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Dendrimer-triglycine-EGF nanoparticles for tumor imaging and targeted nucleic acid and drug delivery

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Abstract

We designed an epidermal growth factor (EGF)-containing polyamidoamine (PAMAM) Generation 4 dendrimer vector labeled with quantum dots for targeted imaging and nucleic acid delivery. ¹H-NMR, SDS-PAGE, and western blotting were applied to characterize the synthesized G4.0-GGG-EGF nanoparticles. Targeting efficiency, cell viability, proliferation, and intracellular signal transduction were evaluated using HN12, NIH3T3, and NIH3T3/EGFR cells. We found that EGF-conjugated dendrimers did not stimulate growth of EGFR-expressing cells at the selected concentration. Consistent with this, minimal stimulation of post-receptor signaling pathways was observed. These nanoparticles can localize within cells that express the EGFR in a receptor-dependent manner, whereas uptake into cells lacking the receptor was low. A well characterized vimentin shRNA (shVIM) and siRNA YFP were used to test the delivery and transfection efficiency of the constructed targeted vector. Significant knockdown of expression was observed, indicating that this vector is useful for introduction of nucleic acids or drugs into cells by a receptor-targeted mechanism.

Keywords

dendrimer; EGF; gene delivery; imaging; siRNA; gene knockdown; HNSCC; nanoparticles; quantum dots; RNAi; vimentin; YFP

Introduction

Dendrimers have emerged as the most versatile nanostructured platform for drug delivery because of their well-defined highly branched architecture and numerous surface sites that enable a high drug payload and/or assembly of a variety of functional moieties 1-2. Polycationic

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Conflicts of Interest Statement

None Declared

dendrimers have been extensively studied for gene delivery because they aid efficient internalization of DNA following endocytosis and membrane destabilization, and facilitate escape of gene/dendrimer polyplexes from endosomes and lysosomes as a result of their well-known proton-sponge feature 3^{-10} . Covalent coupling of targeting ligands to the dendrimer is a viable approach to develop efficient targeted therapeutic modalities for drug delivery. Epidermal growth factor receptor (EGFR) overexpression occurs in multiple human solid tumors, including cancers of the head and neck, lung, breast, colon, and brain¹¹. EGF 12 and anti-EGFR antibody such as Cetuximab 13 have been used as targeting ligand to selectively enhance cellular uptake of drug-carrying vehicles by human carcinomas.

EGFR signaling regulates cell growth, survival, differentiation, and motility. Since EGFR-targeted drug delivery systems possibly utilize the ligand-receptor interaction for drug delivery, it is important to determine the cellular response to EGFR ligation by targeted nanoconjugates, to ensure that stimulation of pro-oncogenic properties does not occur. To date, considerable attention has been paid to confirmation of the enhanced uptake of ligand-carrying dendrimers by cells. Nonetheless, subsequent intracellular signal transduction mediated by EGF-conjugated dendrimers and impact on therapeutic efficacy has not been well studied. One report indicated the possibility that use of EGF-conjugated nanoparticles may enhance cell growth¹² whereas, in another study, the authors reported a synergistic growth inhibitory effect on EGFR-overexpressing breast cancer cells by EGF-conjugated polyethylene glycol-poly(ϵ -caprolactone) block copolymer loaded with ellipticine¹⁴. It should also be noted that, while the normal response of keratinocytes to EGF is proliferation, many tumor cells do not display this and may even be growth-inhibited by EGF¹⁵. Thus, biochemical and biological effects may vary, depending upon the reagent used and the nature of the target cell, and should be considered in design of EGFR-targeted vectors. In this study, the synthesis and characterization of EGF-conjugated dendrimers are discussed.

