

Affinity Switching for Lysozyme and Dual-responsive Microgels by Stopped-flow Technique: Kinetic Control and Activity Evaluation^{*}

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Abstract The use of proteins as therapeutics in nanomedicine is an emerging research field and has developed rapidly. However, proteins are always vulnerable to renal excretion or digestion by the proteolytic system *in vivo*, which limits their usage to a large extent. Although biocompatible polymers have been covalently linked to proteins to protect them from recognition by the immune system and prolong their circulation time, the biological activity of them is sometimes decreased. To fill this gap, physical isolation, wrapping, or encapsulation techniques are employed. Up to now, various mature examples were reported, but the whole time scales for guest molecules loading and releasing, especially the initial rapid loading process, were rarely mentioned. Herein, a series of dual-responsive poly(*N*-isopropylacrylamide-*co*-methacrylic acid) (P(NIPAM-*co*-MAA)) microgels were synthesized and employed to investigate the kinetics of *in situ* complexation and release of lysozyme under external stimuli modulation upon a stopped-flow apparatus, which was suitable for rapid dynamic monitoring. Close inspection of the adsorption kinetics during the early stages (< 50 s) revealed that the initial microgel collapse occurred within ~1 s, with more rapid transitions being observed when higher lysozyme concentrations were targeted. All the dynamic traces could be well fitted with a double exponential function, suggesting a fast (τ_1) and a slow (τ_2) relaxation time, respectively. Then, the kinetics of releasing bound lysozyme from microgels was carried on by utilizing the pH-responsive property, and the evaluation of the activity of released lysozyme was synchronously measured in a *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell suspension. The corresponding relaxation time (τ) was also calculated by fitting the recorded dynamic traces. We speculate that this work can provide basic dynamics data and theoretical basis for microgels based nanocarriers to be used for protein delivery, controlled release, and possible chemical separation.

Keywords Microgels; Stimuli-responsive; Lysozyme; Adsorption; Kinetics

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INTRODUCTION

Artificial synthetic polymeric nanoparticles (NPs) that have a specific affinity for guest molecules, such as small molecule compounds^[1], biomacromolecules^[2], or proteins^[3], have aroused considerable interest due to their potential applications in biomedical domain. Not only that, the capability of uptake and subsequent controlled release of targeted guest molecules to adapt to special occasions is an expected feature. To meet this demand, stimuli-responsive polymers^[4–7], which can selectively respond to external stimuli (*e.g.*, temperature, pH, light, enzyme, redox *etc.*) through the changes of their chemical or physical properties, are gaining considerable attention and have been explored to regulate the process and execute the task at desired time and site in the field

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of nanomedicine.

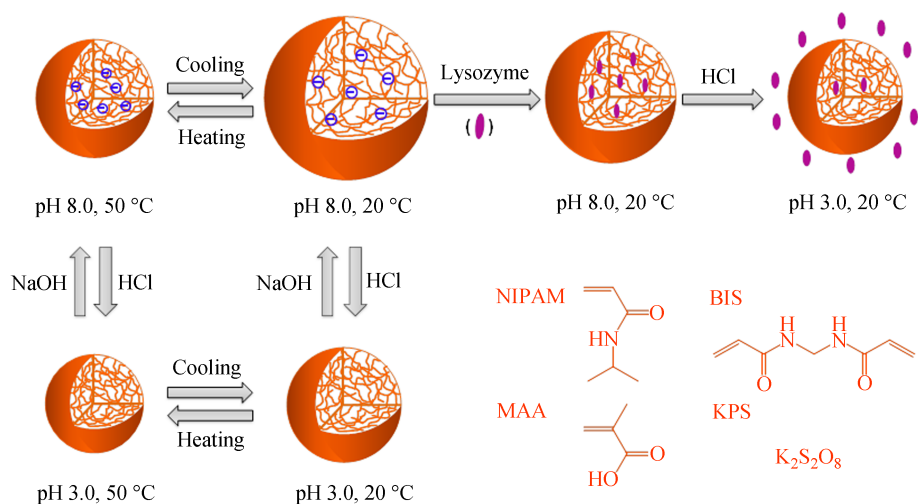
Microgels are cross-linked latex particles which can swell appreciably in a good solvent and stay monodispersed. Compared to macroscopic gels, microgels exhibit much faster rates of volume phase transitions (VPT) in response to external stimuli^[8, 9]. Moreover, the cross-linked network exhibits excellent structural stability in certain conditions, such as physiological condition, different with the conventional non-cross-linked micelles, which cannot retain the original entire structure and perhaps result in premature leaking of encapsulated cargos once subject to long time circulation in the body. For the biocompatible gels, they are expected to take more important role in the fields of regenerative medicine, since this kind of support can efficiently mediate cell attachment and proliferation which are key issues for tissue regeneration^[10]. The introduction of multiple environmentally responsive monomers will make microgels sensitive to the microenvironment of the tumor (*e.g.*, lower internal pH, abnormal temperature, and abundant reactive oxygen species)^[11, 12]. The uptake and release of cargoes into and out of stimuli-responsive microgels have been widely studied and have tremendous potential application in drug loading, delivery, and release. Among them, pH-responsive microgels have been the subject of significant interest. For example, Zhang *et al.*^[13] designed methotrexate contained pH-responsive chitosan-based microgels; once the microgels were exposed to low pH environments, the microgels became swollen and released the encapsulated drug subsequently.

