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Method for amine–amine attachment in nanodevice preparation

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Abstract A process for covalent attachment of amines to other amines is described for nanodevice preparation. This method utilizes a linker with aldehyde and *N*-hydroxysuccinimide ester terminals separated by a heptyl spacer. Nanodevices consisting of amine or hydrazide-containing molecules (Lucifer Yellow, fluorescein-5-thiosemicarbazide, and 2-hydrazinopyridine) attached to the surface of a generation 4 amine-terminated poly(amidoamine) dendrimer were prepared as model compounds. In spite of the numerous amine groups present on the dendrimer, aggregate formation was negligible, yielding well-defined nanoconstructs. This negligible aggregation was attributed to the reversible imine bonds formed between the surface amine groups of the dendrimer and the aldehyde group of the linker. We also prepared a dual fluorophore-dendritic nanodevice in which fluorescein was attached to the dendrimer surface via the linker while rhodamine was directly bound to the dendrimer surface. Fluorescence, UV-visible spectrophotometry, size exclusion chromatography, electrophoresis, and dynamic light scattering were used to analyze these nanodevices.

Introduction

Progress in nanoscience and nanotechnology has led to an explosive growth in the young and immature, inter-disciplinary field of nanomedicine. Syntheses of precise, biocompatible macromolecules with nanometer dimensions have been reported. Perhaps, one of the most monodispersed polymers that is commercially available is the poly(amidoamine) (PAMAM) dendrimer [1–3]. This molecule has a core from which branches emanate in a regular, well-defined fashion. The resulting water-soluble macromolecule has a high density of reactive groups on its surface with cavities in its interior. The unique architecture of a dendrimer allows attachment of various molecules on its surface or inclusion within its cavities. Attachment of molecules such as fluorescent probes, drugs, imaging agents, oligonucleotides, cell-specific targeting agents, or antibodies to a dendrimer creates nanodevices [4–10]. These nanoparticles can be controlled and manipulated for numerous potential applications in medicine [11, 12].

The availability of a wide variety of biocompatible linkers for construction of nanodevices is critical for their successful application in nanoscience and nanomedicine. A number of commercially available heterobifunctional bioconjugation linkers have been used in preparation of various nanodevices [10]. In this study, we describe the use of a nine-carbon long hydrophobic linker for conjugation of amine or hydrazide containing fluorescent dyes to the surface of an amine-terminated, generation-4 (G4) PAMAM dendrimer. Fluorescein, which displays a yellowish-green emission, was conjugated to a G4 dendrimer via the linker in a one-pot reaction. A second fluorescent molecule, rhodamine with a red emission was directly attached to the dendrimer surface. Such a nanodevice could have potential applications as sensors. Replacing fluorescein and rhodamine with cell targeting agents and drug molecules, respectively, will provide a cell-specific drug carrier. In this case, the long aliphatic non-polar linker would reduce potential steric hindrance between targeting agent and membrane receptor. Unlike many linkers that have two hydrolytically unstable terminal functional groups, our

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linker containing an aldehyde and *N*-hydroxysuccinimde (NHS) ester has only one terminus (NHS ester side) that is unstable in aqueous media. Although unstable, the NHS active ester reacts rapidly with an amine and forms a stable amide bond after conjugation.

Methods and materials

General

PAMAM dendrimer G4 was purchased from Dendritic Nanotechnologies (Mt. Pleasant, MI). All other chemicals were obtained from Sigma Aldrich (Milwaukee, WI). Fluorescence spectra were taken on a Model: LS 50B fluorescence spectrophotometer (Perkin Elmer, Waltham, MA). UV spectra were obtained on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Dynamic light scattering (DLS) measurements were obtained with a Protein Solutions DynaPro Model LSR (Wyatt Technology Corporation, CA).