Materials and methods

Materials

PAMAM dendrimer generation 4.0, triglycine (GGG), N-hydroxysuccinimide (NHS), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), N,N'-disuccinimidyl carbonate (DSC), triethylamine (TEA) and vimentin antibody were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human EGF was purchased from Austral Biologicals (San Ramon, CA). Antibodies that recognize EGFR (sc-03), ERK2 (sc-54), p-ERK (sc-101760), phosphotyrosine (sc-508), GFP (sc-9996) and actin (sc-1616) were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Anti-p-AKT (4058) was obtained from Cell Signaling Technology (Danvers, MA). Anti-AKT1 (559028) was purchased from BD Biosciences Pharmingen (Mississauga, ON Canada). Horseradish peroxidase-conjugated secondary antibodies were obtained from MP Biomedicals (Aurora, OH). Qdot® 525 ITK™ amino (PEG) quantum dots were purchased from Invitrogen (Carlsbad, CA). TransIT keratinocyte transfection reagent was obtained from Mirus Bio (Madison, WI). siRNA targeting yellow fluorescent protein (YFP) was purchased from Qiagen (Valencia, CA).

Synthesis of EGF-conjugated dendrimer derivatives

As illustrated in Fig. 1A, the synthesis of EGF-conjugated dendrimers involves two steps—introducing a triglycine spacer to the dendrimer, and coupling EGF to the dendrimer *via* the spacer.

Step 1) Introducing triglycine spacer to the dendrimer—Triglycine was activated into an active ester by using NHS/EDC in 0.1M sodium phosphate buffer (pH5.5), where the feed molar ratio of triglycine: NHS: EDC was 1:1.2:1.2¹⁶. The resulting N-

hydroxysuccinimide (NHS)-activated triglycine (i.e., NHS-GGG) was slowly added to the G4.0 PAMAM dendrimer-containing bicarbonate buffer solution (pH 8.5) and the reaction proceeded for 2h, where the feed molar ratio of NHS-GGG-NH₂/G4.0 was 64:1. The resultant G4.0-GGG was purified by dialysis against deionized water and then lyophilized.

Step 2). Coupling EGF to G4.0-GGG—Recombinant human EGF was activated using NHS/EDC for 15min with a feed molar ratio of 1:2:3 for EGF: NHS: EDC in 0.1M sodium phosphate buffer (pH=5.5). Afterwards, G4.0-GGG-NH₂ was slowly added to the solution for an overnight coupling reaction at ambient temperature, where the feed molar ratio of EGF to G4.0-GGG-NH₂ was 5:1. The resulting G4.0-GGG-EGF was ultrafiltered 4 times using a Centriprep® centrifugal filter unit (30,000 NMWL) (Nominal Molecular Weight Limit), (Millipore, Billerica, MA) and then lyophilized.

Labeling dendrimers with Quantum dots (Qdots)

Qdots were linked to the dendrimer via a long PEG spacer to minimize interference of fluorophores with assembled functional entities on the dendrimer surface. As shown in Fig. 1B, Qdot® 525 ITC™ amino (PEG) quantum dots were coupled to the dendrimer via triglycine using a DSC/TEA coupling method¹⁷, where the feed molar ratio of Qdot to G4.0 PAMAM was 1:1. Briefly, Qdots (1 equivalent) dissolved in DMF were activated by adding DSC (1 equivalent) and TEA (1 equivalent). After an overnight reaction with stirring, the resulting Qdot-NHS was then precipitated with cold ether and vacuum dried. A 2h coupling reaction between Qdot-NHS esters and G4.0-GGG (1 equivalent) or G4.0-GGG-EGF (1 equivalent) was carried out in a pH8.5 bicarbonate buffer solution. The resulting Qdot -labeled G4.0 nanoparticles were purified by dialysis against deionized water and lyophilized.

¹H-NMR spectroscopy

¹H-NMR spectra of the synthesized polymers were recorded on a Varian superconducting Fourier-transform NMR spectrometer (Mercury-300). Deuterium oxide (D₂O, 99.9%) was used as the solvent. The chemical shift for D₂O is 4.8ppm.

SDS-PAGE assay

Tris-glycine-SDS polyacrylamide gel electrophoresis was carried out by standard procedures, using 12% resolving gels.

Cell culture

Culture conditions for HN12, NIH3T3 and NIH3T3/EGFR cells have been described previously^{18–21}.

Immunostaining

Immunofluorescent detection of cellular proteins was carried out as previously described²².

Cell proliferation assays

Measurement of cell growth was carried out by MTT assay and by cell counting assays, as described previously²³.

