MULTIFUNCTIONAL NANOCARRIERS FOR ENHANCED TUMOR DELIVERY

Doctoral Thesis Presented
By

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ABSTRACT

A tumor is an abnormally growing mass of cells. Ordinary chemotherapeutic agents used to prevent tumor growth produce more side effects and less than desired therapeutic effect. Targeted drug-delivery systems are necessary to provide and maintain an optimum drug concentration in target tissues and cells. Long-circulating nanoparticles have the ability to passively and actively target tumors and deliver the anticancer drugs. Passive targeting can be achieved by prolonged circulation and retention of the drug-loaded nanoparticles in the tumor, via enhanced permeability and retention (EPR) effect. Active targeting is possible by attaching various ligands to the nanoparticles to specifically target the tumors. These multifunctional particles help in improving the pharmacokinetic profile of the drug, improve the drug bioavailability and reduce undesired side-effects in the non-target organs. The main long-term goal of this project is the development of multifunctional micelles and liposomes that increase the delivery of anticancer drugs to the tumor.

Passive targeting of nanoparticles can be achieved by using polyethylene glycol-phosphatidylethanolamine (PEG-PE) coating to prevent uptake of the nanoparticles by macrophages and make the particle long-circulating. For active targeting, several moieties can be attached to the particle surface. Anti-nucleosome monoclonal antibody 2C5 recognizes a broad variety of tumor cells via the tumor cell surface-bound nucleosomes. It can recognize murine and human tumor cells, but not normal cells. Cell-penetrating peptides (CPPs) have shown enhanced transport of cargoes through the plasma membrane into the cells. Stimuli-sensitive PEG-hydrazone (Hz)-PE conjugate
(with a low pH-sensitive hydrazone bond between PEG and PE) which is stable at normal pH and can shield the CPP function.

In this study, micelles and liposomes were modified with a CPP-PEG-PE conjugate to improve the transport of these nanocarriers into the cells. Since CPPs are susceptible to enzymatic degradation and non-specific cellular interactions, they were shielded by the PEG-Hz-PE conjugate. At normal pH (as found in circulation), the PEG-Hz-PE shielded the CPP and at low pH (as found in tumors), when this polymer underwent hydrolysis, PEG detached to expose the CPP and increased the internalization of the micelles and liposomes. CPP modified pH-sensitive micelles loaded with anticancer drug paclitaxel, improved tumor cell association and cytotoxicity in vitro. Intratumor injections of these micelles demonstrated apoptosis in tumors developed subcutaneously in mice and established this proof of concept.

Liposomes were further modified with mAb 2C5-PEG-PE. Recognition of the tumor cells during the first phase of delivery was imparted by the attached mAb 2C5. During the second phase of delivery, the exposure of the shielded TATp upon hydrolysis of pH-sensitive polymer at the slightly acidic pH of the tumor cell environment, improved the uptake of drug-loaded liposomes by tumor cells. Thus, tumor cell recognition and uptake of the liposomes was obtained in a controlled fashion. Moreover, the growth of subcutaneously developed drug-resistant and drug-sensitive tumors in nude mice was inhibited and substantial decrease in the mean tumor weights was obtained by intravenous injection of these drug-loaded multifunctional liposomes. In this way, nanoparticles can be modified with multiple functionalities to improve tumor targeting and enhance the effect of an anticancer agent.
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1. INTRODUCTION

1.1. STATEMENT OF THE PROBLEM

1.1.1. Rationale for target-specific delivery.

A major problem associated with the use of a drug is its tendency to distribute uniformly in the body. This usually leads to undesirable side-effects on otherwise healthy organs and tissues, possible inactivation of the drug and low bioavailability in the organ of interest [1]. As a result, a larger amount of drug is required to achieve the high local concentration needed for a therapeutic effect but will exacerbate the side effects.

Many targeted drug delivery systems have been developed to overcome these problems. Use of such delivery systems can reduce the quantity of the drug required, deliver the drug specifically to the targeted pathological site, improve therapeutic outcome and reduce the side effects.

Over the past few decades, nanoparticles have come into wide-spread practice as drug delivery systems. Some examples include polymeric and metallic nanoparticles, dendrimers, microcapsules, micelles and liposomes. They can be used for diagnosis, treatment and monitoring of cancer. Some nanoparticles that are commercially available/in clinical trials are summarized in the following table:

~ 1 ~
Table 1. *Nanoscaled systems for systemic cancer therapy* [2]

Micelles and liposomes have been much studied and have repeatedly been shown to provide significant *in vitro* and *in vivo* anti-tumor effects of the loaded drug.

1.1.2. Passive targeting of nanoparticles.

An important characteristic of the nanoparticles is their tailorability to suit the needs peculiar to a tumor’s physiology or other particular features that make the tumor targetable. However, while manipulating their physical characteristics, the effect of a nanoparticle on *in vivo* behavior needs to be taken into consideration [3]. Some of the important features of the tumor microenvironment include: its non-uniform arrangement of vasculature, hypoxia and high metabolic activity [4], [5]. The frequency of cell
division is very high compared to the adjoining tissues and delivery of oxygen and other nutrients is not adequate. To support its growth, tumor tissue recruits development of new blood vessels from the already existing ones, a process called angiogenesis [6]. The arrangement of these vessels is non-uniform and unorganized, with loops and trifurcations in the vessels. Endothelial cell junction gaps of 100-700 nm diameter increase the vascular leakiness [7], [8]. This ‘leaky vasculature’ enables nanoparticles such as micelles and liposomes with molecular weights of ≤ 40 KDa and sizes between 10-400 nm to extravasate into the tumor interstitium. This, in addition to the poor lymphatic drainage found in the tumors, leads to retention of the nanoparticles in the tumor, a phenomenon known as the ‘enhanced permeability and retention effect’[9], [10]. Passive targeting of the drugs is thus possible with these nanoparticles (Figure 1).

![Figure 1. Schematic of enhanced permeability and retention effect for passive targeting of nanoparticles.](image)

To accumulate optimally in the target area, micelles and liposomes should also circulate for an extended period of time in comparison to the free drug [11]. To make these nanoparticles long-circulating, coating them with PEG or “PEGylation” is necessary.
which can be achieved with PEG-PE, a conjugate of polyethylene glycol and phosphatidylethanolamine [12],[13]. The hydrophilic PEG organizes a tight network of water around the nanoparticle and prevents its recognition, uptake by macrophages and clearance by the RES (reticulo-endothelial system) organs such as liver, spleen and kidneys [14]. This “stealth” property imparted by the PEG makes the nanoparticle long-circulating [15], [16] and results in a 10-100 fold increased tumor accumulation compared to the non-PEGylated nanoparticle [11, 17-18]. Repeated passages through the tumor vasculature increase the half-life of the drug in the circulation.

1.1.3. Active targeting of nanoparticles.

To “actively” target nanoparticles towards the tumor tissue and enhance the effects of drugs delivered by these systems, several modalities can be attached to their outer surface. Cell penetrating peptides (CPPs) increase the intracellular penetration of these carriers [19]. However, these peptides are susceptible to enzymatic degradation and non-specific cellular interactions [20]. In order to confine their function to the tumor cell surface, CPPs can be shielded with a pH-sensitive polymer that degrades at the slightly more acidic extracellular pH of tumor cells. To impart a tumor recognition capability, various ligands and antibodies can be added to the surface of the nanoparticle [21-22].

In conclusion, an optimized multifunctional carrier should be long-circulating with attached moieties that can actively target the tumor cells to improve the delivery of the drug-containing carrier.
1.2. REVIEW OF THE LITERATURE.

1.2.1. Tumor physiology and lowered extracellular pH.

Tumor cells typically undergo rapid cell division which reduces the levels of oxygen and other nutrients to inadequate levels. The presence of hypoxia and low nutrient levels stimulates the cells to increase glycolysis for the production of ATP which leads to the generation of elevated amounts of lactic acid [23], [4]. Thus, intracellular proton concentration increases, and the affected cells respond by pumping out these excess protons. The ion pumps/ ion exchangers/ proton pumps present on the membrane are known to be particularly efficient in cancer cells and help in the removal of protons into the extracellular space [24], [4]. Tumor vasculature is abnormal and the blood flow distribution is therefore heterogeneous and relatively slow in some areas. This leads to the variable accumulation of protons and metabolic acids in the extracellular space [5].

All these factors contribute to the lowered pH of the tumor extracellular space (pHe). The range of pHe observed in tumors was 5.66-6.78 and depended upon the type of tumor and its location [25].

1.2.2. Micelles and liposomes for drug delivery to tumors

Ideally, pharmaceutical nanocarriers should be cheap, easy to prepare, biodegradable, biocompatible, non-toxic, non-immunogenic, small in size, high in drug loading capacity, show prolonged circulation and accumulate in the desired site [26]. Micelles and
liposomes can fulfill these criteria and provide a surface that can be modified for particular requirements of a pathological site.

Micelles are colloidal particles with a size in the range of 5-50 nm. Amphiphilic molecules that make up micelles self-assemble spontaneously in an aqueous environment where the hydrophobic fragment forms the core and hydrophilic fragment forms the corona of the micelle [27]. Since the core of the micelle is hydrophobic, poorly water soluble drugs can be encapsulated in the core and be protected from exposure to the aqueous phase, thus enabling enhanced drug delivery of water-insoluble drugs [28].

Micelles made from diacyllipids conjugated to PEG are more stable than the conventional amphiphilic polymeric micelles because of an increase in the hydrophobic interactions between the lipid chains of the micelle core (Figure 2). This contributes to the development of critical micelle concentration (CMC) values of $10^{-5}$ to $10^{-6}$ M for micelles prepared from diacyllipid-PEG conjugates [29] which are significantly lower (at least 100-fold) than conventional plain micelles. A low CMC denotes that micelle integrity will be maintained upon dilution in blood and hence will show good drug retention [30].

Modification of micelles with PEG makes them long-circulating and a high concentration of drug can be maintained in the blood circulation for a longer period of time. This also enhances drug targeting to areas with low blood supply or with low amount of surface antigen where a longer circulation time is essential for the drug activity [30-31].

An important characteristic of micelles is their delivery of drugs to tumors with a smaller vascular pore cut-off size when the larger liposomes are not able to deliver the drugs [32].
**Figure 2.** Self assembling PEG-PE micelles with (1) hydrophobic core, (2) hydrophilic corona, (3) nonpolar drugs solubilized within the micelle core, (4) polar drugs adsorbed on micelle surface, (5) substances with intermediate polarity distributed between the core and the corona [30].

Liposomes are phospholipid nanoparticles with a size range of 100-1000 nm and consist of a lipid bilayer with an aqueous core (**Figure 3**). Hydrophilic compounds can be entrapped in the core and hydrophobic compounds within the lipid bilayer, thus limiting the pharmaceutical’s side-effects and premature inactivation in the blood circulation [33]. They can also be made long-circulating by the incorporation of PEG chains on their surface [34].

Liposomes and micelles can be modified with a number of targeting moieties, including but not limited to antibodies and their Fab’ fragments, proteins/peptides, polysaccharides and low-molecular weight ligands for specific recognition and binding to a targeted site.
**Figure 3.** Structure of liposome with (1) hydrophilic drug encapsulated in the aqueous core and (2) hydrophobic drug entrapped in the lipid bilayer [30].

An example of successful clinical application of a PEG-coated liposome is Doxil®, a doxorubicin-loaded long-circulating PEGylated liposome. The half life of doxorubicin in this formulation is approximately 45 hrs in humans as compared to 10 hrs for free doxorubicin. Both, the clearance rate and the volume of distribution are low, with more than 90% of the drug remaining encapsulated while in the circulation [35]. The side-effects associated with free doxorubicin are strongly diminished which makes this formulation clinically useful.

Liposomes modified with TAT-peptide on the surface enhance delivery of drugs and genes in vivo [36]. Micelles can also be modified with cell-penetrating peptides. For example, drug-loaded micelles with TATp attached to the surface improved delivery to tumors and enhanced apoptosis of the tumor cells [37]. Monoclonal antibody 2C5/2G4 have been attached to liposomes and micelles to specifically target them to tumors and to ischemic myocardium [11, 38-44]. Folate receptor-targeted liposomes encapsulating anticancer drugs to target tumor cells have been prepared. pH-responsive micelles encapsulating anticancer drugs have been formulated as well [45-47]. In response to the low pH found in tumors, the pH-sensitive polymer degrades and the drug is released.
Various combinations of these targeting moieties can generate a multifunctional nanoparticle.

In this study, in order to obtain an improved drug delivery to tumors, micelles and liposomes have been developed with a cell-penetrating peptide, a pH-sensitive polymer and monoclonal antibody 2C5 on their surface.

1.2.3. *In vivo* longevity of nanoparticles.

1.2.3.1. Importance of PEG coating.

As stated before, PEGylation of nanoparticles increases their circulation time by preventing opsonization by macrophages and accumulation in RES organs. The other important characteristics of such PEGs include low toxicity and immunogenicity, high polymer chain flexibility and minimum interference with the biological properties of the drug. Since it is possible to modify only one group of the PEG chain to either add ligands or make any other conjugate, the resulting compound does not form cross-linked aggregates [30]. PEG is available in various molecular weights and the length of the head group can be selected according to specific needs.

Nanoparticles can be made long-circulating by addition of PEG since it remains stable in the circulation [34]. It is removed unchanged by filtration via the kidneys.

Studies have shown that plain, non-PEGylated liposomes follow saturable and non-linear kinetics whereas PEGylated liposomes follow dose-independent, non-saturable, log-linear kinetics which makes them better candidates for drug delivery [48-49].
1.2.3.2. PEGylation of liposomes.

Theoretically, the amount of PEG-lipid able to incorporate into vesicles is directly correlated with the membrane elasticity, in this case, the cholesterol content of the bilayers and the PEG chain length [50]. Leroux and co-workers showed that increasing the mol% of PEG (up to 15 mol %) did not increase the carrier’s size and did not influence their removal from the bloodstream [51].