Preparation of linker, 9-oxo-nonanoic acid 2, 5-dioxo-pyrrolidin-1-yl ester (**6**)

Oleic acid (9.00 g, 31.86 mmol) was treated with formic acid (100 mL) and 30% hydrogen peroxide (20 mL) to generate **2** (8-(3-octyl-oxiranyl)-octanoic acid). The reaction was stirred at room temperature for 17 h. Hydrogen peroxide and formic acid were removed at 25 °C with a rotary evaporator connected to a vacuum pump. After confirming a new spot by TLC ($R_f = 0.28$ (hexane/acetone = 5:1), the dried substance was dissolved in 100 mL of 1 M-NaOH and stirred for 24 h at room temperature. Then, the reaction mixture was heated at 60 °C for 30 min, 30 °C for 1 h, and then allowed to cool to room temperature. The pH was adjusted to 1 using 6 M-HCl. A white, foamy compound was produced and the white compound was extracted with dichloromethane (3 × 200 mL). Saturated aqueous NaCl was added to aid in phase separation. The combined dichloromethane layers were dried in vacuo to yield 8.12 g (81%) of a white flaky solid product, a crude vicinal alcohol, **3** ($R_f = 0.69$ in hexane/ethyl acetate = 2:1). In turn, an aliquot of **3** (3.53 g, 11.17 mmol) was dissolved in THF (150 mL). Aqueous 0.09 M-$NaIO_4$ solution (125 mL) was added. The mixture was stirred until TLC showed new spots which were positive for aldehydes by Schiff reagent (about 3 h): R_f of **4** = 0.32 and R_f of **5** = 0.13 in hexane/ethyl acetate = 2:1. Subsequently, the reaction was quenched with THF (100 mL) to precipitate the $NaIO_4$. Precipitated $NaIO_4$ was filtered off. *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC)-hydrochloride (2.42 g, 12.62 mmol) was added to the reaction mixture at room temperature. The solution was stirred for 30 min, followed by the addition of NHS (1.49 g, 12.95 mmol). The reaction was allowed to run for 40 h at room temperature. THF was removed under reduced pressure at 30 °C resulting in the formation of a precipitate. The precipitate was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate (2 × 100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was purified using flash column chromatography with a gradient hexane–ethyl acetate eluent (7:1 → 2:1). $R_f = 0.13$ (hexane/ethyl acetate = 2:1); Yield = 1.22 g (41%); Light yellow oil; ^{1}H-NMR (300 MHz, $CDCl_3$): δ1.22–1.45 (m, 6H), 1.55–1.65 (p, J = 7.2 Hz, 2H), 1.70 (p, J = 7.5 Hz, 2H), 2.36–2.42 (dt, J = 7.3, 1.5 Hz, 2H), 2.50–2.59 (t, J = 7.5 Hz, 2H), 2.80 (s, 4H, NHS), 9.70(s, 1H, C<u>H</u>O); ^{13}C-NMR (300 MHz, $CDCl_3$): δ 22.1, 24.7, 25.8, 28.7, 28.9, 29.0, 31.0, 43.9, 168.0, 169.2, 203.1; FT-IR(neat): 3515(w), 2924(s), 2857(s), 2725(s), 1815(s), 1789(s), 1738(s), 1462(w), 1430(w), 1371(s), 1205(s), 1068(s), 872(s), 648(s); MS: m/z 292.126 (M + Na^+).

Preparation of G4 PAMAM dendrimer-linker-dye (2-HP, Lucifer Yellow, or FTSC) conjugates

Generation 4 PAMAM dendrimer with 64 amine terminal groups and a diaminobutane core (MW = 14,243) was obtained commercially in methanol. Its purity was confirmed by C18 reversed-phase HPLC and polyacrylamide gel electrophoresis. It showed one major peak on HPLC and a sharp band on the PAGE gel (a very weakly stained slower migrating band was also observed). The solvent (methanol) was first evaporated and the resulting dendrimer was dissolved in phosphate-buffered saline (PBS). To the stirred solution of dendrimer was added the linker dissolved in DMSO (3 mol linker **6**/1 mol dendrimer). The reaction was performed overnight (20 h) with constant stirring at room temperature. The second molecule (3 equivalents fluorescein-5-thiosemicarbazide (FTSC), 2-hydrazinopyridine (2-HP) or Lucifer Yellow/G4 equivalent, giving a molar ratio, $r = 3$) was then added to the reaction mixture. FTSC was made in DMF while Lucifer Yellow and 2-HP solutions were made in water. After 30 min, sodium cyanoborohydride (1.2 equivalents/G4 equivalent) was added in case of Lucifer Yellow, and the reaction mixture was stirred in the dark overnight at room temperature. The reaction mixture was then ultrafiltered and washed with 1 M-NaCl (containing 20% DMF) using a 3000 MWCO centrifugal device (Nanosep 3 K Omega, Pall Life Sciences). The washing step was repeated until the filtrate was colorless. The last wash was carried out with PBS.

