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ORIGINAL PAPER

Aptamer-labeled PLGA nanoparticles for targeting cancer cells

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Abstract Cancer is one of the leading causes of death in most parts of the world and is a very serious cause of concern particularly in developing countries. In this work, we prepared and evaluated the aptamer-labeled paclitaxel-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles (Apt-PTX-PLGA NPs) which can ameliorate drug bioavailability and enable accurate drug targeting to cancer cells with controlled drug release for cancer therapy. Paclitaxel-loaded PLGA nanoparticles (PTX-PLGA NPs) were formulated by a single-emulsion/solvent evaporation method and were further surface-functionalized with a chemical cross-linker *bis*(sulfosuccinimidyl) suberate (BS3) to enable binding of aptamer on to the surface of the nanoparticles. The prepared nanoparticles were characterized by atomic force microscopy, scanning electron microscopy, and X-ray photoelectron spectroscopy. Cytotoxicity studies were carried out using normal human mammary epithelial cells (HMEC cells) and human glial cancer cells (GI-1 cells) by methylthiazolyldiphenyl-tetrazolium bromide assay and Alamar blue assay, which confirmed that PTX-PLGA NPs with aptamer conjugation (Apt-PTX-PLGA NPs) were comparatively non-toxic to HMEC cells while toxic to GI-1 cancer cells. Cellular uptake of PTX-PLGA NPs with and without aptamer conjugation was studied using GI-1 cells and monitored by confocal microscopy and phase contrast microscopy. Our studies demonstrated significant internalization and retention of nanoparticles inside the cells, inducing apoptosis. The preferential accumulation of PTX-PLGA NPs within the cancer cells were also confirmed by flow cytometry-based uptake studies. The results indicated that Apt-PTX-PLGA NPs could be a promising targeted therapeutic delivery vehicle for cancer treatment.

Keywords PLGA nanoparticles · Aptamers · Cancer therapy · Drug delivery · Paclitaxel

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1 Introduction

Targeted drug delivery is considered to be a very potential and desired requirement in cancer chemotherapy. Most of the anti-cancer drugs are non-specific and can also cause injury to normal tissues (Sahoo and Labhasetwar 2003). Nanotechnology has introduced new pathways for improved therapeutic delivery using different types of nanoparticles to eradicate tumor population without inducing toxicity to normal tissues (Koo et al. 2005; Farokhzad and Langer 2009; Ferrari 2005; Praetorius and Mandal 2007). Diverse biocompatible nanodrug carriers with controlled drug release have already been developed (Ravichandran 2009; Bob 2004; Sahoo et al. 2007; Rawat et al. 2006). Success of such carriers depend on their efficiency to encapsulate a cytotoxic dose of chemotherapeutic drug and their capability to functionalize a tumor specific ligand which can target the tumor cells while sparing the surrounding normal tissues (Suri et al. 2007; Wang et al. 2009; Davis 1997; Haley and Frenkel 2008).

Nowadays, cancer drug delivery research is being focused on developing innovative and effective tumor-targeted therapies by utilizing various nanoparticles as drug carriers and antibodies/peptides/aptamers as targeting moieties (Ray and

White 2010; Toti et al. 2010; Arruebo 2009; Nobs et al. 2004). Nanoparticles can accumulate within tumor cells by passive or active process. Passive targeting implies that nanoparticles smaller than the fenestrations of endothelial cells extravagate at the leaky tumor vasculatures, penetrate, and get internalized into the tumor interstitium. Active targeting involves drug delivery to a specific site based on molecular recognition of the receptor on the target cell by the targeting ligand coupled to nanoparticles (Labhasetwar and D 2005; Domı et al. 2004; Brannon-Peppas and Blanchette 2004).

Aptamers are a class of therapeutic oligonucleotides that bind to cell surface receptors with high affinity and specificity (Levy-Nissenbaum et al. 2008). This property of aptamers has been exploited for developing targeted drug carriers which can deliver variety of cargoes into cells. Aptamers possess several unique properties which make them attractive tools for use in a wide array of molecular biology applications and as potential pharmaceutical agents (Cerchia and de Franciscis 2010; Ray and White 2010). Aptamers bind to targets with high affinity and are structurally stable across a wide range of temperature and storage conditions. These can be chemically synthesized, reducing the cost and intensive work of monoclonal antibody production (Ireson and Kelland 2006; Mairal et al. 2008; Mallikaratchy et al. 2009; Lee et al. 2006).

A 26-nucleotide guanosine-rich (G-rich) DNA sequence (AS1411) discovered serendipitously by Bates et al. was found to have anti-proliferative activity and subsequently found to bind nucleolin (Bates et al. 2009). Nucleolin is a predominantly nuclear and cytoplasmic phosphoprotein, which is also over-expressed on the plasma membrane of some tumor cells compared with that of normal cells. Nucleolin expression is correlated with cell proliferation. Compared with normal cells, the nucleolin levels are higher in tumor cells Soundararajan et al. (n.d.). Moreover, cell surface nucleolin functions as a receptor to various growth factors like midkine and pleiotrophin which is required for the cells to grow efficiently, however if over expressed can transform the cells. Therefore, the functional blockage of the cell surface nucleolin represents a potential target for the development of anti-cancer therapeutics. It was proved that AS1411 inhibited the pro-survival NF–κB signaling pathway and thus blocked DNA-replication and induced cell cycle arrest and apoptosis. Furthermore, studies conducted by Soundararajan et al. concluded that anti-nucleolin aptamer inhibited the binding of nucleolin to bcl-2 mRNA. This resulted in the destabilization of the mRNA with a consequent decrease in the level of anti-apoptotic bcl-2 protein in the breast cancer cells. Currently, the anti-nucleolin aptamer AS1411 is in phase II clinical trials for acute myeloid leukemia and renal cell carcinoma. Various in vitro and in vivo studies conducted so far have demonstrated that the anti-nucleolin aptamer can target nanoparticles to cancer cells expressing nucleolin on their cell surface and can potentially be used as a non-invasive imaging tool for the diagnosis of cancer (Soundararajan et al. 2009; Ireson and Kelland 2006; Ozalp et al. 2011; Ray and White 2010; Jim and L 2011).

Paclitaxel (PTX) is a potent anti-cancer drug isolated from the bark of *Taxus brevifolia*, which arrests the cell division through stabilization of the microtubuli. PTX disrupts the dynamic equilibrium within the microtubule system and blocks cells in the late G2 phase and M phase of the cell cycle, thereby inhibiting cell replication (Mallikaratchy et al. 2009). PTX is a very hydrophobic compound, and to enhance its solubility and allow parenteral administration, commercial formulation of paclitaxel (Taxol) is currently formulated at a concentration of 6 mg/ml in a vehicle containing non-ionic surfactant cremophor (polyoxyethylated castor oil) and ethanol as solubilizing agents at a ratio of 1:1. However, Cremophor® EL is correlated with various hypersensitivity and toxicity issues like nephrotoxicity and neurotoxicity; it can also alter endothelial function causing vasodilatation, labored breathing, lethargy, and hypotension (De et al. 2005; Jin et al. 2009; Gradishar et al. 2005).

