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Research Letter

Loading and release of a model high-molecular-weight protein from temperature-sensitive micro-structured hydrogels

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Abstract

The authors prepared a micro-structured, thermosensitive hydrogel with *N*-isopropylacrylamide microgels with a lower critical solution temperature (LCST) of 32 °C dispersed on a matrix of *N*-isopropylacrylamide-co-dimethylacrylamide with an LCST at 40 °C. Incubation of the hydrogel at 33 °C in a solution of fluorescein-albumin induced loading of the protein. The protein was not loaded at a temperature below the LCST of the microgels (4 °C), suggesting that the shrinkage of the microgels followed by the formation of micropores within the hydrogel matrix is a prerequisite for protein loading. A sustained and complete release of the loaded protein was obtained at 37 °C.

Introduction

Proteins have become important therapeutic agents for the treatment of diverse pathologies.[1] However, most therapeutic proteins lack stability because of the high risk for proteolytic and chemical degradation, as well as denaturation and aggregation. This has resulted in an increased demand for materials that are able to protect proteins from hostile environments and to control their release.[2–8] *N*-isopropylacrylamide (NIPAAm) hydrogels present a swollen-to-unswollen transition at an LCST of 32 °C.[9] NIPAAm hydrogels have been studied for the loading and release of insulin (5.8 kDa) and albumin (66.5 kDa). Such studies demonstrated the complete release of insulin; nevertheless, physical entanglement precluded the complete release of albumin from the hydrogels, either at a constant temperature or with oscillating swelling–deswelling in response to temperature cycling.[10]

We have previously reported on the properties of micro-structured NIPAAm hydrogels prepared by dispersing NIPAAm microgels (MGs, with a hydrodynamic diameter (D_h) of around 600 nm) in the pre-gelling solution of NIPAAm. The resulting materials presented a very fast swollen–unswollen transition when the temperature of the medium changed from 25 to 42 °C. We hypothesized that the fast response occurs because the MGs shrink much faster than the hydrogel matrix,[11,12] generating pores for expelling the water when the hydrogel matrix collapses, at a slower rate.[13] We were able to load cytochrome C (12 kDa) by diffusion into the swollen micro-structured hydrogel at 4 °C. At this temperature, MGs and hydrogel matrix were at their maximum swelling. This system presented some temperature control of the release; however, subsequent attempts to load green fluorescent protein (27 kDa) were unsuccessful due to size exclusion of this protein (unpublished data).

In this work, we prepared a micro-structured, thermosensitive hydrogel composed of NIPAAm MGs with an LCST of 32 °C dispersed on a matrix of NIPAAm co-polymerized with dimethylacrylamide (DMA) with an LCST tuned to 40 °C. The LCST of 40 °C was selected in a previous work for the positive control release of drugs; this is the release of cargo by squeezing the hydrogels at temperatures above the body temperature. The triggering high temperature can be caused by fever or can be induced.[14] In the current work, the LCST of 40 °C for the hydrogel matrix was selected first: to have a gap with the LCST of the MGs (32 °C), in such way that pores are formed at 33 °C, which was the temperature selected for protein loading and second: to have a swollen hydrogel matrix at 37 °C that allows protein diffusion out of the micro-structured hydrogel. The morphology and size/weight evolution with temperature of the systems produced (MGs and a micro-structured hydrogel) were characterized by

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a field emission scanning electron microscope (FESEM), dynamic light scattering (DLS), and gravimetry. The loading of fluorescein-labeled albumin was studied by confocal microscopy and UV–Vis spectroscopy, at 4 and 33 °C, respectively. The protein release was performed at 37 °C in phosphate-buffered saline (PBS, pH 7.4).

Methods

Materials and methods

NIPAAm (TCI, Tokyo, Japon 98%) was recrystallized with *n*-hexane. Ethyleneglycoldimethylacrylate (EGDMA, 98%) (Aldrich, St. Louis, MO, USA) was purified with a column packed with an inhibitor removing resin (Aldrich). *N*-methylenbisacrylamide, DMA, *N*,*N*-tetramethylethylendiamine, FITC-albumin, and ammonium persulfate were from Aldrich and used as received. Distilled water was used to prepare the solutions used.