Preparation of G4 PAMAM dendrimer-linker-FTSC-rhodamine conjugate

The dendrimer-linker-FTSC conjugate in PBS was reacted with three equivalents of NHS-rhodamine for 1 h in the dark at room temperature. The resulting dendrimer-linker-FTSC-rhodamine was then washed as described earlier.

Size exclusion chromatography

SEC was performed on a Hitachi system with an auto-sampler (L-7200), pump (L-7100), UV-visible detector (L-7420), and an interface (D-7000) with a 20-μL loop. The size exclusion column used was a BioSep SEC S2000 (300 × 7.8 mm) with a GFC 2000 guard cartridge purchased from Phenomenex (Torrance, CA). The eluant was 20 mM phosphate buffer, pH 3 with 0.5 M-NaCl. The flow rate was 0.5 mL/min. Samples were filtered through a 0.45 μm syringe filter. Injection volume was 20 μL. Unless otherwise mentioned, the chromatograms were obtained at 214 nm. The column was calibrated with various generations of PAMAM dendrimers.

Polyacrylamide gel electrophoresis

PAGE of dendrimers and their conjugates was performed under acidic conditions as described previously [13]. Samples from the reaction mixture were applied directly onto the gels. After electrophoresis, the gel was washed with 0.1 M bicarbonate buffer and placed on a UV trans-illuminator for fluorescence image capture (in case of FTSC and Lucifer Yellow). The gel was then stained with Coomassie blue and destained with CH_3OH/acetic acid/water.

Results and discussion

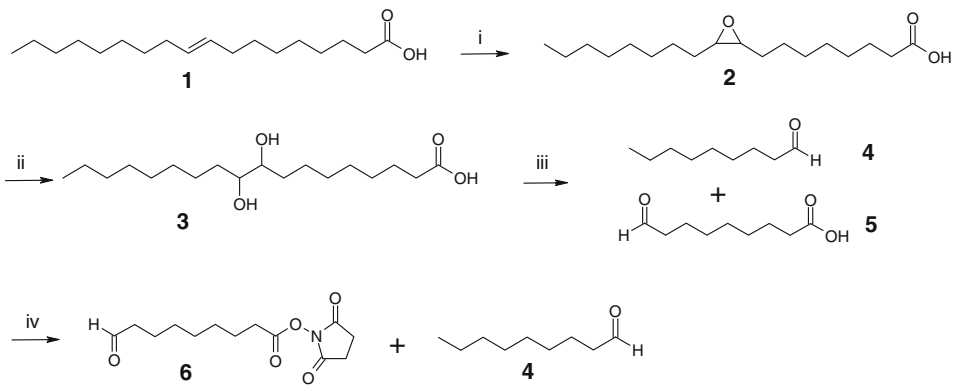
Synthesis of linker

Oleic acid (**1**) was derivatized to an epoxide (**2**) using formic acid and hydrogen peroxide (Scheme 1). The crude intermediate **2** was treated with aqueous sodium hydroxide solution to form 9,10-dihydroxyoleic acid (**3**) [14, 15]. After the diol compound (**3**) was confirmed with MALDI-TOF (m/z 339.220, $M + Na^+$) and FT-IR, the vicinal alcohol of **3** was cleaved to generate nonanal (**4**) and 9-oxo-nonanoic acid (**5**) using sodium periodate ($NaIO_4$) [16]. In the oxidative cleavage reaction, the concentration of $NaIO_4$ was kept at less than 0.1 M and a quantitative amount was used to achieve high efficiency as suggested in the literature [17, 18]. By quenching the reaction with THF, leftover $NaIO_4$ and water soluble by-products could be removed by precipitation. Water-soluble *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) was used to convert **5** to its *N*-hydroxysuccinimide (NHS) ester derivative (**6**). The combined yield of the last two steps was 41%. The low yield is partly due to the use of intermediates, which were not purified.

Preparation of various dendrimer conjugates

In the event that both molecules to be attached contain the same organic functional group like amine, one can use "homobifunctional linker" at the expense of forming homo-conjugation products that need to be removed. Most amine-reactive homobifunctional linkers have both termini that form stable bonds with amines. Our linker **6** allows avoiding such uneconomical conjugation. The linker **6** carries two different amine-reactive functional groups (an aldehyde group at one end and an NHS ester at the other terminus) with different reactivity; the NHS ester forms a stable amide bond while the aldehyde forms an unstable Schiff base with an amine.

