Design and Application of Niosomal Drug Delivery Systems

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Dedication

I dedicate this dissertation to my parents Feride and Ismail, my brother Ibrahim and the meaning of my life Muharrem.Without your support, trust and love my success would not have been possible.

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Abstract

Cancer is currently a serious health problem leading cause of death worldwide. The most used treatment for cancer is chemotherapy and the therapeutic efficacy of many anticancer drugs is limited by their poor penetration into tumor tissue and by their side effects on healthy cells. Nanotechnology in cancer diagnosis and therapy provides new aspects by developing novel nanomaterials such as nanoparticles that can deliver the loaded agents to the tumors without damaging healthy cells.

Niosomes are non-ionic surfactant vesicles having a bilayer structure with biodegradable and nonimmunogenic feature. They can be produced at low costs and exhibit high stability making them very attractive drug carriers in drug delivery studies. Especially, easy modification of their composition and surface allows development of novel targeted drug delivery systems.

Within the scope of this thesis, the design of different niosomal drug delivery systems and their applications in targeted cancer therapy were evaluated. Firstly, polyethylene glycolated niosomes were synthesized as a drug carrier and anticancer drug doxorubicin was encapsulated into the niosomes. To ensure an effective targeted drug delivery, an aptamer specifically binding to cell surface protein MUC1 protein was conjugated to a cell penetrating peptide followed by binding to the surface of the niosomes. In a second approach, polyethylene glycolated niosomes were used to develop a targeted co-drug delivery system against brain cancer. Curcumin and doxorubicin were entrapped into the niosomes and the niosomal surface was decorated with a tumor homing and penetrating peptide, which penetrates tumor cells via Neuropilin-1 receptor mediated endocytosis.

In conclusion, in the present work two new different targeted niosomal drug delivery systems against ovarian and brain cancer were designed, synthesized, characterized and applied *in vitro*. These studies may provide new insights for the development of effective targeted therapy in cancer.

Key words: *niosomes, structure and characterization, targeted drug delivery, co-drug delivery*

Kurzfassung

Krebs ist derzeit ein ernstes Gesundheitsproblem und die führende Todesursache weltweit. Die am häufigsten verwendete Behandlung von Krebs ist die Chemotherapie. Die therapeutische Wirksamkeit vieler Krebsmedikamente ist auf Grund ihrer schlechten Durchdringung in das Tumorgewebe und durch ihre Nebenwirkungen auf normale, pathologisch unveränderte Zellen begrenzt. Hier bietet die Nanotechnologie neue Asätze in der Krebsdiagnose und -therapie, indem neuartige Nanomaterialien wie z.B. Nanopartikel entwickelt und eingesetzt werden. Sie können mit Arzneimittel beladen werden und diese direkt den Tumorzellen zuführen, ohne die gesunden Zellen zu beschädigen. Niosome sind nichtionische Tensid-Vesikel mit einer Doppelschichtstruktur, die biologisch abbaubar und nicht-immunogen sind und mit niedrigen Kosten sowie in hoher Stabilität hergestellt werden können. Das macht sie zu sehr attraktiven Arzneimittelträgern in drug delivery-Studien. Eine einfach darstellbare Modifikation ihrer Zusammensetzung und Oberfläche ermöglicht die Entwicklung neuartiger, individualisierter und zielgerichteter *drug delivery*-Systeme, die sowohl Patienten- als auch krankheitsspezifisch desingt werden können.

Im Rahmen dieser Arbeit wurde unterschiedliche niosomale Arzneimittelabgabesysteme erstmalig selbst hergestellt, modifiziert, Oberflächen-dekoriert, physiko-chemisch charakterisiert und deren Design und Anwendungen im gezielten in-vitro Biotesting evaluiert. Polyethylenglykol-Niosome wurden als Arzneimittelträger synthetisiert, in die das Antikrebsmedikament Doxorubicin eingekapselt war. Um eine zielgerichtete Arzneimittelabgabe gegen Eierstocktumore zu gewährleisten, wurde ein an das Zelloberflächenprotein MUC1 spezifisch bindendes Aptamer über einen Peptidlinker, der die Zellpenetration ernöglicht, an die Oberfläche der Niosomen gebunden. In einem zweiten Ansatz wurden Polyethylenglykol-Niosome verwendet, um ein Ko-Arzneimittelabgabesystem zielgerichtet gegen Hirntumore zu entwickeln. Die beiden Wirkstoffe Curcumin und Doxorubicin wurden in die Niosome eingeschlossen und an die Niosomoberfläche wurde ein Tumor-Homing und tumordurchdringendes Peptid gebunden, das über eine Neuropilin-1-Rezeptor vermittelte Endozytose in die Tumorzellen eindringt.

In der vorliegenden Arbeit wurden somit *in vitro* zwei neue, hochspezifische niosomale Arzneimittelabgabesysteme gegen Eierstock- und Hirntumor selbst entwickelt, charakterisiert und angewendet. Diese Studien können neue Erkenntnisse zur zukünftigen Entwicklung effektiver, gezielter Krebstherapien liefern.

Stichwörter: *Niosomen, Struktur und Charakterizierung, zielgerichtete Arzneimittelabgabe, Ko-Arzneimittelabgabesystem*

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List of Abbreviations

AFM	Atomic force microscopy
BBB	Blood-brain barrier
BCSF	Blood-cerebrospinal fluid barrier
BS3	Bis(sulfosuccinimidyl)suberate
CNS	Central nervous system
CPP	Cell penetrating peptide
Cys-TAT	Transactivator of transcription peptide with cysteine residue
DAPI	4,6-diamino-2-phenylindol
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DOX	Doxorubicin
DSPE	1,2-distearoyl-snglycero-3-phosphoethanolamine
EDC	N-[3-(dimethylamino)propyl]-Nethylcarbodiimide hydrochloride
EDXD	Energy-dispersive X-ray diffraction
EE	Entrapment efficiency
EIM	Ether injection method
EPR	Enhanced permeability and retention
FCS	Fetal calf serum
HCPT	Hydroxycamptothecin
HeLa	Cervical cancer cells
HLB	Hydrophilic-lipophilic balance
hMSC	Human mesenchymal stem cells
HPLC	High-performance liquid chromatography
HPRT	Housekeeping gene
IC50	Inhibitory concentration
LDL	Low-density lipoprotein
LOD	Limit of detection
LOQ	Limit of quantification
LUV	Large unilamellar vesicles

mAb	Monoclonal antibody
MCF-7	Breast cancer cell line
MDR	Multidrug resistance
MLV	Multilamellar vesicles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MUC1	Mucin1 aptamer
NPG	*N*-Palmitoylglucosamine
NRP-1	Neuropilin-1
P/S	Penicillin/Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBCA	Poly(butyl cyanoacrylate)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEGNIO	Polyethylene glycolated niosome
PDI	Polydispersity index
PLGA	Poly(lactic-co-glycolicacid)
PTX	Paclitaxel
REV	Reverse phase evaporation method
Rh6g	Rhodamine 6G
SAXS	Small angle X-ray scattering
$scCO_2$	Supercritical carbon dioxide fluid
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
Span	Sorbitan monostearate
SUV	Small unilamellar vesicles
T_c	Transition temperature
TEM	Transmission electron microscopy
TFH	Thin-film hydration method
TfR	Transferrin receptor
UV	Ultraviolet
U87	Human glioblastoma cells
VIP	Vasoactive intestinal peptide

1. Introduction

One of the most active research areas of nanotechnology is nanomedicine, that applies the knowledge and tools of nanotechnology to the prevention, diagnosis, and treatment of the diseases. Drug delivery systems (DDS) are defined as formulations or devices enabling the transportation of a drug in the desired part of the body. By using nanoscale delivery vehicles, multiple tasks could be performed simultaneously, such as the controlling the delivery of the therapeutic agents to the target side and enhancing bioavailability of poorly soluble drugs. Therefore, the efficiency of the agents can be improved.

Multifunctional nanoparticles, acting as a drug vehicle, can be precisely fabricated with appropriate physicochemical properties. Their size, shape, composition and physical parameters can be finely tuned. Nanoparticles can be constructed from various organic and inorganic materials resulting in liposomes, niosomes, carbon nanotubes, quantum dots, micelles, metal nanoparticles, or dendrimers. By coating of their surface with hydrophilic polymers, their circulation time in the body could be enhanced through decrease of enzymatic degradation. Polyethylene glycol (PEG) is one of the most used non-toxic, non-immunogenic, non-antigenic, and highly water-soluble coating polymer for drug delivery studies. Due to these favorable properties, PEGylation plays an important role in the development of novel drug delivery devices.

In the treatment of cancer, delivery systems attempt to improve the therapeutic index of anticancer agents by efficiently directing them to tumor cells. This can be achieved by active targeting. Receptors overexpressed or specifically expressed by cancer cells are unique targets. The surface of the nanoparticles may be functionalized by using biomolecules such as antibodies, aptamers, peptides and small molecules that bind specifically to these receptors. Thus, high specificity and penetration capabilities that are crucial for cancer treatment can be gained. Aptamers are single stranded oligonucleotides and one of the most promising targeting ligands that bind specifically to cancer cell surface receptors with high affinity. This property of aptamers has been utilized for developing targeted drug carriers which can deliver several types of cargos into the cells. On the other hand, cell penetrating peptides (CPPs) have been widely employed as delivery vectors for the import of the molecules that otherwise cannot cross the plasma membrane of eukaryotic cells. The combination of targeting ligands and penetration enhancing peptides could open new perspectives.

In the recent years, niosomes have gained great attention in drug delivery studies. Niosomes are vesicular systems which are mostly formed by non-ionic surfactants and cholesterol. They exhibit low toxicity and high biocompatibility with the biological systems. Besides, their simple preparation, low cost, and high stability make them outstanding candidates for drug delivery. Because of their hydrophobic bilayer and hydrophilic core, niosomes can load both hydrophobic and hydrophilic therapeutic agents. Furthermore, with the PEGylation of niosomes their surfaces are functionalized easily for biomolecule conjugation. Hereby, stability, bioavailability, as well as therapeutic efficiency of the encapsulated agent are improved by using a protecting and targeting vehicle. Especially for the treatment of cancer, the prolonged and specific delivery of chemotherapeutic agents plays a key role to reduce side effects and increase the therapeutic efficacy. In this context, PEGylated niosomes facilitate the design of various targeted niosomal drug delivery systems bearing great potential for commercialization.

2. Aim and Scope

Development of multifunctional nanoparticles offers great hope to overcome some challenges faced in diagnosis, therapy, and monitoring of many types of cancer. Nanoparticles provide an opportunity to reduce toxicity and enhance therapeutic efficiency. Vesicular nanoparticles such as niosomes are the leading structures that are used in drug delivery applications. In this thesis design, development, and application of novel niosomal drug delivery systems for targeted cancer therapy were aimed. In the theoretical part, the structure and preparation methods of niosomes are summarized by considering characterization techniques. The recent studies on niosomal drug delivery systems are discussed with special focus on brain targeting. Then the design and production of targeted niosomal drug carriers are presented in the experimental section.

In the first experimental part, the development of an effective carrier with targeting moiety to overcome penetration problem in solid tumors was aimed. For this aim, PEGylated niosomes were synthesized to obtain nanostructures. To test the applicability of cell penetrating peptide and aptamer, the surface of niosomes were modified with CysTAT peptide and MUC1 aptamer binding to MUC1 protein found on the cell membrane. Doxorubicin (DOX) as a cancer model drug was encapsulated into the platform and niosomes were characterized in detail. *In vitro* studies were carried out on MUC1 positive HeLa and negative U87 cells to investigate the specificity of the designed niosomal system as well as the influence of drug-loaded niosomes on the viability of selected cancer cells.

In the second part of experimental section, a novel drug delivery system was developed against brain tumors. The therapeutic efficacy of many anticancer drugs is limited because of the blood-brain barrier (BBB). Therefore, development of novel therapy methods is quite important. Targeted co-drug delivery could be a promised strategy as was shown in recent studies. The objective of this study was to create a novel niosomal co-drug delivery system targeted to glioblastom. Accordingly, doxorubicin and curcumin were loaded into PEGylated niosome and the surface of niosome was modified with tumor homing and penetrating peptide (tLyp-1). The characterization studies were performed for niosomal formulations. *In vitro* studies were carried out by using glioblastoma (U87) and human mesenchymal stem cells (hMSC) cells to investigate the specificity and cytotoxicity of the tLyp-1 targeted co-drug loaded niosomal formulation.

3. Theoretical Background

This chapter is divided into two subchapters. Chapter 3.1 (review article) provides fundamental information about niosomes including the structure, synthesis and applications in drug delivery. Chapter 3.2 (book chapter) focuses on the targeting strategies and potential of niosomes for brain targeting.

3.1 Review Article - Niosomes as nanoparticular drug carriers: Fundamentals and recent applications

This chapter comprises the review article 'Niosomes as Nanoparticular Drug Carriers: Fundamentals and Recent Applications' by D. Ag Seleci, M. Seleci, A. Jochums, J.G. Walter, F. Stahl and T. Scheper, Journal of Nanomaterials, Article ID 7372306. It was reprinted by permission of Hindawi Publishing Corporation.

3.1.1 Summary

Drug delivery is a process of administration of pharmacological molecules to create a therapeutic effect, preferably in the diseased area. However, conventional drug delivery systems suffer from limitations such as lack of specificity, poor solubility, and overdose toxicity. Nanoparticles as drug carriers have aroused a great interest in drug delivery studies because of their multifunctional character. This character enabled delivery of therapeutic load with a controlled rate and high specificity. Niosomes are one of the most promising vesicular drug delivery systems that have a bilayer structure composed of non-ionic surfactants and cholesterol.

In the first part of this review article, material composition of niosomes, their effects on vesicle structure and stability are summarized. Varieties of techniques are used to synthesize niosomes. All these methods are explained in detail. The physicochemical properties of niosomes such as size, morphology, zeta potential, and stability as well as their characterization techniques are described. The effects of niosomes' physicochemical properties in biological applications are also evaluated. Furthermore, the applications of niosomes as drug carrier for various types of chemotherapeutic agents are described via review of recent studies. The combination of ligand-mediated active targeting strategies with niosomal drug delivery systems is listed in this present review. Besides, the importance of co-drug delivery systems for the development of effective cancer therapy and the application potential of niosomes as a co-drug carrier are discussed.

Finally, recent applications of niosomes in drug delivery are summarized.

3.1.2 Abstract

Drug delivery systems are defined as formulations aiming for transportation of a drug to the desired area of action within the body. The basic component of drug delivery systems is an appropriate carrier that protects the drug from rapid degradation or clearance and thereby enhances drug concentration in target tissues. Based on their biodegradable, biocompatible, and nonimmunogenic structure, niosomes are promising drug carriers that are formed by self-association of nonionic surfactants and cholesterol in an aqueous phase. In recent years, numerous research articles have been published in scientific journals reporting the potential of niosomes to serve as a carrier for the delivery of different types of drugs. The present review describes preparation methods, characterization techniques, and recent studies on niosomal drug delivery systems and also gives up to date information regarding recent applications of niosomes in drug delivery.

3.1.3 Introduction

Delivering drug with a controlled rate and targeted delivery received much attention in recent years. The application of nanotechnology to medicine has provided the development of multifunctional nanoparticles that, acting as drug carriers, can be loaded with different drugs. Nanocarriers present a great approach in drug delivery with promising features such as protection of drug from degradation and cleavage, controlled release, and in case of targeted delivery approaches the delivery of drug molecules to the target sites [1]. Niosomes are one of the promising drug carriers that have a bilayer structure and are formed by self-association of nonionic surfactants and cholesterol in an aqueous phase. Niosomes are biodegradable, biocompatible, and nonimmunogenic. They have long shelf life, exhibit high stability, and enable the delivery of drug at target site in a controlled and/or sustained manner [2]. In recent years, the potential of niosomes as a drug carrier has been extensively studied [3–5]. Various types of nonionic surfactants have been reported to form niosomes and enable the entrapment of a large number of drugs with a wide range of solubility [6–8]. The composition, size, number of lamellae, and surface charge of niosomes can be varied and optimized to enhance the performance of niosomes for drug delivery. The aim of this review is to present the fundamentals of niosome preparation and characterization as well as a description of their use in drug

delivery, with particular attention to more recent studies. This review will provide an overview on the increasing interest on niosomes in the field of drug delivery.

3.1.4 Structure and components of niosomes

The main components of niosomes are nonionic surfactants, hydration medium and lipids such as cholesterol. The list of materials used in the preparation of niosomes has been shown in Table 3.1. The self-assembly of nonionic surfactants in aqueous media results in closed bilayer structures (Figure 3.1). A high interfacial tension between water and the hydrophobic tails of the amphiphile causes them to associate. The steric and hydrophilic repulsion between the head groups of nonionic surfactant ensure that hydrophilic termini point outwards and are in contact with water. The assembly into closed bilayers usually requires some input of energy such as mechanical or heat. Niosomes can be categorized in three groups according to their sizes and bilayers. Small unilamellar vesicles (SUV) (10–100 nm), large unilamellar vesicles (LUV) (100–3000 nm), and multilamellar vesicles (MLV) where more than one bilayer is present.

Table 3.1 The materials used in niosome preparation

Non-ionic surfactants	Examples	References
Alkyl ethers		
• Alkyl glycerol ethers	Hexadecyl diglycerol ether (C16G2)	[9]
• Polyoxyethylene glycol alkyl ethers (Brij)	Brij 30, Brij 52, Brij 72, Brij 76, Brij 78	[10-12]
Crown ethers	Bola	[13,14]
Alkyl esters		
• Sorbitan fatty acid esters (Spans)	Span 20, Span 40, Span 60, Span 80, Span 65, Span 85	[15-18]
• Polyoxyethylene sorbitan fatty acid esters (Tweens)	Tween 20, Tween 40, Tween 60, Tween 80, Tween 65, Tween 85	[7,19,20]
Alkyl amides		
• Glycosides	C-glycoside derivative surfactant (BRM-BG)	[21]
• Alkyl polyglucosides	Octyl-decyl polyglucoside (OrCG110), Decyl polyglucoside (OrNS10)	[22]
Fatty alcohols or fatty acids		
• Fatty alcohols	Stearyl alcohol, Cetyl alcohol, Myristyl alcohol	[23]
• Fatty acids	Stearic acid, Palmitic acid, Myristic acid	[23]
Block copolymer		
• Pluronic	Pluronic L64, Pluronic 105	[24,25]
Lipidic components		
Cholesterol		[26]

l-α-Soya phosphatidyl choline		[27]
Charged molecule		
Negative charge	Diacetyl phosphate, Phosphotidic acid, Lipoamine acid, Dihexadecyl phosphate	[28,29]
Positive Charge	Stearylamine, Steary piridinium chloride, Cetyl pyridinium chloride	[29]

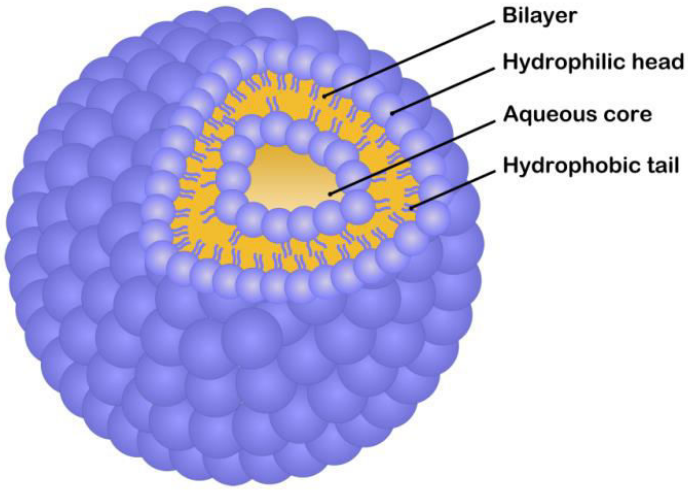

Figure 3.1 Structure of niosomes.

Nonionic surfactants

Nonionic surfactants are a class of surfactants, which have no charged groups in their hydrophilic heads. They are more stable and biocompatible and less toxic compared to their anionic, amphoteric, or cationic counterparts [41]. Therefore, they are preferred for formation of stable niosome for *in vitro* and *in vivo* applications. Nonionic surfactants are amphiphilic molecules that comprise two different regions: one of them is hydrophilic (water-soluble) and the other one is hydrophobic (organic soluble). Alkyl ethers, alkyl esters, alkyl amides, fatty acids are the main nonionic surfactant classes used for niosome production. The hydrophilic-lipophilic balance (HLB) and critical packing parameter (CPP) values play a critical role in the selection of surfactant molecules for niosome preparation.

Hydrophilic-lipophilic balance (HLB)

HLB is a dimensionless parameter, which is the indication of the solubility of the surfactant molecule. The HLB value describes the balance between the hydrophilic portion to the lipophilic portion of the nonionic surfactant. The HLB range is from 0 to 20 for nonionic surfactants. The lower HLB refers to more lipophilic surfactant and the higher HLB to more hydrophilic surfactant. Surfactants with a HLB between 4 and 8 can be used for preparation of vesicle [42]. Hydrophilic surfactants with a HLB value ranging from 14 to 17 are not suitable to form a bilayer membrane due to their high aqueous solubility [43]. However, with the addition of an optimum level of cholesterol, niosomes are indeed formed from polysorbate 80 (HLB value = 15) and Tween 20 (HLB value = 16.7) [44, 45]. Tween 20 forms stable niosome in the presence of equimolar cholesterol concentration. The interaction occurs between the hydrophobic part of the amphiphile next to head group and the 3-OH group of cholesterol at an equimolar ratio and this interaction could explain the effect of cholesterol on the formation and hydration behavior of Tween 20 niosomal membranes [46, 47].

Drug entrapment efficiency of the niosomes is also affected by HLB value of surfactant [48]. Shahiwala et al. have incorporated nimesulide into niosomes using lipid film hydration technique by changing the HLB. They found that as the HLB value of surfactant decreases from 8.6 to 1.7, entrapment efficiency decreases [43, 49].

Critical packing parameter (CPP)

During the niosomal preparation, the geometry of the vesicle depends upon the critical packing parameter. On the basis of the CPP of a surfactant, the shape of nanostructures formed by self-assembly of amphiphilic molecules can be predicted. Critical packing parameter depends on the symmetry of the surfactant and can be defined using following equation [50, 51]:

$$CPP = \frac{v}{Ic \times a_{\circ}}$$

where V is hydrophobic group volume, $l\ c$ is the critical hydrophobic group length, and a_0 is the area of hydrophilic head group. If CPP ≤ 1/3 corresponding, for example, to a bulky head group, small hydrophobic tail spherical micelles may form. Nonspherical micelles may form if 1/3 ≤ CPP ≤1/2, and bilayer vesicles can occur if 1/2 ≤ CPP ≤ 1. Inverted micelles form if CPP ≥ 1 when the surfactant is composed of a voluminous tail

and a small hydrophobic tail [47]. CPP could be considered as a tool for realizing, rationalizing, and predicting the self-assembled structure and its morphological transition in amphiphilic solutions [52].

Cholesterol

In the bilayer structure of niosomes, cholesterol forms hydrogen bonds with hydrophilic head of a surfactant [19, 53]. Cholesterol content of niosomes thereby influences the structures of niosomes and physical properties such as entrapment efficiency, long time stability, release of payload, and biostability [17, 46]. Cholesterol improves the rigidity of vesicles and stabilizes niosomes towards destabilizing effects induced by plasma and serum components and decreases the permeability of vesicles for entrapped molecules thus inhibiting leakage [54]. Drug entrapment efficiency plays an important role in niosomal formulations and it can be altered by varying the content of cholesterol. Agarwal et al. demonstrated that cholesterol improves the stability of enoxacin loaded niosome with increasing cholesterol content, resulting in increases of entrapment efficiency [55]. The effect of cholesterol on flurbiprofen entrapment was studied by Mokhtar et al. and cholesterol was found to have little effect on the flurbiprofen entrapment into Span 20 and Span 80 niosomes. However, a significant increase in entrapment efficiency of flurbiprofen was obtained when 10% of cholesterol was incorporated into niosomes prepared from Span 40 and Span 60 followed by a decrease in encapsulation efficiency of the drug upon further increase in cholesterol content [56]. According to the reported results, the addition of cholesterol and its amounts needs to be optimized depending on the physical-chemical characteristic of surfactants and loaded drugs.

Charged molecule

Charged molecules increase the stability of the vesicles by the addition of charged groups to the bilayer of vesicles. They increase surface charge density and thereby prevent vesicles aggregation. Dicetyl phosphate and phosphatidic acid are most used negatively charged molecules for niosome preparation and, similarly, stearyl amine and stearyl pyridinium chloride are well-known positively charged molecules used in niosomal preparations. Normally, the charged molecule is added in niosomal formulation in an amount of 2.5–5 mol %. However increasing the amount of charged molecules can inhibit niosome formation [29].

3.1.5 Methods of preparation

Thin-film hydration method (TFH)

Thin-film hydration method is a simple and well-known preparation method. In this method, the surfactants, cholesterol, and some additives such as charged molecules are dissolved in an organic solvent in a round bottomed flask. Then the organic solvent is removed using a rotary vacuum evaporator to obtain thin film on the inside wall of the flask. An aqueous solution of drug is added and the dry film is hydrated above the transition temperature (Tc) of the surfactant for specified time with constant shaking [57, 58]. Multilamellar niosomes are formed by this method.

Ether injection method (EIM)

In ether injection method, the surfactants with additives are dissolved in diethyl ether and injected slowly through a needle in an aqueous drug solution maintained at a constant temperature, which is above the boiling point of the organic solvent. The organic solvent is evaporated using a rotary evaporator. During the vaporization, the formation of single layered vesicles occurs [59–61].

Reverse phase evaporation method (REV)

In this method, niosomal ingredients are dissolved in a mixture of ether and chloroform and added to aqueous phase containing the drug. The resulting mixture is sonicated in order to form an emulsion and the organic phase is evaporated. Large unilamellar vesicles are formed during the evaporation of the organic solvent [62–64].

Microfluidization method

The microfluidization method is based on submerged jet principle. In this method, the drug and the surfactant fluidized streams interact at ultra high velocities, in precisely defined micro channels within the interaction chamber. The high-speed impingement and the energy involved leads to formation of niosomes. This method offers greater uniformity, smaller size**,** unilamellar vesicles, and high reproducibility in the formulation of niosomes [65, 66].

Supercritical carbon dioxide fluid ($scCO_2$)

Manosroi et al. have described the supercritical reverse phase evaporation technique for niosome formation [67, 68].They added Tween 61, cholesterol, glucose, PBS, and ethanol into the view cell and the CO_2 gas was introduced into the view cell. After magnetic stirring until equilibrium, the pressure was released and niosomal dispersions were obtained [67]. This method enables one step production and easy scale-up.

Proniosome

Proniosome technique includes the coating of a water-soluble carrier such as sorbitol and mannitol with surfactant. The coating process results in the formation of a dry formulation. This preparation is termed “Proniosomes” which requires to be hydrated before being used. The niosomes are formed by the addition of the aqueous phase. This method helps in reducing physical stability problems such as the aggregation, leaking, and fusion problem and provides convenience in dosing, distribution, transportation, and storage showing improved results compared to conventional niosomes [69].

Transmembrane pH gradient

In this method, surfactant and cholesterol are dissolved in chloroform and evaporated to form a thin lipid film on the wall of a round bottomed flask. The film is hydrated with a solution of citric acid (pH = 4) by vortex mixing and the resulting product is freeze-thawed for niosome formation. The aqueous solution of drug is added to this niosomal suspension after that phosphate buffer is added to maintain pH between 7.0 and 7.2 [70]. According to this method, the interior of niosome has a more acidic pH value than the outer medium. The added unionized drug passes through the niosome membrane and enters into the niosome. The drug ionizes in an acidic medium and cannot escape from the niosomal bilayer [71].

Heating method

This is a patented method, which was created by Mozafari et al. [72, 73]. Surfactants and cholesterol are separately hydrated in buffer and the solution is heated to 120°C with stirring to dissolve cholesterol. The temperature is reduced and surfactants and other additives are then added to the buffer in which cholesterol is dissolved while stirring continues. Niosomes form at this stage, are left at room temperature, and then are kept at 4-5°C under nitrogen atmosphere until use [53].

The "bubble" method

In this method, surfactants, additives, and the buffer are added into a glass flask with three necks. Niosome components are dispersed at 70°C and the dispersion is mixed with homogenizer. After that, immediately the flask is placed in a water bath followed by the bubbling of nitrogen gas at 70°C. Nitrogen gas is passed through a sample of homogenized surfactants resulting in formation of large unilamellar vesicles [74].

3.1.6 Characterization of niosomes

The characterization of niosome is essential for the clinical applications. Characterization parameters have a direct impact on the stability of niosomes and a significant effect on their *in vivo* performance. Therefore, these parameters such as morphology, size, polydispersity index (PI), number of lamellae, zeta potential, encapsulation efficiency, and stability must be evaluated.

Size and morphology

Dynamic light scattering (DLS) [75], scanning electron microscopy (SEM) [76], transmission electron microscopy (TEM) [77], freeze fracture replication electron microscopy (FF-TEM) [68], and cryo-transmission electron microscopy (cryo-TEM) [67] are the most used methods for the determination of niosome sizes and morphology. DLS provides simultaneously cumulative information of particle size and valuable information on the homogeneity of the solution. A single sharp peak in the DLS profile implies existence of a single population of scatterers. The PI is helpful in this respect. It less than 0.3 corresponds to a homogeneous population for colloidal systems [75]. The microscopic approaches are generally used to characterize the morphology of the niosomes.

Zeta potential

Surface zeta potential of niosomes can be determined using zetasizer and DLS instruments. The surface charge of niosome plays an important role in the behavior of niosomes. In general, charged niosomes are more stable against aggregation than uncharged vesicles. Bayindir and Yuksel prepared paclitaxel loaded niosomes and investigated the physicochemical properties such as zeta potential of niosomes. They found that negative zeta potential values ranging between −41.7 and −58.4mV are sufficiently high for electrostatic stabilization of niosomes [12].

Bilayer characterization

Bilayer characteristics of niosomes have an importance on drug entrapment efficiency. The number of lamellae can be determined by AFM, NMR, and small angle X-ray scattering (SAXS) for multilamellar vesicles [54]. Membrane rigidity of niosomal formulations can be measured by means of the mobility of fluorescence probe as a function of temperature [20]. DPH (1,6 diphenyl-1,3,5-hexatriene) is most used fluorescent probe and added to niosomal dispersion. DPH normally exists in hydrophobic region in the bilayer membrane. The microviscosity of niosomal membrane is determined by fluorescence polarization. High fluorescence polarization means high micro viscosity of the membrane [78]. Moreover, the bilayer thickness can be characterized using the latter method, together with the *in situ* energy-dispersive X-ray diffraction (EDXD) [79].

