Poly(amidoamine) dendrimer-erythromycin conjugates for drug delivery to macrophages involved in periprosthetic inflammation

Admira Bosnjakovic, MSa,1, Manoj K. Mishra, PhDa,1, Weiping Ren, PhDb,2, Yunus E. Kurtoglu, PhDa, Tong Shi, BSb, Dianna Fan, MSb, Rangaramanujam M. Kannan, PhDa,b,⁎,2

aDepartment of Chemical Engineering and Materials Science, Wayne State University, Detroit, Michigan, USA
bDepartment of Biomedical Engineering, Wayne State University, Detroit, Michigan, USA

Received 30 July 2010; accepted 27 October 2010

Abstract

Erythromycin (EM), an antibiotic that has been used for infectious diseases, is now gaining attention because of its novel anti-inflammatory effects. We explore a dendrimer-EM nanodevice for sustained treatment of orthopedic inflammation. To sustain pharmacological activity, EM was conjugated to poly(amidoamine) dendrimer (PAMAM) through an ester bond. A bifunctional PAMAM dendrimer was prepared having neutral hydroxy and reactive amine groups on the surface and was reacted with EM prodrug (EM-2′-glutarate). The cytotoxicity, efficacy and antibacterial properties were evaluated on macrophages (RAW 264.7 cells) associated with periprosthetic inflammation. The conjugate is noncytotoxic and showed significant reduction of nitrite level (by 42% as compared with untreated cells and free EM). The zone of inhibition of the conjugate on bacterial growth at different concentrations showed similar activity compared to free EM. The anti-inflammatory properties of EM combined with the targeting potential of the dendrimer can lead to sustained and targeted intracellular delivery.

From the Clinical Editor: In this study, a specific dendrimer-erythromycin conjugate nanodevice is investigated for the treatment of periprosthetic inflammation. The anti-inflammatory properties of erythromycin combined with the targeting potential of the dendrimer can lead to sustained and targeted intracellular delivery.

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Key words: PAMAM dendrimer; Erythromycin; Drug delivery; Periprosthetic inflammation; Macrophages

Total joint replacement has been very successful in restoring function and mobility to millions of patients worldwide since its advent more than 30 years ago. With improvements in prophylaxis against infection, the fatigue strength of the components, and skeletal fixation, wear debris-induced periprosthetic membrane inflammation has become the primary limitation on the longevity of total joint replacements.¹ There is currently no cure for aseptic loosening in patients with osteolysis except revision surgery, primarily due to a lack of safe or effective antibiotic candidate(s).² A recent approach to limiting osteolysis has focused on reducing periprosthetic inflammation and enhancing periprosthetic bone quality.²-⁴ Erythromycin (EM) has been used against infectious disease for over 50 years.⁵ For the last decade, EM has attracted a great deal of attention because of its novel anti-inflammatory effects far beyond antibiotics.⁶,⁷ EM demonstrates a unique "phagocyte-targeted delivery" property, favorably concentrating in monocyte/macrophages.⁸-¹¹ Evidence is accumulating, including ours,¹² that EM exerts its anti-inflammatory effects through targeting to NF-κB signaling.¹³-¹⁵ Data from our previous studies showed that EM inhibits wear debris-induced inflammation and osteolysis (both in vitro and in vivo),¹²,¹⁶-¹⁹ suggesting that EM represents an appropriate drug candidate to prevent or treat periprosthetic membrane inflammation. However, delivering adequate levels of EM to the site of periprosthetic inflammation without undesirable systemic side effects presents a considerable challenge. The long-term goal is to develop and validate intravenously administered dendrimer-EM nanodevices that can target the periprosthetic tissue and reduce local inflammation in a sustained manner.

