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

Yi Yao, Jian-Hui Wu, Shi-Jie Cao, Bi-Yi Xu, Jia-Tao Yan, Di Wu, Wen Li*, and Afang Zhang*

Department of Polymer Materials, School of Materials Science & Engineering, Shanghai University, Shanghai 200444, China

Electronic Supplementary Information

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_{cp} s), 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

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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 multi-valent 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.^[21–25] 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 authors, E-mail: wli@shu.edu.cn (W.L.)

E-mail: azhang@shu.edu.cn (A.Z.)

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corresponding nanogels (NGs) with different particle sizes were prepared. These non-cytotoxic dendronized copolymer nanogels carry multiple positively charged guanidine pendants under a wide range of pH conditions ($pK_a \approx 12.5$), which should offer optimal recognition properties through ionic interactions toward negatively charged nucleic acids.^[7,26] 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 $0.264 \mu\text{g}\cdot\mu\text{L}^{-1}$. The sequence information of siRNA is 5'-CGUACGCGGAUACUUCGAdTdT-3' (sense); 5'-UCGAAGUAUCCGCGUACGdTdT-3' (antisense). Ribonuclease A (RNase A) was purchased from Thermo Fisher Scientific. Azobisisobutyronitrile (AIBN) was recrystallized twice from methanol. Ethylene glycol dimethacrylate (EGDMA) was passed through a basic alumina column before use. *N,N*-dimethylformamide (DMF) was distilled from CaH_2 . Dichloromethane (DCM) was dried by refluxing over sodium. Pure water was redistilled before use. Other reagents and solvents were of reagent grade and used without further purification. Macherey-Nagel pre-coated TLC plates (silica gel 60 G/UV254, 0.25 mm) were used for thin-layer chromatography (TLC) analysis. Silica gel 60 M (Macherey-Nagel, 0.04–0.063 mm, 200–300 meshes) was used as the stationary phase for column chromatography.

Instrumentation

¹H-NMR spectra (500 MHz) were recorded on a Bruker AV 500. Gel permeation chromatography (GPC) measurements were carried out on a Waters GPC e2695 instrument with DMF as the eluent. Cloud points (T_{cp} s) were measured on a UV-Vis spectrometer (Lambda 35) equipped with a thermostatically regulated bath. T_{cp} was determined as the temperature at which the transmittance at $\lambda = 700 \text{ nm}$ reached 50% of its initial value. Atomic force microscopy (AFM) experiments were carried out

on a Bruker Nanoscope VIII Multi-Mode operated by Peak Force. Dynamic light scattering (DLS) measurements were performed on DynaPro Nanostar (Wyatt Technology Corporation). Fluorescent (FL) experiments were carried out on Horiba Jobin Yvon Fluorolog^R-3 with FluorEssenceTM.

Synthesis of Copolymers

Dendronized copolymer **P1-Boc**

MG1 (200 mg, 0.284 mmol), **MGu** (11.80 mg, 0.028 mmol), and AIBN (1.0 mg) were dissolved in dry DMF (0.3 mL) in a Schlenk tube. The solution was deoxygenated and stirred at 65 °C for polymerization. After 4 h, the mixture was cooled to room temperature. The polymer was purified by column chromatography (silica gel, DCM as eluent) to yield the polymer as a colorless oil (348 mg, 66%). ¹H-NMR (DMSO- d_6 , δ , ppm): 0.88 (br, CH_3), 1.09 (t, CH_3), 1.39 (br, CH_3), 3.44–3.69 (m, CH_2), 4.01 (m, CH_2), 4.81 (d, CH_2) 6.63 (br, Ar-H).

Dendronized copolymer **P1**

P1-Boc (120 mg) was dissolved in a mixture of DCM/ $\text{CF}_3\text{CO}_2\text{H}$ (10 mL, 1/1, V/V) solution and stirred at room temperature. After 3 h, the mixture was evaporated to dryness to give **P1** as a colorless oil (92 mg, 76%). ¹H-NMR (DMSO- d_6 , δ , ppm): 0.84 (br, CH_3), 1.05–1.15 (m, CH_3), 1.28 (br, CH_2), 1.80 (br, CH_3), 3.35–4.17 (m, CH_2), 4.42 (br, CH_2), 4.80 (br, CH_2), 6.62 (m, CH_2).