Western blot analysis

Western blotting of total cellular protein was carried out by standard procedures, as described previously¹⁵.

Nucleic acid delivery

HN12 cells or YFP-expressing HN12 cells were seeded in 6-well culture plates and allowed to proliferate until 40% confluent. To prepare vector/DNA complexes, 5 µg of G4-GGG-EGF, G4, or 2 µL of TransIT were mixed with 2 µg of shVIM plasmid DNA or YFP siRNA in 50 µL H₂O, gently vortexed and allowed to stand at room temperature for 20 min. The solution was centrifuged briefly, plated in duplicate wells, and incubated for 48 h. Vimentin or YFP expression was quantified by western blot.

Statistical analysis

Data analysis was performed using GraphPad Prism v4.00 for Windows (GraphPad Software Inc., San Diego, CA). *P* values < 0.05 were considered statistically significant.

Results and Discussion

Structural characterization of EGF-conjugated dendrimer derivatives

Spectroscopic and bioanalytical assays were applied to characterize dendrimer derivatives. According to the ¹H-NMR spectrum (Supplementary Figure S1), nearly 64 triglycine spacer molecules were conjugated to the dendrimer surface, indicating 100% surface site modification. SDS-PAGE analysis further confirmed that the surface sites were completely modified with triglycine. As shown in Figure 2A, the major band (solid arrowhead) of G4.0-GGG conjugates falls into the range of 25 kDa -37 kDa, as predicted for G4.0 carrying 64 triglycine spacer molecules. In addition, two bands (open arrowheads) above 50 kDa are attributed to the aggregation of G4.0-GGG conjugates. Previously, glutaric anhydride was utilized as a spacer between EGF and dendrimer, which introduced negatively charged carboxylate groups to the dendrimer surface¹². Although this may not be a concern for delivery of drugs through covalent conjugation, the reduction of amine surface groups will diminish the ability of dendrimer-based vectors to complex with nucleic acids, which is based on electrostatic interaction, hence our use of triglycine. The C-terminus of triglycine was used for conjugation with G4.0 PAMAM dendrimers, making available the N-terminus for subsequent coupling with EGF and Qdots, and maintaining sufficient numbers of amine groups on the dendrimer for complexation with nucleic acids. Purity of G4.0-GGG-EGF conjugates was assessed by western blot, which also indicated the molecular weight of G4.0-GGG-EGF conjugates and the number of EGF moieties per dendrimer, together with the ¹H-NMR and SDS-PAGE results. Based on molecular size, it was found that G4.0-GGG-EGF conjugates had an average of one EGF molecule per dendrimer according to our western blot assay (Fig. 2B). These results also demonstrated that the chemical synthesis was robust. Multiple batches of G4.0-GGG-EGF were synthesized. As shown in Fig. 2B, no detectable free EGF band was found in the purified product obtained from two different syntheses, indicating its suitability for use in subsequent studies of cell growth and signal transduction.

Targeting ability of Qdot-labeled EGF-conjugated dendrimers

Overexpression of epidermal growth factor receptor (EGFR) occurs in up to 80% to 90% of HNSCC, of which the EGFR is 38% to 47% overexpressed compared to normal cells^{24,25}. To examine EGFR expression prior to study of targeting ability of EGF-conjugated dendrimers, immunostaining was performed. NIH3T3 fibroblasts express low levels of EGFR (Supplementary Fig. S2A). In contrast, NIH3T3/EGFR cells showed high expression of EGFR at the cell membrane (Supplementary Fig. S2B). The EGFR is mostly localized at the cell surface, but is also internalized into endosomes after ligand binding. EGFR was also found in the cytoplasm and nucleus in the HN12 cells, but with strong immunoreactivity predominantly in the cell membrane (Supplementary Fig. S2C). The levels of EGFR in these cell lines indicate their suitability for use in our study. Since both NIH3T3/EGFR and HN12 cells express a high

level of EGFR, we used them to evaluate the biological properties of the delivery system in order to ascertain its generality in interacting with EGFR-expressing cell lines.