It has been observed that PEG attached to the liposome surface reduces van der Waals forces of attraction and increases repulsive forces (steric and electrostatic) [52-53]. The conformation of PEG chains on the liposomal surface depends on the PEG chain length and on the density of PEG chains covering the nanoparticle surface [54] and [55]. A study of the interactions between proteins and PEGylated surfaces shows that a high surface PEGylation density is more important than the chain length in the prevention of protein interactions with the surface. Grafted PEG can assume two conformations (mushroom and brush) depending on the concentration of the surface PEG. At a low density of PEG (up to 4 mol %), the PEG chains of the same length will acquire the “mushroom” conformation. The same PEG molecules will have a “brush” conformation if the density of PEG chains on the nanoparticle is above 4 mol%. The “mushroom” conformation of PEGs results in contraction of PEG chains and increases the likelihood of creating “gaps” in the PEG shell, while the movements of PEG in “brush” configuration are more restricted. Previous experimental data indicates that a brush conformation provides the most effective opsonin repulsion [56]. With more overlap of the PEG chains and increasing PEG chain surface density, repulsion of proteins becomes
increasingly efficient [57]. Also, longer chains fill the gaps between less densely grafted PEG chains [58].

Increasing the molar ratios of methoxy PEG: phospholipid to 15 mol %, increases the circulation time of liposomes. However there is an upper limit to the amount of methoxy PEG-lipids that can be incorporated into lipid bilayers. Above this, mixed micelles are formed and the bilayer collapses [59].

Garbuzenko, et al have used scaling mathematical models to describe the effect of up to 25 mol% of PEG<sub>2k</sub>-DSPE in the presence or absence of cholesterol and the effect of phosphatidylcholine saturation on the size and the lipid bilayer packing of large unilamellar vesicles [60]. They concluded that when a matrix lipid (PC), cholesterol and PEG<sub>2k</sub>-DSPE were combined, the optimal biological stability of the vesicles was achieved at 7±2 mol % PEG-DSPE. Their results also clearly show that, with increased PEG-DSPE mol% a decrease in the packing parameter of the vesicles is obtained. However, the combination of phosphatidylcholine, cholesterol and grafted PEG maintains the vesicle as a lipid bilayer for higher mol % of PEG (as seen for PC: PEG-DSPC liposomes). These results correlate with the findings of Hristova and Needham, who incorporated 15 mol% PEG<sub>5k</sub>-lipid in phospholipid/cholesterol vesicles [50]. Furthermore, Allen and co-workers [61] have described and characterized liposomal formulations, containing DOPE/CHEMS with up to 20 mol% of mPEG. In order to increase the molar ratio of mPEG-lipid on the liposomal surface, they added, on top of the pre-formed vesicles already containing 10 mol% of mPEG-DSPE, an additional 10 mol% mPEG-DSPE in the form of micelles using the post-insertion technique. Approximately 20 mol% mPEG-DSPE was present in the outer leaflet of the liposomes,
confirming that a high mol% of PEG is achievable [61]. While some investigators find destabilization of lipid lamellar structures only after the incorporation of more than 30-40 mol% of mPEG-lipid, others have mentioned much lower amounts [59, 62-65]. An explanation for this wide disparity of PEG mol% ranges is probably the different carriers/techniques used for PEG surface incorporation.

1.2.3.3. Use of detachable PEG.

Despite the attractive properties of PEG, there are several drawbacks. Firstly, the PEGylated nanoparticles can accumulate in the tumors but if the drug is not released from this stable formulation at the required rate/amount; tumor cell killing is not possible. Secondly, for a nanoparticle taken up by endocytosis, the PEG coat may prevent endosomal escape and release of drug in the cytoplasm [30]. Therefore, detachable PEGs have been used as an alternative to avoid this problem. Here, the chemistry has been developed in such a way that the PEG is stable in the circulation, increases the half-life of the encapsulated drug and detaches from the nanoparticle just before it enters the tumor cell to obtain controlled drug release [66].

pH- cleavable bonds are used in polymers to make them detachable when pH changes. Typically, there is a drop in the pH from the normal value of 7.4 in systemic arterial blood to 6 or lower in extracellular fluid of a tumor cell and in endosomes, infarcts and inflammatory areas. Such a polymer is stable at normal pH but degrades at this low pH.
PEG-PE conjugates can be made pH-sensitive by introduction of cleavable bonds such as vinyl-esters, double esters and hydrazones which are stable at pH 7.4 but degrade at lower pH [67-68]. This destabilization may be due to swelling of the polymer, increase/decrease in the permeability of the polymer, degradation of the pH-sensitive bond or changes in the network of pH-sensitive bonds [69], [70].

Thus, different types of systems can be developed for drug delivery using pH-sensitive polymers containing PEG:

i. The pH-sensitive conjugate of a long chain PEG is incorporated in a drug-loaded nanoparticle. In this way, certain functions present on the surface of the nanoparticle are kept ‘hidden’ in the PEG coat and exposed only near the tumor cells where necessary.

ii. The drug is attached to the polymer by a pH-sensitive bond and, as the bond breaks, the attached drug is released.

iii. The drug is encapsulated in the polymer and is released when the polymer degrades at lower pH.

iv. The pH-sensitive polymers/ lipids undergo destabilization at the low pH of endosomes, fuse with the endosomal membrane and cause endosomal leakage that releases the encapsulated drug/DNA in the cytoplasm and prevents the lysosomal degradation of the carrier’s contents.
1.2.4. Ligands for active targeting

1.2.4.1. Anti-nucleosome monoclonal antibody, 2C5.

Nucleosomes are a complex of a DNA subunit and four pairs of histone proteins. They are the repeating subunits of chromatin. Apoptically dying tumor cells release nucleosomes which bind to the neighboring live tumor cells via their nucleosome-specific receptors.

These nucleosomes are the antigens for the anti-nucleosome autoantibody, 2C5 [71-72]. It is a monoclonal antibody belonging to the class of antibodies of the IgG2a isotype. It recognizes murine and human tumor cells through their surface-bound nucleosomes, with a very low affinity for the normal cells [71]. It inhibits tumor growth of both murine and human origin. Studies using mAb 2C5 alone demonstrated accumulation in various unrelated human tumors in vivo with a tumor-cell to normal-cell ratio ranging from 2.7 to 13.4 indicating higher tumor accumulation compared to normal cells [73]. Prophylactic and established tumor therapy studies have demonstrated significant tumor growth suppression in nude mice.

This mAb 2C5 can be conjugated to the surface of micelles or liposomes via incorporation of pre-formed 2C5-PEG-PE micelles [74]. In this method, pNP-PEG-PE (PEG-PE with the activated para nitrophenyl carbonyl group) bind to the amino group of mAb 2C5 to form 2C5-PEG-PE conjugate [74]. This conjugate forms micelles which are then incorporated via a post-insertion technique into preformed micelles and liposomes. This post-insertion technique has previously shown to yield a stable association of
proteins, peptides and other polymers with liposomes [75-76]. Approximately 70-80 mAb 2C5 molecules can be attached to a single liposome [22].

When used at a sub-therapeutic concentration, mAb 2C5 can be a targeting moiety for recognizing tumor cells. When attached to the surface of micelles or liposomes, it specifically targets the tumor cells and delivers drug locally, further minimizing non-specific interactions. The target of mAb 2C5 can be found on various types of tumors and is not restricted to a specific tumor antigen, which makes it a “universal” tumor-targeting antibody [71-72].

Previous studies have reported preparation of 2C5-conjugated Doxil using a sub-therapeutic amount of mAb 2C5 without any significant loss of doxorubicin [77]. This preparation had the ability to recognize nucleosomes and bound 3 to 8-folds greater to tumor cells in vitro compared to non-modified Doxil and IgG-Doxil (IgG was used as a non-specific antibody, which as expected showed no preferential attachment with the tumor cells). With both human and murine tumor cells, 5 to 8-folds higher IC50 values were obtained compared to Doxil alone [38, 78].

Although 2C5-Doxil demonstrated a 3-fold higher tumor accumulation compared to Doxil and IgG modified Doxil, an increase in clearance and reduction in half-life was observed compared to plain Doxil [79]. This was because of the Fc-mediated uptake and clearance by RES which occurs when the whole antibody e.g. 2C5 is attached to the liposome as compared to the use of only Fab’ portion of the antibody, where Fc-mediated uptake is not possible. The circulation time of 2C5-Doxil was 16 hours while that for Doxil was 21 hrs. However, 2C5-Doxil was still long-circulating, demonstrated preferential accumulation in tumor and had a rapid therapeutic effect [79]. The
accumulation of 2C5-Doxil was less EPR-dependent and took fewer passages within 4-6 hr window and so resulted in a higher tumor accumulation. Marked reduction in tumor volume was obtained within a week after the onset of therapy. Additionally, an enhanced anti-cancer effect was obtained compared to the original, un-modified Doxil. 2C5-Doxil also demonstrated significant therapeutic efficacy against intracranial human brain tumor xenografts in nude mice [80].

Similarly, specific binding and a preferential accumulation of mAb 2C5-micelles was observed in tumors developed in mice. A greater inhibition of tumor growth in mice was observed with paclitaxel-loaded 2C5-micelles than with paclitaxel-loaded plain micelles [81].

These studies provided background support for the development of multifunctional liposomes with mAb 2C5 attached to the surface, the development of which will be discussed in the following sections.

1.2.4.2. Tumor-specific ligands.

Tumor cells over express certain proteins and receptors, e.g. epidermal growth factor receptor (EGFR), transferrin receptor (TfR), folate receptor (FR), vasoactive intestinal peptide (VIP) receptor, integrins, matrix metalloproteinases (MMP), HER2 (Herceptin) to name a few.

Fab’ fragment of monoclonal antibody, C225 (Cetuximab®) or EMD72000 (Matuzumab®), which binds to EGFR was covalently linked to drug-loaded liposomes and this preparation showed enhanced cell association and cytotoxicity compared to non-targeted liposomes in EGFR over-expressing cancer cell line HCT116 [82]. Enhanced
therapeutic effect of these targeted, drug-loaded immuno-liposomes was demonstrated *in vivo* in MDA-MB 468 tumor models developed in nude mice [83].

Transferrin and anti-transferrin antibody can be conjugated to drug-loaded nanoparticle surface to impart tumor-specific targeting and enhanced cytotoxicity in transferrin over-expressing cancer cells. Anti-Bcr-Abl siRNA/ asDNA loaded nanoparticles have shown increased cell transfection and lowered cell viability in chronic myeloid leukemia cell lines which express higher amounts of transferrin [21]. Tumor targeting via folate-modified liposomes is an approach used to target tumor cells over-expressing folate receptor. Doxorubicin and 5-fluorouracil loaded-liposomes targeting folate receptor have shown enhanced *in vitro* and *in vivo* cytotoxicity [84]. Anti-HER2 antibody labeled Doxil has shown enhanced anti-cancer effect compared to Doxil alone [85].

Similarly, reduction in tumor growth was observed in mice treated with MMP2-labeled nanoparticles and nanoparticles modified with Fab’ fragment of anti-β1-integrin antibody [86-87].

Many other tumor-specific ligands have been made use of for active targeting of the nanoparticles towards tumor cells and obtain a better effect than the non-targeted ones. The type of tumor to be treated plays an important role in the choice of the targeting ligand.
1.2.4.3. **Cell-penetrating peptides.**

Cell penetrating peptides (CPPs) are short peptides which, when attached to cargoes can transport them through the plasma membrane into the cells. These proteins contain domains called protein transduction domains (PTD), which are mainly involved in the transport. TAT peptide [88] [89], penetratins [90], transportan [91], amphiphilic model peptide [92], signal sequence-based peptides [93] and synthetic polyarginines (e.g. Arg9) are examples of CPPs. TAT peptide (TATp) is derived from the transactivator of transcription (TAT) protein encoded by human immunodeficiency virus type I (HIV-I). Residues 47-57 (YGRKKRRQRRR) constitute the PTD of this peptide [89].

The exact mechanism of entry of TATp is somewhat controversial, since there are many studies that support various methods of internalization. One group showed that lipid-raft-dependent endocytosis is the mode of entry of TATp [94]. According to another group, the mode of entry of full length TAT protein is mediated by heparan sulfate proteoglycans (HSPG), whereas short TAT peptide (TAT basic peptide) does not involve binding to HSPG [95]. It has also been demonstrated that full length-TAT and heterologous proteins fused to the transduction domain of TAT enter by a caveolae-mediated pathway [96]. The mechanism of entry also seems to depend on the size of cargo/ molecule attached to TATp [97]. When TATp is attached to small molecules, electrostatic interactions promote cell penetration [98] [99], and when attached to larger cargoes, the mode of entry is via macropinocytosis [100].

TATp conjugation to antisense oligonucleotides increased the delivery of these oligonucleotides when compared to the unconjugated oligonucleotides [101]. PEGylated nanoparticles (NLPs) encapsulating a plasmid DNA and with TATp attached to the
surface, had a significantly higher transfection than NLPs without the TATp moiety [102]. Micelles [37], [103] and liposomes [104], [36] with attached TATp enhanced the uptake as compared to their non-TATp conjugated counterparts. Levchenko, et al demonstrated that TATp-liposome uptake was independent of temperature and the localization of these liposomes occurred initially in the cytoplasm and moved later to the perinuclear region [105]. TATp-liposomes when injected intratumorally, improved gene delivery to intracranial human brain tumor xenografts in nude mice [106].

However, this peptide is unstable because of its composition which consists mainly of positively charged amino acids (six arginines and two lysines). This composition makes it highly susceptible to degradation by proteolytic enzymes. The sequence of TATp can be cleaved at six sites by trypsin. Analysis of stability of TATp in plasma showed that TATp is cleaved in human plasma due to plasma enzymes [107]. As a result, its potential as a mediator of cellular delivery is restricted [107],[108].

In the present studies, micelles were prepared with a short length PEG-PE, TAT peptide conjugated to PEG_{1000} -PE and PEG_{2000}-Hz-PE. Under these conditions, the TATp conjugate remains shielded under normal pH conditions by the long pH-sensitive polymer and it is exposed under acidic pH conditions, such as at a tumor, where the pH sensitive bond is degraded. Thus, TAT peptide is protected from the proteolytic enzymes in the circulatory system and, after being exposed at the target tissue, can exert its effect.