Dendrimers are treelike globular molecules (~2–10 nm) with well-defined structure, tailored surface functionality, and...
multivalency, that are extensively investigated for various biomedical applications\textsuperscript{20,21} such as drug delivery systems,\textsuperscript{22-24} antiviral agents, and magnetic resonance imaging contrast agents.\textsuperscript{25,26} Poly(amideamine) dendrimer (PAMAM) dendrimers have also been studied in isolation as topical antibacterial agents to treat intrauterine infection.\textsuperscript{27} In the past decades dendrimers have been investigated as novel diagnostic nanodevices.\textsuperscript{28,29} Recently we have reported a dendrimer-based enzyme-linked immunosorbent assay for detection of the cytokine interleukin-6, which is over expressed in infected intra-amniotic fluid.\textsuperscript{30} It is well known that the functional surface groups on dendrimers can affect their physicochemical properties, making them hydrophilic. Conjugation of poorly water-soluble drugs to PAMAM dendrimer can increase the bioavailability as well as drug solubility, and decrease the dose frequency.\textsuperscript{31-33} Drug molecules can be either encapsulated, complexed or covalently attached to the dendrimers through their functional groups.\textsuperscript{34,35} The most common covalent linkage of the drugs to the surface functional groups of dendrimers can be achieved through the ester or amide bond, which can be cleaved hydrolytically or enzymatically.\textsuperscript{36} In the recent past disulfide bonds have also been demonstrated to be cleaved in the presence of intracellular glutathione.\textsuperscript{37} Ligands can be attached to dendrimers to increase the accumulation of drug at the targeted site.\textsuperscript{38} Neutral PAMAM dendrimers with no targeting ligands have been shown to have an intrinsic ability to localize in cells associated with neuroinflammation in a rabbit model of cerebral palsy.\textsuperscript{39} The cellular uptake of dendrimers is also affected by their size, structure, surface functional groups, and charge.\textsuperscript{40,41} Premature or lack of drug release from the dendrimer-drug conjugate before the appropriate time can decrease the in vivo effectiveness.

In this article we describe the synthesis and characterization, cytotoxicity and in vitro efficacy of hydroxyl-terminated fourth-generation PAMAM dendrimer-EM conjugate (PAMAM G4-OH). PAMAM G4-OH was partially functionalized with amine groups using amino-valeric acid and conjugated to EM-2'-glutarate. A zone-of-inhibition study was performed to determine the levels of \textit{Staphylococcus aureus} activity inhibited by dendrimer-EM conjugate. The effect of the conjugate on lipopolysaccharide (LPS)-induced nitrite (NO$_2$) production in the RAW 264.7 macrophage cell line was assessed.

### Methods

#### Chemicals and reagents

PAMAM G4-OH with ethylenediamine core and 64 surface groups (MW 14,279) in methanol was purchased from Dendritech Inc. (Midland, Michigan). N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC), 4-(dimethylamino) pyridine (DMAP), glutaric anhydride, 5-(tert-butoxycarbonyl-aminoo) valeric acid [5-(Boc-amino)valeric acid], \(N,N\)-dimethylacetamide (DMA), diethyl chlorophosphate, and LPS (\textit{Escherichia coli} O55:B5) were purchased from Sigma-Aldrich (St Louis, Missouri). Triethylamine (TEA) and \(N\)–\(N\)-butoxycarbonyl-amino) \(N\)-dimethylacetamide (DMF), and dimethyl sulfoxide (DMSO) were purchased from EMD Chemicals (Gibbstown, New Jersey). All anhydrous solvents DMSO, DMF, acetonitrile (ACN), and dichloromethane (DCM) were purchased from Acros Organics (Morris Plains, New Jersey). Regenerated cellulose dialysis membrane (MW cutoff 1000 Da) was obtained from Spectrum Laboratories (Rancho Dominguez, California). Dulbecco’s modified Eagle medium (DMEM) was purchased from Gibco-BRL Life Technologies (Gaithersburg, Maryland). Fetal bovine serum was purchased from HyClone Laboratories (Logan, Utah).

Thin-layer chromatography was performed on silica gel GF$_{254}$ Plates, and the spots were visualized with ultraviolet (UV) light and 2% H$_2$SO$_4$ in ethanol.

#### NMR spectra analysis

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA 400 spectrometer (Varian, Palo Alto, California) using commercially available deuterated solvents. Proton chemical shifts are reported in parts per million (ppm, δ), and tetramethylsilane was used as internal standard. Coupling constants (\(J\)) are reported in hertz (Hz).

#### ESI and MALDI-TOF MS

Electrospray ionization (ESI) mass spectra were recorded on a Waters Micromass QZ spectrometer (Waters Corporation, Milford, Massachusetts). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm) (Bruker Daltonics Inc., Billerica, Massachusetts), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2,5-dihydroxybenzoic acid. Cytochrome c (MW 12,361 g/mol), and apomyoglobin (MW 16,952 g/mol) were used as external standards. A dendrimer solution was prepared by dissolving 2 mg of dendrimer in 1 mL of DMSO. The matrix solution was prepared by dissolving 20 mg of matrix in 1 mL of the 1:1 mixture of deionized water and ACN (0.1% TFA vol/vol). Analytical samples were prepared by mixing 10 μL of dendrimer solution with 100 μL of matrix solution, followed by deposition of 1 μL of sample mixture onto a MALDI plate. This mixture was allowed to air dry at room temperature (22-25°C).

