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Poly(ethyl cyanoacrylate) colloidal particles tagged with Rhodamine 6G: preparation and physicochemical characterization

Research Article

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Abstract: This paper describes the preparation and characterization of poly(ethyl cyanoacrylate) colloidal particles loaded with the organic fluorophore Rhodamine 6G. We studied the physicochemical properties of the colloidal particles: morphology, size-distribution, ζ-potential, fluorescent properties and photobleaching upon UV-light illumination. The properties of the obtained colloidal particles, as well as the dye loading efficiency, were found to depend on the concentrations of ethyl cyanoacrylate monomer and Rhodamine 6G in the polymerization medium. The fluorophore release from the colloidal particles in aqueous buffer is also studied.

Keywords: *Poly(ethyl cyanoacrylate) • Colloidal particles • Rhodamine 6G • Fluorescence • Photobleaching.*

1. Introduction

Rhodamine 6G (R6G) is a cationic lipophilic dye, which is among the most stable organic fluorophores. It is mostly applied in laser technology, with application in fluorescent bioimaging as well [1-5]. Owing to its lipophilic character, R6G is also known as a distinct stain for lipids and phospholipid-based polymers [6]. The R6G fluorophore has been loaded onto different colloidal systems, such as polystyrene latexes [7], nanoaggregates of poly(ethylene oxide)-*b*-polymethacrylate [8], poly(methyl methacrylate) particles [9], *etc*. Such fluorescent colloidal systems may be useful for different biological and technological applications. For example, polymer particles incorporating dyes are applicable to cell labelling [10], sensitive diagnostic reagents [11,12], flow tracing [13] and electronic inks [14]. The applications of polymeric colloids as drug carrier systems requires understanding of the mechanisms of particle-cell interactions, as well as the mechanisms of particle internalization and transportation inside living cells. The fluorescent tagging of polymeric particles offers a possibility for visual tracking of the fluorescently stained particles in such biomedical experiments. For example, such a strategy has been applied in revealing the particle transcytosis through the endothelium cells of brain capillaries [15], as well as for visualization of the skin penetration by polymer colloids [16].

The colloidal particles of poly(alkyl cyanoacrylates), PACA, are perspective colloidal drug carriers, which becomes clear from the intensive research in this area [17-21]. Fluorescent tagging of such colloids could allow heir visualization by fluorescence microcopy, which may reveal details about the mechanisms of particle-cell interactions in future biomedical tests. Fluorescent PACA particles are usually prepared by utilization of fluorescently labelled colloidal stabilizer (dextrane) [15] or by the incorporation of fluorophore in the polymer matrix during the particle preparation by polymerization [16]. Inorganic fluorescent materials, such as semiconductor nanocrystals (known as QDs), have been incorporated in poly(butyl cyanoacrylate), PBCA, colloids for applications in bioimaging, which allowed successful observation of the QD-tagged colloids by fluorescence microscopy [22].

Here, we describe the preparation and physicochemical characterization of poly(ethyl cyanoacrylate) (PECA) submicron particles, loaded with R6G. PECA colloidal particles have been studies as carrier systems for enhanced intravesical drug delivery [23]. The fluorescent tagging of such colloids could reveal the tissue distribution of the particles after *in vivo* application,

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as shown for epirubicin-loaded PECA [23]. Previous investigations show that PECA colloidal particles are cytotoxic against L929 cells at concentrations higher than 25 μg mL [24]. However, cytotoxicity of materials depends on the type of cells, the coating of material and other factors, which should be taken into account in each particular case. It is known that the cytotoxicity of PACA colloids correlates with the rate of their degradation and toxicity of the obtained degradation products [24]. The degradation of PACA materials may involve hydrolysis of the ester bond [25], however a mechanism involving the so-called "unzipping of the monomer" may also take place [26]. PECA degrades relatively faster than other PACA materials with longer alkyl chains (such as butyl, isohexyl, octyl, *etc.*), which may result in higher toxicity. However, in cases of multiple dosing utilization of rapidly degrading PECA could appear more appropriate than overloading the organism with slowly degradable materials. On the other hand, R6G can be used to stain living cells at concentrations as low as low as 0.3 μM [27]. The LD_{50} for R6G has been found to be 6.15 mg kg^{-1} when applied *via* intraperitoneal route on mice [28].