These studies represent examples where simple modifications with tumor targeting ligands and cell-penetrating peptides provide a successful drug delivery system that can improve the original formulation.
1.2.5. Multifunctional nanocarriers.

1.2.5.1. Concept of multifunctionality.

In response to the continued push to bring about a more effective therapeutic outcome, proposals are being advanced to develop better drug delivery systems (DDS). An *in vivo* benefit of such a nanocarrier is the ultimate goal of a DDS. Several DDS have shown good effects clinically, but the challenge of overcoming unwanted side-effects remains.

To make a formulation better targeted to the appropriate pathological site, various functional moieties can be attached to a nanocarrier for simultaneous or sequential but rapid accumulation, and a therapeutic or diagnostic action.

In this context, the goal for such a micelle or liposome is a greater circulation half-life, better tumor targeting, increased tumor accumulation, responsiveness to stimuli such as low pH or high temperature, increased tumor cell penetration and marked suppression of tumor growth compared to the non-modified formulation. In other words, a micelle or liposome should be made multifunctional by addition of multiple functionalities to their surface [109]. The active targeting moieties that have been mentioned before, namely TATp and mAb 2C5 can be attached to micelles and/or liposomes to make them multifunctional.

1.2.5.2. Development of multifunctional nanocarriers.

The development of multifunctional nanocarriers is a challenge because the individual moieties having different functions must be attached in such a way that their individual
action is maintained and can be made useful in a controlled fashion. Engineering of the moieties to make them act in a coordinated way can be accomplished by attaching them to the micelle/ liposome surface by attachment of the targeting ligands via PEG spacer arms of differing lengths. In this way, the ligand can be outside the PEG coat and available for binding its target [30].

The distal terminal of PEG can be functionalized so that an activated functional group of the ligand can be attached. The ligand-PEG-PE conjugate can be added during the preparation of micelles or liposomes or via co-incubation of micelles formed from the conjugate with the liposomes via post-insertion method as described before [74].

In the present studies, in a first step towards the development of multifunctional nanocarriers, micelles and liposomes were developed with TATp attached to a short length PEG-PE (Figure 4). The TATp exposed on the surface of the micelles and liposomes promoted enhanced cell penetration. But since this peptide is non-specific and prone to proteolytic degradation as studied in vitro by analytical and biological techniques, in the second step, we have introduced another moiety, a shielding pH-sensitive PEG.

Figure 4. Structure of a TATp-modified nanoparticle with TATp groups exposed on the particle surface.
In this scheme, micelles containing PEG\textsubscript{1000}-PE, TAT peptide conjugated to PEG\textsubscript{1000} -PE and PEG\textsubscript{2000}-Hz-PE were prepared. While the TATp conjugate remained shielded by the longer PEG\textsubscript{2000}-Hz-PE at physiological pH, it is ‘exposed’ under acidic pH conditions, characteristic of a tumor, where the pH sensitive bond is degraded. The pH-sensitive PEG increases the circulation time and prevents proteolysis of the TATp but degrades at the target site to expose the cell penetrating TAT peptide. When exposed at the target tissue, TATp increases micelle penetration and delivers more drug to the tumor, thus inducing increased cell killing compared to the plain micelles (Figure 5).

\textbf{Figure 5.} Schematic diagram depicting the shielding of TATp by the long PEG chains in the pH-sensitive-TATp-micelle and the exposure of TATp as a result of the hydrolysis of hydrazone bond of PEG-Hz-PE at acidic pH.
Finally, in order to develop a multifunctional liposome that shows better therapeutic
effect than the commercially available preparation, multifunctional Doxil® containing
TAT-peptide moieties, sterically shielded with a degradable pH-sensitive hydrazone bond
between long shielding PEG chains and PE (PEG$_{2000}$-Hz-PE conjugate) was prepared
(Figure 6). The nucleosome-specific antibody (mAb 2C5), was attached to a longer PEG
chain (2C5-PEG$_{3400}$-PE). During the first phase of delivery, PEGylated liposomes can
accumulate in the tumor via the EPR effect (a “passive” targeting effect) and additionally
in the tumor cells via the mAb 2C5 (“active” targeting). In the second phase of delivery,
within the acidified milieu (typical of solid tumors or ischemic tissues) these carriers can
lose their PEG coating by hydrolysis of a hydrazone-containing pH-sensitive polymer,
and penetrate inside the cells via the action of exposed TATp moieties. Thus, specific
tumor targeting can be achieved along with an improved drug delivery which helps to
achieve a better therapeutic effect than the commercial doxorubicin liposomes.
**Figure 6.** Schematic of the effect of low pH on TATp-modified pH-sensitive immunoliposomes composed of a pH-degradable PEG\textsubscript{2000}-Hz-PE with a long PEG block, TATp-PEG\textsubscript{1000}-PE with a short PEG block, and mAb2C5-PEG\textsubscript{3400}-PE.
1.2.6. Tumors and the drug resistance phenomenon.

Multidrug resistance (MDR) is a phenomenon whereby the tumor becomes resistant to the anticancer drug and higher doses of drug are necessary for the same cytotoxic/therapeutic effect. Various mechanisms implicated in this phenomenon include altered metabolism of drug, changes in the amount/structure of drug targets present on the tumor cells, increased DNA repair activity and reduction in drug accumulation due to reduced uptake or increased drug efflux [110-111]. One of the major pathways for MDR is the enhanced drug efflux caused by over expression of P-gp (P-glycoprotein).

P-gp belongs to ATP-binding cassette transporter superfamily and is encoded by a MDR1 gene [112]. Anticancer drugs such as doxorubicin and several other compounds are removed from the cells by this efflux pump which leads to decreased cytotoxic activity of the drug [111].

Approaches to overcome MDR have included use of:

1. PgP blockers/modulators which can inhibit the drug efflux [113-114].

2. Inhibitors of MDR gene expression [115-117].

3. Chemical modification of the drug [118-121].

4. Novel drug delivery systems such as nanoparticles [122-128].

In this case, encapsulation in nanoparticles has been used/ adapted to overcome drug resistance (Figure 7).

Free drug enters a cell by diffusion. Being closer to the membrane where P-gp pumps are located, it is spatially available for drug efflux. When encapsulated in a nanoparticle, the
drug undergoes endocytosis and the contents of the nanoparticle are released near the nuclear region and hence the drug is less prone to efflux by P-gp [127, 129].

**Figure 7.** Schematic of P-gp efflux of drug and prevention of P-gp drug efflux by encapsulation in a nanoparticle.

Other possible mechanisms by which these multifunctional nanocarriers can be expected to overcome P-gp efflux are:

i. Prolonged circulation in the tumor can be provided by the PEG.

ii. Higher accumulation at the target cells can be obtained by the combined effect of mAb 2C5 and the cell penetrating peptide.

iii. mAb2C5-Doxil specifically internalized by tumor cells and can help in localization of drug load inside the cells near the nucleus [38].

Based on these previous findings and proposed mechanisms, multifunctional liposomes were developed for the treatment of multidrug resistant tumors.
2. OBJECTIVES AND SPECIFIC AIDS

OBJECTIVE:
To develop multifunctional nanocarriers for efficient tumor targeting and drug delivery using
- TATp
- pH-sensitive polymer
- mAb 2C5

SPECIFIC AIDS:
1. To study cleavage properties of TATp using trypsin as a model enzyme, its effect on TATp-mediated cell association of micelles and liposomes and the effect produced upon shielding of TATp.

2. To prepare and characterize TATp-modified pH-sensitive micelles containing paclitaxel and study their cell targeting and cytotoxic effect on tumor cells in vitro and in vivo.

3a. To develop and characterize doxorubicin loaded multifunctional liposomes modified with TATp, pH-sensitive polymer and mAb 2C5 and to evaluate their cell targeting and cytotoxic effect on tumor cells in vitro.

3b. To evaluate the cell targeting and cytotoxic effects of the multifunctional liposomes on drug-sensitive and drug-resistant tumor cells in vitro and in vivo.
3. EXPERIMENTAL DESIGN AND METHODS

3.1. Materials.

Diacyllipid-polyethylene glycols (PEG$_{750}$-PE, PEG$_{1000}$-PE, PEG$_{2000}$-PE, PEG$_{5000}$-PE), 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DPPE-SH), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), NHS-PEG$_{1000}$-Maleimide, fully hydrogenated soy phosphatidylcholine (HSPC) and L-$\alpha$-phosphatidylcholine (egg), were purchased from Avanti polar lipids (Alabaster, AL). mPEG$_{2000}$-SH was from Laysan Bio Inc, 4-(4-N-Maleimidophenyl) butyric acid hydrazide hydrochloride (MPBH) was obtained from Pierce Biotechnology Inc, and 4-acetyl phenyl maleimide was from Acros organics. TATp (12 mer: CysTyrGlyArgLysLysArgArgGlnArgArgArg; mol. mass 1663 Da) was synthesized by Tufts University Core Facility, Boston, MA. Trypsin from porcine pancreas (Type IX-S, 13000-20000 units/mg) and trypsin-chymotrypsin inhibitor from soybean were purchased from Sigma (St. Louis, MO). Triethylamine (TEA), 9-Fluorenylmethyl chloroformate (FMOC-Cl) and cholesterol were purchased from Sigma-Aldrich. Poly-oxyethylene3400-bis (p-nitrophenyl carbonate) [PEG (pNP)$_2$] was purchased from Laysan Bio Inc (Alab, Alabama). Fluoromount-G was from Southern Biotechnology Associates Inc (Birmingham, AL), and Cell Titer Blue cell viability assay kit was from Promega (Madison, WI). The mAb 2C5 was produced in ascites via I.P. injection of 1.5×$10^6$ hybridoma cells/ml into pristine primed 4 week old Balb/C male mice. The production and the purification of the mAb 2C5 were carried out by Harlan Bioproducts (Indiannapolis, IL) using the cell line from our laboratory. Control bovine antibody IgG was obtained from MP Biomedicals LLC (Ohio, USA). Doxil®, a commercially available preparation of doxorubicin in PEGylated liposomes (ALZA
Corp.), was purchased from Pharmaceutics Inc. (West Roxbury, MA). Lipodox ®, a
generic of Doxil was purchased from SunPharma, India. Trypsin from porcine pancreas
(type IX-S), chymotrypsin-trypsin inhibitor, heparinase-I from Flavobacterium
heparinum, and lyophilized human plasma (reconstituted with deionized water) were
purchased from Sigma-Aldrich (St. Louis, MO). Cell lines (B16-F10, HeLa, NIH 3T3,
MCF-7 and SKOV-3 drug-resistant and drug-sensitive) were purchased from the
American Type Culture Collection (Manassas, VA). All cell culture media, heat-
inactivated fetal bovine serum (FBS), and concentrated solutions of
penicillin/streptomycin stock solutions were from Cellgro® (Herndon, VA). All other
chemicals and solvents were of analytical grade, purchased from Fischer Scientific and
used without further purification. TATp-PEG₁₀₀₀-PE (TATp-conjugate), PEG₂₀₀₀-Hz-PE
(pH sensitive polymer) and pNP-PEG₃₄₀₀-PE were prepared in-house.

Methods:

3.2. TATp CLEAVAGE PROPERTIES AND SHIELDING METHODOLOGIES.

3.2.1. Synthesis of TATp-PEG₁₀₀₀-PE.

TATp-PEG₁₀₀₀-PE conjugate was synthesized as described previously [47] with some
modifications. Briefly, DOPE was reacted with ~ 1.5-fold molar excess of NHS-PEG₁₀₀₀-
maleimide by stirring for 2 h in chloroform at room temperature with a 3-fold molar
excess of triethylamine. A 2-fold molar excess of TATp-Cys was then added, and the
reaction was continued with stirring overnight. The solvent was evaporated, and the
product was freeze-dried overnight. The excess of TATp-Cys was separated from the
product by gel filtration chromatography. The solvent was evaporated, product was
freeze-dried overnight, and the dried product was dissolved in 0.5 mL of water and loaded onto a Sephadex G25 column (length, 10–15 cm; diameter, 0.5–1 cm). Fractions were collected and monitored by TLC using silica plates (mobile phase of chloroform/methanol 80:20% v/v), and TATp-PEG-PE was visualized with phosphomolybdic acid and Dragendorff spray reagents.

3.2.2. Preparation of TATp-modified micelles (TM) and TATp-modified liposomes (TL).

A lipid film was made by mixing chloroform solutions of PEG\textsubscript{1000}-PE and TATp-PEG\textsubscript{1000}-PE in the mole ratio 95:5. When necessary, 1 mol % of the fluorescent probe, dipalmitoyl phosphatidylethanolamine (lissamine-rhodamine B) (Rh-PE) was added. The final concentration of the lipids was 3.3 mg/ml. This dried film was suspended in phosphate buffered saline, PBS, pH 7.4. The hydrated mixture was vortexed for 5 min. TM form spontaneously. TATp liposomes were prepared by hydrating a dried lipid film of egg phosphatidylcholine, cholesterol and TATp-PEG\textsubscript{1000}-PE in the mole ratio 65:30:5. This film was lyophilized overnight and hydrated with PBS, pH 7.4. The solution was extruded 21 times through polycarbonate filters of size 200 nm, using a hand-held extruder (Avanti, Alabaster, AL). The final concentration of the lipids was 2.5 mg/ml.

3.2.3. Characterization and determination of cleavage of TATp in TM and TL.

The micelles and liposomes were divided into three groups, the first group was treated with trypsin (0.1mg/ml), the second group was treated with a mixture of trypsin+ trypsin
inhibitor and the third group was kept as is. All the solutions were incubated at 37°C for 10 min. These formulations were characterized by dynamic light scattering (DLS) using a N4 submicron particle system (Coulter Corporation, Fl) and Zeta Phase Analysis Light Scattering (PALS) using a Zeta potential analyzer (Brookhaven, Holtsville, NY).

The cleavage of TATp was followed by HPLC using the proteolytic enzyme, trypsin. An aliquot of 10 µl was removed from micelles or liposomes. One µl of trypsin solution in PBS, pH 7.4 (1mg/ml) was added to this aliquot to get a final trypsin concentration of 0.1 mg/ml. This solution was incubated at 37°C for 10 min, following which, 1 µl of borate buffer pH 10.0 and FMOC-chloride solution (0.4 mg/ml) were added. This mixture was kept at room temperature for a further 10 min. It was diluted with 400 µl water and analyzed by following the RR fragment resulting from trypsinolysis of TATp using the HPLC method already established in the lab [107].

