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Bioconjugative polymer nanospheres studied by isothermal titration calorimetry

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Abstract

Active ester surfmers are polymerizable monomers with a reaction centre for interaction with biomolecules. These molecules enable for the preparation of nanoscale polymer beads (surfmer nanoparticles) via emulsion polymerization of styrene (St) or methyl methacrylate (MMA). The reactivity of such fabricated surfmer nanoparticles was determined by immobilization of remazol-brilliant blue and streptavidin. The interaction of a *p*-(11-(acrylamido)undecanoyloxy)phenyldimethylsulfonium methylsulfate (AUPDS) and the surfmer nanoparticle (poly(St-co-AUPDS)) with remazol-brilliant blue was monitored by isothermal titration calorimetry (ITC). The binding properties and hydrolysis stability of surfmer nanoparticles was quantified via (ITC). The enthalpy of the reaction of AUPDS with remazol-brilliant blue amounts to $-32.1\,\mathrm{kJ\,mol^{-1}}$, the enthalpy of the reaction of poly(St-co-AUPDS) with remazol-brilliant blue to $-38.8\,\mathrm{kJ\,mol^{-1}}$. Time and temperature dependence investigations of the surfmer nanoparticles displayed their long-ranging storage stability at 6 °C in aqueous solution. The faster hydrolysis of the surfmer nanoparticle surface is the reason for the long-term instability at room temperature.

Keywords: Isothermal titration calorimetry; Surfmer nanoparticles; Temperature dependence; Streptavidin

1. Introduction

Polymer beads and latex particles have become important tools in modern biotechnology [1–4]. They are used as solid phase carrier displaying a high surface area for immobilization and handling of proteins or peptides. For bioconjugation with a protein, the particles must provide a specific chemical function at their surface. The presently used preparation of polymer colloids for bioconjugation is quite circuitous and vulnerable to failures. Active ester surfmers feature a resort here. Active ester surfmers are polymerizable monomers with a reaction centre for interacting with biomolecules. These molecules make the preparation of nanoscale polymer beads (surfmer nanoparticles) via emulsion polymerization of styrene (St) or methyl methacrylate (MMA) possible. Ultrasensitive isothermal titration calorimetry (ITC) is one technique for thermodynamically monitoring chemical reactions initiated by the addition of a component to a reaction mixture. ITC has become the technique of choice for characterizing biomolecular interaction and recognizing reactions in this respect. Unlike other techniques, ITC does not require immobilization or modification of the starting reactants. Furthermore, there are no molecular weight restrictions since the heat of binding is a naturally occurring phenomenon. Several papers and books have recently been published which describe the application of isothermal titration calorimetry for the study of biological and biomolecular recognition reactions [5–9]. Only few papers describe the interaction of molecules with specially designed nanoparticles [10,11].

In our earlier publications [12–14], we described a one-stage preparation for nanoparticles with activated ester groups on their surface. These groups are able to react with aromatic or aliphatic amines to the corresponding amide under weakly basic (pH 7.5–8.5) conditions. Up to now the determination of the yield of this acylation reaction and the amount of immobilized compounds is strongly depended on the kind of immobilized compound.

In this work we present a thermo-chemical method, which offers a more general reaction analysis and determines the reactive sites on polymer particles. We will describe

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in detail an isothermal titration method in which polymer particles are titrated with the amine-containing compound remazol-brilliant blue at 25 °C. With this method, we will determine the affinity constant K_a, the reaction enthalpy ΔH and the concentration c_S of the functional groups on the surface of the particles. Further interest is focused on the immobilization capacity of the polymer particles. Besides we will show experimental results on temperature dependency of the binding site stability on the polymer surface.

2. Experimental

2.1. Materials

Styrene (≤99%, Fluka, Buchs, Switzerland) was distilled before use to remove the inhibitor. The dye remazol-brilliant blue (1-amino-9,10-dihydro-9,10-dioxo-4((3-((2-(sulfooxy) ethyl)-sulfonyl)phenyl)-amino)-2-anthracenesulfonic acid disodium salt, Acros Organics, Geel, Belgium) and 2, 2′-azobis (2-methyl-propionamidin)-dihydrochlorid (≥98°% AIBA, Fluka) were used without further purification. Buffers were prepared by using sodium-hydroxide (≥99%, Merck, Darmstadt, Germany) and potassium dihydrogen-phosphate (≥99.5%, Merck, Darmstadt, Germany). Streptavidin (SAv) was purchased from Boehringer, Mannheim, Germany. Ultra pure water with a resistance higher than 18.0 MΩ cm^{-1} was used.