Proteins with high specificity and selectivity possess specialized functions in biological reactions and therefore are ideal candidates for various applications in biotechnology^[14]. Protein-containing complexes have been readily used for controlled delivery of therapeutic proteins^[15]. Bioconjugates fabricated from protein-polymer conjugates are always prepared either by polymerizing from initiation sites on protein or coupling reaction between protein and polymers^[16–20]. However, the reaction point is nonspecific and the resultant conjugates are heterogeneous in the number and placement of the polymer chains, leading to a decreased protein activity^[21], although the covalent linkage can enhance the stability of the conjugates. Alternatively, the “complex coacervation”^[22–24], “solid-like precipitates”^[25], and “core-shell”^[26] architectures had been reported by simply mixing linear charged polyelectrolytes with oppositely charged proteins. Polymeric nanoparticles carrying active cargos internal are one such type of nanocarriers, which can not only solubilize and stabilize functional cargoes within them, but also protect them from damage and degradation during the transport process. For example, Yoshimatsu *et al.*^[3] reported the synthesis and applications of multifunctional poly(*N*-isopropylacrylamide) (PNIPAM) based nanoparticles containing acrylic acid (AA) and *N*-*tert*-butylacrylamide (TBAm) as functional comonomers. They demonstrated that these nanoparticles had selective protein affinity and could be modulated by temperature to catch and release lysozyme (a basic protein with a molecular weight of 14 kDa and an isoelectric value of ~11.2^[27]) over other major egg-white proteins. The effect of balance between hydrophobic and negatively charged groups in the nanoparticles and nanoparticle concentrations on the lysozyme activity were further systematically investigated. The repeated catch and release cycles did not lead to the denaturation of lysozyme and had no impact on the lysozyme activity. By using the same batch of microgels, Shea group further applied them for binding and protecting a target enzyme from irreversible activity loss upon exposure to heat (85 °C, 30 min)^[28]. On cooling the solution, the enzymes could autonomously release from microgels and maintain 80% of their activity after subjected to heat. These results perfectly confirm that physical encapsulation is an excellent method to not only stabilize proteins but also successfully protect their activity.

However, it should be noted that although many protein-polymer conjugate examples were reported, and the equilibrium swelling ratio, the critical transition pH or temperature, as well as the cargo loading efficiency of bulk gels or microgel particles were systematically investigated, the whole time scales for guest molecules loading and release, especially the initial rapid processes, were rarely mentioned. In spite of its importance in both academic research and practical applications, the kinetics of adsorption and desorption of guest molecules into and out of microgels seems especially important. Considering the rapid VPT rate for microgels, the kinetics study for microgels were performed upon a stopped-flow apparatus, which was suitable for rapid dynamic monitoring. For example, Dupin *et al.*^[29] and Yin *et al.*^[30] studied the acid-induced swelling and deswelling kinetics of a series of near monodisperse sterically stabilized poly(2-vinylpyridine) latex particles, respectively,

by using the stopped-flow technique. The characteristic times for the transition were within the time scale of milliseconds, which were in fairly good agreement with that predicted by the Tanaka equation^[31].

Herein, starting from NIPAM and methacrylic acid (MAA) that were selected as functional monomers, a series of monodispersed and random copolymerized dual-responsive poly(*N*-isopropylacrylamide-*co*-methacrylic acid) (P(NIPAM-*co*-MAA)) microgels with different feed recipe and crosslinking density were synthesized by emulsion polymerization at 70 °C. These microgels exhibited completely reversible temperature- and pH-responsive swelling or deswelling behavior in water due to the internal properties from PNIPAM and PMAA (Scheme 1)^[32, 33]. Not only that, the excellent solubility of PNIPAM at low temperature endows the microgel to be a nanosized soluble complex other than macroscopic precipitates. At pH 8.0 and 20 °C, positively charged lysozymes could be readily internalized into the negatively charged P(NIPAM-*co*-MAA) microgels by means of electrostatic interactions, resulting in the formation of dynamic P(NIPAM-*co*-MAA)/lysozyme complexes and concomitant microgel shrinking. The adsorption and release studies were performed under different conditions in a batch system, *i.e.*, solution pH, protein concentration, and temperature. This method is highly effective for protecting the activity of biomolecules that the released lysozyme exhibited similar activities to that of the free one, meaning that the loading process was not detrimental to the enzyme. The entire loading dynamic process was then studied by stopped-flow equipment as a function of lysozyme concentration. By carefully inspecting the adsorption dynamic traces during the early stages (< 50 s), it was found that the initial microgel collapse occurred within ~1 s, with more rapid transitions being observed when higher lysozyme concentrations were used. The dynamic traces could be well fitted with a double exponential function, and two characteristic relaxation times (τ) were calculated: τ_1 was correlated with the nonequilibrium complex formation within the microgel shell, while τ_2 was consistent with the formation of final equilibrium complexes in the microgel internal. Then, the kinetics of releasing bound lysozyme from microgels was realized by utilizing the pH-responsive property, and the evaluation of the activity of released lysozyme was synchronously measured in a *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell suspension^[34]. We speculate that this work can provide basic dynamics data and theoretical basis for microgel based nanocarriers, and, thus, endow them to be a potential versatile nanocarrier system for protein delivery, controlled release, and chemical separation.



