Conditional Ultrasound Sensitivity of Poly[(*N*-isopropylacrylamide)-*co*-(vinyl imidazole)] Microgels for Controlled Lipase Release

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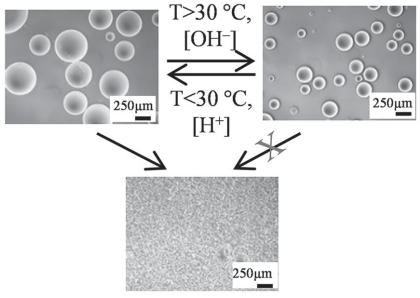
Triggering the release of cargo from a polymer network by ultrasonication as an external, non-invasive stimulus can be an interesting concept for on-demand release. Here, it is shown that, in pH- and thermosensitive microgels, the ultrasound sensitivity of the polymer network depends on the external conditions. Crosslinked poly[(*N*-isopropylacrylamide)-*co*-(vinyl imidazole)] microgels showed a volume phase transition temperature (VPTT) of 25–50 °C, which increases with decreasing pH. Above the VPTT the polymer chains are collapsed, while below VPTT they are extended. Only in the case of maximum observed swelling, where the polymer chains are expanded, the microgels are mechanically fragmented through ultrasonication. In contrast, when the polymer chains are partially collapsed it is not possible to manipulate the microgels by ultrasound. Additionally, the ultrasound-induced on-demand release of wheat germ lipase from the microgels could be demonstrated successfully. The principle of conditional ultrasound sensitivity is likely to be general and can be used for selection of matrix–cargo combinations.



1. Introduction

Ultrasound (US) has been explored as an external stimulus for the release of a cargo since it is non-invasive and can penetrate deep into the body.[1,2] Micellar structures,[3] core-shell particles,[4] and microbubbles were investigated as US sensitive carriers.[5] In the case of triggering release from polymer networks using US, the cleavage of covalent bonds of the carrier might be necessary.[6] From a mechanistic point of view, US-induced pressure variations in a solvent lead to gas bubble formation. Their growing and collapse transitions cause turbulent fluid movements, which generate solvodynamic shear.[7] Polymer segments of a polymer network in the vicinity of the shearing forces move faster than the ones located further away, resulting in tension along the polymer backbone, molecular stretching, and finally bond scission.[8] It could be shown that linear and stretched polymer chains degrade faster upon US irradiation compared to coiled ones.[9] Therefore, we hypothesized that polymer networks with environmentally dependent swelling can be used to realize a conditional US sensitivity, which only is present when the matrix displays maximum swelling (Figure 1a). Potentially, poly(*N*-isopropyl acrylamide) (PNIPAM)-based networks show such a desired

