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Biodegradable Nanoparticles for Cytosolic Delivery of Therapeutics

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Abstract

Many therapeutics require efficient cytosolic delivery either because the receptors for those drugs are located in the cytosol or their site of action is an intracellular organelle that requires transport through the cytosolic compartment. To achieve efficient cytosolic delivery of therapeutics, different nanomaterials have been developed that consider the diverse physicochemical nature of therapeutics (macromolecule to small molecule; water soluble to water insoluble) and various membrane associated and intracellular barriers that these systems need to overcome to efficiently deliver and retain therapeutics in the cytoplasmic compartment. Our interest is in investigating PLGA and PLA-based nanoparticles for intracellular delivery of drugs and genes. The present review discusses the various aspects of our studies and emphasizes the need for understanding of the molecular mechanisms of intracellular trafficking of nanoparticles in order to develop an efficient cytosolic delivery system.

Keywords

Biodegradable polymers; Nanoparticles; Sustained release; Gene delivery; Drug delivery; Cellular uptake; Endocytosis

Introduction

Effective intracellular drug delivery is important for therapeutic agents which have specific molecular targets inside a cell. The targets can be located in the cytoplasm (glucocorticoid receptors, proteins, siRNA), nucleus (DNA, antisense oligonucleotides, DNA intercalating agents such as doxorubicin), mitochondria (anti-oxidants) or other subcellular compartments of a cell. Further, cytosolic delivery is desirable for drugs which undergo extensive efflux from the cell by the efflux transporters such as multidrug resistance proteins (MRP) and P-glycoproteins (P-gp) [1]. Macromolecular drugs such as recombinant proteins and plasmid DNA usually have their site of action in the cytoplasm and nucleus, respectively. The ultimate goal of gene delivery can be fulfilled only if the plasmid DNA is able to localize and integrate with the nuclear or mitochondrial DNA. Further, these therapeutic entities are highly susceptible to enzymatic degradation and delivery systems need to be designed in order to ensure the protection of proteins/plasmid DNA from proteases and nucleases.

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Drug carrier systems for cytosolic delivery of therapeutics

A number of drug carrier systems (liposomes, cell penetrating peptides, cationic polymer conjugates, polymeric nanoparticles) have been explored for intracellular delivery of therapeutics. These are required to cross a series of membrane barriers in order to reach the site of drug action in the cells and during this process lose a significant portion of the drug molecules at each successive barrier. These barriers include the cellular association and internalization of the drug-carriers by endocytosis; intracellular trafficking and release of drug or drug-carrier into the cytoplasm; cytoplasmic translocation of drug or drug-carrier to nucleus or any other cellular organelle; and the nuclear/organellar uptake. Figure 1 depicts a typical intracellular trafficking pathway for nanoparticles (NPs) and other colloidal drug carrier systems. The cell contains several intracellular organelles with specific functions. Intracellular targeting of therapeutics to these specific organelles is not only expected to significantly enhance the therapeutic efficacy but also reduce non-specific effect and hence toxicity. Therefore, there is significant interest in achieving intracellular target-specific delivery of therapeutics using different carrier systems.

The efficiency of drug-carriers for cytosolic delivery of therapeutics is limited mainly by their interaction with cell membranes and the endosomal release of carriers. Most of the carriers including liposomes and polymeric NPs associate with the cell membrane and are internalized into cells by means of endocytic mechanisms. Cell recognition and association of drug-carriers with cell membrane can be enhanced by the use of targeting ligands which can bind to specific receptors on cell membranes. This promotes not only the association and binding of drug-carriers to the cell membrane but also can increase the cellular internalization by means of receptor-mediated endocytosis. Another bottleneck for cytosolic drug delivery is the sequestration of drug-carriers within the endosomal compartment, following endocytosis. This has opened numerous avenues for research into development of strategies to enhance the endosomal escape of drug-carriers, in order to improve the efficiency of cytosolic drug delivery.

Liposomes

Liposomes have been extensively investigated as a potential drug carrier system for cytosolic delivery due to the enormous diversity of structure and compositions that can be achieved. Different formulation strategies have been developed to increase the ability of liposomes to mediate cytosolic delivery of therapeutics. These include the development of ‘fusogenic’ and ‘pH-sensitive liposomes’. Fusogenic lipids are included in liposomes since they undergo a phase transition under acidic conditions. This facilitates an interaction and fusion or destabilization of liposomes with the endosomal membranes, resulting in release of the encapsulated therapeutic in the cytoplasm. pH-sensitive liposomes are stable at physiological pH (pH 7.4) but undergo destabilization, and acquire fusogenic properties under acidic conditions. Different hypothetical mechanisms have been proposed for the endosomal escape of pH-sensitive liposomes: (i) destabilization of pH-sensitive liposomes at acidic pH triggers destabilization of the endosomal membrane by pore formation leading to cytosolic delivery; (ii) upon destabilization of liposomes, the encapsulated molecules diffuse to the cytoplasm through the endosomal membranes; and (iii) fusion between the liposomes and endosomal membranes leading to cytosolic delivery [2,3].

Various strategies have been proposed for the formulation of pH-sensitive liposomes, depending on the mechanism of triggering pH-sensitivity. The most common one involves the combination of phosphatidylethanolamine (PE) with compounds containing an acidic group (e.g. carboxylic groups) that act as a stabilizer at neutral pH [4,5]. Recently, liposomes have been prepared with novel pH-sensitive lipids and synthetic fusogenic peptides either encapsulated [6,7] or incorporated in lipid bilayers [8-10]. Liposomes undergo binding to the

cell membrane and are internalized through the endocytic pathway with or without the involvement of clathrin coated vesicles. It has been shown that pH-sensitive liposomes are internalized more efficiently than non-pH-sensitive liposomes [11,12]. PE-containing liposomes have the tendency to form aggregates due to the poor hydration of its head-groups, and thus exhibit high affinity to adhere to cell membranes [13]. Immunoliposomes (prepared by conjugating antibodies to the liposome) are internalized more efficiently by the cells using a receptor-mediated endocytic process than the non-specific endocytic process used by unmodified liposomes. However, although the non-pH-sensitive immunoliposomes are internalized as extensively as the pH-sensitive-immunoliposomes, they have much lower capacity to mediate cytosolic delivery of the encapsulated therapeutic molecules [14,15]. These studies suggest that the endosomal escape is a critical step for efficient cytosolic delivery of therapeutics using liposomes.

pH-sensitive liposomes have been shown to promote the cytoplasmic delivery of encapsulated calcein and fluorescent dextrans [15]. Cytoplasmic delivery was observed by the emergence of diffuse cytoplasmic fluorescence. It was shown to require metabolic energy, and was partially inhibited by chloroquine and monensin, which increase the pH of the endosomal vesicles. However, the *in vivo* efficacy of liposomes upon intravascular injection is limited by the aggregation of liposomes in the presence of plasma proteins and the rapid clearance of liposomes from the blood stream by means of the reticuloendothelial system (RES) [16]. “Stealth” or long-circulating liposomes designed to evade detection by the RES, are potential drug-carriers for *in vivo* drug delivery [17]. Such formulations can be prepared by including cholesterol [18] or other amphiphilic stabilizers [19,20], or phosphatidylinositol and gangliosides [21] in the liposomes, or by producing a hydrophilic surface by grafting polyethylene glycol (PEG) chains on the liposome surface [22]. These sterically stabilized liposomes act as long-circulating drug reservoirs and enable targeting of drugs to non-RES target sites. Ligands such as antibodies can be conjugated to the PEG chains on PEG stabilized liposomes to achieve specific tissue targeting.