2.2. Surfmer synthesis

p-(11(Acrylamido)undecanoyloxy)phenyldimethylsulfonium methylsulfate (AUPDS, 1) (see Fig. 1) was synthesized starting from 11-acryloylamino-undecanoic acid and 4-hydroxy-phenyl-dimethylsulfonium methylsulfate according to the method previously described [13].

2.3. Latex preparation and characterization

Preparation of the latex was carried out as described elsewhere [13]. The typical procedure of the latex preparation was as follows: A 100 mL three-necked-flask was equipped with a reflux condenser, with a magnetic stirrer bar and with an argon supply-line. A total of 30 ml of water was three times degassed by altering vacuum and argon atmosphere by bubbling argon through the liquid for 30 min.

Hundred milligram AUPDS (0.2 mmol) dissolved in 2 ml degassed water and 2 ml (34 mmol) co-monomer styrene were added by a syringe through a rubber-septum. The emulsion was stirred (400 U min^{-1}) and heated to the reaction temperature of 60 °C when the water soluble initiator was added. After two hours, the latex solution was heated to 65 °C for 10 min., then removed from the heat and the product cooled down to room temperature. The yield was determined by weighing the solid content from a dried aliquot of the latex.

Three times the latex was purified by centrifugation and subsequent redispersion with water before further application. The solid content of the latex was adjusted to 5 wt.-% by controlled dilution at the last redispersion step.

To determine the storage stability of the activated ester functions on the particle surface, two aliquots of freshly prepared poly(St-co-AUPDS) latex were stored either in a refrigerator at 6 °C or in a thermostat with water circulation at 25 °C.

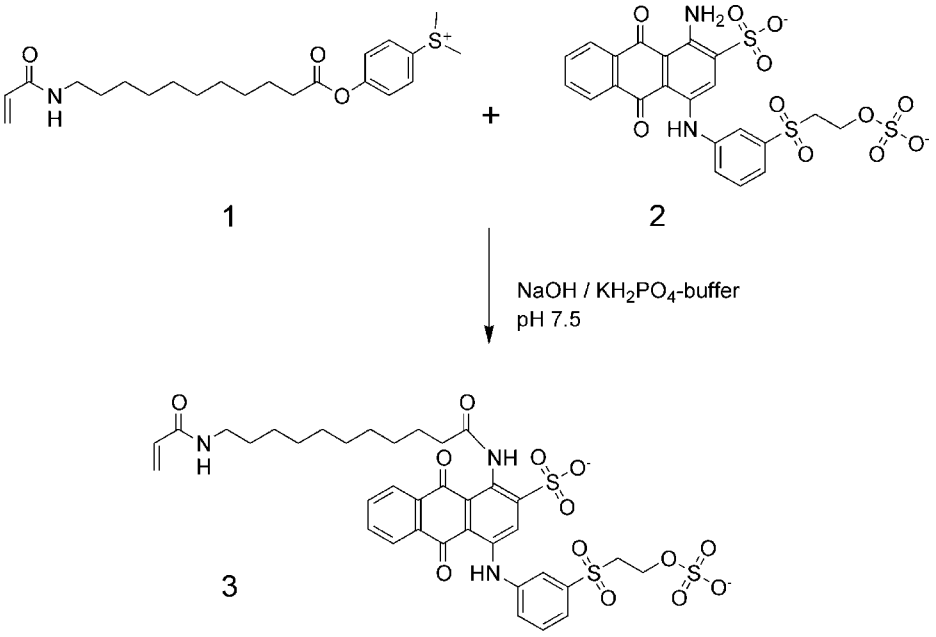

Fig. 1. Reaction scheme of the activated ester surfmer AUPDS (1) with remazol-brilliant blue (2) under aqueous conditions. The reaction product (3) contains a newly built up amide function.

The shape of the particles was determined by using scanning force microscopy (Parc Scientific Instrument SFM with Ultralever tip, radius 10 nm). Therefore we prepared a particle sample on a silicone wafer and scanned in the non contact mode with a rate of 1 Hz.

