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Comparative Endocytosis Mechanisms and Anticancer Effect of HPMA copolymer- and PAMAM Dendrimer-MTCP Conjugates for Photodynamic Therapy

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Abstract

Polymer architecture can influence biodistribution and the mode of presentation of bioactive agents to cells. Herein, we examined delivery, loading efficiency and mode of cellular entry of polymer conjugates of the photosensitizer Meso-Tetra (4-Carboxyphenyl) Porphyrine (MTCP) when attached to hyper-branched amine terminated poly(amido amine) (PAMAM) dendrimer or random coil linear *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer containing free amines in the side chains. The *in vitro* dark cytotoxicity and phototoxicity of MTCP and related conjugates were assessed on mouth epidermal carcinoma (KB) and human adenocarcinoma alveolar basal epithelial (A549) cells. Phototoxicity of polymeric conjugates increased by approximately 100 and 4,000 fold in KB and A549 cells compared with non-conjugated MTCP. The increase in phototoxicity activity was shown to result from increased rate of cellular uptake, whereas, cellular internalization of MTCP was negligible in comparison with the conjugated forms. Our results suggest the superiority of amine-terminated HPMA copolymer vs PAMAM dendrimer under study for delivery of MTCP. Treatment with various pharmacological inhibitors

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Supporting Information. UV/vis and fluorescence spectrum, H-NMR and Mass spectra is available from the Wiley Online Library

Conflict of Interest. The authors declare no conflict of interest.

Supporting Information:

Figure.S.1: UV/visible and fluorescence spectrum of photosensitizer and polymeric nano-carrier conjugates.

Figure.S.2: Proton Nuclear Magnetic Resonance (H-NMR) spectrum of compounds.

Figure.S.3: ESI and MALDI-TOF Mass spectrum of compounds.

of endocytosis showed that polymer architecture influenced the mechanism of cellular uptake of the conjugated photosensitizer. Results show that polymeric conjugates of MTCP improved solubility, influenced the route and the rate of cellular internalization and drastically enhanced the uptake of the photosensitizer.

Graphical abstract

The association of photosensitizer molecules with polymeric carriers can improve solubility and influenced the route and the rate of cellular internalization and drastically enhance the uptake of the photosensitizer. Cartoon depicting the hypothesis that random coil HPMA copolymer-MTCP conjugates have more chance for physical association with cell membrane than spherical hyper-branched PAMAM dendrimer-MTCP conjugates. Negatively charged MTCP might be taken up via lipid raft domain on the cell membrane surface.

Keywords

Polymeric architecture; Endocytosis; Photosensitizer; HPMA; PAMAM

1-INTRODUCTION

Photodynamic therapy (PDT) is a minimally invasive method which damages the cells and tissues locally exposed to light in the presence of oxygen by generating reactive oxygen species, with minimal damage to the surrounding tissues.^[1] Photosensitizers play essential roles in the development and applications of modern PDT treatments.^[1, 2] Meso-Tetra (4-Carboxyphenyl) Porphyrin (MTCP) is a member of the family of porphyrin-based photosensitizers.^[3] Porphyrins are effective chromophores of visible light which have the ability to produce singlet oxygen and free radicals and have been successfully used for PDT.^[2] A major drawback for these photosensitizers is their poor solubility in aqueous solvents, limited accumulation at the target cells, and multidrug resistance.^[2, 4] Water-soluble polymers have been used as carriers of photosensitizers to overcome these challenges and deliver the photosensitizer “payload” to the tumor site.^[5–7]

Poly(amido amine) (PAMAM) dendrimers are a class of star-like structural macromolecules featured with a central core, a low-density interior formed by repeating monomer units and a large-density exterior terminated with surface functional groups for conjugation of drugs, targeting moieties and imaging agents.^[8,9] The ability of PAMAM dendrimers to deliver different photosensitizers with different strategies such as encapsulation of protoporphyrin IX (PpIX) and rose Bengal (RB)^[10], using 5-Aminolevulinic acid (5-ALA) PS as a scaffold to generate a dendrimer^[11], and chemical conjugation of hydrophobic Chlorin e₆ (Ce₆)^[12] has been investigated. These studies showed improved delivery of the photosensitizers to target cells, increased singlet oxygen generation efficiency, lower probability of aggregation of the PS, enhanced lysosomal targeting and lack of dark cytotoxicity.

Copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) are linear hydrophilic drug carriers^[13] that have been used for delivery of photosensitizers such as meso chlorin e₆ mono (N-2-aminoethylamide), for photodynamic therapy.^[14] It has been shown that

combination of chemotherapy and photodynamic therapy with HPMA conjugated drug and photosensitizers result in a synergistic response.^[15] While these studies show promise for improved delivery of PS, little is known about the influence of polymer architecture on cellular uptake rate and mechanisms of photosensitizers. It is known that polymer architecture influences biodistribution, tumor localization, cellular delivery and pharmacological activity.^[16–19] The influence of polymer architecture on loading of photosensitizers, their presentation to target cells, mechanisms of uptake, subcellular trafficking and efficacy however are largely unexplored. In this manuscript we report the influence of polymer architecture on delivery, mode of cellular entry and efficacy of hyperbranched PAMAM- and random coil HPMA copolymer-MTCP conjugates for photodynamic therapy.

2- EXPERIMENTAL METHODS

2-1- Materials

Commercially available ethylene diamine-core fourth generation poly(amido amine) (PAMAM) dendrimers were purchased from Dendritech (Midland, US). Meso-Tetra (4-Carboxyphenyl) porphyrine was purchased from Frontier Scientific (UT, US). *N*-(2-hydroxypropyl)methacrylamide (HPMA) comonomer was synthesized by procedure previously described.^[20] Chlorpromazine, Dynasore, Methyl- β -cyclodextrin, Genistein, Nystatin, Wortmannin, LY294002, Cholchicine, and Elacridar were obtained from Sigma (St. Louis, MO). Tariquidar was purchased from APExBIO (TX, US). EMEM, F12K, and FBS were all purchased from ATCC (VA, US). TrypLE™ Express was from Gibco (US). Wheat germ agglutinin CF™488A conjugate was purchased from VWR (US). Hoechst, AIBN, APMA, NHS, DCC, HBSS, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2-2- Methods

