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SHORT COMMUNICATION

Coating of nanoparticles bearing amino groups on the surface with hydrophilic HPMA-based polymers

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Abstract Two hydrophilic polymer systems, multivalent *N*-(2-hydroxypropyl)methacrylamide-based copolymers bearing thiazolidine-2-thione (TT) reactive groups randomly distributed along the polymer chain and monovalent semitelechelic pHPMA with the TT end group, were designed for surface modification of gene delivery vectors, namely, DNA polyelectrolyte complexes (PECs) and adenoviruses. In this study, the amino group-modified polystyrene latex nanoparticles were selected as a suitable model of a nanoparticulate delivery system bearing NH_2 groups on the surface. The coating process was monitored by changes in molecular weight and hydrodynamic parameters of the nanoparticles by light scattering methods. It was shown that for study of the coating process the model latex particles are more suitable than the original PEC vectors due to better chemical and physical stability of latexes. The results obtained in the model study (reaction conditions, methods of evaluation) suggest an optimal polymer structure and a method of efficient and complete coating of nanoparticle surface well applicable to the real gene delivery vectors.

Keywords Polyelectrolyte complex · Latex · Nanoparticle · Coating · Light scattering

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Abbreviations

DNA	sodium salt of deoxyribonucleic acid from calf thymus
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
PEC	polyelectrolyte complex
pHPMA	poly[*N*-(2-hydroxypropyl)methacrylamide]
PLL	poly(L-lysine hydrobromide)
TT	thiazolidine-2-thione
M_w	weight-average molecular weight
M_{wa}	apparent weight-average molecular weight
R_h	hydrodynamic radius (nm)
ζ	zeta potential of particles (mV)
c	concentration (g ml^{-1})
dn/dc	refractive index increment
φ	positive/negative charge ratio
d_{ct}	surface density of coating polymer (g nm^{-2} mol^{-1})
n_{ct}	number of coating molecules fixed on particles surface

Subscripts and superscripts

cp	coating polymer
pc	uncoated polyelectrolyte complexes (PEC)
cc	coated PEC
ct	coating layer of latexes and PECs
lx	uncoated latex particles
cl	coated latex particles

Definitions

$$\Delta M_w^{ct} = M_w^{cc} - M_w^{pc}$$
$$\Delta R_h = R_h^{cc} - R_h^{pc}$$

Introduction

Chemical coating of DNA delivery vectors such as viruses or DNA polyelectrolyte complexes (PECs) with hydrophilic polymers has been generally accepted as a strategy enabling prolonged blood circulation of the vectors and protection from nonspecific interaction with plasma blood components and from liver uptake during their transport to the target cells [1]. Recently, new highly hydrophilic random copolymers of *N*-(2-hydroxypropyl)-methacrylamide (HPMA) and semitelechelic HPMA-based polymers containing thiazolidine-2-thione (TT) reactive groups have been developed [2, 3] as biocompatible polymer precursors intended for efficient chemical coating of PECs and viruses bearing amino groups (NH_2) on the surface. Properties of the new TT-polymers and the efficiency of the coating procedure were compared with those of previously described 4-nitrophenoxy (ONp) groups-containing copolymers [4–7] successfully used for surface modification of both viral and PEC systems. Optimization of the coating reaction conditions using the new TT groups-containing polymers required detailed study of the course of reaction carried out in aqueous environment, in particular the TT groups with amino groups, competition between aminolysis and hydrolysis, and effects of polymer concentration [8]. The course of the coating reaction was evaluated directly on DNA/poly (L-lysine) (DNA/PLL) complexes by monitoring changes in the weight-average molecular weight (M_w) and hydrodynamic radius (R_h) of the complexes using light scattering methods. However, while addition of reactive coating polymers to the freshly prepared DNA/PLL complexes caused a significant increase in M_w and R_h, the coating efficiency of complexes used after 4 days was by an order of magnitude lower (see Fig. 1). Thus, the PECs are not a good system for verification of suitability of various reactive polymers intended for coating reactions of amino-group-bearing gene carriers like viruses. Moreover, the complexes prepared by self-assembling due to Coulombic forces are very sensitive to environment changes such as pH, ionic strength, etc. Therefore, we were looking for more stable and commercially available model nanoparticles bearing amino groups on the surface. These requirements were fulfilled by amino-group-modified polystyrene latex nanoparticles (Sigma Chemical). In addition to amino groups, the latex particles were also labeled with fluorescent probes, which make their bioapplicability even more interesting.

