Review

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Polymeric Nanoparticle Engineering: From Temperature-Responsive Polymer Mesoglobules to Gene Delivery Systems

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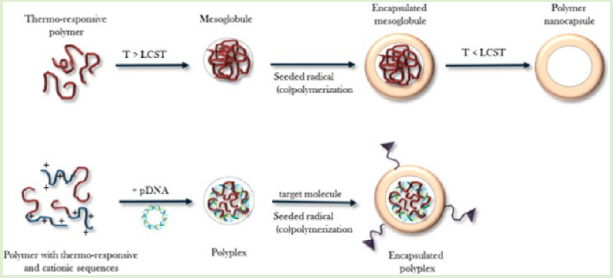
ABSTRACT: A novel approach for the preparation of nano- and microcapsules in aqueous solutions by using thermoresponsive polymer (TRP) templates (mesoglobules) is described. The method comprised three steps: formation of mesoglobules, coating the templates by seeded radical copolymerization, followed by core dissolution and core removal upon cooling. When mesoglobule entraps biomacromolecules during the process of their formation, it makes it possible to load a controlled amount of bioactive compounds without covalent attachment. Special attention is paid to the mesoglobule dissolution upon cooling, as well as their loading efficiency. Details on the outer shell formation and the possibilities for targeting ligands incorporation and control of the shell porosity are discussed. Finally, the seeded radical copolymerization was used for covering DNA complexes with cationic copolymers bearing TRP blocks. This Review is an attempt to convince researchers of the promising perspectives for using mesoglobules as potential reservoirs, carriers, and transferring agents for biologically active substances.



INTRODUCTION

Polymeric capsules are composed of a hollow core and a polymeric shell. Due to the large compartment for hydrophilic load and a membrane with easy to modify properties, they could find applications as drug carriers and microreactors.[1−3] In contrast to polymeric micelles, polymeric capsules can accommodate large quantities of cargo molecules and, importantly, large-size molecules such as therapeutic polypeptides, proteins, as well as RNA and DNA.[4] Two of the most widely used approaches for the synthesis of polymeric capsules are the template-free and template-assisted techniques. The template-free technique is based on self-assembly of amphiphilic block copolymers with appropriate composition. In this way, vesicular structures with an aqueous core enclosed by a bi- or multilayer membrane analogous to liposomes are formed, which are called polymersomes. They have advantages over liposomes in terms of superior stability and toughness, reduced permeability, restricted chain mobility within the membrane, and better resistance to dissolution.[5] The biggest drawback of polymersomes is their polydispersity in size, which hinders their application as drug carriers. In contrast to template-free method, the use of a matrix allows production of capsules having much narrower particle size distribution. For the past 20 years the template-assisted layer-by-layer (LbL) deposition technique has been widely used.[6−8] The clear advantage of the LbL approach is the precise control over the outer shell properties, especially the shell thickness and the shell morphology. A serious deficiency of this method is that the templates have to be sacrificed in a final step to prepare capsules. The core removal process strongly depends on the nature of the template such as Au-nanoparticles, SiO_2, $CaCO_3$, polystyrene, or melamine formaldehyde particles.[9] The elimination of the core materials can require tedious multistep procedures: hydration, enzymatic degradation, or wet etching with toxic or corrosive chemicals, such as HF, KCN, or EDTA complexes. Moreover, in most cases, the LbL technique requires multiple polymer adsorption steps, which is time and material consuming. Another drawback of most LbL capsules is their micrometer scale size, which sets limits to their application as intravenous drug delivery systems. Furthermore, in most cases, the cargo can be loaded in and released from the capsules only by diffusion, which makes loading/release processes difficult to control.

Most chemotherapeutic treatments require the drug nanocarriers to be administered intravascularly. The injection site is usually quite far from the target (e.g., tumor location), which requires the polymeric nanocarrier to travel through the circulatory system, perhaps for a long time, before coming in contact with the target. Obviously, the two approaches for preparation of hollow particles described here do not fully meet the essential requirements for biomedical applications, namely, narrow-size distribution, ability to carry and release biologically

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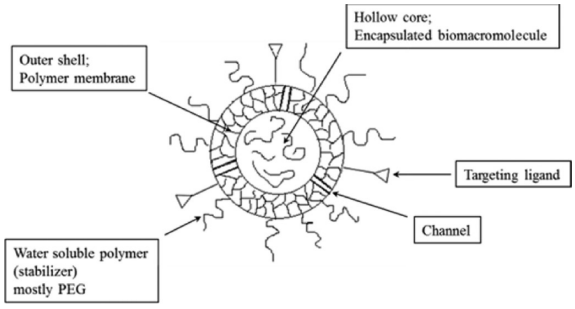
active substances, an outer membrane (shell) with complex functionality and tuned porosity, appropriate mechanical strength, as well as biocompatibility, biodegradability, and lack of toxicity.

So far, biodrugs, particularly peptides and proteins, have been most commonly encapsulated by using organic solvents.[10] It is desirable, however, that the processes of preparation of nanocarriers and encapsulation of biomacromolecules payload are carried out in an environmentally benign medium such as water without application of significant shear stress, which might contribute to disruption of the higher structural hierarchy order of proteins. The high requirements for the quality of the nanocarriers as a promising tool to treat cancer and other diseases have led scientists to work on improving the already existing methods for their preparation as well as to search for new synthetic approaches. Scheme 1 presents the desired structure of a polymeric capsule to be used as a drug nanocarrier. Particularly important features are the inclusion of a predetermined amount of biomacromolecules as payload, carrying out the reactions in an aqueous medium, and preparation of strongly elongated, rod-like nanocarriers.

Scheme 1. Desired Structure of Polymeric Nanocapsules for Incorporation of Biomacromolecules



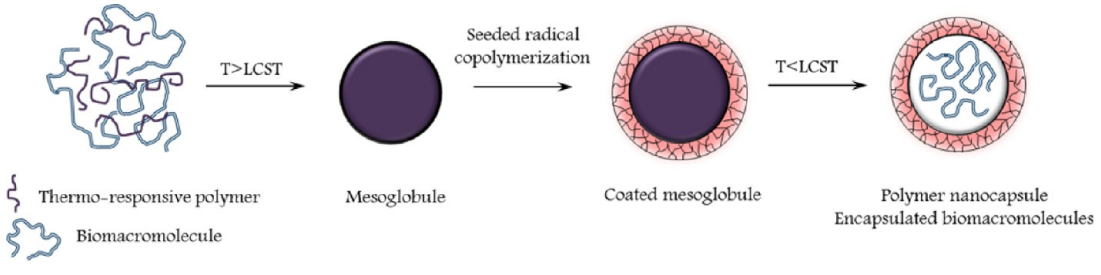
Based on the above requirements, we looked into a novel approach for preparation of nano- and microcapsules using thermoresponsive polymer (TRP) templates. TRPs are soluble in cold water and phase separate upon heating above the phase transition temperature (PTT) or lower critical solution temperature (LCST). It is well-known that, in dilute aqueous solutions (in most cases below 0.5 g/L), the TRPs form stable, monomodal spherical nanoaggregates, called mesoglobules, which disaggregate and dissolve at temperatures lower than the LCST.[11,12] The mesoglobules can be prepared in a very narrow size distribution with dimensions ranging from ca. 50 to 400 nm, making them potential templates for fabrication of nanocapsules. The size of mesoglobules strongly depends on the initial concentration, the heating protocol, and frequently on the molar mass of the individual macromolecules. The ability of the mesoglobules to disintegrate and dissolve upon cooling is the basis of our new approach. If the mesoglobules are coated with a water-swollen cross-linked polymeric membrane (i.e., as a result of the formation of core–shell particles), it can be expected that the core will start to dissolve upon cooling down below the LCST of the TRP, and the dissolved macromolecules will diffuse through the polymeric membrane, thus creating empty spaces in the interior of the particles. Complete removal of the TRP can be achieved by centrifugation or extensive dialysis, thus producing polymeric nanocapsules. The outer polymeric membrane can be constructed by heterophase copolymerization on the surface of the mesoglobules (e.g., seeded radical copolymerization). Scheme 2 represents the different synthetic stages of this new method. The most valuable advantage of the process is that all operations are carried out sequentially in one pot and only in an aqueous medium by simply changing the temperature, which is of particular importance as far as biomacromolecules are concerned. The possibility of adjusting the size of the template as well as the ease of formation of membranes of desirable thickness, porosity, and functional characteristics are other benefits of the proposed method.

This contribution highlights a thorough examination of both the successes and the problems associated with each of the stages of preparation of hollow nanospheres by using the method depicted in Scheme 2. In the first two parts, the preparation and characteristics of mesoglobules of different TRP and the control of their dimensions and loading efficiency are discussed. Next, details for the formation of an outer membrane and the possibilities for integrating vectors or important functional groups are presented followed by dissolution and extraction of the template core by dialysis at low temperatures. Results on encapsulation of gene transfection agents in the last section reveal the feasibility of the new approach.

MESOGLOBULES AS TEMPLATES FOR PREPARATION OF POLYMERIC NANOCAPSULES

In this part we will discuss in more detail the mechanism of aggregation and the reverse process of disintegration and dissolution in aqueous solution of the three most commonly studied TRPs: poly(*N*- isopropylacrylamide) (PNIPAM), poly(2-isopropyl-2-oxazoline) (PIPOX), and polymers of oligoethylene glycol (meth)acrylates (Figure 1). Bearing amide functionalities, PNIPAM and PIPOX are representatives of the largest group of TRPs. Both polymers are structural isomers and can be considered as simple models for protein denaturation in aqueous solution. PNIPAM has a nonpolar carbon backbone and hydrophilic amide groups in its side

Scheme 2. Schematic Presentation of the New Method for Preparation of Nanocapsules



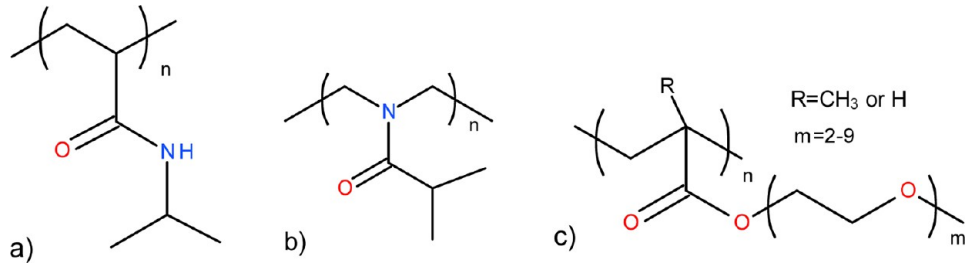
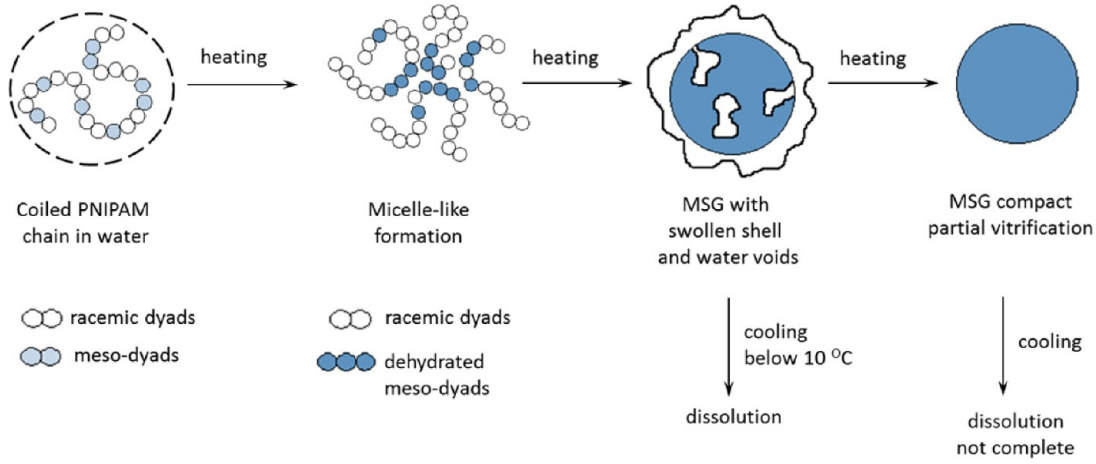


Figure 1. Structural formulas of poly(*N*- isopropylacrylamide) (PNIPAM) (a), poly(2-isopropyl-2-oxazoline) (PIPOX) (b), and polymers of poly(oligoethylene glycol (meth)acrylates) (POEG(M)A) (c).

