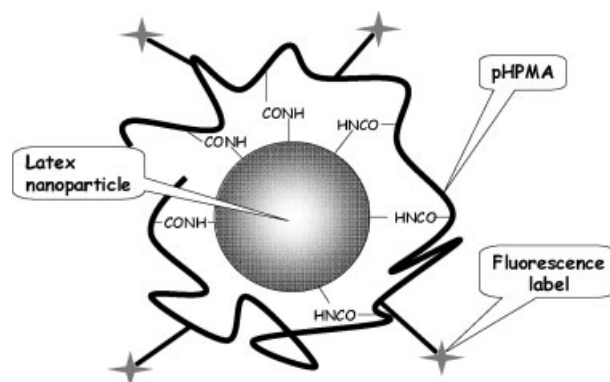


Investigation of Nanoparticle Coating by Fluorescence Correlation Spectroscopy

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Protecting gene delivery vectors by hydrophilic copolymer coating is a promising approach in targeted gene therapy. Herein we demonstrate the usefulness of FCS in characterizing the coating process of a virus model – latex beads with coumarin-labeled *N*-(2-hydroxypropyl)-methacrylamide copolymer containing thiazolidine-2-thione groups. FCS gives an estimation of the average number of polymer chains coating the latex bead, which agrees with light scattering results, giving an evidence of quality of the vector surface modification. FCS is advantageous because it decreases the sample consumption by a factor of 1 000. The coumarin label showed advantages over the previously used fluorescein-based one.



Introduction

Gene therapy could form a third pillar in addition to radiotherapy and chemotherapy in the standard cancer arsenal, while avoiding some debilitating problems. The therapy success relies on the ability of gene vectors to selectively deliver therapeutic genes to a sufficient number of target cells. The main issues restricting the effective gene delivery are incapacibilities of vectors to sufficiently protect the genetic material against degradation in bloodstream and to satisfactorily recognize the target cells. One of the most successful strategies, which reliably overcomes these barriers, consists of the formation of a hydrophilic polymeric layer surrounding the gene delivery vector and

protecting it efficiently from any unwanted biological interactions.^[1] Therefore, we have developed new hydrophilic polymers and copolymers based on *N*-(2-hydroxypropyl)methacrylamide (pHPMA) containing thiazolidine-2-thione (TT) reactive groups for efficient chemical coating of viral and non-viral gene delivery vectors. The coating efficiency of the copolymers was tested on model nanoparticles, polyelectrolyte DNA/poly(L-lysine) complexes^[2] and latex particles bearing amino groups on the surface.^[3–5] The coating reaction carried out in aqueous solution was evaluated by monitoring changes in the weight-average molecular weight, \bar{M}_w , and hydrodynamic radius, R_h , of the nanoparticles using light scattering methods.^[2–5] However, in the case of coated viruses, no conclusions can be made about the coating density from the light scattering intensity data. An indirect evidence comes only from changes of the hydrodynamic radius, R_h , of viruses, which increased in the course of the coating reaction by ca. 10 nm (Adenovirus WT2).^[6] The viruses have a similar surface area to DNA/PLL complexes, but about 10 times larger \bar{M}_w ; therefore, the relative change in the \bar{M}_w due to surface polymer layer is only several percent, which is within experimental error. No quantitative conclusions can be drawn under such conditions.

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In order to overcome this stalemate situation, we decided to apply highly sensitive fluorescence correlation spectroscopy (FCS) to investigate the coating process of viral model – latex beads with pHMPMA polymer. The main aims were as follows: (1) to determine the quality of the nanoparticle “stealthing”; (2) to determine the ratio of free and latex-bound polymer fractions; (3) to correlate the results with dynamic light scattering (DLS) data. First measurements performed with fluorescein-labeled polymer showed that FCS is capable of characterizing coated particles in 10 000 times less concentrated samples than DLS (data not shown). However, systematic studies revealed that photochemical artifacts (such as pH dependence or fluorescence quenching) can raise a serious obstacle to quantitative data analysis. This motivated the synthesis of a new coumarin-labeled coating polymer, which overcomes the problems observed for the fluorescein label.