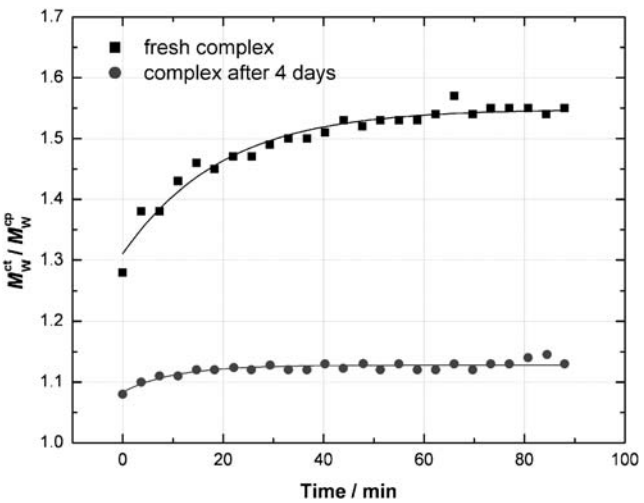

Fig. 1 The kinetics of coating reactions of freshly prepared (*filled squares*) and 4-day-old (*filled circles*) PLL/DNA complexes prepared by standard procedure at φ=2 with multivalent copolymer **I**

The goal of this work is to investigate surface modifications of model latex nanoparticles bearing amino groups on the surface by the reaction with hydrophilic polymers aimed at finding reaction conditions suitable for coating of real gene delivery vectors. The multivalent and semitelechelic HPMA copolymers containing TT reactive groups, with very similar molecular weight and chemical composition as in our previous study [8], were used for chemical coating of model latex nanoparticles. The changes in the weight-average molecular weight, the hydrodynamic size of model particles, and the ζ-potential were tested using light scattering methods.

Experimental

Materials

Calf thymus DNA (CT-DNA) sodium salt was purchased from Sigma; before use, the purity of DNA was checked on spectrophotometer using the absorbance ratio A_{260}/A_{280}. Poly (L-lysine hydrobromide) (PLL, 134,000) were obtained from Sigma. A latex bead water suspension solution (amine-modified polystyrene: 2.5% solids, fluorescent blue, 50 nm in diameter) was obtained from Sigma; the concentration of latex was checked by weighing lyophilized solution. Methacryloyl chloride, 1-aminopropan-2-ol, glycylglycine, thiazolidine-2-thione, *N,N*'-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), 2,2′-azobisisobutyronitrile (AIBN) 4,4′-azobis(4-cyanopentanoic acid) (ABIK), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and dimethyl sulfoxide (DMSO) were from Fluka. All other chemicals and solvents were of analytical grade. The solvents were dried, purified by conventional procedures, and distilled before use.

Synthesis of initiator and monomers

ABIK-TT radical initiator (3,3′-[azobis(4-cyano-4-methyl-1-oxobutane-4,1-diyl)]bis(thiazolidine-2-thione)) was prepared as described in [8]. Briefly, 4,4′-azobis-(4-cyanopentanoic

acid), thiazolidine-2-thione, and 4-(dimethylamino)pyridine were dissolved in THF. DCC dissolved in THF was added to this mixture and allowed to react (−10 °C for 1 h and then at 5 °C for 24 h). The desired product was obtained by crystallization of the evaporated oily residue from dichloromethane-diethyl ether solution.

The *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-(methacryloylglycyl)glycine (Ma-GlyGly-OH) monomers were prepared according to the procedures published earlier [9, 10]. The 3-[*N*-(methacryloylglycyl)glycyl]-thiazolidine-2-thione (Ma-GlyGly-TT) was prepared by the DMAP-catalyzed reaction of Ma-GlyGly-OH with thiazolidine-2-thione in the presence of DCC [2].