Scheme 3. Schematic Presentation of Chain Aggregation and Dissolution of PNIPAM in Water during a Heating and Cooling Cycle[a]



[a]Modified with permission from ref 18. Copyright 2006 American Chemical Society.

chains, whereas PIPOX has polar backbone and amide group as a part of the main chain (Figure 1). The formation of >C═O···H−N intrachain hydrogen bonds plays an important role in the temperature responsiveness of PNIPAM in aqueous solution, whereas such bonds do not exist in PIPOX aqueous solution. As it will be discussed below, the presence or absence of hydrogen bonding during the aggregation process leads to significant differences in the mechanism of formation of mesoglobules and their morphology and properties.

PNIPAM Mesoglobules. *Aggregation (Demixing) of PNIPAM.* Water is a good solvent for TRPs at temperatures lower than the LCST. Even at lower temperatures (i.e., at least 20 °C below PPT) and in dilute solutions, the TRP dissolves as individual chains. It has been found for PNIPAM that an average number of 11 water molecules per monomer unit are attached to the polymer chain.[13] As a result, at least three hydration layers are formed around the chain: direct >C═O···H−O−H bonding to amide groups, bridges between the bound water molecules, and outer hydration layer. When aqueous solutions of PNIPAM are heated above their LCST, two processes simultaneously occur: intrachain contraction, leading to collapse of individual chains from water-swollen coils to compact globules, and interchain association, resulting in aggregation. Both interactions compete with each other for dehydration in the heating process. It is very important to note that the dehydration starts already at least 15−20 °C below the phase transition. The driving force for this transition is associated with the temperature-dependent molecular interactions, such as hydrogen bonding and hydrophobic association. The mode of heating is of particular importance for the mesoglobule formation and reverse dissolution, because in terms of kinetics, the chain folding and association cannot follow the temperature change. A proper adjustment of the rates of interchain association and intrachain contraction leads to formation of mesoglobules with rather different sizes and structures. A nonequilibrium, fast heating leads to formation of small aggregates due to more pronounced intrachain contraction and less interchain association. Furthermore, it favors the vitrifying of the polymer-rich phase.[14] At slow rates of heating, the chains start to associate with each other before their collapse, thus forming larger size mesoglobules.

Once formed, the PNIPAM mesoglobules neither precipitate nor disintegrate upon dilution. According to Wu et al.[15] the interchain aggregation occurs before the individual chains reach their fully collapsed globule state. Upon increasing temperature above the LCST, micelle-like structures are initially formed, due to the partial selective dehydration of the macromolecules (Scheme 3). This assumption is in accordance with the fact that PNIPAM meso-dyads are more hydrophobic than the racemo-dyads.[16] It has been shown that the phase separation behavior strongly depends on the PNIPAM tacticity.[17] From transmittance measurements, it was revealed that the LCST tends to decrease with increasing meso-diad content of PNIPAM. Similarly to the amphiphilic copolymers, for a very narrow temperature range, one can consider meso- and racemic dyads as hydrophobic and hydrophilic monomer units, respectively randomly distributed along the polymer chain (see Scheme 3). The first step is their aggregation into loose or molten

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mesoglobules, meaning that initially the latter are not dense and fully packed and contain a significant amount of water (up to 70%) in the form of "bound water" and "peripheral water". The polymer chains are not uniformly distributed inside the particle, and they keep certain mobility. Moreover, to form stable particles, the outer shell should be hydrophilic.[11] Presumably, the mesoglobule periphery is composed of small loops of partially hydrated PNIPAM sequences, implying the formation of rough surface. Scheme 3 represents chain association (demixing) and dissolution (remixing) of PNIPAM macromolecules during a heating and cooling cycle.

Obviously, the heating rate and the initial polymer concentration are essential for the PNIPAM mesoglobule size. The most suitable for the preparation of mesoglobules are dilute solutions of the TRP below the overlapping concentration (typically in the 0.1−1.0 g/L range). The addition of a surfactant,[19] ionic liquids,[20] or osmolyte[21] results in a significant size reduction (Figure 2).

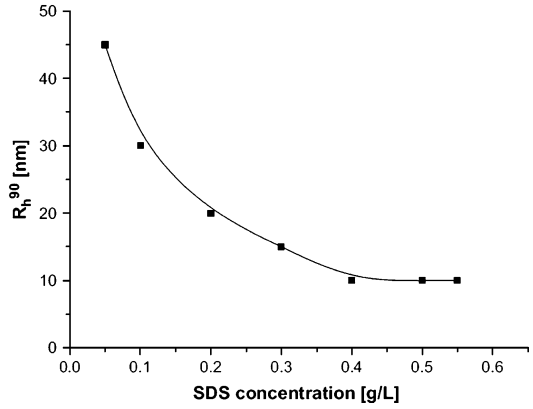

Figure 2. Hydrodynamic radius of PNIPAM mesoglobules as a function of surfactant (SDS) concentration, reprinted from ref 19. Copyright 2008, with permission from Elsevier.

Evidently, at a specified condition, one may generate reproducibly spherical PNIPAM nanoparticles with desired size. It is noteworthy that the prolonged incubation at elevated temperatures produces templates that dissolve much more slowly and incompletely in cold water due to chain entanglement and partial vitrifying.

Dissolution (Remixing) of PNIPAM Mesoglobules. The dissolution is largely influenced by the thermal history of the preceding phase separation events. The rate of remixing of PNIPAM mesoglobules is always slower than the rate of demixing.[22] A retardation of the effects of disintegration and dissolution of mesoglobules (hysteresis) is typically observed, implying that additional intrachain hydrogen bonds are formed upon chain collapse. Actually, the dissolution of PNIPAM mesoglobules involves two processes: disruption of hydrogen bonding, formed in the collapsed state, and dissolution of collapsed and entangled chains due to rehydration.[23] Upon cooling, the interchain hydrogen bonds are progressively replaced by hydrogen bonds between water molecules and monomer units. The interchain hydrogen bonds act as physical cross-linkers and may impart gel-like behavior of the polymer. It is noteworthy that a portion of them remains active even upon cooling below the LCST.[18] Therefore, initially swelling associated with a large increase in hydrodynamic dimensions (Figure 3) instead of direct dissolution is observed.[19] The sharp

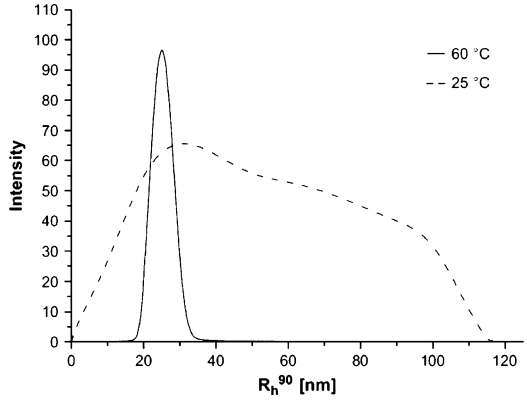

Figure 3. Hydrodynamic radius R_h^{90} distribution of PNIPAM mesoglobules at 60 and 25 °C; $[c]_{PNIPAM}$ = 1 g/L, SDS/PNIPAM = 0.2. Reprinted from ref 19. Copyright 2008 with permission from Elsevier.

increase in R_h (swelling) upon cooling could be a problem when PNIPAM mesoglobules are used as templates for preparation of nanocapsules (Scheme 2). It imparts enormous strain on the outer shell, which may cause deformation or even rupture of the latter.

The polymer chains in mesoglobules are strongly entangled since the TRP is in a condensed phase state. The higher the molar mass, the more entangled the chains. Hence, one would expect that the polymer matrix in the mesoglobules would disintegrate rapidly if it is composed of a TRP of lower molar mass. Another reason for the hysteresis in the cooling−heating cycles is that PNIPAM mesoglobules are partially vitrified, consequently, longer time for disintegration is required for complete remixing.[14] Importantly, the inter- and intrachain interactions are so strong that they can be completely overcome only by deep cooling to 4 °C, when water becomes an extremely good solvent for PNIPAM.

PIPOX Mesoglobules. PIPOX is often considered as a structural isomer of PNIPAM. The two polymers share many similarities from the macroscopic viewpoint. Both polymers are soluble in cold water, and their aqueous solutions undergo a phase transition upon heating. The LCST of PIPOX ranges from 36 to 63 °C depending on polymer molar mass and concentration. The crucial difference between PNIPAM and PIPOX is in the mechanism of heat-driven phase transition. This difference can be explained with respect to the strength of hydrogen bonds between the monomer units and water molecules that those polymers form. Weaker hydrogen bonds are formed in the aqueous solutions of PIPOX, as the hydrogen bond-forming amide groups are absent in PIPOX (Figure 1). The clouding of aqueous PIPOX solutions resembles the formation of two liquid phases. This liquid/liquid phase separation is characteristic for PIPOX solutions.[24] Within the polymer-rich phase, the polymer chains adopt exclusively trans-conformation.[25] This conformation facilitates interchain dipolar interactions between amide groups, which promote partial organization of the chains, leading to crystallization of PIPOX. Crystallization occurs by nucleation, which requires introduction of thermal energy. Therefore, prolonged heating at (50−70 °C) temperatures of PIPOX aqueous solutions has been found to induce crystallization.[26,27] Scheme 4 clearly shows the mechanism of change in the conformation of the individual phases of the heating process.

Scheme 4. Changes in the Conformation of PIPOX in Water as a Function of Temperature[a]

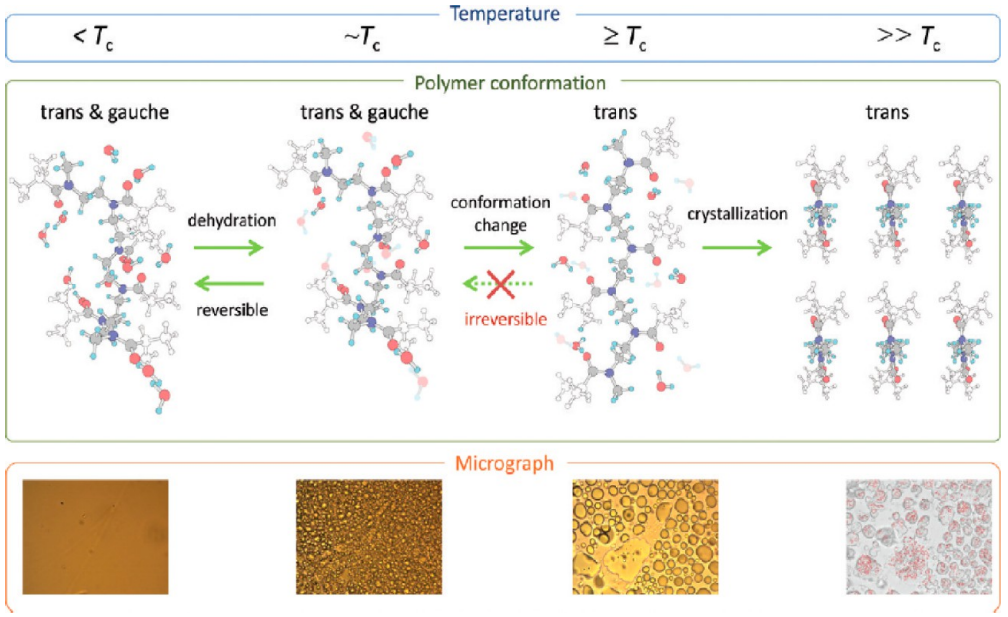

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Table 1. Experimental conditions, molecular characteristics and properties of mesoglobules prepared from different TRPs

polymer	M_n (Da)	C (g/L)	T (°C)	additive (SDS) s/p[b]	mean size (R_h, nm)	heating mode	references
PNIPAM	15500	0.08	50	-	108	gradual	11, 12, 19
	160000[a]	0.25	50	-	86		
	84000	1.0	60	-	50		
		1.0	60	0.2	25		
PVCL	330000[a]	0.2	50	-	129	gradual	11, 12
PVME	9400	0.02	50	-	150	gradual	11, 12
	14000	0.02	50	-	200		
PIPOX	3660	0.5	80	-	490	abrupt	37
				0.5	400		
	5540	0.1	70	-	350		
				0.5	150		
	8940			0.5	123		
PDEGMA	6400	0.5	70	-	81	abrupt	33, 34
				0.3	39		
	11400			-	92		
				0.3	41		
	14000	0.5		-	165		
	112000	0.1		0.3	50		
				0.5	16		
				-	960	gradual	
				-	520		
PETEGA	7000	1.0	70	-	50	gradual	32
			40		90		
	20000		70		35		
	40000		70		25		
			40		50		

[a]M_w. [b]s/p - surfactant to polymer weight ratio.