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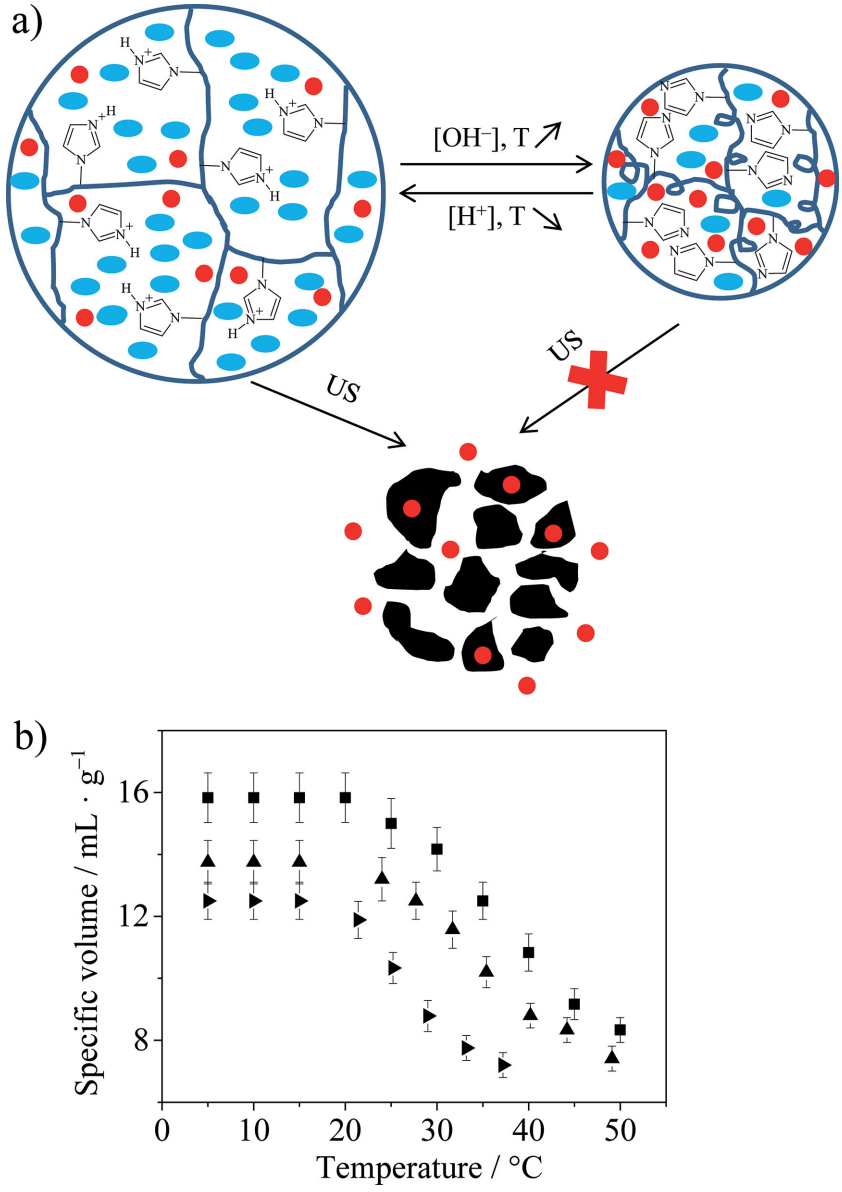

Figure 1. a) Concept scheme (●-enzyme, ●-fragmented MGs, ●-water molecules,); b) Volume-temperature dependence of MGs at different pH (pH 4.7 (■); pH 7.4 (▲); pH 8.2 (▶)).

behavior, as they exhibit a volume phase transition temperature (VPTT) due to hydrophobic collapse as hydrogen bonds are destabilized at increased temperatures.[10] In order to tune the VPTT, PNIPAM can be copolymerized with monomers carrying acidic or basic moieties.[11] If the comonomer has an acidic functional group, a pH increase shifts the VPTT to higher temperatures,[12] while a basic comonomer leads to increased VPTT with decreasing pH.[13] In both cases, the charged repeating units increase the hydrophilicity of the matrix. In such systems, swelling can be adjusted by the combination of two stimuli: temperature and pH.

For the US dependent release of bioactive compounds, their diffusion rate in the matrix has to be drastically increased. Ultrasonication of a polymer network swollen in aqueous medium will certainly cause a temperature increase, which, in general, can enhance mobility of molecules loaded in the network and consequently their diffusion rate. However, for polymers displaying a VPTT this is not the case since the temperature increase will cause collapse of the network counterbalancing the increase of diffusion rate caused by the temperature elevation. In contrast, the low diffusion rates of large molecules, such as proteins, embedded in a polymer matrix with a VPTT could be increased by fragmentation of the covalent polymer network. For a model bioactive compound to be incorporated into the matrix, a class of proteins should be chosen that is tolerant to organic solvents required for the preparation of the microgels (MGs), such as lipases. Additionally, electrostatic interactions between the polymer matrix and a cargo may have a significant influence on the cargo's embedding into the matrix, its release, and on the stimuli-sensitivity of the network.[14,15]