The fluorescent R6G-PECA colloidal particles, prepared in our experiments, may be useful for researchers in the field of bioimaging (for example, such colloids can be used for visualization of the particles entering living cells and their transportation within the cells), as well as in laser technology (R6G is a classical laser dye). However, the most important question concerning a tag is how to keep the main particle properties intact. Unfortunately, the complete answer to this fundamental question is still missing in literature, despite of the vast number of investigations in the area. To answer it, we outline here a very simple procedure of loading PECA colloids with the organic fluorophore Rhodamine 6G. The PECA is a hydrophobic polymer and it could therefore be successfully loaded with the relatively lipophilic R6G fluorophore. The dye is loaded in the particles during their preparation by emulsion polymerization of ethyl cyanoacrylate in aqueous medium. Furthermore, we study quantitatively the physicochemical properties of as-tagged colloids: morphology, size-distribution, zeta-potential and optical properties. The loading efficiency of the fluorophore and the kinetics of its release from the fluorophore-loaded particles are studied. The photobleaching of Rhodamine 6G loaded in colloids is also investigated. In such a way we prescribe the range of parameters, which should be observed in order to maintain the difference between tagged and pure PECA colloids.

2. Experimental procedure

2.1. Materials and reagents

The ethyl cyanoacrylate (ECA) monomer was from Special Polymers Ltd (Bulgaria). Rhodamine 6G (for fluorescence) was from Fluka. Phosphate buffered saline (PBS, tablets), acetone and Pluronic F68 were from Sigma. Distilled water was used for all preparations. All other reagents were of analytical grade.

2.2. Preparation of pure and R6G-tagged PECA colloidal particles

The pure PECA colloidal particles were prepared by emulsion polymerization technique in acidic aqueous solution. Distilled water (8.0 mL), HCl (0.1 M, 1.0 mL) and Pluronic F68 (5% w/w, 1.0 mL) were mixed together in a 25-mL beaker in order to prepare the polymerization medium. The Pluronic F68 is a polymeric amphiphile (PEG-PPO-PEG triblock copolymer), which is used as an emulsifier in these experiments. Then, ECA monomer (50, 65, 75 and 85 mL in different nanoparticle preparations) was dissolved in dry acetone (1.0 mL) and dropwise added to the polymerization medium at room temperature upon intensive stirring. Colloid dispersion was formed within the first minutes, which was left to polymerize for 3 hours and was then neutralized with NaOH (0.1 M, 1.0 mL) and buffered at pH 7.4 by the addition of 1.0 mL phosphate buffer (0.1 M phosphate, 1.37 M NaCl, 0.027 M KCl). The residue of acetone was removed by rotary evaporation and the volume of the resulting dispersion was made 10 mL by addition of distilled water. Samples were analyzed by dynamic and electrophoretic light scattering 24 h after the preparation.

The fluorophore-tagged (R6G-PECA) colloidal particles were prepared by the same procedure as described above for pure PECA colloidal particles. However, a water solution of R6G was used instead of distilled water for the preparation of the polymerization medium. Experiments with different concentrations of ECA (5.0-8.5 μl/mL) and R6G ($2.0\times10^{-5}\div1.3\times10^{-4}$ M) were carried out.

2.3. Characterization of the colloidal particles

The as-prepared colloidal particles were imaged by a scanning electron microscope (SEM) JSM-5510 (JEOL) operated at 10 kV of acceleration voltage. The samples for SEM were prepared by centrifugation of the as-obtained particle dispersion, washing the particles with

distilled water and evaporation of a drop of the purified particle dispersion on a glass substrate. The sample was then coated with gold by JFC-1200 fine coater (JEOL).

Fourier transform infrared (FTIR) spectra were taken in the interval 400-4400 cm^{-1} (Bruker Tensor 27 spectrometer). For that purpose, the particles were centrifuged, washed twice with distilled water and dried under vacuum to obtain white powder material. The sample for FTIR analysis was prepared using the KBr tablet technique.

