Intracellular Delivery: A Multifunctional and Modular Approach

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Abstract Intracellular delivery of drugs and nucleic acids has become one of the most widely explored areas of research. However, it has become increasingly evident that it is also necessary to control the nanocarrier's disposition within a cell. Much attention has been paid nowadays to control the distribution of the nanocarrier within the cell by using organelle targeted nanocarriers. In this review we have described various approaches developed in our laboratory for intracellular delivery of drugs and nucleic acids with lipid-based nanocarriers.

Keywords Cell penetrating peptides • Nanocarrier • Intracellular delivery • pH-sensitive • Organelle targeting

Abbreviations

EPR	enhanced permeability and retention
PEG	polyethylene glycol
PEG-PE	polyethylene glycol-phosphatidyl ethanolamine
CPPs	cell penetrating peptides
LL	Lipofectin® lipids
PTDs	protein Transduction Domains
HIV-1	human immunodeficiency virus type 1
pNP-PEG-PE	(*p*-nitrophenyl) carbonyl-PEG-PE
QDs	quantum dots
APC	antigen presenting cells
PC	egg phosphatidylcholine
Chol	cholesterol

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Rh	rhodamine
DDS	drug delivery system
STPP	stearyl triphenyl phosphonium
Rh-123	rhodamine-123
PCL	paclitaxel
LSD	lysosomal storage diseases
ERT	enzyme replacement therapy
RhB	octadecyl derivative of rhodamine B
C_{12}FDG	5-dodecanoylamino fluorescein di-β-D-galactopyranoside

1 Introduction

It is now well understood that nanocarrier-mediated drug delivery can control the disposition of a drug within the body. The term 'targeting' in nanocarrier-mediated drug delivery often involves binding of the nanocarrier to a cell-surface receptor (receptors that are preferentially expressed/over-expressed on the target cell) followed by internalization of the nanocarrier via the endocytic pathway. The problem, however, is that any nanocarrier entering the cell via the endocytic pathway becomes entrapped in the endosome and eventually ends up in the lysosome, where active degradation under the action of the lysosomal enzymes takes place. This problem is particularly critical for nucleic acids, peptidic drugs that are sensitive to degradation. As a result, only a small fraction of such an unaffected substance appears in the cell cytoplasm. So far, multiple but only partially successful attempts have been made to bring various macromolecular drugs and drug-loaded pharmaceutical carriers directly into the cell cytoplasm, bypassing the endocytic pathway. Methods such as microinjection or electroporation used for the delivery of membrane-impermeable molecules in cell experiments are invasive in nature and can damage the cellular membrane (Chakrabarti et al. 1989; Arnheiter and Haller 1988). Non-invasive methods such as the use of pH-sensitive carriers, including pH-sensitive liposomes (which at low pH inside endosomes destabilize endosomal membrane and liberate the entrapped drug into the cytoplasm) (Straubinger et al. 1985; Torchilin 2005b) and cell-penetrating molecules (Torchilin 2005b, 2007b; Sawant and Torchilin 2010) are much more efficient. These approaches assume that just the cell cytosol delivery is adequate for the final action of a drug or nucleic acid.

However, it has become increasingly evident that it is also necessary to control the nanocarrier's disposition within the cell. Many drugs must be delivered to specific cell organelles such as nuclei (the target for gene and antisense therapy), lysosomes (the target for the delivery of deficient lysosomal enzymes in therapy of lysosomal storage diseases), and mitochondria (the target for pro-apoptotic anticancer drugs) to exert their therapeutic action. Thus, the focus has now moved towards targeting the nanocarrier or its cargo to an individual cell organelle.

Liposomes (mainly, for water soluble drugs) and micelles (for poorly soluble drugs) can be considered as prototype nanocarriers. Liposomes are artificial

phospholipid vesicles with sizes varying from 50 to 1,000 nm and greater, which can be loaded with a variety of drugs (Lasic 1993; Torchilin 2005b). Further, the addition of a polyethylene glycol (PEG) coating renders these liposomes long-circulating (Lasic and Martin 1995), which allows them to accumulate in various pathological areas with compromised (leaky) vasculature, such as tumors or infarcts. It has been shown that (Torchilin et al. 1996) the long-circulating liposomes can be made 'targeted', if antibodies or other specific binding molecules (ligands) have been attached to the water-exposed tips of the PEG chains (Torchilin et al. 2001a).

Micelles, including polymeric micelles, are also a popular and well-investigated pharmaceutical carrier due to their small size (10–100 nm), *in vivo* stability, ability to solubilize water insoluble anticancer drugs, and prolonged blood circulation times (Torchilin 2001, 2007a). The typical core-shell structure of polymeric micelles is formed by the self-assembly of amphiphilic block-copolymers consisting of hydrophilic and hydrophobic monomer units in aqueous media (Torchilin 2001). The use of special amphiphilic molecules as micelle-building blocks can also introduce the property of micelle extended blood half-life. Block-copolymer micelles can also be used to target their payload to specific tissues through either passive or active means. The passive targeting is due to the small micellar size which allows in spontaneous penetration into the interstitium of body compartments with a leaky vasculature (tumors and infarcts) by the enhanced permeability and retention (EPR) effect (Maeda et al. 2000; Torchilin 2001, 2007a; Maeda et al. 2009; Lukyanov et al. 2004). Active targeting of micelles can also be achieved by attachment of target-specific ligands to their surface (Torchilin 2001, 2007a).

We have been specifically interested in micelles made of PEG-phosphatidyl ethanolamine (PEG-PE), where, the use of lipid moieties as hydrophobic blocks capping hydrophilic polymer (such as PEG) chains provides the additional advantage of particle stability when compared with conventional amphiphilic polymer micelles due to the existence of two fatty acid acyls, which contribute considerably to an increase in the hydrophobic interactions between the polymeric chains of the micelle core (Lukyanov and Torchilin 2004). Such PEG-PE micelles demonstrate good stability, longevity in the blood and the ability to accumulate in the areas with a damaged or leaky vasculature (Lukyanov et al. 2004; Lukyanov and Torchilin 2004).

The liposomes and micelles can be assembled in 'modular fashion' by addition of various components such as cationic lipids, intracellular peptide-conjugated lipids, ligand-modified lipids and organelle-targeted lipid conjugates for tumor targeted or intracellular delivery. Various components of these 'modular system' can be further incorporated in one carrier (either liposomes or micelles) to build a 'multifunctional system' (e.g. combination of cell penetrating function, cancer cell targeting antibody and stimuli-sensitivity in one system) to perform various functions simultaneously or in orchestrated fashion.

In this review, we will discuss the approaches successfully developed in our laboratory for intracellular delivery of liposomes and lipid-core micelles, particularly, the use of cationic lipids, cell penetrating peptides (CPPs), and organelle-targeting ligands.

2 Intracellular Delivery of Lipid-Core Micelles with Cationic Lipids

Polymeric micelles cannot diffuse through the cell membrane but rather are internalized by endocytosis. Detailed reviews of the endocytotic pathways and endocytosis of nanocarriers can be found in (Conner and Schmid 2003; Mukherjee et al. 1997; Bareford and Swaan 2007). Following cell uptake, micelles are contained within acidic endosomes and are further directed to various transport pathways including fusion with lysosomes or exocytosis. Therefore, it is necessary to further improve the efficiency of drug-loaded micelles by enhancement of their intracellular delivery to compensate for excessive drug degradation in lysosomes as a result of the endocytosis-mediated capture of micelles by cells.

