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Polyamidoamine Dendrimer Microgels: Hierarchical Arrangement of Dendrimers into Micrometer Domains with Expanded Structural Features for Programmable Drug Delivery and Release

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Abstract

In this paper, we report on the fabrication of micron-sized dendrimer hydrogels (μ DHs) using the water-in-oil (w/o) inverse microemulsion method coupled with the highly efficient *aza*-Michael addition. EDA core polyamidoamine (PAMAM) dendrimer G5 (10 w%) and polyethylene glycol diacrylate (PEG-DA, $M_n = 575$ g/mol) (the molar ratio of amine/acrylate = 1/1) were dissolved in the water phase and added to hexane in the presence of surfactants span 80/tween 80 (5/1, w/w) (volume ratio of hexane to surfactants: 70:1) to form w/o microemulsions, in which PAMAM G5 cross-links with PEG-DA via the *aza*-Michael addition reaction. The resulting microgels are within 3–5 μ m with relatively narrow size distribution. μ DHs are pH-responsive degradable. They show good cytocompatibility and do not cause acute toxicity in vivo. Furthermore, they can realize a high loading of the hydrophobic drug CPT and enter the cells in the form of particles. The CPT and CPT/dendrimer complex can be slowly released following the zero-order release kinetics. Taken together, μ DHs possessing hierarchically ordered dendrimers in micron domains represent a new class of microparticles with expanded structural features for programmable drug delivery and release.

TOC Graphic

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Notes

The authors declare no competing interest.

ASSOCIATED CONTENTS

Supporting Information

Keywords

dendrimer; microgel; inverse microemulsion; *aza*-Michael addition; camptothecin; degradation; fluorescent dendritic micro-hydrogel

INTRODUCTION

Polyamidoamine (PAMAM) dendrimers are best-known for their well-defined hyperbranched structures and have been intensively studied to construct nanoparticulate delivery systems.^{1–4} We pioneered an unconventional concept of using PAMAM dendrimers to form a cross-linked network with polyethylene glycol (PEG), i.e., dendrimer hydrogel (DH). DHs possess unique spatial structure and configuration with tunable physiochemical properties and high flexibility in delivering drugs of different types for therapeutic applications.^{5–9} We made the first generation of DHs using photoinitiated polymerization. We found photoinitiator residue and UV-induced free radicals during the gel formation affect the AKT signaling molecule and its phosphorylated form, a biomarker indicative of proliferation and stress of cells, raising safety issue for long-term application.¹⁰ Therefore, we sought new methods to make safer formulations. We successfully made two new types of DHs with the strain-promoted azide-alkyne cycloaddition (SPAAC) bioorthogonal chemistry (commonly known as copper-free click chemistry)⁸ and the *aza*-Michael addition (aMA) reaction.^{9, 11} Although both reactions proceed efficiently in water at room temperature without the use of a catalyst, the SPAAC approach requires coupling of a strained alkyne, e.g., DBCO, to the dendrimer before the click reaction. In contrast, the aMA method directly utilizes the existing functional groups (amines of dendrimers to react with acrylate groups in PEG-DA) to form a hydrogel. We can control gel solidification (from minutes to hours) and degradation kinetics by varying reactant concentration or converting dendrimer surface amines to non-reactive acetyl (Ac) groups.¹¹ The aMa is environmentally friendly (green), efficient, and pre-functionalization of dendrimers is not needed for non-acetylated dendrimer-based formulation. Furthermore, dendrimer cryogels prepared using the aMa method shows superelasticity.¹²