#### High-performance liquid chromatography (HPLC)

HPLC characterization was carried out using Waters HPLC instrument (Waters Corporation, Milford, Massachusetts) equipped with dual pumps, an autosampler and dual UV detector interfaced to Breeze software. The HPLC chromatogram was monitored at 210 and 238 nm simultaneously using the dual UV absorbance detector. Freshly prepared, filtered, and degassed H$_2$O/ACN (0.14% w/w TFA) was used as mobile phase. Symmetry300 C$_{18}$ reverse-phase column (5 μm particle size, 25 cm × 4.6 mm; length × internal diameter) was used for characterization of the conjugates. HPLC analysis was done using 90:10 to 30:70 (H$_2$O/ACN) gradient flow in 30 minutes with flow rate of 1 mL/min.

#### Release study protocol

The release studies were performed in 0.1 M phosphate buffered saline (PBS) solution (pH 7.4). The conjugate was...
dissolved in preheated buffer (2.5 mg/mL) in triplicate and were stirred continuously in a water bath (dual-action shaker; Polyscience, Niles, Illinois) at 37°C. At appropriate time intervals, samples were withdrawn and immediately analyzed by HPLC (conditions explained in HPLC section) to determine the EM concentrations.

**Dynamic light scattering and zeta potential**

The particle size and zeta potential of G4-OH and dendrimer conjugates were determined by dynamic light scattering method using Zetasizer Nano ZEN3600 instrument (Malvern Instruments, Worcestershire, United Kingdom). The samples were dissolved in deionized water (18.2 Ω) and filtered using AccuSpin Micro 17 ultracentrifuge (Fisher Scientific, Pittsburgh, Pennsylvania). Dynamic light scattering measurements were performed at a 173-degree scattering angle at 25°C.

**RAW 264.7 cell culture stimulation and dendrimer treatment**

Murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, Virginia). RAW 264.7 cells were maintained in DMEM (GIBCO-BRL) containing 10% fetal bovine serum and antibiotics (100 units/mL of penicillin-G and 100 μg/mL of streptomycin) at 37°C in a humidified incubator with 5% CO₂. RAW 264.7 cells were plated at a density of 1 × 10⁵ cells per well, in 96-well plates in the presence of LPS (1 μg/mL) with dendrimer-EM conjugate at different concentrations of EM for 48 hours.

**Cell toxicity assay**

Cell toxicity was determined by measuring the release of lactate dehydrogenase (LDH) from dead or dying cells into the culture medium using a Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer’s instructions. After 48 hours of treatment with dendrimer-EM conjugate at different concentrations, the conditioned media were collected and used for the measurement of LDH activity. Briefly, 100 μL of culture medium was added into a plate of 96 wells, mixed with 100 μL working solution, and incubated at room temperature for 30 minutes in the dark. Then, the plate was read under the UVmax colorimeter (Molecular Devices, Sunnyvale, California) at optical density (OD) 490 nm. Blank culture medium was used as a control, and the total cell lysate was used as a positive control. LDH activity was expressed as absorbance (OD) per milligram of protein.

**Detection of nitrite production**

RAW 264.7 cells (passage 10) (5 × 10⁵ cells per well) were cultured in triplicate in 24-well plates and stimulated with 1 μg/mL LPS (positive control), and 1 μg/mL LPS [+dendrimer, or +D-EM] for 48 hours. The concentrations of free EM were 0.1, 1.0, 5.0, and 10.0 μg. They correspond to the concentration of EM in the dendrimer-EM conjugate. The production of nitric oxide (NO) was determined by assaying culture supernatant for NO₂⁻, a stable reaction product of NO. In brief, 100 μL of culture supernatant were mixed with an equal volume of Griess reagent (Cayman, Ann Arbor, Michigan) [1% sulfanilamide and 0.1% N-(naphthyl)ethylene-diamine dihydrochloride in 2.5% H₃PO₄] at room temperature for 10 minutes. Absorbance was measured at 540 nm in a microplate reader. NO₂⁻ concentration was calculated from an NaNO₂ standard curve.