Synthesis of micro-structured hydrogels

MGs were prepared by dispersion polymerization.[15] Briefly, NIPAAm and EGDMA (5 mol%) were dissolved in water (10 mg monomer/mL water). The mixture was heated to 70 °C and then the initiator ammonium persulfate was added to the solution (APS, 3 mol% with respect to NIPAAm). The reaction was run for 45 min. The MGs produced were dialyzed against water for 5 days and then freeze-dried. The D_h was obtained by DLS using a Zetasizer instrument (DTS1060; Malvern Instruments, Miami, FL, USA). The effect of temperature on the MGs was studied from 20 to 50 °C. Scanning transmission electron microscopic (STEM) images of the MGs were acquired with an FESEM, Model JSM-7800F Prime. To this end, a droplet of microparticulate dispersion (0.4 wt%) was spread onto the surface of a 400-mesh copper carbon grid. Two droplets of uranyl acetate (2 wt%) were added to the copper carbon grid. The samples were vacuum-oven dried at 22 °C for 24 h. The dried specimens were clamped onto a STEM specimen rod, inserted into the sample chamber, and observed at 25 kV.

The micro-structured hydrogel was prepared with NIPAAm (1 M), DMA (20 mol%), MGs (15 wt%; with respect to monomers), and as a crosslinker *N*-methylenebisacrylamide (5 wt%). Monomers and MGs were dispersed in water and stirred for 15 min and then APS (3 mol%) and *N*, *N*-tetramethylethylenediamine (3 mol%) were added. The solution was placed between two 10 × 10 cm silanized glass plates for 24 h at 4 °C. The resulting micro-structured hydrogel was cut into 1 cm diameter discs, which were washed with water for 5 days. The structure and morphology of the lyophilized samples of the hydrogel were studied by FESEM employing the instrument described above.

Loading and release of FITC-albumin

Micro-structured hydrogel discs were placed in a solution containing 30 mg/mL of FITC-albumin at either 4 or 33 °C, in PBS. After 72 h, the hydrogel discs were removed from the solution and dried on a Petri dish overnight at room temperature. The hydrogel discs were washed for 2 min with PBS to eliminate the protein precipitated on the surface of the discs and then dried again. The amount loaded into the hydrogels was calculated from the concentration in raffinate.

Confocal images of the micro-structured hydrogels were obtained with a LEICA TCS-SP8 confocal microscope, operating in the fluorescence and bright field modes (LEICA Microsystems, Heidelberg GmbH, Germany). Macroscopically different zones were recorded, preferentially at the center of the specimens, in order to depict representative images. Images in both acquisition modes were generated employing an excitation laser of a wavelength of 488 nm. The fluorescence detection window was 495–545 nm (green channel, corresponding to the emission of the FITC-labeled protein). 3D images were processed from the *z*-stack reconstruction of successive *xy* images, employing the LAS X software (LEICA).

For the release experiments, a protein-loaded micro-structured hydrogel disc was placed in 3 mL of PBS in a water bath at 37 °C, with shaking at 50 rpm. At predetermined intervals, 1 mL of samples were extracted and replaced with fresh PBS. The concentrations of the released protein were determined by UV–Vis spectroscopy at 280 nm. The experiment was performed by triplicate.

The release data were fitted to the Korsmeyer–Peppas model, up to 60% release, according to the following equation:

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

where M_t is the amount of protein released at time t; M_∞ the total protein released over a long time period; k the kinetics constant, and n the mechanism of drug release. For the case of cylindrical-shaped matrices, $0.45 \leq n$ corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-Fickian transport, $n = 0.89$ to case II (relaxational) transport, and $n > 0.89$ to super case II transport.[16]

Results

MGs were obtained with a D_h of 585 nm (Polydispersity Index = 0.136) at 25 °C. An STEM image of the MGs is shown in Fig. 1(a). Spherical structures of about 200 nm, corresponding to dry MGs, are observed. FESEM of the micro-structured hydrogel is presented in Fig. 1(b). It shows a low microporous structure.

An LCST of 32 °C was determined for the MGs, measured as the maximum change in the D_h versus temperature, shown in Fig. 2.

For the micro-structured hydrogel, an LCST of 40 °C was determined gravimetrically as the maximum change in the degree of swelling (Q = weight of wet micro-structured hydrogel/weight of dry micro-structured hydrogel) versus temperature (also shown in Fig. 2).

Loading of FITC-albumin was negligible at 4 °C, even when the micro-structured hydrogels are at maximum swelling.

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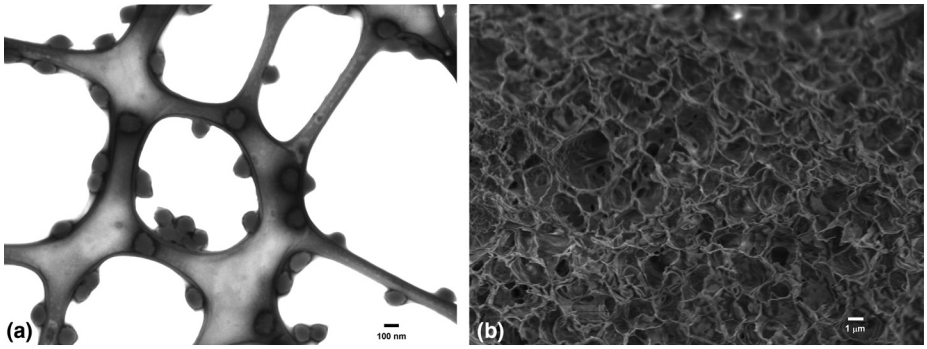


Figure 1. (a) Transmission electron microscopy of MGs. (b) FESEM of micro-structured hydrogels.