Dynamic light scattering (DLS) system Malvern 4700C (Malvern Instruments, UK) was used to measure the nanoparticle size-distribution in water at 25°C. Electrophoretic light scattering (ELS) system ZETASIZER IIC (Malvern Instruments, UK) was applied to measure the ζ-potential of colloidal particles in physiological phosphate-buffered saline (PBS, 0.01 M with respect to phosphates; 0.137 M NaCl) at pH 7.4.

The R6G-PECA colloidal particles were imaged by fluorescent microscopy, equipped with a CCD-camera. The examination was performed in epi-illumination system using a mercury lamp (365 nm, 100 W) as a light source. The fluorescence spectra of samples were measured by using a monochromator (Carl Zeiss) equipped with a photomultiplying tube (the excitation wavelength was 365 nm). Fluorescence spectra are measured at wavelengths longer than 500 nm. For the fluorescence measurements, the unloaded R6G was removed from the dispersion of R6G-PECA particles by centrifugation and washing with distilled water. Fluorescence measurements were performed on aqueous dispersions of R6G-PECA particles immediately after washing in order to avoid confusion from possible release of R6G from the particles.

2.4. Loading and release of R6G

The amount of R6G loaded in polymer colloidal particles was determined by centrifugation of the as prepared R6G-PECA dispersions at 14,000 rpm for 30 min. The concentration of R6G in the obtained optically clear supernatant was measured by UV-vis spectrophotometer (Jenway 6400). The measurements were carried out at 523 nm. The loading efficiency (L, %) is defined by Eq. 1.

$$L = 100 \times \frac{C_i - C_s}{C_i} \quad (1)$$

Here, C_i is the total (initial) amount of R6G and C_i is the amount of R6G in the dispersing medium (unloaded R6G).

The kinetics of R6G release was studied in phosphate buffer. For that purpose, R6G-PECA colloidal particles were prepared by using 75 μl ECA and R6G of initial concentration in the polymerization medium 6.0×10^{-5} M (loading efficiency 64%). Then, 2 mL of the as-prepared R6G-PECA dispersion (phosphate-buffered at pH 7.4) was centrifuged and washed with distilled water to remove the unloaded R6G. Then, PBS (0.01 M, 10 mL, pH 7.4, 37 ± 1°C) was added to disperse the colloidal particles in a closed glass vessel. The vessel was then put into water bath (37 ± 1°C) under gentle stirring of the dispersion. Aliquots (1 mL) were taken from the dispersion at regular time intervals and centrifuged (14000 rpm, 15 min). The amount of released R6G in the supernatant was determined spectrophotometrically at 523 nm.

2.5. Photobleaching

To study the photostability of the fluorophore, incorporated in polymer colloidal particles, water dispersions of the R6G-PECA particles (used as-obtained) were illuminated with UV-light (wavelength 365 nm; the light power density at the sample position was 2.1 mW cm^{-2}) for 72 h at room temperature. The light radiation was measured with Research Radiometer (Ealing Electro-optics, Inc.). The respective control sample was kept in dark. The fluorescence spectra of the UV-illuminated and control samples were measured at regular time intervals in order to follow the photobleaching kinetics.

3. Results and discussion

3.1. Chemistry of the polymerization process

In our study, the polymerization of ECA is carried out in aqueous solutions. Previous investigations showed that at similar conditions the hydroxide anions (OH^-) from water initiate anionic polymerization at room temperature [29]. The process is usually carried out in acidic solutions (decreased concentration of hydroxide ions) in order to obtain controllable polymerization, which leads to particle formation. Previous investigations of the mechanism of polymerization of butyl cyanoacrylate in aqueous medium and formation of the corresponding particles showed that the process leads to the formation of polymeric molecules, which have “acidic” H-atom that could be easily dissociated and the polymerization re-initiated [29]. This mechanism (in the case of polymerization of ethyl cyanoacrylate) is illustrated in Fig. 1. The step-wise mechanism of particle formation has been supposed by Behan *et al*. [29] as follows: i) initiation of the polymerization in the monomer droplet (alkyl cyanoacrylates are insoluble in water and form micron-sized droplets after addition to the polymerization medium; acetone is used in our experiments as a co-solvent in order to improve the dispersion of the

