CELL PENETRATING PEPTIDE AMPHIPHILE INTEGRATED LIPOSOMAL SYSTEMS FOR ENHANCED DELIVERY OF CARGO TO TUMOR CELLS

A THESIS

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MASTER OF SCIENCE

By

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August, 2013

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

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ABSTRACT

CELL PENETRATING PEPTIDE AMPHIPHILE INTEGRATED LIPOSOMAL SYSTEMS FOR ENHANCED DELIVERY OF CARGO TO TUMOR CELLS

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M.S. in Materials Science and Nanotechnology

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Liposomes have been extensively utilized as effective nanocarriers due to their enhanced solubility, higher stability and greater ability to facilitate the slow release of encapsulated drugs compared to free drug administrations. Liposomes are also preferred as drug vectors due to their non-toxic nature, biodegradability and structural resemblance to the cell membrane. However, their low internalization efficiencies pose an important challenge for their use in drug delivery applications. Internalization issues inherent in many liposomal systems can be circumvented by the use of cell penetrating peptides, which non-covalently attach on the liposome surface and greatly enhance liposomal uptake in a receptor- and charge-dependent manner.

In this study, we examined the liposomal dynamics effected through the integration of an amphiphilic cell penetrating peptide into a simple liposome system. Peptide amphiphiles with a cell penetrating arginine-rich domain were incorporated into liposomal membranes formed by negatively charged dioleoylphosphoglycerol (DOPG) phospholipids in the presence of cholesterol. Throughout the present study, we sought to analyze the effect of peptide incorporation on (a) the physical characteristics, such as size, surface potential

and membrane polarity, of the liposomal membrane, (b) the alterations in the encapsulation and delivery mechanisms of hydrophilic (Rhodamine B) and hydrophobic (Nile Red) drug models and (c) the enhancement of therapeutic capability in liposomes loaded with the drugs Doxorubicin-HCl and Paclitaxel. Our results revealed that the modification of liposomes by cell penetrating peptide amphiphiles results in the improvement of cargo delivery and the enhancement of the therapeutic effects of the anti-cancer drugs Doxurubicin and Paclitaxel.

Keywords: Cell Penetrating Peptide, Liposome, Cancer, Drug Delivery

ÖZET

LİPOZOMLARIN HÜCRE DELİCİ PEPTİTLERLE MODİFİYE EDİLİLEREK ANTİKANSER İLAÇ GÖNDERİMİNİN GELİŞTİRİLMESİ

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İlaçlara kattığı yüksek çözünürlük, stabilite ve zaman salınımı gibi özellikleri olması itibariyle liposozmlar etkili nano taşıyıcılar olarak yaygınca kullanılmaktadır. Toksik etki göstermemeleri, biyobozunurluğu ve hücre zarına benzer yapıda olmaları da lipozomların tercih edilmesindeki diğer sebeplerdir. Buna rağmen lipozomların hücre içine alınımının kısıtlı olması, lipozomların ilaç taşıyıcısı olarak kullanımında önemli sorunlar teşkil etmektedir. Bu durumun üstesinden gelme amacıyla lipozomları reseptör ve yük bağıntılı işlev gören hücre delici peptitlerle modifiye edip lipozomların hücre içine girişini desteklemek kayda değer bir stratejidir.

Bu çalışmada, liposomları tek adımda modifiye eden bir sistem geliştirilmiş olup, entegre olan amfifilik hücre delici peptitlerin lipozomların etkinliği üzerindeki rolü test edilmiştir. Arjinin miktarı itibariyle zengin hücre delici bölümü ve hidrokarbon zincirine sahip hücre delici peptitler DOPG ve kolesterolden oluşan eksi yüklü lipozomlara entegre edilmiştir. Çalışmada modifiye edilmiş lipozomların (a) boyut, yüzey potansiyeli ve zar polaritesi gibi fiziksel özellikleri, (b) hidrofilik Rhodamin B ve hidrofobik Nile Red gibi ilaç modellerinin enkapsülasyon ve iletim etkinliği, (c) Paclitaxel ve Doxurobicin-HCl gibi

anti kanser ilaçlarla yüklenmesi sonrası terapütik etkinliği araştırılmıştır. Sonuçlar ışığında amfifilik hücre delici peptitlerle kovalent bağ olmaksızın modifiye edilmiş lipozomların ilaç iletimi yönünden daha iyi olup, anti kanser ilaçlarla yüklendiğinde terapütik etkinliğini arttığını göstermiştir.

Anahtar Kelimeler: Hücre delici peptitler, Lipozomlar, Kanser, İlaç İletimi

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CHAPTER 1

INTRODUCTION

1.1 CANCER

The World Health Organization (WHO) lists cancer as the second most prevalent cause of death in developed countries, after cardiovascular diseases. In the developing world, cancer is ranked as the third most common lethal medical condition. In addition to being a terminal illness, cancer poses an immense economic and psychological burden on the affected and their families. Factors like aging, unhealthy lifestyles (malnutrition, smoking etc.) and pollution increase the incidence and mortality rates of cancer. By 2020, 16 million new cases are estimated to emerge around the world. Due to the frequency of incidence, high mortality rates and difficulty of treatment associated with many cancers, efficient treatment strategies against this disease are urgently necessary.

1.2 STRATEGIES FOR CANCER TREATMENT

Cancer can be managed with variety of options, such as cytoreductive surgery, radiotherapy, chemotherapy and immunotherapy. The selection among these therapies depends on parameters such as the type (e.g. whether the tumor in question is metastatic), location, size and structure of the malignant tumor. In all treatments, the primary goal is the removal of all cancerous cells with minimum collateral damage to the native, healthy body tissue. However, current treatment technologies are rarely capable of eliminating all malignant cells while keeping normal cells perfectly healthy. When tumors are structurally distinct and less invasive, or located in non vital organs, it may be possible to

remove all cancer tissue from body by surgery. But this is not a common case, since many types of malignancies either occur in vital organs or have a natural tendency to invade vital tissues and form malignant structures that cannot be separated from native tissue by mechanical means. In addition, cancer cells can spread to very distant tissues via metastasis, or may recur in the same location and necessitate the resumption of treatment. Chemotherapeutic agents and radiotherapeutic applications are often capable of eliminating cancer cells to a greater extent than invasive surgery, but also cause systemic side effects and result in the death of healthy cells. Immunotherapy is not associated with particularly severe side effects, yet it is also slow to act and provides enough time for tumor cells to evolve defensive mechanisms.

A variety of cancer treatment options, each with their distinct sets of advantages and disadvantages, are available for specific types of cancers in specific degrees of progress. We will discuss these treatment options briefly to develop a broad understanding of cancer treatment before detailing advanced anticancer drug delivery strategies.

1.2.1 Cytoreductive Surgery

Local tumors with non-invasive characteristics can be ectomized by basic surgery. This procedure involves the removal of either the tumor tissue or the entire organ in which the tumor is nested. Removal of excessive prostate tissue in prostate cancer and mastectomy in breast cancer are examples of such surgical procedures. The main concern in anti-cancer surgeries is the elimination of leftover cancer cells on the tumor site. Since cancer cells reproduce at rates much greater than their healthy equivalents, even a single remaining malignant cell may well end up forming a new tumor.

Against metastatic cancers, surgery can only provide short term relief and cannot serve as a conclusive treatment. Even if the majority of the tumor mass is removed from its initial location, surviving cancer cells frequently establish metastatic tumors throughout the body by traveling through blood and lymph streams. The development of these new tumors almost inevitably necessitates further treatment.

1.2.2 Radiotherapy

Cancer cell irradiation is an effective way to kill target cells. In this procedure, radio waves are focused on target tissues and cancer cells are exposed to ionizing radiation. The main rationale of this treatment is to disrupt cell metabolism via radiation-induced DNA damage. When the cell physiology is disrupted, survival and division rates decrease and the target tissue shrinks in mass. The main problem associated with radiotherapy is the exposure of healthy cells to ionizing radiation. Compared to tumor cells, healthy cells have more efficient DNA repair mechanisms, and are much more resistant to radiation. However, the failure to repair a single incident of oncogenic mutation may result in the occurrence of cancer in any tissue exposed to radiation therapy. Furthermore, as with surgery, radiotherapy alone is not a sufficient treatment method against metastatic tumors. Local tumors can be destroyed efficiently, yet new treatments will be necessary when metastasized cancer cells form new tumor masses throughout the body.

1.2.3 Immunotherapy

Cancer cells can be eliminated by manipulating the immune system to fight against tumor cells. For this purpose, cytokines and interferons are frequently utilized to enhance the immune alertness of body. Since cancer cells divide often and have weak DNA repair mechanisms, they are likely to produce mutated antigens that can be recognized when

immune alertness is high. When these tumor antigens are recognized by T cells and natural killer cells, the tumor tissue is rapidly invaded by the immune system and apoptotic signals are produced to initiate cell death in tumor cells.

Cell based therapies, which involve the recruitment or priming of immune cells against malignant tumors, are also among available methods. Perhaps the most striking example of such a treatment approach is dendritic cell therapy, which utilizes the priming of these cells against tumor antigens.[1-2] In this procedure, isolated dendritic cells are co-cultured with tumor cells and primed against tumor antigens. When dendritic cells are transplanted to the patient, they activate the immune system against cancer cells, which eventually leads tumor shrinkage. This procedure is capable of increasing life expectancies by up to several years, even with conditions normally associated with very poor prognoses, such as pancreas cancer.

Overall, immunotherapy is an efficient way to manage cancer, yet there are several weaknesses associated with it. First, a boost to the immune system may trigger the development of autoimmune disorders, which will harm healthy tissue along with cancer cells. Further, cancer cells are capable of rapidly adapting to hostile conditions and surviving cells may be able to escape from enhanced immune surveillance by producing new antigens. These survivors can then continue to divide and restore the tumor to its original size.

1.2.4 Chemotherapy

Chemotherapy is use of cytotoxic chemical substances against cancer cells, and primarily relies on drugs that display more pronounced effects against frequently dividing cells. By

interfering with specific cellular processes (e.g. DNA replication or microtubule dynamics), these drugs are able to cause cytotoxic damage or trigger growth inhibition. For example, drugs like Doxorubicin, Cisplatin and Daunomycin intercalate with DNA to impair transcription and replication.[3-4] Other drugs, like Paclitaxel, Taxanes and Epothilones, inhibit the movement of microtubules, which is essential for cell division.[5-6] These drugs share the common ability to block the cell cycle at a certain point, triggering a cell cycle arrest that will eventually result in cell death. Since such drugs are administered directly into the bloodstream, they can reach every part of body, which renders them highly effective against metastatic tumors.

While chemotherapeutics are effective against fast-dividing cancer cells, they are also associated with severely detrimental side effects due to the considerable cytotoxicity they exhibit against healthy cells. Since chemotherapeutics are widely distributed throughout the body, they are capable of significantly altering the physiology of systems with high cell turnovers, usually for the worse. Disruption of hematopoietic stem cell division and adult neurogenesis are examples of side effects caused by these treatments, which are much more severe than the hair and nail losses associated with other treatments. In order to reduce side effects and increase treatment efficiency, sophisticated drug administration methods need to be developed. These strategies must focus on delivery vehicles with accurate tumor targeting and effective internalization capacities. As the focus of the present work is the delivery of chemotherapeutic agents using advanced nanocarrier systems, the next section will be devoted to an in-depth discussion of nanocarrier-based drug delivery systems to further detail the opportunities and restrictions this novel nanomedical approach presents.

1.3 NANOSCALE DELIVERY OF ANTICANCER AGENTS

The uptake inefficiency, misdistribution and metabolic interference of therapeutic agents such as pharmaceuticals, peptides or nucleic acids, continue to represent major challenges in their medical applications. The cell membrane acts as an impermeable barrier to a wide range of these therapeutic substances and, by blocking their entry into cells, prevents them from manifesting their physiological impact. For many years it has been a great challenge to increase the transportation rate of therapeutics through the cell membrane. To this end, the discovery of liposomes around half a century ago served as an attractive opportunity for pharmaceutical applications. Later still, natural and synthetic cell penetrating peptides, which enhance the translocation of materials across the cell membrane, were discovered, and this finding opened new possibilities in biomedical research.

Integration of nanotechnological applications into medical research revolutionized the area of cancer therapeutics. A variety of nanocarrier systems can be designed to accurately deliver drugs into target cells with little to no loss of efficiency. The ideal nanoscale delivery agent ensures that the anticancer drug is accurately localized in the tissue(s) of interest without loss of function or efficiency.[7] In order to achieve the greatest therapeutic efficiency, nanocarriers should be able to a) improve the half-life of the drug cargo by protecting it from degradation b) prevent non-specific localization and action of drugs c) prevent the premature escape of the drug from circulation d) improve the absorption of the drug by the target tissue and/or e) improve the cellular uptake of the drug.[8] An ideal nanocarrier should be capable of fulfilling all of these criteria.

Many parameters may alter the effectiveness of nanocarrier dynamics. The size of nanoparticles may determine their localization patterns in the body: Small particles, for

example are capable of crossing biological barriers easily and can be excreted quickly by kidneys.[9] Surface charge is another important criterion, and positively charged nanoparticles in particular may have a higher capacity for cellular uptake due to their affinity for the negatively charged cell membrane.[10] On the other hand, using nanoparticles with negative surface charges can prevent non-specific internalization events and improve selectivity when utilized alongside targeting residues. Another important feature of nanocarriers is surface hydrodynamics. Nanocarriers with hydrophobic surfaces tend to aggregate and are eventually captured by immune cells. Hydrophilic polymer coats therefore improve solubility and circulation times.[11]

In addition to operational requirements, biocompatibility and biodegradability are key properties of any nanoscale agent in internal medicine.[7] The carrier has to be metabolized into smaller nontoxic components that can be cleared from circulation by means of renal excretion or further metabolization. Therefore, both composition and size of nanocarriers are important parameters. For instance, liposomes are perfectly biocompatible since they consist of natural or synthetic lipids that can easily be metabolized. Metal-based carrier systems, such as gold and iron oxide nanoparticles, cannot be metabolized by cells and have a size-dependent tendency to accumulate in various body parts.[12] Thus, in the case of inorganic nanoparticles, it is necessary to adjust particle sizes such that renal excretion is feasible. As stated earlier, the size of the nanocarrier is important in crossing biological barriers, such as the blood brain barrier or the nephrons of the kidney.[9]

Despite displaying significant advantages over free drugs, current nanocarrier systems are far from having ideal features all across the board. There are various types of nanocarrier

systems with individual strengths and weaknesses. In this section, we will review these nanocarrier systems to develop a broader understanding of nanomaterial applications on cancer therapeutics.

1.3.1 Polymer Based Nanocarriers

Polymeric nanoparticles are sub-micron level carriers that can be synthesized from natural or synthetic polymers. Depending on the polymer type and synthesis procedure, particles with different sizes, shapes and encapsulation capacities can be obtained. The critical feature of polymer based nanoparticles is that they are suitable for surface modifications to facilitate tumor targeting or controlled release.[13] An abundance of end groups on the nanocarrier surface allows the efficient chemical modification of these nanoparticles with various targeting or penetration-enabling residues. In order to control the release dynamics, drugs can be absorbed or encapsulated within the polymer based structure. Structural changes within the drug-carrier complex may also alter treatment efficiency.[14]

Polymers like PLGA, PLA, chitosan, guar gum and gelatin are commonly used for nanocarrier fabrication. Various methods of synthesis and modification can be utilized to improve drug carriage and facilitate targeted therapy. For instance, a study by Sharma et al., reports guar gum nanoparticles functionalized by folate and methotrexate to specifically target colon cancer tissue.[15] Like most polymer based nanocores, guar gum particles have an abundance of functional groups amenable for modification, which were utilized for the attachment of a great number of targeting residues. *In vivo* studies done by this group confirmed the preferential uptake of nanoparticles to the large intestine. In another study, paclitaxel loaded gelatin nanoparticles were used for the intravascular

treatment of bladder cancer.[16] It is notable that gelatin based nanoparticles displayed targeting abilities without any modification. Since tumors produce high amount of unstructured collagen, gelatin based materials may preferentially attach to cancerous tissue via strand invasion. In this particular study, nanoparticles were administered to the bladder itself and the nanocarriers were thus protected from proteolytic destruction, which would have happened if they were given directly into the bloodstream.

1.3.2 Inorganic Nanospheres

A large range of inorganic nanocarrier systems can be used for the delivery of therapeutics such as DNA, proteins and chemotherapeutic agents. However, inorganic nanoparticles have to be modified with biological (or at least biofunctional) materials to operate safely and efficiently. Additionally, these modifications have to improve the interaction between the nanocarrier and the therapeutic agent in order to increase drug stability and prevent premature leakage.[17] Many inorganic nanocarrier materials are inert, display low solubilities and cannot be efficiently metabolized by body. Surface modification of these particles must be adjusted depending on the material type, therapeutic agent and target tissue. For instance, gold nanoparticles are frequently modified with thiol groups, while carbon based nanostructures are commonly functionalized with carboxyl groups.[17]

In order to produce efficient and clinical grade inorganic nanocarriers, two main problems must be solved. First, a suitable chemical interaction must occur between the nanocarrier and the therapeutic agent. Drug-carrier interactions have to be in optimal levels to provide controlled release. Interactions between nanocarriers and drugs are mainly mediated by hydrophobicity or electrostatic forces. Since inorganic nanocarriers generally have dense cores, they cannot be used for the encapsulation of therapeutics. As such, surface

modifications must be performed to facilitate the adsorption of therapeutic agents onto the inorganic core surface. For instance, in a study done by Kneuer et al., silica based nanoparticles were modified with N-(2-aminoethyl)-3-aminopropyltrimethoxysilane or N-(6-aminohexyl)-3-aminopropyltrimethoxysilane to create a positive surface charge on the silica surface.[18] This surface, due to its high positive charge, was able to adsorb DNA molecules and form a DNA carrier system.

The second challenge associated with inorganic nanospheres is to attach a variety of functional groups to improve therapeutic dynamics of the drug-carrier complex without compromising the core-drug interaction. Improvements on cellular penetration and targeting capabilities are common objectives for the secondary modification of nanocarriers. For example, it has been shown that magnetic nanoparticles modified with polyethylene glycol and folic acid display enhanced internalization by breast cancer cells compared to nanoparticles modified with only polyethylene glycol.[19] In another study, iron oxide nanoparticles were modified with arginine-glycine-aspartate (RGD) peptide and chlorotoxin, which target several types of integrins and matrix metalloproteins that are overexpressed in variety of cancer tissues.[20] By doing so, they were able to achieve cancer-specific targeting capacity and tumor shrinkage.

1.3.3 Liposomes

Liposomes are biomimetic delivery systems that have been used to administer various therapeutic and imaging agents. Compared to free drugs, they are significantly advantageous in terms of pharmacokinetics, efficiency and management of side effects. In addition to their innately superior physicochemical properties, they can be modified for specific purposes such as tissue targeting or improved cell penetration. For these reasons,

liposomal chemotherapeutics have been used as advanced anti cancer agents for several decades. There are many forms of liposomal chemotherapeutics that are already being used by clinics, such as Doxil and Marqibo.[21-22] Many more such drugs, like MBP-426 and ThermoDox, are in development and will soon be ready for clinical trials.[23-24] Since liposomes are the main focus in this thesis, the following sections will encompass an extensive review of liposomal drug delivery. We will first detail the physicochemical properties of liposomes, and then continue with their surface modification potential and anti cancer applications.

1.3.3.1 Physical Properties of Liposomes

Liposomes are nanosized vesicular structures artificially produced from natural or synthetic lipids and cholesterol. When combined under suitable conditions, lipids align to form bilayered spherical structures in water, which eventually stabilize as liposomal vesicles. Since lipids have both hydrophobic and hydrophilic sites, they are highly disposed towards a configuration where their hydrophilic regions face outwards and their hydrophobic sections collapse together.