Whereas 2.4 mg/g hydrogel of FITC-albumin was loaded at 33 °C (MGs are unswollen, but hydrogel matrix is swollen). Figure 3 presents photographs and 3D reconstructions from confocal micrographs of the micro-structured hydrogels upon incubation with the protein at 4 and 33 °C. Confirming the UV–Vis results, this figure demonstrates that incubation at 33 °C favored loading of the protein, to a high extent, as evidenced by the yellow staining of the hydrogel sample resulting from its illumination with white light. The particular color observed with the naked eye stems from the light emission of the FITC tag conjugated to the protein (Fig. 3(b)). In contrast, the sample incubated at 4 °C did not present the yellow staining at all, revealing that protein loading under these conditions is rather negligible (Fig. 3(a)). A plausible explanation for the observed outcome comes from the 3D reconstruction of confocal images, which reveal that the hydrogel incubated at 33 °C presented the formation of microscopic pores along its inner structure, inside of which the FITC-labeled protein was found to be confined (emitting green fluorescence; Fig. 3(b), confocal panel). Our interpretation of the previous results takes into consideration that the 33 °C incubation temperature is just a bit higher than the LCST of the MGs (32 °C) but lower than the LCST of the hydrogel matrix (which was tuned to 40 °C); these conditions generate the microscopic pores which are presumably formed upon shrinking of the MGs incorporated at certain locations of the hydrogel matrix, leaving behind empty spaces (this is in good agreement with the size evolution of the MGs determined by the DLS experiments, see Fig. 2). In contrast, the sample incubated at 4 °C did not show any visible appearance of micropores nor the green fluorescence expected from protein loading (Fig. 3(a), confocal panel). Furthermore, this finding is line with the notion that the amount of protein loaded at this temperature is negligible.

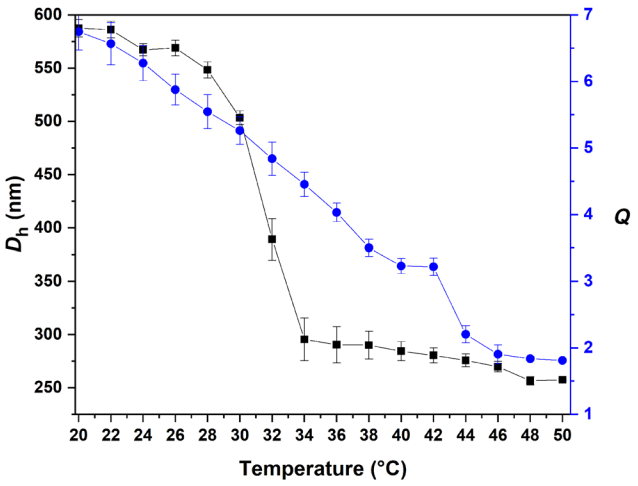


Figure 2. Left: D_h versus temperature for MGs; right: Q versus temperature for the micro-structured hydrogel.

The release kinetics at 37 °C is shown in Fig. 4. A sustained release is observed, with the complete release of FITC-albumin in about 5 h. The release mechanism parameter, n, from Eq. (1) was 0.88 ($R^2 = 0.98$) corresponding to an anomalous (non-Fickian) mechanism, a combination of swelling of the material and diffusion of the protein.

As mentioned previously, conventional NIPAAm hydrogels have the disadvantage that protein is not completely retrieved due to strong interactions between protein and polymer.[10] The results in this work indicate that no entanglement of the loaded protein occurs within the micro-structured hydrogel, probably due to the diffusional pathway provided by shrinking of the MGs at the loading temperature.

