Thermoresponsive Nanogels from Dendronized Copolymers for Complexation, Protection and Release of Nucleic Acids

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Abstract   A series of thermoresponsive cationic dendronized copolymers and their corresponding nanogels containing dendritic oligoethylene glycol (OEG) units and guanidine groups were prepared, and their complexation, protection, and release of nucleic acids were investigated. The dendritic OEGs endow these copolymer materials with good biocompatibility and characteristic thermoresponsiveness, while cationic guanidine groups can efficiently bind with the nucleic acids. The dendritic topology also affords the copolymers specific shielding effect which plays an essential role in protecting the activity of nucleic acids. At room temperature, dendronized copolymers and the corresponding nanogels could efficiently capture and condense the nucleic acids, while above their cloud points ($T_c$), more than 75% of siRNA could be released in 1 h triggered by ATP. More importantly, the copolymer showed protective capability to siRNA, while nanogels exhibit even better protection when compared to the copolymers due to the synergetic effect from the three-dimensional cross-linked network and high density of dendritic units in vicinity. This kind of smart dendronized copolymer nanogels form a novel class of scaffolds as promising materials for biomedical applications.

Keywords  Dendronized polymer; Guanidine; Nanogels; Thermoresponsive; Bioactive protection

INTRODUCTION

Biomacromolecules, including proteins, peptides, and nucleic acids, play pivotal roles in a range of biological processes, and can be effective components of drugs for a variety of diseases.[1] However, most of them can lose their biological activity easily in extracellular environments.[2] Recent researches suggest that formation of hybrid conjugates with synthetic polymer materials via covalent and/or non-covalent methods is an efficient way to improve the stability of these bioactives.[3] In particular, modification via supramolecular interactions such as electrostatic interactions can not only enhance the stability of proteins but also provide possibility of releasing biomacromolecules from the polymers under the designated conditions, for instance, with temperature,[4] pH,[5] redox,[6] or ATP[7] as stimulus.

A variety of polymer materials have been developed as conjugated matrix, such as hyperbranched polymers,[8] dendrimers,[9] polymer brushes,[10] hydrogels,[11] nanoparticles,[12,13] and nanogels[14] to improve the stability of biomacromolecules. Among them, nanogels with stimuli-responsive property have aroused more and more attention due to the following advantages: (1) high moisture contents and desirable mechanical features, showing great priority to encapsulate and shield guest molecules; (2) a high surface area for multivalent bioconjugation, and an internal 3D network for entrapment of bioactives, which also provides an intracellular-like microenvironment for protection of them in deleterious conditions;[15,16] (3) the stimuli-sensitivity, affording them release of bioconjugates at the desired conditions. Till now, most of the smart nanogels were constructed by linear polymers with less tunable structures and properties. Alternatively, dendronized polymers are constructed by combination of a linear polymer backbone and densely packed dendritic pendants,[17,18] which show excellent property as bioconjugates.[19,20] However, smart nanogels as conjugation matrix from dendronized polymers are rarely reported.

In a previous work, we found that oligoethylene glycol (OEG)-based dendritic polymers not only show excellent thermoresponsive behavior around physiological temperature, but also contribute tunable shielding effect due to the densely packed dendritic units along the side chains. They show great advantages for encapsulating and shielding guest molecules, which is favorable to protection of biomacromolecules.[21-23] Here, we report on one kind of stimuli-responsive nanogels from the OEG-based dendronized copolymers containing cationic guanidine moieties, and their properties when used as smart gene carriers. The corresponding dendronized copolymers were also prepared for comparison. As illustrated in Fig. 1, dendronized copolymers (P1) and their
corresponding nanogels (NGs) with different particle sizes were prepared. These non-cytotoxic dendronized copolymer nanogels carry multiple positively charged guanidine pendant units under a wide range of pH conditions (pKₐ ≈ 12.5), which should offer optimal recognition properties through ionic interactions toward negatively charged nucleic acids [27,28]. Therefore, their capability of loading, protection, and release of siRNA, a promising drug for a wide range of therapies for disease, was investigated in details.