Liposomes can be produced in various forms. Parameters like size, lamellarity and composition significantly influence various features, such as stability, drug containment capacity, and solubility. If need be, synthesis conditions can be altered to adjust these properties. Liposomes can be multilamellar or unilamellar. Briefly, multilamellar liposomes have multiple concentric bilayers within each other while unilamellar liposomes consist of a single lipid bilayer. Unilamellar liposomes are also classified further with respect to their size. Liposomes ranging from 20-40 nm in diameter are classified as small unilamellar vesicles, while these that with 40-80 nm diameter sizes are

called medium unilamellar vesicles.[25] Any liposomal vesicle larger than 100 nm is considered a large unilamellar vesicle. Multilamellar liposomes tend to be larger, since larger volumes are necessary to build up their concentric series of bilayers. Figure 1 demonstrates the basic structural differences between unilamellar and multilamellar liposomes.

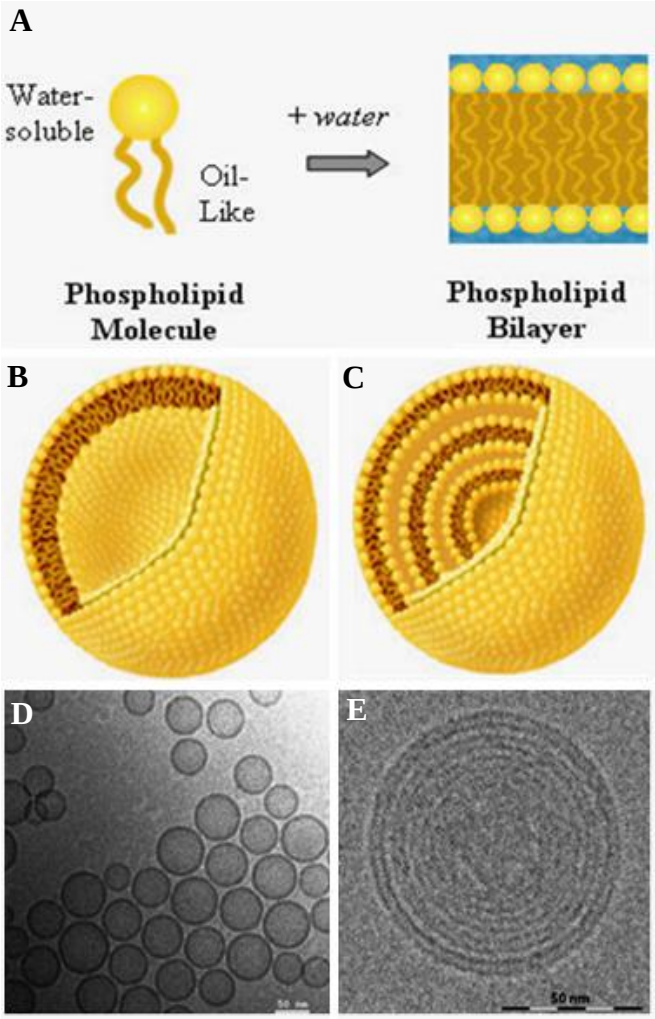

Figure 1 Physical properties of unilamellar and multilamellar liposomes. A. Schematics of basic lipids and a lipid bilayer. B. Schematic of unilamellar liposomes. C. Schematic of multilamellar liposomes. D. Cryo-TEM image of unilamellar liposomes. Adopted from Battersby et al.[26] E. Cryo-TEM image of a multilamellar liposome. Image acquired from vironova.com. Scale bars are 50 nm. All schematics adopted were from encapsula.com.

Phosphatidyl Glycerol (PG), Phosphatidyl Serine (PS) and Phosphatidyl Choline (PC) are natural lipids found in such products as animal fat and eggs, while lipids like Distareol Phosphatidyl Choline (DSPC), Dipalmitoyl Phosphatidyl Choline (DPPC), Dioleoyl Phosphatidyl Glycerol (DOPG) and Dipalmitoyl Phosphatidyl Serine (DPPS) are synthesized artificially.

These lipids differ in size, shape and/or charge, and provide different physicochemical characteristics to the liposomes they form. These characteristics are significant enough to alter the native behavior of these liposomes throughout the body. A study done by Gabizon et al. clearly demonstrates this effect.[27] In this study, researchers prepared a variety of liposomes with similar physical properties yet different lipid compositions. PG-PC, PG-DSPC, DPPG-DSPC, HPI-DSPC and GM-DSPC liposomes were prepared in the presence of cholesterol, and the biodistribution of liposomes were analyzed following intravenous administration. After 24 h of exposure, high accumulations of PG-PC-Chol, GM-DSPC-Chol and DPPG-DSPC-Chol liposomes were observed in liver and spleen. In terms of passive tumor targeting, HPI-DSPC-Chol and GM-DSPC-Chol liposomes were superior compared to others.

In addition to phospholipids, cholesterol is a staple as a liposome constituent. Cholesterol has no capacity to form a bilayer or vesicular structure by itself, and yet has drastic effects on liposome properties when used in conjunction with phospholipids. The addition of cholesterol improves the rigidity and stability of the vesicle membrane and reduces the permeability of water-soluble molecules.[25] The use of cholesterol is therefore clearly advantageous, especially when the drug load is hydrophilic and leaky.

Effects of cholesterol content to liposome structure were extensively studied by Kirby and colleagues.[28] In this study, the stability of negatively or positively charged small unilamellar liposomes with varying cholesterol contents was tested both *in vivo* and *in vitro*. Cholesterol rich liposomes were found to be capable of remaining stable in both circulation and *in vitro* environment regardless of their surface charge. In contrast, cholesterol-poor and cholesterol-free liposomes degraded very quickly when exposed to whole blood, plasma or serum. When injected intraperitoneally, high cholesterol, neutral charged liposomes demonstrated the greatest stability.

Beside its ability to provide stability to the supramolecular structure of liposomes, cholesterol is also capable of improving the stability of the cargo. Research by Lee et al. demonstrated that liposomes containing high amounts of cholesterol are more efficient in terms of stabilizing retinol, a lipophilic compound.[29] Two properties of cholesterol allow this molecule to increase drug stability. First, by bridging strong bonds between lipids, cholesterol prevents the leakage and eventual loss of cargo. In addition, by forming strong bonds with hydrophobic molecules, cholesterol further blocks the dissipation of material and acts as a stabilizing barrier against diffusible reactants from outside.

1.3.3.3 Characterization of Liposomes

The development of accurate quality assessment criteria for liposomes is crucial to enable the objective comparison between different liposome formulations. Since many physicochemical properties have vital roles in liposome dynamics, many properties have to be measured accurately to gain a thorough understanding of the liposome's ability to deliver a given drug. The most important aspects of liposomes are their lamellarity, size, surface charge, encapsulation efficiency and release profile. In this section, we will review

why these characteristics are important, and how they can be quantified.

Determination of Lamellarity:

Lamellarity is an important factor on liposomal dynamics due to its influence on the encapsulation and release of drugs.[30] Additionally, after endocytosis, it is likely that the fate of the liposome and its contents is significantly affected by the number of lamellae. As such, an accurate analysis of lamellarity is crucial.

Lamellarity can be measured with a variety of complex methods, such as NMR (by estimating intravesicular lipid content) and small angle x-ray scattering (by Fourier Transformation of scattered light, which will give the a probability distribution for the distances between electrons).[30] More direct and simple methods, such as freeze fractured TEM, can also be used for lamellarity analysis. In this technique, small amounts of sample are loaded into the imaging chamber, and freeze-shock is applied. Liposomes keep their morphology due to the high speed cooling, and can be imaged via electron microscopy. Acquired images can be analyzed and statistically consistent lamellarity rates can be obtained using this technique.

Size Measurement:

Size is an essential aspect of liposomes in clinical use. As mentioned previously, localization, longevity and fate of liposomes are heavily affected by size. There are several techniques available to calculate the exact size of, or make comparative size analyses between, liposomes. These include microscopy techniques and chromatographic methods.

Any microscopy technique that allows liposomes to maintain their size and shape can be used for size measurement. The most important concern in image-based size assessment methods is to keep liposomes from size and shape changes, which is usually accomplished by structurally fixing them prior to imaging. When this criterion is fulfilled, any method with proper resolution and magnification power can be used. As with lamellarity analysis, freeze fractured TEM can be used for the size assessment of liposomes. Other variations of electron microscopy systems, such as cryo-EM or EM with negative staining also provide the required information. Other than electron beam dependent systems, atomic force microscopy (AFM) is also used in liposome size assessment.[31-32]

In addition to microscopy based methods, chromatographic techniques also can be applied in order to compare and quantify liposomal size. Size exclusion chromatography is an ideal method for the separation of materials with distinct sizes, including liposomes. HPLC with size exclusion chromatography is commonly utilized in order to separate liposomes and non encapsulated materials.[30] However, it must be kept in mind that liposomes may get stuck in column during the progress of this technique, which can render it difficult to retrieve them. Depending on the type of lipid and surface modification, the interaction between the separation column and liposomes may be strong enough to interfere with both size measurements and liposome retrieval.

Determination of Surface Charge:

Surface charge is one of the most important aspects of liposomes, and plays a crucial role in determining the solubility and cell-liposome interactions of any given drug delivery vesicle. It may be affected from a variety of parameters, such as the type(s) of lipid used, surface modifications and cargo. Particles with low surface charges tend to pull each other

and aggregate. When we consider the relatively large initial size of liposomes, *in vivo* aggregations may result in tremendous damage throughout the body. Particles with high surface charges can repel each other through electromagnetic interactions, and are therefore more likely to dissolve and diffuse than to aggregate.

In order to quantify surface charge, zeta potential measurements can be performed. This approach relies on the detection of minute changes in light scattering patterns following the application of electromagnetic forces on the sample. The scattering pattern fluctuates much more with highly charged materials compared to neutral and low charged materials.[30]

Encapsulation Efficiency:

Liposomes cannot encapsulate all the cargo dissolved in preparation media. A fraction will remain in media, while rest is successfully loaded. The ratio between loaded and unloaded cargo is an important parameter that enables us to estimate the amount of cargo per carrier molecule. By doing so, it is possible to adjust treatment doses to utilize optimal concentrations of drug and carrier. The main principle underlying the calculation of encapsulation efficiency is to separate unloaded material from liposomes and quantifying fractions. This can be achieved through various methods. Liposomes can be filtered through membranes with small pores to obtain a solution that contains only the unloaded material. Liposome solutions can be dialyzed with a membrane of appropriate size. Liposome pellets can be destructed with detergents and the cargo encapsulated by liposomes can be exposed for subsequent measurement. Finally, size exclusion chromatography can be applied in order to separate the unloaded cargo from the liposomes.[33] After separation, the amount of loaded and unloaded material can be

measured via absorbance or fluorescence, depending on the nature of the subject material.

Profiling Liposomal Release:

Profiling release dynamics is a crucial effort to estimate the time dependent efficiency of the drug-carrier complex. Vesicles with high release rates are predisposed towards aggressive behavior in shorter periods of time. Liposomes with low release rates tend to provide a sustained long term effect. Release rates, and therefore therapeutic dynamics, are affected by various conditions such as pH and temperature. Considering these parameter in release profiling experiments allows researchers to further understand and predict the behavior of liposomes in the body. Release dynamics of liposomes can be determined using an appropriate dialysis method.[34] Briefly, after separating liposomes from unloaded cargo materials, liposomes are placed in a dialysis bag. As liposomes release their contents, the concentration of the subject material will be increased in the solution outside the membrane. By acquiring small aliquots from this solution, time-based changes in the concentration of the released material can be observed. As stated earlier, the precise procedure for concentration measurements depends on the nature of the subject material. This method can also be applied at different pH or temperature values to understand release dynamics in various environmental conditions.

1.3.3.4 Functionalization of Liposomes

Surface modification is an essential tool for the functionalization of liposomes for specific purposes. Site specific drug delivery is possible by using different combinations of surface modifications. A variety of materials, such as carbohydrates, inorganic polymers, proteins, peptides and metals, can be attached to liposome surfaces to improve liposomal efficiency with respect to a specific purpose. For instance, several polymers (such as PEG) can be

used to improve the stability of liposomes and extend their circulation times. Antibodies can be conjugated to facilitate target-specific attachment. Used alongside a series of magnetic tools, some metals (such as iron oxide) can be used for active targeting to a subject tissue. The use of peptides with specific affinities (such as cell penetrating properties) is another method to improve liposomal dynamics by enhancing cellular uptake. Figure 2 demonstrates schematics of various liposomal surface modifications and functions. In the next section, we will discuss several types of liposome modifications and the therapeutic benefits associated with each modification type.

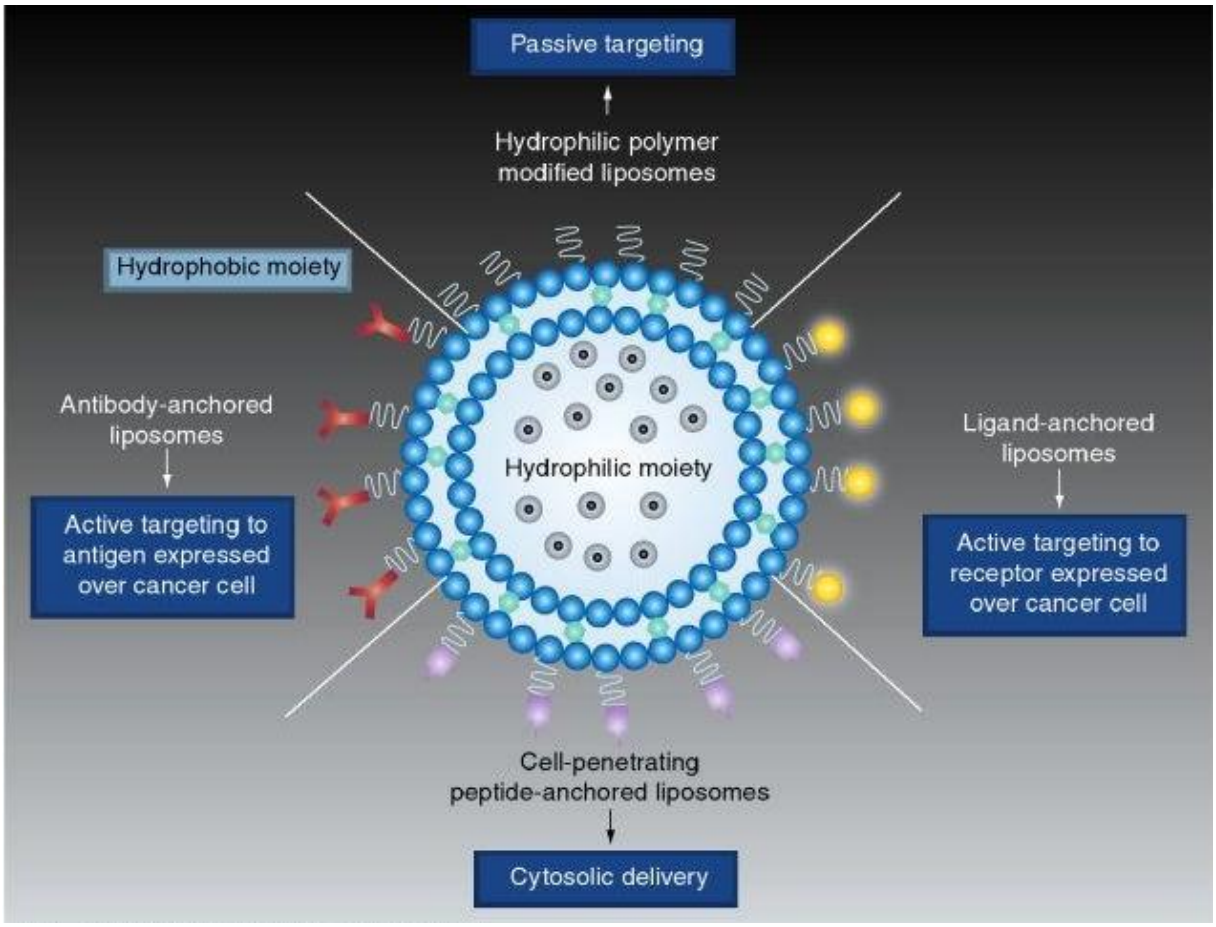

Figure 2 Strategies for liposome modification. (Top) Liposome modification with hydrophilic non bioactive polymers is frequently used for passive tumor targeting. (Bottom) Modification with cell penetrating peptides improves cellular uptake and drug delivery. (Left) Modification with antibodies is utilized to achieve target-specific attachment. (Right) Modification with bioactive small molecules is utilized to achieve ligand specific active targeting. Image adopted form Paliwal et al. [35]

1.3.4 Liposome Surface Modifications

1.3.4.1 Antibodies

As mentioned earlier, gene expression profiles of cancer cells are different from their healthy counterparts. In some cases, this difference in gene expression results in alterations in surface antigen productions. Changes in surface antigen populations, allows cancer cells to be targeted via specific antibodies, which can be anchored to the liposome surface. For example, it is known that many types of breast cancer cells overexpress HER2, which is a growth factor receptor located on the cell surface.[36] Overexpression of this antigen provides an appropriate immunologic target for drug delivery applications. Using various forms of immunoliposomes, many studies have reported efficient targeting, accumulation and multiple drug delivery to HER2 over expressing tumors.[37-41] Up to sixfold higher tumor accumulations were observed with liposomes modified with HER2 antibodies compared to their non modified equivalents.

Liposomal targeting by immunological modification can also be applied to other forms of cancer. A study done by Sapre et al. reported that the efficiency of immunoliposomes against human B cell lymphoma.[42] Cancerous human lymphoid B cells overexpressing the surface antigen CD19 were targeted via liposomes modified with anti-CD19. Following modification, researchers observed longer circulation times and higher cytotoxicities against these cancer cells in blood.

Another modification strategy is to target the vascularization of tumors. Vascular Endothelial Growth Factor (VEGF) is the primary regulator of angiogenesis and the expression of VEGF receptor (VEGFR) generally corresponds to the level of vascularization in a specific tissue.[43] As cancer cells grow continuously and tend to

form bulk tumors, they must induce angiogenesis to remain connected with blood circulation. Therefore, solid tumors have to overexpress signals capable of inducing the neovascularization required. As stated above, the extent of VGEF signaling corresponds to the vascularization of a given tissue, and the components of this signaling pathway are therefore abundantly expressed in many solid tumors. Thus, it is reasonable to utilize targeting strategies involving materials with an affinity to VEGFR or VEGF itself. Many studies reported improvement in tumor localization and cytotoxicity following the use of VEGF targeting antibodies.[44-45]

1.3.4.2 Small Molecules

Small molecules displaying high affinities to cancer cells can be used for liposome modification and may facilitate tumor specific targeting. For example, folic acid plays a crucial role in DNA biosynthesis, cell division and other metabolic events.[46] As cancer cells divide and replicate continuously, many forms of cancer cells overexpress folate receptors. Since receptor expression patterns for cancer and healthy cells are different, the increased affinity between folate and its receptor in tumor cells can be used to achieve liposomal targeting. Several studies have demonstrated the *in vivo* and *in vitro* efficiency of liposomes modified with folic acid-PEG conjugations, and have found that folate-modified liposomes increase cancer cell cytotoxicity while doing little to no damage to healthy tissues.[47-48]

Estrogen is another small molecule that can be used to target some types of cancer cells. 60-80 percent of all breast cancer cells overexpress estrogen receptor (ER).[49] As such conjugating estrogen to liposome surface is a reasonable approach for tumor recognition. Several studies utilized this approach to specifically target breast cancer cells.[50-51] A

study by Paliwal et al. reported the improvement of treatment efficiency following the estrogen modification of doxorubicin-loaded stealth liposomes on tumor cytotoxicity.[50] *In vitro* cytotoxicity of liposomes to ER positive and negative cells was studied comparatively along with *in vivo* biodistribution and tumor growth inhibition. Estrogen modified liposomes achieved higher cytotoxicity against ER positive cancer cells compared to non modified liposomes. In addition to improved cytotoxicity, estrogen modified liposomes demonstrated distinctively higher drug stability and better tumor accumulation, such that drug half life was 50% higher and tumor accumulation six fold higher in liposomes with estrogen modification compared to non modified ones.