**Antibacterial assay**

*S. aureus* strain was used for the present study. A zone-of-inhibition study was performed using the modified Kirby-Bauer method to determine the levels of *S. aureus* activity inhibited by dendrimer-EM conjugate release in the sample eluents. Growth of the organism and diffusion of the EM commence simultaneously, resulting in a circular zone of inhibition in which the amount of dendrimer-EM conjugate exceeds inhibitory concentrations. The diameter of the inhibition zone reflects the function of the dendrimer-EM conjugate amount in the eluents. A disk paper, soaked with the dendrimer-EM PBS solution with known concentration, was put into a bacterial agar plate (inoculated with the bacteria strain of *S. aureus* for the zone-of-inhibition test. This strain of *S. aureus* is the main bacterium causing bone infection. The bacteria plates were incubated for 24 hours, and then the diameters of the zone of growth inhibition around each disk to the nearest whole millimeter were measured. The results were expressed as the size of zone of inhibition. All the tests were performed in triplicate and were repeated two times.

**Preparation of bifunctional PAMAM dendrimer (G4-OH-Link-NH₂, 2)**

To a solution of 5-(Boc-amino)valeric acid (168.9 mg, 0.7774 mmol) in DMSO (10 mL), EDC (447 mg, 2.33 mmol) and DMAP (10.0 mg, 0.0818 mmol) were added under nitrogen. The mixture was stirred for 1 hour at room temperature, and then the obtained reaction mixture was then lyophilized, yielding 328.0 mg (0.0194 mmol) of intermediate 1 (G4-OH-Link-Boc). ¹H NMR (DMSO-d₆, 400 MHz): δ 8.04 (bs, CO-NH, Boc), 7.92 (bs, CO-NH, G4-OH), 7.77 (bs, CO-NH, G4-OH), 7.12 (bs, CO-NH, G4-OH), 4.69 (bs, OH, G4-OH), 3.96 (m, CH₂OCHO), 3.37–3.18 (896H, m, aliphatic protons of G4-OH and CH₂ of linker), 2.01 (m, CH₂, methylene protons of linker), 1.45 (m, 2×CH₂, methylene protons of linker), 1.34 (s, 3×CH₃, methyl groups of Boc). To obtain free amine groups, 6.0 mL of TFA/DCM (1:1) were added to 328.0 mg G4-OH conjugate containing Boc groups at 0°C and stirred for 1 hour. The solvent was removed under reduced pressure. The mixture was redissolved in DMSO and dialyzed with DMSO (membrane MW cutoff 1000 Da) for 24 hours and with deionized water for 6 hours. The obtained reaction mixture was then lyophilized, yielding 2.96 mg (0.0017 mmol) of intermediate 2 (G4-OH-Link-NH₂, 2).
Synthesis of erythromycin-2′-glutarate (EM-2′-glutarate, 3)

Glutaric anhydride (31.09 mg, 0.272 mmol) and TEA (30 μL) were added to a solution of EM (100 mg, 0.136 mmol) in 80:20 (vol/vol) anhydrous DMA/DMF (10 mL). The reaction mixture was stirred for 24 hours under nitrogen, and an additional 15.54 mg (0.136 mmol) of glutaric anhydride and TEA (30 μL) were added, followed by stirring for 72 hours. The reaction mixture was evaporated under reduced pressure, and pure EM-2′-glutarate was isolated by flash column chromatography on silica gel using methanol/ethyl acetate/hexanes (5:2:2) as mobile phase (100 mg, 86% yield) having Rf value 0.4 (methylithy/ethyl acetate/DMC = 5:2:2). 1H NMR (DMSO-d6, 400 MHz): δ 5.07 (dd, J1 = 11.6 Hz, J2 = 2.4 Hz, 1H), 4.72 (dd, J1 = 4.8 Hz, J2 = 0.9 Hz, 1H), 4.60 (d, J = 7.6, 1H), 4.52–4.49 (m, 2H), 4.01–3.96 (m, 2H), 3.88 (d, J = 9.6 Hz, 1H), 3.69 (s, 1H), 3.67 (dd, 1H), 3.44–3.36 (m, 2H), 3.22 (s, 3H), 2.89 (d, J = 8.8 Hz, 1H), 2.84 (d, J = 7.6 Hz, 1H), 2.61 (dd, J1 = 11.2 and J2 = 3.2 Hz, 1H), 2.28–2.13 (m, 8H), 2.11 (s, 6H), 1.86 (t, J = 6.8 Hz, 1H), 1.80–1.74 (m, 1H), 1.71–1.63 (m, 4H), 1.59–1.46 (m, 2H), 1.39–1.31 (m, 1H), 1.23 (s, 3H), 1.15–0.97 (m, 1H, 0.81 (d, J = 7.2 Hz, 3H), 0.72 (t, J = 7.2 Hz, 3H). ESI MS (m/z): calculated for C42H52O16 [M-H]− 846.4, found 846.8.