Experimental Part

Materials

An aqueous suspension of latex beads [amine-modified polystyrene: 2.5% solids, $R_h = (27 \pm 1)$ nm] was obtained from Sigma-Aldrich (Munich, Germany). The fluorescent probe 7-(diethylamino)-*N*-succinimidyl coumarin-3-carboxylate (CU-OSu), methacryloyl chloride, triethylamine (Et_3N), trifluoroacetic acid (TFA), 1-aminopropan-2-ol, 4,5-dihydrothiazole-2-thiol, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP) and 2,2'-azobisobutyronitrile (AIBN) were purchased from Fluka (Sigma-Aldrich, Co.). Solvents and other chemicals were of analytical quality (Lachema Co., Czech Republic). The fluorescence calibration standard Alexa Fluor 405 was purchased from Molecular Probes (Eugene, OR, USA).

Synthesis of Monomers

N-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized by the reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane in the presence of sodium carbonate.^[7] *N*-methacryloylglycylglycine (Ma-GlyGly-OH) was prepared by the Schotten-Baumann acylation of glycylglycine with methacryloyl chloride in an aqueous alkaline medium.^[8] 3-(*N*-Methacryloylglycylglycyl)thiazolidine-2-thione (MaGlyGly-TT) was prepared by the reaction of Ma-GlyGly-OH with 4,5-dihydrothiazole-2-thiol using the DCC method in DMF in the presence of a catalytic amount of DMAP.^[9]

Synthesis of 7-(Diethylamino)-*N*-(2-methacrylamidoethyl)coumarin-3-carboxamide (Ma-ED-CU)

N-[2-[(*tert*-butoxycarbonyl)amino]ethyl]methacrylamide (Ma-ED-Boc) was prepared by the reaction of 2-[(*tert*-butoxycarbonyl)amino]ethylamine with methacryloyl chloride in chloroform in the presence of triethylamine.^[10] The Boc protection group was

removed by treatment of Ma-ED-Boc (0.065 g, 2.8×10^{-4} mol) with an excess of trifluoroacetic acid in the presence of the octylpyrocatechol inhibitor. The deprotected monomer Ma-ED.TFA was diluted with methanol, the excess of TFA was removed by evaporation in a vacuum, and the monomer was dried in a vacuum. The solid residue was dissolved in *N,N*-dimethylformamide (DMF) (1 mL) and Cu-OSu (0.1 g, 2.8×10^{-4} mol) was added, followed by the addition of Et_3N (78 μL , 5.6×10^{-4} mol) in two portions within 10 min. The reaction mixture was stirred for 2 h at room temperature. DMF was evaporated in a vacuum, and the oily residue was dissolved in methanol and purified by chromatography on a Sephadex LH-20.

The product was chromatographically pure (HPLC, Tessek SGX-18) with a melting point of 215–220 °C.

Synthesis and Characterization of Terpolymer P-1

The terpolymer P-1, poly(HPMA-*co*-Ma-GlyGly-TT-*co*-Ma-ED-CU) (Figure 1), was prepared by the solution radical terpolymerization of HPMA (0.5 g, 3.5×10^{-3} mol) with Ma-GlyGly-TT (0.118 g, 3.9×10^{-4} mol) and Ma-ED-CU (0.015 g, 3.9×10^{-5} mol) in DMSO (4.25 g) at 60 °C for 6 h. The AIBN concentration in the polymerization mixture was 1.5 wt.-%. After polymerization, terpolymer P-1 was precipitated into an acetone-diethyl ether (3:1) mixture, filtered off, washed with acetone and diethyl ether and dried in a vacuum.

The content of TT reactive groups in polymer P-1 (8.55 mol-%) was determined by UV spectrophotometry (methanol): $\lambda_{\text{max}}(\epsilon) = 305$ ($10\,860 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). After aminolysis of TT reactive groups with 1-aminopropan-2-ol, the weight-average molecular weight ($\overline{M}_w^p = 49\,400$) and polydispersity index ($\overline{M}_w^p/\overline{M}_n^p = 2.35$) of polymer P-1 were determined by SEC on an HPLC (Shimadzu Co.) with a Superose 6 column equipped with UV, Optilab rEX and multiangle light scattering detector DAWN[®] 8TM (Wyatt Technology Corp., USA) using 0.3 M sodium acetate buffer (pH = 6.5) containing 0.5 g · L⁻¹ sodium azide as the mobile phase at a flow