2-2-1- Preparation and characterization of polymers—The obtained dendrimers were purified by dialysis against deionized water under sink conditions for 3 days using cellulose membrane dialysis bag with the molecular weight cut off size 3.5 kDa (Spectrum Labs, US) to remove small molecular weight impurities. The dialyzed dendrimers were lyophilized and absence of small molecular weight impurities was verified by size exclusion chromatography (SEC) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare, Piscataway, NJ) equipped with XK 26/70 column packed with Superose 6 prep grade media (GE Lifesciences, Buckinghamshire, U.K.) at a flow rate of 1.0 ml/min of PBS (pH 7.4) with a 254 nm filter. HPMA copolymer-APMA was synthesized by free radical precipitation copolymerization with Azobisisobutyronitrile (AIBN) as initiator. Briefly, *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-(3-aminopropyl)methacrylamide (APMA) comonomers were combined with AIBN in anhydrous methanol as a solvent in nitrogen purged sealed glass ampule. The mole% feed ratios of comonomers was kept constant at 80:20 HPMA:APMA. The ampule was placed in 50°C oil bath for 24h, after which the resulting polymer was collected by precipitation and washed three times with cold acetone. The resulting white solid product was dissolved in deionized water and subsequently dialyzed using a 10 kDa molecular weight cut off (MWCO) cellulose

membrane dialysis bag. The purified copolymers' average molecular weight (MW) and polydispersity (Mw/Mn) were estimated by SEC using HPMA homopolymers of known molecular weight as standards by UV detector on the FPLC. Sizes were also confirmed using the multi-angle laser light scatter (MALS) and refractive index detectors. The absence of small molecular weight impurities was checked with SEC. Molecular weight and the number of functional groups on the surface of PAMAM dendrimer was theoretically calculated based on perfect dendrimer synthesis. The number of amine functional groups on the side chains of HPMA-APMA copolymers was calculated based on the molecular weight of HPMA-APMA copolymer and the percent of feed ratio mass of APMA (20%) divided by APMA molecular weight (142.9 gr/mole) yielding mole% ratio of amine groups in the polymer side chains.

2-2-2- Synthesis and characterization of PAMAM-MTCP and HPMA-MTCP conjugates—MTCP-polymer conjugates were prepared according to Scheme 1.

Carboxylic groups of MTCP were activated via a carbodiimide reaction with DCC/NHS. Briefly, MTCP (10-fold excess molar ratio of MTCP to polymer) was dissolved in 3ml DMSO and reacted with DCC (4:1 molar ratio DCC: MTCP) for 1 hour. Then, NHS (1:1 molar ratio NHS: DCC) was added to the reaction mixture in the presence of a N,N-diisopropylethylamine (DIPEA). The activated MTCP was added dropwise to the polymer solution in DMSO. The reaction was stirred for 3 days at room temperature and kept in dark. Unreacted MTCP was removed by membrane dialysis using DMSO buffer for 7 days. The dialysis buffer was replaced with deionized water gradually for 10 days. The contents were lyophilized, stored at -80°C and protected from light. Size exclusion chromatography was used to confirm the purity and homogeneity of the final products. The chromatographic elution profiles of MTCP and MTCP-conjugates under study were obtained using FPLC (as described above) in order to evaluate relative elution volumes and to check for the absence of small molecular weight impurities. The mobile phase for elution was phosphate buffer saline (PBS) (pH=7.4) at a flow rate of 1.0 ml/minute. The conjugates were further characterized for hydrodynamic radius (R_h) using a Dynamic Light Scattering (DLS) detector (Heleos II) attached to the FPLC system and analyzed using Astra™ 5.3.4.13 software (Wyatt Technologies, Santa Barbara, CA). The zeta potential of compounds dispersed in distilled (DI) water at a concentration of 1.0 mg/ml and pH=7, was measured using a Malvern Instruments Zetasizer Nano ZS (Westborough, MA). Optical absorption and fluorescence spectra were recorded by SpectraMax M2 microplate reader (Molecular Devices, CA, US). The structure of the polymer-MTCP conjugates was examined using H-NMR (Varian INOVA 500, US). The molecular weight of MTCP was investigated by Electro Spray Ionization (ESI) mass spectrometry (Micromass QToF-2, US). For large mass polymer conjugates Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectra (MALDI-TOF) was acquired using Bruker Daltonics Ultraflex extreme with 2,5-dihydroxybenzoic acid matrix. The average number of MTCP present per conjugated polymer chain or dendrimer were calculated based on the calibration curves of MTCP (data not shown) and correlated with H-NMR. The molecular weights of the HPMA-MTCP and PAMAM-MTCP conjugates were determined theoretically based on the estimated molecular weight of polymer and the average number of MTCP molecules attached to the polymers.

2-2-3- Cell Culture—Mouth Epidermal Carcinoma Cells (KB), and Human adenocarcinoma alveolar basal epithelial (A549) cells were obtained from American Type Tissue Collection (ATCC) (Manassas, VA, USA) and maintained in the recommended media (Kiaghn's Modification of Ham's F-12 medium with 2mM L-glutamine and 1500 mg/L sodium bicarbonate, for A549 cells; Eagle's Minimum Essential Medium (EMEM) 2mM L-glutamine, 1mM sodium pyruvate, and 1500 mg/L sodium bicarbonate; for KB cells) supplemented with 10% Fetal Bovine Serum (FBS). Cell cultures were incubated at 37 °C in 5% CO₂ and 95% humidified air and kept in logarithmic phase of growth throughout all experiments. All experiments were done in the passage numbers 6–10.

2-2-4- Determination of Cell Viability; Cytotoxicity—The *in vitro* dark cytotoxicity of photosensitizer and related conjugates were assessed by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technology, US). Briefly, A549 cells (4×10^3 per well) and KB cells (4.5×10^3 per well) were seeded in 96-well microliter plates for 36 hours before the treatment. Cells were exposed to fresh-prepared different concentrations of compounds and incubated for 7 hours. The drug containing media was removed and cells washed with PBS (pH 7.4) twice. Then cells were incubated in fresh complete media for another 48 hours in dark. The media was removed and 100 ml of medium solution containing 10% (v/v) CCK-8 reagent was added. The cells were incubated at 37°C for a further 1–2 hours. The absorbance was obtained by scanning with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) at 450nm. Each experiment was performed at least three times in triplicate. Data were analyzed by Excel 2010 (Microsoft, USA) and GraphPad Prism 5 (GraphPad Software, Inc., USA). Control experiments were performed using cells treated with complete medium involving no particles as negative control and 0.01% (v/v) Triton X-100 as positive control. **Phototoxicity:** The same experiment as cytotoxicity was conducted for phototoxicity assays where cells were illuminated with Actinic aquarium lamp (Coralife-China) (maximum emission wavelength at 420nm, energy dose 10 mW/cm²). Cells were exposed to the same range of concentrations of compounds and incubated for 7 hours. After removing the drug and washing with PBS (pH 7.4) cells were illuminated in fresh media for 10 minutes. Then, cells were incubated in fresh complete media for another 48 hours in dark and viability was assessed by CCK-8 as mentioned above.