Liposomes have also been actively pursued as a potential tool for targeted gene delivery to specific cells. Cationic liposomes can be complexed with polyanionic plasmid DNA to form highly compact nanostructures with a net positive charge called the “lipoplexes.” Labeling of lipoplexes with asialofetuin and protamine sulfate has documented improved *in vivo* efficiency due to efficient targeting to nucleus by the nuclear localization signal (NLS) present in protamine sulfate [23]. The addition of protamine sulfate to asialofetuin-lipoplexes increased the *in vivo* gene expression levels by 75 fold compared to those by conventional lipoplexes. The smaller size of the lipoplexes produced in combination with protamine and the possible nuclear targeting by NLS present in protamine are responsible for better gene delivery.

Despite significant progress made so far in the formulation of pH-sensitive and ligand-conjugated liposomes, there are still two major obstacles, including the stability of liposomes in the presence of plasma proteins and obtaining a sustained cytosolic delivery of the therapeutic agent.

Cell-penetrating peptides

As cell membrane constitutes a major barrier for intracellular delivery of large hydrophilic proteins, peptides and oligonucleotides[24], cell penetrating peptides (CPPs) have been explored to overcome this barrier [25,26]. These CPPs can ferry molecules or colloidal drug carrier systems that are tagged to them across the cell membrane, into the cytoplasm and to the nucleus [27,28]. The characteristic CPPs is attributed to the presence of a stretch of 9-16 cationic amino acid residues [29]; the most commonly studied CPPs include HIV-1 Trans-activating transcriptional activator (TAT) peptide, HSV VP-22 (Herpes Simplex virus type-1 transcription factor) peptide and penetratin [30,31]. Several theories have been proposed to

determine the exact mechanism by which these CPPs enter the cells. For example, TAT penetration through the cell membrane has been shown to be independent of receptors and transporters, and has been suggested to enter the cell by forming an inverted micelle by destabilizing the phospholipid bilayer [32]. The main benefit of TAT coupling is that, along with efficient delivery of molecules, biological activity of the coupled molecule is preserved, and the size of the molecule being transported is also not a rate-limiting factor.

TAT has been suggested not only to enhance intracellular delivery, but also nuclear delivery and hence has been investigated for nucleic acid delivery. TAT peptide conjugated to antisense oligonucelotides was shown to deliver the oligonucleotide to the nucleus [33]. After being internalized, TAT peptide has also been found to colocalize inside the golgi along with BODIPY-ceramide, which is a marker for golgi [34]. Therefore, it is quite possible that there is direct trafficking from the early endosome to the golgi body without entering the late endosome [34]. A secretory pathway could be present where the peptide enters the cytosol from the endoplasmic reticulum.

Gene therapy has demonstrated a significant potential in the treatment of genetic, acquired and neurodegenerative disorders [35-37]. Amongst non-viral gene delivery methods, various drug delivery systems and polymers are being investigated such as liposomes, cationic lipid-DNA, polymer complexes [38,39]. To overcome relatively inefficient cellular uptake of non-viral gene expression vectors, [40] TAT peptide conjugation to vectors have been explored [41]. Kleeman et al. have demonstrated gene expression in alveolar basal epithelial cells with polyethylenimine (PEI) covalently coupled to TAT through a polyethylene glycol (PEG) spacer which demonstrated higher transfection efficiencies *in vivo* in mice lung following intratracheal administration than unconjugated PEG complex [42]. In a similar study by Rudolph et al., solid lipid particles conjugated to dimeric HIV-1 TAT have demonstrated enhanced gene delivery to the lungs [43].

Biodegradable nanoparticles

Although the above mentioned drug carrier systems result in relatively higher efficiency for cytosolic delivery, they are incapable of maintaining therapeutic drug concentrations for a prolonged time [44]. Therefore, the focus recently has shifted to the development of drug delivery systems which not only target the drug to its site of action but also maintain the drug concentrations at therapeutically relevant levels for a sustained period of time. We are investigating biodegradable nanoparticles (NPs) formulated with poly-($_{D,L}$-lactide-*co*-glycolide) (PLGA) for cytoplasmic delivery of drugs, therapeutic proteins, and plasmid DNA [45,46]. Poly-lactic acid (PLA) and PLGA polymers are biodegradable and biocompatible polymers, which are approved for human use by the US Food and Drug Administration. Polymeric NPs can be formulated to encapsulate various types of therapeutic agents including low molecular weight drugs, and macromolecules such as proteins or plasmid DNA. PLGA-NPs with entrapped therapeutics are of special interest for intracellular drug delivery owing to their biocompatibility, biodegradability and ability to sustain therapeutic drug levels for prolonged periods of time. The polymeric matrix prevents the degradation of the drug, and also allows precise control over the release kinetics of the drug from NPs. Moreover, the duration and levels of drug released from the NPs can be easily modulated by altering formulation parameters such as drug: polymer ratio, or polymer molecular weight and composition. PLGA-NPs are generally formulated using ‘water-in-oil-in-water’ double emulsion solvent evaporation techniques, using poly vinyl alcohol (PVA) as an emulsifier [47]. Since the NP comes in direct contact with the cell membranes, the surface properties of NPs are critical in determining the internalization mechanism and intracellular disposition of NPs.