Scheme 1 Schematic illustration of the dual-responsive P(NIPAM-*co*-MAA) microgels and charge neutralization-induced deswelling resulted from the complex with lysozyme, as well as its subsequent pH-induced release

EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPAM, 97%, Tokyo KaseiKagyo Co.) was purified by recrystallization from a mixture

of benzene and *n*-hexane (1/3, *V/V*). Potassium persulfate (KPS) and *N,N'*-methylene-bis(acrylamide) (BIS) were recrystallized from methanol and ethanol, respectively, and stored at $-20\text{ }^{\circ}\text{C}$ prior to use. Nonionic surfactant, polyoxyethylene sorbitan monolaurate (Tween 20), was purchased from Amersco and used as received. Lysozyme and *Micrococcus lysodeikticus* (*M. lysodeikticus*) were purchased from Sigma-Aldrich and used as received. Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of $18.4\text{ M}\Omega\cdot\text{cm}$.

Preparation of P(NIPAM-co-MAA) Microgels

Synthetic protocols for the preparation of P(NIPAM-co-MAA) microgels were in accordance with the previously reported literatures with slight modification^[35, 36]. Typically, into a 250 mL three-neck round-bottom flask equipped with a mechanical stirrer, reflux condenser, and a nitrogen gas inlet, NIPAM (0.95 g, 8.4 mmol), MAA (0.32 g, 3.7 mmol), BIS (38.9 mg, 0.25 mmol), Tween 20 (50 mg, 0.04 mmol), and deionized water (100 mL, pH 3.0) were charged. After heating to $70\text{ }^{\circ}\text{C}$ and degassing by bubbling with nitrogen for 30 min, KPS (25 mg, 94 μmol) dissolved in 1.0 mL of deionized water was injected under mechanical stirring at 400 r/min to start the copolymerization. The copolymerization was conducted under stirring for 8 h. The dispersion was passed through glass wool in order to remove particulate matter and then dialyzed against water at pH 8.0 (MW cutoff $\sim 14\text{ kDa}$) for 3 days. Fresh water was replaced approximately every 6 h until there was no further change in the conductivity. The purified dispersion (P(NIPAM-co-MAA) microgels; S3 in Table 1) was further diluted and stored at $4\text{ }^{\circ}\text{C}$ for further use. Following the same procedure, control sample (PNIPAM microgels; S1 in Table 1) and other kinds of P(NIPAM-co-MAA) microgels (S2 and S4 in Table 1) with different feed recipe and crosslinking density were also prepared. The physical parameters of these microgels in various conditions are summarized in Table 1.

Table 1 Dynamic laser light scattering data obtained for poly(*N*-isopropylacrylamide-co-methacrylic acid) (P(NIPAM-co-MAA)) microgels at different temperatures and pH values as well as after complex formation with lysozyme

Sample	Monomer content ^a (mol%)			$\langle R_h \rangle$ ^b (nm) pH 8.0		$\langle R_h \rangle$ ^b (nm) pH 3.0		$\langle R_h \rangle$ ^b (nm) $20\text{ }^{\circ}\text{C}$, pH 8.0
	NIPAM	MAA	BIS	$20\text{ }^{\circ}\text{C}$	$50\text{ }^{\circ}\text{C}$	$20\text{ }^{\circ}\text{C}$	$50\text{ }^{\circ}\text{C}$	[Lysozyme]/[MAA] = 1/1
S1	98	0	2	288	125	285	126	288
S2	88	10	2	344	186	270	124	312
S3	68	30	2	480	366	260	125	386
S4	65	30	5	450	352	258	120	364

^a The amount of monomer in the feed recipe was relative to the sum of all monomers and cross-linker; ^b Intensity-average hydrodynamic radius, $\langle R_h \rangle$, was determined by dynamic light scattering with scattering angle at 90° , the estimated error associated with each value was $\pm 2\text{ nm}$.

Lysozyme Activity Evaluation

The activities of the lysozymes were evaluated using standard assays that measured the lytic ability of the lysozyme with respect to the *Micrococcus lysodeikticus* (*M. lysodeikticus*)^[34]. Lysozyme solution ($5\text{ }\mu\text{g/mL}$) and *M. lysodeikticus* suspension ($125\text{ }\mu\text{g/mL}$) were prepared in advance. Then, $100\text{ }\mu\text{L}$ of *M. lysodeikticus* suspension and $100\text{ }\mu\text{L}$ of lysozyme solution were mixed. The decrease in optical density (OD) at 450 nm (OD_{450}) for the first 1 min was used as a measure of lysozyme activity.

Capability Evaluation for Lysozyme to Microgels

Lysozyme solution ($24\text{ }\mu\text{g/mL}$; relative to the amount of MAA moieties) and microgel dispersions (0.1 g/L , pH 8.0) were prepared in advance. After stirring at $20\text{ }^{\circ}\text{C}$ overnight, the mixture was subjected to centrifugation (3000 relative centrifugal force (rcf) for 10 min) to separate the microgel/lysozyme complex and free lysozyme. The lysozyme activity in the supernatant was determined following the above mentioned method. The absorption capability was calculated correlative to $(\text{OD}_{450})_0$.

Characterization

Potentiometric titrations

The microgels were dispersed in deionized water at pH 10. The dispersion was titrated by the dropwise addition of 0.1 mol/L HCl and the solution pH was monitored with a Corning Check-Mite pH meter (precalibrated with pH 4.0, 7.0, and 10.0 buffer solutions).