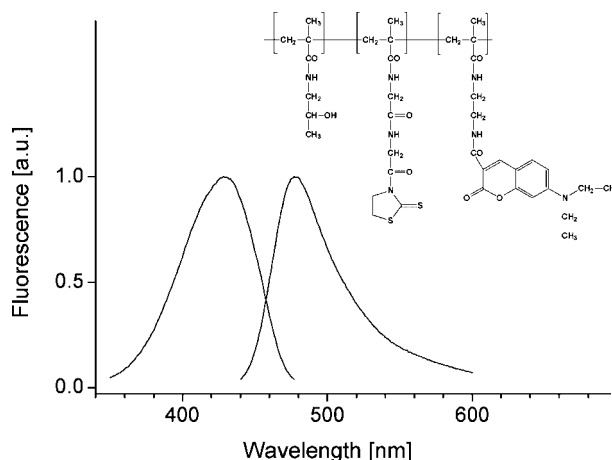


Figure 1. Structure of a coumarin-labeled HPMA copolymer containing reactive thiazolidine-2-thione groups used for pH-triggered chemical coating of latex beads. The fluorescent spectra were measured in HEPES buffer (pH = 8.2) at 25 °C.

rate of $0.5 \text{ mL} \cdot \text{min}^{-1}$. The structure of the coating copolymer is shown in Figure 1.

Coating of Latex Particles

0.5 mL ($0.301 \text{ mg} \cdot \text{mL}^{-1}$) of the latex bead suspension and 20 μL of polymer solution containing the required amount of a coating polymer (freshly prepared in $10 \times 10^{-3} \text{ M}$ HEPES, $\text{pH} = 7.4$) were mixed and then 20 μL of 1 M HEPES, $\text{pH} = 8.7$ was added in order to adjust the pH to 8.2. The final concentrations of coating polymers in solution ranged from 0.062 to $4 \text{ mg} \cdot \text{mL}^{-1}$. The solution was stirred thoroughly, incubated for 30 min, diluted 1 000 times with HEPES, $\text{pH} = 8.2$, and finally 0.2 mL of the solution was transferred onto the chamber slide (Lab-Tek, Nunc, USA). Samples were measured immediately (within 1 h after the coating started). The solutions for DLS were prepared in the same way but in higher amounts (2 mL of latex suspension) and used without any dilution.

Fluorescence Correlation Spectroscopy

FCS measurements were carried out on a MicroTime 200 fluorescence lifetime microscope system (PicoQuant, Germany) comprising a modified inverted epifluorescence microscope IX81 with a water immersion objective ($\text{NA} = 1.2$, $60\times$) (Olympus Optical Co.) and a single molecule detection system (PicoQuant, Germany). FCS autocorrelation curves $[G(\tau)]$ were fitted with the model of three-dimensional free diffusion of two particle components, taking into account the triplet state formation:

$$G(\tau) = 1 + \frac{1 - T(1 - e^{-\tau/\tau_0})}{N(1 - T)} \cdot \left(\frac{F_1}{1 + \tau/\tau_1} \cdot \frac{1}{\sqrt{1 + S^2\tau/\tau_1}} + \frac{F_2}{1 + \tau/\tau_2} \cdot \frac{1}{\sqrt{1 + S^2\tau/\tau_2}} \right) \quad (1)$$

where N is the mean effective number of fluorescent particles in the confocal element, τ_1 and τ_2 are the mean diffusion times through the confocal element of the first and second components, respectively, F_1 and F_2 are the fluorescence intensity fractions of the first and second components, respectively ($F_1 + F_2 = 1$), T is the fraction of molecules converted to the triplet state, τ_0 is the average effective lifetime of the triplet state; S is the structure parameter r/z , where r is the radius and z is half of the height of the confocal element. We assumed that the first component is a longer

diffusing one ($\tau_1 > \tau_2$). The size of the confocal element (r and z) was evaluated by measuring the average diffusion time of the standard (Alexa Fluor 405). Knowing the diffusion coefficient $D = r^2(4\tau_i)^{-1}$ ($i = 1, 2$), the hydrodynamic radius R_h can be calculated: $R_h = kT(6\pi\eta D)^{-1}$, where k is the Boltzmann constant, T is the absolute temperature and η is the solvent viscosity. The total number concentration of fluorescent particles, N_{FCS} , was calculated from the amplitude of the autocorrelation function: $N_{\text{FCS}} = N/V$, where the confocal volume $V = 2\pi r^2 z$. An excellent and extensive description of this method can be found in ref.^[11]