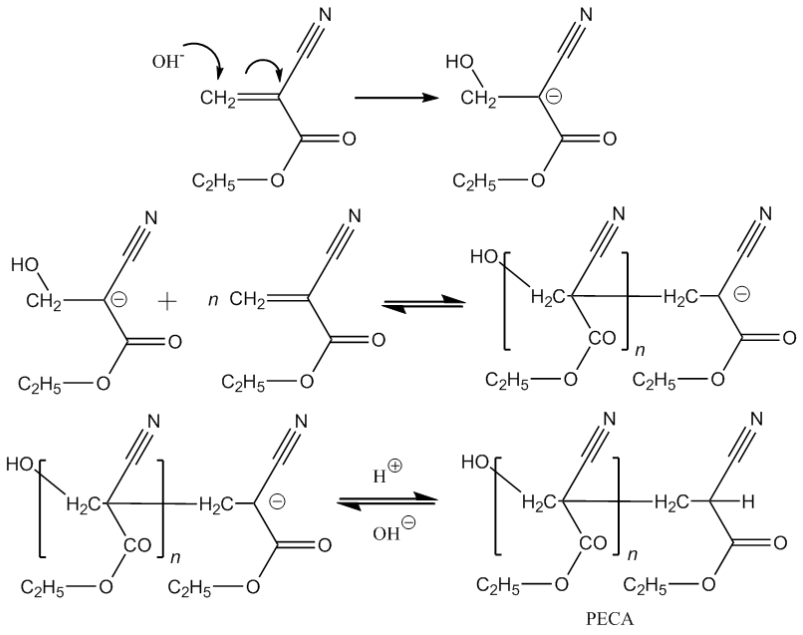

Figure 1. Anionic polymerization of ethyl cyanoacrylate initiated by hydroxide ions and terminated by hydrogen cations.

monomer); ii) the polymerization is terminated by hydrogen cations and/or acidic inhibitors in the monomer; iii) aggregation of the oligomeric units and formation of submicron particles, followed by their swelling with monomer; iv) *in situ* re-initiation of terminated oligomers by live chains followed by further polymerization until reaching given molecular mass distribution. The R6G molecules are probably extracted in hydrophobic interior (R6G is a lipophilic dye) of the micelles during the polymerization (the ECA monomer is also lipophilic and insoluble in water) and thus become entrapped in the polymer matrix of the PECA particles. The alkyl cyanoacrylate monomers can also polymerize according to zwitterionic mechanism. In this case, the compounds carrying nucleophilic groups (such as amino –NH_2 group) could act as polymerization initiators, leading to covalent attachment of the respective compound to the polymer backbone. The R6G molecule contains one NH-group, directly attached to the aromatic nuclei, as well as one imine NH-group, which is protonated as hydrochloride. However, more detailed investigations may be carried out in the future in order to reveal possible association of the dye molecules with the polymer backbone.

The FTIR spectrum of PECA is shown in Fig. 2. The spectrum is similar to previously reported FTIR studies of PECA [26,30,31]. It shows the characteristic absorption bands for the carbonyl C=O ester (1750 cm^{-1}), C≡N groups (2251 cm^{-1}) and C-H (2890-3050 cm^{-1}). The broad absorption at 3490 cm^{-1} is attributed to O-H vibrations. The insignificant absorbance at 1626 cm^{-1} is from C=C stretching vibration of unreacted

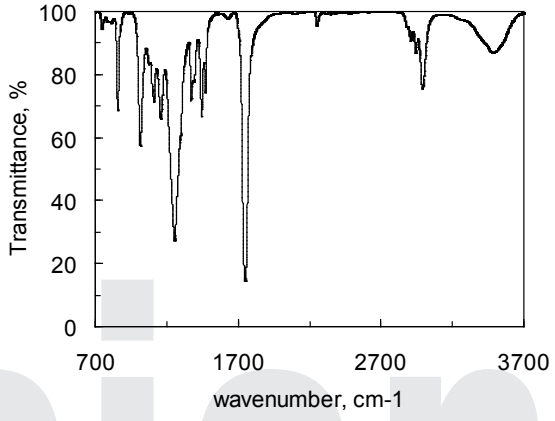

Figure 2. FTIR spectrum of PECA.

monomer (ECA), but its very low intensity indicates that the polymerization of ECA is almost complete. The spectrum of R6G-PECA (not shown here) is similar, with no significant absorbance from R6G because of the very low amount of loaded dye.