2.4. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed at 25 °C on a thermal activity monitor calorimeter (Thermometrics AB, Järfälla, Sweden) equipped with a high performance titration unit and a nanowatt amplifier. Prior to each measurement, each solution and suspension was degassed to remove air bubbles. 0.8 ml of the AUPDS solution ($c_{\mathrm{AUPDS}} = 199\,\mu\mathrm{mol\,l^{-1}}$) was titrated by adding $25 \times 3\,\mu$l aliquots of remazol-brilliant blue in the same buffer ($c_{\mathrm{remazol\text{-}brilliant\,blue}} = 6.09\,\mathrm{mmol\,l^{-1}}$, see Fig. 2). The same amount of the latex was titrated by adding $30 \times 4\,\mu$l aliquots of remazol-brilliant blue in the same buffer

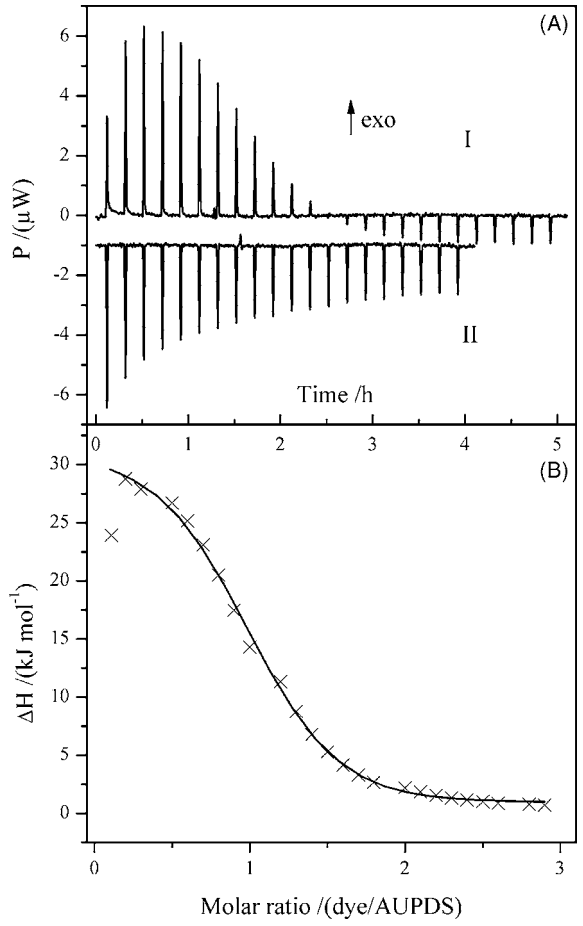

Fig. 2. Experimental titration curve for the titration of the activated ester surfmer AUPDS at 25 °C (I) and the corresponding control dilution experiment (II). (A) Measured heat power versus time. An aqueous solution of 95.42 mg remazol-brilliant blue in 25 ml of a pH 7.5 buffer of sodium hydroxide/potassium dihydrogen phosphate, $c_{\mathrm{phosphate}} = 0.01\,\mathrm{mol\,l^{-1}}$ was titrated into a solution of $199\,\mu\mathrm{mol\,l^{-1}}$ AUPDS in the same buffer. (B) Observed titration heat q_{obs} vs. molar ratio remazol-brilliant blue to AUPDS (the first value was excluded from analysis).

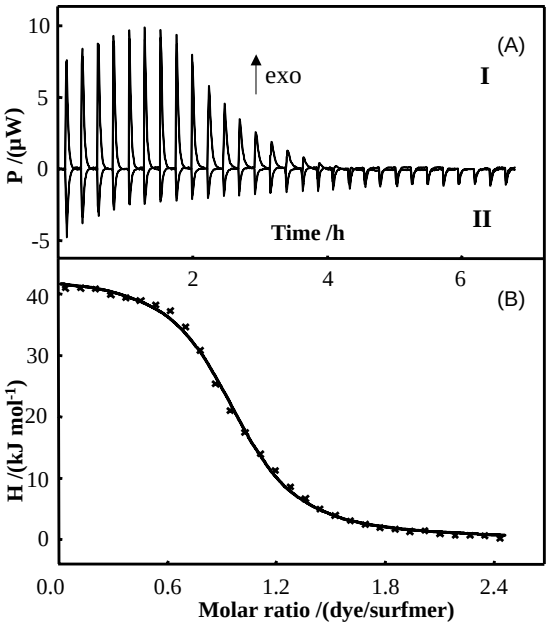

Fig. 3. Experimental titration curves for the titration of the of poly(St-co-AUPDS) latex at 25 °C (I) and the corresponding control dilution experiment (II). (A) Measured heat power versus time. An aqueous solution of 62.85 mg remazol-brilliant blue in 10 ml of a pH 7.5 buffer of sodium hydroxide/potassium dihydrogen phosphate, $c_{\mathrm{phosphate}} = 0.01\,\mathrm{mol\,l^{-1}}$ was titrated into a suspension of $760\,\mu\mathrm{mol\,l^{-1}}$ poly(St-co-AUPDS) latex in the same buffer. (B) Observed titration heat q_{obs} versus molar ratio remazol-brilliant blue to poly(St-co-AUPDS) latex (the first value was excluded from analysis).