Cellular internalization of NPs: mechanism and factors

We have demonstrated that PLGA-NPs are internalized into cells through a concentration and time-dependent endocytic process [48]. Cellular internalization of PLGA-NPs is partly through fluid phase pinocytosis and in part through clathrin-coated pits in vascular smooth muscle cells (VSMCs). It was further demonstrated that NPs rapidly escape the endo-lysosomes and enter the cytoplasm within 10 minutes of incubation with cells, under *in vitro* cell culture conditions. PLGA-NPs exhibit a negatively charged surface at neutral pH owing to the presence of uncapped end carboxylic acid groups of the polymer. We have previously demonstrated that biodegradable PLGA NPs following cellular internalization (via endocytosis) undergo surface charge reversal (anionic to cationic) in the acidic pH of endo-lysosomes. This facilitates an interaction of NPs with the vesicular membranes, leading to transient and localized destabilization of the membrane, thereby resulting in the escape of NPs into the cytosol [49]. A significant fraction of NPs undergoes exocytosis and only 15% of the internalized NPs escape into the cytosolic compartment. However, the fraction of NPs that escapes the endosomal compartment seems to remain in the cytoplasmic compartment and release the encapsulated therapeutic in a sustained manner as the polymer degrades slowly.

The efficiency of cytoplasmic delivery and intracellular retention of NPs is therefore governed by the ease and rapidity of escape of NPs from endo-lysosomes. Thus, the surface properties of NPs play an important role in their intracellular trafficking and can potentially influence the efficiency of cytosolic drug delivery. These include the generally overlooked aspect of surface associated PVA in NPs, hydrophilicity and the surface charge (zeta potential) of the NPs. It has been shown that a fraction of PVA used in the formulation of NPs remains associated with the NP surface, and cannot be removed even by multiple washings [50]. This residual PVA on NP surface can alter its physical properties and affect the cellular uptake of NPs. NPs with lower amount of surface associated PVA show about 3 fold higher cellular uptake in VSMCs than the NPs with higher residual PVA. This could be due to shielding of the surface charge reversal of NPs by the presence of higher amount of surface associated PVA, which could affect the endosomal escape of NPs. Further, the amount of PVA associated with the NP surface depends on the amount of PVA, the molecular weight and degree of hydroxylation of PVA used as emulsifier in the formulation [47]. Thus, formulation of NPs can be altered to achieve different surface properties, which in turn affect the cellular uptake and the intracellular disposition of NPs.

Cellular internalization of NPs also depends on their particle size and has been shown to affect the gene transfection efficiency of plasmid DNA-loaded NPs. The smaller size (less than 100 nm) NPs showed 27-fold higher gene transfection than the larger size (more than 100 nm) NPs [51]. However, this difference in gene transfection was not related to the surface properties, cellular uptake or the release of DNA from the NPs. Thus, the smaller size with a uniform particle size distribution is expected to increase the gene transfection efficiency of plasmid DNA loaded NPs. Other important formulation parameters which influence the cytosolic delivery of drugs include the molecular weight of polymer, molecular weight and degree of hydrolysis of PVA [47]. NPs formulated with higher molecular weight PLGA polymer showed enhanced gene transfection. This was attributed to the relatively higher DNA loading and its release from NPs prepared with high molecular weight polymer. Higher viscosity and better emulsifying properties of the polymer solution facilitate higher loading of DNA in NPs and also leads to lower particle size of NPs. Polymer composition can affect its hydrophobicity and thus can influence the loading and release of DNA from the NPs. NPs prepared using more hydrophobic polymers (poly lactides) demonstrated lower transfection than those formulated using copolymers of poly lactide and glycolide [47]. The slow rate of release of DNA from the relatively more hydrophobic polymeric matrix may be responsible for the lower levels of gene

transfection. Therefore, cellular internalization and subsequent efficacy of the delivered drugs is dependent on the size, surface properties and formulation of PLGA NPs.

Cytosolic delivery of drugs/plasmid DNA

We have shown previously that the NPs which escape into the cytoplasmic compartment are retained intracellularly and undergo slow degradation, thus acting as an intracellular depot. Figure 2 depicts the localization of NPs inside the cells even after 14 days. The therapeutic efficacy of drugs with cytoplasmic targets depends on the intracellular drug levels and their maintenance for a sustained period of time. We have reported efficient cytoplasmic delivery of dexamethasone and sustained intracellular drug levels by using biodegradable NPs [45]. Dexamethasone exerts its pharmacological actions by binding with glucocorticoid receptors present in the cytoplasmic compartment of cells. Vascular smooth muscle cells (VSMCs) were treated with dexamethasone in solution or dexamethasone-loaded NPs for 3 days, following which no further dose of drug was added. NPs could be detected inside the cytoplasm of cells even 14 days after nanoparticle incubation with cells. Thus, it was hypothesized that NPs escape endo-lysosomes and then release dexamethasone in a sustained manner while present in the cytoplasm, thus maintaining intracellular drug levels. The efficacy of drug also depends on the release rate of drug from NPs. Figure 3a depicts the difference in intracellular dexamethasone levels with two different formulations of NPs that release the encapsulated drug at different rate. The formulation of NPs that releases the drug at higher rate and for a longer duration demonstrated higher drug levels and for longer duration than one which has drug release over a shorter duration of release. In a further study, equal doses of two formulations of dexamethasone-loaded NPs releasing different doses of the encapsulated drug were evaluated for the anti-proliferative activity in VSMCs. Dexamethasone-loaded NPs showed significantly greater and more sustained inhibition of cell proliferation as compared to the drug solution. NP formulation which released a higher dose of dexamethasone showed greater and sustained anti-proliferative effect in the cells, as compared to NPs which released a lower dose of drug. Thus our study suggests that the dose of the drug and its retention at the intracellular site of action determine the magnitude and duration of the therapeutic effect of the drug [45].

In another study, we have demonstrated sustained intracellular localization of plasmid DNA with NPs as compared to transient localization of DNA in cells transfected with naked DNA alone. Breast cancer cells (MDA-MB-435S) transfected with *wt*-p53 plasmid DNA loaded NPs showed significantly higher and more sustained (7 days) intracellular DNA levels as opposed to transfection with naked DNA and DNA-Lipofectamine™ complex [46]. This has significant implications in cancer gene therapy, where sustained gene expression is of importance for greater therapeutic benefit. Thus, biodegradable NPs can not only be used for efficient cytoplasmic delivery of low molecular weight drugs but also for macromolecules like DNA. One of the key features of PLGA NP mediated gene delivery is the ability to achieve sustained gene expression. Though the levels of gene expression with NPs are lower than that achieved with lipid based gene delivery, they are sustained for a prolonged period of time. Further NP-mediated gene transfection is not affected by the presence of serum in the cell culture media, and thus PLGA NPs constitute a potential gene delivery vector for *in vivo* gene delivery. Prabha and Labhasetwar have shown slow intracellular release of plasmid DNA from the PLGA NPs. Figure 4a depicts the sustained intracellular release of DNA which is labeled with TOTO dye (red color) and encapsulated in NPs while the cells transfected with plasmid DNA showed its transient intracellular retention. Sustained intracellular release of DNA with NPs also resulted in sustained expression gene expression. As shown in Figure 4b, *wt-p53* gene-loaded NPs resulted in sustained mRNA levels for p53 in MDA-MB-435S breast cancer cells [46]. The sustained p53 expression levels resulted in greater and sustained inhibition of cell proliferation as compared to plasmid DNA alone (Figure 4c). Cohen et al. has shown that despite the lower transfection levels observed *in vitro* with NPs as compared to liposomal formulations, the *in*

vivo gene transfection with NPs was 1-2 orders of magnitude greater than the liposomes, 7 days after an intra-muscular injection in rats [52]. Their studies demonstrated gene expression sustained over 28 days *in vivo* with a single dose of intramuscular injection of NPs. Such sustained gene expression is advantageous especially if the half-life of the expressed protein is very short and/or a chronic gene delivery is required for better therapeutic efficacy.