Entrapment efficiency

Entrapment efficiency (EE%) is defined as the portion of the applied drug which is entrapped by the niosomes. Unencapsulated free drug can be removed from the niosomal solution using centrifugation [80], dialysis [24], or gel chromatography [81]. After this step, the loaded drug can be released from niosomes by destruction of vesicles. Niosomes can be destroyed with the addition of 0.1% Triton X-100 or methanol to niosomal suspension. The loaded and free drug concentration can be determined by a spectrophotometer [82] or high-performance liquid chromatography (HPLC) [83].

Stability

The stability of niosomes can be evaluated by determining mean vesicle size, size distribution, and entrapment efficiency over several month storage periods at different temperatures. During storage the niosomes are sampled at regular intervals of time and the percentage of drug, which is retained into the niosomes, is analyzed by UV spectroscopy or HPLC methods [82, 84].

In vitro release

One often applied method to study *in vitro* release is based on using of dialysis tubing. A dialysis bag is washed and soaked in distilled water. After 30 mins, the drug loaded niosomal suspension is transferred, into this bag. The bag containing the vesicles is immersed in buffer solution with constant shaking at 25°C or 37°C. At specific time intervals, samples were removed from the outer buffer (release medium) and replaced

with the same volume of fresh buffer. The samples are analyzed for the drug content by an appropriate assay method [17].

3.1.7 Niosomes as drug carriers

Niosomes are very promising carriers for the delivery of numerous pharmacological and diagnostic agents. A number of publications have reported the preparation, characterization, and use of niosomes as drug carriers. Because of their nonionic nature, they offer excellent biocompatibility and low toxicity. The unique structure of niosomes allows the development of effective novel drug delivery systems with ability of loading both hydrophilic and lipophilic drugs. Hydrophilic drugs and lipophilic drugs are entrapped into the aqueous core and membrane bilayer of niosome respectively (Figure 3.2).

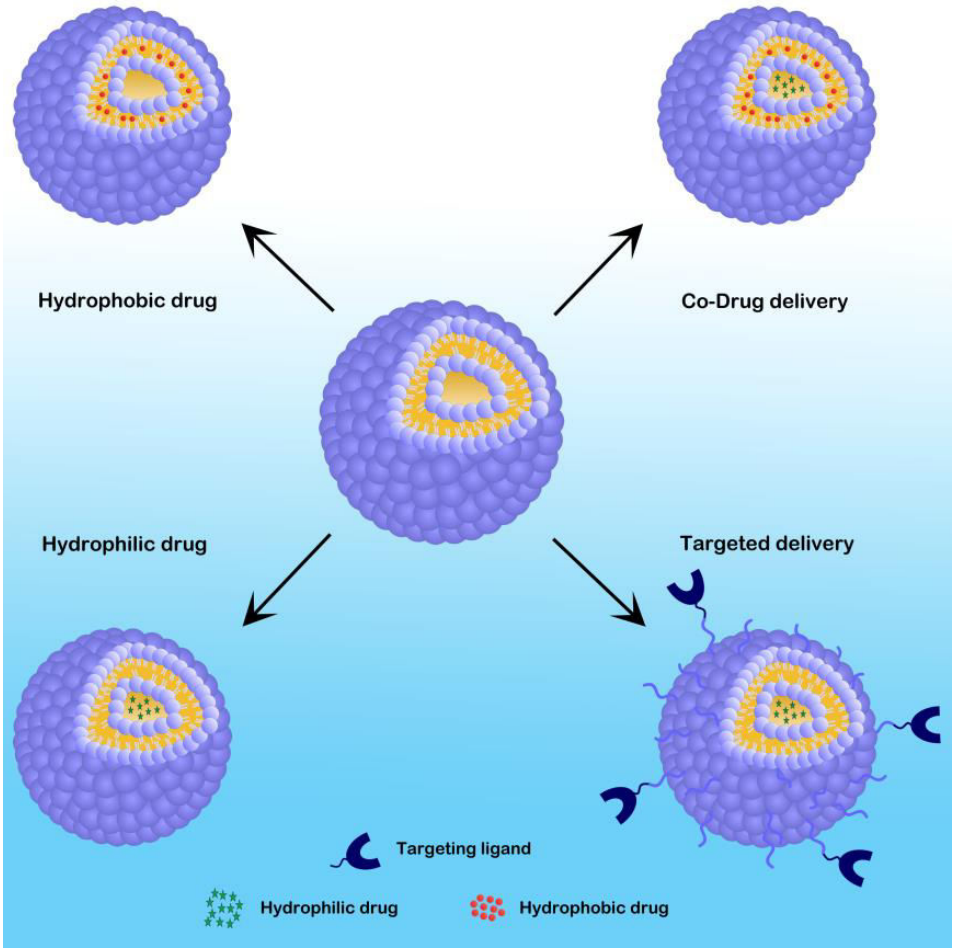

Figure 3.2 Niosomes in drug delivery

Anticancer drug delivery

The current treatment for cancer is usually chemotherapy. The therapeutic efficacy of many anticancer drugs is limited by their poor penetration into tumor tissue and by their severe side effects on healthy cells. Various attempts have been made to overcome these drawbacks, including the use of niosomes as a novel drug delivery system.

Melanoma

Artemisone is a 10-amino-artemisinin derivative exhibiting antimalarial activity and also possessing antitumor activity. Dwivedi et al. encapsulated artemisone in niosomes using thin-film hydration method. The formulations showed highly selective cytotoxicity towards the melanoma cells with negligible toxicity towards the normal skin cells [85]. 5-Fluorouracil (5-FU), largely used in the treatment of different forms of skin cancers, was encapsulated in an innovative bola-niosomal system made up of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (bola-surfactant), Span 80, and cholesterol. The percutaneous permeation of 5-FU-loaded bola-niosomes was evaluated by using human stratum corneum and epidermis membranes. Bola-niosomes provided an increase of the drug penetration of 8- and 4-fold with respect to free drug aqueous solution [13]. The use of cisplatin is limited due to its severe toxic effects. Gude et al. synthesized niosomal cisplatin by using Span 60 and cholesterol and investigated the antimetastatic activity in experimental metastatic model of B16F10 melanoma. Their results suggest that cisplatin encapsulated in niosomes has significant antimetastatic activity and reduced toxicity when compared to free cisplatin [86].

Breast cancer

5-FU-loaded polyethylene glycol- (PEG-) coated and uncoated bola-niosomes were prepared by Cosco et al. and were tested on breast cancer cell lines (MCF-7 and T47D). Both bola-niosome formulations provided an increase in the cytotoxic effect with respect to the free drug. *In vivo* experiments on MCF-7 xenograft tumor SCID mice models showed a more effective antitumor activity of the PEGylated niosomal 5-FU at a concentration ten times lower (8 mg/kg) than that of the free solution of the drug (80 mg/kg) after a treatment of 30 days [87]. Cantharidin entrapped niosomes were prepared by injection method. Their potential in enhancing the antitumor activities of the drug and reducing its toxicity was evaluated on human breast cancer cell line MCF-7. Moreover, *in vivo* therapeutic efficacy was investigated in S180 tumor-bearing mice. Mice treated

with 1.0 mg/kg niosomal cantharidin showed the most effective antitumor activity, with an inhibition rate of 52.76%, which was significantly higher than that of the same concentration of free cantharidin (1.0 mg/kg, 31.05%) [88]. Recently, tamoxifen citrate niosomes were prepared by film hydration technique for localized cancer therapy through *in vitro* breast cancer cytotoxicity as well as *in vivo* solid antitumor efficacy. The optimized niosomal formulation of tamoxifen showed significantly enhanced cellular uptake (2.8-fold) and exhibited significantly greater cytotoxic activity on MCF-7 breast cancer cell line. *In vivo* experiments showed enhanced tumor volume reduction induced by niosomal tamoxifen when compared to free tamoxifen [89].

Ovarian cancer

Uchegbu et al. prepared doxorubicin loaded niosomes. The activity of doxorubicin in hexadecyl diglycerol ether (C16G2) and Span 60 niosomes was studied against a human ovarian cancer cell line and its doxorubicin resistant subline. According to the results, there was a slight reduction in the IC50 against the resistant cell line when the drug was encapsulated in Span 60 niosomes in comparison to the free drug in solution [90].

Lung cancer

Adriamycin was encapsulated into the niosome using a monoalkyl triglycerol ether by Kerr et al. and the activity of niosomal adriamycin compared with free adriamycin solution on human lung tumor cells grown in monolayer and spheroid culture and in tumor xenografted nude mice. The growth delay (i.e., the time taken the tumor volume to double) was significantly longer for adriamycin (15 days) and niosomal adriamycin (11 days) than for control (5.8 days). It is possible that the therapeutic ratio of adriamycin could be further enhanced by administration in niosomal form [91]. In another study, pentoxifylline loaded niosomes were prepared by lipid film hydration method. Intravenous administration of niosomal pentoxifylline (6 mg/kg and 10 mg/kg) resulted in significant reduction in lung nodules in an experimental metastatic B16F10 model suggesting accumulation of pentoxifylline in a distant target. Light microscopic observation of histologic sections showed a decrease in number of tumor islands in the lung [92].

Targeted delivery

The efficiency and particularly the specificity of cellular targeting of niosomal drug delivery systems can be further improved by active targeting for tumor therapy, by using a ligand coupled to the surface of niosomes, which could be actively taken up, for example, via a receptor-mediated endocytosis. Niosome surfaces can be conjugated with small molecules and/or macromolecular targeting ligands to enable cell specific targeting [93]. Proteins and peptides, carbohydrates, aptamers, antibodies, and antibody fragments are the most commonly used molecules that bind specifically to an overexpressed target on the cell surface [94–96]. Bragagni et al. developed brain targeted niosomal formulation using with the glucose derivative as a targeting ligand. They formulated niosomal doxorubicin composed of span: cholesterol: solulan: Npalmitoylglucosamine. Preliminary *in vivo* studies in rats showed that intravenous administration of a single dose of the developed targeted-niosomal formulation with respect to the commercial one was able to significantly reduce the hearth accumulation of the drug and to keep it longer in the blood circulation and also to allow the achievement of well detectable doxorubicin brain concentrations [30]. Moreover, an efficient tumor-targeted niosomal delivery system was designed by Tavano et al. Niosomes were prepared from a mixture of Pluronic L64 surfactant and cholesterol and doxorubicin was entrapped into the niosome. After the preparation, transferrin was conjugated to niosomes surface using EDC (N-[3-(dimethylamino)propyl]-Nethylcarbodiimide hydrochloride) chemistry. Doxorubicin loaded niosome anticancer activity was achieved against MCF-7 and MDA-MB-231 tumor cell lines, and a significant reduction in viability in a dose and time-related manner was observed [24].The information about some recent studies on niosomal targeted drug delivery is summarized in Table 3.2.

Table 3.2 Niosomes in targeted drug delivery

Targeted Tissue	Loaded Therapeutic Agent	Composition	Preparation Method	Surface Modification	Targeting Molecule	References
Brain	Doxorubicin	Span 60, cholesterol, solulanC24 and N-palmitoyl glucosamine	TLE-paddle method	-	N-palmitoyl glucosamine	[86]
	Dynorphin-B	Span 60, cholesterol, solulanC24 and N-palmitoyl glucosamine	Sonication	-	N-palmitoyl glucosamine	[26]

	Vasoactive intestinal peptide	Span 60, cholesterol, solulanC24 and N-palmitoyl glucosamine	Sonication	-	N-palmitoyl glucosamine	[87]
Breast cancer	Doxorubicin	Oxidate pluronic L64, cholesterol	Thin-film hydration	EDC Chemistry	Transferrin	[24]
Chronic myelogenous leukemia	Doxorubicin	Tween60, pluronic L64	Thin-film hydration	-	Magnetite	[88]
Epidermoid carcinoma	Hydroxycamptothecin	Span 60, cholesterol	Thin-film hydration	Periodate oxidation	Transferrin	[89]
	Doxorubicin	Span 60, cholesterol, solulanC24 and N-palmitoyl glucosamine	Sonication	-	N-palmitoyl glucosamine	[90]
Melanoma	Doxorubicin	Span 60, cholesterol, dicetyl phosphate, N-lauryl glucosamine	Ethanol injection method	-	N-lauryl glucosamine	[91]

Co-drug delivery

In recent years, nanoparticles have emerged as a promising class of carriers in co-delivery of multiple drugs for combination therapy [97]. Combinational therapies enhance therapeutic efficacy and decrease dosage while obtaining equal or greater levels of efficacy and reducing drug resistance [98]. Anticancer drugs often have serious side effects. With multidrug delivery system Pasut et al. achieved higher anticancer activity for carcinoma cells, whereas multi drug delivery system decreased cytotoxicity against endothelial cells and cardiomyocytes, with respect to free drug treatment. In their system, they have developed simultaneous anticancer drug epirubicin and nitric oxide carrying system, in which nitric oxide and epirubicin were covalently conjugated to each terminal of PEG. Nitric oxide acts as not only protecting reagent against anthracycline induced cardiomyopathy but also sensitizer of anticancer drug treatment. In order to increase anticancer efficacy and enhance cardiocyte protecting ability of co-delivery system, they used branched PEG as polymer backbone instead of linear one [99]. Multidrug resistance (MDR) of malignant neoplasm is the survival ability of cancer cells under the treatment with structurally and functionally diverse anticancer drugs. Increased drug efflux is mostly mediated by ATP-driven extrusion pump proteins of the ATP-binding cassette (ABC) superfamily, such as P-glycoprotein (P-gp) encoded by MDR-1, multidrug

resistance (MDR) proteins (MRPs/ABCC) and breast cancer resistance protein (BCRP/ABCG2). These drug efflux pumps noticeably decrease the intracellular concentration of numerous therapeutic agents [100]. Chemosensitizers, such as Verapamil, Elacridar, Tariquidar, and cyclosporine A mainly act as antagonist for P-gp and suppress drug efflux and consequently recover chemosensitivity of MDR cancer cells. Paclitaxel was co-encapsulated with cyclosporine A within actively targeted polymeric lipid-core micelles. P-gp inhibition with cyclosporine A caused an enhanced cytotoxicity of paclitaxel. Micelles loaded with this dual cargo demonstrated significantly higher cytotoxicity in the MDCKII-MDR1 cells than micelles loaded with paclitaxel alone [101]. Niosomes are promising nanocarriers in multi drug delivery applications [102]. Recently Sharma et al. reported the dual encapsulation of hydrophobic curcumin and hydrophilic doxorubicin in niosomes for cancer multi drug delivery [44]. Results showed that dual-drug loaded niosomes had higher cytotoxicity on HeLa cells when compared to free drugs. In another study, gallic acid, ascorbic acid, curcumin, and quercetin were encapsulated into the niosome as single agents or in combination and the effect of the drugs co-encapsulation on the physicochemical properties of the carriers, on their antioxidant properties and capability to release the encapsulated materials, was evaluated [103]. Furthermore, Marianecci et al. prepared, characterized, and applied multi drug niosomes using lidocaine and ibuprofen. Results suggest the potential application of niosomes in dermal administration of the two drugs at the same time in the same pharmaceutical formulation, as useful carriers for the treatment of various skin diseases, such as acute and chronic inflammations in presence of pain [104].

Antibiotics

Niosomal carriers are also suitable for the delivery of antibiotics and anti-inflammatory agents. These carriers have been used extensively to improve poor skin penetration and as well as enhance skin retention of the drugs. Begum and coworkers designed rifampicin, a broad spectrum antibiotic, encapsulated in a niosomal delivery system.They investigated the activity of this system in *in vitro* conditions and this study showed that niosomal formulation of rifampicin is able to provide consistent and prolonged release of the drug [105]. In another study to increase efficacy of the antibiotics and reduce the dose, Akbari et al. synthesized ciprofloxacin loaded niosomes using different nonionic surfactants and cholesterol in various concentrations by film hydration method. Drug

release through bilayers and antibacterial activity of the niosomes were examined. The results showed that cholesterol content and phase transition temperature of the surfactants influenced the performance of niosomes. Besides, all formulations presented more antibacterial activity as compared to free ciprofloxacin [106]. Vesicular systems, niosomes and liposomes, are mostly used in ophthalmic controlled delivery. Abdelbary and El-Gendy examined the feasibility of the niosomes as a carrier for the ophthalmic controlled delivery of gentamicin antibiotic. Various surfactants (Tween 60, Tween 80, or Brij 35) were combined with cholesterol and a negative charge inducer dicetyl phosphate in different molar ratios. The ability of these vesicles to entrap the selected drug was evaluated and the obtained results showed that entrapment efficiency and the release rate of gentamicin is affected by cholesterol content, type of surfactant, and the presence of charge inducer. Gentamicin loaded niosomes composed of Tween 60, cholesterol, and dicetyl phosphate were the most effective in terms of prolongation of *in vitro* drug release [107].

Anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) loaded niosomes have been prepared by several groups. These drugs may cause adverse effects such as mucosal irritation. Topically applied NSAIDs loaded niosomes can substantially improve drug permeation. To investigate the potential application of the niosomes for delivery of anti-inflammatory agents, Marianecci et al. synthesized ammonium glycyrrhizinate (AG) loaded niosomes using several surfactants and cholesterol at various concentrations. Drug entrapment efficiency, anisotropy, cytotoxicity and skin tolerability, and some further analysis have been performed for characterization. The AG-loaded niosomes demonstrated no toxicity and good skin tolerability and were able to improve the anti-inflammatory activity in mice. Moreover, an enhancement of the anti-inflammatory activity of the niosome delivered drug was observed on chemically induced skin erythema in humans [7].

Antiviral drugs

Niosomes have also demonstrated the capability to deliver various antiviral agents. Ruckmani and Sankar synthesized zidovudine, which is the first anti-HIV compound approved for clinical use, encapsulated niosomes, and examined their entrapment efficiency and as well as sustainability of release. The niosomes were formulated by combining the proportions of Tween, Span, and cholesterol. Niosomes composed Tween

80 entrapped large amounts of zidovudine and the addition of dicetyl phosphate enhanced drug release for a longer time [108]. The drug leakage from Tween 80 formulations stored at room temperature was significant compared to niosomes stored at 4∘C for 90 days. Besides, the results of a pharmacokinetic study in rabbits also confirmed that Tween 80 formulations with dicetyl phosphate were cleared from the circulation within five hours [109].

3.1.8 Recent studies

Over the past three decades, niosomes have been successfully used as drug carriers to overcome some major biopharmaceutical problems such as insolubility, side effects, and poor chemical stability of drug molecules [110]. Table 3.3 summarizes the most recent applications of niosomes as drug delivery systems.

Table 3.3 Recent studies on niosomes in drug delivery

Type of the Drug	Name of the Drug	Composition	Experimental Model	Year	References
Angiotensin receptor blockers	Candesartan cilexetil	Span60, cholesterol, dicetyl phosphate, maltodextrin	*In vitro* dissolution test for proniosomal tablets, *in vivo* evaluation of proniosomal tablets, pharmacokinetic analysis	2016	[106]
Anti-inflammatory	Naproxen	Tween80, tween20, cholesterol	*In vitro* drug release study, preformulation study	2016	[107]
	Dexamethasone	Span60, cholesterol	Characterization of niosomes, *in vitro* release studies, stability test	2015	[108]
Anti-bacterial	Moxifloxacin	Tween60, cholesterol	*In vitro* release studies, antimicrobial activity	2016	[109]
	Cefixime	C-glycoside derivative surfactant, cholesterol	*In vitro* release study, biocompatibility and bioavailability studies using experimental animals	2016	[21]
Anti-cancer	Doxorubicin	Span60, cholesterol, dicetyl phosphate, N-lauryl glucosamine	Optimization studies for formulation, skin irritancy and histopathological investigation of rat skin	2016	[91]

	Paclitaxel	Span40, cholesterol, dicetyl phosphate	Formulation studies, Pharmacokinetic and tissue distribution studies	2015	[6]
Anti-viral	Nevirapine	Tyloxapol, cholesterol	Diffusion kinetics of drug, microviscosity studies, *in vitro* release study	2015	[8]
H_2 receptor antagonist	Famotidine	Span60, cholesterol	Kinetic analysis of drug-release profiles, *ex vivo* permeability study	2016	[110]

3.1.9 Strengths and limitations of niosomes in drug delivery

One of the most important strengths of niosomes compared with liposomes is their chemical stability. Niosomes are more stable against chemical degradation or oxidation and have long storage time compared to liposomes [51].The surfactants, which are used for niosomes preparation, are biodegradable, biocompatible, and nonimmunogenic [83]. Handling and storage conditions of surfactants do not need any specifications. Moreover, composition, size, lamellarity, stability, and surface charge of niosomes can be controlled by the type of preparation method, surfactant, cholesterol content, surface charge additives, and suspension concentration [66]. On the other hand, niosomes show physical stability problems. During storage of dispersion niosomes are at risk of aggregation, fusion, drug leakage, or hydrolysis of encapsulated drugs. Furthermore, the sterilization of niosomes needs much effort. Heat sterilization and membrane filtration are unsuitable for niosomes. Thus, these areas need further research to produce commercially niosomal preparations.

3.1.10 Conclusion

Niosomes are novel nano drug carriers to design effective drug delivery systems. They offer a great opportunity for loading hydrophilic, lipophilic drugs, or both drugs together. Numbers of studies have been performed with different types of niosomes in delivery of the anticancer agents, anti-inflammatory agents, anti-infective agents, and so forth. The relevant studies demonstrated that niosomes improve the stability of the entrapped drug, reduce the dose, and enable targeted delivery to a specific type of tissue. The structural properties and characteristics of the niosomes can be enhanced by using novel

preparations, loading, and modification methods for particular routes of administration. Thus, niosomes present itself as promising tools in commercially available therapeutics.

Competing interests

The authors declare that there are no competing interests regarding the publication of this paper.

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3.1.11 References

[1] M. Seleci, D. Ag Seleci, R. Joncyzk, F. Stahl, C. Blume, and T. Scheper, "Smart multifunctional nanoparticles in nanomedicine,"*BioNanoMaterials*, vol. 17, no. 1-2, pp. 33–41, 2016.

[2] N. B. Mahale, P. D. Thakkar, R. G. Mali, D. R. Walunj, and S. R. Chaudhari, "Niosomes: novel sustained release nonionic stable vesicular systems—an overview," *Advances in Colloid and Interface Science*, vol. 183, pp. 46–54, 2012.

[3] L. Tavano, L. Gentile, C. Oliviero Rossi, and R. Muzzalupo, "Novel gel-niosomes formulations as multicomponent systems for transdermal drug delivery," *Colloids and Surfaces B: Biointerfaces*, vol. 110, pp. 281–288, 2013.

[4] K. B. Bini, D. Akhilesh, P. Prabhakara, andK. Jv, "Development and characterization of non-ionic surfactant vesicles (niosomes) for oral delivery of lornoxicam," *International Journal of Drug Development and Research*, vol. 4, no. 3, pp. 147–154, 2012.

[5] Q. Li, Z. Li, W. Zeng et al., "Proniosome-derived niosomes for tacrolimus topical ocular delivery: in vitro cornea permeation, ocular irritation, and in vivo anti-allograft rejection," *European Journal of Pharmaceutical Sciences*, vol. 62, pp. 115–123, 2014.

[6] Z. S. Bayindir, A. Bes¸ikci, and N. Y¨uksel, "Paclitaxel-loaded niosomes for intravenous administration: pharmacokinetics and tissue distribution in rats," *Turkish Journal of Medical Sciences*, vol. 45, no. 6, pp. 1403–1412, 2015.

[7] C. Marianecci, F. Rinaldi, M. Mastriota et al., "Anti-inflammatory activity of novel ammonium glycyrrhizinate/niosomes delivery system: human and murine models," *Journal of Controlled Release*, vol. 164, no. 1, pp. 17–25, 2012.

[8] S. K. Mehta and N. Jindal, "Tyloxapol niosomes as prospective drug delivery module for antiretroviral drug nevirapine," *AAPS PharmSciTech*, vol. 16, no. 1, pp. 67–75, 2014.

[9] P. Arunothayanun, M. S. Bernard, D. Q. M. Craig, I. F.Uchegbu, and A. T. Florence, "The effect of processing variables on the physical characteristics of non-ionic surfactant vesicles (niosomes) formed froma hexadecyl diglycerol ether," *International Journal of Pharmaceutics*, vol. 201, no. 1, pp. 7–14, 2000.

[10] A. Pardakhty, J. Varshosaz, and A. Rouholamini, "In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin," *International Journal of Pharmaceutics*, vol. 328, no. 2, pp. 130–141, 2007.

[11] M. Manconi, D. Valenti, C. Sinico, F. Lai, G. Loy, and A. M. Fadda, "Niosomes as carriers for tretinoin: II. Influence of vesicular incorporation on tretinoin photostability," *International Journal of Pharmaceutics*, vol. 260, no. 2, pp. 261–272, 2003.

[12] Z. S. Bayindir and N. Yuksel, "Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery," *Journal of Pharmaceutical Sciences*, vol. 99, no. 4, pp. 2049–2060, 2010.

[13] D. Paolino, D. Cosco, R. Muzzalupo, E. Trapasso, N. Picci, and M. Fresta, "Innovative bola-surfactant niosomes as topical delivery systems of 5-fluorouracil for the treatment of skin cancer," *International Journal of Pharmaceutics*, vol. 353, no. 1-2, pp. 233–242, 2008.

[14] D. Paolino, R. Muzzalupo, A. Ricciardi, C. Celia, N. Picci, and M. Fresta, "In vitro and in vivo evaluation of Bola-surfactant containing niosomes for transdermal delivery," *Biomedical Microdevices*, vol. 9, no. 4, pp. 421–433, 2007.

[15] T. Yoshioka, B. Sternberg, and A. T. Florence, "Preparation and properties of vesicles (niosomes) of sorbitan monoesters (Span 20, 40, 60 and 80) and a sorbitan triester (Span 85)," *International Journal of Pharmaceutics*, vol. 105, no. 1, pp. 1–6, 1994.

[16] V.C.Okore,A.A.Attama, K. C.Ofokansi,C.O.Esimone, and E. B. Onuigbo, "Formulation and evaluation of niosomes," *Indian Journal of Pharmaceutical Sciences*, vol. 73, no. 3, pp. 323–328, 2011.

[17] D. Akhilesh, K. B. Bini, and J. V. Kamath, "Review on span-60 based non-ionic surfactant vesicles (niosomes) as novel drug delivery," *International Journal of Research in Pharmaceutical and Biomedical Sciences*, vol. 3, pp. 6–12, 2012.

[18] C. P. Jain and S. P. Vyas, "Preparation and characterization of niosomes containing rifampicin for lung targeting," *Journal of Microencapsulation*, vol. 12, no. 4, pp. 401–407, 1995.

[19] S. Mandal, C. Banerjee, S. Ghosh, J. Kuchlyan, and N. Sarkar, "Modulation of the photophysical properties of curcumin in nonionic surfactant (Tween-20) forming micelles and niosomes: a comparative study of different microenvironments,"*The Journal of Physical Chemistry B*, vol. 117, no. 23, pp. 6957–6968, 2013.

[20] L. Di Marzio, C. Marianecci, M. Petrone, F. Rinaldi, and M. Carafa, "Novel pH-sensitive non-ionic surfactant vesicles: comparison between Tween 21 and Tween 20," *Colloids and Surfaces B: Biointerfaces*, vol. 82, no. 1, pp. 18–24, 2011.

[21] M. Imran, M. R. Shah, F.Ullah et al., "Glycoside-based niosomal nanocarrier for enhanced in-vivo performance of Cefixime," *International Journal of Pharmaceutics*, vol. 505, no. 1-2,pp. 122–132, 2016.

[22] M. Manconi, C. Sinico, D. Valenti, F. Lai, and A. M. Fadda, "Niosomes as carriers for tretinoin: III. A study into the in vitro cutaneous delivery of vesicle-incorporated tretinoin," *International Journal of Pharmaceutics*, vol. 311,no. 1-2, pp. 11–19, 2006.

[23] P. Bandyopadhyay and M. Johnson, "Fatty alcohols or fatty acids as niosomal hybrid carrier: effect on vesicle size, encapsulation efficiency and in vitro dye release," *Colloids and Surfaces B:Biointerfaces*, vol. 58, no. 1, pp. 68–71, 2007.

[24] L. Tavano, R.Muzzalupo, L.Mauro, M. Pellegrino, S. And`o, and N. Picci, "Transferrin-conjugated Pluronic niosomes as a new drug delivery system for anticancer therapy," *Langmuir*, vol. 29, no. 41, pp. 12638–12646, 2013.

[25] R.Muzzalupo, L. Tavano, R. Cassano, S. Trombino, T. Ferrarelli, and N. Picci, "A new approach for the evaluation of niosomes as effective transdermal drug delivery systems," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 79,no. 1, pp. 28–35, 2011.

[26] M. Bragagni, N. Mennini, S. Furlanetto, S. Orlandini, C. Ghelardini, and P.Mura, "Development and characterization of functionalized niosomes for brain targeting of dynorphin-B," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 87, no. 1, pp. 73–79, 2014.

[27] S. P. Vyas, R. P. Singh, S. Jain et al., "Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B," *International Journal of Pharmaceutics*, vol. 296, no. 1-2, pp. 80–86, 2005.

[28] A. Sankhyan and P. Pawar, "Recent trends in niosome as vesicular drug delivery system," *Journal of Applied Pharmaceutical Science*, vol. 2, no. 6, pp. 20–32, 2012.

[29] V. B. Junyaprasert, V. Teeranachaideekul, and T. Supaperm, "Effect of charged and non-ionic membrane additives on physicochemical properties and stability of niosomes," *AAPS PharmSciTech*, vol. 9, no. 3, pp. 851–859, 2008.

[30] M. Bragagni, N. Mennini, C. Ghelardini, and P. Mura, "Development and characterization of niosomal formulations of doxorubicin aimed at brain targeting," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 15, no. 1, pp. 184–196, 2012.

[31] C. Dufes, F. Gaillard, I. F. Uchegbu, A. G. Schatzlein, J.-C. Olivier, and J.-M. Muller, "Glucose-targeted niosomes deliver vasoactive intestinal peptide (VIP) to the brain," *International Journal of Pharmaceutics*, vol. 285, no. 1-2, pp. 77–85, 2004.vol.

[32] L. Tavano, M.Vivacqua,V.Carito, R.Muzzalupo, M. C.Caroleo, and F. Nicoletta, "Doxorubicin loaded magneto-niosomes for targeted drug delivery," *Colloids and Surfaces B: Biointerfaces*, vol. 102, pp. 803–807, 2013.

