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Cationic branched polymers for cellular delivery of negatively charged cargo

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Abstract

Receptor-independent cellular uptake of small molecule therapeutics is limited by their physical interaction with the negatively charged surface of cellular membranes. Passive diffusion through the hydrophobic membrane bilayer follows this process. Unless specific carriers exist in the biological membrane, such interactions limit therapeutics to those that are hydrophobic with modest positive charge at physiological pH. Small negatively charged molecules are therefore not efficient as therapeutics. To enable delivery of such molecules into eukaryotic cells, cationic branched polymers with tetraalkylammonium pendant groups were synthesized by copolymerization of a functional monomer (glycidyl methacrylate) with degradable and non-degradable divinyl crosslinkers in the presence of an efficient chain transfer agent, CBr₄, followed by reaction of the multiple pendant epoxide groups and most of the alkyl bromide chain ends with amines. Cationic branched polymers with covalently attached fluorescent labels entered human cancerous and non-cancerous cells. The non-labeled analogues were able to carry anionic cargo (carboxyfluorescein) into the cells, while no uptake was observed in the absence of the cationic carriers. Most of the polymers were not significantly toxic at the concentrations used. This pilot study showed that cellular uptake of anionic small molecules can be promoted even in the absence of natural uptake mechanisms.

Keywords

hyperbranched macromolecules; anionic small molecule delivery; cationic polymers; cellular anion delivery

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Introduction

Cationically-charged polymers have garnered major interest for a number of important therapeutic applications, including drug delivery, tissue engineering, and gene delivery.¹ The cationic moieties are useful for complexation with negatively charged groups found in certain drugs and the backbones of nucleic acids (formation of polyplexes).²⁻⁵ In the case of gene delivery, cationic polymers are also useful for mediating transfection via nucleic acid condensation and protection from enzymatic degradation.⁶⁻⁹ In order to be useful, a cationic polymer should have an appropriate architecture that best expresses its functionalities. Due to the presence of multiple chain-ends (and therefore multiple identical or different chain-end functionalities), macromolecules with branched architectures are ideal, including star-shaped polymers and nanoparticles with multiple grafted arms, and crosslinked micelles, as well as dendrimers and (hyper)branched polymers. Star polymers¹⁰ and crosslinked micelles¹¹ derived from block copolymers have already found numerous applications in the drug-delivery field; materials with multiple cationic groups have already been successfully employed for siRNA delivery^{12, 13}. Dendrimers¹⁴ are promising materials for drug and gene delivery^{15, 16, 17} that provide well-defined core-shell structures with great functionality. However, there are drawbacks to using dendrimers, including difficult multi-step syntheses requiring tedious purification procedures following each step and limited size due to steric restrictions.¹⁸ Branched, including hyperbranched, polymers resemble dendrimers in many respects, but have more irregular structures.^{19, 20} Importantly, (hyper)branched polymers can be synthesized using simpler one-pot methods²¹ and provide a facile way to produce materials with high expression of functionality. Hyperbranched polymers are more poorly defined than dendrimers and can be rather disperse in terms of molecular weight distribution and degree of branching, but are significantly easier to synthesize and are therefore suitable for fast screening in order to identify compositions that are appropriate for specific desired applications.

With demand for such materials growing over the years, a variety of methods have been developed for their synthesis.²² Initially, step-growth approaches were developed, utilizing AB_x ($x \geq 2$) and $A_x + B_y$ ($x \geq 2$ and $y > 2$) type monomers (in the latter case, soluble branched polymers are only formed prior to network formation). These methods proved to be simple and robust. However, considering the nature of polycondensation reactions and the involvement of functional groups present in the material synthesis (A must react with B only), the scope of compatible end functionalities is quite limited. To answer this limitation, chain-growth methods were developed, allowing for the incorporation of functionalities that are independent of the polymer synthesis and retained in the resulting polymer. One popular chain-growth approach is self-condensing vinyl polymerization (SCVP), where inimers (compounds containing both an initiating and a polymerizable group in the same molecule) are used.^{23, 24} This method has wide applicability, even being recently used in conjunction with controlled radical polymerization (e.g., atom transfer radical polymerization (ATRP)) to form highly branched polymers with disulfide and ester group containing backbones.²⁵ Methods have been reported that allow for the synthesis of hyperbranched polymers with narrow molecular weight distributions using the inimer approach.²⁶⁻²⁸ However, the synthesis of inimers is typically a multistep process, which can involve numerous

purification steps,²⁹ rendering SCVP impractical for situations where simplicity and cost-effectiveness are important. It has been recently demonstrated that inimers can actually be formed *in situ* from carboxylate monomers via exchange reactions with “ligands” attached to hypervalent iodine centers,³⁰ but such approaches have limited applicability.