Improving cytosolic delivery: functionalization of the NP surface

The surface of NPs can be functionalized and conjugated to different ligands to (i) overcome rapid opsonization and phagocytosis of NPs, (ii) enhance cellular uptake, and (iii) target NPs to specific cellular organelles.

Affecting biodistribution of NPs

The polymeric NPs can be surface modified and functionalized to improve their biodistribution and also conjugated to targeting ligands which can direct NPs to specific cells/tissues where drug delivery is desired. Surface modification of NPs is achieved either by adsorbing amphiphilic excipients onto pre-formed NPs or by covalently linking excipients to the core forming polymer prior to NP formulation. Incorporation of additional excipients such as polyethylene oxide (PEO) has been attempted to prevent the generation of an extremely acidic microenvironment inside the NPs on polymer degradation [53]. PEO/PEG has been used to coat the polymeric NPs to provide a protective hydrophilic sheath, which prevents the rapid opsonization of the otherwise hydrophobic NPs by the RES and thus prolong the circulation time of NPs in the bloodstream [54]. The hydrophobic part of PEO/PEG polymers can adsorb to NP surface while the hydrophilic chains protrude towards the aqueous medium. PEG coating on the NP surface also provides an attractive opportunity to chemically conjugate active-targeting ligands to the NP surface [55,56]. These coatings can modify the biodistribution of NPs when injected into the systemic circulation. However, it has been argued that some of these polymers can be easily displaced by serum proteins, which can lead to aggregation of NPs [57]. Thus, alternative approaches of synthesizing co-polymers of PLA/PLGA with PEG [58,59] and co-encapsulation of PEG with plasmid DNA inside PLA NPs have been tried [60].

Enhancing cellular uptake

Ligands can be chemically coupled to NP surface to increase the cellular uptake of NPs. Various cell penetrating peptides including synthetic peptides, protein transduction domains (PTD), and membrane-translocating sequences (MTS) have been described for the intracellular delivery of drugs especially macromolecules [61]. In general, these peptides are made up of 30 amino acids, are positively charged and have the ability to translocate the cell membrane and deliver drugs into the cytoplasm or the nucleus. HIV protein derived transactivating regulatory protein (HIV-TAT) and penetratin are the most extensively investigated peptides for intracellular delivery of drugs. Efficient gene transfection has been demonstrated by means of TAT-conjugated liposomes both *in vitro* and *in vivo* [62]. The conjugation of TAT on the surface of liposomes was found to influence their intracellular uptake. The TAT conjugated liposomes take a non-endocytic route for cellular entry and the uptake was observed to be energy independent. However, the mechanism of cell penetration of such peptides is yet to be elucidated completely and thus the issue of cell specificity by means of these peptides is still unresolved [61].

As previously described the intracellular retention of PLGA-NPs is limited by the process of rapid exocytosis of NPs, which seems to occur due to the inefficient escape of NPs from the endosomal compartment to the cytosol of the cell. Strategies can be devised to design the NP formulation to facilitate greater intracellular retention of NPs and reducing their exocytosis.

One such approach is to conjugate ligands such as transferrin to the NP surface, which function in two ways: first to target the NPs to specific cell populations rich in the receptors for these ligands, and second to enhance the cellular uptake and retention of the NPs. Transferrin (Tf) conjugated NPs would be internalized into the cells using Tf-receptor mediated endocytosis, unlike the unconjugated NPs which internalize by a non-specific endocytic process. This difference in the endocytic pathway has been shown to result in a lower exocytosis and therefore greater intracellular retention of Tf-conjugated NPs as compared to the unconjugated NPs [63]. As shown in Figure 5a, Tf-conjugated NPs demonstrated a 2-fold greater cellular uptake than unconjugated NPs in MCF-7 cells. Further, 75% of the internalized unconjugated NPs were shown to undergo exocytosis as compared to 50% Tf-conjugated NPs (Figure 5b). These studies thus showed that Tf-conjugated NPs result in greater cytoplasmic localization of the entrapped drugs as compared to the unconjugated NPs. The therapeutic efficacy of paclitaxel loaded NPs was studied in drug sensitive MCF-7 cells and in resistant MCF-7/Adr cells [63]. The effect of drug retention on anti-proliferative activity was greater in the resistant cell line than in the nonresistant cell line. After 8 days of treatment, Tf-conjugated paclitaxel loaded NPs demonstrated about 80% inhibition in cell growth whereas unconjugated NPs demonstrated 40% and paclitaxel solution demonstrated only 20% inhibition when compared to untreated cells. Based on these studies, it appears that the duration of drug retention in cancer cells, especially in resistant cell line is critical to overcome the problem of drug resistance [63]. We have also demonstrated increased efficacy of paclitaxel-loaded NPs on conjugation with transferrin in a murine model of prostate cancer. Tf receptors are overexpressed by 2-10 fold in tumor cells than in normal cells and thus transferrin and/or transferrin antibodies have been used for targeting drugs to tumor cells. Figure 6 shows the efficacy of Tf-conjugated NPs over unconjugated NPs in prostate cancer model. In this study, a single-dose intra-tumoral injection of Tf-conjugated paclitaxel NPs produced a complete regression and a significantly higher survival rate than the unconjugated NPs or drug dissolved in Cremophor EL in a murine model of prostate cancer [64]. The mechanism of greater efficacy of Tf-conjugated NPs was determined to be due to greater cellular uptake and sustained intracellular retention of the encapsulated drug than that with drug in solution or unconjugated NPs.