Synthesis and characterization of reactive polymers

Reactive HPMA polymers were prepared by two polymerization procedures. Multivalent random copolymer **I** bearing TT reactive groups was prepared by radical solution copolymerization of HPMA with Ma-GlyGly-TT in DMSO, initiated with AIBN at 60 °C as described in [2]. A reactive semitelechelic pHPMA polymer with TT end groups was prepared by radical polymerization of HPMA initiated with ABIK-TT in DMSO at 50 °C [8]. The characteristics of the polymers are summarized in Table 1. The content of TT groups was determined by UV absorption with a HEλIOS α (Thermochrom) spectrophotometer (ε_{305}=10,700 l mol^{-1} cm^{-1}, methanol). Weight- and number-average molecular weights were determined by size exclusion chromatography on a Shimadzu high-performance liquid chromatography (Shimadzu, Japan) equipped with a multiangle light scattering detector DAWN EGS 8 (Wyatt Technology, Santa Barbara, CA, USA) and UV and RI detector, using a Superose 12 column and 0.3 M sodium acetate buffer, pH 6.5 as a mobile phase [2, 3].

Table 1 Characteristics of HPMA (co)polymers used for nanoparticles coating

Coating polymer	Structure	M_w	M_w / M_n	R_h [nm]	Reactive groups (mol%)
I	pMa-GlyGly-TT	28,000	2.11	4.8	7.40
II	pHPMA-TT	45,100	2.16	6.7	–

En dash indicates that coating polymer **II** contains 4.52×10^{-5} mol g^{-1} TT reactive groups.

Preparation of nanoparticles and their chemical coating

All complexes were prepared in 0.01 M HEPES buffer, pH 7.4, by rapid addition of PLL solution (M_w=134,000; concentration of charged monomer units c=0.01 mol l^{-1}) to a stirred solution of DNA (c=20 μg ml^{-1}) so that the ratio of positive to negative charges (φ) was equal to 2.0 [11]. The stock solution of latex beads was diluted with 0.01 M HEPES buffer, pH 7.4, to a final concentration of 0.3 mg ml^{-1}.

A freshly prepared aqueous solution of coating polymers containing TT groups were then added to a stirred solution of nanoparticles bearing amino groups followed by addition of 1 M HEPES buffer (pH 8.7) to reach the final pH 8.2. The reaction was carried out at room temperature for sufficient time to react (0.5–1.0 h). High excess of polymer TT groups over free amino groups on nanoparticle surface was used; the concentrations of coating polymers ranged from 0.2 to 4.0 mg ml^{-1}; unreacted TT groups were hydrolyzed.

Static and dynamic light scattering

Static light scattering (SLS) measurements were carried out with a homemade instrument equipped with a 30-mW He–Ne laser in the angular range 30–140°. The obtained data were analyzed using the Zimm plot procedure. Calculation of the amount of bound coating copolymer on the particle surface was made by the procedure used in the previous paper [8]. The accuracy of the M_w measurements was about 1%. The refractive index increments of polystyrene latex particles (0.257 ml g^{-1}) and coating copolymers (0.167 ml g^{-1}) were taken from literature [8, 12, 13].

Polarized dynamic light scattering measurements were performed within the same instrument as mentioned above (SLS) with an ALV 5000 multibit autocorrelator. Data were analyzed using the GENDIST program. The experimental error of R_h-determination was typically about 3%. Kinetics of coating processes was monitored through changes of the particle hydrodynamic radius (R_h) and scattering intensity (I_s) at the scattering angle 173° on a Nano-ZS, Model ZEN3600 (Malvern Instruments, UK) zetasizer.

Electrophoretic light scattering

Measurements of the ζ-potential were made using a Nano-ZS, Model ZEN3600 (Malvern Instruments, UK). At least ten measurements of each sample were carried out to check for reproducibility. The measurements of the electrophoretic