Synthesis of erythromycin-2′-glutarate-N-succinimidyl ester, 4

EM-2′-glutarate (70.00 mg, 0.0825 mmol) was dissolved in 70:30 (vol/vol) anhydrous ACN/DCM (5 mL), and TEA (50 μL) and N-hydroxysuccinimidyl-phosphate (SDPP) (39.00 mg, 0.1238 mmol) were added to the solution at 0°C. SDPP was synthesized following the literature procedure.42 After 6 hours of stirring, thin-layer chromatography (MeOH/ethyl acetate/DCM = 1:3:2, Rf = 0.48) showed that reaction was complete. The reaction mixture was isolated by flash column chromatography on silica gel using methanol/ethyl acetate/hexanes (5:2:2) as mobile phase (70:30 (vol/vol) anhydrous ACN/DCM (5 mL), and TEA (50 μL) and N-hydroxysuccinimidyl-phosphate (SDPP) (39.00 mg, 0.1238 mmol) were added to a solution of EM (100 mg, 0.136 mmol) in 80:20 (vol/vol) anhydrous DMA/DMF (10 mL). The reaction mixture was evaporated under reduced pressure, and pure EM-2′-glutarate was isolated by flash column chromatography on silica gel using methanol/ethyl acetate/hexanes (5:2:2) as mobile phase (100 mg, 86% yield) having Rf value 0.4 (methylithy/ethyl acetate/DMC = 5:2:2). 1H NMR (DMSO-d6, 400 MHz): δ 5.07 (dd, J1 = 11.6 Hz, J2 = 2.4 Hz, 1H), 4.72 (dd, J1 = 4.8 Hz, J2 = 0.9 Hz, 1H), 4.60 (d, J = 7.6, 1H), 4.52–4.49 (m, 2H), 4.01–3.96 (m, 2H), 3.88 (d, J = 9.6 Hz, 1H), 3.69 (s, 1H), 3.67 (dd, 1H), 3.44–3.36 (m, 2H), 3.22 (s, 3H), 2.89 (d, J = 8.8 Hz, 1H), 2.84 (d, J = 7.6 Hz, 1H), 2.61 (dd, J1 = 11.2 and J2 = 3.2 Hz, 1H), 2.28–2.13 (m, 8H), 2.11 (s, 6H), 1.86 (t, J = 6.8 Hz, 1H), 1.80–1.74 (m, 1H), 1.71–1.63 (m, 4H), 1.59–1.46 (m, 2H), 1.39–1.31 (m, 1H), 1.23 (s, 3H), 1.15–0.97 (m, 1H, 0.81 (d, J = 7.2 Hz, 3H), 0.72 (t, J = 7.2 Hz, 3H). ESI MS (m/z): calculated for C42H52O16 [M-H]− 846.4, found 846.8.

Results

Synthesis and characterization of bifunctional PAMAM dendrimer, 2

PAMAM-G4-OH was reacted with 5-(Boc-amino)valeric acid in the presence of EDC and DMAP to yield intermediate 1 (Figure 1, A). The intermediate 1 was confirmed by proton NMR chemical shift and integration of Boc-protected groups (s, 1.34 ppm, 9H), as well as chemical shifts of methylenes that belong to the linker (m, 1.45 ppm, 4H) and dendrimer ester methylenes (m, 3.96 ppm, CH2OCO) that appear after conjugation (Supplementary Figure S1, which can be found in the online version of this article). The integration of characteristic peak for Boc groups obtained from NMR suggested that 13 molecules of Boc-linker were conjugated to the dendrimer. The deprotection of Boc-protected amino groups in mild conditions, TFA/DCM (1:1), gave bifunctional dendrimer 2, which consists of 54 hydroxyl and 10 amine groups. The slightly lower number of NH2 groups (10 instead 13) is most probably due to hydrolysis of the ester bond. The number of amine groups was determined based on proton NMR using characteristic peak for methylene protons that belong to the linker (m, 1.45 ppm, 4H) (Supplementary Figure S2).