**EXPERIMENTAL**

**Materials and Reagents**

Monomers MG1 [27], MGu, and MGu [28,29] were synthesized according to previous reports. siRNA was obtained from GenePharma Co., Ltd. (Shanghai) and configured as a solution of according to previous reports. siRNA was obtained from GenePharma Co., Ltd. (Shanghai) and configured as a solution of.

**Preparation of Nanogels (NGs)**

NGs were prepared by emulsion polymerization in water using monomers MGu and MG1 with a feed molar ratio of 10:1, 2,2′-azobis(2-methylpropionamide) dihydrochloride (AIBA) as the initiator, and EGDMA as the crosslinker. The product was purified by dialysis with an excess amount of pure water for 5 days.

**Cytotoxicity Assay/Test**

The cytotoxicity of polymers and nanogels against Ins-1 cells was evaluated by a cell counting kit-8 (CCK-8) assay. Cells were seeded in 96-well tissue culture plates with a concentration of 1000 cells/well in 100 μL culture medium. The cells were cultured at 37 °C in a 5% CO₂ atmosphere for 48 h. Then, samples of different concentrations in PBS buffer (2 μL) were added and incubated with the cells for 48 h. After that, 10 μL of CCK-8 solution was added to each well and the cells were

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**Fig. 1** Cartoon representation of loading/release of siRNA using cationic dendronized copolymers and the corresponding nanogels mediated by their thermoresponsive properties.
incubated for another 2 h in a cell culture incubator. The absorbance was then measured at 450 nm using a microplate reader. The relative cell viability (%) was directly proportional to the absorbance of formazan dye.

siRNA Binding by the Copolymers

**Gel electrophoresis method**

Briefly, 1 μL of siRNA solution and the copolymers/nanogels were incubated at different N/P ratios (1 to 50) for 1 h at 25 °C to form stable complexes for the subsequent experiments. Then complexes were mixed with 1 μL of nucleic acid loading dye and loaded into a 2% agarose gel containing TBE buffer and stained with ethidium bromide (EB). The gels were run in 0.5× TBE buffer for about 10 min at 120 V and then visualized and imaged using a Bio-Rad UV transilluminator.

**Fluorescence analysis**

The complex solution of siRNA and copolymers/nanogels was prepared according to the gel electrophoresis method. Subsequently, 1 μL of EB solution (0.1 mg·mL\(^{-1}\)) was added to the complex solution. Then fluorescence intensity of the solution with EB and siRNA complexes was measured using F-4500 FL spectrophotometer under the excitation wavelength of 470 nm and the fluorescence emission at 590 nm was recorded. Experiments were performed in triplicate, and the results were averaged. Binding ratio of siRNA = \([I_r - I_s]/I_s\) × 100% (\(I_r\) is the intensity of sample, \(I_s\) is the intensity of free siRNA, and \(I_0\) is the intensity of free polymers/nanogels).

**Assay of siRNA Release**

The complex solutions at different N/P ratios were incubated at 37 °C, which is above their \(T_{cs}\), for 1 h in the absence of presence of ATP (100 μmol·L\(^{-1}\)). Subsequently, the fluorescence intensity of the complex solution with EB was measured according to the fluorescence analysis method. Release ratio of siRNA = \([I_r - I_s]/I_s\) × 100% (\(I_r\) and \(I_s\) are the intensities of samples before and after the siRNA release, respectively).

**Protection Ability of P1/NGs to siRNA**

The complex solutions were incubated with RNase A at indicated concentrations and time intervals. After the scheduled incubation time, 1 μL of ethylenediaminetetraacetic acid (EDTA, 50 mmol·L\(^{-1}\)) was added to the mixture and incubated for 10 min to stop the enzymatic reaction. Then, 1 μL of heparin sodium salt (26.4 μg·μL\(^{-1}\)) was added and incubated at 25 °C for 1 h till the complete release of residual siRNA in the copolymers/nanogels. Subsequently, the fluorescence intensity of the complex solution with EB was measured according to the fluorescence analysis method. Residual ratio of siRNA = \([I_r - I_s]/I_s\) × 100%.