In general, the thermal response of aqueous PIPOX solutions is faster than that of PNIPAM solutions, both for aggregation and disintegration, which is most likely due to the relatively weaker hydrogen bonding capacity of PIPOX and the absence of partial vitrifying during mesoglobule formation. The larger dimensions of PIPOX mesoglobules (see Table 1) are probably due to different thermal transition behavior. Importantly, prolonged heating at higher temperatures should be avoided in order to prevent crystallization of PIPOX and to prepare mesoglobule templates that easily dissolve upon cooling down to room temperature.

Mesoglobules of Oligoethylene Glycol Acrylate and Methacrylate. Recently, polymers of oligo(ethylene glycol) acrylate and methacrylate (POEGA or POEGMA) have been

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extensively studied as thermoresponsive polymers.[28] These polymers are composed of a hydrophobic polymethacrylate or polyacrylate backbone, grafted with short hydrophilic poly(ethylene glycol) chains. The thermoresponsive properties are governed by the balance between hydrophobic and hydrophilic moieties. The most important advantage, as compared to PNIPAM, is the fully reversible phase transition without marked hysteresis. The range of application of these polymers is extremely broad as shown by the excellent reviews, which have recently appeared.[29,30] Since our goal is to identify the most suitable templates for preparing polymeric capsules, we will focus primarily on the mode of formation and properties of colloidal mesoglobules from POEGA and POEGMA.

In spite of the considerable interest in POEGA and POEGMA, only a few studies have reported on the formation of mesoglobules.[31−34] When dilute (0.1 g/L − 1g/L) aqueous solutions of POEGMA or POEGA are heated above their LCST, stable colloidal spherical mesoglobules of narrow size distribution are formed. Their dimensions depend on the polymer molar mass, rate of heating, and solution concentration. Importantly, the hydrophilic PEG moieties are located preferably at the periphery of the mesoglobules, thus improving their stability in aqueous media.

Usually, the slow, gradual heating produces large mesoglobules from hundreds of nanometers to more than 1 μm in radius. In all cases, their dimensions, formed at higher concentrations, are larger than those formed at lower concentrations of TRP. Well-defined mesoglobules with considerably smaller dimensions are obtained when a shock, abrupt heating, is used (Figure 4). Besides the heating protocol,

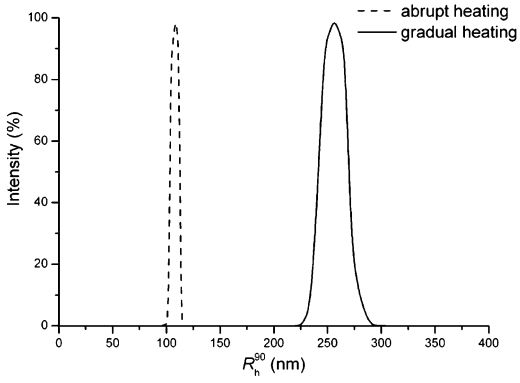


Figure 4. Size distribution of PDEGMA mesoglobules, (PDEGMA M_n = 112 000) prepared via abrupt and gradual heating of aqueous solution with a concentration 0.1 g/L. Reprinted from ref 34. Copyright 2012 Wiley Periodicals, Inc. With permission from John Wiley and Sons.

the polymer molar mass and surfactant addition can also influence the size of the mesoglobules as shown by Toncheva et al.[32] for templates prepared from poly(ethoxy triethylene glycol acrylate) (PETEGA; Figure 5). Recent light scattering studies have clearly shown that the mesoglobules remain highly hydrated[34] implying that hydrophilic biomolecules can be effectively loaded using an appropriate protocol. This suggestion was supported by recent studies[35] showing that the pentapeptide−PDEGMA conjugate readily forms stable mesoglobules.

Importantly, Hu and co-workers[36] demonstrated that POEGMA mesoglobules self-assemble into a colloidal crystalline phase at higher polymer concentrations between 3.8 and 6.3 wt %. While this finding is promising in microelectronics, it is not desirable in the use of mesoglobules as templates for polymeric capsules, since the formation of crystal phase makes it difficult to dissolve the template under cooling.

Summary and Setting Preferences for the Templates. The size of the template is crucial for the capsule engineering. Table 1 summarizes data for dimensions of mesoglobules, prepared from different TRPs, experimental conditions for their preparation, as well as other data such as molar mass, and the presence and quantity of additives. It is quite obvious that via selection of a proper preparation protocol one can control the size of the templates. In all cases, the temperature, the heating rate, and the initial polymer concentration are essential for the particle size. The most suitable conditions are dilute solutions of the TRP, that is, below the overlapping concentration. With the exception of PNIPAM, the higher the molar mass, the more compact (with smaller sizes) the mesoglobules. The most pronounced molar mass dependence of mesoglobules dimensions is observed for PDEGMA polymers (Table 1). In all cases, the slow (equilibrium) heating results in aggregates of larger size. More compact (smaller dimensions) and better defined (narrower size distribution) particles are obtained by abrupt, shock heating. Size reduction is observed upon addition of a surfactant,[19] ionic liquids,[20] and osmolyte.[21]

The ideal system for preparation of nanocapsules via mesoglobule templates should possess the following characteristics:

- The aqueous colloidal dispersions of mesoglobules should be stable in time even in the absence of surfactants. The mesoglobules should be uniform in shape with a maximum size of 100 nm and narrow size distribution.
- The mesoglobules should be able to quickly dissolve upon cooling, without any significant increase in volume due to swelling.
- Low molar mass polymers (preferably below 10 000) with narrow dispersity and flexible chains without functional groups able to interact with the membrane would facilitate diffusion of the polymer chains through the membrane and, hence, their more effective removal from the interior of the particles.
- The process of mesoglobule formation should not be hampered by interactions with molecules that are to be loaded in the nanocapsules.

CAN THE MESOGLOBULES BE LOADED WITH BIOMACROMOLECULES?

The main objective of the present contribution is to show the feasibility of creating scaffolds that would keep biomolecules, such as peptides, proteins, or nucleotides, in a fully functional state during their storage and delivery to a specific target. Chemically conjugated biological species cannot be applied in this case, because conjugation leads to a substantial decrease of the biological activity.[38] Therefore, it is essential that the incorporation of biomolecules is based on physical entrapment into the mesoglobules in aqueous solution, that is, in the absence of harsh organic solvents, acids, bases, or other toxic compounds. Detailed studies of incorporation of biomolecules during the process of formation of the template would contribute to achieve controlled loading. As already mentioned, an important advantage over other methods is that the loading

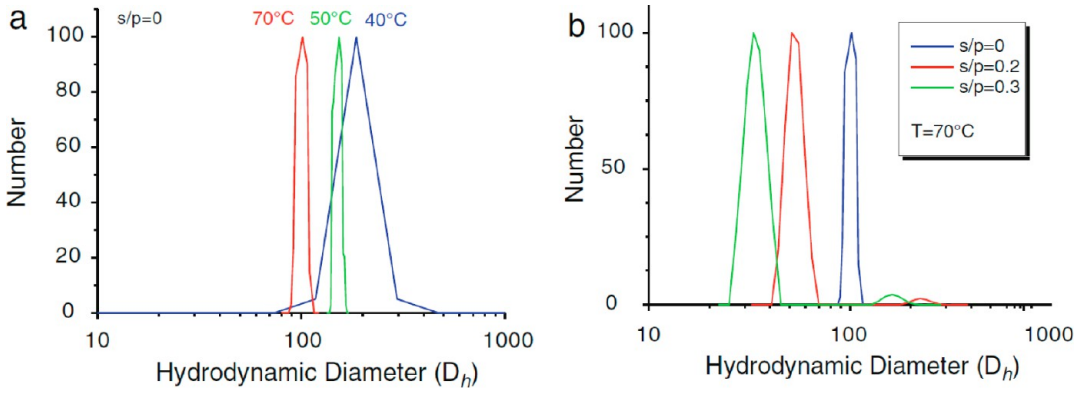


Figure 5. Influence of preparation protocol on the mesoglobule size: (a) heating temperature; (b) SDS as additive. PETEGA M_n = 8000, polymer concentration 1 g/L. Reprinted from ref 32. Copyright 2011 Springer-Verlag. With kind permission from Springer Science and Business Media.

Table 2. Loading Efficiency of Mesoglobules and Their Higher Aggregates Towards Different Proteins and Dyes

polymer	loaded molecule	loading efficiency 25 °C	loading efficiency >37 °C	protocol	mean size (D_h) and shape	references
PNIPAM cross-linked	lysozyme	132 mg/g	4.91 mg/g	water, penetration and adsorption	sphere, 80 μm	43
	ovalbumin	120 mg/g	6.92 mg/g			
PNIPAM	Nile Red	fluorescent analysis		water, two dye populations	na	44
PNIPAM hydrogel	α-lactalbumin	2.5 mg/g	7.0 mg/g	water, adsorption desorption	sphere, 1500 nm (25 °C), 400 nm (40 °C)	45
	lysozyme	3.5 mg/g	3.0 mg/g			
	myoglobin	3.0 mg/g	19.5 mg/g			
PEO-*b*-PtNEA	FITC-Lys	-	46 mg/g	phosphate buffer, heating	sphere, 450 nm	46
poly(NIPAM-*co*-AAm)	blue dextran	10%	-	water, penetration	sphere, 160–200 μm	47
poly(NIPAM-*co*-BMA-*co*-AA)	FITC-insulin	-	91%	water, phosphate buffer	sphere, 2–4 mm	48
	cytochrome C	-	44%			
	angiotensin II	-	33%			

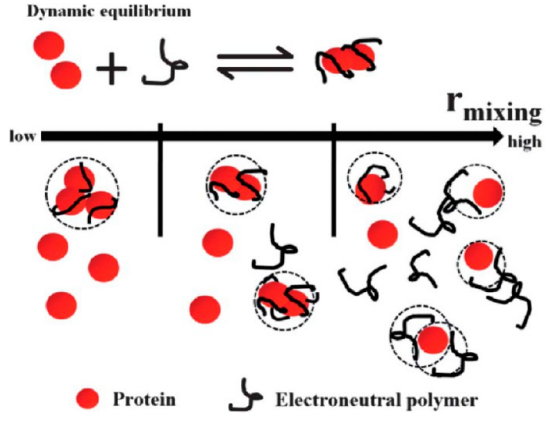
operations can be performed entirely in aqueous medium in a mild and nondestructive fashion.

Loading of Biomolecules by Physical Entrapment. For the last two decades, peptides and proteins have been available in sufficient quantities and purity to use them successfully as therapeutic agents. Their nature and short-time activity make it difficult to be delivered to the desired site in the human body. Very often they can be easily eliminated from the systemic circulation due to enzymatic degradation and excretion. Therefore, efficient delivery systems are required, and various approaches are tested. Many researchers use well-known drug carriers such as polymeric micelles, nanogels, or polymersomes.[4]

Undoubtedly, our approach can be applied only if mesoglobule templates are able to incorporate sufficient amounts of biomolecules during the phase transition process. However, studies on the incorporation of biodrugs into mesoglobules via heating of mixed aqueous solution at temperatures above the LCST of the TRP are very few (see Table 2).