($c_{\mathrm{remazol\text{-}brilliant\,blue}} = 10.04\,\mathrm{mmol\,l^{-1}}$, Fig. 3). The first aliquot was used to correct volume errors on the first injection, which was caused by inserting the filled syringe into the cell. This injection was taken into consideration in the titrant concentration scale, but the corresponding heat was excluded from the evaluation. The precise shape of the binding isotherm contained all information necessary to completely characterize the binding reaction [15–17]. Data were analyzed using the proprietary software DIGITAM 4.1 supplied by Thermometric AB (Järfälla, Sweden) with the instrument.

2.5. Immobilisation of streptavidin

An experiment was performed by adding 20 μl of an aqueous streptavidin solution ($c_{\mathrm{SAv}} = 13.4\,\mu\mathrm{mol\,l^{-1}}$) and 10 μl suspension of poly(St-co-AUPDS) nanoparticles were added to 970 μl of a pH 7.5 buffer (sodium hydroxide/potassium dihydrogen phosphate, $c_{\mathrm{phosphate}} = 0.01\,\mathrm{mol\,l^{-1}}$). The particle suspension was shaken over night at room temperature and then separated into solid and aqueous phase by centrifugation. Streptavidin determination was performed as described earlier [12,18].

3. Results

After the preparation of the surfmer, we are able to investigate the reaction between the nucleophilic group of the dye

Table 1
Probe for ITC measurements with remazol-brilliant blue, affinity constants, molar enthalpies, concentration and molality of the activated ester groups per mass of the polymer particles

Sample	K_a (M^{-1})	ΔH ($kJ\,mol^{-1}$)	c_S ($\mu mol\,l^{-1}$)	b_S ($\mu mol\,g^{-1}$)
poly(St-co-AUPDS) latex	96000 ± 9000	38.8 ± 2.1	760 ± 40	15.2 ± 0.8
AUPDS	65000 ± 3000	32.1 ± 1.4	199 ± 12	–

remazol-brilliant blue and the activated ester of the AUPDS (see Fig. 1) by isothermal titration calorimetry.

It was shown by our experimental work that the nucleophilic reaction of the amine group of remazol-brilliant blue with the activated ester group of AUPDS is an exothermic reaction. Fig. 2A (I) shows the heat detected upon gradual injection of a remazol-brilliant blue into an initially pure solution of AUPDS versus time. The corresponding dilution control experiment is shown in Fig. 2A (II). Integration and normalization of the peaks yielded the observed heat per mole of injectant q_{obs} as a function of the molar ratio of the reaction partners (Fig. 2B. The results (ΔH, K_a) of the fit to a 1:1 model are listed in Table 1. The enthalpy ΔH amounts to -32.1 ± 1.2 kJ/mol and the affinity constant K_a to $65{,}000 \pm 3000\,M^{-1}$.

The reaction of the dye remazol-brilliant blue and the activated ester groups on the surface of the poly(St-co-AUPDS) particles was investigated and the results of the ITC experiments indicate a reaction on the particle surface. Fig. 3A shows the heat detected upon gradual injection of remazol-brilliant blue into an initially pure suspension of poly(St-co-AUPDS) latex versus time (Fig. 3A (I), large peaks).