One of our objectives of synthesizing linker **6** was to attach one or two cell-targeting molecules (containing an amine or hydrazide functionality) separated by a spacer to an amine terminated PAMAM dendrimer. The presence of a spacer would allow the targeting agent to be located away from the dendrimer's surface so that it may bind to its receptor without any steric hindrance. Under our reaction conditions and linker-dendrimer mole ratio ($r = 3$), reaction



Scheme 1 Synthesis of linker. Reagents: (i) H_2O_2 and Formic acid; (ii) 1 M-NaOH; (iii) $NaIO_4$, THF, and H_2O; and (iv) EDAC, NHS, and THF

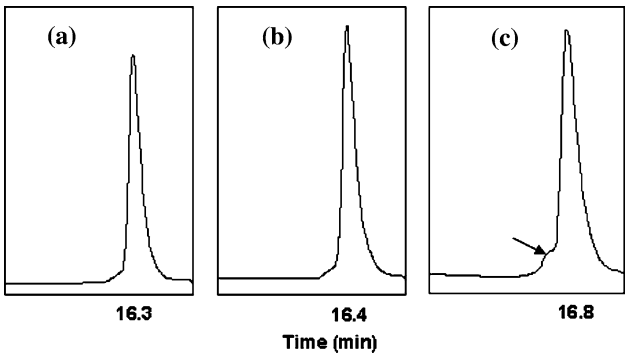
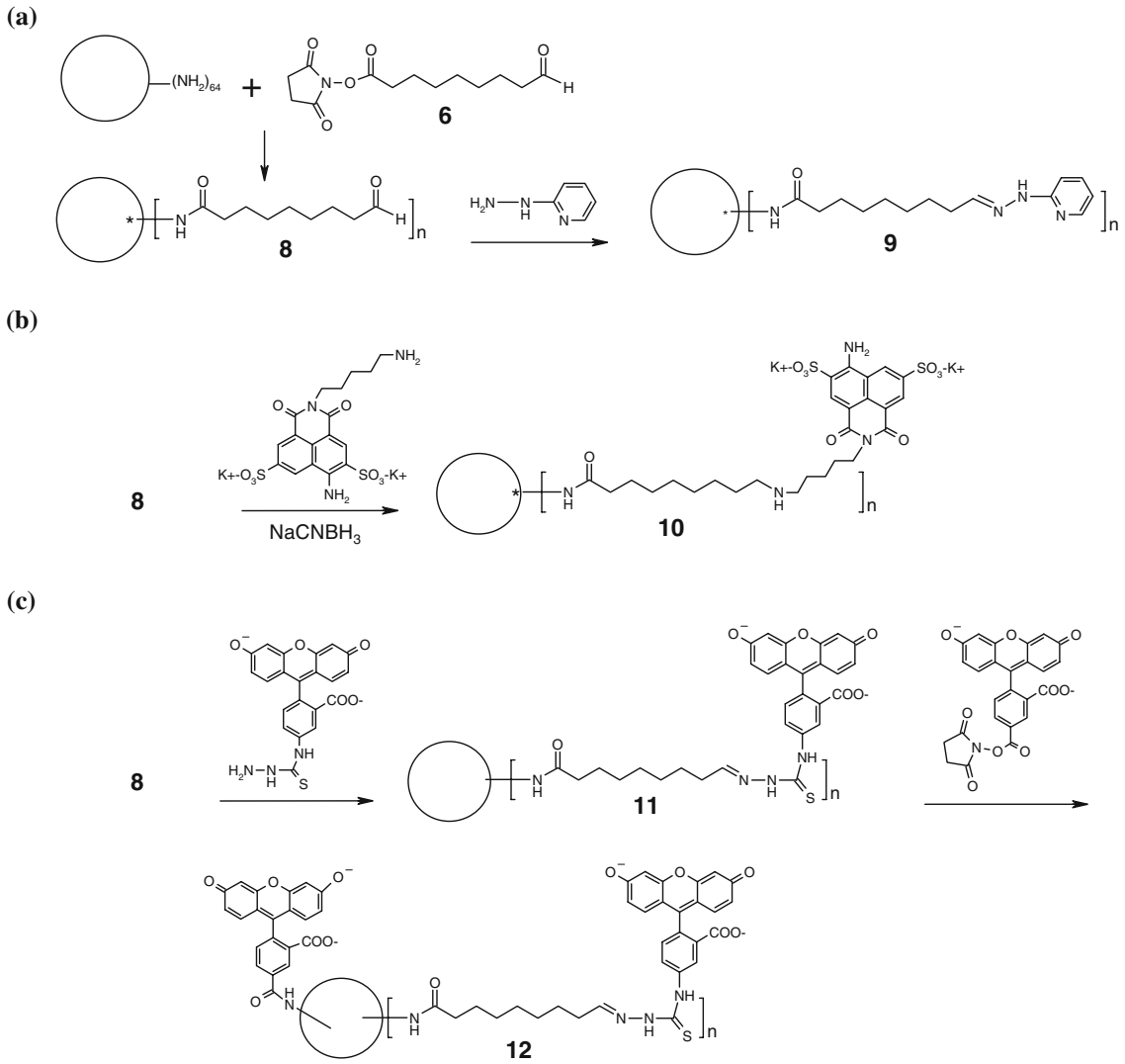


