi/mmol, Izotop, Russia) by the standard procedure [32]. ^{14}C-DNA was sonicated at 22 kHz with an UZDN-2T disintegrator (Russia) for 15 min, with 30-s intervals after each minute of sonication, at 0°C. In total, ten sonication cycles were performed. As a result of this procedure, 4.6kb fragments were obtained (electrophoretic data). To obtain preparative amounts of the pCMV-SPORT-β-Gal plasmid (BioLifeTech, catalogue no. 10586-04), *E. coli* XL-1 cells transformed with this construct were cultured in a fermenter (shaker) at 37 ± 0.5°C for 14–16 h (night culture) in a Luria-Bertani liquid microbiological medium (ratio, 800 ml of medium per 4 l of air) supplemented with 50 mg/ml ampicillin as a selective component of cells carrying the plasmid with the corresponding marker gene.

To form nucleoliposome complexes (lipoplexes), ^{14}C-adenosine-labeled or plasmid DNA was mixed with liposomes and incubated for 30 min. Experiments were performed with four- to six-month-old inbred ICR mice weighing 36–40 g. Lipoplexes containing 80 μg of ^{14}C- adenosine-labeled DNA (65000 cpm per mouse) and 160 μg of phosphatidylcholine/lactosolipid liposomes were injected to anesthetized mice through a glass capillary into the portal vein of the liver. One day after injection, operated animals were euthanized; their internals were extracted, weighed, and lyzed in 0.6 N KOH at 37°C. Lysates were neutralized with 0.6 N $HClO_4$ and loaded on filters. Then, filters were dried and placed into flasks with scintillation liquid. The radioactivity trapped on the filters was measured in a Rakbeta counter. Polybilayer liposomes used to transfect mice *in vivo* were formed from a mixture containing phosphatidylcholine (70 mol %), lactosolipid (20 mol %), and dicholenim (10 mol %) by evaporation from reverse phase, as described [27]. Solutions of original lipids were stored at –80°C and liposomes were stored at 4°C under nitrogen. Liposomes were used within two weeks.

2.3.2. Cells, cell survival and genotoxicity determination

Rat Gasserian ganglion neurinoma (RGGN) cells were cultured in the RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum, and 50 μg/ml gentamycin. RGGN cells were seeded after the treatment with 0.02% EDTA (24-well plates) for their growing and DNA synthesis measurements. The initial cell density was

5×10^4 per well. RGGN cells were incubated unsealed in CO_2-incubator (5% CO_2, 37°C), liposomes were added 24h after cell passing [18, 33].

^{14}C-Thymidine (56 mCi/mmol, "IZOTOP", Russia) (5 mcCi) was added to 1 ml of the culture medium 24 hrs after liposomes. Cells were washed with cold Hanks medium 2 hrs after labeling, and fixed overnight with a cold mixture ethanol/"ice" acetic acid (9:1) to remove the unbound ^{14}C-thymidine. The cell monolayer was stained with 0.2 % crystal violet in 2 % aq. ethanol solution, the stained cells were washed with water, and the dye was eluted with 10% aq. acetic acid. Cell number was measured as the optical density value at 595nm with $O.D.^{595}$ value equaled to 0.1 corresponding to 32,500 cells [34]. Then the cells were lysed with 0.3 N KOH overnight at 37°C, the pH value of the mixture was adjusted to 7, and the radioactivity value was counted using Bray's solution.

2.3.3. Animal experiments

Animals that were injected with the complex through the portal vein were euthanized two days after injection. For histochemical analysis, organs were frozen at 80°C immediately after their extracting from mice. Sections of these organs (25 μm thick), obtained using a cryostat microtome, were mounted on slides. Then, 200 μl of PBS (pH 7.5) containing X-Gal (6 mg/ml), 1 mM $MgSO_4$, 4 mM $K_4[Fe(CN)_6]$, and 4 mM $K_3[Fe(CN)_6]$ were poured over slides. X-Gal (6 mg) was preliminarily dissolved in 200 μl of DMSO. Slides were placed in a thermostate (37°C) in a moist chamber. The time required for the development of blue staining as a result of X-Gal degradation (30-50 min) was determined [4]. Thereafter, slides with sections were incubated in 2.5% glutaric aldehyde at 4°C for 2 h. To visualize cell structures (predominantly nuclei), sections were additionally stained with hematoxylin. Then, after successive dehydration in 70, 96, and 100% ethanol, a mixture of ethanol and xylol (1:1), and o-xylol, sections were embedded into Canada balsam drops under cover slips. For spectrophotometric detection of β-galactosidase activity in organs *in vivo*, they were homogenized on ice in PBS (pH 8.0) containing 1 mM Mg^{2+} and 10 mM K^+. Then, 1 ml of the homogenate was mixed with 100 μl of a substrate (chlorophenol-red-β-*D*-galactopyranoside) and 100 μl of mercaptoethanol. The mixture was stirred on a Vortex and divided into two parts (the experimental and the control). The experimental part was incubated in a thermostate at 37°C for 30 min (the optimal time for color development for 0.2 g aliquots), and the control part was incubated on ice. Then, both tubes were centrifuged at 11000 rpm for 7.5 min. The supernatant was collected and stored in the cold. Then, 200 μl of the reaction mixture were added to cuvettes with PBS. The specific activity of β-galactosidase was determined using the standard enzyme (Sigma, catalogue no. 9031-11-2) at different dilutions, by the optical density at 280 nm (D_{580}), which corresponded to the absorption maximum of the reaction product in the visible part of the spectrum. The values of optical density of the standard samples were used to plot a calibration curve that was then used to determine the activity of β-galactosidase in homogenates of organs. The coefficient used for calculation was determined by approximation to linear direct proportionality by the least squares

method using the MS Excel software. The values of D_{580} for homogenates of organs incubated at 37°C were measured relative to the matching samples that were incubated on ice (the control). The activity of transgenic β-galactosidase in organs was determined by the difference in the activity of the enzyme in the experimental and control samples. Using the calibration curve, the activity of β-galactosidase was recalculated to the international units of enzymatic activity (IU) and expressed in IU per gram of organ.

2.4. Modified Chitosan, VII

All chemical reagents used (L-α-phosphatidylcholine, α-tocopherol ester of succinic acid, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and p-nitrophenyl phosphate (Sigma); polyethylenimine (PEI) were of analytical grade.

2.4.1. Plasmids

All recombinant DNA manipulations (transfection, purification of plasmid DNA) were performed according to the protocols described earlier [35]. The following plasmids driven by the IE CMV promoter were used: pEQ176 with bacterial β-Gal gene (a gift from Dr. J. Overbaugh, University of Washington, Seattle, U.S.A.) and pCSEAP plasmid with secreted alkaline phosphatase gene (a gift from Dr. K. Doronin, University of Sant Luis, U.S.A.).

2.4.2. Synthesis

Chitosan preparations containing secondary and tertiary amino groups were prepared by G.G. Krivtsov using two-stage synthesis, intermediate product not being isolated. Initial chitosan preparation (reagent grade, m.m. 312 kDa, polydispercity 6.7, deacetylation degree 85%, 15% of N-acetylglucoseamine residues) was a gift from Dr. D.B. Freiman ("Sonat" Company, Moscow, Russia). We used reductive amination reaction [36] on the first stage to get chitosan preparation containing 20–25% of N-ethylated primary amino-groups. Chitosan (1% solution in 1% aq. acetic acid) was treated by 2% aq. solution of acetaldehyde in the presence of excess of sodium cyanoborhydride for 12 hours at 20°C. Resulted N-ethylated chitosan preparation was precipitated by 4% aq. sodium hydroxide solution, and was washed by water. On the second stage, Eshveiler-Clark reaction [36] was carried out: ethanol (85%), formic acid and formaldehyde (37%) were added to the final N-acetylated chitosan residue the CH_2O and HCOOH to primary NH_2 groups molar ratio being 2:2:1, and reaction mixture was heated 3 hours at 75°C as pointed earlier [37]. After that reaction mixture was left to reach room temperature, and was dialyzed exhaustively against 0.5% aq. acetic acid solution. Resulting N-ethylated (secondary) and N-dimethylated (tertiary) chitosan preparation was lyophilized and analyzed. Primary (40%), secondary (25%) and tertiary (20%) amino groups contents were measured by potentiometric titration. Characteristic viscosity was decreased from 492 $cm^3.g^{-1}$ (for initial chitosan) to 256 $cm^3.g^{-1}$ (for resulting mCHIT). Molecular mass of mCHIT is 60 kDa (gel filtration data).

2.4.3. pH-sensitive amphiphilic liposomes and lypoplex preparation

Liposomes were formed from a mixture containing egg yolk L-α-phosphatidyl choline (Sigma), (Fluka) (or α-tocopherol ester of succinic acid, Sigma) (9:1, mol. %) using reverse phase evaporation technique [27]. by the addition of lipid fraction ether solution to water (55°C, 1 ml/min), followed by exhaustive removal of organic solvent by evaporation under reduced pressure and *in vaccuo*. Nitrogen gas was passed through liposome suspension (conc. 2 mg/ml), liposomes prepared were stored at 4°C under nitrogen and used during three-week period. Plasmid DNA was mixed with liposome suspension (1:10, w/w) to form lipoplex, and magnesium chloride was added to lipoplex complex to reach final volume of 50 μL and Mg (II) ion concentration – of 50 mM.

2.4.4. Cell lines, transfection, and plasmid DNA

Human melanoma cell line (MeWo) and human tumor T-lymphocyte line (Jurcat) were obtained from ATCC bank. HeLa, human osteosarcoma (HOS-1) and human immortalzied premonocyte (U937) cell lines were kindly provided by Dr. T.I. Ponomareva (Institute of Agricultural Biotechnology, Moscow, Russia). HeLa, HOS-1 and human melanoma MeWo cells were grown at 37°C and 5% CO_2 in MEM (HyClone) medium suplemented with 10% fetal calf serum (HyClone), 2 mM L-glutamine, and 50 μg/ml of gentamycin. Immortalized premonocyte U937 and transformed lymphocyte Jurcat cells were grown at 37°C and 5% CO_2 in RPMI 1640 (HyClone) medium suplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 mcg/ml of gentamycin. Plasmid DNAs were finally purified by two cycles of centrigfugation in a CsCl gradient. The cells were transfected with plasmid DNA using a number of techniques: Ca $(PO_4)_x$ method [38], amphiphylic liposome- [28, 39, 40] and PEI- [41] mediated transfection. DNA concentration was estimated by measuring the absorbance at 260 nm, and horizontal agarose electrophoresis as well.

2.4.5. pEQ176 plasmid expression testing

Cells were washed with phosphate buffer solution and then fixed with 0.25% glutaraldehyde and 2% formaline solution for 5 mins at 4°C. Cells were covered *in situ* with coloured solution (5 mM yellow blood salt, 5 mM red blood salt, 2 mM $MgCl_2$ and 1% X-Gal indigogenic substrate), prepared in phosphate-salt buffer, after two-fold washing, and cells were incubated in the solution for 2 hrs. The expression level was observed by microscopic study counting blue-coloured cells, and calculated as a percentage of coloured cells from total amount of cells.

2.4.6. Testing of pCSEAP expression level in culture medium

Aliquotes of cultural medium (80 μL) harvested from cell monolayer 4 days after transfection were centrifugated 14,000 rpm, 2 min and heated at 65°C for inhibition of endogenic alkaline phosphatase activity. Equal volume of reactive buffer solution (0.5 M Na_2HCO_3, 0.5 mM $MgCl_2$; pH 9.8) was added to every aliquote, and the

mixture was incubated 10 min at 37°C. 50 μL of 60 mM p-nitrophenylphosphate solution (Sigma) (37°C) was added to every probe, and mixture was incubated for 20–30 min. Optical density at 405 nm was measured with rider "Titertek" (Flow).

3. RESULTS AND DISCUSSION

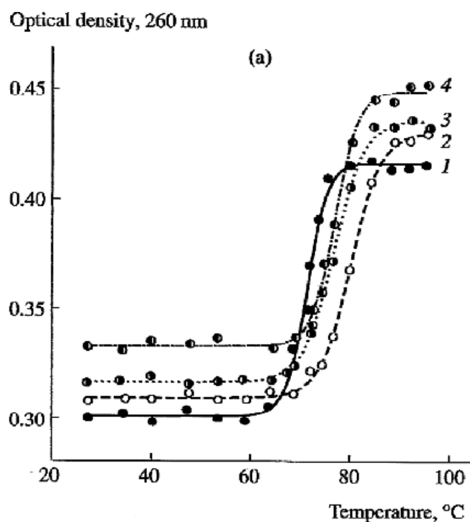
3.1. Cholenims

3.1.1. DNA encapsulation

To determine the relationship between the structure of cholenims and cholenim-based lipoplexes and their effectiveness in gene transfer, it was necessary to study the interaction between these compounds and nucleic acids, as well as their effect on DNA structure. For this purpose, we used the following physicochemical methods: fluorescence probes, spectrophotometry, circular dichroism spectroscopy, and electron microscopy. The hydrophilic moiety of cholenims includes the groups which are characteristic of the structure of natural polyamines spermine and spermidine, which exhibit affinity to and stabilize DNA helix [42], as well as polyethyleneimine, which display activity in gene transfer [41]. Due to complexity of the melting curves of plasmid DNA, we studied the effect of cholenims on the melting curves of genomic DNA.

Figure 1 (upper field) shows the melting curves of fragments of genomic DNA and its complexes with cholenims. Analysis of these curves showed that the complexes formed by DNA and compounds I, II, or III have a higher melting temperature (by 8, 5, and 4°C, respectively) compared to pure DNA fragments. Thus, these compounds stabilize the DNA helix, with their stabilizing effect decreasing in the following order: compound I > compound II > compound III. The affinity of colenims for the double helix of DNA is different due to different positive charges of their hydrophilic groups and different hydrophobicity/hydrophilicity ratios. Apparently, electrostatic interactions between the amino groups of compounds I and II and the negatively charged phosphate groups of the polynucleotide chain are important of stabilizing complexes. There is a good correlation between the ΔT_{melt} value and the charge of cholenim: the greater the charge, the greater the stabilizing effect (Table 1). Analysis of circular dichroism spectra of the pCMV-SPORT-β-Gal plasmid and its complexes with compounds I–III led us to conclude that they are practically identical and that these compounds do not affect the structure of double helix of DNA, which retains B-conformation (spectra not shown). As a fluorescent probe we used pyrene, whose oscillatory structure of emission spectra is highly sensitive to polarity of its microenvironment. Due to this property, pyrene is widely used in studies of membranes, micelles, and hydrophobic clusters [22, 23].

As seen from the results, the value of this ratio almost did not depend on the concentration of cholenim up to the threshold value; further increase in cholenim concentration results in a sharp increase in the I_3/I_1 ratio (in the absence of DNA, this parameter did not depend on the concentration of cholenims within the concentration range analyzed). These values, different for compounds I (6.0×10^{-5} M), II (8.6×10^{-5} M), and III



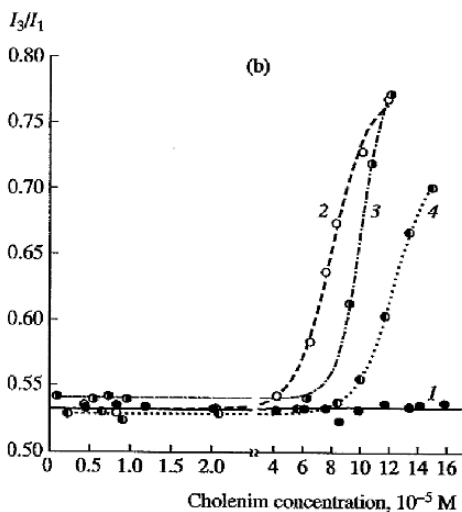


Figure 1. Physicochemical characteristics of the complexes (lipoplexes) DNA-CHOLENIMS. (a) UV melting curves of salmon roe DNA in the buffer containing 10 mM NaCl and 1 mM Tris-HCl (pH 7.2) *(1)* in the control and in the presence of *(2)* monocholenim, *(3)* dicholenim, and *(4)* tricholenim (1.0±0.2). 10^{-4} M. (b) Dependence of pyrene emission spectrum (the I_3/I_1 index) on the concentration of *(2)* monocholenim, *(3)* dicholenim, and *(4)* tricholenim. Curve *1* shows DNA spectrum in the absence of cholenims.

Designations: I_1 and I_3, amplitudes of oscillatory lines of emission spectra of monomeric pyrene at 383 and 372 nm, respectively, in the presence of salmon sperm DNA (45μM by phosphate)

Table 1. Properties of hydrophobic oligocation CHOLENIMS and their lipoplexes

Cholesterol derivatives	T melt., (°C)	ΔT	CMC, M	Charge*	EM, diameter (nm)	Transfection efficacy against PC-12 cells	
						DNA/ cholenim ratio	Picog protein per 10^5 cells
Monocholenim	80	+8	$6.0{*}10^{-5}$	+2	100–130	3:1	105
						2:1	187
						0,7:1	36
Dicholenim	77	+5	$8.6{*}10^{-5}$	+1	200–250	3:1	100
						2:1	113
						0,7:1	14
Tricholenim	76	+4	$1.0{*}10^{-4}$	0	300–340	3:1	56,5
						2:1	31,3
						0,7:1	7,5

Note: ΔT designates an increase in melting temperature of DNA samples in the complex with an oligocation; CMC, critical micelle concentration; EM, diameter of particles of the corresponding complexes with plasmid DNA or DNA fragments (electron microscopy data).
* Calculated for the amino groups at pH 7.0.

(1.0×10^{-4} M), correspond to formation of complexes between these compounds and DNA, which contain hydrophobic clusters where pyrene molecules are inserted, and may be regarded as critical micelle concentrations (CMC). There is a good correlation between the CMC value and the decrease in the total positive charge of polar groups of cholenims. Thus, it can be postulates that cholenims bind with DNA to form a hydrophobic coat around the helix, and that the disadvantageous (in terms of energy) contact between hydrophobic cholesterol residues with aqueous environment at certain concentration results in a decrease in solubility of complexes.

Electron-microscopic study showed that the complexes between genomic DNA and the plasmid with cholenims represent spherical particles with a diameter of 100 to 300 nm. Condensation of 4–6 kb DNA fragments and compound II showed that the size of particles significantly varies. The fact that the size of DNA/cholenim particles is large and almost does not depend on the molecular weight of DNA is unusual for a simple micellar structure. Figure 1 (lower field) shows the dependence of the spectral parameter I_3/I_1, which is the most sensitive to hydrophobicity of microenvironment, on the concentration of cholenims at a constant DNA concentration (I_1 and I_3 are the amplitudes of oscillatory lines of emission spectra of the monomeric form of pyrene at 383 and 372 nm).

3.1.2. Gene transfer and delivery

The results of transfection of PC-12 cells with the complexes containing the pCMV-SPORT-β-Gal plasmid and cholenims are summarized in Table 1. The greatest effectiveness of transfection of PC-12 cells was reached when DNA/cholenim

genosomes were used at a ratio of 2:1. However, significant effectiveness of transfection was also observed at DNA/cholenim ratio of 3:1. Similar results were obtained for the dicholenim-based complex. The effectiveness of transfection in the case of DNA/dicholenim genosomes at ratios of 2:1 and 3:1 was considerably higher than at the ratio 0.7:1 and comparable with the effectiveness of transfection for the DNA/cholenim ratio at the ratio 3:1. Tricholenim was much less effective in gene delivery compared to the other two compounds. In this case, the effectiveness of transfection markedly decreased as the proportion of tricholenim in genosomes increased. The effectiveness of transfection of RGGN-1 cells was 30 and 32 pg protein per 10^5 cells for the DNA/monocholenim complex and 14 and 23 pg protein per 10^5 cells for the DNA/dicholenim complex (ratio, 2:1 and 1:1, respectively). Although this index for RGGN-1 cells in general was considerably lower compared to the effectiveness of transfection of PC-12 cells, this finding also supports the fact that monocholenim and dicholenim may be used as gene carriers *in vitro*. However, it should be noted that, in the case of *in vivo* transfection, there might be another relationship between the effectiveness of gene transfer and qualitative and quantitative composition of cholenim-based complexes. Amphiphilic liposomes consisting of phosphatidylcholine and dicholenim at the ratio 1:1 (w/w) were used to transfer the β-galactosidase gene using intravenous injection at the lipid/DNA ratio 1.6:1 (w/w). Sections of organs were incubated with the substrate X-Gal, which in the presence of β-galactosidase is degraded, yielding the bright blue dye indigo. In preparations analyzed, the reporter DNA was expressed predominantly in endothelial cells of pulmonary vessels and in neighboring cells, which provides evidence that vascular endothelial cells are permeable for our complexes (Figure 2).

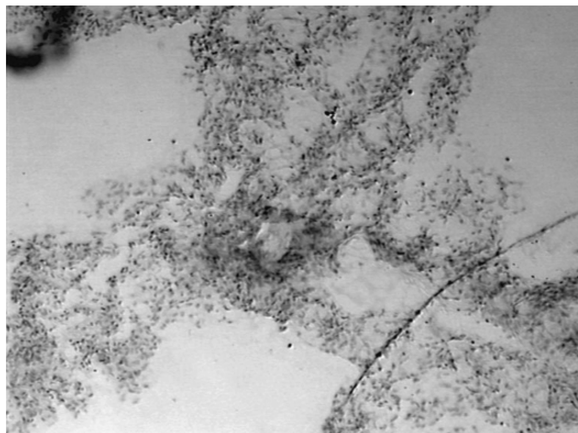


Figure 2. Histochemical preparation of ICR mouse lung after injection into the portal vein of the liver of lipoplexes formed by the pCMV-SPORT-β-Gal plasmid and liposomes PC/DICHOLENIM (1:1). Staining around the blood vessel is the result of degradation of the substrate X-Gal by bacterial β-galactosidase. Magnification, 200; computer processing; AXIOSKOP 20 Carl Zeiss

This distribution pattern is characteristic of cationic liposomes injected intravenously. Thus, the introduction of the cholesterol fragment into the structure of oligoethylene imines improves the characteristics of the corresponding complexes: increases the hydrophobicity/hydrophilicity ratio, stabilizes the lipoplex, and ensures optimal CMC values. Our data confirm the existence of stable DNA/cholenim complexes and electrostatic interaction in them of positively charged groups with negatively charged phosphate groups of DNA, with the deoxyribose phosphate backbone being apparently involved in the stabilization of genosomes. Compounds I–III interact with DNA to form a hydrophobic coat around its double helix. The high effectiveness of DNA/cholenim lipoplexes in gene transfer *in vitro* is probably determined by their complete dissociation in the cytosol before the nuclear membrane, because this ability of lipoplexes is a key characteristic required for transfection [43].

3.2. Glycoclip

3.2.1. Cyto- and genotoxicity

Potential cyto- and genotoxicity of GLYCOCLIP/DOPE and CLIP/PC liposomes were estimated in experiments with a cultured glyal cell line [44], which is very sensitive to any influence, as described earlier [4]. The influence of the former liposomal preparation on the growth (24hrs) of RGNN cells and the DNA synthesis in these cells was studied. The preparations have almost no effect on cell growth at both concentrations used: 6μg/ml (number of cells survived after 24 hrs incubation was 110.5 +/−3.1% (M+/−s) comparing to the control one) and 60μg/ml (98.6+/−9.3%). The influence on DNA synthesis was evaluated as the extent of incorporation of ^{14}C-thymidine into RGNN cell genomic DNA. It had equally essential effect on the DNA synthesis at both concentrations (6 or 60 μg/ml): the values of the DNA synthesis were 58.5+/−7.8% and 66.3+/−9.2% comparing to the control ones, correspondingly. The influence of CLIP liposomes on DNA synthesis in RGGN cells was not so pronounced (in the range of experimental error), as found for the GLYCOCLIP ones. The CLIP/PC liposomal preparation has no effect on either the cell survival, or the DNA synthesis in RGGN cells at 6μg/ml level. The number of cells survived after 24 hrs incubation was 100.0 +/−5.0% comparing to the control, and the value of the DNA synthesis in the cells was 101.8 +/−7.0% comparing to the control one. Only 10-fold dose of CLIP/PC liposomes (60μg/ml) had an effect on the DNA synthesis: 55.5 +/−11.7% ($p < 0.05$) comparing to the control one. CHOLENIM preparation itself appeared to be completely non-toxic at the range of concentrations used [18].

3.2.2. Gene encapsulation and delivery in vitro

Gene transfer activity of the liposomes based on GLYCOCLIP was studied with the commonly used reporter gene transfer system: transfection of pCMV-Luc plasmid into CHO cells followed by gene transfer efficiency testing using luminometer assay [45]. Figure 3 represents data on reporter gene (pLuc) transfer efficiency with liposomal preparations of compounds I and II into CHO cells in comparison with

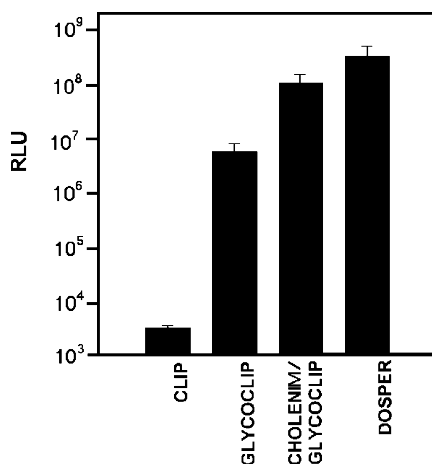


Figure 3. Transfection efficiency of lipoplex and glycolipoplex preparations formed of various cationic lipids, GLYCOCLIP and pCMV-Luc reporter plasmid against CHO cells: 1-CHOLENIM I/GLYCOCLIP V; 2-CLIP IV; 3-GLYCOCLIP VI; 4-Lipofectin; 5-Dosper

corresponding data for commercial gene transfer agent DOSPER. As follows from the results the introduction of a triacetyl-glucose moiety into the structure of a cationic lipid enhances remarkably the transfection: RLU value of GLYCOCLIP/DOPE liposomes equals to 7.10^6 (compare GLYCOCLIP/DOPE and CLIP/PC values).

The GLYCOCLIP-based liposomes' RLU values are only slightly less than those of DOSPER mediated gene transfer. Our data on the inhibition of DNA synthesis in RGGN cells after 24hrs incubation with GLYCOCLIP/DOPE liposomes corresponds to the data testifying to the toxicity of many cationic liposomes during *in vitro* experiments [14]. However, it was demonstrated that the efficiency of gene transfer with cationic liposomes is not directly connected with the degree of their toxicity [46], so one may get high transfection efficiency with the use of gene transfer agents demonstrating a certain toxicity *in vitro*. It is possible that lowering the concentration of GLYCOCLIP/DOPE liposomes used for transfection will help to avoid their influence on DNA synthesis. It cannot be excluded that this effect will not appear during *in vivo* transfection. The fact that GLYCOCLIP/DOPE liposomes don't influence the cell growth at least during the first 24hrs is also promising. Thus, partial glyconylation of polylysine has been shown to increase the efficiency of transfection with its participation, and the conjugation of modified polylysine with a few lactose moieties causes appearance of genosome's specificity to cell surface lectin [47, 48]. A series of amphiphilic dendritic galactosides were synthesized to be used for selective targeting of liposomes to the hepatic asialoglycoprotein receptor [49]. Introduction of carbohydrate moieties into the structures involved in lipoplex formation increases the efficacy and the specificity (hepatocytes) of transfection. Lipoplexes composed of galactosylated peptides demonstrate tropicity to hepatocytes [50].

DOPE and PC represent helper lipids, which enhance transfection efficiency being included into liposomes and lipoplex composition [24,51]. The presence of a helper lipid and the difference between the helper lipids (DOPE or PC) in GLYCOCLIP and CLIP liposomal formulations used can give no strong influence on the gene transfer efficiency in the case of CHO cells, because of the endocytotic way of the lipoplex internalization into this cell line [51]. Therefore the enhanced transfection efficiency of GLYCOCLIP liposomes compare to CLIP liposomes can be explained by the presence of carbohydrate (glucose) moiety in the first one. Introduction of CHOLENIM preparation into glycolipoplex composition facilitates the elaboration of DNA from a lipoplex in perinuclear space. That is the main reason for increasing transfection efficiency of GLYCOCLIP/ CHOLENIM/DOPE liposomes comparing to GLYCOCLIP/DOPE ones. Another reason is the higher value (3.2) of $+/-$ ratio. It appeares that mechanism of gene transfer with the glycolipoplex includes both adsorbic endocytosis usual for lipoplex formulations, and receptor-mediated gene transfer characteristic for carbohydrate ligand-mediated gene transfer. Our results represent one of the first examples of the use of a cationic glycolipid, its liposomal formulations, and genosomes/lipoplexes composed of GLYCOCLIP as gene transfer agents. We believe that glycocationic lipids of this type will be effective especially for *in vivo* studies due to the affinity of carbohydrate structures to the cell surface and the vessell's endothelium as well.