Fluorophores such as FITC can be directly conjugated to amine-terminated dendrimers to facilitate visualization²⁶. Indeed, we have already prepared FITC-labeled dendrimers and evaluated testing toxicity and cellular uptake. However, photostability and loading density can not be controlled well due to the presence of many reactive end groups on the dendrimer surface. Since our G4.0-GGG-EGF conjugates are intended for gene delivery, direct coupling of fluorophores to the dendrimer may impair the ability of dendrimer to complex with nucleic acids. Therefore, we used Qdots to provide long-term photostability to EGF-conjugated dendrimers, which can be potentially used for targeted live-cell imaging and dynamics studies^{27,28}.

According to our previous report, N-terminal conjugation of triglycine to G4.0 significantly reduced dendrimer toxicity¹⁶. Here, the number of amine surface groups remained unchanged after C-terminal triglycine conjugation. The toxicity of G4.0 modified with triglycine was similar to that of unmodified G4.0. Because of the negligible toxicity of dendrimers at concentrations of 0.2 μ M or below as identified, we evaluated the synthesized conjugates at or below 0.2 μ M to exclude potential toxic effects.

Receptor-mediated uptake of EGF-conjugated dendrimers was demonstrated using HN12 and NIH3T3/EGFR cells, using Qdot-labeled dendrimer-EGF conjugates. We chose the concentration of EGF from our previous studies which is optimal for inducing a downstream signaling response in the cells used^{15,29}. As shown in Fig. 3A, EGF-conjugated dendrimers were efficiently taken up into EGFR-expressing cells, whereas uptake by NIH3T3 controls was minimal. Further experiments compared the effect of the EGF moiety on internalization. NIH3T3/EGFR cells were incubated in the presence of Qdot-G4.0-GGG-EGF, or Qdot-G4.0-GGG, and Fig. 3B shows that Qdot-labeled EGF-conjugated dendrimers are detectable in NIH3T3/EGFR cells within 1h, and this becomes more profound with a longer incubation period (14h). In contrast, uptake of Qdot-labeled dendrimers lacking EGF is minimal. Similar data were obtained with HN12 cells (Supplementary Fig. S3). These data suggest that EGF-conjugated dendrimers can be taken up efficiently by cells in an EGFR-dependent manner. In addition, the contrast between EGFR-positive and -negative cells strongly indicates that the EGF conjugated to the nanoparticles is active in terms of receptor binding ability. Since EGF retained its targeting ability after conjugation, the chemistry employed was satisfactory.

Effect of EGF-conjugated dendrimers on cell proliferation and post-receptor signaling

As EGF is a potent mitogen for normal and some transformed epithelial cells, we determined the effect of EGF-conjugated dendrimers on cell proliferation. Cells were seeded in 12-well culture plates, serum-starved and exposed to equimolar amounts of G4.0-GGG, G4.0-GGG-EGF, or EGF as control. After three days, viability was determined by MTT assay. Fig. 4A shows combined results from four experiments run in triplicate using HN12 cells. No significant difference was observed between cells treated with EGF-conjugated or unconjugated dendrimers ($p = 0.62-0.99$).

To determine possible biochemical events that occur in cells exposed to EGF-conjugated dendrimers, we treated cells as above, prepared protein lysates and western blotted these with antibodies that recognize phosphorylated (active) forms of ERK and AKT, protein kinases that are activated in a wide range of cells following EGFR ligation. Robust phosphorylation (activation) of ERK was found in EGF-treated cells, as predicted (Fig. 4B), whereas the EGF-conjugated dendrimer induced minimal ERK activation. Similar observations were made in NIH3T3/EGFR cells (Supplementary Fig. S4). Taken together, these data suggest that the EGF-

conjugated dendrimers used in these studies do not activate common proliferation-associated signaling pathways in EGFR-expressing cells, consistent with the results of cell growth assays.