**RESULTS AND DISCUSSION**

**Synthesis of Cationic Dendronized Copolymers and Corresponding Nanogels (NGs)**

Dendronized copolymers composed of ethoxyl-terminated OEG dendrons and guanidine moieties were designed and synthesized (Scheme 1). The ethoxyl-terminated OEG-based dendritic macromonomer (MG1) was used as major constituents for the copolymers to mediate their cloud points (\(T_{cs}\)) around physiological temperature. The guanidine monomer (MGu) was selected to generate cationic functional groups for nucleic acid recognition. Dendronized copolymer P1-Boc was synthesized through free radical copolymerization of MG1 and MGu with the molar feed ratio of 10:1. The corresponding copolymer P1 with positively charged guanidinium was obtained through deprotection of P1-Boc with trifluoroacetic acid (TFA). Furthermore, three NGs with different particle sizes were prepared from the same monomers to get similar compositions as the dendronized copolymers. They were obtained through emulsion polymerization in water from MG1 and MGu (the molar ratio is 10:1) in the presence of EGDMA as the crosslinker and hexadecyl trimethyl ammonium bromide (CTAB) as the surfactant.

The chemical structure of copolymer P1 was characterized by \(^1\)H-NMR spectroscopy (Fig. S1 in the electronic supplementary information, ESI), and the composition ratio of [MG1]/[MGu] was analyzed to be 9.7:1 according to the cor-

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**Scheme 1** Synthetic routes to P1 and NGs: (a) AIBN, DMF, 65 °C, 3 h; (b) TFA, DCM, 25 °C, 4 h; (c) CTAB, AIBA, H\(_2\)O, 65 °C, 4 h.
responding proton integrations, which is consistent with the feed ratio of the comonomers with small deviation. The molecular weight of P1 was determined by GPC to be 30.4 kDa. The molar ratios of [MG1]/[MG1]+ for NGs were determined by elemental analysis, which were 12.9 for NG-1, 12.1 for NG-2, and 12.5 for NG-3. Additionally, hydrodynamic radii of these nanogels (0.05 mg·mL⁻¹ in water) were measured by DLS to be about 54, 95, and 194 nm for NG-1, NG-2, and NG-3, respectively (Fig. 2a). Their microscopic morphologies were further investigated by AFM. As shown in Figs. 2(b) and 2(c), nanogels with spherical shape showed a relatively narrow size distribution. The radius was 55 ± 8 nm for NG-1, 105 ± 20 nm for NG-2, and 205 ± 45 nm for NG-3. Above results suggested that positively charged dendronized copolymer and nanogels with three different diameters were successfully prepared through conventional polymerization techniques.

Thermoresponsive Behavior of Copolymers and the Nanogels
Copolymer P1 and NGs were all dispersed well in Tris-HCl buffer (pH 7.0) at room temperature, but their solutions transferred into opaque upon heating, indicating the typical thermoresponsiveness inherited from OEG-based dendritic polymers. The thermoresponsive behavior in Tris-HCl buffer was further investigated by UV-Vis spectroscopy, and their turbidity curves are shown in Fig. 3(a). T_{cp} of P1 was determined to be 35.5 °C. Its thermally induced phase transition was sharp (temperature window Δ ~ 1.0 °C), and very small hysteresis (< 2 °C) was observed between heating and cooling processes, indicating its fully reversible dehydration/hydration process. Three NGs also exhibited characteristic thermoresponsive behavior. Their T_{cp}s were 36.3 °C for NG-1, 35.1 °C for NG-2, and 34.9 °C for NG-3, which are close to body temperature. These nanogels with 3D-networks showed less sharp thermally induced phase transitions (Δ ~ 2 °C) than that of P1. The thermoresponsive behavior of NGs was further followed by DLS at different temperatures. As shown in Fig. 3(b), after temperature increased quickly to 38 °C, which is above their T_{cp}s, and held for 10 min, large aggregates formed with sizes of 104, 233, and 972 nm for NG-1, NG-2, and NG-3, respectively. However, they could recover to their original size after cooled quickly to 25 °C and stabilized for 10 min. This process could be repeated for multiple cycles, indicating that these NGs exhibited excellent reversibility for the thermally induced aggregations and dis-aggregations.