[33] M.Hong, S. Zhu, Y. Jiang, G. Tang, and Y. Pei, "Efficient tumor targeting of hydroxycamptothecin loaded PEGylated niosomes modified with transferrin," *Journal of Controlled Release*, vol. 133, no. 2, pp. 96–102, 2009.

[34] C. Dufes, J.-M. Muller, W. Couet, J.-C. Olivier, I. F. Uchegbu, and A. G. Schatzlein, "Anticancer drug delivery with transferrin targeted polymeric chitosan vesicles," *Pharmaceutical Research*, vol. 21, no. 1, pp. 101–107, 2004.

[35] S. Pawar and P. Vavia, "Glucosamine anchored cancer targeted nano-vesicular drug delivery system of doxorubicin," *Journal of Drug Targeting*, vol. 24, no. 1, pp. 68–79, 2016.

[36] N.Yuksel, Z. S. Bayindir, E. Aksakal, and A.T.Ozcelikay, "In situ niosome forming maltodextrin proniosomes of candesartan cilexetil: in vitro and in vivo evaluations," *International Journal of Biological Macromolecules*, vol. 82, pp. 453–463, 2016.

[37] N. Shah, "Characterization, optimization and formulation of niosome containing naproxen," *Journal of Biomedical and Pharmaceutical Research*, vol. 5, no. 1, pp. 1–6, 2016.

[38] M. A. Mavaddati, F. Moztarzadeh, and F. Baghbani, "Effect of formulation and processing variables on dexamethasone entrapment and release of niosomes," *Journal of Cluster Science*, vol. 26, no. 6, pp. 2065–2078, 2015.

[39] S. Sohrabi, A. Haeri, A. Mahboubi, A. Mortazavi, and S. Dadashzadeh, "Chitosan gel-embedded moxifloxacin niosomes: an efficient antimicrobial hybrid system for burn infection,"*International Journal of BiologicalMacromolecules*, vol. 85, pp. 625–633, 2016.

[40] V. J.Mokale, H. I. Patil, A. P. Patil, P. R. Shirude, and J. B.Naik,"Formulation and optimisation of famotidine proniosomes: an in vitro and ex vivo study," *Journal of Experimental Nanoscience*, vol. 11, no. 2, pp. 97–110, 2016.

[41] J. Jiao, "Polyoxyethylated nonionic surfactants and their applications in topical ocular drug delivery," *Advanced Drug Delivery Reviews*, vol. 60, no. 15, pp. 1663–1673, 2008.

[42] I. F. Uchegbu and A. T. Florence, "Non-ionic surfactant vesicles (niosomes): physical and pharmaceutical chemistry," *Advances in Colloid and Interface Science*, vol. 58, no. 1, pp. 1–55, 1995.

[43] A. Shahiwala and A. Misra, "Studies in topical application of niosomally entrapped nimesulide," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 5, no. 3, pp. 220–225, 2002.

[44] V. Sharma, S. Anandhakumar, and M. Sasidharan, "Self degrading niosomes for encapsulation of hydrophilic and hydrophobic drugs: an efficient carrier for cancer multi-drug delivery,"*Materials Science and Engineering: C*, vol. 56, pp. 393–400, 2015.

[45] G. Caracciolo, D. Pozzi, R. Caminiti et al., "Effect of hydration on the structure of solid-supported Niosomal membranes investigated by in situ energy dispersive X-ray diffraction," *Chemical Physics Letters*, vol. 462, no. 4-6, pp. 307–312, 2008.

[46] B. Nasseri, “Effect of cholesterol and temperature on the elastic properties of niosomal membranes,” *International Journal of Pharmaceutics*, vol. 300, no. 1-2, pp. 95–101, 2005.

[47] C. Marianecci, L. Di Marzio, F. Rinaldi et al., “Niosomes from 80s to present: the state of the art,” *Advances in Colloid and Interface Science*, vol. 205, pp. 187–206, 2014.

[48] G. P. Kumar and P. Rajeshwarrao, “Nonionic surfactant vesicular systems for effective drug delivery-an overview,” *Acta Pharmaceutica Sinica B*, vol. 1, no. 4, pp. 208–219, 2011.

[49] S. Biswal, P. N. Murthy, J. Sahu, P. Sahoo, and F. Amir, “Vesicles of non-ionic surfactants (niosomes) and drug delivery potential,” *Internatinal Journal of Pharmaceutical Sciences and Nanotechnology*, vol. 1, no. 1, pp. 1–8, 2008.

[50] J. N. Israelachvili, *Intermolecular and Surface Forces, 1985*, Academic Press, New York, NY, USA, 1985.

[51] I. F. Uchegbu and S. P. Vyas, “Non-ionic surfactant based vesicles (niosomes) in drug delivery,” *International Journal of Pharmaceutics*, vol. 172, no. 1-2, pp. 33–70, 1998.

[52] R. A. Khalil and A.-H. A. Zarari, “Theoretical estimation of the critical packing parameter of amphiphilic self-assembled aggregates,” *Applied Surface Science*, vol. 318, pp. 85–89, 2014.

[53] S. Moghassemi and A. Hadjizadeh, “Nano-niosomes as nanoscale drug delivery systems: an illustrated review,” *Journal of Controlled Release*, vol. 185, no. 1, pp. 22–36, 2014.

[54] T. Liu, R. Guo, W. Hua, and J. Qiu, “Structure behaviors of hemoglobin in PEG6000/Tween 80/Span 80/H2Oniosome system,” *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 293, no. 1–3, pp. 255–261, 2007.

[55] S. Agarwal, V. Bakshi, P. Vitta, A. P. Raghuram, S. Pandey, and N. Udupa, “Effect of cholesterol content and surfactant HLB on vesicle properties of niosomes,” *Indian Journal of Pharmaceutical Sciences*, vol. 66, no. 1, pp. 121–123, 2004.

[56] M. Mokhtar, O. A. Sammour, M. A. Hammad, and N. A. Megrab, “Effect of some formulation parameters on flurbiprofen encapsulation and release rates of niosomes prepared from proniosomes,” *International Journal of Pharmaceutics*, vol. 361, no. 1-2, pp. 104–111, 2008.

[57] S. Bhaskaran and P. K. Lakshmi, “Comparative evaluation of niosome formulations prepared by different techniques,” *Acta Pharmaceutica Sciencia*, vol. 51, no. 1, pp. 27–32, 2009.

[58] A. J. Baillie, A. T. Florence, L. R. Hume, G. T. Muirhead, and A. Rogerson, “The preparation and properties of niosomes non-ionic surfactant vesicles,” *The Journal of Pharmacy and Pharmacology*, vol. 37, no. 12, pp. 863–868, 1985.

[59] A. Marwa, S. Omaima, E. L. G. Hanaa, and A.-S. Mohammed, “Preparation and in-vitro evaluation of diclofenac sodium niosomal formulations,” *International Journal of Pharmaceutical Sciences and Research*, vol. 4, no. 5, pp. 1757–1765, 2013.

[60] A. Rogerson, J. Cummings, N. Willmott, and A. T. Florence, “The distribution of doxorubicin in mice following administration in niosomes,” *Journal of Pharmacy and Pharmacology*, vol. 40, no. 5, pp. 337–342, 1988.

[61] S. Srinivas, Y. A. Kumar, A.Hemanth, andM. Anitha, “Preparation and evaluation of niosomes containing aceclofenac,” *Digest Journal ofNanomaterials and Biostructures*, vol. 5, no. 1,pp. 249–254, 2010.

[62] S.Moghassemi, E. Parnian, A. Hakamivala et al., “Uptake and transport of insulin across intestinal membrane model using trimethyl chitosan coated insulin niosomes,” *Materials Science and Engineering C*, vol. 46, pp. 333–340, 2015.

[63] A. Budhiraja and G. Dhingra, “Development and characterization of a novel antiacne niosomal gel of rosmarinic acid,” *Drug Delivery*, vol. 22, no. 6, pp. 723–730, 2015.

[64] H. Kiwada, H. Niimura, Y. Fujisaki, S. Yamada, and Y. Kato, “Application of synthetic alkyl glycoside vesicles as drug carriers. I. Preparation and physical properties,” *Chemical and Pharmaceutical Bulletin*, vol. 33, no. 2, pp. 753–759, 1985.

[65] A. S. Zidan, Z. Rahman, and M. A. Khan, “Product and process understanding of a novel pediatric anti-HIV tenofovir niosomes with a high-pressure homogenizer,” *European Journal of Pharmaceutical Sciences*, vol. 44, no. 1-2, pp. 93–102, 2011.

[66] S. Verma, S. K. Singh, N. Syan, P. Mathur, and V. Valecha, “Nanoparticle vesicular systems: a versatile tool for drug delivery,” *Journal of Chemical and Pharmaceutical Research*, vol. 2, no. 2, pp. 496–509, 2010.

[67] A.Manosroi, R. Chutoprapat, M. Abe, and J.Manosroi, “Characteristics of niosomes prepared by supercritical carbon dioxide ($scCO_2$) fluid,” *International Journal of Pharmaceutics*, vol. 352, no. 1-2, pp. 248–255, 2008.

[68] A.Manosroi,W. Ruksiriwanich, M.Abe, H. Sakai,W.Manosroi, and J. Manosroi, “Biological activities of the rice bran extract and physical characteristics of its entrapment in niosomes by supercritical carbon dioxide fluid,” *The Journal of Supercritical Fluids*, vol. 54, no. 2, pp. 137–144, 2010.

[69] V. R. Yasam, S. L. Jakki, J. Natarajan, and G. Kuppusamy, “A review on novel vesicular drug delivery: proniosomes,” *Drug Delivery*, vol. 21, no. 4, pp. 243–249, 2014.

[70] L.D. Mayer,M. B. Bally, and P. R. Cullis, “Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient,” *Biochimica et Biophysica Acta (BBA)—Biomembranes*, vol. 857, no. 1, pp. 123–126, 1986.

[71] A. K. Verma and J. C. Bindal, “A vital role of niosomes on controlled and novel drug delivery,” *Indian Journal of Novel Drug Delivery*, vol. 3, pp. 238–246, 2011.

[72] M. R. Mozafari, “A new technique for the preparation of nontoxic liposomes and nanoliposomes: the heating method,” in *Nanoliposomes: From Fundamentals to Recent Developments*, pp. 91–98, Trafford Publishing, Oxford, UK, 2005.

[73] M. R. Mozafari, C. J. Reed, and C. Rostron, "Cytotoxicity evaluation of anionic nanoliposomes and nanolipoplexes prepared by the heating method without employing volatile solvents and detergents," *Die Pharmazie*, vol. 62, no. 3, pp. 205–209, 2007.

[74] H. Talsma, M. J. Van Steenbergen, J. C. H. Borchert, and D. J. A. Crommelin, "A novel technique for the one-step preparation of liposomes and nonionic surfactant vesicles without the use of organic solvents. Liposome formation in a continuous gas stream: the 'bubble' method," *Journal of Pharmaceutical Sciences*, vol. 83, no. 3, pp. 276–280, 1994.

[75] L. Tavano, R. Aiello, G. Ioele, N. Picci, and R. Muzzalupo, "Niosomes from glucuronic acid-based surfactant as new carriers for cancer therapy: preparation, characterization and biological properties," *Colloids and Surfaces B: Biointerfaces*, vol. 118, pp. 7–13, 2014.

[76] A. Priprem, K. Janpim, S. Nualkaew, and P. Mahakunakorn,"Topical niosome gel of *Zingiber cassumunar* Roxb. extract for anti-inflammatory activity enhanced skin permeation and stability of compound D," *AAPS PharmSciTech*, vol. 17, no. 3, pp.631–639, 2016.

[77] W. Hua and T. Liu, "Preparation and properties of highly stable innocuous niosome in Span 80/PEG 400/H2Osystem," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 302, no. 1, pp. 377–382, 2007.

[78] A. Manosroi, P. Wongtrakul, J. Manosroi et al., "Characterization of vesicles prepared with various non-ionic surfactants mixed with cholesterol," *Colloids and Surfaces B: Biointerfaces*, vol. 30, no. 1-2, pp. 129–138, 2003.

[79] D. Pozzi, R. Caminiti, C.Marianecci et al., "Effect of cholesterol on the formation and hydration behavior of solid-supported niosomal membranes," *Langmuir*, vol. 26, no. 4, pp. 2268–2273, 2010.

[80] D. Pando, G. Guti´errez, J. Coca, and C. Pazos, "Preparation and characterization of niosomes containing resveratrol," *Journal of Food Engineering*, vol. 117, no. 2, pp. 227–234, 2013.

[81] M. Tabbakhian, S. Daneshamouz, N. Tavakoli, and M. R. Jaafari, "Influence of liposomes and niosomes on the in vitro permeation and skin retention of finasteride," *Iranian Journal of Pharmaceutical Sciences*, vol. 1, no. 3, pp. 119–130, 2005.

[82] S. K. Mehta and N. Jindal, "Formulation of Tyloxapol niosomes for encapsulation, stabilization and dissolution of antitubercular drugs," *Colloids and Surfaces B: Biointerfaces*, vol. 101, pp. 434–441, 2013.

[83] A. Y. Waddad, S. Abbad, F. Yu et al., "Formulation, characterization and pharmacokinetics of Morin hydrate niosomes prepared from various non-ionic surfactants," *International Journal of Pharmaceutics*, vol. 456, no. 2, pp. 446–458, 2013.

[84] Y. Hao, F. Zhao, N. Li, Y. Yang, and K. Li, "Studies on a high encapsulation of colchicine by a niosome system," *International Journal of Pharmaceutics*, vol. 244, no. 1-2, pp. 73–80, 2002.

[85] A. Dwivedi, A. Mazumder, L. du Plessis, J. L. du Preez, R. K. Haynes, and J. du Plessis, "In vitro anti-cancer effects of artemisone nano-vesicular formulations on melanoma cells," *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol. 11, no. 8, pp. 2041–2050, 2015.

[86] R. P. Gude, M. G. Jadhav, S. G. A. Rao, and A. G. Jagtap, "Effects of niosomal cisplatin and combination of the same with theophylline and with activated macrophages inmurine B16F10 melanoma model," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 17, no. 2, pp. 183–192, 2002.

[87] D. Cosco, D. Paolino, R. Muzzalupo et al., "Novel PEG-coated niosomes based on bola-surfactant as drug carriers for 5- fluorouracil," *Biomedical Microdevices*, vol. 11, no. 5, pp. 1115–1125, 2009.

[88] W. Han, S.Wang, R. Liang et al. "Non-ionic surfactant vesicles simultaneously enhance antitumor activity and reduce the toxicity of cantharidin," *International Journal of Nanomedicine*, vol. 8, pp. 2187–2196, 2013.

[89] D. S. Shaker, M. A. Shaker, and M. S. Hanafy, "Cellular uptake, cytotoxicity and *in-vivo* evaluation of Tamoxifen citrate loaded niosomes," *International Journal of Pharmaceutics*, vol. 493, no. 1-2, pp. 285–294, 2015.

[90] I. F. Uchegbu, J. A. Double, L. R. Kelland, J. A. Turton, and A. T. Florence, "The activity of doxorubicin niosomes against an ovarian cancer cell line and three in vivo mouse tumour models," *Journal of Drug Targeting*, vol. 3, no. 5, pp. 399–409, 1996.

[91] D. J. Kerr, A. Rogerson, G. J. Morrison, A. T. Florence, and S. B. Kaye, "Antitumour activity and pharmacokinetics of niosome encapsulated adriamycin in monolayer, spheroid and xenograft," *British Journal of Cancer*, vol. 58, no. 4, pp. 432–436,1988.

[92] S. Y. Gaikwad, A. G. Jagtap, A. D. Ingle, S. G. A. Ra, and R. P. Gude, "Antimetastatic efficacy of niosomal pentoxifylline and its combination with activated macrophages in murine B16F10 melanoma model," *Cancer Biotherapy & Radiopharmaceuticals*, vol. 15, no. 6, pp. 605–615, 2000.

[93] M. Kong, H. Park, C. Feng, L. Hou, X. Cheng, and X. Chen, "Construction of hyaluronic acid noisome as functional transdermal nanocarrier for tumor therapy," *Carbohydrate Polymers*, vol. 94, no. 1, pp. 634–641, 2013.

[94] A. Narang and R. Mahato, *Targeted Delivery of Small and Macromolecular Drugs*, CRC Press, 2010.

[95] D. Ag, R. Bongartz, L. E. Dogan et al., "Biofunctional quantum dots as fluorescence probe for cell-specific targeting," *Colloids and Surfaces B: Biointerfaces*, vol. 114, pp. 96–103, 2014.

[96] M. Seleci, D. A. Seleci, M. Ciftci et al., "Nanostructured amphiphilic star-hyperbranched block copolymers for drug delivery," *Langmuir*, vol. 31, no. 15, pp. 4542–4551, 2015.

[97] S. Gadde, "Multi-drug delivery nanocarriers for combination therapy," *Med Chem Comm*, vol. 6, no. 11, pp. 1916–1929, 2015.

[98] B. Al-Lazikani, U. Banerji, and P. Workman, "Combinatorial drug therapy for cancer in the post-genomic era," *Nature Biotechnology*, vol. 30, no. 7, pp. 679–692, 2012.

[99] G. Pasut, F. Greco, A.Mero et al., "Polymer-drug conjugates for combination anticancer therapy: investigating the mechanism of action," *Journal of Medicinal Chemistry*, vol. 52, no. 20, pp. 6499–6502, 2009.

[100] Y. D. Livney and Y. G. Assaraf, "Rationally designed nanovehicles to overcome cancer chemoresistance," *Advanced Drug Delivery Reviews*, vol. 65, no. 13-14, pp. 1716–1730, 2013.

[101] C. Sarisozen, I. Vural, T. Levchenko, A. A. Hincal, and V. P. Torchilin, "PEG-PE-based micelles co-loaded with paclitaxel and cyclosporine A or loaded with paclitaxel and targeted by anticancer antibody overcome drug resistance in cancer cells," *Drug Delivery*, vol. 19, no. 4, pp. 169–176, 2012.

[102] M. Thakkar and S. Brijesh, "Opportunities and challenges for niosomes as drug delivery systems," *Current Drug Delivery*, vol. 13, pp. 1–15, 2016.

[103] L. Tavano, R. Muzzalupo, N. Picci, and B. De Cindio, "Coencapsulation of antioxidants into niosomal carriers: gastrointestinal release studies for nutraceutical applications," *Colloids and Surfaces B: Biointerfaces*, vol. 114, pp. 82–88, 2014.

[104] C. Marianecci, F. Rinaldi, L. D. Marzio, A. Ciogli, S. Esposito, and M. Carafa, "Polysorbate 20 vesicles as multi-drug carriers: in vitro preliminary evaluations," *Letters in Drug Design and Discovery*, vol. 10, no. 3, pp. 212–218, 2013.

[105] K. Begum, A. F. Khan, H. K. Hana, J. Sheak, and R. U. Jalil, "Rifampicin niosome: preparations, characterizations and antibacterial activity against staphylococcus aureus and staphylococcus epidermidis isolated from acne," *Dhaka University Journal of Pharmaceutical Sciences*, vol. 14, no. 1, pp. 117–123, 2015.

[106] V. Akbari, D. Abedi, A. Pardakhty, and H. Sadeghi-Aliabadi, "Release studies on ciprofloxacin loaded non-ionic surfactant vesicles," *Avicenna Journal of Medical Biotechnology*, vol. 7, no. 2, pp. 69–75, 2015.

[107] G. Abdelbary and N. El-Gendy, "Niosome-encapsulated gentamicin for ophthalmic controlled delivery," *AAPS Pharm-SciTech*, vol. 9, no. 3, pp. 740–747, 2008.

[108] K. Ruckmani and V. Sankar, "Formulation and optimization of zidovudine niosomes," *AAPS PharmSciTech*, vol. 11, no. 3, pp. 1119–1127, 2010.

[109] K. Ruckmani, V. Sankar, and M. Sivakumar, "Tissue distribution, pharmacokinetics and stability studies of zidovudine delivered by niosomes and proniosomes," *Journal of Biomedical Nanotechnology*, vol. 6, no. 1, pp. 43–51, 2010.

[110] H. Abdelkader, A. W. G. Alani, and R. G. Alany, "Recent advances in non-ionic surfactant vesicles (niosomes): selfassembly, fabrication, characterization, drug delivery applications and limitations," *Drug Delivery*, vol. 21, no. 2, pp. 87–100, 2014.

3.2 Book Chapter - Niosomes for brain targeting

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3.2.1 Summary

Several nanocarriers have been used in drug delivery applications tracking the progress in nanotechnology. One of the main purposes of using these structures is to overcome the current limitations such as poor penetration through the blood brain barrier (BBB) and lack of specificity of conventional therapies for brain targeting. A number of comprehensive studies have been carried out with niosomes, non-ionic surfactant vesicles as a drug carrier. High chemical stability and relatively low cost of the materials for niosome preparations make them promising candidates to produce commercial products for brain delivery.

This chapter focuses on design and application potential of niosomal carriers for brain targeting. In the introduction part, the difficulties that have been encountered to penetrate the BBB in the treatment of central nervous diseases and the properties of the niosomes are presented. Afterward, the role of coating the nanoparticles surfaces with non-ionic surfactants to facilitate crossing the BBB is discussed. Brief information about structure and preparation methods of niosomes is provided. Physicochemical characterization parameters of niosomes and the techniques to determine these parameters are reviewed. The application potential of niosomes in drug delivery and brain targeting is discussed. Moreover, two different targeting strategies (active and passive) to enhance the specificity of cancer therapy are described. Especially, the most commonly used active targeting ligands for brain targeting are listed. The combination of niosomal drug delivery systems with active targeting ligands for the specific delivery of drugs to the brain is evaluated in detail.

3.2.2 Abstract

Delivering drug to the brain has still many obstacles. Especially crossing the brain barriers is a big challenge. The application of nanomaterials to medicine has provided the development of novel drug carriers, can facilitate the delivery of drugs to the brain. Niosomes are non-ionic surfactant based vesicles and has been used as a nanocarrier for different types of drugs. Moreover, niosome surfaces can be modified with targeting ligands to enable cell specific targeting. Due to these promising features, niosomes have a great potential using as a carrier for the delivery of drugs to the brain. The present chapter provides the fundamental information about niosomal drug delivery systems and their recent applications brain targeting.

3.2.3 Introduction

A number of impediments are present for the effective treatment of central nervous system (CNS) diseases. The blood–brain barrier (BBB), which plays a key role in protecting and maintaining the homeostasis of the brain, prevents most drugs from entering the CNS from the bloodstream. Additionally, the blood-cerebrospinal fluid barrier (BCSF) and other specialized CNS barriers hinder efficient delivery of therapeutic and diagnostic agents to diseased areas of the brain. Recent advances in nanotechnology caused a growing interest using nanomaterials in medicine to solve a number of problems associated with BBB [1]. The development of a wide range of nanomaterials provides many advantages and new scientific approaches in CNS disease diagnosis, treatment, monitoring, and prevention. Multifunctional nanomaterials are able to penetrate the BBB and can be easily modified by active and passive targeting to enhance the concentration of the drug molecule inside the specific area of the brain [2]. Furthermore, the drug is protected by nanocarrier from degradation and/or interaction with the biological environment, before reaching the target tissue [3].

Among various nanocarriers, vesicular systems have received growing attention in the recent years for brain drug delivery [4]. They can enhance bioavailability of encapsulated drug and provide therapeutic activity in a controlled manner for a prolonged period. Niosomes (non-ionic surfactant vesicles) are one of the promising vesicular drug carriers that have a bilayer structure and are assembled by self-association of non-ionic surfactants in an aqueous phase. In recent years, a variety of non-ionic surfactants has been described to form niosomes and enable the encapsulation of numerous drugs with a wide range of solubility [5-7]. The non-ionic nature of non-ionic surfactants offers high

biocompatibility and low toxicity that are important parameters for drug delivery applications. Moreover, niosomes can be produced with lower costs, and have greater stability, longer shelf life and wider formulation versatility in comparison with traditional liposomes. These superiorities and advantages of niosomes, compared to other drug delivery devices, make them promising tools for brain targeting to produce commercially available therapeutics.

This chapter describes the using of niosomes as potential drug delivery systems and provides up to date information regarding recent applications of niosomes for brain targeting.

3.2.4 Non-ionic surfactants: Enhanced penetration through Bbb

Non-ionic surfactants are amphiphilic molecules that are composed of two different regions: one of them is hydrophilic (water-soluble) and the other one is hydrophobic (organic-soluble). They are a class of surfactants that have no charged groups in their hydrophilic heads. Alkyl ethers, alkyl esters, alkyl amides, and fatty acids are the main non-ionic surfactant classes used for niosome production. They are generally less toxic, less hemolytic and less irritating to cellular surfaces compared to their anionic, amphoteric, or cationic counterparts. Non-ionic surfactants are used extensively in the chemical industry in such areas as detergents, health and personal care, coatings and polymers as solubilizers, wetting agents, emulsifiers, and permeability enhancers [8].

Apart from being a part of niosomes, surfactants play a key role in coating of the nanoparticle surface. Surface modifications lead to an increase of nanocarrier circulation time in the blood and facilitate penetration of nanoparticles through the BBB via recognizing cellular receptors [9]. Especially polysorbate 80 (Tween 80) coated nanoparticles are able to transport the loaded drugs across BBB, which cannot cross the BBB without Tween 80 [10-12]. First *in vivo* experiments were performed by Kreuter et al. to enlighten the mechanism of this transport system [13]. They investigated the possible involvement of apolipoproteins that bind to lipoprotein receptors on the brain capillary endothelial cells, in the transport of nanoparticle-bound drugs into the brain. Different apolipoproteins were adsorbed directly onto uncoated or Tween 80-precoated dalargin-loaded poly(butyl cyanoacrylate) (PBCA) nanoparticles. After intravenous injection of these samples to mice, the antinociceptive effect was measured. Results showed that especially apolipoproteins E and B yielded high antinociceptive effects that were similar to Tween 80-coating alone and even higher after Tween 80 coating plus

apolipoprotein E and B overcoating. According to results, it was concluded that the Tween 80-coated PBCA nanoparticles adsorb apolipoproteins E and B from the blood, and these proteins stimulate receptor-mediated endocytosis of the particles by the endothelial cells forming the BBB. Moreover, coating PBCA with the non-ionic surfactant poloxamer 188 also enhanced the antitumor action of doxorubicin against intracranial glioblastoma [14, 15]. Consequently, these systems facilitate the delivery of the drug into the brain.

3.2.5 Niosomes: Structure, preparation, and characterization

Niosomes are primarily composed of non-ionic surfactants, cholesterol and hydration medium. The self-assembly of non-ionic surfactants in aqueous media results in the formation of closed bilayered vesicles. Application of additional energy such as mechanical (physical shaking, ultrasound) or heat is needed for the formation of niosomes. Cholesterol interacts with non-ionic surfactants via hydrogen bonds in the bilayer and plays an important role in the physical properties and structure of niosomes. It prevents the vesicle aggregation and improves the rigidity of niosomes [16]. Besides, charged molecules such as dicetyl phosphate, phosphatidic acid and stearylamine are added to the bilayer to increase the stability of vesicles [17]. The addition amount of charged molecule to niosomal formulation needs to be 2.5–5.0 mol%. Adding the high amount of charged molecules may inhibit niosome formation. These vesicles can be categorized into three groups according to their size and number of bilayers: small unilamellar vesicles (SUV, 10–100 nm), large unilamellar vesicles (LUV, 100–3000 nm), and multilamellar vesicles (MLV, 1000-more nm) where more than one bilayer is present (Figure 3.3).

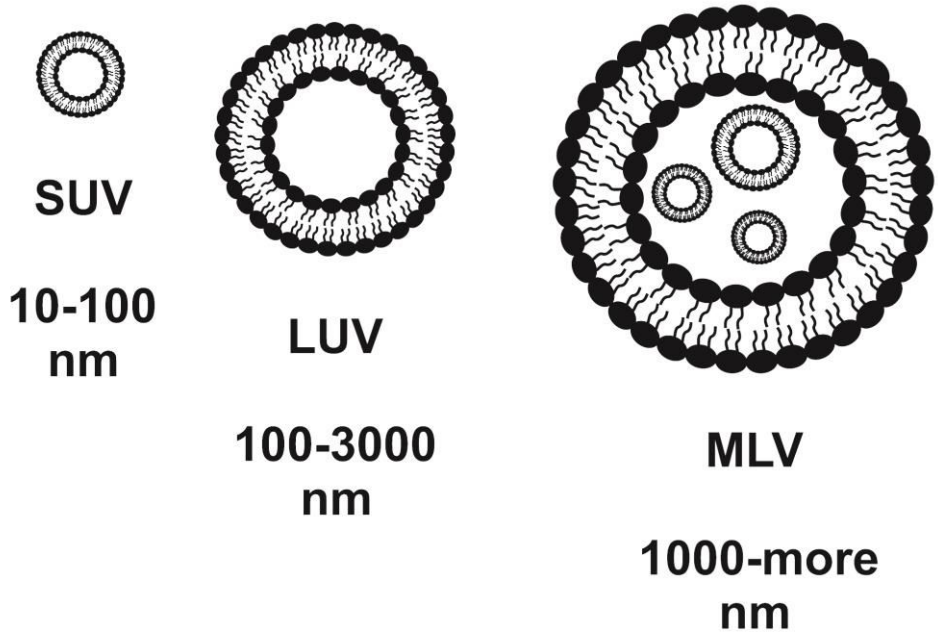


Figure 3.3 Structure and categorization of niosomes

Preparation of niosomes requires simple methods. Preparation techniques comprise generally the hydration of a mixture of surfactant/lipid at elevated temperature using hydration medium including drug. Subsequently, size reduction methods such as sonication and extrusion are applied to obtain a colloidal dispersion. Finally, the unentrapped drug is removed from the niosomal dispersion by centrifugation, gel filtration, or dialysis. The well-known protocols for niosome preparation are: thin-film hydration [18, 19], ether injection [20], reverse phase evaporation [21], trans membrane pH gradient [22] and proniosome [23].

Physicochemical characterization parameters of niosomes are vesicle size, morphology, size distribution, zeta potential, number of lamellae. These parameters have a direct impact on the stability of niosomes. The characterization methods of niosomes are summarized in Table 3.4. Furthermore, entrapment efficiency (EE%), stability and drug release are critical factors for medical applications of niosomes. EE% is the percentage of the drug entrapped in niosomes referred to the initial amount of drug that is present in the non-purified sample. It is affected by niosome contents, physicochemical properties of drug, and preparation methods [24]. The stability of niosomes can be tested by measuring mean vesicle size and size distribution or determining the entrapment efficiency over several month storage periods at different conditions. Sustained drug release from niosomes is another quite important issue to minimize side effects of the drug in the human body and enhance the effects of drug at the target location. The release rate of the drug from niosomes is generally determined via dialysis method.