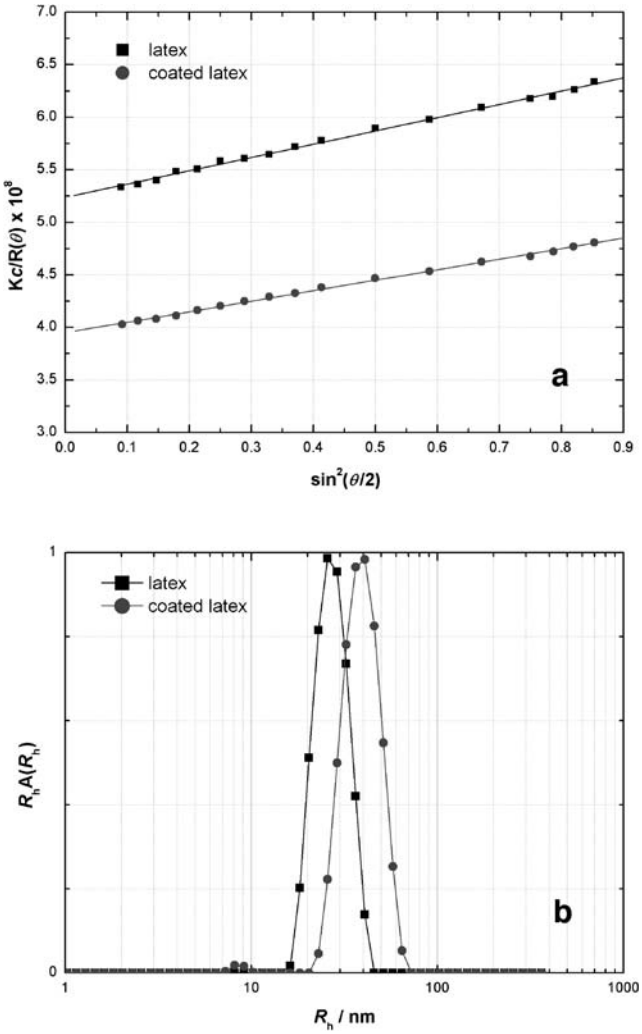

Fig. 2 Zimm plots (**a**) and R_h distributions (**b**) for latex particles (*filled squares*) and coated latex particles (*filled circles*) after 5 h incubation in HEPES buffer solution, pH 8.2; multivalent copolymer **I**

mobility were converted to ζ-potential (mV) using the Smoluchowski approximation.

Results and discussion

Two reactive TT-polymers based on pHPMA (semitelechelic and multivalent) were used for chemical coating of DNA polyelectrolyte complexes and amine-modified polystyrene latex beads. The course of the coating reaction carried out in aqueous solutions was evaluated by monitoring changes of the weight-average molecular weight, hydrodynamic radius, and ζ-potential of the latex nanoparticles using light scattering methods. The sensitivity of the methods is demonstrated in Fig. 2a and b, where Zimm plots (a) and R_h-distribution functions (b) for uncoated and coated latex particles (after 5 h) are shown for multivalent copolymer **I**. As the widths of R_h-distributions of coated and uncoated latexes are the same, no aggregation of latexes takes place in the course of coating reaction. Contrary to PECs, where the reaction kinetics could be well monitored [8], the coating of latexes was very fast. Steady-state conditions were achieved in several minutes. The results of light scattering measurements obtained for both of the coating copolymers (after 6 h) are shown in Table 2. Freshly prepared DNA/PLL complexes had to be used for the study of surface modification of PECs; the coating efficiency of 4-day-old complexes was by an order of magnitude lower than that of the fresh ones, as shown in Fig. 1. This phenomenon could be explained by an additional slower reorganization of segments of polycation chains in the polyelectrolyte complex so that after a certain period of time the resulting structure of PECs contained a higher ratio of compensated phosphate groups coming from DNA than in the freshly prepared complex. This proposal should be supported by ζ-potential measurement of fresh (16.3±1.7 mV) and 4-day-old (8.5±0.7 mV) DNA/PLL complex; the ζ-potential of freshly prepared complex is about twice higher than that measured after 4 days. Characteristics of PECs coated with copolymers having very similar molecular weights and chemical compositions [8] as those used in this study are also shown for comparison in Table 2. The molecular weights of each

Table 2 Coating of DNA/PLL complexes prepared by the standard method of self-assembling of DNA with PLL (M_w=134,000) at φ=2 and of polystyrene latexes with reactive HPMA (co)polymers (c_{cp}= 2.0 mg ml^{-1}); changes of the weight-average molecular weights and hydrodynamic radii