3.3. Glycolipid

3.3.1. Gene delivery in vivo

The first stage in the study of the effectiveness of gene transfer using liposomes containing phosphatidylcholine and GLYCOLIPID VI included the determination of the pattern of distribution of ^{14}C-adenosine-labeled eukaryotic DNA in mouse organs. The maximal DNA level (recalculated per gram tissue) was detected in the kidneys (6000–8000 cpm per gram) and liver (4000 cpm per gram). Note that the content of ^{14}C–DNA in the liver was three times greater than in the lungs (Figure 4). It is known that intravenous injections of the complexes of cationic liposomes with DNA are usually characterized by "the effect of the first passage," i.e., the majority of injected liposome with bloodstream get from the heart to the lungs [52]. When using liposomes containing GLYCOLIPID VI, this effect was not observed. In our experiments, we observed certain affinity of the complex of these ^{14}C-DNA-containing liposomes for the liver and kidneys.

The maximal level of ^{14}C-DNA in the kidneys is probably due to the fact that it might have been eliminated as early as 24 h after injection, because kidneys are excretory organs. Then, we studied the expression of the β–galactosidase gene in mouse organs in the case of delivery of the pCMV-SPORT-β-Gal plasmid (100 μg) in the complex with mixed liposomes consisting of phosphatidylcholine, GLYCOLIPID, and dicholenim (160 μg).

When this lipoplex was injected into the portal vein, the *Lac Z* gene was expressed predominantly in hepatocytes. However, despite the presence on the surface of

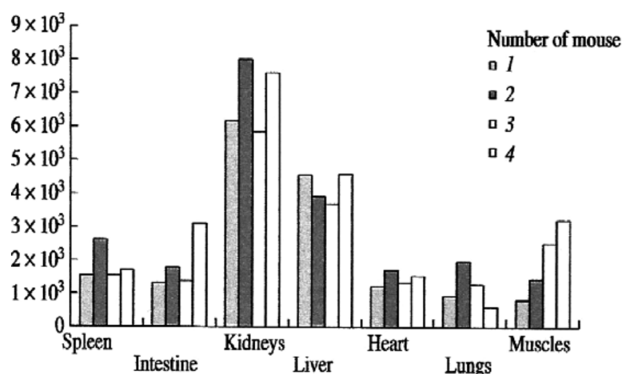


Figure 4. Distribution of the lipoplex formed by ^{14}C-adenosine-labeled DNA and liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim in mouse organs after injection into the portal vein of the liver (cpm/min per gram organ; $n = 4$)

liposomes of a lactose residue, which exhibits affinity for the lectin located on the surface of hepatocytes, the degree of expression was low, and expression was observed mostly in epithelium of blood vessels and in the immediate vicinity of them. This fact is indicative of a low permeability of tissues for such complexes. A more long-term incubation with the substrate led to appearance of the dye indigo in the form of small (less than 1 μm) bright blue granules both in the control and experimental liver section. It can be assumed that this phenomenon may be accounted for by location of the endogenous enzyme in lysosomes or other compartments of the cytoplasm of hepatocytes. In the lungs and spleen, the level of expression of the *Lac Z* gene (reaction with X-Gal) was high (Figure 5).

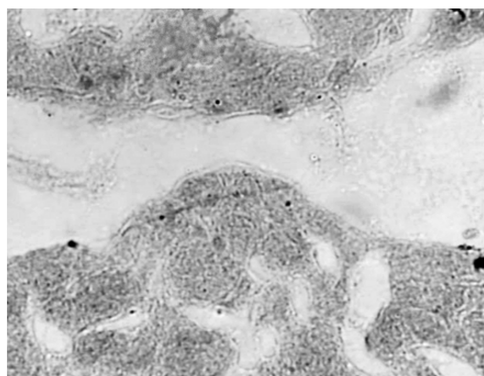


Figure 5. Histochemical assessment of expression of the *LacZ* gene (the pCMV-SPORT-β-Gal plasmid) in the ICR mouse spleen after injection of the lipoplex based on the pCMV-SPORT-β-Gal plasmid and liposomes composed of phosphatidylcholine/GLYCOLIPID/dicholenim (ratio 1:1.6, w/w) into the portal vein of the liver. Dark areas indicate the sites of the highest expression (magnification ×200)

A high endogenous activity of β-galactosidase was detected in the kidneys, which hampered the assessment of the effectiveness of the exogenous enzyme. For quantitative estimation of expression of the β-galactosidase gene in mouse organs after injecting the complex of the plasmid with the liposomes consisting of phosphatidylcholine, lactosolipid, and dicholenim, the activity of this enzyme in tissues was determined spectrophotometrically.

The maximal activity of the enzyme was observed in the spleen (data are not shown), and equal activity was detected in the lungs and liver. A high level of endogenous activity of β-galactosidase in some organs hampers quantitative assessment of expression. Thus, the results of this study showed that GLYCOLIPID VI containing a lactose residue, which was used in the form of liposomes to transfer ^{14}C-adenosine-labeled or plasmid DNA, determined the affinity of lipoplexes for kidney, liver, and spleen tissues. The effect of the first passage, characteristic of cationic complexes, was not observed when ^{14}C-DNA was injected in the complex with liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim, was considerably decreased when the plasmid was injected in the complex with liposomes comprised of phosphatidylcholine, lactosolipid, and dicholenim. In the last case, the expression of β-galactosidase was maximum in the spleen. GLYCOLIPID VI, which determines the affinity of lipoplexes for tissues, as well as glycolipids on the whole, is a prospective tool for designing on its basis of nonviral vectors of a new generation for targeted gene delivery to tissues.

3.4. Modified Chitosan (mCHIT)

We used natural polycationic polysaccharide, chitosan, which can usually be prepared by deacetylation of chitin – linear poly– (N-acetyl-glucosamine) to gene transfer against cultured cell lines. Chitosan macromolecule represents linear polymer of glucosamine, part of whose primary amino-groups (normally 5–20%) are still acetylated. It is well–known that chitosan being one of the most widespead biomass represents non-toxic, biocompatible biopolymer [53, 54], which is suitable for gene delivery purpose [14–16],[55–57]. However, in our preliminary study we also worked to get reporter gene transfer of transformed cells using non-modified chitosan preparations. After the data on efficient transfection of 3T3 and HepG2 cells with complexes of plasmid DNA and polyethylenimine (PEI) were published [41], Dr. G.G. Krivtsov decided to introduce the secondary and tertiary aminogroups into chitosan structure to use modified chitosan preparations (mCHIT) for gene transfer. The matter is that PEI contains the secondary amino groups along with the primary and tertiary ones. He synthesized the chitosan preparation, containing N-ethyl- (the secondary one) and N,N-dimethyl amino (the tertiary one) groups to facilitate ionic interaction of chitosan with the DNA and to increase transfection efficiency against different transformed cell lines, especially the suspension blood cell ones. The latter topic is an acquit area of research now, and is also very important for development of non-viral delivery systems for *ex vivo* gene therapy of variety of genetic diseases and cancer pathologies [58–60].

A number of papers on the usage of different chitosan preparations and nanospheres as transfection agents have been published [14–16],[55–57]. It was reported that unmodified chitosan has ability to condense DNA and form small discrete particles [57]. They can transfect HeLa cells (β-gal [14] or Luc [57] genes) independently of the presence of 10% fetal serum. Gene expression gradually increased with time, being at 96 hours 10 times more efficient, than polyethylenimine [57]. It was suggested that non-ionic interactions between chitosan macromolecule and cell surface might play an important role in chitosan-mediated transfection [56]. pH-sensitive endosomolytic peptide enhanced gene expression in COS-1 cells by factor 4, but during in vivo experiments on rabbits (intestine and colon) gene expression appeared to be still low [15]. Hydophobically modified chitosan (containing five deoxycholic acyl moieties per 100 anhydroglucose units) was prepared, its aggregates being 162 $+/-18$ nm in diameter [14]. Transfection of COS-1 cells using self-aggregates/plasmid DNA complexes at $+/-$ charge ratio 4 was reported. Nanospheres composed of cDNA and gelatin or chitosan (200–750 nm) were used for in vitro transfection, efficiency being lower than in the case of lipofectamine-mediated and Ca–phosphate ones [55]. Method for oral DNA delivery with N-acetylated chitosan was reported [16].

All groups that have been working with chitosan preparations as gene delivery systems used non-N-alkylated chitosan samples containing only primary aminogroups along with N-acetyl moiety. These preparations usually represent particles of small size (80 nm) as measured by variety of techniques [14–16],[55–57]. Chitosan preparation hydrophobized with deoxycholeic acyl moieties (5%) forms self-aggregates of medium size (200 nm) itself. Nanosheres formed of chitosan are even bigger: 200–700 nm [14, 15],[55–57]. These chitosan preparations are characterized with ability to form DNA aggergates with supercoiled plasmid like cationic liposomes and other polycations usually do [39]. The size of these aggregates is even bigger. All known chitosan preparations tested for gene delivery in vitro and/or in vivo are far from being as effective as any commercial gene transfer ones, e.g. PEI™. We usually obtained low transfection efficiency values with non-modified chitosan preparations. The reason for these, by our opinion, is unsufficient ability of polysaccharide bearing only primary glucosamine moieties and forming big aggregates to be as stable as to survive in endosome-lyzosomal complex. There are very few reasons to add any hydrophobic moieties (like choleic acid) into glucosamine residue, as chitosan biomacromolecule having well-known hydrophobic properties is able to bind 10-fold amount (w/w) of fat molecules [61].

Transfection was carried out with two various reporter gene plasmids: pEQ176 (β-galactosidase) and pCSEAP (secreted alkaline phosphatase) (under IE CMV promoters) against transformed cell lines with different ethiology: three adherent cell lines (MeWo, HeLa, and HOS-1) and two suspension cell cultures (U937 and Jurkat) as well. Transformed blood cell lines had been cultured by conventional methods. A number of transfection techniques (Ca-phosphate; pH-sensitive amphiphylic liposomes/Ca^{2+}- and PEI-mediated gene transfer) were used for comparing results of mCHIT glycoplex transfection. Glycoplex composition was choosen with mCHIT

nitrogen/DNA phosphorus ratio equalling to 10:1 which corresponds to +/− charge ratio 8. At other ratio values we got a decrease of efficacy by decreasing the ratio, and an increase of toxicity by increasing the ratio in the case of both mCHIT and PEI (data are not shown).

It follows from data on efficiency of transfection of pEQ176 plasmid into selected transformed cell lines, that mCHIT and PEI preparations demonstrated maximum transfection activity (up to 100%) for human melanoma cell line (MeWo). However, gene transfer efficacy appeared to be lower for HeLa and HOS-1 cell lines: from 2 to 5% of bacterial β-gal gene expressed cells, which is in the connection with the results of liposomes/Ca ions-mediated [28, 39] and Ca-phosphate transfection method [38]. Gene transfer activity of mCHIT preparation against immortalized premonocytes (U937) (10% of cells are expressing bacterial β-galactosidase gene) was higher than PEI activity by factor 10. mCHIT demonstrated also the ability to transfect transformed lymphocyte cell line (Jurkat), which is very difficult to be transfected, 10 fold higher then PEI (0.01% and 0.001% β-gal expressing cells, correspondingly).

The similar results were obtained in the case of transfection experiments with another plasmid, pCSEAP, containing secreted alkaline phosphatase gene with one exception. We were not able to detect expression of SEAP gene after transfection with Ca-phosphate precipitates. PEI and mCHIT preparations showed the same level of SEAP gene transfer activity against adherent cell cultures. Lowest level of transfection was found for HeLa cells, twice higher - for HOS-1 cells, and 8 fold higher – for melanoma cells MeWo. Ca-phosphate precipitate transfection demonstrated the same level of gene transfer efficiency for all adherent cell lines, as mCHIT and PEI-mediated showed in the case of HeLa cells. Amphiphilic PC liposomes in the presence of Ca ions (>15 mM) [28, 39] were active only in the case of MeWo, but twice more effective than Ca-phosphate technique. Glycoplex preparation was twice more effective against U937 cells higher than PEI. pH-sensitive PC liposomes/Ca^{2+} also showed sufficient transfection in the case of U937 cells (6 fold lower than mCHIT).

Remarkable gene transfer properties of mCHIT glycoplex preparation, which contains secondary and tertiary amino groups, compare to PEI (one of the most powerful gene transfer agent now) appear to be connected, first, with enhanced endocytosis of glycoplex particles through mono- and lymphocyte cytoplasmatic membrane (probably, receptor-mediated transfer). N-acetylglucosamine residues (N-AGA), which are normally present in any commercial chitosan preparation, can be considered as the most probable candidate for a ligand in receptor-mediated endocytosis. Corresponding fraction of immunoglobulins was found in patient's blood. Those proteins are also exposed on cytoplasmatic membrane, their nature being different for various cell types. Second, mCHIT bearing secondary and tertiary amino groups and being higher positively charged can form more tough and stable complexes permitting plasmid DNA to survive through endosome-lyzosome complexation [43]. Third, mCHIT preparations, being not so highly positively charged as quarternary cationic lipids, provide the type of DNA complexation with

mCHIT which resembles the interaction of DNA with PEI and facilitate an easy escape of DNA from the complex at nuclear membrane or/and perinuclear space to be transcribed in the nuclei [62].

The mCHIT preparation demonstrated the highest gene transfer activity for all types of cells used and for both of β-Gal and CSEAP plasmids. It appears that the data obtained reflect a difference in value and structural homogenity of negative potential/charge of cytoplasmatic membrane of transformed cells of different tissue genesis. This issue can be supported by transfection efficiency data for two suspension cultures of white blood cells. The most important result we got is the comparatively high efficiency of transfection of suspension cell lines, especially for Jurkat transformed lymphocyte cell line, which is usually very difficult to be transfected with any delivery sytem.

Gene transfer with amphiphilic liposomes containing pH-sensitive agent α-tocopherol ester of succinic acid and complexed with plasmid DNA in the presence of high concentration of Me (II) ions (20 mM Ca ions and higher concentrations) [28,39,40] appeared to be even more active than Ca-phosphate precipitate technique. The former one is promising for targeted delivery in combination with the use of addressing groups. Reporter genes can be easily substituted in GLYCOPLEX by therapeutic genes, e.g. suicide genes, ADA gene, because of still big size (up to 8–10 kb) for the purpose of *ex vivo* gene therapy.

4. CONCLUSIONS

Introducing the cholesterol moiety into the structure of oligoethylene imines improves the characteristics of the corresponding complexes: increases the hydrophobicity/hydrophilicity ratio, stabilizes the lipoplex, and ensures optimal CMC values. The existence of stable DNA/CHOLENIM complexes and electrostatic interaction of positively charged groups with negatively charged phosphate groups of DNA are confirmed, the deoxyribose phosphate backbone being apparently involved in the stabilization of genosomes. CHOLENIMS interact with DNA to form a hydrophobic coat around its double helix. CHOLENIM-based lipoplex provides reporter DNA retard circulation in blood. Mono-, di-, and tri CHOLENIMS-based lipoplexes are characterized by various tissue distributions in animal experiments.

The enhanced transfection efficiency of GLYCOCLIP V liposomes compare to CLIP liposomes can be explained by the presence of carbohydrate (glucose) moiety in the first one. Introduction of CHOLENIM preparation (as helper lipid) into glycolipoplex composition facilitates the elaboration of DNA from a lipoplex in perinuclear space. It appeares that mechanism of gene transfer with the glycolipoplex includes both adsorbic endocytosis usual for lipoplex formulations, and receptor-mediated gene transfer characteristic for carbohydrate ligand-mediated gene transfer. We believe that glycocationic lipids of this type will be effective especially for *in vivo* studies due to the affinity of carbohydrate structures to the cell surface and the vessell’s endothelium as well.

It is shown that GLYCOLIPID VI containing a lactose residue, which was used to form liposomes for gene delivery into tissues of ^{14}C-adenosine-labeled or plasmid DNA, expessed the affinity of corresponding lipoplexes for kidney, liver, and spleen tissues. GLYCOLIPID VI is a prospective tool for designing on its basis of nonviral vectors of a new generation for targeted gene delivery to tissues. The mCHIT preparation demonstrated high gene transfer activity (β-Gal and CSEAP plasmids) against both monolayer and suspension cell lines.

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CHAPTER 3

ARTIFICIAL IMPLANTS – NEW DEVELOPMENTS AND ASSOCIATED PROBLEMS

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Abstract: Implanted short-term and long-term medical devices have been exhibiting extreme promises in promoting quality of life while increasing life expectancy of affected individuals. The risk of bacterial infections associated with open surgery or the implementation of these devices remains to be a major drawback. The primary causes of infections associated with medical devices are Staphylococcus epidermidis and Staphylococcus aureus. The two potential interventions to bacterial infections associated with medical devices include the development of materials that could discourage bacterial adherence and exhibit antimicrobial activity. The preventional methods ranged from the development of anti adhesive polymers comprising the implant to impregnating implant cements with antibiotic devices that extend the therapeutic response due to slow release effect. New areas of implant research include the use of liposomal antibiotics as coatings for implants. In this communication, we will review the chemical nature of commonly used implants, the source of infections, as well as the preventional measures of coatings and the antibiotics employed to reduce infection due to different implants and medical devices

Keywords: artificial implants, infections, bacteria, antibacterial, anti-adhesion, antibiotics, polymers, IRI, implant coating, biomaterials, bone cements

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1. INTRODUCTION

The integration of artificial implants in biological environments is an outstanding advancement in medicine that allows for increased mobility, improved sight, as well as enhanced delivery of food and drugs. Although this vast range of artificial implants can improve the quality of life by restoring compromised physiological functions, they may also carry such health risks as biocompatibility and microbial infections that impede a successful implantation.

Microorganisms may cause device-related infections by: a) colonizing the implant through direct inoculation at the time of implantation; b) reaching the implants by hematogenous seeding during bacteremia or; c) through direct continuous spreading from an adjacent infectious focus. Infections caused by Staphylococcus epidermidis and S. aureus are more common, making up some 70–90% of the implant related infections [1]. Some serious complications of implant-related infections include: abscesses, endocarditis and septicemia [2]. Infections caused by these bacteria generally are preceded by protein adsorption [2] onto the surface of implants and the resultant "film" formation that supports bacterial adherence and colonization.

Aseptic techniques and decontamination of the surgical site are common prophylactic approaches to infection. In addition, a relatively new approach to reduce the risk of microbial infection and inflammation due to an artificial implant involves the coating of the implants with free- or encapsulated- antibiotics in lipids (i.e. liposomes) or polymers. Such alterations in implant composition should preserve the implant integrity while allowing its integration into the host system and diminishing adverse reactions.

In the following paragraphs, we will review recent developments in several medical implants that have had profound impact on modern medicine. We will also elaborate on the potential bacterial contaminations of particular implants and the new approaches to address the infection and inflammation problems. The specific implants that will be dealt with include dental implants, catheters, stents, orthopedic implants, intraocular lenses, as well as skin grafting. Finally, we will briefly discuss the implications of respiratory and cardiac implants and related complications.

2. DENTAL IMPLANTS

Dental implants provide a restorative tool to support crowns, bridge abutments, and removable dentures. Osseointegrated implants are titanium posts that are surgically implanted in alveolar bone. A tight immobile bond (osseointegration) forms between bone and titanium, and prosthetic and restorative fixtures are attached to the implants. Titanium implants differ from natural teeth, which may make them more susceptible to mechanical stress. Small proportions of implants are not successful and may fail due to infection. Bacterial adhesion on titanium implant surfaces has a strong influence on healing and long-term outcome of dental implants. Reducing the risk of infection is particularly more important and often more difficult to accomplish because the mouth is exposed to many unsanitary conditions. Two of

the most common sources of infection in dental implants are Streptococcus mutans and Streptococcus sangus [3]. Streptococci and Actinomyces species appear to be the initial colonizers of artificial dental implants and plaque formation. Attachment of these microbes, in turn, encourages other anaerobic bacteria including Fusobacterium, Capnocytophaga, and Prevotella to invade and colonize dental implants resulting in periodontitis [3].

Dental implants are available in different shapes and materials with diverse surface characteristics to enhance their clinical performances. For instance, titanium implants appear to resist the adhesion of the primary colonizers Streptococcus mutans and Streptococcus sangus. Modification of titanium implant surfaces by titanium nitride (TiN) or zirconium nitride (ZrN) coatings may further reduce bacterial adherence and improve their clinical performance [3]. Studies on the effect of different surface treatments of titanium implants employed in oral surgery emphasized the importance of interactions between microbes and implants. For example, highly polished titanium surfaces tend to discourage bacterial adhesion [4] but their usefulness is restricted because the polished neck of dental implants does not osseointegrate as do textured surfaces. Likewise, titanium implants coated with a hard ceramic resulted in a moderate reduction in plaque formation [5]. An implant with titanium zirconium-oxide on the ondosseous section with titanium-niobium-oxinitride covering the supragingival area indicated antimicrobial and anti-adhesion properties while was very resistant to wear [6]. Generally speaking, titanium alloys appear to be more effective on inhibiting plaque formation because they hide the highly reactive surface of the titanium.

The role of antibiotics in reducing dental implant related infections have been investigated as well and it was found that Tetracycline (TC) is an effective and widely used antimicrobial agent against periodontal infections for several reasons. These include: i) TC has the ability to delay plaque formation and to reach and react towards root surface bacteria; and ii) TC exhibits anti-collagenase activity, hence works against a wide variety of periodontal bacteria [7]. The antimicrobial effects of antibiotics impregnated into a polyurethane dental implant have been reported against Porphyromonas gingivalis. The antibiotic is released and starts working as soon as the bacterial enzyme begins degrading the implant. The use of biodegradable polymers such as poly (-hydroxybutyrate-co-hydroxyvalerate) PHBV and PVA (polyvinyl alcohol) incorporated TC are more attractive because they negate the necessity for a second surgery to remove the capsules or sphere. Although considerable advances have been made to improve the applications of dental implants in the context of bacterial infection, more research is needed to effectively reduce or even eliminate bacterial infections associated with these medical devices.

3. CATHETERS

Catheters are used in a wide range of applications varying from urinary catheters implanted for relatively short periods to venous catheters that are permanent at times. As with all medical implants, one of the major complications is microbial

infections that result from bacterial adhesion to the catheters. More than 150 million venous catheters are utilized every year in the USA alone, with a contamination rate of approximately 4% [8]. Catheter-related infections of the venous system are often referred to as CRBIs (catheter related bloodstream infections). Majority of CRBIs are caused by the organisms that colonize the skin (70–90%). These bacteria are primarily responsible for short-term infections. Long-term infections (those persisting for longer than 8 days), however, are primarily caused by the bacteria of the lumen where the catheter is implanted. As with many implants, the most common bacteria responsible for catheter-related infections are Staphylococcus aureus and Staphylococcus epidermidis. The initial bacterial adhesion to the surfaces of implants is generally directed by van der Waals forces, electrostatic interactions, and by hydrophobic interactions between bacterial membrane components and biomaterial surfaces [9, 10]. Bacteria can also adhere to catheter surface more strongly by methods other than the ones indicated above. For example, S. aureus and S. epidermidis express adhesin receptors that strongly bind to the glycoproteins, collagen, or laminin of the extracellular matrix surrounding the implant [11]. The stronger binding of S. aureus to the extracellular matrix materials surrounding the implants is attributed to the expression of more adhesin receptors compared to that of S. epidermidis [12].

There are two main strategies aimed at preventing catheter-related infections. One is the creation of anti-adhesive biomaterials and the other is the incorporation of antimicrobial or antiseptic agents into the polymer matrix. Of the materials used for catheter construction, plastic catheters have a higher rate of infection than the steel [13]. Common plastic materials used in catheters are polyvinyl chloride (PVC), Teflon, siliconized latex, poly urethane, and Vialon. Studies indicated that PVC and siliconized latex show significant bacterial adhesion, while polyurethane exhibits the best anti-adhesive properties [14, 15]. Teflon coating on catheters have been shown to reduce bacterial colonization, but one problem with Teflon is that it doesn't stick well to the polyurethane, a common composite of catheters [16]. It is also shown that implant matrices containing heparin or polyurethane oxide have better anti-bacterial adhesive properties [17]. Like wise, the use of a heparin coating, when attached to the IV catheter via benzalkonium chloride, proved very effective as an anti-bacterial adhesion agent [18]. Silver/collagen cuffs were also proposed as a coating for central venous catheters, but the research showed no reduction in the incidence of infection [19]. Although silver is a good antibacterial agent, serum components such as albumin renders it inactive by binding and precipitating it. A catheter coating composed of exidine and silver sulfadiazine, however, reduces short-term venous infection [20]. A possible explanation is that the silver compounds resist or reduce the precipitation of silver by serum proteins.

Other coatings used to reduce catheter infections include steryl polyethylene oxide-co-4,4'-methylene diphenyl diisocyanate-co-steryl polyethylene oxide (MSPEO) and chitosan, both of which are bioabsorbable and bacteriostatic. MSPEO works well against bacteria because it does not adsorb plasma components due to its steric repulsion, but it has problems forming stable attachment on implant surfaces.

Chitosan, on the other hand, attaches well to catheter materials and can tightly be incorporated with bacterial cell wall, but is slightly haemostatic [7]. Combination of the two products referred to as chi-MSPEO, however, proved to be a less toxic and effective anti-bacterial coating that adheres well to polyurethane catheters [21]. Thrombosis, a major concern associated with catheterization of the venous system, was absent in the studies using this mixture.

Antibiotics coated catheters have been investigated in catheter related infections. This is an attractive approach because of their expected rapid and local antibacterial effects. However, this approach is often problematic because the antimicrobial drugs elude from the catheter too quickly, hence do not exhibit prolonged bacterial inhibition. To address this problem, tridodecylmethylammonium chloride (TDMAC), a cationic surfactant, was used to coat the catheter and was shown to greatly increase retention of anionic antibiotics [22]. In this study, several antibiotics and antimicrobial agents including cefazolin, teicoplanin, cancomycin, silver, chlorhexidine-silver sulfadiazine (C-SS) and minocycline-rifampin (M-R), were investigated for their ability to inhibit bacterial colonization on these catheters. The data indicated that cefazolin conjugated to catheter with TDMAC and C-SS showed the lowest amount of colonization (2.1% and 2% respectively). The highest degree of colonization was seen in silver impregnated catheters (45.1%) and vancomycin conjugated with TDMAC (62%). A significant advantage of C-SS and M-R coated catheters is that they do not evoke antimicrobial resistance in bacteria [23, 24]. Hence, the Hospital Infection Control Practices advisory committee recommended the short-term use of these catheters [25].

Several investigators have also explored application of liposomal antibiotics in prevention of catheter-associated bacterial infections [26]. Application of ciprofloxacin encapsulated in DPPC-PEG-DSPE (Dipalmitoyl phosphatidylcholine – polyethylene glycol – distearoyl phosphatidyl ethanolamine) – gelatin liposome formulation on a silicon catheter completely eliminated bacterial adhesion and effectively inhibited the growth of Pseudomonas aeruginosa [26]. The liposomal antibiotic coating showed a slow but constant antibiotic release over a 94 hour time period. The hydrogel that shielded liposomes during insertion was composed of gelatin nitrophenyl carbonate activated PEG. Likewise, application of rifampicin entrapped in a PDMS-based polyurethane (PU) grafted with monomethoxy polyethylene glycol (MPEG) minimized catheters-associated urinary tract infections. The data indicated a great repulsion of E. coli and S. epidermidis adherence. The drug release kinetics showed a gradual release of rifampicin from the PU-MPEG coatings for 45 days. This slow release of the antibiotic retains an adequate concentration of the drug at the sites of infection and eliminates the need for the frequent systemic antibiotic therapy and reduces drug toxicity as well [27].