3.2.4. Cell interaction studies.

Fluorescence microscopy studies:

Murine melanoma (B16F10) and human cervical cancer (HeLa) cells were grown at 37°C, 5% CO₂ using Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% v/v Fetal Bovine Serum and 1% v/v antibiotics. These cells were grown in 6 well plates containing a sterile coverslip in each well to about 70% confluency. The formulations prepared were Rh-PE labeled plain micelles or liposomes and Rh-PE labeled TATp-micelles or TATp-liposomes. Two solutions of trypsin (2mg/ml each) were prepared in PBS, pH 7.4. One solution was incubated with trypsin- chymotrypsin inhibitor (20 mg/ml) in 1:1 v/v ratio and another solution was incubated with PBS 1:1 v/v
ratio, at $37^\circ$C for one hour, so as to have both the trypsin solutions in the same concentration and in the same experimental conditions. Trypsin solution without the inhibitor was added to one of the micelle/liposomal formulations. The final concentration of trypsin on the micelles/liposomes was 0.1 mg/ml. Trypsin solution incubated with inhibitor was added to another micelle/liposome solution (with the final concentration of inhibitor 1 mg/ml and trypsin concentration 0.1 mg/ml). These formulations were incubated for one hour at $37^\circ$C, for maximum action of trypsin on TATp containing formulations. A third sample of TAT micelles/ liposomes was kept as is. The plain micelle/liposome sample was also left untreated. After one hour, the samples were added to the respective wells of the 6 well plates, in serum free media, so that the final concentration of lipids was 0.3 mg/ml. HOECHST 33342 was used to stain the cells at a concentration of 5 µg/ml for 15 min. The media was then removed, cells were washed and the coverslips were mounted cell side down on glass slides using the Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) mounting media and observed using a Nikon Eclipse E400 microscope under epifluorescence with a Rhodamine/TRITC filter [47].

**FACS analysis:**

For FACS analysis, the micelles/liposomes were treated in a similar way with the trypsin/inhibitor solutions. They were then added to the respective wells in 6-well plate and incubated for one hour at $37^\circ$C. The media was removed; the cells were washed with PBS. They were harvested using a 0.25% trypsin, 0.1% EDTA solution, centrifuged at 1000 rpm and the supernatant was removed. The pellet was resuspended in cold PBS, pH
7.4. The cellular uptake/attachment efficiency was determined by FACScan™ (Beckton Dickinson biosciences, San Jose, CA) acquiring 10,000 events per sample.

3.2.5. **In vitro cytotoxicity studies with TATp liposomes.**

For these studies, Doxil®, a commercially available doxorubicin loaded formulation was modified with TATp by using post-insertion method, where in brief, TATp-PEG1000-PE micelles were prepared and incubated with Doxil overnight for complete incorporation of the phosphatidylethanolamine part of micelles in the liposomes. The cytotoxicity of these TATp-modified liposomes, loaded with doxorubicin (TATp-Doxil), against B16-F10 and HeLa cells was studied. Liposomal formulations were pre-treated with trypsin (0.1 mg/ml) or trypsin (0.1 mg/ml) with trypsin inhibitor (1 mg/ml) for 1 h before addition to cells. Cells were grown in DMEM (10% FBS) and transferred into 96-well microplates at a density of $5 \times 10^3$ cells/well. Following 24 h incubation and growth to ~ 50% confluency, trypsin-treated formulations containing doxorubicin were added to cells in triplicate to give the final concentration of 5 μg/ml. After 24 h incubation at 37°C, 5% CO2, plates were washed three times with PBS, followed by the addition of 20 μl/well of CellTiter Blue solution to assess viability. After 1 h incubation at 37°C, 5% CO2, the cell survival was estimated by measuring the fluorescence intensity using a microplate reader (Synergy HT multimode microplate reader, BioTek Instrument, Winooski, VT) with 525/590 nm excitation/emission wavelengths.
3.2.6. Preparation and characterization of shielded, non pH-sensitive micelles.

A lipid film was made by mixing chloroform solution of PEG_{750}-PE as the main component, with 5 mol % TATp-PEG_{1000}-PE. This formulation was the non-shielded formulation. To prepare shielded formulations, increasing mol % of PEG_{5000}-PE or PEG_{2000}-PE ranging from 0 % to 95 % were added to the above micelles to gradually replace PEG_{750}-PE, at the same time keeping the molar concentration of the lipids constant. This dried film was suspended in phosphate buffered saline, PBS, pH 7.4. The hydrated mixture was vortexed for 5 min. Micelles formed spontaneously. We analyzed the cleavage of TATp Arg-Arg groups and the effect of increasing PEGylation by fluorenylmethyl chloroformate (FMOC) labeling using fluorescence detection with HPLC.
3.3. TATp MODIFIED pH-SENSITIVE MICELLES.

3.3.1. Synthesis of hydrolysable PEG conjugate (PEG_{2000}-Hz-PE).

An aldehyde-derived hydrazone-based PEG_{2k}-Hz-PE conjugate (pH-sensitive conjugate) was synthesized by a two step method as previously described [130] with modifications. For step I (synthesis of acyl hydrazide-PEG derivative) 40 μmol of mPEG-SH in chloroform were mixed with two molar excess of the acyl hydrazide cross-linker MPBH in the presence of five molar excess of triethylamine over lipid. Following 2hr stirring at room temperature, the product was dialyzed (Spectra/Por 6 dialysis membrane, MWCO 1K, Spectrum Laboratories, Rancho Dominguez, CA) against deionized water for two hours, analyzed by TLC, freeze-dried and stored as chloroform solution at -80°C. For step II of the synthesis (activation of phospholipid with 4-acetyl phenyl maleimide) forty micromoles of 4-acetyl phenyl maleimide were reacted with 27 mmol of 1, 2-dipalmitoyl-sn-glycero-3-phosphothioethanolamine (DPPE-SH) in presence of triethylamine overnight with continuous stirring. The activated phospholipid was separated on a silica gel column using chloroform: methanol mobile phase (9:1 v/v). The fractions containing the product were identified by TLC analysis, pooled, concentrated, freeze-dried and stored as chloroform solution at -80°C. The final product, PEG_{2000}-Hz-PE, was synthesized by reacting the hydrazide activated PEG derivative with 1.5M excess of the activated phospholipid overnight under the constant stirring at room temperature. The conjugate was separated and purified by size exclusion gel chromatography using Sepharose-CL4B media.
3.3.2. Preparation and characterization of TATp-modified pH-sensitive micelles.

TATp-modified pH-sensitive micelles were prepared by mixing PEG\textsubscript{2000}-Hz-PE, PEG\textsubscript{1000}-PE and TAT-PEG\textsubscript{1000}-PE in the mol % ratio of 77.5:20:2.5. When necessary, 1mol % of Rh-PE and/or Paclitaxel (1.5 wt %) was added to the lipid mixture. The organic solvent was evaporated under argon and a lipid film was formed which was freeze-dried overnight. This film was hydrated with PBS, pH 7.4 and divided into two groups- one was at pH 7.4 and to the other group, 0.1N HCl was added to adjust the pH to 5.0. These conditions were maintained for 1-2 hrs at 37\textdegree C. The particle size and zeta potential were analyzed.

3.3.3. Determination of kinetics of hydrolysis of hydrazone bond and TATp in TATp-modified pH-sensitive micelles.

Micelles were prepared as mentioned above. From the samples incubated at pH 5.0, a 10 \( \mu \text{L} \) aliquot was removed immediately, to which 1 \( \mu \text{L} \) of phosphate buffer pH 9.5 and 1 \( \mu \text{l} \) of trypsin (0.01 mg/ml) was added. After incubating this mixture at 37\textdegree C for 10 min, the samples were subjected to FMOC-Cl derivatization [107]. This mixture was diluted with 400 \( \mu \text{L} \) of deionized water and subjected to HPLC analysis [107]. Aliquots were removed after 15 min, half hour and one hour, treated in the same way as described above and analyzed by HPLC. The kinetics of the hydrolysis of PEG-Hz-PE bond and exposure of TATp (present in the pH sensitive micelles) to trypsinolysis, followed the same pattern. An aliquot of 10 \( \mu \text{L} \) was removed from the sample incubated at pH 7.4, processed and analyzed in the same way as mentioned above.
3.3.4. Cell interaction studies.

Fluorescence microscopy:

B16F10 (murine melanoma) cells and HeLa (human cervical cancer) cells were grown on coverslips in 6-well plates in DMEM supplemented with 10% fetal bovine serum to 70% confluency. Rhodamine PE-labeled micelles were prepared. One sample was incubated at pH 5.0 and the other at pH 7.4 at 37°C for one hour. The media was replaced by serum-free media, and the cells were treated with these preincubated micelles (2 ml/well, 0.3 mg total lipid/ml). After a 1 h incubation period, the samples were processed as described above and observed for differences in fluorescence between samples preincubated at acidic and at neutral pH, under Nikon Eclipse E400 microscopy with fluorescence [47].

FACS analysis:

For FACS analysis, B16F10 and HeLa cells were grown to 70% confluency in 6-well plates in DMEM (with 10% fetal bovine serum). Samples were processed in the same way as above and added to serum-free media in the respective wells. After one hour, the medium was removed and the cells were washed three times with medium. The cells were processed for FACS analysis as mentioned previously and analyzed by flow cytometric analysis.
3.3.5. *In vitro* cell viability studies.

TATp-modified pH-sensitive micelles containing paclitaxel were prepared. B16F10 cells were grown in DMEM (10% FBS) and transferred to a 96-well plate (1000 cells/well). After 24 hr incubation in 5% CO$_2$ at 37°C, cells were treated with TATp-modified pH-sensitive micelles preincubated for 1 hour at pH 5.0 and pH 7.4, and kept for 72 hours in DMEM (with 10% FBS). The media was removed, the wells were washed with fresh media, replaced with 100 μL media and 20 μL Cell Titer Blue (Promega, Madison, WI) was added to each well. Cell viability was evaluated after 1.5 hours of incubation at 37°C, 5% CO$_2$ by measuring the fluorescence produced by resorufin at 550$_{ex}$/590$_{em}$ using Synergy HT multidetection microplate reader (Biotek, Winooski, VT).

3.3.6. *In vivo* studies- Determination of apoptosis by TUNEL assay.

Female C57/BL6 mice (Charles River Laboratories, Wilmington, MA) were used for the experiments. A protocol approved by Northeastern University Institutional Animal Care and Use Committee in accordance with “Principles of Laboratory Animal Care” (NIH publication # 85-23, revised in 1985) was followed. Mice were divided into three groups with 3 animals in each group. Approximately, 1 x $10^6$ B16F10 cells were injected subcutaneously over the left flank. Tumor growth, which is expected to be optimal in size after about 15 days of inoculation, was checked regularly by palpation. After the tumor reached a size of 0.5 cm$^3$, mice were injected intratumorally at three sites with either paclitaxel solution in methanol, TATp-micelles loaded with paclitaxel and TATp-modified pH-sensitive micelles loaded with paclitaxel at a dose of 1mg paclitaxel/kg.
Tumors were harvested after 72 hrs and fixed in freezing media. Tumor sections were stained for a terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay. Apoptotic cells produced on treatment with these various micelle formulations were detected under fluorescence microscope.
3.4. MULTIFUNCTIONAL TATp-CONTAINING, pH-SENSITIVE, mAb 2C5-
MODIFIED LIPOSOMES

3.4.1. Synthesis of pNP-PEG$_{3400}$-PE and 2C5 mAb modification.

In order to attach mAb 2C5 to the liposomal preparations, we first conjugated the mAb to
the distal ends of PEG blocks via p-nitrophenylcarbonyl (pNP) groups (using a pNP-
PFG$_{3,4k}$-PE conjugate) to form immuno-micelles. Using the post-insertion method of
micelles [72-74], [93-94], [111] we were able to form ligand-coupled liposomes (will be
discussed below).

First, we synthesized and purified pNP-PEG$_{3400}$-PE according to an established method in
our laboratory as previously described [74]. Briefly, the synthesis includes the interaction
of PE with a 10-fold molar excess of PEG$_{3400}$-(pNP)$_2$ in chloroform in the presence of
triethylamine. Organic solvents were removed, pNP-PEG$_{3400}$-PE micelles were formed
and separated from free PEG and pNP on a CL-4B column. pNP- PEG$_{3400}$-PE product
was freeze-dried, extracted with chloroform and stored at -80°C.

For antibody conjugates with PEG$_{3400}$-PE (mAb 2C5 or non-specific IgG), 40 molar
excess of pNP-PEG$_{3400}$-PE dispersed in 10 mg/mL micellar solution in 5 mM Na-citrate,
150 mM NaCl, pH 5.0, was added to an equal volume of a 1 mg/mL solution of mAb
2C5/IgG in 100mM Tris-buffered saline (TBS), pH 8.5. The mixture was incubated with
stirring at pH 8.5 overnight at 4 °C.
3.4.2. Preparation of multifunctional liposomes.

A formulation, mimicking the commercially available Doxil®, was made using chloroform solutions of fully hydrogenated soy Phosphatidylcholine (9.58 mg/ml), cholesterol (3.19 mg/ml) and \(N\)-{\[
\text{(carbonyl-methoxypolyethylene glycol 2000)-1,2-
\text{distearoyl-Sn-glycero-3-phosphoethanolamine sodium salt (mPEG-DSPE, 3.19 mg/mL)}
\]}
(HSPC-Chol-PEG). These lipid components and their concentrations were similar to Doxil®.

Rhodamine-PE (1 mol %) was incorporated for fluorescence microscopy experiments. A lipid film was prepared by evaporating the chloroform under \(N_2\) gas, hydrated using PBS, pH 7.4 and vortexed for 5 minutes. The contents were then subjected to a sonication cycle of 11 W for 30 min using a probe sonicator to form liposomes of a uniform size distribution.

Due to shortage of the Doxil® supply, a generic, Lipodox® was used after physico-chemical, cytotoxicity and cell interaction studies confirmed that its activity was similar to Doxil.

