Nano-Templates from Thermoresponsive Poly(ethoxytriethyleneglycol acrylate) for Polymeric Nano-Capsules

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Summary: Temperature responsive poly(ethoxytriethyleneglycol acrylate) (PETEGA) of Mn = 8000 and $M_w/M_n = 2.30$ was synthesized by ATRP. Dilute aqueous solutions of PETEGA exhibit lower critical solution temperature (LCST) at around 34 °C. We found that PETEGA can form nano-sized uniform colloidal aggregates (50-200 nm) above LCST either with or without an additional surfactant. Therefore PETEGA nano-aggregates were used as templates for the seeded free radical copolymerization of acrylamides or methacrylates together with a cross-linker to form acrylamide or methacrylate based core-shell particles. The formation of the PETEGA templates was investigated by dynamic light scattering (DLS) in order to find optimal conditions for obtaining narrow dispersed aggregates of desired sub-micron dimensions. Core-shell particles were characterized by DLS and scanning electron microscopy.

Keywords: LCST; nano-templates; poly(ethoxytriethyleneglycol acrylate); seeded free radical polymerization; stabilized core-shell particles

Introduction

Recently, micro- and nano-sized hollow particles have been a subject of intense interest because of their unique properties and potential application as capsules.[1] Compared to polymer micro-spheres or micelles, polymeric nano-containers with a structure of hollow sphere are able to encapsulate both larger quantities and bigger guest molecules within their ''empty'' interior. They are also mechanically more stable than the liposomes made of naturally occurring phospholipids and the polymer vesicles, which can undergo undesirable structural changes resulting from, for instance, uncontrollable changes in the environmental conditions, chemical reactions, etc. Therefore the polymeric nano-containers have many potential applications, such as micro-reactors for template synthesis, vessels for confined reactions, drug carriers, protective shells for cells and enzymes, and artificial cells.

A convenient method to fabricate hollow particles is to coat a sacrificial core particle with a shell followed by decomposition or dissolving of the core. Recently, a layer by layer (LbL) self-assembly technique has been utilized by Caruso et al. to coat submicron polymer particles.[2] Silica particles are most frequently used as sacrificial core material because HF can easily dissolve them. In addition, SiO_2 spheres can be easily and reproducibly synthesized applying the Stöber process.[3] Thus, a wide range of capsules have been prepared using various cores and oppositely charged polyelectrolyte couples, and they have been extensively characterized.[4]

However, most of the polymer nano-containers reported so far can load and release guest molecules from their interior only by diffusion. It is therefore rather difficult to control the loading and releasing

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process, especially in the case of encapsulation of biomacromolecules. This drawback may be overcome if bio-macromolecules are loaded during the preparation step of the hollow nano-particles, provided that the conditions of the loading process are sufficiently mild to preserve the structure and functionality of the biomacromolecules. This problem is of significant importance as the stability of the biomacromolecules is limited by chemical degradation and denaturation.

Recently a novel strategy for the preparation of hollow nano- and microparticles in aqueous medium has been developed, which involves the following three steps: (i) formation of a core template by temperature induced phase transition of thermo-responsive polymer poly(N-isopropyl acrylamide) (PNIPAM) above its lower critical solution temperature (LCST); (ii) shell formation by seeded radical copolymerisation; (iii) removal of PNIPAM from the core by dialysis at low temperature.[5] This opens a new pathway for the preparation of mechanically stable polymeric capsules of predefined dimensions. The major advantage of this method is that it allows the PNIPAM template to be removed from the matrix under very mild conditions. Nano- and microcapsules of different sizes would be readily obtained by tuning the size of the template particles, which can be controlled by the addition of surfactants.

The present work is focused on the advantages of the method described in our prevous study.[5] Our objective is to optimize the method, which will allow us to use a wide range of thermo-responsive polymers and copolymers, and to prove the concept for real applications. This article addresses/discusses the following issues: (i) the recent progress in the development of the new method; (ii) formation of core-shell nano-particles by using another temperature-responsive polymer, namely poly(ethoxytriethylenglycol acrylate) (PETEGA)[6]; (iii) our vision for the future applications of the method. In this way our study will induce further developments in the area of controlled loading of biologically active substances.