Every injection of 4 μl a remazol-brilliant blue solution ($c_{\text{remazol-brilliant blue}} = 10.04\,mmol\,l^{-1}$) increased the concentration of binding sites on the surface of the nanospheres within the sample cell by about 40 nmol. The corresponding dilution control experiment is shown in Fig. 3A (II, small peaks). The integration of the peaks of the dilution experiment results in decreasing negative values. The integrated peaks of the titration experiment of poly(St-co-AUPDS) latex with remazol-brilliant blue (Fig. 3A (I)) are showing decreasing positive values at the first six injections. After summation and normalization of the integrated values the observed heat per mole of injectant q_{obs} was yielded as a function of the molar ratio of the reaction partners (Fig. 3B). It can be seen that the first seven values of this function are now nearly equal. The solid line was a fit of the above mentioned experimental data, which allowed for the determination of the enthalpy ΔH, the affinity constant K_a and the concentration of the available reaction centres c_S on the nanoparticle. The enthalpy of this reaction was exothermic and corresponded to $-38.8 \pm 1.7\,kJ\,mol^{-1}$ for the titration of a poly(St-co-AUPDS) latex with remazol-brilliant blue. The affinity constant K_a yielded to $96{,}000 \pm 9000\,M^{-1}$. We calculated the concentration of the available reaction centres on the nanoparticle surface to $760 \pm 40\,\mu mol\,l^{-1}$ in this titration experiment. Regarding the solid content of the titrated latex there are 15.2 ± 0.8 μmol reactive ester groups per 1 g particles.

To investigate the reaction activity and the storage stability against hydrolysis of the activated ester on the surface of the particles, we repeated the titration experiments with samples stored under different conditions. Thus, we divided one sample into two portions. One sample was stored at 25 °C in a thermostat with water circulation, the other in the refrigerator at 6 °C. After sequent time intervals, shown in Fig. 4, we

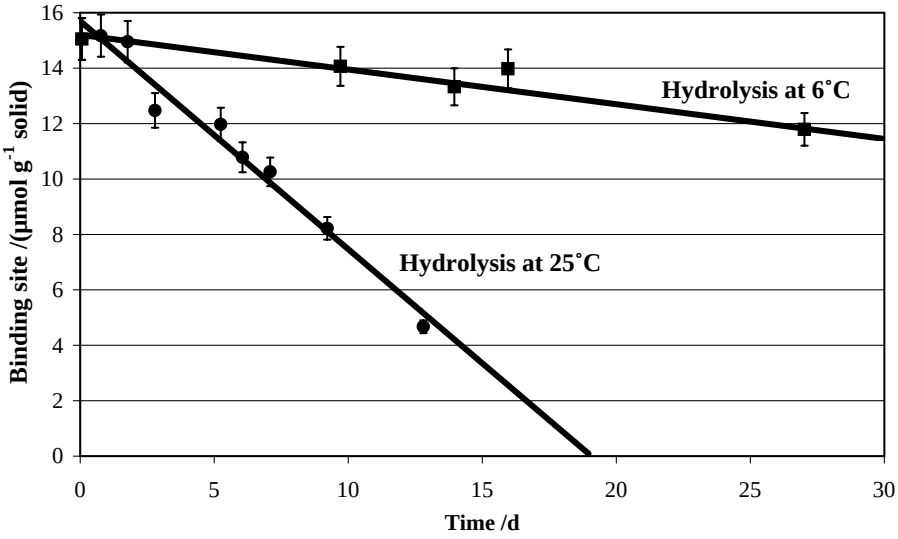

Fig. 4. Temperature and time depending hydrolysis of surfmer binding sites on the particle surface. The stability against hydrolysis increases drastically, if the poly(St-co-AUPDS) latex particles are stored at 6 °C (squares). There is only a short-term stability at a storage temperature of 25 °C (circles). The solid lines are linear regressions of the data sets.

titrated an aliquot of the different samples to determine the concentration of the non-hydrolyzed activated ester groups on the particle surface. The method, which was used for the consecutive ITC measurements, is the same method as described above for the titration of poly(St-co-AUPDS). The time dependence of the concentration of available binding sites is shown in Fig. 4. The site concentration of the sample which was stored at 6 °C shows a smaller decrease (squares, Fig. 4) in comparison to the sample stored at room temperature (diamonds, Fig. 4). The solid lines in this Fig. 4 are linear regressions of the data sets and are shown to visualize the gradients of the hydrolysis.

The bioconjugation of poly(St-co-AUPDS) with a protein was carried out as described in materials and methods (Section 2.5). Therefore, streptavidin was immobilised onto freshly prepared poly(St-co-AUPDS). The determination via fluorescence titration yielded a protein amount of 3.0 ± 0.5 mg tetrameric streptavidin per gram particles (details will be published elsewhere).