Facilitating endosomal escape

Most of the viruses and toxins have evolved well-defined machinery to infect the cells and to integrate with the host cell genome. Now-a-days scientific efforts are focused on synthesizing bio-inspired polymeric materials which can mimic some of the viral mechanisms of cellular entry. These include the use of membrane disruptive or viral fusogenic peptides. Wagner has listed some of these membrane active peptides which are under extensive investigation for enhancing gene transfection by non-viral methods [65]. Synthetic peptides derived from N-terminus of influenza virus haemagglutinin or artificial amphipathic peptides (for example GALA and KALA) have been used for improving the intracellular delivery of DNA-polyplexes [66]. Specific interaction of these peptides with the endosomal membranes is due to the presence of acidic residues (aspartic and glutamic acid). At neutral pH, the negatively charged carboxylic groups destabilize the alpha-helical structure of peptide; however acidic endosomal pH promotes formation of amphipathic helices of peptide, which further allows multimerization and membrane interaction of peptides. Peptides can be incorporated into PLL-DNA complexes by covalent linkage to PLL [67] or by non-covalent ionic interaction of negatively charged peptides with positively charged polyplexes [68].

Another strategy to enhance cytosolic delivery of therapeutics is to design and synthesize multi-functional pH-responsive polymers. Murthy et al. have described such a polymer, where the backbone is relatively hydrophobic, membrane-disruptive polymer that is masked by grafted PEG chains at neutral pH [69]. The PEG chains are grafted to the polymer backbone using two linkers: first- disulfide groups and then acid-degradable acetal groups. As the polymer

formulations enter the cell, acetal groups are designed to degrade in acidic pH of endosome, thereby unmasking the membrane disruptive backbone. This leads to the disruption of endosomal membrane and release into the cytosol. Due to the reductive environment in the cytosol, the disulfide groups are reduced, thereby releasing the conjugated drug/plasmid DNA. The authors have shown successful intracellular delivery of antisense oligonucleotides in cell culture using these polymers.

Targeting of NPs to intracellular organelles

For drugs with the nucleus as the site of action (such as plasmid DNA or DNA intercalators), crossing the nuclear membrane poses another barrier to drug delivery. Nuclear uptake of plasmid DNA is the rate limiting step in efficient transfection and successful gene therapy [70]. Molecules smaller than 40-45 kDa and less than 100 nm have been shown to cross the nuclear membrane by passive transport, while other molecules interact with cytosolic factors to cross the nuclear membrane through the nuclear pore complexes [71]. Certain peptide sequences, known as nuclear localization signals (NLS) have been reported to specifically interact with the cytoplasmic factors which can then target molecules to the nucleus. NLS are peptides with no general consensus sequence, however mostly composed of basic amino acids. NLS present in the SV-40 large T antigen was the first and the most extensively studied NLS [72]. Various strategies have been devised to target plasmid DNA to the nucleus of a cell, including conjugation of NLS peptide to the end of a linear DNA molecule and non covalent modification of a plasmid DNA with NLS among others [71]. Liposomes and NPs can be surface modified and conjugated to the identified NLS sequences, to effect nuclear targeting. In pioneering work, gold nanoparticles coupled with SV-40 large T antigen on their surface were shown to efficiently translocate into the nucleus, upon microinjection into the cells [73]. A recent study proposed enzymatically digested low molecular weight protamine as a non-toxic and efficient gene carrier. Plasmid DNA complexed with low molecular weight protamine was found to efficiently translocate into the cell and then entered the nucleus of cell owing to the structural similarity of protamine with HIV-TAT peptide [74]. Another evolving concept is that of utilization of the viral proteins for nuclear targeting of molecules or the design of synthetic viruses. However, two or more targeting peptides may be used to target the NPs to specific cells and then translocate the drug to the nucleus. One such approach using a combination of cell penetrating and nuclear targeting peptides has been explored with gold NPs [75].

Conclusions

The above mentioned studies clearly demonstrate that NP-mediated intracellular delivery is a dynamic process; involving endocytosis, exocytosis, and sorting into different intracellular compartments. It appears that the NP surface and its interaction with cell surface controls the uptake and intracellular trafficking of NPs, and hence that of the encapsulated therapeutic agents. With this intention, our laboratory has developed a model to study the dynamics of intracellular trafficking of NPs, particularly to determine the influence of different surface properties of NPs on their sorting into different intracellular compartments following cellular uptake. Thus, a better understanding of pathways of cellular uptake and its influence on intracellular distribution of NPs could be critical for developing intracellular target-specific NPs.

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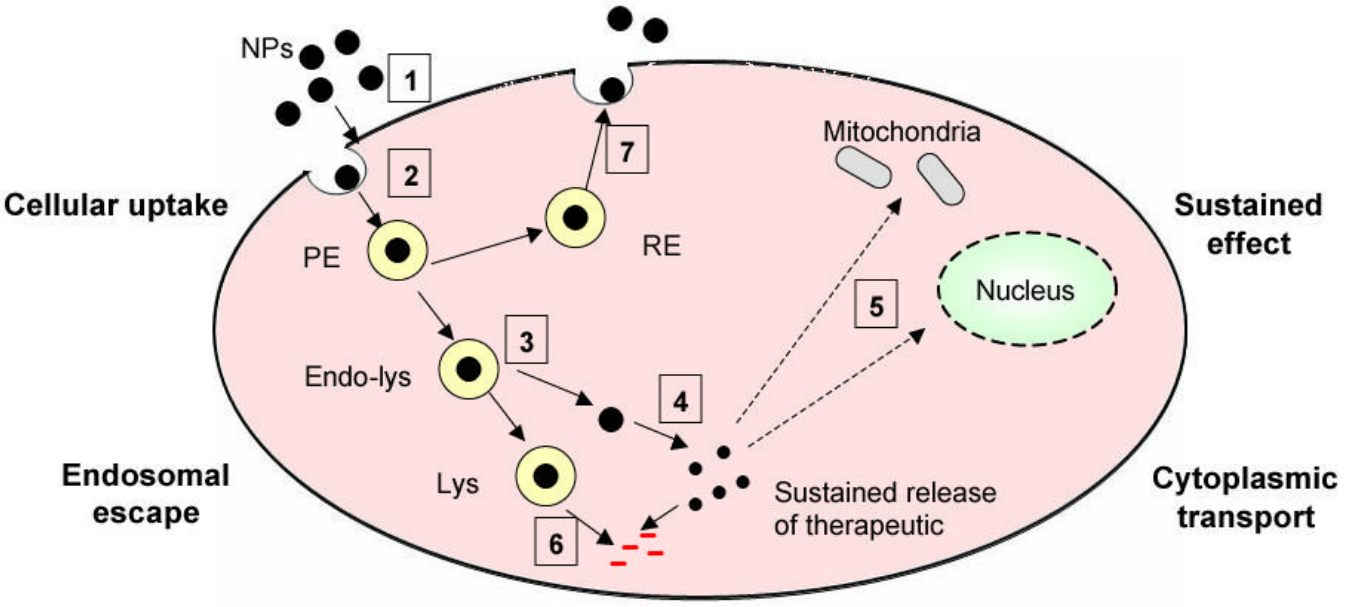