Experimental Part

Materials.
Monoethoxy triethylene glycol (90%), acryloylchloride (Aldrich, 98%), triethyl amine (TEA, 99%), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 99%), CuBr (99.99%), ethyl 2-bromo propionate (EBP, 99%), and sodium dodecyl sulfate (SDS, 99%) were purchased from Aldrich and used as received. 2-Hydroxyethyl methacrylate (HEMA) (Fluka, 98%) was purified by vacuum distillation. HEMA was then dissolved in bi-distilled water (10 vol%) and stored in a refrigerator for further use. N-isopropylacrylamide (Aldrich, 97%, NIPAM) was re-crystallized three times from hexane. N,N'-methylenebisacrylamide (Merk, 98%, BIS) was re-crystallized from methanol. Potassium persulfate (Aldrich, 99% ACS grade, KPS) was re-crystallized from bi-distilled water.

Ethoxytrietyleneglycol acrylate (ETEGA) was synthesized from monoethoxy triethylene glycol and acryloylchloride in methylene chloride in the presence of TEA as described by Jiang et. al. [6]

Poly(ethoxytrietyleneglycol acrylate) (PETEGA) of $M_n = 8000$ and $PDI = 2.3$ (from SEC with polystyrene standards) was obtained by ATRP of ETEGA monomer initiated by EBP in acetone (30 vol%) at 90 °C, similarly to [6], but acetone was used instead of anisole as a reaction medium. EBP:CuBr:PMDETA molar ratio was 1:1:1, and $M_{n(theor)}$ was 10000. Polymerization time was 16 hours and the yield was 72%, determined gravimetrically. PETEGA was purified from copper compounds and the ligand by passing its acetone solution through a short column with basic Al_2O_3 filament, followed by precipitation in hexane repeated three times.

Aqueous dispersion of PETEGA nanotemplates 0.2 g of PETEGA was initially dissolved in 100 ml bi-distilled water at 4 °C

for 24 hours to form a 2.0 g/l stock solution. Part of the stock solution (25 ml) was later diluted with 75 ml bi-distilled water into a single necked round bottom flask equipped with a stir-bar and kept at room temperature. A known amount of SDS was added and the flask was then immersed in an oil bath and stirred at 70 °C. After a minute of stirring at c.a. 600 rpm, slight to moderate opalescence of the solution appeared.

Synthesis of core-shell particles with PHEMA shells.

In a typical procedure 0.5 ml of a 10% aqueous solution of HEMA, and 0.01 g of BIS were added to a 100 ml aqueous dispersion of 0.05 g PETEGA nano-templates stirred at 600 rpm at 70 °C. The solution was bubbled with high purity argon via stainless steel needle for 30 minutes then 0.05 g of KPS were added. The flask was sealed with SubaSeal rubber septum and the reaction mixture was stirred for 24 hours. The reaction flask was opened to the air while still at 70 °C and then cooled down to room temperature. The solution was placed in dialysis tubes with a cut-off value of 12000 Da and dialyzed for 24 hours against distilled water to remove low molecular weight impurities and a part of the PETEGA core-template.

Synthesis of core-shell particles with PNIPAM shells.

The procedure was performed similarly in exactly the same way as that with HEMA, except NIPAM monomer was used instead of HEMA.

Characterization

Cloud point measurements.

Cloud points of 1 g/L aqueous solution of PETEGA were determined by cooling and heating cycles on a Jasco V-530 UV/VIS spectrophotometer switched to transmittance regime at constant wavelength of 500 nm. The cuvette compartment was thermostated by Medson MTC-P1 thermocontroller with a stability of 0.05 °C.

Dynamic light scattering (DLS).