1.3.4.3 Proteins

Several proteins have been found to be useful for the manipulation of anticancer pharmacodynamics in liposomes, antibodies and small molecules. The main principle behind protein-based modifications is similar to previously mentioned approaches. Modification with transferrin (TF) protein can be given as an example. TF is an iron binding glycoprotein that stabilizes ferric iron and facilitates endocytosis via TF receptor (TFR).[52] It has been previously reported to be overexpressed in several cancer types, being associated with metastasis in particular.[53] As such, TF can be used as a targeting moiety in order to improve the therapeutic effect of various drug delivery systems against TFR-overexpressing cancer cells. Yue et al. functionalized Paclitaxel loaded vesicles with TFR-recognition capabilities and characterized the resulting improvements in delivery pharmacodynamics.[54] They have demonstrated that, following TF modification, the functionalized liposomes accumulated earlier in tumor tissue, and facilitated a greater extent of tumor shrinkage. In another study, Wu et al. developed TF modified liposomes encapsulating multiple drugs and reported that TF modification could improve the uptake

and cytotoxicity of liposomes.[55]

1.3.4.4 Sugars

Several types of sugars can be used for liposome functionalization. For instance, various cancer types, such as stomach, colon, breast, ovarian cancers and leukemia, overexpress the hyaluronic acid (HA) receptor CD44.[56] Eliaz et al. reported improvements in the targeting capacity of in HA-modified liposomes against CD44-overexpressing cancer cells.[57] In this study, researchers demonstrated that HA modified liposomes could selectively bind to CD44-overexpressing cancer cell lines. When loaded with Doxorubicin, HA modified liposomes achieved significantly better delivery rates, such that IC50 value of nonmodified liposomes was around 175 fold higher compared to HA modified liposomes. Active targeting of CD44 by HA modification was also reported in several other studies.[58-60]

1.3.4.5 Peptides

Peptides can be used to modify liposomesfor a variety of purposes, such as targeting tumors or improving pharmacokinetics. Peptides are small chains of amino acids that display distinct and adjustable chemical properties depending on their sequences. Peptides are advantageous in surface modification applications since they are easy to synthesize, structurally stable and can be tailored to serve specific purposes. By using different peptide sequences, it is possible to achieve targeting or improved cell penetration.

Integrins are cell surface proteins involved in homodimeric cell to cell interactions mediating attachment, migration and division, and have been popular target molecules for drug delivery applications.. It has been previously reported that several types of integrins

are overexpressed in cancer cells.[61] This difference in expression can be utilized for active tumor targeting purposes. Peptides with RGD motifs have an affinity for native integrin proteins and are frequently used as tumor targeting moieties. In one study, for instance, liposomes modified with PEG-RGD conjugates were shown to target angiogenic tumors better and enhance tumor shrinkage, demonstrating higher accumulation in the tumor site and greater cytotoxicity compared to non-modified liposomes.[62]

In addition to targeting, a family of peptide sequences can be used to improve cellular uptake. These positively-charged sequences are called cell penetrating peptides (CPP), and display a high capacity to facilitate entry into cells, rendering them particularly useful for the effective delivery liposomes into cytosolic or nuclear compartments. Following the derivation of the CPP from Human Immunodeficiency Virus proteins (HIV), many unifying features of CPPs have been characterized, and both natural and artificial CPP sequences continue to to provide new strategies for drug delivery applications.[63] As cell penetrating peptides are the main focus of this thesis, they will be discussed separately in the following section.

1.3.4.6 Cell Penetrating Peptides

Many different approaches, such as viral vectors, nanoparticles and liposomes, have been used for the delivery of DNA, drugs or other agents into cells. However, these methods all invariably display a number of undesirable properties, ranging from immune reactivity to inefficiency of delivery. The discovery of cell penetrating peptides (CPP), which improve cellular internalization and endosomal escape of macromolecules, has allowed the development of new strategies for drug delivery. Due to their ability to facilitate cellular internalization, CPPs offer a substantial opportunity to improve cargo delivery into target

cells. Thus, CPPs are an important focus on gene and drug delivery studies.

CPPs are short peptide sequences that mainly consist of positively charged amino acids. These low molecular weight, highly cationic peptides are rich in basic amino acids like arginine or lysine.[64] In addition to their charge, their penetration capacity is based on their sequence and target cell type, since compatible membrane-CPPs interaction is crucial to achieve efficient penetration.[65]

CPPs are capable of entering into cells via receptor mediated and/or non receptor mediated mechanisms. Although the internalization mechanisms are not fully understood, the coexistence of different uptake types is frequently suggested.[66] Since CPPs mainly consist of cationic amino acids, electrostatic interactions are important for the transduction of CPPs (and their cargo) through the cell membrane.[67] In addition to electrostatic interactions, receptor mediated macropinocytosis and subsequent endosomal release also have primary roles in the accumulation of CPPs in the cytoplasm.[68] Coexistence of various internalization mechanisms may help explain the delivery efficiency of CPPs.

Several different strategies have been developed to integrate CPPs on liposome surfaces. This integration can be facilitated either through the efficient linkage of CPPs with reactive residues on the surface, or via non-covalent integration, as we have achieved in our study. One commonly used approach is the addition of cysteine to the CPP sequence to facilitate the formation of strong disulphide bonds with liposome surface residues.[69] Spacer molecules are also used to connect CPPs and liposomes. In addition to the covalent linkage of CPPs and liposomes, CPPs can be integrated into the lipid bilayer by non

covalent interactions. In our study, we modified CPPs with a hydrophobic lauric acid, which is both chemically and structurally similar to fatty acid chains of membrane lipids. By doing so, we allowed the non covalent incorporation of our CPP during liposome formation, such that our modified CPPs were added to the structure of liposomes due to their similarity to the other lipids within the liposome matrix.

1.4 MOTIVATION AND GOALS

Peptide amphiphiles (PA) possess both a bioactive “head group” and a lipid-like alkyl tail in one molecule, which renders them highly suitable for the functionalization of liposome-based drug carrier systems. PAs can be designed and chemically synthesized with high yield and specificity and, more importantly, are easy to incorporate into liposomes without experiencing activity loss or requiring laborious chemical functionalization steps, primarily due to the amphipathic properties that they share with lipids.[64, 70] Here, we designed and incorporated a poly-arginine cell penetrating amphiphilic peptide segment into a liposomal system, and examined the integration of this arginine-rich peptide amphiphile into liposomes formed by negatively charged dioleoylphosphoglycerol (DOPG) phospholipid molecules in the presence of cholesterol. We aimed to elucidate the changes in the physical characteristics of the resulting liposomes in terms of size, surface potential and membrane polarity, and characterized their encapsulation capacities using hydrophilic and hydrophobic dyes Rhodamine B and Nile Red. After optimizing the encapsulation efficiencies of liposomes using fluorescent dyes, we then profiled the dynamics of liposome uptake. Furthermore, we investigated the possible augmentation of anti-cancer properties in known cancer drugs, doxorubicin-HCl and paclitaxel, loaded into cell penetrating peptide amphiphile-modified liposomes. This study not only analyzes the effect of peptide amphiphile integration on the physical properties of liposomes, but also discusses the *in vitro* therapeutic effect of peptide amphiphile integrated liposomes on MCF7 breast cancer cells in terms of cellular uptake and cytotoxicity.

CHAPTER 2

CELL PENETRATING PEPTIDE MODIFICATION FOR IMPROVED LIPOSOMAL DELIVERY OF ANTI CANCER THERAPEUTICS

This part of thesis is based on article "Cell Penetrating Peptide Amphiphile Integrated Liposomal Systems for Enhanced Delivery of Anticancer Drugs to Tumor Cells" Published in RSC Faraday Discussions

2.1 INTRODUCTION

Liposomes are vesicles with varying diameters and contain lipid bilayers surrounding aqueous compartments. The self-organization of lipid molecules into bilayer in aqueous environment through their amphipathic character is responsible for the formation of spherical vesicles. Liposomes have been considered as potential drug delivery agents for several decades due to their biocompatibility, biodegradability and their resemblance to cell membrane. They have a long history of use to improve the delivery of many therapeutics such as vaccines, antibiotics and anticancer drugs.[71] Hydrophobic materials can be incorporated into the lipid bilayer of liposomes, while hydrophilic components can be encapsulated within aqueous lumen.

Liposomes happen to be reliable drug delivery vehicles due to the following properties:

- They are biocompatible, biodegradable and similar to cell membrane which makes their administration safe.[72]
- They increase the longevity of cargo which will be exposed to metabolic interference of humorous environment unless it is protected.
- They can be modified for specific purposes with a wide spectrum of functional

materials.[73]

Phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and dioleoyltrimethylammonium propane (DOTAP) are commonly used lipids which can be isolated from natural resources or produced synthetically. PC is used for preparation of uncharged liposomes while PG, PE or DOTAP are used to introduce charges. Liposomes ordinarily contain cholesterol to improve stability and prevent leakage of cargo.

Various approaches have been practically used to functionalize liposomes including chemical coupling of lipid molecule and ligand of interest before liposome formation, covalent conjugation of biologically active segments to the liposome surface and non-covalent association of liposome constituents.[74-75] Their versatile nature enables design of various functional liposomal systems decorated with a wide range of bioactive molecules such as antibodies, viral proteins, carbohydrates, peptides, aptamers, and vitamins for therapeutic delivery.[71, 76-77] The easiness of functionalization together with their extensive encapsulation capacity make them attractive tools for the development of systems which deliver the cargo to the target location with enhanced *in vivo* stability and circulation time. Besides their facile integration, the versatility of modifications enables diverse biofunctionality to the liposomal carrier.

Amphiphilic peptides comprised of a bioactive peptide sequence and a hydrophobic segment have great potential for functionalization of liposomal carriers. They can be designed and chemically synthesized with high yield and specificity and more importantly, they can be easily incorporated into liposomes noncovalently due to their lipid-like amphipathic property with minimized activity loss or without laborious

chemical functionalization steps.[78-81] The easiness of functionalization together with their extensive encapsulation capacity make them attractive tools for development of carrier systems, which can deliver cargo to the target with enhanced *in vivo* stability and circulation time.[82] Besides their facile integration, the versatility of peptide sequences provides diverse biofunctionality to the liposomal carriers.

The importance of cell penetrating peptides including HIV-Tat derived peptides, oligoarginines, and chimeric cell penetrating peptides have been emphasized in several works for delivery of therapeutic agents to target cells.[83-84] Arginine-rich peptides were also synthesized and investigated for enhanced cellular uptake efficiency by conjugating with large molecules such as fatty acids.[85]

Herein, we designed and synthesized an arginine-rich, cell penetrating peptide amphiphile molecule and examined its integration into liposomal formulation of 1,2-dioleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (DOPG) phospholipid in the presence of cholesterol. We studied size, surface potential, and membrane polarity of the resulting liposomes with and without peptide amphiphile incorporation. Encapsulation capacities of these carriers were examined by using hydrophilic and hydrophobic dyes, Rhodamine B and Nile Red, respectively. After optimization of the encapsulation efficiencies of liposomes, *in vitro* uptake profile and cytotoxicity of cancer drugs including Doxorubicin-HCl and Paclitaxel entrapped in liposomes with and without peptide amphiphile molecules were examined on MCF7 human breast cancer cell line.

2.2 MATERIALS & METHODS

2.2.1 Chemicals and Solutions

Lipids:

1,2-dioleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (DOPG) was purchased from Avanti Polar Lipids and Cholesterol from AppliChem.

Peptides:

9-Fluorenylmethoxycarbonyl (Fmoc) and *tert*-butoxycarbonyl (Boc) protected amino acids, [4-[α-(2',4'-dimethoxyphenyl)Fmoc-aminomethyl]phenoxy]acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) and Lauric acid were purchased from NovaBiochem, ABCR and Merck. Piperidine, Acetic anhydride, Dichloromethane (DCM) and Dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA) : triisoproplysilane (TIS) were purchased form Sigma.

Cell Culture Reagents:

Dulbecco's Modified Eagle Medium (DMEM), Penicillin/streptomycin (PS) antibiotic combination and fetal bovine serum (FBS) were purchased from Invitrogen Gibco.

Liposome Contents:

Anticancer drugs Doxorubicin-HCl (DOX) and Paclitaxel (PTX) were acquired from Applichem. Our tracking materials, Nile Red (NR) and Rhodamine B (RHB) were purchased from Alfa Aesar.

2.2.2 Peptide Synthesis and Characterization

C_{12}-PPPPRRRR-NH_2 peptide amphiphile was constructed on MHBA Rink Amide resin. Amino acid couplings were performed with 2 equivalents of Fmoc protected amino acid, 1.95 equivalents of HBTU and 3 equivalents of DIEA in DMF for 3 h. Fmoc deprotections were performed with 20% piperidine/DMF solution for 20 min. Cleavage of the peptides from the resin was carried out with a mixture of TFA:TIS:H_2O in the ratio of 95:2.5:2.5 for 2 h. Excess TFA was removed by rotary evaporation. The remaining viscous peptide solution was treated with ice-cold diethyl ether and the resulting white pellet was freeze-dried. The peptide amphiphiles were identified and analyzed by reverse phase HPLC on an Agilent 6530 accurate-Mass Q-TOF LC/MS equipped with an Agilent 1200 HPLC. An Agilent Zorbax SB-C8 4.6 mm x 100 mm column was used as stationary phase and water/acetonitrile gradient with 0.1% volume of formic acid was used as mobile phase to identify peptide amphiphile.

2.2.3 Liposomes

2.2.3.1 Liposome Preparation

Liposomes were prepared via curvature tuned preparation method reported previously.[86] 50 mg of DOPG and Cholesterol combination was directly rehydrated in PBS buffer (10 mM, pH 7.4, 3% glycerol, with cargo material) at 65 °C under nitrogen supply while the mixture was continuously stirred. Further, solution was treated with a rapid pH jump (pH 7.4→pH 11 →pH 7.4) continuing with an equilibrium period of 25 min, where lipid clusters curl into encapsulating nanosized liposomes. The resulting liposomes were purified using a G50 sephadex column and stored at 4 °C. For NR delivery experiments empty liposomes were prepared with DOPG - Cholesterol combination and NR was incorporated into liposomes after preparation.

2.2.3.2 Characterization of Liposomes

Particle size and Zeta-Potential by Dynamic Light Scattering*:*

The mean diameter and zeta potential of liposomes were measured using a Malvern Zetasizer nano-ZS ZEN 3600 (Malvern Instruments, USA) instrument with detector angle of 173°. Standard deviations were calculated from the mean of the data of a series of experiments ($n \geq 3$). Zeta measurement was carried out using a dip cell 35 electrode in quartz cuvettes.

Transmission Electron Microscopy:

Using a glass pipette, a drop of sample was added to a 200 mesh copper grid with a thin film of formvar polymer and carefully dried using a filter paper. Then, sample was left at room temperature to dry until a dried film was obtained. Transmission Electron Microscopy (TEM) analyses were performed using with FEI Tecnai G2 F30 operated at 100 keV.

Nile Red Polarization Studies:

A 1.5 mM stock solution of Nile Red was prepared in ethanol and further diluted in water to 0.01 mM. Nile Red fluorescence intensity maxima switches with respect to the polarity of the environment and thus Nile Red dye solubility in different types of liposomes was measured and compared in between the formulations w/o peptide amphiphile. For this, final concentration of 3 μM, Nile Red was introduced in the liposome dispersions by injection of accurate amount of stock solution (450 μL) into 1.050 mL of 2x diluted liposome (2.5 mg/mL) or PBS. Samples were left to equilibrate for 24 h at 4 ° C before measurements were taken. To determine micelle character, fluorescence intensity of

liposomes in PBS (λ excitation = 520 nm, λ emission = 525-700 nm) was measured and compared.

Quantification of the number of membrane integrated peptide amphiphiles:

Protein concentration determination procedure provided by Thermo Scientific was followed measuring the absorbance at analysis wavelength of 205 nm with extinction coefficient of 31 mg/mL at 1 cm path length. For this, peptide amphiphile solutions were prepared at different concentrations in the range of 0.6 mg/mL to 0 mg/mL by 2 fold dilution. Liposomes were purified from unbound peptide amphiphiles using Millipore Microcon Centrifugal Filter Units, with 50K MW cut-off, 3 to 4 times. Further, the waste was collected in each step and peptide concentration was measured via Thermo Scientific NanoDrop 2000. Peptide amphiphile integrated liposome concentration was then calculated indirectly by substituting the amount of unbound PA from initial PA. This information was later used for calculation of number of lipids per liposome.

Determining encapsulation capacities of liposomes:

A- Hydrophilic molecules: Rhodamine B (RHB) was used as tracker dye as model hydrophilic molecule and encapsulated at two initial concentrations of 10 μM and 100 μM using previously described liposome preparation procedure. Then, liposomes were purified from unbound RHB by using Centrifugal Filter Units with 50 kDa cut-off, 4 times. RHB concentration was determined by fluorescence measurements (λ excitation= 540 nm, λ emission= 575 nm) following the destruction of liposome integrity by use of EtOH (100 μL in 1900 μL EtOH) and % encapsulated RHB concentration was then calculated from previously prepared standard curve. Same procedure was followed for hydrophilic drug Doxorubicin HCl which was encapsulated with an initial concentration

of 0.2 mg/mL. Standard curve was obtained by using λ excitation of 480 nm and λ emission of 588 nm to measure fluorescence intensities of DOX with known concentrations.

B- Hydrophobic molecules: NR was used as tracker dye as model hydrophobic drug. Dye encapsulation was tracked by measuring the increased emission (λ excitation = 476 nm, λ emission = 633 nm) following the administration of 0.53 μM NR to previously prepared liposomes. After 24 h, all samples reached to a maximum. At that point, liposomes were lysed by suspending 100 μL of sample in 1900 μL ethanol and encapsulated NR concentration was calculated by substituting the value of measured fluorescence to the standard curve. For hydrophobic drug, PTX, HPLC procedure optimized by Wang *et al*.[87] was used to measure encapsulation capacities and efficiencies of liposome formulations. PTX was extracted from the liposomes with acetonitrile (100 μL of liposome in 900 μL ACN), then filtered with a 0.2 mm syringe filter and the concentrations were analyzed using HPLC-1200S (Agilent). 10 μL sample solution was injected into Eclipse XDB-C18 (4.6 x 150 mm, 5 μm) column, water and acetonitrile in the volume ratio of 53:47 was used as mobile phase. Elution rate was 1.0 mL/min and the PTX detection wavelength was set at 229 nm. The drug concentration of PTX was calculated from standard curves.