Post-insertion technique was used to incorporate into HSPC-Chol-PEG/ Doxil®/Lipodox® liposomes, a variety of polymers/polymer conjugates. Basically, micelles composed of a combination of one or more of the following polymers were prepared: TATp-PEG\(_{1000}\)-PE (2.5 mol %), PEG\(_{2000}\)-Hz-PE (15 mol %), 2C5-PEG\(_{3400}\)-PE (2.5 mol %), IgG-PEG\(_{3400}\)-PE (2.5 mol %) and Rhodamine-PE (1 mol %). They were incubated with the above liposomes overnight. They were subsequently dialyzed overnight against DI water, using a Spectra Por dialysis membrane with a cutoff of 300KDa.
3.4.3. Determination of the optimum shielding of TATp with long chain PEG\textsubscript{2000}-Hz-PE.

Liposomes containing HSPC: cholesterol: PEG\textsubscript{2000}-PE/ Doxil ® / Lipodox ® were incubated with micelles containing 2.5 mol\% TAT\textsubscript{p}-PEG\textsubscript{1000}-PE and PEG\textsubscript{2000}-Hz-PE (2.5-15 mol \%) overnight. They were then analyzed for their particle size and zeta potential. In order to confirm that the effect on zeta potential was due to the shielding of TATp and not the effect of PEG\textsubscript{2000}-Hz-PE itself, liposomes were prepared without TATp-PEG\textsubscript{1000}-PE but containing PEG\textsubscript{2000}-Hz-PE (2.5-15mol\%). The optimal formulation selected contained TATp-PEG\textsubscript{1000}-PE: PEG\textsubscript{2000}-Hz-PE: 2C5-PEG\textsubscript{3400}-PE (2.5:15:2.5) molar ratio added to base liposomes composed of HSPC-Chol-PEG/ Doxil ®/ Lipodox ®.

3.4.4. Characterization of liposomes.

3.4.4.1. Measurement of particle size and zeta potential.

Liposomes were analyzed for their particle size distribution by dynamic light scattering (DLS) using a Coulter® N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL) and by transmission electron microscopy (TEM) (Jeol, JEM-1010, Tokyo, Japan). Zeta potentials of various liposomal formulations were analyzed using 90 PLUS particle size analyzer with ZETA PALS (Phase Analysis Light Scattering) System, Brookhaven Corp. (Holtsville, NY) at 25 °C.
3.4.4.2. Specific activity of mAb 2C5 on liposomal surface by ELISA.

The preservation of specific activity of the monoclonal antibody 2C5 attached to the surface of the liposomes using PEG₃₄₀₀-PE as the linker was determined using indirect ELISA assay. Calf thymus nucleohistones (Worthington Biochemical, Lakewood, USA) were used as the binding substrate/antigen. Goat anti-mouse IgG antibody linked to HRP (horse radish peroxidase) (ICN Biomedical, Aurora, USA) was used as the secondary antibody. The intensity of the color developed on addition of the substrate, K-blue TMB peroxidase, was read at a dual wavelength of 620 nm with the reference filter set at 492 nm using a Labsystems Multiscan MCC/340 microplate reader installed with Genesist-lite.

3.4.5. Cell interaction studies.

**Fluorescence microscopy studies:**

Human breast cancer (MCF-7) cells, normal human fibroblasts (NIH 3T3), human ovarian cancer cells (SKOV3- drug-resistant and drug-sensitive) and human cervical cancer (HeLa) cells were grown at 37°C and 5% CO₂ in Dulbecco’s modified eagle’s medium DMEM containing 10% v/v FBS and 1% v/v antibiotics. These cells were grown in 6 well plates containing a sterile coverslip in each well to about 70% confluency. Multifunctional liposomes labeled with 1 mol % rhodamine-PE (preincubated for 30 minutes at pH 5 and pH 7.4) were incubated with the cells for one hour at 37°C. Hoechst nuclear stain (2.5μg/ml) was added for the last 30 minutes of incubation. Cells were washed three times with PBS and mounted individually cell-side down on glass slides using fluorescence-free glycerol based mounting medium (Fluoromount-G).
mounted slides were studied with a Nikon Eclipse E400 microscope under fluorescence using a Rh/TRITC filter for Rh-labeled liposomes (red) and a UV-2B filter for Hoechst labeled nuclei (blue).

**Fluorescence activated cell sorting (FACS) analysis:**

Quantitative analysis of the interaction of multifunctional liposomes with the aforementioned cell lines was done using FACS. Cells were grown in 6 well plates to 70% confluency at 37 °C and 5% CO₂. The media was then replaced with fresh medium and multifunctional Doxil®/ Lipodox ® pre-incubated for 60 minutes at pH 5 and/or pH 7.4 were added to the cells. The required control liposomes were also added to the respective wells. The final concentration of the lipids was 0.3 mg/ml for each formulation. After one hour, the formulation was removed; the cells washed with PBS, pH 7.4, and detached using trypsin (0.25%). They were then washed with cold PBS, pH 7.4, and centrifuged to form a pellet. The pellet was resuspended in cold PBS and the cell interaction was determined by FACScan™ (Beckton Dickinson biosciences, San Jose, CA) acquiring 10,000 events per histogram.

3.4.6. *In vitro* cell viability studies.

The cytotoxicity of Doxil/Lipodox-modified multifunctional immunoliposomes against SKOV3 (drug-resistant and drug-sensitive cells) and HeLa cells was studied. 3×10⁴ cells/well cells were seeded into 96 well plates. Multifunctional immuno-Doxil formulations were pre-incubated at pH 5 and/or 7.4 for 60 minutes prior to their addition to cells. After 12 hr incubation at 37°C, 5% CO₂, plates were washed three times with fresh media, and cells were placed in the incubator with fresh media for additional 48
hours. Finally, cytotoxicity of the various formulations was evaluated by the addition of 20μl/well CellTiter Blue solution to 100 μl fresh medium to assess the cell viability. After 1 hr incubation at 37°C, 5% CO₂, the cell survival was estimated by measuring the fluorescence intensity using a microplate reader (Synergy HT multimode microplate reader, BioTek Instrument, Winooski, VT) with 525/590 nm excitation/emission wavelengths.

3.4.7. Determination of P-gp levels in SKOV3 drug-resistant and sensitive cells.

SKOV-3 drug-resistant and sensitive cells were treated with FITC labeled P-gp specific antibody (UIC2). Both cells were grown in T75 flask to 80 % confluency. A pellet was obtained and resuspended in PBS, pH 7.4 to get 300,000 cells/ 100 μl. They were incubated with 5 μl of FITC labeled P-gp specific antibody (UIC2) for 1 hr on ice. The cells were washed one hour later with cold PBS and analyzed by FACS technique using the green fluorescence intensity (FL1) filter.

3.4.8. In vivo studies of multifunctional liposomes in SKOV-3 drug-resistant and sensitive tumors

Female nu/nu mice (n= 50) purchased from Charles River Laboratories were equally divided into two groups- 25 mice for inoculation with SKOV3 drug-resistant and 25 with drug-sensitive cells. They were inoculated subcutaneously in the left flank with approximately 4 x 10⁶ SKOV-3 (sensitive) cells or 7 x 10⁶ cells (resistant) cells mixed in a 1:1 v/v ratio with Matrigel HC [126, 131], according to the protocol approved by the Institutional Animal Care and Use Committee at Northeastern University. When tumors reached a volume of approximately 150 mm³, 14 days after tumor inoculation, mice in each group were sorted randomly into 5 sub-groups for treatment with either: Saline,
plain-Lipodox, Lipodox-TATp, Lipodox-2C5 and Multifunctional Lipodox. Tumor volumes were measured every alternate day using vernier calipers and the percent change in tumor volume was calculated by the formula: \(\frac{(V_n-V_0)}{V_0} \times 100\), where \(V_n\) is the mean volume on day ‘n’ following the treatment and \(V_0\) is the mean volume on day ‘0’ (beginning of the experiment). Twenty days after the first injection, when saline-administered tumors reached a mean tumor volume of 1000 mm\(^3\), mice were sacrificed by CO\(_2\) and tumors excised. Tumor weights were measured at the end of the study after excision. Mouse body weight was taken every three days to monitor the safety of the formulations.

The excised tumors were fixed in freezing media and stored at -80 °C. Approximately 8 µm thick cryostat sections were made and the sections were stained for a terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay. Apoptotic cells produced on treatment with these various micelle formulations were detected under fluorescence microscope.

In previous studies by our group, a dose of 2 mg/kg of doxorubicin given every 5 days for a total 4 doses, equivalent to a total dose of 8 mg/kg, was used to evaluate the efficacy of mAb 2C5 –Doxil [38, 42]. Other groups have reported effects at higher doses equivalent to 18 mg/kg. However, the dose rate used previously in the lab corresponds to 6 half-lives of Doxil and 7 half-lives of mAb 2C5- Doxil. For our in vivo studies that involve Lipodox (a generic of Doxil), mAb 2C5-Lipodox, TATp-Lipodox and multifunctional immuno-Lipodox, this sub-therapeutic dose (8 mg/kg i.e. 2mg/kg/ 5days /4 times) was chosen so that statistically significant differences between various formulations could be evaluated.
4. RESULTS AND DISCUSSION

4.1. TATp CLEAVAGE PROPERTIES AND SHIELDING METHODOLOGIES.

4.1.1 Preparation and characterization of TATp micelles and TATp liposomes.

The results of the zeta potential measurements are presented in Table 2, which shows the effect of trypsin on zeta potential values of TATp-modified liposomes/micelles. Our results suggest that the positive charge of the TATp-modified particles shifts to neutral following the incubation with 0.1 mg/ml of trypsin for 10 min at 37°C. Since TATp is rich in arginine, which contributes to its cationic properties, it is also the main contributor to the total positive charge of both micelles and liposomes. The decrease in zeta potential values from $-6.1 \pm 2.7$ mV to $-18.4 \pm 2.8$ mV for TATp-modified micelles and from $4.3 \pm 2.7$ mV to $-15.9 \pm 2.0$ mV for TATp-modified liposomes supports our conclusion that TATp was cleaved by trypsin. When particles were incubated simultaneously with trypsin and trypsin inhibitor, the positive charge of TATp-modified particles was retained, confirming that trypsinolysis resulted in TATp cleavage and charge decrease. Particle size analysis of both micelles and liposomes showed no significant changes following incubation with trypsin, retaining a magnitude of 20 nm and 100 nm for micelles and liposomes, respectively. Thus, TATp degradation does not affect the size characteristics of the peptide-modified particles.
Table 2  Zeta Potential of TATp-modified micelles and liposomes (Values are mean± SD, n=5, p <0.05 as calculated by Student’s T-test and one way ANOVA)

4.1.2 Determination of cleavage properties of TATp in TM and TL

TATp undergoes cleavage at its carboxyl terminus, on treatment with trypsin and the first and the most intense proteolysis fragment formed is the arginine- arginine (RR) dimer, (YGRKKRRQR↓RR) which can be followed using HPLC. In this experiment, TM and TL were treated with trypsin. This was followed by incubation with FMOC-chloride, a fluorescent label. A chromatographic peak of FMOC-Cl labeled RR dimer was obtained at 1.5 minutes. Figure 8 is a representative of the RR peak obtained using the HPLC analysis.

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Micelles</th>
<th>Liposomes</th>
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<tbody>
<tr>
<td></td>
<td>Mean ±SD (mV)</td>
<td>Mean ±SD (mV)</td>
</tr>
<tr>
<td>Plain</td>
<td>-18.1± 1.4</td>
<td>-14± 2.1</td>
</tr>
<tr>
<td>TATp-modified</td>
<td>-6.1± 2.7</td>
<td>4.3± 2.7</td>
</tr>
<tr>
<td>TATp-modified + trypsin</td>
<td>-18.4± 2.8</td>
<td>-15.9± 2</td>
</tr>
<tr>
<td>TATp-modified + trypsin+inhibitor</td>
<td>-8± 3.1</td>
<td>-4.4± 1.2</td>
</tr>
</tbody>
</table>
4.1.3 Cell interaction studies with Fluorescence microscopy and FACS analysis

Trypsin was used to confirm that when TATp is attached to the surface of micelles or liposomes, TATp-to-cell interaction is influenced by TATp exposure to proteolytic enzymes. TATp-modified micelles and liposomes, treated with trypsin for 1 h, were analyzed for their cell interaction capabilities by fluorescence microscopy and FACS techniques.

TATp-modified liposomes were tested for their interaction with cells and for the effect of trypsin on this interaction. Fluorescence microscopy showed that rhodamine-labeled TATp-modified liposomes were significantly internalized by HeLa and B16-F10 cells (Figure 9). Diminished cell internalization was observed following the pre-incubation of formulations with trypsin (0.1 mg/ml for 1 h). When trypsin inhibitor (1 mg/ml) was

Figure 8. RR fragment obtained on treatment of TATp with trypsin, as analyzed by HPLC. The retention time is 1.5 min.
added during the pre-incubation time, a significant signal recovery occurred, indicating prevention of TATp cleavage. Cell interaction of the TATp-modified liposomes was also confirmed using FACS (Figure 9). As expected, the analysis of the geometric mean fluorescence revealed a 15–20-fold stronger binding of TATp-modified liposomes to cells compared to plain liposomes. As obtained with fluorescence microscopy, liposomes pre-treated with trypsin (0.1 mg/ml for 1 h) showed a dramatic reduction in cell binding. Again, when trypsin inhibitor (1 mg/ml) was added during the pre-incubation time, a significant increase in fluorescence intensity occurred, indicating prevention of TATp cleavage.