DLS measurements were performed on a Brookhaven BI-200 goniometer with vertically polarized incident light of wavelength $\lambda = 632.8$ nm supplied by a helium- neon laser operated at 75 mW and a Brookhaven BI-9000 AT digital auto-correlator. Measurements of scattered light from the polymer aqueous solutions were made at angle $\theta = 90^{\circ}$ to the incident beam in temperature range from 25 °C to 70 °C. The autocorrelation functions from DLS were analyzed by the constrained regularized CONTIN method to obtain apparent hydrodynamic diameters, D_h.[7]

DLS measurements at ambient temperature were done on a Zetasizer 3 by Malvern Instruments. Results from Brookhaven and Malvern instruments were in a very good agreement.

Scanning electron microscopy (SEM).

The diameters of particles were measured on a JEOL JSM-6390 SEM unit, operating at 5 kV. Samples for SEM were prepared by putting a drop of water solution of core-shell nano-particles before and after dialysis on a glass substrate, dried under vacuum and coated with gold for 20 s.

Results and Discussion

The original method for the preparation of core-shell particles used in our previous study[5] was based on a system consisting of submicron PNIPAM templates as seed particles for the radical copolymerization of HEMA and polyethyleneglycol dimethacrylate cross-linker initiated by KPS. Additionally SDS was used as a surfactant. During the formation of the crosslinked PHEMA shell, secondary nucleation was avoided due to the large specific surface area of the seed particles, which were at moderately low concentrations in the range of 0.5–1.0 g/l. Evidently, because of the hydrophilicity of HEMA the polymerization starts in the aqueous phase resulting in HEMA oligomers with increasing hydrophobicity since water is a poor solvent for PHEMA. This causes adsorption of hydrophobic oligoHEMA on the hydrophobic PNIPAM template stabilized with SDS. Furthermore, the first PHEMA layer attracts and dissolves more monomer and

cross-linker. In this way the polymerization site is limited to the surface of the seeding particles. As a result a shell is formed.

We came across certain difficulties in our attempts to prepare hollow PHEMA nanoparticles by dissolving the PNIPAM cores at a low temperature. The PNIPAM chains were only partially released from the interior of the particles. This could have been due to any of the following reasons:

1. PNIPAM and respectively the PNIPAM cores do not completely dissolve at temperatures lower than the temperature of the phase transition.
2. The large dimensions and the reduced flexibility of the PNIPAM core-forming macromolecules hinder their release.
3. HEMA partially swells into the PNIPAM core thus forming interpenetrating networks.
4. The shell acts as a membrane, and therefore the shell permeability defined by the density and thickness of the crosslinked PHEMA does not allow the template macromolecules to leave the interior of the core-shell particle.

The last two factors can be controlled by varying the polymerization procedure for the formation of the shell, while the first two depend on the individual properties of the thermo-responsive polymer. The initially used commercial PNIPAM forms nano-templates, which do not completely dissolve when needed.[5] Therefore the use of other thermo-responsive polymers is currently investigated.

PETEGA might be a good candidate for thermo-sensitive polymer for template formation. PETEGA is a polymer with LCST of 34 °C (Figure 1), which is very similar to that of PNIPAM. PETEGA has fully reversible agglomeration-dissolution cycle at temperatures that are either higher or lower than 34 °C. Additionally PETEGA has a low T_g (approximately −60 °C), which makes it much more flexible than PNIPAM (T_g of around 130 °C) and thus it is more likely to escape the confined environment provided by the PHEMA shell. The phase

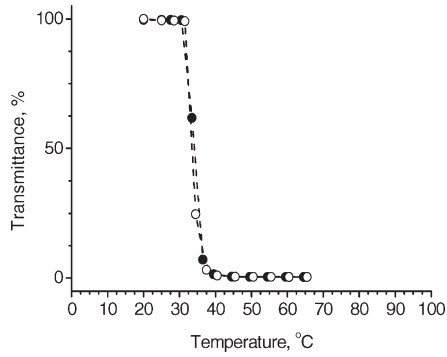


Figure 1.
Cloud point curves of 1.0 g/l aqueous PETEGA solution. Black circles are points obtained from heating, hollow circles are from cooling.