Another class of chain-growth techniques for synthesizing highly branched polymers involves the radical copolymerization of monovinyl with di- or even multivinyl monomers. This method has a major benefit in that it is cost-effective due to the ability to use commercially available monomers. When employing this method, it is important to consider that in order to delay macrogelation, the number of pendant polymerizable groups per chain should be limited. This can be accomplished by either using a very small amount of crosslinker, or by using larger amounts of crosslinker while limiting the length of the chains. The former approach is not ideal because it cannot yield very highly branched materials and is not suitable for the incorporation of multiple functionalities originating from the crosslinker per single branched macromolecule. Therefore, efficient ways to limit the molecular weights of the polymers are preferred. Various approaches can be used to limit polymer chain length under a variety of mechanisms. For instance, reagents capable of reversibly deactivating propagating radicals,^{31, 32} chain transfer agents,³³⁻³⁶ and excessive amounts of radical sources (initiators)³⁷⁻⁴⁰ have been successfully used to synthesize multifunctional branched macromolecules. One particularly interesting chain transfer agent is CBr₄ because the chain transfer reactions afford alkyl bromide chain ends, which can be employed in further functionalizations, including nucleophilic substitutions and chain extension reactions under ATRP conditions.⁴¹⁻⁴³ This approach allows for the generation of polymers that not only have functional groups at the chain ends directly originating from the transfer agent, but also functional groups at the branching points introduced with the crosslinker (e.g., degradable functionalities),^{41, 43, 44} and backbone functionalities derived from the monomer(s) (e.g., reactive pendant groups).

The presence of reactive pendant functionalities along the backbone of a highly branched polymer can be exploited via post-polymerization modifications to further functionalize the material at hand. One such functional group that is widely used and quite robust is the epoxide. Epoxides can easily react with a variety of nucleophiles⁴⁵⁻⁴⁹ to afford useful products with drastically different properties than those of the original materials. This high diversity of transformations that epoxides can undergo is particularly useful since a systematic library can be developed rather efficiently, with all resulting materials being derived from the same starting material. Some synthetically useful examples of these transformations within the scope of polymer chemistry include the synthesis of polymers with pendant azide groups,⁵⁰ polymers functionalized via thiol “click” reactions,⁵¹ as well as star polymers with cationic, hydrophilic cores.⁴³

The synthesis of cationic, hydrophilic, highly branched polymers by copolymerization of crosslinkers in the presence of chain transfer agents combined with epoxide transformation chemistry is presented in this work. We also present initial toxicity studies using human cancerous and noncancerous cells in culture. Finally, the cellular uptake of a membrane-impermeable, negatively charged, small molecule fluorescent dye in the presence, but not absence of the synthesized cationic polymers is shown. These studies are of importance for

the future development of positively charged carrier molecules that mitigate uptake of negatively charged biological effector molecules into cells where no naturally occurring uptake mechanism exists.

Experimental Section

Materials

The polymerization inhibitor was removed from glycidyl methacrylate (GMA, 97%, Aldrich) by passing the neat monomer through a short column of neutral alumina. The same procedure was used to purify ethylene glycol dimethacrylate (EGDMA, 98%, Alfa Aesar), but using basic alumina. Azobisisobutyronitrile (AIBN, 98%, Aldrich) was recrystallized from ethanol. Bis(2-methacryloyloxyethyl) disulfide ((MAOE)₂S₂) was synthesized as described in the literature.⁵² All other reagents, CBr₄ (98%, Acros), *N,N'*-dimethylethanolamine (DMEA, 99%, Acros), *N,N'*-dimethylbutylamine (DMBA, 99%, Aldrich), fluorescein isothiocyanate (FITC, 90%, Acros), tetramethylsilane (TMS, >99.9%, Aldrich), paclitaxel (>99%, Acros), trypan blue (>60%, Acros), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, MP Biomedicals), 7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (resazurin, Acros), fetal bovine serum (BioWest), penicillin and streptomycin (Corning), and the solvents: anisole (PhOMe; 99%, Aldrich), *N,N'*-dimethylformamide (DMF; >99.8%, EMD), acetone (99.5%, EMD), methanol (MeOH; 99.8%, EMD), tetrahydrofuran (THF; 99%, Fisher), CDCl₃ (99.8% D, Cambridge Isotope), and DMSO-*d*₆ (99.9% D, Cambridge Isotope) were used as received. Working solutions of MTT (5 mg/mL), resazurin (440 μM) and trypan blue (250 μg/mL) were prepared in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and sterile filtered through 0.22 μm nylon filters before use. Cell culture materials were purchased from Corning Inc. unless otherwise stated. Origin™ 7.0 was used for plotting toxicity data in Figure 2.