Nucleic acid delivery

The efficiency of EGF-conjugated dendrimers to deliver nucleic acids was tested by examining RNA interference-mediated inhibition of vimentin and YFP. To exclude any potential interference of Qdots with nucleic acid delivery, EGF-conjugated dendrimers without Qdots were used. A previously used vimentin shRNA (shVIM) plasmid²² or YFP siRNA were used to transiently transfect HN12 cells or YFP-expressing HN12 cells, respectively. As indicated in Fig. 5A, vimentin expression was moderately reduced by 20% or 23% in HN12 cells treated with shVIM delivered by unconjugated dendrimer or TransIT. However, a significant 40% reduction in vimentin expression was found in HN12 cells transfected with shVIM delivered by EGF-conjugated dendrimers (Fig. 5A). An appreciable reduction (70%) of YFP expression mediated by G4.0-GGG-EGF delivery of siRNA was observed in HN12/YFP cells (Fig. 5B). This confirms enhanced nucleic acid delivery by EGF-conjugated dendrimers and suggests that EGFR-targeted delivery may be a viable approach for efficient delivery of nucleic acids such as shRNA plasmids and siRNA.

In summary, we have developed EGF-conjugated dendrimer nanoparticles, using a triglycine spacer for conjugation of EGF. These nanoparticles were further labeled with Qdots to afford a targeted imaging modality. They localized intracellularly in an EGFR-dependent manner, whereas uptake into cells lacking the receptor was low. EGF-conjugated dendrimers did not stimulate growth of EGFR-expressing cells, and minimal stimulation of post-receptor signaling pathways was observed. The efficiency of the constructed targeted delivery system was demonstrated through the delivery of vimentin shRNA plasmid and YFP siRNA. The data indicate that this may be a useful nanoscale vector for introduction of nucleic acids or drugs into cells by a growth factor-targeted mechanism, and for targeted cell imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A **B** **Fig.1. Synthetic Schemes**

A. Synthesis of EGF-triglycine-dendrimer conjugates. **B.** Labeling EGF-triglycine-dendrimer conjugates with Qdots coated with amine-derivatized PEG.

Fig.2.

A. *SDS-PAGE analysis of PAMAM dendrimer conjugates* G4.0-GGG conjugates were applied to 12% SDS-polyacrylamide gel, resolved and stained with Coomassie R-250. Molecular size markers are indicated. **B.** *Western blot analysis of EGF-conjugated dendrimers.* G4.0-GGG-EGF nanoparticles were electrophoresed, together with the indicated amounts of recombinant EGF standards, then western blotted and probed with anti-EGF antibody. #1, first synthesis; #2, second synthesis.

A

Fig.3. EGFR-dependent uptake of Qdot-labeled PAMAM dendrimer G4.0 derivatives

A. NIH3T3/EGFR cells, or NIH3T3 as control, were exposed to G4.0-GGG-EGF nanoparticles for 24h, then fixed, counterstained with DAPI and imaged as described in Methods. **B.** NIH3T3/EGFR cells were exposed to Qdot-G4.0-GGG (Q-G4) or Qdot-G4.0-GGG-EGF (Q-G4-E) nanoparticles for the indicated times, then processed as in (A). Original magnification, $\times 400$.

Fig.4. EGF-dendrimer conjugates show minimal effect on cell proliferation

A. HN12 cells were cultured in triplicate in the presence of the indicated compounds and the number of viable cells determined by MTT assay. Bar=SD. **B.** HN12 cells were serum starved, then treated with G4.0-GGG, G4.0-GGG-EGF, or EGF as a control. Total cellular protein lysates were prepared and western blotted with the indicated antibodies.

Fig.5. RNAi-mediated gene knockdown using EGF-conjugated dendrimers

A. HN12 cells plated in 6-well plates at 40% confluence were treated with 2 μ g of shVIM plasmid DNA complexed with G4.0-GGG-EGF (5 μ g), G4.0 (5 μ g), TransIT (2 μ L), or untreated. Vimentin expression was quantified by using Quantity One (Bio-Rad) and normalized to actin of each group. Vimentin expression was then expressed with vimentin expression in untreated cells being 100%. **B.** YFP-expressing HN12 cells plated in 6-well plates at 40% confluence were untreated, treated with 2 μ g of YFP siRNA complexed with 2 μ L of TransIT, 5 μ g of G4.0, or 5 μ g of G4.0-GGG-EGF. Proteins were extracted, western blotted with the indicated antibodies and YFP expression calculated relative to actin as an internal standard.