Urological stents coated with antibiotics encapsulated in polymers have also been tested in the context of catheter-associated infections. For instance, studies by Multanen et al [28] indicate that ofloxacin coating bioreabsorble self-reinforced L-lactic acid polymer (SR-PLLA) reduces bacterial adhesion with the exception of E. faecalis, which is naturally resistant to ofloxacin. A liposomal ciprofloxacin

containing hydrogel for external coating of silicone Foley catheters has been developed by Pugach et al [29]. This particular coating offered several advantages in rabbits catheterized with liposomal ciprofloxacin hydrogel coated catheters compared with untreated controls [29]. For instance, catheters coated with liposomal encapsulated ciprofloxacin hydrogel showed a significant increase ($p = 0.04$) in protection from the development of bacteriuria compared to controls (untreated or hydrogel coated) and increased median time (from 3.25 days in untreated catheters to 6.25 days treated catheters) to development of bacteriuria in rabbits. Recently, Schinabeck et al [30] developed a rabbit model of catheter-associated infection with C. albicans biofilms and showed that antifungal lock therapy with liposomal amphotericin B is an effective treatment strategy for such infections. In this study a silicone catheter was surgically placed in New Zealand White rabbits and animals were infected with C. albicans and treated with saline (untreated controls), liposomal amphotericin B lock, and fluconazole lock. Quantitative cultures revealed that catheters treated with liposomal amphotericin B yielded 0 cfu, which was significantly better when compared to the untreated controls ($P < 0.001$) and the fluconazole-treated group ($P = 0.0079$) [30].

Chronic urinary catheters exhibit even greater problems with an infection rate of nearly 100% [31]. Phosphorylcholine (PC), an effective anti-thrombotic IV catheter coating, drastically reduces adsorption of fibrinogen to implant surfaces. This, in turn, discourages adherence of several bacterial species including S. aureus [13], E. coli, and Proteus mirabilis adhesion to the urinary catheters. In summary, many advances in different fronts have been made in an effort to reduce catheter-associated bacterial infections and the resultant morbidity and mortality. However, more work needs to be done in this area to completely eradicate the problem. Towards this end, a possible solution would be to develope controlled release formulations of antibiotics designed specifically for catheter coating.

4. STENTS

Medical stents are designed to maintain the lumen of a body tube and are commonly used instead of or along with angioplasty. Stents, the hollow cylinders that keep the lumen open, are very useful devices but have their own share of problems that may result in rejection of the implant. Restenosis is a serious problem with stent implants as they can completely close off the opening that was maintained by stents. In addition, stents can develop post insertion infections, which will result in removal of the device and may increase morbidity and mortality. The review of recent publications reveals several approaches to minimize bacterial colonization of the stent. Coating of the stents with liposomal antibiotics proved to be effective therapeutic measures as they are for urinary tract catheters.

Hydrogels can be used to cover metallic stents for controlled drug release and gene transfection. A photoreactive material consistingof a gelatin macromer (multiple

styrene–derivatized gelatin) and carboxylated camphorquinone (photo-initiator) can be used as the coating material. A few minutes of visible light irradiation of a stent after dip-coating of an aqueous solution of the photoreactive material results in the formation of a homogeneously cross-linked gelatinous layer on the entire exterior surface of the metal stent. Rhodamine-conjugated albumin as a model drug or the adenoviral vector expressing bacterial beta-glactosidase (AdLacZ) as a model transfection vector was photo-immobilized in the gelatinous layer. Results showed effective gene transfection and drug release from gel after three weeks of implantation [32].

Another stent used for study was composed of polytetraflouroethylene (PTFE) and coated in liposomes containing PC (phosphatidylcholine) and CHOL (cholesterol). This liposomal coating showed that less than 30% of the liposome remained attached to the stent 72 hours after preparation. Upon incubation of the same composite in urine, $50 \pm 5\%$ of the drug was released from the stent over a 48 hour time period [33]. These release kinetics can be found to be beneficial in preventing infection associated with urinary stent implantation. Medical stents are very important in maintaining functional passageways for constituents of the body and there are a wide variety of coatings used on a wide variety of stents to ensure integration in the biological system. Much of the research described, however, only show effective results over a relatively short period of time (less than three weeks). Therefore, more long-term studies are clearly needed to prolong the presence and effectiveness of antimicrobial drugs in the body as stents are often left in the body for very long periods of time.

5. ORTHOPEDIC IMPLANTS

Orthopedic implants are the most widely utilized and researched medical devices. Their applications range from hip and knee replacement to cranial implants. These implants are of particular concern and often exhibit the largest risk of rejection and removal because they are generally much larger than other medical implants. For instance, acute infection and chronic myelitis occur in 5 to 33% of the open fracture implant replacements [34, 35] and 1 to 3% of orthopedic surgeries [36]. Studies indicate that most total knee and total hip arthroplasty patiens (58%) with surgical site infections (SSI) develop post-surgery deep wound infections (DWI). Hematoma and post-operative drainage appear to increase SSI [37]. Financial burden of post-surgical infection-related complications in the USA alone is about \$ 3.4 x 10^8 per year. S. aureus is isolated in 90% of primary abscesses while Gram negative bacteria comprise 10 to 20% of the implant related infections [38]. E. coli is the most common cause of secondary infections followed by Enterobacteriaciae and P. aeruginosa. New advances in materials used in cranial implants include the use of hydroxy appetite cements (HAC). Hydroxy appetite (HA) comprises 80 to 90 % of the calcified skeletons [39]. Hydroxy appetite cement, however, is a better

alternative to ceramic HA because it hardens within the body instead of being done in the lab. The best use for HAC appears to be the skull implants because of its biocompatibility and that it requires no special tools (i.e. screws, micro plates, etc.) for integration into the skull [40]. Furthermore, HAC is osteoconductive, infection resistant, and adheres well to the surrounding bones.

As previously mentioned, microorganisms such as S. aureus, S. epidermidis, Enterobacteriaciae and P. aeruginosa are commonly associated with orthopedic implants. Early treatments of these infections include the systemic administration of antibiotics cefazolin and ciprofloxacin or gentamicin and penicillin G to manage Gram–positive and Gram–negative bacteria, respectively [34]. The systemic antibiotic therapy is relatively effective, but as mentioned earlier, requires more frequent administration and higher dosages that could result in drug toxicity. In addition, one of the biggest problems associated with orthopedic implants is the production of antibiotic impermeable biofilms around the implant. Biofilms are produced by bacteria and often result in the removal of the implant in order to cure the infection. An effective and alternative antimicrobial approach is the use of antibiotic loaded polymethyl methacrylate (PMMA) beads at the infection sites [41]. Several drawbacks are associated with the application of the polymeric beads [34–42]. These include inadequate antibiotic concentration that may result in antibiotic resistant strains and the fact that PMMA is not biodegradable and therefore requires a second surgery to remove the beads. However, coating of stainless steel implants with gentamicin encapsulated in the biodegradable polylactide–co–glycolide (PLGA) showed an optimum release kinetic and maintained adequate levels of antibiotic for three weeks. This antibiotic carrier system eliminated infections caused by S. aureus at the implant site [41].

Other research groups have employed antibiotic carrier systems composed of less biodegradable materials that mimic the structure and functions of bones. These include calcium phosphate gelatin (with a Ca/P ratio of 2.3) impregnated with gentamycin, which showed an initial burst of antibiotic release followed by an essentially constant release for 3 months in vitro [43]. However, upon implantation into rabbit tibia the release duration was substantially shortened to about 4 weeks. This shortening of gentamicin release was attributed to the degradation of gelatin. Histological findings showed that this bone composite was biocompatible as no chronic lymphocytic infiltrates nor areas of macrophages or foreign body giant cell formation observed, therefore, this formulation may have a great potential as a bone substitute material [43].

Finally, Yagamurlu and co-workers [44] utilized a conjugate composed of the biodegradable material poly (3-hydroxybutyrate -co-3- hydroxyvalerate) (PHBV) and sulfactam-cefoperazone to inhibit the growth of S. aureus. This treatment was very effective in inhibiting bacterial growth and in the prevention of implant-related osteomyelitis (IRO). Despite the advances outlined above, more work needs to be done as no universal composite has been developed that could be utilized with regard to many problems that are associated with orthopedic implants.

6. LENSES

Bacterial contaminations of lenses during or after surgery are extremely important because infection-related complications could result in blindness. One study showed that the PC coating of an intraocular lens (IOL), composed of silicone, decreased adherence of S. epidermidis by 20–fold [45]. A further 20–fold decrease in adhesion of the bacteria was achieved when the IOLs were composed of PMMA. Heparin has also been used for coating the silicone IOLs. These heparin modified silicone (HMS) lenses display a 15–fold reduction in silicone oil adherence, which has been linked to the presence of vitreoretinal disease [46]. As for PMMA lenses, heparin coating resulted in a significant decrease in adherence of S. epidermidis, which can cause implant-associated bacterial endophthalmitis [47]. The coating of intraocular lenses has also been proven to reduce inflammation in and around the eye [48].

7. SKIN GRAFTS

Skin grafts and tissue repairs are becoming a common practice in modern medicine. The fragile nature of the skin and tissues, in comparison to implants, and the important protective role of the skin in infection and inflammation are challenging aspects of these operations. As for infection control measures, liposomal delivery systems have been utilized to prevent infections and expedite healing process [49, 50]. For instance, polyvinyl-pyrrolidone-iodine liposome hydrogel improves wound healing by a combined moisturizing and antiseptic action, when compared to conventional antiseptic chlorhexidine [49]. Encapsulation of silver sulfadiazine (SSD), the drug of choice for topical treatments of infected burns, has also improved its efficacy by allowing a slow release of the antibacterial drug over 24 hours [50]. As with other implants, the use of antibiotic grafted polymers have been proven to be far more effective than traditional methods in preventing infections and accelerating tissue repair.

8. RESPIRATORY IMPLANTS

Intubation or implantation of artificial devices into the respiratory system are often necessary in order to overcome respiratory problems ranging from ventilation of a defective lung to intubation of a newborn with immature respiratory system.

The most common types of respiratory implants, however, are endotracheal tubes (ET). ETs allow oxygenation and positive pressure ventilation, but prolonged post-surgical procedures are associated with bacterial infections and increased mortality [31]. Introduction of the patients own throat flora during endotracheal intubation and exposure of the secretion pool around the tube cuff to nosocomial microbes are the major risk of pneumonia in intubated patiens [13]. P. aeruginosa is one of the commonly encountered and recognized bacteria associated with respiratory intubations [51]. The following measures are suggested to reduce infections related to catheters and ETs:

1) Anti infective coated catheters: Polyurethane catheters that are impregnated with minute quantities of silver sulphadiazine and chlorhexidine indicated a significant reduction in catheter-related infections in clinical trials. Hexetidine may prevent infections by biofilm forming bacteria as it has anti-plaque forming activity [52]. Likewise, preclinical studies with silver hydrogel coated ETs exhibited a significantly longer onset time for P. aeruginosa [51].

2) Antibiotic coated catheters: Several antibiotic coated catheters including minocycline-rifampin-coated catheters have proven to be superior to antiseptic coated catheters because, unlike the older types of antiseptic catheters, both external and internal surfaces of the catheter are coated. In addition, the combination of minocycline and rifampin exhibits superior surface activity against staphylococci [24] versus chlorhexidine-silver sulphadiazine. The use of higher concentration of chlorhexidine-silver sulphadiazine on the external and internal surfaces of the catheters is now being evaluated in a multicenter trial [25]. The major theoretical drawbacks with antibiotics coated catheters are: a) the ineffectiveness of antibiotics against antibiotic-resistant bacteria and yeasts; b) the risk of promoting bacterial resistance with long-term topical use; and c) risk of hypersensitization. Future studies are needed to evaluate the impact of anti-infective-coated devices on the emerging nosocomial bacterial resistance [26–28]. Avoiding the risk factors that increase the need for prolonged intubation or reintubation will reduce the risk of infections associated with intratracheal catheters.

9. CARDIAC IMPLANTS

Another development in the area of artificial implants is the replacement of heart components with artificial devices, primarily pacemakers and prosthetic cardiac valves. These devices serve to maintain cardiac function without the need for total heart replacement. These techniques greatly reduce the risk of immunological rejection, but bring with them the risk of infection. Endocarditis and sepsis are two very unfavourable and potentially lethal complications associated with cardiac valve replacement. Prosthetic valve endocarditis (PVE) occurs in 0.5–1% of the operations with a high mortality rate of 50% [53, 54].

A treatment modality for PVE is designed and patented by the St. Jude Medical Inc. It is a silver-coating sewing ring commercially known as Silzone®. The Silzone® incorporates silver to Dacron implant fibers in an effort to utilize antimicrobial activity of silver without leaching into the cardiovascular system [55]. The Artificial Valve Endocarditis Reduction Trial (AVERT) was then designed to evaluate the efficacy of the Silzone® in reducing PVE in the absence of the concerned device-associated thrombosis. Although the study confirmed Silzone's anti-PVE activity in the absence of thrombosis, it revealed a higher rate of paravalvular leakage in the Silzone® study arm [54]. Consequently, this device was debunked, but the concept has since been evaluated by others with mixed results [56–58].

Infections of prosthetic heart valves generally occur at the sewing cuff-tissue interface [59]. In vivo efficacy of antimicrobial-fabric impregnated with

minocycline-rifampin or direct coating of the prosthetic heart valves with these antibiotics has been confirmed against S. aureus and S. epidermitis [58, 60]. Likewise, studies by other investigators indicate that the coating of the cardiac valve prevents infections caused by S. epidermidis (with a greatest inhibition), S. aereus, E. faecelis, P. aeruginosa, and Candida albicans [60]. The broader spectrum of MR antimicrobial activities and the fact that the combination therapy will less likely select resistant strains comparing to that of rifampin alone make the MR approach more attractive.

Fungal endocarditis associated with valve replacement is a rare but potentially dangerous complication with 8% fatality rate [27]. Common causative agents include C. albicans, Aspergillus, and C. parapsilosis [61–63]. Systemic applications of liposomal amphotericin B along with flucytosine are effective treatment modalities. Direct application of these antibiotics on prosthetic cardiac valve appears to be another option but there is no data available at this time [64, 65].

10. CONCLUSIONS

As this paper has shown, there has been a great deal of work on the developing new and better implant composites as well as many coatings, rods, spheres, beads and separate implants that attempt to ward off bacterial adhesion and to act as bacteriocidal. These implants range from the skin to the teeth to joint replacement and even the repair of skull defects and the replacement of intraocular lenses. The trend in these materials is to develop new, better, and more cost effective biodegradable polymers that will allow for slow absorption of the material by the body thereby negating addition invasion procedures to remove part or all of the implants. Much research has also been done on the bacteria and microorganisms causing the infection; and often eventual removal of implants is required to find the best strategies to fight these microbes. Although a great deal of work has been done in the area of medical implants, there is no device or technique better than simple sterility during an operation and still no practice of implant preparation to completely eliminate the existence of infection in a surgery as invasive as implantation of a foreign device. Consequently in the end it can be said that although the research community is close to finding the perfect device and materials and antimicrobials for implantation, more research is left to be done in hope that implantation related infections could be completely eliminated.

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CHAPTER 4

NIOSOMES AS NANOCARRIER SYSTEMS

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Abstract: Nonionic surfactant vesicles (niosomes) result from the organized assembly of sufficiently insoluble surfactants in aqueous media. The low cost of ingredients and manufacture, possibility of large-scale production, stability and the resultant ease of storage of niosomes have led to the exploitation of these nanocarriers as alternatives to other micro and nano-encapsulation technologies. Niosomes are an already established encapsulation technology in different areas including food, biotechnology, cosmetics and pharmaceutics. This article reviews general properties of niosomes along with recent trends in their preparation methods and their applications in the encapsulation and delivery of bioactive agents via different routes

Keywords: niosomes, liposomes, non-ionic surfactants, drug delivery, nanocarriers, encapsulation technology

1. INTRODUCTION

Colloidal drug delivery systems such as liposomes and niosomes have distinct advantages over conventional dosage forms. These systems can act as drug reservoirs and provide controlled release of the active substance. In addition, modification of their composition or surface can allow targeting.

Niosomes are non-ionic surfactant based vesicles that had been developed as alternative controlled drug delivery systems to liposomes in order to overcome the problems associated with sterilization, large-scale production and stability. The first niosome formulations were developed and patented by L'Oreal in 1975. They are liposome-like vesicles formed from the hydrated mixtures of cholesterol, charge inducing substance, and nonionic surfactants such as monoalkyl or dialkyl polyoxyethylene ether. Basically, these vesicles do not form spontaneously. Thermodynamically stable vesicles form only in the presence of proper mixtures of surfactants and charge inducing agents.

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The mechanism of vesicle formation upon use of nonionic surfactants is not completely clear. The most common theory is that nonionic surfactants form a closed bilayer in aqueous media based on their amphiphilic nature (Figure 1). Formation of this structure involves some input of energy, for instance by means of physical agitation (e.g. using the hand-shaking method; *see* Baillie et al 1985) or heat (e.g. using the heating method; *see* Mozafari 2005a). In this closed bilayer structure, hydrophobic parts of the molecule are oriented away from the aqueous solvent whereas the hydrophilic head comes in contact with the aqueous solvent. It resembles phospholipid vesicles in liposomes and hence, enables entrapment of hydrophilic drugs. The low cost, stability and resultant ease of storage of nonionic surfactants has led to the exploitation of these compounds as alternatives to phospholipids.

Niosomes can entrap hydrophilic drugs and other bioactives upon encapsulation or hydrophobic material by partitioning of these molecules into hydrophobic domains. These vesicles can be formulated either unilamellar or multilamellar in structure. Moreover, niosomes possess great stability, cost-effectiveness, and simple methodology for the routine and large-scale production without the use of hazardous solvents.

The superiorities and advantages of niosomes, compared to other micro and nano encapsulation technologies can be summarized as follows:

- Compared to phospholipid molecules used in liposome formulations, the surfactants used in the formation of niosomes are more stable;
- Simple methods are required for manufacturing and large–scale production of niosomes;

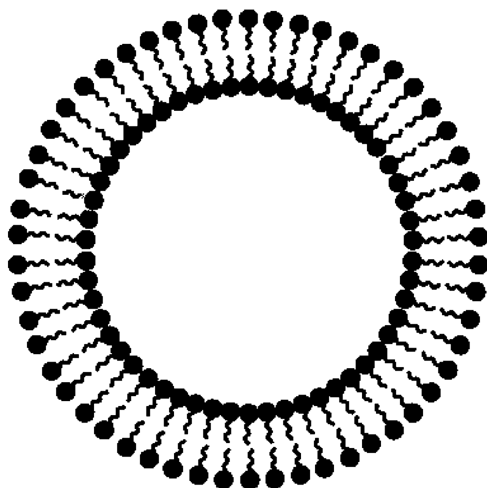

Figure 1. Schematic representation of a noisome. Dark circles represent polar head groups and lines are apolar tails of the surfactant molecules

- As the excipients and equipments used for production are not expensive, niosome manufacturing process is cost-effective;
- Niosomes possess longer shelf-life than liposomes and most other nanocarrier systems;
- Unlike liposomes, they are stable at room temperature and less susceptible to light.

2. FACTORS AFFECTING THE FORMATION OF NIOSOMES

2.1. Type of Surfactants

Type of the surfactants influences encapsulation efficiency, toxicity, and stability of niosomes. The first niosomes were formulated using cholesterol and single-chain surfactants such as alkyl oxyethylenes. The alkyl group chain length is usually from C_{12}–C_{18}. The hydrophilic- lipophilic balance (HLB) is a good indicator of the vesicle forming ability of any surfactant. Uchegbu et al (1995, 1998) reported that the sorbitan monostearate (Span) surfactants with HLB values between 4 and 8 were found to be compatible with vesicle formation. Polyglycerol monoalkyl ethers and polyoxylate analogues are the most widely used single-chain surfactants. However, it must be noted that they possess less encapsulation efficiency in the presence of cholesterol. Etheric surfactants have also been used to form niosomes. These types of surfactants are composed of single-chain, monoalkyl or dialkyl chain. The latest ones are similar to phospholipids and possess higher encapsulation efficiency. Esther type amphyphilic surfactants are also used for niosome formulation. They are degraded by estherases, triglycerides and fatty acids. Although these types of surfactants are less stable than ether type ones, they possess less toxicity. Furthermore, glucosides of myristil, cethyl and stearyl alcohols form niosomes.

2.2. Surfactant/Lipid and Surfactant/Water Ratios

Other important parameters are the level of surfactant/lipid and the surfactant/water ratio. The surfactant/lipid ratio is generally 10–30 mM (1–2.5% w/w). If the level of surfactant/lipid is too high, increasing the surfactant/lipid level increases the total amount of drug encapsulated. Change in the surfactant/water ratio during the hydration process may affect the system's microstructure and thus, the system's properties.

2.3. Cholesterol

Steroids are important components of cell membranes and their presence in membranes brings about significant changes with regard to bilayer stability, fluidity and permeability. Cholesterol, a natural steroid, is the most commonly used membrane additive (Figure 2) and can be incorporated to bilayers at high molar

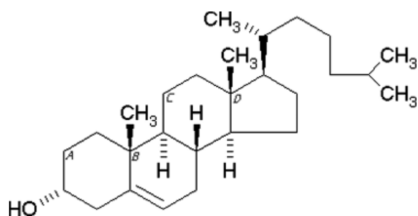

Figure 2. Chemical structure of cholesterol

ratios. Cholesterol by itself, however, does not form bilayer vesicles. It is usually included in a 1:1 molar ratio in most formulations to prevent vesicle aggregation by the inclusion of molecules that stabilize the system against the formation of aggregates by repulsive steric or electrostatic effects. It leads to the transition from the gel state to liquid phase in niosome systems. As a result, niosomes become less leaky.

2.4. Other Additives

As is the case with liposomes, charged phospholipids such as dicethylphosphate (DCP) and stearyl amine (SA) have been used to produce charge in niosome formulations. The former molecule provides negative charge to vesicles whereas the later one is used in the preparation of positively charged (cationic) niosomes.

2.5. Nature of the Drug

One of the overlooked factors is the influence of the nature of the encapsulated drug on vesicle formation (Table 1). The encapsulation of the amphipathic drug doxorubicin has been shown to alter the electrophoretic mobility of hexadecyl diglycerol ether ($C_{16}G_2$) niosomes in a pH dependent manner, indicating that the amphipathic drug is incorporated in the vesicle membrane.

Table 1. The effect of the nature of the drug on the formation of niosomes

Nature of the drug	Leakage from the vesicle	Stability	Other properties
Hydrophobic drug	Decreased	Increased	Improved transdermal delivery
Hydrophilic drug	Increased	Decreased	–
Amphiphilic drug	Decreased	–	Increased encapsulation, altered electrophoretic mobility
Macromolecular drug	Decreased	Increased	–

3. PREPARATION OF NIOSOMES

Niosomes can be prepared using non-ionic surfactants. As the number of double layers, vesicle size and its distribution, entrapment efficiency of the aqueous phase, and permeability of vesicle membranes are influenced by the way of preparation, these parameters should be taken into account while making a decision on selecting the optimum methodology for formulation.

Most of the experimental methods consist of the hydration of a mixture of the surfactant/lipid at elevated temperature followed by optional size reduction to obtain a colloidal dispersion. Subsequently, the unentrapped drug is separated from the entrapped drug by centrifugation, gel filtration or dialysis. Only a couple of methods could be found in the literature on the preparation of niosomes on an industrial scale (Novasome®, heating method). In the Novasome® method, niosomes are prepared upon injection of the melted surfactants/lipids into a large volume of well-agitated, heated aqueous solutions. The novel heating method and other well-known procedures for niosome preparation are summarized below.

3.1. Ether Injection Method

This method is essentially based on slow injection of an ether solution of niosomal ingredients into an aqueous medium at high temperature. Typically a mixture of surfactant and cholesterol (150 μmol) is dissolved in ether (20 mL) and injected into an aqueous phase (4 mL) using a 14-gauge needle syringe. Temperature of the system is maintained at 60°C during the process. As a result, niosomes in the form of large unilamellar vesicles (LUV) are formed (Baillie et al 1985; Vyas and Khar 2002).

3.2. Film Method

The mixture of surfactant and cholesterol is dissolved in an organic solvent (e.g. diethyl ether, chloroform, etc.) in a round-bottomed flask. Subsequently, the organic solvent is removed by low pressure/vacuum at room temperature, for example using a rotary evaporator. The resultant dry surfactant film is hydrated by agitation at 50–60°C and multilamellar vesicles (MLV) are formed (Baillie et al 1985; Varshosaz et al 2003).

3.3. Sonication

Typically the aqueous phase is added into the mixture of surfactant and cholesterol in a scintillation vial. Then, it is homogenized using a sonic probe. The resultant vesicles are of small unilamellar (SUV) type niosomes (Baillie et al 1986). The SUV type niosomes are larger than SUV liposomes (i.e. SUV niosomes are >100 nm in diameter while SUV liposomes are <100 nm in diameter).

It is possible to obtain SUV niosomes by sonication of MLV type vesicles, obtained for example through the film method explained above. For small volume samples probe type sonicator is used while for larger volume samples bath type sonicator is more appropriate.

3.4. Method of Handjani–Vila

Equivalent amounts of synthetic non-ionic lipids are mixed with the aqueous solution of the active substance to be encapsulated and a homogenous lamellar film is formed by shaking. The resultant mixture is homogenized employing ultracentrifugation and agitation at a controlled temperature (Handjani-Vila 1990).

3.5. Reverse Phase Evaporation

Reverse phase evaporation technique is being used to prepare different carrier systems including archaeosomes, liposomes, nanoliposomes and niosomes. Typically surface-active agents are dissolved in chloroform, and 0.25 volume of phosphate saline buffer (PBS) is emulsified to get water in oil (w/o) emulsion. The mixture is then sonicated and subsequently chloroform is evaporated under reduced pressure. The lipid or surfactant first forms a gel and then hydrates to form niosomal vesicles (Kiwada et al 1985a, 1985b; Vyas and Khar 2002).

Alternatively, hydrogenated or nonhydragenated egg phosphatidylcholine (ePC) is dissolved in chloroform and PBS. The mixture is sonicated under low pressure, forming a gel. The gel is subsequently hydrated. Free drug or other bioactives to be encapsulated (un-entrapped material) is generaly removed by dialysis or centrifugation. Protamine is added prior to centrifugation process to achieve phase separation.

3.6. Heating Method

This is a non-toxic, scalable and one-step method and is based on the patented procedure of Mozafari (2005b). Mixtures of non-ionic surfactant, cholesterol and/or charge inducing molecules are added to an aqueous medium (e.g. buffer, distilled H_2O, etc.) in the presence of a polyol such as glycerol. The mixture is heated while stirring (at low shear forces) until vesicles are formed (Mozafari 2005b).

3.7. Post-Preparation Processes

The main post-preparation processes in the manufacture of niosomes are downsizing and separation of unentrapped material. After preparation, size reduction of niosomes is achieved using one of the methods given below:

1. Probe sonication results in the production of the niosomes in the 100–140 nm size range.
2. Extrusion through filters of defined pore sizes.

3. Combination of sonication and filtration has also been used to obtain niosomes in the 200nm size range (e.g. doxorubicin niosomes).
4. Microfluidization yielding niosomes in sub-50 nm sizes.
5. High-pressure homogenisation also yields vesicles of below 100nm in diameter.

As in most cases 100% of the bioactive agent cannot be encapsulated in the niosomal vesicles, the unentrapped bioactive agent should be separated from the entrapped ones (Kiwada et al 1985a, 1985b). In some instances, this provides an advantage since this drug delivery system (or generally speaking bioactive carrier system) gives an initial burst to initiate therapy followed by a sustained maintenance dose.

Most commonly used methods for separating unentrapped material from niosomes are as follows:

- Dialysis;
- Gel filtration (e.g. Sephadex G50);
- Centrifugation (e.g. 7000 × *g* for 30 min for the niosomes prepared by hand-shaking and ether injection methods);
- Ultracentrifugation (150000 × *g* for 1.5 h).

4. ENTRAPMENT EFFICIENCY

Both the yield and the entrapment efficiency of niosomes depend on the method of preparation. Niosomes prepared by ether injection method have better entrapment efficiency than those prepared by the film method or sonication. Addition of cholesterol to non-ionic surfactants with single- or dialkyl-chain significantly alters the entrapment efficiency. However, surfactants of glycerol type lead to reduction in entrapment capacity as the amount of cholesterol increases.

Employing film method and a subsequent sonication results in formation of liquid crystal and gel type niosomes. Niosomes in the form of liquid crystals possess better entrapment efficiency than gel type vesicles as observed in liposomes as well. Urea niosomes are the best example for gel type niosomes and exhibit 10% entrapment capacity. This can be improved by the addition of cholesterol.

5. STABILITY OF NIOSOMES

Vesicles are stabilized based upon formation of 4 different forces:

1. van der Waals forces among surfactant molecules;
2. repulsive forces emerging from the electrostatic interactions among charged groups of surfactant molecules;
3. entropic repulsive forces of the head groups of surfactants;
4. short-acting repulsive forces.

Electrostatic repulsive forces are formed among vesicles upon addition of charged surfactants to the double layer, enhancing the stability of the system.

Biological stability of the niosomes prepared with alkyl glycosides was investigated by Kiwada et al (1985a, 1985b). They reported that niosomes were not stable

enough in plasma. This may be due to single–chain alkyl surfactants. SUVs were found to be more stable.