	Coating polymer	
	I[a]	II[b]
Complexes (data from [2])		
M_w^{lx} x 10^{-7}	3.42	2.78
M_w^{cl} x 10^{-7}	5.34	3.57
$\Delta M_w^{ct}/M_w^{pc}$	0.56	0.28
R_h^{pc} (nm)	38.2	37.0
R_h^{cc} (nm)	52.8	48.1
ΔR_h^{ct}	14.6	11.0
Latexes		
M_w^{lx} x 10^{-7}	2.19	2.23
M_w^{cc} x 10^{-7}	2.75	2.57
$\Delta M_w^{ct}/M_w^{pc}$	0.26±0.01	0.15±0.01
R_h^{lx} (nm)	27.1	27.2
R_h^{cl} (nm)	38.3	36.6
ΔR_h^{ct}	11.2	9.4

[a] M_w 24,000, 7.0 mol% of TT
[b] M_w 45,200, TT groups 4.67×10^{-5} mol g^{-1}

TT-polymer (ΔM_w^{ct}) fixed on the latex surface were estimated from the apparent molecular weights of coated and uncoated latexes by a procedure described in the previous paper [8] and the obtained values of $\Delta M_w^{ct}/M_w^{1x}$ (ratio of the molecular weight of a coating polymer to that of the uncoated particle) are also given in Table 2. A higher $\Delta M_w^{ct}/M_w^{1x}$ was found for multivalent copolymer **I** than for semitelechelic polymer **II**, in agreement with the findings obtained for PECs (see Table 2). $\Delta M_w^{ct}/M_w^{1x}$ values obtained for latexes are about twice smaller than those for DNA/PLL complexes (Table 2). This is probably due to the lower concentration of amino groups exposed on the surface of latexes than on the surface of polyelectrolyte complexes freshly prepared from DNA in excess of amino-group-containing polymer. An increase in the hydrodynamic radius of latexes (ΔR_h^{ct}) due to coating is about $2R_h^{cp}$ for both of the copolymers (see Table 1). Thus, the thickness of coating layers is close to that of monolayers of corresponding coating copolymers. If ΔM_w^{ct} is known, the surface density of coating copolymer per square nanometers, d_{ct} ($d_{ct} = \Delta M_w^{ct}/4\pi(R_h^{pc})^2$), and the number of pHPMA molecules fixed on the surface of latex particle, n_{ct} ($n_{ct} \approx \Delta M_w^{ct}/M_w^{cp}$), can be estimated. The results are shown for both of the DNA/PLL complexes [8] and latexes in Table 3. Again, values d_{ct} and n_{ct} are lower for semitelechelic copolymer **II** than for multivalent **I**. d_{ct} is lower for latex particles than for PECs, but the difference is small especially for semitelechelic copolymers. If we know the surface density of coating copolymer d_{ct}, a hypothetical surface area covered by a coating macromolecule can be estimated as a ratio of M_w^{cp}/d_{ct}. The surface covered by one polymer chain is 120 nm^2 for the semitelechelic polymer **II**, which is a value close to the calculated value of coil cross-section, $\pi(R_h^{cp})^2$ = 140 nm^2. In agreement with previous results [8], the semitelechelic polymer molecules cover the latex surface only with a low overlap with neighboring molecules. In the case of multivalent copolymers **I**, the covered surface with a molecule is 46 nm^2, and the coil cross-section is 72 nm^2. Therefore, the overlap and entanglement of coating molecules is more pronounced for the multivalent copolymer **I**, which covers the latex surface denser (a better particle protection in bioactive environment can be expected) than the semitelechelic polymer **II**. The effect of copolymer concentration on the efficiency of coating latex particles was also tested. The results are shown in Fig. 3, where $\Delta M_w^{ct}/M_w^{1x}$ values are plotted as a function of copolymer concentration (c_{cp}). While $\Delta M_w^{ct}/M_w^{1x}$ is practically independent of c_{cp} for multivalent copolymer **I** in the c_{cp} range 0.2–4.0 mg ml^{-1}, a small increase in $\Delta M_w^{ct}/M_w^{1x}$ was found for semitelechelic polymer **II** with an implying saturation above c_{cp} ≥4.0 mg ml^{-1}. ζ-potentials of coated latexes and PECs measured after 6 h of coating are shown in Table 3. While the ζ-potentials of uncoated latexes and PECs (φ=2) are positive (ζ=31±1 and 24±3 mV, respectively), coating of latexes and complexes with multivalent copolymers results in a ζ-potential drop to slightly negative ζ-potential values. The negative ζ-potential of coated complexes results from negative charges of carboxylic groups formed in a side reaction (hydrolysis) of a part of reactive groups of the multivalent copolymers incubated in aqueous solution at pH 8.2. The slightly negative ζ-potentials of nanoparticles together with the neutral highly hydrophilic polymer-coated surface allow us to presume an efficient elimination of interactions of the modified particles with blood proteins (especially with negatively charged albumin) during their circulation and transport to target. The ζ-potentials of latexes and PECs coated with semitelechelic polymers at the c_{cp} concentration 2.0 mg ml^{-1} are slightly positive. This means that only a part of amino groups on the surface of particles was acylated by the coating copolymers and some groups remained free probably due to steric hindrance to the polymer access to the particle surface.