Poly(lactide-co-glycolide) (PLGA) was chosen as a drug carrier for paclitaxel due to its biodegradability and biocompatibility properties and its approval by the FDA. PLGA has been extensively used in the formulation of particles for various drug delivery applications (Esmaeili and Hossein 2008; Jin et al. 2009; Dong and Feng 2007; Feng et al. 2002). BS3 cross-linker (Thamake et al. 2011) was added to allow functionalization of aptamer on to the surface of the nanoparticle. In this study, we synthesized PTX-loaded PLGA-based nanoparticles which showed an enhanced cytotoxic profile in vitro when compared with commercial Taxol® (Xu et al. 2009; Vicari et al. 2008). We also found that PTX-PLGA NPs conjugated with AS1411 aptamer would target the tumor cells alone and would further enhance the anti-tumor efficacy of PTX as compared with non-targeted PTX-PLGA nanoparticles (Dhar et al. 2008).

2 Materials and methods

The 50:50 PLGA(poly lactic-co-glycolic acid), MW 30–70 kDa with an inherent viscosity of 0.59 dL/g, polyvinyl alcohol (PVA), MW 12–23 kDa, 87–89% hydrolyzed, *N*-hydroxysuccinimide (NHS), EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide), and penicillin/streptomycin solution were obtained from Sigma-Aldrich (St. Louis, MO). Ethyl acetate and all other reagents used were supplied by Fisher Scientific (Fairlawn, NJ). Paclitaxel was obtained from Wako Chemicals. All reagents were of analytical grade. Cell cultures chemicals-DAPI, trypan blue, trypsin (0.25%), and MTT assay kit were purchased from Sigma-Aldrich. Alamar blue stain was supplied by Invitrogen.

Aptamer AS1411 (NH_2-5′-(GGTGGTGGTGGT TGTGGTGGTGGTGG)-3′) and non-specific aptamer

(NH_2 -5′-(CCTCCTCCTCCTTCTCCTCCTCCTCC) - 3′) were purchased from Operon.

2.1 Formulation of paclitaxel-loaded nanoparticles

Effective encapsulation of chemotherapeutic drug within the nanoparticle depends on various factors like preparation method, polymer and drug composition, solvent and drug solubility, and stabilizer composition (Fonseca et al. 2002). We have adopted the nanoprecipitation-solvent evaporation method to synthesis paclitaxel-loaded PLGA nanoparticles with minor modifications. PVA, a widely known stabilizer for PLGA nanoparticles (Feng 2001), was employed in the synthesis along with a hydrophobic eight-carbon spacer chain of homobifunctional chemical cross-linker, *bis*(sulfosuccinimidyl) suberate (BS3). This carbon spacer chain will align itself on the nanoparticle surface with its COOH groups on the terminal hydrophilic side extending towards the outside of the nanoparticle surface (Thamake et al. 2011). Paclitaxel, a highly potent chemotherapeutic drug widely used against various tumors, showed limited clinical success owing to its low therapeutic efficiency and low solubility in many pharmaceutical solvents (Jin et al. 2009). Upon incorporation within PLGA nanoparticles, paclitaxel demonstrated enhanced therapeutic index of the drug and lack of the toxic effects caused by its commercial adjuvant Cremophor®EL (Gradishar et al. 2005).

Nanoparticles were prepared using a single-emulsion technique/solvent evaporation method that has been reported elsewhere with slight modification (Thamake et al. 2011; Cartiera et al. 2010). Briefly, 65 mg of PLGA dissolved in 1 ml of ethyl acetate was added to 2.2% aqueous solution of PVA containing 0.5 mg ml^{-1} of BS3. This mixture was sonicated at room temperature using an ultrasonic processor UP200H system (Hielscher Ultrasonics GmbH, Germany) at 40% amplitude for 2 min in continuous mode. The excess solvent was evaporated by continuous stirring for 45 min to 1 h followed by centrifugation at 10,500 rpm for 15 min to remove excess of aqueous solution. The separated nanoparticles were washed by resuspending in water three times. One milligram of paclitaxel in 1 ml of ethyl acetate was added along with the polymer/solvent mixture to prepare drug-loaded nanoparticles. Surface morphology and size were also determined by high-resolution scanning electron microscopy and atomic force microscopy.

2.2 Preparation of aptamer-conjugated drug-loaded nanoparticles

In order to achieve an aptamer–nanoparticle bio-conjugate capable of targeting desired cells, the binding protocol must preserve the biological activity of the aptamer (Balamurugan et al. 2008). Hence, special care was taken in choosing a suitable covalent binding procedure for functionalizing the aptamer on nanoparticle surface with effective binding by maintaining its biological activity (Janas and Janas 2011). Amine-modified AS1411 aptamer was conjugated on the carboxyl group carrying nanoparticles using the common conjugation strategy of carbodiimide chemistry (cross-linking of the carboxylic acid group on the nanoparticle surface and the amine group of the aptamer to form an amide linkage). The carboxyl groups on the nanoparticle surface were converted to its succinimide by using EDC and NHS, which was then allowed to react with NH_2-AS1411 aptamer. This method is well studied by many researchers which effectively carried out the conjugation of aptamer on to the polymer nanoparticles (Davies et al. 2010; Ling et al. 2011; Farokhzad et al. 2004; Dhar et al. 2008).

PTX-PLGA NPs (10 μg/μL) was washed three times with 250-μL aliquots of a 10 mM phosphate buffered saline (PBS) (pH 7.4) and incubated with 200 μL of 400 mmol/L EDC and 200 μL of100mmol/L NHS for 15 min at room temperature with gentle shaking. The resulting NHS-activated particles are covalently linked to amine-modified AS1411 aptamer (1 μg/μL). The sample was allowed to react for 2 h with constant mixing at room temperature, and three final washes were performed using the 20 mM Tris–HCl, 5 mM $MgCl_2$ at pH 8.0. The resulting aptamer–nanoparticle bioconjugates were resuspended and preserved in suspension form in DNase–RNase-free water at 4°C before use.