2.2.4 Cell Culture

2.2.4.1 Cytotoxicity Tests

Cytotoxicity of samples was evaluated using Alamar Blue™ assay. MCF7 cells were seeded in DMEM supplemented with 10% FBS and 1% Pen/Strep under conditions of 5% CO_2 at 37 ^{0}C. They were cultured in 96-well plate in 200 μL medium per well with a

density of 8000 cells/well for 24 h. 50 µL of liposome or peptide formulations were administered with final peptide concentrations of 250 µM, 25 µM and 12.5 µM in culture (n=4 for all groups). After 4 and 24 h incubation with liposomes, culture media replaced with serum free DMEM supplemented with %1 pen/strep and %10 Alamar Blue™ solutions and cells were incubated for additional 4 h. Cell viability was quantified spectrophotometrically by measuring fluorescence at excitation of 540 nm and emission of 590 nm with microplate reader (Molecular Devices Spectramax M5). The data exhibited percentages of viable cells compared to the survival of a control group (1X PBS treated cells as controls of 100% viability).

Cytotoxicity of anticancer agents was determined via a similar manner. MCF7 cells were plated in 96-well plates at a cell density of 8000 (for DOX treatment) and 4000 (for PTX treatment). Then, the cells were treated with serial dilutions of drug loaded liposomes and free drug for another 24 h. After rinsing the cells with fresh media, viability of cells were quantified via Alamar Blue™ assay as described previously. For time response experiments, cells were exposed to drug loaded liposomes and free drug, of which the final drug concentrations were adjusted to 10 µM for DOX and 30 µM for PTX, for 1, 3 and 6 h. Afterwards cells were rinsed 2 times with fresh medium and incubated in standard cell culture medium for 24 h. Finally, cytotoxicity levels and inhibition of the cell proliferation were determined by Alamar Blue assay.

2.2.4.2 Liposome Uptake Quantification

MCF7 cells were seeded at a density of 30.000 cells per well in 24 well plates. 100 µL of liposome formulations were then administered and 500 µL of total culture volume achieved. For RHB loaded liposomes, administration was optimized to a final RHB

concentration of 4.5 μM. After 3 h of incubation, culture media was removed and cells were washed 2 times with pre-warmed PBS. Afterwards, cells were lysed using 100 μL of 0.5 M NaOH for 15 min with vigorous shaking in dark. Lysates were collected and centrifuged at 15000 rpm for 5 min. Rhodamine B concentration was measured from supernatants by fluorescence measurement by Nanodrop 3300 (Ex. 540 nm, Em. 590 nm). Measured fluorescence values were then normalized to protein concentrations of samples in order to calculate relative uptake score. Protein concentration of lysates was measured through Bradford protein assay (Roche). In the case of Nile Red (NR) loaded liposomes, sample concentrations were adjusted to obtain 10 μM of final NR concentration. After 3 h of incubation with either NR loaded liposomes or free NR, cells were cleaned as stated above and lysed with 300 μL of 90 % ethanol for 15 min with vigorous shaking. Afterwards, lysates were centrifuged and fluorescence of supernatants was measured at excitation/emission maxima of 476/633 nm. Measured fluorescence units were used to estimate the relative uptake of liposomes loaded with NR.

2.2.2.4 Imaging

MCF7 cells were seeded at a density of 10.000 cells per well in 24 well plates with 1.5 mm glass sphere. 100 μL of sample solution was administered with a final NR concentration of 10 μM. Final culture volume of 500 μL achieved. Following 24 h incubation, cells were washed two times in pre-warmed PBS and directly mounted onto glass slides. 20X and 40X images were taken by Zeiss AxioCam^{TM} fluorescence microscope. All imaging parameters were same for all experimental groups.

2.3 RESULTS & DISCUSSION

2.3.1 Synthesis and Characterization of Peptides and Liposomes

2.3.1.1 Peptides

Liposome functionalizations with cell penetrating peptides have been previously achieved through chemical conjugation of a peptide to the lipids before or after liposome preparation. Both strategies not only require additional steps and decrease the peptide integration efficiency but might also result in the loss of biological activity.[88] Prolonged steps of modification reactions and purification processes severely decrease the end yield of the final product as well. Thus, development of noncovalent strategies is preferred to avoid chemical modifications and subsequent extra procedures with low fidelity.[89] Use of amphiphilic peptides is a promising approach due to their chemical resemblance to phospholipids.[90-91] Therefore, we designed and synthesized an arginine-rich CPP amphiphile molecule consisting of a lauryl hydrocarbon chain group (C_{12}), linker domain of poly proline and cell penetrating poly arginine domain, which gave rise to C_{12}-PPPPRRRR-NH_2, as shown in figure 3.

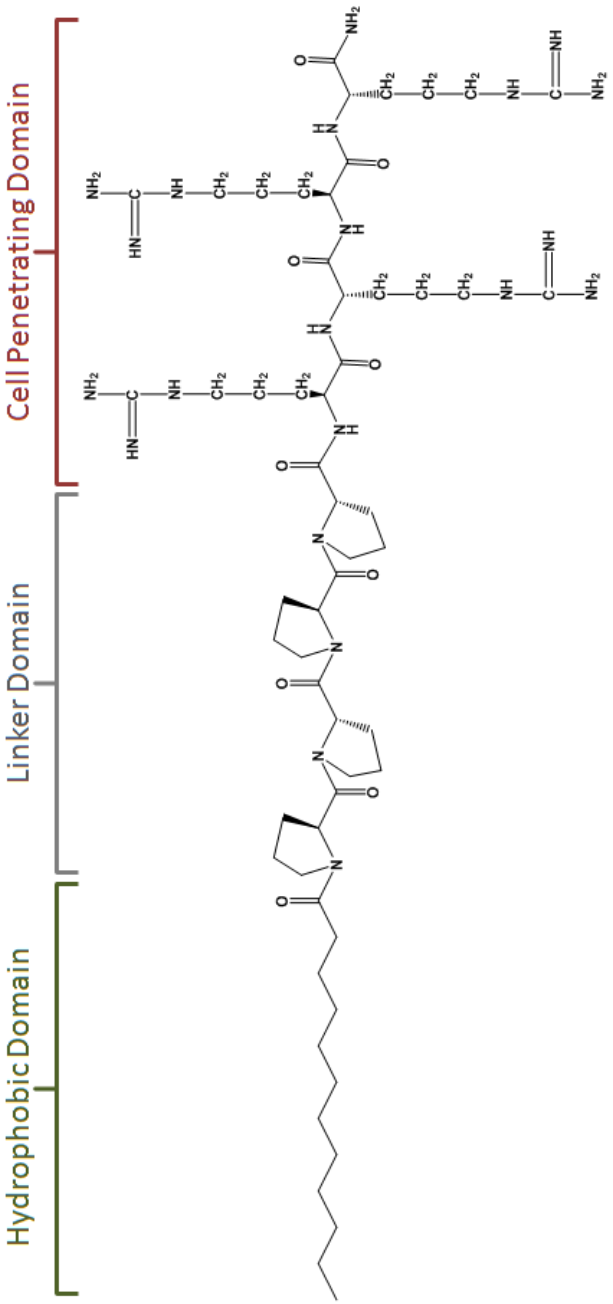

Figure 3 Chemical representation of C_{12} (Lauryl)-PPPPRRRR-NH_2, cell penetrating peptide amphiphile. Peptide consists of 3 main domains. C_{12} forms hydrophobic domain and facilitates incorporation with bilayer. Poly-Proline at linker domain prevents β-sheet formation and allows Poly-Arginine tip to reach bilayer surface. Poly-Arginine sequence at cell penetrating domain improves internalization and endosomal escape.

As mentioned in the materials and methods section, C_{12}-PPPPRRRR-Am peptide amphiphile was constructed on MHBA Rink Amide resin which contains Fmoc protected amine groups. Fmoc protections were removed via 20% piperidine/DMF solution at each step. Subsequent to Fmoc deprotection, amino acid couplings were performed with Fmoc protected amino acid, HBTU and DIEA in DMF. After amino acid coupling, any possible unprotected amine groups were acetylated with %10 acetic anhydrate in DMF to block further elongation form these residues. This cycle including Fmoc deprotection, amino acid coupling and acetylation process was repeated with desired amino acids or C_{12} until all synthesis was completed. At the end of synthesis, we obtained amphiphilic peptides attached to Rink Amide resin. Cleavage of the peptides from resin was carried out with a mixture of TFA:TIS:H_2O which breaks bonds between amide and the resin. Excess TFA was removed by rotary evaporation since overexposure to TFA may result damage on peptide. The remaining viscous peptide solution was treated with ice-cold diethyl ether and the resulting white pellet of peptides was obtained while small molecules and byproducts dissolved in ether. Acquired pellet was dissolved in water and freeze dried for further purification from organic solvents. Then we purified peptide by HPLC and analyzed the purity of peptide via LC-MS and liquid chromatography. As the liquid chromatogram below (Figure 4A) demonstrates, there is only one major product peak, which means only one type of material exists in sample solution.

Subsequent to observing single peak, we analyzed sample with LC-MS in order to ensure the peak is belongs to C_{12}-PPPPRRRR-NH_2. Observed mass spectra ensured the purity of the peptide. As shown in spectra below, no contamination was observed. (Figure 4B)

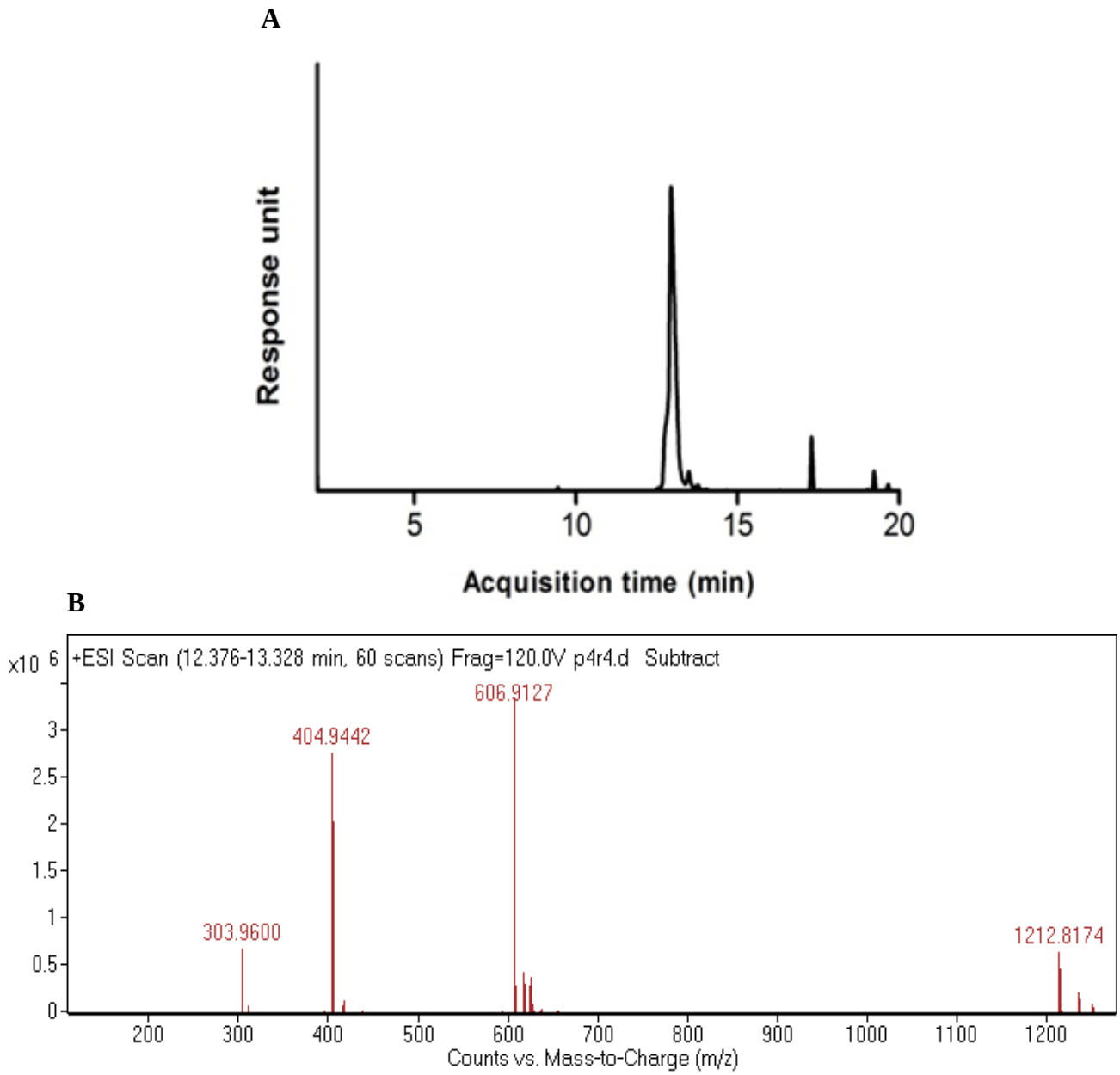

Figure 4 A. Liquid chromatogram of Lauryl-PPPPRRRR-NH_2. **B.** Mass spectrum of corresponding peptide molecule. Mass data $[M+H]^+$ (calculated) = 1212.54, $[M+H]^+$ (observed) = 1212.82 (observed $[(M+2H)/2]^+$= 606.91, $[(M+3H)/3]^+$= 404.94, $[(M+4H)/4]^+$= 303.96)

2.3.1.2 Liposomes

Cell penetrating arginine-rich peptide amphiphile integrated and bare liposomes were prepared according to curvature tuned liposome preparation (CTLP) method, where phospholipid residues are forced to be ordered in a nanostructure with the synergistic effect of a sudden pH jump and constant temperature.[86] By using this method, it is possible to integrate amphiphilic CPPs on liposome membrane via a single step and without any covalent reaction taking place. Addition of an amphiphilic molecule to liposomal membrane can change the fluidity and curvature of the membrane, as well as the physical properties of the resulting liposome such as particle size, zeta potential and morphology. In this study, negatively charged phospholipids, DOPG, was used as the main component of the liposomes.[92] Previously, it was observed that DOPG liposomes prepared by CTLP methodology with a size of 20 nm in diameter were not large enough to develop an efficient drug delivery system.[93-94] Therefore, cholesterol was incorporated into the formulation to reduce the membrane curvature, which resulted in formation of larger liposomes and provided intermediate membrane fluidity and prolonged circulation time due to delayed renal filtering.[95] As shown in Table 1, we obtained uniform liposomes with a size of 63.5±8.2 nm in diameter and a net negative charge of -41.4±3.9 mV when liposomes were formed by DOPG and cholesterol at a molar ratio of 50:50. After peptide amphiphile integration, whether low or high amount of peptide was integrated, particle sizes of liposomes were measured to be 95.26±7.03 nm and measurements revealed that disparity between liposomes were quite trivial, so that only observed standard deviations were low.

Table 1 Physical properties of liposomes

Liposome Formulation	Size (nm)	Zeta-Potential (mV)	Peptide Integration %	Peptide # per Liposome
DOPG:Chol	63.5±8.2	-41.4±3.9	-	-
DOPG:Chol:PA*	95.26±7.03	-30.4±2.57	75.4	4568
DOPG:Chol:PA* (Low)	98.49±4.9	-33.2±1.71	77.24	2706

*Difference between DOPG:Chol:PA and DOPG:Chol:PA(Low) is amount of CPPA integrated. Ratios were 7:6:1 for first one while 7:6.5:0.5 for DOPG:Chol:PA low.

In addition to size measurements, we sought to understand the change in electrical charge of liposomes upon PA modification. Zeta-potential of membrane, which mainly consist of negatively charged DOPG and neutral cholesterol, increased to -30.4 mV from -41.4 mV due to positive net charge possessed by the poly-arginine region of CPP amphiphile molecule.

In order to further characterize physical characteristics of our liposomes, we conducted transmission electron microscopy imaging (Figure 5). Our main purpose with this imaging was to understand whether our liposomes were unilamellar or not. This is important since bilayer amount per liposome is a crucial parameter on cargo capacity. Acquired images revealed that resulting liposomes were unilamellar in both PA modified and non modified groups. In addition to liposome lamellarity, observed sizes of liposomes were consistent with Zeta-sizer measurements which were around 50 nm for non modified ones versus 100 nm for PA modified liposomes. Particle size of the liposomes stored at 4 °C was monitored, and no significant change was observed in samples for over 3 weeks.

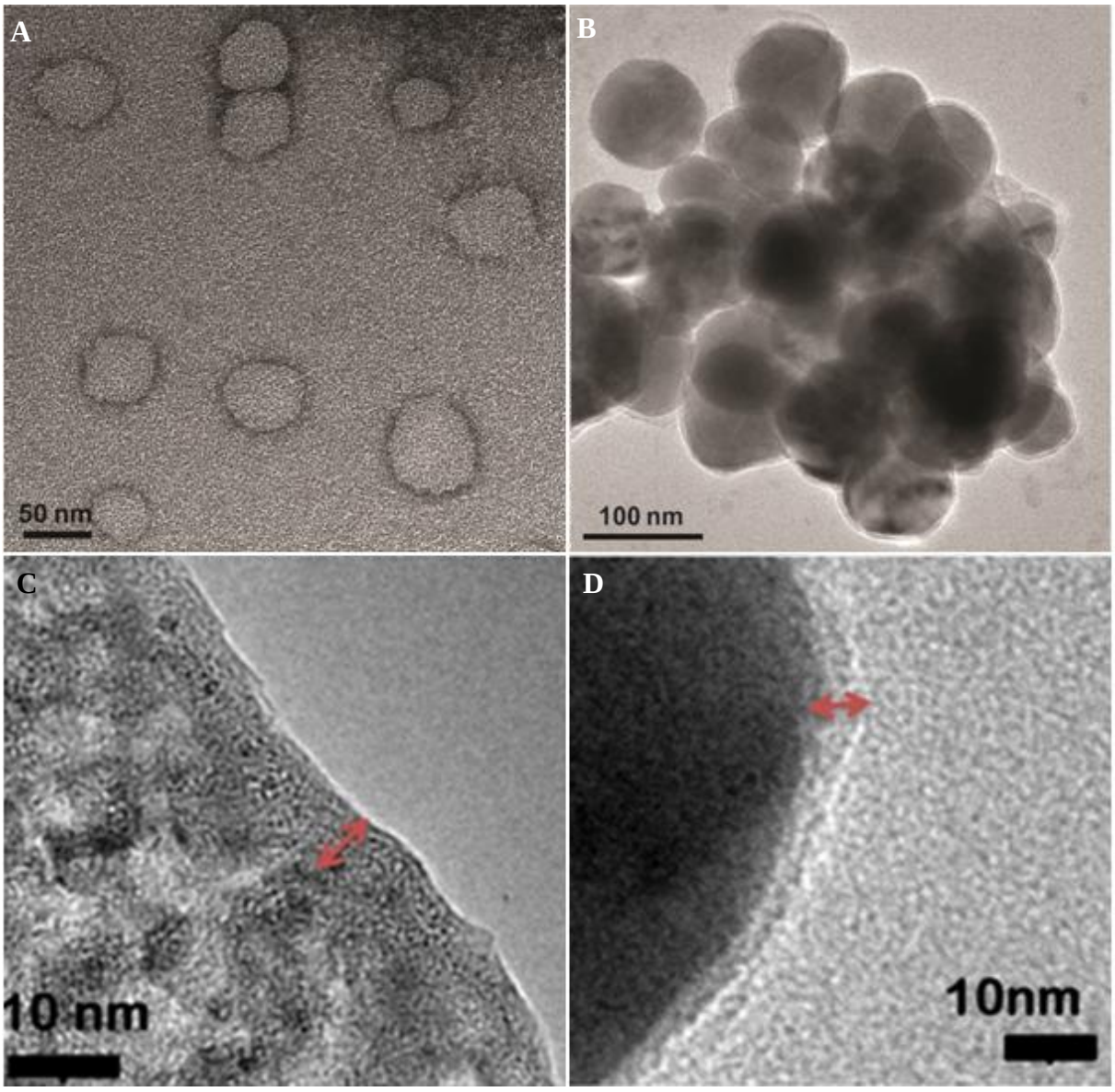

Figure 5 Transmission electron microscope images of liposomes. **A & C**. DOPG:Chol, **B & D.** DOPG:Chol:PA. **C & D** are close section of single liposomes and red arrows point the unilamellar membrane. Scale bars = 10 nm, 50 nm and 100 nm, respectively.