2-2-5- Cellular Uptake Quantification, and Time-dependent Uptake by Flow Cytometry—The rate of uptake of photosensitizer and related conjugates by cultured cells was visualized by flow cytometry. Cells were grown on 12-well imaging plates at a density of approximately 17,000 cells/cm². After 36 h, cells reached the confluency of 70%; cells were then incubated with compounds (same equivalent of MTCP) for various time points (15min to 10 hours). Following incubation, cells were rinsed with PBS buffer, washed twice and then detached using TrepLE. Cells were centrifuged at 1,600 rpm for 7min to obtain cell pellet, and washed again with ice cold PBS containing 2%FBS (pH=7.4). Cells were spun down twice and re-suspended in the same buffer to obtain a single-cell suspension, and subsequently analyzed by flow cytometry (Cytek DxP analyzer, US). For each sample, 30,000 events were collected by list-mode data that consisted of side scatter, forward scatter and emitted light resulting from MTCP-conjugated nanoparticles was detected by the Red-FL2 detector. To calculate the background fluorescence of unlabeled cells, cells without

addition of conjugates were carried along as untreated control in every measurement. For whole-cell analysis 30,000 cells were counted. The percent cell entry (considering the fluorescence intensity at 1 h as 100% for treated cell with drug without inhibitor) was plotted against time. Data analysis was performed with Flowjo software (version10). Average median fluorescent intensity was calculated for each experiment using at least three independent sets. All cell experiments were performed in the dark.

2-2-6- Confocal Laser Scanning Microscopy—Cells were grown on Lab-Tek™ Chambered Coverglass for 24 hours. After incubation, cells were washed with Hank's Balanced Salt Solution (HBSS) buffer three times and cell membranes were stained with wheat germ agglutinin-CF™488A conjugate and fixed with 4% formalin in PBS. Cell nuclei were stained with Hoechst according to the manufacturer's protocol. Fluorescent images of fixed cells were taken under a confocal laser scanning microscope (Olympus FV1000). The intensity of the laser beam and the photodetector sensitivity were kept constant in order to compare the relative fluorescence intensities between experiments. Acquisition conditions for all images or treatments were identical, and images were analyzed using Olympus FV1000 software.

2-2-7- Energy-Dependent Mechanism of Entry—Cells were grown in 12-well plates at a density of 17,000 cells/cm², 36 hours before experiment. Cells were incubated for 2 hours at 4°C, then the media containing cold drug solution (containing the same equivalent of photosensitizer) was added and incubated for 1 more hour. The fluorescence intensity of internalized conjugates was measured by flow cytometry as mentioned in Section 2-2-5.

2-2-8- Chemical Inhibitors of Endocytosis and p-Glycoprotein—The relative amount of conjugate uptake in the presence of different endocytosis and P-glycoprotein inhibitors (Table 1) was assessed via flow cytometry. Cells were grown on 12-well plates at a density of 17,000 cells/cm² for 36 hours. They were pre-incubated with inhibitors prior to 1h incubation with photosensitizer and related conjugates. To study the effect of various inhibitors on uptake, the cells were pre-incubated with the following inhibitors at concentrations which were not toxic to the cells. CCK-8 cytotoxicity measurement revealed no cytotoxicity for the inhibitors for both cell types being analyzed (data not shown). Concentrations for inhibitors were obtained from reported literature values^{25, 28–32}; (I) Chlorpromazine (28 uM), (II) Methyl-β-cyclodextrin (10 mM), (III) Genistein (200 uM), (IV) Nystatin (20 uM), (v) Wortmannin (50 nM), (VI) LY294002 (60 uM), (VII) Cholicine (5 uM), (VIII) Dynasore (80 uM), (IX) Elacridar (1 uM), (X) Tariquidar (150 nM). Conjugate-only treated cells were utilized as control and compared with inhibitor plus conjugate-treated cells.

2-2-9- Statistical Analysis—Analysis for each instrument was performed with associated software products as listed below: GraphPad Prism 5 for cytotoxicity; Flowjo (Version 10) for flow cytometry; Olympus FV1000 for confocal microscopy; Astra™ 5.3.4.13 (Wyatt Technologies, Santa Barbara, CA) for SEC; VNMR J4.2A for NMR analysis; MassLynx 4.1 for ESI/MS and FlexControl 3.4 for MALDI-TOF analysis. For all methods statistical analyses were performed with Excel 2010 software (Microsoft, USA). Student's t test (two

tailed, unpaired) was performed for statistical analysis. All experiments were performed in triplicate, and the results were presented as mean/median $\pm$ standard deviation. The difference between values was considered significant at the level of $p < 0.05$, unless stated otherwise.

3- RESULTS AND DISCUSSION

3-1- Synthesis and Characterization

Amine terminated PAMAM- and HPMA copolymer-MTCP conjugates were successfully synthesized and characterized (Table 2). The absence of small molecular weight impurities was verified by size exclusion chromatography. PAMAM-MTCP conjugate was synthesized with PS loading degree of 1.53 ± 0.07 mole MTCP per one mole PAMAM polymer. Under similar reaction conditions, HPMA copolymers containing amine groups in the side chains had more MTCP per mole of HPMA copolymer chain (7.02 ± 0.29 mole MTCP/mole HPMA). There were approximately 64 and 69 amine groups on PAMAM-G4 dendrimer and HPMA-APMA copolymers, respectively. However, compared with linear HPMA copolymers, the compact spherical architecture of PAMAM dendrimers probably posed steric hindrance on the surface of these structures due to crowded functional groups, thus limiting the payload for the MTCP amphipathic photosensitizer molecules having hydrophobic center with negative exterior carboxylic charges.

The conjugation of MTCP to PAMAM dendrimers was verified by UV/vis spectroscopy (Figure S.1), H-NMR (Figure S.2) and Mass spectrometry (Figure S.3). Polymer-MTCP conjugate showed UV/vis spectrum and fluorescence intensity similar to free MTCP indicating that the optical properties of the PS molecules would not be affected by chemical conjugation (Figure S.1). The hydrodynamic radius of the linear HPMA copolymer was about 4 nm, similar to that of hyper-branched PAMAM dendrimers (Table 2). The conjugation of MTCP to the polymeric carriers did not have a significant effect on their hydrodynamic size. After conjugation of PS molecules to polymers, the zeta potential of PAMAM-MTCP conjugate at pH 7 decreased from about +45.71 mV to +20.12 mV which was possibly due to the contribution of MTCP molecules for decreasing the overall charge of the PAMAM-MTCP conjugates. Although HPMA copolymer-MTCP conjugate had almost 4.5 fold more loaded MTCP molecules per mole of polymer in comparison with PAMAM-MTCP conjugate, the zeta potential of HPMA-MTCP conjugate increased from approximately +24.78 mV to +32.35 mV at the same pH after MTCP conjugation. This could be due to increased intra- and inter-polymer interactions leading to exposure of positively charged polymers to the surface with negatively charged hydrophobic MTCP in the core, or increasing effects of MTCP on pKa of the HPMA amine groups leading to the exhibition of more positive charges on HPMA.