2.3 Surface morphology characterization

The shape and surface morphology of paclitaxel-loaded PLGA NPs were analyzed using a scanning electron microscope (SEM) (FESEM, JSM-6700F, JEOL, Japan) at an accelerating voltage of 3–5 kV. Nanoparticles were fixed to sample stubs with double-sided carbon tape and sputter-coated with platinum which was carried out by an Auto Fine Coater (JEOL, Tokyo, Japan) for 50 s for viewing by SEM. For atomic force

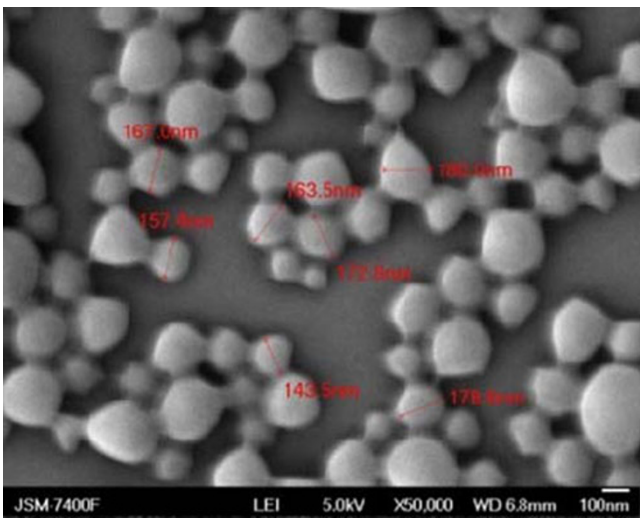

Fig. 1 SEM image of PLGA-PTX nanoparticles

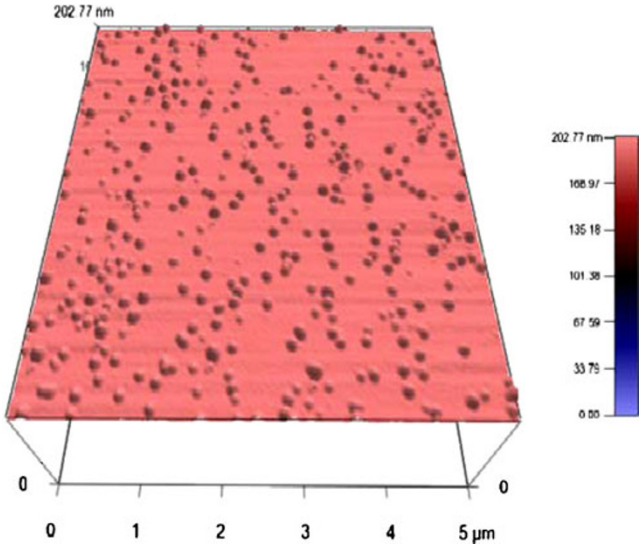

Fig. 2 AFM image of PLGA-PTX NPs

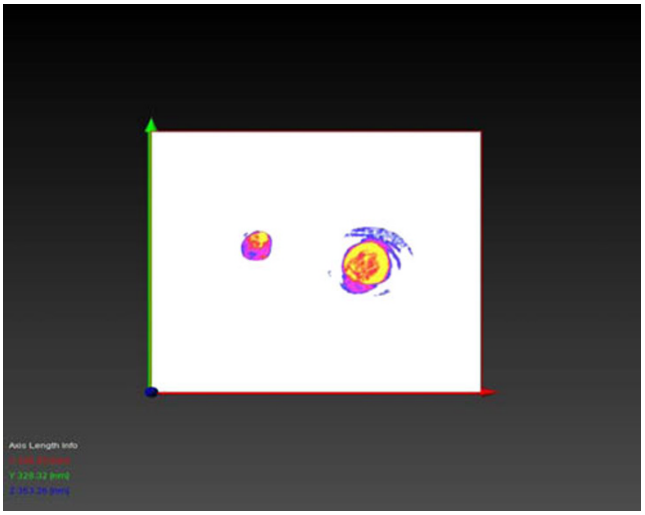

Fig. 4 2D-sliced image obtained from 3D-TEM imaging of PTX-PLGA NPs

microscopy (AFM), drug-loaded PLGA solution (200 μL) was deposited on a glass surface and vacuum-dried. The sample was characterized by AFM (Digital Instruments 3000AFM) in tapping mode. Three-dimensional imaging of the drug-loaded nanoparticles was done using transmission electron microscopy (TEM, JEM 2200 FS, JEOL, Japan). One drop of the sample solution was deposited onto a carbon-coated copper grid that had been previously hydrophilized under UV light and air-dried at room temperature prior to examination under TEM.

2.4 Surface chemistry characterization

The aptamer labeling on the surface of paclitaxel-loaded PLGA NPs was confirmed from the surface chemistry measured by X-ray photoelectron spectroscopy (XPS, AXIS His-165 Ultra, Kratos Analytical, Shimadzu Corporation, Japan). Five microliters of the sample was applied on a clean silicon substrate and dried in vacuum. The binding energy spectrum was recorded from 0 to 1,000 eV with pass energy of 80 eV under the fixed transmission mode.

2.5 Cell culture studies

GI-1 cells obtained from Riken Bio Resource Center, Japan, were cultured in monolayers to 80% confluence by maintaining in Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution in a 5% CO_2-humidified atmosphere at 37°C. Normal human mammary epithelial cells obtained from Gibco were maintained in HuMEC-

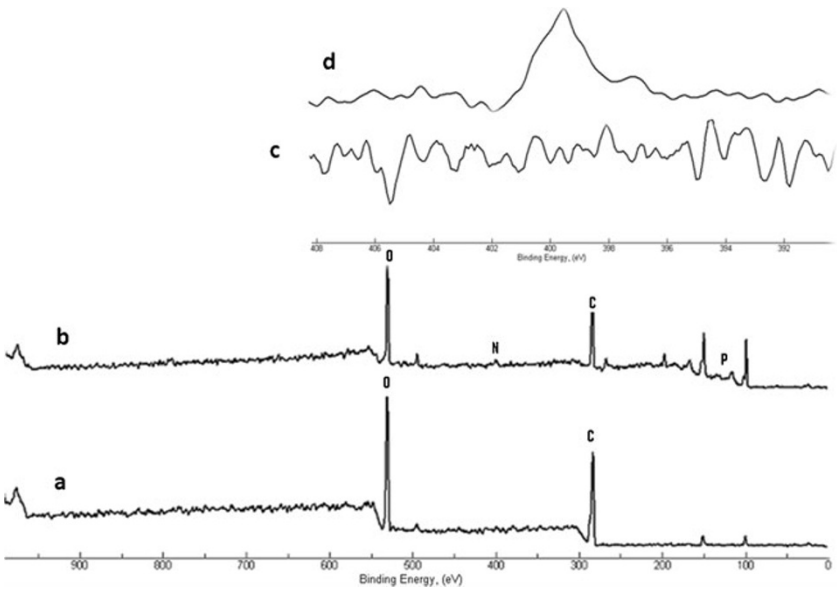

Fig. 3 XPS spectra of **a** PTX-PLGA NPS, **b** Apt-PTX-PLGA NPs, *above* N spectra of **c** PTX-PLGA NPS and **d** Apt-PTX-PLGA NPs