Synthesis of G4-PAMAM dendrimer-erythromycin conjugate (dendrimer-EM, 5)

The amino groups of bifunctional dendrimer 2 were conjugated to EM-2′-glutarate through an amide bond (Figure 1, B). Before conjugation of the dendrimer with EM-2′-glutarate, EM-glutarate-N-succinimidyl ester was prepared.42 EM consists of a 14-membered lactone ring with 10 asymmetric centers, and glycosylated in two positions with desosamine and cladinose sugars.43 EM also contains four hydroxyl groups that differ in their reactivities.44,45 The order of reactivity was established by acetylation. The most reactive hydroxyl group is at the 2′ position, where acetylation occurs first because of the proximity of a tertiary amine that acts as an autocatalyst.44,45 When a limiting amount of acetic anhydride is used, the 2′-monoaceteate would be the only product, and it can be obtained without any catalyst. The tertiary amine group also increases the rate of 2′-monoacate hydrolysis, which can be achieved by quenching the reaction with methanol. The second most reactive hydroxyl group is at the 4′ position, and the third one is at the 11 position. The reaction of EM with glutaric anhydride in the presence of TEA occurred at the 2′ position, which gave EM-2′-glutarate (3) and was characterized by mass, proton NMR, and HPLC. The molecular weight of 3 from mass spectrum [M-H]− was found to be 846.8, which is in agreement with the...
calculated one, 846.4 (Supplementary Figure S3). The chemical shifts of H-1′ (d, 4.60 ppm, J = 7.6 Hz), H-2′ and H-3′ (m, 4.52–4.49 ppm) of desosamine as well as overlapping signals for methylene protons that belong to the glutarate moiety and EM (m, 1.71–1.63 ppm and m, 2.28–2.13 ppm) confirmed the formation of EM-2′-glutarate (3, Supplementary Figure S4). The HPLC chromatograms showed that the retention times for EM and EM-2′-glutarate were 22.08 and 23.66 minutes, respectively (Supplementary Figure S5). EM-2′-glutarate was then reacted with SDPP42 in the presence of TEA to obtain activated ester of erythromycin (4). The ester was characterized by proton NMR showing a characteristic peak for methylene protons of succinimide ester (s, 2.63 ppm, 4H) (Supplementary Figure S6). We achieved better yield when SDPP was used compared with N-hydroxysuccinimide. The activated ester of EM was coupled with bifunctional dendrimer to yield dendrimer-EM conjugate, 5.

The HPLC analysis of conjugates (Figure 2, A) showed that the retention time of bifunctional dendrimer (7.96 minutes) was very close to that of G4-OH (8.13 minutes), because there is no significant difference in chemical structure. However, the dendrimer-EM and G4-OH-Link-Boc intermediate gave very distinct broad peaks at 11.69 and 13.13 minutes, respectively. The broad peaks are due to the larger size and nonpolar character of the conjugates compared with G4-OH and bifunctional dendrimer.

The proton NMR spectrum for the dendrimer-EM conjugate is shown in Figure 2, B. Comparison of the integration of amide peaks of dendrimer and methyl groups of EM revealed that four molecules of EM were attached to the surface of the dendrimer. MALDI-TOF MS spectra of dendrimer-EM were
Figure 2. Characterization of dendrimer-EM conjugate and its precursors. (A) HPLC chromatograms of G4-OH, bifunctional dendrimer G4-OH-Link-NH₂ (2), Dendrimer-EM (5), and G4-OH-Link-Boc (1) at 210 nm. (B) Proton NMR spectrum of dendrimer-EM (5) in DMSO-\textit{d}_6. (C) MALDI-TOF MS spectra of (a) dendrimer-EM (5), and (b) G4-OH dendrimer.
Particle size and zeta potential of G4-OH, bifunctional dendrimer

dendrimer/nanoparticle to the receptor. We have investigated
They also influence the binding properties of the drug and
play a major role in targeting and delivery to the specific site.