In the following the preparation and characterization of MGs based on crosslinked poly[(*N*-isopropylacrylamide)-*co*-(vinyl imidazole)] (P(NIPAM-*co*-VIm)) is described. VIm was employed as a comonomer in order to introduce pH sensitivity to the polymer network. The influence of temperature and pH on swelling of the MGs in water and consequently their US sensitivity were investigated. Additionally, it was explored whether such systems can be used for controlled release of lipases and how the electrostatic interactions between enzyme and matrix influence this process. For this purpose, two lipases with different isoelectric points (IEP) were investigated: *rhizopus oryzae* lipase (ROL) with an IEP of 7.6[16] and wheat germ lipase (WGL) with an IEP of 5.4.[17]

2. Experimental Section

2.1. Materials

All listed chemicals were purchased from Sigma Aldrich (Seelze, Germany) if not otherwise specified. *N*-isopropylacrylamide (NIPAM, ≥99%) was recrystallized two times from *n*-hexane. Vinylimidazole (VIm, ≥99%) was redistilled under high vacuum

at 72 °C. *N,N'*-methylene-bis-acrylamide (BIS, 99%), *N,N,N',N'*-tetramethylethylenediamine (TEMED, ≥99.5%), ammonium peroxodisulfate (APS, 98%), sorbitan monooleate 80 (SPAN 80), polysorbate 80 (Tween 80), lipase from *Rhizopus oryzae* (ROL), lipase from wheat germ (WGL), *n*-heptane (≥99%), and acetone (Th. Geyer, Renningen, Germany) were used without further purification. A micro bicinchoninic acid (BCA) assay (Thermo Scientific, Dreieich, Germany) was used to quantify lipase release. Deionized water was obtained from an Ultra Clear UV/UF TM system (Evoqua, Barsbuettel, Germany).

2.2. Synthesis

The synthesis of the crosslinked P(NIPAM-*co*-VIm) unloaded MGs, in brief, was performed by emulsifying an 7.5 mL aqueous solution (1.35 g NIPAM, 0.125 g VIm, 0.025 g BIS, and 0.27 g APS) and a 124 mL *n*-heptane solution (1.25 g SPAN 80 and 0.25 g Tween 80) through stirring at 600 rpm in an ice bath. 88 mg TEMED in 1 mL *n*-heptane was added, the reaction was run for 4 h, and the formed MGs were extracted with acetone.[18] To prepare MGs loaded with a lipase, a similar procedure was used. 0.225 g of ROL or 0.150 g of WGL was added to the system by dissolution in water, together with NIPAM, VIm, BIS, and APS. In the purification step, following washing with acetone, these samples were treated with additional 50 mL of deionized water. Further details, as well as methods of characterization, can be found in the Supporting Information.

2.3. Ultrasonication

The ultrasonication was performed using a Sonopuls HD 2070 (Bandelin, Berlin, Germany) with a microtip MS 73, at a nominal power of 70 W and a frequency of 20 kHz. The US experiments were performed by immersing the microtip 2 cm into polypropylene centrifuge tubes containing 50 mL of dispersion at three different starting temperatures of 10, 20, and 37 °C.

3. Results and Discussion

3.1. Microgel Synthesis and Characterization

Crosslinked MGs were synthesized via a redox initiated reverse phase emulsion polymerization of NIPAM (89.0 mol%), VIm (9.8 mol%) and BIS as a crosslinker (1.2 mol%). The reaction was performed at room temperature to avoid deactivation of the thermosensitive lipase. After extracting the particles with acetone, a gel content of 95 wt% was determined. The extract was dried and the remains analyzed by ^{1}H-NMR spectroscopy, which showed the presence of surfactants, but no residual monomers (Supporting Figure S1). Thus the MGs composition was judged to correspond to the monomer feed ratio.

