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Synthesis and in vitro Characterization of a Dendrimer-MORF Conjugate for Amplification Pretargeting

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Abstract

Amplification pretargeting can play an important role in molecular imaging by significantly increasing the accumulation of signal in target tissues. Multiple-step amplification pretargeting offers the potential to greatly improve target localization of effector molecules through the intermediate use of polymers conjugated with multiple copies of complementary oligomers. In this study, PAMAM dendrimer generation 3 (G3) was conjugated with multiple copies of a phosphorodiamidate morpholino (MORF) oligomer. Characterization of the conjugation by native-PAGE and SE-HPLC demonstrated that the conjugation was successful. The average numbers of MORF groups in the G3-MORF conjugate, both the attached and the accessible to the ^{99m}Tc labeled complementary MORF (cMORF), were determined. The antitumor antibody CC49 was conjugated with both MORF and cMORF (collectively (c)MORF) at an average of about one group per molecule. Nine of the 32 carboxyl groups of the dendrimer were modified with MORF, of which 90% percent were accessible in solution to ^{99m}Tc -cMORF. After purification, the G3-MORF was radiolabeled with tracer ^{99m}Tc -labeled cMORF (i.e. G3-MORF/ ^{99m}Tc -cMORF) and added to the antibody CC49 previously conjugated with cMORF (i.e. CC49-cMORF/G3-MORF/ ^{99m}Tc -cMORF), the complex demonstrated a single peak on SE-HPLC as evidence of complete hybridization between G3-MORF/ ^{99m}Tc -cMORF and CC49-cMORF. The CC49-(c)MORF were bound to both Protein G and Protein L coated plates, and G3-MORF was added to hybridize with CC49-cMORF before the ^{99m}Tc -cMORF was added to test amplification pretargeting. Compared to conventional pretargeting without the G3-MORF, the signal was amplified about 6 and 14 times respectively, showing that the G3-MORF participated in amplifying the signal. Further amplification studies using the CC49-(c)MORF for LS174T tumor cell in tissue culture also demonstrated clear evidence of signal amplification.

Keywords

Dendrimer; Amplification; Pretargeting

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INTRODUCTION

Conventional nuclear medicine imaging with radiolabeled tumor specific agents such as antitumor antibodies can provide high tumor/nontarget ratios but usually with slow signal localization and clearance. Pretargeting is one approach that provides useful tumor/nontarget radioactivity ratios more rapidly by placing the effector carrying the radioactivity or other label on a small molecule designed to clear from the circulation and whole body rapidly (1). Pretargeting requires at least two steps in which the targeting macromolecule, usually an antibody, is administered first followed by the radioactive effector. The pretargeting approaches that have been reported thus far use (strep)avidin, bispecific antibodies, or oligonucleotides (2–5). One advantage of (strep)avidin for pretargeting is the fourfold valency of this protein for biotin, providing the potential of modest signal amplification (2). However, a polymer conjugate with multiple copies of oligomers such as peptide nucleic acids (PNAs) or phosphorodiamidate morpholinos (MORFs), administered intermediately between the antitumor antibody and the small effector, provides a potential for amplification far in excess of four (6–8). Compared to conventional pretargeting, the three-step amplification pretargeting approach is obviously more complex, but with the potential to greatly increase the localization of radioactivity in the target. Multivalent bispecific antibody and enzyme catalytic system have also been considered for signal amplification (9,10).

The choice of polymer is critical for successful amplification pretargeting. It should be large enough to carry sufficient numbers of oligomers; after conjugation it should be water soluble and stable in vivo; it should have favorable pharmacokinetics; and the oligomers must be arranged on the polymer in such a way that they can be easily accessed by their effector. In our previous amplification pretargeting studies, polylysine (PL) and poly(methyl vinyl ether-alt-maleic acid) (PA) and other linear polymers provided lower amplification factors than expected. However, the concept has now been shown to be feasible (6–8), although further studies for optimization are required.