Microgels are particles with a micrometer-scale three-dimensional network.^{13–15} Microgels not only inherit the properties of macroscopic hydrogels including highly hydrated and tunable mechanical and chemical properties^{16–21} but also exhibit particle features, possessing greater structural flexibility for drug delivery.^{22–29} Microgels can be fabricated

using various methods such as microemulsion, microfluidics, and nano-precipitation.^{23, 30–46} In this work, we prepared micron-sized dendrimer hydrogels (μ DHs), i.e., dendrimer microgels, using the inverse microemulsion *aza*-Michael addition (IMaMA) method. Both PAMAM dendrimer and polyethylene glycol diacrylate (PEG-DA) are dissolved in water and which then forms micro-droplets in a continuous organic phase in the presence of surfactants. The microdroplets quickly gelate to form gel particles as a result of cross-linking of dendrimer and PEG via the highly efficient *aza*-Michael addition reaction. We investigated μ DHs' degradation behaviors at various pHs and cytocompatibility. We found that μ DHs degrade via pH-dependent aminolysis. We also prepared μ DHs loaded with the anticancer drug camptothecin (CPT) by solubilizing CPT into PAMAM dendrimer in the water phase before the IMaMA procedure. We studied the kinetics of CPT release from μ DHs and found that CPT release kinetics was zero-order, the highly desirable sustained drug release profile. In addition, we found μ DHs facilitated CPT cellular uptake and potentiated CPT toxicity. μ DHs represent an advanced platform for programmable drug delivery and release.

MATERIALS AND METHODS

Materials.

EDA-core PAMAM dendrimer generation 5 (G5) was purchased from Dendritech (Midland, MI, USA). PEG-DA ($M_n = 575$ g/mol), span 80, tween 80, and cell proliferation reagent WST-1 were purchased from Sigma-Aldrich (St. Louis, MO). Camptothecin (CPT) was obtained from AK Scientific (Union City, CA). Hexane, sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), and phosphate buffered saline (PBS, 10 $\times$) were purchased from Fisher Scientific (Pittsburgh, PA). SnakeSkin dialysis tubings (3500 and 50 000 MWCO) were obtained from Thermo Scientific (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA (0.25%), and penicillin–streptomycin (10,000 U/mL) were purchased from Life Technologies (Carlsbad, CA).

Preparation of CPT-encapsulating G5 solution.

CPT in excess amounts (20 mg) was added to 1 mL water containing 100 mg G5. The mixture was vortexed briefly, sonicated for 1 h, and then left in the dark overnight. The saturated CPT/G5 solution was obtained by removing undissolved CPT via centrifugation (10 000 rpm, 3 min). CPT concentration in the CPT/G5 solution was determined at 365 nm by using an Evolution 201 UV-Visible spectrophotometer.

Preparation of μ DHs.

μ DHs were prepared by using the inverse microemulsion method. Hexane was selected as the continuous oil phase, and a mixture of span 80/tween 80 (5/1, w/w) was used as the surfactant. The oil phase was composed of hexane and surfactant (hexane/surfactants=70:1 by volume). The aqueous solution was prepared by mixing 20 mg of G5 and 25.5 mg of PEG-DA (the molar ratio of amine/acrylate = 1/1) in 180 μ L of deionized water followed by thorough vortexing for 10 s. Then the aqueous solution was immediately added to 7 mL of the oil phase and stirred at 30 000 rpm for 1 min by using an IKA disperser. The formed

microemulsions were stirred at 500 rpm for 2 h, during which G5 and PEG-DA reacted to form μ DHs within the stabilized emulsions. The emulsion solution was then centrifuged at 3000 rpm for 3 min, and the upper surfactant and hexane layer was discarded. The remainder was washed with deionized water three times to obtain surfactant-free μ DHs. Following the same method, CPT loaded μ DHs (μ DH^{CPT}) and FITC-labeled μ DHs (μ DH^{FITC}) were also prepared using CPT-encapsulating G5 (CPT/G5) and FITC-labeled G5 (**Scheme 1a**), respectively. Fourier transform infrared spectroscopy (FTIR) spectra of PAMAM G5, PEG-DA, and μ DHs were recorded on Nicolet iS5 (ThermoFisher Scientific) equipped with iD7 ATR.

Morphology, ζ Potential, size and size distribution.

SEM images of μ DH particles were taken under a scanning electron microscope (Hitachi FE-SEM Su-70, Japan). The μ DH suspension in water was dropwise added onto a silicon wafer. The samples were air dried and sputter-coated with a thin layer of Pt-Au for 90 s to make the sample conductive before testing. Size distributions based on SEM results were analyzed using ImageJ2 software. The analysis was performed on a minimum of 100 particles. Morphology of μ DHs in suspension was recorded on an Eclipse Ti-U inverted microscope (Nikon Instruments Inc., Melville, NY). ζ Potential, hydrodynamic size and size distribution of μ DHs in suspensions (1 mg/mL) were determined by using a Malvern Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, U.K.).