Figure 1. Schematic drawing of steps involved in cytosolic delivery of therapeutics using polymeric nanoparticles (NPs)

(1) Cellular association of NPs, (2) Internalization of NPs into the cells by endocytosis, (3) Endosomal escape of NPs, (4) Release of therapeutic in cytoplasm, (5) Cytosolic transport of therapeutic agent, (6) Degradation of drug either in lysosomes or in cytoplasm, (7) Exocytosis of NPs. Major barriers include: (A) Cellular uptake of NPs, (B) Endosomal escape of NPs, (C) Cytoplasmic transport of therapeutic/NPs, (D) Sustained therapeutic benefit. [PE: Primary endosomes, RE: Recycling endosomes, Endo-lys: Endo-lysosomes, Lys: Lysosomes, Solid circles represent polymeric NPs].

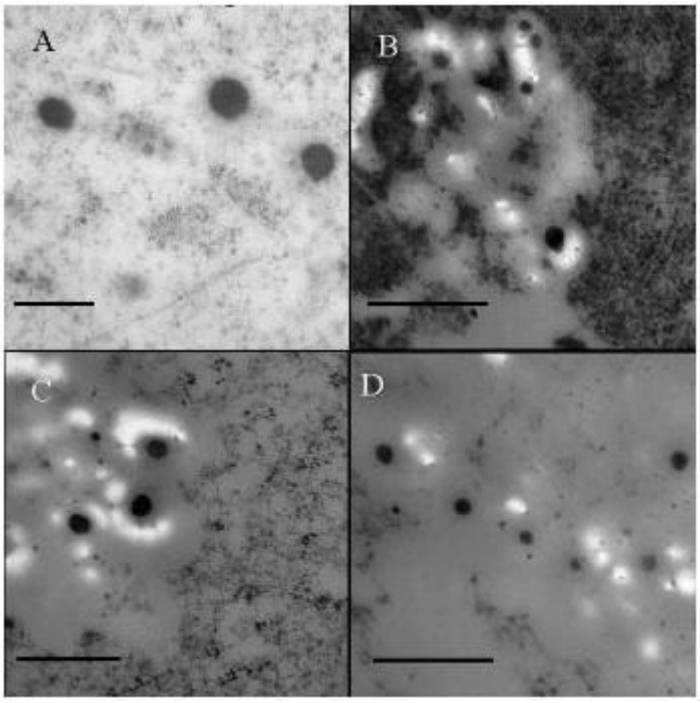

Figure 2. Prolonged retention of NPs in the cytoplasm of cells

Transmission electron microscopic pictures demonstrating the presence of NPs in the VSMCs on day 1 (A), 3 (B), 10 (C), and 14 (D) post-incubation. The bar is 500 nm long. NPs are indicated black spherical structures in the cytosol. These NPs were loaded with osmium tetraoxide for the purpose of contrast. Reprinted with permission from ref [45]. Copyright (2004) American Chemical Society.

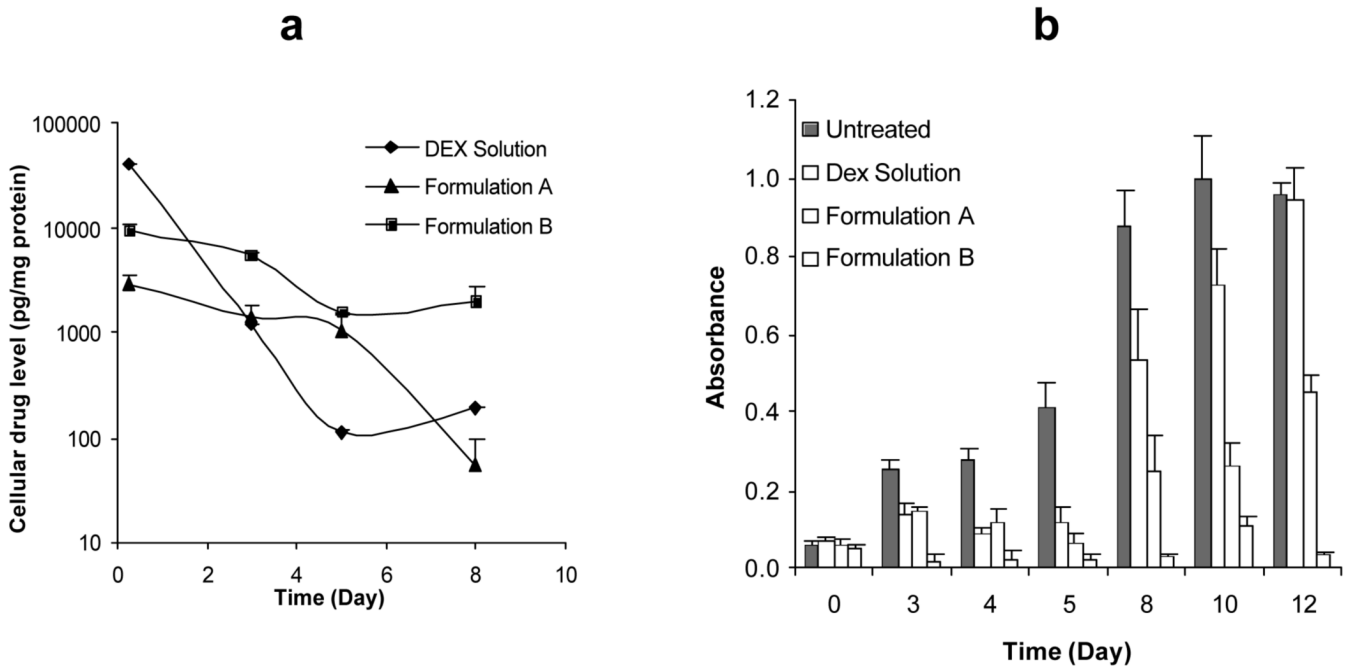

Figure 3. Sustained cytoplasmic delivery of drugs with NPs

(a) Intracellular dexamethasone levels following treatment with tritiated dexamethasone in solution or in nanoparticles (formulations A and B). Data are means ± the standard error of the mean (n=3). The two formulations of NPs differed only in their ability to release different amounts of dexamethasone. (b) Inhibition of VSMC proliferation with dexamethasone in solution and encapsulated in NP formulations. Cell proliferation was measured using a MTS assay (CellTiter 96® AQueous, Promega, Madison, WI). MTS is chemically reduced by cells into formazan, which is soluble in tissue culture medium. The measurement of the absorbance of the formazan was carried out using 96 well microplates at 492nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. Data are means ± the standard deviation (n= 6). Reprinted with permission from ref [45]. Copyright (2004) American Chemical Society.