Unloaded MGs had an average diameter of 120 ± 20 µm in the dry state. Their diameter in the swollen state depended on the pH and the temperature. At room temperature and acidic pH, the average diameter of MGs was larger (280 ± 40 µm at pH 4.7) compared to the diameter at basic pH (160 ± 25 µm at pH 7.4 and 140 ± 20 µm at pH 8.2). This can be rationalized by increasing the protonation of VIm moieties with decreasing pH, which led to higher water uptake.[19] In contrast, PNIPAM MGs had an average diameter of 110 ± 30 µm at all pH values.

The MGs containing NIPAM undergo volume phase transition with increasing temperature, because of the changes of the hydrogen bond pattern and finally hydrophobic collapse of polymer chains.[20] The influence of both pH and temperature on the volume is depicted in Figure 1b (and Supporting Figure S2). VPTT of the MGs shifted to higher values with decreasing pH. The highest swelling of MGs was observed at a pH of 4.7. At all other conditions, the volume phase transition of the polymer had already started and MGs were partially collapsed.

3.2. US Sensitivity of MGs

The US sensitivity of MGs was examined at three different initial temperatures: 10, 20 and 37 °C. The ultrasonication was carried out for 20 min and during the treatment, the temperature increased by 9, 8 or 6 °C, respectively, even though the samples were externally temperature-controlled during the experiments. Only MGs swollen at pH 4.7 showed US sensitivity at initial temperatures of 10 °C or 20 °C (Figure 2). At all other conditions, MGs fragmentation was not observed (Supporting Figure S3). It can be concluded that only MGs with maximum observed swelling were sensitive to ultrasonication.

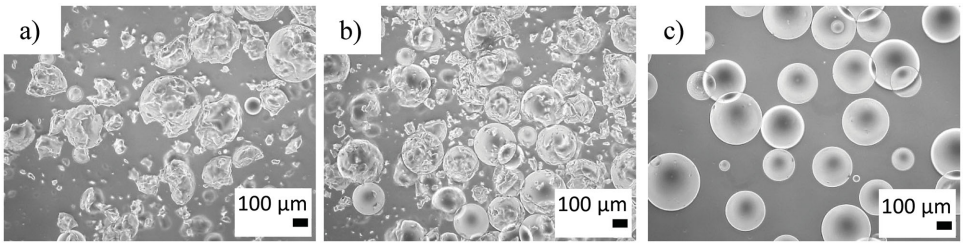

Figure 2. Phase contrast microscope images of US treated MGs for 20 min at pH 4.7 and different initial temperatures: a) 10 °C; b) 20 °C; c) 37 °C.