Similar results were obtained with rhodamine-PE labeled TATp-micelles using fluorescence microscopy and FACS analysis (Figure 10). FACS analysis revealed that TATp-micelles had a 1.3-fold higher binding with the cells when compared to plain micelles or TATp-micelles pre-treated with trypsin (Figure 10).
Figure 9. Fluorescence microscopy (upper panel) and flow cytometry analysis (lower panel) showing interaction of rhodamine-PE labeled TATp- liposomes upon pretreatment with trypsin / trypsin inhibitor (0.1 mg/ml and 1 mg/ml respectively) for 1 hr at 37°C followed by incubation with B16F10 and HeLa cells for 1 hr. (*p≤ 0.05 compared to plain liposomes and TATp-liposomes pre-treated with trypsin, n=3, mean ± SD)
**Figure 10.** Fluorescence microscopy (left panel) and flow cytometry analysis (right panel) showing interaction of rhodamine-PE labeled TATp-micelles upon pretreatment with trypsin / trypsin inhibitor (0.1 mg/ml and 1 mg/ml respectively) for 1 hr at 37\(^\circ\)C followed by incubation with B16F10 cells for 1 hr. (*p \leq 0.05 compared to plain micelle and TATp-micelles pre-treated with trypsin, n=3, mean ± SD)

These results suggest that proteolytic enzymes, like trypsin, will prevent or reduce the interaction of TATp-modified liposomes with cells by degrading TATp moieties on their surface and that those carriers should be shielded in order to maintain their cell transport activity.
4.1.4. *In vitro* cytotoxicity of doxorubicin-loaded TATp liposomes.

The cytotoxicity of TATp-modified liposomes, loaded with doxorubicin (TATp-Doxil), against B16-F10 and HeLa cells was studied. Liposomal formulations were pre-treated with trypsin (0.1 mg/ml) or trypsin (0.1 mg/ml) with trypsin inhibitor (1 mg/ml) for 1 h and diluted with media to neutralize trypsin before addition to cells. Cells were seeded into 96-well microplates and grown to 50% confluency. Trypsin-treated formulations containing doxorubicin were added to cells in triplicate to give the final concentration of 5μg/ml. After 24 h incubation at 37°C, cell viability was assessed using Cell-Titer Blue solution (Figure 11).

**Figure 11.** In vitro cytotoxicity of TATp-Doxil formulations toward B16-F10 (Left) and HeLa (Right) cells. The formulation was pre-treated with 0.1mg/ml trypsin or trypsin (0.1 mg/ml)+1mg/ml trypsin inhibitor for 1 hr followed by 24 hrs incubation with cells at 37°C before the viability assay. Data represented are at a 5 μg/ml final doxorubicin concentration. (*p≤0.05 TATp-Doxil + trypsin vs. TATp-Doxil, # p≤0.05 TATp-Doxil + trypsin vs.TATp-Doxil+ trypsin+ inhibitor (n= 3, mean±SD)
The interaction between TATp-modified liposomes and cells is necessary for increased penetration of the liposomes in the cells. This results in an increased cytotoxic effect of TATp-modified Doxil as seen above. Since pre-incubation with trypsin degrades TATp, cell interaction is reduced and the cytotoxic effect is diminished. Trypsin inhibitor inhibits trypsin activity, facilitates the interaction between the cells and TATp-Doxil, and thus increases the cytotoxic effect of the drug-loaded formulation.

Degradation of CPPs before reaching their target, whether in the cytoplasm, nucleus, or extracellular fluids, must be avoided for CPPs to serve as an efficient moiety that promotes intracellular delivery of a carrier or cargo. Steric shielding of CPPs is essential to ensure its effective in vivo function.

4.1.5. Characterization of non pH-sensitive steric shielding of TATp-modified micelles.

Steric shielding of TATp from proteolytic degradation is essential to maintain its biological activity. PEGylated TATp- micelles were prepared as described in materials and methods. The percent cleavage of TATp produced by trypsin was evaluated by following the formation of arginine-arginine dimer by FMOC-Cl labeling using an HPLC method established previously in the lab [107] (Figure 12).
Figure 12. PEG shielding of TATp-micelles. TATp RR fragment analysis of TATp-modified micelles, shielded with increasing mol% of long PEG2k-PE or PEG5k-PE blocks as followed by HPLC. TATp enzymatic cleavage was measured following incubation of TATp-modified micelles with trypsin (0.1mg/ml) and presented as percent of cleavage. Particles were incubated with enzyme for 10 min in PBS, pH 7.4, at 37°C. (n=3, mean±SD)

In the design of TATp-PEGylated nanoparticles, it is necessary that the system provides maximum protection to the peptide. Long chain polymers, such as PEG2000-PE and PEG5000-PE were used here to represent polymers which show such shielding and the cleavage of TATp produced by trypsin was quantified. Increasing the molar concentration of PEG had an inverse linear relationship with enzymatic cleavage of TATp indicating successful shielding with PEG.
These results suggest that stimuli-sensitive architecture of intelligent drug delivery platforms should also take into consideration the need for the shielding of TATp from enzymatic cleavage.

Since these particles are designed to be delivered to tumor cells, we took the advantage of lowered pH, typical of inflamed and neoplastic tissues and shielded TATp with pH-sensitive polymer, PEG$_{2000}$-Hz-PE to enable the protection of the peptide at a neutral pH and its exposure at the tumor cell surface after degradation of the pH-sensitive polymer at lower pH.
4.2. TATp-MODIFIED pH-SENSITIVE MICELLES

4.2.1 Preparation and characterization of TATp-modified pH-sensitive micelles.

pH-sensitive micelles were prepared from PEG<sub>2000</sub>-Hz-PE, PEG<sub>1000</sub>-PE and TATp-PEG<sub>1000</sub>-PE in the mol % 77.5:20:2.5. In order to mimic the acidic conditions of the tumor extracellular environment, samples were briefly incubated at pH 5.0. For the normal pH conditions, samples were incubated at pH 7.4. Zeta potential value for micelles pre-incubated at pH 5 was less negative compared to micelles pre-incubated at pH 7.4, but was comparable to those of TATp-micelles (Table 3). This result supports our conclusion that pH-sensitive polymer degrades at acidic pH and “exposes” TATp. The particle size of the micelles was in the range 7.4-20 nm.

TEM image shows uniform distribution of the TATp-modified pH-sensitive micelles (Figure 13).

<table>
<thead>
<tr>
<th>Micelles</th>
<th>Particle Size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain micelles</td>
<td>15 ± 2</td>
<td>-22 ± 1.4</td>
</tr>
<tr>
<td>TATp micelles</td>
<td>20 ± 4</td>
<td>-9 ± 2.2</td>
</tr>
<tr>
<td>TATp pH-sensitive at pH 5.0</td>
<td>12 ± 4.8</td>
<td>-10 ± 3</td>
</tr>
<tr>
<td>TATp pH-sensitive at pH 7.4</td>
<td>20 ± 3</td>
<td>-24 ± 4</td>
</tr>
</tbody>
</table>

Table 3 Size range and zeta potential of TATp-modified pH-sensitive micelles at pH 5.0 and pH 7.4 (values of zeta potential are mean ± SD, n = 5, p < 0.05, as analyzed by Student’s T-test)
4.2.2 Determination of hydrolysis of hydrazone bond and cleavage of TATp in TATp-modified pH-sensitive micelles.

TATp-modified pH-sensitive micelles were prepared as above. Samples were preincubated at pH 5.0 or pH 7.4. They were then subjected to treatment with 0.1 mg/ml trypsin, followed by FMOC-Cl labeling and analysis by HPLC as described in materials and methods.

Figure 14 shows the cleavage of TATp and hydrolysis of hydrazone bond in TATp-modified pH-sensitive micelles. When incubated at acidic pH (pH 5.0), the hydrolysis of pH-sensitive polymer occurs and the resulting fragment of the polymer, PEG-Hz-NH₂ when labeled with FMOC-Cl appears at RT 6.9 min, the exposed TATp in turn gets cleaved which is seen by the formation of the RR peak. However, incubation of the micelles at pH 7.4 fails to produce any increase in the RR peak which indicates that the
protective hydrazone polymer shields the TATp and prevents it from degradation by the added trypsin.

(a)

pH 5
**Figure 14.** HPLC analysis of the cleavage of TATp and hydrolysis of hydrazone bond in TATp-modified pH-sensitive micelles, at pH 5.0 (a) and pH 7.4(b), 37°C. The analysis was performed following the exposure of TATp to trypsin digest and monitored by the RR fragment from its appearance at 1.5 min and PEG-Hz-NH₂ fragment at 6.9 min.

The kinetics of hydrolysis of hydrazone bond and degradation of TATp were followed (Figure 15). It was observed that the hydrolysis of hydrazone bond and cleavage of TATp follow the same kinetic pattern. With increasing time of incubation, hydrolysis of pH-sensitive polymer increases along with increased exposure of TATp as seen by
increase in AUC of PEG-Hz-NH₂ and RR dimer (Figure 15). Complete hydrolysis of pH-sensitive polymer was obtained within one hour, while longer incubation times did not produce significant increase in the AUC of PEG-Hz-NH₂.

![Image of chromatograms showing cleavage](image)

**Figure 15.** Kinetics of the cleavage of TATp and hydrolysis of pH sensitive hydrazone bond in TATp-modified pH-sensitive micelles pre-incubated at pH 5.0 for various time-points at 37 °C, was determined by HPLC analysis from the RR dimer at 1.5 min and hydrazone bond at 6.9 min.

**4.2.3. In vitro studies.**

**4.2.3.1. Cell interaction studies:**

For cell interaction studies with B16F10 and HeLa cells, rhodamine-PE labeled micelles were used. In this case, we studied cell interaction of TATp-modified pH-sensitive micelles at pH 7.4 or after a brief pre-incubation at pH 5. An increase in the cell
interaction was observed when the micelles were pre-incubated at pH 5 but not at pH 7.4 indicating that pH-sensitive PEG shielding was lost at pH 5. Figure 16 shows that plain micelles and the TATp-modified pH-sensitive micelles pre-incubated at pH 7.4 showed only a marginal interaction with the cells, while those pre-incubated at pH 5.0 showed a significantly higher interaction with the cells, indicating exposure of the TATp. Unshielded TATp-micelles used as a control also showed increased cell interaction.

In agreement with the fluorescence microscopy experiment, when analyzed by flow cytometry, the fluorescence associated with B16F10 and HeLa cells was higher in case of cells treated with TATp-micelles and TATp-modified pH-sensitive micelles pre-incubated at pH 5 in comparison to those pre-incubated at pH 7.4. These studies again demonstrate the loss of pH-sensitive polymer at acidic pH and availability of TATp for cell interaction.
Figure 16. Fluorescence microscopy (upper panel) and flow cytometry analysis (lower panel) showing the internalization of rhodamine-PE-labeled TATp-modified pH-sensitive micelles pre-incubated at pH 5 or pH 7.4 for 1 hr at 37 °C followed by incubation for 1 hr with B16-F10 (mouse melanoma) or HeLa (human cervical carcinoma) cells. The pH was neutralized before addition to the cells. (*p≤ 0.05, n=3, mean ± SD).
4.2.3.2. Cell viability studies.

The cytotoxicity of TATp-modified pH-sensitive micelles loaded with paclitaxel against B16F10 cells was studied. Micelles were pre-incubated at pH 5 or pH 7.4 for 1 hour. After one hour, the pH was neutralized and the micelles were added to the cells in triplicate. Plain, non-drug containing micelles, paclitaxel in methanol solution, paclitaxel-loaded plain micelles and paclitaxel-loaded TATp micelles were used as controls. Cytotoxicity was determined after 72 hours of incubation with the micelles. Micelles pre-incubated at pH 5 and the non-shielded TATp micelles showed comparable cytotoxicity which was significantly higher than that of the micelles pre-incubated at pH 7.4 as well as other formulations (Figure 17). Since TATp was exposed, improved drug delivery and enhanced cytotoxicity was produced.
**Figure 17.** *In vitro* cytotoxicity of PCT loaded TATp-modified pH-sensitive micelles preincubated at pH 5.0 or pH 7.4 at 37 °C, on B16F10 cells (*p <0.05, mean ± SD, n= 3).

### 4.2.4. *In vivo* studies- Determination of apoptosis using TUNEL assay.

The next question was, whether this deshielding of polymer and exposure of the TATp would occur *in vivo* to improve drug delivery and enhance apoptosis. In other words, an *in vivo* “proof-of-concept” study was conducted and hence, to prevent unwanted distribution, different paclitaxel-loaded micelles were injected intratumorally. Tumors were harvested after 72 hours. TUNEL assay was carried out using the DNA fragmentation kit to confirm the presence of apoptosis in the tumor tissue. DAPI stained the nuclei of the cells present in the tissue. The TUNEL method revealed apoptotic bodies showing green fluorescence of FITC-labeled TdT. Since TUNEL method stains
fragments produced by apoptosis alone, the cell nuclei showing green fluorescence were apoptotic. TUNEL staining showed considerable apoptotic activity in the sections obtained from tumors treated with paclitaxel-loaded TATp-modified pH-sensitive micelles and TATp-modified micelles. Few apoptotic cells were seen in paclitaxel-loaded, non-modified, plain micelles (Figure 18). These results show that TATp-modification and pH-sensitive shielding work efficiently in vivo and the increased delivery of drug on account of the presence of TATp promotes apoptosis. The data obtained in this experiment is in line with the in vitro results.

Figure 18. Detection of apoptotic activity by fluorescence microscopy in B16F10 tumor sections as shown by TUNEL staining. Intratumor injections of plain micelles, TATp-micelles and TATp-pH-sensitive micelles loaded with paclitaxel (1 mg/kg) were given. Left panel shows DAPI staining while right panel shows TUNEL staining.
So far we have seen how a cell penetrating peptide can be shielded at normal pH and made to act at the typical acidic pH of a tumor cell environment after the pH-sensitive shielding is removed.