Synthetic procedures

Highly branched polyGMA (hb-polyGMA^{EST}-Br_x)—GMA (6.0 mL, 45 mmol), EGDMA (426 μL, 2.25 mmol, 5 mol % vs. GMA), AIBN (0.0372 g, 227 μmol), CBr₄ (2.2464 g, 6.8 mmol, 30 eq. vs. AIBN), and PhOMe (6 mL) were added to a 25-mL round-bottom flask equipped with a magnetic stir bar. The flask was capped with a rubber septum (pre-washed with acetone and dried), and the solution was deoxygenated by bubbling with nitrogen for 45 min while cooling the reaction flask in an ice bath. The flask was then immersed in an oil bath set at 60 °C. Samples were taken periodically and analyzed. Final polymer was isolated by diluting the reaction mixture with acetone (ca. 1 : 1 by volume) and precipitation in diethyl ether. The polymer was redissolved in a minimal amount of chloroform and treated dropwise with a 10 % (v/v) solution of Br₂ in chloroform until a slight yellow-orange color remained, indicating complete conversion of any remaining alkenyl groups. The polymer was then finally purified by reprecipitation in diethyl ether followed by drying *in vacuo*. This same procedure was used for the synthesis of hb-polyGMA^{DS}-Br_x, with use of (MAOE)₂S₂ instead of EGDMA (569 μL, 2.25 mmol, 5 mol % relative to GMA).

Modification of hb-polyGMA^{EST}-Br_x with DMBA and DMEA—Dried hb-polyGMA^{EST}-Br_x (142 mg, 1.0 mmol epoxide groups) was weighed into a small test tube equipped with a magnetic stir bar and dissolved with 0.5 mL DMSO-*d*₆. In a separate test tube, DMBA (168 μL, 1.2 mmol) and concentrated HCl (86 μL, 1.0 mmol) were mixed thoroughly. Once mixed, 0.25 mL DMSO-*d*₆ was added and the entire contents transferred to the test tube containing the epoxide-containing polymer. DMSO-*d*₆ (0.25 mL) was used to wash out the amine/acid test tube and added to the reaction vessel. The test tube was capped with a rubber septum (pre-washed with acetone and dried) and immersed in an oil bath set at 60 °C. Samples were taken at timed intervals, diluted with DMSO-*d*₆, and analyzed by NMR to determine reaction progress. The final polymer (hb-polyBuAmm^{EST}-Z_x (the adopted nomenclature is explained in the Results and Discussion section)) was purified by precipitation in acetone (3×), followed by redissolving in water, dialysis in water (MWCO = 2,000 Da) to remove any excess amine, and lyophilization. The same procedure was followed for the (bio)degradable analogue (hb-polyGMA^{DS}-Br_x) to form hb-polyBuAmm^{DS}-Z_x.

Hb-polyGMA^{EST}-Br_x and hb-polyGMA^{DS}-Br_x were also modified with DMEA under similar conditions as previously reported⁴³ to produce polymers with hydroxyethylammonium salt repeat units (hb-polyHOEAmm^{EST}-Z_x and hb-polyHOEAmm^{DS}-Z_x, respectively).

Modification of hb-polyHOEAmm^{EST}-Z_x with FITC—Dried hb-polyHOEAmm^{EST}-Z_x (50 mg, 187 μmol -CH₂CH₂OH groups) and fluorescein isothiocyanate (7.3 mg, 18.7 μmol, FITC) were weighed into a small vial and dissolved with 0.7 mL DMSO-*d*₆. The vial was then capped, sealed with parafilm, and placed in an oil bath set at 50 °C for 6.5 h. The reaction mixture was then slowly diluted with 5 mL of a H₂O/EtOH (1:1) solution and transferred to a dialysis bag (MWCO = 2,000 Da). This was dialyzed in H₂O/EtOH (1:1) solution for 4 days (changing solution 3 times per day) and the final polymer (hb-polyFLEAmm^{EST}-Z_x) was obtained after lyophilization.