PEG-PE micelles carry a net negative charge (Lukyanov and Torchilin 2004), which can hinder their internalization by cells. Modification of PEG-PE micelles with positively charged lipids may improve the uptake of drug-loaded micelles by cells. Such positively charged micelles could also more readily escape from endosomes and enter the cytoplasm. To test these ideas, we have prepared paclitaxel-loaded micelles from mixture of PEG-PE and positively charged Lipofectin® lipids (LL) (Wang et al. 2005). The intracellular fate of paclitaxel-loaded PEG-PE/LL micelles and micelles prepared without the addition of the LL was investigated by fluorescence microscopy with BT-20 breast adenocarcinoma cells. Both fluorescently-labeled PEG-PE and PEG-PE/LL micelles were endocytosed by BT-20 cells (Fig. 1). However, with PEG-PE/LL micelles, endosomes appeared to be partially disrupted and released drug-loaded micelles into the cell cytoplasm, a result of the

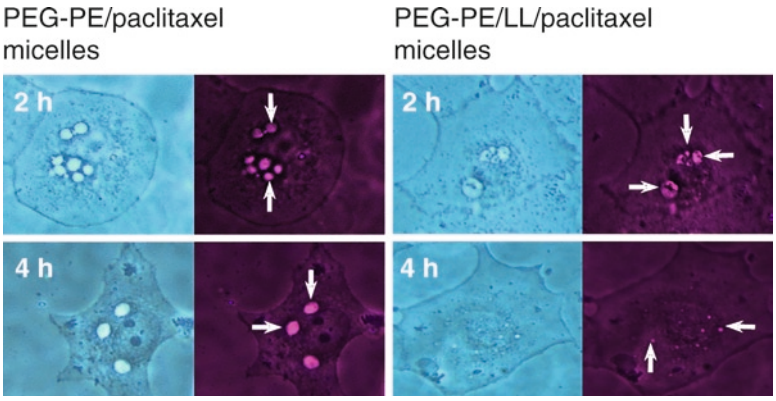

Fig. 1 Microscopy of BT-20 cells incubated with PEG-PE/ paclitaxel micelles and PEG-PE/LL/paclitaxel micelles for 2 and 4 h. Bright-field (*left images* in each pair) and fluorescence (*right images* in each pair). *Arrows* indicate fluorescent endosomes in cells incubated with PEG-PE/paclitaxel micelles for 2 h; partially degraded endosomes in cells incubated with PEG-PE/LL/paclitaxel micelles for 2 h; punctuate fluorescent structures in cells incubated with PEG-PE/LL/paclitaxel micelles for 4 h; larger (fused) endosomes in cells incubated with PEG-PE/paclitaxel micelles for 4 h (Modified from Torchilin 2005a)

de-stabilizing effect of the LL component on the endosomal membranes. After 4 h incubation, larger, fused fluorescent endosomal structures became apparent in the case of LL-free micelles, whereas cells incubated with PEG-PE/LL micelles had smaller punctuate fluorescent structures in the cytoplasm. Increased cytoplasmic delivery of paclitaxel was confirmed by the results of *in vitro* cytotoxicity studies against BT-20 cells (human breast carcinoma) and A2780 cells (human ovarian carcinoma). The paclitaxel-loaded PEG-PE/LL micelles were significantly more cytotoxic compared to that of free paclitaxel or paclitaxel delivered using noncationic LL-free PEG-PE micelles: in A2780 cancer cells, the IC50 values for free paclitaxel, paclitaxel in PEG-PE micelles, and paclitaxel in PEG-PE/LL micelles were 22.5, 5.8 and 1.2 μM, respectively. In BT-20 cancer cells, the IC50 values of the same preparations were 24.3, 9.5 and 6.4 μM, respectively.

However, use of cationic lipids has sometimes been associated with cytotoxicity, especially when used in the high amounts usually used for gene delivery (Torchilin et al. 2003a). Therefore, it is necessary to find a novel ligand that enhances both the cellular uptake and the escape from lysosomal degradation without cytotoxicity or immunogenicity.

3 Intracellular Delivery of Nanocarriers Using Cell Penetrating Peptides (CPPs)

A promising approach for the intracellular delivery that has emerged over the last decade is the use of CPPs (Schwarze et al. 1999). Many different short peptide sequences have been identified that promote transport of a variety of cargoes across the plasma membrane and deliver their payload intracellularly. This process is termed “protein transduction”. Such proteins or peptides contain domains of less than 20 amino acids and are referred to as Protein Transduction Domains (PTDs) or CPPs, which are highly enriched with basic residues (Schwarze and Dowdy 2000).

TATp, the most frequently used CPP, is derived from the transcriptional activator protein encoded by human immunodeficiency virus type 1 (HIV-1) (Jeang et al. 1999). Authors Green (Green and Loewenstein 1988) and Frankel (Frankel and Pabo 1988) demonstrated that the 86-mer trans-activating transcriptional activator, Tat, protein encoded by HIV-1, was efficiently internalized by cells *in vitro* when introduced in the surrounding media. Later it was shown that residues 49–57 were responsible for membrane translocation, with the positive charge contributing largely to the transduction ability of TAT (Park et al. 2002).

TATp-mediated cytoplasmic uptake of plasmid DNA (Astriab-Fisher et al. 2002; Nguyen et al. 2008), nanoparticles (Zhao et al. 2002; Lewin et al. 2000; Rao et al. 2008), liposomes (Torchilin et al. 2001b; Fretz et al. 2004; Levchenko et al. 2003; Torchilin 2001; Zhao et al.) and micelles (Sethuraman and Bae 2007; Sawant et al. 2008) has been reported. A variety of uptake mechanisms appear to be involved in different systems, and in some cases, the mechanism is cell-type or cargo-specific (Zorko and Langel 2005). Smaller molecules attached to TATp seem to transduce

directly into cells by energy independent electrostatic interactions and hydrogen bonding (Vives et al. 2003), but the larger cargos get into cells by an energy dependent macropinocytosis pathway (Wadia et al. 2004). Some examples relating to the intracellular delivery of pharmaceutical nanocarriers by CPPs are presented in Table 1.

Table 1 Examples of nanoparticles delivered using CPPs

Particle and CPP used	Result	References
Liposomes, TAT, antennapedia, octaarginine	Increased intracellular targeting of airway cells.	Cryan et al. (2006)
Liposomes, lipid-modified TATp	Increased cellular uptake in various cells.	Yagi et al. (2007)
Sterically stabilized liposomes, 200 nm, TATp coupled to the linker	Increased transfection in vitro and in vivo.	Torchilin et al. (2003a)
Liposomes, polyarginine	siRNA in R8-liposomes effectively inhibited the targeted gene and significantly reduced the proliferation of cancer cells.	Zhang et al. (2006)
Folic acid targeted, paclitaxel loaded, TATp-modified polymeric liposomes (FA-TATp-PLs)	Increased intracellular uptake of FA-TATp-PLs in both KB and A549 cells and superior toxicity in vivo in SCID mice bearing KB nasopharyngeal cancer.	Zhao et al. (2010)
CLIO (MION) particles, 41 nm, TATp	Mouse lymphocytes, human natural killer, HeLa, human hematopoietic CD34+, mouse neural progenitor C17.2, human lymphocytes CD4+, T- cells, B-cells, macrophages, immune cells, stem cells. For intracellular labeling, MRI, magnetic separation of homed cells, cell imaging.	Josephson et al. (1999), Lewin et al. (2000), and Dodd et al. (2001)
PEG-Polylactic acid micelles, 20–45 nm, TATp	Increased cytotoxicity of doxorubicin at acidic pH.	Sethuraman et al. (2008)
pH-sensitive polymeric micelles, 20–45 nm, TATp	Increased uptake at pH 6.6 compared to pH 7.4.	Sethuraman and Bae (2007)
Gold particles, 20 nm, TATp	Increased intracellular localization.	Tkachenko et al. (2004) and de la Fuente and Berry (2005)
Quantum dot-loaded polymeric micelles, 20 nm, TATp coupled to a linker	Concurrent imaging and distinguishing tumor vessels from perivascular cells and matrix made possible.	Stroh et al. (2005)
Nanocomplexes of PEI and DNA, TATp	Increased transfection in SH-SY5Y cells.	Suk et al. (2006)

(continued)

Table 1 (continued)