Recently, there has been interest in exploring dendrimers as potential drug delivery vehicles (11–19). Dendrimers are branched polymers with highly reactive pendant functional groups (Figure 1) that can be used for covalent conjugation of drugs, ligands, and antibodies for targeted delivery (20–34). Unlike the linear PA and PL polymers, steric hindrances diminishing the accessibility of the conjugated oligomers to their complements should be minimal in the case of dendrimers because of their spherical geometry. The goal of this investigation was to use a dendrimer to achieve a higher degree of amplification pretargeting. As the first step towards this goal, a small dendrimer, generation 3 (G3) with 32 carboxyl groups on its surface, was conjugated with MORF and the in vitro properties of the conjugated polymer evaluated for amplification pretargeting.

EXPERIMENTAL PROCEDURES

Materials and Instruments

The 18-mer MORF and its complement, cMORF (collectively (c)MORF), were purchased from Gene-Tools (Philomath, OR) with 3'-amine modification and with the same base sequences used in this laboratory in connection with conventional pretargeting (35,36). The base sequences and molecular weights were: MORF: 5'-TCTTCTACTTCACAACTA-3-C-linker-amine, 6,059 Da; cMORF: 5'-TAGTTGTGAAGTAGAAGA-3-C-linker-amine, 6,317 Da. The cDNA phosphorothioate oligomer was purchased from IDT (Coralville, IA). PAMAM-succinamic acid dendrimer generation 3 (G3), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDC-methiodide) and anti-mouse F(ab')₂ fragment-R-phycoerythrin antibody were purchased from Sigma-Aldrich (St. Louis, MO). *N*-hydroxysuccinimide *S*-acetylmercaptoacetyltryglycine (NHS-MAG₃) was synthesized in

house (37) and its structure was confirmed by elemental analysis, proton magnetic resonance, and mass spectrometry. The human colon cancer cell line (LS174T) was obtained from the American Type Culture Collection (Manassas, VA). Microcon YM-30 centrifugal filter devices were purchased from Millipore Corporation (Bedford, MA). Tris-glycine gradient precast polyacrylamide gels (4–20%) were purchased from Bio-Rad Laboratories (Hercules, CA). The antiTAG-72 IgG antibody CC49 was prepared by Strategic Biosolutions (Newark, DE) from the CC49 murine hybridoma cell line, a gift from Jeffery Schlom PhD (Laboratory of Tumor Immunology and Biology, Center for Cancer Research, NCI, NIH, Bethesda, MD). The Hydralink kit was purchased from Solulink (San Diego, CA). The P-4 resin (Bio-Gel P-4, medium) was purchased from Bio-Rad and the Sephadex G-100 resin from Pharmacia Biotech (Piscataway, NJ). Protein G and Protein L coated plates were obtained from Pierce (Rockford, IL). All other chemicals were reagent grade and were used without purification. The ^{99m}Tc -pertechnetate was eluted from a ^{99}Mo - ^{99m}Tc generator (Bristol-Myers Squibb Medical Imaging, N. Billerica, MA). SE-HPLC system is consisting of one Waters 515 pump connected with one 2487 dual λ absorbance detector and one radioactivity detector. A Superose 12/30 HR column was purchased from GE Healthcare (Piscataway, NJ) and mobile phase is 0.2 M phosphate buffer (pH 7.0).

Preparation of ^{99m}Tc -labeled cMORF

The conjugation of cMORF with MAG_3 and subsequent labeling was as previously described (38). Briefly, the cMORF was first conjugated with S-acetyl NHS- MAG_3 via the 3'-derivatized amine in pH 8.0 HEPES buffer. After purification on a P-4 column with NH_4OAc buffer, the peak fractions were pooled and underwent a preliminary purification procedure consisting of the labeling procedure described below but without radioactivity followed by another P-4 column purification. For radiolabeling, 10 μL of ^{99m}Tc -pertechnetate generator eluate was introduced into solution consisting of 3 μL (0.88 μg) of MAG_3 -cMORF in pH 5.2 NH_4OAc buffer, 4 μL of 50 $\mu\text{g}/\mu\text{L}$ sodium tartrate in a pH 9.2 buffer, and 1.5 μL of 4 $\mu\text{g}/\mu\text{L}$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in ascorbate-HCl solution (1 $\mu\text{g}/\mu\text{L}$ sodium ascorbate in 10 mM HCl). The solution was heated to 100 $^\circ\text{C}$ for 20 min. The labeling efficiency was routinely greater than 95% as determined by SE-HPLC.