Table 3.4 Characterization methods of niosomes

Parameters	Methods used to determine the parameter	References
Size and morphology	Dynamic light scattering (DLS), Nanoparticle tracking analysis (NTA), Transmission electron microscopy (TEM), Scanning electron microscopy (SEM), Cryo-transmission electron microscopy (cryo- TEM), Atomic force microscopy (AFM)	[25-28]
Size distribution	DLS	[5]
Surface charge and zeta potential	Zetasizer, Microelectrophoresis, DLS, pH-sensitive fluorophores	[29]
Bilayer characteristics	Small angle X-ray scattering (SAXS), *In situ* energy-dispersive X-ray diffraction (EDXD)	[25,30]

3.2.6 Niosomal drug delivery systems

Side effects, poor solubility and chemical stability are the main problems of conventional drugs passing through different environments in the human body on their way to the target location. These cause inefficient therapeutic effect. Niosomes have been used for the delivery of several pharmacological and diagnostic agents to overcome these problems. Due to their biocompatibility, low toxicity, and unique structure, they allow the development of effective novel drug delivery systems [31]. They are able to load both hydrophilic and lipophilic drugs. Hydrophilic drugs are encapsulated into the aqueous core and lipophilic drugs are incorporated in the membrane bilayer of niosome. Moreover, they offer a great opportunity for loading both drugs together in one nanocarrier (Figure 3.4). Another feature of the niosomes is the fluidity of their membrane, which allows the controlled release of a compound without destroying the vesicular structure. The drug release occurs by passive transport of the drug through the niosomal membrane bilayer. Physicochemical parameters of niosomes can be arranged to obtain the desired drug delivery system. Besides, the surface of niosomes can be easily modified to create targeted niosomes.

Figure 3.4 Encapsulation drugs in niosome

The application of niosomal technology is widely varied and can be used to treat a number of diseases. They have been used in pulmonary delivery [32], transdermal delivery [33], ophthalmic delivery [34], vaccine delivery [35], gene delivery [36], protein and peptide delivery [37] and delivery of chemotherapeutics. The concept of loading anti-cancer drugs into niosomes for a better delivery of the drug to specific target location is widely investigated by researchers. Anti-cancer drugs such as doxorubicin [38, 39], paclitaxel [27], methotrexate [40], 5-fluorouracil [41] were successfully entrapped in niosomes and characterized in detail to develop efficient drug carrier systems for cancer therapy. To test the efficiency and specificity of niosomal anti-cancer drugs, *in vitro* and *in vivo* investigations were performed [42, 43]. Results from a number of studies suggest that niosomes have great potential in the application of several types of cancer therapy [44, 45]. Furthermore, antibiotics [46], anti-inflammatory [47], and antiviral drugs were entrapped in niosomes to improve their stability and reduce the dose of the drug.

3.2.7 Targeting strategies

Transporting of drugs to the target site is a major drawback in the treatment of many diseases. Numbers of conventional drugs have limited effectiveness, poor biodistribution and a lack of selectivity. Especially, effective and specific delivery of drugs to the brain is a big challenge since most drugs cannot pass the BBB. Nanoparticles are promising tools to deliver drugs to the desired part of the body. There are two different strategies for targeting of the nanoparticles: passive and active targeting.

Passive targeting

Tumor tissue has highly disorganized vascular architecture, irregular blood flow, and reduced lymphatic drainage. These properties provide the enhanced permeability and retention (EPR) effect for nanoparticles that can enhance the intracellular concentration of the drugs in cancer cells. The strength of EPR effect is influenced by two factors. First, angiogenic tumors produce vascular endothelial growth factors. These growth factors increase the permeability of newly formed vessels associated with the tumor and cause infiltration of circulating particles. Second, due to the reduced lymphatic drainage of tumors, the permeating nanocarriers are not removed efficiently and thus are retained in the tumor tissue, which leads to the accumulation of nanoparticles.

Passive targeting of nanoparticles also enables the drugs to pass the BBB via different pathways [1]. A wide range of CNS drugs may enter into the brain with nanocarriers.

Active targeting

Active targeting is based on targeting ligands such as antibodies, peptides, aptamers and small molecules that bind specifically to an overexpressed target on the cell surface and trigger receptor-mediated endocytosis after binding. Nanoparticles are conjugated with targeting ligands thereby allowing accumulation of the drug within tissues or intracellular organelles specifically.

Active targeting of the BBB and brain tumors represent a promising non-invasive approach for enhanced drug delivery to the brain. The identified and commonly-targeted receptors for brain targeting are: transferrin, insulin, low-density lipoproteins (LDL), leptin, glutathione, folic acid, and neuropilin. Summary of brain targeting ligands and their receptors are listed in Table 3.5.

Table 3.5 Targeting ligands and their receptors for active brain targeting

Targeting ligand	**Targeted receptor**	**References**
Folic acid	Folate receptor	[48,49]
Transferrin	Transferrin receptor (TfR)	[50,51]
Anti-TfR monoclonal antibody (mAb), 7579	TfR	[52]
Angiopep-2	Low-density lipoprotein receptor (LDR)	[53,54]

Anti-insulin receptor monoclonal antibody (29B4)	Insulin receptor	[55]
Glutathione	Glutathione receptor	[56]
Peptides comprising amino acid residues 70–89 of leptin ($Lep_{70–89}$)	Leptin receptor (ObR)	[57]
tLyp-1 peptide	Neuropilin receptor	[58,59]

3.2.8 Niosomes for brain targeting

In the literature, the advantages of niosomes have been already used to obtain efficient delivery of therapeutic agents to the brain. Morin hydrate (MH), which has a neuroprotective effect in Parkinson's disease, was encapsulated in niosomes composed of nonionic surfactants, cholesterol and dicetyl phosphate. Niosomal formulations were optimized and injected to mouse via lateral tail vein to take real time images. A non-invasive real-time imaging technique was applied to understand the *in vivo* biodistribution of MH niosomes. The *ex vivo* imaging of the excised organs demonstrated the capability of MH niosomes to cross the BBB [60]. Varshosaz et al. prepared niosomal formulation of α-tocopherol and ascorbic acid for enhanced brain delivery of these drugs in preventing neuronal cell damages during ischemia-reperfusion disorders. After characterization studies, neuroprotective effects of the niosomal formulations were investigated in an ischemiare-perfusion model in male rats. *In vivo* results showed that the effectiveness of the formulated new drug delivery system in protection of cerebral tissue against elevation in oxygen free radical concentration during cerebral ischemia-reperfusion course was more than the free ascorbic acid [61].

Moreover, delivery of the drugs to the brain via nasal route provides some more advantages. Folic acid is a water soluble vitamin having difficulty in crossing the BBB and the low blood level of folic acid is the main cause of depression in Alzheimer's disease. Ravouru et al. developed niosomal nasal drug delivery systems by using folic acid to target the brain. *Ex vivo* perfusion studies were carried out using a rat model and results showed that about 48.15% of the drug was absorbed through nasal cavity at the end of 6 hrs [62].

Recently, niosomes containing cationic lipids were used as a carrier for gene delivery in retina and brain. Niosome-DNA vectors (nioplexes) were prepared and characterized in detail. *In vitro* experiments were performed to evaluate transfection efficiency and cell viability in different cell lines. Subsequently, nioplexes were administrated to rat retina

via intravitreal and subretinal injections and to rat brain with in cerebral cortex. *In vivo* results demonstrated that after injections of nioplexes, the cells in rat retina and brain were transfected successfully [63].

These outcomes provide new insights for the development of niosome based delivery systems for brain targeting. Furthermore, several approaches were used for active targeting of the niosomal drugs to the brain that are explained below.

3.2.9 Modification of niosomes with targeting ligands

Glucose derivatives

The large energetic demand of the brain is provided almost by β-D-glucose. Glucose in the blood must cross the BBB's luminal and abluminal membranes to reach neural tissue. It is transported to the brain via transporters, enzymes, and cell signaling processes. Glucose transporter (GLUT1) enables glucose transport across the BBB and it is overexpressed on BBB cells. Therefore, glucose derivatives are promising targeting ligands for drug transport through the BBB.

Dufes et al. synthesized *N*-Palmitoylglucosamine (NPG, the glucose-derivatized surfactant) niosomes entrapping vasoactive intestinal peptide (VIP) by shaking a mixture of NPG, non-ionic surfactants (Span 60 and Solulan C24) and cholesterol in PBS at 90 ºC for 30 min, followed by probe sonication for 5 min [64]. VIP was entrapped into niosomes by probe sonicating them in 125I-VIP and unlabelled VIP solution. VIP and 125I-VIP-loaded glucose-bearing niosomes were intravenously injected to mice. After administration of VIP in solution or encapsulated in glucose-bearing niosomes or in control niosomes, brain uptake was determined by measuring the radioactivity of 125I-labeled VIP. Results indicated that VIP encapsulation within glucose-bearing niosomes mainly allowed a significantly higher VIP brain uptake compared to control niosomes. In another study, Bragagni et al. investigated the development and characterization of a niosomal formulation functionalized with NPG to obtain a potential brain targeted delivery system for the anticancer drug doxorubicin [65]. The developed doxorubicin NPG-niosomal formulation was injected in rats, in comparison with a commercial solution of drug in order to evaluate its effectiveness in enhancing doxorubicin brain delivery. After administration, significantly higher doxorubicin plasma levels were obtained with the NPG-niosomal dispersion with respect to the commercial solution at the same drug dosage. Their results showed that the developed niosomal formulation was

able to keep the drug longer in the blood circulation system compared to the commercial drug solution. Moreover, NPG functionalized niosomal formulation was also used to targeted delivery of dynorphin-B, which is an endogenous neuropeptide with relevant pharmacological activities on the central nervous system [66]. The optimized niosomal formulation with entrapped dynorphin-B was administered intravenously to mice. The antinociceptive effect of this niosomal formulation and a simple solution of the peptide were investigated. A significantly higher antinociceptive effect was obtained for targeted niosomal dynorphin-B, than for peptide solution. It can be concluded that encouraging and promising results were obtained in the previous studies by using niosomes bearing NPG as a drug carrier and this may trigger the usage of this system in further studies.

Transferrin

Transferrin receptor, which is an iron binding transmembrane protein and facilitates iron uptake in cells, is highly expressed in brain endothelial cells. Several types of nanoparticles were functionalized with TfR binding ligands such as peptides [67], antibodies [68], or transferrin [69] to deliver therapeutics to the brain.

TfR is also overexpressed in tumor cells. Niosomes were coupled with transferrin to improve tumor therapy. Hydroxycamptothecin (HCPT) was loaded into polyethylene glycolated niosomes (PEG-niosomes) and transferrin was conjugated to the surface of PEG-niosomes. Compared with HCPT injection, transferrin conjugated PEG-niosomes demonstrated stronger anti-tumor activity in mice [70]. In another study, niosomes were prepared from Pluronic L64 surfactant and cholesterol by Tavano et al. After the preparation, transferrin was conjugated to niosomes and rhodamine was loaded the vesicles. The specific uptake of rhodamine-loaded transferrin conjugated-niosomes was evaluated on tumor cells via confocal microscopy. Results demonstrated that transferrin conjugated-niosomes were specifically uptaken by tumor cells [71]. However, transferrin-bearing niosomes have not been applied for brain targeting yet, they have a great potential for future studies.

tLyP-1 peptide

Neuropilin-1 (NRP-1) is a transmembrane protein overexpressed on the surface of both glioma and endothelial cells of angiogenic blood vessels [72-74]. tLyp-1 (tumor homing and penetrating peptide) peptide with 7 amino acid (CGNKRTR), is as a ligand targeted to the NRP-1 receptor with high affinity and specificity. Hence, tLyp-1 has been used as

a targeting ligand for the delivery of drugs to the brain tumor [58]. Recently, polyethylene glycolated niosomes (PEGNIO) were synthesized and doxorubicin and curcumin were encapsulated in niosomes via thin film hydration method. The surface of co-drug loaded PEGNIO was modified with tLyp-1. After characterization studies, *in vitro* investigations were carried out on human glioblastoma and human mesenchymal stem cells. The results clearly indicated that the strategy by co-administration of doxorubicin and curcumin with tLyp-1 functionalized niosomes could significantly improve anti-glioma treatment [75].

3.2.10 Conclusions and future directions

The treatment of brain related diseases presents a major challenge. Using nanoparticles may enable to overcome the difficulties of delivering therapeutic agents to specific regions of the brain. Niosomes are one of the promising drug carriers to design novel drug delivery systems for brain disease treatment. Their unique structure provides loading hydrophilic, or lipophilic drugs, or both drugs together in the same vesicle at the same time. Besides, their surface can easily be functionalized and modified with ligands. These features of the niosomes have been already applied to deliver different types of agents to the brain. However, presently there are no commercial niosomal drugs available for brain targeting. Therefore, further research studies needed to be performed. Especially, the design of new targeted and co-drug loaded niosomal delivery systems for brain targeting may contribute to producing commercially available products.

Key words

Nonionic surfactants, niosomes, drug delivery, passive and active targeting, targeting ligands, brain targeting

3.2.11 References

1. Bhaskar, S.; Tian, F.; Stoeger, T.; Kreyling, W.; de la Fuente, J. M.; Grazú, V.; Borm, P.; Estrada, G.; Ntziachristos, V.; Razansky, D. Multifunctional Nanocarriers for diagnostics, drug delivery and targeted treatment across blood-brain barrier: perspectives on tracking and neuroimaging. *Part. Fibre Toxicol.* 2010, 7, 3.

2. Srikanth, M.; Kessler, J. A. Nanotechnology—novel therapeutics for CNS disorders. *Nat. Rev. Neurol.* 2012, 8, 307-318.

3. Seleci, M.; Ag Seleci, D.; Joncyzk, R.; Stahl, F.; Blume, C.; Scheper, T. Smart multifunctional nanoparticles in nanomedicine. *BioNanoMat.* 2016, 17, 33-41.

4. Lai, F.; Fadda, A. M.; Sinico, C. Liposomes for brain delivery. *Expert Opin Drug Del.* 2013, 10, 1003-1022.

5. Tavano, L.; Mauro, L.; Naimo, G. D.; Bruno, L.; Picci, N.; Andò, S.; Muzzalupo, R. Further evolution of multifunctional niosomes based on pluronic surfactant: dual active targeting and drug combination properties. *Langmuir* 2016, 32, 8926-8933.

6. Begum, K.; Khan, A. F.; Hana, H. K.; Sheak, J.; Jalil, R. U. Rifampicin niosome: preparations, characterizations and antibacterial activity against staphylococcus aureus and staphylococcus epidermidis isolated from acne. *Dhaka University Journal of Pharmaceutical Sciences* 2015, 14, 117-123.

7. Marianecci, C.; Rinaldi, F.; Mastriota, M.; Pieretti, S.; Trapasso, E.; Paolino, D.; Carafa, M. Anti-inflammatory activity of novel ammonium glycyrrhizinate/niosomes delivery system: human and murine models. *J. Control. Release* 2012, 164, 17-25.

8. Kumar, G. P.; Rajeshwarrao, P. Nonionic surfactant vesicular systems for effective drug delivery—an overview. *Acta Pharm Sin B.* 2011, 1, 208-219.

9. Kreuter, J. Nanoparticulate systems for brain delivery of drugs. *Adv Drug Deliver Rev.* 2001, 47, 65-81.

10. Kreuter, J.; Alyautdin, R. N.; Kharkevich, D. A.; Ivanov, A. A. Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). *Brain Res.* 1995, 674, 171-174.

11. Sun, W.; Xie, C.; Wang, H.; Hu, Y. Specific role of polysorbate 80 coating on the targeting of nanoparticles to the brain. *Biomaterials* 2004, 25, 3065-3071.

12. Schroeder, U.; Sommerfeld, P.; Sabel, B. A. Efficacy of oral dalargin-loaded nanoparticle delivery across the blood–brain barrier. *Peptides* 1998, 19, 777-780.

13. Kreuter, J.; Shamenkov, D.; Petrov, V.; Ramge, P.; Cychutek, K.; Koch-Brandt, C.; Alyautdin, R. Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood-brain barrier. *J. Drug Target.* 2002, 10, 317-325.

14. Petri, B.; Bootz, A.; Khalansky, A.; Hekmatara, T.; Müller, R.; Uhl, R.; Kreuter, J.; Gelperina, S. Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly (butyl cyanoacrylate) nanoparticles: revisiting the role of surfactants. *J. Control. Release* 2007, 117, 51-58.

15. Gelperina, S.; Maksimenko, O.; Khalansky, A.; Vanchugova, L.; Shipulo, E.; Abbasova, K.; Berdiev, R.; Wohlfart, S.; Chepurnova, N.; Kreuter, J. Drug delivery to the brain using surfactant-coated poly (lactide-co-glycolide) nanoparticles: influence of the formulation parameters. *Eur. J. Pharm. Biopharm.* 2010, 74, 157-163.

16. Bouwstra, J. A.; van Hal, D. A.; Hofland, H. E.; Junginger, H. E. Preparation and characterization of nonionic surfactant vesicles. *Colloids Surf., A* 1997, 123, 71-80.

17. Uchegbu, I. F.; Vyas, S. P. Non-ionic surfactant based vesicles (niosomes) in drug delivery. *Int. J. Pharm.* 1998, 172, 33-70.

18. Balakrishnan, P.; Shanmugam, S.; Lee, W. S.; Lee, W. M.; Kim, J. O.; Oh, D. H.; Kim, D.-D.; Kim, J. S.; Yoo, B. K.; Choi, H.-G. Formulation and in vitro assessment of minoxidil niosomes for enhanced skin delivery. *Int. J. Pharm.* 2009, 377, 1-8.

19. Shirsand, S.; Para, M.; Nagendrakumar, D.; Kanani, K.; Keerthy, D. Formulation and evaluation of Ketoconazole niosomal gel drug delivery system. *Int J Pharm Investig.* 2012, 2, 201.

20. Marwa, A.; Omaima, S.; Hanaa, E.-G.; Mohammed, A.-S. Preparation and in-vitro evaluation of diclofenac sodium niosomal formulations. *Int J Pharm Sci Res.* 2013, 4, 1757.

21. Guinedi, A. S.; Mortada, N. D.; Mansour, S.; Hathout, R. M. Preparation and evaluation of reverse-phase evaporation and multilamellar niosomes as ophthalmic carriers of acetazolamide. *Int. J. Pharm.* 2005, 306, 71-82.

22. Moghassemi, S.; Hadjizadeh, A. Nano-niosomes as nanoscale drug delivery systems: an illustrated review. *J. Control. Release* 2014, 185, 22-36.

23. Yasam, V. R.; Jakki, S. L.; Natarajan, J.; Kuppusamy, G. A review on novel vesicular drug delivery: proniosomes. *Drug delivery* 2014, 21, 243-249.

24. Rajera, R.; Nagpal, K.; Singh, S. K.; Mishra, D. N. Niosomes: a controlled and novel drug delivery system. *Biol. Pharm. Bull.* 2011, 34, 945-953.

25. Hua, W.; Liu, T. Preparation and properties of highly stable innocuous niosome in Span 80/PEG 400/H 2 O system. *Colloids Surf., A* 2007, 302, 377-382.

26. Manosroi, A.; Chutoprapat, R.; Abe, M.; Manosroi, J. Characteristics of niosomes prepared by supercritical carbon dioxide (scCO 2) fluid. *Int. J. Pharm.* 2008, 352, 248-255.

27. Bayindir, Z. S.; Yuksel, N. Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery. *J. Pharm. Sci.* 2010, 99, 2049-2060.

28. Sharma, V.; Anandhakumar, S.; Sasidharan, M. Self-degrading niosomes for encapsulation of hydrophilic and hydrophobic drugs: an efficient carrier for cancer multi-drug delivery. *Mater. Sci. Eng., C* 2015, 56, 393-400.

29. Liu, T.; Guo, R.; Hua, W.; Qiu, J. Structure behaviors of hemoglobin in PEG 6000/Tween 80/Span 80/H 2 O niosome system. *Colloids Surf., A* 2007, 293, 255-261.

30. Pozzi, D.; Caminiti, R.; Marianecci, C.; Carafa, M.; Santucci, E.; De Sanctis, S. C.; Caracciolo, G. Effect of cholesterol on the formation and hydration behavior of solid-supported niosomal membranes. *Langmuir* 2009, 26, 2268-2273.

31. Ag Seleci, D.; Seleci, M.; Walter, J.-G.; Stahl, F.; Scheper, T. Niosomes as Nanoparticular Drug Carriers: Fundamentals and Recent Applications. *J Nanomater* 2016, 2016, Article ID 7372306.

32. Moazeni, E.; Gilani, K.; Sotoudegan, F.; Pardakhty, A.; Najafabadi, A. R.; Ghalandari, R.; Fazeli, M. R.; Jamalifar, H. Formulation and in vitro evaluation of ciprofloxacin containing niosomes for pulmonary delivery. *J. Microencapsul.* 2010, 27, 618-627.

33. Muzzalupo, R.; Tavano, L.; Lai, F.; Picci, N. Niosomes containing hydroxyl additives as percutaneous penetration enhancers: effect on the transdermal delivery of sulfadiazine sodium salt. *Colloids Surf., B* 2014, 123, 207-212.

34. Khalil, R. M.; Abdelbary, G. A.; Basha, M.; Awad, G. E.; El-Hashemy, H. A. Design and evaluation of proniosomes as a carrier for ocular delivery of lomefloxacin HCl. *J Lipos Res.* 2017, 27, 118-129.

35. Vyas, S.; Singh, R.; Jain, S.; Mishra, V.; Mahor, S.; Singh, P.; Gupta, P.; Rawat, A.; Dubey, P. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. *Int. J. Pharm.* 2005, 296, 80-86.

36. Puras, G.; Mashal, M.; Zárate, J.; Agirre, M.; Ojeda, E.; Grijalvo, S.; Eritja, R.; Diaz-Tahoces, A.; Navarrete, G. M.; Avilés-Trigueros, M. A novel cationic niosome formulation for gene delivery to the retina. *J. Control. Release* 2014, 174, 27-36.

37. Pardakhty, A.; Varshosaz, J.; Rouholamini, A. In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin. *Int. J. Pharm.* 2007, 328, 130-141.

38. Seleci, D. A.; Seleci, M.; Jochums, A.; Walter, J.-G.; Stahl, F.; Scheper, T. Aptamer mediated niosomal drug delivery. *RSC Adv.* 2016, 6, 87910-87918.

39. Tavano, L.; Vivacqua, M.; Carito, V.; Muzzalupo, R.; Caroleo, M. C.; Nicoletta, F. Doxorubicin loaded magneto-niosomes for targeted drug delivery. *Colloids Surf., B* 2013, 102, 803-807.

40. Lakshmi, P.; Devi, G. S.; Bhaskaran, S.; Sacchidanand, S. Niosomal methotrexate gel in the treatment of localized psoriasis: phase I and phase II studies. *Indian J. Dermatol. Venereol. Leprol.* 2007, 73, 157.

41. Paolino, D.; Cosco, D.; Muzzalupo, R.; Trapasso, E.; Picci, N.; Fresta, M. Innovative bola-surfactant niosomes as topical delivery systems of 5-fluorouracil for the treatment of skin cancer. *Int. J. Pharm.* 2008, 353, 233-242.

42. Kassem, M. A.; El-Sawy, H. S.; Abd-Allah, F. I.; Abdelghany, T. M.; Khalid, M. Maximizing the Therapeutic Efficacy of Imatinib Mesylate–Loaded Niosomes on Human Colon Adenocarcinoma Using Box-Behnken Design. *J. Pharm. Sci.* 2017, 106, 111-122.

43. Liu, F.-r.; Jin, H.; Wang, Y.; Chen, C.; Li, M.; Mao, S.-j.; Wang, Q.; Li, H. Anti-CD123 antibody-modified niosomes for targeted delivery of daunorubicin against acute myeloid leukemia. *Drug delivery* 2017, 24, 882-890.

44. Shaker, D. S.; Shaker, M. A.; Hanafy, M. S. Cellular uptake, cytotoxicity and in-vivo evaluation of Tamoxifen citrate loaded niosomes. *Int. J. Pharm.* 2015, 493, 285-294.

45. Dwivedi, A.; Mazumder, A.; Du Plessis, L.; Du Preez, J. L.; Haynes, R. K.; Du Plessis, J. In vitro anti-cancer effects of artemisone nano-vesicular formulations on melanoma cells. *Nanomedicine* 2015, 11, 2041-2050.

46. Mahdiun, F.; Mansouri, S.; Khazaeli, P.; Mirzaei, R. The effect of tobramycin incorporated with bismuth-ethanedithiol loaded on niosomes on the quorum sensing and biofilm formation of Pseudomonas aeruginosa. *Microb. Pathog.* 2017, 107, 129-135.

47. Leelarungrayub, J.; Manorsoi, J.; Manorsoi, A. Anti-inflammatory activity of niosomes entrapped with Plai oil (Zingiber cassumunar Roxb.) by therapeutic ultrasound in a rat model. *Int J Nanomedicine* 2017, 12, 2469.

48. Kuo, Y.-C.; Chen, Y.-C. Targeting delivery of etoposide to inhibit the growth of human glioblastoma multiforme using lactoferrin-and folic acid-grafted poly (lactide-co-glycolide) nanoparticles. *Int. J. Pharm.* 2015, 479, 138-149.

49. Kang, C.; Yuan, X.; Li, F.; Pu, P.; Yu, S.; Shen, C.; Zhang, Z.; Zhang, Y. Evaluation of folate-PAMAMfor the delivery of antisense oligonucleotides to rat C6 glioma cells in vitro and in vivo. *J Biomed Mater Res B.* 2010, 93, 585-594.

50. Li, Y.; He, H.; Jia, X.; Lu, W.-L.; Lou, J.; Wei, Y. A dual-targeting nanocarrier based on poly (amidoamine) dendrimers conjugated with transferrin and tamoxifen for treating brain gliomas. *Biomaterials* 2012, 33, 3899-3908.

51. Cui, Y.; Xu, Q.; Chow, P. K.-H.; Wang, D.; Wang, C.-H. Transferrin-conjugated magnetic silica PLGA nanoparticles loaded with doxorubicin and paclitaxel for brain glioma treatment. *Biomaterials* 2013, 34, 8511-8520.

52. Xu, G.; Wen, X.; Hong, Y.; Du, H.; Zhang, X.; Song, J.; Yin, Y.; Huang, H.; Shen, G. An anti-transferrin receptor antibody enhanced the growth inhibitory effects of chemotherapeutic drugs on human glioma cells. *Int. Immunopharmacol.* 2011, 11, 1844-1849.

53. Xin, H.; Jiang, X.; Gu, J.; Sha, X.; Chen, L.; Law, K.; Chen, Y.; Wang, X.; Jiang, Y.; Fang, X. Angiopep-conjugated poly (ethylene glycol)-co-poly (ε-caprolactone) nanoparticles as dual-targeting drug delivery system for brain glioma. *Biomaterials* 2011, 32, 4293-4305.

54. Huang, S.; Li, J.; Han, L.; Liu, S.; Ma, H.; Huang, R.; Jiang, C. Dual targeting effect of Angiopep-2-modified, DNA-loaded nanoparticles for glioma. *Biomaterials* 2011, 32, 6832-6838.

55. Ulbrich, K.; Knobloch, T.; Kreuter, J. Targeting the insulin receptor: nanoparticles for drug delivery across the blood–brain barrier (BBB). *J. Drug Target.* 2011, 19, 125-132.

56. Birngruber, T.; Raml, R.; Gladdines, W.; Gatschelhofer, C.; Gander, E.; Ghosh, A.; Kroath, T.; Gaillard, P. J.; Pieber, T. R.; Sinner, F. Enhanced Doxorubicin Delivery to the Brain Administered Through Glutathione PEGylated Liposomal Doxorubicin (2B3-101) as Compared with Generic Caelyx,®/Doxil®—A Cerebral Open Flow Microperfusion Pilot Study. *J. Pharm. Sci.* 2014, 103, 1945-1948.

57. Tamaru, M.; Akita, H.; Fujiwara, T.; Kajimoto, K.; Harashima, H. Leptin-derived peptide, a targeting ligand for mouse brain-derived endothelial cells via macropinocytosis. *Biochem. Biophys. Res. Commun.* 2010, 394, 587-592.

58. Hu, Q.; Gao, X.; Gu, G.; Kang, T.; Tu, Y.; Liu, Z.; Song, Q.; Yao, L.; Pang, Z.; Jiang, X. Glioma therapy using tumor homing and penetrating peptide-functionalized PEG–PLA nanoparticles loaded with paclitaxel. *Biomaterials* 2013, 34, 5640-5650.

59. Miao, D.; Jiang, M.; Liu, Z.; Gu, G.; Hu, Q.; Kang, T.; Song, Q.; Yao, L.; Li, W.; Gao, X. Co-administration of dual-targeting nanoparticles with penetration enhancement peptide for antiglioblastoma therapy. *Mol. Pharm.* 2013, 11, 90-101.

60. Waddad, A. Y.; Abbad, S.; Yu, F.; Munyendo, W. L.; Wang, J.; Lv, H.; Zhou, J. Formulation, characterization and pharmacokinetics of Morin hydrate niosomes prepared from various non-ionic surfactants. *Int. J. Pharm.* 2013, 456, 446-458.

61. Varshosaz, J.; Taymouri, S.; Pardakhty, A.; Asadi-Shekaari, M.; Babaee, A. Niosomes of ascorbic acid and α-tocopherol in the cerebral ischemia-reperfusion model in male rats. *Biomed Res Int.* 2014, 2014.

62. Ravouru, N.; Kondreddy, P.; Korakanchi, D. Formulation and evaluation of niosomal nasal drug delivery system of folic acid for brain targeting. *Curr. Drug Discov. Technol.* 2013, 10, 270-282.

63. Ojeda, E.; Puras, G.; Agirre, M.; Zarate, J.; Grijalvo, S.; Eritja, R.; Martinez-Navarrete, G.; Soto-Sánchez, C.; Díaz-Tahoces, A.; Aviles-Trigueros, M. The influence of the polar head-group of synthetic cationic lipids on the transfection efficiency mediated by niosomes in rat retina and brain. *Biomaterials* 2016, 77, 267-279.