3-2- In Vitro Phototoxicity of the Conjugates

Cytotoxicity assays for the conjugates with CCK-8, showed time- and dose-dependent effect. The photodynamic killing of KB and A549 cancer cells, as a model of epidermal cancer cells, was dependent on pre-incubation time, irradiation time (light dose) and MTCP concentration. The cytotoxicity of MTCP after light illumination (i.e. MTCP phototoxicity)

increased 100 and 1,600 fold respectively when compared with non-illuminated counterparts in KB and A549 cells. This ratio increased significantly in carrier-MTCP constructs. Phototoxicity of PAMAM-MTCP was 1,600 and 11,300 fold, and for HPMA-MTCP more than 5,000 and 67,000 fold higher than their dark cytotoxicity in KB and A549 cells, respectively. As expected, the phototoxicity of MTCP increased by approximately 100 and 4,500 fold with the copolymer conjugate (with same equivalent of PS) when compared with non-conjugated photosensitizer in KB and A549 cells, respectively (Table 3). The phototoxic effect of all conjugates was in the nontoxic concentration range of the amine terminated polymeric carriers without MTCP.

Conjugation of MTCP to both carriers changed the dose of MTCP cellular toxicity three orders of magnitude. To investigate the reason for increased toxicity, we evaluated the cellular internalization of conjugates by confocal microscopy. Following 7 hours incubation with drugs there was no observed free MTCP uptake in KB and A549 cells. However, there was a greater degree of uptake of PAMAM-MTCP and HPMA-MTCP inside the cells (Figure 1). This confirmed that polymer-conjugated photosensitizers were taken up to a higher extent than free MTCP.

To compare the effect of polymer architecture, cytotoxicity studies revealed that 0.83 nM of HPMA-MTCP (equivalent to 5.82 nM MTCP) had the same phototoxicity as 7 nM of PAMAM-MTCP (equivalent to 10.71 nM MTCP) in KB cells. In A549 cells, results showed that 0.07 nM of HPMA-MTCP (equivalent to 0.51 nM MTCP) and 2.79 nM PAMAM-MTCP (equivalent to 4.28 nM MTCP) had the same phototoxicity (Table 3). Therefore, conjugation to linear HMPA copolymer significantly increased the phototoxicity of MTCP, 1.83 and 8.5 times more than PAMAM-MTCP in KB and A549 cells, respectively (Table 3). Based on these results, it can be concluded that although the cytotoxicity of PAMAM-MTCP in dark is more than HPMA-MTCP; at same equivalent of MTCP concentrations, lower concentrations of linear HPMA-MTCP conjugate was needed to show the same phototoxicity as higher concentrations of hyper-branched PAMAM-MTCP conjugate. Therefore HPMA copolymers with amine termination in the side chains are more efficient for MTCP delivery compared to the amine terminated PAMAM dendrimer investigated in this study. This increase in phototoxicity was shown to result from increased rate of cellular uptake as discussed below. Our results also showed A549 cell line is more sensitive to MTCP photosensitizer and related conjugates than KB cells. The cell-type dependent cytotoxicity effect might be a result of the threshold of reactive oxygen species (ROS) generation to cause cell death in each cell line and also may depend on cell defense mechanisms against drugs.

3-3- Time Dependent Cellular Uptake

In an attempt to assess the relative mode of uptake of these constructs, a time point assay was performed to determine the uptake saturation point. The uptake profiles of MTCP and its polymeric conjugates (with same equivalent of MTCP) were time- and dose-dependent, however the internalization rate was polymer architecture dependent. A comparison of the time-dependent fluorescence intensity levels indicates that the rate of cellular uptake was significantly higher for HPMA-MTCP conjugate, followed by PAMAM-MTCP conjugates

and MTCP respectively (Figure 2). The uptake of polymer conjugated-MTCP was substantially higher than that of free MTCP. HPMA-MTCP conjugate was about 54 and 155 fold more toxic than MTCP in KB and A549 cells respectively after 7 hours of treatment. The uptake of PAMAM-MTCP was approximately 27 and 110 times more than MTCP in KB and A549 cells, respectively (Table 4). The key factor influencing the phototoxicity of polymeric conjugates was the increased cellular uptake of the conjugates compared with free MTCP. One possible explanation for the large difference in uptake between free MTCP and polymer-conjugated MTCP may be related to the degree of association with cell membrane and/or overcoming multidrug resistance pump after conjugation to polymeric carriers. HPMA-MTCP conjugate showed a much higher uptake than PAMAM-MTCP conjugate. Linear HPMA-MTCP conjugate showed 2 and 1.5 fold increase in cellular uptake than hyper-branched PAMAM-MTCP conjugate after 7 hours of incubation. Both polymer conjugates had similar size, but the zeta potential of HPMA-MTCP was much higher than PAMAM-MTCP. The flexible backbone of linear HPMA-MTCP with the random coil structure with higher positive zeta potential allows increased interaction with the cell surface leading to the increased uptake compared to the more compact and spherical structure of PAMAM-MTCP with lower positive zeta potential and limited interaction with cell surface.

3-4- Energy-Dependent Mechanism of Uptake

To determine if the mechanisms of uptake were energy dependent or due to passive transport across the cell membranes, cells were incubated at 4°C and 37°C to evaluate the relative level of conjugate internalization. On reducing the incubation temperature, the cell uptake of MTCP and polymer-MTCP conjugates reduced significantly in a time dependent manner (Figure 3). The reduction in cellular uptake was significantly higher in MTCP treated cells, after 1–8 hour incubation at 4°C (in comparison with drug treated cells at 37°C), followed by HPMA-MTCP conjugates and finally PAMAM-MTCP conjugates in KB and A549 cells (Table 5). Thus, all polymer conjugates and free MTCP molecules appear to be taken up by an active process. It was surprising to us that the uptake of smaller MTCP amphipathic molecules with hydrophobic center became negligible on reducing the incubation temperature, which suggests that the entry of MTCP is more energy dependent than the conjugated forms.

3-5- Mechanism of Uptake

Many entry pathways into cells have been identified, which vary in the cargoes they transport and in the protein machinery that facilitate the endocytosis process. These mechanisms can be divided into two major categories, namely phagocytosis and pinocytosis. Pinocytosis is classified as clathrin-dependent and clathrin-independent. Clathrin-independent pathways can be classified as caveolae-mediated endocytosis, non-clathrin non-caveolae dependent endocytosis, and macropinocytosis.^[21] A screening of a series of chemical inhibitors was performed to investigate the uptake mechanisms of the different conjugates. Below, we describe the results of inhibitors that were used for each endocytosis pathways investigated for the polymer-MTCP conjugates.