G3-MORF Conjugation

To 2.4 mg of EDC-methiodide (8.1 μmol) was added 10 μL of 1% PAMAM-succinamic acid dendrimer G3 (10 nmol). The mixture was gently vortexed at ambient temperature for 2 min and a 1 nmol/ μL solution of MORF in distilled water was added at five different MORF/dendrimer molar ratios. Each conjugation mixture was incubated at ambient temperature overnight.

Nonreducing polyacrylamide gel electrophoresis (PAGE) conditions similar to those described elsewhere for analysis of dendrimers were used for the analysis of G3-MORF polymers (31, 39,40). The free MORF, free dendrimer, and one of the G3-MORF conjugation reaction mixtures (prepared at a 16:1, MORF/dendrimer molar ratio) before purification were analyzed using a 4–20% Tris-glycine gradient gel under native conditions. The running buffer was 2.5 mM Tris and 19.2 mM glycine at pH 8.2. Since MORF is uncharged, negatively charged complementary DNA (cDNA) was added to form a charged duplex that would migrate in the gel and also can be stained with ethidium bromide. Ten microliters of each of the three samples were mixed with 10 μL PBS containing equal molar cDNA at room temperature for 20 min. To each mixture, 10 μL of 50% sucrose and 1% bromophenol blue was added. The gel was run at low voltage (80 V) on a vertical gel electrophoresis system and then stained with ethidium bromide solution (0.5 $\mu\text{g}/\text{mL}$) at ambient temperature in the dark. After staining, the gel was first imaged with UV light on a Kodak Imaging Station 440CF (Eastman Kodak Co., New Haven, CT). The gel was then stained with 2.5% Coomassie Blue-R250 solution in 10% acetic

acid and 50% methanol at room temperature for 2 h and destained in 10% acetic acid with 50% methanol for 4 h at room temperature and then in water over night.

The absorbance at 265 nm of an aliquot of the conjugation reaction mixture before purification was used to determine the average number of MORFs bonded to each dendrimer (i.e., groups of MORF per conjugate molecule (GPM)) by SE-HPLC analysis. Since the dendrimer does not appreciably absorb in the UV at 265 nm, unlike MORF, the UV peak areas of free MORF and the MORF coupled dendrimer were used to calculate the attached GPM. Since GPM accessible to radiolabeled cMORF is a more important parameter than the attached GPM, the average number of MORFs accessible in solution was determined by a SE-HPLC shift assay in which ^{99m}Tc -cMORF at trace concentration was added to the conjugation reaction mixture before purification. The hybridization was carried out for 20 min at room temperature followed by SE-HPLC analysis to measure the radioactivity bound to free MORF and that bound to the G3-MORF. The areas of G3-MORF/ ^{99m}Tc -cMORF and MORF/ ^{99m}Tc -cMORF peaks on the radioactivity trace were compared with the corresponding peaks on the UV trace. Since accessibility of the ^{99m}Tc -cMORF to free MORF is assumed to be 100%, the identical ratio of G3-MORF to free MORF by radioactivity and UV detection would imply that the accessibility of the ^{99m}Tc -cMORF to G3-MORF was also 100%.