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The amount of the peptide molecules incorporated into the liposomal membrane was calculated by measuring absorbance of free peptide molecules collected during the purification process.[91] As shown in Table 1 previously, about four thousand peptide amphiphile molecules were estimated to be integrated per liposome. Peptide amphiphile insertion was also monitored by fluorescence assay by using Nile Red as a polarity sensitive probe.[96] Nile Red dispersion is usually colorless in aqueous conditions, however, changes in the environment of the hydrophobic dye revealed a switch from polar to nonpolar in the presence of phospholipid and caused increased fluorescence emission. In the case of peptide-integrated liposomes, a blue shift was observed indicating a slight decrease in membrane polarity, indicating that the amphiphilic CPPs were integrated into membrane (Figure 6).

Along with size and zeta-potential results, Nile Red polarity studies also confirmed that peptide amphiphile molecules were embedded into the liposomal membrane, and increased the nonpolar character of the membrane, which can be considered as an advantage for encapsulation of hydrophobic molecules.

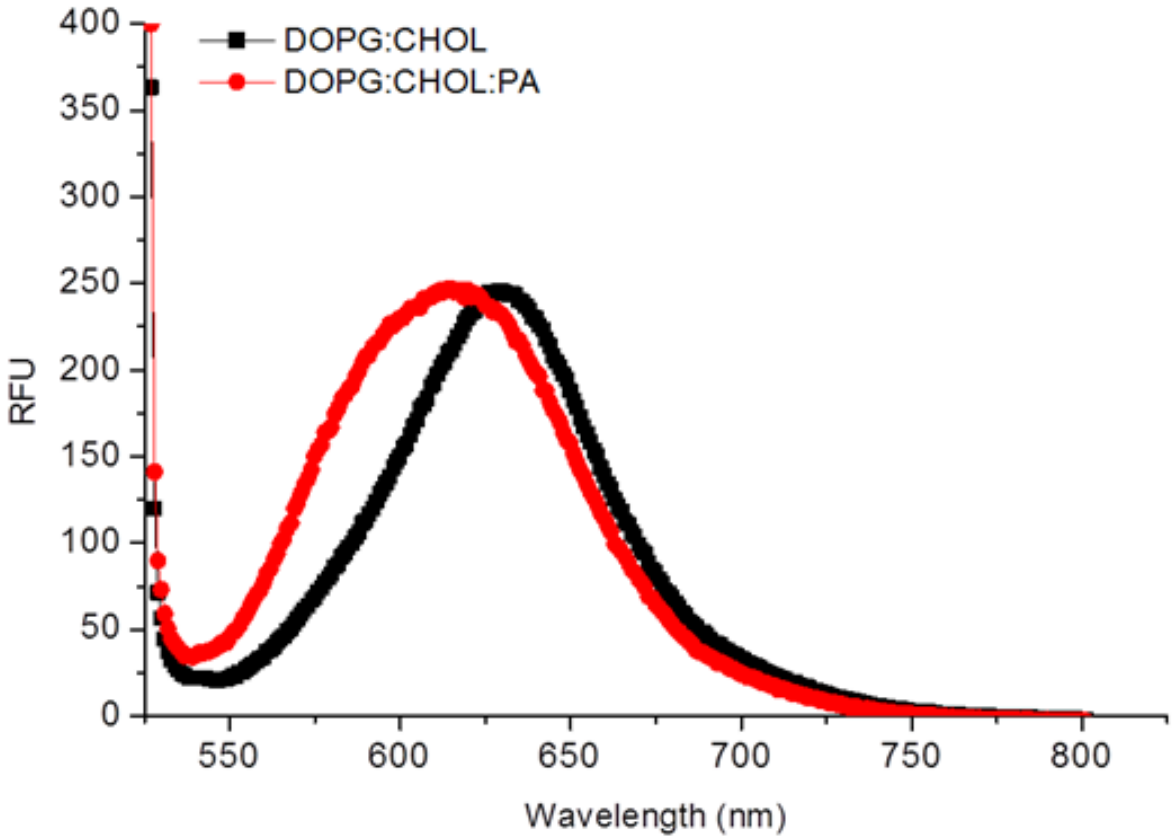

Figure 6 Polarity change in DOPG:Chol liposomes from polar towards nonpolar when positively charged cell penetrating peptide amphiphiles were integrated to their membrane. **Black** DOPG:Chol liposomes. **Red** DOPG:Chol:PA liposomes.

2.3.2 Safety of Liposomal Carriers

In order to evaluate the biocompatibility of liposomal formulations, MCF7 cells were treated with bare liposomes, PA integrated liposomes and PA molecules. Peptide amphiphiles molecules did not cause significant change in cell viability in either liposomal or free form after 4 h and 24 h of treatment (Figure 7A&B).

On the contrary, liposomal formulations had trivial but positive effect on cell proliferation after 24 h, which could be due to use of liposomes as nutritional source by cells. Providing empty liposomes into culture media may enhance the fitness of cells within cell culture medium since cells were provided with readily available lipids and can build up more membranes (and eventually new cells) with fewer burdens on lipid biosynthetic pathways.

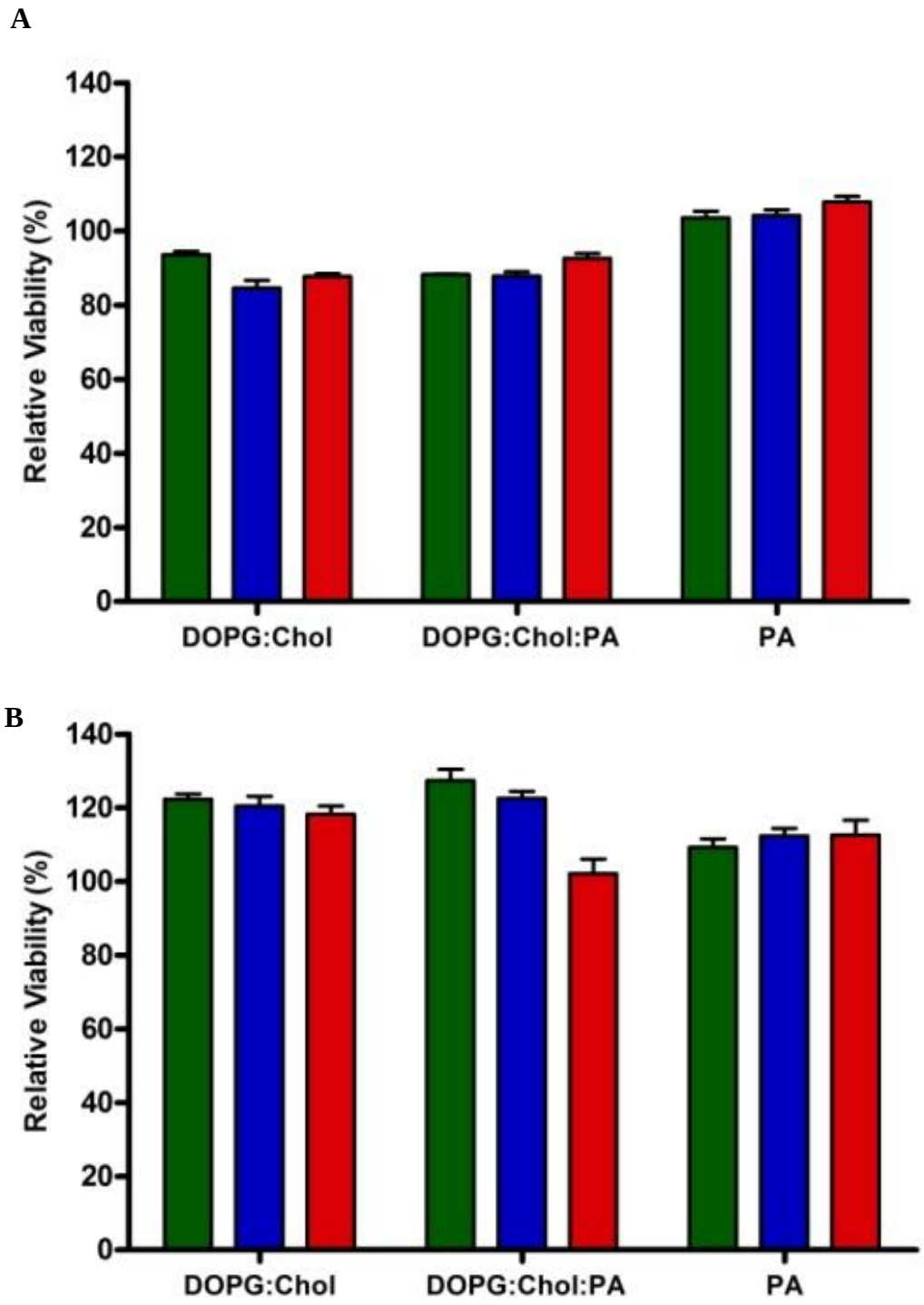

Figure 7 Viability of MCF7 cells after treatment with drug free liposomes with PA, without PA and only PA in free form for **A.** 4 h and **B.** 24 h. Samples were optimized to final peptide concentrations of 250 (green), 25 (blue) and 12.5 (red) µM. Results were normalized to untreated cells in PBS. (n=4)

2.3.3 Enhanced Uptake via CPP Modification

2.3.3.1 Liposomes with Hydrophilic Content

Rhodamine B (RHB), a hydrophilic fluorescent dye, was used to probe uptake of liposomes prepared with and without peptide amphiphile molecules. Since it is fluorescently active and non toxic, we used RHB to track liposome internalization and cargo delivery dynamics. RHB was loaded into liposome during synthesis phase by adding into solution that will eventually form the whole liposome. Since it is hydrophilic, RHB cannot be loaded into lipid bilayer after liposomes were formed. After preparation, we observed that initial RHB concentration as well as peptide integration affected encapsulation capacities of the liposomes. Although encapsulated dye concentration decreased slightly from 36.3 mM to 30.5 mM after PA integration, they encapsulated almost four times higher amount of Rhodamine B compared to PA free liposomes (Table 2). Since peptide modified liposomes have approximately two fold greater diameters (Table 1), which will eventually make them have approximately four times larger internal volume. Therefore peptide modified liposomes can entrap much more RHB. As shown in table 2, RHB per liposome is around four times greater when compared to non modified ones.

Table 2 Encapsulation of model dyes

	Encapsulated Rhodamine B (μM)		**Number of Rhodamine B Molecule / Liposome**	
Liposome Formulation	*10 μM**	*100 μM**	*10 μM**	*100 μM**
DOPG:Chol	7.90±0.8	36.3±3.9	4.15×10^4	19.1×10^4
DOPG:Chol:PA	7.00±0.4	30.5±2.2	12.4×10^4	54×10^4

*Represents initial concentration of Rhodamine B in environment where liposomes were synthesized. As mentioned in methods section, Rhodamine B was loaded into liposome during production phase. Therefore initial concentration of Rhodamine B is an important parameter defining the amount of cargo loaded into liposome.

Delivery efficiency of liposomes was evaluated via measuring the amount of fluorescent reagents uptaken by MCF7 cells. Tracking RHB uptake is an efficient way to quantify liposomal uptake rates.[97-98] Upon 3 h of treatment of equal amounts of RHB either with PA modified liposome, bare liposome or free form, use of PA integrated liposomes resulted in five folds more Rhodamine B uptake compared to both native liposomes and free RHB ($p < 0.001$) (Figure 8). Although amount of dye encapsulated via peptide modified liposomes were larger, observed relative uptake increase was not caused by enhanced dye entrapment, since cells were treated with equal amount of RHB in every form. Therefore amount of peptide modified liposomes were quite few and these few liposomes performed very well in terms of cargo delivery.

Additionally, although it was previously published that vehicle size and *in vitro* uptake rates are inversely proportional[99], we observed that cell penetrating PA integrated liposomes, which were larger than bare liposomes, showed enhanced delivery of hydrophilic dye into MCF7 cells. On the other hand, no significant difference was detected between uptake of free RHB and bare liposomes. These results revealed that noncovalent integration of cell penetrating PAs to liposomes improved cellular uptake of hydrophilic cargo by facilitating the liposome internalization rate. Cationic guanidine group of arginine side chain was previously shown to be important for the membrane translocation property[100], which might have enhanced internalization of PA functionalized liposomes.

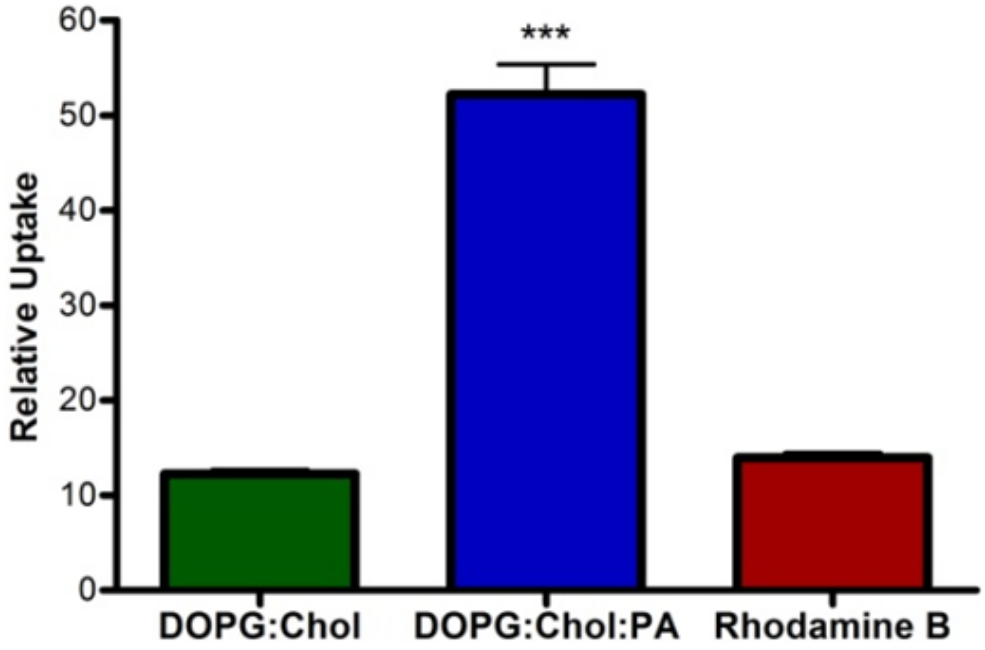

Figure 8 Uptake of 4.5 μM Rhodamine B within DOPG:Chol and DOPG:Chol:PA liposomes by MCF7 breast cancer cells after 3 h of treatment. Free Rhodamine B was used as control. Acquired signals were normalized to protein concentration of samples to calculate relative uptake value. (*** stands for $p < 0.001$) (n=4)

2.3.3.2 Liposomes with Hydrophobic Content

Nile Red (NR) was used as a hydrophobic model molecule to observe uptake of PA integrated and bare liposomes**.** The hydrophobic dye encapsulation was performed after liposomes were prepared. Since NR is a hydrophobic dye and tends to interact with lipids rather than dissolving in aqueous environment. Hydrophobic dye hardly dissolved in water and liposomes provide a suitable environment for NR dye to embark. After loading process, in order to analyze amounts of loaded NR, free NR was firstly removed and encapsulated material was quantified by fluorescence spectroscopy along with calibration curve, after destruction of liposomes in ethanol. Due to slightly lower polarity and larger lipophilic surface area, amphiphilic peptide integrated liposomes entrapped four times higher amount of NR with respect to the bare liposomes (Table 3).

The number of encapsulated dye per liposome was found to be 7.9×10^5 and 3.13×10^6 for bare and peptide integrated liposomes, respectively. Since modified liposomes have larger diameter and eventually much more lipids on bilayer structure, it was expected for CPP modified liposomes would have higher NR carriage capacity.

Table 3 Encapsulation of tracking dyes by liposomes

Liposome Formulations	Encapsulated Nile Red (μM)	Number of Nile Red Molecule/ Liposome
DOPG:Chol	150.4±5.2	7.90×10^5
DOPG:Chol:PA	176.7±6.7	31.3×10^5

Several anticancer drugs including Paclitaxel (PTX), Cyclosporine, and Amphotericin B have hydrophobic properties and are absorbed in the membrane of liposome in addition to its lumen.[101] Therefore, NR is a hydrophobic fluorescent dye and is a suitable model molecule to track the behavior of the lipophilic cargo in liposomes. In order to track uptake efficiency of liposomes MCF7 cells were treated with free NR or native or PA integrated liposomes for 3 h. Liposomes and dyes, which were not uptaken by cells, were removed via several washing steps. Then fluorescent images were taken with equal imaging parameters such as exposure time and gain. Fluorescent images indicated that hydrophobic cargo delivery rates of DOPG:Chol liposomes were slightly improved upon peptide modification in contrast to unmodified liposome. (Figure 9) In order to observe dose dependent improvement facilitated by peptide modification, we also used two different modified liposomes that bears different amount of peptides. Images also indicated that liposomes modified with high amount of amphiphilic CPP performed slightly better than liposomes modified with relatively low amount of amphiphilic cell penetrating peptides.

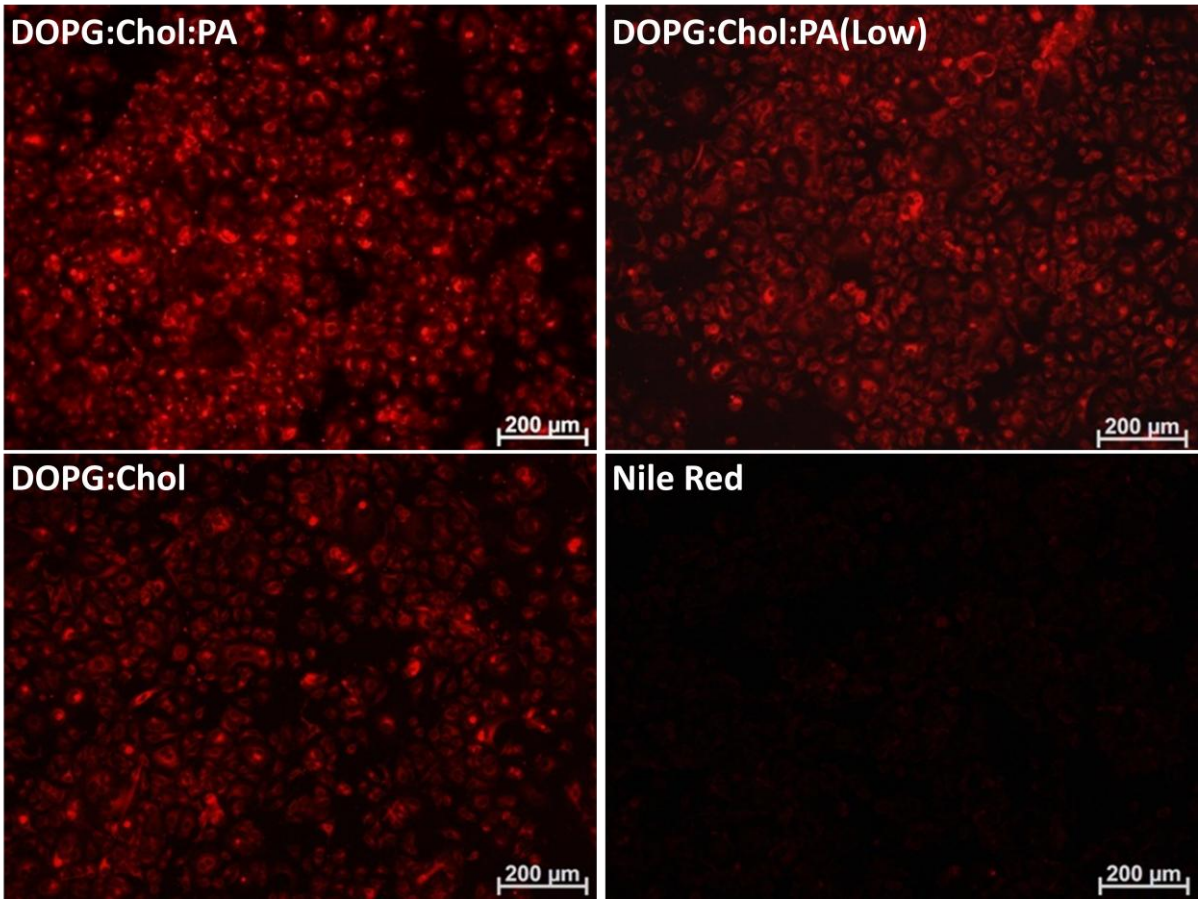

Figure 9 Uptake of 10 µM Nile Red by MCF7 cells. NR was administrated in free or liposome encapsulated form for 3 h. Fluorescent microscopy images of cells following liposomal DOPG:Chol:PA, DOPG:Chol:PA (Low), DOPG:Chol, and free Nile Red administration.