Static (SLS) and Dynamic (DLS) Light Scattering

Static light scattering measurements were carried out with a home-made instrument equipped with a 30 mW He-Ne laser in the angular range $30\text{--}140^\circ$. The obtained data were analyzed using the Zimm plot procedure. Calculation of the amount of the coating copolymer bound to the particle surface was carried out using a procedure used in previous papers.^[2,3] The accuracy of the \overline{M}_w measurements was about 1%. The refractive index increments of polystyrene latex particles ($0.257 \text{ mL} \cdot \text{g}^{-1}$) and the coating copolymer ($0.167 \text{ mL} \cdot \text{g}^{-1}$) were taken from the literature.^[2,12,13]

Dynamic light scattering was measured with the same instrument using an ALV 5000, multibit, multi-tau autocorrelator covering approximately 10 decades of the delay time τ . Data were analyzed using the GENDIST program.^[14]

Results and Discussion

The multivalent reactive TT-polymer based on HPMA labeled with coumarin was used for the chemical coating of amine-modified polystyrene latex beads throughout the paper.

Dynamic Light Scattering Results

The coating in aqueous suspension was monitored by following the weight-average molecular weight, \overline{M}_w , and hydrodynamic radius, R_h , of the latex nanoparticles using light scattering methods. The concentration of coating copolymer, $c_p = 2 \text{ mg} \cdot \text{mL}^{-1}$, was selected for the measurement since it provides fully coated latex particles (see below). The results are collected in Table 1. Molecular

Table 1. Parameters of coated and uncoated latex particles; $c_p = 2 \text{ mg} \cdot \text{mL}^{-1}$.

Sample	c	$\overline{M}_w^{\text{I}}$	$\overline{M}_w^{\text{CL}}$	$\Delta\overline{M}_w^{\text{C}}$	$\Delta\overline{M}_w^{\text{C}}/\overline{M}_w^{\text{I}}$	R_h
	$\text{mg} \cdot \text{mL}^{-1}$	$10^7 \text{ g} \cdot \text{mol}^{-1}$	$10^7 \text{ g} \cdot \text{mol}^{-1}$	$10^6 \text{ g} \cdot \text{mol}^{-1\text{a}}$		nm
Latex (L)	0.301	2.26 ± 0.04	—	—	—	27.7 ± 0.8
Coated latex (CL)	0.279	—	2.83 ± 0.05	5.7 ± 0.2	0.250 ± 0.015	39.1 ± 1.2

^{a)} $\Delta\overline{M}_w^{\text{C}} = \overline{M}_w^{\text{CL}} - \overline{M}_w^{\text{I}}$; see text for details.

weights of the TT-polymer covering the latex surface were estimated: $\Delta\bar{M}_w^C = \bar{M}_w^{CL} - \bar{M}_w^L$, where \bar{M}_w^{CL} and \bar{M}_w^L are the apparent molecular weights of the coated and uncoated latexes, respectively.^[2] The number of pHPMA molecules fixed on the latex surface, $n_C \approx \Delta\bar{M}_w^C / \bar{M}_w^P$ (\bar{M}_w^P : polymer molecular weight) was estimated to be 110 ± 7 .

Fluorescence Correlation Spectroscopy (FCS) Results

Spectral properties of the coumarin labeled polymer are shown in Figure 1. The fluorescent spectra did not change considerably upon the coating process.

Samples containing the same amount of latex particles ($c_L = 0.28 \mu\text{g} \cdot \text{mL}^{-1}$), but different concentrations of coating polymer ($c_P = 0.062\text{--}4.0 \mu\text{g} \cdot \text{mL}^{-1}$), were measured at 25°C .

Both the fluorescence intensity (I_F) and the effective number concentration of fluorescence particles, N_{FCS} , increased with increasing polymer concentration, reflecting an increasing number of fluorescent particles in the samples (Figure 2).

The number concentration N_{FCS} is called effective here since it is calculated using the effective number of particles, N , which depends on the fluorescence intensity of the diffusing species. N can be attributed to the real number of particles only for a single component system. Whenever more particles species are present, the relationship between N and the actual particle number can be complex.