Analyses—Monomer conversions were determined by ¹H NMR spectroscopy on a Bruker Avance DRX 400 spectrometer operating at 400 MHz. Apparent molecular weights (*M*_{n,app}) and dispersities (*M*_w/*M*_n) were determined by size exclusion chromatography (SEC) using linear poly(methyl methacrylate) standards on a Tosoh EcoSEC HLC-8320 system equipped with a series of 4 columns (TSK gel guard Super HZ-L, Super HZM-M, Super HZM-N, and Super HZ2000) with THF as the eluent (0.35 mL min⁻¹, 40 °C). Part of the samples withdrawn from the reaction mixtures were diluted with THF, filtered through 0.2 μm PTFE Acrodisc filters, and the obtained solutions were directly injected into the SEC without purification.

Cell lines and cell culture

HFL1 (a human fetal lung fibroblast cell line)⁵³, MDA-MB-231 (a human breast cancer cell line)⁵⁴, and DU145TXR (a multidrug resistant human prostate cancer cell line)⁵⁵ were cultured as described in⁵⁴ and⁵⁵ in media supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) in humidified incubators

at 37 °C and 5% CO₂. HFL1 cells were cultured on collagen coated surfaces (0.1 mg/mL rat tail collagen) to promote attachment. DU145TXR were maintained in media containing 10 nM paclitaxel, which was replaced with paclitaxel-free media at least 24 h before experiments.

Cytotoxicity

Cells were seeded in 96-well plates at densities of 3,000 to 4,000 cells/well in 150 µL media. 24 h after seeding, serial dilutions of polymer stock solutions (10 mg/mL to 0.5 µg/mL in DMSO) were further diluted in media and 50 µL were added to triplicate wells. The MTT assay was used to evaluate cytotoxicity to HFL1 and DU145TXR cells as described previously.^{56, 57} Briefly, media containing polymers was removed after 48 h incubation and the cells were rinsed twice with PBS before 200 µL fresh media were added. After 24 h incubation in a humidified incubator at 37 °C and 5 % CO₂, 20 µL MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated for 4 h. After carefully aspirating the media, the formazan crystals were dissolved in DMSO and the absorbance at 570 nm was measured using a BioTek Eon™ plate reader. The absorbance measured at the reference wavelength of 720 nm was subtracted from all wells as previously described.⁵⁸ Viability was calculated as the absorbance ratio of polymer treated wells to vehicle (DMSO) treated control wells.

The resazurin reduction assay is similar to the MTT assay, but does not require a solubilization step since the pink fluorescent resorufin produced by metabolically active cells is soluble in culture media.^{59, 60} Cytotoxicity of polymers to MDA-MB-231 human breast cancer cells was determined using the resazurin reduction assay, following the same protocol and exposure procedures as described above for the MTT assays. 40 h after adding polymers to cells in 96-well plates, 20 µL of resazurin solution was added to each well and the plates were incubated in a humidified incubator at 37 °C and 5% CO₂ for 8 h before reading their fluorescence using a Gemini™ XPS Microplate Spectrofluorometer set at 560 nm excitation and 590 nm emission wavelengths. Viability was expressed as the fluorescence ratio of polymer treated wells to vehicle (DMSO) treated control wells.

Cellular uptake

Polymers and carboxyfluorescein were diluted in PBS and 100 µL were added to cells seeded in 96-well plates. The mixture was incubated for 25 minutes at 37 °C in humidified incubators at 5% CO₂. Following incubation, the liquid was aspirated and the cells were washed three times with PBS to remove excess polymers and/or dye. Extracellular fluorescence and any fluorescence signal from dead or damaged cells was quenched by addition of 100 µL/well of 250 µg/mL trypan blue.⁶¹⁻⁶³ Images were acquired with an EVOS® FL Digital Inverted Microscope (Advanced Microscopy Group, Bothell, WA) or with a Cytation™ 5 (BioTek Instruments, Winooski, VT) imaging multi-mode reader. Quantitative signal intensity data for each image were recorded as the mean intensity of pixels in the respective observed wells of the 96 well plates using the Cytation™ 5. The signal intensity is reported in relative intensity units (RIU).