3.2. Morphology and size distribution

The investigation with SEM shows that both PECA and R6G-PECA colloidal particles are spherical in shape with monomodal size distributions (Figs. 3 and 4). The DLS measurements show that the size distribution is lognormal, which is typical for colloidal particles. The average sizes of both types of particles (R6G-PECA and PECA) do not depend significantly on the concentration of ECA in the polymerization medium (Table 1), which is

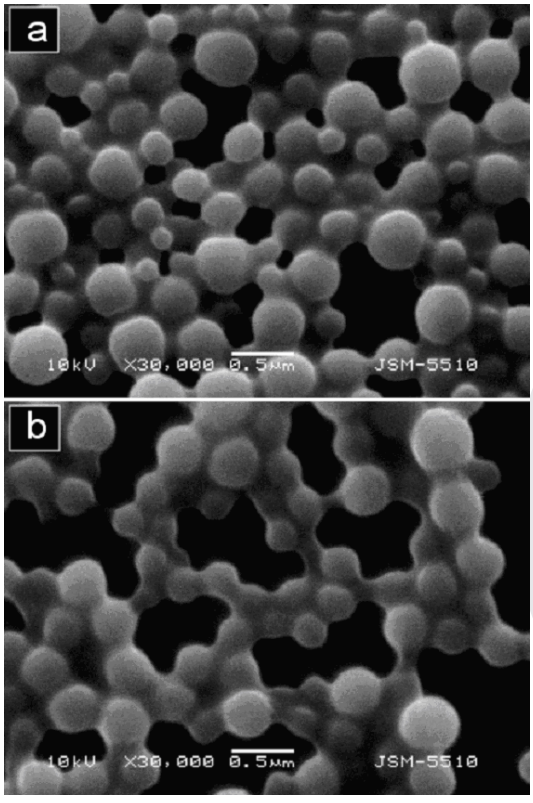

Figure 3. Representative SEM images of: (a) pure PECA particles, and (b) R6G-PECA particles. The particles are prepared at ECA concentration 7.5 mL mL^{-1}. The concentration of R6G in the polymerization medium in case (b) is 6.0×10^{-5} M, respectively.

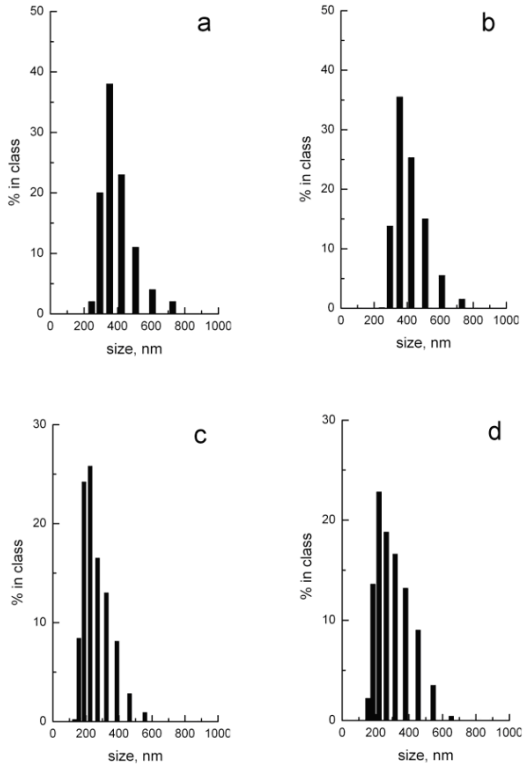

Figure 4. Representative size distributions (measured by DLS) of: (a) PECA particles, prepared at ECA concentration 5.0 μL mL; (b) PECA particles, prepared at ECA concentration 8.5 μL mL^{-1}; (c) R6G-PECA particles, prepared at ECA and R6G concentrations 5.0 μL mL^{-1} and 6.0×10^{-5} M, respectively; (d) R6G-PECA particles, prepared at ECA and R6G concentrations 8.5 μL mL^{-1} and 6.0×10^{-5} M, respectively.

a net result of the emulsion polymerization technique. The average sizes of PECA particles are 400-500 nm (Table 1). In contrast, the size of R6G-PECA particles is found to be about 200-300 nm. The exact reasons for the effect of loaded compounds on the average size of PACA particles are not clear. Similarly, poly (butyl cyanoacrylate) particles, loaded with ciprofloxacin have been found to be smaller (~240 nm) than the respective particle of pure PBCA formed at other conditions (~170 nm) [32]. Previous preparations by polymerization in the presence of 2 mM HCl (pH~2.7) resulted in the formation of slightly smaller in size PECA particles (~380 nm) [31].