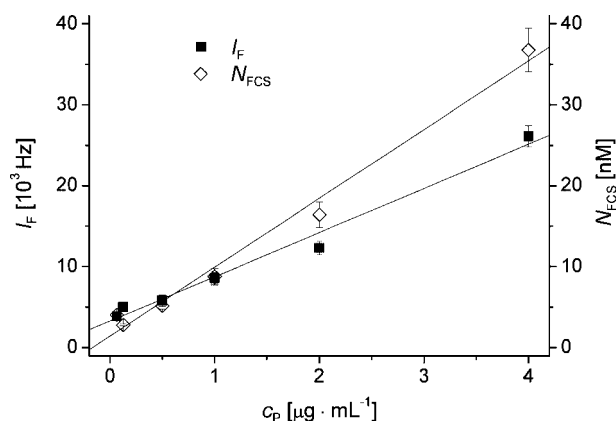


Figure 2. Average fluorescence intensity I_F (filled squares) and the total number concentration N_{FCS} (open diamonds) obtained from FCS autocorrelation curves fitted with a 2 component model [Equation (1)] plotted as a function of concentrations of the coumarin labeled HPMA coating copolymer (c_P). The concentration of latex particles was $0.28 \mu\text{g} \cdot \text{mL}^{-1}$ and the solution was at $\text{pH} = 8.2$. Measurements were performed at 25°C using a 405 nm laser as the excitation source. Data points are fitted with linear functions for eye guidance. Error bars represent standard deviation.

The ratio I_F/N_{FCS} (often referred to as cpm, “counts per molecule”)^[11] is not constant but increases with increasing c_P (not shown). This is evidence for the coating process. The polymer molecules attached to latex surfaces diffuse together with the particles, which consequently decreases the number of independently diffusing host particles (N_{FCS}) in the suspension. Thus, as a result of the coating process, for the increasing polymer concentration, the number concentration of fluorescent particles N_{FCS} increases more slowly than the integrated fluorescence intensity, I_F .

Knowing the molecular weight of the polymer, the number concentration of the polymer molecules N_P that were added to the suspension of latex particles can be calculated from the corresponding weight concentration c_P : $N_P = c_P / \bar{M}_n^P$. We can compare this value with the number concentration of fluorescence particles obtained from FCS (N_{FCS}). The N_{FCS}/N_P ratio is plotted versus c_P in Figure 3.

The lower the ratio, the more polymer molecules are attached to latex particles. It is interesting to note that at very small concentrations of the polymer (e.g., $c_P = 0.062 \mu\text{g} \cdot \text{mL}^{-1}$) the ratio is higher than unity, which is probably the result of segmental motions of the polymer spacers to which the coumarin label is attached. It is well known that for long polymers such motion contributes to the FCS autocorrelation function and is often responsible for the appearance of the fast diffusing component.^[15] An increase in the polymer concentration causes an abrupt drop of the ratio, which reaches about 0.2 for a polymer concentration of $1 \mu\text{g} \cdot \text{mL}^{-1}$. This is in agreement with the scenario for the changes in the I_F/N_{FCS} ratio mentioned above (I_F should be, in general, proportional to N_P);

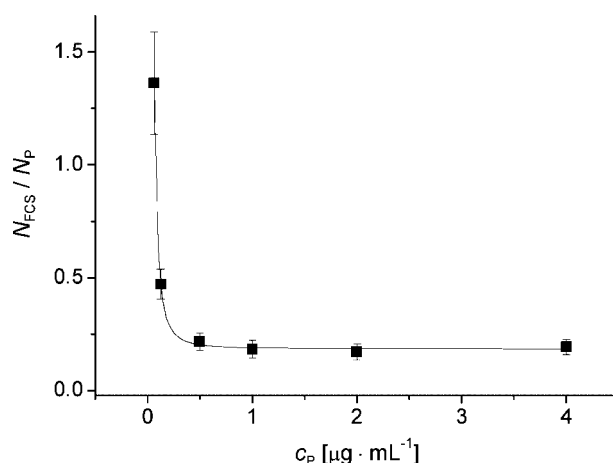


Figure 3. Ratio N_{FCS}/N_P of the number concentrations plotted as a function of c_P . N_{FCS} values were taken from Figure 2, and N_P values for coumarin-labeled HPMA copolymer molecules were calculated from $N_P = c_P / \bar{M}_n^P$. Points are fitted with a hyperbola for eye guidance. Error bars are calculated from the standard deviations of N_{FCS} and the error of N_P (estimated as 10%).

polymer molecules coating latex bead diffuse together with latexes, which lowers the N_{FCS} value. Above $1 \mu\text{g} \cdot \text{mL}^{-1}$, the coating process seems to be saturated. The latex particles are fully coated and, thus, no longer accessible to excess polymer molecules. The $N_{\text{FCS}}/N_{\text{p}}$ ratio should increase again for high polymer concentrations (some indication of this behavior can be observed in Figure 3 at $c_{\text{p}} = 4 \mu\text{g} \cdot \text{mL}^{-1}$).