Results and Discussion

Synthesis of highly branched epoxide-containing polymers

A facile, cost-effective synthesis of highly branched cationic polymers as well as establishing potential use as cellular delivery agents was the subject of this work. As previously mentioned, in addition to increased numbers of chain-end functionalities in highly branched polymers, pendant reactive functionalities are useful to include along the interconnected polymer chains. Such moieties allow for the introduction of post-polymerization modifications that are otherwise difficult to incorporate during polymerization due to incompatibility with the reaction mixture components. The epoxide group was chosen as it can undergo a wide variety of chemical transformations that are useful for this purpose.

Inclusion of pendant reactive epoxide groups was undertaken by using glycidyl methacrylate (GMA) as the monomer. The divinyl crosslinker, ethylene glycol dimethacrylate (EGDMA), was then copolymerized with GMA in the presence of a radical initiator, i.e., azobisisobutyronitrile (AIBN), and an efficient chain transfer agent, CBr_4 , to afford a highly branched, epoxide-containing polymer ($\text{hb-polyGMA}^{\text{EST}}\text{-Br}_x$), as shown in Scheme 1. The superscript EST is used to denote the ester groups present at the branching points. By replacing EGDMA with bis(2-methacryloyloxyethyl) disulfide ($(\text{MAOE})_2\text{S}_2$), and introducing an reductively cleavable disulfide group at the branching points of the polymer, a biodegradable analogue of this polymer was also made ($\text{hb-polyGMA}^{\text{DS}}\text{-Br}_x$). The superscript DS stands for branching point disulfide groups. During the syntheses of these polymers, special care was taken to prevent crosslinking of the resulting materials during future storage. Since divinyl crosslinkers were used, a certain number of unreacted pendant vinyl functionalities were inevitably present. To remove these pendant vinyl groups, each polymer that was initially purified by precipitation in diethyl ether and was then treated with a 10 % (v/v) chloroform solution of Br_2 , which added to the double bonds quantitatively. The resulting polymers were finally purified by precipitation in diethyl ether and drying *in vacuo*.

Post-polymerization modifications of epoxides with tertiary amines

As previously mentioned, epoxides are capable of undergoing a number of robust transformations and, when applied to polymer synthesis, can be quite useful in creating new materials with tailored properties. One of the most useful of these transformations is the conversion of an epoxide to a quaternary ammonium salt using a tertiary amine. This produces a moiety that is cationic and hydrophilic by means of both its ionic nature and the presence of a newly formed $-\text{OH}$ group.

Two different tertiary amines were used in pendant epoxide conversions of polyGMA, namely dimethylbutylamine (DMBA) and dimethylethanolamine (DMEA) as shown in Scheme 2. To accomplish this efficiently, both an amine and a proton source are required, where the former is responsible for opening the epoxide ring, and the latter is used to protonate the produced alkoxide anion (capable of opening further epoxide groups), thus preventing crosslinking *in situ*. A mixture of free and protonated amine was made by mixing

amine (1.2 eq. vs. epoxide) and a proton source, e.g., HCl, (1 eq. vs. epoxide). The small excess of free amine opens the epoxide ring, while the protonated amine transfers a proton to the alkoxide anion and is itself converted to free amine, which participates in a reaction with another epoxide group. We recently reported kinetics for reactions between epoxide and amines in linear polyGMA, providing insight as to the efficiency of these reactions.⁴³ Modification of hb-polyGMA^{EST}-Br_x with DMBA was completed at a similar rate to other studied amines, particularly DMEA (Figure 1). The multiple end groups in the branched polymers (originally alkyl bromide) are likely converted to ammonium groups under the reaction conditions, i.e., in the presence of nucleophilic amines, although some HBr elimination leading to the formation of carbon-carbon double bonds is also feasible due to the pronounced basicity of the utilized amines. The analysis of the end groups in the modified polymers by NMR spectroscopy was not performed because of its complexity. This was partially a consequence of the low concentration of end groups, and partially (as mentioned above) due to the presence of various end groups, including some unreacted alkyl bromide chain ends. The end groups in the modified polymers are thus designated with the collective symbol Z in this text. The resulting polymers were purified by precipitation in acetone, dissolving in water, dialysis in water (MWCO = 2,000 Da) to remove any excess amine, and lyophilization.

The choice of tertiary amines used in the described modifications was specific to the biological aspect of this work. DMEA was chosen specifically for its terminal -OH group to enhance water solubility and for its availability as a reactive functional group. By reacting the isothiocyanate group of FITC (employed here as a fluorescent tag for cell-based studies) with the pendant -OH group of a repeat unit, an *O*-thiocarbamate is formed, producing a polymer (polyFLEAmm^{EST}-Z_x) with covalently bound fluorescent groups (Scheme 2). Due to the high fluorescence intensity of the dye and to conserve the polymer characteristics, only 10 mol % of FITC relative the β-hydroxyethylammonium groups was employed.