Since both the excess free MORF (MW 6 KDa) and coupling reagent EDC-methiodide (MW < 400 Da) are low molecule weight, they could be removed from the high molecular weight product by filtration (MWCO 30 KDa). The reaction mixture was repeatedly centrifuged using the Microcon YM-30 centrifugal filter devices to near dryness with water added between centrifugations until there was no evidence of free MORF or coupling agents by SE-HPLC. Stability of the conjugation mixture at room temperature in water was measured by monitoring the UV signal on SE-HPLC for more than one week.

Preparation of CC49-(c)MORF

The CC49 antibody was conjugated with either MORF or cMORF using a commercial Hydralink linker following the instruction of the manufacturer and experiences in this laboratory (41). Briefly, the antibody was first modified with succinimidyl 4-hydrazinonicotinate acetone hydrazone, while the (c)MORF was modified with succinimidyl 4-formylbenzoate. After purification of the conjugates, combining the hydrazine-modified antibody and the benzaldehyde-modified (c)MORF resulted in hydrazone formation. Purification of the antibody-(c)MORF from the free (c)MORF was achieved on Sephadex G-100 1×30 cm Econo-column using 100 mM PBS (pH 7.2) as eluent. The attached GPM was determined by SE-HPLC using the UV signal at both 265 and 280 nm. Under the assumption that the absorption coefficients of CC49 (280 nm) and (c)MORF (265 nm) do not change after conjugation, the concentration of CC49 and the (c)MORF GPM was calculated as described previously (41,42).

Preserved immunoreactivities of CC49-(c)MORF were confirmed by flow cytometry with native CC49 as the positive control and the incubation medium as the negative control. Briefly, 2×10^5 LS174T cells in 100 μL of PBS were incubated with each antibody (3 μg) at room temperature for 30 min in the dark. The cells were washed three times using PBS, then gently resuspended in ice cold PBS. And phycoerythrin labeled secondary antibody, sheep anti-mouse F(ab')_2 , in 100 μL of 3% BSA/PBS was added. After 30 min incubation at room temperature in the dark, the cells were washed with PBS three times and resuspended in ice cold 3% BSA/PBS for analysis of immunoreactive fraction on a Becton-Dickinson FACSCalibur system (San Jose, CA). Background fluorescence was measured on a set of cells treated with secondary antibody alone. The immunoreactive fraction was used here to refer to percentage of immunoreactivity retained after conjugation compared with unmodified antibody. The

percentage of fluorescence shift in the FACS study was used as an index to calculate the immunoreactive fraction.

Hybridization in Solution

To confirm that the G3-MORF can link the CC49-cMORF and ^{99m}Tc -cMORF together in solution, the G3-MORF was first labeled by hybridization with tracer ^{99m}Tc -cMORF. The G3-MORF (4.75 μg , 2.5 μL) was mixed with 5 μL of ^{99m}Tc -cMORF (0.13 μg , 82.5 μCi) at room temperature and, after 30 min, 2 μL of the mixture was added to 40 μL of CC49-cMORF (4.3 μg). After a further 30 min, the mixture was analyzed by SE-HPLC by monitoring both radioactivity and UV signals.