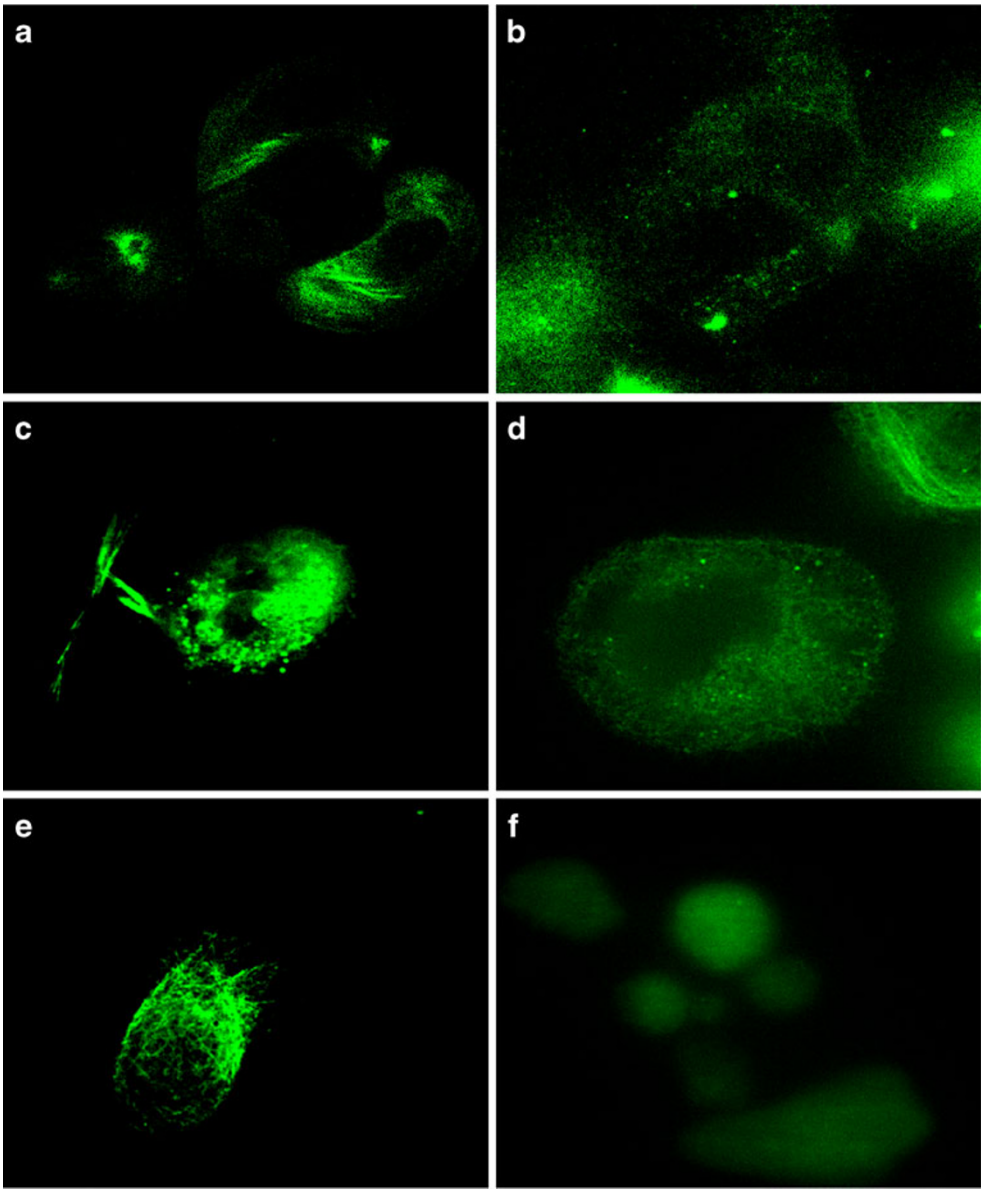

Fig. 5 Confocal images of (**a**) HMEC cells (**b**) GI-1 cells treated with Apt-PTX-PLGA NPs after 24 hours (**c**) HMEC cells (**d**) GI-1 cells treated with Apt-PTX-PLGA NPs after 72 hours (**e**) HMEC (**f**) GI-1 cells treated with Apt-PTX-PLGA NPs after 120 hours

ready medium (Gibco) supplemented with growth supplements and antibiotics in 5% CO_2-humidified atmosphere at 37°C. For use in experiments, 1×10^4cells/ml per well were seeded in glass-based dish for confocal studies; approximately 5,000–8,000 cells were seeded in 96-well plates for cytotoxic studies; 3×10^4 cells were plated in a 25-mL flask for phase contrast studies, and 2.5×10^6 cells per well was seeded in glass plate for flow cytometry studies.

2.6 Confocal microscopy

GI-1 cells were seeded in glass-based bottom well dish at a density of 1×10^4 cells/ml. The plates were incubated at 37° C and grown to 70% confluency. Cells were treated with a fixed concentration, i.e., 100 μg/ml, of aptamer-conjugated Nile red dye-tagged paclitaxel-loaded PLGA NPs (Apt-NR-PTX-PLGA NPs) and aptamer-conjugated paclitaxel-loaded PLGA nanoparticles (Apt-PTX-PLGA NPs) for different time periods. The cells were incubated with the dye-loaded particles and subjected to confocal microscopy after 2 and 120 h. The anticancer drug-loaded PLGA nanoparticles were incubated with the cells and subjected to confocal microscopy at 24, 72, and 120 h.

At the end of the incubation period, the cell monolayers were rinsed three times with 1 ml of PBS buffer (0.01 M, 7.4) to remove excess nanoparticles or free dye. Apt-NR-PLGA NPs-treated cells were stained with lysotracker (Sigma) to mark the location of endosomes within the cells. Nanoparticles gain entry into the cells by means of endosome-mediated transport. The Apt-PTX-PLGA NPs treated cells were stained with tubulin marker to selectively mark the micro-spindles. This is to evaluate the action of