Let us consider the mode of interaction between a protein and a TRP in aqueous solution below the PTT by referring to recent studies.[39,40] When a protein such as bovine serum albumin (BSA) interacts with an uncharged water-soluble polymer such as PNIPAM at temperatures below the LCST of the latter, the main driving forces are hydrogen bonding and hydrophobic interactions.[40] Because of the weak protein–polymer interactions, the binding is in a reversible dynamic equilibrium state as shown in Scheme 5. With increasing

Scheme 5. Schematic Illustration of the Complex Formation of the BSA with PNIPAM at Temperatures Lower than the LCST of PNIPAM[a]



[a] r_{mixing} is the molar ratio of PNIPAM to BSA. Reproduced from reference 40. Copyright 2013, with permission of The Royal Society of Chemistry. http://dx.doi.org/10.1039/c3ra43146k.

PNIPAM/BSA molar ratio, r_{mixing}, the interchain interactions of PNIPAM are enhanced, which hinders the binding affinity between BSA and PNIPAM. It is noteworthy that the authors performed their experiments at 25 °C, a temperature very close to the LCST of PNIPAM at which water is no longer a good solvent for PNIPAM. As a result, two interactions competitive to BSA-PNIPAM, namely, polymer intrachain contraction and

polymer−polymer interchain association, took place. In this case, PNIPAM showed well-pronounced temperature-dependent protein absorption and adsorption.

For better understanding of the process of protein loading, it must be considered that the properties of mesoglobules very much resemble those of the smart polymeric nanogels (PNGs), because they represent a gel phase, which is finely dispersed.[41] The essential difference is that the PNGs are chemically cross-linked materials and, hence, not able to disintegrate. High protein-loading capacities have been found for hydrophilic PNGs. The main reason for this is that the swollen PNGs, in which the water content prevails, provide a large cargo space for incorporation of biomolecules. The microphase separation, which takes place inside the mesoglobules, results in the formation of hydrophilic and hydrophobic domains, similarly to the structure of PNG. This assumption has been confirmed by Junk et al.[41] who found that the mesoglobules from dendronized TRPs, formed by slow heating at high polymer concentrations, were composed of a dense polymeric layer (skin barrier) at the periphery of the particle thus entrapping considerable amount of water (Figure 6). The release of

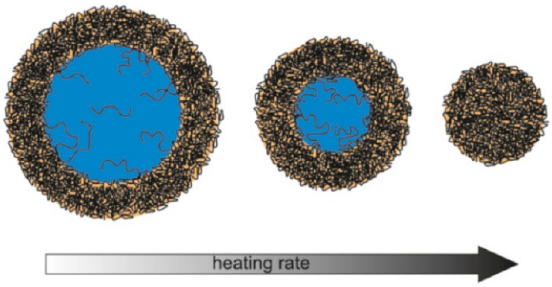


Figure 6. Simplified sketch on the effect of heating rate on morphology of mesoglobules. Reprinted with permission from ref 41. Copyright 2011 American Chemical Society.

molecules, accommodated in the aqueous core is obviously greatly hindered. Detailed insights into the impact of the heating rate on the mesoglobules morphology provide important information about how to tailor their structure. As a general rule, hydrophobic guest molecules can be loaded into completely collapsed aggregate, whereas uptake of hydrophilic molecules is facilitated by a slow heating rate leading to formation of an aqueous core (Figure 6) or aqueous voids as shown in Scheme 3.

In most cases hydrophilic protein or hydrophilic peptide sorption into PNIPAM mesoglobules does not occur, although even the compact mesoglobules are highly hydrated. Instead, adsorption on the surface is predominant, resulting in covering of mesoglobules by protein (or peptide) molecules. This is in agreement with the aggregation behavior of a peptide-conjugated TRP, which formed nanoparticles composed of a compact core of TRP moieties, surrounded by a thin peptide shell.[35,42] On the contrary, hydrophobic peptides occupy the mesoglobules' interior, where they can be protected from enzymatic hydrolysis.[49] PNIPAM showed a temperature dependence of protein adsorption. Importantly, most of the proteins adsorbed at temperatures higher than the PTT can be desorbed by lowering temperature. Adsorption of flexible proteins is preferable, since the process is reversible, due to the fact that flexible proteins can easily acquire the structural rearrangement into the native state.

It is logical to assume that mesoglobules can be used for entrapment of nucleotides. Encapsulation of DNA or oligonucleotides into mesoglobules has not been observed yet. Chen et al.[50] have recently shown that the DNA conformation in an aqueous PNIPAM solution can be controlled by changing the solution temperature as presented in Figure 7. Experimental results show that not all compacted DNA condensates dissolve because some are observed also after the heating (not shown in Figure 7). Importantly, the particles formed at low and higher temperatures are similar in size (200 nm) and shape (spherical). In their study Chen et al. have not mentioned whether some PNIPAM macromolecules are incorporated into the DNA condensates. Partial PNIPAM incorporation onto the template can be suggested from the heterogeneous surface composition. The mechanism of DNA compaction by PNIPAM should be much different from that with polycationic polymers as ionic interactions between PNIPAM and DNA are unlikely, since there are no positively charged groups in the PNIPAM macromolecules. Two mechanisms of binding have been considered:[50] (i) weak binding analogous to DNA−polyamide systems[51] and (ii) compaction of DNA in a crowding environment of PNIPAM, similar to the effect of PEGs.[52] Importantly, analogous to peptide−PNIPAM systems, DNA and PNIPAM are compatible only in chilled aqueous medium. This was confirmed by recent studies of Moura et al.[53] and Martinho et al.[54] who were able to immobilize oligonucleotide (OND) and DNA on thermoresponsive shell of PNIPAM, copolymerized with a small amount of positively charged comonomer, aminoethyl methacrylate hydrochloride, in order to increase the absorption of negatively charged DNA and OND. At temperatures lower than PTT, the nucleotides are distributed into the interior of the shell. By increasing the temperature above the phase transition, DNA and OND are anchored in the dense surface layer, not located inside the shell but oriented toward the water phase. Other results, published elsewhere,[53,54] suggest that the incorporation into the TRP of small amounts of hydrophobic, weakly acidic, or basic comonomers is an important factor for successful biomedical and biotechnological applications. In that case, the electrostatic interactions were the dominant force. Thus, the loading efficiency of proteins, peptides, or nucleotides would be significantly enhanced.

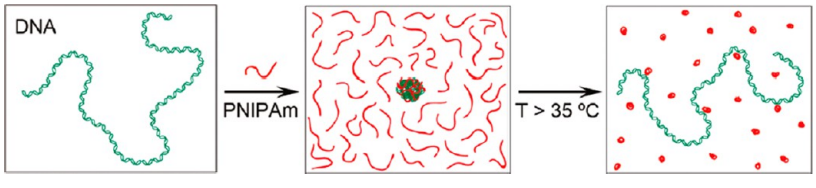


Figure 7. Schematic presentation of DNA compaction and temperature-induced decompaction in PNIPAM aqueous solutions. Reprinted with permission from ref 50. Copyright 2010 American Chemical Society.

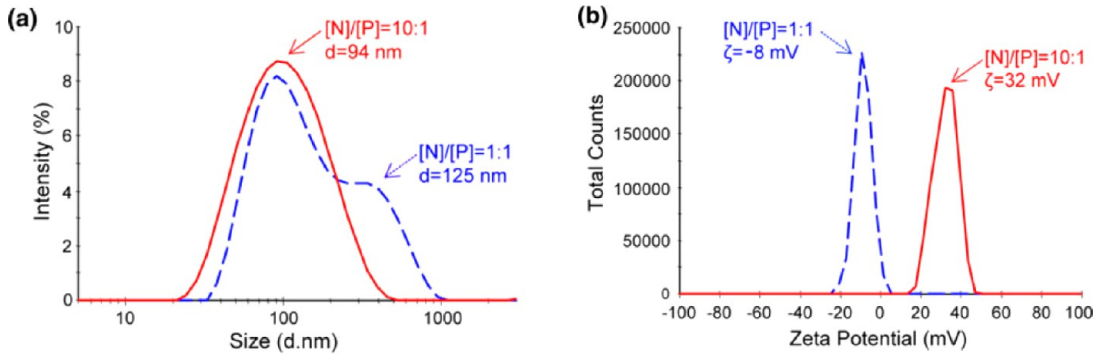


Figure 8. Effects of the N/P ratio on (a) average diameters and (b) zeta potentials of polyplexes. Reprinted from ref 56. Copyright 2012 Springer Science+Business Media Dordrecht. With kind permission from Springer Science and Business Media.

Table 3. Polyplexes Based on TRP

polymer	M_n (kDa)	DNA	mean size (D_h, nm)	additional remarks/properties	references
PNIPAM	20−25	T4DNA	200	DNA compaction/decompaction controlled by temperature	50
poly(NIPAM-*co*-DMAEMA)	138	pDNA	200	TRP mask cytotoxicity, aspecific transfection efficiency	58
poly(NIPAM-*co*-DMAEMA-*co*-BMA)	40−200	pDNA	na	transfection efficiency increase by hydrophobic unit incorporation, thermally regulated gene expression	57, 59
CS(-*g*-PDMAEMA)-*g*-PNIPAM	25−50	pDNA	100−150	enhanced transfection efficiency, thermally modulated	61
4-branched PDMAPAAm-*b*-PNIPAM	36	pDNA	100	used as adsorbent material	60
PEI-*g*-PNIPAM	65−85	pDNA	160−240	temperature-dependent transgene expression, cold-shock effect	62
PEI-*g*-PNIPAM	na	pDNA	150−300	temperature-dependent toxicity and transfection	63
P(NIPAM-*co*-DMAEMA-*co*-BMA)	80−100	pDNA	na	polyplex formation/dissociation modulated by temperature alternation	55
PNIPAM-*b*-PLLys	14	ssDNA	60	DNA encapsulation into biodegradable polymer shell decorated with targeting functions, transfection effectiveness	64, 65
		pDNA	163		
(PNIPAM-*g*-PEG)-*b*-PLLys	25	ssDNA	100	high ability to condense DNA, low cytotoxicity, transfection effectiveness	56, 65
		pDNA	166		
PDMAEMA-PPO-PDMAEMA	8.1	pDNA	195	rod-like polyplexes, DNA encapsulation into biodegradable polymer shell, transfection effectiveness	66

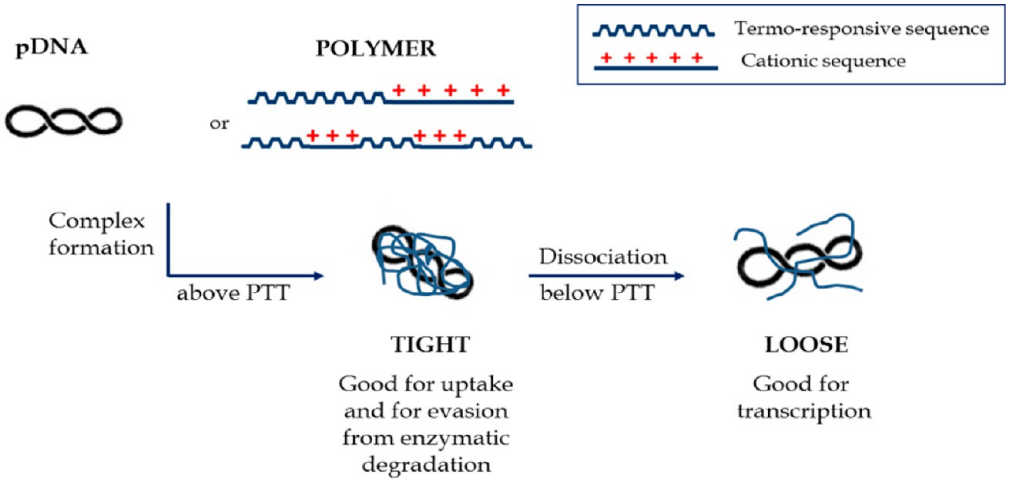


Figure 9. Formation of loose and tight complexes due to the presence of TRP sequences in polyplexes. Modified from ref 57. Copyright 2000 with permission from Elsevier.