Signal Amplification on Plates

The experimental procedure is outlined in Table 1. Both the CC49-(c)MORF antibodies were first immobilized on both the Protein G and Protein L coated 96-well flat-bottomed plates, via their Fc and VL regions respectively, according to the manufacturer's instructions and the plates were rinsed three times using 200 μL of washing buffer (0.1 M PBS, pH 7.2, with 0.05% Tween-20). To two sets of wells was added 100 μL of each CC49-(c)MORF solution (about 3.3 $\mu\text{g}/\text{mL}$) containing the same amount of (c)MORF (2 pmol). As controls, to an equal number of wells only PBS was added in place of the antibodies. The plates were incubated for 60 min at room temperature and then rinsed with 3×200 μL of washing buffer. To the wells containing CC49-cMORF and one set of PBS control wells, 100 μL of G3-MORF (2.4 $\mu\text{g}/\text{mL}$ in PBS, pH 7.2, containing 1% BSA) was added. The plates were incubated for 30 min at room temperature and then rinsed with 3×200 μL of washing buffer. To each well, 50 μL of ^{99m}Tc -cMORF (2 μCi in PBS, pH 7.2, containing 1% BSA) was added. The plates were incubated for another 30 min at room temperature and the free ^{99m}Tc -cMORF was removed with 3×200 μL of washing buffer. The radioactivity bound to the plates was removed with 2×200 μL of 1% sodium dodecyl sulfate (SDS) in 0.2 N of NaOH and 200 μL of 2 M hydrochloride for counting in a NaI(Tl) well counter. To the wells containing CC49-MORF rather than CC49-cMORF and one set of PBS control wells was added 100 μL of PBS (pH 7.2, containing 1% BSA). The plates were incubated for 30 min at room temperature before the wells were rinsed with 3×200 μL of washing buffer. To each well 50 μL of ^{99m}Tc -cMORF was added and the studies performed as described above. The amplification factor (AF) was calculated from the percentage of bound radioactivity removed from the wells of all four studies. $\text{AF} = (\text{Amplification pretargeting} - \text{Nonspecific amplification}) / (\text{Conventional pretargeting} - \text{Nonspecific pretargeting})$. An identical study was performed on the Protein L coated plate.

Signal Amplification on Cells

The cell amplification pretargeting was performed similarly to that described in Table 1 for amplification pretargeting on the Protein G and Protein L coated plates. Cells were suspended in Minimum Essential Medium (MEM) with 10% FBS and were seeded in 12-well flat-bottomed plates at 1 mL/well. The cells were used when 80% confluence was reached and the medium was changed to MEM with 1% FBS. Firstly, 100 μL of CC49-(c)MORF solution (about 3.3 $\mu\text{g}/\text{mL}$) containing the same amount of (c)MORF (2 pmol) was added to each well in the two groups followed by incubation at 4 $^{\circ}\text{C}$ or 37 $^{\circ}\text{C}$ for 1 hour (the 4 $^{\circ}\text{C}$ incubations were included to evaluate the influence of internalization). Thereafter, 100 μL of G3-MORF (2.4 $\mu\text{g}/\text{mL}$ in PBS, pH 7.2, containing 1% BSA) was added to the CC49-cMORF wells and to one set of PBS control wells; 100 μL of PBS without G3-MORF was added to the CC49-MORF wells and one set of PBS control wells. After incubation for 30 min, the cells were rinsed with 2×0.5 mL of MEM with 1% FBS and 2 μCi of ^{99m}Tc -cMORF in 50 μL of PBS (containing 1% BSA) was added to each well followed by 30-min incubation. The cell medium was then carefully removed, and the cells were rinsed with 2×0.5 mL with cold PBS. The

cells were then lysed with 1% sodium dodecyl sulfate (SDS) in 0.2 N of NaOH, and the radioactivity in the medium and cells was measured in an auto gamma counter.

RESULTS

G3-MORF Conjugation

In the SE-HPLC UV traces of G3-MORF before purification shown in Figure 2, in each case, the peak at early retention time (18.9–22.9 min) is due to the conjugated G3-MORF while the peak appearing later is due to free MORF. The conjugate prepared at higher molar ratio demonstrated shorter retention time on the SE-HPLC indicating higher molecular weight. Since these traces were obtained before purification, the ratio of peak areas is a measure of the conjugation efficiency (i.e. GPM). As shown, the average GPM increased with increasing molar ratio of MORF to dendrimer as expected. However, molar ratios higher than 16:1 did not further increase the average GPM (data not shown). The possibility that the MORF was bound to the dendrimer noncovalently even in part was excluded by the results of an identical study without coupling agent under the same conditions that demonstrated only individual peaks corresponding to unconjugated dendrimer and free MORF. The conjugate was stable at room temperature for at least one week as monitored by SE-HPLC.