Cytotoxicity

Previous reports of significant cytotoxicity associated with many polycations^{64, 65} necessitated the evaluation of the synthesized polymers for potential toxicity in living cells. *In vitro* cytotoxicity was assessed in a non-cancerous human cell line, HFL1⁵³, as well as two human cancer cell lines, MDA-MB-231⁵⁴ and DU145TXR⁵⁵ using two different cell viability assays^{56, 57, 60}. A decrease in the number of metabolically active cells due to cytotoxicity of an added compound is thus reflected in the decrease in formazan or resorufin production and therefore reduction of the resultant absorbance or intensity of fluorescence. As shown in Figure 2, 48-hour incubation with the cationic polymers described here did not display significant toxicity, indicated by greater than 90% metabolic activity of the non-cancerous HFL1 cells as determined by the MTT assay (Figure 2A). Little to no toxicity was also observed for the breast cancer cells, MDA-MB-231, at polymer concentrations below 5 µg/mL using the resazurin assay (Figure 2B). The polymer hb-polyHOEAmm^{DS}-Z_x displayed slight toxicity to HFL1 at the relatively high concentration of 25 µg/mL and 50 µg/mL, resulting in 12 % and 23 % decrease in metabolic activity, respectively (Figure 2A). All three polymers displayed slightly increased cytotoxicity to the breast cancer cell line MDA-MB-231 at 25 µg/mL and 50 µg/mL, but did not have significant impact on metabolic

activity at lower concentrations (Figure 2B). In similar experiments with a multidrug resistant prostate cancer cell line, DU145TXR⁵⁵, all three polymers displayed some toxicity at concentrations of 5 µg/mL and 25 µg/mL after a 48-hour incubation as determined by the MTT assay, see Table 1. The nondegradable polymer hb-polyBuAmm^{EST}-Z_x and the reductively degradable hb-polyBuAmm^{DS}-Z_x reduced metabolic activity by 20% and 25% at both 5 µg/mL and 25 µg/mL concentrations. At 5 µg/mL the toxicity of hb-polyHOEAmm^{DS}-Z_x was the same as that of the other polymers (20% reduction in metabolic activity), but at 25 µg/mL, hb-polyHOEAmm^{DS}-Z_x resulted in over 60% reduction in cell metabolic activity.

Cellular uptake of polymers

To determine whether the synthesized polymers are capable of entering living cells, the cellular uptake of an intrinsically fluorescent hb-polyFLEAmm^{EST}-Z_x that resulted from a post-polymerization modification of hb-polyHOEAmm^{EST}-Z_x with the fluorescent dye FITC (Scheme 2) was first investigated. All uptake experiments were performed with incubation times of less than 1 h. Following incubation with 10 µg hb-polyFLEAmm^{EST}-Z_x, the media containing the fluorescent polymer or vehicle (DMSO) control was removed and the wells were washed three times with PBS. Trypan blue solution (100 µL) was then added to each well. Because the absorption spectra of trypan blue overlaps with the green emission spectra of FITC and carboxyfluorescein (below), trypan blue quenches the green fluorescence emitted from any fluorescein moieties in its immediate proximity⁶¹. The plasma membrane of living cells is impermeable to trypan blue and therefore the fluorescence signal of cell-internalized fluorescein fluorophores is preserved. In contrast, the fluorescence of fluorophores that are extracellularly located or localized to dead cells is quenched by trypan blue.^{62, 63}

The appearance of fluorescent cells after incubation with the FITC-labeled polymer hb-polyFLEAmm^{EST}-Z_x (Figure 3A-C) indicated that all three tested human cell lines accumulated the material. For bright field overlaid images, see Supplemental Figure 3A-C. Cells incubated with solvent (DMSO) control (no added polymer) showed no discernable green fluorescence (Supplemental Figure 1A-C), confirming that the fluorescence observed in Figure 3 is not due to autofluorescence of the cell lines.