transition process followed by cloud point measurements also shows lack of hysteresis (Figure 1) in contrast to PNIPAM.[8]

Formation of PETEGA nano-templates. Another similarity between PETEGA and PNIPAM is their ability to spontaneously form nano-aggregates when dilute aqueous solution of the polymer is heated above LCST. The formation of PETEGA

Table 1.
DLS data for PETEGA nano-templates formed at different temperatures and at 0.5 g/l with different amounts of SDS.

No	s/p	T (°C)	Dh (nm)	Dispersity
1	0	40	177	0.16
2	0	50	132	0.03
3	0	60	97	0.142
4	0	70	91	0.132
5	0.1	40	110	0.062
6	0.1	50	173	0.244
7	0.1	60	111	bimodal
8	0.1	70	70	0.234
9	0.2	40	n.d.*	n.d.
10	0.2	50	125	0.106
11	0.2	60	80	0.112
12	0.2	70	60	0.168
13	0.3	40	n.d	n.d.
14	0.3	50	171	bimodal
15	0.3	60	38/152	bimodal
16	0.3	70	20/105	bimodal
17	0.4	40	n.d.	n.d.
18	0.4	50	42/189	bimodal
19	0.4	60	17/94	bimodal
20	0.4	70	10/71	bimodal

*Not determined, a very low intensity of scattered light was observed due to absence of aggregates.

aggregates upon heating was followed by DLS. Data is collected in Table 1. PETEGA nano-aggregates are being formed at around 40 °C and are stable at temperatures as high as 70 °C. During heating up the solution dimensions of PETEGA aggregates tend to get smaller (Table 1, entries 1–4). Upon addition of the ionic surfactant SDS smaller aggregates are being formed at the respective temperature and this tendency remains with the increase in temperature. When increasing the SDS content the aggregates become smaller and a bimodal distribution of scattered light is observed due to the appearance of small particles with diameters in the range of 10–50 nm. Particles of sub 100 nm diameters were obtained at 70 °C (entries 4, 8, and 12, Table 1). It should be pointed out that the solutions were not stirred during measurements. Therefore the observed dependencies of diameters versus temperature and amount of SDS can be considered only as qualitative since during the syntheses of the core-shell particles a much larger volume of the PETEGA template solution was used (100 ml) and to avoid inefficient heat transfer the solution had to be stirred (at 600 rpm). The heating rate should also have a certain effect on the formation of the PETEGA nano-templates, but it was not investigated in the present study as solutions were instantly exposed to high temperatures.

Synthesis and characterization of core-shell particles.

The PETEGA nano-aggregates were later used as templates for the seeded radical copolymerization of HEMA or NIPAM together with BIS cross-linker either in the presence or absence of SDS at 70 °C. In all cases clear to slightly opalescent reaction solutions were observed at the end of the shell formation depending on the resulting particle size. After dialysis of the crude reaction solution to remove low molecular weight compounds, particle sizes and morphology were investigated by DLS and SEM, and the results are collected in Table 2. All runs were performed at similar conditions, only the amount of SDS was varied. The amount of added SDS has a significant effect on the dimensions of the resulting particles. Without SDS or at the lowest s/p (surfactant/polymer) ratio of 0.1 the formed particles had fairly large diameters of approximately 300 to 500 nm (entries p4, p10, p11, and N6

Table 2.
Formation of core-shell particles of PETEGA cores and PHEMA or PNIPAM shells; Reaction volume is 100 ml, reaction temperature is 70 °C, reaction time is 24 hours. Concentration of PETEGA, HEMA, and KPS is 0.5 g/l. Concentration of NIPAM is 1.0 g/l. BIS is 20% of the quantity of HEMA or NIPAM.