3.3. ζ – potential

The ζ-potential of the particles is determined in physiological phosphate-buffered saline (PBS, pH 7.4). Generally, all of the as-obtained particles exhibit considerably low absolute values of the ζ-potential (Table 1), which is probably a result of the relatively high concentration of electrolytes in the PBS solution. Indeed, previous investigations of PECA particles showed that higher concentration of electrolyte results in decrease of the absolute value of the ζ-potential [31]. As seen from the results in Table 1, the R6G-PECA particles have slightly lower absolute values of the ζ-potential than the PECA ones. The PECA polymer, as well as the non-ionic emulsifier used (Pluronic F68) does not contain ionic functional groups, but it is possible that some of the ester bonds of the polymer (PECA) may be hydrolyzed to acrylic carboxyl groups. Ionized carboxyl groups and additional adsorption of ions from the dispersing medium result in the observed negative ζ-potential. Despite of the low absolute value of ζ-potential, the colloidal dispersion is stable due to steric repulsion between the molecules of Pluronic F68, which are adsorbed on the particle surface. Negative ζ-potential is typical for pure PACA particles stabilized with non-ionic surfactants. For example, pure PECA particles have been found to exhibit negative ζ-potential, increasing in absolute value in the pH interval range of 4-9 between 0 to -60 mV (in the presence of 1 mM KNO_3).

Table 1. Average size (measured by DLS) and ζ-potential (mV) of pure PECA and R6G-PECA colloidal particles prepared at various ECA and R6G concentrations. The measurements of ζ-potential were carried out in phosphate-buffered saline (pH=7.4, 0.137 M NaCl).

Particles	Concentrations		Average size, nm	ζ-potential, mV
	[ECA], μL mL^{-1}	[R6G], M		
PECA	5.0	-	430 ± 25	-2.7 ± 0.4
	6.5	-	438 ± 14	-3.4 ± 0.2
	7.5	-	534 ± 17	-3.1 ± 0.3
	8.5	-	405 ± 23	-2.8 ± 0.2
R6G-PECA	5.0	6.0×10^{-5}	250 ± 15	-2.2 ± 0.4
	6.5	6.0×10^{-5}	250 ± 7	-2.5 ± 0.4
	7.5	6.0×10^{-5}	337 ± 15	-2.3 ± 0.4
	8.5	6.0×10^{-5}	288 ± 11	-2.0 ± 0.1
	7.5	2.0×10^{-5}	420 ± 26	-2.0 ± 0.2
	7.5	4.5×10^{-5}	350 ± 20	-1.4 ± 0.5
	7.5	1.0×10^{-4}	320 ± 15	-2.2 ± 0.1

3.4. R6G loading and release

The R6G is incorporated into the PECA particles by dissolution in the polymerization medium prior to the monomer addition. We study the effect of experimental variables such as the concentrations of ECA and R6G on the loading efficiency. The concentration of ECA is in the range 5.0-8.5 μL mL^{-1}. The increase in ECA concentration results in a progressive increase of the loading efficiency (Fig. 5a). Higher loading efficiency means larger fraction of the initial fluorophore loaded in the particles. In this case the ECA concentration is important with respect to the final loading efficiency and constitutes a suitable variable for obtaining colloidal particles with predetermined R6G content. Particles with higher content of R6G could be obtained at higher initial R6G concentration, however the loading efficiency is lower. A decrease in the loading efficiency is observed at concentrations above 2.0×10^{-5} M (Fig. 5b), following the increase of R6G concentration at constant amount of ECA. Similar tendencies have been previously observed during investigation of the effect of concentrations of monomer and drug in the polymerization medium on the entrapment efficiency for ciprofloxacin in PBCA particles [32].