3-5-1- Caveolae mediated pathway—To study caveolae dependent endocytosis three different inhibitors, Methyl- β -cyclodextrin, nystatin and genistein were used. Methyl- β -

cyclodextrin (M β CD) is a cyclic oligomer of glucopyranoside and a member of the cyclodextrins family, which, due to the presence of relatively hydrophobic cavity, can be used to extract cholesterol from cell membranes and inhibits cholesterol-dependent endocytic processes.^[22] Cholesterol plays a broad regulatory role in endocytosis. Depleting cells of cholesterol reversibly inhibits a variety of plasma membrane contour changes. These include macropinocytosis, caveolae dependent endocytosis, non-clathrin non-caveolae dependent endocytosis, membrane ruffling, cell spreading and filament actin reorganization.^[23, 24] The uptake of MTCP into KB and A549 cells was reduced significantly by approximately 97.14% and 74.65% respectively. There was no significant reduction in the uptake of HPMA-MTCP conjugates after pre-incubation with M β CD in KB cells and the A549 cell line. The PAMAM-MTCP uptake decreased after inhibition with M β CD by 24.84% in KB cells, without any significant inhibition in A549 cells (Figure 4 and Table 5).

Nystatin is a fungal metabolite that disrupts lipid rafts by binding to cholesterol within the plasma membrane.^[25] Pre-incubation of both cell lines with 20 μ M nystatin before treatment reduced the uptake of MTCP into KB cells by approximately 21.39% without any effect on the MTCP uptake into A549 cells. PAMAM-MTCP uptake was diminished by about 21.27% and 14.89% into A549 and KB cells, respectively after pre-incubation with nystatin. Nystatin reduced the HPMA-MTCP conjugate cellular uptake by almost 16.56% into A549 with no significant inhibition on KB cells (Figure 4 and Table 5).

Genistein is a tyrosine-kinase inhibitor that causes local disruption of the actin network at the site of endocytosis and inhibits the recruitment of dynamin II, both known to be indispensable events in the caveolae-mediated uptake mechanism.^[26] However, this does not imply that genistein can be used as a selective inhibitor of caveolae function. Genistein can inhibit uptake of clathrin-coated pit of receptors that need tyrosine phosphorylation for accumulation in clathrin coated pits. It has also been reported that it can inhibit F-actin requirement to clathrin-coated pits.^[27] Genistein decreased the uptake of MTCP by approximately 23.92% in A549 cells and 20.97% in KB cells. The uptake of polymer-MTCP conjugates was not influenced after pre-incubation of cells with genistein, except the significant reduction in uptake of PMAM-MTCP into KB cells that was decreased by about 15.28% (Figure 4 and Table 4).

3-5-2- Clathrin mediated pathway—Two different endocytosis inhibitors, namely chlorpromazine and dynasore were used to study clathrin mediated endocytosis of MTCP and its related conjugates.

Chlorpromazine is a cationic amphipathic drug that causes the assembly of adaptor proteins and clathrin on endosomal membranes thus depleting it from the plasma membrane, leading to a block in clathrin mediated pathway.^[28] However chlorpromazine also causes an inhibition of receptor recycling and clathrin independent mechanism in some cells.^[29] Dynasore was the first compound identified to block dynamin's GTPase activity in a biochemical assay and it has been shown to rapidly and reversibly block clathrin-mediated pathway in cells.^[30] The cellular uptake of MTCP into KB and A549 cells decreased partially in the presence of chlorpromazine (~27–32%) (Figure 4 and Table 5). The effect of

this inhibitor on inhibition of PAMAM-MTCP uptake was approximately 30.27% and 16.16% on A549 and KB cells, respectively. We observed a slightly lower degree of inhibition for HPMA copolymer-MTCP treated A549 and KB cells (~19% and 8.69%, respectively) (Figure 4 and Table 5). On the other hand, the uptake of MTCP and its related conjugates was not significantly affected after treatment with 80 μ M of dynasore, with the exception of the uptake of PAMAM-MTCP conjugates into A549 cell that decreased by approximately 22.4% (Figure 4 and Table 5).

3-5-3- Macropinocytosis—Wortmannin and LY294002 prevent fluid phase endocytosis (macropinocytosis) and phagocytosis by inhibiting multiple isoforms of phosphatidylinositol-3-kinases. LY294002 is a reversible inhibitor of PI3K whereas wortmannin acts irreversibly.^[31,32] The uptake of MTCP was diminished by 16–29% in the presence of these two inhibitors. The effect of these inhibitors on cellular uptake of HPMA-MTCP was negligible. PAMAM-MTCP cellular uptake decreased by approximately 14% in the A549 cells treated with Wortmannin and KB cell treated with LY294002 (Figure 4 and Table 5). Colchicine is another phagocytic and macropinocytic inhibitor. Colchicine inhibits microtubule polymerization, both of which reduce membrane ruffling.^[33] Colchicine treatment reduced the uptake of MTCP (~24–37.5%) and PAMAM-MTCP (~27–30%) more than HPMA copolymer-MTCP (~11–14%) in both cell lines (Figure 4 and Table 5).

Here, various pharmacological inhibitors of endocytosis were used, with a view to characterize the cellular uptake mechanisms of conjugates. These endocytosis inhibitors have a broad spectrum and are rarely selective with respect to specific trafficking pathways. They may also inhibit different endocytic pathways in different cell types as the efficacy of endocytosis inhibitors are highly cell line-dependent.^[21] Cellular entry pathways may be differently present or even totally absent in selected cell types.^[21] Our results show that at least one of either clathrin and caveolae dependent inhibitors can inhibit MTCP cellular uptake into both cell lines. It was surprising that the highest degree of inhibition was observed after removing cholesterol with Methyl- β -cyclodextrin in MTCP treated cells (74–97% reduction). One effect of cholesterol depletion with M β CD on cells is disrupting lipid rafts.^[34] Membrane lipid rafts are highly ordered membrane domains that are enriched with cholesterol, sphingomyelins and gangliosides. However, there are some reports that show lipid raft is not only important for caveolae-mediated uptake, but also for other mechanisms such as macropinocytosis.^[24] M β CD was used to determine whether endocytosis is dependent on the integrity of lipid rafts. It was rather surprising that nystatin by binding to cholesterol did not work in both cell lines. MTCP is a small amphipathic compound having hydrophobic center and negative exterior charges, hence cells may employ multiple pathways for its uptake. It has been suggested that photosensitizer molecules may permeate the cells by diffusion both across and along the membranes, by non-specific endocytosis or even by pinocytosis.^[35] Large aggregates or PS-carrying particles can also be internalized via phagocytosis.^[35] Overall, it is believed that photosensitizers which are less hydrophobic and have more than two negative charges (MTCP has four carboxylic groups, therefore it can have four negative charges at physiological pH) tend to be too polar to diffuse across the plasma membrane, and are taken up by endocytosis.^[1] It is possible that the most important factor to engulf negatively charged MTCP is the lipid raft domains on the cell membrane

which can provide the opportunity for MTCP to associate with cell membrane. The presence of sphingomyelins, with positively charged choline group, in lipid rafts may be the main cause of MTCP's tendency for binding to the plasma membrane. It may also be possible that changes in physicochemical properties of cellular membranes such as permeability and fluidity after cholesterol depletion by M β CD, is the cause of decreased MTCP uptake.