Field-emission scanning electron microscopy (FE-SEM)

FE-SEM observations were conducted on a high-resolution JEOL JSM-6700 field-emission scanning electron microscope. The samples for SEM observations were prepared by placing 10 μ L of microgel solutions on copper grids successively coated with thin films of Formvar and carbon.

Laser light scattering (LLS)

A commercial spectrometer (ALV/DLS/SLS-5022F) equipped with a multita digital time correlator (ALV5000) and a cylindrical 22 mW UNIPHASE He-Ne laser ($\lambda_0 = 632$ nm) as the light source was employed for dynamic LLS measurements. Scattered light was collected at a fixed angle of 90° for duration of ~ 10 min. Distribution averages and particle size distributions were computed using cumulants analysis and CONTIN routines. All data were averaged over three measurements.

Circular dichroism (CD) and UV-Vis spectra

CD and UV-Vis were performed on JASCO J1500 and UNIC 4802 UV/Vis double beam spectrophotometers, respectively. Quartz cells were used in both measurements.

Stopped-flow with light-scattering detection

Stopped-flow studies were conducted using a Bio-Logic SFM300/S stopped-flow instrument equipped with three 10 mL step-motor-driven syringes (S1, S2, and S3), which can be operated independently to carry out single- or double-mixing. This stopped-flow device was attached to a MOS-250 spectrometer and kinetic data were fitted using the Biokine program provided by Bio-Logic. For light scattering detection at a scattering angle of 90° , both the excitation and emission wavelengths were adjusted to 335 nm with 10 nm slits. Using either FC-08 or FC-15 flow cells, typical dead times are 1.1 and 2.6 ms, respectively. The solution temperature was maintained at 20°C by circulating water around the syringe chamber and observation head. Prior to loading into the motor-driven syringes, all aqueous solutions were clarified by passing through $2.0\ \mu\text{m}$ Millipore Nylon filters. For pH jump experiments, the final pH was controlled by varying the mixing ratio of the microgel dispersion to that of acidic solution. The mixing ratios were calculated based on titration results, and the final pH after stopped-flow mixing was monitored with a Corning Check-Mite pH meter.

RESULTS AND DISCUSSION

All of the reported microgels were synthesized *via* free radical emulsion copolymerization of NIPAM and MAA monomers at pH 3.0 and 70°C in the presence of *N,N'*-methylene-bis(acrylamide) (BIS; 2.0 mol%; served as a cross-linker) and potassium persulfate (KPS; served as a thermally decomposed initiator) in water (with a fixed solid content of 1.0 wt%) that containing a small amount of polyoxyethylene sorbitan monolaurate (Tween 20; 5 mol%). After the synthesis, the microgels were purified by dialysis against deionized water (pH 8.0), and the water was replaced several times until there was no further change in the conductivity. Although these microgels differed in the content of functional monomers and cross-linkers, the particle sizes were all in the range of 280–300 nm with narrow distribution, as revealed by SEM images (Fig. 1).

For lightly cross-linked microgels, the shift of apparent pK_a value was confirmed, because of the increasing crosslinking density^[37]. Here, a typical acid titration curve for one of the microgels (S3 in Table 1) used in this study is shown in Fig. S1(a) (in supporting information, SI). Addition of 0.1 mol/L HCl to this aqueous dispersion (pH 10.0) leads to the deionization of MAA residues. Using these titration data, the pK_a of this sample could be calculated to be ~ 6.47 (Fig. S1b in SI), which is shifted to higher values compared with the

homopolymers of PMAA in dilute aqueous solution^[38]. Below this critical value, PMAA chains are insoluble due to the deionization of pendent carboxyl groups. Therefore, slightly alkaline condition (pH 8.0) was employed to ensure the existence of positively charged lysozymes and negatively charged PMAA chains. In addition, the circular dichroism (CD) spectra of lysozyme showed that the signals had no significant changes no matter at acidic (pH 3.0) or alkaline (pH 8.0) conditions, which provided the conclusion that the structural integrity of lysozyme was not perturbed in a wide pH range (Fig. S2 in SI).

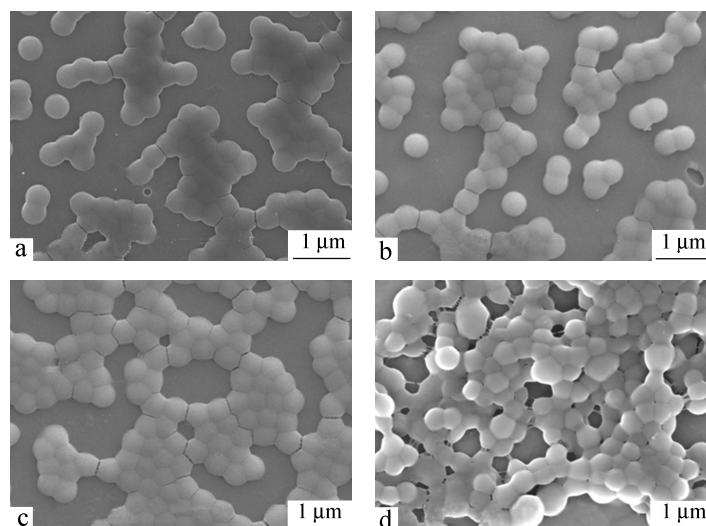


Fig. 1 SEM images recorded for the morphologies of P(NIPAM-*co*-MAA) microgels with different feed recipes: (a) S1, (b) S2, (c) S3, and (d) S4 once dried from aqueous dispersion (pH 3.0, 5.0 g/L) at room temperature