This is a significant step towards the development of a multifunctional nanocarrier containing TATp shielded by a pH-sensitive PEG-PE. The addition of mAb 2C5 for enhanced targeting would potentially give a therapeutic benefit over the non-modified counterpart. We will be modifying a commercial preparation Doxil, using these targeting moieties and studying if the modified multifunctional Doxil provides an enhanced therapeutic benefit over plain Doxil.
4.3. MULTIFUNCTIONAL TATp-CONTAINING, pH-SENSITIVE, mAb 2C5-CONTAINING LIPOSOMES

4.3.1. Concept of multifunctional TATp-modified pH-sensitive immuno-liposomes.

In this study, a surface-modified multifunctional liposomal carrier with 2C5 monoclonal antibody, the cell penetrating peptide TATp and pH-sensitive PEG-hydrazone-PE polymer as an effective TATp steric shield was designed. mAb 2C5 was attached to a long PEG chain (2C5-PEG$_{3400}$-PE). TATp was conjugated with a short PEG$_{1000}$-PE spacer and the “shielding” effect of TATp by long PEG$_{2000}$ chains was optimized. This nanocarrier was designed in such a way that during the first phase of its delivery, the 2C5 mAb moieties are available, targeting the carrier specific to cancer cells. At normal pH, surface TATp moieties are “shielded” by the long PEG blocks. Upon the exposure to lowered pH, degradation of the hydrazone bond and removal of the long PEG chains occur, TATp moieties are exposed and enhanced penetration of TATp-modified carrier through the cell membrane occurs.
**Figure 19.** Schematic of the effect of low pH on TATp-modified pH-sensitive immunoliposomes composed of a pH-degradable $PEG_{2000}$-Hz-PE with a long PE block, TATp-$PEG_{1000}$-PE with a short PEG block, and mAb2C5-$PEG_{3400}$-PE.

### 4.3.2. Preparation and characterization of multifunctional liposomes.

In this study, the outer surface of liposomes was modified by incorporating $PEG_{2000}$-Hz-PE, TATp-$PEG_{1000}$-PE and mAb 2C5-$PEG_{3400}$-PE into pre-formed liposomes using a post-insertion technique [132].

Previous studies have shown that the amount of PEG that can be incorporated into vesicles depends upon the content of cholesterol [50]. Several groups have shown that 15 mol\% of PEG did not increase the particle size nor their removal from the circulation [51]. Based on this data reported earlier by various groups, it was decided that liposomes with $PEG_{2000}$-Hz-PE shielding up to 15 mol \% would be tested for their particle size and zeta potential to determine the optimal amount that could be used.
4.3.2.1. Determination of optimum PEG$_{2000}$-Hz-PE shielding.

In this study we have modified the commercially-available liposomal preparations Doxil. Lipodox (generic of Doxil) was used later in place of Doxil because of shortage in Doxil supply and was modified similarly.

To determine the amount of PEG$_{2000}$-Hz-PE that could efficiently shield TATp, zeta potentials of the TATp-modified liposomal preparations with different mol % of longer PEG blocks were measured. TATp was conjugated to short PEG$_{1000}$-PE block. The longer PEG$_{2000}$-Hz-PE was used as the shielding polymer containing longer PEG chains. TATp-Doxil and TATp-Lipodox were coated with increasing molar ratios of PEGs from 2.5 mol% to the highest shielding at 15 mol% (Figure 20).

![Zeta potentials of TATp-modified liposomes shielded with increasing quantity of PEG$_{2000}$-Hz-PE. Plain liposomes (▲), TATp-modified liposomes (■), PEG$_{2000}$-Hz-PE shielded liposomes (◆). n=5, results are mean ± SD.](image-url)
The zeta potential parameter reflects the degree of exposure of TATp moieties on the carrier’s surface and allows determining the effective PEG shielding content needed. Higher PEG shielding resulted in lower zeta potential values suggesting effective shielding of TATp in a dose dependent manner (Figure 18). The zeta potential of liposomes coated with 15mol% approached that of plain liposomes indicating optimum shielding with 15 mol% PEG2000-Hz-PE. No significant change in zeta potentials was observed for plain, unmodified liposomes that were PEGylated at increasing molar ratios (up to 15 mol%).

The size of the liposomal formulations prepared in this study following the increase in the molar ratios of PEG-PE block polymer was within 100nm interval as confirmed by dynamic light scattering analysis (Figure 21).

![Figure 21. Particle size of liposomes with increasing mol % of PEG coating. (n=3, mean ± SD)](image)

Results obtained from particle size and zeta potential analysis suggest that different liposomal formulations could be efficiently coated with additional PEG blocks that did not drastically change the size of the liposomes and shielded TATp as well. For the further experiments, we used PEG2000-Hz-PE (pH cleavable conjugate) at 15 mol%.
4.3.2.2. Determination of particle size and zeta potential

The multifunctional liposomes eventually used included TATp-PEG$\text{1000}$-PE (2.5 mol %), 2C5-PEG$\text{3400}$-PE (2.5 mol %) and PEG$\text{2000}$-Hz-PE (15 mol %). Doxil and Lipodox were modified with these functionalities and characterized for particle size and zeta potential (Figure 22, Table 4).

![Doxil Particle Size Graph]

(a)
Figure 22. Particle sizes of modified Doxil (a) and Lipodox (b) preparations and a TEM image of Doxil and multifunctional immuno-Doxil.

Particle size of the formulations was approximately 90 nm. These results were confirmed using transmission electron microscopy.
Table 4 presents zeta potentials of modified Doxil and Lipodox preparations.

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Doxil</th>
<th>Lipodox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain Liposomes</td>
<td>-48.8 ± 2.9</td>
<td>-45.6 ± 3.1</td>
</tr>
<tr>
<td>TATp Liposomes</td>
<td>-8.5 ± 2.2</td>
<td>-8.3 ± 1.3</td>
</tr>
<tr>
<td>2C5 Liposomes</td>
<td>-38.6 ± 3.4</td>
<td>-37 ± 2.1</td>
</tr>
<tr>
<td>TATp+Hz+2C5 (Multifunctional), pH 7.4</td>
<td>-42 ± 3.5</td>
<td>-40 ± 1.9</td>
</tr>
<tr>
<td>TATp+Hz+2C5 (Multifunctional), pH 5</td>
<td>-10 ± 1.5</td>
<td>-7 ± 1.8</td>
</tr>
</tbody>
</table>

Table 4. Zeta potential of liposomal formulations.

TATp moieties on the carrier surfaces contributed to the positive charge of the carriers, compared to plain Doxil and plain Lipodox (Table 4). While the original Doxil and Lipodox had zeta potential values of -48.8±2.9 mV and -45.6±3.1mV, TATp-modified liposomes had value of -8.5±2.2 mV and -8.3 ±1.3 mV respectively. These positive values are due to the TATp’s content of positively charged amino acids, arginine and lysine. Multifunctional immuno-Doxil and Lipodox possess zeta potentials (mV) of -42 ±3.5 mV and -40±1.9 for preparation incubated at pH 7.4. When pre-exposed to pH 5, multifunctional nanocarriers showed more positive zeta value: -10± 1.5 mV and -7± 1.8 mV confirming our proposed mechanism for the TATp shielding and de-shielding effect.
4.3.2.3. Specific activity of modified mAb 2C5 by indirect ELISA

To confirm the presence and activity of mAb 2C5 on the surface of liposomes, an indirect enzyme-linked immunosorbent assay (ELISA) was performed as described previously [75]. A water-soluble fraction of calf thymus nucleohistone was used as the antigen and horseradish peroxidase-linked goat anti-mouse IgG was used as secondary antibody to confirm the presence of mAb 2C5 on Doxil and Lipodox. IgG alone and IgG-modified liposomes were used as non-specific controls. mAb 2C5 at 2.5 mol% was conjugated to these liposomes (Figure 23).
**Figure 23.** Specific activity of mAb 2C5 on the surface of Doxil (a) and Lipodox (b) by indirect ELISA.

Immunoreactivity of mAb 2C5-PEG\textsubscript{3400}-PE upon incorporation in the membrane of Doxil and Lipodox was determined. Although the activity of mAb 2C5 upon conjugation to the liposomes was preserved considerably, there was some loss of the activity (**Figure 23**). This loss was probably due to the steric hindrance of PEG on the liposome surface and to some loss during conjugation. Unlike the case with free mAb 2C5 molecules in solution, the whole surface of the antibody is not available for interaction with the nucleosome antigen. This loss can be compensated by the multi-point attachment of mAb 2C5 present on liposomes. Standard IgG antibody, IgG modified liposomes and plain liposomes used as controls showed no activity.

Thus Doxil and Lipodox showed that mAb 2C5 could be incorporated using the “post-insertion” technique on its surface and its activity could be maintained after incorporation.
HSPC (fully hydrogenated soy Phosphatidylcholine): cholesterol: PEG liposomes were also modified in a similar way and labeled with Rhodamine-PE for cell interaction studies.

4.3.3 Cell interaction studies.
Fluorescence microscopy:

Cell interaction of multifunctional immuno-liposomes with NIH-3T3 (normal cells), MCF-7 (human breast cancer cells), and HeLa (human cervical cancer cells) was studied visually using fluorescence microscopy. Plain liposomes, TATp liposomes and 2C5 liposomes were used as controls. Rhodamine-PE (1 mol %) was used to fluorescently label all liposomes. All liposomes were made from HSPC-Cholesterol-PEG.

Figure 24. Fluorescence microscopy of rhodamine-PE labeled plain liposomes, TATp liposomes, mAb 2C5 liposomes or TATp-2C5-Hz liposomes pre- incubated at pH 5 or pH 7.4 with NIH-3T3, MCF-7 and HeLa cells.
TATp-liposomes were internalized by all the cell lines (Figure 24). However, 2C5-modified liposomes were taken up only by cancer cells and not by NIH-3T3 which are normal cells and which do not over-express nucleosomes. The internalization of multifunctional immuno-liposomes pre-incubated at pH 5 was significantly higher than those pre-incubated at pH 7.4. These results indicate that mAb 2C5 on the surface of liposomes targets the cancer cells and that the TATp exposed upon degradation of pH-sensitive polymer at an acidic pH further increases the cellular interaction.

**FACS analysis:**

Quantitative analysis of cell interaction of liposomes was performed using Doxil, TATp-modified Doxil, 2C5-modified Doxil and multifunctional immuno-Doxil (Multi-Dox, pre-incubated at pH 5 or pH 7.4). The innate fluorescence of doxorubicin in these liposomes was used to help determine the changes in geometric mean fluorescence intensity upon binding of different liposomes to the cells.
**Figure 25.** Flow cytometry. Increase in the geometric mean fluorescence intensity (vs. non-modified liposomes) of multifunctional immuno-Doxil incubated for 1 hour with NIH-3T3, MCF-7 and HeLa cells. Multifunctional carriers were incubated with cells following their pre-exposure to normal or acidic conditions for 1hr [*p ≤ 0.05, by Student’s T-test] (n=3, mean ± SD).

A two-fold stronger binding of Doxil-2C5 was observed in MCF-7 and HeLa cell lines, but not in NIH-3T3 cells which do not over-express nucleosomes. Doxil-TATp had a 6.5-fold stronger binding with MCF-7 cells, a 6-fold stronger binding for HeLa cells and a 3.6-fold stronger binding with NIH-3T3 cells, in comparison to Doxil alone. Multifunctional Doxil pre-incubated at pH 5 generated a 3.7 fold, 7-fold and 7.5-fold stronger binding with NIH-3T3, MCF-7 and HeLa cells respectively, while
multifunctional Doxil pre-incubated at pH 7.4 showed 2-fold stronger binding for MCF-7 and HeLa cells but not with NIH-3T3 (Figure 25).

Based on the results, it can be concluded that the deshielding of TATp at acidic pH and increased cell association with mAb 2C5 both promote interaction of multifunctional Doxil with MCF-7 cells and HeLa cells, while improved interaction with NIH-3T3 cells occurs through the action of TATp alone, owing to the non-specific interaction of TATp with all cells.

4.3.4. *In vitro* cytotoxicity studies.

Cytotoxicity of multifunctional immuno-Doxil and controls was determined against HeLa cells.
Figure 26. In vitro cytotoxicity of plain Doxil, TATp-modified Doxil, mAb 2C5-modified Doxil and multifunctional TATp-modified, pH-sensitive, immuno-Doxil (Multi-Dox) preincubated at pH 5.0 or pH 7.4 for 1 hr at 37°C. Upper panel shows the cytotoxicity of the different preparations over a range of concentrations of doxorubicin whereas lower panel compares survival of cells at a representative concentration of 12.5μg/ml of doxorubicin (mid-concentration of all the concentrations tested). [*p ≤ 0.05, Student’s t-test n=3, mean ± SD].

In vitro cytotoxicity revealed highest toxicity of TATp-Doxil in HeLa cells which is in agreement with the highest cell interaction of TATp-Doxil with these cells (Figure 26). The next most effective formulation showing significantly higher cytotoxicity was multifunctional-Doxil pre-incubated at pH 5. The highest cell viability was observed in plain, unmodified Doxil treated cells. Though TATp remains shielded in multifunctional immuno-Doxil incubated at pH 7.4, reduction in cell viability can be attributed to the presence of mAb 2C5 on the surface which imparts cell targeting property to Doxil, also
seen in case of Doxil-2C5. Cytotoxicity of control, doxorubicin-free plain liposomes, TATp-liposomes, 2C5-liposomes and multifunctional liposomes was conducted with a lipid concentration similar to the Doxil modified liposomes. No significant difference from untreated cells was observed and hence these formulations were not used for further cytotoxicity studies.

Table 5 provides the IC$_{50}$ values of Doxil ® modified with various functionalities. The IC$_{50}$ values of Doxil-TATp were the lowest, followed by multifunctional immuno-Doxil pre-incubated at pH 5. HeLa cells showed similar IC$_{50}$ values for Doxil-2C5 and multifunctional immuno-Doxil pre-incubated at pH 7.4.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>HeLa IC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil</td>
<td>100.0</td>
</tr>
<tr>
<td>Doxil-TATp</td>
<td>1.8</td>
</tr>
<tr>
<td>Doxil-2C5</td>
<td>65.0</td>
</tr>
<tr>
<td>Multi-Dox pH 7.4</td>
<td>70.0</td>
</tr>
<tr>
<td>Multi-Dox pH 5</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Table 5. IC$_{50}$ values of unmodified and surface modified Doxil multifunctional immuno-liposomes with HeLa cells.

In conclusion, multifunctional Doxil helps increasing the liposomal association due to the presence of mAb 2C5. When this formulation is pre-incubated at an acidic pH, the TATp is de-shielded and with the further help of mAb 2C5, improves interaction with the cells and promotes increased tumor cell killing.
The next step was to determine the effectiveness of this formulation in multi drug resistant cells.

