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Preparation of Reduction-sensitive Nanogels with a Large Swelling Capacity by a Surfactant-free Precipitation Method

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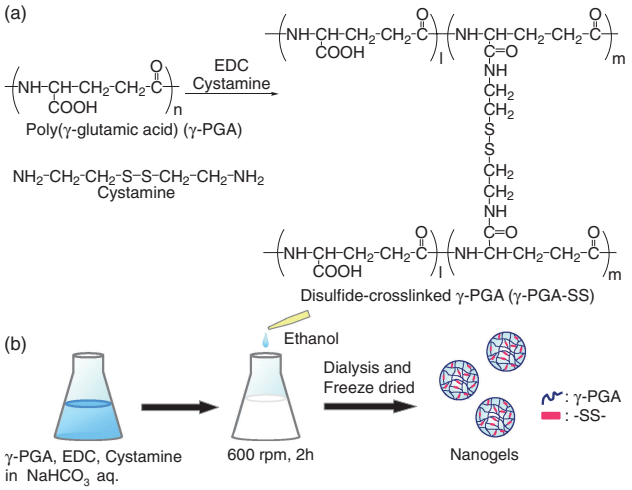
Reduction-sensitive nanogels with a large swelling capacity were successfully prepared by the crosslinking reaction of biodegradable polymers in water, followed by the insolubilization of the polymers with the gradual addition of ethanol. The obtained nanogels were ca. 200–300 nm in diameter, and had a high water content, over 99%. Moreover, these nanogels were easily decomposed by the cleavage of the disulfide crosslinkages to their thiol groups using a reductant. These biocompatible and biodegradable nanogels prepared without toxic surfactants will be useful as novel carriers for drug delivery systems.

Polymeric micelles, nanospheres, and capsules have been widely studied as carriers for drug delivery systems (DDS) and for diagnosis.[1] Among them, nanosized hydrogels (nanogels) have attracted increasing attention because of their high water content analogous to biological systems, facile immobilization of target drugs and proteins, and flexible design for stimuli responsiveness to temperature, pH, or biomolecules.[2] Although nanogels are conventionally prepared using surfactant-stabilized oil in water (O/W) emulsions,[3] these methods are limited in the biomedical field due to the multistep procedures and toxic surfactants. Recently, a self-assembling system of amphiphilic graft copolymers was developed using a surfactant-free method, but these nanogels have a limitation in that the size of the obtained nanogels was below 50 nm.[4] Nanocarriers with diameters over 200 nm are desirable for vaccination systems, because the ideal size for uptake by dendritic cells is known to be over 200 nm.[5] Therefore, a simple and nontoxic approach for the preparation of biocompatible nanogels with diameters of several hundreds of nm in size is desirable in DDS, especially for vaccination.

Poly(γ-glutamic acid) (γ-PGA) is a naturally occurring polymer secreted by a *Bacillus subtilis* strain, and has biocompatible and biodegradable properties.[6] Recently, we reported that disulfide-crosslinked γ-PGA (γ-PGA-SS)-based materials, such as hydrogels and nonwovens,[7] could be decomposed by biological reductants, cysteine or glutathione via the cleavage of the disulfide bond to the thiol groups. Therefore, if nanogels composed of γ-PGA-SS polymers can be prepared by a simple and nontoxic method, then these nanogels can be useful as novel DDS carriers due to their biocompatibility and reduction sensitivity.

Herein, reduction-sensitive γ-PGA-SS nanogels with a large swelling capacity were prepared by a surfactant-free precipitation method consisting of the gradual addition of a poor solvent, ethanol, into a reaction solution containing γ-PGA, a disulfide-crosslinker, and a condensation reagent during the crosslinking reaction (Scheme 1). To the best of our knowledge, this is the first example of the preparation of biocompatible nanogels possessing diameters of ca. 200–300 nm without using any surfactants.



Scheme 1. (a) Synthesis of disulfide-crosslinked γ-PGA (γ-PGA-SS) and (b) illustration of the preparation method of γ-PGA-SS nanogels.

Three wt% of γ-PGA and cystamine were dissolved in 5 mL of 0.5 M sodium hydrogen carbonate ($NaHCO_3$) solution in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and the crosslinking reaction proceeded for 2 min at room temperature (Scheme 1a). The feed ratio of the amine groups of cystamine to the carboxyl groups of γ-PGA ([NH_2]/[COOH]) was adjusted to 1:1, 2:1, and 4:1, respectively. Next, ethanol, which is a poor solvent for γ-PGA, was gradually added to the reaction solution under magnetic stirring at 600 rpm for 2 h. After this reaction, the obtained solution became cloudy. The unreacted compounds were removed by dialysis for 3 days in ultra pure water, and then the resultant solution was freeze-dried for 3 days to obtain the γ-PGA-SS nanogels (Scheme 1b). The formation of the nanogels was observed under the conditions of a [NH_2]/[COOH] ratio = 1:1 and 2:1, whereas the rapid aggregation was formed under the conditions of [NH_2]/[COOH] = 4:1 (Table S1).[10] The crosslinking of γ-PGA with cystamine was confirmed by FT-IR spectroscopy (Figure S1).[10] The shift of the IR peaks assigned to the amide I and II groups ($\nu_{C=O}$: from 1580 to 1630 cm^{-1} and δ_{N-H}: from 1537 to 1527 cm^{-1}) was observed after the reaction, confirming the amide bond formation between γ-PGA and cystamine. Scanning electron microscopic (SEM) observation clearly indicated the successful preparation of monodispersed nanogels under the conditions of [NH_2]/[COOH] = 2:1, and their mean diameter was 270 ± 8 nm (Figure 1a and Table S1).[10] Surpris-

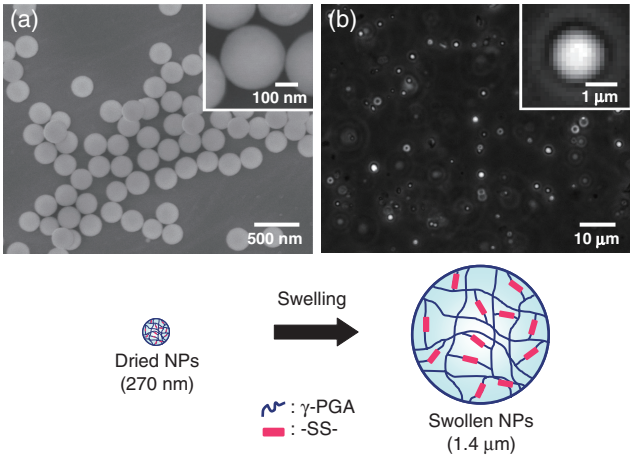


Figure 1. Scanning electron microscopic and differential interference contrast images of nanogels prepared at a $[NH_2]/[COOH]$ ratio = 2:1 in the (a) dried and (b) swollen state.

ingly, when the nanogels were immersed in water, the diameter drastically and rapidly increased over 5-fold to 1390 ± 300 nm (Figure 1b). The estimated water content of the swelling gel particles was over 99.3% (water content ratio = $(V_s - V_d)/V_s$, V_s and V_d were the volume of the reswollen and dried particles, respectively), and the gel particles were stable in aqueous solution for more than 1 week. Micrometer-sized freeze-dried gel particles of 2.6 ± 0.7 μm diameter were obtained at a $[NH_2]/[COOH]$ ratio = 1:1, and these particles also revealed a high swelling capacity in aqueous solution (Table S1).[10] However, these obtained microgels were unstable in water, and the collapse of the gel particles and the ejection of the polymer chains from the particles were observed (Figure S2).[10] Accordingly, the γ-PGA-SS nanogels prepared at $[NH_2]/[COOH]$ = 2:1 were used for further studies.

Since the γ-PGA-SS nanogels were crosslinked with disulfide bonds, reduction-sensitive degradability was expected in response to biological reductants such as cysteine or glutathione. This specific degradation property is useful for functional DDS carriers. We selected dithiothreitol (DTT) as a model reductant because it is widely employed for reduction experiments.[7,8] When γ-PGA-SS nanogels were immersed in 20 mM DTT solution at room temperature, the white turbidity of the nanogel solution gradually disappeared, and the solution became transparent after 48 h of incubation. The size distribution and particle morphology were then evaluated by dynamic light scattering (DLS) measurements and SEM observation (Figure 2). The monodispersed distribution was drastically changed to polydispersity, and SEM observations clearly revealed fibrous constructs after the treatment, supporting the decomposition of the nanogels. To investigate this decomposition behavior in detail, the time-dependent morphological changes of the nanogels were observed by SEM (Figure S3).[10] The spherical morphology gradually changed to a fibrous structure depending on the incubation time, and no nanogels could be observed after 48 h of DTT treatment.