The absorption capability of lysozyme into microgels was evaluated by coincubating lysozyme (24 $\mu\text{g/mL}$; relative to the amount of MAA moieties) and P(NIPAM-*co*-MAA) microgels (0.1 g/L) in water at pH 8.0. The concentration of free lysozyme in the filtrate was estimated by its activity measurements in a *M. lysodeikticus* suspension. The results of uptake are summarized in Fig. 2(a). It showed that a large amount of lysozyme ($\sim 73.2\%$) was absorbed into the S3 samples with 68 mol% of NIPAM, 30 mol% of MAA, and 2 mol% BIS. The loading contents of lysozyme into other samples were only 5.6%, 33.4%, and 55.2% for S1, S2, and S4, respectively. These results suggest that the strong electrostatic interaction between negatively charged microgels and positively charged lysozyme made an important contribution to the lysozyme capture. Moreover, the MAA content also played an important role, but the higher cross-linking density in S4 sample blocked the access of lysozyme into the interior of microgels. It should be noted that although the amount of MAA in the S1 microgel was 0, the slight adsorption of lysozyme into S1 (5.6%) still happened. Two reasons can be attributed to explain this phenomenon: 1) once the lysozyme solution was added into S1 dispersion, the abruptly generated osmotic pressure will drive the diffusion of lysozyme into microgels; 2) the possible physical absorption or physical interaction between PNIPAM and lysozyme will also lead to the same results. Based on these results, S3 sample was selected for further investigation.

The pH-responsive property is an important basic physical parameter of microgels in this work. Thus, the pH-dependent intensity-average hydrodynamic radius ($\langle R_h \rangle$) and scattered light intensities (SLI) for S3 at 20 $^{\circ}\text{C}$ were studied by dynamic laser light scattering (LLS) (Fig. 2b). Upon the pH values increasing from 3.0 to 9.0, $\langle R_h \rangle$ increased from ~ 260 nm to ~ 480 nm at 20 $^{\circ}\text{C}$, about 6.3 times higher in microgel hydrodynamic volumes. Accordingly, the SLI increased with an opposite tendency as $\langle R_h \rangle$. This pH-responsive character and VPT exhibited by MAA contained microgels were arose from the changes in charge density, which led to the hydrophilic to hydrophobic transition of PMAA moieties and subsequent microgels shrinking induced higher

refractive index difference relative to pure water. In the next step, the $\langle R_h \rangle$ and SLI obtained as a function of [lysozyme]/[MAA] for S3 at pH 8.0 and 20 °C are shown in Fig. 2(c). In the present condition, negatively charged S3 and positively charged lysozymes were obtained and their strong electrostatic interaction endowed S3 to be an efficient nanocarrier for lysozyme. Dynamic LLS in Fig. 2(c) revealed the minimum $\langle R_h \rangle$ (385 nm) and maximum SLI values for S3 microgels when the [lysozyme]/[MAA] value reached 0.2, and no obvious changes were observed at a higher value (> 1.0). Here, the difference between the size change of S3 samples upon addition of NaOH (Fig. 2b) and lysozyme was noted. In principle, the abundant positive charges within lysozyme at pH 8.0 should guarantee the higher adsorption capacity of the negatively charged S3 and result in the more screening of opposite charges and smaller size. However, the significant exclusion volume of lysozyme substantially hinders the diffusion and deep penetration into the microgel inner cores, although it possesses greater interaction with microgel particles. Anyway, these results confirmed that the possibility to apply PMAA contained microgels as protein nanocarriers is feasible. Moreover, the temperature-dependent optical transmittance at 700 nm obtained for S3 micellar dispersion at pH 8.0 and 3.0 are shown in Fig. S3 (in SI). As can be seen, the volume phase transition of S3 was completed at 35 °C when the pH was adjusted to 3.0 and 50 °C at pH 8.0. The fully reversible nature of temperature-induced switching of swelling/deswelling at different pH values (8.0 and 3.0) was also verified (Fig. 2d), confirming the excellent temperature sensitivity.