Application as carriers of negatively charged cargo

The ability of polymers hb-polyBuAmm^{EST}-Z_x, hb-polyHOEAmm^{DS}-Z_x, and hb-polyBuAmm^{DS}-Z_x to interact with negatively charged cargo molecules and bring them inside cells was evaluated next. Carboxyfluorescein is an anionic fluorescent dye that is unable to permeate cellular membranes⁶⁶ and is therefore unable to enter cells on its own. In the experiments presented in Figure 3, DU145TXR had shown the most prominent increase in emission when the cells were incubated with the intrinsically fluorescent polymer hb-polyFLEAmm^{EST}-Z_x (Figure 3C). When the same cells were incubated with 10 µg carboxyfluorescein alone under identical conditions, no significant fluorescence was observed (Figure 4A). Similar experiments using the noncancerous HFL1 and the breast cancer MDA-MB-231 cell lines confirmed that the anionic carboxyfluorescein fluorophore was not internalized to a significant degree by any of the cell lines tested (Supplemental

Figure 2A-C). In the presence of cationic polymers (hb-polyHOEAmm^{DS}-Z_x (Figure 4C) and hb-polyBuAmm^{DS}-Z_x (Figure 4D)), however, a dramatic increase in the number of fluorescing cells was observed. The presence of 10 µg hb-polyBuAmm^{EST}-Z_x and 10 µg carboxyfluorescein (Figure 4B) resulted in only moderate cellular fluorescence, indicative of lower uptake of the dye into the cells. To ensure that the observed fluorescence was the result of cellular uptake of carboxyfluorescein in the presence of the cationic polymers, all images presented were obtained after adding trypan blue to quench extracellular fluorescence.^{61, 62} The GFP overlaid brightfield images are shown in Supplemental Figure 4.

Although all three polymers allowed the cell-impermeable anionic dye, carboxyfluorescein, to be internalized, the addition of the different polymers resulted in different numbers of fluorescent cells and different emission intensities (Figure 4B-D). The use of 5 µg of carboxyfluorescein in the presence of hb-polyBuAmm^{DS}-Z_x resulted in nearly saturated fluorescence signal (Figure 4D). When the amount of carboxyfluorescein was decreased to 2 µg in the presence of hb-polyBuAmm^{DS}-Z_x (Figure 5C), the fluorescence signal was still comparable to that produced in the presence of hb-polyBuAmm^{EST}-Z_x and 10 µg carboxyfluorescein (Figure 4B). Enlarged images, digitally zoomed into the center of the wells are presented in Supplemental Figure 5.

The mean fluorescence intensities of the images acquired when using 10 µg carboxyfluorescein and 10 µg of each polymer were 9,607 for hb-polyBuAmm^{EST}-Z_x (Figure 4B), 18,134 for hb-polyHOEAmm^{DS}-Z_x (Figure 4C), and as much as 32,328 for hb-polyBuAmm^{DS}-Z_x (Figure 4D), compared to only 3,572 for 10 µg carboxyfluorescein alone (Figure 4A). When the amount of carboxyfluorescein was reduced to 2 µg, the mean fluorescence was 3,304 without polymers (5A), 7,557 in the presence of hb-polyHOEAmm^{DS}-Z_x (5B), and 11,434 in the presence of hb-polyBuAmm^{DS}-Z_x (Figure 5C). The fact that the addition of five times the amount of carboxyfluorescein resulted in less than 1.5-fold increase in mean fluorescence, suggests that polymer hb-polyBuAmm^{EST}-Z_x may have been close to saturation in the presence of 2 µg of the anionic cargo.

Alternatively, the produced dye-polymer complex may not be as membrane permeable as that formed at lower anionic dye concentrations. In contrast, hb-polyBuAmm^{DS}-Z_x, which contains the same DMBA-derived moiety as hb-polyBuAmm^{EST}-Z_x but also contains the biodegradable disulfide linkages, exhibited significantly increased mean fluorescence when comparing the addition of 2 µg versus 10 µg carboxyfluorescein as cargo. This suggests that the hb-polyBuAmm^{DS}-Z_x polymer may have greater binding capability for the cargo molecule and/or enhanced cellular uptake characteristics.