As shown in Figure 3, one conjugated dendrimer (prepared at a 16:1 MORF/dendrimer molar ratio) was also analyzed by native PAGE following the addition of cDNA and staining with Coomassie Blue (panel A) or ethidium bromide (panel B). The migration of the conjugated dendrimer (lanes 2) was distinctly slowed compared to that of the free dendrimer (lanes 1) indicating a higher molecular weight after conjugation with MORF. The somewhat broad band for the G3-MORF indicates that the conjugate consisted of several G3-MORFs with different GPMs. Both results also demonstrate that there was no unreacted dendrimer left after conjugation. The multiple bands for the free MORF (lane 3) may indicate impurities in the commercial product.

Analysis of the conjugation mixture prepared at the 16:1 molar ratio on SE-HPLC is shown in Figure 4. UV chromatogram (top trace) indicates that this G3-MORF contained an average of 9 GPM. Since there are 32 carboxyl groups on the surface of the G3 dendrimer, the average conjugation efficiency was 28%. After adding trace level of ^{99m}Tc -cMORF to the conjugation mixture, the ratio of radioactivity peak intensity of G3-MORF/ ^{99m}Tc -cMORF to MORF/ ^{99m}Tc -cMORF was compared with that of the UV trace. Since the accessibility of the ^{99m}Tc -cMORF tracer to free MORF may be assumed to be 100%, the comparison shows that about 90% of the MORF groups on the dendrimer formed a duplex with ^{99m}Tc -cMORF. Thus about 8 of the 9 MORF groups in the polymer were accessible to ^{99m}Tc -cMORF in solution.

Preparation of CC49-(c)MORF

The CC49-(c)MORF was successfully synthesized with an average GPM in the range of 1.0–1.3 by the method described previously (41). The flow cytometry results of Figure 5 show that both the CC49-MORF and CC49-cMORF retained more than 90% of the immunoreactive fraction of the native CC49.

Hybridization in Solution

The SE-HPLC trace for the CC49-cMORF/G3-MORF/ ^{99m}Tc -cMORF complex prepared by mixing the ^{99m}Tc -cMORF tracer with the G3-MORF followed by hybridization to the CC49-cMORF is shown in Figure 6. The radioactivity profile shows only a single peak with a high molecular weight retention time of 16.8 min and no evidence of free G3-MORF/ ^{99m}Tc -cMORF signifying complete hybridization of the G3-MORF/ ^{99m}Tc -cMORF to the CC49-cMORF

antibody. The UV profile also shows only a single peak with the identical 16.8 min retention time (data not presented) and therefore provides further evidence of complete hybridization.

Signal Amplification on Plates

Figure 7 presents histograms showing the percentage of radioactivity bound to wells used for amplification pretargeting and conventional pretargeting on a Protein G coated plate (panel A) and Protein L coated plate (panel B) as described above.

As shown in Figure 7, on Protein G, the amplification factor was about 6 while on Protein L this factor was about 14. The differences between amplification and controls in both cases are significant (Student's *t*-Test, $P < 0.05$). In both cases the G3-MORF therefore played an important role in the accumulation of radioactivity.

Signal Amplification on Cells

The results of the cell signal amplification studies are shown in Figure 8 for incubations at both 4 °C and 37 °C. Cell accumulations of ^{99m}Tc -cMORF by amplification pretargeting was significantly higher (Student's *t*-Test, $P < 0.05$) at both temperatures by up to 2.5 times that of conventional pretargeting without the G3-MORF. The percentage of radioactivity increased in all study groups except for amplification pretargeting when incubation temperature was increased to 37 °C.