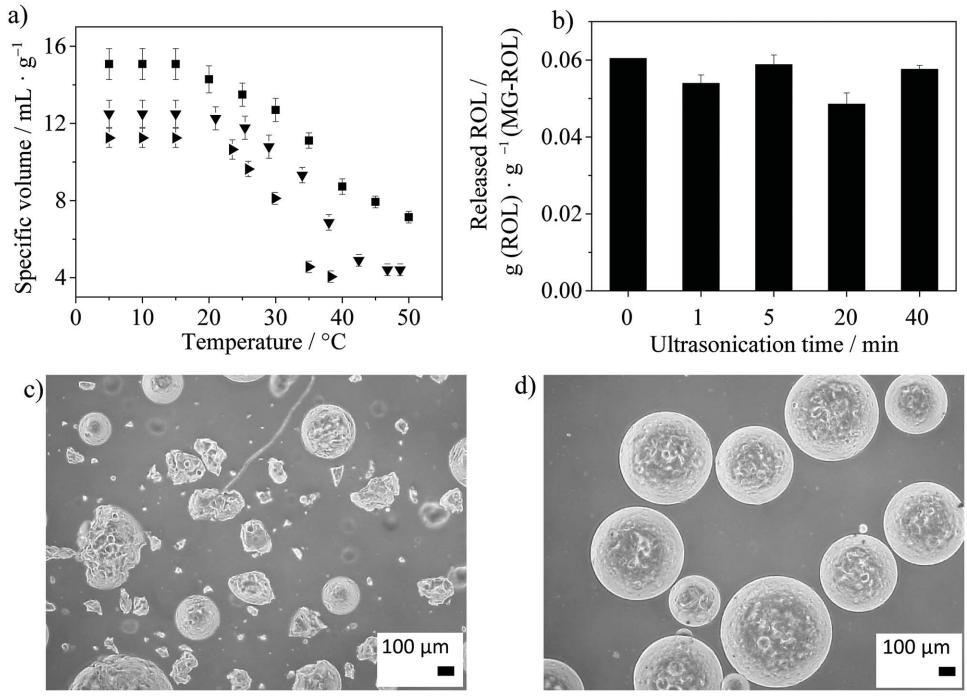

Figure 3. a) Volume-temperature dependence for MG-ROL (at pH 4.7 (■); at pH 7.4 (▲); at pH 8.2 (▶)); b) ROL released from MGs (determined with BCA assay) as a function of US time (US treatment at initial temperature of 20 °C and pH 4.7). Phase contrast microscope images of MG-ROL treated with US for 20 min at pH 4.7 and starting temperature of: c) 20 °C; d) 37 °C.

Additionally, at pH 4.7 and a starting temperature of 20 °C, the degree of fragmentation of MGs was enhanced with increasing ultrasonication time. After 1 min of ultrasonication almost no fragmentation occurred, while after 5 min some debris of fragmented MGs could be observed. When US was carried out for 20 min, most of MGs were fragmented, but intact MGs could also be noticed. After 40 min treatment, no intact MGs were observed in the dispersion (Supporting Figure S4).

3.3. Loaded MGs and Controlled Lipase Release

The presence of a lipase during the synthesis influenced the size of MGs, which may be explained by the protein's influence on the emulsification process, as proteins also may act as surfactants. MGs containing ROL (MG-ROL) and the MGs containing WGL (MG-WGL) had average diameter in the dry state of 160 ± 30 µm and 50 ± 10 µm, respectively (Supporting Table S1).

MG-ROL showed similar volume response to temperature and pH as the unloaded MGs (Figure 3a), and accordingly US sensitivity was also similar. At pH 4.7 and temperatures up to 25 °C the material was most swollen and ultrasonication at 10 °C or 20 °C fragmented MG-ROL into smaller pieces, whereas at 37 °C no fragmentation of MGs was observed (Figure 3c and 3d). Even though the extent of fragmentation of MGs can be tuned by varying the US time, an influence on the ROL release was not found. The amount of released lipase from particles treated with US at 20 °C was the same regardless of US time (1, 5, 20, 40 min). Moreover, the same amount of the lipase was released even without ultrasonication (Figure 3b). This may be related to unfavorable interactions between the matrix and the protein at pH 4.7, which under these conditions are likely both protonated and therefore positively charged. Lipase activity was not changed upon US exposure (Supporting Figure S5).

When lipase release from MG-WGL was tested at pH 6.2, no release of WGL was observed. At this pH value the polymer was anticipated to be positively charged, while the enzyme should be negatively charged (because the pH was higher than the IEP = 5.4 of WGL). This could result in pronounced attractive forces between matrix and cargo hindering lipase release even upon US irradiation. In contrast, at pH 4.7 WGL was not released from the MGs without US treatment, but upon US release was successful (Figure 4). Furthermore, with increasing ultrasonication time, enhanced enzyme release was observed. An increase of the ultrasonication time from 5 to 20 min raised the amount of released lipase by a factor of 4. US treatment longer than 20 min did not cause a substantial increase of released WGL, because the majority of particles were already fragmented during the first 20 min, which already had led to a complete release of the entrapped lipase. Interestingly, the loading capacity of the MGs for WGL was higher (10 wt%) than for ROL