The release kinetics of R6G in aqueous medium at pH 7.4 is followed for 22 hours (Fig. 6). The particles keep their colloidal stability during this period. After that period the particles tend to form aggregates and there are no good sink conditions for valuable release studies for longer periods. The leaking of fluorophore within the first few hours is very low and allows a relatively long-term fluorescent staining of the PECA particles. The released 40% are most probably dye molecules that have been adsorbed on and/or entrapped close to

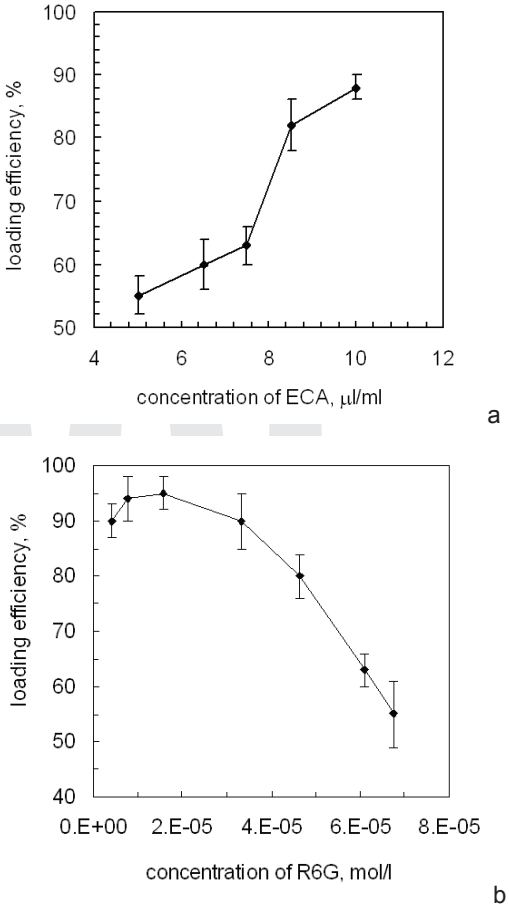

Figure 5. Loading efficiency for R6G in PECA colloidal particles as a function of the: (a) ECA concentration at constant R6G concentration 6.0×10^{-5} M; (b) R6G concentration at constant ECA concentration 7.5 μL mL^{-1}.

the surface of the particles. The rest of the dye remains strongly associated with polymer inside the particles and its release is very slow. The fluorophore release may involve diffusion of R6G molecules out of the particle as well as some partial hydrolysis and erosion of the polymer. It has been previously shown that PACA could undergo hydrolysis of the ester bond [25], which may result in enhanced release of loaded substances. Analysis of the release kinetics could help to reveal the mechanism of release of the loaded compound, but it is known that systems with fundamentally different physicochemical behaviour and mechanisms of release may display similar release profiles [33]. For that reason, distinguishing between the different mechanisms requires considerable accuracy of the experimental data. Moreover, the release experiments are subjects to many potential errors, which must be evaluated and overcome in future experiments before theoretical models can be thoroughly tested.

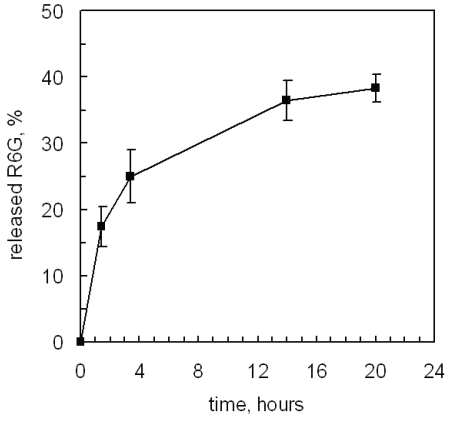

Figure 6. Release kinetics of R6G from R6G-PECA colloidal particles. The initial content of R6G is 5.5×10^{-6} mmol mg^{-1} PECA.

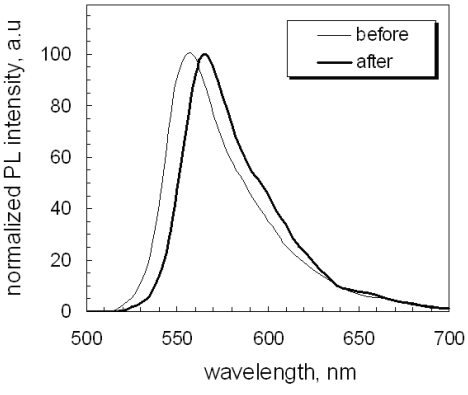

Figure 7. Fluorescence spectra of R6G in the polymerization medium in concentration 6.0×10^{-5} M before and after entrapment in PECA particles.