In vitro Cytotoxicity
Considering possible applications of these dendronized copolymer materials in biomedical fields, in vitro cytotoxicity of these polymeric materials was checked first. Briefly, INS-1 cells were incubated for 48 h with P1 and NGs of different concentrations, and cell viability was determined by CCK-8 assay. As shown in Fig. 4, cell viability reduced slightly when concentrations of samples increased from 25 μg·mL⁻¹ to 100 μg·mL⁻¹. When the concentration was as high as 100 μg·mL⁻¹, cell viability could still retain 95% for P1 and above 90% for NGs, indicating these guanidine-containing dendritic polymeric materials at given concentrations are safe to the cells.

Fig. 2  (a) The hydrodynamic radius of NGs by DLS in water at 25 °C, and atomic force microscopy images of (b) NG-1, (c) NG-2, and (d) NG-3 on mica from aqueous solutions of 0.02 mg·mL⁻¹.
siRNA Binding and Release
To check the potential bio-applications of these dendronized copolymeric materials, the binding ability of P1 and NGs with siRNA was evaluated with a fluorescence spectrophotometer using EB as indicator. For all materials, the binding ratio increased dramatically with increase of N/P ratio and then tended to be stable after the N/P ratio was over 40/1 (Fig. 5a). P1 (9.1 mol% of Gu+) showed a binding ratio of 54.2% even at a high N/P ratio of 50. In contrast, NGs exhibited better binding ability compared to P1 at the same N/P ratio, and the binding ratio increased with an increase of particle size (65.1% for NG-1, 81.6% for NG-2, and 98.7% for NG-3). In particular, NG-3 with the largest size showed the highest binding ability, indicating its enhanced binding affinity for siRNA. The high binding ability of NGs was also confirmed by agarose gel electrophoresis as illustrated in Fig. S2 (in ESI). Considering NGs had similar Gu+ content to P1, such remarkable performance could be attributed to their particle sizes, spherical morphology, and crosslinked 3D-network structure. Compared to P1 that adopted worm-like morphology in solutions with sizes of 5−15 nm,[22,25,27] NGs contained crosslinked polymers with significantly larger sizes (radius between 50−200 nm), which may be advantageous for condensation of siRNA. Besides, the synergistic effects from the densely packed OEG dendrons within the NGs from different polymer chains should also have enhanced the encapsulation of guest siRNA, as demonstrated in our previous work on related OEG-based dendronized copolymers.[22,32]

Thermally induced collapse of OEG-based dendronized copolymers have been proven before by our group to show characteristic behavior in mediating guest molecules to reversibly shift or encapsulate within the polymer matrix.[22,25,27] Therefore, release of siRNA from the positively charged copolymer materials during phase transition was examined. As shown in Fig. 5(b), for P1 and NGs complexed with siRNA, at temperature of 37 °C which is above their T_{cp}, siRNA...
can be released but with a ratio of less than 40% (15.0% for P1, 15.1% for NG-1, 25.2% for NG-2, and 36.9% for NG-3). The low release ratios of siRNA may be attributed to the strong binding interaction between guanidine moieties (Gu−) and siRNA. In order to increase release efficiency of siRNA, ATP with a concentration of extracellular ATP in tumor tissue (100 μmol·L−1) was added for detachment of siRNA that adhered to Gu− and promotion of its release. It was found that in the presence of ATP, all polymers could release more than 40% of siRNA at 25 °C (below their Tre5) (Fig. S3 in ESI). Interestingly, when increasing the temperature to 37 °C (above their Tre5), more than 78% of siRNA could be released in 1 h for P1 and all NGs. The release of siRNA from NG-3 could even reach 99.6%. These results indicated that efficient release of siRNA could be realized at a temperature above their Tre5 and triggered with ATP. We believe this finding can be interesting for targeted release of siRNA to be controlled simultaneously through two stimuli cooperatively.