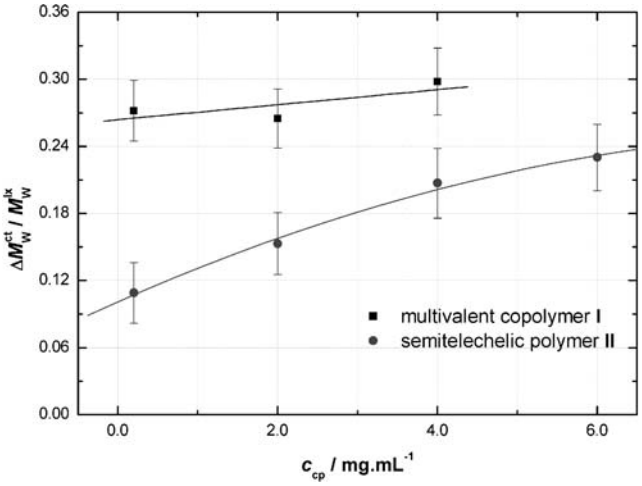

Fig. 3 The dependence of coating polymer concentration (c_{cp}) on the surface modification efficiency $\Delta M_w^{ct}/M_w^{1x}$ of the latex particles for multivalent copolymer **I** (*filled squares*) and semitelechelic polymer **II** (*filled circles*)

Table 3 Characteristics of coated PECs prepared by the standard method of self-assembling of DNA with PLL (M_w=134,000) at φ=2 and coated polystyrene latexes; concentration of a (co)polymer used for coating was 2.0 mg ml^{-1}

Coating copolymer	ΔM_w^{ct} x 10^6	d_{ct} (g nm^{-2} mol^{-1})	n_{ct}	ζ_{ct}(6 h) (mV)
I[a]	19.2	1,040	790	−3.3±0.2
II[b]	7.8	440	170	+1.1±0.2
I	5.7	610	205	-9.4±0.3
II	3.4	370	77	+0.4±0.7

Latex ζ = +30.7±1.0 mV
[a] M_w 24,000, 7.0 mol% of TT
[b] M_w 45,200, TT groups 4.67×10^{-5} mol g^{-1}

Conclusion

Coating of model latex particles bearing amino groups on their surface by the covalent coupling with acylating TT reactive groups incorporated in structures of highly hydrophilic multivalent and semitelechelic HPMA (co) polymers was successfully demonstrated by changes in nanoparticle parameters. The ratios of molecular weight of a coating polymer to molecular weight of the uncoated particle ($\Delta M_w^{ct}/M_w^{lx}$) in polymer-coated latex particles are about twice smaller than those in freshly prepared DNA/PLL complexes (1 h after preparation). The surface density of coating copolymer per square nanometers (d_{ct}) is also smaller for latex particles than for freshly prepared PECs, particularly if semitelechelic polymers were used for coating reaction, but the difference is not significant. On the other hand, the coating efficiency of reactions carried out with latexes is substantially higher than that found for coating of 4-day-old PECs. Moreover, chemical and time stability, high coating reproducibility, and commercial availability make the latex particles a suitable model for testing of coating reactions definitely a better model than unstable complexes. Latex particles labeled with fluorescence probes and covered with biocompatible nanocoatings can be also directly used in a wide range of medicinal and diagnostic applications.

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