Loading by Electrostatic Interactions. Temperature-Responsive, Mesoglobule-like Polyplexes. Polyion complexes (polyplexes) with plasmid DNA have a great potential to act as nonviral gene delivery vectors. However, their poor solubility combined with nonspecific interactions with components of the reticulo-endothelial system (RES), may result in rapid bloodstream clearance. Another serious problem, when using polyplexes, is their relatively low cell transfection efficiency.[55] Polyplexes are formed by mixing DNA (or OND) and a cationic polymer (or a copolymer bearing cationic moieties) in aqueous solutions at different molar ratios between the cationic groups of the (co)polymer and the phosphate

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groups of DNA (N/P). The size of the polyplexes strongly depends on the N/P ratio (Figure 8). The increased zeta potential at increased N/P ratios can be ascribed to gradual covering of the negatively charged nucleotide by the positively charged polymer.

The polyplexes must overcome a series of obstacles in the gene transfection process, including passing through the cell membrane, escaping from the endosome, and entering the nucleus. In the first two stages, the polyplex should be stable and compact to protect the plasmid from being degraded. When DNA is transported into the nucleus, however, the polyplex should be loose enough to allow DNA release. Obviously, traditional cationic polymers cannot satisfy these contradictory requirements at the same time. A promising approach is to use copolymers comprising temperature-responsive and cationic moieties (Table 3).

The incorporation of a thermoresponsive polymer entity (e.g., PNIPAM) leads to the formation of a tight and stable complex at 37 °C due to the collapse of the PNIPAM chains. Reducing the temperature below the LCST weakens the ionic interaction. As a result, the complex can be easily dissociated, which would allow the DNA transcription into the nucleus (Figure 9). Thus, by changing temperature, it is possible to control the transition of the polyplex from tight or compact (prevents DNA degradation) to weakly bound or loose (suitable for transfection). The so-called temperature cycling or cold shock was employed by incubation above the LCST, then below the LCST, and again above the LCST. These reversible DNA-binding structures generated in a controlled manner are expected to result in more efficient gene transfection. For example, the temporary lowering of the incubation temperature to 20 °C in a post-transfection period of the culture resulted in a substantial increase of gene expression levels in comparison to cultures incubated at 37 °C,[59] as shown in Figure 10.

Importantly, many polyplexes based on TRPs showed a poor stability at 37 °C and a tendency to aggregate. This was observed by Zhou et al.[60] who studied polyplexes from a star-like copolymer comprising cationic poly(*N*,*N*-dimethylamino-propylacrylamide) and PNIPAM moieties (Figure 11). Since aggregated species are much larger than 200 nm (size of 200 nm or less is a prerequisite for efficient transfection[58]), they cannot be taken up by the cells. Coating the polyplexes with a polymeric membrane, which is discussed in the next section, might prevent their aggregation.

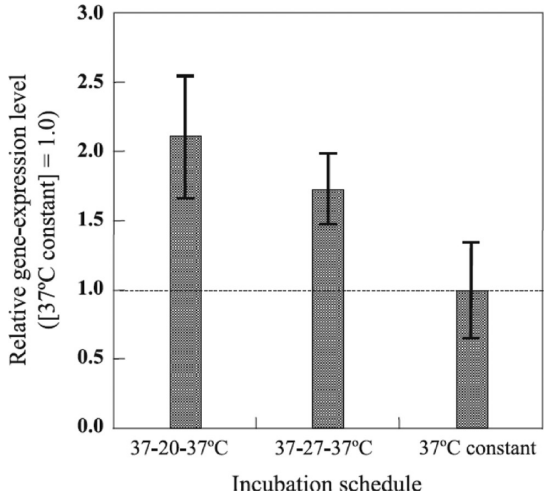


Figure 10. Thermal dependence of β-galactosidase expression in poly(NIPAM-DMAEMA-BMA)/pDNA polyplex. Reprinted from ref 59. Copyright 2004 with permission from Elsevier.

OUTER SHELL MEMBRANE FORMATION BY MEANS OF SEEDED HETEROPHASE COPOLYMERIZATION

In the following sections we focus on the development of long-circulating polyplexes prepared through the formation of a polymeric membrane/shell on the polyplex surface, which would provide additional stabilization and stealth properties. In particular, our expectations are that

- the polymeric membrane would greatly reduce protein and cellular interactions, leading to extension of the plasma circulation time, thus improving the therapeutic strategies;
- the high (positive or negative) values of zeta potential of the initial polyplex particles would be greatly reduced, thus avoiding the salt-induced aggregation;
- specific ligands could be easily attached in order to achieve targeted delivery and transfection that would improve the therapeutic efficiency;
- undesirable aggregation of thermoresponsive polyplexes, as shown in Figure 11, could be avoided.

The idea to cover polyplex nanoparticles with a polymeric membrane was first proposed in 2009 by Dimitrov et al.[67] All stages of the polyplex encapsulation are schematically presented in Figure 12.

The strategy for preparation of core−shell nanoparticles based on mesoglobule templates, used as seeds, provides a good control of the size and polydispersity of the final composite nanoparticles. As mentioned above, the size of mesoglobules can be easily adjusted, which is of great importance, since the particle size and size distribution are crucial for medical applications. The method of outer shell formation by seeded polymerization technique (radical surface copolymerization) is quite flexible and offers a highly efficient approach for the synthesis of advanced polymer colloids.[68] This introduces a new pathway for preparation of mechanically stable polymeric capsules with a wide range of sizes, engineered functionality, and controlled porosity of the outer shell. The proper selection of monomers and cross-linkers used for the outer shell formation as well as the type of initiator is of great importance.

The first attempt to build an outer hydrophilic shell on the mesoglobule surface was done by Weda et al.[19] HEMA and PEG-DMA as a cross-linker were used to coat PNIPAM mesoglobules. The selection of the comonomers was based on the expectations that the $-CH_2CH_2OH$ group from PHEMA and the PEG moiety from PEG-DMA would prevent flocculation of the particles. The polymerization was initiated by the hydrophilic radical initiator KPS and was carried out in aqueous solution at 60 to 80 °C, which is well above the LCST of the core-forming PNIPAM. Initially, the polymerization proceeded entirely in the aqueous phase resulting in the formation of HEMA oligomers. As water is a poor solvent for linear PHEMA, the hydrophobicity of HEMA oligomers constantly increased with increasing molar mass. When the oligomers became hydrophobic enough, they adsorbed on the mesoglobule surface, thus forming a PHEMA layer. The latter was able to react with molecules of both the monomer and cross-linker from the aqueous phase so that the polymerization

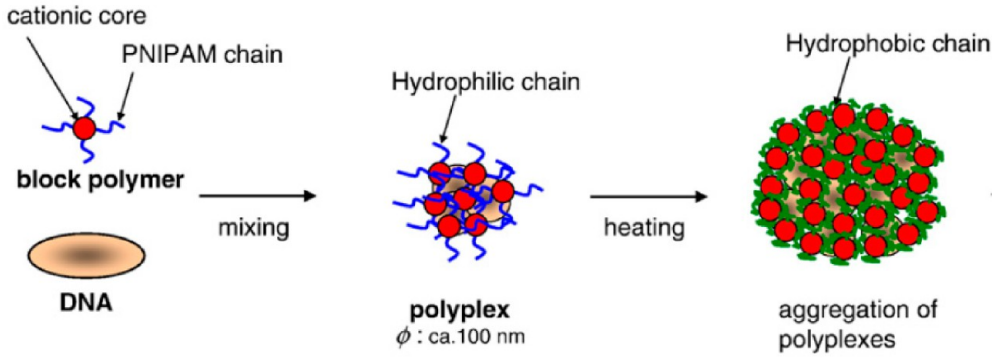


Figure 11. Aggregation of thermoresponsive polyplexes after prolonged heating. Reprinted from ref 60. Copyright 2007 with permission from Elsevier.

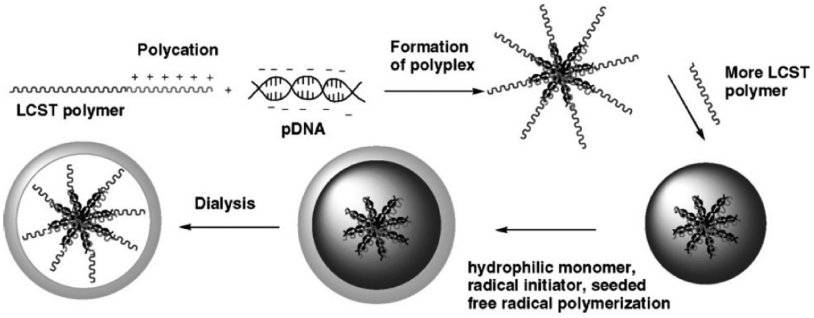


Figure 12. Scheme of polyplex encapsulation. Reprinted from ref 67. Copyright 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. With permission from John Wiley & Sons.

continued within the layer and the shell thickness grew. A correlation between the initiator to monomer ratio and shell thickness was found, thus providing an effective control over the thickness of the polymeric membrane. Importantly, the spherical shape of the resulting core−shell particles was preserved.

An essential advantage of a membrane, which is built from a thermoresponsive polymer, is the fact that its hydrophilicity and permeability can be easily tuned by changing the temperature. The latter is a desired function for both loading and release of biologically active substances.

In the case of core-forming PIPOX and the shell-forming PNIPAM, rather unexpectedly, raspberry-like morphology was observed as shown in Figure 13.[37] The anisotropy arises from the immiscibility of the monomers and polymers when forming the cross-linked membrane.[69]

When an oil-soluble initiator AIBN is added to the suspension of hydrophobic seed particles in water, radicals are generated on the mesoglobule surface. In this case the partition between the monomer and the seed particle depends strongly on the interfacial tensions of the particle and the aqueous phase $\gamma_{P,A}$, the particle and the monomer, $\gamma_{P,M}$, and the monomer and the aqueous phase $\gamma_{M,A}$ as shown by Mock et al.[69] The shape of the monomer droplets is determined by the monomer/polymer wetting properties, kinetics of the polymer network contraction, and kinetics of monomer polymerization (Scheme 6).

When a hydrophilic initiator such as KPS was used, NIPAM oligomers were initially formed in the aqueous phase. At a certain moment, they collapsed and began to adsorb on the PIPOX mesoglobules surface forming a nodule and, by trapping monomer and cross-linker from the aqueous phase, further evolved as polymeric particles attached to the surface. The

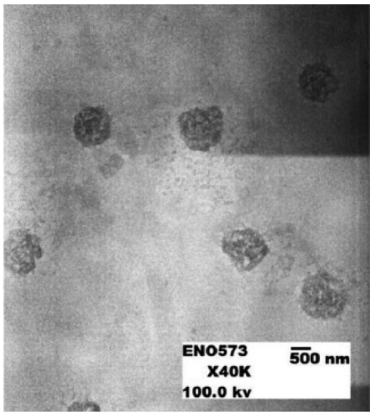
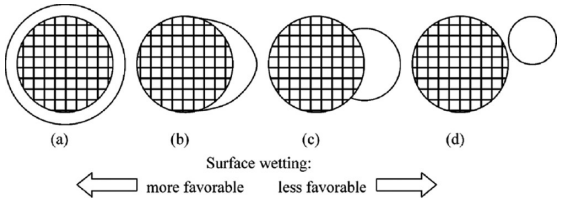


Figure 13. TEM image showing raspberry-like morphology of the PIPOX(core)/PNIPAM(shell) particles. Reprinted from ref 37. Copyright 2013, with permission from Elsevier.