64. Dufes, C.; Gaillard, F.; Uchegbu, I. F.; Schätzlein, A. G.; Olivier, J.-C.; Muller, J.-M. Glucose-targeted niosomes deliver vasoactive intestinal peptide (VIP) to the brain. *Int. J. Pharm.* 2004, 285, 77-85.

65. Bragagni, M.; Mennini, N.; Ghelardini, C.; Mura, P. Development and characterization of niosomal formulations of doxorubicin aimed at brain targeting. *J. Pharm. Pharm. Sci.* 2012, 15, 184-196.

66. Bragagni, M.; Mennini, N.; Furlanetto, S.; Orlandini, S.; Ghelardini, C.; Mura, P. Development and characterization of functionalized niosomes for brain targeting of dynorphin-B. *Eur. J. Pharm. Biopharm.* 2014, 87, 73-79.

67. Dixit, S.; Novak, T.; Miller, K.; Zhu, Y.; Kenney, M. E.; Broome, A.-M. Transferrin receptor-targeted theranostic gold nanoparticles for photosensitizer delivery in brain tumors. *Nanoscale* 2015, 7, 1782-1790.

68. Ulbrich, K.; Hekmatara, T.; Herbert, E.; Kreuter, J. Transferrin-and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB). *Eur. J. Pharm. Biopharm.* 2009, 71, 251-256.

69. Sonali; Singh, R. P.; Singh, N.; Sharma, G.; Vijayakumar, M. R.; Koch, B.; Singh, S.; Singh, U.; Dash, D.; Pandey, B. L. Transferrin liposomes of docetaxel for brain-targeted cancer applications: formulation and brain theranostics. *Drug delivery* 2016, 23, 1261-1271.

70. Hong, M.; Zhu, S.; Jiang, Y.; Tang, G.; Pei, Y. Efficient tumor targeting of hydroxycamptothecin loaded PEGylated niosomes modified with transferrin. *J. Control. Release* 2009, 133, 96-102.

71. Tavano, L.; Muzzalupo, R.; Mauro, L.; Pellegrino, M.; Andò, S.; Picci, N. Transferrin-conjugated pluronic niosomes as a new drug delivery system for anticancer therapy. *Langmuir* 2013, 29, 12638-12646.

72. Li, P.; Rossman, T. G. Genes upregulated in lead-resistant glioma cells reveal possible targets for lead-induced developmental neurotoxicity. *Toxicological Sciences* 2001, 64, 90-99.

73. Nasarre, C.; Roth, M.; Jacob, L.; Roth, L.; Koncina, E.; Thien, A.; Labourdette, G.; Poulet, P.; Hubert, P.; Cremel, G. Peptide-based interference of the transmembrane domain of neuropilin-1 inhibits glioma growth in vivo. *Oncogene* 2010, 29, 2381-2392.

74. Hu, Q.; Gao, X.; Gu, G.; Kang, T.; Tu, Y.; Liu, Z.; Song, Q.; Yao, L.; Pang, Z.; Jiang, X. Glioma therapy using tumor homing and penetrating peptide-functionalized PEG-PLA nanoparticles loaded with paclitaxel. *Biomaterials* 2013, 34, 5640-5650.

75. Ag Seleci, D.; Seleci, M.; Stahl F.; Scheper, T. Tumor homing and penetrating peptide-conjugated niosomes as multi-drug carriers for tumor-targeted drug delivery. *RSC Adv*. 2017, 7, 33378–33384.

4. Experimental Investigations

The purpose of this study is to develop novel niosomal formulations for targeted drug delivery and to investigate their application potential under *in vitro* conditions. For this purpose, several targeting moieties including an aptamer and a peptide were combined with the drug loaded-niosomes for targeting of cancer cells. The experimental part of this dissertation consists of two chapters. In chapter 4.1, PEGylated niosomes, which have high stability and bioavailability, were synthesized and characterized. The surface of the niosomes was modified with MUC1 aptamer-CysTAT peptide conjugate and their applicability for targeted therapy was examined. The second chapter 4.2 aims to develop an efficient drug delivery platform specifically for brain tumors. The effects of combined loading of curcumin and chemotherapeutic agent to niosomes on glioblastoma were investigated by using tumor-homing peptide tLyp-1, which has high affinity and specificity to neuropilin receptor (NRP-1).

4.1 Aptamer mediated niosomal drug delivery

The results of this chapter were published as D. Ag Seleci, M .Seleci, A. Jochums, J.G. Walter, F. Stahl, and T. Scheper, Aptamer mediated niosomal drug delivery, RSC Advances, 2016, 6, 87910- 87918. The article was reproduced with permission of Royal Society of Chemistry.

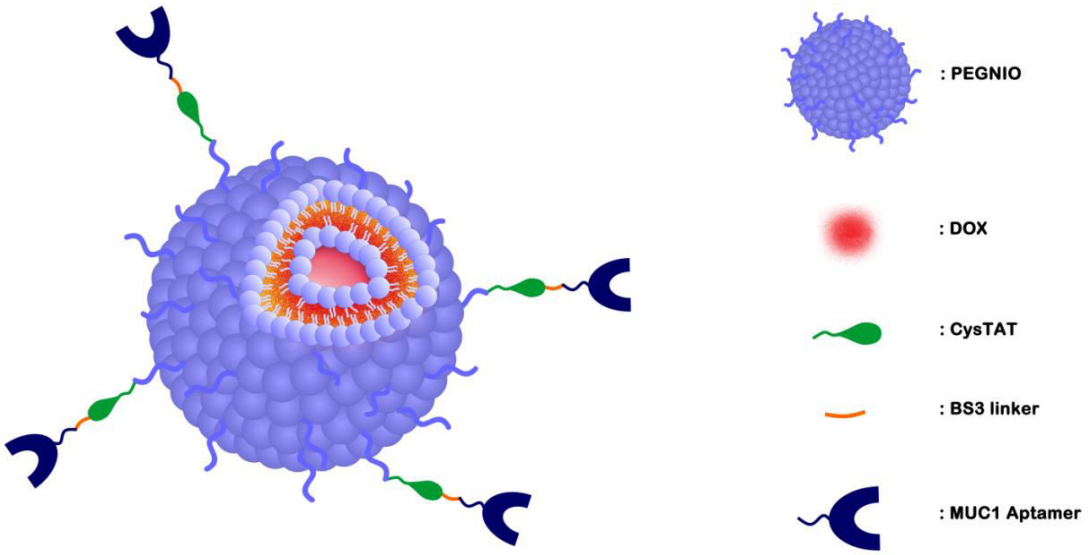
4.1.1 Summary

Vesicular nanocarriers such as liposomes and niosomes are remarkable tools for nanomedical applications. In general, vesicles composed of phospholipids are called liposomes whereas niosomes consist of non-ionic surfactants that are more economical and chemically more stable when compared to the phospholipids. Cholesterol can also be used to improve membrane rigidity as well as stability. The surface of the nanoparticles can be modified with biological molecules to enhance the therapeutic effect within the targeted tissue. Moreover, the attachment of polyethylene glycol (PEG) molecules to the surface can increase the circulation time in the blood stream long enough to allow transport to target tissues by decreasing recognition of nanoparticles by the immune system. In the present study, a novel niosomal drug carrier was designed. PEGylated niosomes were prepared by the thin film layer hydration technique, using Span60, Cholesterol and DSPE-PEG(2000)Maleimide. Cell penetrating peptide (CysTAT) and cell specific MUC1 (S2.2) aptamer were conjugated to each other by using BS3 homo-bifunctional crosslinker and conjugates were characterized via gel electrophoresis as well as HPLC. Then the niosome surface was modified with the conjugate, CysTAT-MUC1 to allow active targeting by binding to the target cell surface. As a selected model anticancer drug, doxorubicin (DOX) was encapsulated into the niosomes. The hydrodynamic size of the niosomes were determined to be approximately 150 nm with a relatively low polydispersity index (~0.25). After the conjugation of CysTAT-MUC1, increasement in size was of around 14 nm was observed. Drug release profile was monitored in physiologic pH 7.4 and pH 5.6 to mimic acidic tumor microenvironment over 60 h. As expected DOX release was higher at pH 5.6. MUC1 gene and receptor expression levels of the cells were investigated by PCR and flow cytometry, respectively. HeLa (cervical cancer cells) showed considerably higher expression levels and were therefore selected as positive cell line, whereas U87 (human glioblastoma cells) cell exhibited much lower intensity and were used as a control. Cellular uptakes of the niosomal formulations and free dye were evaluated on both cell

lines by flow cytometric analysis. DOX loaded targeted niosomes were taken up more efficiently by HeLa cells than free DOX after 2 h. However, the uptake level of free DOX was higher than for niosomal formulations for U87 cells. The internalization was also observed through fluorescence microscopy. The cytotoxicity of niosomal formulations and free DOX was investigated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay 24 and 48 h after treatment. According to the results, bare niosomes were almost nontoxic to HeLa and U87 cells with relative cell viabilities above 80% for both 24 and 48 h. The targeted drug-loaded niosomes showed a stronger cytotoxic effect on MUC1 receptor overexpressing HeLa cells.

Overall, the results presented here demonstrate the potential of an aptamer targeted niosomal drug delivery system. Biocompatible PEGylated niosomes were synthesized with well defined physicochemical properties and their surface was decorated with peptide-aptamer conjugate. Taking into account the obtained results, the designed platform has an application potential for delivery of agents to MUC1-overexpressing tumor cells.

Table of Contents Graphic



4.1.2 Abstract

Development of nanoscale carrier systems for targeted drug delivery is crucial for cancer treatment. The current methods of drug delivery exhibit some problems such as lack of therapy efficiency at the desired parts of the body, degradation of the drug before reaching the desired tissue and limitations in cellular penetration. In this work, a novel drug delivery platform was developed to overcome these problems and to enable specific and efficient uptake into the cells. The surface of the synthesized polyethylene glycolated niosomes (PEGNIO) was modified with cell penetrating peptide (CPP) and cell specific MUC1 (S2.2) aptamer, and doxorubicin (DOX) as a cancer model drug was encapsulated in this platform. Fluorescence microscopy and flow cytometry analysis were used to investigate the cellular uptake and intracellular distribution of the DOX loaded niosomal formulation. *In vitro* cytotoxicity studies were carried out using MUC1 positive HeLa and negative U87 cells. Moreover, dynamic light scattering (DLS), zeta potential measurements and fluorescence absorption spectroscopy were performed to determine the vesicle size, as well as charge and spectroscopic properties of the conjugates. From these results, this novel aptamer mediated niosomal drug delivery platform may have application potential in targeted drug delivery towards MUC1-overexpressing tumors.

4.1.3 Introduction

A drug delivery system (DDS) is described as a formulation that enables the introduction of drug molecules into the body with improved efficacy and safety. Due to the rapid progress of nanotechnology, numerous nanocarriers have been developed to securely deliver drugs into target sites. New targeting agents, including aptamers, short peptides, and small molecules have recently become promising targeting ligands to design novel drug delivery systems. The current treatment options for cancer are surgical operation, radiation and chemotherapy or a combination. The therapeutic efficacy of many anticancer drugs is limited by their poor penetration into tumor tissue and by their side effects on healthy cells.[1] To overcome these limitations, development of a novel carrier platform for specific drug uptake into the cell with an optimal dose at high efficiency is important. Therefore, anticancer drugs can be conjugated with biomolecules exhibiting potential for cellular targeting and penetration and can be delivered to the desired site of action by multi-functional carrier platforms. Vesicular nanocarriers have received great attention as potential drug carrier systems. Nonionic surfactant based vesicles “niosomes” are one class of vesicular nanocarriers which can accommodate a large

number of drugs with a wide range of solubility.[2–4] Due to their low cost, long term storage stabilities and lower toxicity of niosomes have been used for drug delivery.[5–7] The addition of polyethylene glycol (PEG) to the nanocarrier surface increases the steric stabilization of the nanoparticle and allows for further surface modifications to design specific drug delivery systems. It is generally thought that PEGylation protects the delivery systems against the immune system and thereby prolong circulation life times.[8] In solid tumors, the penetration of the tissue by the anticancer drug is limited which causes reduced efficacy and the development of drug resistance.[9] A promising approach to overcome the cellular barrier is based on the use of certain peptides namely cell penetrating peptides (CPPs), able to translocate across the cell membrane and deliver their payload intracellularly within minutes.[10] CPPs consist of small cationic or amphipathic peptides that aid the uptake of attached cargos into living cells. A wide variety of small molecules and biomolecules including plasmid DNA, siRNA, oligonucleotides and peptide nucleic acid molecules have been attached to these peptides and were subsequently internalized.[11–13] The ability of CPPs to translocate biologically active molecules into cells makes these peptides promising candidates for theranostic applications.[14] TAT is one of the smallest polycationic CPPs composed of arginine and lysine residues. Studies on the binding affinities of cationic TAT peptides indicate that these peptides strongly bind electrostatically to the various anionic species (e.g. heparan sulphate proteoglycans) present at the extracellular surface of cell membranes.[15,16] The exact molecular mechanism of cellular entry of CPPs is currently not fully understood. Former studies indicated that in general uptake occurs by endocytosis (or more specifically macropinocytosis) and direct membrane translocation.[17] The lack of cell specificity remains the major drawback for the clinical application of CPPs.[18] Receptors that are over-expressed in many cancer cells are suitable targets to achieve a more specific delivery. MUC1 is a large transmembrane glycoprotein overexpressed in most malignant adenocarcinoma, including ovarian, lung, pancreatic, prostate, and breast cancers, making it an ideal target molecule for chemotherapeutics.[19] Aptamers are short oligonucleotides that are capable to selectively bind their corresponding target.[20–22] These reagents are selected by an *in vitro* process called SELEX, (systematic evolution of ligands by exponential enrichment).[23] Several MUC1 aptamers were developed by Ferreira et al. and S2.2 is a 25-nucleotide truncated version of the original MUC1 aptamer. It binds MUC1 protein with high specificity and affinity with a KD of 0.135 nM.[24,25] S2.2 has been used in a few targeted delivery systems. Yu et al. used MUC1

aptamer to target paclitaxel (PTX) loaded poly(lactic-co-glycolicacid) (PLGA) nanoparticles.[26] Furthermore PEG-modified MUC1 targeting doxorubicin (DOX) was designed and the aptamer–doxorubicin complex was prepared by intercalation of the aptamer with DOX by Tan et al.[27] Recently, Liu et al. synthesized vinorelbine (VRL) loaded and MUC1 aptamer modified lipid–polymer hybrid nanoparticles.[22] In this study, polyethylene glycolated niosomes (PEGNIO) were prepared from span60, cholesterol and 1,2-distearoyl-snglycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)-2000] (DSPE-PEG(2000)Maleimide). DOX was encapsulated into the PEGNIO. The niosomes were characterized with respect to size, morphology and drug encapsulation efficiency. Cysteine-modified cell penetrating peptide (CysTAT) was conjugated to the amine group of MUC1 aptamer in the presence of a crosslinking agent bis(sulfosuccinimidyl)suberate (BS3). Subsequently, CysTAT–MUC1 conjugate was attached to DOX encapsulated PEGNIO (PEGNIO/DOX) via the formation of a thioether linkage. The anticancer activity of DOX-loaded targeted vesicles was studied in HeLa (cervical cancer cells) and U87 (human glioblastoma cells) cell lines by evaluating the cellular uptake and cytotoxicity. The designed nanoparticular drug delivery system composed of aptamer–CPP–PEGNIO was first fabricated in this study. Our findings suggest that this platform can serve as a delivery vehicle for cancer cells overexpressing MUC1.

4.1.4 Materials and methods

Materials

Amine and Cy5 modified MUC1 aptamer S2.2 (50-NH2-GCA GTT GAT CCT TTG GAT ACC CTG G-30), (50-Cy5-GCA GTT GAT CCT TTG GAT ACC CTG G-30) were purchased from Integrated DNA Technologies. CysTAT with CYGRKKRRQRRR–NH2 sequence was obtained from GenScript. DSPE-PEG(2000) Maleimide was provided by Avanti. Span60, cholesterol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), 4,6-diamino-2-phenylindol (DAPI) and Dulbecco's Modified Eagle Medium (DMEM) were ordered from Sigma Aldrich. RNAtidy G and sodium dodecyl sulfate (SDS) were purchased from Applichem. BS3 was ordered from Covachem. Doxorubicin (hydrochloride) was purchased from Cayman Chemical. GeneRuler 100 bp DNA ladder and dNTP Set (100 mM solutions) were obtained from Fermentas. M-MLV reverse transcriptase and its M-MLV RT 5-buffer as

well as GoTaq polymerase and its 5-Green GoTaq reaction buffer were provided from Promega. PCR primers were synthesized by Life Technologies.

Preparation of PEGylated niosomes

Multi lamellar vesicles (MLVs) of PEGNIO were prepared by the thin film hydration method.[28] Span 60, cholesterol, and DSPEPEG(2000) Maleimide were dissolved in 1.0 mL chloroform in a round-bottom flask with the molar ratio of 4.95 mM : 4.95 mM : 0.1 mM. The solvent was evaporated with constant rotation at 38 °C under reduced pressure to form a thin lipid film. Then the thin film was hydrated with 1.0 mL of distilled water for preparing empty niosomes, or a doxorubicin solution (0.22×10^{-3} M in water) to obtain PEGNIO/DOX conjugate at 60 °C for 60 min. Afterward, the niosomal suspension was equilibrated at room temperature overnight, to complete annealing and partitioning of the drug between the lipid bilayer and the aqueous phase.[5] Small unilamellar vesicles (SUVs) were prepared starting from MLVs by extruding the niosomes 11 times through 0.4 mm and 0.1 mm pore size polycarbonate filters using mini extruder set (Avanti polar lipids). Niosomes were purified by dialysis against water for 3 h using 6–8 kDa dialysis bag.

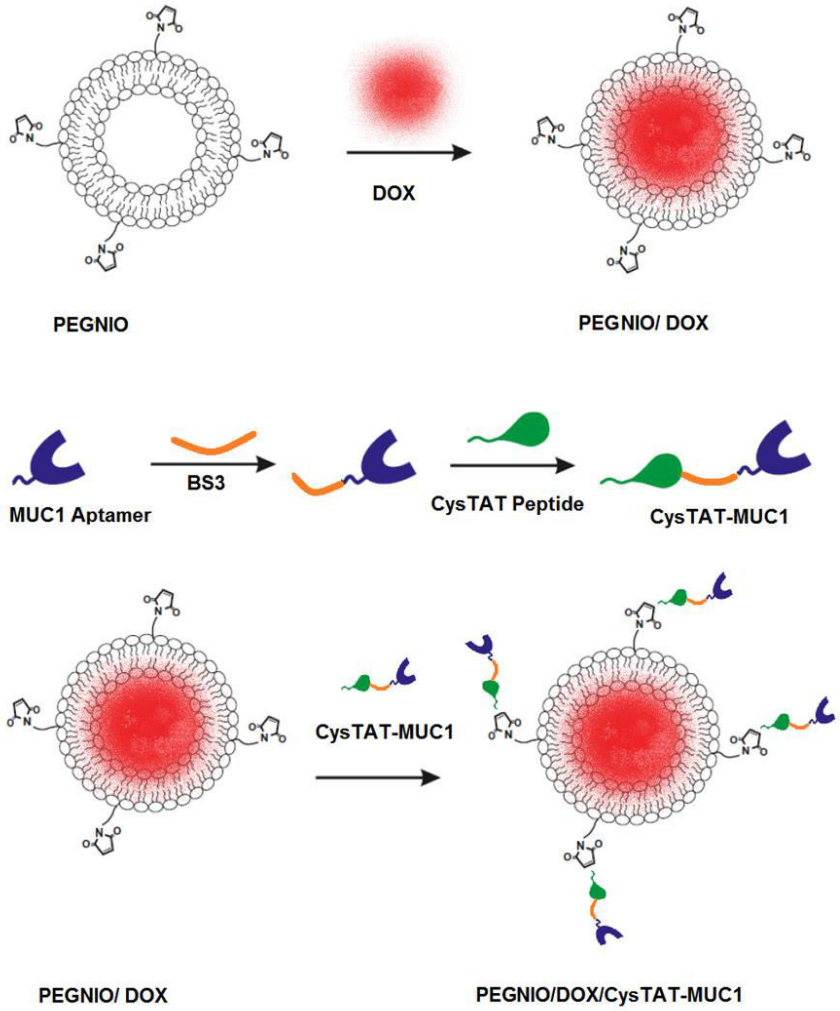
Synthesis and characterization of CysTAT–MUC1 conjugate

The conjugation between CysTAT and amine modified MUC1 aptamer was performed using an amine to amine crosslinker BS3. 35 µL containing 5 nmol amine modified MUC1 aptamer, 30 µL containing 20 nmol CysTAT peptide and 150 µL containing 1.75 mmol BS3 were mixed in 85 µL 0.1 M sodium phosphate including 0.15 M sodium chloride at pH 7.4 and incubated at room temperature for 1 h. Once the reaction was completed, 300 µL 1.0 M Tris buffer was added to quench the reaction for 20 minutes at room temperature. Unreacted peptides and quenched crosslinkers were removed through dialysis against PBS (pH 7.2) using 3.5 kDa dialysis bag. During this reaction aptamer–aptamer and peptide–peptide conjugation can occur. Denaturing urea polyacrylamide gel electrophoresis (Urea PAGE) and HPLC were used for the characterization of CysTAT–MUC1. Urea PAGE was performed according to manufacturer's instructions (QIAGEN). Briefly, 15% acrylamide/urea gel was prepared and run for 30 min at 200 V. The samples were heated at 95 °C for 2 min and then they were immediately transferred onto ice. The samples were loaded onto the gel and the gel was run for 1.5 h at 200 V. Afterward the gel was stained with methylene blue solution and documented using an INTAS UV documentation system. HPLC measurements and carried out using VWR Hitachi

Chromaster. For the analysis, a DAD detector and Kinetix 2.6 mm C8 100 °A, 150x4.6 mm (Phenomenex) column were used. Detection was performed at 214 nm at room temperature. The mobile phase consisted of 0.065% trifluoroacetic acid (TFA) in water and 0.05% TFA in acetonitrile with a flow rate of 1.0 mL min^{-1}.

Conjugation CysTAT–MUC1 to PEGNIO/DOX

CysTAT–MUC1 was conjugated to PEGNIO/DOX via thiol group of cysteine to maleimide group on PEGNIO resulting in the formation of a thioether linkage.[29,30] Maleimide group reacts specifically with thiol-groups in the pH range 6.5–7.5. 100% of the prepared CysTAT–MUC1 conjugate in PBS (pH 7.2) and 50% of the prepared PEGNIO/DOX were mixed and incubated overnight at room temperature. After completing the reaction between sulfiydryl group of CysTAT and maleimide group of DSPE-PEG(2000) Maleimide, the final PEGNIO/DOX/CysTAT–MUC1 conjugate was purified using 14 kDa dialysis bag to remove unbound CysTAT–MUC1. Schematic representation of niosome synthesis, drug encapsulation and the bioconjugation processes are shown in Scheme 1.



Scheme 1. Schematic representation of drug the encapsulation and the bioconjugation process.

Measurement of particle size, distribution and zeta potential

Size, size distribution and zeta potential of niosomes were determined by dynamic light scattering (DLS) analysis using Malvern Zetasizer Nanoseries-Nano-ZS. The polydispersity index (PDI) was used as a measure of the width of size distribution. PDI less than 0.3 corresponds to a homogeneous population for colloidal systems.[31] Each sample was measured three times.

Stability

The stability of DOX loaded niosomal formulations was tested via DLS analysis. After the synthesis of PEGNIO/DOX and PEGNIO/DOX/CysTAT–MUC1, these conjugates were stored at 4 °C in the dark. The particle size and PDI values were measured for 2

months. Additionally, the particle size of PEGNIO/DOX/CysTAT–MUC1 was measured in cell culture media before and after the incubation at 37 °C for 24 h.

Entrapment efficiency

The drug encapsulation efficiency was determined using the dialysis technique.[32] According to this method, directly after the preparation 1.0 mL of PEGNIO/DOX and 1.0 mL of PEGNIO/DOX/CysTAT–MUC1 dispersions were dropped into two dialysis bags (12–14 kDa) and immersed in 100 mL of distilled water with magnetic stirring at 100 rpm. Samples were dialyzed for 3h. The percent of encapsulation efficiency (E%) was expressed as the percentage of the drug entrapped in niosomes (and thus not removed via dialysis) referred to the initial amount of drug that is present in the nondialyzed sample. It was determined by diluting 50 µL of dialyzed and 50 µL of nondialyzed niosomes in 1.0 mL of methanol. This step is essential for breaking the niosomal membrane, thereby releasing the entrapped DOX. Subsequently, the amount of DOX was determined by HPLC using a C18 column (Phenomenex Kinetix, 4.6 x100 mm, 2.6 mm) at 35 °C and a UV detector was conducted at 254 nm. The mobile phase consisted of methanol and water (60/40, v/v) containing 0.1% formic acid and 0.1% ammonia solution (25%) with a flow rate of 1.0 mL min^{-1}.[33] The stock solutions of DOX were prepared at 1.0 mg mL^{-1} in methanol and further diluted with methanol in the concentration range 1.0–200 µgmL^{-1}. The amount of encapsulated DOX was calculated according to the calibration curve (y = 120 777x - 67 040, R^2 = 0.9984). The limit of detection (LOD) and limit of quantification (LOQ) for DOX were found to be 6.12 µgmL^{-1}, 18.55 µgmL^{-1} respectively based on 3.3σ/slope and 10σ/slope formulations.[34]

Drug release

Drug release experiments were performed using the dialysis method. The DOX-loaded niosome solutions were prepared and transferred into a dialysis membrane tubing (Thermo, Slide-ALyzer MINI Dialysis Devices, 10K MWCO). The tubing was immersed in 10 mL of the PBS buffer (pH 5.6 and 7.4), placed in an incubator at 37 °C and stirred at 100 rpm. At specific time intervals, 0.5 mL samples were removed from the release medium and replaced with the same volume of fresh buffer. A calibration curve was established with a known concentration of free DOX by fluorescence emission measurements at 595 nm using NanoDrop3300. The amount of released DOX was

calculated according to the calibration curve (y=254.93x-30.74, R^2 = 0.9960, LOD=0.61 µg/mL, LOQ= 1.84 µg/mL).

Cell culture

HeLa and U87 cell lines were provided from German Collection of Microorganisms and Cell Cultures (DSMZ). Both cell lines were grown in DMEM containing 10% fetal calf serum (FCS) and 1.0% penicillin/streptomycin (P/S). All cells were cultivated in medium and incubated with samples and reagents at 37°C in a humidified environment with 5.0% CO_2.

MUC1 expression on cell surfaces

PCR and flow cytometry analysis were used to confirm expression of the MUC1 receptor in HeLa and lack of expression in U87 cells. Total cellular RNA of the cells was isolated using Trizol reagent (Invitrogen) and transcribed into cDNA. The primers were designed with Lasergene Primer Select Soft ware using the NCBI reference mRNA sequence for Homosapiens mucin 1, cell surface associated (MUC1), transcript variant 1 (NM-002456.5). The primer sequences are as follows: MUC1 forward 5´-TAC CGA TCG TAG CCC CTA TG-3´ and reverse 5´-CCA CAT GAG CTT CCA CAC AC-3´. The human housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was additionally used to prove the successful synthesis of cDNA. The primer sequences are: HPRT forward 5´-AAG CTT GCT GGT GAA AAG GA-3´ and reverse 5´-AAG CAG ATG GCC ACA GAA CT-3´. The protocol described in our previous publication was used in the polymerase chain reactions.[35] The annealing temperature of 60 °C was used for MUC1 and HPRT during PCR experiments. PCR products were separated in 1.5% agarose gel in TAE buffer and stained with 5.0 µL/100 mL buffer Roti-Safe Gel Stain ready to use by Thermo. The gel was run using the Thermo EC electrophoresis unit at 100 V for 60 min and documented using an INTAS UV documentation system. For flow cytometry studies, 5×10^5cells were collected. 100 µL of 5.0 µM Cy5 labelled MUC1 aptamer in PBS were added to the cells and the cell suspension was shaken at room temperature for 1 h with 500 rpm in the dark. The cells were washed once in 300 µL of PBS to remove unbound aptamer. Before flow cytometric analysis, cells were resuspended in 500 µL of PBS and the stained cells were analyzed in a BD Accuri C6 flow cytometer. At least 20 000 gated events were observed in total and living cells were

gated in a dot plot of forward versus side scatter signals. For drawing dot plots and histograms the BD Accuri C6 software was used.

Cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to determine cytotoxicity of the niosomal formulations. Cells (8×10^3) were seeded out in 96-well tissue plates (Sarstedt, USA) in a volume of 200 µL and cultivated for three days. After this cultivation time cells were washed once in PBS and treated with SUVs (PEGNIO, PEGNIO/DOX, PEGNIO/DOX/CysTAT-MUC1) and free DOX for 24 h and 48 h. The equivalent concentration of free DOX was used in niosomal formulations.Then the samples were removed and cells were incubated in 110 µL/well 10% MTT solution (5.0 mg/mL in PBS) in medium for 4 h. During this incubation time, formazan complex was produced by the cells. 100 µL SDS solution (1.0 g SDS in 10 ml 0.01 M HCl) was added to each well to release the purple colored salt from the cells. After 24 h of incubation, UV–*vis* absorption was measured at 570 nm to 630 nm as the reference wavelength using a microplate reader Epoch Biotek.

Cellular uptake and internalization

The DOX uptake by HeLa and U87 cells for different DOX formulations was analyzed by flow cytometry. HeLa and U87 cells were treated with PEGNIO/DOX, PEGNIO/DOX/CysTAT-MUC1 and free DOX for 2 h and treated cells were washed two times with PBS, and then analyzed in a BD Accuri C6 flow cytometer.

Cellular internalization of PEGNIO/DOX/CysTAT-MUC1 was determined via fluorescence microscopy studies. HeLa and U87 cells were cultivated for 2 days on the chamber slides (µ slides 8 well purchased at ibidi GmbH) in a volume of 200 µL of the medium. PEGNIO/DOX/CysTAT-MUC1 was diluted with medium and then added to the cells. The cells were incubated for 4 h at 37°C and washed once in PBS. Afterward 100 µL DAPI solution (1.0 µg/mL) was added to the cells and incubated for 15 min. After DAPI staining, the cells were washed with PBS once. Images were taken using an OLYMPUS BX41 fluorescence microscope equipped with an OLYMPUS SC30 camera.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat statistical software. All experiments were repeated three times. The paired t-test was performed. The difference

between two groups was considered to be significant when the P value was less than 0.05.