Particle size and zeta potential

Zeta potential and size of the nanoparticle or drug molecule
play a major role in targeting and delivery to the specific site.
They also influence the binding properties of the drug and
dendrimer/nanoparticle to the receptor. We have investigated
particle size and zeta potential of G4-OH, bifunctional dendrimer
(2) and dendrimer-EM conjugate (5), which are shown in Table 1.
There is no large variation in size between G4-OH, G4-OH-Link-
Boc, and bifunctional dendrimer (2); this may be due to minimal
difference in molecular weight of these molecules. The
dendrimer-EM conjugate is slightly larger (4.98 nm) compared
with the other compounds listed in Table 1. G4-OH has a
slightly positive zeta potential (4.54 mV), which is due to the
presence of tertiary amines in its core despite its supposed
neutrality. There is little difference between G4-OH and G4-
OH-Link-Boc, but there is a difference between G4-OH and G4-
OH-Link-NH₂ (2). This is due to an increase in the number of
primary amine groups (12–13) on the surface of the dendrimer
after deprotection of Boc groups (6.29 mV). Because of the
tertiary nitrogen groups of EM, the zeta potential of dendrimer-
EM conjugate is 8.08 mV, which is slightly higher compared
with G4-OH and bifunctional dendrimer.

Release studies

The drug release rate of the conjugate was carried out in PBS
buffer pH 7.4 and analyzed by HPLC. The ester linkage used for
conjugation of EM was susceptible to hydrolysis, and about 90%
of the EM was released within 10 hours (Figure 3). EM release
from the conjugate was completed within 20 hours. There are
two ester links in the dendrimer-EM conjugate, and the release
study clearly showed that the ester bond in the 2′ position
releases preferentially. This is consistent with the previous
studies in which EM-2′-monoacetate easily hydrolyzes when the
reaction is quenched with methanol as a result of the proximity of
a tertiary amine group. This tertiary amine group acts as an
autocatalyst for the hydrolysis of the ester bond.44,45 To confirm
that the EM release was due to hydrolysis of the ester linkage,
another control experiment was carried out. The conjugate was
dissolved in anhydrous DMSO, and the stability was analyzed
for 24 hours. The conjugate was completely stable in the
anhydrous condition and did not release any EM. The drug
release from the conjugate followed pseudo first-order reaction
kinetics (k’ = 0.36 hr⁻¹, t₁/₂ = 1.9 hours) as determined by plotting
the natural logarithm of ester-linked EM against time
(Figure 3). The rate constant was determined from the linear
regression of this graph, where the slope of the line is the reaction
rate constant. This release profile is appropriate for the present
application, and it is anticipated that the dendrimer will transport
the drug into cells and release it over a period of several hours.

The cytotoxicity of dendrimer-EM conjugate toward
RAW 264.7 macrophages

To assess the efficacy of the dendrimer-EM conjugate, it was
important to show that the conjugate was nontoxic. The
concentrations of EM in dendrimer-EM conjugate is represented
in x-axis (Figure 4). The free-dendrimer concentration was based
on the amount of dendrimer present in the dendrimer-drug
conjugate, at the indicated EM concentration. For example, the
highest concentration of EM (10 μg/mL) is equivalent to ~60
μg/mL of dendrimer-EM conjugate. Therefore, 50 μg/mL of the
free dendrimer was used as control. As shown in Figure 4, free
dendrimer, EM, and dendrimer-EM conjugate did not show
appreciable cytotoxicity in LPS-activated RAW 264.7 cells at
concentrations up to 10 μg/mL for a treatment period of 48 hours,
based on the LDH assay. Because these are the same activated
cells on which the efficacy is tested, the nontoxic behavior of the
compounds suggests that the efficacy reported is primarily
due to the anti-inflammatory activity.