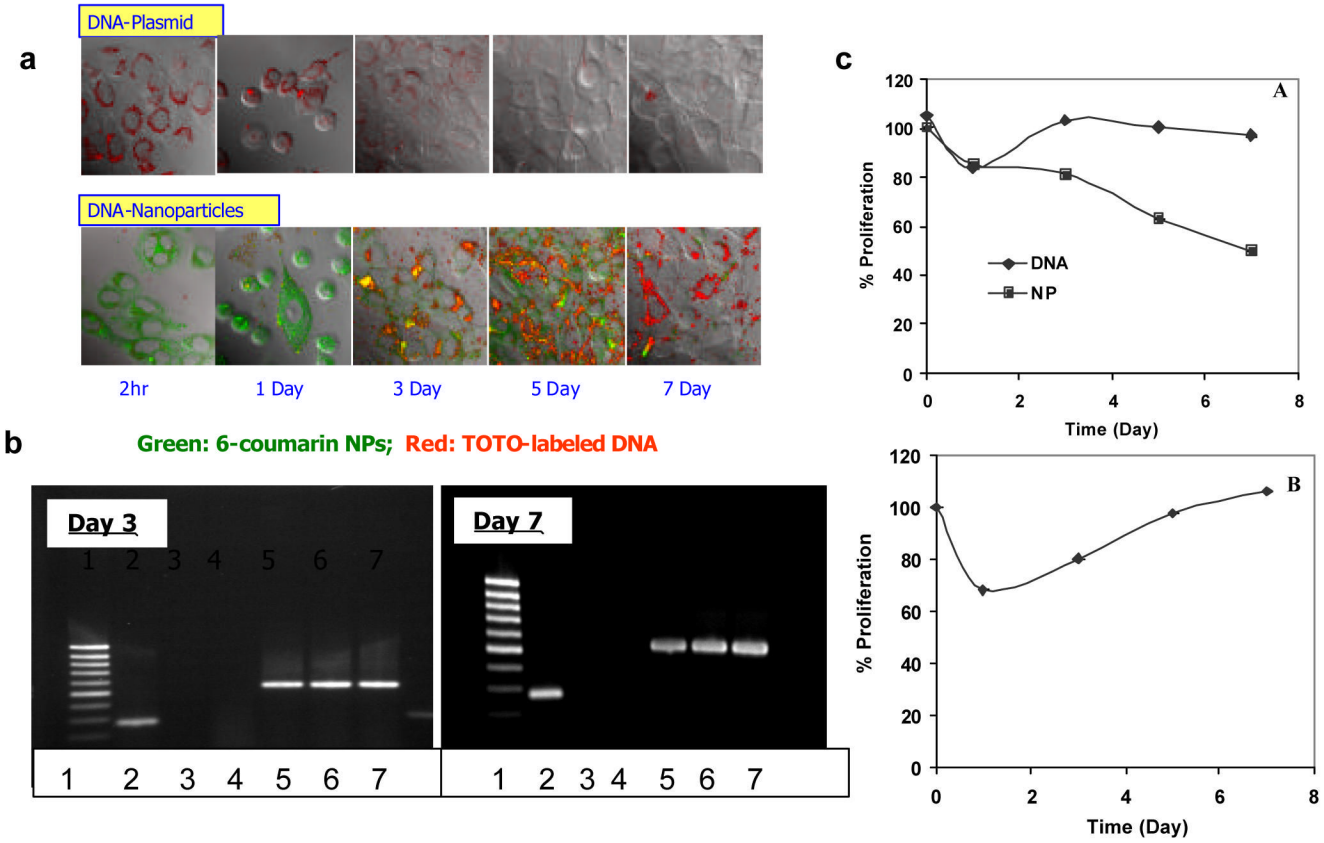

Figure 4.

a) Intracellular DNA delivery. DNA was labeled with TOTO (red) and nanoparticles contained a fluorescent dye (6-coumarin, green). Single-dose of DNA (either alone or in nanoparticles) was added to cells, the medium was changed at 2 days and then on every alternate day thereafter. Cells were observed under confocal microscope. Red color is due to DNA that is released from nanoparticles, green color is due to nanoparticles, and yellow color is due to co-localization of released DNA (red) and nanoparticles (green). b) RT-PCR data of cells transfected with *wt*-p53 gene: Lane 1: Molecular weight marker, Lane 2: p53 DNA-loaded NPs, Lane 3: p53(-ve) DNA loaded NPs, Lane 4: p53 DNA only; Lane 5: β-actin for p53 DNA loaded nanoparticles, Lane 6: β-actin for p53(-ve) DNA loaded nanoparticles, Lane 7: β-actin for p53 DNA. c) Antiproliferative activity of *wt*-p53 DNA: MDA-M435S cells were treated either with A: *wt*-p53-plasmid DNA or *wt*-p53 DNA-loaded nanoparticles (NP) or B: DNA-Lipofectamine™ complex. Reprinted with permission from ref. [46]. Copyright (2004) American Chemical Society.