4.1.5 Results

Doxorubicin is one of the most extensively used broad-spectrum anticancer drugs. It accumulates inside the cell nucleus where it intercalates into DNA and inhibits the progression of topoisomerase II to cause DNA damage and cleavage.[36,37] Long treatment durations and toxic side effects are inconvenient in the use of conventional chemotherapeutics.[38] Recent studies show that, biocompatible nanoparticles, with an increased surface area to volume ratio can overcome non-cellular and cellular-based mechanisms of resistance and increase the selectivity of drugs towards cancer cells, while reducing their toxicity towards normal tissues.[39] The addition of PEG to the nanoparticle surface prolongs vesicles residence time in blood and accumulation at the pathological sites.[5] Taking this into consideration, we decided to use PEGylated niosomes for obtaining aptamer targeted-cell penetrating vesicular systems.

Conjugation and characterization of CysTAT–MUC1

BS3 is an amine reactive, homobifunctional, sulfo-NHS ester, crosslinking reagent.[40] The reactivity of the sulfo-NHS esters is highly reactive toward amines in the pH range of 7–9. The amine modified MUC1 aptamer was reacted with the –NH2 group in the CysTAT peptide in the presence of BS3 to produce the CysTAT–MUC1 conjugates. Besides the desired conjugate CysTAT–MUC1, the reaction can also result in dimers of MUC1 aptamer and peptide. In order to investigate the product spectrum of the crosslinking reaction, gel electrophoresis was performed. Free MUC1 aptamer and CysTAT–MUC1 were applied to Urea PAGE. A single band was observed for free aptamer. In the case of CysTAT–MUC1 conjugate double bands were observed (Fig. S4.1). In the double bands, first band indicates CysTAT–MUC1 conjugate and the second one exhibits unbound aptamers in the conjugate. Additionally, the conjugation of aptamer and peptide was proven by HPLC analysis. For this aim, chromatograms of MUC1 aptamer, BS3 crosslinker, Cys-TAT peptide were compared with chromatogram of CysTAT–MUC1 conjugate (Fig. S4.2). No peaks were observed for MUC1 aptamer in this HPLC method. CysTAT peptide and BS3 crosslinker have different retention times 7.75 and 8.67 respectively, showed that there is no interference during the analysis of the CysTAT–MUC1 conjugates. The conjugation efficiency was about 82% in

accordance with the integrated areas of CysTAT peptide before and after conjugation. Besides CysTAT–MUC1, the conjugation can also result in aptamer and peptide dimers. Nonetheless, since these side products are not able to bind to maleimide group of PEGNIO (due to the lack of –SH group), we used to the as prepared conjugate with no additional purification.

Synthesis and characterizations of DOX loaded niosomal formulations

PEGylated niosomes were prepared by the thin film layer hydration technique, using span60:cholesterol:DSPE-PEG(2000)Maleimide. 0.22 mM doxorubicin solutions were used in the preparation of loaded vesicles. CysTAT–MUC1 was conjugated to PEGNIO/DOX to obtain targeted drug delivery system. The size of nanocarriers is very important for effective accumulation in tumor by enhanced permeability and retention effect (EPR) and cellular internalization. The mean diameters of empty and doxorubicin-loaded niosomal formulations, along with the corresponding polydispersity index (PDI), doxorubicin entrapment efficiency (E%) values, and zeta potential values are listed in Table 4.1. The empty vesicle size was analyzed to be 151.0 ± 36nm. The hydrodynamic diameter did not change after DOX loading (152.7 ±34 nm) but after conjugation with CysTAT–MUC1 it increased to 164.5±40 nm. The stealth niosomes showed zeta potential values close to 0 mV but the surfaces of nanoparticles was grafted with PEG to improve water solubility and avoid aggregation.[5,41,42] Due to the presence of PEG in the niosome structure, no aggregation was observed. DOX loading influenced the zeta potential of the vesicles. The change in the zeta potential may be a result of DOX intercalation in the vesicle membrane.[5,43,44] Conjugation of CysTAT–MUC1 increased niosome size, corresponding with the presence of the aptamer on the niosomal surface increasing the hydrodynamic diameter. Moreover, the negatively charged DNA aptamer reduced the surface potential of the niosome.[29] PDI ranged from 0.275 to 0.214, demonstrating that the vesicle population is relatively homogeneous in size. The DOX entrapment efficacy (E%) was calculated to be around 39% and 37% for PEGNIO/DOX and PEGNIO/DOX/CysTAT–MUC1 respectively. The stability of niosomal DOX formulations was tested via DLS analysis and no changes were observed in the size and PDI values after two months storage at 4 °C in the dark (data not shown). Additionally, PEGNIO/DOX/CysTAT–MUC1 sample was diluted in cell culture media and was incubated at 37 °C for 24 hours. The size of the sample was measured before and after incubation and no changes were observed.

Table 4.1 Characterization of PEGNIO formulations incorporating DOX

Samples	Size (nm) Intensity (%) (Mean±SD)	Poly-dispersity Index (PDI)	Zeta Potential (mV)	Entrapment Efficiency E%
PEGNIO	151.0 ± 36	0.244	-4.96 ± 0.43	-
PEGNIO/DOX	152.7 ± 34	0.214	-3.56 ± 0.27	39.52 ±1.8
PEGNIO/DOX/ CysTAT-MUC1	164.5 ± 40	0.275	-8.62 ± 0.50	37.48 ±2.1

Drug release

Sustained drug release is one of the important properties of nanoscale drug delivery systems that will minimize side effects of the drug. In vesicular drug carrier systems, the drug release occurs by passive transport of the drug through the membrane bilayer.[45] The release of DOX from PEGNIO/DOX/CysTAT–MUC1 was investigated using dialysis methods at pH 7.4, which was chosen in accordance with physiological conditions and in an acidic environment (pH 5.6). The solutions were taken out at specific intervals and measured by fluorescence emission measurements at 595 nm to determine the amount of DOX that has been released. The *in vitro* DOX release profiles from PEGNIO/DOX/CysTAT–MUC1 showed the faster release of DOX under acidic environments than that at neutral pH (Fig. 4.1). Within 48 h, the release of DOX was 30% and 52% at pH 7.4 and pH 5.6 respectively. This can be explained by the higher solubility of DOX at the lower pH.[46] Especially, the pH change from 7.4 to 5.6 corresponds to the pH change from the normal physiological environment in body circulation to the intracellular tumor tissue. According to results, this conjugate is expected to be a promising drug delivery system for the tumor targeted delivery of DOX.

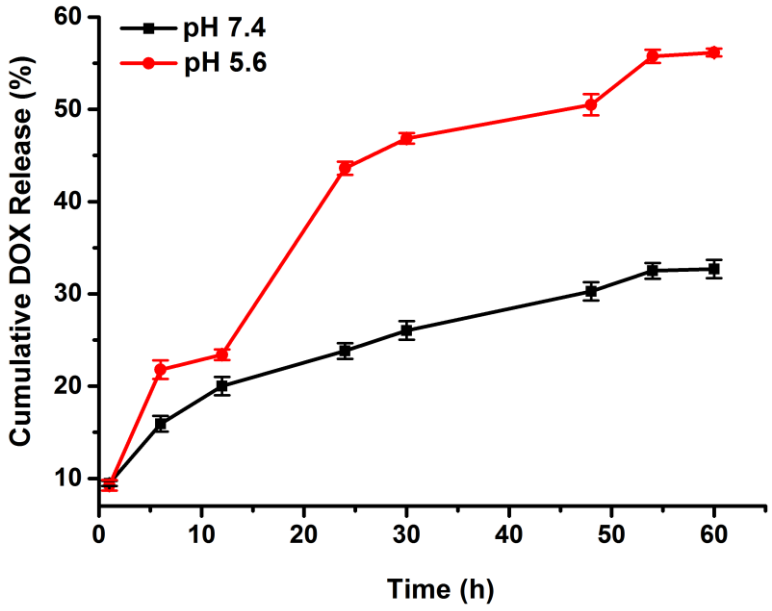


Figure 4.1 *In vitro* cumulative release of DOX from PEGNIO/DOX/CysTAT-MUC1 at pH 7.4 and 5.6.

Confirmation of MUC1 receptor expression on the cell surface

To test PEGNIO/DOX/CysTAT–MUC1 as a targeted drug delivery system *in vitro*, first the expression of MUC1 was evaluated in HeLa and U87 cells. Gene expression of MUC1 was investigated at the mRNA level using polymerase chain reaction (PCR) and at the cell surface protein level using flow cytometry. Agarose gel analysis with the results of PCR experiments is shown in Fig. 4.2. The housekeeping gene (HPRT) was measured as a control for both HeLa and U87 cells (Fig. 4.2a and b). Bands corresponding to housekeeping genes were observed for both cell types (263 bp) confirming the success of RNA extraction and PCR. Fig. 4.2a demonstrates that HeLa cells show a high level of transcription of theMUC1 gene, which results in a strong band at the expected base pair length of 283 bp. No corresponding band was observed in U87 cells, thereby indicating the lack of expression of MUC1. The PCR results were also confirmed by using Cy5 labeled MUC1 aptamer in flow cytometry. Cells were treated with a Cy5 labeled MUC1 aptamer. After the treatment, the mean fluorescence intensity was measured to be 28 523 for U87 cells, and 57 993 for HeLa cells (Fig. 4.2c). Both methods confirmed that MUC1 expression was considerably higher in HeLa cells than in U87 cells. These results are in agreement with the literature.[47,48]

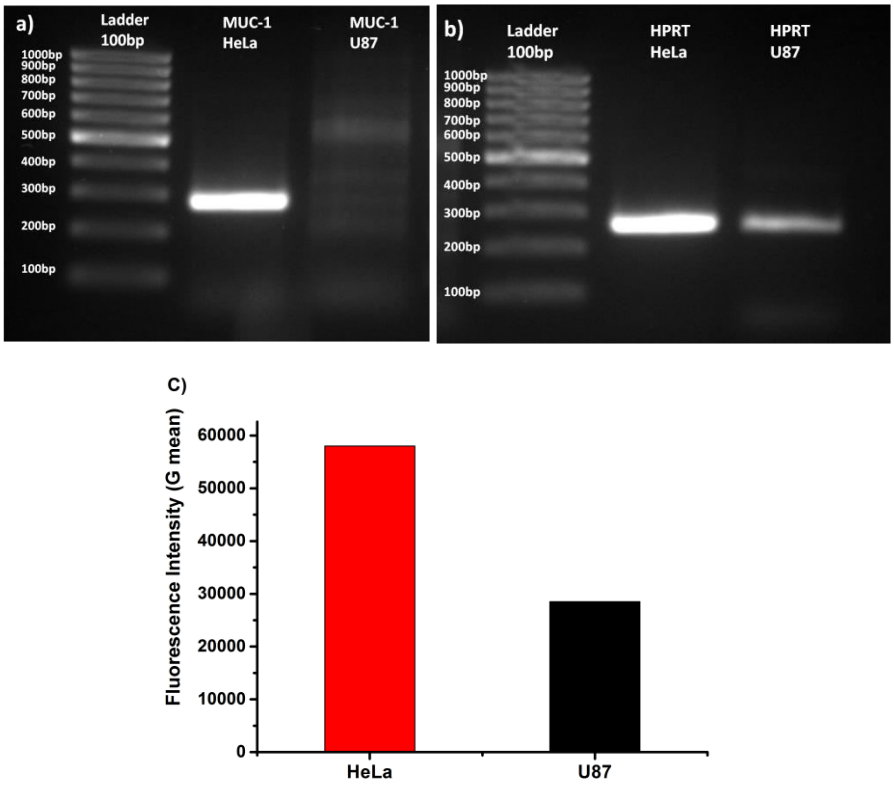


Figure 4.2 Image of 1.5% agarose gel with results of PCR for HPRT gene and MUC1 expression in HeLa and U87 cells (a, b). Flow cytometry analysis of MUC1 expression on HeLa and U87 cells using Cy5-labelled aptamer S2.2 (c).

Cellular uptake and internalization

Flow cytometry was used to investigate the total DOX uptake by HeLa and U87 cells for different DOX formulations and to evaluate receptor mediated cell targeting. The cells were treated with samples for 2 h. Untreated control cells and treated cells were analyzed using a BD Accuri C6 flow cytometer. As shown in Fig. 4.3a, the cellular DOX level for PEGNIO/DOX/CysTAT–MUC1 in HeLa cells was higher than that of PEGNIO/DOX and free DOX. Free DOX enters the cells by diffusion, leading to higher drug levels than found with the PEGNIO/DOX. After encapsulation of DOX in PEGNIO, the DOX uptake by diffusion is reduced.[49] In the case of U87 cells, the uptake of free DOX was higher than PEGNIO/DOX/CysTAT–MUC1 (Fig. 4.3b). Fig. 4.3c indicates that the synthesized PEGNIO/DOX/CysTAT–MUC1 conjugate bound to MUC1 positive HeLa cell specifically. Nonspecific binding to U87 cells was also observed, but the fluorescence signals were lower than for HeLa cells. Cellular internalization of PEGNIO/DOX/CysTAT–MUC1 obtained by fluorescence microscopy analysis. DOX is

a fluorescent drug and, it localizes to the nucleus in tumor cells.[50] Both cell lines were treated with PEGNIO/DOX/CysTAT–MUC1 for 4 h. The synthesized conjugate bound to HeLa cells, resulting in high fluorescence of the cell nucleus, thereby demonstrating successful internalization. In contrast, fluorescence was

significantly lower for U87 cells, indicating some nonspecific uptake in U87 cells (Fig. 4.4a and b). Fluorescence microscopy images show results similar to flow cytometry analysis.

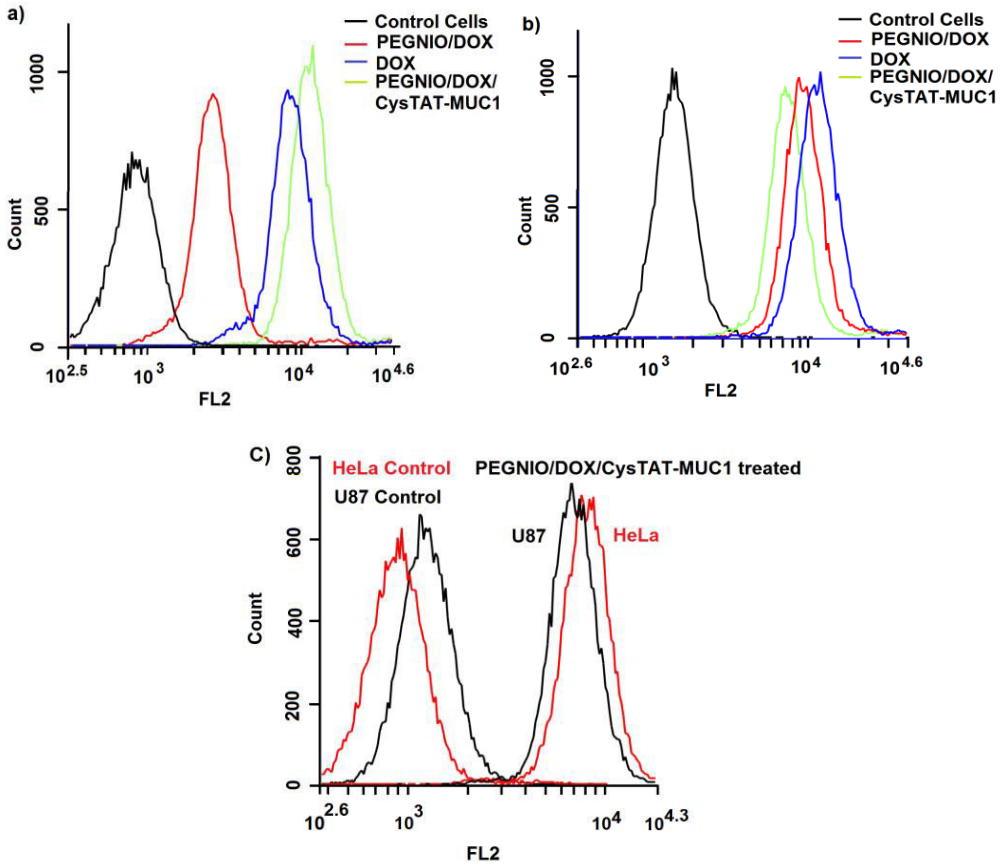


Figure 4.3 Flow cytometric measurement of DOX uptake by Hela (a) and U87 cells (b) after incubating with PEGNIO/DOX, PEGNIO/DOX/CysTAT-MUC1 and free DOX. Histogram of binding of PEGNIO/DOX/CysTAT-MUC1 to MUC1 positive HeLa cells and MUC1 negative U87 cells (c).

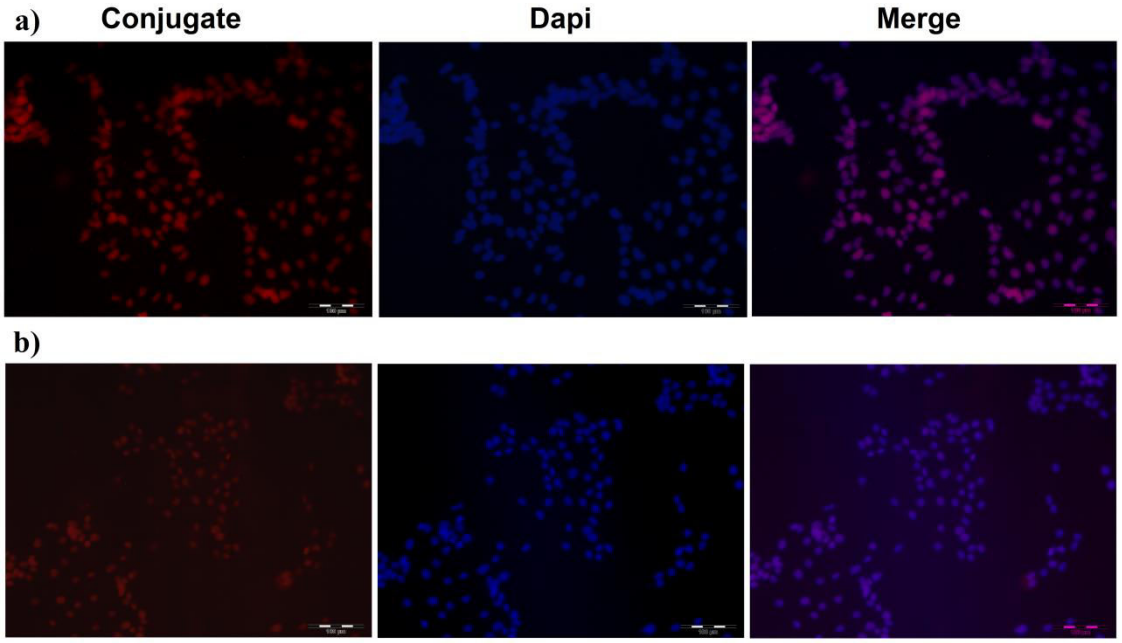


Figure 4.4 Fluorescence microscopy images of HeLa (a) and U87 cells (b). PEGNIO/DOX/CysTAT-MUC1 was incubated with the cells for 4 h at 37 °C.

Cytotoxicity

The cytotoxicity of bare niosomes, drug loaded formulations and free DOX was investigated by MTT assay using HeLa and U87 cells. PEGNIO was practically nontoxic to HeLa and U87 cells with relative cell viabilities above 80% for both 24 and 48 h (Fig. 4.5). PEGNIO/DOX was less toxic than free DOX on both cell lines after 24 and 48 h. This can be explained by the reduced diffusive uptake of PEGNIO/DOX in comparison to free DOX (Fig. 4.3a and b).[49,51] Due to the conjugation of the targeting ligand to PEGNIO/DOX, PEGNIO/DOX/CysTAT–MUC1 was more toxic to HeLa cells than to U87 cells after 24 and for 48 h. Both, after 24 and 48 hours PEGNIO/DOX/Cys-TAT–MUC1 had less cytotoxic effect of on U87 cells in comparison with free DOX ($p<0.01$ and $p<0.05$ respectively). PEGNIO/DOX/CysTAT–MUC1 increased the cytotoxicity for HeLa cells in comparison to PEGNIO/DOX for 24 and 48 hours ($p < 0.01$ and $p < 0.05$ respectively). Moreover, in comparison to free DOX, PEGNIO/DOX/CysTAT–MUC1 showed a significantly increased toxic effect on HeLa cells after 48 h. According to obtained results, it is clear that the aptamer conjugated niosomal formulation acted as a targeted DOX delivery platform for MUC1 expressing tumor cells.

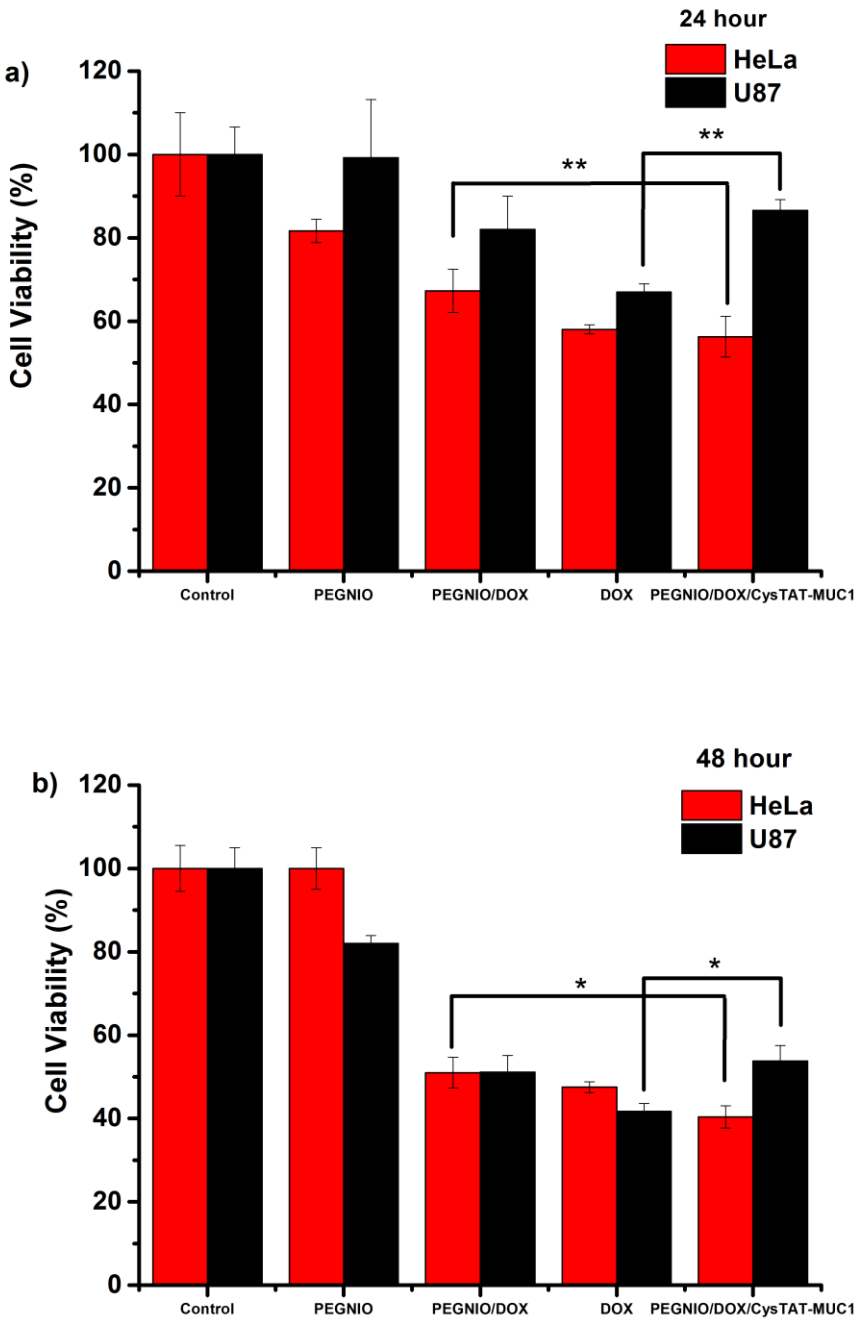


Figure 4.5 Cytotoxicity of the free drug and niosomal formulations on HeLa and U87 cells. Cells were incubated with PEG/NIO, PEGNIO/DOX, PEGNIO/DOX/CysTAT-MUC1 and free DOX (equivalent concentration of loaded DOX) for 24 h (a) and for 48 h (b). MTT assay was applied. Error bars represent the standard deviation from the mean (N=3). Data were analyzed using paired t-test, and *$p < 0.05$, **$p < 0.01$ was considered significant and very significant respectively.

4.1.6 Conclusions

The objective of this study was to develop an efficient aptamer targeted niosomal drug delivery system. For this aim, PEGNIO was successfully synthesized by the thin film hydration method. The model drug DOX was encapsulated into the vesicles, and the surface of the vesicles was decorated with cell penetrating peptides and MUC1 aptamer as a targeting ligand. The drug loaded niosomes exhibit great potential as targeting drug carriers. The targeted drug-loaded nanoparticles show stronger cytotoxicity of the MUC1 receptor overexpressed HeLa cells. As a conclusion, the formulation PEGNIO/DOX/CysTAT–MUC1 might be a promising and efficient strategy for the delivery of DOX to MUC1 overexpressed tumor cells.

Acknowledgements

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4.1.7 References

1 L. Brannon-Peppas and J. O. Blanchette, Adv. Drug Delivery Rev., 2004, 64, 1649–1659.

2 A. Sankhyan and P. Pawar, J. Appl. Pharm. Sci., 2012, 02, 20–32.

3 D. Ag Seleci, M. Seleci, J.-G. Walter, F. Stahl and T. Scheper, J.Nanomater., 2016, 13.

4 F. B. Barlas, B. Demir, E. Guler, A. M. Senisik, H. A. Arican, P. Unak and S. Timur, RSC Adv., 2016, 6, 30217–30225.

5 M. Hong, S. Zhu, Y. Jiang, G. Tang and Y. Pei, J. Controlled Release, 2009, 133, 96–102.

6 R. Muzzalupo, L. Tavano and C. La Mesa, Int. J. Pharm., 2013, 458, 224–229.

7 L. Tavano, R. Muzzalupo, L. Mauro, M. Pellegrino, S. Ando and N. Picci, Langmuir, 2013, 29, 12638–12646.

8 J. Park, P. M. Fong, J. Lu, K. S. Russell, C. J. Booth, W. M. Saltzman and T. M. Fahmy, Nanomedicine, 2009, 5, 410–418.

9 A. I. Minchinton and I. F. Tannock, Nat. Rev. Cancer, 2006, 6, 583–592.

10 E. Koren and V. P. Torchilin, Trends Mol. Med., 2012, 18, 385–393.

11 W. L. L. Munyendo, H. Lv, H. Benza-Ingoula, L. D. Baraza and J. Zhou, Biomolecules, 2012, 2, 187–202.

12 M. Lindgren, K. Rosenthal-Aizman, K. Saar, E. Eiriksdottir, Y. Jiang, M. Sassian, P. Östlund, M. Hallbrink and Ü. Langel, Biochem. Pharmacol., 2006, 71, 416–425.

13 M. Seleci, D. A. Seleci, M. Ciftci, D. Odaci Demirkol, F. Stahl, S. Timur, T. Scheper and Y. Yagci, Langmuir, 2015, 31, 4542–4551.

14 S. Deshayes, M. C. Morris, G. Divita and F. Heitz, Cell. Mol. Life Sci., 2005, 62, 1839–1849.

15 M. Mano, C. Teodosio, S. Simoes, d. L. M. Pedroso and A. Paiva, Biochem. J., 2005, 390, 603–612.

16 N. Schmidt, A. Mishra, G. H. Lai and G. C. L. Wong, FEBS Lett., 2010, 584, 1806–1813.

17 B. R. Liu, Y.-W. Huang, H.-J. Chiang and H.-J. Lee, J. Nanosci. Nanotechnol., 2010, 10, 7897–7905.

18 E. Vives, J. Schmidt and A. Pelegrin, Biochim. Biophys. Acta, Rev. Cancer, 2008, 1786, 126–138.

19 J. Taylor-Papadimitriou, J. Burchell, D. W. Miles and M. Dalziel, Biochim. Biophys. Acta, Mol. Basis Dis., 1999, 1455, 301–313.

20 Z. Cao, R. Tong, A. Mishra, W. Xu, G. C. L. Wong, J. Cheng and Y. Lu, Angew. Chem., Int. Ed., 2009, 48, 6494–6498.

21 T. Chen, M. I. Shukoor, Y. Chen, Q. Yuan, Z. Zhu, Z. Zhao, B. Gulbakan and W. Tan, Nanoscale, 2011, 3, 546–556.

22 Z. Liu, H. Zhao, L. He, Y. Yao, Y. Zhou, J. Wu, J. Liu and J. Ding, RSC Adv., 2015, 5, 16931–16939.

23 A. Ozer, J. M. Pagano and J. T. Lis, Mol. Ther.–Nucleic Acids, 2014, 3, e183.

24 C. S. M. Ferreira, C. S. Matthews and S. Missailidis, Tumor Biol., 2006, 27, 289–301.

25 Y. Hu, J. Duan, Q. Zhan, F. Wang, X. Lu and X.-D. Yang, PloS One, 2012, 7, e31970.

26 C. Yu, Y. Hu, J. Duan, W. Yuan, C. Wang, H. Xu and X.-D. Yang, PLoS One, 2011, 6, e24077.

27 L. Tan, K. G. Neoh, E. T. Kang, W. S. Choe and X. Su, Macromol. Biosci., 2011, 11, 1331–1335.

28 M. N. Azmin, A. T. Florence, R. M. Handjani-Vila, J. F. B. Stuart, G. Vanlerberghe and J. S. Whittaker, J. Pharm. Pharmacol., 1985, 37, 237–242.

29 Z.-X. Liao, E.-Y. Chuang, C.-C. Lin, Y.-C. Ho, K.-J. Lin, P.-Y. Cheng, K.-J. Chen, H.-J. Wei and H.-W. Sung, J. Controlled Release, 2015, 208, 42–51.

30 J. Takasaki and S. M. Ansell, Bioconjugate Chem., 2006, 17, 438–450.

31 L. Tavano, R. Aiello, G. Ioele, N. Picci and R. Muzzalupo, Colloids Surf., B, 2014, 118, 7–13.

32 S. B. Shirsand, M. S. Para, D. Nagendrakumar, K. M. Kanani and D. Keerthy, Int. J. Pharm. Invest., 2012, 2, 201–207.

33 G. Wei, S. Xiao, D. Si and C. Liu, Biomed. Chromatogr., 2008, 22, 1252–1258.

34 A. Shrivastava and V. B. Gupta, Chron. Young Sci., 2011, 2, 21– 25.

35 R. Bongartz, D. Ag, M. Seleci, J.-G. Walter, E. E. Yalcinkaya, D. O. Demirkol, F. Stahl, S. Timur and T. Scheper, J. Mater. Chem. B, 2013, 1, 522–528.