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% CO_2 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

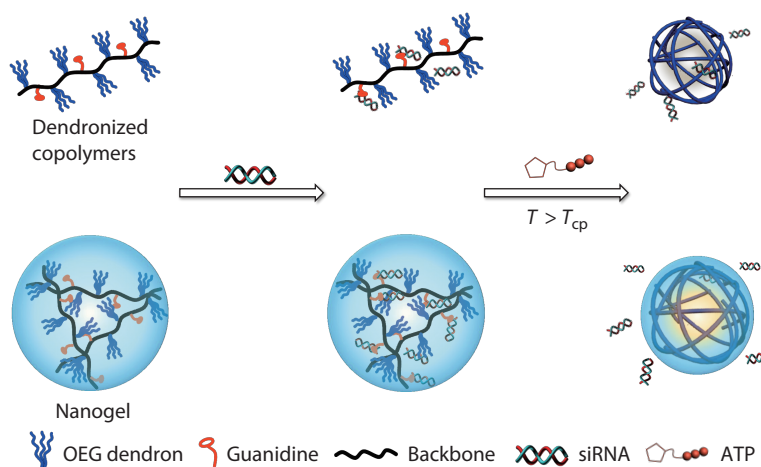


Fig. 1 Cartoon representation of loading/release of siRNA using cationic dendronized copolymers and the corresponding nanogels mediated by their thermoresponsive properties.

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 $^{\circ}\text{C}$ 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 $\times$ TBE buffer for about 10 min at 120 V at room temperature 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 $\text{mg}\cdot\text{mL}^{-1}$) 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 = $[(I_r - I_s)/(I_r - I_0)] \times 100\%$ (I_s is the intensity of sample, I_r 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 $^{\circ}\text{C}$, which is above their T_{cp} s, for 1 h in the absence or presence of ATP (100 $\mu\text{mol}\cdot\text{L}^{-1}$). Subsequently, the fluorescence intensity of the complex solution with EB was measured according to the fluorescence analysis method. Release ratio of siRNA = $[(I'_s - I_s)/(I_r - I_0)] \times 100\%$ (I'_s 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

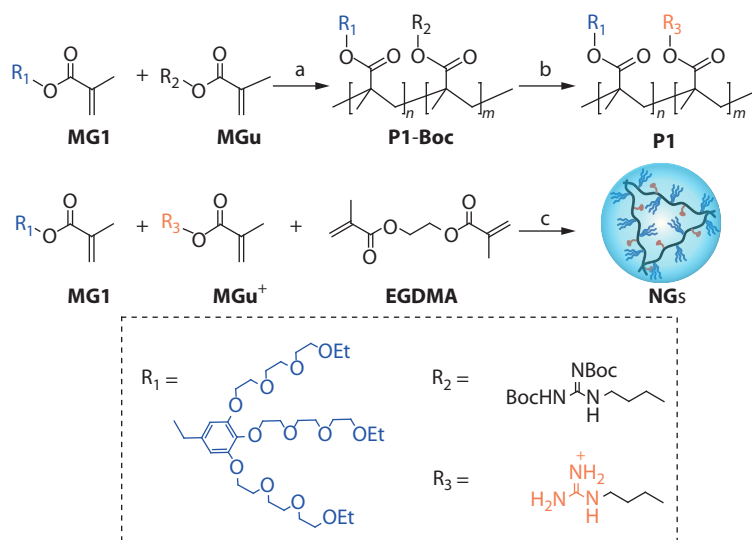
indicated concentrations and time intervals. After the scheduled incubation time, 1 μL of ethylenediaminetetraacetic acid (EDTA, 50 $\text{mmol}\cdot\text{L}^{-1}$) was added to the mixture and incubated for 10 min to stop the enzymatic reaction. Then, 1 μL of heparin sodium salt (26.4 $\mu\text{g}\cdot\mu\text{L}^{-1}$) was added and incubated at 25 $^{\circ}\text{C}$ 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 = $[(I_s - I_0)/(I_r - I_0)] \times 100\%$.

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_{cp} s) 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 ^1H -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-



Scheme 1 Synthetic routes to **P1** and **NGs**: (a) AIBN, DMF, 65 $^{\circ}\text{C}$, 3 h; (b) TFA, DCM, 25 $^{\circ}\text{C}$, 4 h; (c) CTAB, AIBA, H_2O , 65 $^{\circ}\text{C}$, 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]/[MGu⁺] 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.^[30] Their 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 $\Delta \sim 1.0$ °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 ($\Delta \sim 2$ °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.