The maximum inhibition of uptake for PAMAM-MTCP conjugate was obtained by colchicine treatment (Figure 4 and Table 5), suggesting the microtubules may play a more important role in the internalization pathways responsible for nanoparticle uptake. On the other hand, clathrin dependent inhibitors, chlorpromazine, dynasore, and nystatin, and all used caveolae dependent inhibitors, reduced the uptake of PAMAM-MTCP conjugates into A549 cells, significantly. In KB cells, all caveolae dependent inhibitors in parallel with chlorpromazine and LY294002 decreased the uptake of PAMAM conjugated photosensitizer. Therefore it can be concluded that both clathrin and caveolae-mediated pathways were involved in the uptake of PAMAM-MTCP conjugate. Our previous results show that cationic dendrimers are transported by an energy dependent process and dendrimer internalization and intracellular trafficking occurs by a classical endocytosis pathway that can be inhibited in the presence of colchicine.^[36,37] We also showed previously that a lower generation amine-modified dendrimer (G2) entered cells primarily through clathrin-mediated endocytosis, while the higher generation dendrimer (G4) utilized multiple routes.^[36,37] It has also been shown by other groups that even though all the dendrimers are taken up by fluid-phase endocytosis, significant differences in uptake mechanisms exist depending on the dendrimer's surface functionalities.^[38] The amine-modified cationic dendrimers or carboxylate-modified anionic PAMAM dendrimers were shown to utilize multiple routes of entry to Caco-2 and B16f10 melanoma cells.^[37, 39] Oddone and coworkers reported PAMAM G4-FITC is taken up by clathrin-mediated endocytosis with endosomal and lysosomal localization⁴⁰. Our findings for PAMAM-MTCP conjugate are in contrast to other studies that have shown anionic dendrimers to be mainly taken up by caveolae mediated endocytosis in A549 lung epithelial cells, while cationic and neutral dendrimers appear to be taken up by a non-clathrin non-caveolae mediated mechanism that may be by electrostatic interactions or other non-specific fluid-phase endocytosis.^[38]

HPMA-MTCP conjugate had an increased rate of uptake, while the influence of most inhibitors on their uptake was more limited. Only methyl- β -cyclodextrin and colchicine showed about 10.5–11.5% inhibition in the uptake of this conjugate into KB cells and these changes were not significant (Figure 4 and Table 5). In A549 cells, chlorpromazine followed by nystatin and then colchicine decreased the uptake of linear HPMA copolymer-MTCP conjugate. This was expected, since this carrier had more positive charge with flexible amphipatic MTCP arms that can be associated with the cell membranes. Therefore based on the available molecular pathways and cell phenotypes, cells can use any of the pathways to engulf this conjugate. If one pathway was inhibited, cells likely used the other available pathways to take up the conjugate, and therefore the overall uptake did not change. It was also reported previously that cell entry and subsequent intracellular trafficking of HPMA-based copolymers are strongly dependent on the physicochemical characteristics of the carrier, such as charge and molecular weight.^[41] These studies showed that the copolymers were internalized by the cells through multiple endocytic pathways: positively charged

copolymers robustly engaged clathrin-mediated endocytosis, macropinocytosis and dynamin-dependent endocytosis, while weakly negatively charged copolymers weakly employed these pathways; strongly negatively charged copolymers only mobilized macropinocytosis.^[41]

3-6- Overcoming multi drug resistance pump

Tariquidar (XR9576) and Elacridar (GF120918; GW0918) are potent and selective noncompetitive inhibitors of P-glycoprotein and breast cancer resistance protein (BCRP), an important membrane protein that pumps many foreign substances out of cells.^[42] Here, we focused on MTCP as a photosensitizer, which has also been extensively evaluated to combat MDR.^[43] The limited cellular uptake of MTCP may have been caused by increased MDR pump activity. The rate of cellular uptake of MTCP was investigated in the presence of these two inhibitors. Figure 5 shows there is no significant difference in the uptake of MTCP with or without inhibitors. Therefore, there is no possibility of overcoming multidrug resistance pump after conjugation to polymeric carriers that cause the large difference in uptake between free MTCP and its polymer-conjugated forms. The most important factor inhibiting the uptake of MTCP is the low physical association with the cell membrane because of the superficial negative charge of MTCP.

4- CONCLUSIONS

In this study the dark- and photo-toxicity of polymeric conjugates of the photosensitizer MTCP on KB and A549 cancerous cells was studied by paying particular attention to the mode of cell entry as a function of polymer architecture. Our results suggest the superiority of amine-terminated HPMA copolymers vs PAMAM dendrimers for delivery of MTCP. Free MTCP had much more limited effect compared to polymeric conjugates. Our studies suggest that charge and the macromolecular nature of the constructs are probably the most important factors in cellular uptake. As to which pathway is used for delivery of the conjugates our results indicate that it would be unwise to ascribe a specific endocytotic pathway for each of these conjugates. The inhibitors used in our studies had different effects depending on the cell type. Overall these studies suggest the utility of HPMA copolymer-APMA constructs for delivery of MTCP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HPMA

N-(2-hydroxypropyl)methacrylamide

PAMAM-NH2 G4	Amine terminated generation 4 poly(amido amine) dendrimer
MTCP	Meso-Tetra(4-Carboxyphenyl) Porphyrine
APMA	N-(3-aminopropyl)methacrylamide
AIBN	Azobisisobutyronitrile
PDT	Photodynamic Therapy
PBS	Phosphate Buffer Saline
M_n	number average molecular weight
M_w	weight average molecular weight
MWCO	molecular weight cut-off
PDI	Poly Dispersity Index
SEC	Size Exclusion Chromatography
FPLC	Fast Protein Liquid Chromatography
H-NMR	Proton Nuclear Magnetic Resonance
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight
DMSO	Dimethyl Sulfoxide
NHS	N-Hydroxysuccinimide
DCC	N,N'-Dicyclohexylcarbodiimide
SD	Standard Deviation
EMEM	Eagle's Minimum Essential Medium
KB	Mouth Epidermal Carcinoma Cells
A549	Human adenocarcinoma alveolar basal epithelial cells
CCK-8	Cell Counting Kit-8
FBS	Fetal Bovine Serum
FK12	Kiaghn's Modification of Ham's F-12 medium
MeOH	Methanol
DCU	N,N'-Dicyclohexylcarbodiimide
HBSS	Hank's Balanced Salt Solution
MDR	Multi Drug Resistance