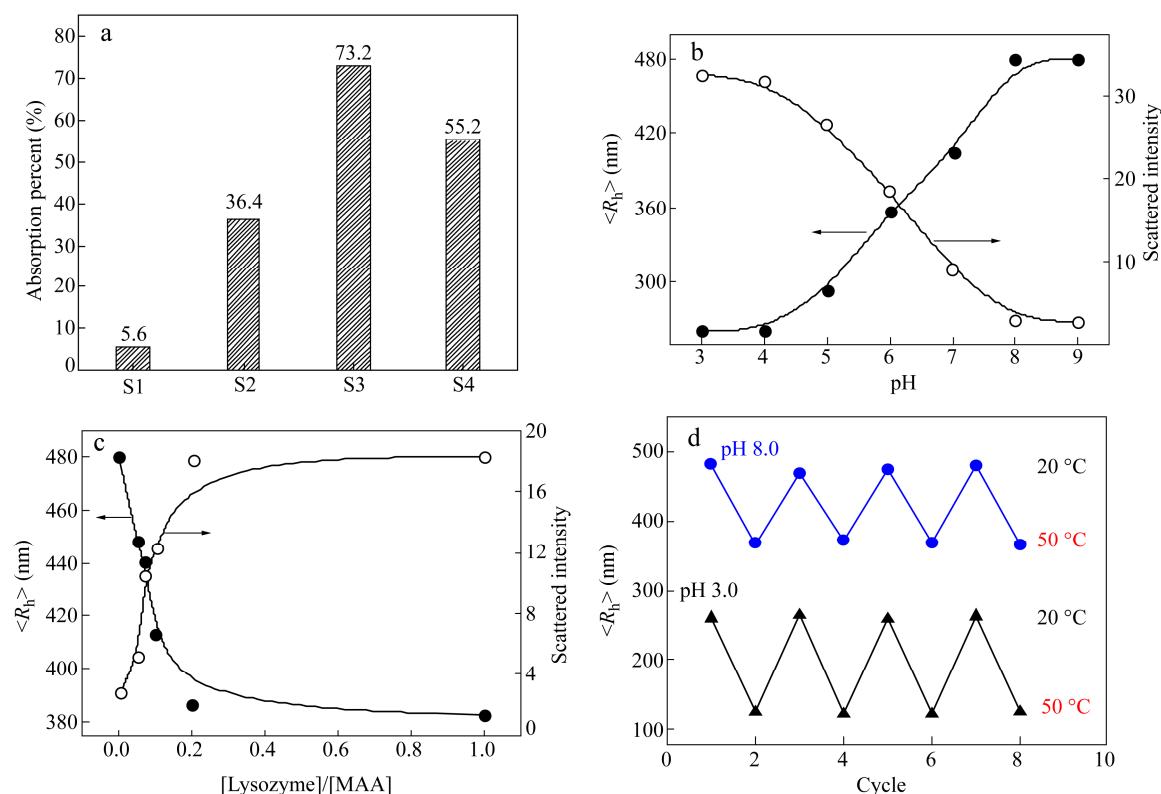


Fig. 2 (a) The absorption capability of lysozyme (24 $\mu\text{g/mL}$; relative to the amount of MAA moieties) to microgels (S1, S2, S3, and S4 in Table 1) in aqueous dispersion (pH 8.0) at 20 °C (The concentrations of microgels were fixed at 0.1 g/L.); (b) pH-dependent intensity-average hydrodynamic radius, $\langle R_h \rangle$, and scattered intensities obtained for aqueous dispersion of P(NIPAM-co-MAA) microgels (S3 in Table 1) at 20 °C; (c) $\langle R_h \rangle$ and scattered intensities obtained as a function of [lysozyme]/[MAA] for P(NIPAM-co-MAA) microgels at pH 8.0 and 20 °C (The scattering angle was fixed at 90°.); (d) Change in the $\langle R_h \rangle$ values of S3 at different pH values when microgel dispersion temperature was cycled between 50 and 20 °C

It has been known that the adsorption of polyelectrolyte chains into oppositely charged microgels will get through a first fast process (nonequilibrium complex within microgel shell) and a second slow process (equilibrium complex through polyion neutralization reactions in the inner part), leading to the formation of a collapsed microgel and corresponding two characteristic relaxation time (τ_1 and τ_2 ; $\tau_1 < \tau_2$)^[35]. Inspired by this work, the entire dynamic process for the uptake of lysozyme with various concentration into S3 microgels was monitored by stopped-flow kinetic technique. Figures 3(a) and 3(b) show the time-dependent SLI for S3 microgel dispersions (0.01 g/L) after mixing with varying concentrations of lysozyme upon a stopped-flow technique at pH 8.0 and 20 °C in long and short time ranges, respectively. Without lysozyme ([lysozyme]/[MAA] = 0; reference in Fig. 3a), the dynamic trace of SLI maintained as a straight line, and no relaxation process was discernible. Once the [lysozyme]/[MAA] value was above 1/20, relaxation processes with quite large positive amplitudes could be observed, revealing an abrupt increase of SLI within the first 50 s and then reaching a plateau value gradually (Fig. 3b). This result was ascribed to the strong electrostatic attraction between S3 and lysozyme that dramatically weakened the repulsive forces between neighbouring chains and led to the collapse of microgel network. Similarly to the polyelectrolyte chains, the charge neutralization in the microgel surface occurred first, which led to the formation of a collapsed, relatively dense outer shell and an abrupt increase of SLI^[35]. Then, a slow neutralization process of the swollen inner parts was followed. Moreover, the absorption of lysozyme into S3 also increases the overall mass of microgels, having an additional contribution in SLI increase. The shrunk microgel size and enhanced scattered light intensity after lysozyme complex are well in accordance with the static data (Fig. 2c). Double-exponential function was used for fitting these kinetics curves. The corresponding results are shown in Figs. 3(c) and 3(d). The characteristic relaxation