Conclusions

Drug delivery has been focus of much research over the last years. Several types of vehicles have been assessed for delivery or targeted delivery of small molecules or nucleic acids into cells, see ^{1-3, 8-10, 67, 68} just to mention a few. Most of these vehicles, although successful to a degree, still have some shortcomings with respect to feasibility of synthesis, leakiness of cargo, immunogenicity, cost of production and shelf-life. The work presented here was intended as a pilot study to test the capability of a new type of potential delivery vehicles in the form of hyperbranched cationic polymers to shuttle anionic small molecule cargo into

cells. The results clearly indicate that the cationic hyperbranched polymers developed in this study are capable of entering cancerous and noncancerous cells in culture while exhibiting limited toxicity even upon extended exposure to the cells. There appears to be increased uptake in the cancer cell lines (Figure 3B-C) compared to the noncancerous cell line (Figure 3A), which warrants further investigation. The study further shows that the cationic nature of the polymers allows for targeted import of otherwise cell-impermeable negatively charged cargo. The biodegradable disulfide linkage resulted in an increase in intracellular carboxyfluorescein fluorescence compared to the corresponding non-degradable (with ester branch point links) polymer hb-polyBuAmm^{EST}-Z_x. It is not clear whether the latter observation is due to decreased cellular uptake of hb-polyBuAmm^{EST}-Z_x or decreased association of hb-polyBuAmm^{EST}-Z_x with anionic cargo, carboxyfluorescein. Our results may open the path for cellular delivery of negatively charged drugs that cannot otherwise enter cells due to lack of import mechanisms. Further studies to test utility of the polymers for biomedical or biological application will be of interest.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evolution of NMR spectra of the reaction between DMBA and hb-polyGMA^{EST}-Br_x in DMSO-d₆ at 60 °C (left). Epoxide protons are denoted by “e.” Kinetics of epoxide transformations using both DMBA and DMEA (right). Data shown for DMEA is for linear polyGMA, adapted from ref ⁴³.

Figure 2.

Cytotoxicity of polymers hb-polyBuAmm^{EST}-Z_x (black squares), hb-polyHOEAmm^{DS}-Z_x (red circle), hb-polyBuAmm^{DS}-Z_x (green up triangle), hb-polyFLEAmm^{EST}-Z_x (blue down triangle) to HFL1 (A) and MDA-MB-231 (B) cells. Data represents the mean $\pm$ SD of at least six replicates per concentration.

Figure 3.

Non-cancerous HFL1 (A), MDA-MB-231 breast cancer (B) and DU145TXR prostate cancer (C) cells incubated with 10 μg hb-polyFLEAmm^{EST}-Z_x, the polymer modified by covalent attachment of the fluorescent dye FITC. Following incubation, liquid was aspirated and wells were washed three times with PBS. Extracellular fluorescence was quenched by addition of 100 μL trypan blue before imaging on an EVOS® FL Digital Inverted Microscope at 20 $\times$ objective magnification and green fluorescence protein (GFP) fluorescence filter.

Figure 4. DU145TXR cells incubated with 10 μg carboxyfluorescein alone (A) or in the presence of 10 μg polymers hb-polyBuAmm^{EST}-Z_x (B), hb-polyHOEAmm^{DS}-Z_x (C), or hb-polyBuAmm^{DS}-Z_x (D). The cells were rinsed with PBS, and extracellular fluorescence was quenched by addition of trypan blue before imaging. Images were acquired on a BioTek Cytation5 at 4× objective magnification. Scale bar represents 1000 μm.

Figure 5.

DU145TXR incubated with 2 μg carboxyfluorescein alone (A) or in the presence of 10 μg hb-polyHOEAmm^{DS}-Z_x (B), or 10 μg hb-polyBuAmm^{DS}-Z_x (C). The cells were rinsed with PBS, and extracellular fluorescence was quenched by addition of trypan blue before imaging. Images were acquired on a BioTek Cytation5 at 4 $\times$ objective magnification. Scale bar represents 1000 μm .

Scheme 1.
Synthesis of highly branched, multibrominated epoxide-containing polymers

Scheme 2.

Post-polymerization modifications of hb-polyGMA-Br_x with DMEA and DMBA and modification of polyHOEAm^{EST}-Z_x with FITC.

Table 1

DU145TXR viability determined by MTT assay after 48 hour incubation with 5 µg/mL or 25 µg/mL experimental polymers. Data represents the mean ± standard deviation from at least 6 data points per concentration.

	DU145TXR Viability		
	<u>hb-polyBuAmm^{EST}-Z_x</u>	<u>hb-polyHOEAmm^{DS}-Z_x</u>	<u>hb-polyBuAmm^{DS}-Z_x</u>
5 µg/mL	82.0% ± 4.5%	77.9% ± 4.9%	78.3% ± 2.9%
25 µg/mL	79.7% ± 2.5%	37.0% ± 3.4%	76.4% ± 1.9%