Drug loading and entrapment testing.

To estimate CPT loading content in μ DH^{CPT}, pre-weighed μ DH^{CPT} particles were suspended in PBS until gel particles completely degraded. CPT in the solution was quantified based on its absorbance at 365 nm using UV-Vis spectrometry on Evolution 201 UV-Visible spectrophotometer (Thermo Scientific). The drug loading efficiency was also determined based on the percentage of CPT loaded into μ DH particles.

μ DH degradation studies.

Pre-weighed μ DH particles (w_0) were suspended in pH 5.3 or pH 7.4 PBS and incubated at 37 °C for different lengths of time up to 72 h. Each incubation solution was centrifuged at 1×10^4 rpm, and the supernatant was discarded. The residues were washed with DI water twice, freeze-dried, and weighed (w_t). Mass remaining (%) is determined as $w_t/w_0 \times 100$. The particles collected at different time points were imaged using SEM. The completely degraded μ DH was analyzed by a Waters high performance liquid chromatography (HPLC) system equipped with a Waters 1515 isocratic HPLC pump, a Waters 2487 dual λ absorbance detector, and a Waters 717 plus autosampler. The mobile phase was the mixture of acetonitrile and water (acetonitrile/water = 1/1 by volume). The UV detector monitored the eluents at 220 nm.

Drug release studies.

μ DH^{CPT} particles with known quantities were suspended in pH 5.3 or pH 7.4 PBS and loaded into a dialysis tube (MWCO 3500). The dialysis tube was immersed in 30 mL of PBS and incubated at 37 °C. At pre-determined time intervals, 1 mL of PBS outside of the

dialysis tube was withdrawn for quantification of the released free CPT using UV-Vis spectrometry. One mL of fresh PBS was added to maintain a constant volume and sink condition. A control group of CPT/PBS was tested under the same condition. The release of total CPT including those in free form or complexed with G5 was studied using a dialysis tube of MWCO 50,000. All experiments were performed in triplicate.

Cytotoxicity assessment.

HN12 head and neck squamous cell carcinomas were seeded in a 96-well plate at a density of 1×10^4 cells/well. Following overnight culture in DMEM supplemented with 10% serum in 95% air/5% CO₂ at 37 °C, the culture medium in each well was replaced with 200 µL of medium containing various amounts of µDHs (final concentrations 200, 500, and 1000 µg/mL) or µDH^{CPT} (final concentrations 20, 100, and 200 µg/mL). After another 48 h of culture, cell viability was determined by using the WST-1 assay.

Cellular uptake studies.

HN12 cells were seeded in a 12-well plate at a density of 7.5×10^4 cells/well. After 24 h of cell attachment, the culture medium in each well was replaced with 2 mL of medium containing 25 µg/mL µDH^{FITC}. Following incubation for 6, 24, and 48 h respectively, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by permeation with 0.1% Triton X-100 for 10 min. Nuclei were counterstained with DAPI. The cells were imaged under an Eclipse Ti-U inverted microscope (Nikon Instruments Inc., Melville, NY).

In vivo safety assessment.

C57BL/6 mice were randomly divided into four groups (n=3) and injected intravenously with 50 µL of PBS solution containing different concentrations of µDH particles (0.1, 1, and 5 mg/mL). At 24 h post-administration, blood was collected from mice through cardiac puncture after euthanization. A blood sample taken from the untreated mice was included as a control. Inflammatory cytokines (IL-1β and TNF-α) in the blood were measured using BD OptEIA kits (BD Biosciences, San Jose, CA) following the manufacturer's protocol.

RESULTS AND DISCUSSION

Characterization of dendrimer microgels.