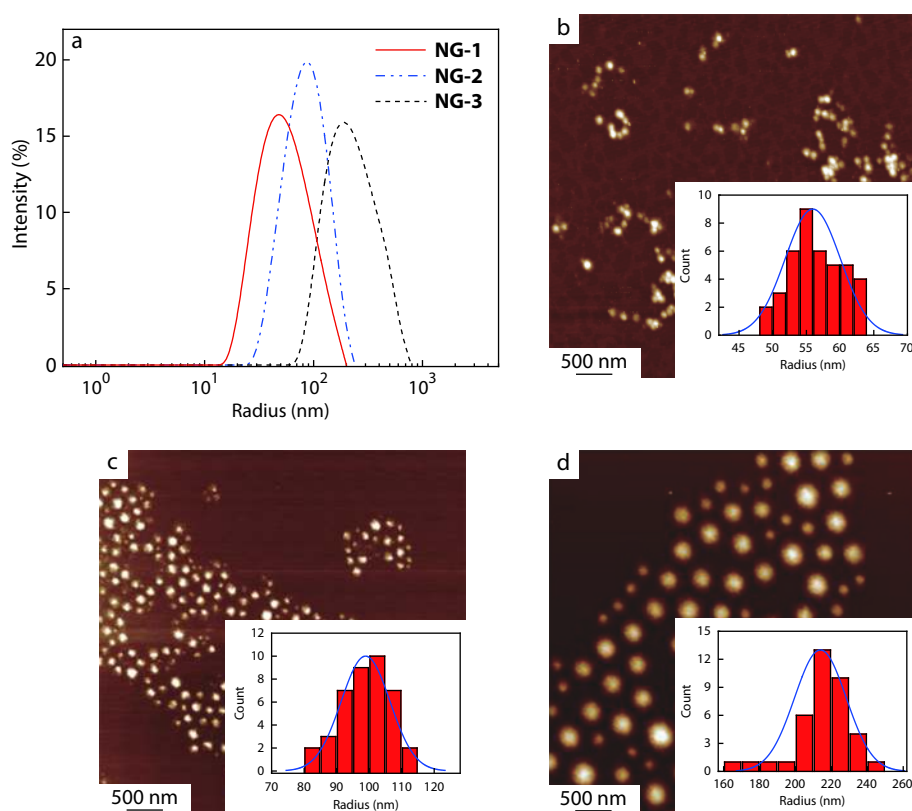


Fig. 2 (a) The hydrodynamic radius of **NGs** by DLS in water at 25 °C, and atomic force microscopy images of (b) **NG-1**, (c) **NG-2**, and (d) **NG-3** on mica from aqueous solutions of 0.02 mg·mL⁻¹.

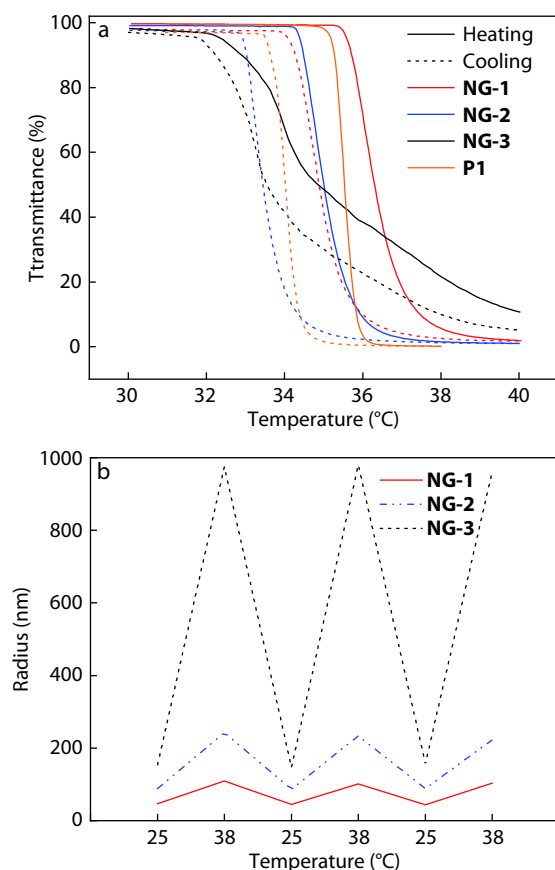


Fig. 3 (a) Plots of transmittance versus temperature for **P1** and **NGs** in Tris-HCl buffer (pH 7.0, 20 mmol·L⁻¹) at 0.5 mg·mL⁻¹; (b) Hydrodynamic radii of **NGs** between 25 and 38 °C. $c = 0.05$ mg·mL⁻¹.

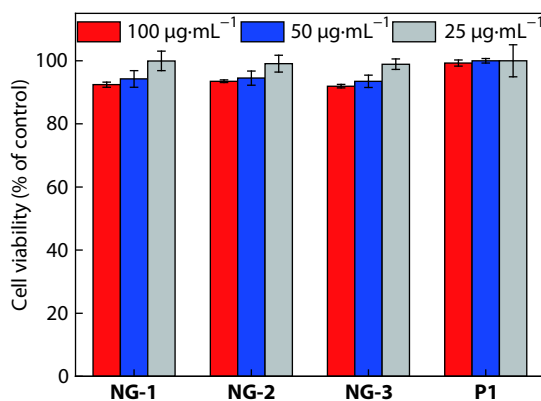


Fig. 4 Cell viability of INS-1 cells after incubation with cationic copolymer **P1** or **NGs** at different concentrations.