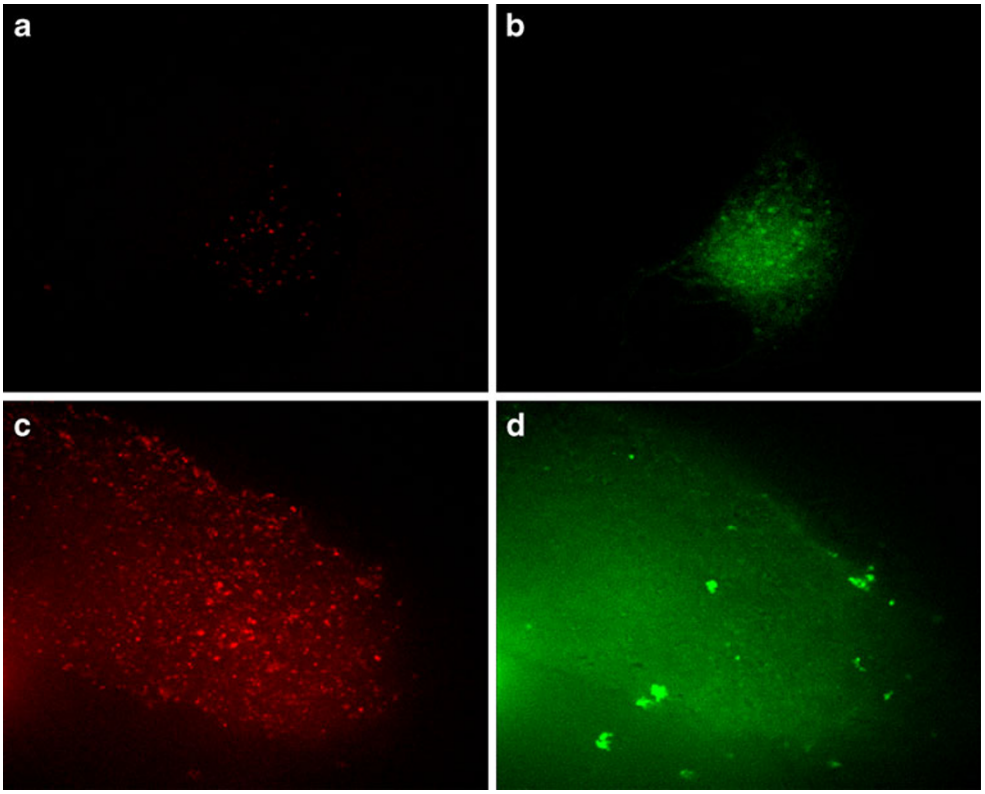

Fig. 6 Confocal images of GI-1 Cells treated with Apt-PTX-PLGA NPs after 24 hours (**a**) NR-tagged Apt-PTX-PLGA particles (**b**) Lysotracker stained lysosomes. Images of GI-1 Cells treated with Apt-PTX-PLGA NPs after 120 hours (**c**) NR-tagged Apt-PTX PLGA particles and (**d**) Lysotracker stained lysosomes

paclitaxel released from the PLGA nanoparticles. Fresh PBS (0.01 M, pH 7.4) buffer was added to the plates, and the cells were viewed and imaged under a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems GmbH, Germany) equipped with an argon laser using FITC filter (Ex 488 nm, Em525 nm) and Red filter (Ex 561 nm). The images were processed using Leica Application Suite software.

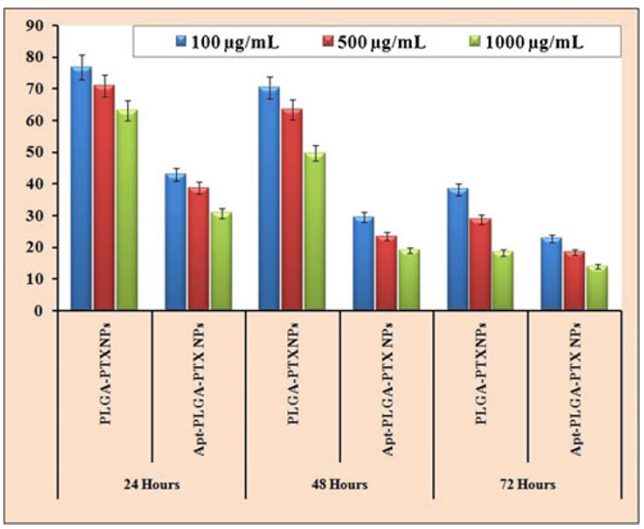

Fig. 7 Alamar blue assay analysis of PTX-PLGA NPs and Apt-PTX-PLGA at time intervals 24, 48, 72 h

2.7 In vitro cell viability assay

Bioassay of cell viability was investigated by means of the cellular mitochondrial activity (using methylthiazolyldiphenyl-tetrazolium bromide or MTT assay). GI-1 cell line and normal HMEC cells were exposed to plain paclitaxel(Taxol), plain PLGA nanoparticles(Plain-PLGA NPs), paclitaxel-loaded PLGA nanoparticles(PTX-PLGA), specific aptamer-labeled paclitaxel-loaded PLGA nanoparticles (AS1411-PTX-PLGA NPs), and non-specific aptamer-labeled paclitaxel-loaded PLGA nanoparticles (NS-PTX-PLGA NPs) at a concentration of 100 µg/ml for a 24-h duration. The NPs were sterilized with UV irradiation for 30 min before use. At given time interval, the cultured cells were assayed for cell viability with MTT (Sigma). The wells were washed twice with PBS, and 10 µl of MTT (5 mg/mL, Sigma) supplemented with culture medium was added. After 4 h incubation in the incubator, the culture medium was removed, and the precipitate (formazan crystals) was dissolved in 100 µL of dimethylsulfoxide. Relative percentage of metabolically active cells relative to untreated controls was then determined on the basis of the mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to formazan by cellular mitochondrial dehydrogenase present in viable cells. The amount of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide that is converted to formazan indicates the number of viable cells. The results were assessed in a 96-well format

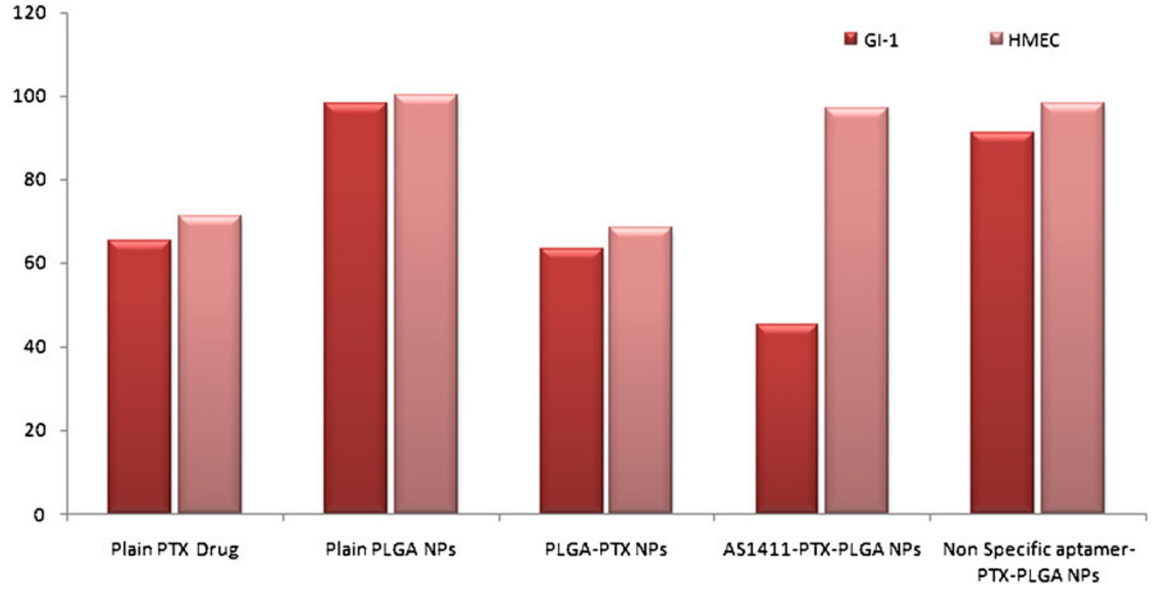

Fig. 8 MTT cytotoxicity assay results of Plain PTX, plain PLGA NPs, PTX-PLGA NPs, AS1411-PTX-PLGA NPs and non-specific Apt-PTX-PLGA NPs

micro plate reader by measuring the absorbance at a wavelength of 490 nm.