In addition to fluorescent images we also quantified amounts of dye internalized after 3 h of treatments. Uptake levels were quantified by NR extraction method, where non internalized liposomes were washed away and cells were lysed with ethanol. Since ethanol provide appropriate polar environment, Nile red dye becomes fluorescently active and can be measured with fluorescence spectroscopy. Quantification analysis revealed that PA integrated DOPG:Chol liposomes demonstrated enhanced uptake compared to bare liposomes (Figure 10).

Difference between liposomes with high and low amounts of CPP modification was also observed here. Uptake level of free Nile Red was significantly lower than that of both PA modified and bare liposomes. Uptake levels of each group were significantly different from each other, consistent with uptake images shown above. Considering the number of NR per liposome values, higher encapsulation capacity of PA integrated liposomes enables administration of less amount of liposome compared to bare liposomes for delivering equal concentration of Nile Red (Table 3). Thus, our results suggested that PA integration enhanced the hydrophobic Nile Red uptake not only by increasing the encapsulation capacity of DOPG:Chol liposomes but also by enhancing liposome internalization.

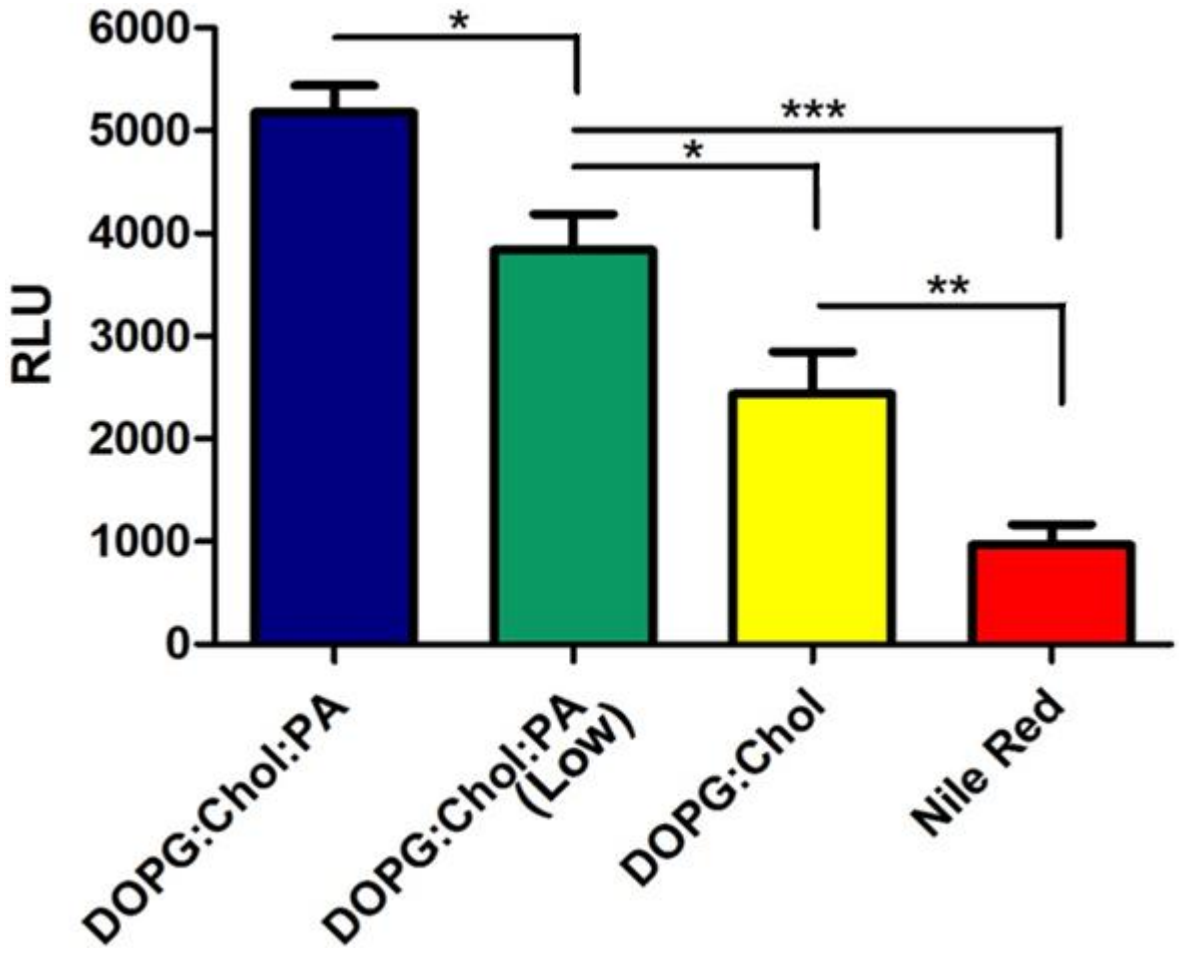

Figure 10 Uptake quantification of 10 μM Nile Red by MCF7 cells. NR was administered in free or liposome encapsulated form for 3 h. DOPG:Chol:PA and DOPG:Chol:PA(Low) differs in terms of amount of CPP incorporated. Uptake levels quantified via ethanol lysis method prior to fluorescence spectroscopy.

2.3.3 Release Dynamics of Liposomes

To understand the effect of PA integration on *in vitro* release of the entrapped Rhodamine B, liposomes suspended in 10% FBS containing PBS medium were dialyzed at pH 5.5 and pH 7.4 against PBS at 37 °C. The rationale underlying use of different pH s is to mimic different environments within body. pH 7.4 represents the blood circulation and extracellular environment, where it is better for liposomes to keep their content. pH 5.5 represents acidic intracellular environment that liposomes are expected to release their contents there. As shown in figure 9, DOPG:Chol:PA liposomes were stable for over 72 h with no apparent release (< 2%), while DOPG:Chol liposomes released 7.5% of the encapsulated RHB. On the other hand, both modified and non modified liposome formulations released just above 15 % of their content at pH 5.5.

These results indicated that both formulations showed stability at both pH conditions and in serum containing PBS medium. Yet around 20 % of NR was released when intracellular environment was mimicked. We hypothesize that further NR integration into cells from liposomes occurs via lysis and destruction of liposome within cell. Due to its hydrophobicity and low solubility in water, we did not measure the release dynamics of Nile Red loaded liposomes.

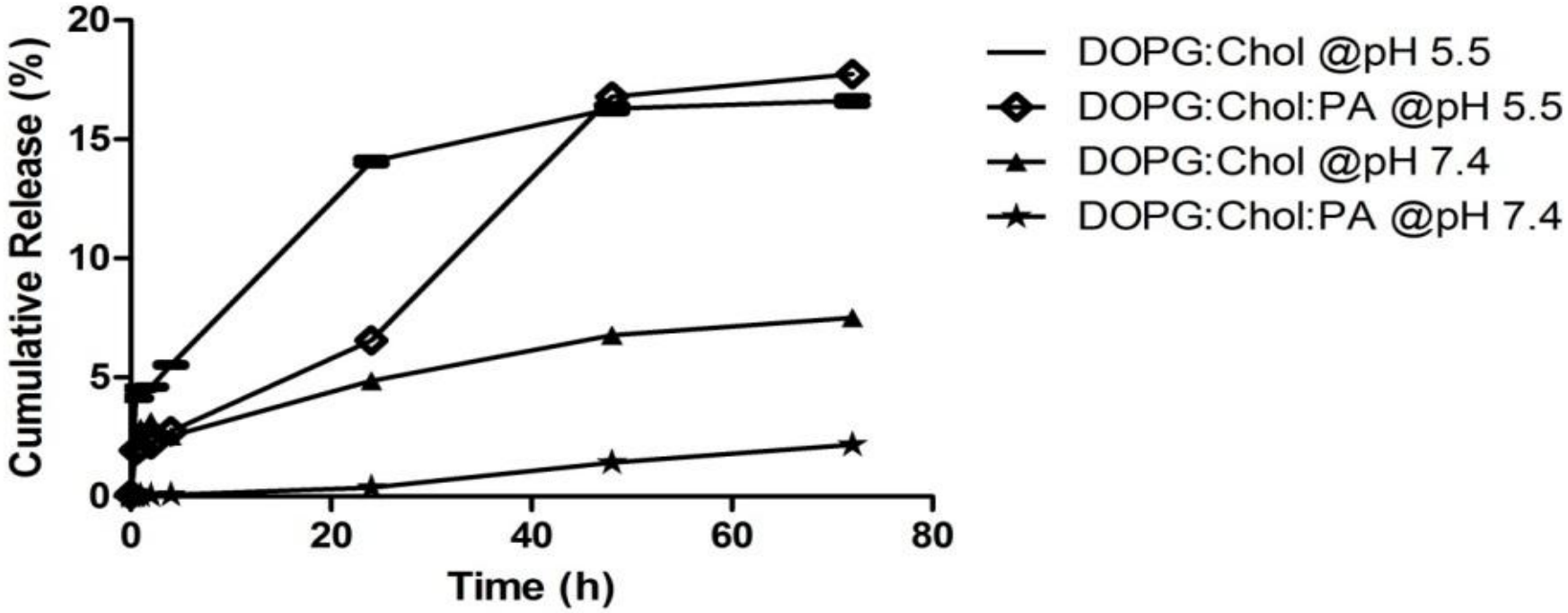

Figure 11 *In vitro* release profile of DOPG:Chol and DOPG:Chol:PA liposomes at pH 7.4 and pH 5.5. Both liposome formulations were stable at physiological condition while they both showed slow release slightly triggered by acidic pH.

2.3.4 Therapeutic Effects of CPP Modified Liposomes

2.3.4.1 Encapsulation of Doxorubicin and Paclitaxel

After observing the improvement in terms of uptake, the effect of arginine-rich cell penetrating peptide integration on liposomal delivery was investigated by using two well-known cancer drugs, hydrophilic Doxorubicin-HCl (DOX) and hydrophobic Paclitaxel (PTX). For DOX and PTX encapsulations, 1:25 and 1:15 (w/w) liposomal content to drug ratio were used, respectively. Both liposome formulation and the liposome preparation methods are important parameters affecting the encapsulation capacity.[102-103] Therefore, effect of peptide integration on encapsulated DOX amount was determined by using fluorescence spectroscopy, and PTX carrying liposomes were analyzed by HPLC to quantify entrapment efficiency. Results are presented in Table 4 and Table 5.

Doxorubicin is a hydrophilic anticancer drug that is commonly used as cancer therapeutics. Since it is highly toxic and highly diffusible, proper delivery is essential. When we loaded DOX into native and PA integrated liposomes, amounts encapsulated were 0.26 mM and 0.23 mM, respectively. Since similar methods with Rhodamine B encapsulation were used, similar drug load per liposome was observed when we compared modified and non-modified liposomes.

Paclitaxel is a well known hydrophobic drug, which is currently used as dissolved in 50:50 (v/v) mixture of Cremophor® and ethanol. Drawbacks of current formulation, such as Cremophor® associated serious side effects and possible precipitation of PTX in aqueous media, requires development of new carrier systems with high encapsulation efficiency.[104] Similar to Nile Red encapsulation results, PA integrated liposomes showed higher encapsulation efficiency for PTX compared to bare liposomes.

Table 4 DOX encapsulation capacities

Liposome Formulation	Encapsulated DOX (mM)	Dox # per Liposome	Encapsulation Efficiency (%)
DOPG:Chol	$0.258 \pm 2.6 \times 10^{-4}$	1.36×10^{3}	75.0
DOPG:Chol:PA	$0.227 \pm 1.2 \times 10^{-3}$	4.03×10^{3}	65.9

Table 5 PTX encapsulation capacities

Liposome Formulation	Encapsulated PTX (mM)	PTX # per Liposome	Encapsulation Efficiency (%)
DOPG:Chol	0.092	4.85×10^{5}	49.33
DOPG:Chol:PA	0.117	20.7×10^{5}	62.6

Larger size and eventually larger membrane of PA integrated liposome membrane compared to bare liposomes might be the reason for enhanced encapsulation of PTX. The concentration of encapsulated Paclitaxel was found to be 92 μM in native liposomes and 117 μM in PA integrated liposomes (Table 5). When the number of encapsulated molecules per liposome for each drug was calculated, the PA integrated liposomes showed superior encapsulation efficiency over bare liposomes, as table 4 and 5 demonstrates.

2.3.4.2 Therapeutic Effects of Doxorubicin Loaded Liposomes

In vitro therapeutic effects of anticancer drug loaded PA integrated and PA free DOPG:Chol liposomes were evaluated via cytotoxicity assays and determination of cell proliferation rates by using MCF7 breast cancer cell line. The activity of Doxorubicin-HCl on tumor cells is mainly mediated by oxidative DNA damage and topoisomerase II inhibition in the nucleus, which results in apoptosis.[105] Here, dose response of DOX loaded DOPG:Chol liposomes and free DOX were evaluated by quantification of total metabolic activity of MCF7 cells after 24 h of exposure (Figure 12).

Half maximal inhibitory concentration (IC_{50}) values were calculated as 2.58 μM, 2.48 μM and 0.88 μM for DOX loaded DOPG:Chol, DOPG:Chol:PA liposomes and free drug. When used at low concentrations, liposomal DOX was previously shown to have lower toxicity compared to free form.[106] As we see from the graph, free doxorubicin performed better than liposomes within concentration ranges of 0.5 to 2.5 μM. This condition may seem contrary to results shown in Rhodamine B uptake (Figure 3) where free Rhodamine B uptake was significantly lower when compared to peptide modified liposomes. Yet, cytotoxicity is a combination of parameters much more than cellular uptake. Liposomal DOX system is taken into the cell by endocytosis and is slowly released to the cytoplasm, which results in increased

number of barriers and slower therapeutic effect in contrast to free DOX.[106-107]

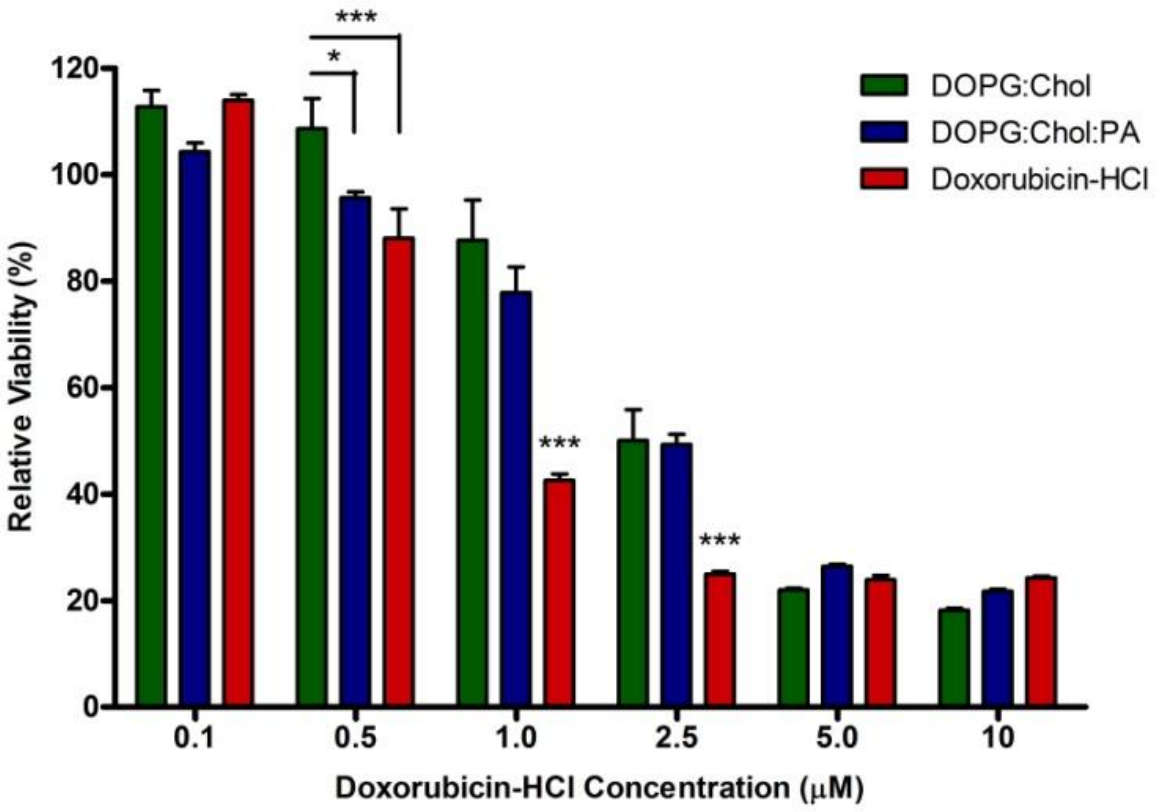

Figure 12 Dose response of MCF7 cells against free Doxorubicin-HCl and Doxorubicin-HCl loaded DOPG:Chol and DOPG:Chol:PA liposomes. After 24 h of exposure to Doxorubicin-HCl, viability of cells was measured by Alamar Blue. Results were normalized to untreated cells in PBS. (*** stands for $p < 0.001$, ** stands for $p < 0.01$, * stands for $p < 0.05$) (n=4)

Even though its uptake is less, free doxorubicin is able to diffuse directly into cell, and therefore it can demonstrate cytotoxic effect earlier. This condition is also consistent with the release dynamics of liposomes as shown in figure 9. According to release data, liposomes released just 20 percent of cargo. Overall, these results showed that increasing the concentration of DOX enhanced the effectiveness of liposome-DOX systems.

Following dose response studies, time dependent response of MCF7 cells to DOX treatment was evaluated at 1, 3 and 6 h exposure times (Figure 11). In this experiment, cells were treated with doxorubicin either in free or liposomal form for 1, 3 and 6 h, then incubated for 24 h without drug. We aimed to understand the effect of drug entered within given time periods only. As demonstrated in figure 10, 24 h exposure to drugs may not allow to analyze cytotoxic differences between delivery systems, since system is saturated with drugs and cytotoxicity levels were close to maximum achievable.