DLS

Dynamic Light Scattering

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Figure 1. Cellular uptake study by Confocal Microscopy

Cells were grown on Lab-Tek™ Chambered Coverglass for 24 hours. After incubation for with drugs ((MTCP 0.158 mM, PAMAM-MTCP 20.61 μ M, HPMA-MTCP 4.49 μ M)) for 7hours, cell membranes were stained with Wheat germ agglutinin CF™488A conjugate and cell nuclei were stained with Hoechst according to the manufacturer's protocol. Cells fixed with 4% para-formaldehyde solution in PBS. Red dots are represented to polymer-conjugated MTCP. Fluorescent images of fixed cells were taken under a confocal laser scanning microscope (Olympus FV1000 - US).

performed with Flowjo software version 10. **A)** The rate of uptake of PAMAM-MTCP, HPMA-MTCP and MTCP in different cell lines. **B)** The graphs in Figure 2A were overlapped for each cell line to compare the rate of uptake of MTCP and related conjugates with the same equivalent of MTCP in KB and A549 cells separately. Values are median $\pm$ SD (n=3, * p<0.05, *** p<0.0001).

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Figure 3. Energy dependent uptake

To determine if the mechanisms of uptake were due to endocytosis or other energy-dependent mechanisms, cells were grown in 12-well plates at a density of 17000 cells/cm², 36 hours before experiment. Cells were incubated for 2 hours at 4°C, then the media containing cold drugs solution (contain same equivalent of photosensitizer MTCP 31.53 μM, PAMAM-MTCP 20.61 μM, HPMA-MTCP 4.49 μM) was added and incubated for 1–8 hours in 4°C. The fluorescence intensity of internalized nanoparticles was measured by flow cytometer (Cytex DXP analyzer, US). Values are median ± SD (n=3, * p<0.05, **p<0.001, *** p<0.0001).

Figure 4. Effect of different endocytosis inhibitors on the uptake of MTCP, PAMAM-MTCP and HPMA-MTCP into KB and A549 cells

Cells were pre-incubated with inhibitors for 1h at 37°C (2 hours at 4°C for energy dependent mechanism study), following by a 1h incubation with drugs (MTCP 31.53 μ M, PAMAM-MTCP 20.61 μ M, HPMA-MTCP 4.49 μ M) in the previous temperature. After incubation, cells were spun down twice and resuspended in PBS containing 2%FBS to obtain a single-cell suspension, and subsequently analyzed on Cytex flow cytometer (DxP analyzer, US). Graphs are represented as percentage of uptake or the background provided by FACS analysis of cells incubated with drugs without the respective inhibitor (CON). The abbreviations are listed below: Chlorompamazine (CPZ), Methyl- β -cyclodextrin (M β C), Genistein (GNS), Nystatin (NYS), Wortmannin (WRM), LY294002 (LY), Cholchine (COL), Dynasore (DYN). Values are median $\pm$ SD (n=3, * p<0.05, **p<0.001, ***p<0.0001).

Figure 5. Multi Drug Resistance study

The rate of cellular uptake of MTCP was investigated in the present of two multi-drug resistance inhibitors, Tariquidar (TRQ) (150nM) and Elacridar (ELC) (1uM), that are a potent and selective noncompetitive inhibitor of P-glycoprotein BCRP. The rate of MTCP (0.158mM) uptake by cultured cells was visualized by flow cytometry and data analysis was performed with Flowjo software. There is no significant difference in the uptake of MTCP with or without MDR inhibitors in the range of time. Values are median $\pm$ SD.

Scheme 1. Polymerization of HPMA-APMA copolymer and synthesis of HPMA-MTCP and PAMAM-MTCP conjugates

Carboxylic groups of MTCP was activated in carbodiimide reaction with DCC/NHS.

Polymerization of HPMA-APMA copolymer was done in free radical reaction using AIBN as initiator. The feed ratio of comonomers were kept constant at 80:20 (mol%)

HPMA:APMA. Conjugation of activated-MTCP to polymeric nano-carriers was done in DMSO solution for 3 days. All reactions was done in dark. Figures are not up to scale.

Table 1

List of inhibitors of Endocytosis and p-Glycoprotein.

Mechanism	Inhibitor	Concentration
Caveolea mediated pathway	Methyl- β -cyclodextrin	10 mM
	Genistein	200 μ M
	Nystatin	20 μ M
Clathrin mediated pathway	Chlorpromazine	28 μ M
	Dynasore	80 μ M
Macropinocytosis	Wortmannin	50 nM
	LY294002	60 μ M
	Colchicine	5 μ M
Multi Drug Resistance	Elacridar	1 μ M
	Tariquidar	150 nM

Table 2
Physicochemical characteristics of photosensitizer, nano-polymeric carriers and related conjugates.

compounds	Physicochemical characteristics [#]					
	Hydrodynamic Radius(Rn) (nm) [‡]	Zeta Potential (mV) (pH=7.4)	Elution Volume [†]	Number of Functional Groups [*]	Molecular Weight (gr/mol) [£]	Molar Ratio of MTCP
MTCP	N/A	-13.16 ± 1.54	21.55	4-COOH	790.8	N/A
PAMAM(G4)	4.04±0.39	45.71 ± 3.59	18.64	64-NH2	14214.19	N/A
HPMA-MTCP	4.5±0.42	32.35 ± 1.41	15.61	N/A	54,975.63	7.023±0.29
HPMA-APMA	4.86±0.05	24.78 ± 2.84	15.05	69-NH2	49,421.59	N/A
PAMAM-MTCP	4.016±0.24	20.12 ± 0.74	19.03	N/A	15,424.11	1.53 ± 0.07

[#] Values are mean ± SD (n=3).

[‡] The hydrodynamic sizes of nanoparticles were characterized using Dynamic Light Scattering detector attached to the *FPLC* systems.

[†] The elution volume peaks of particles were detected at the wavelength 254nm by Size Exclusion Chromatography.

^{*} The number of functional groups on the surface of *MTCP* and *PAMAM* molecules is theoretical value based on perfect particle synthesis. The number of amine functional groups on the side chain of *HPMA-APMA* copolymer was calculated based on feed ratio during polymerization as described in "Results and discussion".

[£] Molecular weight of *PAMAM* is theoretical values based on perfect dendrimer synthesis. Molecular weight of *HPMA* copolymer was estimated on the same *FPLC* setup using *HPMA* homopolymer standards of known molecular weights. The molecular weight of conjugates were determined theoretically based on the molecular weight of polymers and the number of ligands (*MTCP*) on average attached to the polymer surface.