Scheme 6. Possible Swelling Geometries at Different States of the Monomer−Seed Particle Systems: (a) Very Favorable; (b) Moderately Favorable; (c) Not Favorable; (d) Extremely Unfavorable[a]



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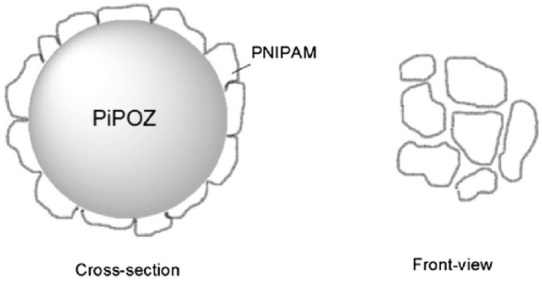
immiscibility between PIPOX and PNIPAM revealed by DSC led to the formation of an inhomogeneous layer with raspberry-like morphology, as shown in Scheme 7.

Scheme 7. Schematic Presentation of the Cross-Section and Front View of Core-Shell Nanoparticles with Raspberry-Like Morphology, Formed by Seeded Copolymerization of NIPAM and BIS on the Surface of PIPOX Mesoglobules[a]



[a]Reprinted from ref 37. Copyright 2013, with permission from Elsevier.

A strong advantage of the approach of using heterophase seeded radical polymerization is the possibility to incorporate monomer units bearing specific functions so that the resulting particles meet a set of preferable properties such as the following:

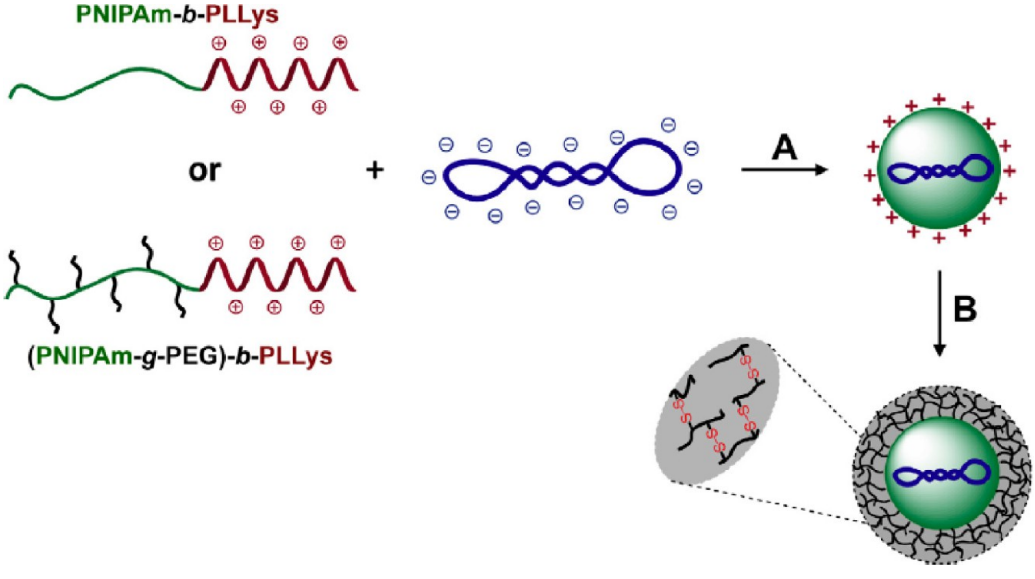
- Stimuli-responsiveness of the shell. Monomers such as NIPAM, acrylic acid, oligoethylene glycol methacrylate, other acrylate monomers, etc. have been successfully used. Due to the collapse of the shell chains under stimuli, channels can be formed. Glucose and temperature dual-responsive nanospheres were fabricated by incorporation of glucose-responsive monomer (3-acrylamidophenylboronic acid) (APBA) as reported by Du et al.[70]
- Biodegradability of the shell. By using biodegradable cross-linking agents such as *N*,*N*′-bis(acryloyl) cystamine (BAC), disulfide bonds were incorporated in the network of the shell.[64−66] They can be degraded under reducing conditions or via thiol−disulfide exchange occurring in the cells.
- Attachment of cell recognizable targeting ligands. This was demonstrated by Dimitrov[64] by incorporating a folate-terminated PEO-macromonomer.

Seeded radical copolymerization has been successively used for encapsulation of temperature-responsive polyplexes with a biodegradable cross-linked shell.[64−66] For this purpose, first plasmid or linear DNA was compacted via complex formation with cationic diblock copolymer (PNIPAM-*b*-PLLys or (PNIPAM-*g*-PEG)-*b*-PLLys[64,65] (Scheme 8) or via interaction of plasmid DNA with cationic worm-like micelles comprising a poly(propylene oxide) core and a PDMAEMA corona[66] (Scheme 9). The polyplex particles exhibit good colloidal stability at high temperature (up to 70 °C), allowing formation of a cross-linked shell via copolymerization of NIPAM and *N*,*N*′-bis(acryloyl) cystamine. The formation of the outer shell was confirmed by the increase of apparent hydrodynamic diameter and change in zeta potential from highly positive to less positive or from highly negative to less negative (Table 4).

CORE RELEASE: FORMATION OF HOLLOW NANOPARTICLES

According to our approach, nanocapsules are fabricated by dissolution and removal of the core template. When core−shell particles are cooled down to a temperature below $LCST_{core}$, the polymer chains of the core dissolve and go into the water phase by penetrating through the cross-linked shell into the water phase. The outer polymeric shell (membrane) acts as a barrier to the diffusion of macromolecules from the core to the particle/water interface. Therefore, to obtain hollow structures (capsules), construction of a permeable outer membrane is mandatory. However, systematic information on factors affecting the membrane permeability is not available. Factors,

Scheme 8. Polyplex Formation from Thermoresponsive PNIPAM−PLLys Block Copolymers Followed by Coating with Biodegradable Polymer Layer[a]



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Scheme 9. Schematic Illustration of the Formation and Encapsulation of the Polyplex between pDNA and Cationic Micelles[a]

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Table 4. Preparation of Polymeric Capsules and Encapsulated Polyplexes via Seeded Interfacial Copolymerization

				hollow particles (D_h, nm)			
polymer core	mean size of core (D_h, nm)	polymer shell	shell thickness (nm)	25 °C	>40 °C	additional remarks	references
PNIPAM	50−100	PHEMA, PEG-DMA	3−25	300	125	capsules like deflated balloon	19
P(St-*co*-NIPAM)	40−230	PNIPAM, BIS	30−90	460	175	hollow microgels with temperature sensitivity	71
PNIPAM	-	PNIPAM, APBA, DVB	80−100	300−400	200−300	temperature and glucose responsive capsules	70
PIPOX	300−2000	PNIPAM,BIS	40−70	345	705	raspberry-like shell	37
PNIPAM	108−285	PNIPAM,BIS	80−135	1400	300	core made via semicontinuous heterophase polymerization, fully soluble mesoglobules	72
PDEGMA	100−350	PNIPAM, BIS	10−35	100−250	-	raspberry-like shell	73
		PHEMA, BIS	20	131	-	smooth shell	
P(G-*co*-EGC)	225−350	PNIPAM,BIS	25−30		-	no dissolution of the core because of too high MW	74
P(PNIPAM-*b*-PLLys)/ssDNA	80−150	PNIPAM,BIS, PEG-DA or BAC	15−90		-	for the first time encapsulated polyplex, folic acid used as targeting vector	64
P(PNIPAM-*b*-PLLys)/pDNA	163	PNIPAM, PEG-A, BAC	15		-	encapsulated polyplex, reduced transfection efficiency compared to the corresponding polyplexes	65
P(PNIPAM-*g*-PEG)-*b*-PLLys/pDNA	116	PNIPAM, PEG-A, BAC	25		-	encapsulated polyplex, reduced transfection efficiency compared to the corresponding polyplexes	65
PDMAEMA-PPO-PDMAEMA/ pDNA	195	PNIPAM, BAC	10		-	encpsulated polyplex protecting shell decreased the cytotoxicity	66

generally considered to govern the shell permeability are given below.

- chemical composition and the hydrophilic/hydrophobic balance of the shell
- cross-linking density
- thickness
- formation of channels within the membrane
- charged/uncharged state of the shell as well as the encapsulated macromolecules (core polymer)
- molecular weight and dispersity of the core-polymer
- flexibility of the core-polymer
- the disintegration rate of the core template

Some of the above factors, e.g. chemical composition, cross-linking density, membrane thickness, are quite obvious. Others are discussed below.

The seeded copolymerization allows preparation of shell membranes with controllable thickness and permeability. The thickness increases with increasing monomer to initiator ratio as well as the reaction time. Obviously, a thicker shell would hinder polymer chain diffusion, which would result in a reduced release rate of the core polymer during the core-removal process. The flexibility of the core-polymer is of significant importance as well: a unique feature of the flexible macromolecules is their ability to permeate the pores despite the fact that the coil size can be larger than the pore size.[75]

It should be noted that the hindered transport of core-forming polymers is greatly influenced by the molecular weight and dispersity. Low MW TRPs with narrow MW distribution are easily permeating through the shell membrane, while large macromolecules are retained.[70,73] Thus, the fact that permeability is limited to a certain molecular weight allows efficient encapsulation of large biomacromolecules, which are not able to leave the core.

The formation of channels within the membrane is beneficial since it may accelerate the release of the template. It should be noted, however, that the macromolecule diffusion in the channel is restricted as shown by a model study for a polymer in a tube.[76] The size and the pore geometry can be adjusted by several approaches. The first approach consists of an appropriate selection of polymers forming the core and the shell. The core is invariably composed of mesoglobules of a TRP or copolymers comprising thermoresponsive sequences. The cross-linked shell, however, can be constructed by polymers with properties that are quite different from those of the core-forming polymer. In recent articles, thermoresponsive PNIPAM[37,70,7,73] or the hydrogel-forming PHEMA[19,73] have been used for construction of the membrane. As discussed above, upon cooling, the mesoglobules swell and then dissolve (Figure 3). The presence of a cross-linked polymeric shell on their surface, however, may prevent the mesoglobules from swelling to equilibrium (e.g., effect of core compression). PNIPAM and PHEMA form membranes with a different morphology and mechanical strength, which inevitably leads to a different mechanism and kinetics of core-TRP extraction upon cooling. The membrane composed of cross-linked PNIPAM is raspberry-like and uneven in contrast to that of cross-linked PHEMA, which is smooth, uniform in thickness,

and homogeneous. The raspberry-like morphology of the hollow particles is shown in Figure 14.

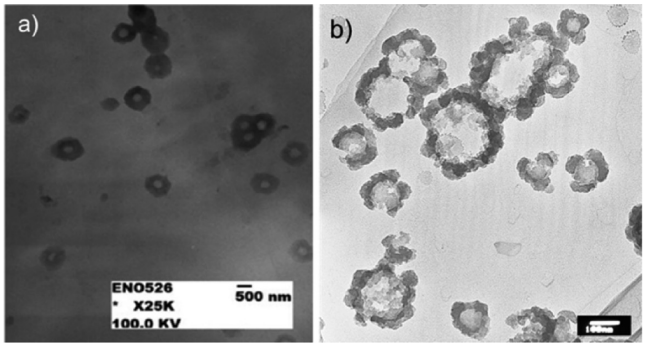


Figure 14. TEM images of hollow nanoparticles composed of PNIPAM membrane. The particles were obtained using templates from (a) PIPOX mesoglobules[37] and (b) PDEGMA mesoglobules.[73] Reprinted from refs 37 (Copyright 2013) and 73 (Copyright 2014), with permission from Elsevier.

Let us consider the cases where the core and the shell are composed of different TRP characterized by $LCST_{core}$ and $LCST_{shell}$. In the case of $LCST_{core} > LCST_{shell}$, when the temperature is below $LCST_{core}$ and close to or higher than $LCST_{shell}$ (Scheme 10b), the collapse of the shell results in the formation of channels, which greatly facilitates the diffusion of the core polymer and the trans-membrane transport.[37] In the case when dialysis is carried out to remove the core polymer at temperatures lower than the LCST for both polymer and $LCST_{core} \leq LCST_{shell}$ (Scheme 10c), water is a good solvent for all components of the system. At such conditions, the initial core−shell particles consist of swollen core and shell that are cross-linked physically and chemically, respectively. The core dimensions increase until an equilibrium between the force exerted by the shell and the resisting elastic force developed in the core is reached. In the next step, the macromolecules from the mesoglobules are expected to pass through the cross-linked shell hydrogel. However, due to the increase in the macromolecule dimensions, the process of core release is slow and frequently incomplete.