Another 96-well plate of GI-1 cells (5,000 cells/100 μl per well) was plated to make a comparison of the anti-proliferative effect of PTX-PLGA NPs and Apt-PTX-PLGA NPs on the cancer cells. Assays based on the cellular metabolic activity (using Alamar blue or AB), was performed after treating the cells with varying concentrations (0.001 μg–1 μg/ml) of the PTX-PLGA NPs and Apt-PTX-PLGA NPs for 24, 48, and 72 h. Alamar blue assay evaluates the proliferation and metabolic activity of cells. In living cells, the mitochondrial reductase enzymes are active and reduce Alamar blue to form a different-colored product from the blue dye. This reducing ability of the cells explains the active metabolism taking place within the cells. When the samples added to the cells are toxic in nature, the reducing ability of the cells to reduce the dye decreases. By measuring the fluorescence intensity of Alamar blue dye at 590–620 nm, the cell viability was determined. This colorimetric cell proliferation assays allow for easy and reliable colorimetric determination of viable cell numbers with excellent sensitivity. All the experiments were repeated in triplicate. Just before adding MTT reagent, representative phase contrast microscope images of cells were taken using an Olympus BX 41 microscope (Olympus, Center Valley, PA, USA).

2.8 Flow cytometry

GI-1 cells and HMEC cells were plated at a density of 2.5×10^6 cells per well in DMEM medium and HuMEC-Ready medium, respectively, and incubated at 37°C until it attained 70% confluence. The culture medium was replaced with Apt-NR-PLGA NPs suspension medium for 1–3 h at 37°C. The incubated cells were washed three times with cold PBS and trypsinized. The pellet was washed with PBS three times and fixed with 1% (*w/v*) *para*-formaldehyde solution. DAPI staining was done to stain the nucleus. The cellular uptake of nanoparticles by the cells was investigated by flow cytometry (FACScan, Becton Dickinson).

3 Results and discussion

3.1 PLGA drug delivery carriers suitable for aptamer functionalization

The surface morphology of the paclitaxel-loaded PLGA nanoparticles was analyzed by SEM and AFM. Under SEM observation, we found that the morphology of paclitaxel-loaded PLGA nanospheres fabricated with PVA had fine spherical shape with a smooth surface (Fig. 1) while the size of the particles ranged from 100 nm to 1 μm; most of the nanosphere sizes were skewed to about 200 nm. For cell culture work, we optimized the size of the particles to below 200 nm by filtering the particle solution through 0.2 μm Millipore syringe filter. Fig. 2 shows the AFM image of the filtered nanoparticles taken under high resolution. It was observed that the particles were of smooth surface and had a uniform size distribution of 200 nm.

3.2 Aptamer conjugation and surface chemistry analysis

Surface chemistry of the nanoparticles prepared by the solvent extraction/evaporation technique was analyzed by