Particle and CPP used	Result	References
PEG-PEI conjugates, TATp	Increased transfection in mice.	Kleemann et al. (2005)
Boron carbide nanoparticles, TATp	Increased translocation in murine EL4 lymphoma cells, B16F10 melanoma cells. For boron neutron capture therapy.	Mortensen et al. (2006)
TATp liposomes DNA complexes	Increased transfection in vitro and in vivo.	Gupta et al. (2007)
TATp liposomes DNA complexes.	Increased transfection in vitro of the antigen presenting cells.	Pappalardo et al. (2009)
Low cationic liposomes–plasmid DNA complexes (lipoplexes) modified with TATp and/or with anti-myosin monoclonal antibody 2G4 (mAb 2G4) specific toward cardiac myosin	Increased transfection of hypoxic cardiomyocytes in vitro by the mAb 2G4-modified TATp lipoplexes. Increased accumulation of mAb 2G4-modified TATp lipoplexes in the ischemic rat myocardium and significantly enhanced transfection of cardiomyocytes in the ischemic zone in vivo.	Ko et al. (2009)

3.1 TATp-Modified Liposomes and Lipid-Core Micelles for Drug Delivery and Imaging

We have successfully modified liposomes and micelles with TATp using (*p*-nitrophenyl) carbonyl-PEG-PE (pNP-PEG-PE) (Torchilin et al. 2001a, 2003b). pNP-PEG-PE can be readily incorporated into liposomes and micelles *via* its phospholipid moiety, and it reacts easily with any amino group-containing substrate compound *via* its water-exposed pNP group to form a stable and non-toxic carbamate bond (Fig. 2). The reaction between the pNP group and the amino group of the ligand proceeds easily and quantitatively at pH 8.0, while excessive wfree pNP groups are readily eliminated by spontaneous hydrolysis. Some possible ways to attach CPPs to the micellar and liposomal surface are shown in Fig. 3.

An early study of liposomal delivery with a TATp showed that modification with TATp (47–57), enhanced delivery liposomes intracellularly to different cells, such as murine Lewis lung carcinoma (LLC) cells, human breast tumor (BT20) cells and rat cardiac myocytes (H9C2) (Torchilin et al. 2001b). The liposomes were tagged with TATp via the spacer, pNP-PEG-PE, at a density of a few hundreds of TATp per 200 nm liposome vesicle. These preparations of TATp-liposomes, which allowed for the direct contact of TATp residues with cells, displayed an enhanced liposome uptake by the cells. This suggested that the translocation of TATp-liposomes into cells requires direct, free interaction of TATp with the cell surface. Further studies on the intracellular trafficking of rhodamine-labeled TATp-liposomes loaded with FITC-dextran revealed that

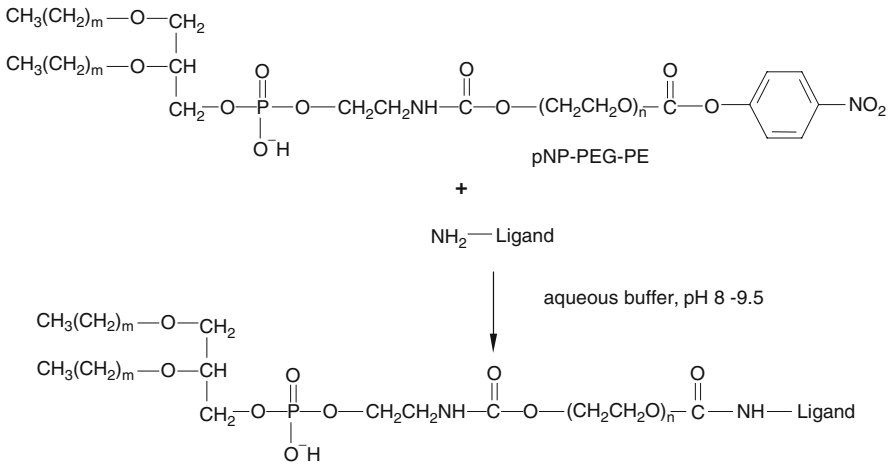

Fig. 2 Schematic of amino-group-containing ligand, such as peptide attachment using pNP-PEG-PE

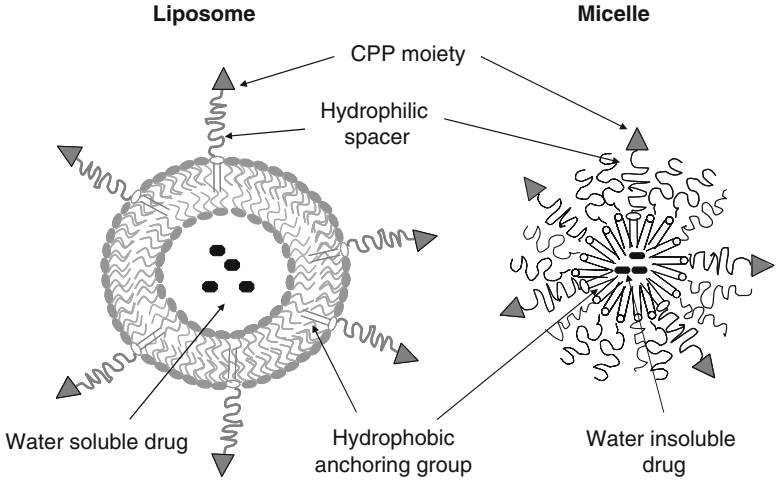

Fig. 3 CPP attached to liposomes and micelles by the insertion into the hydrophobic phase of liposome membrane or micelle inner core via a spacer arm (linker) with a hydrophobic "anchor"

TATp-liposomes remained intact in the cell cytoplasm at 1 h of translocation since the fluorescence of the intraliposomal (FITC-dextran) and membrane (rhodamine-PE) labels coincided (Fig. 4). After 2 h, they had migrated into the perinuclear zone and after several hours, the liposomes had completely disintegrated (Torchilin et al. 2003a).

One of the major obstacles to the use of TATp-mediated intracellular delivery of pharmaceutical nanocarriers is the lack of selectivity of TATp. This non-selectivity has generated concern about drug-induced toxic effects towards normal tissues. This suggested that intratumoral administration of TATp containing

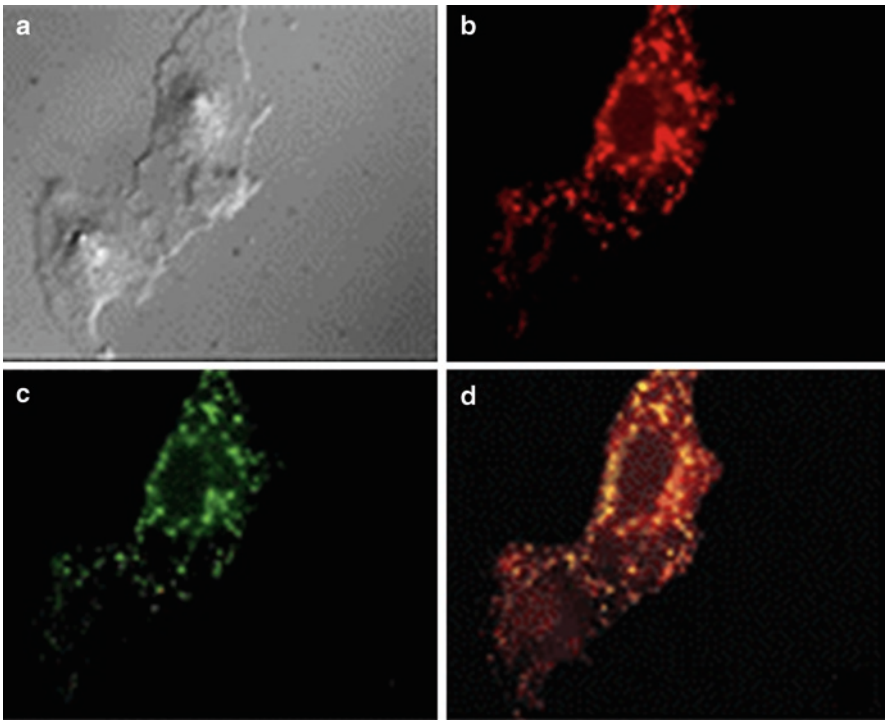

Fig. 4 Intracellular trafficking of rhodamine-PE- labeled and FITC-dextran-loaded TATp-liposomes within BT20 cells. Typical patterns of intracellular localization and integrity of TATp-liposome after 1 h. (**a**), DIC light; (**b**), DIC with an rhodamine filter; (**c**), DIC with an FITC filter; (**d**), DIC composite of (**b**)–(**c**). (Magnification, ×400) (Modified from Torchilin 2005a)

nanocarriers may serve as good solution to this problem for delivery of anticancer drugs at least in certain cases. We prepared and studied paclitaxel-loaded TATp containing PEG-PE micelles (Sawant and Torchilin 2009). Such paclitaxel-loaded micelles were prepared using PEG_{750}-PE as the main micelle-forming component with the addition of 2.5 mol% TATp-PEG_{1000}-PE to promote direct unhindered contact with cells.