As expected, for both free and liposomal DOX systems, viability decreased with increasing exposure time. After 1 h of treatment, we observed that PA modified DOPG:Chol liposomes and free DOX had almost two fold lower viability with respect to PA free DOPG:Chol liposomes ($p < 0.001$). Higher cytotoxicity of PA integrated liposomal Doxorubicin-HCl demonstrated improvement in delivery efficiency caused by arginine-rich cell penetrating peptide incorporation into native liposomes. Bare liposomes exhibited a dramatic decrease in cell viability when exposure time was increased from 1 h to 3 h ($p < 0.001$) while PA integrated liposomes and free DOX did not show any significant difference. However, at 6 h of treatment, there was significant decrease in cell viability for PA integrated liposomal and free DOX ($p < 0.001$ for DOPG:Chol:PA and $p < 0.01$ for free Doxorubicin-HCl) compared to earlier time points. Statistically significant cell viability decrease was also observed for PA

free liposomes ($p < 0.05$) after 6 h of drug exposure compared to earlier time points.

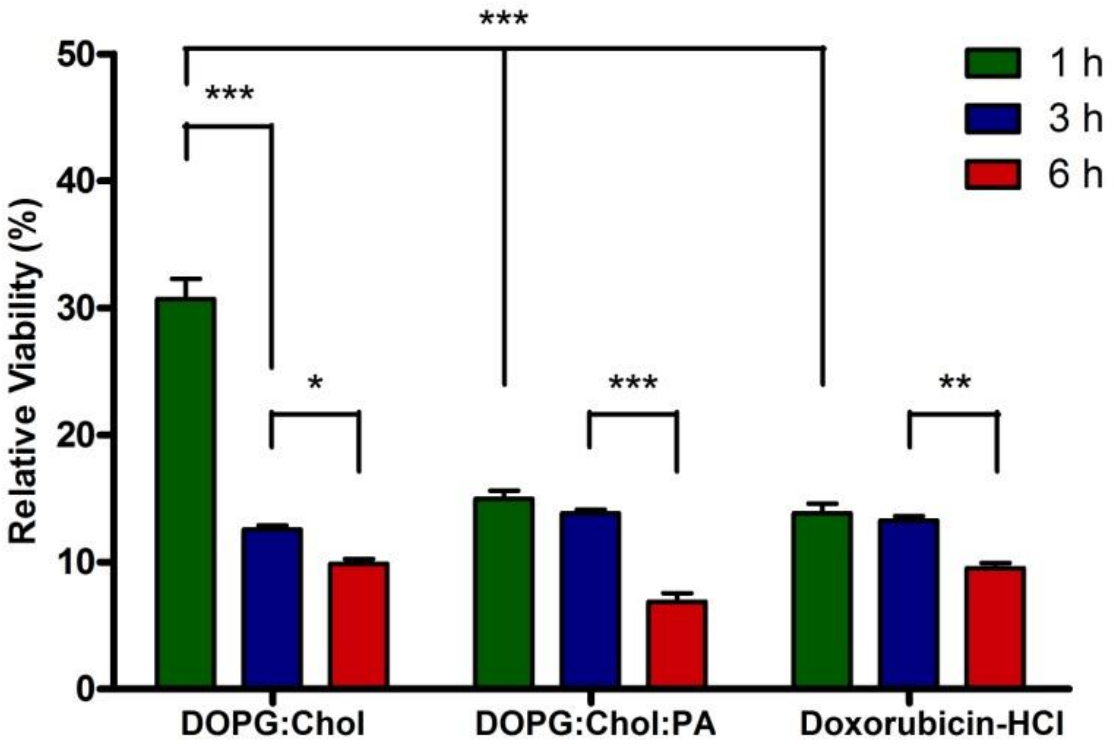

Figure 13 Time response of MCF7 cells to 1, 3 and 6 h of 10 μM free or liposomal Doxorubicin-HCl treatment. Following administration, cells were incubated in fresh media for further 24 h and viability of cells was measured. (*** stands for $p < 0.001$, ** stands for $p < 0.01$, * stands for $p < 0.05$) (n=4)

2.3.4.3 Therapeutic Effects of Paclitaxel Loaded Liposomes

Paclitaxel was used as a hydrophobic drug to examine *in vitro* therapeutic effect of liposome encapsulated and free drug. PTX mainly acts as G2/M cell cycle inhibitor and impedes cell proliferation by inhibiting microtubule dynamics.[108] Therefore it acts slowly, since it causes cell cycle arrest not as direct as doxorubicin which intercalates with DNA and disrupts central dogma. We investigated responses of MCF7 cells against free and liposomal PTX in both time and dose dependent manners by determining cell proliferation inhibition and fitness of the MCF7 cell culture. Dose dependent cytotoxicity studies demonstrated that PA integrated liposomes resulted in enhanced therapeutic effect at all doses from 0.2 nM to 2 μM of PTX ($p < 0.001$) compared to free PTX (Figure 12). Superiority of peptide modified liposomes was clear almost in every concentrations of PTX we administered.

As a result of 10 μM PTX administration with DOPG:Chol and DOPG:Chol:PA, viability of MCF7 cells was equally affected and decreased to about 50%. At this concentration, the inhibitory effect was two folds higher for both PA integrated and native liposomes than that of free PTX. The results revealed that both liposomes caused proliferation inhibition since average doubling time of MCF7 cells are 24 h and untreated cell number (control group) was doubled at given time.[109] These results show that enhanced cell growth inhibition via PA incorporated liposomes was observed for PTX treatment with various concentrations. In the case of free form, we did not observe consistent decrease in cell viability through increasing PTX concentration on treatment media. This condition is probably due to low solubility of PTX that is not capable of diffusing freely in the media beyond 200 nM. Of course it was possible to increase its solubility by using chemicals like DMF or DMSO, yet cytotoxic properties of these solvents are too significant to ignore.

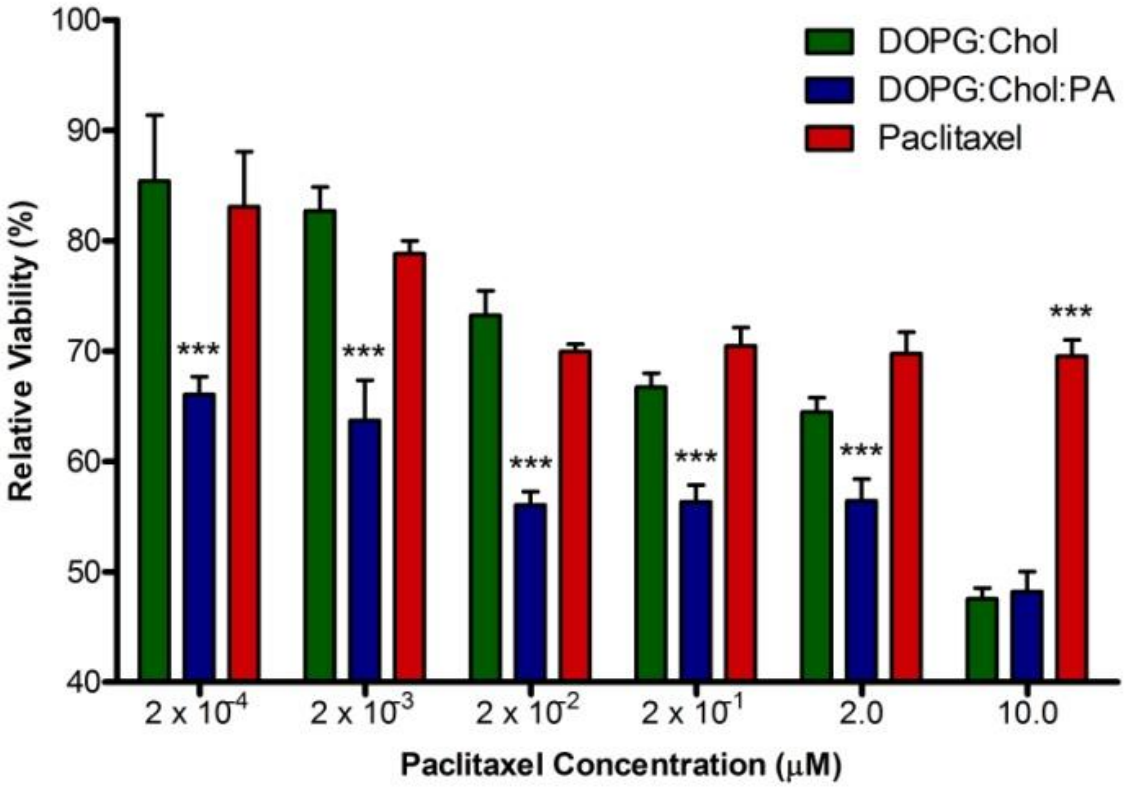

Figure 14 Dose response of MCF7 cells against free Paclitaxel and Paclitaxel loaded DOPG:Chol and DOPG:Chol:PA liposomes within spectrum of concentrations ranging from 0.2 nM to 10 μM final concentration. Subsequent to 24 h of exposure to Paclitaxel, viability of cells was measured. Cell proliferation in the presence of DOPG:Chol:PA liposomes was significantly lower ($p < 0.001$) than both DOPG:Chol liposome and free Paclitaxel at all Paclitaxel concentrations except 10 μM. At 10 μM Paclitaxel concentration, free Paclitaxel showed significantly lower effect compared to both DOPG:Chol and DOPG:Chol:PA liposomes ($p < 0.001$). (n=4)

In addition, response of MCF7 cells was evaluated by administering 30 μM of PTX at 1, 3 and 6 h. 24 h of drug free incubation was done to observe the effect of drug internalized only in given time periods. Results demonstrate that, peptide modified liposomes performed better in terms of inhibiting MCF7 cell culture after longer periods of treatment as shown in figure 14.

At longer liposomal PTX exposure, cell viability decreased by 25 percent in peptide integrated liposomes, while it did not change and remained at 75 percent in native liposomes. In the case of free Paclitaxel, therapeutic effect was not improved and the cell viability was close to native liposomes. Both native and PA modified liposomes had time dependent cytotoxic response. Cells treated with free paclitaxel did not respond to changing exposure time, probably due to low solubility. Overall, our results suggest that PA integrated liposomes provide a more efficient delivery method for Paclitaxel compared to PA-free liposomes and free drug.

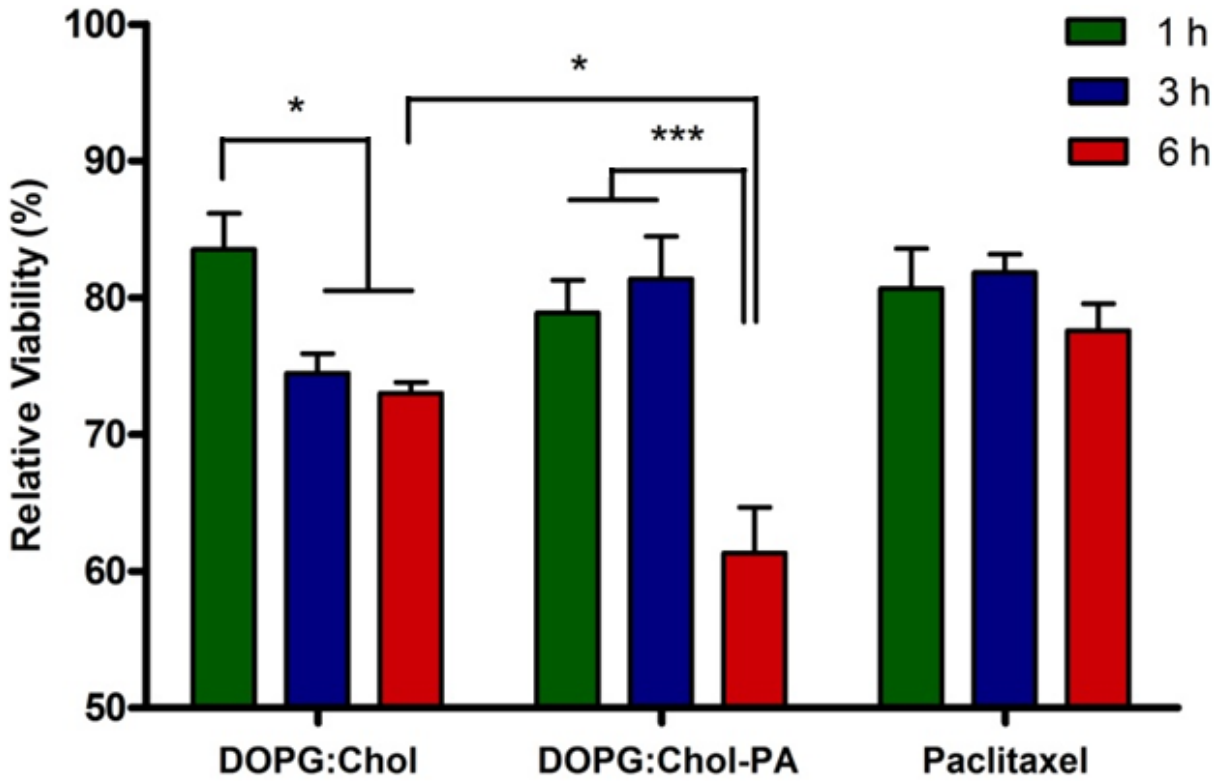

Figure 15 Time response of MCF7 cells to 1, 3 and 6 h of 30 μM free or liposomal Paclitaxel exposure. Cytotoxic effects of Paclitaxel loaded DOPG:Chol and DOPG:Chol:PA liposomes. All results were normalized to viability level of nontreated cells. (*** stands for $p < 0.001$, ** stands for $p < 0.01$, * stands for $p < 0.05$) (n=4)

2.4 CONCLUSION & FUTURE PERSPECTIVES

In summary, we detailed the development of a liposomal carrier system and its functionalization with cell penetrating arginine-rich peptide amphiphile molecules by the use of amphipathicity as an alternative functionalization method for chemical linkage. The resulting liposomes were found to be nontoxic in the absence of cargo and had high encapsulation capacities for both hydrophobic (Nile Red) and hydrophilic (Rhodamine B) dye models. We also showed that these liposomes had very slow *in vitro* release rates, which enable the sustained and prolonged exposure of a given target tissue to the drug cargo. Fluorescence spectroscopy measurements indicated that the integration of cell penetrating peptide amphiphiles onto the liposome surface enhanced the uptake of both hydrophobic and hydrophilic model reagents by MCF7 breast cancer cells with respect to both native liposomes and free reagents. In order to analyze the therapeutic potency of these liposomes in the delivery of common cancer drugs, Doxorubicin-HCl and Paclitaxel were loaded into liposomes and studied *in vitro*. Cytotoxicities of the drug-liposome complex were observed to depend on drug concentration. Time response studies showed that cell penetrating PA incorporation into DOPG:Chol liposomes improved liposomal delivery and enhanced the therapeutic effect of both hydrophilic (Doxorubicin-HCl) and hydrophobic (Paclitaxel) anticancer agents on MCF7 breast cancer cells. By using of this noncovalent functionalization technique, peptide epitopes can be easily incorporated into liposomal systems in a single step without requiring any additional chemical reagents, and the loss of activity normally associated with such modification methods is minimized by avoiding anchorage.

The use of cell penetrating peptide amphiphiles was the simplest type of modification, and only aimed to enhance basic drug uptake rates and the therapeutic effect. By utilizing this

method, we developed a simple production method to facilitate liposome synthesis and modification in a single step. By using this approach, many types of advanced liposome modifications can be utilized. As reviewed in the first section, a variety of materials, such as bioactive peptides and small molecules, were used to bestow tumor targeting and improved uptake capacities to liposomes. By utilizing this method, a variety of modifications with different characteristics can be utilized on the same delivery vector. As mentioned earlier, many types of cancer cells have high affinities to RGD peptide, due to the overexpression of various integrins. It follows that an amphiphilic peptide incorporating this sequence would be an efficient modification to augment liposomal tumor targeting capacities. Another potential application could be use of amphiphilic peptides conjugated with estrogen. Many cancer types overexpresses estrogen receptors, and therefore readilyprovide a distinctive targeting moiety. Liposomes modified with estrogen-PA would be efficient nanovesicles for these types of cancer. Many more examples can be provided. The fact that their sequences can be altered to suit specific applications, amphiphilic peptides is coveted as surface modification materials. It is possible to mimic a wide range of molecules by changing aminoacid sequences or attaching various functional groups to a base peptide sequence. By modifying a novel peptide with proper chemical groups, it is always possible to create new opportunities to improve cargo delivery or achieve high specific targeting efficiencies.

Overall, our strategy offers a high yield and efficient liposome modification method by which liposomes can be modified easily and efficiently with bioactive amphiphilic peptides. However, to fully evaluate the efficiency of our strategy, the targeting capacities of liposomes modified with different amphiphilic peptides need to be studied. *In vivo* studies can then be performed with the appropriate cancer model(s).

REFERENCES

1. Wang, B., et al., *Targeting of the non-mutated tumor antigen HER2/neu to mature dendritic cells induces an integrated immune response that protects against breast cancer in mice.* Breast Cancer Research, 2012. **14**(2): p. R39.
2. Trumpfheller, C., et al., *Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity.* Journal of Internal Medicine, 2012. **271**(2): p. 183-192.
3. Fornari, F.A., et al., *Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells.* Molecular Pharmacology, 1994. **45**(4): p. 649-656.
4. Momparler, R.L., et al., *Effect of Adriamycin on DNA, RNA, and Protein Synthesis in Cell-free Systems and Intact Cells.* Cancer Research, 1976. **36**(8): p. 2891-2895.
5. Brito, D.A., Z. Yang, and C.L. Rieder, *Microtubules do not promote mitotic slippage when the spindle assembly checkpoint cannot be satisfied.* The Journal of Cell Biology, 2008. **182**(4): p. 623-629.
6. Goodin, S., M.P. Kane, and E.H. Rubin, *Epothilones: Mechanism of Action and Biologic Activity.* Journal of Clinical Oncology, 2004. **22**(10): p. 2015-2025.
7. Malam, Y., M. Loizidou, and A.M. Seifalian, *Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer.* Trends in pharmacological sciences, 2009. **30**(11): p. 592-599.
8. Peer, D., et al., *Nanocarriers as an emerging platform for cancer therapy.* Nature Nanotechnology, 2007. **2**(12): p. 751-760.
9. Brannon-Peppas, L. and J.O. Blanchette, *Nanoparticle and targeted systems for cancer therapy.* Advanced Drug Delivery Reviews, 2004. **56**(11): p. 1649-1659.
10. Shenoy, D.B. and M.M. Amiji, *Poly(ethylene oxide)-modified poly(ε-caprolactone) nanoparticles for targeted delivery of tamoxifen in breast cancer.* International Journal of Pharmaceutics, 2005. **293**(1-2): p. 261-270.
11. Brigger, I., C. Dubernet, and P. Couvreur, *Nanoparticles in cancer therapy and diagnosis.* Advanced Drug Delivery Reviews, 2002. **54**(5): p. 631-651.
12. Varna, M., et al., *In Vivo Distribution of Inorganic Nanoparticles in Preclinical Models.* Journal of Biomaterials and Nanobiotechnology, 2012(3): p. 269-279.
13. Sanvicens, N. and M.P. Marco, *Multifunctional nanoparticles – properties and prospects for their use in human medicine.* Trends in Biotechnology, 2008. **26**(8): p. 425-433.
14. Kumari, A., S.K. Yadav, and S.C. Yadav, *Biodegradable polymeric nanoparticles based drug delivery systems.* Colloids and Surfaces B: Biointerfaces, 2010. **75**(1): p. 1-18.
15. Sharma, M., et al., *Folic acid conjugated guar gum nanoparticles for targeting methotrexate to colon cancer.* Journal of Biomedical Nanotechnology, 2013. **9**(1): p. 96-106.
16. Lu, Z., et al., *Paclitaxel-Loaded Gelatin Nanoparticles for Intravesical Bladder Cancer Therapy.* Clinical Cancer Research, 2004. **10**(22): p. 7677-7684.
17. Xu, Z.P., et al., *Inorganic nanoparticles as carriers for efficient cellular delivery.* Chemical Engineering Science, 2006. **61**(3): p. 1027-1040.
18. Kneuer, C., et al., *Silica nanoparticles modified with aminosilanes as carriers for plasmid DNA.* International Journal of Pharmaceutics, 2000. **196**(2): p. 257-261.
19. Kohler, N., G.E. Fryxell, and M. Zhang, *A Bifunctional Poly(ethylene glycol) Silane Immobilized on Metallic Oxide-Based Nanoparticles for Conjugation with Cell Targeting Agents.* Journal of the American Chemical Society, 2004. **126**(23): p. 7206-7211.
20. Chen, F., et al., *Functionalization of iron oxide magnetic nanoparticles with targeting ligands: their physicochemical properties and in vivo behavior.* Nanomedicine, 2010. **5**(9): p. 1357-1369.
21. Barenholz, Y., *Doxil® — The first FDA-approved nano-drug: Lessons learned.* Journal of Controlled Release, 2012. **160**(2): p. 117-134.
22. O'Brien, S., et al., *Phase II study of marqibo in adult patients with refractory or relapsed*