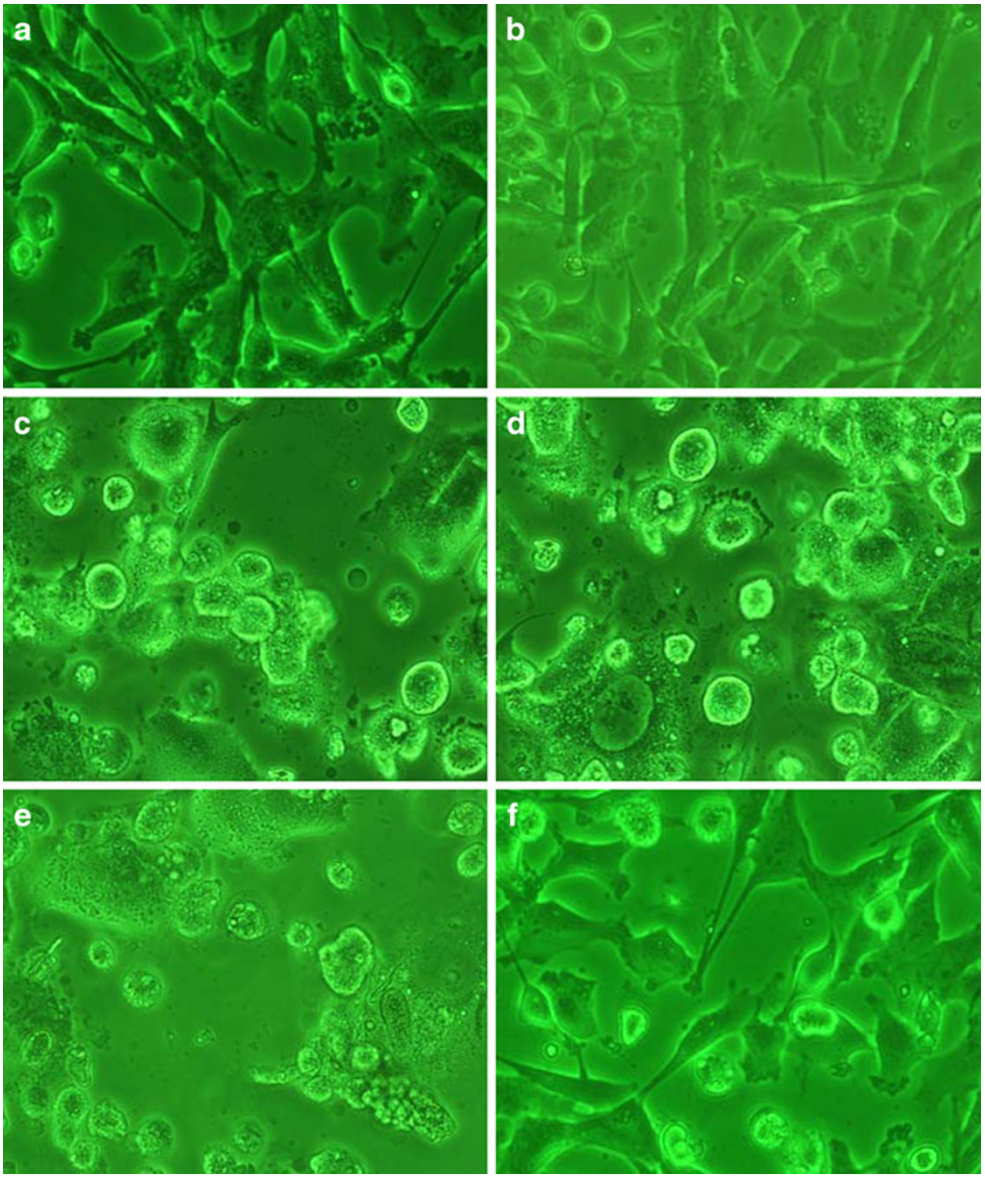

Fig. 9 Phase contrast images of (**a**) Control GI-1 cells (**b**) GI-1 cells treated with Plain PLGA NPs (**c**) GI-1 cells treated with Plain PTX (**d**) GI-1 cells treated with PTX-PLGA NPs (**e**) GI-1 cells treated with Apt AS1411-PTX-PLGA NPs (**f**) GI-1 cells treated with non-specific aptamer-PTX-PLGA NPs

XPS. Paclitaxel is the only substance which contains nitrogen in the nanoparticles prepared with PVA as emulsifier (Feng 2001). Therefore, nitrogen can be the characteristic element of paclitaxel. While scanning PTX-PLGA NPs, the scan of nitrogen failed to detect the existence of N1s core-level signal on the exterior. This fact may be attributed to the very low solubility of paclitaxel in water, which makes the drug tend to stay inside polymeric nanoparticle rather than diffuse from one to another (Feng et al. 2002).

Fig. 3 explains the N 1 s XPS spectra of the surface of the PTX-PLGA NPs with and without aptamer conjugation. Paclitaxel contains nitrogen element, but there is no N 1 s signal detected from the surface of the PTX-PLGA NPs, which proved that the drug was completely encapsulated inside the polymeric matrix of the NPs. The successful aptamer conjugation on the NP surface can be confirmed by the presence of the N 1 s region in the XPS spectra of Apt-PTX-PLGA NPs. XPS analysis supported the conclusion of successful conjugation of the aptamer on the polymeric nanoparticles by showing the nitrogen peak, which was completely absent in the PTX-PLGA NPs without aptamer treatment.

The encapsulation of drug within the PLGA NPs was further confirmed by means of 3D TEM imaging. From Fig. 4, we can clearly distinguish the inner core and outer surface of the drug-loaded nanoparticles leading to the confirmation of successful drug encapsulation within the nanoparticles. Further in vitro cytotoxic cell studies conducted for the drug-loaded PLGA nanoparticles supports the drug being entrapped within the particles.

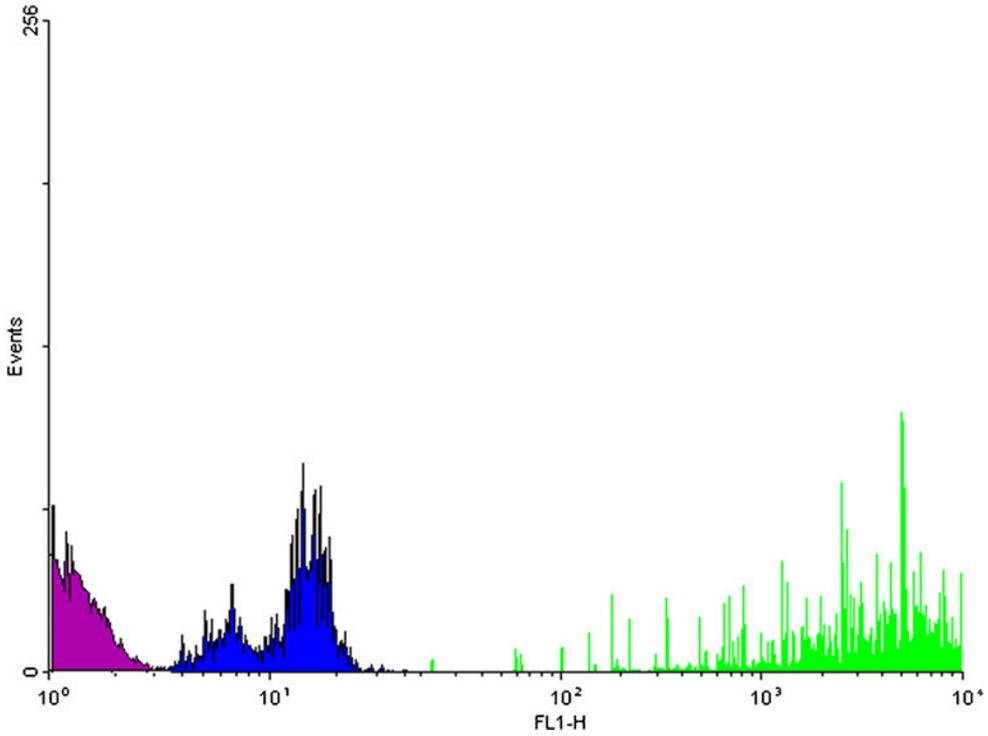

Fig. 10 Flow cytometry analysis —control cells (*purple*); normal cells treated with Apt-NR-PLGA NPs (*blue*) and tumor cells treated with Apt-NR-PLGA NPs (*green*)

3.3 In vitro cellular uptake: confocal microscopy studies

Confocal microscopy of normal HMEC cells and GI-1 tumor cells exposed to Apt-PTX-PLGA NPs demonstrated increased cytotoxic effect on tumor cells alone with the increase in the time of incubation. The cells were subjected to confocal microscopy after treating with the particles for 24, 72, and 120 h. At each interval, the cells were washed with PBS, stained with tubulin marker to mark the micro-spindles. Paclitaxel, a microtubule-targeted anti-cancer drug exerts its anticancer effects by causing abnormal stabilization of the dynamic microtubule polymerization leading to the failure of mitotic cell cycle and induction of apoptotic cell death (Alexandre et al. 2007). The normal HMEC cells grew normally in general culture conditions and were not affected by the presence of drug-loaded nanoparticles. In tumor GI-1 cells, by utilizing the tumor vasculature and receptor-mediated endocytosis, Apt-PTX-PLGA NPs was capable of entering the cells and exerting anti-proliferative effect and induced apoptosis (Fig. 5a–f).