Fig. 1 SEC of dendrimer-linker conjugates. SEC chromatograms were obtained at 214 nm for the dendrimer (**a**) and the dendrimer-linker reaction mixtures at $r = 3$ (**b**) and $r = 16$ (**c**). Arrow points a dimer peak

of the PAMAM dendrimer (G4) with linker **6** showed no aggregate formation in SEC chromatograms (Fig. 1a, b). However, it should be noted that at higher linker/dendrimer ratios ($r = 16$), aggregate formation was possible (Fig. 1c). Although NHS ester reacts rapidly with amines under aqueous conditions, the reaction between the dendrimer and the linker was allowed to proceed overnight to ensure complete reaction or hydrolysis of the linker in order to avoid potential interference in the next step. The dendrimer-linker conjugate (**8** in Scheme 2a) was then reacted with amine (Lucifer Yellow) or hydrazide containing molecules (2-HP and FTSC). In the case of Lucifer Yellow, a reducing agent (sodium cyanoborohydride) was used to stabilize the Schiff's linkage (this was not necessary for the hydrazides,



Scheme 2 Preparation of dendrimer conjugates. **a** Reaction with 2-HP; **b** Reaction with Lucifer Yellow; and **c** Reaction with FTSC, followed by rhodamine

2-HP, and FTSC). Reactions were performed overnight in the dark. Controls were also run in which the amine molecule was added to the dendrimer (without linker) under identical experimental conditions. All reaction mixtures were then exhaustively washed with NaCl–DMF solution. The salt was used to destroy any non-specific electrostatic interactions between the second molecule and the dendrimer while the DMF aided in removal of dye from the dendrimer cavities. The resulting solutions were clear and showed no visual particulates or signs of aggregate formation by PAGE, SEC, or DLS. SEC chromatograms obtained were similar to those in Fig. 1. Since the amine molecules possess absorption peaks not found in the dendrimer or linker, SEC chromatograms were also obtained at these wavelengths. An example of such a chromatogram is shown for the dendrimer-linker-2HP conjugate (**9** in Scheme 2a), which was obtained at 300 nm (Fig. 2). A small peak at 16.2 min was obtained for the control, while the conjugate showed a fivefold larger peak at 16.5 min. The presence of a peak in the control sample suggests that there is some non-specific binding between 2-HP and the dendrimer in spite of the extensive washing. This non-specific binding is likely due to inclusion of 2-HP into the non-polar dendrimer cavity, rather than electrostatic interactions. DLS showed a hydrodynamic radius of 2.1 nm for the dendrimer-linker-2HP conjugate. PAGE was also used to analyze the products. All reacted samples showed only one major band similar to the dendrimer when stained by Coomassie Blue (data not shown). 2-Hydrazinopyridine is not fluorescent but it has a UV-absorption band at 300 nm not seen in the dendrimer or the linker. UV-spectra of 2-HP, and 2-HP reacted dendrimer samples are shown in Fig. 3. As mentioned before, non-specific binding was observed for the control (dendrimer reaction with 2-HP, without linker). The