Niosomes in the form of liquid crystal and gel can remain stable at both room temperature and 4°C for 2 months. No significant difference has been observed between the stability of these two types of niosomes with respect to leakage. Even though no correlation between storage temperature and stability has been found, it is recommended that niosomes should be stored at 4°C. Ideally these systems should be stored dry for reconstitution by nursing staff or by the patient and when rehydrated should exhibit dispersion characteristics that are similar to the original dispersion.

Simulation studies conducted to investigate physical stability of these niosomes during transportation to the end-user revealed that mechanical forces didn't have any influence on physical stability. It is assumed that the reason behind the stability of niosomes may be due to the prevention of aggregation caused by steric interactions among large polar head groups of surfactants.

The factors which affect the stability of niosomes are as following:

- type of surfactant;
- nature of encapsulated drug;
- storage temperature;
- detergents;
- use of membrane spanning lipids;
- the interfacial polymerization of surfactant monomers in situ;
- inclusion of a charged molecule.

6. TOXICITY OF NIOSOMES

Unfortunately, there is not enough research conducted to investigate toxicity of niosomes. Researchers measured proliferation of keratinocytes in one of the topical niosome formulations (Hofland et al 1991). The effect of surfactant type on toxicity was investigated. It was determined that the ester type surfactants are less toxic than ether type surfactants (Hofland et al 1991, 1992). This may be due to enzymatic degradation of ester bounds. In general, the physical form of niosomes did not influence their toxicity as evident in a study comparing the formulations prepared in the form of liquid crystals and gels. However, nasal applications of these formulations caused toxicity in the case of liquid crystal type niosomes.

In some instances, encapsulation of the drug by niosomes reduces the toxicity as demonstrated in the study on preparation of niosomes containing vincristine (Parthasarathi et al 1994). It decreased the neurological toxicity, diarrhoea and alopecia following the intravenous administration of vincristine and increased vincristine anti-tumor activity in S-180 sarcoma and Erlich ascites mouse models.

7. APPLICATIONS OF NIOSOMES

7.1. Transdermal Applications

It is well-known fact that transdermal applications provide a great advantage of protecting drugs from the hepatic first pass effect. However, stratum corneum layer of skin forms a barrier, resulting in a slow absorption at the application site.

The fact that in the manufacture of niosomes nonionic surfactants are used to form vesicles makes them good candidates for transdermal drug delivery. Sentjurc and co-workers (1999) investigated transport of liposome-entrapped spin labelled compounds into skin by electron paramagnetic resonance imaging methods. In addition, the mechanistic aspects of cyclosporin-A skin delivery were assessed. Niosomes containing urea formulations have been prepared and being treated by the cosmetic industry, as almost magical ingredients.
Two mechanisms are suggested for transdermal absorption of vesicles:

i) diffusion of nisomes from the stratum corneum layer of skin as a whole, or:
ii) forming new vesicles by each individual component (re-formation of vesicles). The later one takes place only at certain regions of stratum corneum where water content is high. Many researchers agree upon the second mechanism since the diameter of vesicles is larger than the lipid lamellar spaces of the stratum corneum.

7.2. Parenteral Applications

Niosomes in sub-micron size are used for parenteral administration. Niosomal vesicles up to 10 μm are administered via i.p. or i.m. Florence and Cable (1993) prepared ^{59}Fe-deferroxamine trioxyethylene cholesterol vesicles for i.v. use and reported that the distribution of such vesicles depends upon vesicle size as evident from the data indicating greater distribution in liver and spleen.

Uchegbu et al (1996, 1997, 1998) investigated the effect of dose on plasma drug concentration by comparing doxorubicin-containing niosomes with free drug in mouse upon i.p. administration. The data revealed that plasma drug concentration is influenced by dose. Niosomes enhance plasma drug concentration. Furthermore, they conducted experiments for toxicity and determined that there is a positive correlation between dose and toxicity. However, Florence and Cable (1993) indicated that the preparation of doxorubicin in the form of niosomes reduces its cardiac toxicity upon i.v. administration.

7.3. Peroral Applications

The oral use of niosomal formulations was first demonstrated by Azmin et al (1985) in a study involving 100 nm methotrexate $C_{16}G_3$ niosomes. Significantly higher levels of methotrexate were found in the serum, liver and brain of PKW mice following oral administration of a niosomal formulation. It thus appears that there is enhanced drug absorption with these niosomal formulations.

Rentel et al (1996) prepared niosome-based ovalbumin vaccines by two different types of surfactants and administered p.o. to mouse. In comparison to the conventional vaccines, niosome-based vaccines resulted in increased antibody titer. However, type of surfactant didn't have any influence on antibody production.

7.4. Radiopharmaceuticals

The first applications of niosomes as radiopharmaceuticals have been achieved by Erdogan et al in 1996. They prepared ^{131}I labeled iopromide niosomes with positive charge in order to enhance contrast during CT in rats (Erdogan et al 1996). The formulations were in the form of gel or liquid crystal. They were found more in kidneys and maintained their activity over 24 hours. In another study, Korkmaz et al (2000) used ^{99m}Tc- labeled DTPA containing niosomes and found that DTPA was accumulated in liver and spleen in large quantities. The gamma sintigraphic images of mouse were better with ^{99m}Tc-DTPA niosomes [N1 formulation: SurI: SA: CHOL (10:1:4)]. Similarly, gel type ^{99m}Tc-labelled niosomes of DMSA accumulated in liver, kidneys, and spleen in mouse and maintained the activity for 24 hours. Niosome formulation also provided better stability in comparison to conventional solutions of DMSA as they are less susceptible to light, temperature and oxidation.

7.5. Ophthalmic Drug Delivery

There is only a single study on the use of niosomes for ophthalmic drug delivery to date (Saettone et al 1996). Saettone et al (1996) reported on the biological evaluation of a niosomal Cyclopentolate delivery system for ophthalmic delivery. Polysorbate 20 and cholesterol were used for niosome formulations. It was determined that cyclopentolate penetrated the cornea in a pH dependant manner within these niosomes. Optimum pH for peak permeation values was pH 5.5. Permeation decreased at pH 7.4. However, in vivo data revealed that there was increased mydriatic response with the niosomal formulation irrespective of the pH of the formulation. In short, the increased absorption of cyclopentolate may be the result of the altered permeability characteristics of the conjuctival and scleral membranes. Niosomes >10 μm are suitable for drug administration to eye.

8. PRONIOSOMES

Proniosomes are prepared by hydration and agitation in hot water for a short period of time. They offer a versatile vesicle delivery concept with the potential for drug delivery via the transdermal route. They form niosomes following topical application under occlusive conditions, due to hydration by water from the skin itself.

Alsarra et al (2005) prepared topical niosomes of Ketorolac tromethamine (KT) as an alternative noninvasive mode of delivery, as transdermal delivery certainly seemed to be an attractive route of administration to maintain the drug blood levels

of KT for an extended period of time. Using a wide-mouth glass tube, KT was mixed with surfactant, lecithin, and cholesterol in absolute ethanol. Then, the open-end of the glass tube was covered with a lid and the tube was warmed in a water bath at 65 ± 3°C for 5 min. After that, PBS was added and the mixture was further warmed in the water bath for about 2 min until a clear solution was obtained. The mixture was allowed to cool to room temperature until a proniosomal gel was formed. The proniosomal gel was then mixed with one of several 2% polymeric gels (HPMC, CMC, or Carbopol) to give a final concentration of 0.5% KT. The resultant vesicles were characterized with respect to shape, surface morphology, and size by means of SEM.

The formulations prepared with Span 60 and Tween 20 gave the highest entrapment efficiency. This may be due to the fact that the highly lipophilic portion of the drug is housed within the lipid bilayer of the niosomes. Type of surfactant influenced the vesicle size. The niosomes prepared with Tween 20 were larger than those prepared with Span 60. The reason behind that may be the decrease in surface energy with increasing hydrophobicity of the surfactant. Span is more hydrophobic than Tween. Although increasing the amount of cholesterol or reducing lecithin increased hydrophobicity, they didn't change the vesicle size significantly. SEM analysis revealed that most of the vesicles are spherical and discrete with sharp boundaries. Ex vivo release studies indicated that inclusion of an optimum ratio of surfactant/lecithin in the vesicles may play a more important role than cholesterol plays in modulating drug permeation.

In order to achieve drug release through skin, proniosomes should be hydrated to form niosomal vesicles before they permeate across the skin. Drug transfer across skin is achieved by several mechanisms including adsorption and diffusion of niosomes onto the surface of skin, facilitating drug permeation, tendency of the vesicles to act as penetration enhancers, reducing the barrier properties of the stratum corneum and the lipid bilayers of niosomes forming a rate-limiting membrane barrier for drugs.

9. CONCLUSIONS

Niosomes have been proven to be useful controlled drug delivery systems for transdermal, parenteral, oral, and ophthalmic routes. They can be used to encapsulate anti-infective agents, anti-cancer agents, anti-inflammatory agents and fairly recently as vaccine adjuvants. Niosomes may enable targeting certain areas of the mammalian organisms and may be exploited as diagnostic imaging agents.

Niosomes are superior systems when compared to other carriers with respect to stability, toxicity and cost-effectiveness. The problem of drug loading remain to be addressed and although some new approaches have been developed to overcome this problem, it is still necessary to increase encapsulation efficiencies as it is important to maintain the biological potential of the formulations.

As type of surfactant is the most important parameter affecting the formation of the vesicles, as well as their toxicity and stability, the surfactants with the higher

phase transition should be selected as they yield more desirable permeability and toxicity profiles.

Transdermal, peroral, parenteral and ophthalmic routes are suitable for niosomal applications. Recently, the use of niosomes as vaccines and radiodiagnostic agents have been studied and found to be promising areas of application.

Selection of a suitable drug to be delivered by niosomes should be made taking into account that niosomes are capable of encapsulating both hydrophobic and hydrophilic drugs.

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CHAPTER 5

STARCH – A POTENTIAL BIOMATERIAL FOR BIOMEDICAL APPLICATIONS

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Abstract: The unique physicochemical and functional characteristics of starches isolated from different botanical sources such as corn, potato, rice and wheat make them useful for a wide variety of biomedical and pharmaceutical applications. Starch properties such as swelling power, solubility, gelatinization, rheological characteristics, mechanical behaviour and enzymatic digestibility are of utmost importance while selecting starch source for distinctive applications such as bone fixation and replacement. Starches can also be used as carriers for the controlled release of drugs and other bioactive agents. The chemically modified starches with more reactive sites to carry biologically active compounds are useful biocompatible carriers, which can easily be metabolized in the human body. This chapter reviews the physico-chemical, morphological and thermal characteristics of different starches that may be of importance during their use in specific biomedical and pharmaceutical applications

Keywords: starch; biomaterial; biomedical; pharmaceutical; rheological; digestibility; chemical modification

1. INTRODUCTION

The physico-chemical and functional characteristics of starch systems and their uniqueness in various products vary with starch biological origin (Svegmark & Hermansson, 1993). Starches from various plant sources, such as wheat, corn, rice and potato have received extensive attention in relation to structural and physico-chemical properties. Starch is widely used in food, pharmaceutical and biomedical applications because of its biocompatibility, biodegradability, non-toxicity, and abundant sources. The role of starch for tissue engineering of bone, bone fixation, carrier for the controlled release of drugs and hormones; and as hydrogels has already been recognized (Mano & Reis, 2004; Won et al, 1997; Lenaerts et al., 1998; Pal et al., 2006; Pereira et al., 1998; Chakraborty et al., 2005). Starch-based

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biodegradable bone cements are highly advantageous because they can provide for immediate structural support and, as they degrade from the site of application, allow the ingrowth of new bone for complete healing of bone fracture (Domb et al, 1996; Pereira et al, 1998). Starch nanoparticles, nanospheres, and nanogels have also been used as base materials for nanoscale construction of sensors, tissues, mechanical devices, and drug delivery systems (Chakraborty et al., 2005). Starches of different sources have been studied extensively in relation to their structural, physico-chemical and functional properties, and it has been suggested that the extent of variation in these properties depends on the source of starch (Tester & Karkalas, 2002; Singh et al, 2002, 2003, 2004; Kaur et al, 2002; Yusuph et al, 2003).

Native starch characteristics, their correlation with different properties of starch based products and their interactions with different ingredients during product development have been studied (Singh et al, 2002a, 2002b; Kaur et al, 2005; Azizi & Rao, 2005). Many techniques and methods for the characterization of starch have been developed that are suitable for screening of starches from different sources (Singh & Singh, 2001, 2003; Kim et al, 1995). Industrial interest in new value-added products has resulted in many studies being carried out on the characterization of starches isolated from different genotypes and novel sources (Singh et al, 2006, 2007a; Kim et al, 1995; Romero-Bastida et al, 2005; Taveres et al, 2005; Wang et al, 2005). The native starch isolated from different sources has limitations such as low shear resistance, thermal resistance, thermal decomposition and high tendency towards retrogradation which limits its use in some industrial applications. Starch modification, which involves the alteration of the physical and chemical characteristics of the native starch to improve its functional characteristics, can be used to tailor starch to specific applications (Singh et al, 2007b; Kaur et al, 2006; Hermansson & Svegmark, 1996). Starch modification is generally achieved through derivatization such as etherification, esterification, cross-linking and grafting of starch; decomposition (acid or enzymatic hydrolysis and oxidization of starch) or physical treatment of starch using heat or moisture etc. Chemical modification involves the introduction of functional groups into the starch molecule, resulting in markedly altered physico-chemical properties. Such modification of native granular starches profoundly alters their swelling, gelatinization, retrogradation, pasting, and digestibility properties.

The physico-chemical, morphological and thermal properties; as well as the enzymatic digestibility of the starches from different sources have been discussed in detail in this chapter. An account of the different types of chemical modifications, which are important to tailor the starch characteristics for a particular biomedical or pharmaceutical use, is given in the final section of this chapter.

2. PHYSICO-CHEMICAL CHARACTERISTICS OF STARCHES

Starch is the major reserve polysaccharide of plants and is present in the form of discrete granules comprised of amylose and amylopectin. Amylose is a linear polymer composed of glucopyranose units linked through α-D-(1$\rightarrow$4) glycosidic linkages while the amylopectin is a branched polymer with one of the highest

molecular weights known among naturally occurring polymers (Karim et al, 2000). Amylopectin is the major component with an average molecular weight of the order 10^7–10^9 (Aberle et al, 1994). It is composed of linear chains of $(1\rightarrow4)$-α-D–glucose residues connected through $(1\rightarrow6)$-α-linkages. A slight degree of branching (9–20 branch [α-$(1\rightarrow6)$] points per molecule) has been reported for amylose (Hoover, 2001). The extent of branching has been shown to increase with the molecular size of amylose (Greenwood & Thomson, 1959). The characterization of starch/starch based biomaterials for use in biomedical applications is important due to their different swelling, solubility and surface characteristics. The conversion of starch from powder to gel form is required for their use in pharmaceutical applications and this transformation is achieved through gelatinization (gelatinization is discussed in detail in the gelatinization and retrogradation section). During and after gelatinization, the amylopectin has stabilizing effects, whereas amylose forms gels and has a strong tendency to form complexes with lipids and other components (Singh et al, 2003).

Amylopectin and amylose are therefore preferred for many food and pharmaceutical applications, respectively. By genetic engineering, using, antisense technique, it has been possible to modify the botanical source so that it produces granular starch practically without amylose/amylopectin (Hofvander et al, 1992; Talberg et al, 1998). Starch properties depend on the physical and chemical characteristics such as granule size and size distribution, amylose/amylopectin ratio and mineral content (Madsen & Christensen, 1996). The amylose content of the starch granule varies with the botanical source of starch and is affected by climatic conditions and soil type during growth (Juliano et al, 1964; Morrison et al, 1984; Asaoka et al, 1985; Morrison & Azudin, 1987). Amylose content of potato starch varies from 23% to 31% for different genotypes (Kim et al., 1995; Wiesenborn et al., 1994). Amylose content of rice is specified as waxy, 0–2%; very low, 5–12%; low, 12–20%; intermediate, 20–25%; and high 25–33% (Juliano, 1992).

The amylose content of wheat starch varies from 18 to 30% (Deatherage et al, 1955; Medcalf & Gilles, 1965; Soulaka & Morrison, 1985). Phosphorus is one of the important non-carbohydrate constituents present in the starches, which vary from 0.003% in waxy corn starch to 0.09% in potato starch (Schoch, 1942a). Phosphorus at such a low concentration has been reported to significantly affect the functional properties of starches. Phosphate is present as phosphate monoesters and phospholipids in starches. The phosphate monoesters affect starch paste clarity and viscosity while the presence of phospholipids results into opaque and lower viscosity pastes (Schoch, 1942a,b; Craig et al, 1989). Phosphate groups esterified to the amylopectin fraction of potato starch contribute to the high viscosity and also to a high transparency, water binding capacity and freeze thaw stability (Craig et al., 1989; Swinkels, 1985). Phospholipids present in starch have a tendency to form complex with amylose and long branched chains of amylopectin, which results in limited swelling. Wheat and rice starches have higher phospholipids content and produce starch pastes with lower transmittance as compared to the corn and potato starches with lower phospholipids content. Free fatty acids in rice and maize starches

contribute to their higher transition temperatures and retrogradation (Davies et al., 1980), which is due to amylose-lipid complex formation.

Potato starch with higher phosphate monoester content resulted into paste with higher light transmittance. More than 90% of the lipids inside wheat starch granules are lysophospholipids and have been thought to occur in the form of inclusion complexes with amylose (Morgan et al, 1993). ^{31}P-nuclear magnetic resonance has been used to locate the phosphorylations in modified wheat and corn starches and in native potato and taro starches (Muhrbeck & Tellier, 1991; Jane et al, 1992). This technique has also been used to determine the locations of phosphodiester cross-linkages in corn starch (Kasemsuwan & Jane, 1994). Starch phosphate-monoesters in native potato starch are mainly found on amylopectin which contains one phosphate-monoester group per 317 glucosyl residues. The phosphorus in potato starch is located densely in the granule core together with amylopectin. Wheat starch lipids constitute 1% of the granular weight, having surface lipids to the extent of 0.05% (Eliasson et al, 1981). The lipids are present at lower levels and significantly affect the swelling of wheat starch (Morrison et al, 1993). It has also been reported that surface lipids oxidize and contribute to the so-called cereal odor of wheat starch.

Swelling power and solubility provide evidence of the magnitude of interaction between starch chains within the amorphous and crystalline domains. The extent of this interaction is influenced by the amylose/amylopectin ratio, and by the characteristics of amylose and amylopectin in terms of molecular weight/distribution, degree and length of branching, and conformation (Hoover, 2001). Swelling/water absorption capacity of starches is very important in biomedical and pharmaceutical applications such as implants and drug-delivery systems because the equilibrium degree of swelling influences: (a) the solute diffusion coefficient through the starch based hydrogels, (b) the surface properties and surface mobility, and (c) its mechanical properties (Peppas, 1996; Pereira et al, 1998). Swelling power and solubility of the starches from different sources differ significantly. Potato starch has much higher swelling power and solubility than other starches (Singh et al, 2002). Corn starch exhibits higher swelling power than wheat starch but lower than potato starch (Singh et al., 2002). The high swelling powers and solubility of potato starches might be due to higher content of phosphate groups on amylopectin-repulsion between phosphate groups on adjacent chains will increase hydration by weakening the extent of bonding within the crystalline domain (Galliard & Bowler, 1987). The presence of lipids in starch may have a reducing effect on the swelling of the individual granules (Galliard & Bowler, 1987). Since corn, rice and wheat starch granules contain lipids contrary to potato starch granules; this may possibly explain the difference in the swelling power of these starches. The differences in swelling power and solubility of starches from different sources may also be due to the difference in morphological structure of starch granules. Water Binding and solubility of starch depend on damage starch content (Evers & Stevens, 1985). The damage starch content in rice starch has been reported to depend on starch isolation method. The damage starch was observed to be lower in the starch isolated by the

protease digestion as compared to alkaline steeping method (Wang & Wang, 2001). Starch isolated with alkaline steeping method with 0.1–0.2% sodium hydroxide had 73–85% yield (on dry starch basis, dsb), 0.07–0.42% residual protein, and 0.07–2.6% damaged starch (Yang et al, 1984; Lumdubwomg & Seib, 2000). Granules continue to swell as the temperatures of the suspension are increased above the gelatinization range. According to Hermansson and Svegmark (1996) corn and wheat granules may swell up to thirty times their original volume and potato starch granules up to hundred times their original volume, without disintegration. It has been suggested that amylose plays a role in restricting initial swelling because this form of swelling proceeds more rapidly after amylose has been exuded. The increase in starch solubility, with the concomitant increase in suspension clarity is seen mainly as the result of the granule swelling permitting the exudation of the amylose. The granules become increasingly susceptible to shear disintegration as they swell, and they release soluble material as they disintegrate. The hot starch paste is a mixture of swollen granules and granule fragments, together with colloidally and molecularly dispersed starch granules. The mixture of the swollen and fragmented granules varies with the botanical source of the starch.

2.1. Morphological Characteristics and Granular Structure

Starch is laid down in the form of granules that function as an energy reserve. The granules vary in size and shape based on their botanical origin. Tuber starch granules are generally voluminous and oval shaped with an eccentric hilum. Cereal starch granules such as maize, oats, and rice have polygonal or round shapes. High amylose maize starch exhibits filamentous granules (budlike protrusions). Legume seed starch granules are bean-like with a central elongated or starred hilum. The hilum is not always distinguishable, especially in very small granules. The semi-crystalline structure of a starch granule can be identified at the light microscope level and through characteristic X-ray diffraction patterns. Microscopy (predominantly optical and scanning electron microscopy) is mainly used for looking at the whole granule. Under polarized light in a microscope, a typical birefringence cross is observed as two intersecting bands (the “Maltese cross”). It indicates that the starch granule has a radial orientation of crystallites or there exists a high degree of molecular order within the granule. An examination of these granules under optical or electron microscopy reveals pronounced concentric rings (French, 1984). At higher levels of organization, the semi-crystalline rings are composed of stacks of alternating crystalline lamellae (Yamaguchi et al, 1979; Kassenbeck, 1978). The combined repeat distance of crystalline and amorphous lamellae accounts for the peak observed in small angle X-ray and neutron scattering experiments (Oostergetel & Van Bruggen, 1989). The currently accepted crystalline structure consists of a radial arrangement of clusters of amylopectin. Cameron and Donald (1992) have developed a model, which allows quantification of the various parameters needed to describe this complex model. The starch granule structure is modeled as a finite number of lamellae of alternating electron density

embedded in a background region of a third electron density, assumed to correspond to the amorphous growth ring. X-ray scattering is another approach that has been frequently used in starch granule structure investigation. Wide-angle X-ray diffraction (WAXD) has revealed the packing within the crystals of the granule, enabling a detailed analysis of the different polymorphs (Imberty & Perez, 1988). Cereal starches typically exhibit the A polymorph, where as tubers show the B form and legumes exhibit the mixed state polymorph C. The V type can only be found in amylose helical complex starches after starch gelatinization and complexing with lipid or related compounds. The X-ray diffraction pattern of starch could be altered by heat-moisture treatment. For example, B-type of potato starch can be converted to A or C type using heat/moisture treatment. WAXD essentially deals with the interatomic distances. Less extensively used is small-angle X-ray scattering (SAXS) which, due to the reciprocal relationship between spacings in real space and in the scattering pattern, probes larger length scales than WAXS (Donald, 2001). Lenaerts et al. (1998) carried out the solid- state ^{13}C NMR on cross-linked high amylose starch powders, tablets and hydrated tablets with different cross-linking degrees. They reported the predominance of V type of single helix arrangement of amylose in the dry state, which changed to B type double helix arrangement upon hydration, in low cross-linking degree homologues. They therefore hypothesized that the tendency of amylose to undergo the V to B transition is an important factor in controlling water transport and drug release rate.

Morphological characteristics of starches from different plant sources vary with the genotype and cultural practices. The variation in the size and shape of starch granules may be due to the biological origin (Svegmark & Hermansson, 1993). The morphology of starch granules depends on the biochemistry of the chloroplast or amyloplast, as well as physiology of the plant (Badenhuizen, 1969). The granular structure of potato, corn, rice and wheat starches show significant variation in size and shape when viewed by scanning electron microscope (SEM). The average granule size ranges between 10 and 100 μm for potato starch granules. The average size of individual corn and wheat starch granules ranges between 5 and 25 μm. The rice starch granules are smaller in size and ranges between 3–5 μm. Potato starch granules have been observed to be oval and irregular or cuboidal in shape. The starch granules are angular shaped for corn, and pentagonal and angular shaped for rice. At maturity, wheat endosperm contains two types of starch granules: large (A-granules) and small (B-granules). A-granules are disk like or lenticular in shape with diameter range between 10–35μm. On the other hand, B-starch granules are roughly spherical or polygonal in shape, ranging between 1–10 μm in diameter. Each amyloplast of wheat contains one large A-granule and a variable number of B-granules (Parker, 1985). The A-granule forms soon after anthesis and may continue to grow throughout grain filling, while the B-granules are initiated some days after anthesis and remain considerably smaller (MacLeod & Duffus, 1988). There have been reports of a third class of very small C-granules that are initiated at very late stage of grain filling (Bechtel et al, 1990). The small B-granules have a particular impact on the processing quality of the wheat (Stoddard, 1999). The higher

surface-to-volume ratio of the B-granules has been associated with a higher rate of water absorption than that of A-granules, affecting the mixing of the dough and the baking properties of the final products (Bechtel et al, 1990). The surfaces of the granules from corn, rice and wheat appear to be less smooth than potato starch granules. The individual granules in case of rice starch develop in compact spherical bundles or clusters, known as compound granules, which fill most of the central space within the endosperm cells. Physico-chemical properties like percent light transmittance, amylose content, swelling power and water binding capacity were significantly correlated with the average granule size of the starches separated from different plant sources (Singh & Singh, 2001; Zhou et al, 1998). Recent research has illustrated the potential of microscopy for elucidating the phenomena underlying starch functionality. Light microscopes and confocal scanning laser microscopes can be used to obtain information about features such as distribution of granules, degree of swelling of granules, and the general distribution of amylose rich and amylopectin rich phases, where as electron microscopes are required to reveal fine details of the granules and for the studies of the supramolecular structures of macromolecular dispersions (Hermansson & Svegmark, 1996).

3. GELATINIZATION AND RETROGRADATION CHARACTERISTICS

The gelatinization of the native starch granule is required in almost all culinary and industrial uses of starch (Blanshard, 1987). Gelatinization leads to a change in the organization of granules. The phase transitions involved are only slowly being discovered, in a large part hampered by the lack of understanding of the native granule structure (Waigh et al, 1997). The crystalline order in starch granules is often the basic underlying factor influencing its functional properties. Collapse of crystalline order within the starch granules manifests itself as irreversible changes in properties such as granule swelling, pasting, loss of birefringence, and starch solubility (Atwell et al., 1988). Many techniques, including differential scanning calorimetry (DSC), X-ray scattering, light scattering, optical microscopy, thermo-mechanical analysis (TMA) and NMR spectroscopy have been employed to study these events in an attempt to understand the precise structural changes underlying gelatinization (Jenkins & Donald, 1998).

The starch granule is a semicrystalline, and gives rise to birefringence when viewed under polar light in the microscope. As the starch granule gelatinizes and its structure is disrupted, this birefringence is lost. Many studies have attempted to characterize the point at which all birefringence is lost for a sample studied under an optical microscope. This point is termed the birefringence end point temperature. The order-disorder transitions that occur on heating an aqueous suspension of starch granules have been extensively investigated using DSC. This technique has been widely used to study the thermal behavior of starches, including gelatinization, glass transition temperature and crystallization. Stevens and Elton (1971) first reported the application of DSC to measure the heat of gelatinization of starch. Donovan

(1979) reported that there are two endothermic peaks when heating wheat and potato starches with 27% water to 150°C, and suggested that two kinds of structures or two different environments may be present. Eliasson (1980) observed three peaks when a wheat starch/water mixture with water content in the interval 35–80% was heated to 140°C and concluded that DSC could not explain the second peak. Shorgen (1992) studied the gelatinization of corn starch with 11–50% water and reported that the starch gelatinized (melted) at 190–200°C in the range of water content of 11–30%. Starch transition temperatures and gelatinization enthalpies by DSC may be related to characteristics of the starch granule, such as degree of crystallinity (Kruger et al, 1987). This is influenced by chemical composition of starch and helps to determine the thermal and other physical characteristics. Starches from different botanical sources, differing in composition exhibited different transition temperatures and enthalpies of gelatinization.