4.3.5. Development of drug-resistance in sensitive cell lines.

SKOV3 cells were grown in a medium containing 50 ng/ml of paclitaxel for 11 passages. IC\textsubscript{50} values of sensitive and resistant strains were calculated periodically to determine the extent of resistance.

P-gp efflux pump is one of the most widely characterized proteins responsible for development of resistance. Cross resistance to structurally and functionally unrelated compounds is a characteristic of P-gp. It has already been reported to efflux compounds such as doxorubicin, paclitaxel, colchicine and etoposide. So, the resistant strains developed from incubation with paclitaxel were used for the studies involving doxorubicin. A 50-fold difference was obtained in the IC\textsubscript{50} values calculated for doxorubicin in sensitive vs. resistant SKOV3 cell line.

Additionally, P-gp expression studies were also carried out to confirm that this protein was over-expressed in a higher concentration in the resistant cells as compared to sensitive cells.
4.3.6. P-gp expression studies to determine/confirm drug-resistant and sensitive cells.

To differentiate drug-sensitive cells from drug-resistant cells, the levels of P-gp were measured. An assay of P-gp expression on the cell surface using FITC labeled P-gp specific antibody (UIC2) was conducted.

**P-gp expression levels:**

![Graph showing P-gp expression levels](image)

*Figure 27. Pgp expression in SKOV3 drug-sensitive and drug-resistant cells. Results are mean ± SD, n=3, *p≤ 0.05 indicates significant difference.*

P-gp expression in drug-resistant and sensitive cells was analyzed by flow cytometry. SKOV-3 sensitive cells showed 8% increase in the fluorescence intensity whereas resistant cells had 73 % increase in the fluorescence intensity when compared to untreated cells, indicating the presence of higher amounts of P-gp in the resistant cells (Figure 27).

Thus the presence of P-gp was confirmed as the basis for multi-drug resistance.
4.3.7. Cell interaction studies with SKOV3 drug-resistant and sensitive cells

**Fluorescence microscopy:**

Cell interaction was determined by the method described earlier.

![Fluorescence microscopy of various liposome types with SKOV3 cells](image)

*Figure 28.* Fluorescence microscopy of rhodamine-PE labeled (HSPC-Chol-PEG) plain liposomes, TATp liposomes, mAb 2C5 liposomes or TATp-2C5-Hz liposomes pre-incubated at pH 5.0 or pH 7.4 with SKOV3 drug-sensitive and resistant cells.

*Figure 28* shows the uptake of various rhodamine-PE labeled HSPC: Cholesterol: PEG liposomes by SKOV3 drug-resistant and drug-sensitive cells. TATp liposomes and multifunctional immuno-liposomes pre-incubated at pH 5.0 show a higher interaction in both sensitive as well as resistant cell lines. 2C5 liposomes also conferred cell-interaction property to the liposomes in both the cell lines. These results indicate that mAb 2C5 on
the surface of liposomes helps in targeting the cancer cells and TATp exposed upon degradation of pH-sensitive polymer at acidic pH, further increases the cellular interaction in both cell lines.

**FACS analysis:**

Quantitative analysis of cell interaction of liposomes was performed with Lipodox®, TATp-modified Lipodox®, 2C5-modified Lipodox® and multifunctional immuno-Lipodox® as described earlier. The innate fluorescence of doxorubicin in these liposomes was used to determine the changes in binding of different liposome formulations to the cells.
Figure 29. Flow cytometry. Increase in the geometric mean fluorescence intensity of SKOV3 drug-sensitive (upper panel) and resistant (lower panel) cells incubated for 1 hour with various formulations. Multifunctional carriers were incubated with cells following their pre-exposure to normal/acidic conditions for 1hr. (*p ≤ 0.05, n=3, mean ± SD).

Results obtained in FACS analysis were similar to that obtained from fluorescence microscopy. Lipodox-TATp and multifunctional immuno-lipodox pre-incubated at pH 5 had an overall increased fluorescence intensity compared to Lipodox alone (Figure 29).
The pattern of interaction of each formulation remained the same in both type of cells, indicating that the TATp and 2C5 moieties attached to the surface of the liposomes provide increased interaction of the liposomes with the resistant cells as well.

**4.3.8. *In vitro* cytotoxicity studies.**

Cytotoxicity was determined by the method described earlier.

---

**Figure 30.** *In vitro* cytotoxicity of plain Lipodox®, TATp-modified Lipodox®, mAb 2C5-modified Lipodox® and multifunctional TATp-modified, pH-sensitive, immuno-Lipodox®, preincubated at pH 5.0 or pH 7.4 for 1 hr at 37°C. Upper panel shows the cytotoxicity of the different preparations over a range of concentrations of doxorubicin. The lower panel compares survival of cells at a concentration of 12.5μg/ml of doxorubicin. (*p ≤ 0.05, n=3, mean ± SD).
In vitro cytotoxicity revealed lowest cell viability with Lipodox-TATp, as expected, in both cell lines. The next formulation showing significantly lower cell viability was multifunctional-Lipodox pre-incubated at pH 5. The highest cell viability was observed in plain Lipodox treated cells. Reduction in cell viability produced by Lipodox-2C5 and multifunctional immuno-Lipodox incubated at pH 7.4 can be attributed to the presence of mAb 2C5 on the surface which imparts cell targeting property to Lipodox (Figure 30).

The uptake and hence the cytotoxicity of all the formulations was higher in sensitive cells in comparison with the resistant cells as estimated by the differences in the IC_{50} values for the formulations (Table 6).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>SKOV3 sensitive IC_{50} (µg/ml)</th>
<th>SKOV3 resistant IC_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipodox</td>
<td>6.25</td>
<td>100</td>
</tr>
<tr>
<td>Lipodox-TATp</td>
<td>0.36</td>
<td>3.12</td>
</tr>
<tr>
<td>Lipodox-2C5</td>
<td>4.68</td>
<td>40</td>
</tr>
<tr>
<td>Multifunctional Lipodox, pH 5</td>
<td>0.78</td>
<td>12.5</td>
</tr>
<tr>
<td>Multifunctional Lipodox, pH 7.4</td>
<td>3.125</td>
<td>37.5</td>
</tr>
</tbody>
</table>

Table 6. IC_{50} values of unmodified and surface modified Lipodox multifunctional immuno liposomes with SKOV3 sensitive and resistant cells.

Multifunctional Lipodox pre-incubated at pH 5 increased cell interactions and hence improved the drug delivery and cytotoxicity as compared to Lipodox alone in drug-resistant cell line as well.
4.3.9. *In vivo* studies of multifunctional liposomes in drug-resistant and drug-sensitive tumors.

4.3.9.1. Tumor growth inhibition studies.

![Graph showing tumor growth inhibition studies for SKOV-3 Sensitive tumors. The graph includes lines for Saline, Lipodox, Lipodox-TATp, Lipodox-2C5, and Multifunctional-Lipodox treatments. The x-axis represents days post treatment, and the y-axis represents % change in tumor volume. Significant differences are indicated by asterisks: *** for Saline, ** for Lipodox-TATp, and * for Multifunctional-Lipodox.]
Figure 31. Effect of i.v. administration of liposomal preparations on tumor growth in nude mice bearing SKOV-3 drug sensitive (upper panel) and drug resistant (lower panel) tumors shown as percentage change in tumor volume from initial, calculated as \((\text{V}_n - \text{V}_0) / \text{V}_0 \times 100\), where \(\text{V}_0\) is the tumor volume on day zero of drug administration and \(\text{V}_n\) is the volume on day “n” after drug administration has begun. (\(n=5\), mean + S.D.). *** \(p \leq 0.0001\), ** \(p \leq 0.001\), * \(p \leq 0.05\) calculated from day 10 to day 20, by ANOVA using Tukey’s Post-Hoc test. For SKOV-3 sensitive tumors, * \(p \leq 0.05\) is from day 15 to day 20 between multifunctional Lipodox and Lipodox-2C5. Arrows indicate day of treatment with the formulations given after tumor size of 100 mm\(^3\) was reached.

Multifunctional-Lipodox had an enhanced therapeutic efficacy in vivo in comparison to all controls. The tumor volume in animals treated with this preparation was at least two-fold smaller compared to Lipodox alone in drug-resistant as well as in drug-sensitive SKOV3 tumors (Figure 31). At the end of the treatment, mice treated with multifunctional-Lipodox showed 55% reduction in tumor volume compared to the initial
volume when the treatment was started on SKOV3 drug-sensitive tumors. This was the maximum reduction in tumor volumes when compared to any other formulation treatment. In mice with SKOV3 drug-resistant tumors, treatment with multifunctional-Lipodox increased the tumor volume by only 12% which was the smallest increment when compared to other treatment groups in drug-resistant tumor group. In both drug-sensitive and resistant tumors, multifunctional Lipodox showed significantly reduced tumor volume when compared to Lipodox alone.

Unlike the in vitro data where Lipodox-TATp showed the highest cell interaction and cytotoxicity, this formulation showed no significant difference in reducing the tumor volumes when compared to Lipodox alone. Previous in vitro studies have shown that the peptide gets degraded by proteolytic enzymes in the body which affects its cell interaction as well as cytotoxic effect. Other groups have noted that TATp when conjugated to a drug or to the surface of nanoparticles doesn’t show the expected in vivo results, mainly improved tumor reduction. The effect of human plasma on TATp has shown to reduce the stability of this peptide [107]. TATp mediated drug delivery is also hindered by metabolic degradation in body fluids [133-137].

On treatment with Lipodox-2C5, the tumor volumes were reduced by 3% when compared to the initial tumor volume. Similarly, even though the tumor volumes in SKOV3 resistant tumors increased when treated with Lipodox-2C5, they were significantly smaller as compared to Lipodox alone indicating higher activity of Lipodox-2C5 (Figure 31).
4.3.9.2. Analysis of changes in body weight of mice

An important part in the development of a drug formulation is to determine its safety. Anticancer drugs are known to produce weight loss in patients. Formulations in the form of nanoparticles have shown preferential toxicity to the tumor and low side-effects. As a result it is expected that the body weight will not drop drastically when animals are treated with these formulations.

**Figure 32.** Effect of intravenous administration of liposomal preparations on the body weight in nude mice bearing SKOV-3 drug-sensitive and resistant tumors shown as percentage change in body weight (from initial). (n=5, mean ± S.D, *P ≤ 0.05)

Mice were weighed every 3 days (Figure 32). There was no significant change in the body weight of mice in all treatment groups supporting the absence of gross toxicity.
4.3.9.3. Determination of tumor weight.

The study was ended when the tumors in the mice treated with saline started to exceed a volume of 1000 mm$^3$. Tumors were isolated from the body of the sacrificed mice and weighed.
Figure 3. Final tumor weights in nude mice bearing SKOV-3 sensitive and resistant tumors measured on day 20, following intravenous administration of liposomal preparations as described previously. (n=5, mean ± S.D., *P<0.05)

The final tumor weight determined at the end of the study confirmed that the multifunctional-Lipodox® in both sensitive and resistant tumors served as the most effective treatment (Figure 3). Lipodox treated tumors weighed significantly less than saline treated group indicating activity of the drug. A further increase in the drug’s activity was imparted by the presence of multiple moieties, TATp and mAb 2C5.

4.3.9.4. Determination of apoptosis by TUNEL assay:

TUNEL assay was carried out using the DNA fragmentation kit to confirm the presence of apoptosis in the tumor tissue. DAPI stained the nuclei of the cells present in the tissue. The TUNEL method revealed apoptotic bodies showing green fluorescence of FITC-
labeled TdT. Since TUNEL method stains fragments produced by apoptosis alone, the cell nuclei showing green fluorescence were apoptotic. TUNEL staining showed considerable apoptotic activity in the sections obtained from tumors treated with MultiLipodox, Lipodox-2C5 and Lipodox-TATp. Few apoptotic cells were also seen in non-modified, plain Lipodox. These results show that modification with mAb 2C5 and TATp with pH-sensitive shielding work efficiently in vivo to promote apoptosis (Figure 34, 35).

**Figure 34.** Detection of apoptotic activity by fluorescence microscopy in SKOV-3 drug-sensitive tumor sections as shown by TUNEL staining. Left panel shows DAPI staining while right panel shows TUNEL staining.
Figure 35. Detection of apoptotic activity by fluorescence microscopy in SKOV-3 drug-resistant tumor sections as shown by TUNEL staining. Left panel shows DAPI staining while right panel shows TUNEL staining.

Thus drug-loaded multifunctional immuno-liposomes had an enhanced anti-cancer effect in both drug-resistant and drug-sensitive tumors, indicating that modification of liposomes with active targeting moieties can provide a more prominent therapeutic effect than the conventional unmodified liposomes for the treatment of cancer.
5. CONCLUSIONS

A. Presence of TATp on the surface of nanoparticles increases their interaction with the cells.

B. Degradation of TATp occurs in the presence of proteolytic enzymes and can be prevented by shielding it with a long PEG block.

C. Drug-loaded micelles modified with TATp on the surface shielded by a pH-sensitive long PEG block show enhanced cell interaction and drug delivery when the pH-sensitive bond breaks at the typical acidic pH of a tumor cell environment and TATp is exposed. Enhanced in vivo tumor cell apoptosis is observed with this system indicating that the system is effective in vivo.

D. Drug-loaded multifunctional TATp-modified, pH-sensitive, mAb 2C5 liposomes show enhanced cell uptake and cytotoxicity in comparison to non-modified liposomes in various tumor cell lines including doxorubicin-resistant and sensitive cells.

E. In vivo studies with these liposomes show remarkable reduction of both drug-resistant and sensitive tumors in mice confirming that our system is successful in showing improved therapeutic efficacy over the commercially available unmodified liposomes.

This effect is provided by the multiple functionalities. mAb 2C5 on the surface specifically targets cancer cells. pH-sensitive PEG helps in longer circulation of
the liposome and at the extracellular acidic pH, degradation of this polymer exposes TATp which increases cell penetration.

F. Thus nanoparticles modified with multiple functionalities enhance the effect of an anticancer drug. There are numerous ways to modify the particles. The pathophysiology of the organ should be taken into consideration while developing multifunctional nanoparticles for treatment of the particular disease under research.
6. REFERENCES


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7. APPENDIX

7.1. List of Publications

Some of the data in this thesis has been already published in the following publications:
