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pH-sensitive nanoparticles self-assembled from a novel class of biodegradable amphiphilic copolymers based on chitosan

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Abstract A new type of biodegradable amphiphilic graft copolymers, PEG-*g*-hexanoyl chitosan, was synthesized by a facile scheme. The self-assemble properties of the copolymers were studied by TEM, fluorometry and dynamic light scattering. It was found that spherical nanoparticles of the copolymers could be formed through dialysis method. With the increase of PEG content in the copolymers, the average diameter of the nanoparticles decreased from about 180 to 40 nm. PEG fraction in the copolymers has little effect on the copolymer CAC. The micellization of the copolymers was strongly dependent of the medium pH. When pH was lowered from 7.2 to 6.8, the average diameter of the nanoparticles dramatically changed from about 180 to 60 nm.

1 Introduction

Biodegradable nanoparticles have been paid wide attention in the field of controlled drug release during the last decade due to their nanosized characteristic, capacity of solubilization of hydrophobic drugs and passive targeting of cancerous or inflamed tissues through the enhanced permeation and retention effect etc. [1, 2]. By tailoring the polymer structure, intelligent nanoparticles which can response to the changes in temperature [3–5], light [6, 7] or pH [8–11] can be obtained. Among them, pH-sensitive nanoparticles that can dissociate and release the incorporated drugs at more acidic conditions have been studied extensively. Since the pH value is 6.0–7.0 around the solid tumors in patients and about 5.0 in endosome of normal cells, these vehicles may be useful for the delivery of therapeutic agents in the vicinity of tumor mass and for the delivery of therapeutic agents that must escape endosomal compartmentalization prior to fusion with lysosomes [12]. The rationale for designing pH-sensitive nanoparticles was based on either the protonation/deprotonation of polycations [8–11] or acid-sensitive bonding between drugs and amphiphilic copolymers [13]. Several types of polycation-based copolymers have been used for constructing pH-sensitive nanoparticles, including poly(histidine) [9], poly(2-(diisopropylamino)ethyl methacrylate) [10] and poly(β-amino ester) [11] etc.

Chitosan (CS) is composed of β-(1 → 4)-2-amino-2-deoxy-D-glucopyranose residues with little or no β-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose units. As the *N*-deacetylated derivative of chitin, chitosan is not only naturally abundant but also has many distinctive properties such as biocompatibility, biodegradability, antimicrobial activity and remarkable affinity to proteins [14]. However, the poor solubility of chitosan in both water and organic solvents limits its effective utilization in many fields [15]. Previously, several derivation methods were proposed for solubilizing chitosan in either water or common organic solvents. For example, *N*,*O*-acylated chitosan can be conveniently prepared by acylation reaction using $MeSO_3H$ as a solvent [16, 17]. The resultant chitosan derivative can be soluble in dimethylformamide (DMF) or chloroform. In addition, acylation of amino groups carried by chitosan can be partially avoided without the need of any protection–deprotection processes.

In this communication, poly(ethylene glycol) (PEG) was conjugated to hexanoyl chitosan to obtain amphiphilic graft copolymers. The self-assembly properties of the

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copolymers were studied by several techniques. The results shown in this work indicate that the copolymers may represent a novel class of biomaterials for constructing pH-sensitive nanoparticles.

2 Experimental

2.1 Materials

Chitosan (MW 300,000, deacetylation degree 91.15%) was purchased from Qingdao Haihui Biotech. Co. (Qingdao, China). Methylsulfonic acid was obtained from Shanghai Xingao Chem. Co. (Shanghai, China). Hexanoyl chloride and poly(ethylene glycol) monomethyl ether (Me–PEG–OH, M_n 2,000) were supplied by Aldrich-Sigma (Milwaukee, WI). Triphenyl phosphite and methyl iodide were from Yuhuan Biochem. Reagent Ltd. (Yuhuan, China). Hexanoyl chloride was distilled before use. Other chemicals were used as received.

2.2 Synthesis of poly(ethylene glycol) monomethyl ether iodide (Me–PEG–I)

Me–PEG–I was synthesized according to the method previously reported [18]. In brief, Me–PEG–OH (50 g, 25 mmol) was dissolved in toluene (100 ml) and trace of water was removed by azeotropic distillation. Triphenyl phosphite (19.7 ml, 75 mmol) and methyl iodide (4.67 ml, 75 mmol) were added into the remaining PEG and the mixture was stirred at 120°C for 6 h in darkness. The reaction mixture was cooled, dissolved in 100 ml of toluene and precipitated to 2,000 ml of ethyl ether. The product was recovered by filtration and dried in vacuum at room temperature. ^{1}H NMR ($CDCl_3$): 3.26 (t, $OCH_2C\mathbf{H}_2$–I), 3.38 (s, $C\mathbf{H}_3O$), 3.56 (m, $CH_3OC\mathbf{H}_2C\mathbf{H}_2O$), 3.64 (t, $C\mathbf{H}_2C\mathbf{H}_2O$), 3.75 ppm (t, $OC\mathbf{H}_2$–CH_2–I).

2.3 Synthesis of hexanoyl chitosan (HC)

Hexanoyl chitosan (HC) was synthesized according to the method previously reported [16]. One gram of chitosan (5.88 mmol calculated as glucosamine unit) was dissolved in $MeSO_3H$ (20 ml) at room temperature. Certain amount of hexanoyl chloride was introduced (3 equiv/glucosamine unit of chitosan). The mixture was stirred at room temperature for 5 h, precipitated into 200 ml of ice water and dialyzed against distilled water with a cellulose membrane (cut off MW 8,000) for 1 day. The dialyzed mixture was neutralized with NaOH aqueous solution to pH 8.0. Followed by dialysis against distilled water for 3 days, HC was obtained by freeze-drying. IR (KBr): ν 1739 (ester), and 1630, 1540 (amide). ^{1}H NMR (DMSO-d6): 0.87 ($C\boldsymbol{H}_3CH_2$ from hexanoyl groups), 1.27 ($CH_3C\boldsymbol{H}_2C\boldsymbol{H}_2$ from hexanoyl groups), 1.62 ($OC(O)CH_2C\boldsymbol{H}_2CH_2$ from hexanoyl groups), 2.34 ($OC(O)C\boldsymbol{H}_2CH_2$ from hexanoyl groups), 2.77 ($C\boldsymbol{H}$–NH_2 from chitosan backbone), 3.10–5.20 (other protons from chitosan backbone).

2.4 Synthesis of PEG-*g*-HC

Hexanoyl chitosan (1 g) was dissolved in 50 ml of DMF. Certain amount of Me–PEG–I was added. The mixture was heated to 60°C with stirring for 10 h under nitrogen atmosphere, and then precipitated into diethyl ether. The precipitate was collected by centrifugation, re-dispersed in distilled water and dialyzed with a cellulose membrane (cut off MW 8,000) for 2 weeks. The final product was obtained by freeze-drying. IR (KBr): ν 1741 (ester), 1657 and 1544 (amide). ^{1}H NMR (DMSO-d6): 0.85 ($C\boldsymbol{H}_3CH_2$ from hexanoyl groups), 1.25 ($CH_3C\boldsymbol{H}_2C\boldsymbol{H}_2$ from hexanoyl groups), 1.50 ($OC(O)CH_2C\boldsymbol{H}_2CH_2$ from hexanoyl groups), 2.02 ($(O)CC\mathbf{H}_3$ from chitosan), 2.29 ($OC(O)C\boldsymbol{H}_2CH_2$ from hexanoyl groups), 2.66 ($C\boldsymbol{H}$–NH_2 from chitosan backbone), 3.10–5.20 (other protons from chitosan backbone), 3.50 ($C\mathbf{H}_2C\mathbf{H}_2O$ from PEG backbone). Since the peaks of PEG methylene overlaps with those of H-2,3,4,5,6 and 6′ (H b–f shown in Fig. 1b) monosaccharide residues, PEG grafting level in PEG-*g*-HC was estimated according to the method described by Sugimoto et al. [19]. In brief, the peak intensity of PEG methylene was corrected using the following equation:

$$\text{Corrected peak intensity of PEG methylene} = \frac{\text{whole intensity of 2.6 to 4.2 ppm}}{\text{H} - 1 \text{ intensity of 4.3 to 4.6 ppm}} - 6 \quad (1)$$

$$\text{PEG grafting level} = \frac{\text{corrected peak intensity of PEG methylene}}{\text{number of protons in PEG}} \quad (2)$$

2.5 Characterization

Infrared (IR) spectra were recorded on a Bruker Vector Spectrometer. Samples were either film cast in chloroform onto NaCl plates or pressed into KBr pellets. NMR spectra were obtained on a Bruker DMX-500 NMR Spectrometer operating at 500 MHz. The morphology of the nanoparticles was observed by TEM (JEM-1230). The samples for TEM observation were prepared by directly depositing the nanoparticle solution onto copper grids. The samples were dried in a vacuum oven for 24 h at room temperature prior to TEM imaging. The average diameter of the nanoparticles was determined by Brookhaven 90Plus Particle Size Analyzer (Brookhaven, USA).

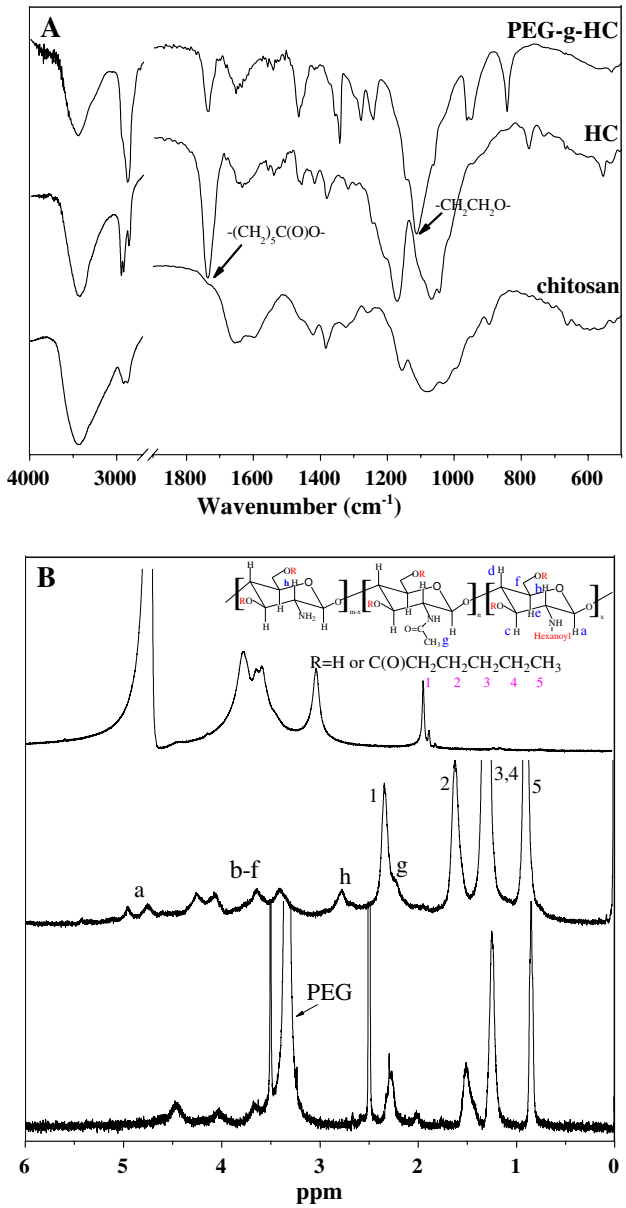

Fig. 1 **a** IR spectra of chitosan, HC and PEG-*g*-HC. **b** ^{1}H NMR spectra of chitosan, HC and PEG-*g*-HC

2.6 Preparation of PEG-*g*-HC nanoparticles

About 0.04 g of PEG-*g*-HC was dissolved in 10 ml of DMF. The solution was filtered with 0.45 μm filter to remove possible dust. The solution was dialyzed against aqueous media with a cellulose membrane (cut off MW 8,000) at room temperature for 3 days.

2.7 Determination of the copolymer CAC

The copolymer CAC was determined using pyrene as a fluorescent probe. Steady-state fluorescence spectra were recorded on a Shimadzu RF-5301PC spectrofluorophotometer. Excitation spectra were monitored at 372 nm. Sample solutions were prepared by adding certain amount of pyrene into the copolymer aqueous solution, which were obtained by dialysis method. The solutions were allowed to stand for 1 day for equilibration. The concentration of pyrene was kept at 6×10^{-7} M. The dependence of I_{339}/I_{335} on the solution concentration was plotted. The CAC is taken as the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low polymer concentration.

3 Results and discussion

3.1 Synthesis of PEG-*g*-HC

The synthetic scheme of PEG-*g*-HC was shown in Scheme 1. Through two-step reactions, the amphiphilic graft copolymers composed of hydrophobilized chitosan and hydrophilic PEG could be conveniently prepared. The chemical structures of the copolymers were confirmed by IR and ^{1}H NMR (Fig. 1). The presence of ester bands at 1,730 cm^{-1} in the FTIR spectrum of HC indicates the successful introduction of hexanoyl groups to chitosan. The grafting of PEG to HC results in strong absorption at about 1,100 cm^{-1} due to the increase of C–O–C bond in the FTIR spectrum of PEG-*g*-HC. The substitution degree of hexanoyl goups in HC was estimated from the integral of the peak at 0.87 against 2.5–5.5 ppm in ^{1}H NMR spectra [16]. It was found that HC could be soluble in both acidic aqueous solution and common organic solvents when substitution degree was below 1.14, while became insoluble in aqueous media as the substitution degree was above 1.14. For that reason, HC with substitution degree of hexanoyl groups being 1.14 was selected for the subsequent study. The percentage of the amine groups remaining after conjugation of hexanoyl groups to chitosan was determined from δ 2.7–2.8 (peak h) vs. δ 3.2–5.2 (peaks a–f) [16] and found to be 62%. PEG grafting level in PEG-*g*-HC was calculated using Eqs. 1 and 2. The detailed structural information of the copolymers was listed in Table 1. The amphiphilic graft copolymers were all soluble in DMF and DMSO.

3.2 Self-assembly property of PEG-*g*-HC

The micellization behavior of the copolymers was studied by fluorescence probe methods. By examining the (0, 0) band in the pyrene excitation spectrum and comparing the intensity ratio (I_{339}/I_{335}), the critical aggregation concentration (CAC) can be determined. At low polymer concentrations, I_{339}/I_{335} takes the value characteristic of pyrene in water, and at high concentrations it takes the value of pyrene in a hydrophobic environment. As shown

Scheme 1 Synthetic route of PEG-*g*-hexanoyl chitosan

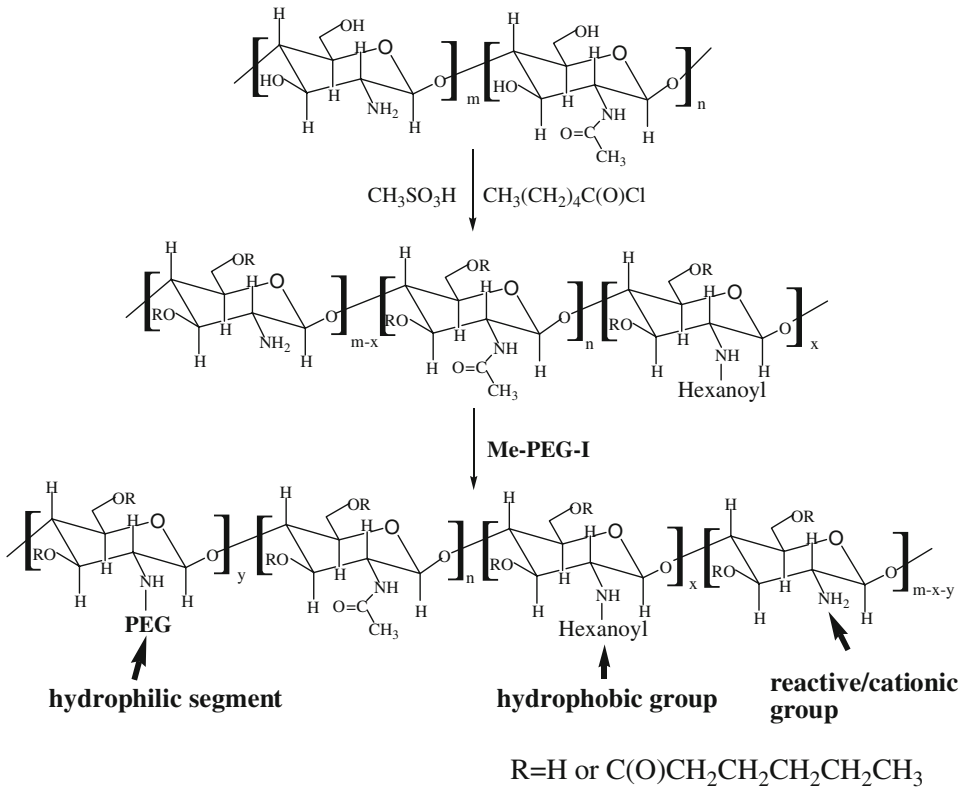

Table 1 Typical characteristics of PEG-*g*-HC

Polymer	PEG-*g*-HC-1	PEG-*g*-HC-2	PEG-*g*-HC-3
PEG/HC feed ratio[a]	3	4	6
PEG grafting level[b]	6.5	8.5	11.3
CAC (g/ml)[c]	6.61×10^{-6}	6.68×10^{-6}	6.76×10^{-6}
D (nm)[d]	180	120	40

[a] In weight

[b] Expressed as PEG molecules per 100 monomer units of HC

[c] Determined by fluorescent method

[d] Average diameter of the copolymer nanoparticles, determined by dynamic light scattering

in Fig. 2, a plot of I_{339}/I_{335} versus log C is flat at low polymer concentrations and sigmoidal in the crossover region. The CAC value thus obtained was 6.61×10^{-6} g/ml when PEG grafting level was 6.5%. The effect of PEG grafting level on CAC was not significant.

The morphology of the nanoparticles was observed by TEM and the results were shown in Fig. 3. In all the cases, spherical nanoparticles could be obtained. The average diameter of the nanoparticles was about 180 nm when PEG grafting level was 6.5%, and 40 nm when the PEG grafting level reached 11.3%, which are consistent with those determined by dynamic light scattering (Fig. 4). It should be noted that the D_h of the nanoparticles measured by DLS are a little larger than those observed by TEM since the nanoparticles are swollen in water, while TEM observation shows the dried aggregates.

The dependence of the copolymer micellization on the medium pH was investigated by measuring the average diameter of the nanoparticles in the media with various pH (Fig. 5). It can be seen that the average diameter of the PEG-*g*-HC-1 nanoparticles dramatically decreased from about 180 nm to 60 nm when the medium pH changed from 7.2 to 6.8. As the medium pH reaches 1.8, complete dissociation of the nanoparticles could be observed. Similar results could also observed for the other two types of the copolymer nanoparticles.

4 Conclusion

Amphiphilic graft copolymer based on chitosan, PEG-*g*-HC, could be synthesized through a convenient scheme. The copolymer could be self-assembled to spherical nanoparticles by dialysis method. The average diameter of the nanoparticles could be modulated by PEG grafting level. The micellization of the copolymers strongly depends on the medium pH. With the decrease of the medium pH, there was a dramatic decrease in the average diameter of the nanoparticles.

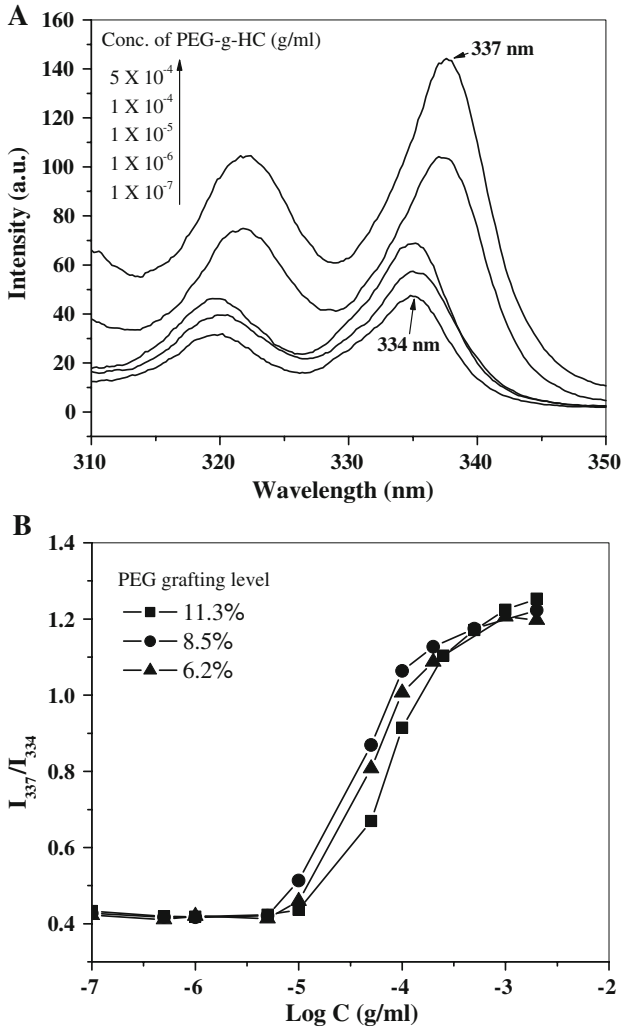

Fig. 2 **a** Steady-state fluorescence excitation spectra monitored at 373 nm for the pyrene probe in an aqueous solution of PEG-*g*-HC at room temperature. The concentration of pyrene is 6.0×10^{-7} M. **b** Plots of the I_{339}/I_{335} ratio of pyrene excitation spectra in water as a function of PEG-*g*-HC concentration at room temperature

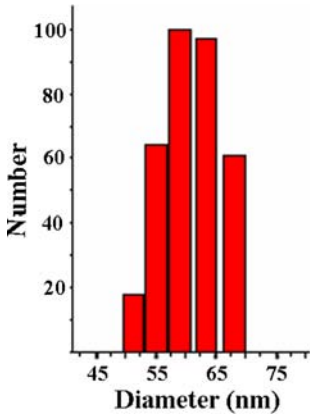

Fig. 4 The size distribution of PEG-*g*-HC nanoparticles. PEG grafting level was 11.3%

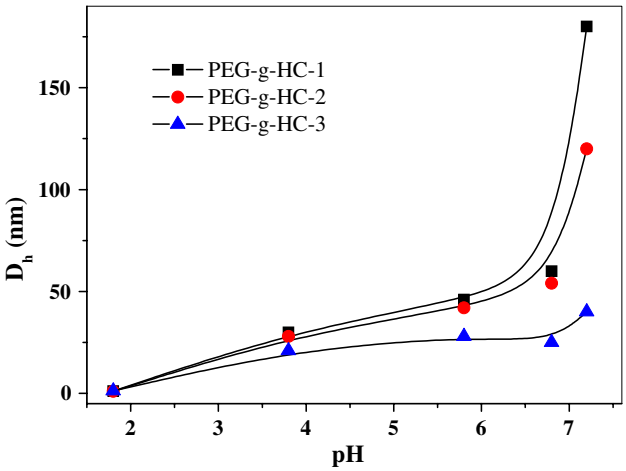

Fig. 5 The dependence of average diameter of PEG-*g*-HC nanoparticles on the media pH

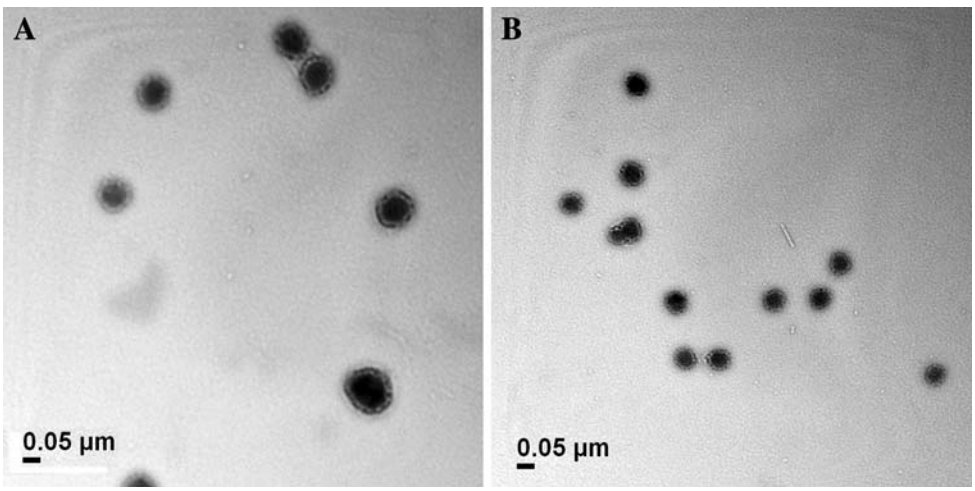

Fig. 3 Nanoparticles observed by TEM for PEG-*g*-HC. PEG grafting level was (**a**) 6.5% and (**b**) 11.3%

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References

1. G.S. Kwon, T. Okano, Adv. Drug Deliv. Rev. **21**, 107 (1996). doi:10.1016/S0169-409X(96)00401-2
2. R. Haag, Angew. Chem. Int. Ed. **43**, 278 (2004). doi:10.1002/anie.200301694
3. D. Neradovic, C.F. van Nostrum, W.E. Hennink, Macromolecules **34**, 7589 (2001). doi:10.1021/ma011198q
4. O. Soga, C.F. van Nostrum, A. Ramzi, T. Visser, F. Soulimani, P.M. Frederik, P.H.H. Bomans, W.E. Hennink, Langmuir **20**, 9388 (2004). doi:10.1021/la048354h
5. J.X. Zhang, L.Y. Qiu, K.J. Zhu, Y. Jin, Macromol. Rapid Commun. **25**, 1563 (2004). doi:10.1002/marc.200400180
6. A.P. Goodwin, J.L. Mynar, Y.Z. Ma, G.R. Fleming, J.M.J. Frechet, J. Am. Chem. Soc. **127**, 9952 (2005). doi:10.1021/ja0523035
7. J.Q. Jiang, X. Tong, Y. Zhao, J. Am. Chem. Soc. **127**, 8290 (2005). doi:10.1021/ja0521019
8. S. Yusa, A.K. Sakakibara, T. Yamamoto, Y. Morishima, Macromolecules **35**, 5243 (2002). doi:10.1021/ma0200930
9. E.S. Lee, H.J. Shin, K. Na, Y.H. Bae, J. Control. Release **90**, 363 (2003). doi:10.1016/S0168-3659(03)00205-0
10. C. Giacomelli, L. Le Men, R. Borsali, J. Lai-Kee-Him, A. Brisson, S.P. Armes, A.L. Lewis, Biomacromolecules **7**, 817 (2006). doi:10.1021/bm0508921
11. D. Shenoy, S. Little, R. Langer, M. Amiji, Mol. Pharm. **2**, 357 (2005). doi:10.1021/mp0500420
12. E.S. Lee, K. Na, Y.H. Bae, Nano Lett. **5**, 325 (2005). doi:10.1021/nl0479987
13. Y. Bae, S. Fukushima, A. Harada, K. Kataoka, Angew. Chem. Int. Ed. **42**, 4640 (2003). doi:10.1002/anie.200250653
14. K.A. Janes, P. Calvo, M.J. Alonso, Adv. Drug Deliv. Rev. **47**, 83 (2001). doi:10.1016/S0169-409X(00)00123-X
15. M.N.V.R. Kumar, R.A.A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A.J. Domb, Chem. Rev. **104**, 6017 (2004). doi:10.1021/cr030441b
16. H. Sashiwa, N. Kawasaki, A. Nakayama, E. Muraki, N. Yamamoto, H. Zhu, H. Nagano, Y. Omura, H. Saimoto, Y. Shigemasa, S.I. Aiba, Biomacromolecules **3**, 1120 (2002). doi:10.1021/bm0200478
17. M.E.I. Badawy, E.I. Rabea, T.M. Rogge, C.V. Stevens, G. Smagghe, W. Steurbaut, M. Hofte, Biomacromolecules **5**, 589 (2004). doi:10.1021/bm0344295
18. N. Gorochovceva, R. Makuska, Eur. Polym. J. **40**, 685 (2004). doi:10.1016/j.eurpolymj.2003.12.005
19. M. Sugimoto, M. Morimoto, H. Sashiwa, H. Saimoto, Y. Shigemasa, Carbohydr. Polym. **36**, 49 (1998)