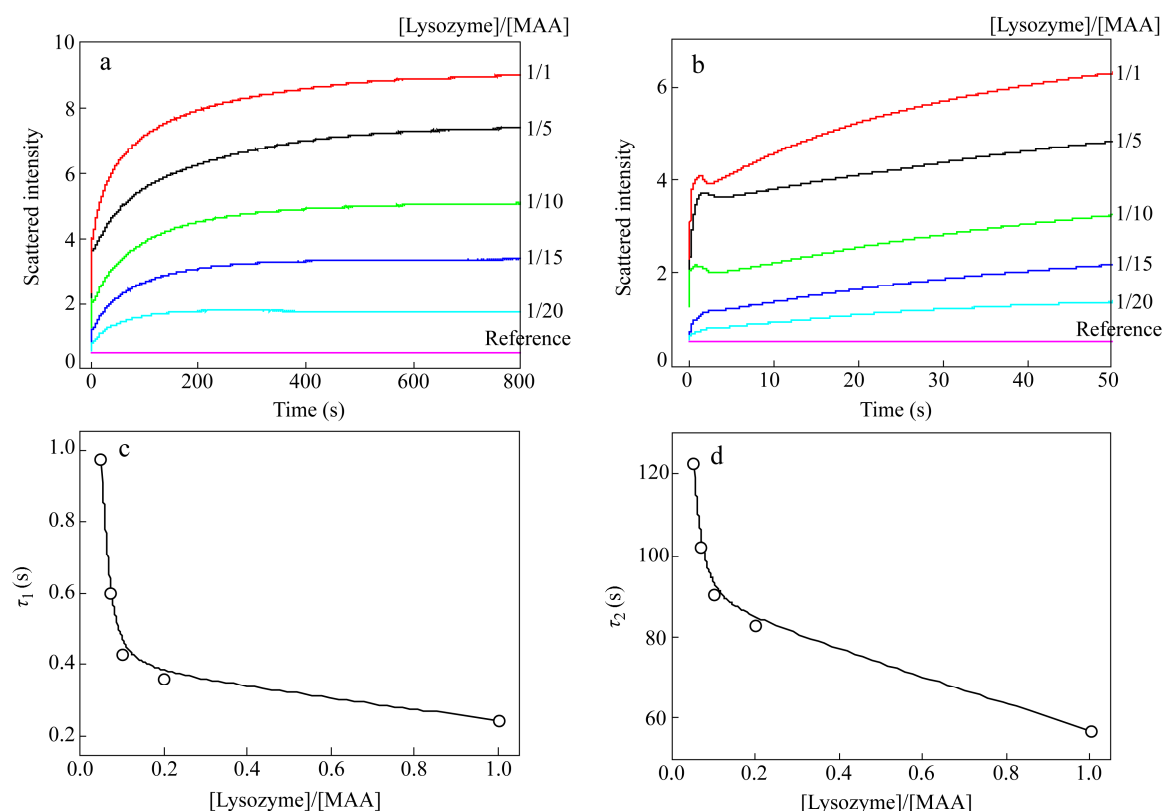


Fig. 3 Variation in scattered light intensity for (a) long time scales and (b) short time scales for the aqueous dispersion of P(NIPAM-co-MAA) microgels (0.01 g/L, pH 8.0; sample 3 in Table 1) after mixing with different concentration of lysozyme at 20 °C; The characteristic relaxation times (c) τ_1 and (d) τ_2 obtained from double exponential fitting of dynamic traces

time τ_1 decreased from 0.97 s to 0.25 s and τ_2 decreased from 122 s to 57 s when the [lysozyme]/[MAA] value increased from 1/20 to 1/1, respectively. In other words, a higher driving force (lysozyme concentration) resulted in a faster absorption. Similar observations were reported by Shibayama and Nagai^[39] that faster shrinking process was detected for macroscopic PNIPAM gels at higher temperatures. On the other hand, considering the large molecular weight of lysozyme, it would be impossible for all of them to enter into the interior of the microgel. The absorption capability as well as the residual amount of lysozyme was further measured as ζ -potential values in aqueous solutions (Fig. S4 in SI). As expected, lysozyme solution led to a strongly cationic character with a zeta potential value of ~ 22.0 mV because of the abundant charges. However, after complex with microgels, the zeta potential value of residual supernatant after centrifugation was tested to be ~ 5.5 mV; about 75% of lysozyme was adsorbed by microgels, consistent with the result in Fig. 2(a) ($\sim 73.2\%$). Different from the pH-induced microgel deswelling^[30], although no HCl was added directly, the small molecules, such as NaCl, were still generated. Nevertheless, compared to the large amount of NaCl generated by *in situ* acid (HCl)-base (NaOH) neutralization, lysozyme induced small molecules generation was slightly effected. Hence, after the initial increasing in SLI, no subsequent gradual decrease in SLI was observed over longer time scales in current situation, and the dynamic traces increased from the beginning until the final equilibrium.