The *in vitro* cell interaction of the TATp-bearing PEG-PE micelles was confirmed by fluorescence microscopy with 4T1 cells (Fig. 5). Plain micelles composed of PEG_{750}-PE demonstrated limited interaction with the cells (Fig. 5a). However, the use of the TATp-bearing PEG_{750}-PE micelles resulted in a strong interaction with the cells (Fig. 5b). This enhanced interaction also resulted in increased *in vitro* cytotoxicity against MCF-7 and 4T1 cells with paclitaxel-loaded TATp-bearing micelles compared to paclitaxel-loaded micelles without TATp at both 5 and 50 nM paclitaxel concentrations.

For *in vivo* studies, to avoid any unwanted distribution of paclitaxel-loaded TATp-micelles, micelles were injected intratumorally in mice and tumors were harvested after 48 h. Nuclear DNA fragmentation in tumor sections undergoing

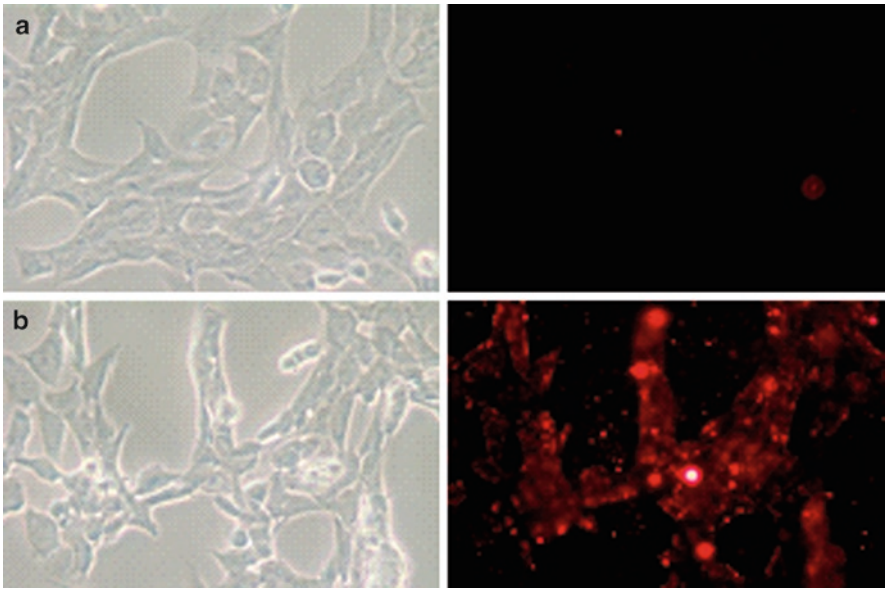

Fig. 5 *In vitro* interaction of rhodamine-PE labeled PEG-PE micelles with 4 T1 cells. Left panel shows the bright field and right panel shows the fluorescent microscopy of 4 T1 cells treated with rhodamine-PE labeled micelles. (**a**), rhodamine-PE :PEG_{750}-PE micelles; (**b**), rhodamine-PE: PEG_{750}-PE: TATp-PEG_{1000}-PE micelles. Magnification ×40 objective (Modified from Sawant and Torchilin 2009)

apoptosis was observed using the TUNEL assay with a DNA fragmentation kit. Very few TUNEL-positive cells were observed in tumors injected with free paclitaxel and paclitaxel-loaded micelles (Fig. 6c). However, significant apoptotic cell death was observed in tumors treated with paclitaxel-loaded TATp-bearing micelles (Fig. 6d).

Another application of CPPs involves the labeling of cells with semiconductor nanocrystals or quantum dots (QDs). QDs are now more popular than standard fluorophores for the study of tumor pathophysiology since they are photostable, more robust stable light emitters, and relatively insensitive to the wavelength of the excitation. They are also capable of distinguishing tumor vessels from both the perivascular cells and the matrix, with concurrent imaging. QDs trapped within PEG-PE micelles bearing a TATp-PEG-PE linker were used to label mouse endothelial cells *in vitro*. For *in vivo* tracking, bone marrow-derived progenitor cells labeled with TATp-bearing QD-containing micelles *ex vivo*, were injected in mice bearing a tumor in a cranial window model. It was possible to track the movement of labeled progenitor cells to the tumor endothelium, that may provide a path towards the understanding of the fine details of tumor neovascularization (Stroh et al. 2005).

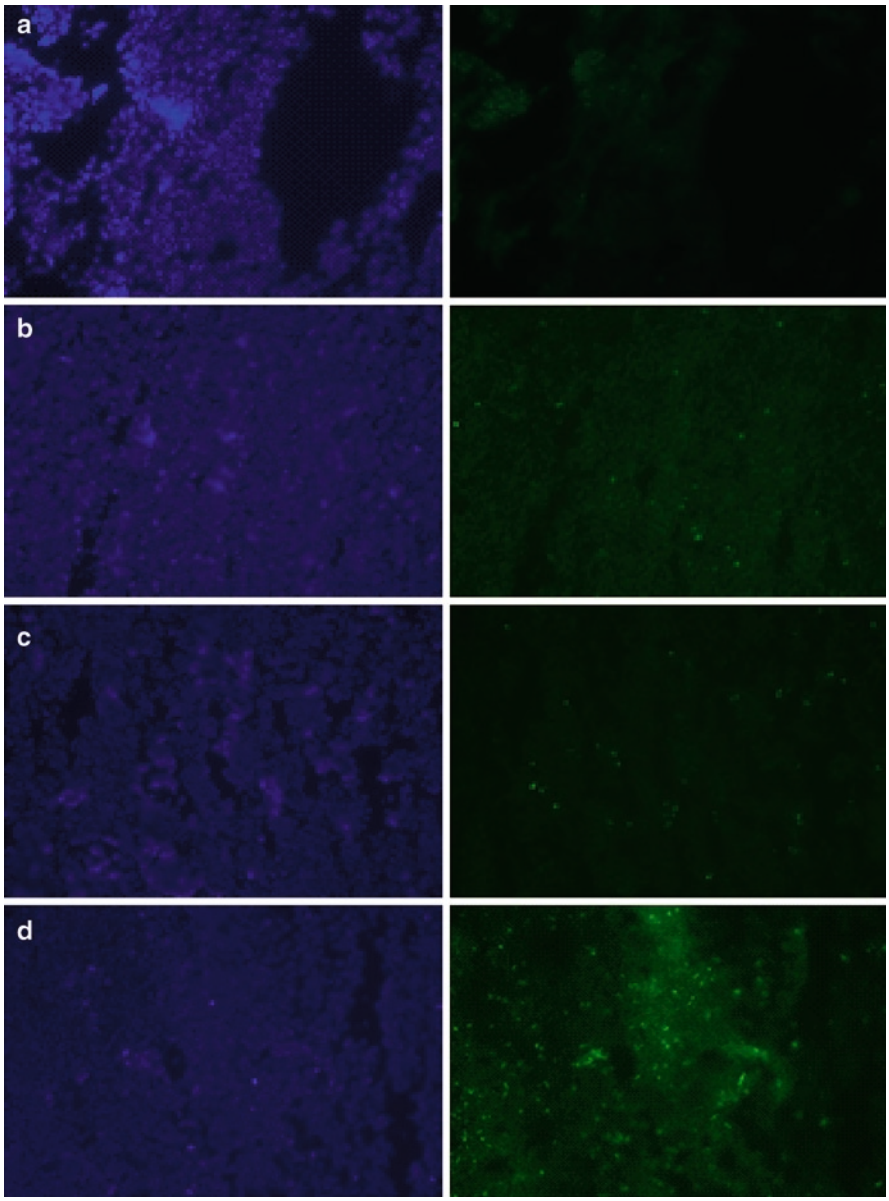

Fig. 6 Detection of apoptotic cells by fluorescence microscopy of frozen tumor sections. Apoptosis was determined by TUNEL. *The left panel* shows the sections stained with DAPI and the *right panel* shows TUNEL. (**a**), Negative control; (**b**), free paclitaxel; (**c**), paclitaxel-loaded micelles without TATp; (**d**), paclitaxel-loaded micelles with TATp. Magnification ×20 objective (Modified from Sawant and Torchilin 2009)