Cytotoxicity and phototoxicity

Table 3

A. The fifty percent Lethal Dose concentration (LC50) value of MTCP, PAMAM-MTCP and HPMA-MTCP conjugates in two cell lines, KB and A549 cells, are listed under non-illumination (cytotoxicity) and illumination (phototoxicity) conditions. All LC50 values are based on equivalent molar of MTCP in conjugated-polymer. The safety concentration was reported as the maximum concentration of drugs that do not have any toxicity in dark (viability>99%). **B.** Comparison of cytotoxicity with phototoxicity of free MTCP and polymer conjugated-MTCP. It was calculated by dividing the LC50 of particles in non-illumination condition versus illumination counter-part. **C.** Comparison of the phototoxicity of different polymer-MTCP conjugates with free MTCP and PAMAM-MTCP with HPMA-MTCP conjugates. It was calculated by dividing the LC50 value (based on molar of MTCP) of drug in column 2 by LC50 value of the drug in column 1. Values are mean $\pm$ SD (n=3).

Components	50% Lethal Dose Concentration(μ M)				Dark Safety Concentration(mM)	
	KB Cells		A549 Cells		KB	A549
	DARK	LIGHT	DARK	LIGHT		
PAMAM	19.8 $\pm$ 1.01	20.2 $\pm$ 2.11	6.97 $\pm$ 0.89	6.93 $\pm$ 0.27	<0.002 $\pm$ 0.001	<0.0015 $\pm$ 0.002
MTCP	165 $\pm$ 8.02	1.477 $\pm$ 0.05	3720.12 $\pm$ 8.3	2.315 $\pm$ 0.0072	<0.065 $\pm$ 0.021	<1.24 $\pm$ 0.0401
PAMAM-MTCP	17.69 $\pm$ 1.01	0.0107 $\pm$ 0.0009	48.34 $\pm$ 2.04	0.00428 $\pm$ 0.0006	<0.001 $\pm$ 0.0008	<0.009 $\pm$ 0.0005
HPMA-MTCP	>338.71 $\pm$ 21.9	0.00582 $\pm$ 0.00017	>341.40 $\pm$ 14.09	0.0005 $\pm$ 0.00001	<0.0047 $\pm$ 19.37	<0.0051 $\pm$ 0.0008
HPMA-APMA	N/A	N/A	N/A	N/A	<0.05 $\pm$ 0.0054	<0.07 $\pm$ 0.017

Phototoxicity 1	Toxicity Ratio		Cytotoxicity 2
	KB cells	A549 cells	
MTCP	100	1600	MTCP
PAMAM-MTCP	1650	11300	PAMAM-MTCP
HPMA-MTCP	>5817	>67300	HPMA-MTCP

Phototoxicity 1	Toxicity Ratio		Phototoxicity 2
	KB cells	A549 cells	
PAMAM-MTCP	138	540	MTCP
HPMA-MTCP	93	4500	MTCP

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C	1	Toxicity Ratio		2
	Phototoxicity	KB cells	A549 cells	Phototoxicity
	HPMA-MTCP	1.83	8.5	PAMAM-MTCP

Table 4
Comparison of the rate of cellular uptake of polymer-conjugated-MTCP with MTCP, and HPMA-MTCP with PAMAM-MTCP in two cell lines

Cell were treated with compounds (MTCP 31.53 μ M, PAMAM-MTCP 20.61 μ M, HPMA-MTCP 4.49 μ M) for 7hours and the cellular uptake was checked by Flow cytometry. The numbers were calculated by dividing the median of fluorescence intensity of the drug in column 1 with drug in column 2. Values are median $\pm$ *SD* (n=3).

1	Times more taken up		2
	KB cells(FR+)	A549(FR-)	
HPMA-MTCP	54.18 $\pm$ 4.10	155.43 $\pm$ 3.58	MTCP
PAMAM-MTCP	27.11 $\pm$ 1.14	110.29 $\pm$ 2.69	MTCP
HPMA-MTCP	1.99 $\pm$ 0.14	1.5 $\pm$ 0.046	PAMAM-MTCP

Table 5

Endocytosis inhibitor study

List of inhibitors used for testing the different pathways and their effect in inhibition of uptake of particles. The values are the percent inhibition of cell entry by considering the fluorescence intensity as 100% for treated cell with drug and without inhibitors. Values are mean $\pm$ SD (n=3).

Mechanism	compounds	MTCP		PAMAM-MTCP		HPMA-MTCP	
		A549	KB	A549	KB	A549	KB
Energy dependent endocytosis	Inhibitor						
	4 °C	90.85 $\pm$ 2.59	94.27 $\pm$ 3.26	47.11 $\pm$ 5.32	55.87 $\pm$ 2.76	41.14 $\pm$ 5.69	69.99 $\pm$ 3.4
Caveolea mediated pathway	Methyl- β -cyclodextrin	74.65 $\pm$ 3.19	97.14 $\pm$ 4.30	6.93 $\pm$ 0.27	24.84 $\pm$ 2.85	10.22 $\pm$ 0.35	10.645 $\pm$ 1.13
	Genistein	23.92 $\pm$ 3.64	20.97 $\pm$ 3.28	9.37 $\pm$ 1.94	15.28 $\pm$ 0.59	8.71 $\pm$ 1.46	8.61 $\pm$ 2.14
	Nystatin	0.93 $\pm$ 0.19	21.39 $\pm$ 0.06	21.27 $\pm$ 0.34	14.89 $\pm$ 0.16	16.56 $\pm$ 1.50	5.68 $\pm$ 0.35
Clathrin mediated pathway	Chlorpromazine	27.53 $\pm$ 3.78	31.73 $\pm$ 3.98	30.27 $\pm$ 2.97	16.16 $\pm$ 1.29	19.00 $\pm$ 1.36	8.69 $\pm$ 0.25
	Dynasore	6.77 $\pm$ 0.1	13.30 $\pm$ 0.16	22.40 $\pm$ 0.94	9.13 $\pm$ 0.60	5.68 $\pm$ 0.03	0.32 $\pm$ 0.016
Macropinocytosis	Wortmannin	16.56 $\pm$ 1.28	11.36 $\pm$ 1.01	14.52 $\pm$ 2.01	5.62 $\pm$ 0.19	4.70 $\pm$ 0.37	3.37 $\pm$ 0.11
	LY294002	7.13 $\pm$ 0.99	28.92 $\pm$ 4.52	8.72 $\pm$ 0.13	14.78 $\pm$ 0.33	4.32 $\pm$ 0.003	5.31 $\pm$ 0.18
	Colchicine	24.45 $\pm$ 1.3	37.46 $\pm$ 3.12	27.53 $\pm$ 1.56	29.90 $\pm$ 2.36	14.26 $\pm$ 0.67	11.77 $\pm$ 0.89