Dendrimer-EM conjugate inhibited nitrite production

RAW 264.7 cells were cultured with LPS (1 μg/mL) in the
presence of dendrimer-EM for 48 hours, a concentration-
dependent inhibition of NO₂ generation was observed (Figure 5).
These macrophages are the in vivo targets in the orthopedic
applications envisaged for these conjugates. Stimulation by LPS
causes the macrophages to release the inflammatory markers
(NO), the suppression of which would be a measure of efficacy.
Dendrimer-EM significantly inhibited NO₂ release, as compared
with untreated cells, and EM-treated cells at concentrations of 1–10 μg/mL. Interestingly, we also noted that dendrimer-EM demonstrated much stronger NO2– production inhibition than free EM in the dosage range of 5–10 μg/mL. The improved in vitro activity of the dendrimer-EM conjugate where the drug and the dendrimer are ester-linked is significant, because polymer-drug conjugates for cancer applications typically show lower activity in vitro, compared with free drug.24,35 This suggests that enabling an effective intracellular drug release profile (as shown in Figure 3) is crucial for improved efficacy. The improved anti-inflammatory activity of the conjugate is qualitatively comparable to the improvements in the activity of dendrimer-N-acetyl cysteine conjugates with glutathione-sensitive linkers.37

**Dendrimer-EM conjugate preserves its antibacterial activity**

The antibacterial activity of the dendrimer-EM conjugate on gram-positive *S. aureus* at different concentrations is shown in Figure 6. Based on a zone-of-inhibition evaluation, the dendrimer-EM conjugate showed similar activity, as compared to free EM with the same concentration. Furthermore, free dendrimer showed no effect of bacterial inhibition at...
concentrations up to 0.2 mg/mL. This suggests that either conjugation to dendrimers did not affect the antibacterial activity of EM, or free EM was released from the conjugate within the 24-hour period. The latter explanation is most likely, because the drug is released from the conjugate appreciably over this period (as shown in the release data), and because the cell wall of this bacteria is thick. Of course, it is not clear if the EM is released outside the bacteria in the agar medium and is taken up as free EM, or if the conjugate is internalized and the drug is released inside the cell. The results suggest that the conjugation to dendrimer did not negatively affect the antibacterial activity, while producing a superior anti-inflammatory activity.

Discussion

In recent years dendrimer-based nanocarriers have been extensively used for drug and gene delivery. The drugs are attached to dendrimer surface functional groups either directly or via spacers, and very often with a targeting moiety (ligand to receptor). The most common bonds are ester and amide, which can be hydrolyzed inside the cell by endosomal or lysosomal enzymes. The drug release due to hydrolysis is governed by the pH, and ester bonds are more labile toward hydrolysis as compared with amide bonds. Studies have shown that ester-linked conjugates can be hydrolyzed by esterase enzymes in human plasma, whereas amide bonds are quite stable. A key challenge for dendrimer-drug conjugates is to engineer systems wherein the drug is released over a time scale appropriate for the application.

In this study a neutral PAMAM dendrimer-EM conjugate was prepared and evaluated for its anti-inflammatory and antibacterial activity. The hydroxy-terminated PAMAM dendrimers are more biocompatible and significantly less cytotoxic than the amine-terminated counterparts. Based on the structure of the dendrimer and the drug, and the reactivities of the groups involved, EM was modified to contain a reactive acid group, while the G4-OH dendrimer was functionalized to contain a few reactive amine groups. The spacer was included to provide steric relief for enabling drug release. PAMAM G4-OH was reacted to a protected amine linker, followed by deprotection to obtain a bifunctional dendrimer through an amide bond. A combination of HPLC, proton NMR, and MALDI-TOF MS analyses showed that the conjugates were “pure,” with a drug payload of ~16% by weight, which is relatively high for polymer conjugates, yet were very soluble in PBS buffer. The conjugates released the drug effectively, with more than 90% of free drug over a period of 10 hours. The efficient release of drug from the 2′-position of EM preferentially is due to a tertiary amine group present in the vicinity of the 2′ position in EM. The conjugates were not cytotoxic to RAW 264.7 macrophages (the target cells for anti-inflammatory activity) and showed improved efficacy over free drug. In LPS-activated macrophages the conjugates were more effective in reducing NO2 production as a marker for NO,a measure of anti-inflammatory activity) as compared with free drug. This suggests that (i) the dendrimer enabled more drug to be transported into cells, and (ii) the conjugate released the drug at an appropriate time scale. However, the antibacterial activity of the conjugate was comparable to free drug. This may be because the gram-positive S. aureus has a thick cell wall, and the neutral dendrimer may not provide a significant intracellular transport advantage to the free drug.

Our study indicated that the dendrimer-EM conjugates have a high drug payload (16%), improve the solubility of the drug, and could lead to improved activity. These attributes are especially important in delivering a sufficient amount of EM to the site of periosteal inflammation and reducing local inflammation in a sustained manner.

Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.nano.2010.10.008.

References