siRNA Protection
Biomacromolecules are prone to be degraded by nucleases or enzymes from plasma and tissue, which is a challenge for their use as drugs. Protection ability of P1 or NGs towards siRNA in solutions was therefore investigated by incubating with RNase A, an endoribonuclease that specifically hydrolyzes siRNA. Firstly, protection ability under pH 5.5 (Tris-HCl buffer, 20 mmol·L−1) was tested. Stability of the naked siRNA was checked for comparison, and high enzyme activity was observed and all siRNA degraded completely within 1 h (Fig. 6a). In contrast, siRNA encapsulated in nanogels showed much better stability. After 3 h, 28.7%, 35.6%, and 47.6% of siRNA was left for NG-1, NG-2, and NG-3, respectively. These indicate significant protection effect of the dendronized polymeric nanogels for siRNA from the enzyme, which is much enhanced when compared to the worm-like polymer P1 (less than 14.6%). Interestingly, nanogels with larger size showed better protection tendency to siRNA. It suggests siRNA may need longer time to diffuse out of the larger particles due to the enhanced encapsulation. Even after 6 h, there was still over 16% (16.1% for NG-1, 26.2% for NG-2, 35.4% for NG-3) of siRNA left in these nanogels.

The protection ability of nanogels to siRNA under different concentrations of the enzyme was further tested. As shown in Fig. 6b), the amount of siRNA residual in all NGs was decreased but still more than 20% was retained even when enzyme concentration was increased from 0.5 mg·mL−1 to 8 mg·mL−1 (16 times). However, when compared to those in NGs, siRNA in the solution of copolymer P1 was decreased by more than 20% and was retained even when enzyme concentration was increased to 8 mg·mL−1. Similar to the above, the siRNA residual amount in nanogels increased with increase of the particle size. These results suggested that all nanogels, especially those with larger particle sizes, could effectively protect siRNA from nuclease degradation. We ascribe this to the synergistic effect of shielding ability from dendritic architecture of OEG-based dendritic moieties together with the nanogels morphology. As proved previously, these densely packed OEG dendrons along a polymer backbone provide confined microenvironments to encapsulate the guest molecules and even can prevent their protonation, coordination, or interaction with other reactants.

Fig. 6 Quantitative residual content of siRNA in P1 and NGs after incubated with 0.5 mg·mL−1 of RNase A, (a) at different time intervals and (b) with different concentrations of RNase A for 1 h.

CONCLUSIONS
In summary, a novel kind of thermoresponsive OEG-based cat-ionic dendronized copolymers and their corresponding nanogels were successfully synthesized and prepared. They showed high binding ability to siRNA at room temperature, but could release them efficiently when triggered simultaneously by ATP and thermally induced collapse. These dendronized polymers also provided characteristic protection to siRNA under different pH, temperature, and even in the presence of RNAzymes. Especially, nanogels showed better binding and protection capability when compared with the corresponding worm-like copolymers, benefitting from cooperative effects of the spherical morphology, 3D-network, and high OEG density in vicinity. We therefore believe these stimuli-responsive dendritic materials can be promising candidates as “smart” drug carriers.

Electronic Supplementary Information
Electronic supplementary information (ESI) is available free of charge in the online version of this article at https://doi.org/10.1007/s10118-020-2452-4.

ACKNOWLEDGMENTS
We sincerely thank Dr. Hongmei Deng from the Instrumental
Analysis and Research Center of Shanghai University for her assistance with NMR measurements. This work was financially supported by the National Natural Science Foundation of China (Nos. 21971161, 21971160, and 21574078), Shanghai Pujiang Program (No. 19PJ1403700), and Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning.

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https://doi.org/10.1007/s10118-020-2452-4