Temperature-responsive systems that gel at body temperature have been explored for in situ gelation. The gels formed once injected in the body are useful to control the release of proteins and other macromolecules.[17–19] For example, copolymers of NIPAAm and poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) undergo reverse thermogelation at body temperature and remain in a flowable state at room temperature. These materials have been tested for the release of proteins.[3] However, these systems have limitations, such as relatively low mechanical strength and poor physical stability, which cause premature protein release. The stability of temperature responsive systems may be improved by adding additional covalent crosslinks after

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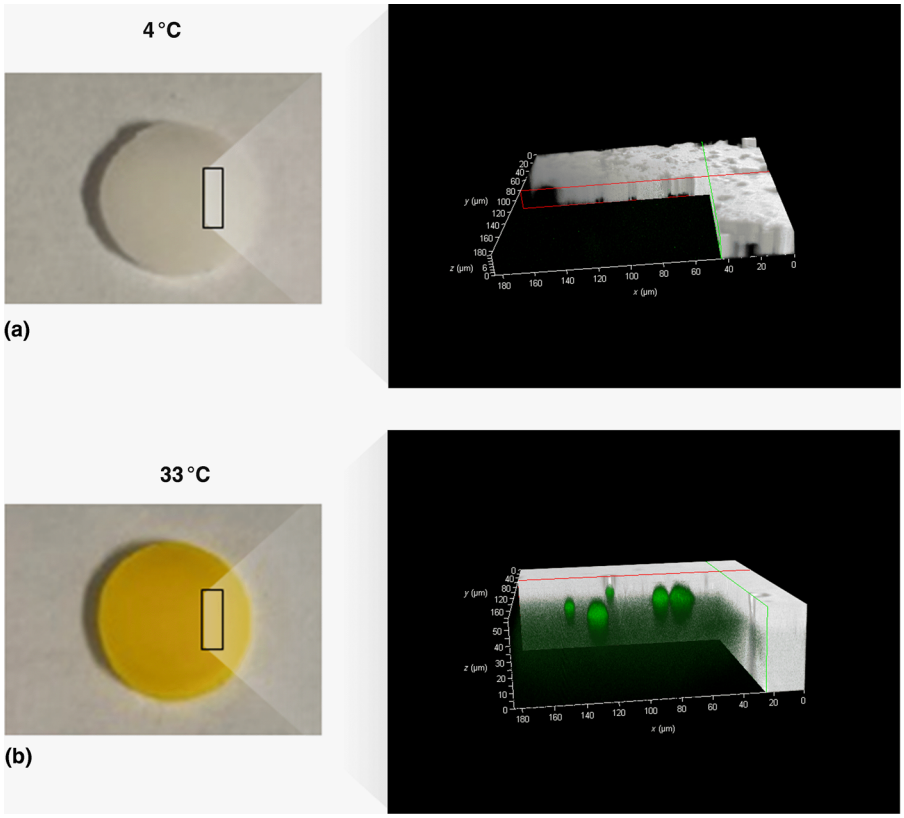


Figure 3. Photographs and the corresponding confocal micrographs of micro-structured hydrogels upon incubation with FITC-albumin at (a) 4 °C and (b) 33 °C.

initial gelation.[7,20] Examples of such methods are photopolymerization or the Michael addition reaction. These strategies requires the copolymerization of NIPAAm with materials that undergo chemical modifications to include reactive moieties for in situ crosslinking.[21]

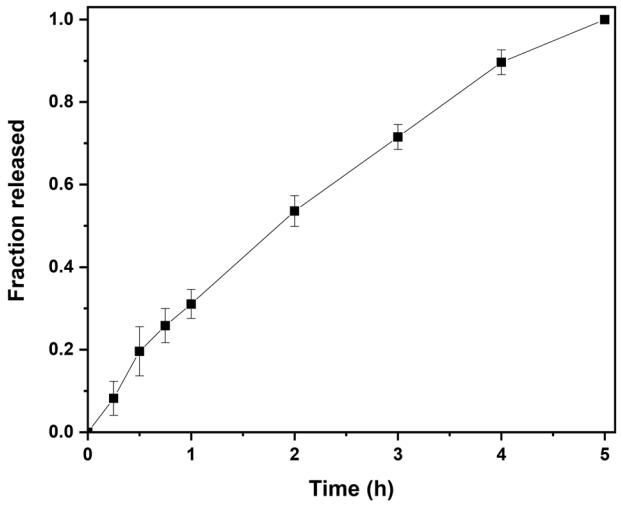


Figure 4. Release of FTIC-albumin at 37 °C, in PBS (pH 7.4), 50 rpm.

In our case, the materials that are produced through facile and scalable reactions, mostly in water, are environmentally friendly. Besides, MGs act as crosslinking points giving good mechanical properties to micro-structured hydrogels.

Conclusion

We present here a simple method for loading high-molecular-weight proteins. The system consists of micro-structured NIPAAm hydrogels where the continuous phase (hydrogel matrix) has a tuned LCST at 40 °C which is above the LCST of the incorporated MGs (32 °C). In this way, at 33 °C, micro-pores are formed due to the reduction in the diameter of the MGs, allowing the diffusion of the high-molecular-weight model protein into the micro-structured hydrogel. A complete release of the protein is achieved in 5 h at 37 °C (pH 7.4), indicating that the protein does not suffer entanglement in the micro-structured hydrogel.

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