Discussion

In this investigation, a generation 3 PAMAM dendrimer was conjugated with multiple copies of MORF and used in combination with the CC49 antibody conjugated with cMORF and with the cMORF labeled with ^{99m}Tc for in vitro amplification pretargeting. For this investigation, the PAMAM dendrimer, anionic at neutral pH, was selected because anionic dendrimers have shown lower toxicity and a longer clearance time from circulation than cationic dendrimers (43–46) and because the carboxyl groups may be readily modified with amine-modified MORF using carbodiimides. Furthermore, PAMAM dendrimers are commercially available in a variety of generations. In addition, the spherical geometry of dendrimers may decrease steric hindrances compared to linear polymers. In this investigation, we determined that increasing the conjugation molar ratio of MORF to dendrimer beyond 16:1 did not increase the number of attached GPM, possibly due to steric hindrance. The maximum average number of MORF that was attached (i.e. GPM) was 9 under the conditions of this investigation. Higher generation dendrimers may therefore be required if higher GPM values are needed. Adding *N*-hydroxysulfosuccinimide (Sulfo-NHS) to improve the degree of conjugation was unsuccessful.

The conjugation was confirmed by PAGE analysis. As expected, the broad band on native PAGE in Figure 3 indicates that the G3-MORF used in this investigation consisted of conjugates with a range of GPM with an average of 9. Analysis of the dendrimer after conjugation but before purification, when stained with ethidium bromide, provided a G3-MORF band that is much brighter than that of the unconjugated MORF which suggests a high degree of conjugation. The average molecular weight of G3-MORF was about 64 KDa or about 7.2 KDa per MORF, and therefore lower than that of the PA (9.1 KDa/MORF) and PL (10.7 KDa/MORF) polymers studied previously. Although the experimental protocols were very different, that the spherical dendrimer demonstrated higher conjugation efficiency (28%) than the linear PA (19%) or PL (11%) polymers supports a suggestion that the spherical geometry of the dendrimer may be helpful in reducing steric hindrances. Possibly for the same reason, accessibility of the MORF in the G3-MORF conjugate was higher at 90% compared to 50–60% for PL-MORF and PA-MORF conjugate (7). As shown in Figure 4, when added at a 1:1 molar ratio, the ^{99m}Tc -cMORF completely hybridized to the G3-MORF and MORF mixture

and the G3-MORF completely hybridized to the CC49-cMORF as shown in Figure 6 demonstrating uninhibited accessibility in solution in both cases.

The results presented in Figure 7 clearly demonstrates that the G3-MORF participated in signal amplification on both Protein G and Protein L coated plates in that significantly more ^{99m}Tc -cMORF effector accumulated in the amplification studies compared with the conventional pretargeting studies and provided an amplification factor of about 14 in the case of the Protein L coated plate. Because the molecular volume of G3-MORF is fairly large, steric hindrance may restrict its hybridization to CC49-cMORF. That the CC49 antibody was immobilized on Protein G and Protein L coated plates via Fc and VL regions respectively should result in different orientations of the bound antibodies, which in turn could expose different aspects of the antibody-bound cMORF for G3-MORF binding and may explain the differences in amplification between the two plates.

While the results of comparable studies in cell culture showed that signal was also amplified, the amplification factor was not as high as that on the plates. Studies subsequent to those reported herein are suggesting that accessibility of the cMORF on antibody to G3-MORF after binding to the antigen may be restricted on the cell membrane compared to the accessibility on either Protein G or Protein L on the plate although further studies will be required to establish the reason for this observation. As shown in Figure 8, performing the amplification pretargeting at 4 °C to eliminate any influence of internalization made no significant difference to suggest that internalization was not a significant factor in this study.

Measurements of specific binding to plates or cells in wells are usually complicated by the unavoidable contribution of nonspecific binding. It was for this reason that multiple controls were necessary in this investigation. Thus in the plate study, the nonspecific amplification binding of the ^{99m}Tc -cMORF effector to the intermediate G3-MORF was evaluated by leaving out the CC49-cMORF and the nonspecific conventional pretargeting binding of the ^{99m}Tc -cMORF to the well surface was evaluated by leaving out the G3-MORF and the antibody, as described in Table 1. Fortunately as shown in Figure 7, the nonspecific binding of the G3-MORF in the amplification pretargeting to both Protein G and Protein L coated plates was much lower than the amplification binding. As shown in Figure 8, the identical controls were used in the cell study. However, the G3-MORF did not increase the nonspecific binding on cells.