To confirm the cleavage of the disulfide bond of the nanogels after DTT treatment, the thiol groups in the decomposed samples were detected by UV–vis spectroscopy using Ellman's method (Figure S4a).[9,10] The decomposed samples

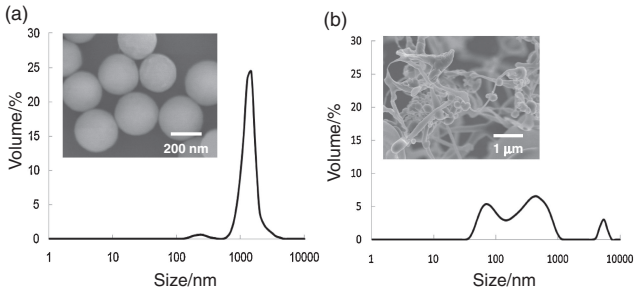


Figure 2. Size distributions and SEM images (insets) of nanogels ($[NH_2]/[COOH]$ = 2:1) (a) before and (b) after incubation in 20 mM DTT solution for 48 h.

clearly showed absorbance at 412 nm, indicating the formation of thiol groups, whereas the original γ-PGA and γ-PGA-SS nanogels did not show any absorbance. Furthermore, the appearance of an IR peak assigned to the S–H stretching at 2540 cm^{-1} (ν_{S-H}) of the decomposed nanogels also revealed thiol formation after the decomposition (Figure S4b).[10] These results indicated that the γ-PGA-SS nanogels were decomposed by cleavage of the disulfide bonds in response to the reductant.

In conclusion, we successfully prepared reduction-sensitive nanogels with a large swelling capacity of 200 nm diameter by a surfactant-free precipitation method. The obtained nanogels showed high stability in aqueous solution, and reduction-sensitive degradability. These γ-PGA-SS nanogels would be useful as novel functional carriers in nanoparticle vaccine systems.

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References and Notes

1 a) K. Kataoka, A. Harada, Y. Nagasaki, *Adv. Drug Delivery Rev.* **2001**, *47*, 113. b) T. Akagi, M. Baba, M. Akashi, *Polymer* **2007**, *48*, 6729. c) X. W. Lou, L. A. Archer, Z. Yang, *Adv. Mater.* **2008**, *20*, 3987.
2 a) S. Nayak, L. A. Lyon, *Angew. Chem., Int. Ed.* **2005**, *44*, 7686. b) S. Balthasar, K. Michaelis, N. Dinauer, H. von Briesen, J. Kreuter, K. Langer, *Biomaterials* **2005**, *26*, 2723. c) N. Morimoto, X.-P. Qiu, F. M. Winnik, K. Akiyoshi, *Macromolecules* **2008**, *41*, 5985. d) H. Hayashi, M. Iijima, K. Kataoka, Y. Nagasaki, *Macromolecules* **2004**, *37*, 5389. e) V. Lapeyre, I. Gosse, S. Chevreux, V. Ravaine, *Biomacromolecules* **2006**, *7*, 3356.
3 a) K. Landfester, *Angew. Chem., Int. Ed.* **2009**, *48*, 4488. b) K. McAllister, P. Sazani, M. Adam, M. J. Cho, M. Rubinstein, R. J. Samulski, J. M. DeSimone, *J. Am. Chem. Soc.* **2002**, *124*, 15198. c) J. K. Oh, D. J. Siegwart, H. Lee, G. Sherwood, L. Peteanu, J. O. Hollinger, K. Kataoka, K. Matyjaszewski, *J. Am. Chem. Soc.* **2007**, *129*, 5939.
4 a) K. Akiyoshi, S. Deguchi, N. Moriguchi, S. Yamaguchi, J. Sunamoto, *Macromolecules* **1993**, *26*, 3062. b) N. Morimoto, T. Endo, M. Ohtomi, Y. Iwasaki, K. Akiyoshi, *Macromol. Biosci.* **2005**, *5*, 710.
5 H. Kim, T. Uto, T. Akagi, M. Baba, M. Akashi, *Adv. Funct. Mater.*, in press. doi:10.1002/adfm.201000021
6 H. Kubota, Y. Nambu, T. Endo, *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31*, 2877.
7 a) M. Matsusaki, H. Yoshida, M. Akashi, *Biomaterials* **2007**, *28*, 2729. b) H. Yoshida, M. Matsusaki, M. Akashi, *Adv. Funct. Mater.* **2009**, *19*, 1001. c) H. Yoshida, K. Klinkhammer, M. Matsusaki, M. Möller, D. Klee, M. Akashi, *Macromol. Biosci.* **2009**, *9*, 568.
8 a) F. Meng, W. E. Hennink, Z. Zhong, *Biomaterials* **2009**, *30*, 2180. b) J. K. Oh, D. J. Siegwart, K. Matyjaszewski, *Biomacromolecules* **2007**, *8*, 3326.
9 G. L. Ellman, *Arch. Biochem. Biophys.* **1959**, *82*, 70.
10 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