36 G. Minotti, P. Menna, E. Salvatorelli, G. Cairo and L. Gianni, Pharmacol. Rev., 2004, 56, 185–229.

37 P. Mohan and N. Rapoport, Mol. Pharmaceutics, 2010, 7, 1959–1973.

38 B. Haley and E. Frenkel, Urologic Oncology: Seminars and Original Investigations, 2008.

39 T. M. Allen and P. R. Cullis, Science, 2004, 303, 1818–1822.

40 G. T. Hermanson, Bioconjugate Techniques, Academic press, 2nd edn, 2008.

41 Y. Li, M. Kr¨oger and W. K. Liu, Biomaterials, 2014, 35, 8467– 8478.

42 J. V. Jokerst, T. Lobovkina, R. N. Zare and S. S. Gambhir, Nanomedicine, 2011, 6, 715–728.

43 N. O. Sahin, in Nanomaterials and Nanosystems for Biomedical Applications, Springer, 2007, 67–81.

44 M. Bragagni, N. Mennini, C. Ghelardini and P. Mura, J. Pharm. Pharm. Sci., 2012, 15, 184–196.

45 G. A. Hughes, Nanomedicine, 2005, 1, 22–30.

46 J. Qi, P. Yao, F. He, C. Yu and C. Huang, Int. J. Pharm., 2010, 393, 177–185.

47 C. t. S. M. Ferreira, M. C. Cheung, S. Missailidis, S. Bisland and J. Gariepy, Nucleic Acids Res., 2009, 37, 866–876.

48 M. S. Syrkina, M. A. Rubtsov, D. M. Potashnikova, Y. D. Kondratenko, A. A. Dokrunova and V. P. Veiko, Acta Naturae, 2014, 6, 62–70.

49 X.-B. Xiong, Y. Huang, W.-l. Lu, X. Zhang, H. Zhang, T. Nagai and Q. Zhang, J. Controlled Release, 2005, 107, 262–275.

50 A. D. Heibein, B. Guo, J. A. Sprowl, D. A. MacLean and A. M. Parissenti, BMC Cancer, 2012, 12, 381.

51 X. Li, L. Ding, Y. Xu, Y. Wang and Q. Ping, Int. J. Pharm., 2009, 373, 116–123.

4.1.8 Supplementary information

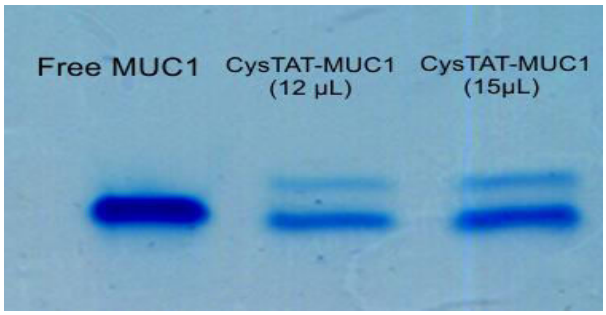


Figure S4.1 Confirmation of conjugation of MUC1 aptamer with CysTAT peptide (Urea PAGE)

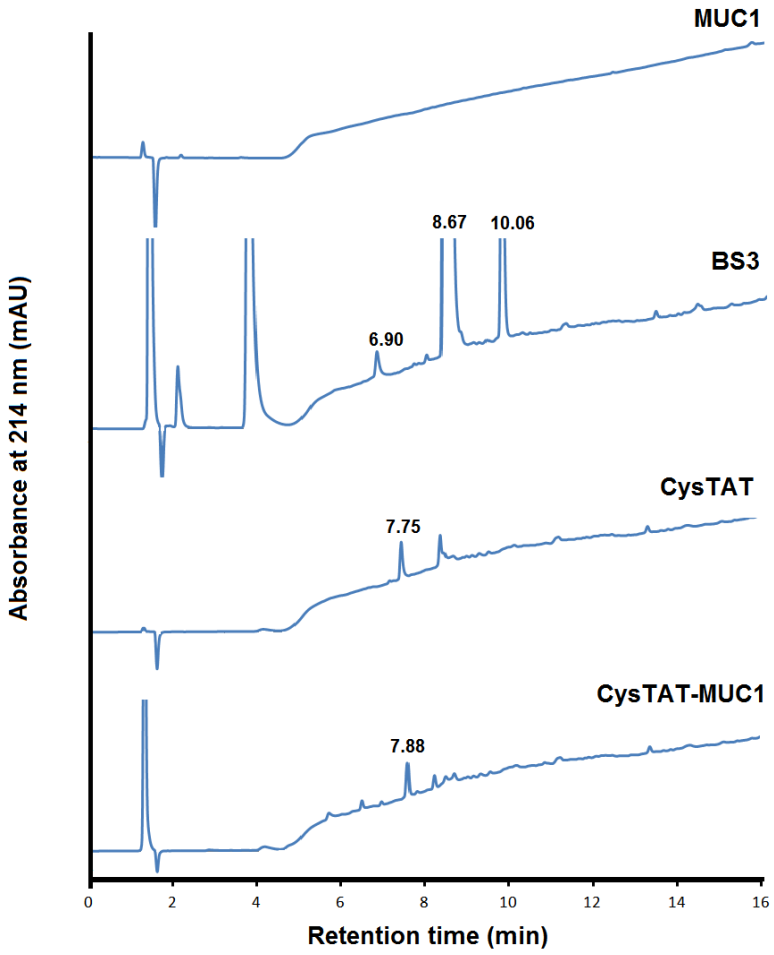


Figure S4.2 HPLC chromatogram of MUC1 aptamer, BS3 crosslinker, CysTAT peptide and CysTAT-MUC1 conjugate.

4.2 Tumor homing and penetrating peptide conjugated niosomes as multi-drug carriers for tumor-targeted drug delivery

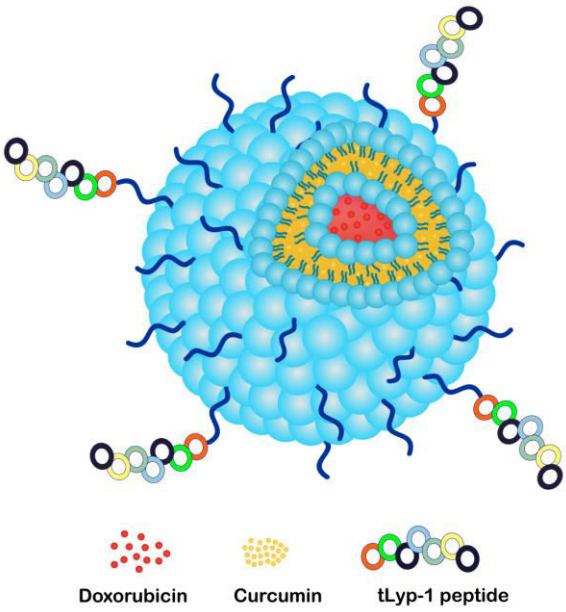
This chapter was published as D. Ag Seleci, M .Seleci, F. Stahl, and T. Scheper, Tumor homing and penetrating peptide conjugated niosomes as multi-drug carriers for tumor-targeted drug delivery, RSC Advances, 2017, 7, 33378–33384. It was reproduced with permission of Royal Society of Chemistry.

4.2.1 Summary

Many types of cancer cannot be treated with only one type of drug due to the pathological complexity of tumor tissues. Using multiple drugs might result in synergistic or additive effects at a lower dose compared with mono-chemotherapy. Furthermore, the low dose combination therapy may reduce adverse effects. Therefore, the combination therapy is a promising approach in clinical chemotherapy. Development of novel delivery systems using several nanomaterials provides to deliver multiple drugs via incorporation in one single carrier. Niosomes are one of the drug carriers exhibiting a bilayer structure and can accommodate hydrophilic drugs in their core and lipophilic drugs in their membrane at the same time. Here, polyethylene glycol modified niosomes (PEGNIO) were prepared from span60, cholesterol and 1,2-distearoyl-snglycero-3-phosphoethanolamine-N-[maleimide (polyethyleneglycol)-2000] (DSPE-PEG (2000) Maleimide) via thin film hydration method. Curcumin (C) and doxorubicin (D) were encapsulated into the PEGNIO. The tumor-homing peptide tLyp-1, which has high affinity and specificity to neuropilin receptor (NRP-1), was conjugated to C and D loaded PEGNIO (PEGNIO/D–C) via the formation of a thioether linkage. Detailed characterization studies were performed. The morphology of large unilamellar PEGNIO was monitored via upright microscopy and results showed that PEGNIOs were spherical and homogeneous in shape. The size of bare niosomes was analyzed to be 150 nm using Dynamic Light Scattering (DLS). After co-encapsulation of doxorubicin and curcumin, a slightly smaller hydrodynamic diameter as 144 nm was obtained. The conjugation of tLyp-1 did not affect the size of the niosomes significantly. The size was measured to be 146 nm for PEGNIO/D–C/tLyp-1. The stability of PEGNIO/D–C/tLyp-1 was tested via measuring the size and no changes were observed in the size after 30 days storage at 4 °C in the dark. Moreover, the entrapment efficacy (E%) was calculated to be 32.6 ±1.9% for curcumin and 23.3 ± 1.6% for doxorubicin. The t-Lyp-1 peptide conjugation efficiency was calculated to be 23.9% using BCA Assay. Dialysis method was used to study the

release of doxorubicin and curcumin from PEGNIO/D–C/t-Lyp-1.Two different pH values were used for release. Here pH 7.4 and pH 5.6 were chosen to mimic physiological conditions and acidic tumor environment respectively. The release profiles of doxorubicin and curcumin from PEGNIO/D–C/t-Lyp-1 showed faster release of both drugs under acidic environments. To test the specificity of t-Lyp-1 targeted niosomes, Rh6g was used as a model fluorescent dye and encapsulated into niosomes. The uptake of the Rh6g loaded niosomal formulations by glioblastoma cells (U87, NRP-1 positive) and human mesenchymal stem cells (hMSC, NRP-1 negative) cells were analyzed by flow cytometry. Results showed that PEGNIO/Rh6g/tLyp-1 was taken up by U87 cells specifically. The cytotoxic effects of niosomal formulations on U87 and hMSC cells were tested via MTT assays. PEGNIO/D–C/t-Lyp-1 was significantly more cytotoxic for U87 cells in comparison to free D–C. Besides, PEGNIO/D–C/t-Lyp-1 was more toxic to U87 cells than to hMSC cells. Furthermore, the effect of free D–C, PEGNIO/D–C and PEGNIO/D–C/tLyp-1 on tumor-like spheroids formed by U87 cells, was evaluated for 6 days. The morphological changes of treated versus non-treated spheroids were monitored via bright field microscopy. PEGNIO/D–C/tLyp-1 treated spheroids became distorted, with many disassociated cells. According to all obtained results, it can be concluded that this targeted and codrug-loaded niosomal delivery system could improve the efficacy of doxorubicin on glioma therapy.

Table of Contents Graphic



4.2.2 Abstract

Development of nanoscale drug delivery systems, which can mediate efficient tumor targeting together with high cellular internalization, is crucial for glioma treatment. Combination of therapeutic agents in nanoparticles provides synergistic effects and allows further surface modifications with targeting ligands for specific glioma therapy. To achieve this goal, both doxorubicin and curcumin were encapsulated in polyethlene glycolated niosomes (PEGNIO). The surface of co-drug loaded PEGNIO was modified with tumor homing and penetrating peptide (tLyp-1). Physicochemical properties were determined via dynamic light scattering (DLS) and spectral analysis. Moreover, flow cytometry studies were performed to examine the specific cellular uptake of the tLyp-1 targeted niosomal formulation. *In vitro* cytotoxicity and inhibition of tumor-like spheroids growth were investigated on human glioblastoma (U87) and human mesenchymal stem cells (hMSC) cells. The results clearly indicated that the strategy by co-administration of doxorubicin and curcumin with tLyp-1 functionalized niosomes could significantly improve anti-glioma treatment.

4.2.3 Introduction

Glioblastoma is a malignant brain tumor and patients diagnosed with glioma have a median survival of less than 2 years. The current treatment for brain cancer is usually chemotherapy. However, the therapeutic efficacy of many anticancer drugs is limited by the existence of the blood-brain barrier (BBB), the blood-brain tumor barrier (BBTB), a relatively weak enhanced permeability and retention (EPR) effect and by their severe side effects on healthy cells.[1]

Combination of therapeutic agents has recently attracted great attention for glioblastoma chemotherapy.[2] It provides synergistic effects and decrease adverse side effects associated with high doses of single anticancer drugs and helps to avoid drug resistance.[3] Over the last decade, the advances in nanomedicine have enabled to develop novel nanocarriers for site-specific drug delivery and to gain access to brain tumors.[4, 5] By taking advantages of these nanocarriers, different combinations of various drugs were co-loaded on nanoparticles for glioma therapy.[6, 7] Dilnawaz *et al.* loaded both curcumin and temozolomide into magnetic nanoparticles (MNPs) and studied their *in vitro* cytotoxic effects on 3D glioma tumor spheroids. The dual drug loaded MNPs formulations demonstrated higher cytotoxic effects than the single drug loaded MNPs formulations as compared to their corresponding native drugs in 2D and 3D culture.6 In

another study Xu *et al.* studied *in vitro* cytotoxicity effects of paclitaxel and temozolomide co-loaded in polymer nanocomposites and the results suggested that the composite gel possessed much higher growth-inhibiting effects as well as apoptosis-inducing rates in glioma cells than other formulations.[8]

Niosomes are drug carriers exhibiting a bilayer structure and are in most cases formed by self-association of non-ionic surfactants and cholesterol in aqueous phase. They can accommodate a large number of drugs with a wide range of solubilities.[9, 10] Hydrophilic drugs and lipophilic drugs can be entrapped into the aqueous core and membrane bilayer of niosome respectively.[11, 12] Therefore, niosomes are promising nanocarriers in multi-drug delivery applications. The efficiency of niosomal multi-drug delivery systems can be further improved by active targeting for tumor therapy by using a ligand coupled to the surface of niosomes. In this way, nanocarriers can be actively taken up, for example, via receptor-mediated endocytosis.[13] Recently, Tavano *et al.* reported the dual encapsulation of hydrophobic curcumin or quercetin and hydrophilic doxorubicin in Pluronic-based niosomes for cancer multi-drug delivery.[14] Besides, the surface of the niosomes was coupled with transferrin and/or folic acid for breast cancer targeting. Results showed the high potential of the dual drug loaded niosomes in breast tumor treatment.

Neuropilin-1 (NRP-1) is a transmembrane protein overexpressed on the surface of both glioma and endothelial cells of angiogenic blood vessels.[15-17] CGNKRTR (tLyP-1) is a homing peptide which penetrates tumor cell through an NRP-1 mediated endocytosis via C-end Rule (CendR) internalization pathway.[18, 19] Therefore, tLyP-1 is a promising targeting ligand for the delivery of therapeutic agents to tumors. Xu *et al.* produced tLyp-1 targeted camptothecin-loaded mesoporous silica nanoparticles and it showed minimal adverse effects on human mesenchymal stem cells (hMSC), whereas significant induction of tumor cell death was observed.[20] Moreover, Hu *et al.* conjugated the tLyp-1 peptide to the surface of paclitaxel (PTX) loaded PEG-PLA nanoparticles *via* a maleimide-thiol coupling reaction for anti-glioma drug delivery. They achieved the longest survival of the mice bearing intracranial C6 glioma treated with PTX-loaded tLyp-1-nanoparticles in comparison to PTX loaded nanoparticles.[17]

In this study, polyethylene glycolated niosomes (PEGNIO) were prepared from span60, cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG (2000) Maleimide) for multi-drug delivery. Curcumin (C) and doxorubicin (D) were encapsulated into the PEGNIO. The tumor-

homing peptide tLyp-1 was conjugated to C and D encapsulated PEGNIO (PEGNIO/D-C) *via* the formation of a thioether linkage. The effect of the PEGNIO/D-C/tLyp-1 on human glioblastoma cells (U87) and hMSC was investigated in detail. Our results suggest that this targeted and co-loaded drug delivery platform could improve the efficacy of doxorubicin on glioma therapy.

4.2.4 Materials and methods

Materials

DSPE-PEG(2000) Maleimide was provided by Avanti (Alabama, USA). Sorbitan monostearate (Span60), cholesterol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), curcumin and rhodamine 6G (Rh6g) were ordered from Sigma Aldrich (Munich, Germany). Alpha Minimum Essential Media (Alpha-MEM) was purchased from Life Technologies (Darmstadt, Germany). Doxorubicin hydrochloride was purchased from Cayman Chemical (Michigan, USA). tLyP-1 peptide (CGNKRTR) was ordered from GeneCust (Ellange, Luxembourg).

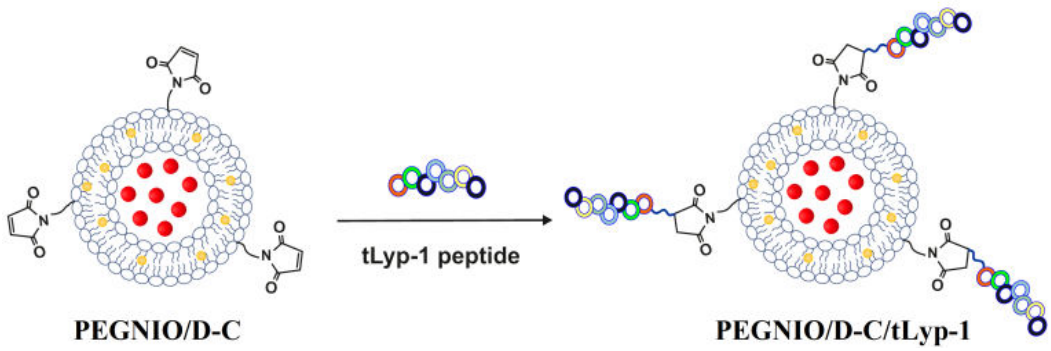
Preparation of niosomes

The thin film hydration method was used to prepare PEGylated niosomes.[21] Span 60, cholesterol and DSPE-PEG(2000) Maleimide were dissolved in 1.0 mL chloroform in a round-bottom flask with the mM ratio (4.95:4.95:0.1). The solvent was evaporated with constant rotation under reduced pressure to form a thin lipid film. Doxorubicin or Rh6g loaded niosomes were obtained by hydrating the thin lipid film with 1.0 mL of doxorubicin or Rh6g (0.22×10^{-3} and 0.42×10^{-3} M, respectively) aqueous solution at 60 °C for 60 min.[14, 22] To obtain doxorubicin-curcumin-loaded niosomes, 200 µL of curcumin solution (2.18×10^{-3} M) was added to the initial chloroform mixture.[14] After vortexing, the solvent was evaporated under reduced pressure, and the obtained film was then hydrated with 1.0 mL of doxorubicin aqueous solution at 60 °C for 60 min. Afterward, the niosomal solution was equilibrated at room temperature overnight to complete annealing and partitioning of the drug between the lipid bilayer and the aqueous phase.[23] Small unilamellar vesicles (SUVs) were prepared starting from multilamellar vesicles (MLVs) by sonication in an ultrasonic bath and following extruding the niosomes through 0.4 µm and 0.1 µm pore size polycarbonate filters (mini-extruder set

Avanti polar lipids, sample volume 1.0 mL). Niosomes were purified by a flow of niosome suspensions across a Sephadex G-25 gel column and volume of eluted niosomes was adjusted to 1.0 mL.

Preparation and characterization of tLyp-1 conjugated niosomes

For the preparation of tLyp-1 targeted niosomes, the thiol group of tLyp-1 was coupled with the maleimide group of PEG chains on niosomes. tLyp-1 peptide was dissolved in 50 mM HEPES buffer, pH 6.5 at 200 µg mL-1 concentration (50 µL) were reacted with niosomes (950 µL) for overnight at room temperature resulting in the formation of a thioether linkage.[24, 25] The products were then purified using a 14 kDa dialysis bag to remove the unconjugated peptides. Schematic representation of co-drug encapsulation and the bioconjugation processes are shown in Scheme 1.



Scheme 1. Schematic representation of co-drug encapsulation and the bioconjugation process (yellow dots: curcumin, red dots: doxorubicin).

The size of the niosomes were measured by dynamic light scattering (DLS) analysis using Malvern Zetasizer Nano ZSP. The polydispersity index (PDI) was used as a measure of the width of size distribution. Each sample was measured three times. The morphology of large unilamellar PEGNIO (before extrusion) was monitored via Olympus BX41 upright microscope. 100X immersion oil objective was used.

To estimate the tLyp-1 conjugation efficiency, BCA Protein Assay was used.[4] 25 µL of BSA standards, PEGNIO (as blank) and PEGNIO/tLyp-1 were added in triplicate wells (96-well plate) and 200 µL of BCA Protein Assay Reagent were added to the samples. After incubation at 37 °C for 30 min, the absorption was measured *via* microplate reader (BioTek Epoch) at 562 nm. Conjugation efficiency was expressed as the percentage of

the peptide bound to the surface of noisome, referred to the amount of peptide that is present initially.

Stability

The stability of PEGNIO/D-C/tLyp-1 was tested via DLS analysis. PEGNIO/D-C/tLyp-1 was stored at 4 °C in the dark. The particle size and PDI values were measured after 30 days. Moreover, the particle size of PEGNIO/D-C/tLyp-1 was measured in cell culture media after the incubation at 37 °C for 24 h.

Entrapment efficiency

After encapsulation, the D-C loaded niosomes were purified using Sephadex G-25 gel column. The percent of encapsulation efficiency (E%) was expressed as the percentage of the drug entrapped in niosomes (and thus not removed via Sephadex column) referred to the initial amount of drug that is present in the non-purified sample (Equation 1). 200 µL of non-purified and purified niosomes diluted in 600 µL of methanol and niosomes were separated by centrifugation at 12,000 rpm for 5 min. This step allows the breaking of niosomal membranes and the release of the encapsulated drug. A calibration curve was derived with a known concentration of free doxorubicin by fluorescence emission measurements at 595 nm using NanoDrop 3300. The stock solutions of doxorubicin were prepared at 1.0 mg mL^{-1} in methanol and further diluted with methanol in the concentration range 1.0–50 µg mL^{-1}. The amount of doxorubicin in purified and non-purified samples was calculated according to the calibration curve (y = 850.94x+12.092, R^2 =0.9973) via measuring fluorescence emission at 595 nm. The limit of detection (LOD) and limit of quantification (LOQ) for doxorubicin were found to be 4.92 µg mL^{-1} and 14.92 µg mL^{-1} respectively, based on 3.3σ/slope and 10sσ/slope formulations. The amount of curcumin in purified and non-purified samples was calculated according to the calibration curve (y=0.1459x+0.0058, R^2=1, LOD=0.15 µg mL^{-1} and LOQ=0.46 µg mL^{-1}), which was established with a known concentration of free curcumin (0.5-10 µg mL^{-1}) by absorbance measurements at 426 nm using a microplate reader (BioTek Epoch).

$$\mathbf{E}\ (\%) = \frac{\textbf{Amount of drug in purified sample}}{\textbf{Amount of drug in non} - \textbf{purified sample}} \times \mathbf{100}$$

Drug release

The *in vitro* release profiles of curcumin and doxorubicin from PEGNIO/D-C/tLyp-1 were studied using a dialysis method. PEGNIO/D-C/tLyp-1 solutions were prepared and transferred into a dialysis membrane tubing (Thermo, Slide-ALyzer MINI Dialysis Devices, 10k MWCO). The tubing was immersed in 10 mL of the PBS buffer (pH 5.6 and 7.4) containing Tween80 (1.0%, v/v), placed in an incubator at 37 °C and stirred at 100 rpm. Here, Tween 80 was added to obtain optimal release conditions since curcumin has limited solubility in PBS.[26] At predetermined time intervals, 0.5 mL samples were removed from the release medium and replaced with the same volume of fresh buffer. The amount of released doxorubicin and curcumin was calculated as % according to the calibration curves and in respect to loading concentration (the drug concentration in niosomes, before starting the release). They were established with a known concentration of free doxorubicin and free curcumin by fluorescence emission measurements at 595 nm using NanoDrop 3300 and absorbance measurements at 426 nm using a microplate reader respectively.

Cell culture

U87 cell lines were provided from German Collection of Microorganisms and Cell Cultures (DSMZ).U87 cells were grown in DMEM containing 10% fetal calf serum (FCS) (Biochrom GmbH, Germany) and 1.0% penicillin/streptomycin (P/S). Human adipose-derived mesenchymal stem cells (hMSCs) were isolated from subcutaneous adipose tissues of 3 different patients scheduled for abdominoplasty after obtaining informed written consent, as approved by the Institutional Review Board, project #2251-2014 on 15th May, 2014. The isolated populations have been extensively characterized as mesenchymal stem cells by surface marker analysis and functional properties (differentiation capacity). hMSCs were cultured in alpha-MEM including 10% human serum (HS) (HS, c.c.pro GmbH, Germany)and 0.5% gentamicin. Both cell lines were cultivated and incubated with samples at 37 °C in a humidified environment with 5.0% CO_2.

Cellular uptake

Rh6g was used as a model fluorescent dye and encapsulated into niosomes. The uptake of the Rh6g loaded niosomal formulations by U87 and hMSC cells was analyzed by flow cytometry. The cells ($4x10^4$) were treated with PEGNIO/Rh6g and PEGNIO/Rh6g/tLyp-

1 for 2 h and treated cells were washed two times with PBS, and then analyzed in a BD Accuri C6 cytometer.

Cytotoxicity

The cytotoxic effects of niosomal formulations and free drugs were tested on U87 and hMSC cells using MTT assay. Cells ($8x10^3$) were seeded out in 96-well tissue plates (Sarstedt, USA) in a volume of 200 µL and cultivated for three days. After this cultivation time, cells were washed once with PBS and treated with PEGNIO/D-C, PEGNIO/D-C/tLyp-1 and free D-C for 24 h. The equivalent concentration of free doxorubicin and curcumin was used in niosomal formulations. Then the samples were removed and cells were incubated in 110 µL per well 10% MTT solution (5.0 mg mL-1 in PBS) in the medium for 4 h. During this incubation time, formazan complex was produced by the cells. 100 µL SDS solution (1.0 g SDS in 10 mL 0.01 M HCl) were added to each well to release the purple colored salt from the cells. After 24 h of incubation, UV-Vis absorption was measured at 570 nm to 630 nm as the reference wavelength using a microplate reader Epoch Biotek. Besides, IC50 values (the drug concentration required for 50% inhibition of cell viability) were calculated for U87 cells using the growth sigmoidal/dose response function of Origin software.

Effect on tumor-like multicellular spheroids

The effect of niosomal formulations was tested on 3D spheroid cultures. U87 cells ($8x10^3$/well) in 100 µL of culture medium were seeded into 96-well round-bottom ultra low attachment plates (Sarstedt, Germany) and incubated for 2 days to form spheroids. Afterward, 100 µL of each sample (D-C, PEGNIO/D-C, PEGNIO/D-C/tLyp-1) was added to the spheroids and incubated further for 2, 4 and 6 days. Bright-field images of U87 tumor-like spheroids treated with the samples were taken with Olympus IX50 inverted light microscope equipped with an Olympus camera (SC30, Japan) by using cellSens Standard software (Olympus Co. Japan).

Statistical analysis

Statistical data analysis was performed using the Student's t-test. The difference between two groups was considered significant when the p value was less than 0.05.

4.2.5 Results and discussion

Characterizations of niosomal formulations

The morphology of large unilamellar PEGNIO was monitored using an upright microscope (Figure S4.3). Figure S4.3 indicated that PEGNIOs were spherical and homogeneous in shape. The mean diameters of empty and co-drug-loaded niosomal formulations, along with the corresponding polydispersity index (PDI) and drug entrapment efficiency (E%) values, are listed in Table 4.2. The empty vesicle size was analyzed to be 150.3 nm. When doxorubicin and curcumin were co-encapsulated, a slightly smaller hydrodynamic diameter as 144.1 nm was obtained. In our previous study, we demonstrated, that the encapsulation of doxorubicin in PEGNIO did not result in an alteration of niosomal size.[22] However, when a hydrophobic drug is encapsulated in the colloidal system, they were claimed to lead to H bonding between their hydroxyl groups and niosomal matrices, resulting in an increase in the niosomal cohesion and then a decrease in the diameter.[14] Here, the decrease in the size can be attributed to the entrapment of hydrophobic curcumin in the bilayer. After the conjugation of tLyp-1, the size of the niosomes did not change significantly. The size was measured to be 146.1 nm for PEGNIO/D-C/tLyp-1. PDI less than 0.3 corresponds to a homogeneous population for colloidal systems.[27] PDI ranged from 0.175 to 0.140 for the niosomal formulations, demonstrating that the vesicle population is relatively homogeneous in size. The entrapment efficacy (E%) was calculated to be 32.6±1.9% for curcumin and 23.3±1.6% for doxorubicin.

Under the determined experimental conditions, the t-Lyp-1 peptide conjugation efficiency was calculated to be 23.9%.

The stability of PEGNIO/D-C/tLyp-1 was tested via DLS analysis and no changes were observed in the size and PDI values after 30 days storage at 4 °C in the dark (data not shown). Furthermore, PEGNIO/D-C/tLyp-1 was diluted in cell culture media and was incubated at 37 °C for 24 hours. The size of the sample was measured and after incubation and no changes were observed.

Table 4.2 Characterization of PEGNIO formulations

Samples	Size (nm) Intensity (%) (Mean±SD)	Poly-dispersity Index (PDI)	Entrapment Efficiency E%
PEGNIO	150.3 ± 51	0.175	-
PEGNIO/D-C	144.1 ± 61	0.152	D:23.3±1.6 C:32.6±1.9
PEGNIO/D-C/tLyp-1	146.1 ± 69	0.140	D:22.0±1.5 C:31.2±1.8

Drug release

Prolonged drug retention and sustained drug release are important properties for nanoscale drug delivery systems that will minimize side effects of the drug.[22] The unique structure of niosomes allows to control the release of the encapsulated drug combinations to increase antitumor activity.[14] The release of doxorubicin and curcumin from PEGNIO/D-C/t-Lyp-1 was investigated using dialysis methods at pH 7.4, which was chosen in accordance with physiological conditions and in an acidic environment (pH 5.6) similar to the tumor. The release profiles of doxorubicin and curcumin from PEGNIO/D-C/t-Lyp-1 showed the faster release of both drugs under acidic environments than that at neutral pH (Fig. 4.6). Within 32 h, the release of doxorubicin and curcumin was 74±1.2% and 62±0.6% at pH 5.6 respectively. At physiological pH, the release of curcumin and doxorubicin was 36±1.7% and 68±2.9%. According to results, this conjugate is expected to be a promising co-drug delivery system for the tumor-targeted therapy.