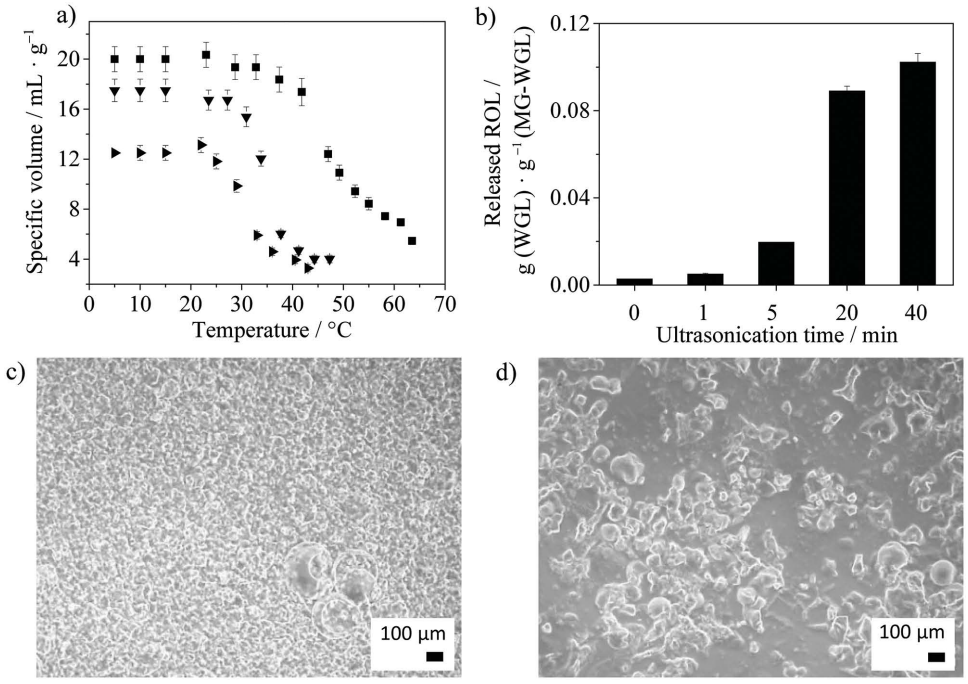

Figure 4. a) Volume-temperature dependence for MG-WGL (at pH 4.7 (■); at pH 7.4 (▲); at pH 8.2 (▶)); b) WGL released from MG-WGL (determined with BCA assay) as a function of US time (US treatment at initial temperature of 20 °C and pH 4.7). The maximum released amount was set to 100%, which corresponds to 10 wt% loading of the dried particles; Phase contrast microscope images of MG-WGL treated with US for 20 min at pH 4.7 and starting temperature of: c) 20 °C; d) 37 °C.

(6 wt%), which likewise can be rationalized by the fact that at the pH conditions used for encapsulation, the ROL and the polymer matrix are positively charged and slightly repulse each other, while WGL is negatively charged so that electrostatic attraction to the matrix can be anticipated.

4. Conclusion

The effective fragmentation of microgels based on crosslinked P(NIPAM-*co*-VIm) by US requires a high degree of swelling. By employing a pH and temperature sensitive matrix, US sensitivity at acidic pH and temperatures up to 25 °C is observed. Under these conditions, the degree of MG breakage could be well controlled by the time of US application. No intact MGs were found after 40 min of US treatment. Accordingly, a conditional US sensitivity was observed. It was found that a controlled release of lipase could be achieved when the lipase isoelectric point is close to the pH value, at which MGs were fragmented, criteria, which are fulfilled by WGL. The MG fragmentation as well as the release of lipase can be directly initiated and regulated upon demand by US. The described conditional ultrasound sensitivity of microgels as a general principle is anticipated to be transferable to other copolymer/protein systems, which gives the opportunity to choose matrix and cargo accordingly for the externally controlled release upon demand.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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