Kim et al (1995) have studied the thermal properties of starches from 42 potato cultivars and correlated these properties with the physicochemical characteristics. Gelatinization occurs initially in the amorphous regions as opposed to the crystalline regions of the granule, because hydrogen bonding is weakened in these areas. Gelatinization temperatures and enthalpies (ΔH_{gel}) associated with gelatinization endotherm varied between the starches from different sources. In wheat starch, onset (T_o), peak (T_p) and final (T_c) temperature values have been found to range between 46–52°C, 52–57°C and 58–66°C, respectively. T_o, T_p and T_c for potato starches range between 59–60°C, 63–64°C and 67–69°C, respectively. T_P gives a measure of crystallite quality (double helix length). Enthalpy gives an overall measure of crystallinity (quality and quantity) and is an indicator of the loss of molecular order within the granule (Tester & Morrison, 1990; Cooke & Gidley, 1992). ΔH_{gel} value for wheat and potato starches range between 14–17 J/g and 12–13 J/g, respectively. DSC endothermic peaks appear between 69 to 78°C, for corn and rice starches, while ΔH_{gel} values range between 9–11 J/g (Singh et al, 2003). The higher transition temperatures for corn and rice starch may be due to the more rigid granular structure and the presence of lipids. Because amylopectin plays a major role in starch granule crystallinity, the presence of amylose lowers the melting point of crystalline regions and the energy for starting gelatinization (Flipse et al., 1996). More energy is needed to initiate melting in the absence of amylose-rich amorphous regions (Kreuger et al, 1987). This correlation indicates that the starch with higher amylose content has more amorphous region and less crystalline, lowering gelatinization temperature and endothermic enthalpy (Sasaki et al., 2000). The gelatinization characteristics of intact A and B type starch granules in mature wheat endosperm have different temperature regimes (Eliasson & Karlsson, 1983; Soulaka & Morrison, 1985). Compared with the A-starch granules, B-granules started gelatinization at a lower T_o, but had higher T_p and T_c (Seib, 1994). A-granules have higher ΔH_{gel} value than B-granules.

Endothermic peak of starches after gelatinization and storage at 4°C appears at lower transition temperatures. Recrystallization of amylopectin branch chains has been reported to occur in less ordered manner in stored starch gels as it is

present in native starches. This explains the observation of amylopectin retrogradation endotherms at a temperature range below that for gelatinization (Ward et al, 1994). The variation in thermal properties of starches after gelatinization and during refrigerated storage may be attributed to the variation in amylose to amylopectin ratio, size and shape of the granules and presence/absence of lipids. The amylose content has been reported to be one of the influential factors on starch retrogradation (Gudmundsson & Eliasson 1990; Chang & Liu 1991; Baik et al 1997; Fan & Marks, 1998). Pan and Jane (2000) reported the presence of higher amount of amylose in large size maize starch granules. A greater amount of amylose has traditionally been linked to a greater retrogradation tendency in starches (Whistler & Bemiller, 1996), but amylopectin and intermediate materials also play an important role in starch retrogradation during refrigerated storage. The intermediate materials with longer chains than amylopectin may also form longer double helices during reassociation under refrigerated storage conditions. The retrogradation has been reported to be accelerated by the amylopectin with longer amylose chain length (Kalichevsky et al 1990; Yuan et al 1993). Shi and Seib (1992) indicated the retogradation of waxy starches was directly proportional to the mole fraction of branches with degree of polymerisation (DP) 14–24, and inversely proportional to the mole fraction of branches with DP 6–9. The high rate of branches with DP-20–30 or DP $\geq$ 35 has been requested to uncleave the retrogradation enthalpy (Sasaki & Matsuki, 1998). The low degree of retrogradation for waxy starches has been attributed to the high proportion of short chain branches of DP 6–9 (Lu et al., 1997). Using SAXS and WAXD simultaneously during gelatinization in water, together with small angle neutron scattering (SANS), it has been possible to probe the processes that occur at both the molecular and supramolecular length scales (Donald, 2001).

4. ENZYMATIC DIGESTIBILITY OF STARCHES

Starch is hydrolyzed to glucose, maltose and malto-oligosaccharides by α- and β-amylase and related enzymes. Glucoamylase, an exo-acting hydrolase, hydrolyses α-(1$\rightarrow$6) branching points, converting starch completely to glucose (Tester et al, 2004). Enzymatic hydrolysis of native starches at low temperature leads to the formation of pitted or porous granules, which could find useful applications in the food, cosmetic and pharmaceutical industries (Morelon et al, 2005). High amylose maize and legume starch granules have unique properties imparting resistance to digestive enzymes. Resistance is probably related to the crystalline order or packing of the glucan chains of amylose and amylopectin. Raw potato starch is an enzyme-resistant starch which is associated with the large granule size, higher phosphate content, B-type crystalline, different chain length and chain length distribution, as well as different molecular weight and weight distribution, as compared to normal cereal and other starches (Jane et al, 1997). However, when the potatoes are cooked for consumption, the starch is gelatinized and becomes susceptible to hydrolysis by α-amylase (Englyst & Cummings, 1987).

Significant differences exist among the hydrolysis rate values for different starches. These differences could be attributed to the interplay of many factors such as starch source, granule size, amylose/amylopectin ratio, extent of molecular association between starch components, degree of crystallinity and amylose chain length (Tester et al, 2004; Hoover & Sosulski, 1985; Ring et al, 1988; Jood et al, 1988; Dreher et al, 1984). The presence of pores on the granule surface may affect the digestibility of starches. Starch granule size has been reported to affect the digestibility of starches (Svihus et al, 2005; Chiotelli & Meste, 2002). The susceptibility of starches towards enzymatic hydrolysis has also been suggested to be affected by the starch granule specific surface area, which may decrease the extent of enzyme binding; and ultimately result in less hydrolysis in large granules than that in small granules (Tester et al, 2004; Cottrell et al, 1995).

5. CHEMICAL MODIFICATION OF STARCHES

Starches from various plant sources, such as wheat, maize and rice, have received extensive attention in relation to structural and physico-chemical properties (Takeda & Preiss, 1993). Limitations like low shear stress resistance, thermal resistance, thermal decomposition and high retrogradation of native starches limit their industrial applications. These shortcomings can be overcome by chemical and physical modification of starches (Fleche, 1985). There are several literature reports describing the use of chemically modified starches for drug delivery systems (Chakraborty et al, 2005). Epichlorohydrin cross linked high amylose has been used for the controlled release of contramid (Lenaerts et al, 1998). A complex of amylose, butan-1-ol, and an aqueous dispersion of ethylcellulose has been used to coat pellets containing salicylic acid to treat colon disorders (Vandamme et al 2002). The modified starches generally exhibit better paste clarity, stability and increased resistance to retrogradation (Agboola et al, 1991). In chemical starch modification, cross-linking and substitution are used to produce modified starches with desired applications. For example, acetylation of starches is an important substitution method that has been applied to the starches that impart the thickening during many food and non food applications. Cross-linked starches have been used as food additives for a long time because of their non-toxicity and low cost. Cross-linking is generally carried out by treating the granular starch with multifunctional reagents that form either ether or ester inter-molecular linkages between hydroxyl groups on the starch molecules (Rutenberg & Solarek, 1984; Wurzburg, 1986). Sodium trimetaphosphate (STMP), monosodium phosphate (SOP), sodium tripolyphosphate (STPP), epichlorohydrin (EPI), phosphoryl chloride ($POCl_3$), a mixture of adipic acid and acetic anhydride, and vinyl chloride are the important food grade cross-linking agents (Wu & Seib, 1990; Yeh & Yeh, 1993; Yook et al., 1993; Woo & Seib, 1997). STMP has been reported to be an effective cross-linking agent at high temperature with semi-dry starch and at warm temperature with hydrated starch in aqueous slurry (Kerr & Cleveland, 1962). EPI is poorly soluble in water and partly decomposes to glycerol, and also EPI cross-links are

likely to be less uniformly distributed than STMP ones (Shiftan et al., 2000). $POCl_3$ is efficient in aqueous slurry at $pH > 11$ in the presence of a neutral salt (Felton & Schopmeyer, 1943). Therefore, the cross-linking agent greatly determines the change in functional behaviour of the modified starches. Starch phosphates have been reported to give clear pastes of high consistency, and are classified into two groups: monostarch phosphates and distarch phosphates (cross-linked starches). Monostarch phosphates (monoesters) can have a higher DS than distarch phosphates (diesters) as even a very few cross-links (in the case of diesters) can drastically change the paste and gel properties of the starch. Starch phosphates are prepared by reacting starch with salts of ortho-, meta-, pyro-, and tripolyphosphoric acids and phosphorus oxychloride (Paschall, 1964; Nierle, 1969). Lenaerts et al (1991) suggested the use of cross-linked starches as an excipient for the production of controlled release solid oral dosage forms of drugs. Drug release rate of the high amylose starch excipients crosslinked using epichlorohydrin has been reported to increase with increasing cross-linking degree of the polymer (Lenaerts et al, 1992). The benefits of high amylose corn starch, gelatinized and treated with between 1 and 10% short chain cross-linking agents are: high active ingredient core loading, possibility to obtain quasi zero-order release profiles, and very low sensitivity of release profiles to manufacturing conditions such as i.e. tableting pressure (Lenaerts et al, 1992; Lenaerts et al, 1998; Mateescu et al, 1995). Pal et al (2006) prepared a starch based hydrogel membrane by crosslinking of polyvinyl alchohol with starch suspension using glutaraldehyde as a crosslinking agent, and proposed that the membrane had sufficient strength to be used as artificial skin.

Acetylated starches are produced with acetic anhydride in the presence of an alkaline agent like sodium hydroxide (Wurzburg 1978). The acetylation of starches depends upon factors such as starch source, reactant concentration, reaction time and pH. The extent of physicochemical property changes in the acetylated starch compared to the native starch is proportional to the degree of acetylation or degree of C=O substitution incorporated into the starch molecules (Phillips et al, 1999). The degree of acetylation in chemically modified starches is calculated by wet chemistry methods that involve separation and titration methods. The wet chemistry methods assume that the modified starch samples have been purified and are free of any residual compounds that could interfere with the titration used to measure the degree of acetylation (Phillips et al, 1999). Infrared and Raman spectroscopy have been recognized as powerful analytical techniques in the industry for many years (Phillips et al, 1999) and can be used to study the level of acetylation in different starches. The methods involve the calibration of a curve for the level of acetylation versus the intensity ratio of the C=O stretch Raman band to a C-C stretch Raman band. The intensity of the Raman peaks increases linearly with the amount of compound present in the sample (Hendra et al, 1991). Betancur et al (1997) studied the physico-chemical, rheological and functional properties of acetylated *Canavalia ensiformis* starch and reported that starch acetylated with 10% acetic anhydride at pH 8.0–8.5 for 30 minutes reached 2.34% acetyl value and compared to native starch these acetylated starches showed lower gelatinization

temperatures, an increased paste and gel clarity, solubility, swelling power and viscosity. Starch has also been used as a carrier for phenethylamines (Weiner et al, 1972), estrone (Won et al, 1997), and acetylsalicyclic acid (Laakso et al, 1987). Won et al (1997) prepared bromoacetylated starch using bromoacetyl bromide to provide more reactive sites for coupling of bioactive estrone and a suitable spacer between the drug carrier and the hormone. The starch-estrone conjugate was then prepared by reacting the modified starch with the sodium salt of estrone. The structures of the modified starch and the conjugate were predicted using FTIR, ^{1}H NMR, ^{13}C NMR, and UV. It would be beneficial if starch esters used as matrices for drug delivery could be prepared so that they are modified at selected positions of the glucose residues (i.e., at only the primary or secondary positions). This is difficult because of the presence of three hydroxyl groups per glucose residue each in different chemical environments. Also, starch should be solubilized in polar aportic solvents to achieve homogeneous modification (Chakraborty et al, 2005). Chakraborty et al. (2005) carried out the selected esterification of starch nanoparticles using *Candida antartica* Lipase B (Cal-B) as a catalyst. Starch nanoparticles were treated with vinyl stearate, ε-caprolactone and maleic anhydride at 40°C to form starch esters with varying degrees of substitution.

6. CONCLUSIONS

Progress in understanding the factors affecting starch functionality, and the results of chemical modification, has enabled the starch industry to produce starches with desired and improved functional characteristics. The physico-chemical characteristics of starches such as granule size distribution, amylose to amylopectin ratio and lipids content provide a crucial basis for understanding the underlying mechanisms of starch functionality in different systems. Recent advances in the field of starch chemistry and technology reflect the potential of starches isolated from various botanical sources for use in biomedical and pharmaceutical applications.

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CHAPTER 6

ALTERNATIVE APPLICATIONS FOR DRUG DELIVERY: NASAL AND PULMONARY ROUTES

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Abstract: For treatment of human diseases, nasal and pulmonary routes of drug delivery are gaining increasing importance. These routes provide promising alternatives to parenteral drug delivery particularly for peptide and protein therapeutics. For this purpose, several drug delivery systems have been formulated and are being investigated for nasal and pulmonary delivery. These include liposomes, proliposomes, microspheres, gels, prodrugs, cyclodextrins and others. In this chapter, nasal and pulmonary drug delivery mechanisms and some of the relevant drug delivery formulations are evaluated

Keywords: drug delivery systems, pulmonary drug delivery, nasal drug delivery, peptide delivery, protein delivery, liposomes, microspheres

1. INTRODUCTION

Only few decades ago, pulmonary and nasal (intranasal) applications of drugs were not as widespread as it is today. In the year 2000, there were 27 products on the U.S. market for intranasal use, with more than half of these having obtained FDA approval between the years 1990 and 2000. With ever-increasing pharmaceutical technology and numerous medicinal opportunities for intranasal administration, its popularity will most likely continue [1].

Pulmonary and intranasal drugs may be administered for local treartment or systemic action based on the therapeutic intention. Physicotropic drugs, hallucinogenes (cocain), snuffs, antibiotics, vasoconstrictors, antihystaminics and local anesthetics are the examples of nasal drugs administered locally in several dosage forms like nasal solutions, ointments and sprays. Recent observations of side effects of intranasally administered antihistaminic and vasoconstrictor drugs have leaded to their systemic use [2]. Intranasal drugs for systemic action include treatments for migraine headaches, calcium supplementation, Vitamin B12 deficiency and pain

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relief as well as other therapeutic indications. In addition to either local or systemic effects, drugs may be intended for acute or chronic treatments [1].

Additionally, delivery of drugs to or via the respiratory tract can offer several advantages over alternative routes of administration. In general, pulmonary administration of drugs is more satisfactory if the intention is to achieve local action within the respiratory tract.

2. ADVANTAGES OF INTRANASAL DRUG ADMINISTRATION

With optimized formulations, intranasal administration presents many benefits when compared to alternative delivery routes (1–3). These include:

- Not only is the nasal cavity easily accessible, it is virtually non-invasive;
- In most cases, intranasal administration is well tolerated;
- Only slight irritation may occur due to the chemical nature of substance delivered;
- Hepatic first-pass metabolism is avoided with intranasal delivery;
- Destruction of drugs by gastric fluid is not a concern;
- Intranasal mucosae has a big number of microvilli, therefore has a high surface area (150 cm^2);
- Subepithelial tissue has a high vascularization;
- It offers lower doses with more rapid attainment of therapeutic blood levels;
- Quicker onset of pharmacological activity;
- Fewer side effects;
- High total blood flow per cm^3;
- Porous endotheliel basement membrane;
- Drug is delivered directly to the brain along the alfactory nerves.

3. WHICH TYPES OF DRUGS ARE ADMINISTERED INTRANASALLY?

Since many years, nasal route has been used for delivery of drugs and similar other bioactive substances such as illicit drugs, psycotrops, snuffs, etc. Generally the following material are being considered for intranasal delivery:

- Drugs hardly absorbed by oral route;
- Drugs metabolized in the GI tract; and
- Drugs exposed to the first-pass effect of liver can be administered intranasally [2, 3].

4. NASAL ANATOMY AND PHYSIOLOGY

Nasal cavity is circulated by cranium base at the bottom, hard palate at the top and nares and pharynx. The distance from the tip of the nose to the pharyngeal wall is about 10–14 cm and has a 160 cm^2 surface area. The nasal septum divides the nose into two nasal cavities, each with a 2–4 mm wide slit opening and contains three distinct functional regions: vestibular, respiratory and olfactory [1, 2, 4].

The respiratory region contains the largest surface area and is located between the vestibular and olfactory regions. The respiratory region is the most important part for drug delivery administered systemically. The vestibular region is located closest to the nasal passage opening, contains long hairs and serves as a filter for incoming particles. The olfactory region is located in the uppermost portion of each cavity and opposite the septum. This region is responsible for smelling [1].

Nasal mucosa has exopeptidases (like aminopeptidase, diaminopeptidase etc.) and endopeptidases (like cerynproteinase, cysteinproteinase, metalloproteinase, etc.). These enzymes cause enzymatic degredation of peptides and proteins during absorption [5].

The primary function of the nose is olfaction – it heats and humidifies inspired air and also filters airborne particles [6]. Consequently, the nose functions as a protective system against foreign material [7]. The vestibular area serves as a buffer system; it functions as a filter of airborne particles [8]. The olfactory epithelium is capable of metabolising drugs [6]. The respiratory mucosa is the region where drug absorption is optimal [2].

5. NASAL ABSORPTION MECHANISMS

Intranasally administered drugs aimed to obtain systemic effect, pass to the circulation via nasal barrier (epithelium).

The epithelium of the respiratory region consists of four different cell types: basal, mucus-containing goblet, ciliated columnar, and nonciliated columnar. The ciliated columnar cell is the most predominant. The cilia beat in a wave-like, coordinated manner to transport mucus and trapped particles to the pharynx area for subsequent ingestion. Cells in the respiratory region are covered by approximately 300 microvilli, which greatly increase the surface area of the nasal cavity. The respiratory region also contains the inferior, middle and superior turbinates. The lamina propria, below the epithelium houses blood vessels, nerves and both serous and mucus secretory glands [1].

A drug may cross the nasal mucosa by three different mechanisms [1,9]:

i. Transfer via transcellular or simple diffusion across the membrane;
ii. Paracellular transport: Movement through the spaces between cells and tight junctions; and:
iii. Transcytosis (particle internalization by vesicles).

5.1 MUCUS

Mast cells contain polymorphonuclear leucocytes and eosynophyls. Mucus consists of salt 2.5–3%, musin 1–2% (sulphurated scyderoprotein) and water 95%. Lysozymes, enzymes and immunoglobulins, in addition to other proteins, may all be found in the mucus. Proteins and carbohydrates are secreted from endoplasmic reticulum and golgi substance, respectively [2]. Mucus is produced about 1–2 l everyday [2,10]. The mucus consists of an outer viscous layer of mucus and watery

layer located along the mucosal surface [1, 10]. The pH of secretions ranges from 5.5 to 6.5 and from 5.0 to 6.7 in adults and children, respectively [1, 11]. The epithelium is covered with new mucus layer approximately every 10 min [10].

Nasal mucosa is covered by cilia, which does not have the same temperature and movement at every point. The optimum temperature is 18–37°C for mucociliar movement and is blocked at 7–12°C [2].

Nose shows a barrier effect for the inspirated particles and viruses reaching it externally. These particles are retained by the mucus covering the epithelium. The viscous layer of mucus, along with entrapped particles, is transported to the nasopharyngeal area for ingestion [2, 12]. The cilia beat at a frequency which is approximately 10–13 Hz [1, 13].

Mucociliar clerance is affected by several factors such as viscoelasticity of mucus, the thickness of mucus layer, gravity and air flux [2].

6. FACTORS AFFECTING NASAL DRUG ABSORPTION

The physicochemical properties of the drug, nasal mucociliary clearance and nasal absorption enhancers are the main factors that affect drug absorption through the nasal mucosa. One of the greatest limitations of nasal drug delivery is inadequate nasal absorption. Several promising drug candidates cannot be exploited via the nasal route because they are not absorbed well enough to produce therapeutic effects. This has led scientists to search for ways to improve drug absorption through the nasal route [3, 14]. The following parameters need to be considered in order to optimize nasal drug delivery.

a) *Physicochemical Properties of the Drug*: The rate and extent of drug absorption may depend upon many physicochemical factors including the aqueaus-to-lipid partititon coefficient of the drug, the pKa, the molecular weight of the drug, perfusion rate and perfusate volume, solution pH and drug concentration [15]. It has been concluded that in vivo nasal absorption of compounds of molecular weight of less than 300, is not significantly influenced by the physicochemical properties of the drug [16]. There is a direct correlation between the proportion of the nasally absorbed dose and the molecular weight [17].

b) *Mucociliary Clearance*: Particles entapped in the mucus layer are transported with it and, thereby, effectively cleared from the nasal cavity. The combined action of mucus layer and cilia is called "mucociliary clearance". This is an important, non-specific, physiological defence mechanism of the respiratory tract to protect the body against noxious inhaled materials [3, 12]. The normal mucociliary transit time in humans has been reported to be 12 to 15 min [18]. The factors that affect mucociliary clearance include physiological factors such as age, sex, posture, sleep, exercise [19, 20]; common environmental pollutants such as sulphur dioxide, sulphuric acid, nitrogen dioxide, ozone, hair spray and tobacco smoke [21]; diseases including asthma, bronchiectasis, chronic bronchitis, cystic fibrosis, acute respiratory tract infection, immotile cilia syndrome, primary ciliary dyskinesia [21]; drugs [22]; and additives [23].

c) *Nasal Absorption Enhancers:* In order to solve the insufficient absorption of drugs, absorption enhancers are employed. The absorption enhancement mechanisms can be grouped into two classes [3]:
   i. Physicochemical Effects: Some enhancers can alter the physicochemical properties of a drug in the formulation. This can happen by alterning the drug solubility, drug partition coefficient or by weak ionic interactions with the drug; and
   ii. Membrane Effects: Many enhancers show their effects by affecting the nasal mucosa surface [24].

Surfactants, bioadhesive polymer materials, drug delivery systems, cyclodextrins, bile salts, phosphatidylcholines and fusidic acid derivatives are known as absorption enhancers [2, 3].

Nasal absorption of peptides and proteins through nasal mucosa is limited by their high molecular weight. Nasal bioavailability of peptides and proteins is affected by mucociliar clearance and enzyme activity in the nasal cavity. Therefore, nasal bioavailability enhancement can be achieved by different approaches such as modification of chemical structure, prodrug use, addition of absorption enhancers/enzymes and use of mucoadhesive dosage form [5].

7. DRUG DELIVERY SYSTEMS ADMINISTERED INTRANASALLY

For the enhancement of nasal bioavailability, a drug delivery system should have the following properties [2]:

- It should adhere to the nasal mucosa;
- It should pass through the mucus;
- It should cause the formation of viscous layer;
- It should have low clearance;
- It should keep the stability of the drug; and
- It should release the drug slowly.

Some of the commonly used drug delivery systems for nasal administration are explained in the following sections.

7.1. Liposomes and Proliposomes

Liposomes have been used extensively for bioactive delivery by several routes. Alpar et al [25, 26] studied the potential adjuvant effect of liposomes on tetanus toxoid, when delivered via the nasal, oral and I.M. routes compared to delivery in simple solution in relation to the development of a non-parenteral immunization procedure, which stimulates a strong systemic immunity. They found that tetanus toxoid entrapped in DSPC liposomes is stable and is taken up intact in the gut [25, 26].

Intranasal administration of calcitonin-containing charged liposomes in rabbits was investigated to evaluate the in vivo calcitonin absorption performance. Significant level of accumulation of positively charged liposomes on the negatively charged nasal mucosa surface was reported [27]. Plasma calcitonin concentration and pharmacokinetic parameters were calculated. Intranasal bioavailability demonstrated an order of calcitonin containing positively charged liposomes > calcitonin containing negatively charged liposomes > calcitonin solution. The significant enhancement of intranasal bioavailability of calcitonin for positively charged liposomes may be due to charge interaction of positively charged liposomes with the negatively charged mucosa. Marked accumulation of positively charged liposomes on the negatively charged nasal mucosa surface caused high retention of positively charged liposomes on the nasal mucosa which resulted in an increase in residence time with high local concentration of calcitonin [27].

The major cause of mortality in patients with cystic fibrosis (CF) is a lung malfunction. A DNA–liposome formulation was delivered to the nasal mucosa of CF patients in repeated doses. It was reported that the DNA containing liposomes can be succesfully re-administered without apparent loss of efficacy for CF treatment [28].

In a comparative permeability study, insulin liposomes have permeated more effectively after pre-treatment by sodium glycocholate when compared to non-encapsulated insulin solution [29].

Goncharova et al [30] have mentioned the importance of nasal mucosa for the immunisation against Tick-Borne encephalitis. To study intranasal immunization against TBE virus, biodegredable micelles, cationic liposomes and live attenuated bacterial/viral vectors were chosen. The results showed the expression of the gene in transfected cells, thereby demonstrating that the liposomal formulations are suitable for mucosal immunization [30].

In another study using nicotine proliposomes, it has been reported that nicotine delivery was prolonged in rats when administered intranasally [31].

7.2. Microspheres

Microspheres of different ingredients have been evaluated as nasal drug delivery systems. Microspheres of starch, albumin, chitosan, and DEAE-dextran have been investigated. Chemical class of the polymer, binding ability, penetration, polymer concentration, pH, and hydration level are among the factors affecting intranasal delivery [1].

Degredable Starch Microspheres (DSM) is the most frequently used microsphere system for nasal drug delivery and has been shown to improve the absorption of insulin in particular and other bioactive compounds in general. Insulin administered in DSM to rats resulted in a rapid dose-dependent decrease in blood glucose [32,33]. In another study in rabbits, apomorphine release from DSM microspheres was compared with CMC and lactose applied intranasally and the fastest absorption was obtained with lactose [34].

Illum et al [35] introduced well-characterized bioadhesive microspheres for prolonging the residence time in the nasal cavity of human volunteers. The slowest clearance was detected for DEAE-dextran, where 60% of the delivered dose was still present at the deposition site after 3h. On the contrary, these microspheres were not successful in promoting insulin absorption in rats [36].

Human growth hormone (hGH)-loaded microparticles prepared by polycarbophil-cysteine (PCP-Cys) in combination with glutathione (GSH) represented a promising tool for the delivery of hGH for nasal bioavalability [37].

In another study, microspheres intended as a sustained release carrier for oral or nasal administration were prepared by polyacrylic acid molecules [38]. A model drug oxyprenolol HCl was chosen and it was found that some of the formulation variables can influence the release characteristics. The internal structure (by X-ray diffraction, thermal analysis and optical microscopy) and release mechanism were investigated. The work revealed the potential of this pharmaceutical system as an alternative controlled-release dosage form for the intranasal administration [38].

7.3. Gels

Chitosan and chitin have been suggested for use as vehicles for the sustained release of drugs. A sustained drug release based on chitosan salts for vancomycin hydrochloride delivery has been investigated by using different chitosan salts like aspartate, chitosan glutamate and chitosan hydrochloride. Vancomycin hydrochloride was used as the peptidic drug, the nasal sustained release of which should avoid first-pass metabolism in the liver. This in vitro study evaluated the influence of chitosan salts on the release behaviour of vancomycin hydrochloride and it has been reported that in vitro release of vancomycin was retarded mostly by chitosan hydrochloride [39]. Similar results were obtained by Tengamuay et al [40].

Vila et al [41] have prepared chitosan nanoparticles by an ionics cross-linking technique and used tetanus oxoid as model antigen. These nanoparticles were administered intranasally to mice in order to study their feasibility as vaccine carriers. In vitro release studies showed an initial burst followed by an extended release of active toxoid. Following intranasal administration, tetatanus toxoid-loaded chitosan nanoparticles elicited an increasing and long-lasting immunogenity as compared to the fluid vaccine. Interestingly, the ability of these nanoparticles to provide improved access to the associated antigen to the immune system was not significantly affected by the chitosan molecular weight. High and long lasting responses could be obtained with low molecular weight chitosan molecules.

Additionally, the response has not been influenced by the chitosan dose. This group concluded that nanoparticles made of low molecular weight chitosan are promising carriers for nasal vaccine delivery [41].

It was observed that the chitosan delivery (microspheres) of a drug had significantly reduced rates of clearance from the nasal cavity as compared to the control

(solution). Chitosan delivery systems have the ability to increase the residance time of drug in the nasal cavity thereby providing the potential for improved systemic medication [42].

Insulin loaded chitosan nanoparticles have been prepared with trehalose as cryoprotectant by freeze-drying method. The in vivo evaluation of chitosan nanoparticles in rabbits revealed that these nanoparticles are able to reduce glucose levels to a greater extent than insulin-chitosan solution when applied intranasally [43,44].