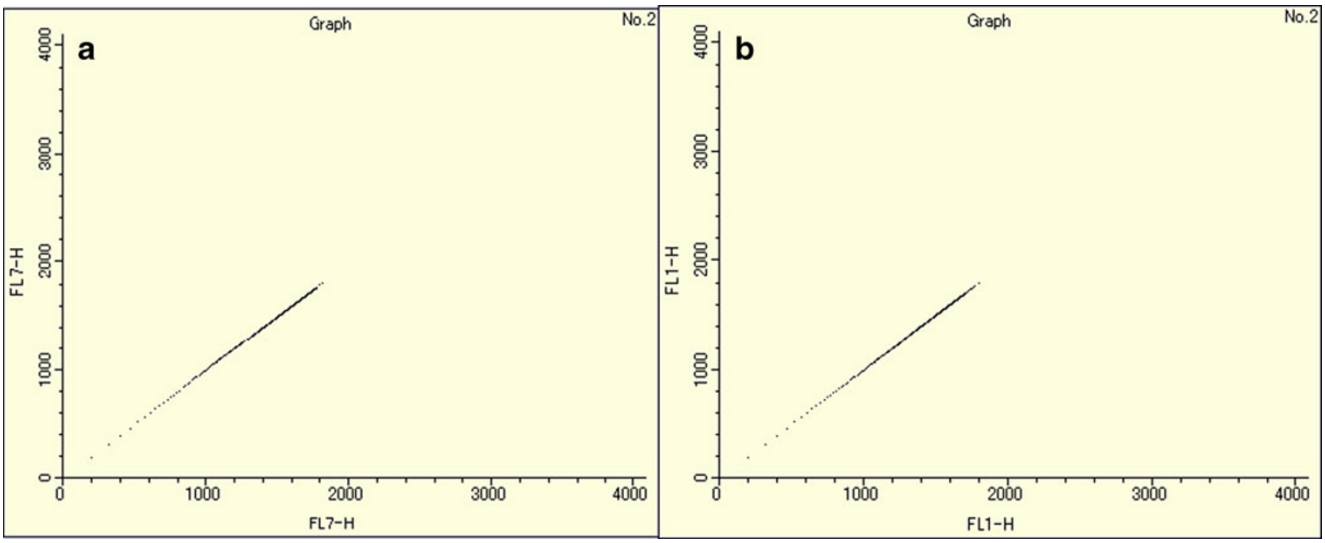

Fig. 11 Flow cytometry analysis of cells treated with particles **a** DAPI filter, **b** Red filter

Figure 6a–b represents the confocal images of cells treated with Apt-NR-PTX-PLGA NPs to study the localization of the particles within the cells. The cells were treated with these particles for 2 and 120 h. At each interval, the cells were washed with PBS and stained with lysotracker. In Fig. 6a, after 2 h incubation of the particles with the cells, we can identify the Apt-NR-PTX-PLGA NPs in the endosomes present in the cytoplasm of the cells. In the case of 120 h incubation of the particles with the cells, due to the action of paclitaxel, the cells undergo apoptosis, and the image clearly shows the scattered cytoplasm and the nanoparticles released from the cells (Fig. 6b).

3.4 In vitro cytotoxicity

The potential of PTX-PLGA formulations to kill the cancer cells is reflected by their cytotoxicity against the cancer cells. MTT assays were performed using equivalent concentrations of commercial paclitaxel, plain PLGA nanoparticles, paclitaxel-loaded PLGA nanoparticles with and without aptamer conjugation, and non-specific aptamer-conjugated paclitaxel-loaded PLGA nanoparticles. A comparative study of cytotoxicity was done using Alamar blue assay for plain PTX-PLGA nanoparticles and AS1411 aptamer-conjugated PTX-PLGA NPs (Fig. 7). Untreated cells served as controls; the assay was terminated at 72 h, and cell viability was determined via colorimetric determination.

Figure 8 shows the in vitro viability of GI-1 cells and HMEC cells after 24 h treatment of paclitaxel, plain PLGA nanoparticles, PTX-PLGA NPs, Apt-PTX-PLGA NPs, and also with non-specific aptamer-conjugated PTX-PLGA nanoparticles at a concentration of 100 μg/mL. From the cytotoxic assays and phase contrast microscopy images of cells treated with particles (Fig. 9a–f), we can infer that (1) the plain PLGA NPs are biocompatible and non-toxic (Fig. 9b); (2) the NP drug formulation demonstrated more efficiency in arresting cell growth than the commercial drug (Fig. 9c–d); and (3) the cellular viability decreased with the incubation period in all cases (Fig. 8).

Moreover, Fig. 7 clearly demonstrates the specific targeting effect of the aptamer conjugation on the PTX-PLGA nanoparticles. It can be seen from Fig. 7 in case of GI-1 cells (1) the cellular viability decreased from 63.16% for PTX-PLGA NP formulation (no aptamer conjugated) to 30.75% for the Apt-PTX-PLGA NP formulation for 24 h incubation; (2) the cellular viability decreased from 49.67% for PTX-PLGA NP formulation to 21.07% for the Apt-PTX-PLGA NP formulation for 48 h incubation; and (3) the cellular viability decreased from 18.36% for PTX-PLGA NP formulation to 15% for the Apt-PTX-PLGA NP formulation for 72 h, respectively. We can thus conclude that the targeting effect of the Apt-PTX-PLGA nanoparticles is highly significant in the targeted drug delivery.

3.5 In vitro cellular uptake: flow cytometry

The relative extent of cellular uptake was qualitatively analyzed by a flow cytometry method. The flow cytometry measures the fluorescence intensity of each cell and plots it against the number of cells. From flow cytometry results as shown in Fig. 10, fluorescence intensity of GI-1 cells incubated with Apt-NR-PTX-PLGA nanoparticles was stronger than that of HMEC cells with Apt-NR-PTX-PLGA NPs or control, indicating that the Apt-NR-PTX-PLGA NPs were specifically recognized by GI-1 tumor cells. In the case of the GI-cells incubated with Apt-NR-PTX-PLGA NPs, there was a shift in the red fluorescence intensity to the right direction, indicating that the cellular uptake of NR-PTX-PLGA nanoparticles was significantly enhanced due to aptamer-mediated endocytic delivery of nanoparticles into the cells. In addition, the overlapping of both DAPI channel and Red filter channel signals indicates the presence of nanoparticles within the same cell (Fig. 11).

4 Conclusions

In this study, the feasibility of targeting nanoparticles to specific tumor cells and cell internalization was demonstrated. Nanoparticles of PLGA polymer loaded with paclitaxel as a model anticancer drug were prepared by the solvent extraction/evaporation single-emulsion method and then functionalized with AS1411 aptamer specific for nucleolin protein for controlled and targeted chemotherapy. The targeting effect was qualitatively and quantitatively investigated by cancer cell uptake of the Nile red-tagged Apt-PTX-PLGA NPs and further confirmed by the cytotoxicity of the cancer cells treated with the Apt-PTX-PLGA NPs for various time intervals. We conclude that the PTX-PLGA nanoparticle formulation has great advantages over the commercial Taxol, and the aptamer conjugation can significantly promote targeted delivery of the drug to the corresponding cancer cells and thus enhance its therapeutic effects and reduced its side effects.

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