PAMAM dendrimers and their analog hyperbranched polymers are ideal building units to prepare hydrogels because of the highly-functionalized surface and well-defined highly branched architecture. We explored the *aza*-Michael addition reaction (**Scheme 1a**) to make in-situ forming PAMAM dendrimer hydrogels with tunable properties and applications. Inverse microemulsions are water microdroplets suspended in a continuous organic phase (w/o) and stabilized with oil-soluble surfactants.^{47, 48} We combined the inverse microemulsion method with the highly efficient *aza*-Michael addition reaction to make dendrimer microgels (µDHs). As shown in **Scheme 1b**, PAMAM dendrimer G5 and PEG-DA were dissolved in the water phase, and hexane was chosen as the continuous oil phase and mixed with span 80/tween 80. Once the two phases were mixed, a strong dispersing force was applied to generate water-in-oil (w/o) microemulsions. The micro-sized water

droplets in this micro-emulsion served as microreactors, in which the *aza*-Michael addition-based cross-linking between G5 and PEG-DA occurred and led to the formation of microgel particles. The disappearance of acrylate signals in FTIR spectra (**Figure S1**) confirms that *aza*-Michael addition-based cross-linking took place between G5 and PEG-DA.

As shown in **Figure 1**, the dendrimer microgels appear as spherical microparticles with mean diameters of $3.3 \pm 0.7 \mu\text{m}$, $4.0 \pm 0.9 \mu\text{m}$, and $3.3 \pm 0.7 \mu\text{m}$ for μDH , $\mu\text{DH}^{\text{CPT}}$, and $\mu\text{DH}^{\text{FITC}}$, respectively, as determined by SEM images. The aggregation of μDH particles shown in the SEM images is due to the volatilization of the solvent during sample preparation. As shown in **Figure 1**, the μDH s in suspension present excellent uniform dispersion. μDH s in suspensions are slightly larger than those dehydrated particles. The mean hydrodynamic diameters of μDH , $\mu\text{DH}^{\text{CPT}}$, and $\mu\text{DH}^{\text{FITC}}$ are $4.0 \pm 0.9 \mu\text{m}$, $4.0 \pm 1.0 \mu\text{m}$, and $3.7 \pm 0.8 \mu\text{m}$, respectively.

pH-Dependent degradation of μDH s.

The positively charged surface of μDH was confirmed by its ζ potential value $24.1 \pm 4.9 \text{ mV}$. It also indicates that even after cross-linking, there are still unreacted amino groups remaining. In addition, the FTIR spectrum of μDH shows the presence of unreacted amino groups (**Figure S1**). The unreacted amino groups in the cross-linked network structure of μDH s can self-trigger aminolysis of the ester bonds in the network (**Scheme 1a**). The aminolysis is highly dependent on pH, which, in turn, controls degradation kinetics of μDH s. μDH s degrade more slowly at pH 5.3 than at pH 7.4 (**Figure 2**). Based on remaining mass tracking, microgels are quite stable within 6 hours at pH 5.3, and no weight loss is observed. Even at pH 7.4, the weight loss within the first 6 hours is minimal (3%). After 24 h incubation, its 33% mass remains at pH 7.4 while its 62% mass remains at pH 5.3. μDH s degrade almost completely in 48 h at pH 7.4. In contrast, μDH s still keep 15% of the mass at pH 5.3 after 72 h incubation. The complete degradation product of the μDH s showed two peaks (1.05 min, 1.52 min) by HPLC analysis. By comparison with the spectra of raw materials (PEG-DA and G5), we believe that the peak with a 1.05 minute is G5 or G5-PEG, while the peak at 1.52 min might be extremely low cross-linked dendrimer-PEG (**Figure S2**).

Particle size reduces along with the mass loss. At pH 7.4, particle size experiences an increase from $2.8 \mu\text{m}$ at 0 h to $3.8 \mu\text{m}$ at 6 h because of swelling. At 24 h, multiple sizes are seen in the degradation product. Approximately half of the particles remain micron-sized, and the half become nanosized (20–70 nm) (**Figure 3a**). After 48 h, only nano-sized particles can be detected. The size reduction process at pH 5.3 is slower than that at pH 7.4 (**Figure 3b**), which agrees well with the mass loss. There is almost no change in both size and size distribution after 24 h incubation at pH 5.3, indicating the stability with 24 h. At 48 h, size reduces to $1.7 \mu\text{m}$ while still being micro-sized. At 72 h, only nano-sized particles are detected, indicating the complete degradation of μDH s.