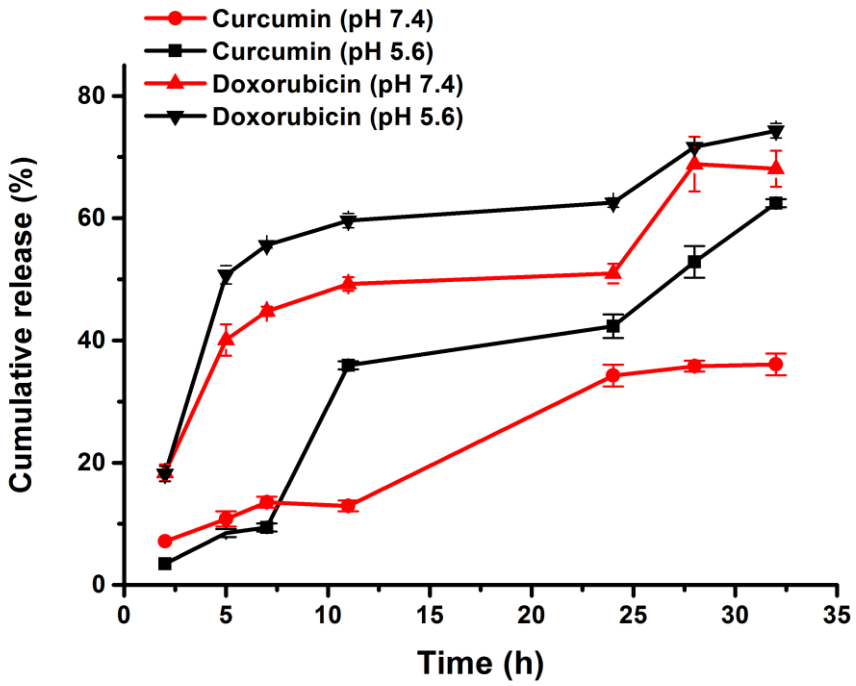


Figure 4.6 *In vitro* cumulative release of doxorubicin and curcumin from PEGNIO/D-C/t-Lyp-1 at pH 7.4 and 5.6.

Cellular uptake

To test the specificity of t-Lyp-1 targeted niosomes, Rh6g was encapsulated in PEGylated niosomes and further t-Lyp-1 was conjugated to PEG chains on niosomes. Flow cytometry was used to investigate the Rh6g uptake by hMSC and U87 cells to evaluate receptor-mediated cell targeting. The cells were treated with PEGNIO/Rh6g for 2 h. Untreated control cells and treated cells were analyzed using a BD Accuri C6 flow cytometer. No differences were observed between targeted and non-targeted niosomal formulations treated hMSC (Fig. 4.7a). In the case of U87 cells, the uptake of PEGNIO/Rh6g/tLyp-1 was higher than PEGNIO/Rh6g (Fig. 4.7b). Fig.4.7c indicates that PEGNIO/Rh6g/tLyp-1 was uptaken by U87 cells specifically. tLyP-1 is able to selectively home in and penetrate into tumor cells mediated NRP-1 receptor which is overexpressed in tumor cells.[20, 28] The expression of NRP-1 receptor, on the surface of the U87 cells would affect the enhanced cellular uptake of PEGNIO/Rh6g/tLyp-1.

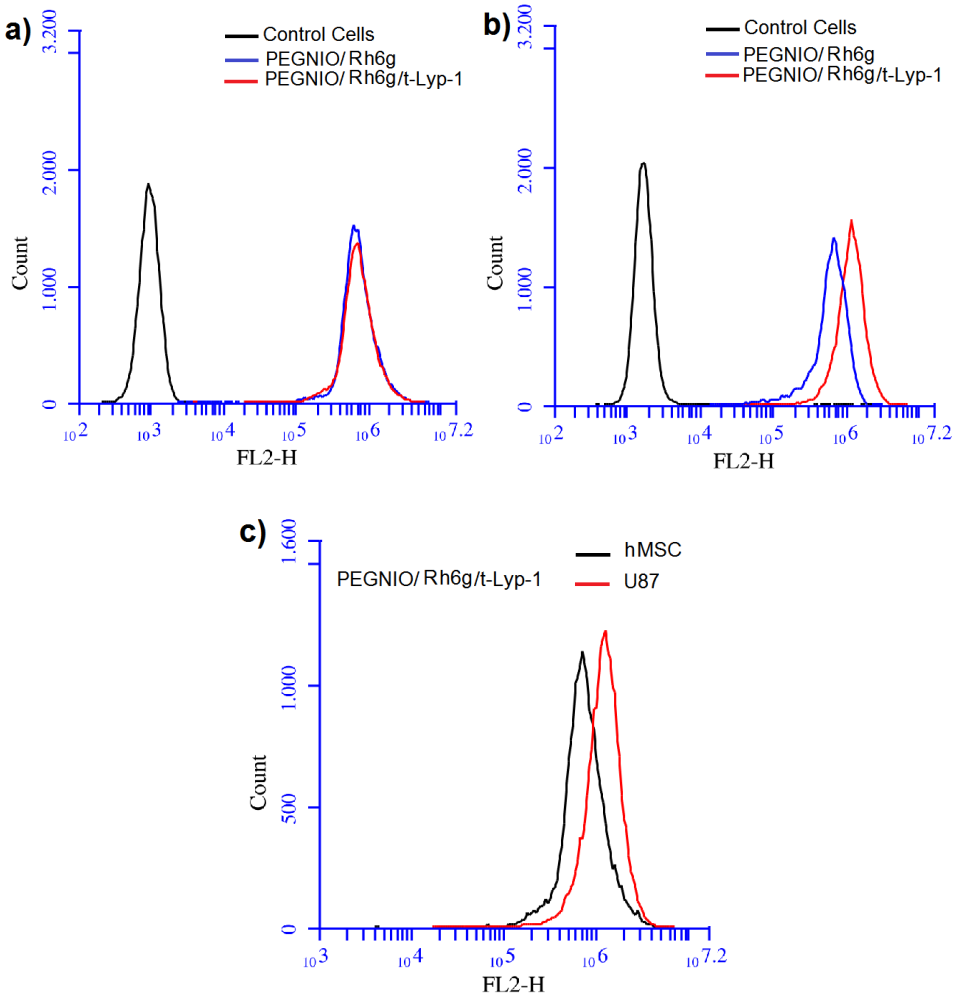


Figure 4.7 Flow cytometry measurements of Rh6g uptake by hMSC (a) and U87 cells (b) after incubating with PEGNIO/Rh6g and PEGNIO/Rh6g/t-Lyp-1. Histogram of binding of PEGNIO/Rh6g/t-Lyp-1 to hMSC and U87 cells (c).

Cytotoxicity

Doxorubicin is classified as a topoisomerase-2 inhibitor and one of the most extensively used broad-spectrum anticancer drugs. However, it has some limitations in clinical use. Long treatment durations cause the development of resistance by tumor cells and toxicity for healthy tissues. Therefore, combinations of two or more agents have been used to overcome toxicity and other side effects of doxorubicin.[29, 30] Curcumin's therapeutic characteristics have been demonstrated against a wide range of cancers.[31-33] The major drawback of curcumin is its poor solubility and stability in water. Thus, here doxorubicin and curcumin were encapsulated in PEGNIO and further the surface of niosome was modified with t-Lyp-1 peptide.

After confirming the NRP-1 receptor-mediated glioma cell binding efficiencies of the PEGNIO/Rh6g/tLyp-1 via flow cytometry, MTT assays were performed to evaluate relative abilities of the various niosomal formulations in inhibiting growth of tumor (U87) and non-cancer (hMSC) cells. PEGNIO/D-C was more toxic than free D-C on both cell lines (Fig. 4.8). Due to the conjugation of the targeting ligand to PEGNIO/D-C, PEGNIO/D-C/t-Lyp-1 was more toxic to U87 cells than to hMSC cells after 24h ($p<0.05$). PEGNIO/D-C/t-Lyp-1 significantly increased the cytotoxicity for U87 cells in comparison to free D-C ($p< 0.05$). Moreover, U87 cells were treated with samples (D-C, PEGNIO/D-C and PEGNIO/D-C/t-Lyp-1) for 48 h at concentration range of doxorubicin (0-50 µg/mL). IC50 values were calculated as to be 0.96 µg/mL, 0.9 µg/mL and 0.76 µg/mL for D-C, PEGNIO/D-C and PEGNIO/D-C/t-Lyp-1 respectively.

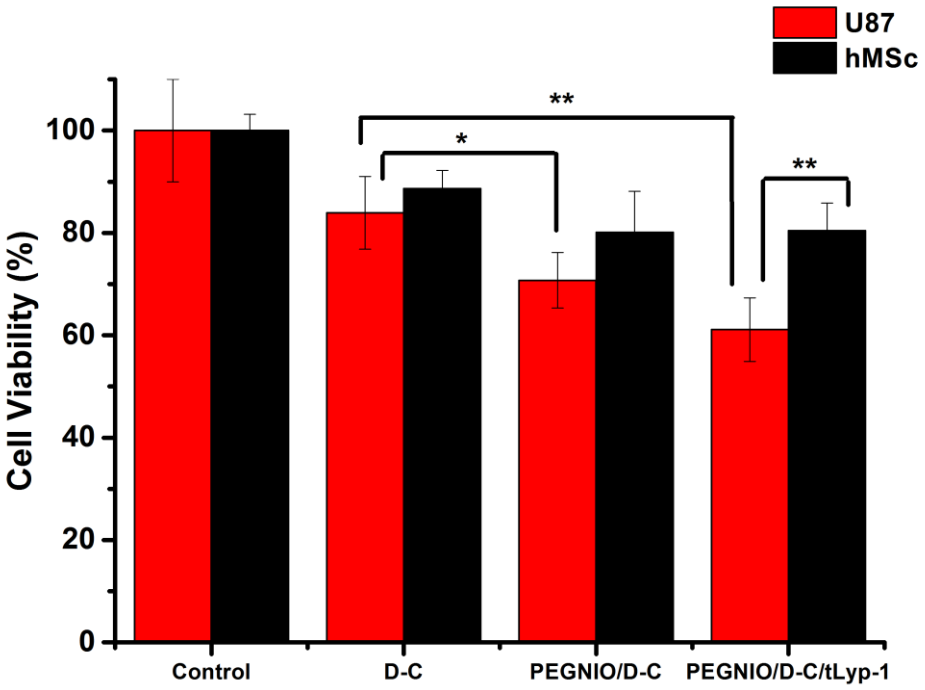


Figure 4.8 Cytotoxicity of the free drug and niosomal formulations on hMSC and U87 cells. Cells were incubated with, PEGNIO/D-C, PEGNIO/D-C/t-Lyp-1 and free D-C (equivalent concentration of loaded D-C) for 24 h. MTT assay was applied. Error bars represent the standard deviation from the mean (N=3). Data were analyzed using t-test, and *$p < 0.05$, **$p < 0.01$ was considered significant and very significant respectively.

Effect on tumor-like multicellular spheroids

According to results obtained in 2D cell cultures, it is clear that the tLyp-1 conjugated niosomal formulation acted as a targeted multi-drug delivery platform for NRP-1 expressing tumor cells. Further, we have used 3D spheroid model which is found to be relevant for therapeutic evaluation, as it reflects better the *in vivo* conditions both in structural and molecular aspects.[34] The effect on tumor-like spheroids was evaluated following the treatment with cell culture media, free D-C, PEGNIO/D-C and PEGNIO/D-C/tLyp-1 for 6 days. As shown in Fig. 4.9a, the tumor spheroids treated with cell culture media retained their morphology (compact spheroids with smooth surface) over entire period of cultivation. The spheroids treated with D-C became smaller in diameter (Fig. 4.9b). The tumor spheroids exposed to niosomal formulations exhibited no more tightly organised structure (Fig. 4.9c, d). Especially, PEGNIO/D-C/tLyp-1 treated spheroids became distorted, with many disassociated cells (Fig. 4.9d). These results demonstrated enhanced effects of PEGNIO/D-C/tLyp-1 on 3D tumor-like tissues in comparison to other formulations.

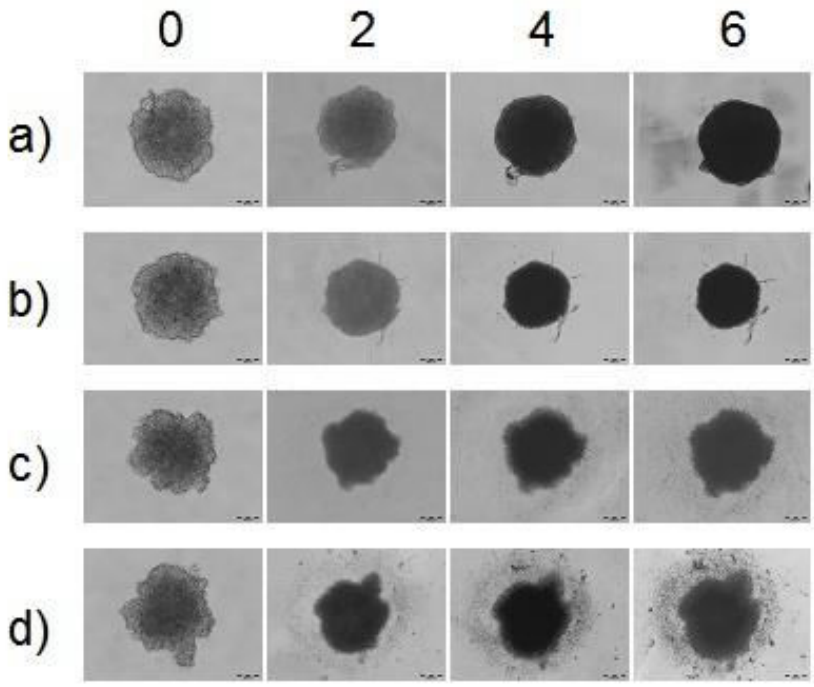


Figure 4.9 Morphology of U87 tumor spheroids treated with cell culture media (a), D-C (b), PEGNIO/D-C (c) and PEGNIO/D-C/t-Lyp-1 (d) on days 0, 2, 4 and 6, respectively.

4.2.6 Conclusion

In this work, a targeted niosomal co-drug delivery system was developed to improve the therapeutic efficacy of anti-glioma drug delivery. Curcumin and doxorubicin were encapsulated into PEGNIO by the thin film hydration method. The surface of PEGNIO was decorated with tLyp-1, which is tumor homing and penetrating peptide. Tumor-like structure dissociation in 3D tumor spheroids, enhanced cellular interaction, and increased cytotoxicity of the drugs in U87 cells were achieved by PEGNIO/D-C/tLyp-1. These data indicated that the formulation PEGNIO/D-C/tLyp-1 might be a promising and efficient strategy for drug delivery in anti-glioma therapy.

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4.2.7 References

1. X. Wei, X. Chen, M. Ying and W. Lu, *Acta Pharma. Sin. B*, 2014, **4**, 193-201.

2. B. Mujokoro, M. Adabi, E. Sadroddiny, M. Adabi and M. Khosravani, *Mat. Sci. Eng. C*, 2016, **69**, 1092-1102.

3. B. Al-Lazikani, U. Banerji and P. Workman, *Nat. biotechnol.*, 2012, **30**, 679-692.

4. Q. Hu, G. Gu, Z. Liu, M. Jiang, T. Kang, D. Miao, Y. Tu, Z. Pang, Q. Song and L. Yao, *Biomaterials*, 2013, **34**, 1135-1145.

5. D. Miao, M. Jiang, Z. Liu, G. Gu, Q. Hu, T. Kang, Q. Song, L. Yao, W. Li and X. Gao, *Mol. pharm.*, 2013, **11**, 90-101.

6. F. Dilnawaz and S. K. Sahoo, *Eur. J. Pharm. Biopharm.*, 2013, **85**, 452-462.

7. Y. Cui, Q. Xu, P. K.-H. Chow, D. Wang and C.-H. Wang, *Biomaterials*, 2013, **34**, 8511-8520.

8. Y. Xu, M. Shen, Y. Sun, P. Gao and Y. Duan, *J. nanosci. nanotechno.*, 2015, **15**, 9777-9787.

9. A. Sankhyan and P. Pawar, *J. Appl. Pharm. Sci.*, 2012, **02**, 20-32.

10. D. Pando, M. Matos, G. Gutiérrez and C. Pazos, *Colloid. Surface B.*, 2015, **128**, 398-404.

11. L. Tavano, R. Muzzalupo, N. Picci and B. de Cindio, *Colloid. Surface B.*, 2014, **114**, 144-149.

12. L. Tavano, L. Gentile, C. O. Rossi and R. Muzzalupo, *Colloid. Surface B.*, 2013, **110**, 281-288.

13. D. Ag Seleci, M. Seleci, J.-G. Walter, F. Stahl and T. Scheper, *J. Nanomater.*, 2016, **2016**.

14. L. Tavano, L. Mauro, G. D. Naimo, L. Bruno, N. Picci, S. Ando and R. Muzzalupo, *Langmuir*, 2016, **32**, 8926-8933.

15. P. Li and T. G. Rossman, *Toxicol. Sci.*, 2001, **64**, 90-99.

16. C. Nasarre, M. Roth, L. Jacob, L. Roth, E. Koncina, A. Thien, G. Labourdette, P. Poulet, P. Hubert and G. Cremel, *Oncogene*, 2010, **29**, 2381-2392.

17. Q. Hu, X. Gao, G. Gu, T. Kang, Y. Tu, Z. Liu, Q. Song, L. Yao, Z. Pang and X. Jiang, *Biomaterials*, 2013, **34**, 5640-5650.

18. E. Ruoslahti, *Adv. mater.*, 2012, **24**, 3747-3756.

19. L. Roth, L. Agemy, V. R. Kotamraju, G. Braun, T. Teesalu, K. N. Sugahara, J. Hamzah and E. Ruoslahti, *Oncogene*, 2012, **31**, 3754-3763.

20. B. Xu, Y. Ju, G. Song and Y. Cui, *J. nanopart. res.*, 2013, **15**, 2105

21. M. N. Azmin, A. T. Florence, R. M. Handjani□Vila, J. F. B. Stuart, G. Vanlerberghe and J. S. Whittaker, *J. Pharm. Pharmacol.*, 1985, **37**, 237-242.

22. D. A. Seleci, M. Seleci, A. Jochums, J.-G. Walter, F. Stahl and T. Scheper, *RSC Adv.*, 2016, **6**, 87910-87918.

23. L. Tavano, R. Muzzalupo, L. Mauro, M. Pellegrino, S. Ando and N. Picci, *Langmuir*, 2013, **29**, 12638-12646.

24. R. Nallamothu, G. C. Wood, C. B. Pattillo, R. C. Scott, M. F. Kiani, B. M. Moore and L. A. Thoma, *AAPS Pharm. Sci. Tech.*, 2006, **7**, E7-E16.

25. Q. Hu, X. Gao, T. Kang, X. Feng, D. Jiang, Y. Tu, Q. Song, L. Yao, X. Jiang and H. Chen, *Biomaterials*, 2013, **34**, 9496-9508.

26. M. Tan, J. Luo and Y. Tian, *RSC Adv.*, 2014, **4**, 61948-61959.

27. L. Tavano, R. Aiello, G. Ioele, N. Picci and R. Muzzalupo, *Colloid. Surface B.*, 2014, **118**, 7-13.

28. B. Xu, Y. Ju, Y. Cui, G. Song, Y. Iwase, A. Hosoi and Y. Morita, *Langmuir*, 2014, **30**, 7789-7797.

29. L. Sun, X. Deng, X. Yang, Z. Li, Z. Wang, L. Li, Q. Wu, F. Peng, L. Liu and C. Gong, *RSC Adv.*, 2014, **4**, 46737-46750.

30. X. Zhao, Q. Chen, Y. Li, H. Tang, W. Liu and X. Yang, *Eur. J. Pharm. Biopharm.*, 2015, **93**, 27-36.

31. M. Singh and N. Singh, *Mol. cell. biochem.*, 2011, **347**, 1-11.

32. N. Dhillon, B. B. Aggarwal, R. A. Newman, R. A. Wolff, A. B. Kunnumakkara, J. L. Abbruzzese, C. S. Ng, V. Badmaev and R. Kurzrock, *Clin. Cancer Res.*, 2008, **14**, 4491-4499.

33. Y. G. Lin, A. B. Kunnumakkara, A. Nair, W. M. Merritt, L. Y. Han, G. N. Armaiz-Pena, A. A. Kamat, W. A. Spannuth, D. M. Gershenson and S. K. Lutgendorf, *Clin. Cancer Res.*, 2007, **13**, 3423-3430.

34. J. Friedrich, C. Seidel, R. Ebner and L. A. Kunz-Schughart, *Nat. protoc.*, 2009, **4**, 309-324.

4.2.8 Supplementary information

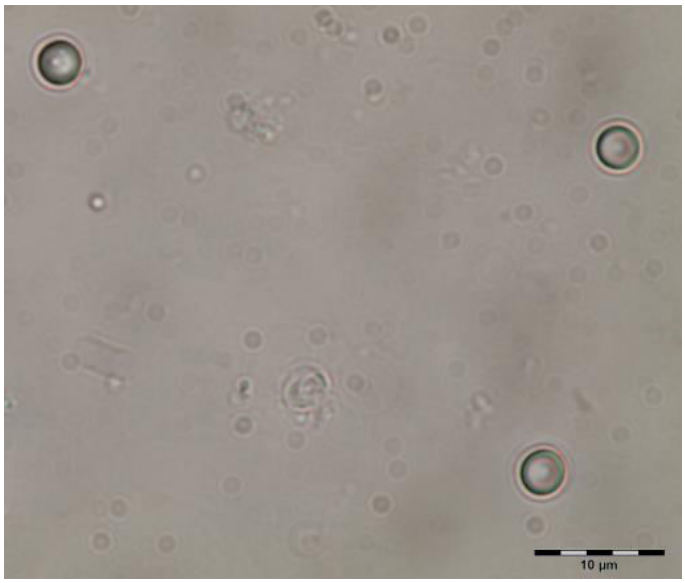

Figure S4.3 Optical micrographs of PEGNIO. Scale bar 10 µm.

5. Conclusions and Outlook

The development of new nanocarriers offers great hope to overcome the limitations in traditional cancer therapy. Non-ionic surfactant based vesicles, termed niosomes, are increasingly remarked nanostructures used in drug delivery studies. Their synthesizing process is considerably cheap and simple. Due to their high stability, controlled release of the loaded agent can be enabled. The main components of the niosomes, surfactants, and lipid molecules are biodegradable and non-immunogenic. The therapeutic efficacy of the therapeutic molecules can be improved by reducing clearance rate, protecting the encapsulated molecule against environmental conditions, and targeting to the specific side of the body. Besides, the surface of niosomes can be coated and functionalized by using the hydrophilic molecules such as polyethylene glycol (PEG). Thus, clearance rate may be reduced and targeting molecules can be conjugated to achieve targeted delivery of loaded agents to the specific side of the body. Aptamers and peptides are some of the most utilized targeting moieties. In the presented studies, these ligands were combined with PEGylated niosomes and their characterizations as well as *in vitro* studies were carried out in detail. To the best of our knowledge, this is the first time that aptamer and peptides were combined and used as a targeting ligand in niosomal drug delivery systems.

In the first study, the development of a niosomal drug delivery system with tumor targeting and penetrating features was aimed. Span60 and cholesterol were used to synthesize PEGylated niosomes via thin-film hydration method. The model drug doxorubicin (DOX) was encapsulated into the niosomes during hydration. On the other hand, a cell penetrating peptide, Cys-TAT and MUC1 aptamer, which can specifically bind to MUC1 glycoprotein expressed on tumor cell surface, were conjugated to each other by using BS3 homo-functional crosslinker. The conjugation was confirmed via gel electrophoresis and HPLC. Then, PEGylated niosome surface was modified with CysTAT–MUC1 targeting moiety through cysteine residue available in peptide sequence. The hydrodynamic size of the targeted drug loaded niosomes was determined around 165 nm with ~0.2 polydispersity index, which indicates homogeneity in the particle size. Drug release profiles were recorded at neutral and mild acidic conditions, 7.4 and 5.6 respectively. Faster release of DOX from niosomes was observed under acidic pH. HeLa (cervical cancer) and U87 (human glioblastoma) cells were selected as the cell lines according to MUC1 protein expression levels by polymerase chain reaction (PCR) and flow cytometry. DOX uptake by HeLa and U87 cells were investigated for

different DOX formulations through flow cytometry. Targeted niosomes showed higher DOX level than nontargeted niosomes and free DOX in MUC1 positive HeLa cells. In contrary, the niosomal formulations, as well as free DOX showed almost the same cellular uptake on MUC1 negative U87 cell line. Fluorescence microscopy images correlate with the flow cytometric results. The cytotoxicity of the niosomal formulations and free DOX were examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) assay on both cell lines for both 24 and 48 h. Plain niosomes indicated almost no toxic effect on both cell lines for all time points. PEGNIO/DOX/CysTAT–MUC1 revealed higher cytotoxicity on HeLa than U87 cells after 24 and 48 h because of the existence of the targeting ligand.

In the second study, the design of targeted niosomal co-drug delivery system for the glioblastoma treatment was aimed. In recent years, the co-delivery of therapeutic agents has attracted great attention for glioblastoma chemotherapy. Especially the combination of curcumin and chemotherapeutics is a promising approach. Here curcumin and DOX were encapsulated in PEGNIO during its synthesis via thin film hydration method. Free drugs were removed by using a Sephadex G-25 gel column. Subsequently, the tumor-homing peptide tLyp-1, which binds to U87 cells specifically, was conjugated to curcumin and DOX encapsulated PEGNIO (PEGNIO/D–C) via the formation of a thioether linkage. All niosomal formulations were characterized in detail. The hydrodynamic size, shape, entrapment efficiencies, and *in vitro* drug release profiles were evaluated. PEGNIO is round in shape and around 150 nm in size. Both drugs were released from niosomes faster under acidic pH. Moreover, to test the specificity of targeted niosomes, rhodamine 6g (Rh6g) was entrapped into the niosomes and t-Lyp-1 was conjugated to PEG chains on niosomes. Flow cytometry was used to investigate the Rh6g uptake by hMSC (human mesenchymal stem cells) and U87 cells to evaluate receptor-mediated cell targeting. The specific uptake of peptide targeted niosomes by U87 cells was obtained. The cytotoxicity of the niosomal formulations and mixture of free drugs were examined via MTT assay on both cell lines for 24 h. tLyp-1 targeted co-drug loaded niosomes showed higher cytotoxicity on U87 than on hMSC cells after 24 h. Furthermore, tumor spheroids formed by U87 cells were treated with niosomal formulations. Spheroids exhibited the structure that there is no more tightly organized. Especially, PEGNIO/D–C/tLyp-1 treated spheroids became distorted, with many disassociated cells.

In conclusion, the fundamental information about niosomes, detailed information about their applications in drug delivery were provided in the theoretical part of this thesis. In the experimental part, two novel different targeted niosomal drug delivery systems for cancer therapy were designed, synthesized, characterized, and applied *in vitro*. Obtained results indicated that PEGNIO is a promising drug carrier for drug delivery studies, which enables the entrapment of different drugs and the conjugation of targeting ligands on its surface. However, *in vivo* applications have to be performed to further evaluate its potential as a commercial product. Currently, there is no commercial niosomal drug available. Therefore, further research studies need to be carried out in this field. The outcomes of this thesis may provide new insights and contribute the development of novel drug delivery devices for cancer therapy.

List of Publications

1. **D. Ag Seleci** (90%), M. Seleci (5%), R. Jonzcyk (5%), F. Stahl, and T. Scheper, Niosomes for Brain Targeting, *Carriers for Brain Targeting*, Apple Academic Press, submitted 30 June 2017

2. **D. Ag Seleci** (95%), M. Seleci (5%), F. Stahl, and T. Scheper, Tumor homing and penetrating peptide-conjugated niosomes as multidrug carriers for tumor-targeted drug delivery*, RSC Advances,* 2017,**7**, 33378-33384.

3. **D. Ag Seleci** (85 %), M. Seleci (10%), A. Jochums (5%), J.G. Walter, F. Stahl, and T. Scheper Aptamer mediated niosomal drug delivery, *RCS Advances*, 2016, 6, 87910–87918.

4. **D. Ag Seleci** (95%), M. Seleci (5%), J.G. Walter, F. Stahl, and T. Scheper Niosomes as Nanoparticular Drug Carriers: Fundamentals and Recent Applications. *Journal of Nanomaterials*, 2016. doi:10.1155/2016/7372306.

5. M. Seleci, **D. Ag Seleci** (10%), F. Stahl, and T. Scheper, Theranostic liposome-nanoparticle hybrids for drug delivery and bioimaging, *International Journal of Molecular Sciences,* 2017, 18, 1415.

6. M. Seleci, **D. Ag Seleci** (5%), R. Joncyzk, F. Stahl, C. Blume, and T. Scheper. Smart multifunctional nanoparticles in nanomedicine, *BioNanoMaterials,* 2016,17, 33–41.

7. M. Seleci, **D. Ag Seleci** (10%), M. Ciftci, D. Odaci Demirkol, F. Stahl, S. Timur, T. Scheper, and Y. Yagci, Nano-structured Amphiphilic Star-Hyperbranched Blockcopolymers for Drug Delivery*, Langmuir*, 2015, 31, 4542–4551.

Poster Presentations

1. **D. Ag Seleci**, M. Seleci, Frank Stahl, and Thomas Scheper, Design of Aptamer Based Drug Delivery, November 9-11th 2015, Crossing Biological Barriers - Advances in Nanocarrier Design for Targeted Drug Delivery, Dresden/Germany

2. M .Seleci, **D. Ag Seleci**, F. Stahl, and T. Scheper, Multifunctional theranostic quantum dot-liposome hybrids for drug delivery and imaging, November 9-11th 2015, Crossing Bioligical Barriers - Advances in Nanocarrier Design for Targeted Drug Delivery, Dresden/Germany

3. M. Seleci, **D. Ag Seleci**, M. Ciftci, D. Odaci Demirkol, F. Stahl, S. Timur, T. Scheper, and Y. Yagci, Nano-structured Amphiphilic Star-Hyperbranched Blockcopolymers for Drug Delivery, March 10-11th 2015, 2nd Conference of Scientific Cooperation between Lower Saxony and Israel, Hanover/Germany

Curriculum Vitae

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