Nasal absorption of nifedipine from gel preparations, PEG 400, aqueous carbopol gel and carbopol-PEG has been studied in rats. Nasal administration of nifedipine in PEG resulted in rapid absorption and high c_{max}; however, the elimination of nifedipine from plasma was very rapid. The plasma concentration of nifedipine in aqueous carbopol gel formulation was very low when administered intranasally. The use of PEG 400 in high concentrations in humans should be considered carefully. This is because PEG 400 is known to cause nasal irritation in concentrations higer than 10% [45].

Nasal absorption of Calcitonin and Insulin from polyacrilic acid gel has been investigated in rats. It has been reported that nasal absorption of insulin is greater from 0.15% (w/v) polyacrylic acid gel than from 1% (w/v) gel. There seem to be an optimum concentratiton and possibly an optimum viscosity for the polyacrilic acid gel base [46].

Ugwoke et al [47] have prepared apomorphine mucoadhesive preparations incorporating Tc-99m labelled colloidal albumin. Drug residence time in rabbit nasal cavity was evaluated by gamma scintigraphy using different agents like Carbopol 971P, CMC and lactose (control), each with or without apomorphine. The use of mucoadhesives such as Carbopol 971P or CMC in nasal gels increases their residence time within the nasal cavity and provides opportunity for sustained nasal drug delivery [47].

7.4. Other Delivery Systems

Phosphatidylcholines are surface-active amphiphilic compounds present in biological membranes and liposomes. Several reports have appeared in the literature showing that these phospholipids can be used for enhancing the systemic nasal drug delivery [48].

Another intensive study has been put on fusidic acid derivatives and among these Sodium Tauro-24, 25-dihydrofusidic acid (STDHF) is the most extensively studied derivative of fusidic acid. STDHF was reported as a good candidate for the transnasal delivery of drugs like insulin, octreotide, and human growth hormone [49–52].

Radioimmunoactive bioavailability of intranasal salmon calcitonin was determined in healthy human volunteers. The nasal absorption of calcitonin was improved by STDHF and it caused a limited transient irritation of the nasal mucosa in some subjects [53].

Didecanoyl-L-phosphatidylcholine (DDPC) has been used as enhancer for intranasal insulin administration in human volunteers. It was observed that intranasal insulin administration was absorbed in a dose dependent manner with slight or no nasal irritation [54]. Another study revealed that Glycyrrhetinic acid derivatives enhance insulin uptake without nasal irritaition or insulin degredation [55].

8. CYCLODEXTRINS

Several compounds have been investigated for their nasal absorption enhancement. Cyclodextrins are observed as the best-studied group of enhancers. The most-studied of them are: α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, methylcyclodextrin and hydroxypropyl β-cyclodextrin. Among these, β-cyclodextrin is being considered for possessing a GRAS (Generally Recognised As Safe) status [56, 57].

Cyclodextrins have been used successfully to increase the absorption of many substances including salmon calcitonin [58, 59], insulin [60] and human growth hormone [61].

9. PRODRUGS

The utility of nasal route for the systemic delivery of 17-beta-estradiol was studied using water-soluble prodrugs of 17-beta-estradiol. This method was examined to determine if it would result in preferential way to the brain. In vivo nasal experiments were carried out on rats. Absorption was fast following nasal delivery of prodrugs with high bioavailability. These products were found to be capable of producing high levels of estradiol in the cerebral spinal fluid and as a result may have a significant value in the treatment of Alzheimer's disease [62].

10. PULMONARY DELIVERY SYSTEMS

Studies on the delivery of drugs to or via the respiratory tract have been carried out in the recent 25 years. This route can offer considerable advantages over other drug dministration ways as listed below [63, 64]:

- Provides local action within the respiratory tract;
- Provides rapid drug action;
- Provides reduced dose;
- Allows for a reduction in systemic side-effects;
- Reduces extracellular enzyme levels compared to GI tract due to the large alveolar surface area;
- Reduces evasion of first pass hepatic metabolism by absorbed drug; and
- Offers the potential for pulmonary administration of systemically active materials.

On the other hand, it has some disadvantages as well [63, 64], which include:

- The duration of activity is often short-lived due to the rapid removal of drug from the lungs or due to drug metabolism; and
- Necessitates frequent dosing.

10.1. Which Types of Drugs are Administered via Pulmonary Route?

Drugs are absorbed from the lungs mainly by the following two mechanims:
i) Passive diffusion; and
ii) Active endocytosis [65].
Drugs for asthma, allergy and chronic obstructive pulmonary diseases are used via pulmonary route. Beta agonists, anticholinergic drugs, mucolytics and corticosteroids are some examples for these drugs [5].

10.2. Pulmonary Anatomy and Physiology

From the trachea, the airways divide dichotomously to form bronchi, respiratory and terminal bronchioles and ultimately alveoli. The role of the airways gradually changes from one of conduction by the large airways to one of gaseous exchange for the peripheral lung (respiratory bronchioles and alveoli) [64].

Nearly 95% of the alveolar cells are Type I cells which are 5 μm in size. Type II cells are 10–15 μm in size and secrete surfactants which are important for the function of the lungs. Phosphatidylcholine and phosphatidylglycerol are the main phospholipids of lung surfactants [65]. Lung surfactants deposit a monomolecular film on the alveoli and prevent pulmonary oedema and provide protection against infections [66].

10.3. Factors Affecting Pulmonary Delivery

The size of inhaled particles is the main factor affecting pulmonary delivery. The important size property for deposition in the lungs is called aerodynamic diameter. It is determined by the actual size of the particle, its shape and its density. The particles in the aerodynamic size range of about 3.5–6.0μm can penetrate, to some extent, at slow inspiratory flow rates beyond the central airways into the peripheral region of the lungs. On the other hand, particles less than 3.5μm and greater than about 0.5μm will mostly bypass the bronchial airways during inhalation and penetrate almost entirely to the deep lung. Larger particles are dominated by their inertial mass and will impact in upper airways due to their inertia. Smaller particles (with aerodynamic diameters less than 0.5μm) are dominated by thermal interactions with the air molecules and will diffuse to the respiratory tract surfaces during inhalation [67].

Diseases of the respiratory tract and hygroscopicity of the powders are the other factors affecting pulmonary delivery [67].

10.4. Pulmonary Drug Delivery Systems

There are three types of conventional methods of inhalation delivery for the treatment of respiratory diseases [67]:
i. Pressurized Metered-Dose Inhalers (MDIs or pMDIs);
ii. Dry Powder Inhalers (DPIs); and
iii. Nebulizers.

The conventional inhalation systems are designed primarily to generate particles of suitable size for topical delivery to the airways.

The lung presents a very attractive route for the invasive delivery of systemically active compounds.

Among the modified-release carrier systems, liposomes are the most frequently used ones. The main advantage of the use of liposomes as drug carriers in the lung is that they can be prepared from phospholipid molecules endogenous to the lung as components of lung surfactant [68]. Secondly, liposomes help to develop controlled release systems for local and systemic delivery. Thirdly, improved pulmonary therapy and lower side-effects can be obtained by liposomal drugs.

Anticancer drugs (ARA-C, 5-fluorouracil), antimicrobials (pentamidin, amikasin, enviroksim), peptides (insulin, calcitonin), enzymes (superoxide dismutase), antiallergic and antihistaminic compounds (salbutamol, metaproterenol), immunosupressive (siklosporin) and antiviral (ribavirin) drugs are some examples of the active compounds used in the pulmonary delivery research (e.g. see Ref. 5). Atropine, benzylpenicillin, carboxyfluorescein, cytarabine, enviroxime, glutathione, glyceryltrinitrite, orciprenaline, oxytocine and pentamidine are other examples of several drugs delivered to the lungs of the animals [64].

Another group of researchers have been studying the delivery of the genetic drugs via the lungs [69, 70] while progress and improvements in the field are ongoing.

11. CONCLUSION

Nasal and pulmonary routes of drug delivery are increasingly gaining importance in drug therapy. Particularly, these routes are considered as alternative ways to parenteral route for peptide and protein therapeutics. It has been shown that intranasal and intratracheal administration to the mucosae are important routes and were found effective for the immunospecific reaction response. It has been reported that various therapeutic and vaccine formulations can be administered successfully by thes nasal and pulmonary routes. However, because of the many hurdles in administration, pulmonary delivery is not usually preferred as yet. In conclusion, nasal and pulmonary drug delivery systems, described in this chapter, seem particularly appropriate techniques for drug delivery with great futuristic potential applications.

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CHAPTER 7

AN OVERVIEW OF LIPOSOME-DERIVED NANOCARRIER TECHNOLOGIES

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Abstract: Lipid-based nanocarrier systems are among the most applied encapsulation, targeting and controlled release technologies. They are being used to incorporate and protect materials with different solubilities and deliver them to the site required inside the body as well as outside the body, in vitro. Among the lipid-based encapsulation systems, liposomes and their derivatives are the most applied and further developed. There are some liposome-derived carriers approved for human use on the market, which mainly utilise oral, transdermal and parenteral delivery routes. Research for the development and optimization of liposomal systems for pulmonary and nasal applications are also ongoing. Methods of preparation of these micro- and nanocarriers have evolved to exclude utilisation of harmful substances such as toxic organic solvents and also enable preparation of safe and efficient systems on industrial scales. In this chapter, an overview of eight different liposome-derived nanocarriers with respect to their characteristics, preparation methods and application is presented

Keywords: Lipidic systems, archaeosomes, multivesicular vesicles, vesicular phospholipid gels, cochleates, virosomes, transferosomes, immunoliposomes, stealth liposomes

1. INTRODUCTION

Liposomal carrier systems are among the most promising encapsulation technologies employed in the rapidly developing field of nanobiotechnology. Liposomes and nanoliposomes are being used successfully as models of biomembranes and also as delivery and controlled release systems for drugs, diagnostics, nutraceuticals, minerals, food material and cosmetics to name but a few (Mozafari & Mortazavi 2005; Mozafari et al 2006). Due to the extra-ordinary success of liposome technology in so many fields, both in research and industry, several liposome-derived systems have been developed in recent years. These carrier systems are

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being made on micro- and nano-scales (from around 20nm to several micrometers) with different levels of complexity to meet specific applications. Some of these carriers are composed of lipids and phospholipids, while some others contain other molecules such as carbohydrates and proteins in their structure.

Compared with other encapsulation strategies such as chitosan- and alginate-based carriers (Anal et al 2003; Anal & Stevens 2005; Bhopatkar et al 2005), liposome-derived encapsulation systems have unparalleled advantages. These include the ability to entrap material with different solubilities, the possibility of being produced using natural ingredients on an industrial scale, and targetability (Mozafari 2004; Yurdugul & Mozafari 2004; Mozafari & Mortazavi 2005; Mozafari 2006). Liposomal carriers can shield an ingredient from free radicals, metal ions, pH and enzymes that might otherwise result in degradation of the bioactive compound. They impart stability to water-soluble material, particularly in high water-activity applications (Gouin 2004). They can accommodate not only water-soluble material, but also lipid-soluble agents and amphiphilic compounds simultaneously, providing a synergistic effect (Suntres & Shek 1996). Another unique property of liposome-based micro- and nano-carriers is the targeted delivery of their content both in vivo and in vitro. In general, these carriers may be targeted to the required site inside the body via active (e.g. by incorporation of antibodies) and passive (e.g. targeting based on particle size) mechanisms (Mozafari & Mortazavi 2005; Mozafari 2006). Some of the main liposome-derived carrier technologies are explained in this chapter.

2. ARCHAEOSOMES

Archaeosomes can be defined as liposomes made from one or more of the polar ether lipids extracted from the domain Archaea (Archaeobacteria). Although Archaea and Bacteria are both prokaryotes, Archaea are more closely related to the domain Eucarya than to Bacteria (Krieg 2001). Many Archaea live in environments including high salt concentrations or low pH values and high temperatures. Hence their membrane lipids are unique and enable them to survive in such hostile conditions. The core lipids (polar head groups removed) of archaea consist of archaeols (diethers) and caldarchaeols (tetraethers), wherein the regularly branched, 5-carbon repeating units forming the isoprenoid chains (usually 20 carbons per chain in archaeols, and 40 carbons per chain in caldarchaeols) are attached via ether bonds at the sn-2,3 position of the glycerol carbons. In contrast to this, the core lipids found in Bacteria and Eucarya consist of unbranched (mostly) fatty acyl chains, often unsaturated, attached via ester bonds to the sn-1,2 glycerol carbons. The polar moieties (archaeols are monopolar and caldarchaeols are bipolar) are similar to those (phospho, glyco, polyol, amino, hydroxyl groups) encountered in ester lipids, but phosphatidylcholine is rarely present in archaeal lipids (Mozafari et al 2005). Although archaeosomes are a recent technology, they have already proven to be a safe delivery system for bioactive agents including drugs and vaccines (Patel & Chen 2006).

Compared with liposomes (which are made from ester phospholipids), archaeosomes are relatively more thermostable, more resistant to oxidation and chemical and enzymatic hydrolysis. They are also more resistant to low pH and bile salts that would be encountered in the gastrointestinal tract (Patel et al 2000). Archaeosomes prepared from the total polar lipid extract or from individual purified polar lipids show promise as adjuvants that promote strong humoral and cytotoxic T-cell responses to encapsulated soluble antigens. Therefore, there is a great potential for using archaeosomes in drug, vaccine and other bioactive material delivery applications. As is the case with liposomes, it is possible to incorporate ligands such as polymers to archaeosomes. It has been shown that incorporation of polyethyleneglycol and Coenzyme Q10 into archaeosomes can alter the tissue distribution profiles of intravenously administered vesicles (Omri et al 2000). Omri et al (2003) have recently reported that intravenous and oral delivery of nanometric-sized archaeosomes to an animal model was well tolerated with no apparent toxicity. The results of these studies are very promising for the utilisation of archaeosomes in the encapsulation and delivery of different bioactive compounds.

3. MULTIVESICULAR LIPOSOMES

Multivesicular liposomes (MVL) - or multivesicular vesicles (MVV) - are composed of several small vesicles entrapped by a single lipid bilayer (Figure 1). MVLs prepared by a multiple emulsion method, possess a unique structure of multiple, nonconcentric, aqueous chambers surrounded by a network of lipid membranes (Kim et al 1983). The structure of MVL has a higher aqueous volume with

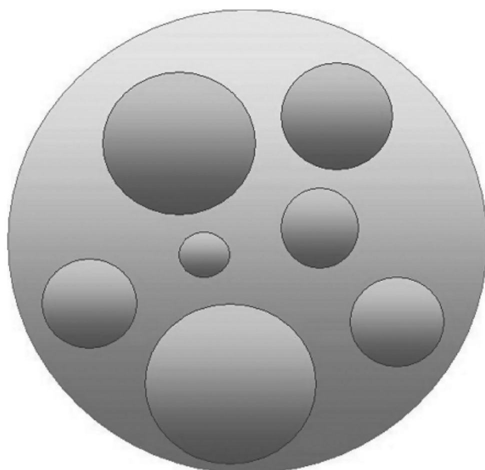

Figure 1. A multivesicular liposome in which several bilayer vesicles are encapsulated by a single bilayer vesicle, mainly composed of phospholipid molecules (From Mozafari and Mortazavi 2005, with permission)

respect to its lipid ratio and much larger particle diameter compared with multilamellar vesicles (MLVs) (Kim et al 1983; Ye et al 2000). Hence, MVLs have high capacity for loading water-soluble compounds with high encapsulation efficiencies. The bioactive agent is encapsulated within the nonconcentric internal aqueous chambers and is released over an extended period of time. The multivesicular nature of MVLs provides sustained release of encapsulated substance since, unlike unilamellar type liposomes, a single breach in the external membrane of a MVL will not result in a total release of the internal aqueous contents (Kim et al 1983; Ye et al 2000). A multivesicular liposome can be prepared by a process comprising the following steps (Kim et al 1983): (i) forming a water-in-lipid emulsion from two immiscible components, i.e. a lipid component (e.g. amphipathic lipids, one or more organic solvents, and a neutral oil such as triolein or trioctanoin) and an aqueous component containing the material to be encapsulated in MVLs; (ii) dispersing the water-in-lipid emulsion into a second aqueous component to form solvent spherules; and then (iii) removing the organic solvent from the solvent spherules to form the multivesicular liposomes suspended in the second aqueous component.

A recent application of multivesicular liposomes was for the encapsulation and release of the antineoplastic agent cisplatin in mice inoculated with a murine carcinoma tumor (Xiao et al 2004). The authors found out that cisplatin-MVLs exhibit high encapsulation efficiency, prolonged sustained release and higher drug accumulation in tumor regions when compared to the un-encapsulated form of the drug (Xiao et al 2004).

4. VIROSOMES

Virosomes (Kara et al 1971; Almeida et al 1975), or artificial viruses, are one type of liposome that contain reconstituted viral proteins in their structure. Unlike viruses, virosomes are not able to replicate but are pure fusion-active vesicles. Due to the presence of the specialized viral proteins on the surface of virosomes, they can be used in active targeting (Mozafari 2006) and delivery/controlled release of their content at the target site. Viruses have developed the ability to fuse with cells during the course of evolution, thus, allowing for release of their contents directly into the cell. This is due to the presence of fusogenic proteins on the viral surface that facilitate this fusion. If these fusogenic viral proteins are reconstituted on the surface of a liposome then the liposome also acquires the ability to fuse with cells. This is an extremely useful tool in active transport because it allows the direct release of the liposomal contents into the cell. As there is no diffusion of the bioactive material involved, it results in a more effective delivery. The most common viruses used in the construction of virosomes are the Sendai, Semliki Forest, influenza, herpes simplex, and vesicular stomatitis viruses. The presence of virus proteins not only allows the liposome to target a particular cell but also allows it to fuse with the cell ensuring direct delivery of the incorporated material (Lasic 1993).

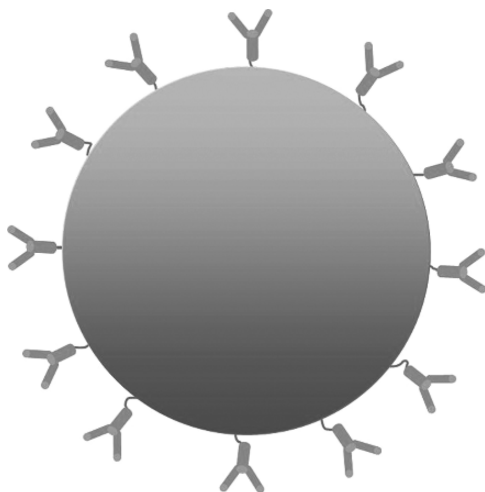

Figure 2. Schematic presentation of an immunoliposome containing antibody molecules on its surface (From Mozafari and Mortazavi 2005, with permission)

5. IMMUNOLIPOSOMES

Another class of lipid vesicles designed for active targeting of their encapsulated/entrapped material inside the body is known as immunoliposomes. The immunoliposomes (Huang et al 1981; Mizoue et al 2002) possess moieties such as antibodies, carbohydrates, and hormones on the outer surface of their membrane (Figure 2). The various ligands can be attached to the outer surface of the lipid vesicles by either insertion into the membrane, adsorption to the surface, via biotin-avidin pair or through the most preferable method, covalent binding (Lasic 1993). These ligands attached to the immunoliposome have a complementary binding site on the target cell. Therefore when the liposome arrives within the area of the target cell it will bind to this cell. Consequently the drug will be released into the surrounding region of the target cell minimising harm and side-effects to healthy cells and tissues. In a recent study, immunoliposomes have been used for gene targeting to human brain cancer cells, which has resulted in a 70-80% inhibition in cancer cell growth (Zhang et al 2002).

6. STEALTH LIPOSOMES

Considerable amount of research and studies have been devoted to develope carrier systems that can avoid phagocytosis and thus circulate longer in the blood. As a result of these studies the so-called "Stealth" particles have emerged. Stealth carriers can be made by covering the surface of the bioactive delivery vehicle with hydrophilic chains which prevent opsonisation. Grafting of poly (ethylene glycol) (PEG) is the most effective method and has been applied

to nanoparticles (Gref et al 1994) and liposomes (Woodle and Lasic 1992) to produce sterically stabilised carriers. Other polymers such as poly (hydroxyethyl L-asparagine) (PHEA) have also been considered to increase liposome circulation time (Metselaar 2003). The sterically stabilised liposomes are involved in passive targeting (Mozafari 2006) of the material they carry.

When sterically stabilised liposomes are injected into an individual, who for instance has either a solid tumour or an internal infection, the vesicles will migrate and accumulate in the tumorous or infected area. As the stealth liposomes become degraded, they will release their drugs into the surrounding area (Allen 1994). This is an example of passive targeting because the stealth liposomes are left to their own devices and yet they migrate and treat the injured area. It has been reported that stealth liposomes with diameters between 70 and 200 nm have longer circulation times (Litzinger et al 1994). Another important consideration when using sterically stabilized liposomes is the size of the coating polymer. If it is too large it may interfere with the ligand-receptor binding of the stealth liposome and the target cell.

7. TRANSFEROSOMES

Delivery of various materials through the skin is highly important in different areas particularly in cosmetics and skin care. For transdermal delivery of bioactive agents using carrier systems, the bioactive compounds must be associated with specifically designed vehicles, in the form of highly deformable particles, and applied on the skin non-occlusively. To meet this end, another type of optimised liposome-based carrier system, called transferosome, has been developed (Cevc and Blume 1992; Cevc 1996). Transferosomes consist of phospholipids, cholesterol and additional surfactant molecules such as sodium cholate. The inventors claim that transferosomes are ultradeformable and squeeze through pores less than one-tenth of their diameter. Therefore 200 to 300nm-sized transferesomes are claimed to penetrate intact skin (Figure 3). Penetration of these particles works best under in vivo conditions and requires a hydration gradient from the skin surface towards the viable tissues.

Insulin-loaded transfersomes, for example, were reported to deliver the drug through the non-compromised skin barrier with a reproducible drug effect that resembles closely that of the ultralente insulin (a long acting insulin used in the treatment of diabetes mellitus) injected under the skin with comparable pharmacokinetic and pharmacodynamic properties (Cevc 2003). It has been suggested that transfersomes can respond to external stresses by rapid shape transformations requiring low energy. This high deformability allows them to deliver drugs across barriers, including skin (Cevc et al 1995). To prepare these vesicles, the so called ‘edge activators’ were incorporated into the vesicular membranes. Surfactants were suggested as examples of such edge activators (Cevc et al 1993), and also sodium cholate or sodium deoxycholate have been used for this purpose (Planas et al 1992; Cevc et al 1995; Paul et al 1995; Lee et al 2005).

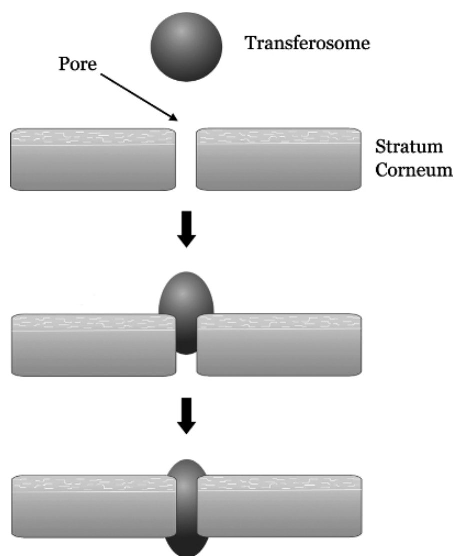

Figure 3. Transferosome penetration through the pores in stratum corneum, the outermost layer of the skin (From Mozafari and Mortazavi 2005, with permission)

8. VESICULAR PHOSPHOLIPID GELS

Vesicular phospholipid gels (VPGs) are highly concentrated phospholipid dispersions of semisolid consistency and vesicular morphology (Brandl et al 1994; Tardi et al 2001). They are under investigation as potential implantable depots for sustained release of bioactive agents (Grohganz et al 2005). VPGs can be prepared by high-pressure homogenisation of high concentrations of phospholipid molecules. Vesicular phospholipid gels can also be prepared by the heating method (Mozafari 2006) without using toxic volatile organic solvents or detergents. Upon dilution, VPGs constitute normal diluted liposome dispersions. During in vitro release tests, Tardi and co-workers found that the incorporated hydrophilic marker (calcein) was released in a sustained manner within periods ranging from several hours up to several days depending on the concentration and composition of the lipids within the matrices (Tardi et al 1998). It appears that vesicular phospholipid gels could be useful as parenteral depot formulations. Alternatively, by mixing with excess buffer, VPGs may be converted to unconcentrated liposome suspensions with small and homogeneous particle sizes possessing high encapsulation efficiencies (Brandl et al 1998). Consequently, VPGs are also useful as intermediates for liposome dispersions, especially those with drugs with high leakage rates and poor storage stabilities such as gemcitabine (Moog 1998). By virtue of the in vitro drug release and the entrapment investigations of VPGs containing bioactive agents such as 5-fluorouracil (Kaiser et al 2003) and chlorhexidine (Farkas et al 2004), good applicability of these carriers is expected as implantable gels or as redispersed liposomes.

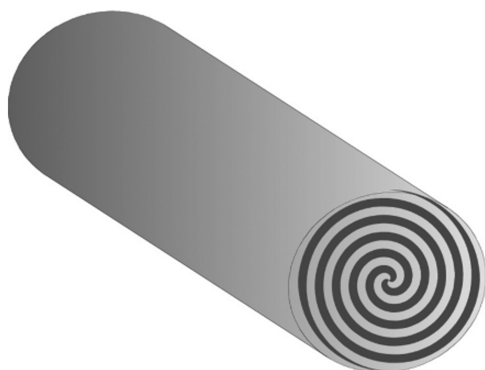

Figure 4. Schematic representation of typical structure of a cochleate

9. COCHLEATES

Cochleates are small-sized and stable lipid-based carriers comprised mainly of a negatively charged lipid (e.g. phosphatidylserine) and a divalent cation such as calcium (Zarif et al 2000; Zarif 2003). They have a cigar-shaped multilayered structure consisting of a continuous, solid, lipid bilayer sheet rolled up in a spiral fashion with little or no internal aqueous space (Figure 4). Hydrophobic, amphiphilic, negatively or positively charged molecules can be delivered by cochleates. Cochleates and their sub-micron versions (i.e. nanocochleates) have been used to deliver proteins, peptides and DNA for vaccine and gene therapy applications (Mannino & Gould-fogerite 1997; Zarif & Mannino 2000). Due to their nanometric size, stability and resistance to degradation in the gastrointestinal tract nanocochleates have revealed great potential to deliver bioactive agents both orally and parenterally (Mannino & Gould-fogerite 1997; Zarif & Mannino 2000; Zarif et al 2000; Zarif 2003). Cochleates containing amphotericin B (AmB) are now in development to enter Phase I clinical trials, for both the oral and parenteral treatment of fungal infections (Zarif 2003). The unique structure and properties of cochleates make them an ideal candidate for oral and systemic delivery of sensitive material including peptide and nucleic acid drugs.

10. SUMMARY

Several liposome-derived bioactive delivery systems have been developed for specialized applications as described in this chapter. Some of these carriers can be employed for active delivery of encapsulant, while others are suitable for passive bioactive delivery. These systems provide a choice of optimized encapsulation and delivery for various applications including systemic and transdermal delivery as well as the choice of short or long-term release. The commercialization of these encapsulation systems is progressing, as is the development of their preparation methods. Safe and reproducible manufacture of these carriers on industrial scales is

now possible. The development of these encapsulation technologies and associated products, for pharmaceutical, cosmetics and food industries, continues to be pursued actively by a number of groups globally. Accordingly, it is reasonable to project that this field will experience steady growth for the foreseeable future.