The results discussed above indicate that, for higher polymer concentrations, a binary mixture of coated latex particles and free polymer molecules is present in suspension. That was the rationale for the application of the two component model to FCS autocorrelation curve fitting. The diffusion times ($\tau_1 = 3.4 \text{ ms}$ and $\tau_2 = 0.07 \text{ ms}$, calculated from values of the hydrodynamic radii obtained from DLS) for coated latex particles and free polymer molecules were used for FCS analysis, giving a satisfactory fit to the measured data. A representative autocorrelation function is plotted together with the best two component fit in Figure 4. The small deviations in the residuals are due to the polydispersity of the components.

The intensity fraction F_1 (see Equation (1)) of the slower component (coated latex particles) is plotted as a function of c_{p} in Figure 5.

F_1 increases with decreasing polymer concentration, reaching a value of 0.75 for $c_{\text{p}} = 0.062 \mu\text{g} \cdot \text{mL}^{-1}$. Please notice that the relation for F_1 , which contains information on both the number of particles and their brightness, is not trivial. Assuming that the fluorescence quantum efficiency is independent of the coating process, the number of polymer molecules fixed on the latex surface, n_{c} , can be obtained from: $(1/F_1 - 1)n_{\text{c}}^2 + n_{\text{c}} - N_{\text{p}}/N_{\text{t}} = 0$ (see Appendix for details). The number concentration of latex particles N_{t}

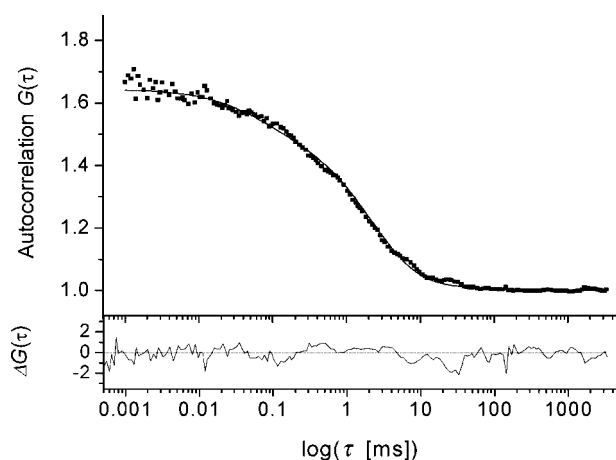


Figure 4. FCS autocorrelation function measured for a mixture of latex particles ($0.28 \mu\text{g} \cdot \text{mL}^{-1}$) and coumarin-labeled HPMA polymer ($2 \mu\text{g} \cdot \text{mL}^{-1}$) at $\text{pH} = 8.2$ and fitted with a 2 component model of free three-dimensional diffusion; the weighted residuals of the fit are shown in the lower panel. Data were measured at 25°C for a 405 nm excitation wavelength.

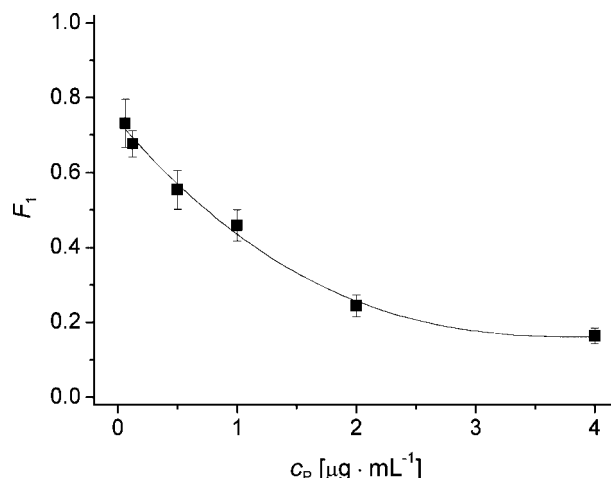


Figure 5. Fluorescence intensity fraction of the slow-diffusing component F_1 , as obtained from the two component fit of FCS autocorrelation curves ($\tau_1 = 3.4 \text{ ms}$; $\tau_2 = 0.07 \text{ ms}$). The samples containing $0.28 \mu\text{g} \cdot \text{mL}^{-1}$ of latex particles ($R_{\text{h}} = 25 \text{ nm}$) and different concentrations of coumarin-labeled HPMA copolymer (c_{p}) were measured at $\text{pH} = 8.2$ and 25°C . The excitation wavelength was 405 nm . Points are fitted with a hyperbola for eye guidance. Error bars represent standard deviation.

and polymer molecules N_{p} were calculated from their weight concentrations using the latex size and density ($R_{\text{h}} = 25 \text{ nm}$, $d \approx 1 \text{ g} \cdot \text{cm}^{-3}$), and polymer molecular weight $\bar{M}_{\text{n}}^{\text{p}}$, respectively. The obtained values of n_{c} are plotted in Figure 6.