An alternative method of preparing porous membranes involves seeded copolymerization in the presence of a particulate porogen matrix such as sucrose or glucose, or glucose comonomers. Dergunov and Pinkhassik[77] successfully used pore forming molecules (glucose pentaacetate and glucose pentabenzoate) to synthesize nanocapsules with pores of controlled size (0.8 ± 0.2 nm). The porous nanocapsules retain molecules larger than the pore size but provide ultrafast access to their interior for molecules and ions smaller than the pore size (Figure 15). Membranes can also become porous in

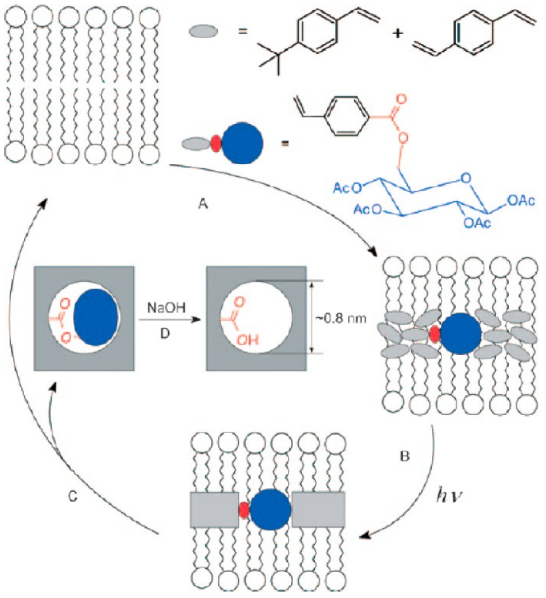
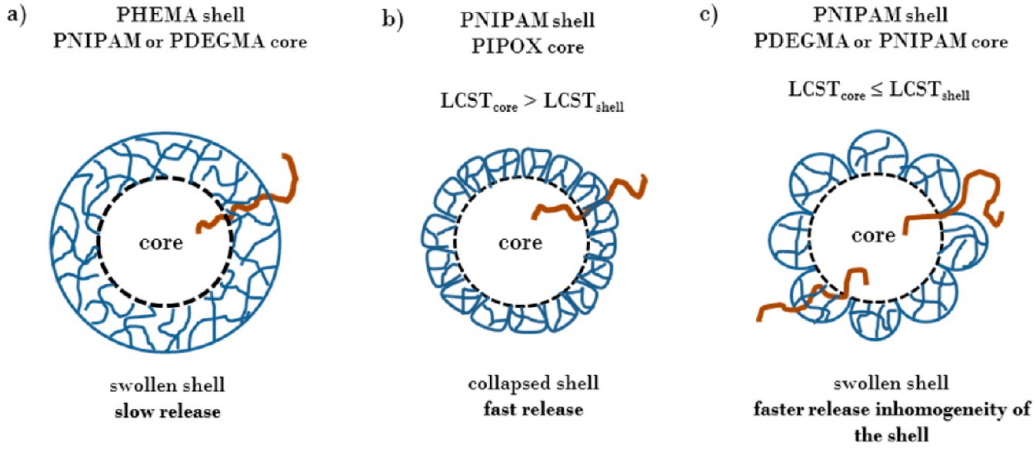


Figure 15. Nanometer thin film with functionalized nanopores. Reprinted from ref 77. Copyright 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. With permission from John Wiley and Sons.

response to the presence of glucose in the medium, when glucose-responsive phenylboronic acid derivative is introduced via NIPAM and 3-acrylamidophenilboronic acid seeded copolymerization as shown elsewhere.[70] Channels can be constructed also when the seeded copolymerization is performed with comonomers creating incompatible (immiscible) domains. For example, channels can be further modified by the formation of a mixed PEG/PNIPAM shell.[78]

Scheme 10. Release of Core Macromolecules from Core/Shell Particles: (a) Synthesized by Weda et al.[19] and Haladjova et al.;[73] (b) Synthesized by Toncheva et al.;[37] (c) synthesized by Du,[70] Chen,[72] and Haladjova et al.[73]



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NONVIRAL GENE DELIVERY BY POLYMER-COATED POLYPLEXES

The method for preparation of core/shell nanoparticles (NP) by surface copolymerization (or seeded radical copolymerization) was used to cover DNA complexes with a desirable polymeric coating as shown in Schemes 8 and 9. Plasmid DNA is unstable in biological media. Therefore, association with a carrier is required to protect it from degradation. Plasmid DNA is generally incorporated into nanoscale formulations by complex formation with cationic lipids (lipoplexes) or cationic polymers (polyplexes), which provide greater stability and functionality.[79] Unfortunately, the therapeutic use of polyplexes is limited mainly due to their instability in vivo and to interactions with cells of the immune system (RES) and blood plasma proteins during their transportation in the bloodstream. Moreover, most of the polyplexes exhibit a high positive surface charge, which causes undesirable responses in the body such as aggregation and tissue damage. Construction of coatings on the polyplex surface via seeded radical copolymerization (the outer shell referred to as the envelope is of the same structure as the membranes of the polymeric nanocapsules) should provide a physical barrier and stealth properties, thus significantly improving stability and increasing circulation time in the system circulation.

It is noteworthy that in the case of a polymer-coated polyplex, it is the membrane that interacts with the cell surface and other cellular compartments and proteins. Hence, understanding the interactions of the polyplex envelope with the cells is crucial for improving their functionality both in vitro and in vivo. A major advantage of the approach is that the seeded surface polymerization process allows preparation of envelopes with properties that can be modulated. For instance, the properties of the membrane can be altered using monomers bearing specific side groups, thus introducing defined ligands. Furthermore, when two monomers are copolymerized, the membranes can be tuned (e.g., hydrophilic/hydrophobic balance) simply by changing the relative ratio between the comonomers.

The polymer coating would first significantly reduce the positive surface potential of the polyplexes resulting in nearly neutral nanoparticles. As the positively charged polyplexes are highly toxic and not approved for clinical applications, the coating would resolve the toxicity problems. Figure 16 clearly shows that the polyplex coated with a polymeric shell is invariably less toxic than the parent (noncoated) polyplex.

The applicability of polyplexes, coated by a biodegradable shell, for nonviral gene delivery was proven in a series of experiments showing successful transfection in HEK 293 cells with a plasmid carrying the enhanced green fluorescence protein (EGFP) gene (Table 5). It should be noted that the proper selection of the initiating system as well as the mild conditions for formation of the coating are very important, since the free radicals generated by initiators such as KPS can damage DNA. Therefore, the surface radical copolymerization was initiated by 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)—a water-soluble, nondestructive for pDNA initiator, and in the presence of *N*,*N*′-bis(acryloyl)cystamine, a cross-linking agent. The use of disulfide-containing BAC imparted biodegradability to the polymer shell in the intracellular environment, which was important for pDNA transfection.

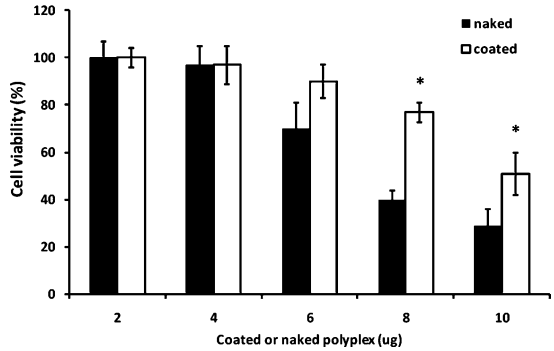


Figure 16. Comparison of HEK 293 cell viability following transfection with naked or coated polyplex, obtained by MTT assay on 24 h. In all experiments P/N = 2. Data are means of three independent experiments, and the error bars represent the SD, $p < 0.05$ against transfection with naked polyplex. Adapted from ref 66 (Supporting Information). Copyright 2013, with permission of The Royal Society of Chemistry. http://dx.doi.org/10.1039/c3ra21890b.

The transfection efficiency strongly depends on the structure of the cationic copolymers used for complex formation with pDNA. Thus, for example, both the parent polyplexes and their coated counterparts, comprising PNIPAm-*b*-PLLys exhibited higher transfection efficiency compared to those formed by the block copolymer containing PEG-grafts (PNIPAm-*g*-PEG)-*b*-PLLys.[65] The results were attributed to the PEG shielding effect and were consistent with other studies, showing that the transfection efficiency of PEG-conjugated polyplexes was generally 2-fold lower compared to non-PEGylated particles due to a decrease in cell binding and uptake.[80,81] On the other hand, for the two systems, the initial noncoated polyplexes displayed higher transfection efficiency as compared to the corresponding coated derivatives (Figure 17).

Obviously, the polymer coating hindered the cellular uptake and decreased the effect. Similar tendency was observed for systems based on $PDMAEMA_{13}$-*b*-PPO_{69}-*b*-$PDMAEMA_{13}$ rod-like micelles.[66] The detected transfection efficiency of the initial polyplex and the coated one at incubation for 24 h are 13% and 8%, respectively (Table 5). After incubation for 48 h, the maximum transfection efficiency of the coated $PDMAEMA_{13}$-*b*-PPO_{69}-*b*-$PDMAEMA_{13}$/pDNA polyplex increased to 27%. Although the transfection efficiency of coated polyplexes was not as high as that of Lipofectamine, these results are encouraging and suggesting that these systems could be considered as potential nonviral gene delivery vectors. It might be expected that the coating reaction would result in aggregation of the polyplex nanoparticles, which is a highly undesirable process, as it prohibits their use as transfection agents. However, as seen from Figure 18, the width of the D_h distribution of the coated polyplexes was slightly smaller than that of the original polyplexes, implying that aggregation did not take place during the coating reaction.

CONCLUSIONS AND FUTURE PERSPECTIVES

A novel synthetic strategy for the preparation of hollow nanoparticles was developed and reported by us in 2008.[19] It involves several steps: temperature-induced PT of TRP, shell formation by seeded radical copolymerization, and subsequent dissolution of TRP by dialysis at lower temperatures, as shown in Scheme 2.

Almost at the same time Zhang and Wang[71] suggested nanocapsule preparation from core−shell particles with

Table 5. Characterization Data and Transfection Efficiency of Polyplexes and the Corresponding Polyplexes Covered by a Polymeric Shell

polyplex	mean size (D_h, nm)	ζ-potential (mV)	transfection (%)	coated polyplex monomer, comonomer, initiator	mean size (D_h, nm)	ζ-potential (mV)	transfection (%)	references
PNIPAM-*b*-PLLys/pDNA (N/P = 20)	163	19.5	22	NIPAM, PEG-DA, BAC, AAPH	195	8.4	12	65
P(NIPAM-*g*-PEG)-*b*-PLLys/pDNA (N/P = 20)	116	27.8	15	NIPAM, PEG-DA, BAC, AAPH	161	11.7	7	65
PDMAEMA-PPO-PDMAEMA/ pDNA (N/P = 0.5; rod-like)	195	−32.3	13	NIPAM, BAC, AAPH	210	−28.3	8 (24 h) 27 (48 h)	66

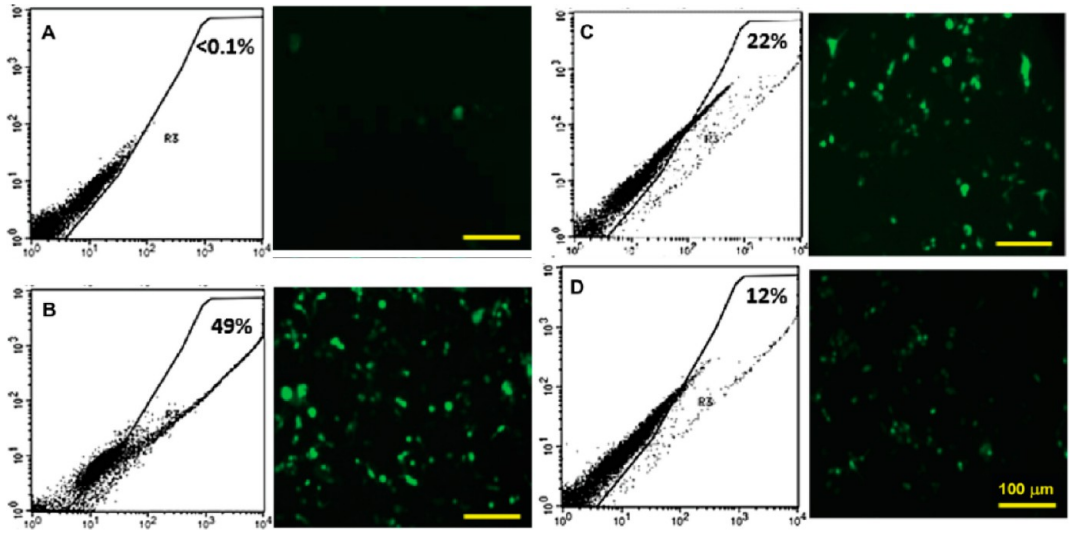


Figure 17. Transfection efficiency of the polyplex formed at N/P = 20 from pDNA and PNIPAm-*b*-PLLys. HEK 293 cells were transfected with pEGFP-C2 containing polyplex for 24 h. 48 h after plasmid transfection the percentage of GFP-positive cells was measured by flow cytometry and assessed by fluorescent microscopy. The GFP positive population was gated in area R3. Data are means of three independent experiments. (A) Non-transfected cells (negative control); (B) cells transfected with Lipofectamine 2000 containing 4 μg of pEGFP-C2 (positive control); (C) transfection of initial polyplex; and (D) after polyplex stealth coating with a biodegradable PNIPAm membrane. Reprinted from ref 65. Copyright 2013, with permission from Elsevier.