philadelphia chromosome negative (Ph-) acute lymphoblastic leukemia (ALL). Journal of Clinical Oncology, 2010. **28**(15).
23. Mita, A., et al., *A phase I pharmacokinetic (PK) study of MBP-426, a novel liposome encapsulated oxaliplatin.* Journal of Clinical Oncology, 2009. **27**(15).
24. Celsion, *Phase 3 study of thermoDox with RadioFrequency Ablation (RFA) in treatment of Hepatocellular Carcinoma (HCC).*
25. Kant, S., *A Complete Review On: Liposomes.* International journal of Pharmacy, 2012. **3**(7).
26. Battersby, B.J., et al., *Evidence for three-dimensional interlayer correlations in cationic lipid-DNA complexes as observed by cryo-electron microscopy.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1998. **1372**(2): p. 379-383.
27. Gabizon, A., et al., *Effect of Liposome Composition and Other Factors on the Targeting of Liposomes to Experimental Tumors: Biodistribution and Imaging Studies.* Cancer Research, 1990. **50**(19): p. 6371-6378.
28. Kirby, C., J. Clarke, and G. Gregoriadis, *Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro.* Biochem J, 1980. **186**(2): p. 591-8.
29. *The Effect of Cholesterol in the Liposome Bilayer on the Stabilization of Incorporated Retinol.* Journal of Liposome Research, 2005. **15**(3-4): p. 157-166.
30. Laouini, A., et al., *Preparation, Characterization and Applications of Liposomes: State of the Art.* Journal of Colloid Science and Biotechnology, 2012. **1**(2): p. 147-168.
31. Jass, J., T. Tjarnhage, and G. Puu, *From liposomes to supported, planar bilayer structures on hydrophilic and hydrophobic surfaces: an atomic force microscopy study*. Biophys J, 2000. **79**(6): p. 3153-63.
32. Palmer, A.F., P. Wingert, and J. Nickels, *Atomic force microscopy and light scattering of small unilamellar actin-containing liposomes.* Biophys J, 2003. **85**(2): p. 1233-47.
33. Du, B., et al., *Preparation, characterization and in vivo evaluation of 2-methoxyestradiol-loaded liposomes.* International Journal of Pharmaceutics, 2010. **384**(1–2): p. 140-147.
34. Nounou MM, E.-K.L., Khalafallah NA, Khalil SA, *In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels.* Acta Pharmaceutica, 2006. **56**(3): p. 311-324.
35. Paliwal, S.R., et al., *Liposomal nanomedicine for breast cancer therapy.* Nanomedicine, 2011. **6**(6): p. 1085-1100.
36. Swede H, M.K., Freudenheim JL, Quirk JT, Muti PC, Hurd TC, Edge SB, Winston JS, Michalek AM, *Breast cancer risk factors and HER2 over-expression in tumors.* Cancer Detection Prevention, 2001. **25**(6): p. 511-519.
37. Kirpotin, D.B., et al., *Antibody Targeting of Long-Circulating Lipidic Nanoparticles Does Not Increase Tumor Localization but Does Increase Internalization in Animal Models.* Cancer Research, 2006. **66**(13): p. 6732-6740.
38. Yang, T., et al., *Antitumor Effect of Paclitaxel-Loaded PEGylated Immunoliposomes Against Human Breast Cancer Cells.* Pharmaceutical Research, 2007. **24**(12): p. 2402-2411.
39. *Bioavailability and therapeutic efficacy of HER2 scFv-targeted liposomal doxorubicin in a murine model of HER2-overexpressing breast cancer.* Journal of Drug Targeting, 2008. **16**(7-8): p. 605-610.
40. Park, J.W., et al., *Anti-HER2 Immunoliposomes: Enhanced Efficacy Attributable to Targeted Delivery.* Clinical Cancer Research, 2002. **8**(4): p. 1172-1181.
41. Mamot, C., et al., *Epidermal Growth Factor Receptor–Targeted Immunoliposomes Significantly Enhance the Efficacy of Multiple Anticancer Drugs In vivo.* Cancer Research, 2005. **65**(24): p. 11631-11638.
42. Sapra, P., et al., *Improved Therapeutic Responses in a Xenograft Model of Human B Lymphoma (Namalwa) for Liposomal Vincristine versus Liposomal Doxorubicin Targeted*

via Anti-CD19 IgG2a or Fab' Fragments. Clinical Cancer Research, 2004. **10**(3): p. 1100-1111.
43. Hoeben, A., et al., *Vascular Endothelial Growth Factor and Angiogenesis.* Pharmacological Reviews, 2004. **56**(4): p. 549-580.
44. Verschraegen, C.F., et al., *Phase II study of bevacizumab with liposomal doxorubicin for patients with platinum- and taxane-resistant ovarian cancer.* Annals of Oncology, 2012.
45. Kuesters, G.M. and R.B. Campbell, *Conjugation of bevacizumab to cationic liposomes enhances their tumor-targeting potential.* Nanomedicine, 2010. **5**(2): p. 181-192.
46. Weinstein, S.J., et al., *Null Association between Prostate Cancer and Serum Folate, Vitamin B6, Vitamin B12, and Homocysteine.* Cancer Epidemiology Biomarkers & Prevention, 2003. **12**(11): p. 1271-1272.
47. *Antitumor effect of folate-targeted liposomal doxorubicin in KB tumor-bearing mice after intravenous administration.* Journal of Drug Targeting, 2011. **19**(1): p. 14-24.
48. Zhang, Z. and J. Yao, *Preparation of Irinotecan-Loaded Folate-Targeted Liposome for Tumor Targeting Delivery and Its Antitumor Activity.* AAPS PharmSciTech, 2012. **13**(3): p. 802-810.
49. CK, O., *Steroid Hormone Receptorsin Breast Cancer Managment.* Breast Cancer Research Treatment, 1998. **51**(3): p. 227-238.
50. SR Paliwal, R.P., N Mishra, A Mehta, SP Vyas, *A Novel Cancer Targeting Approach Based on Estrone Anchored Stealth Liposome for Site-Specific Breast Cancer Therapy.* Current Cancer Drug Targets, 2011. **10**(3): p. 343-353.
51. Paliwal, S.R., et al., *Estrogen-Anchored pH-Sensitive Liposomes as Nanomodule Designed for Site-Specific Delivery of Doxorubicin in Breast Cancer Therapy.* Molecular Pharmaceutics, 2011. **9**(1): p. 176-186.
52. Crichton, R.R. and M. Charloteaux-Wauters, *Iron transport and storage.* European Journal of Biochemistry, 1987. **164**(3): p. 485-506.
53. Cavanaugh PG, J.L., Zou Y, Nicolson GL, *Transferrin receptor overexpression enhances transferrin responsiveness and the metastatic growth of a rat mammary adenocarcinoma cell line.* Breast Cancer Research Treatment, 1999. **56**(3): p. 203-217.
54. Yue, J., et al., *Transferrin-Conjugated Micelles: Enhanced Accumulation and Antitumor Effect for Transferrin-Receptor-Overexpressing Cancer Models.* Molecular Pharmaceutics, 2012. **9**(7): p. 1919-1931.
55. Wu J, L.Y., Lee A, Pan X, Yang X, Zhao X, Lee RJ, *Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil.* Journal of Pharmacological Sciences, 2007. **10**(3): p. 350-357.
56. Herrera-Gayol, A. and S. Jothy, *Effect of Hyaluronan on Xenotransplanted Breast Cancer.* Experimental and Molecular Pathology, 2002. **72**(3): p. 179-185.
57. Eliaz, R.E. and F.C. Szoka, *Liposome-encapsulated Doxorubicin Targeted to CD44: A Strategy to Kill CD44-overexpressing Tumor Cells.* Cancer Research, 2001. **61**(6): p. 2592-2601.
58. Jiang, T., et al., *Dual-functional liposomes based on pH-responsive cell-penetrating peptide and hyaluronic acid for tumor-targeted anticancer drug delivery.* Biomaterials, 2012. **33**(36): p. 9246-9258.
59. Yerushalmi, N. and R. Margalit, *Hyaluronic Acid-Modified Bioadhesive Liposomes as Local Drug Depots: Effects of Cellular and Fluid Dynamics on Liposome Retention at Target Sites.* Archives of Biochemistry and Biophysics, 1998. **349**(1): p. 21-26.
60. Sebastian Taetz, A.B., Claudio Surace, Silvia Arpicco, Jack-Michel Renoir, Ulrich Schaefer, Véronique Marsaud, Saadia Kerdine-Roemer, Claus-Michael Lehr, Elias Fattal, *Hyaluronic Acid-Modified DOTAP/DOPE Liposomes for the Targeted Delivery of Anti-Telomerase siRNA to CD44-Expressing Lung Cancer Cells.* Oligonucleotides, 2009. **19**(2).
61. Taherian, A., et al., *Differences in integrin expression and signaling within human breast cancer cells.* BMC Cancer, 2011. **11**(1): p. 293.

62. Xiong, X.-B., et al., *Enhanced Intracellular Uptake of Sterically Stabilized Liposomal Doxorubicin in Vitro Resulting in Improved Antitumor Activity in Vivo.* Pharmaceutical Research, 2005. **22**(6): p. 933-939.
63. Frankel, A.D. and C.O. Pabo, *Cellular uptake of the tat protein from human immunodeficiency virus.* Cell, 1988. **55**(6): p. 1189-1193.
64. Green, M. and P.M. Loewenstein, *Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein.* Cell, 1988. **55**(6): p. 1179-1188.
65. Koren, E. and V.P. Torchilin, *Cell-penetrating peptides: breaking through to the other side.* Trends in Molecular Medicine, 2012. **18**(7): p. 385-393.
66. Herbig, M.E., et al., *Membrane Surface-Associated Helices Promote Lipid Interactions and Cellular Uptake of Human Calcitonin-Derived Cell Penetrating Peptides.* Biophysical Journal, 2005. **89**(6): p. 4056-4066.
67. Wadia, J.S., *Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis.* Nature Medicine, 2004. **10**: p. 310-315.
68. Zhao, M., et al., *Differential Conjugation of Tat Peptide to Superparamagnetic Nanoparticles and Its Effect on Cellular Uptake.* Bioconjugate Chemistry, 2002. **13**(4): p. 840-844.
69. Bogoyevitch, M.A., *Taking the Cell by Stealth or Storm? Protein Transduction Domains (PTDs) as Versatile Vectors for Delivery.* DNA and Cell Biology, 2002. **21**(12): p. 879-894.
70. Löwik, D., *Non-covalent stabilization of a beta-hairpin peptide into liposomes.* Organic Biomolecular Chemistry, 2003. **1**(11): p. 1827-1829.
71. Woodle, M.C., *Sterically stabilized liposome therapeutics.* Adv. Drug Deliv. Rev., 1995. **16**(2): p. 249-265.
72. Baldeschwieler, J.D. and P.G. Schmidt, *Liposomal drugs: From setbacks to success.* Chemtech, 1997. **27**(10): p. 34-42.
73. Rezler, E.M., et al., *Targeted Drug Delivery Utilizing Protein-Like Molecular Architecture.* Journal of the American Chemical Society, 2007. **129**(16): p. 4961-4972.
74. Kale, A.A. and V.P. Torchilin, *Design, Synthesis, and Characterization of pH-Sensitive PEG−PE Conjugates for Stimuli-Sensitive Pharmaceutical Nanocarriers: The Effect of Substitutes at the Hydrazone Linkage on the pH Stability of PEG−PE Conjugates.* Bioconjugate Chemistry, 2007. **18**(2): p. 363-370.
75. Yoshina-Ishii, C., et al., *General Method for Modification of Liposomes for Encoded Assembly on Supported Bilayers.* Journal of the American Chemical Society, 2005. **127**(5): p. 1356-1357.
76. Rezler, E.M., et al., *Targeted drug delivery utilizing protein-like molecular architecture.* J. Am. Chem. Soc., 2007. **129**(16): p. 4961-4972.
77. Sihorkar, V. and S. Vyas, *Potential of polysaccharide anchored liposomes in drug delivery, targeting and immunization.* J. Pharm. Pharm. Sci, 2001. **4**(2): p. 138-158.
78. D'Errico, G., A.M. D'Ursi, and D. Marsh, *Interaction of a peptide derived from glycoprotein gp36 of feline immunodeficiency virus and its lipoylated analogue with phospholipid membranes.* Biochemistry, 2008. **47**(19): p. 5317-5327.
79. Garg, A., et al., *Targeting colon cancer cells using PEGylated liposomes modified with a fibronectin-mimetic peptide.* Int. J. Pharm., 2009. **366**(1): p. 201-210.
80. Tu, R., K. Mohanty, and M. Tirrell, *Liposomal targeting through peptide-amphiphile functionalization.* American Pharmaceutical Review, 2004. **7**: p. 36-48.
81. Yu, Y.-C., et al., *Structure and dynamics of peptide-amphiphiles incorporating triple-helical proteinlike molecular architecture.* Biochemistry, 1999. **38**(5): p. 1659-1668.
82. Rezler, E.M., et al., *Peptide-mediated targeting of liposomes to tumor cells.* Methods In Molecular Biology, 2007. **386**: p. 269-298.
83. Kokkoli, E. *Engineering Biomimetic Peptides for Targeted Drug Delivery*. in *Frontiers of Engineering: Reports on Leading-Edge Engineering from the 2010 Symposium*. 2011:

National Academy Press.
84. Magzoub, M. and A. Graslund, *Cell-penetrating peptides: smallfrom inception to application.* Q .Rev. Biophys., 2004. **37**(2): p. 147-95.
85. Pham, W., et al., *Enhancing membrane permeability by fatty acylation of oligoarginine peptides.* ChemBiochem, 2004. **5**(8): p. 1148-1151.
86. Genc, R., M. Ortiz, and C.K. O' Sullivan, *Curvature-Tuned preparation of nanoliposomes.* Langmuir, 2009. **25**(21): p. 12604-12613.
87. Wang, F., et al., *Folate-mediated targeted and intracellular delivery of paclitaxel using a novel deoxycholic acid-O-carboxymethylated chitosan–folic acid micelles.* Int. J. Nanomed., 2012. **7**: p. 325-337.
88. García, M., et al., *Liposomes as vehicles for the presentation of a synthetic peptide containing an epitope of hepatitis A virus.* Vaccine, 1999. **18**(3): p. 276-283.
89. Deshayes, S., et al., *Structural polymorphism of non-covalent peptide-based delivery systems: highway to cellular uptake.* BBA-Biomembranes, 2010. **1798**(12): p. 2304-2314.
90. Boato, F., et al., *Synthetic Virus-Like Particles from Self-Assembling Coiled-Coil Lipopeptides and Their Use in Antigen Display to the Immune System.* Angew. Chem. Int. Edit., 2007. **119**(47): p. 9173-9176.
91. Torchilin, V.P., *Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery.* Peptide Science, 2008. **90**(5): p. 604-610.
92. Yanasarn, N., B.R. Sloat, and Z. Cui, *Negatively Charged Liposomes Show Potent Adjuvant Activity When Simply Admixed with Protein Antigens.* Mol. Pharm., 2011. **8**(4): p. 1174-1185.
93. Genç, R.k., M. Ortiz, and C.K. O' Sullivan, *Curvature-Tuned Preparation of Nanoliposomes.* Langmuir, 2009. **25**(21): p. 12604-12613.
94. Genç, R.k., et al., *Green Synthesis of Gold Nanoparticles Using Glycerol-Incorporated Nanosized Liposomes.* Langmuir, 2011. **27**(17): p. 10894-10900.
95. De Meyer, F. and B. Smit, *Effect of cholesterol on the structure of a phospholipid bilayer.* Proceedings of the National Academy of Sciences, 2009. **106**(10): p. 3654-3658.
96. Feitosa, E., et al., *Cationic Liposomes in mixed didodecyldimethylammonium bromide and dioctadecyldimethylammonium bromide aqueous dispersions studied by differential scanning calorimetry, nile red fluorescence, and turbidity.* Langmuir, 2006. **22**(8): p. 3579-3585.
97. Huth, U.S., R. Schubert, and R. Peschka-Süss, *Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging.* J. Control. Release, 2006. **110**(3): p. 490-504.
98. Kikkeri, R., et al., *Design, synthesis and biological evaluation of carbohydrate-functionalized cyclodextrins and liposomes for hepatocyte-specific targeting.* Org. Biomol. Chem., 2010. **8**(21): p. 4987-4996.
99. Machy, P. and L.D. Leserman, *Small liposomes are better than large liposomes for specific drug delivery in vitro.* BBA-Biomembranes, 1983. **730**(2): p. 313-320.
100. Lee, J.S. and C.-H. Tung, *Lipo-oligoarginines as effective delivery vectors to promote cellular uptake.* Mol. Biosyst., 2010. **6**(10): p. 2049-2055.
101. Sharma, A. and U.S. Sharma, *Liposomes in drug delivery: progress and limitations.* Int. J. Pharm., 1997. **154**(2): p. 123-140.
102. Tardi, P.G., N.L. Boman, and P.R. Cullis, *Liposomal doxorubicin.* J. Drug Target., 1996. **4**(3): p. 129-140.
103. Duzgunes, N., *Liposomes*. 2004: Academic Press.
104. Chung, S.-J., C.-K. Shim, and D.-D. Kim, *Enhanced solubility and stability of PEGylated liposomal paclitaxel: in vitro and in vivo evaluation.* Int. J. Pharm., 2007. **338**: p. 317-326.
105. Pan, L., et al., *Nuclear-Targeted Drug Delivery of TAT Peptide-Conjugated Monodisperse Mesoporous Silica Nanoparticles.* J. Am. Chem. Soc., 2012. **134**(13): p. 5722-5725.
106. Hussain, S., et al., *Antitumor activity of an epithelial cell adhesion molecule–targeted*

nanovesicular drug delivery system. Mol.Cancer Ther., 2007. **6**(11): p. 3019-3027.
107. Torchilin, V.P., *Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery.* Peptide Science, 2008. **90**(5): p. 604-610.
108. Zhang, S., Q.-C. Zhang, and S.-J. Jiang, *Effect of trichostatin A and paclitaxel on the proliferation and apoptosis of lung adenocarcinoma cells.* Chinese Med. , 2013. **126**(1): p. 129-134.
109. Sutherland, R.L., R.E. Hall, and I.W. Taylor, *Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells.* Cancer Res., 1983. **43**(9): p. 3998-4006.