The mean number of polymer molecules coating a latex particle increases with increasing c_{p} approaching asymptotically the value ≈ 70 . The obtained values are comparable with the DLS estimates for $c_{\text{p}} = 2 \mu\text{g} \cdot \text{mL}^{-1}$ ($n_{\text{c}} = 110$). The lower value of n_{c} , in the case of FCS measurements, can be due to possible fluorescence quenching on the latex

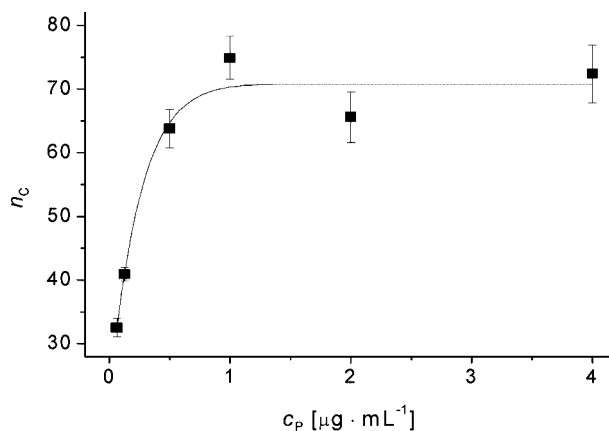


Figure 6. The number of polymer molecules fixed on the latex surface (n_{c}), as calculated from F_1 fractions in Figure 5 using Equation (A9) in the Appendix. Points are fitted with a hyperbola for eye guidance. Error bars calculated using standard deviations of the quantities from Equation (A9). See text for details.

surface and/or to segmental motion of the polymer. Both processes contribute to the fast diffusion component and, hence, would reduce the fluorescence intensity fraction F_1 .

Conclusion

A new developed HPMA copolymer with TT reactive groups labeled with coumarin fluorophore was synthesized. The process of pH-triggered coating of this polymer was monitored and the coated latex nanoparticles were characterized using both DLS and FCS methods. Application of FCS allows determination of the free polymer fraction in solution and, consequently, the amount of polymer sufficient for efficient coating of a single latex particle (surface concentration). We have found that the HPMA copolymer effectively covers the surface of the model nanoparticle. The FCS used for the study gives the advantage of measuring very low concentrations of samples (1 000 times diluted in comparison with DLS). FCS can also profit from fluorescence specificity; e.g., the simultaneous study of two or more components is possible. The coumarin label proved to be well suited for such a quantitative study, in contrast to fluorescein, which was prone to photophysical artifacts caused by its pH sensitivity.

Nomenclature

c_p	concentration of coating copolymer
R_h	hydrodynamic radius
$\overline{M}_w^p, \overline{M}_n^p$	weight-average and number-average molecular weights of polymer
\overline{M}_w^L	molecular weight of uncoated latexes particles (DLS)
\overline{M}_w^{CL}	molecular weight of coated latexes particles (DLS)
$\Delta\overline{M}_w^C$	molecular weight of the polymer coating single latex particle
N_p	number concentration of polymer molecules
N_L	number concentration of latex particles
n_c	number of polymer molecules fixed on the latex surface (coating)

FCS-Related Symbols

$G(\tau)$	autocorrelation function
I_F	fluorescence intensity (count rate)
N	mean effective number of fluorescent particles in the confocal volume
cpm	counts per molecule
N_{FCS}	total number concentration of fluorescent particles calculated as N/V

τ_i	mean diffusion time of i -th component
F_i	fluorescence intensity fraction of the i -th component
T	fraction of molecules converted to the triplet state
τ_0	average effective lifetime of the triplet state
S	structure parameter describing confocal element
r	radius of confocal element
z	half of the height of confocal element
V	volume of confocal element
D	diffusion coefficient
k	Boltzmann constant
T	temperature
η	solvent viscosity

Appendix

Derivation of the Equation Used for Calculating the Average Number of Polymer Molecules that Coat a Latex Bead – n_c