We also imaged μDH degradation products throughout the degradation process (**Figure 4**, **Figure S3**). μDH s after 6 h-incubation at pH 7.4 do not show any apparent changes compared to their initial state. Micron-sized particles and nano-sized particles co-exist at 24

h. Among them, almost all the micro-sized particles soften and collapse. At 48 h, most particles are smaller than 100 nm and only a very small number of particles remain micro-sized, which appear as broken flat discs. In contrast, μ DHs after 24 h- and 48 h-incubation at pH 5.3 do not show noticeable size reduction but only show slight interparticle coalition. At 72 h, nearly all the microparticles disappear, and only nano-sized fragments remain. The DLS, SEM, and microscopy results complementarily illustrated pH-dependent degradation of μ DHs.

Zero-order drug release enabled by μ DHs.

The limited solubility of CPT in aqueous medium has significantly restricted its bioavailability. Based on our measurement, the saturated solubility of CPT in pH 7.4 PBS is approximately 64 μ g/mL. Herein, we prepared CPT/G5 complexes by entrapping the hydrophobic CPT molecules in the interior structure of PAMAM dendrimer G5. The results show that the saturated solubility of CPT increased significantly to 12 mg/mL in 10 wt% G5 PBS solution, suggesting approximately 10 CPT molecules are entrapped in each PAMAM G5 molecule. We used the CPT/G5 saturation solution to prepare μ DH^{CPT} particles. CPT loading capacity was determined to be 225 ± 12 μ g CPT/mg μ DH^{CPT} particles, and CPT encapsulation efficiency was as high as 85 ± 5 %.

CPT release rate is also pH-dependent. CPT release is slower at pH 5.3 than at pH 7.4. Four mathematical models including the zero-order model, first-order model, Higuchi square root of time model, and Hixson and Crowell cube-root model were chosen to fit the release data.^{49, 50} The release of total CPT in free drug form and complexed with G5 follows zero-order release kinetics (**Figure 5a, Table S2**). Majority of the release CPT is found to be associated with G5. At 120 h, only 27% and 12% of free CPT was released at pH 7.4 and pH 5.3, respectively. The release of CPT/G5 is accompanied by the degradation of μ DH. In the process of μ DH degradation, the original solid particles gradually soften, collapse, shrink until disappear, and the building blocks, CPT/G5, is slowly released correspondingly (**Figure 5b**). The degradation rates at pH 7.4 and pH 5.3 affect the release rate (**Figure 5b**). The release of free CPT is based on the diffusion of CPT from CPT/G5 complexes, which is a slow process controlled by the hydrophobic interaction between CPT (**Figure 5a, b**). Even though the release of free CPT is slow, we demonstrate that the CPT/G5 complexes have effective tumor cell inhibition (**Figure S5b**).

Potentiation of anticancer drug potency by μ DHs.

μ DHs show good cytocompatibility over a broad range of concentrations. They do not cause any cytotoxicity at the concentration as high as 1000 μ g/mL (**Figure 6a**). Instead, they promoted cell proliferation as seen in PEG and PEGylated dendrimers.⁵¹ G5 is more tolerated by cells in μ DHs than in free solution form (**Figure S5a**), in large part due to the decreased number of amine groups by cross-linking with PEG. We further studied the in vivo safety of μ DHs. μ DHs of different doses were administered to mice through tail vein injection. Following injection, the mice did not show any apparent signs of discomfort or pain. At 24 h, we analyzed the plasma level of cytokines (IL-1 β and TNF- α) in the treated mice. As shown in **Table 1**, the levels of IL-1 β and TNF- α in the treated mice are comparable to those in the untreated group, indicating μ DHs do not cause acute toxicity. We

further tested the cytotoxicity of $\mu\text{DH}^{\text{CPT}}$ on HN12 cells (**Figure 6b**). The selected three doses of $\mu\text{DH}^{\text{CPT}}$ are within the safety range for both G5 (**Figure S5**) and μDH s. $\mu\text{DH}^{\text{CPT}}$ at 20 $\mu\text{g/mL}$ does not cause cell death, but it causes 36% cell death at 100 $\mu\text{g/mL}$ (corresponding to 3250 nM CPT). $\mu\text{DH}^{\text{CPT}}$ shows the highest cell-killing effect at 200 $\mu\text{g/mL}$ (6500 nM CPT), which causes 71% cell death.