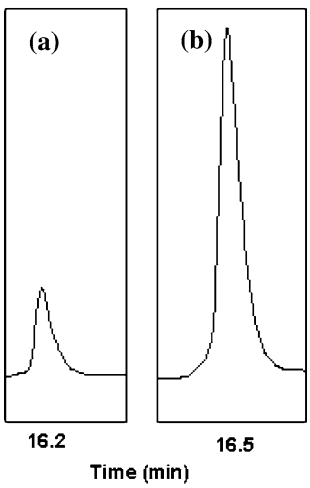


Fig. 2 SEC of dendrimer-linker-2HP conjugate. SEC chromatograms at 300 nm were obtained for the control (**a**) and the dendrimer-linker-2HP ($r = 3$) (**b**)

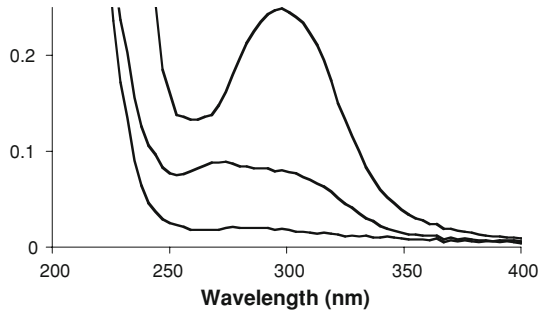


Fig. 3 Absorption spectrum of dendrimer-linker-2HP conjugate. Top curve represents 2-HP, middle curve is the dendrimer-linker-2-HP conjugate and the lower curve is the control

dendrimer-linker-2HP conjugate showed a fourfold increase in absorbance at 300 nm over the control. The absorbance values at 300 nm were used to calculate the number of linkers attached to a G4 dendrimer. The calculated ratio was found to be 1.7 at the theoretical mole ratio of 3.

Samples reacted with Lucifer Yellow were analyzed by fluorescence spectrophotometry (Scheme 2b and Fig. 4). The aldehyde group of the dendrimer-attached linker most likely reacted with the less sterically hindered aliphatic amine present on the end of the pentyl chain in Lucifer Yellow. Reduction of the resulting Schiff bond by sodium cyanoborohydride gave a dye separated from the dendrimer surface by a 15-atom long hydrophobic spacer. As in other control samples, non-specific binding of dye to the dendrimer was apparent. The dendrimer sample with covalently attached Lucifer Yellow showed an almost fourfold increase in fluorescence over the control. The emission peak of Lucifer Yellow and control samples in PBS were at 518 nm. The dendrimer-linker-dye conjugate showed an emission maximum of 514 nm. The small blue shift of the spectrum suggests a slightly more non-polar environment of the fluorescent dye when covalently bound to the dendrimer. This shift may partly be due to the 15-atom long hydrophobic spacer formed by the dye and linker.

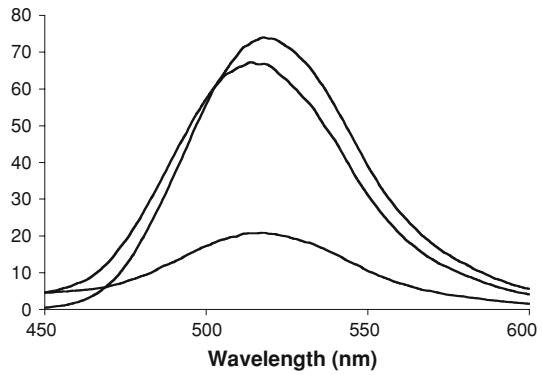


Fig. 4 Fluorescence spectrum of dendrimer-linker-Lucifer Yellow conjugate. Top curve represents Lucifer Yellow in PBS, middle curve is the dendrimer-linker-Lucifer Yellow while the lower curve is the control

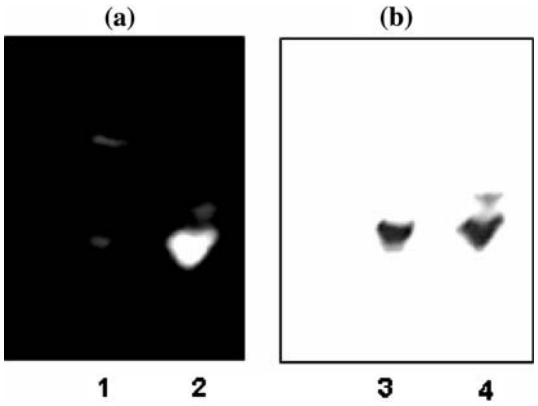


Fig. 5 PAGE of dendrimer-linker-FTSC conjugate. Acidic PAGE on 15% gel (**a**) under UV illumination (lane 1 = control; lane 2 = dendrimer-linker-FTSC); (**b**) stained with Coomassie Blue (lane 3 = control; lane 4 = dendrimer-linker-FTSC)