3.5. Fluorescence properties of R6G-PECA colloidal particles

The R6G dye has a relatively high photostability, high quantum yield, low cost, and its lasing range has close proximity to its absorption maximum (approximately 530 nm). The excitation wavelength in our experiments is 365 nm. The measurements of fluorescence emission are made at wavelengths longer than 500 nm, so no background signal from the excitation source is detected. It is interesting to note the red shift of the fluorescence spectrum of R6G after its entrapment in PECA colloidal particles (Fig. 7). One can suppose that this effect is due to the decreased distance between the dye molecules when they are entrapped in the colloidal particles. The pure PECA polymer does not contain any fluorophore group and the corresponding polymer colloidal particles are not fluorescent at the investigated conditions.

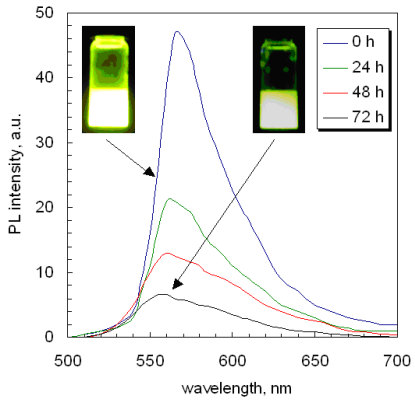

Figure 8. Changes in the fluorescence spectra during UV-illumination of R6G-PECA particles in aqueous dispersion.

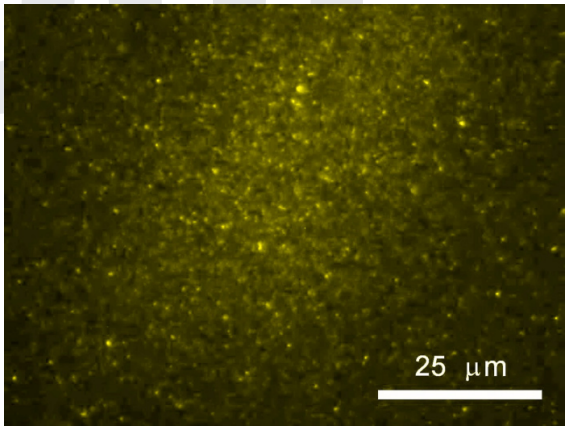

Figure 9. Fluorescence microscopy image of R6G-PECA particles, excited with UV-light (365 nm). The tiny luminescent dots represent the fluorescently tagged particles.

The stability of the as-obtained R6G-PECA colloidal particles against photobleaching upon UV-illumination (365 nm) is illustrated in Fig. 8. As seen, the fluorescence intensity decreases with increasing the illumination time, while the peak shifts to shorter wavelength. These effects result most probably from the transformation of R6G molecule to a form, which does not emit light in the visible region. The investigation of as-prepared R6G PECA particle dispersions by fluorescence microscopy allows observing tiny lighting dots, representing the fluorescently stained polymer particles. (Fig. 9).

4. Conclusions

The emulsion polymerization of ethyl cyanoacrylate in aqueous medium in the presence of Pluronic F68 at pH 2.1 results in the formation of spherical in shape

colloidal particles of average size around 400-500 nm. The organic fluorophore Rhodamine 6G is successfully loaded in the polymer colloidal particles during the polymerization process. The obtained fluorophore-tagged particles are smaller in size (200-300 nm), but with similar values of the ζ-potential (around –3±0.5 mV). The loading efficiency for R6G in the PECA colloidal particles increases with increasing the concentration of monomer in the polymerization medium. The fluorescence spectrum of Rhodamine 6G becomes red-shifted after its entrapment in polymer colloidal particles. The fluorophore is released very slowly in aqueous buffer solution at pH 7.4 and the R6G-tagged PECA colloidal particles could be visually observed by fluorescent microscopy, which is an important prerequisite for their potential applications in bioimaging.

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