3.2 TATp- Modified Liposomes for Delivery of Nucleic Acids

Another exceptionally important yet challenging task is cellular delivery of nucleic acids. Various methods used to deliver these highly negatively charged biomolecules are associated with cellular toxicity or poor efficiency in certain types of cells (e.g. lipofectamnine or microinjection). Currently, liposomes and cationic polymers are used for transfection but they are also less efficient and often accompanied by high levels of toxicity.

Due to the size of plasmids and the high number of negative charges, non-covalent approach have been mostly used. It has been reported that TATp binds to DNA to form complexes which can be internalized through endocytosis (Sandgren et al. 2002). In our laboratory we prepared TATp–liposomes with the addition of a small quantity of a cationic lipid (DOTAP) and incubated with DNA to form stable non-covalent complexes with a model gene encoding for the enhanced-green fluorescent protein (pEGFP-N1) (Torchilin and Levchenko 2003). Such TATp–liposome–DNA complexes when incubated with mouse fibroblast NIH 3T3 and cardiac myocytes H9C2 showed substantially higher transfection *in vitro*, with lower cytotoxicity than the commonly used Lipofectin®.

We have also investigated the potential of TATp-modified liposomes to enhance the delivery of the model gene, pEGFP, to human brain tumor U-87 MG cells *in vitro* and in an intracranial tumor model in nude mice (Gupta et al. 2007). The size distribution of DNA-loaded TATp–liposomes was narrow (around 250 nm) and the DNA complexation was firm at lipid/DNA (+/−) charge ratios of 5 and higher. TATp–lipoplexes demonstrated an enhanced delivery of pEGFP to U-87 MG tumor cells *in vitro* at lipid/DNA (+/−) charge ratios of 5 and 10. *In vivo* transfection of intracranial brain tumors by intratumoral injections of TATp–lipoplexes showed an enhanced delivery of pEGFP selectively to tumor cells and subsequent effective transfection compared to plain plasmid-loaded lipoplexes. No transfection was observed in the normal brain adjacent to the tumor. Thus, TATp–lipoplexes can be used to augment the delivery of genes to tumor cells when injected intratumorally, without affecting the normal adjacent brain.

Another example is gene delivery into immunocompetent cells to modulate immune response. Antigen presenting cells (APC) are among the most important cells of the immune system since they link the innate and the adaptive immune responses, directing the type of immune response to be elicited. However, APC are very resistant to transfection. To increase the efficiency of APC transfection, we used liposome-based lipoplexes additionally modified with TATp for better intracellular delivery of a model pEGFP. pEGFP-bearing lipoplexes made of a mixture of egg phosphatidylcholine (PC): cholesterol (Chol): DOTAP (60:30:10 M ratio) with the addition of 2% mol of PEG-PE conjugate (plain-L) or TATp-PEG-PE (TATp-L) effectively protected the incorporated DNA from degradation. Uptake assays of rhodamine (rh)-labeled lipoplexes and transfections with the EGFP reporter gene were performed with APC derived from the mouse spleen.

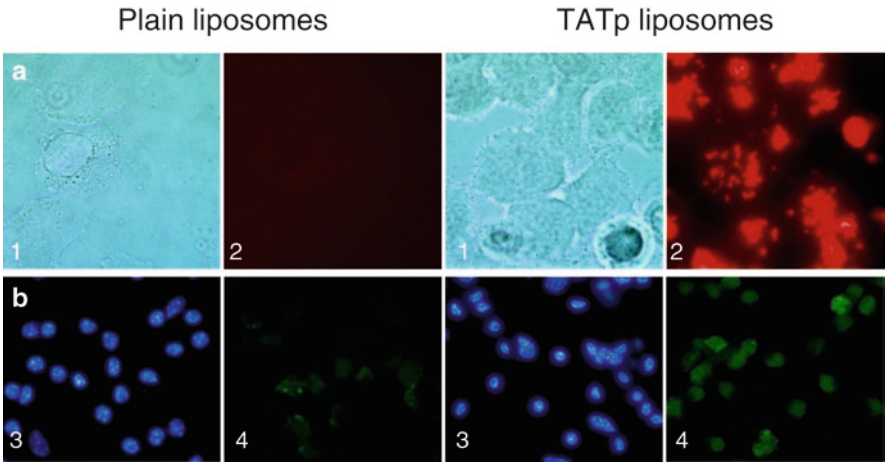

Fig. 7 (**a**) Liposome uptake and internalization of APC cultures incubated for 60 min with Rh-labeled plain-L and TATp-L (100×); (**b**), APC culture fluorescence microscopy 48 h post transfection with EGFP (40×). *1*: bright field, *2*: rh filter, *3*: Hoechst, *4*: EGFP (Modified from Pappalardo et al. 2009)

TATp-L-based lipoplexes significantly enhanced both the uptake and transfection of APC (Fig. 7) (Pappalardo et al. 2009).

We recently reported a double–targeted delivery system simultaneously capable of extracellular accumulation and intracellular penetration for gene therapy in the treatment of myocardial ischemia. We used low cationic liposome–plasmid DNA complexes (lipoplexes) modified with TATp and/or with monoclonal anti-myosin monoclonal antibody 2G4 (mAb 2G4) specific toward cardiac myosin, for targeted gene delivery to ischemic myocardium. *In vitro* transfection of both normoxic and hypoxic cardiomyocytes was enhanced by the presence of TATp determined by fluorescence microscopy and ELISA. The enhanced transfection with TATp-lipoplexes indicated that intracellular delivery mediated by TATp played an important role in the transfection of hypoxic as well as normoxic cells. The *in vitro* transfection was further enhanced by the additional modification with mAb 2G4 antibody in the case of hypoxic, but not normoxic cardiomyocytes. This can be explained by the additional mAb 2G4-mediated targeted delivery of the lipoplexes to the hypoxic cells because of the better binding of the lipoplexes with the hypoxically damaged cells due to exposure of intracellular cardiac myosin. However, we did not observe a synergism between TATp and mAb 2G4 ligands under our experimental conditions. In *in vivo* experiments, we clearly demonstrated an increased accumulation of mAb 2G4-modified TATp lipoplexes in the ischemic rat myocardium and significantly enhanced transfection of cardiomyocytes in the ischemic zone. Thus, the genetic transformation of normoxic and hypoxic cardiomyocytes can be enhanced by using lipoplexes modified with TATp and/or mAb 2G4 (Ko et al. 2009).