Reaction of the dendrimer-linker conjugates with 2-HP or Lucifer Yellow clearly demonstrates that the aldehyde groups on the dendrimer are available for further conjugation. Another fluorescent dye, FTSC was attached to the aldehyde group on the dendrimer (**11** in Scheme 2c). Fluorescence curves obtained with the dendrimer-linker-FTSC conjugate (emission peak at 526 nm) were similar to that of Lucifer yellow (Fig. 4) except that there was no peak shift (data not shown). A PAGE gel of the dendrimer-linker-FTSC conjugate is shown in Fig. 5. Under ultraviolet illumination, the conjugate was fluorescent. The control sample reacted with FTSC showed a very weak fluorescence signal due to non-specific binding (lane 1, Fig. 5). The sample reacted with FTSC showed one bright band and a very weak slower moving band (lane 2, Fig. 5). The control and FTSC conjugate samples stained by Coomassie Blue are shown in lanes 3 and 4, respectively (Fig. 5). The fluorescent bands correspond to the dendrimer bands stained by Coomassie Blue, implying that FTSC was attached to the dendrimer. The results above demonstrate the effective use of linker **6** to couple one or two small molecules such as a cell-targeting agent to a nanomolecule like a G4 PAMAM dendrimer with 64 reactive amine groups without causing self-scrambling of the latter.

A dual fluorophore dendrimer (**12** in Scheme 2c) was prepared by attaching rhodamine to the surface amine groups of the dendrimer-linker-FTSC conjugate (**11** in Scheme 2c). Absorption spectrum of the yellow-green dendrimer-linker-FTSC conjugate (**11**) showed a peak at 500 nm while the red dendrimer-linker-FTSC-rhodamine conjugate (**12**) displayed two peaks at 514 nm and 560 nm (Fig. 6). This dual-fluorophore dendrimer conjugate also showed fluorescence emissions at 526 and 580 nm and SEC peak at 16.4 min similar to the peak obtained for the dendrimer-linker conjugate (Fig. 1b), at both 514 nm and 560 nm (data not shown). These results clearly

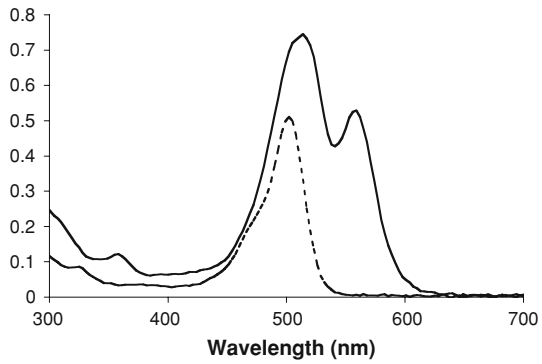


Fig. 6 Absorption spectrum of dendrimer-linker-FTSC-rhodamine conjugate. Top curve represents the dendrimer-linker-FTSC-rhodamine conjugate while the lower curve is the dendrimer-linker-FTSC conjugate

demonstrate the attachment of two different fluorescent dyes at different locations on the G4 PAMAM dendrimer.

Conclusion

We have synthesized a linker with two functional groups (an NHS ester and an aldehyde group) that are reactive toward amines and separated by a long, flexible spacer. In aqueous media, one end with an NHS ester group forms a stable amide bond while the aldehyde end forms a reversible Schiff linkage with amine groups. The linker was made to react with a G4 PAMAM dendrimer to demonstrate its utility in the preparation of nanodevices. It was found that at a low molar ratio of linker and dendrimer, dendrimer-linker conjugates with aldehyde groups were obtained without aggregation of the dendrimer. Molecules containing amine or hydrazide functional groups could then be covalently coupled to the conjugate via aldehyde groups in a one-pot reaction. The presence of a flexible heptyl spacer in the linker enables the attached amine molecule to extend away from the surface of the dendrimer, leaving the dendrimer surface free for attachment of other molecules. Such a nanoconstruct may find potential applications in the preparation of dendrimer-based nanodevices such as targeted drug delivery agents and sensors. Except for the NHS ester group, which is removed after reaction with the amine, the rest of the linker is made up of biocompatible components. This linker may therefore also be useful for biological applications.

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