We studied the cellular uptake of $\mu\text{DH}^{\text{FITC}}$ particles (**Figure 7**). At 6 h, fluorescent dots are seen inside the cells. Given that μDH s do not degrade in the first 6 h of incubation, the presence of the fluorescence signals suggests that μDH s can enter the cells in the form of particles despite their relatively large size. More extended incubation with $\mu\text{DH}^{\text{FITC}}$ (24 h or 48 h) leads to accumulation of stronger fluorescence signals in the cells. This is because $\mu\text{DH}^{\text{FITC}}$ degradation products in the medium continue to be taken up by the cells. In the meantime, the internalized $\mu\text{DH}^{\text{FITC}}$ degrade into nano-sized fragments within the cells as evidenced by a relatively more uniform distribution of fluorescence signals.

CONCLUSIONS

We successfully fabricated micron-sized dendrimer hydrogels (μDH s) using the water-in-oil inverse microemulsion method coupled with the highly efficient *aza*-Michael addition. μDH s are pH-responsive degradable. They show good cytocompatibility and do not cause acute toxicity in vivo. Furthermore, they can realize a high loading of the hydrophobic drug CPT and enter the cells in the form of particles. The CPT and CPT/dendrimer complex can be slowly released following the zero-order release kinetics. Taken together, μDH s possessing hierarchically ordered dendrimers in micron domains represent a new class of microparticles enabling programmable drug delivery and release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SEM image (left panel), optical micrographs of gel particles in suspension (middle panel), and particle size and size distribution (right panel) of μDH , $\mu\text{DH}^{\text{CPT}}$, and $\mu\text{DH}^{\text{FITC}}$.

Figure 2.
Degradation of μ DH at pH 7.4 and pH 5.3.

Figure 3.
Size change of μ DH during incubation at pH 7.4 (a) and pH 5.3 (b).

Figure 4.
SEM images of μ DH after incubation for different lengths of time at pH 7.4 and pH 5.3.

a

b

Figure 5.

(a) In vitro zero-order drug release of free CPT and CPT/G5 complexes from $\mu\text{DH}^{\text{CPT}}$ at pH 7.4 and pH 5.3 at 37 °C (n = 3), the control group is free CPT released from CPT/PBS at pH 7.4. (b) CPT release from $\mu\text{DH}^{\text{CPT}}$ includes burst release of free CPT and release of CPT/G5 complexes driven by pH-dependent μDH degradation.

Figure 6.
Cytotoxicity of μDH and $\mu\text{DH}^{\text{CPT}}$ ($n = 6$).

Figure 7. Cellular uptake and intracellular distribution of fluorescent $\mu\text{DH}^{\text{FITC}}$ in HN12 cells. Scale bar: 20 μm .

Scheme 1.

a) Synthetic routes for 1) FITC labeling, 2) cross-linking reaction, and 3) the self-triggered aminolysis of the μDH degradation process. b) Preparation of dendrimer microgels using the inverse microemulsion aza-Michael addition. CPT can be encapsulated into dendrimer in the water phase and form CPT-loaded dendrimer microgels.

Table 1.

Cytokines (IL-1 β and TNF- α) in plasma at 24 h post-administration of μ DH particles.

Cytokine (ng/mL)	Untreated	μ DH particles (mg/mL)		
		0.1	1	5
IL-1β	18.34 $\pm$ 1.39	17.71 $\pm$ 4.48	18.41 $\pm$ 5.90	20.51 $\pm$ 2.94
TNF-α	11.06 $\pm$ 0.69	16.32 $\pm$ 3.04	14.70 $\pm$ 0.70	15.63 $\pm$ 7.43