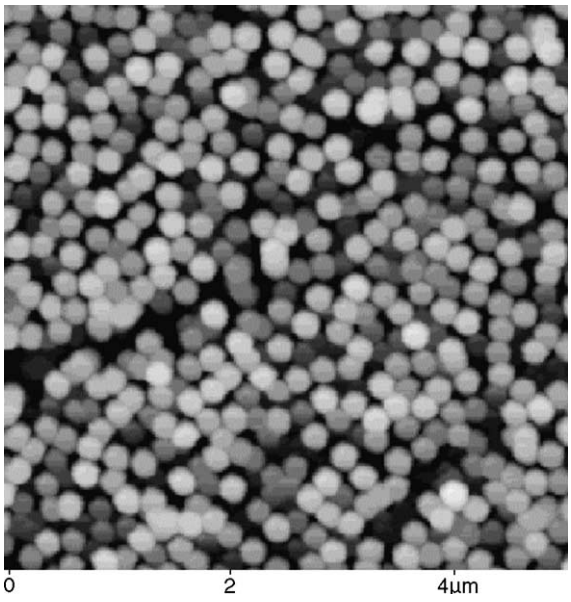

Fig. 5. Scanning atomic force micrograph of poly(St-co-UPDS) particles. The picture shows the nearly monodisperse spherical shape of the particles. The average diameter of the particles is 190 ± 19 nm.

4. Discussion

The exothermic reaction of the dye remazol-brilliant blue and the activated ester of the AUPDS or the activated ester of the prepared poly(St-co-AUPDS) were monitored by isothermal titration calorimetry. The analyzed values of the enthalpies show small differences for both reactions. The reaction of the poly(St-co-AUPDS) is more exothermic as the reaction of the pure AUPDS with the dye remazol-brilliant blue (Figs. 2 and 3). The reaction was successfully carried out under convenient conditions (25 °C, aqueous pH 7.5-buffer sodium hydroxide/potassium dihydrogen phosphate, $c_{\text{phosphate}} = 0.01\,\text{mol}\,\text{l}^{-1}$) and it was possible to directly detect the reactivity of the surface groups on the particles by ITC. Additionally, the regular shape, the small distribution of size (see Fig. 5) and the sedimentation stability of the poly(St-co-AUPDS) nanoparticles allows ITC measurements.

The stability against hydrolysis of the poly(St-co-AUPDS) nanoparticles was also determined by ITC. The usage of the supplied analysing program DIGITAM 4.1 from Thermometrics AB allows the adjustment of the concentrations of the injected sample or the sample in the vessel if one other parameter is fixed. Therefore, we assumed a stoichometric one-to-one reaction and fitted the sample concentration in the vessel. The concentration of the active ester groups on the particle surface could be calculated after the different storage times. The experimental conditions of the calorimetric set-up were unaltered over the whole series of experiments. The input of energy into the sample stored at 25 °C is significantly higher in comparison to the poly(St-co-AUPDS) latex stored at 6 °C. This results in a faster hydrolysis of the active ester groups of the poly(St-co-AUPDS) stored at 25 °C, which caused strongly decreasing concentrations of the binding sites c_S in the calculation of the continuing measurements. The poly(St-co-AUPDS) stored at 6 °C kept nearly 90% of its binding activity over 4 weeks, whereas the binding activity of the sample stored at 25 °C is diminished to 50% after 9 days.

The freshly prepared poly(St-co-AUPDS) nanospheres are able to immobilize 3 wt.% of streptavidin, which proves their potential in bioconjugational chemistry.

5. Conclusion

The novel polymerizable surfactant AUPDS is able to react with the nucleophilic dye remazol-brilliant blue under aqueous conditions. A reaction enthalpy ΔH of $-32.1\,\text{kJ}\,\text{mol}^{-1}$ was determined by ITC. We also presented an ITC-method for the determination of reactive activated ester groups at particle surfaces. This could be realised by the regular shape of the nanoparticles, which built a stable emulsion in the specified buffer. After the successful preparation of poly(St-co-AUPDS) particles, we analyzed the reaction of remazol-brilliant blue at 25 °C with the AUPDS-groups on the particles. A reaction enthalpy ΔH of $-38.8\,\text{kJ}\,\text{mol}^{-1}$ was determined by ITC. We successfully investigated and quantified the stability against hydrolysis by isothermal titration calorimetry. It is necessary to store freshly prepared latex at 6 °C to prevent hydrolysis of the active ester groups. The surfmer nanoparticles provide capabilities for bioconjugation of proteins with amine groups as shown by the immobilization of streptavidin. The prepared nanoparticles are an interesting new tool in nanobiotechnology for immobilization of amine-containing peptides or proteins.

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