3.3 TATp-Modified Liposomes and Micelles: A Multifunctional Approach

An ideal nanoparticular drug delivery system (DDS) should be able to (1) specifically accumulate in the required organ or tissue, and then (2) penetrate target cells to deliver its load (drug or DNA) intracellularly. Organ or tissue (tumor, infarct) accumulation could be achieved by the passive targeting *via* the EPR effect (Maeda et al. 2000) assisted by prolonged circulation of such a nanocarrier (for example, as a result of its coating with protecting polymer such as PEG); or by antibody-mediated active targeting (Torchilin 2004) and (Jaracz et al. 2005), while the intracellular delivery could be mediated by certain internalizable ligands (folate, transferrin) (Gabizon et al. 2004) and (Widera et al. 2003) or by CPPs (Gupta et al. 2005). Ideally, such a DDS should simultaneously carry on its surface various active moieties, i.e. be multifunctional and possess the ability to "switch on" certain functions (such as intracellular penetration) only when necessary, for example under the action of local stimuli characteristic of the target pathological zone (first of all, increased temperature or lowered pH values characteristic of inflamed, ischemic, and neoplastic tissues). These "smart" DDS should be built in such a way that during the first phase of delivery, a non-specific cell-penetrating function is shielded by the function providing organ/tissue-specific delivery (sterically protecting polymer or antibody). Upon accumulating in the target, protecting polymer or antibody attached to the surface of the DDS *via* the stimuli-sensitive bond should detach under the action of local pathological conditions (abnormal pH or temperature) and expose the previously hidden second function to allow for the subsequent delivery of the carrier and its cargo inside cells (Fig. 8).

With this in mind, we prepared targeted long-circulating PEGylated liposomes and PEG–PE-based micelles possessing several functionalities (Sawant et al. 2006; Kale and Torchilin 2007a). First, such systems targeted a specific cell or organ by attaching the monoclonal antibody (infarct-specific antimyosin antibody 2G4 or cancer-specific antinucleosome antibody 2C5) to their surface *via* reactive pNP–PEG–PE moieties. Second, these liposomes and micelles were additionally modified with TATp moieties attached to the surface of the nanocarrier by using TATp-short PEG–PE derivatives. PEG–PE used for liposome surface modification or for micelle preparation was made degradable by inserting a pH-sensitive hydrazone bond between PEG and PE (PEG–Hz–PE). Under normal pH values, TATp functions on the surface of nanocarriers were "shielded" by the long PEG chains (pH-degradable PEG_{2000}–PE or PEG_{5000}–PE) or by long pNP–PEG–PE moieties used to attach antibodies to the nanocarrier (non-pH-degradable PEG_{3400}–PE or PEG_{5000}–PE). At pH 7.5–8.0, both liposomes and micelles demonstrated high specific binding with antibody substrates, but very limited internalization by NIH/3T3 or U-87 cells. However, upon brief incubation (15–30 min) at lower pH values (pH 5.0–6.0) nanocarriers lost their protective PEG shell because of acidic hydrolysis of the PEG–Hz–PE and were effectively internalized by cells *via* TATp moieties (Fig. 9a).

In vivo, TATp-modified pGFP-loaded liposomal preparations have been administered intratumorally in tumor-bearing mice, and the efficacy of tumor cell transfection

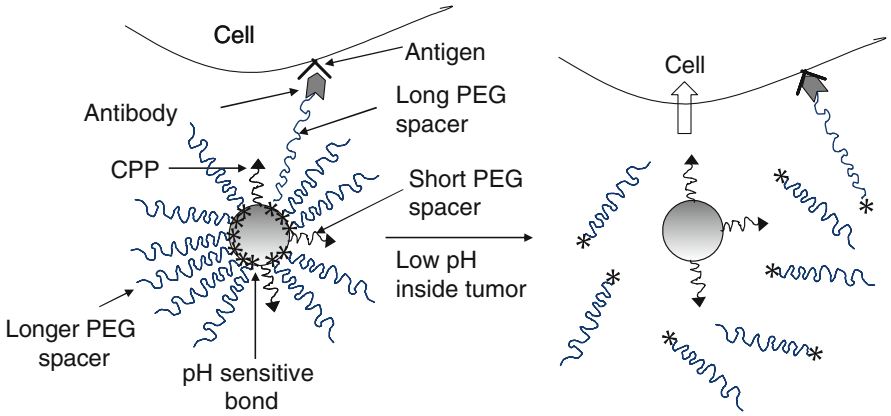

Fig. 8 The principle scheme of the action of stimuli-sensitive double-targeted nanocarriers. The surface of the nanocarrier is modified with a CPP via a relatively short spacer; with longer PEG chains; and with even longer PEG chains decorated at distal termini with a target-specific ligand (antibody). CPP is shielded with longer chains, while these PEG chains and PEG–antibody chains are attached to the surface via pH-sensitive bonds. The whole system is stable in the blood and accumulates in the tumor via the PEG-mediated EPR effect and via antibody-mediated targeting. Inside the tumor, protective PEG chains and PEG–antibody conjugates are detached from the surface because of fast hydrolysis of pH-sensitive bonds at the lowered intratumors pH, CPP becomes exposed and allows for the intracellular delivery

was assessed after 72 h. The administration of pGFP–TATp–liposomes with a non-pH-sensitive PEG coating resulted in minimal transfection of tumor cells because of steric hindrances for liposome-to-cell interaction created by the PEG coat, which shielded the surface-attached TATp. The administration of pGFP–TATp–liposomes with the low pH-detachable PEG resulted in the highly efficient transfection. The removal of PEG under the action of the decreased intratumoral pH led to the exposure of the liposome-attached TATp residues, enhanced penetration of the liposomes inside tumor cells and more effectively delivered the pGFP intracellularly (Fig. 9b) (Kale and Torchilin 2007b).

TATp-modified stimuli-sensitive polymeric micelles with an enhanced ability to interact with cells under acidified conditions have also been described in (Sethuraman and Bae 2007). These results can be considered as an important step in the development of tumor-specific stimuli-sensitive drug and gene delivery systems.

4 Subcellular Targeted Nanocarriers

The next step in the development of targeted nanocarriers would be designing of subcellular or organelle targeted nanocarriers to target at molecular receptor level (Rajendran and Knolker 2010; D'Souza and Weissig 2009; Torchilin 2006). Our focus has been on the development of nanocarrier systems targeted to mitochondria and lysosomes.

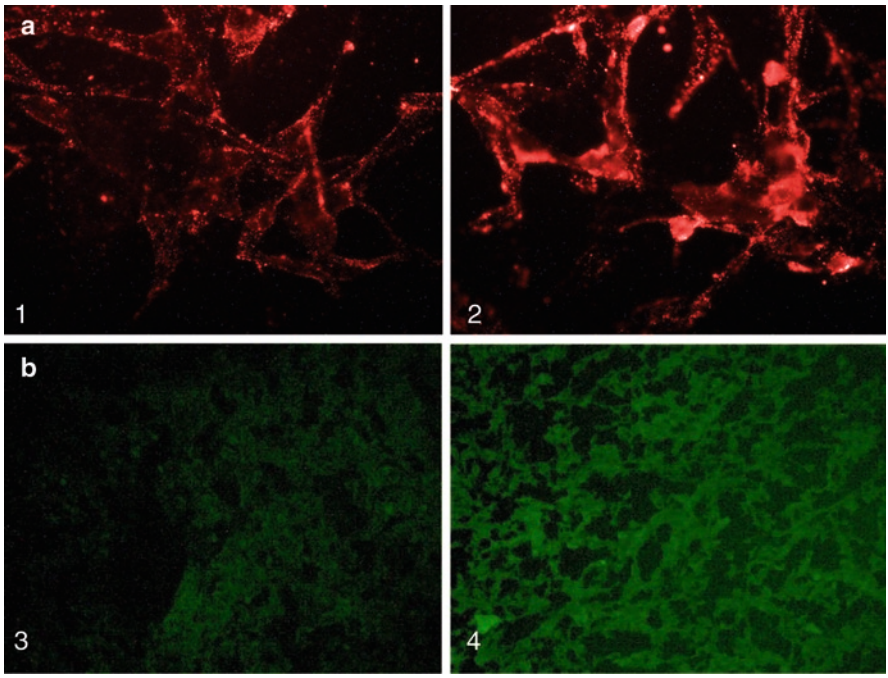

Fig. 9 (**a**), Fluorescence microscopy showing internalization of Rh-PE-labeled-TATp containing liposomes internalization by U87 MG astrocytoma. *1*: 9 mol % pH-non-sensitive PEG-PE at pH 7.4, *2*: 9 mol % pH-sensitive PEG-Hz-PE after incubation at pH 5.0 for 20 min. The cleavable (pH sensitive) PEG-PE-based TATp-containing liposomes kept at pH 7.4–8 show only a marginal association with cells while those preincubated for 20–30 min at pH 5.0 demonstrated a dramatically enhanced association with the cells (higher fluorescence). (**b**), Fluorescence microscopy images of LLC tumor sections from tumors injected with pGFP-loaded TATp-bearing liposomes. *3*: using a pH-non-cleavable PEG coat, *4*: with a low pH-cleavable PEG coat