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CHAPTER 8

UPTAKE STUDIES OF FREE AND LIPOSOMAL SCLAREOL BY MCF-7 AND H-460 HUMAN CANCER CELL LINES

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Abstract: The aim of this study was to investigate the uptake of free and liposomal sclareol and its effect on the growth inhibiting activity against MCF-7 and H-460 human cancer cell lines in vitro. Liposomes composed of EPC/DPPG at molar ratio 9:0.1, used to incorporate sclareol, were prepared by the thin-film hydration method followed by sonication. The final liposomal preparation (EPC/DPPG/Sclareol 9:0.1:5 molar ratio) as well as free sclareol (100μM) were incubated up to 96 hours with both cell lines. Sclareol was extracted from cells using the Bligh-Dyer method and was measured by HPTLC/FID. The results showed that the uptake of free sclareol by both cell lines was faster and higher compared to that of its liposomal form. In both cell lines, free sclareol showed high cytotoxicity, while the liposomal sclareol showed reduced cytotoxicity without affecting its ability to reduce the cell growth rate. These findings suggest that liposomal sclareol may possess chemotherapeutic advantages over its free form and can be used for future in vivo experiments for the treatment of these two types of human cancer

Keywords: Sclareol, liposomes, cytotoxicity, uptake, breast cancer, lung cancer

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Abbreviations: EPC: egg- phosphatidyl choline; DMSO: dimethyl sulfoxide; DPPG: dipalmitoyl phosphatidylglycerol; HPTLC/FID: High Performance Thin Layer Chromatography/Flame Ionization Detector; NCI: National Cancer Institute; NIH: National Institutes of Health, RPMI: Roswell Park Memorial Institute

1. INTRODUCTION

The most common types of cancers in adults are: breast, lung, colon and prostate cancer. Early diagnosis and prompt treatment including chemotherapy still hold out the hope of long-term survival. Breast cancer in women is the leading cause of death in women aged 35–54. Metastases to lung, liver, bone marrow, brain and other sites is the reason of death. Drug therapy for breast cancer includes cytotoxic agents among others like hormonal agents (Pratt *et al*. 1994). Lung cancer is divided into two major types; non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). SCLC differs from NSCLC in that it grows rapidly and responds better to chemotherapeutic agents. NSCLC is heterogeneous aggregate of at least three distinct histologies of lung cancer including epidermoid or squamous carcinoma, adenocarcinoma and large-cell carinoma (Pakunlu *et al*. 2004). It grows slowly and does not respond well to chemotherapy. Treatment depends on a number of factors, including the type of lung cancer (non-small or small cell lung cancer), the size, location, and extent of the tumor, and the general health of the patient. Many different treatments such as surgery, chemotherapy, radiation therapy, photodynamic therapy, and combinations of them may be used to control lung cancer, and to improve quality of life by reducing symptoms (http://www.cancer.gov).

In anticancer therapy and particularly in chemotherapy, side effects depend mainly on the specificity and the dose of the drug used. The anticancer molecules used, due to their cytotoxicity, affect cancer cells and at the same time other cells that divide rapidly (http://www.cancer.gov). Nanotechnology can provide benefits in anticancer chemotherapy by increasing the specificity of drugs and delivering the bioactive molecules to the target site, hence reducing their toxic side effects. The delivery of cytotoxic molecules to tumor cells is an important aspect in the area of anticancer therapy and several delivery systems have been used as adequate for improving the delivery of biologically active molecules to target cells (Books *et al*. 2005; Gupta *et al*. 2005).

In the literature there have been many reports on the use of phospholipid vesicles as carriers for introducing biologically active substances into cells *in vitro* and *in vivo* (Allen *et al*. 1981). Liposomes are nowadays considered as non-toxic lipidic drug carriers and have been proven to be an adequate drug delivery system for lipophilic compounds since they can modulate the pharmacokinetic properties of the encapsulated drugs towards a more beneficial and safer use (Allen *et al*. 1999; Drummond *et al*. 1999). Liposomes or lipid vesicles are spherical self-closed

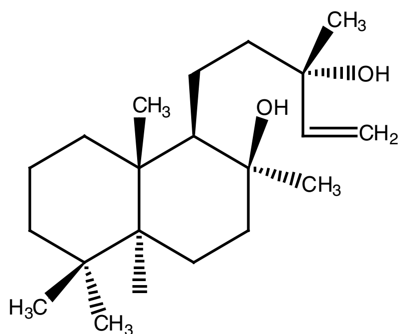


Figure 1. Chemical structure of sclareol

structures composed of curved lipid bilayers, which entrap part of the aqueous medium in which they freely float into their interior. The accumulating evidence from the studies of liposome-cell interactions indicates that liposomes are capable of interacting with cells via several mechanisms occurring simultaneously (Allen *et al.* 1981).

Sclareol (Figure 1) is a labdane diterpene with a structure of a ditertiary alcohol and is found in several plant species (Demetzos *et al.* 2001, 1999, 1990). In previous studies, sclareol exhibited significant cytostatic and cytotoxic effects, mainly in vitro, against several cancer cell lines derived either from leukemia or from solid tumors. It was furthermore found that the compound induced cell cycle arrest and apoptosis, while down regulating the expression of the protooncogene *c-myc*, (Dimas *et al.* 2001, 1999, 1998). Despite its interesting pharmacological actions, sclareol presents high lipophilicity. Additionally, in an attempt to evaluate the anticancer efficacy in vivo, free sclareol found to exhibit significant toxicity when administered intraperitoneally in immunodeficient mice. On the contrary using liposomes we were able to administer in a single cycle a total dose of 1100mg/kg in HCT116 xenografted NOD/SCID mice, which resulted in a significant regression of the tumors (Hatziantoniou *et al.* 2006).

The present study investigates the *in vitro* cytotoxicity of free and liposomal sclareol and the effect on growth rate, based on its uptake by two types of human cancer cells (*i.e.* MCF-7 and H-460).

2. MATERIALS AND METHODS

2.1. Materials

Both cell types (MCF-7 and H-460 cell lines) derived from human tumours, obtained from the NCI (NIH, USA). RPMI 1640, trypsin, L-glutamine, antibiotics, phosphate buffered saline (PBS) and foetal calf serum (FCS) were purchased from Euroclone, U.K. Dyes, salts and buffers as well as sclareol were purchased from Sigma

(Sigma Hellas, Athens). Egg-PC was purchased from Lipoid (Ludwigshafen, Germany), DPPG from Avanti Polar Lipids, Inc. (Alabastar, Alabama, USA) and sucrose from Sigma (St. Louis, MO, USA). All solvents (methanol, ethanol, acetic acid, DMSO) were of analytical grade and purchased from Labscan Ltd. Ireland.

2.2. Methods

Cell culture: Monolayer cultures of MCF-7 and H-460 were adapted to grow in RPMI 1640 medium, supplemented with 5% heat-inactivated FCS, (Euroclone, U.K.), 2 mM L-glutamine and antibiotics (100IU/mL penicillin and 100μg/mL streptomycin). Cells were incubated at 37°C, in a humidified atmosphere with 5% CO_2 (Celis 1994).

Determination of MCF-7 and H-460 cell growth rate: Prior to the application, the lyophilised liposomes were resuspended in deionised water. Free sclareol was diluted in DMSO at a stock of 20mM and kept at 4°C under lightproof conditions. Both were further diluted in supplemented RPMI at a final concentration of 100μM sclareol. Control cultures, in the presence of either DMSO or lipids were added in medium and were run in parallel. No differences in the growth of cells compared to untreated cells were observed in both cases (results not shown). Cells were cultured at plating densities of $3.7*10^6$ and $5*10^6$ cells/dish for H460 and MCF7 respectively, according to their doubling time, for 24h (adaptation time) prior to addition of the drug. After drug addition, the dishes were incubated up to 96h at predetermined time intervals (2, 4, 8, 16, 24, 48, 72 and 96 h). Control cultures received no drug. Cells were then trypsinized and counted using the Trypan blue dye exclusion method (Green and Moehle 1999). The cell growth rate was calculated according to the equation: $(T–C_0/U–C_0)*100$ when $T \geq C_0$ or $(T–C_0/C_0)*100$ when $T < C_0$, where C_0 is the number of viable cells right before adding the drug, T is the number of viable cells treated with sclareol and U is the number of viable cells for the untreated cultures. In that way negative numbers denote cytotoxic activity (Hatziantoniou *et al.* 2006).

Liposome preparation: EPC/DPPG liposomes were prepared by the thin-film hydration method (Hatziantoniou *et al.* 2006). The lipid film was prepared by EPC:DPPG:Sclareol 9:0.1:5 molar ratio and dried under vacuum for 12 h. Multi-lamellar vesicles (MLVs) were prepared by hydrating the lipid film with 0.15 M sucrose (sucrose to lipid ratio 2.24 w/w), above the gel to crystalline phase transition of the lipid (41°C), and stirring for 1 h. The resultant liposomal suspension was subjected to sonication for two 5 min periods interrupted by a 5 min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0.7 – UP 200S, dr. hielsher GmbH, Berlin, Germany). The liposomal suspension was allowed to anneal any structural defects for 30 min and was centrifuged in order to separate the Small Unilamellar Vesicles (SUVs) from MLVs and from the titanium particles contributed from the sonicator probe. Subsequently, the liposomal suspension was freeze-dried and stored at 4°C. Size and ζ-potential of liposomes are the parameters that indicate their physical stability. 100 μl of the liposome dispersion was diluted

10-folds in HPLC-grade water (pH 5.6–5.7) immediately after preparation and mean z-average and ζ-potential of the empty and loaded SUVs were measured in order to determine the effect of sclareol loading on liposomal size and ζ-potential. Samples were scattered (633 nm) at a 90° angle and measurements were made at 25°C in a photon correlation spectrometer (Zetasizer 3000HS, Malvern Instruments, Malvern, UK) and analysed by the CONTIN method (MALVERN software).

The amount of drug trapped in liposomes was evaluated by HPTLC/FID (latroscan MK-5 new, latron Lab. Inc., Tokyo, Japan) (Hatziantoniou and Demetzos 2006; Hatziantoniou *et al.* 2006). Freeze-dried liposomal preparations were reconstituted to half of the initial volume by adding HPLC-grade water, resulting in a sucrose concentration of 300mM. The size and the ζ-potential of reconstituted liposomes were measured as described above. Samples were allowed to anneal for a period of 30 min prior to preparation of the diluted samples in RPMI growth medium.

Sclareol uptake: After treatment of cells up to 96h with free and liposomal sclareol and determination of the cell's growth rate, as noted above, sclareol was extracted from cells by the Bligh-Dyer method (Bligh and Dyer 1959), using $CHCl_3/CH_3OH/H_2O$. The sclareol cellular concentration was determined using HPTLC/FID (Iatroscan $MK5^{new}$; Iatron Lab. Inc., Tokyo, Japan), according to a calibration curve that was previously set up. Hydrogen flow rate was 160mL/min, airflow rate was 1900mL/min, and the scan speed was 30s/scan. As stationary phase silica gel sintered on quartz rods (Chromatorods-SIII; Iatron Lab. Inc.) was used in sets of ten rods (Hatziantoniou and Demetzos 2006; Hatziantoniou *et al.* 2006; Paradissis *et al.* 2005). All results were from three independent experiments. Statistical analysis, for all cell experiments, was done using the Student's t-test. A difference was considered significant if $p<0.05$.

3. RESULTS

The effect of free and liposome-incorporated sclareol on the growth rate of MCF-7 and H-460 cell lines are presented in Figure 2. As it is depicted in Figure 8-2A, free sclareol found to be highly cytotoxic for both cell lines. The growth rate reduced as early as 8 hours upon addition of sclareol.

Liposomal sclareol was substantially less cytotoxic than free sclareol at the same final concentration (100μM), which showed cytotoxicity after 48 hours of continuous incubation of cells. However, as it is clearly represented in Figure 2B, liposomal sclareol significantly reduced the growth rate of cells 24 hours later up on drug's addition. Measurements of sclareol content taken up by both cell types revealed that in the case of free sclareol at the time point that the growth rate was highly reduced (8 hours upon addition of sclareol), cells have already taken up the maximum amount of the drug (Figure 3A). Uptake of free sclareol from cells declined from that time point on and 48 hours later was diminished. The incorporation of sclareol into liposomes resulted in a slower rate of uptake from both cell lines (Figure 3B). The peak of the liposomal sclareol uptake was at 48 hours of incubation for MCF-7 cell line and 72 hours of incubation for H-460 cell line. After that the uptake is declined in both cell lines (Figure 3B).

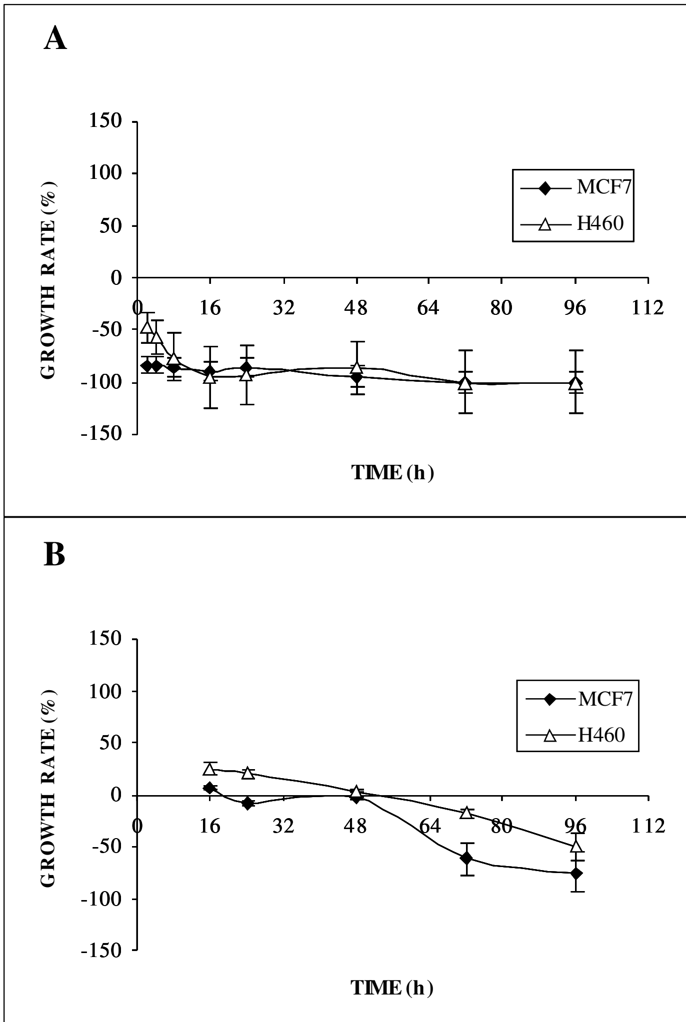
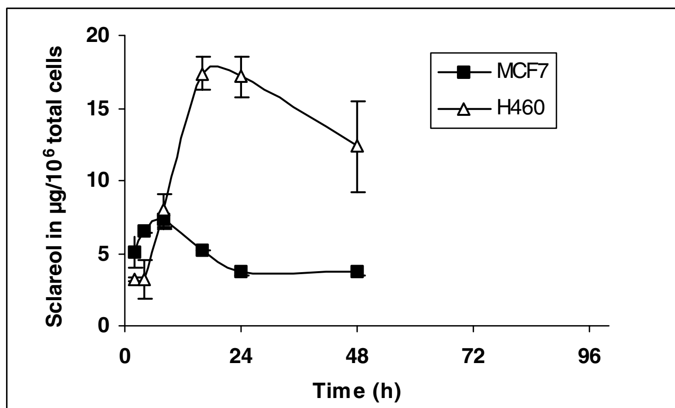


Figure 2. A: Effect of free sclareol on cell growth rate of MCF-7 (black diamonds) and H-460 (triangles) cell lines. Cells were incubated with 100μM of free sclareol. B: Effect of liposomal sclareol on cell growth rate of MCF-7 (black diamonds) and H-460 (triangles) cell lines. Cells were incubated with 100μM of liposomal sclareol

4. DISCUSSION

Extensive literature on the interactions of liposomes with cells has been accumulating over the past several years. However, due to the complex nature of liposome-cell interactions, interpretation of experimental results in terms of liposome-cell interactions has proven to be difficult. None of the mechanisms such as endocytosis,

A



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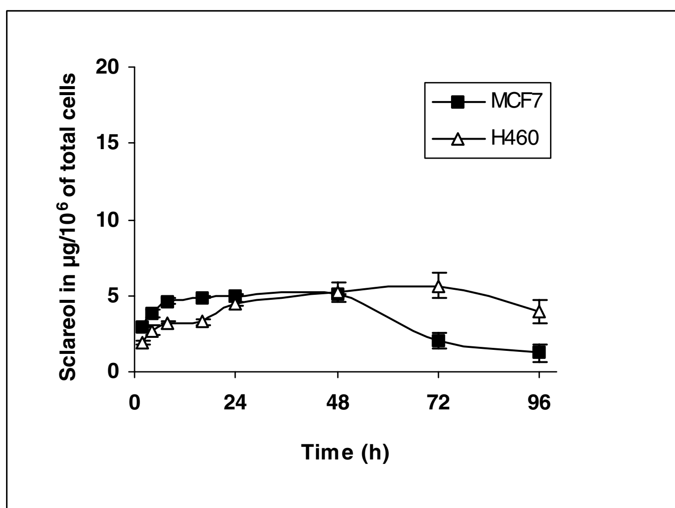

Figure 3. A: Uptake of free sclareol by MCF-7 (black cubes) and H-460 (triangles) cell lines. Cells were incubated with 100μM of free sclareol. B: Uptake of liposomal sclareol by MCF-7 (black cubes) and H-460 (triangles) cell lines. Cells were incubated with 100μM of liposomal sclareol

fusion or absorption of liposomes to cells, which are involved in liposome-cell interactions, are mutually exclusive (Allen *et al.* 1981).

Allen and co-workers (1981) have previously reported that liposome incorporated methotrexate, when tested in cell lines (EMT6 and S49), reduces and mediates the cytotoxicity of the free drug, via the uptake of free drug leaked from liposomes. In another study on the effect of liposomal daunorubicin against leukaemic cells, it has been reported that liposomal daunorubicin was devoid of acute effects such

as ROS production and ATP depletion that resulted in increased necrotic cell death (Liu *et al.* 2002). However, studies on the uptake of cytotoxic compounds by cells are of considerable importance.

Recently published results from our research group showed that sclareol might possess interesting pharmacological properties as it revealed significant cytostatic and cytotoxic effects against leukemic and solid tumor cell lines (Dimas *et al.* 2001, 1999; Hatziantoniou *et al.* 2006). It has been further demonstrated that sclareol induces cell cycle arrest at G0/1 phase of the cycle and kills cells via the mechanism of apoptosis (Dimas *et al.* 2001, 1999). When tested against colon cancer (HCT-116) xenografts developed in NOD/SCID mice, sclareol also exhibited a significant tumor regression in its liposomal form, while the free compound was highly toxic for animals, leading them to death (Hatziantoniou *et al.* 2006). In continuation of our research on sclareol, this work was focused on determining the effect of free sclareol on cell growth rate of human breast (MCF-7) and lung cancer (H-460) cell lines as well as the role of liposomes to alter the pharmakokinetic parameters of sclareol due to its different rate of uptake by cells. The results showed that liposomal sclareol was less cytotoxic at the concentration of 100μM than that of free sclareol at the same final concentration. At that concentration, free sclareol reduced the growth rate of cells while its incorporation into liposomes largely delayed the appearance of cytotoxic effects for both cell lines These experiments revealed that the reduced appearance of cytotoxicity of the liposomal sclareol could be well correlated with a lower accumulation rate of sclareol into cells (Figure 3B).

5. CONCLUSION

The present study was focused on the uptake of a bioactive compound namely sclareol by MCF-7 and H-460 human cancer cell lines. According to the findings, it has been shown that the liposomal sclareol retains significant growth inhibiting activity and alters the pharmacokinetic parameters. These results should be taken into account in feature *in vivo* studies.

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CHAPTER 9

RELEASE ADVANTAGES OF A LIPOSOMAL DENDRIMER-DOXORUBICIN COMPLEX, OVER CONVENTIONAL LIPOSOMAL FORMULATION OF DOXORUBICIN*

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Abstract: Data on the release advantages of a liposomal formulation incorporating a doxorubicin–PAMAM G4 complex in comparison to a liposomal doxorubicin are presented. The liposomes incorporating either doxorubicin-PAMAM complex, or doxorubicin as free drug, were composed of Egg-phosphatidylcholine (EPC): Stearylamine (SA) at a 10:0.1 molar ratio and their size distribution and ζ-potential were characterized. Liposomes incorporating the doxorubicin-PAMAM complex exhibited release properties which were advantageous compared to the conventional type of liposomal doxorubicin in terms of doxorubicin toxicity and its availability to the tumor site. This liposomal formulation may show improved therapeutic properties in vivo

Keywords: Liposome; dendrimer; PAMAM G4; doxorubicin; drug release

1. INTRODUCTION

Liposomes are non-toxic and biocompatible drug delivery systems that have been proven to be very useful in the fight against cancer. Liposomes can increase the therapeutic effectiveness of the encapsulated drugs and decrease their toxicity (Straubinger *et al.* 2004). One of the best-known liposomal drug delivery systems

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*This article is dedicated to the memory of Prof. Demetrios Papahadjopoulos (University of California at San Francisco, UCSF) who was my mentor on liposomal technology and a pioneer of nanotechnology.

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is the liposomal doxorubicin. The high cardiotoxicity of free doxorubicin limits its clinical use, despite its high anticancer activity against a variety of tumours. Liposomal doxorubicin is active against many types of cancer and reduces the toxicity of doxorubicin and it is now in clinical use in USA and Europe (Gabizon 2002). Several clinical trials are currently in progress in order to evaluate the use of doxorubicin liposomes either alone or in combination with other anticancer drugs (Toma *et al.* 2002; Syrigos *et al.* 2002).

Despite several advantages, the therapeutic use of liposomes has limitations, which are related to the release of the encapsulated drug that can be only partially delayed by the modification of the membrane composition. Many attempts are made towards a more effective control of the release of the encapsulated drug, using polymers. One novel approach is the entrapment of liposomes in polymeric microspheres and the progressive release of the intact liposomes from the biodegradable matrix (Stenekes *et al.* 2002). Other approaches are based on the encapsulation of liposomes in microcapsules in order to modulate the release of the encapsulated drug (Dhoot and Wheatley 2003) or to produce liposome-like microspheres (Pan *et al.* 2004).

Dendrimers are highly branched macromolecules that, contrary to traditional "linear" polymers, possess fractal architecture, nanoscaled size and unique physicochemical properties. They are small in size, and exhibit a low polydispersity that can contribute to a reproducible pharmacokinetic behavior. However, the main characteristics of dendrimers are their multiple reactive groups, a well-defined structure, and their ability to encapsulate drugs in their void spaces (Cloninger 2002; Aulenta *et al.* 2003). An ideal dendrimer as drug delivery system must be non-toxic, non-immunogenic and biodegradable (Aulenta *et al.* 2003). The first dendrimer family which has been synthesized, characterized and commercialized is the Poly (amidoamine) (PAMAM) dendrimer. These dendrimers are considered safe regarding toxicity and are non-immunogenic and they have been used in the delivery of drugs, antisense nucleotides and gene therapy, both *in vitro* and *in vivo* (Eichman *et al.* 2001). Dendrimers and dendrons have already been proposed for drug complexation and transport; especially lipidic dendrons that can produce higher order lamellar structures called "dendrisomes" (Khuloud *et al.* 2003) or can aggregate to form nanosystems (Singh and Florence 2005).

In this paper a liposomal formulation composed of egg phosphatidylcholine and stearylamine (EPC:SA 10:0.1 molar ratio) and a doxorubicin-PAMAM complex attached to liposomes is compared to a conventional liposomal formulation with the same composition encapsulating doxorubicin by the pH gradient method (Papagiannaros *et al.* 2005; Papagiannaros *et al.* 2006). The main advantage of the liposomal formulation is the controlled and sustained release of the encapsulated drug; the release of which is controlled by the complexation in the dendrimer's internal cavity. The liposomal membrane employed in the formulation is useful for the biocompatibility of the liposomal system and it offers advantages of the liposomal drug delivery. This liposomal system is compared to that of the conventional liposomes of the same lipid composition with respect to the % release of the

encapsulated drug at 37°C, in 50 RPMI culture medium for 48 h period, in order to assess its possible advantages and evaluate its potential applications in cancer therapy.

2. MATERIALS AND METHODS

2.1. Materials

Egg Yolk Phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (AL, USA). Doxorubicin Hydrochloride was purchased from Pharmacia (NJ, USA). Ammonium sulphate, TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid), PAMAM, Poly (amidoamine) 4th generation, Tris (tris (hydroxymethyl) aminomethane), stearylamine (SA), Sephadex G75, chloroform, absolute ethanol and methanol were of spectroscopic grade and were purchased from Sigma (St. Louis, USA).

2.2. Conventional Liposome Preparation, Characterization and Doxorubicin Encapsulation

Liposomes composed of EPC:SA at 10:0.1 molar ratio, were prepared using the reverse phase evaporation method (Szoka *et al.* 1978) while their size and ζ-potential measurements were performed at 25°C and at an angle of 90° in a photon correlation spectrometer (Zetasizer 3000, Malvern U.K.) and analysed by the CONTIN method (MALVERN software). The liposomes were prepared as follows: EPC, and SA were first dissolved in chloroform / methanol and then transferred into a 100 ml round bottom flask. Then a 150 mM ammonium sulphate (pH=5.3) was added to the flask. The mixture was subsequently sonicated for 15 min in a bath sonicator and the organic solvents were removed using a flash evaporator (Bucchi R-480) at 60°C. The liposomal suspension was finally allowed to anneal at 50°C for 1 hour.

Large Unilamellar Vesicles (LUVs) were prepared by sonicating the liposomal suspension in an ice bath, for two cycles of 5 min each (0.7 cycle and 100% amplitude) interrupted by a 5 min resting period, using a probe sonicator (UP 200S, dr. hielsher GmbH, Berlin, Germany). The 150 mM ammonium sulphate buffer (pH=5.3) of the liposomal suspension was exchanged with a 100 mM TES, 100 mM NaCl buffer (pH=7.5) using a Sephadex G75 column. Doxorubicin was subsequently encapsulated into the liposomes using the pH gradient method (Mayer and Bally 1986). Briefly, 854 μl or 0.015 mmole of doxorubicin was added and the preparation was incubated at 60°C for 30 min. Unentrapped doxorubicin was removed by passing the liposomal suspension through a Sephadex G75 column using 100 mM TES, 100 mM NaCl buffer (pH=7.5).

2.3. Determination of Lipids and Doxorubicin

EPC and SA were determined by high performance thin-layer chromatography coupled with a flame ionization detector (HPTLC-FID, Iatroscan MK-5, Iatron Lab.

Inc. Tokyo, Japan) (Goniotaki *et al.* 2004). Hydrogen flow rate was 160 ml/min, airflow rate 1900 ml/min, scan speed 30 s/scan. As stationary phase Chromarods – SII (Iatron Lab. Inc.) in set of 10 rods was used. Doxorubicin concentration of the liposomal samples was measured on a Perkin Elmer UV-vis spectrometer at λ=481 nm by adding absolute ethanol to the samples. Prior to determination, the samples were purified using column chromatography (Sephadex G75).

2.4. Release of Doxorubicin from Conventional liposomes *in vitro*

Equal volumes of liposomal suspension encapsulating doxorubicin in TES (100 mM) and NaCl (100 mM) buffer (pH: 7.5) and in RPMI 1640 culture medium, were mixed and the liposomes were incubated at 37°C. Aliquots of 300 μl were then withdrawn at various time intervals and passed through Sephadex G-75 column, in order to remove the released doxorubicin. Doxorubicin retained in the liposomes was measured by UV-vis spectrometry at λ=481 nm.

2.5. Incorporation of Doxorubicin in PAMAM Dendrimer and Assessment of Doxorubicin Release

An aqueous solution of doxorubicin (122 μl) was mixed with a PAMAM G4 solution (3:1 and 6:1 molar ratio of doxorubicin-PAMAM) in methanol (2 ml) and the solutions were stirred for 12 hours. The solutions were evaporated to dryness at 30°C in vacuum and the PAMAM dendrimer incorporating doxorubicin was extracted overnight using chloroform. Chloroform was evaporated to dryness, the dry residue was dissolved in TES (10 mM, pH: 7.5) and the absorbance of doxorubicin was measured at λ=481 nm using UV-vis spectrometry. In the later case acidification of the solution and buffering to pH=4.5 was performed before measuring the absorbance. The release of doxorubicin was studied in TES at 37°C using dialysis bags (molecular weight cut off 13,000).

2.6. Incorporation of Doxorubicin-PAMAM Complex in Liposomes

Liposomes were prepared by using the thin film hydration method (Gabizon 2002). The doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) was attached to liposomes, composed of EPC:SA 10:0.1 (molar ratio). Briefly, the lipid film was prepared by dissolving EPC (73.6 μmole), SA (0.736 μmole) and doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) in chloroform. The solvent was slowly evaporated in a flash evaporator to form a lipid film, which was dried under vacuum for at least 12 h. Multilamellar vesicles (MLVs) were prepared by hydrating the lipid film with TES buffer (10 mM, pH=7.5) and stirring for 1 h. Small unilamellar vesicles (SUVs) were prepared from the resultant liposomal suspension, which was subjected to sonication for two 5 min periods interrupted by a 5 min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0,7 – UP 200S, dr. hielsher GmbH, Berlin, Germany). The resultant

vesicles were allowed for 30 min to anneal any structural defects. Non-incorporated doxorubicin-PAMAM complex was removed by passing the liposomal suspensions through a Sephadex G75 column. The size and ζ-potential of liposomes incorporating the doxorubicin-PAMAM complex (3:1molar ratio; 2.1 μmoles of doxorubicin) were measured using photon correlation spectroscopy (Malvern Zetasizer 3000HS). Doxorubicin concentration was measured on a Perkin Elmer UV-vis spectrometer at λ=481nm after the addition of absolute ethanol to the samples.

2.7. Release of Doxorubicin from the Liposomes Incorporating Doxorubicin-PAMAM Complex

The release of doxorubicin from the MLCRS incorporating the doxorubicin-PAMAM complex (3:1 molar ratio; 2.1 μmoles of doxorubicin) was studied in 50% RPMI culture medium and in TES (100 mM), NaCl (100 mM) buffer (pH 7.5), at 37°C, by placing the liposomal formulations in dialysis bag (molecular weight cut off 13,000). The doxorubicin released at various times, up to 48 h was measured using UV-vis at λ=481 nm.

2.8. Statistical Analysis

Statistical analysis of the effect of liposome type on the size and ζ-potential was performed using one-way ANOVA followed by a post hoc Tukey's HSD test (SPSS for Windows release 11). All the results were from four (n=3) independent experiments.

3. RESULTS

3.1. Encapsulation, Physical Properties and Release of Doxorubicin from Conventional Liposomes

Doxorubicin was encapsulated in liposomes composed of EPC:SA (10:0.1 molar ratio) at a doxorubicin to lipid molar ratio of 0.77±0.01 (initial 0.1). The encapsulation efficiency of doxorubicin into liposomes was 99.1%±1.1. Size measurements for liposomes incorporating doxorubicin, indicated an average size of 91.2±0.74 nm and a ζ-potential of –26±3.3 mV (Table 1).

The release of doxorubicin from the conventional liposome EPC:SA 10:0.1 molar ratio in 50% RPMI cell culture medium at 37°C and in TES buffer after 24 hours is quite fast. The liposomes retained 24.5% of the drug in 50% RPMI cell culture medium and 35.5% in buffer at 37°C after 24 hours (Figures 1 and 2).

3.2. Incorporation and Release of Doxorubicin from the Doxorubicin-PAMAM Complex

The doxorubicin-PAMAM complex was formed using two different pH (*i.e.* 10 mM TES buffer at pH: 7.5 or 10 mM acetate buffer at pH: 4.5) and two different molar

Table 1. Physicochemical characteristics of EPC:SA (10:0.1 molar ratio) liposomes encapsulating doxorubicin and of liposomes (EPC:SA 10:0.1 molar ratio), incorporating doxorubicin-PAMAM complex (3:1 molar ratio)

Liposome formulation	Size (nm)	ζ-potential (mV)
Conventional liposomes: EPC:SA 10:0.1 (molar ratio) encapsulating doxorubicin	91.2±0.74	−26.0±3.3
Liposomes incorporating doxorubicin-PAMAM complex: EPC:SA 10:0.1 (molar ratio) encapsulating doxorubicin as doxorubicin-PAMAM complex (3:1 molar ratio)	116.3±7.8	−8.7±1.7

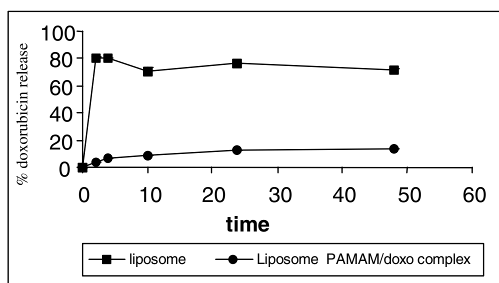

Figure 1. Doxorubicin release from liposomes incorporating doxorubicin-PAMAM complex (•) and from conventional liposomes (■) both composed of EPC:SA 10:0.1 (molar ratio) in 50% RPMI 1640 culture medium at 37°C. Each point represents the mean of three independent experiments (SD never exceeded 5% of the mean value)

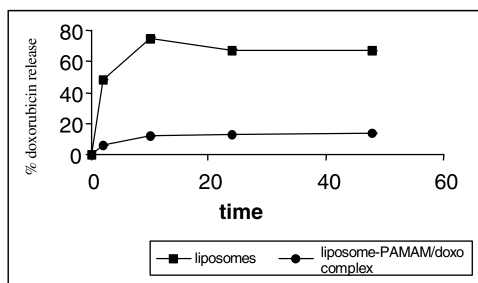

Figure 2. Doxorubicin release from liposomes incorporating doxorubicin-PAMAM complex (•) and from conventional liposomes (■) both composed of EPC:SA 10:0.1 (molar ratio) in TES buffer at 37°C. Each point represents the mean of three independent experiments (SD never exceeded 5% of the mean value)

ratios of doxorubicin to PAMAM (*i.e.* 3:1 and 6:1). The results indicated that a doxorubicin to PAMAM molar ratio of 3:1 was sufficient in order to achieve an almost 97% incorporation of doxorubicin into the dendrimer. Doxorubicin incorporation into PAMAM was higher when the complex was formulated in TES buffer (pH: 7.5) as compared to that of acetate buffer (pH: 4.5). The release of doxorubicin appeared to be quite slow. The lower doxorubicin release (7.4% during 48 h) was observed at a molar ratio of 3:1 of doxorubicin to PAMAM, and the higher (16.5% during 48 h) at molar ratio of 6:1 of doxorubicin to PAMAM in TES buffer (pH: 7.5) at 37°C (Papagiannaros *et al.* 2005).

3.3. Incorporation and Release of Doxorubicin-PAMAM Complex from Liposomes

The incorporation efficiency of doxorubicin-PAMAM complex, (3:1 molar ratio) into liposomes (EPC:SA 10:0.1 molar ratio) was almost 95% while doxorubicin (doxorubicin-PAMAM complex 3:1 molar ratio) to lipid molar ratio was 0.020 (initial 0.028) in TES buffer (pH: 7.5).

The release of doxorubicin (doxorubicin-PAMAM complex 3:1 molar ratio) from the liposomes was quite slow; 13.6% at 37°C (48 h) in TES buffer at pH: 7.5 and 14.0% at 37°C (48 h) in 50% RPMI cell culture medium (Figures 1 and 2).

3.4. Physical Properties of Liposomes Incorporating the Doxorubicin-PAMAM Complex

Size measurements of the doxorubicin-PAMAM complex (3:1 molar ratio) attached to liposomes indicated an average size of 116.3±7.8 nm and a ζ-potential of –8.7±1.7 mV (Table 1). The stability of liposomes was studied for a period up to 26 weeks. The liposomal suspension was kept at 4°C in the dark. No sediment was observed while their average hydrodynamic diameter increased rapidly (>1μm) (Papagiannaros *et al.* 2005).

4. DISCUSSION

A liposome delivery system is proposed for incorporating anticancer drugs, combining the liposomal and dendrimeric technologies. Its ability to modulate the release of the encapsulated drug in a way that is independent of the liposomal membrane but strongly related to the complexation of the drug with the dendrimer, offers advantages over conventional liposomal formulation in terms of the pharmacological activity. The controlled release of the encapsulated cytotoxic drugs is of paramount importance in cancer chemotherapy (Andresen *et al.* 2005). An example is presented in this report, based on the release properties of liposomes encapsulating doxorubicin-PAMAM G4 complex in comparison with the conventional type of liposome encapsulated doxorubicin. This liposomal formulation has shown superior *in vitro* anticancer activity, due to its slow releasing properties

(Papagiannaros *et al.* 2005). It has already been established that the cytotoxic effect of the drug is mediated by the leakage of doxorubicin from the liposomes (Gabizon 2002). However a delayed release of doxorubicin is necessary in order to reduce the toxicity and increase the therapeutic usefulness of the drug (Charrois *et al.* 2004).

The release rate of doxorubicin is an important factor since a slow release is necessary in order to decrease the side effects of doxorubicin and improve its therapeutic index (Gabizon 2002; Horovic *et al.* 1992). A slow release rate can also contribute to the accumulation of the drug in the tumor (Charrois and Allen 2004). The control of the leakage of the encapsulated drug is mainly achieved through modifications in the liposome membrane, mainly by changing the fluidity of the membrane, by addition of cholesterol (Ohvo-Rekila *et al.* 2002) or "rigid state" lipids (Oussoren *et al.* 1998); increasing the rigidity of the liposome membrane also affects the uptake of the encapsulated drug by the tumor cells, therefore reducing the toxicity can also reduce the availability of the drug to the tumor site (Sadzuka *et al.* 2002). On the contrary, doxorubicin incorporated into cholesterol–free liposomes, as a doxorubicin-PAMAM complex, exhibited a slow release rate, at 37°C, after a 48 h incubation period (in 48 hours less than 20% was released). Consequently, it can be expected that this formulation possess reduced doxorubicin side effects. Various drugs encapsulated in dendrimers (Kolhe *et al.* 2005) or incorporated in liposomes together with PAMAM dendrimers (Klopade *et al.* 2002) have shown slow release profiles. The contribution of the doxorubicin-PAMAM complex may not be limited to the delayed release of the encapsulated doxorubicin, since an ibuprofen- PAMAM G4 complex was found to enter lung epithelial cancer cells in 1h (compared to 3h for free ibuprofen) (Kolhe *et al.* 2005), thus the dendrimer could facilitate the cellular entry of the complexed drugs. Furthermore, PAMAM G4 dendrimer conjugated with ibuprofen entered lung carcinoma cells in less than 15 min compared to 1h for free ibuprofen (Kolhe *et al.* 2005) and PAMAM G5 encapsulating methotrexate exhibited four times more activity *in vitro* than the free drug against the KB epidermoidal cancer cell line (Quintan *et al.* 2002).

The encapsulation efficiency of doxorubicin in PAMAM G4 was almost 100%. The presence of dendrimers resulted in a higher encapsulation efficiency and a decreased release rate of the encapsulated drug, although this was achieved by creating a higher and more stable proton gradient across the liposomal membrane (Klopade *et al.* 2002).

Although the average hydrodynamic diameter of the liposomal formulation incorporated doxorubicin-PAMAM complex was almost 116nm immediately after their production, this size increased to the microns (μ) very rapidly with time. This fact was not observed with the conventional liposomal formulation, that does not incorporate the doxorubicin-PAMAM complex, and therefore it might be attributed to the presence of the dendrimer. It has already been observed that dendrimers could facilitate the formation of liposome aggregates (Sideratou *et al.* 2002). The charge of liposomes incorporating doxorubicin – PAMAM complex, did not seem to be involved in the formation of the aggregates suggesting that hydrophobic forces between dendrimers, which are attached to liposomal particles, may be responsible.

Earlier studies using 'dendrons' (partial dendrimers) (Purohit *et al.* 2001) have also reached the same conclusion.

5. CONCLUSIONS

A liposomal drug delivery system incorporating a complex of doxorubicin-PAMAM G4 dendrimers was prepared and compared to conventional liposomal formulation encapsulating doxorubicin with the same lipid composition regarding release properties of the antineoplastic agent. The results suggest that this new controlled release system may be useful in anticancer therapy.

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CHAPTER 10

APPLICATIONS OF LIGHT AND ELECTRON MICROSCOPIC TECHNIQUES IN LIPOSOME RESEARCH

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Abstract: Liposomes and some other vesicular systems are widely used as delivery vehicles for bioactive compounds. Successful applications of these carrier systems in drug delivery, gene therapy and other health related areas depend on comprehensive understanding of their physical properties including polydispersity and morphology. Variations in size and shape of the carrier systems are indications of their stability and shelf life and can guide scientists in improving the therapeutic formulations. Towards this end microscopic techniques can provide vital information on size, configuration, stability and mechanisms of cellular uptake of particles on micro and nanoscales as discussed in this chapter

Keywords: carrier systems, liposomes, niosomes, novasomes, sphingosomes, ufasomes, virosomes, electron microscopy, scanning probe microscopy

1. INTRODUCTION

Liposomes, which are also called lipid vesicles, are spherical, closed–continuous structures (Mozafari et al 2002). They are composed of curved lipid bilayers. These bilayers entrap part of the solvent in which they are dispersed and retain this solvent into their interior. They may have one or more concentric or non-concentric membranes and their size is in between 20nm to several micrometers, while the thickness of the membrane is about 4nm (New 1990; Lasic 1993; Mozafari and Mortazavi 2005).

Liposomes are made mainly from amphiphiles. These amphiphiles are a special class of surfactant molecules and are characterized by having hydrophilic and hydrophobic groups on the same molecule. A liposome-forming molecule has two hydrocarbon chains (hydrophobic or nonpolar tails) and a hydrophilic group (polar

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head). In general, most of these molecules are insoluble in water and they form colloidal dispersions.

Due to their solubility properties, the structure of these aggregates of amphiphilic molecules involves the ordering of lipid molecules and their arrangement in aqeous environments. The hydrophilic part of the amphiphilic molecules tends to be in contact with water whereas the hydrophobic hydrocarbon chains prefer to be hidden from water in the interior of the structures. Lipid bilayer is one of the most frequently seen aggregation structures. On the surface of either side are polar heads, which shield nonpolar tails in the interior of the lamella from water. At higher lipid concentrations these bilayers form lamellar liquid-crystalline phases where two-dimensional planar lipid bilayers alternate with water layers. When diluted, these lipid bilayers seperate, become unstable, curve and form liposomes.

Due to their unique properties – including ease of preparation, versatility in terms of composition, size, charge, fluidity, etc. – and possibility of preparing them using non-toxic, non-immonogenic material on the industrial scales (Lasic and Papahadjopoulos 1998; Mozafari and Mortazavi 2005), liposomes are widely used as controlled release vehicles. For specialized nanotherapeutic and other applications, the lipid vesicles need to be finely tuned and delicately tailored. Morphological and physicochemical studies are strict pre-clinical requirements for successful formulation of liposomal carriers. This chapter reviews commonly used microscopic techniques in the assessment of the lipid vesicles.

2. DIFFERENT TYPES OF MICROSCOPIC VESICLES

The most commonly used microscopic vesicles are *liposomes*. They are in fact synthetic analogues of natural biomembranes. Liposomes are composed of polar lipids such as lecithin. The nanometric versions of liposomes are known as *nanoliposomes* (Mozafari and Mortazavi 2005). There are some other types of microscopic vesicular systems similar to liposomes, namely niosomes, sphingosomes, novasomes, transfersomes, ufasomes and virosomes as explained below.

Niosomes (explained in detail in Chapter 4) are nanometric particles (non-ionic surfactant vesicles) used in the delivery of bioactive compounds and composed of mono or diacyl polyglycerol or (poly) oxyethylene based lipids in mixtures with 0-50 mol % of cholesterol. In general, they are prepared by very similar methods as liposomes (Uchegbu and Vyas 1998; Korkmaz et al 2000).

Sphingosomes are composed of skin lipids and predominantly sphingolipids. They are processed in similar ways as phospholipid liposomes (Brunke 1990; Erdogan et al 2005). In a recent study sphingosomes were used as a drug delivery system to target a model thromboembolic disease in rabbits (Erdogan et al 2005).

Novasomes are paucilamellar (Oligolamellar), nonphospholipid vesicles and made of C_{12}–C_{20} single-chain surfactants bonded via an either esther or peptide bound to polar heads. Double-chained surfactants include palmitoyl or oleayl chains or sterols attached to glycerol phosphorylcholine (Chambers et al 2004).

Transfersomes are another kind of liposomes, which are composed from up to equimolar mixtures of phosphatidylcholine with myristic acid (Cevc and Blume 1992; Cevc 1996) (also see Chapter 7).

In *Ufasomes*, oleic acid is used as single chain surfactant as the amphiphilic molecule and these type of liposomes were prepared long time ago in 1973 (Gebicki and Hicks 1973).

Another derivative of liposomes are *Virosomes* that contain viral proteins in their membranes (Kara et al 1971; Almeida et al 1975). In another words virosomes are reconstituted viral envelopes that retain the receptor binding and membrane fusion activities of the virus they are derived from. Virosomes can be generated by detergent solubilization of the membrane of an enveloped virus, sedimentation of the viral nucleocapsid, and subsequent selective removal of the detergent from the supernatant to produce reconstituted membrane vesicles consisting of the viral envelope lipids and glycoproteins. Size and surface characteristics of virosomes can be studied through microscopic visualization. More information about virosomes are provided in Chapter 7 of this book.

Liposome and its other derivatives are used as models of biological systems (e.g. biomembranes) and in the delivery of drugs and other macromolecules. Depending on the special physico-chemical characteristics of polar lipids and other ingredients of these vesicles, they have a great promise for tissue and cell-specific delivery of a variety of phamaceuticals and biotechnology products.

3. CLASSIFICATION OF LIPOSOMAL VESICLES

Liposomes are classified depending on vesicle size, preparation method and their number of lamella (New 1990; Mozafari and Mortazavi 2005). A multilamellar vesicle (MLV) is a liposome composed of a number of concentric lipidic bilayers. A vesicle composed of several non-concentric vesicles encapsulated within a single bilayer is known as a multivesicular vesicle (MVV). Another type of liposome is known as a unilamellar vesicle (ULV) and contains one single bilayer and one internal (aqueous) compartment. Unilamellar vesicles can be divided into small unilamellar vesicle (SUV, less than 100nm) and large unilamellar vesicle (LUV, larger than 100nm).

The most important liposome characteristics are:

i. Vesicle size;
ii. Number of bilayers and morphology;
iii. Bilayer fluidity; and
iv. Surface characteristics (charge and hydrophilicity).

Vesicle size can be approximately between 0.02 and 10μm. The largest vesicles may have more than 10 bilayers, however, this can be changed by the preparation method. Size is a very important factor playing an important role on the *in vitro* and *in vivo* behaviour of liposomes. Physical stability and biodistribution mainly depend on the liposome size.

Vesicle shape (morphology) is the other significant factor for liposome technology. This is due to the fact that vesicle shape of liposomes provides an idea about their *in vivo* fate and their cellular transition mechanism. Some of the microscopic techniques used in the morphological examinations of liposomes and other vesicular carriers are explained below.

4. MICROSOPY IN LIPOSOME TECHNOLOGY

Methods determining the size of liposomes vary in complexity and degree of sophistication (Talsma et al 1987; New 1990). Microscopy is the oldest but very valuable technique among the others. With light microscopy, the gross view and rough size of the particles can be seen. Undoubtedly, the most precise method is that of electron microscopic examination. Because, it permits visualization of each individual liposome and given time, patience and the required skill, several artifacts can be avoided.

With electron microscopy, one can obtain precise information about the profile of a liposome sample over the whole range of sizes. In addition, electron microscopy can provide information on the configuration of lipid vesicles and their stability in time. However, there are also some disadvantages associated with electron microscopic techniques. These include:

- They can be very time-consuming; and
- Require expensive equipments that may not always be immediately available.

Dynamic Light Scattering, Coulter Counter, Size Exclusion Chromatography and Optical Density method can be mentioned among the other liposomal size measurement techniques. These are mainly used for particle size determination and can not provide information on shape, configuration and presence/absence of aggregation or fusion of vesicular systems, for which microscopic techniques are more appropriate.

Although *Dynamic Light Scattering* is a very simple technique to perform, it has the disadvantage of measuring an average property of the bulk of the liposomes and cannot give detailed deviation, information from the mean value of the size range.

Coulter Counter does not measure the whole range of liposome sizes and uses a rather standard piece of apparatus for which information is available elsewhere (Mosharraf and Nystrom 1995; Gorner et al 2000).

Gel Exclusion Chromatography is a cheaper method than the above–mentioned techniques and it only requires buffer(s) and gel material. This method can be advised if only an approximate idea of the size range of particles is required.

If only relative rather than absolute values are required for the comparison of different liposome formulations, *Optical Density* measurements can be used.

Compared with the aforementioned particle characterization methods, microscopic techniques have the advantage of providing information on both size and shape of the objects. Several electron microscopy (EM) techniques can be employed for liposome research:

a. Scanning Electron Microscopy (SEM);
b. Negative Staining Electron Microscopy (NSEM);
c. Freeze Fracture Transmission Electron Microscopy (FFTEM).

A schematic representation of a scanning electron microscope is depicted in Figure 1. Compared with other electron microscopes, SEM is a less frequently used imaging technique, particularly in liposome research. Nevertheless, several SEM micrographs showing cells with absorbed liposomes have been published, which are very useful in determining mechanisms of cell-liposome interactions (e.g. *see* Vinay et al 1996).

Complicated sample preparation is necessary for all EM techniques due to the fact that sample investigation may require staining, fixation, high vacuum and/or electrical conductiveness. Although staining procedures may vary, almost all EM techniques are based on embedding the vesicles in a thin film of an electron dense "glass". When the films are examined by EM, the relatively electron-transparent vesicles will appear as bright areas against a dark background (hence the term negative stain).

Among the above-mentioned techniques NSEM and FFTEM are the most commonly employed techniques. NSEM is a useful method for clarifying questions related to the size distribution of liposomes. It has several advantages, as it is simple to use and necessitates only limited specialized equipment (that can be found easily at any EM laboratory). However, it requires laborious work in order

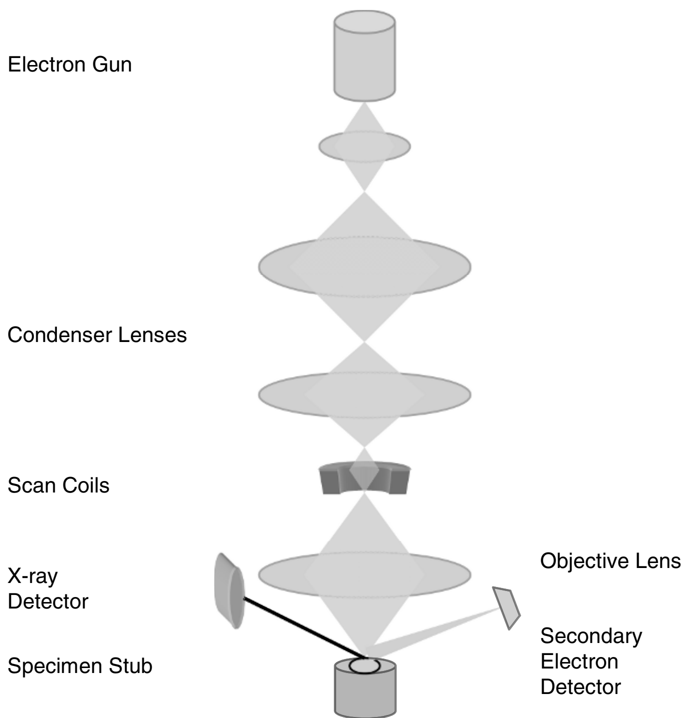

Figure 1. Main components of a scanning electron microscope (SEM) (courtesy of Dr. M. R. Mozafari)

to obtain quantitative data. NSEM was firstly described for visualising viruses, then a wide variety of microorganisms, cells, macromolecules and liposomes. In liposome technology, it provides quantitative data for MLV or ULV type liposomes, niosomes, sphingosomes and the others.

In negative stain methods, a drop of liposome sample at about 0.5–1 $mg.ml^{-1}$ is dried on the microscopic grid coated with special support (carbon film) and stained with an electron dense solution, such as uranyl UO_2^{++} or Tungsten Molybdate.

Two methods are commonly used in NSEM applications: a) Spray Method, and b) Drop Method. The drop method is the technique most commonly used with liposomes and is the easiest to perform. The spray method is not recomended due to the unreliability of the quality of the preparation. Additionally, the shear forces that the specimen must undergo during atomization may alter the size distribution of liposomes. Nevertheless, NSEM still grossly depends on the preparation of the grid, quality of the grid and hydrophilicity of the grid coat itself. Even when an optimal preparation is done, nobody clearly knows that if the vesicles were fractured or thin sectioned in their middle, or how the vesicles collapsed during drying in the negative stain method. In spite of these disadvantages, the methods are widely used and at the magnifications of up to 200,000 offer a resolution about 10–20 A°.

Introduction of cryoelectron microscopy to the science world provided direct observations of liposomes in their hydrated form. A thin film of the sample is vitrified in a few μm in liquid ethane, and the entire film is investigated in a special cryoholder in the microscope, in a similar way to optical microscopy.

In FFTEM methods, even smaller (compared with NSEM) amounts of sample, at higher concentrations, are quickly frozen and fractured. Platinum shadowing produces a replica which is investigated in the electron beam.

Freeze-fracture and freeze-etching technologies were developed gradually as the ultra-fast freezing technologies. Both sample preparation methods have artifacts; either by drying or by cooling, the system may go into gel or liquid-crystalline lamellar lyotropic phase.

Optical microscopy is the other technique employed for liposome technology. Bright-field and particularly phase-contrast microscopy are the most widely employed techniques. Its resolution is below 0.3 μm. It is a powerful technique for LUV, MLV and especially giant unilamellar vesicles if it is equipped with computer. The artifacts of this method are rather few. The sample thickness is important when getting an idea about the multilamellarity of the liposomes. Larger MLVs are very bright between crossed polarizer and analyzer; but below diameters of 1–2μm, the intensity of the circularly polarized light is too low to be observed birefringence.

Direct optical microscopy gives information about size, homogenity of the sample and lamellarity of MLVs. If there is any large liposome contamination with SUVs, optical microscopy is helpful for assessment. Furthermore, several mechanic characteristics of bilayers can be investigated by optical microscopy.

Resolution has been increased by the introduction of a group of microscopic techniques known as Scanning Probe Microscopy (SPM). Two of the most applied

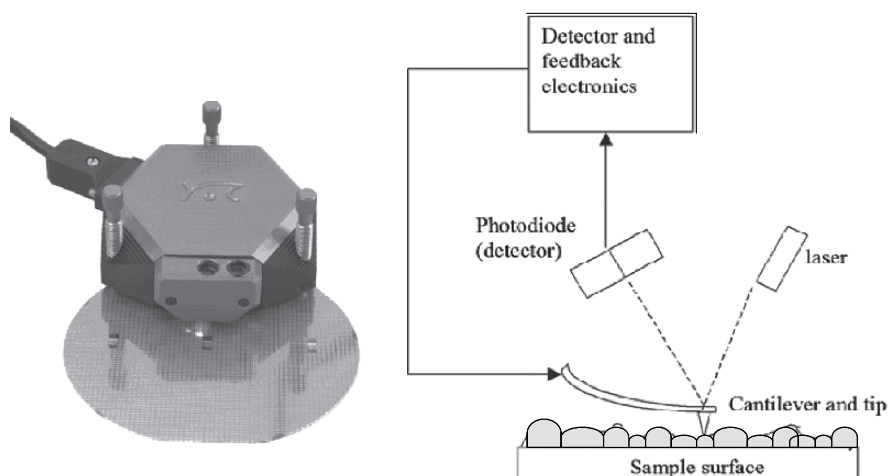

Figure 2. A compact atomic force microscope (AFM) and its main components

SPM techniques are Scanning Tunneling Microscopy (STM) and Atomic Force Microscopy (AFM) (Figure 2). This recent technology gives the possibilty to view various biological and non-biological samples under air or water with a resolution up to 3A°. By this method, monolayers of various lipids and lipid attached molecules such as antibody fragments can be studied (Mozafari et al 2005).

SPM allows the visualization of single biological molecules, such as proteins and nucleic acids, and their complexes with liposomes. In some cases even visualization of the inner details of these complexes is possible. High spatial resolution achieved in SPM techniques is not the only advantage of these methods. Even more important is the possibility to study biological molecules in various environments including air, water, and physiologically relevant solutions, buffers, and organic solvents. External factors such as temperature, pressure, humidity, and salt concentration can be varied during measurements. This gives a unique opportunity to study conformational changes of biomolecules such as proteins and DNA in situ (Kiselyova and Yaminsky 1997). Examination of physical properties of fatty acid multilayer films at the micron and nanometer scale (Martin and Weightman 2000) and micromanipulation of phospholipid bilayers (Maeda et al 2002) are some of the many reported biological applications of SPM. Toward optimization of bioactive delivery formulations, SPM investigations play a crucial role by providing valuable information such as the configuration, size, and stability of the carrier systems.

5. SUMMARY

Several microscopic methodologies have been reviewed in this chapter with respect to their application and importance in the characterization of vesicular carriers of the bioactive compounds. Information such as size, polydispersity, configuration

and mechanisms of cellular uptake of the particles can readily be obtained by microscopic studies. In addition, interaction between vesicles and different molecules can be assessed at nanometric and even angstrom precision. Some microscopic techniques, such as atomic force microscopy, also have the potential of revealing the real-time interaction between the carrier systems and cells. The information obtained through microscopic investigations can assist in the rational design and development of optimal carrier systems for the encapsulation, targeting and controlled release of the bioactive agents.

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