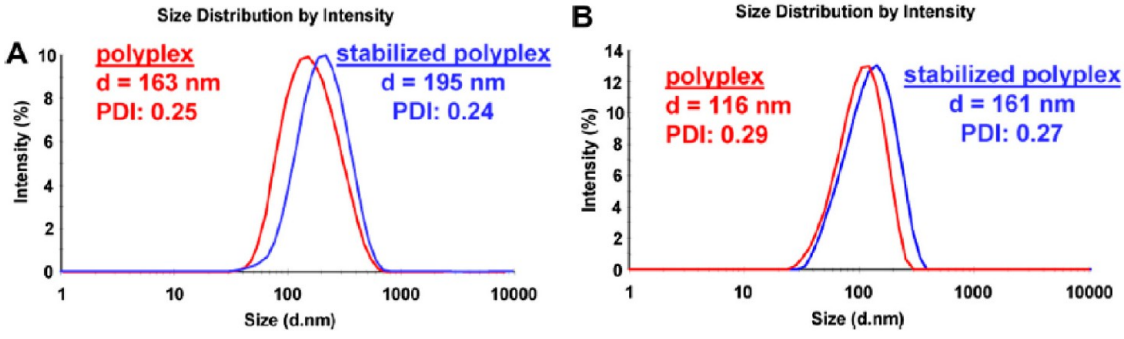


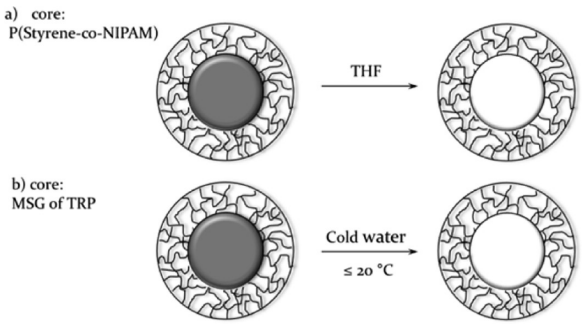
Figure 18. Average diameters and PDI before and after coating of polyplexes, formed at N/P = 20 from pDNA and (A) PNIPAM-*b*-PLLys and (B) (PNIPAM-*g*-PEG)-*b*-PLLys. Reprinted from ref 65. Copyright 2013 with permission from Elsevier.

P(styrene-*co*-NIPAM) core and cross-linked PNIPAM shell. The capsules were obtained by dissolving and removing the P(styrene-*co*-NIPAM) core in tetrahydrofuran. A key advantage of our method over the one reported in ref 71 is that all steps are performed entirely in aqueous medium in a very mild and nondestructive fashion, as shown in Scheme 11.

The aqueous conditions used in our approach are beneficial, since water is the preferred environment for the biomolecules as they are metastable. Water molecules form a solvating layer around the hydrophilic surface residues of proteins and nucleic acids. This water layer has a damping effect on the attractive forces between biomolecules. Moreover, it is well-known that biomacromolecules such as enzymes lose their activity in organic solvents.

Polymer nanocapsules reported so far are able to load and release guest molecules from their interior only by diffusion. Therefore, it is rather difficult to control the loading process. Another drawback of the current methods is that loading can be done only after the formation of the nanocontainer. The load includes mostly low molecular weight compounds, such as doxorubicin, paclitaxel, etc. Our approach overcomes this drawback, as it provides the possibility for incorporation of

Scheme 11. Schematic Illustration of the Differences in the Preparation of Hollow Polymeric Particles: (a) Core Dissolution by Organic Solvent;[71] (b) Core Dissolution in Cold Water Due to Phase Transition[19,37,70,72,73]



biopolymers during the formation of mesoglobule core template. Thus, it enables correct determination of the amount of the entrapped biologically active substance, or, in other words, to achieve a controlled load.

The final goal cannot be achieved unless we know how to include a sufficient amount of biological substance during the process of mesoglobule formation. In all cases a reversible and site-specific interactions are desired. The results, presented in Table 2, show an insufficient loading efficiency. More systematic investigation of the loading process into mesoglobules is needed. We believe that the optimization of mesoglobules properties to achieve a reasonable loading without the so-called nonspecific interactions is feasible. It is quite obvious that better results can be obtained using functionalized TRP, bearing positive or negative charges, hydrophobic, ligating, or polyoxyethylene functions. Moreover, charges play a major role in protein binding since all proteins carry positive or negative charges on their surface. An appropriate example represents the use of poly(NIPAM-*co*-vinylbenzyl iminodiacetic acid) copolymer by Tsai et al.[82] Divalent nickel ions interact with the diacetic acid ligands allowing selective metal affinity attachment to a His6-Cys peptide. Moreover, the peptide can be successfully released upon the addition of the competitive ligand imidazole.

The importance of the mesoglobule morphology for the effective loading of biomolecules should also be noted. Obviously, the "loose" mesoglobule structure is preferable. Unfortunately, the internal voids (see Scheme 3) are not continuous, which will greatly hinder the biomolecule absorption. In the case of a dense mesoglobule, the ligands and charges bound to the TRP are less accessible to the biomolecule.

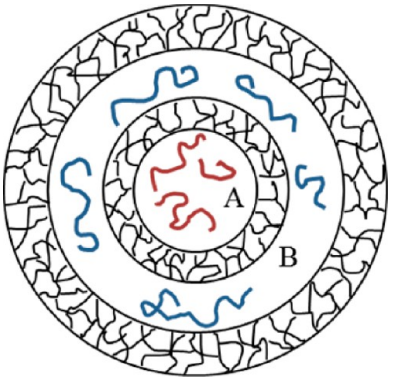
According to our approach, mesoglobule templates are used as seeds (nuclei) for the formation of the shell. The radical copolymerization on the seed surface is a convenient method for the outer layer modification. There are numerous vinyl monomers that can be used to create desired properties, such as stimulus-sensitivity, stealth properties, biodegradability, controlled porosity, tuned hydrophobic/hydrophilic balance, ligand attachment, etc. The copolymerization proceeds at the nanoparticle's surface. The initial mesoglobule grows by the addition of fresh comonomers from the aqueous medium. A serious problem that should be mentioned is the difference in reactivity ratios of the comonomers and the cross-linker. When the reactivity of the first monomer is higher, it is preferably consumed. This leads to inhomogeneity of the outer membrane. When the cross-linker (e.g., BIS) reacts faster than the monomer (e.g., NIPAM), the cross-link density is much higher in the interior of the membrane, giving the outer surface a less dense network structure. In that case, the addition of $NaNO_2$ as a water-soluble inhibitor that is able to deactivate water-soluble radicals is of great importance.[83] The outer shell thickness in most of the experiments is between 10 and 70 nm. The thickness and the overall cross-linked density can be easily controlled. Thus, mesoglobules are protected by polymeric stealth layer that can minimize adsorption of proteins when injected into the bloodstream.

The permeability control of the membrane is still a challenge. Unfortunately, it has not been studied thoroughly. The thickness of the membrane and the cross-linked density are the most obvious parameters that can influence the permeability. While the small molecules can easily cross the membrane, the release of TRP macromolecules is problematic. Obviously, the membrane permeability has to be tuned. Thus, for example the permeability can be temperature-enhanced if the membrane contains thermoresponsive-sequences. A promising approach is the inclusion of pore-forming glucose pentaacetate and glucose pentabenzoate into the membrane structure, as proposed by Dergunow and Pinkhassik.[77] An ideal outer shell would contain a membrane protein as it was already demonstrated with some polymersomes.[84] Thus, the permeability can be selectively tuned depending on the nature of the channel protein incorporated into the membrane.

Finally, mesoglobules comprise a template, able to disintegrate and dissolve at low temperature. Obviously, the membrane can be prepared through any well-known method for nanoparticle coating with a stealth polymer layer. Thus, the radical copolymerization can be successfully replaced by anionic copolymerization of cyanoacrylates or even by the very popular recently layer-by-layer deposition technique. So far this has not been done, but it is a real challenge.

Overall, our approach allows the formation of structures that resemble artificial cell structure or multicompartment architecture as presented in Scheme 12.

Scheme 12. Sketch of Formation of Sphere-in-Sphere Structure with Two Compartments A and B Loaded with Two Different Biomolecules



This review is an attempt to convince researchers of the prospects for further, much deeper study of mesoglobules as potential reservoirs, carriers, and transferring agents for biologically active substances.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A	acrylic acid
AAm	acrylamide
AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
AIBN	α,α′-azoisobutyronitrile
APBA	3-acrylamidophenylboronic acid
BAC	*N*,*N*′-bis(acryloyl)cystamine
BIS	*N*,*N*′-methylenebis(acrylamide)
BSA	bovine serum albumin
BMA	butyl methacrylate
CS	chitosan
DVB	divinylbenzene
FITC	Lys−fluorescein isothiocyanate-lysozome
HEMA	hydroxyethyl methacrylate
KPS	potassium persulfate
LCST	lower critical solution temperature
LbL	layer-by-layer
NIPAM	*N*-isopropylacrylamide
NP	nanoparticle
OND	oligonucleotide
PTT	phase transition temperature
PDI	polydispersity index
PNG	polymeric nanogel
PNIPAM	poly(*N*- isopropylacrylamide)
PHEMA	poly(hydroxyethyl methacrylate)
PDMAEMA	poly(2-dimethylaminoethyl methacrylate)
PEG	poly(ethylene glycol)
PEG-A	poly(ethylene glycol) acrylate
PEG-DA	poly(ethylene glycol) diacrylate
PEG-DMA	poly(ethylene glycol)-dimethacrylate
PETEGA	poly(ethoxy triethylene glycol acrylate)
PIPOX	poly(2-isopropyl-2-oxazoline)
PLLys	poly(L-lysine)
POEGA	poly(oligo(ethylene glycol) acrylate)
POEGMA	poly(oligo(ethylene glycol) methacrylate)
PDEGMA	poly(di(ethylene glycol) methacrylate)
PVCL	poly(*N*-vinyl caprolactam)
PVME	poly(vinyl methyl ether)
PtNEA	poly(*trans*-N-(2-ethoxy-1,3-dioxan-5-yl) acrylamide)
PEI	polyethylene imine
PPO	poly(propylene oxide)
RES	reticulo-endothelial system
SDS	sodium dodecyl sulfate
St	styrene
TRP	thermoresponsive polymer
T_g	glass transition temperature

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