Code	Shell	SDS/PETEGA	Dh[a)]	Diameter
		s/p (g/g)	DLS (nm)	SEM (nm)
P4	PHEMA	0	270	320
P10	PHEMA	0	n.d.	420
P11	PHEMA	0.1	300	312
P12	PHEMA	0.2	130	n.d.
P13	PHEMA	0.4	84	320
N6	PNIPAM	0	160	500
N7	PNIPAM	0.1	130	n.d.
N8	PNIPAM	0.2	110	n.d.

[a)]Hydrodynamic diameter.

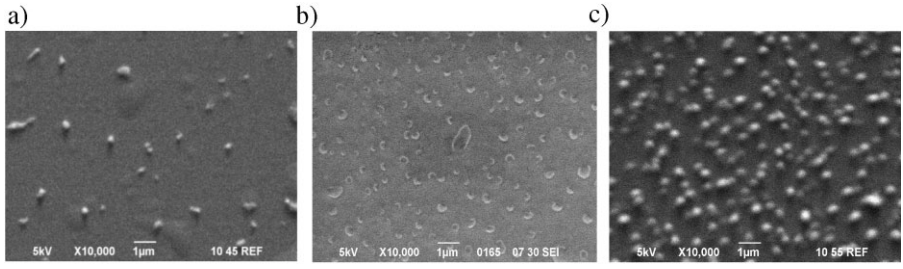


Figure 2.
SEM of core-shell nano-particles of a) p10, s/p = 0; b) P13, s/p = 0.4; and c) N6, s/p = 0.

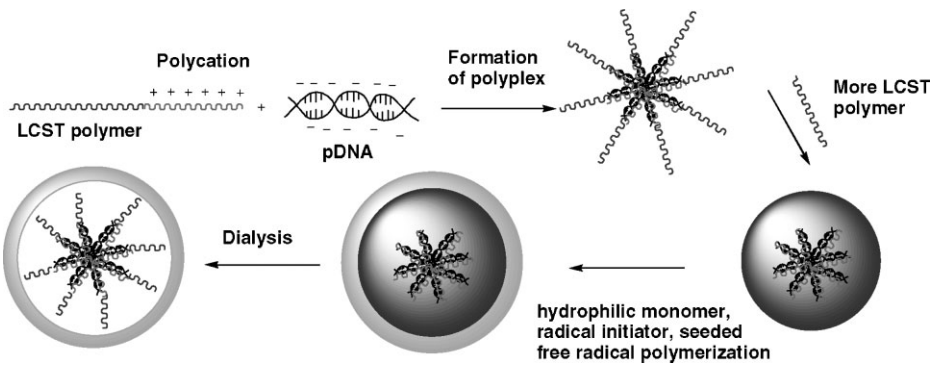
Table 2). With increasing the SDS content the particle diameters became smaller, and at s/p = 0.4 sub-100 nm core-shell particles were formed (entry p13, Table 2). This is a very important result as such particles are suitable for drug/gene delivery systems, where sub-100 nm dimensions are generally preferred for better cell intake.

The morphology of the core shell particles was studied by SEM. Spherical objects of diameters ranging from 300 to 400 nm were frequently observed (Table 2). More unusual shapes were observed only with the sample of the highest s/p content of 0.4, p13, where rather uniform C-like objects appeared (Figure 2b). DLS of p13 gives a hydrodynamic diameter of 84 nm, while diameters measured by SEM are much bigger and reach 300 nm. This could be an indication that during dialysis a certain amount of or the whole PETEGA core had left the PHEMA shell. Thus during the preparation of the sample for SEM the spheres completely dried up and as water was released from the interior the spheres reorganized into folded structures. The latter take a much bigger surface area on the glass surface of the substrate and thus result in SEM measurements of much bigger dimensions. At the present stage of investigations there is no ultimate proof for the appearance of ''hollow'' nano-capsules, but further experiments are planned for clarification.