To exclude the possibility that the differences in amplification factors between the plate and cell well studies may be due to the different media used, an amplification pretargeting was performed on Protein L coated plate as before but using the cell culture medium as in the cell study. Thus one additional control group consisted of L-coated plates incubated with the CC49-MORF was used instead of CC49-cMORF as the first incubation followed by the G3-MORF and finally the ^{99m}Tc -cMORF exactly as before, except now in culture media. The results (not presented) showed that the amplification factor was still about 14 as the former study indicating that the cell culture medium cannot explain the lower amplification factors in the cell study, and that the nonspecific binding of G3-MORF to the antibody was very low.

CONCLUSIONS

A G3 dendrimer-MORF conjugate was synthesized such that 9 of the 32 surface groups were modified with MORF, of which 90% percent were accessible in solution to ^{99m}Tc -cMORF. The conjugated dendrimer was purified and showed unhindered hybridization to ^{99m}Tc -cMORF and CC49-cMORF. On Protein G and Protein L coated plates, the signal was amplified by up to 14 fold that of conventional pretargeting, showing that the dendrimer-MORF conjugate

participated in amplifying the signal. Although not as positive, similar studies in the LS174T tumor cells in tissue culture also demonstrated evidence of signal amplification.

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Figure 1.
Two-dimensional representation of a PAMAM-succinic acid dendrimer generation 3 with 32 carboxyl groups on its surface.

Figure 2. SE-HPLC UV chromatograms at 265 nm of G3-MORF prepared at different molar ratios of MORF to dendrimer: 1:1 (trace A), 2:1 (trace B), 4:1 (trace C), 8:1 (trace D), and 16:1 (trace E). Flow rate was 0.6 mL/min.

Figure 3. Native PAGE of G3-MORF analyzed before purification and after mixing with cDNA and staining with Coomassie Blue (panel A) or ethidium bromide (panel B). Lane 1 contains free dendrimer, lane 2 contains MORF conjugated dendrimer and lane 3 (panel B only) contains free MORF.

Figure 4. SE-HPLC chromatograms with UV 265 nm detection (top trace) and radioactivity detection (bottom trace) of one G3-MORF (prepared at a 16:1 MORF/dendrimer molar ratio) following addition of trace ^{99m}Tc -cMORF. Flow rate was 0.5 mL/min.

Figure 5.

Histograms representing the immunoreactive fractions of native and modified CC49 detected using flow cytometry analysis. A: native CC49; B: CC49-MORF; C: CC49-cMORF. Results are averages, error bars represent one s.d (N = 3).

Figure 6. SE-HPLC radioactivity chromatographic profile for the CC49-cMORF/G3-MORF/ ^{99m}Tc -cMORF complex. The retention times for CC49-cMORF and G3-MORF are indicated by arrows. Flow rate was 0.5 mL/min.

Figure 7.

Histograms presenting the percentage of radioactivity obtained in Protein G (panel A) and Protein L (panel B) coated plates by amplification pretargeting and conventional pretargeting and two control studies in which the antibody or both the antibody and the polymer were eliminated. Results are averages, error bars represent one s.d (N = 5).

Figure 8.

Histograms presenting the percentage of radioactivity accumulated in cells by amplification pretargeting, conventional pretargeting and their control studies without antibodies, performed at 4 °C (solid bars) and 37 °C (hatched bars). Results are averages, error bars represent one s.d (N = 3).

Table 1

Experimental procedures for amplification pretargeting and conventional pretargeting on Protein G and Protein L coated plates

	Amplification pretargeting	Nonspecific amplification	Conventional pretargeting	Nonspecific pretargeting
1 st incubation	CC49-cMORF	PBS	CC49-MORF	PBS
2 nd incubation	G3-MORF	G3-MORF	PBS	PBS
3 rd incubation	^{99m} Tc-cMORF	^{99m} Tc-cMORF	^{99m} Tc-cMORF	^{99m} Tc-cMORF