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

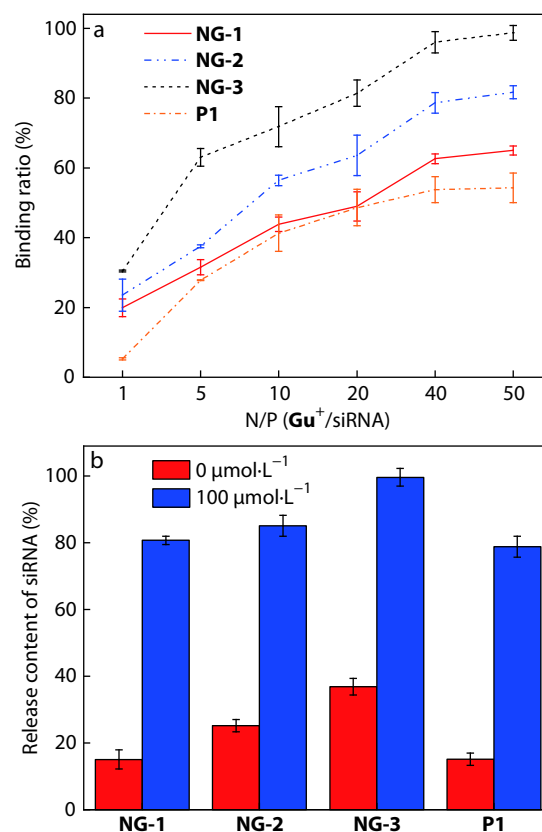


Fig. 5 (a) Binding ratios of siRNA at different N/P ratios (25 °C) for **P1** and **NGs**. (b) Release of siRNA from **P1** and **NGs** at 37 °C in Tris-HCl Buffer (pH 7.0). Column in red: absence of ATP; column in blue: [ATP] = 100 μmol·L⁻¹. N/P ratio = 50.

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,^[31] **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} s, 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 $\mu\text{mol}\cdot\text{L}^{-1}$)^[33] was added for detachment of siRNA that adhered to **Gu**⁺ and promotion of its release.^[34,35] It was found that in the presence of ATP, all polymers could release more than 40% of siRNA at 25 °C (below their T_{cp} s) (Fig. S3 in ESI). Interestingly, when increasing the temperature to 37 °C (above their T_{cp} s), 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 T_{cp} s 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 $\text{mmol}\cdot\text{L}^{-1}$) 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%). Interesting, 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. 6(b), 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 $\text{mg}\cdot\text{mL}^{-1}$ to 8 $\text{mg}\cdot\text{mL}^{-1}$ (16 times). However, when compared to those in **NGs**, siRNA in the solution of copolymer **P1** was more sensitive to the enzyme concentration, and only 6% of siRNA was retained when enzyme concentration was increased to 8 $\text{mg}\cdot\text{mL}^{-1}$. 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.^[27]

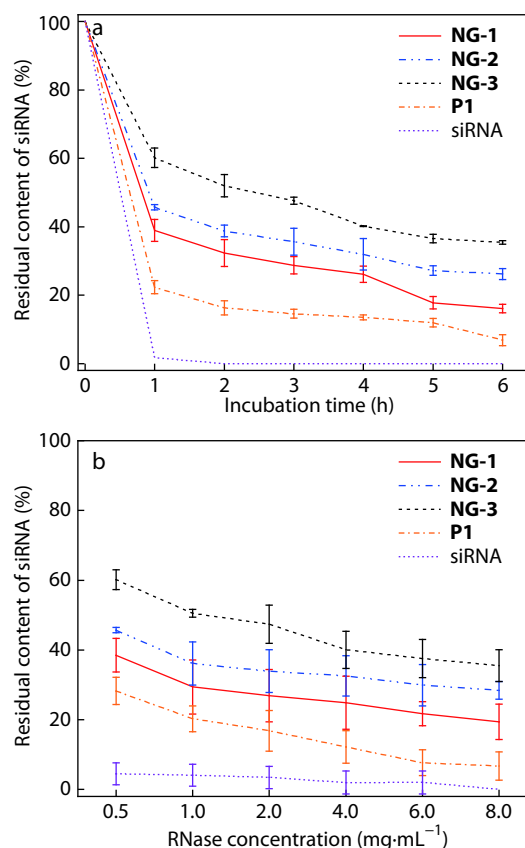


Fig. 6 Quantitative residual content of siRNA in **P1** and **NGs** after incubated with 0.5 $\text{mg}\cdot\text{mL}^{-1}$ of RNase A, (a) at different time intervals and (b) with different concentrations of RNase A for 1 h.

CONCLUSIONS

In summary, a novel kind of thermoresponsive OEG-based cationic 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, benefiting 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>.

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