Mitochondria represent an important target for intracellularly delivered drugs and DNA. Mitochondrial dysfunction contributes to a variety of human disorders, ranging from neurodegenerative diseases, obesity, diabetes, ischemia-reperfusion injury and cancer (Wallace 1999). The number of diseases is also found to be associated with defects of the mitochondria genome has grown significantly over the past decade. Mitochondria also play a key role in the complex apoptosis mechanism. The mechanism of paclitaxel induced apoptosis is believed to be by stabilization of microtubules of cells (Fan 1999; Wang et al. 2000). It has been also observed that there is a 24 h delay between paclitaxel-induced release of cytochrome *c* in intact cells versus cell-free system (Andre et al. 2002). This could be due to only few drug molecules reaching the mitochondria. Hence, we hypothesized that we could improve the apoptosis due to paclitaxel if we could delivery paclitaxel to mitochondria.

The mitochondrion has a major role in the metabolism of eukaryotic cells in the synthesis of ATP by oxidative phosphorylation via the respiratory chain. This process

creates a transmembrane electrochemical gradient, which includes contributions from both a membrane potential (negative inside) and a pH difference (acidic outside). The membrane potential of mitochondria *in vitro* is between 180 and 200 mV, which is the maximum a lipid bilayer can sustain while maintaining its integrity (Murphy 1989). Positively charged molecules are attracted by mitochondria in response to the highly negative membrane potential, but most charged molecules cannot enter the mitochondrial matrix because the inner mitochondrial membrane is impermeable to polar molecules. However, certain amphiphile compounds are able to cross both mitochondrial membranes and accumulate in the mitochondrial matrix in response to the negative membrane potential. It has long been known that amphiphile compounds with delocalized cationic charge can accumulate in mitochondria (Weissig and Torchilin 2001). Rhodamine 123 (Rh-123), a stain for mitochondria in living cells, is the best known representative of this group (Chen et al. 1982). Mitochondrial accumulation of tetraphenylphosphonium chloride and other cationic aryl phosphonium salts was also demonstrated (Rideout et al. 1994). The mitochondrial accumulation and retention of dequalinium (DQA), a single-chain bola amphiphile with two delocalized positive charge centers was also demonstrated (Weissig and Torchilin 2001).

We have modified liposomes with using stearyl triphenyl phosphonium (STPP) to render them mitochondriotropic (Boddapati et al. 2008). *In vitro* STPP liposomes selectively accumulated in mitochondria of living cells. Also when loaded with ceramide as model drug, it elicited strong apoptotic response *in vivo* in 4T1 mammary carcinoma tumor-bearing mice at ceramide doses as low as 6 mg/kg in comparison with the 36 mg/kg or higher reported with non-targeted liposomes.

Recently, we prepared a novel mitochondria-targeted liposomal drug delivery system by the modification of the liposomal surface with Rh-123 (Biswas et al. 2010). A novel polymer was synthesized by conjugating the mitochondriotropic dye Rh-123, with the amphiphilic PEG-PE conjugate. The co-localization study with stained mitochondria (Fig. 10) as well as with the isolation of mitochondria of the cultured cells after their treatment with Rh123-liposomes showed a high degree of accumulation of the modified liposomes in the mitochondria.

To demonstrate that specific delivery of the drug to the desired subcellular compartment can significantly enhance drug action, the mitotic inhibitor, paclitaxel was used. Paclitaxel-loaded Rh123-liposomes (PCL-Rh123-L) produced significantly higher cytotoxicity than free paclitaxel or paclitaxel-loaded plain liposomes. An approximately 35–40% reduction of cell survival was observed with PCL-Rh123-L compared to non-targeted PCL formulations. Thus, Rh-123-modified liposomes target mitochondria efficiently and can facilitate the delivery of a therapeutic payload to mitochondria.

Lysosomes, acidic organelles responsible for recycling of cellular constituents, represent another important intracellular target for diseases such as lysosomal storage diseases (LSD). LSD is associated with the deficiency of certain lysosomal enzymes, which lead to accumulation of corresponding substrates in lysosomes (Futerman and van Meer 2004). These diseases pose a serious medical problem

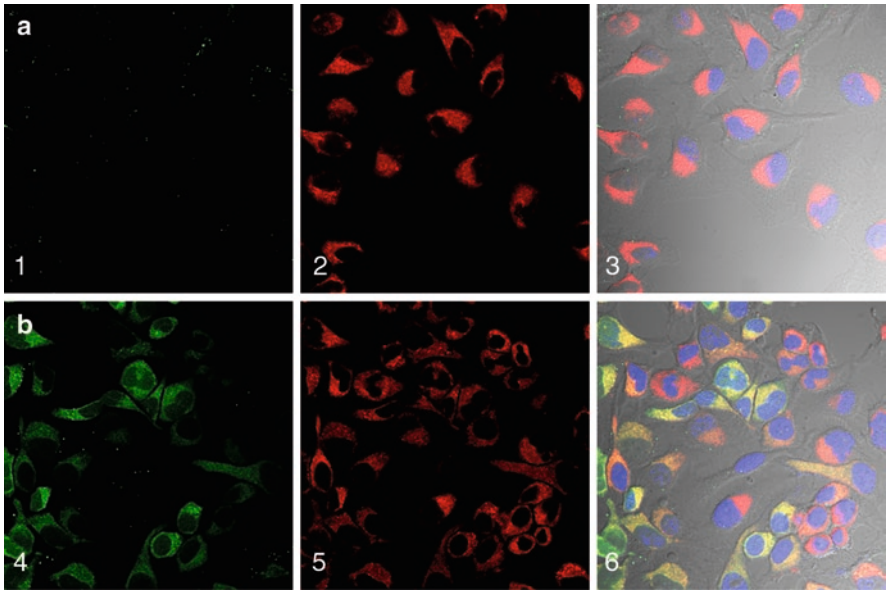

Fig. 10 Intracellular colocalization by fluorescence confocal microscopy. HeLa cells treated with (**a**), NBD-PE labeled plain liposomes; (**b**), Rh123-liposomes. *1*,*4*: Cell treatment with PL or Rh123-L in the green channel (Ex. 505 nm, Em. 530 nm); *2*,*5*: Cell staining treatment for visualization of mitochondria with Mitotracker deep red in the deep red channel (Ex. 644 nm, Em. 665 nm); *3*,*6*: Merged *left* and *middle* panels and Hoechst stained nuclei (Blue channel. Ex. 385 nm, Em,. 470 nm). *Yellow color* indicates co-localization of mitochondria and Rh123-L. Analysis of co-localization (Image J software) confirmed significant accumulation of targeted liposomes in the mitochondria (Pearson's coefficient 0.55, Mander's coefficient 0.75 for Rh123-L compared to Pearson's coefficient −0.083, Mander's coefficient 0.007 for plain liposomes)

(Grabowski 2008; Zarate and Hopkin 2008; van der Ploeg and Reuser 2008). The main approach for the treatment of LSD is enzyme replacement therapy (ERT) based on the administration of exogenous enzymes (Grabowski and Hopkin 2003). This procedure remains limited in use and expensive because of poor delivery and low stability of therapeutic enzymes. The use of liposome-immobilized enzymes for ERT, was understood long ago (Gregoriadis 1978). Lysosomes are also involved in the cellular apoptosis due to the lysosome-dependent cell death pathway (Kirkegaard and Jaattela 2009). Moderate permeabilization of lysosomal membranes can result in apoptosis of cancer cells (Boya et al. 2003). Thus, delivery of lysosome-destabilizing agents that cause cancer cell apoptosis may also benefit from lysosome-targeted carriers.