Conclusions and Outlook

The thermo-responsive polymer used in this study, PETEGA, forms in aqueous medium nano-particle templates of dimensions, predetermined by the amount of the surfactant SDS. Sub-100 nm dimensions of PETEGA templates were obtained at 70 °C at a SDS/PETEGA ratio of 0.4. Therefore PETEGA can be potentially used together with copolymers of PETEGA and polycations to entrap bio-macromolecular polyanions such as nucleic acids (plasmid DNA (pDNA), antisense oligoribonucleotides, or small interfering RNA) during the template forming step (Scheme 1). After covering these templates with stable shells of cross-linked PHEMA or PNIPAM, the entrapped bio-macromolecules would remain within the core of the composite particle, even if PETEGA is removed by dialysis (Scheme 1).

This system allows aqueous loading of nucleic acids or protein drugs while preserving their bioactivity until they are safely delivered to a specific biological site. The results from this study will potentially impact the advancement of gene therapeutics. Future studies of these systems include the use of biodegradable cross-linkers in order to obtain capsules, which are selectively degraded by certain enzymes or pH thus allowing the bio-cargo to be delivered and released when necessary.



Scheme 1.
A possible route for encapsulation of polymer-DNA complexes or polyplexes.

It is also very important to further investigate the possibility of attaching targeting agents to the shell, which will target the nano-capsules toward specific cell types. One interesting possibility is to conjugate/attach ligands to the hydrophilic shell, which specifically bind internalizing cell-surface receptors. In this way, delivery can be restricted mainly to the targeted cells and possible side effects will be substantially diminished. Current concepts allow shell functionalization by introducing biotin-avidin systems,[9] the use of ''click'' chemistry for shell binding with cyclic peptides (e.g. LyP-1),[10] RGD peptides,[11] and others.

The approach described in the current contribution is especially favorable with respect to designing the chemical composition of the shell of the nano-capsules, either by copolymerizing ligands or other moieties bearing polymerizable groups together with HEMA or NIPAM, or by click reactions occurring after the synthesis of the shell.

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[1] T. M. S. Chang, in ''*Polymeric Materials Encyclopaedia*'', J. C. Salamone, Ed., CRC Press, NY *1996*, Vol. *6*, p. 4351.
[2] F. Caruso, *Adv Mater.* **2001**, *13*, 11.
[3] A. Van Blaaderen, J. V. Geest, A. Vrij, *J. Colloid Surf. Sci.* **1992**, *154*, 481.
[4] a) G. B. Sujhorukov, A. A. Antipov, A. Voigt, E. Donath, H. Möhwald, *Macromol. Rapid Commun.* **2001**, *22*, 44. b) G. Ibarz, L. Döhne, E. Donath, H. Möhwald, *Adv. Mater.* **2001**, *13*, 1324.
[5] P. Weda, B. Trzebicka, A. Dworak, Ch. B. Tsvetanov, *Polymer* **2008**, *49*, 1467.
[6] X. Jiang, C. A. Lavender, J. W. Woodcock, B. Zhao, *Macromolecules* **2008**, *41*, 2632.
[7] S. W. Provencher, *Macromol Chem* **1979**, *180*, 201.
[8] J.-F. Lutz, O. Akdemir, A. Hoth, *J. Am. Chem. Soc.* **2006**, *128*, 13046.
[9] C. Cheng, H. Wei, J.-L. Zhu, C. Chang, H. Cheng, C. Li, S.-X. Cheng, X.-Z. Zhang, R.-X. Zhuo, *Bioconjugate Chem.* **2008**, *19*, 1194.
[10] G. von Maltzahn, Y. Pen, J.-H. Park, D.-H. Min, V. R. Kotamraju, J. Jayakumar, V. Fogal, M. J. Sailor, E. Ruoslahti, S. N. Bhatia, *Bioconjugate Chem.* **2008**, on-line.
[11] K. Temming, R. M. Schiffelers, G. Molema, R. J. Kock, *Drug Resistance Updates* **2005**, *8*, 381.