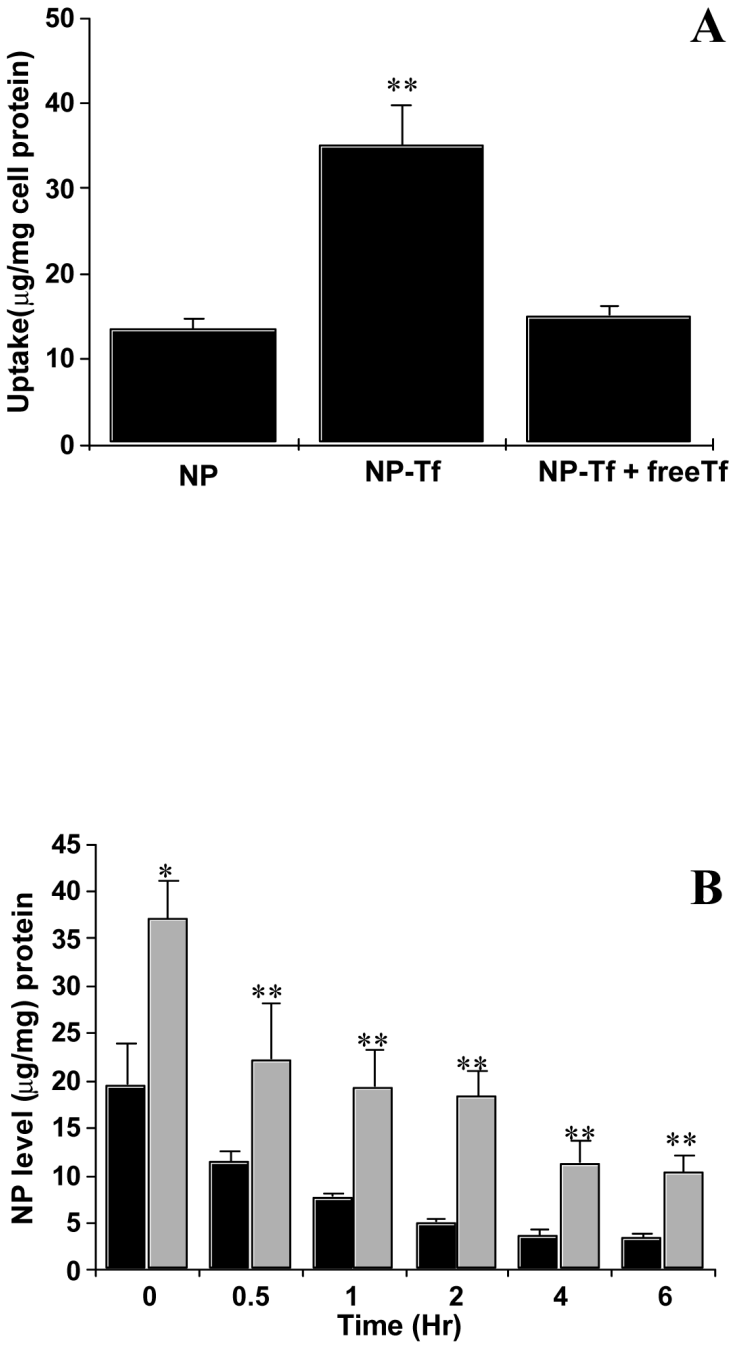

Figure 5. Enhanced cellular uptake and intracellular retention of drug with Tf-conjugated NPs

(A) Uptake of Tf-conjugated NPs (NPs-Tf) and unconjugated NPs (NPs) in MCF-7 cells. To determine the competitive inhibition of uptake of Tf-conjugated NPs, an excess of free Tf was added to the medium prior to incubating cells with Tf-conjugated NPs. Data as mean±SEM (n=6), (*) $p < 0.05$ NPs-Tf + free Tf versus NPs. (**) $p < 0.005$ NPs-Tf versus NPs. (B) Exocytosis of Tf-conjugated and unconjugated NPs in MCF-7 cells. Cells were incubated with Tf conjugated NPs (gray) and unconjugated NPs (black) at 100 μg/mL concentration for 1 h, cells were washed, and then cells were incubated with fresh medium. This NP level was taken as the cellular uptake (0 h time point). In other wells, the cells were washed and incubated with medium, and were processed as above at different time points to determine intracellular

retention of NPs. Reprinted with permission from ref [63]. Copyright (2004) American Chemical Society.

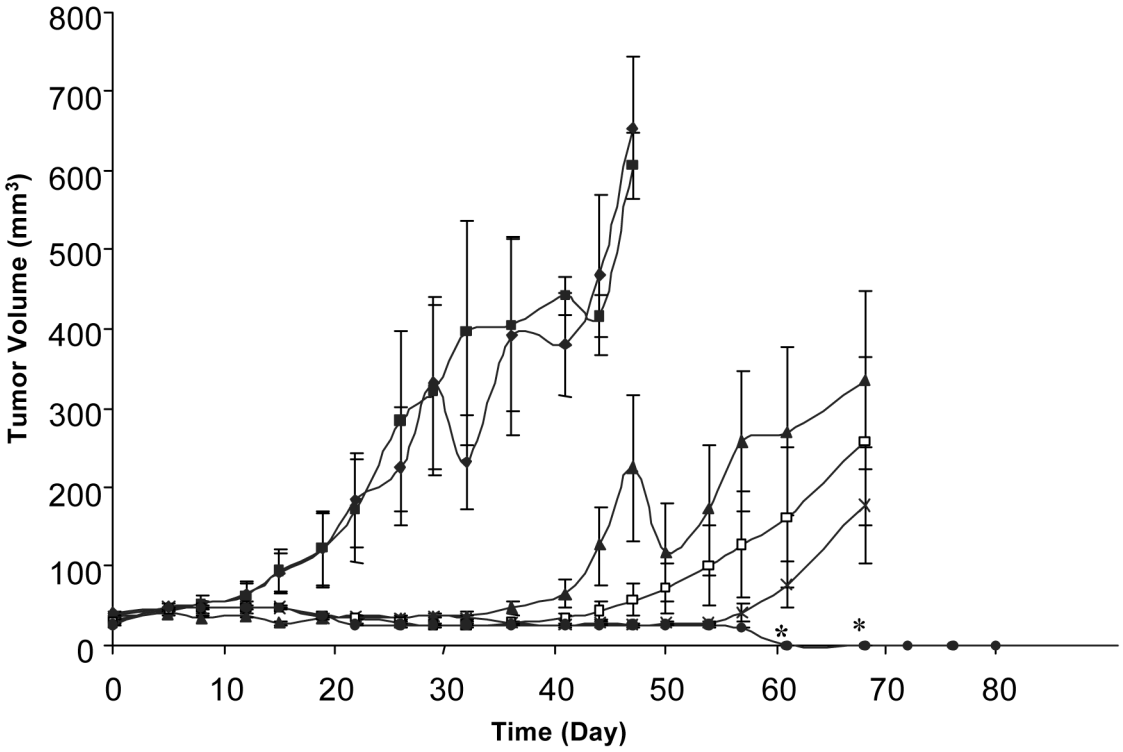

Figure 6. ***In vivo*** **efficacy of Tf-conjugated paclitaxel-loaded NPs**

Antitumor activity of Tx-NPs-Tf in a murine prostate tumor model. PC3 cells (2×10^6 cells) were implanted s.c. in athymic nude mice. Tumor nodules were allowed to grow to diameter of about 50 mm^3 prior to receiving different formulations as a single-dose treatment. Tx-NPs-Tf (•, 24 mg/kg; □, 12 mg/kg), Tx-NPs (×, 24 mg/kg), Tx-Cremophor® EL formulation (▲, 24 mg/kg), (◆) control NPs and (■) Cremophor® EL formulation. Data are means ± s.e.m., n=6. *p <0.005 Tx-NPs-Tf versus Tx-NPs and Tx-Cremophor® EL groups. Reprinted with permission from ref [64], Copyright (2004) Wiley-Liss, Inc., A Wiley Company.