We recently attempted to develop a lysosome-targeted drug delivery system based on liposomes modified with a lysosome-specific ligand octadecyl derivative of rhodamine B (RhB). RhB is used to monitor membrane fusion (Hoekstra et al. 1984) and study lysosomal metabolism (Kuwana et al. 1995). It has been shown to specifically accumulate in the lysosomes of denervated skeletal muscle

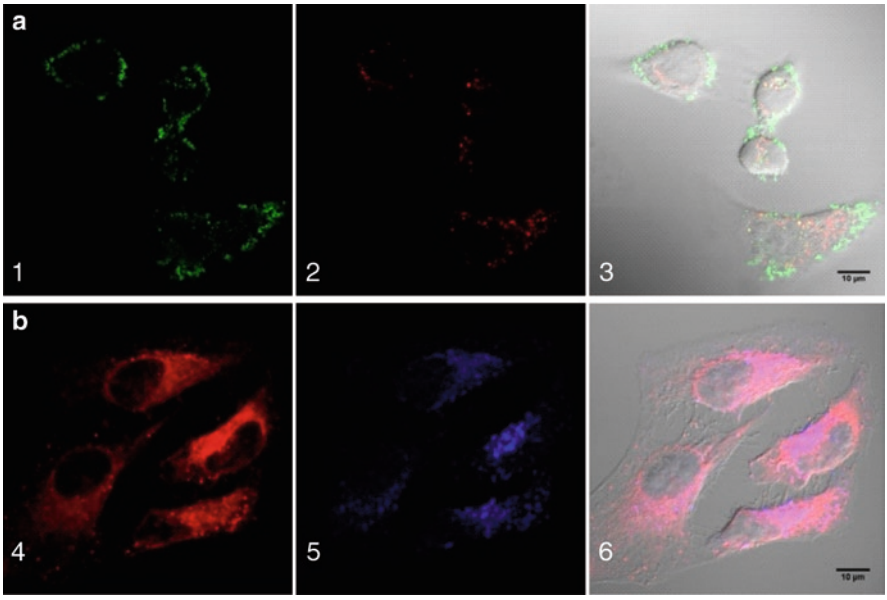

Fig. 11 Confocal microscopy of HeLa cells treated with (**a**), FITC-dextran-loaded liposomes; (**b**), RhB-modified FITC-dextran-loaded liposomes for 4 h. The treated cells were stained with lysosomal markers and analyzed by confocal microscopy. *1*: FITC-dextran-loaded plain liposomes (*green*), *2*: LysoTracker Red-stained lysosomes (*red*), *3*: Overlay of 1 and 2 images with their respective DIC image. *4*: RhB (*red*), *5*: anti-Lamp2 mAb-stained lysosomes (*blue*), *6*: Overlay of 4 and 5 images with their respective DIC image. Bar = 10 μm. Cell incubation with RhB1-modified FITC-dextran-loaded liposomes for 4 h led to the localization of RhB fluorescence mostly in the lysosomes with a high rate of the co-localization with the lysosomal marker (Pearson's correlation coefficient, PCC 0.7; Mander's overlap coefficient, MOP 0.8). The cells treated with the same concentration of FITC-dextran-loaded liposomes demonstrated much lower localization of FITC-dextran in the lysosomes (PCC – 0.1; MOP 0.2)

(Vult von Steyern et al. 1996). Novel acidic fluorescent probes based on rhodamine-B have also been described and used for the optical imaging of the intracellular H^+ (Zhang et al. 2009).

We prepared liposomes loaded with the model compound, FITC-dextran, and modified with RhB (Koshkaryev et al. 2010). Confocal microscopy demonstrated that RhB-liposomes co-localize well with the specific lysosomal markers, unlike plain liposomes (Fig. 11). The comparison of the FITC fluorescence of the lysosomes isolated by subcellular fractionation also showed that the efficiency of FITC-dextran delivery into lysosomes by RhB-modified liposomes was significantly higher compared to plain liposomes.

This was confirmed using 5-dodecanoylamino fluorescein di-β-D-galactopyranoside (C_{12}FDG)-loaded liposomes. C_{12}FDG, is a lipophilic substrate for the lysosomal β-galactosidase (Rotman et al. 1963). It was assumed that upon undergoing the endocytosis, C_{12}FDG-loaded liposomes would eventually deliver their contents into lysosomes, and the intra-lysosomal β-galactosidase will hydrolyze the non-fluorescent

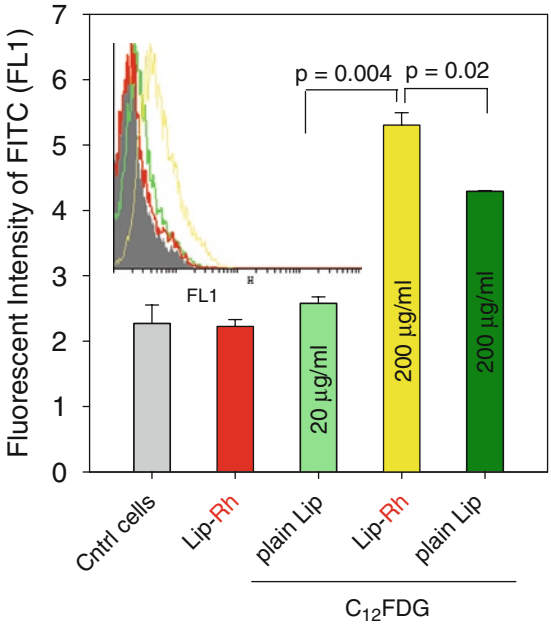

Fig. 12 Flow cytometry of lysosomal targeting by liposomes loaded with C_{12}FDG. HeLa cells were incubated with plain liposomes (20 and 200 μg/ml), Lip-RhB (200 μg/ml). The liposomes were loaded with C_{12}FDG (1.5% mol/mol), a fluorescent substrate for the intralysosomal β-galactosidase. After 4 h incubation with liposomes, the cells were washed and additionally incubated for 20 h with liposome-free DMEM. The fluorescence intensity of FITC (channel FL1) was determined by flow cytometry. Each value is the mean ± SD of 2 experiments

C_{12}FDG into the fluorescent C_{12}FITC, which will be retained inside lysosomes because of its lipophilic moiety. Thus, with a standard flow cytometry procedure, the lysosomal targeting can be quantified by following the fluorescence intensity of live, intact cells. We prepared plain and RhB-modified liposomes loaded with C_{12}FDG. The loading of C_{12}FDG into RhB-modified liposomes was approximately ten times less than into the plain liposomes. This decrease in the C_{12}FDG loading can be attributed to a stoichiometric competition between C_{12}FDG and RhB in the liposomal membrane leading to the partial loss of C_{12}FDG due to its shorter lipophilic moiety. Two different concentrations of plain liposomes (20 μg/ml and 200 μg/ml) were used for cell treatment to achieve the same amount of C_{12}FDG as with RhB-modified liposomes (200 μg/ml). The treatment of cells with different concentrations of C_{12}FDG-loaded plain liposomes (20 and 200 μg/ml) led to a dose-dependent increase in their FITC fluorescence relative to the control (untreated) cells (Fig. 12). These data suggest that endocytosed liposomes actually deliver C_{12}FDG into lysosomes. The cells treated with 200 μg/ml of RhB-modified liposomes demonstrated significantly increased C_{12}FITC fluorescence compared to the cells treated with both 20 and 200 μg/ml of the plain liposomes.

In any case, the development of organelle-specific drug delivery is only in its early stages; however, it might have important clinical future.

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