The two component free diffusion three dimensional model (containing triplet kinetics) described by Equation (1) can be rewritten, as:

$$G(\tau) = 1 + \frac{\alpha_1^2 \langle N_1 \rangle g(\tau, \tau_1) + \alpha_2^2 \langle N_2 \rangle g(\tau, \tau_2)}{[\alpha_1 \langle N_1 \rangle + \alpha_2 \langle N_2 \rangle]^2} \quad (A1)$$

where $\langle N_i \rangle$ is the number concentration and $\alpha_i = Q_i/Q_2$ is a relative fluorescent yield of the i -th component (Q_i, Q_2 are the fluorescent yields of the i -th and some particular component, here the second, in order to retain consistency with the numbering scheme used in the main text).

$$g(\tau, \tau_i) = \frac{1 - T(1 - e^{-\tau/\tau_0})}{N(1 - T)} \cdot \left(\frac{1}{1 + \tau/\tau_i} \cdot \frac{1}{\sqrt{1 + S^2 \tau/\tau_i}} \right), \quad i = 1, 2 \text{ [see Equation (1)]} \quad (A2)$$

The fluorescent intensity fractions F_i of both components obey the relation:

$$\frac{F_i}{N} = \frac{\alpha_i^2 \langle N_i \rangle}{[\alpha_1 \langle N_1 \rangle + \alpha_2 \langle N_2 \rangle]^2} \quad (A3)$$

Since the sum of both the intensity fractions equals 1,

$$\frac{F_1}{N} = \frac{1 - F_2}{N} = \frac{\alpha_1^2 \langle N_1 \rangle}{[\alpha_1 \langle N_1 \rangle + \alpha_2 \langle N_2 \rangle]^2} \quad (A4)$$

The fluorescence yield Q is given by

$$Q = \varepsilon \cdot \Phi_F \cdot \Gamma \quad (\text{A5})$$

where ε is the absorptivity, Φ_F is the fluorescence quantum yield and Γ is the experimental fluorescence collection efficiency.^[11a] We assume that both the quantum yield and the collection efficiency does not change during the coating process. We can also assume that the absorptivity of the individual polymer molecule does not change; however, when they diffuse together in sets on the surface of latex particles, the absorptivity of such coated particles is much higher than for a single polymer molecule. The corresponding absorptivity of this component, ε_1 , can be calculated by multiplying individual absorptivities of the polymer molecules, ε_2 , by the number of polymer molecules on the surface, n_C : $\varepsilon_1 = n_C \cdot \varepsilon_2$. Consequently:

$$Q_1 = n_C Q_2 \Rightarrow \alpha_1 = n_C; \alpha_2 \equiv 1 \quad (\text{A6})$$

Calculating and comparing N from Equation (A3) and (A4), we obtain the relation between N_1 and N_2 :

$$F_2 \alpha_1^2 N_1 = (1 - F_2) \alpha_2^2 N_2 \quad (\text{A7})$$

In our case, $N_1 \equiv N_L$ is the number concentration of latex particles, and N_2 is the unknown concentration of free polymer molecules. The mean number of polymer molecules fixed on latex particles, n_C , can be defined as:

$$n_C = \frac{N_P - N_2}{N_L} \quad (\text{A8})$$

where N_P is the total number concentration of polymer (both the free molecules and those fixed on latex particles). Calculating N_2 from Equation (A7) with α_1 and α_2 from Equation (A6) and substituting into Equation (A8) gives:

$$\frac{1 - F_2}{F_2} n_C^2 + n_C - \frac{N_P}{N_L} = 0 \quad (\text{A9})$$

from which the n_C was calculated.

Acknowledgements: Č. K., V. Š, P. Š. and K. U. acknowledge support by the Grant Agency of the Academy of Sciences of the Czech Republic (grant No. A100500501 and A4050403, KAN 200200651),

the Grant Agency of the Czech Republic (SON/06/E005) within the EUROCORES Programme SONS of the European Science Foundation, which is also supported by the European Commission (Sixth Framework Programme) and by the EU (GIANT, No. 512087). P. J. and M. H. would like to thank the Ministry of Education, Youth and Sports of the Czech Republic (grant No. LC06063).

Received: January 16, 2008; Revised: April 1, 2008; Accepted: April 7, 2008; DOI: 10.1002/macp.200800023

Keywords: coatings; drug delivery systems; fluorescence; light scattering; synthesis

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