To be a nanocarrier, no matter for drugs or proteins, the controlled uptake as well as release of cargos is an important factor and can further evaluate the possibility to practical applications. It is well-established that pH value has a great effect on the charge density and related adsorption extent as well as the final microgel size (Fig. 2b), and the enzymatic activity of lysozyme is not negatively influenced by pH change under the present experimental condition (Fig. S2 in SI). Releasing bound lysozyme from S3 microgels was carried on by utilizing the pH-responsive property, and the evaluation of the activity of released lysozyme was further synchronously measured in a *M. lysodeikticus* cell suspension. As expected, upon cell wall lysis^[40], the mixture will become less turbid, and result in the decrease of SLI. An assistant experiment showed that the whole time scale for cell wall lysis after simple mixing *M. lysodeikticus* (0.25 g/L) with lysozyme (0.05 g/L) upon stopped-flow was within 800 s (Fig. S5 in SI). Based on this result, the influence of pH values on the lysozyme release and subsequent phagocytosis induced SLI changes were examined by the same stopped-flow kinetic technique. Figure 4(a) shows the long time scale (1600 s; long enough for the cell wall lysis process) SLI curves for S3/lysozyme complex upon stopped-flow pH-jump from 8.0 to different final pH values (3.0–7.0) at 20 °C in the presence of 0.02 g/L *M. lysodeikticus*. Comparing the equilibrium times for the traces of pH 3.0 and 7.0, the former was much faster, as evidenced by the detectable SLI loss in the initial time scale (within 400 s). The same tendency of SLI was detected in previous studies for protein-containing micelles, indicating the loss of contrast-rich materials in micelle core^[22]. Moreover, the final SLI of the former was also lower than that of the latter at the same time range, which could be ascribed to the sufficient lysozyme release from S3 microgels and simultaneous phagocytosis between lysozyme and *M. lysodeikticus*. This result agreed well with the transmittance data (Fig. S6a in SI) and digital photographs (Fig. S6b in SI) recorded for the aqueous dispersions of S3/lysozyme/*M. lysodeikticus* mixtures at pH 8.0 and 3.0, that the protein activity completely disappeared in the weak alkali dispersion, suggesting the strong interaction between lysozyme and microgel. However, at acidic pH values, *in situ* deionization would lower the anionic charge density, hence dramatically weaken the electrostatic interaction between microgels and lysozyme, leading to the lysozyme release. The recovered lysozyme activity to *M. lysodeikticus* resulted in a much clearer dispersion due to the occurrence of cell wall lysis (Fig. S6b in SI). The corresponding characteristic relaxation times (τ) were also calculated by fitting the recorded dynamic traces (pH 3.0–5.0), as shown in Fig. 4(b). Upon increasing the pH values from 3.0 to 5.0, the obtained τ generally increased from 105 s to 435 s, indicating that the lower the pH values, the more lysozyme released and the faster phagocytosis proceeded. In control experiment, at 50 °C, the optical transmittance at a wavelength of 700 nm obtained for the aqueous dispersions of S3/lysozyme/*M. lysodeikticus* mixtures was very low no matter at pH 8.0 or 3.0 (Fig. S7 in SI), and this result demonstrated that lysozymes lost their mobility at elevated temperatures due to the microgel collapse, which tightly constrained lysozymes within their network.

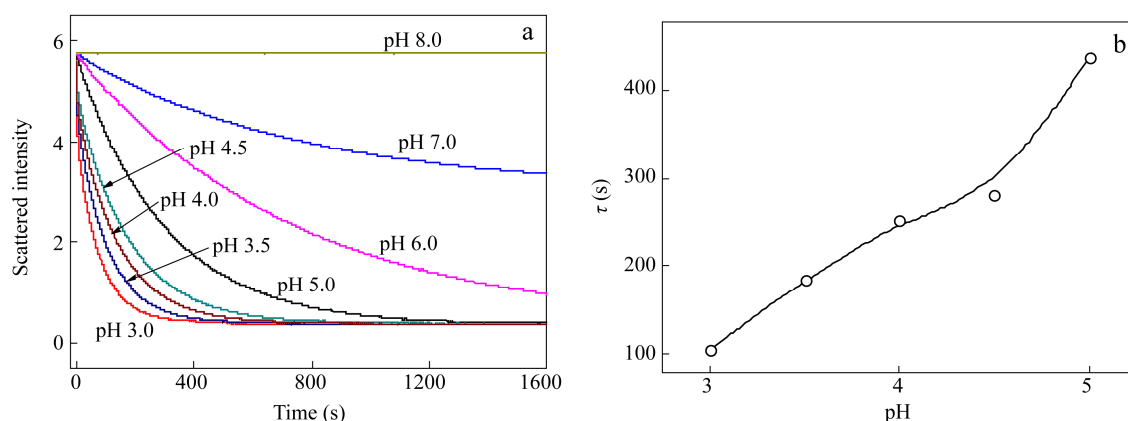


Fig. 4 (a) Time dependent scattered light intensities obtained after mixing *M. lysodeikticus* (0.02 g/L) with the aqueous dispersion of P(NIPAM-*co*-MAA)/lysozyme microgels (microgel: 0.01 g/L, lysozyme: 2.4 μ g/mL; S3 in Table 1) upon stopped-flow pH-jump from 8.0 to different final pH values at 20 $^{\circ}$ C; (b) The characteristic relaxation time, τ , obtained from single exponential fitting of dynamic traces

CONCLUSIONS

In summary, monodispersed dual-responsive P(NIPAM-*co*-MAA) microgels with different feed recipe and crosslinking density were synthesized and employed as packaging material for lysozymes. The reversible microgel-to-latex transition was actuated by pH and temperature regulation. On the basis of the present study, it could be concluded that electrostatic attraction between negatively charged microgels and positively charged lysozymes in weak alkali dispersion led to the formation of P(NIPAM-*co*-MAA)/lysozyme complex, which could be further triggered to disintegration upon acid addition, resulting in the subsequent lysozyme release. Systematic studies of the dynamic process of lysozyme adsorption into microgel interior was carried on by stopped-flow equipment and suggested a fast (τ_1) and a slow (τ_2) relaxation time, respectively. Then, the evaluation of the activity of released lysozyme was measured by a *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell suspension, confirming the physical encapsulation of protein could protect their maximum activity. This simple yet feasible design were not only for protecting and stabilizing the lysozymes, but also providing basic dynamic data, which could be used for guiding the process of controlled uptake and release as well as delivery of therapeutic proteins.

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