Live Monitoring of Cargo Release From Peptide-Based Hybrid Nanocapsules Induced by Enzyme Cleavage

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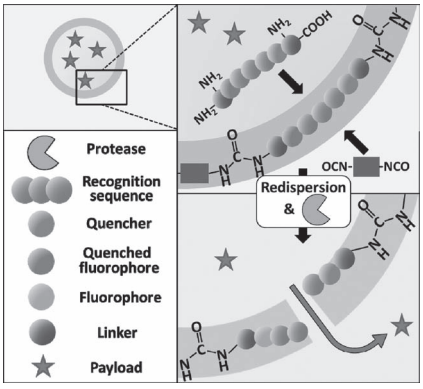
The miniemulsion process is used as a new route for the preparation of enzyme-responsive nanocapsules with payload-release properties. Peptide-based hybrid nanocapsules are prepared via interfacial polyaddition containing a water-soluble dye that is efficiently encapsulated inside. The influence of the synthetic parameters as the functionality of the peptide and the nature of the dispersed phase on the structure of the nanocapsules were investigated. After redispersion in water, the enzymatic cleavage of the peptide sequence and the release of the fluorescent dye are both monitored in real time. This is evidenced because of the quenching FRET system framing the recognition site in the peptide sequence, and the fluorescence recovery of the self-quenched encapsulated dye respectively.



1. Introduction

Encapsulation of bioactive molecules in nanoscaled objects is highly interesting for the delivery of hydrophilic or hydrophobic compounds in living systems.[1] Here, a very desirable property is the possibility to trigger the release of payload via external stimuli. Light-, pH-, or temperature-responsive nanostructures or hydrogels have been presented in the literature.[2–6] Being a response to a local, environmental parameter, the interest for enzymatically triggered delivery has risen because the pioneering work of Duncan, Kopecek, and co-authors,[7–10] who prepared a water-soluble *N*-(2-hydroxypropyl)methacrylamide copolymer with doxorubicin attached to the polymer backbone via a biodegradable peptidyl linker. Later, it was shown that the enzymatic cleavage also proceeds in heterophase with water-insoluble polymers.[11] Suzuki et al.[12] prepared a hydrogel with pending antibiotics attached via peptide linkers where the drug was released only in case of infection. More recently, several types of nanostructured materials have been used to prepare enzyme-triggered release systems. Inorganic and organic materials have been already employed whereby See et al.[13] showed the recovery of fluorescence after the release of a dye connected to, and quenched by gold nanoparticles via a peptide sequence. Mesoporous silica, with pores covered by a grafted peptide, released

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their payload after cleavage of the surrounding peptide layer.[14–16] Concerning organic materials, peptide–polymer conjugates[17–20] are promising candidates. A protein was encapsulated by adsorption and polymerization of monomers around it, including peptide cross-linkers. The protein recovered its activity after enzymatic cleavage of the cross-linking peptide.[21] Maier et al.[22] prepared hydrophobic peptide–polymer nanoparticles, where the peptide recognition sequence was selectively cleaved by a protease. The cleavage was detected by fluorescence recovery of a Förster resonance energy transfer (FRET) pair introduced around (or framing) the recognition sequence. Unstable liposomes, stabilized by a cross-linked polymer shell containing a peptide sequence, were cleaved, and released their content when treated with proteases.[23] Recently, the release of a hydrophobic dye was achieved by oxidation of polysulfide nanoparticles with oxidoreductase enzymes in hydrophilic polysulfones and polysulfoxydes.[24]

The miniemulsion process is a very powerful technique to prepare a wide range of (hybrid) nanostructures[25] and to efficiently encapsulate hydrophobic or hydrophilic, organic or inorganic materials.[26,27] In particular, the inverse miniemulsion was used to synthesize polyurethane and polyurea nanocapsules by interfacial polyaddition.[28] Later, encapsulation of water-soluble dyes[29,30] and pH-, temperature-, and light-triggered release from those nanoparticles was reported.[31] In this work, based on interfacial polyaddition, we present a new approach for the preparation of nanocapsules with specific enzymatically triggered release properties. A peptide, bearing amino functionalities through lysine residues and its N-terminus, was used as the amino component for the polyaddition at the interface of the miniemulsion droplets with diisocyanate. The resulting polyurea thus created the polypeptide-based hybrid nanocapsules. After redispersion of the capsules in water, the peptide sequences were successfully cleaved in heterophase by trypsin. Moreover, a water-soluble dye was efficiently encapsulated in a concentration where self-quenching occurs, and was released during enzymatic degradation. The cleavage of the peptide sequence was monitored in real time by a FRET system while the liberation of encapsulated dye (sulforhodamine 101) was simultaneously observed by fluorescence recovery upon release to aqueous environment. The influence of peptide functionality and amount of cross-linker (toluene diisocyanate) equivalent on the size of the polymer and encapsulation efficiency were studied.

2. Experimental Section

2.1. Peptide Synthesis

(L)-peptides were prepared via microwave-assisted solid-phase peptide synthesis with an automated synthesizer (Liberty, CEM) and characterized with ^{1}H NMR (nuclear magnetic resonance), MALDI-TOF (matrix assisted laser desorption ionisation-time of flight) mass spectrometry, and HPLC (high performance liquid chromatography). Peptides were designed in such a manner that they contained an amino acid recognition sequence, glycine-phenylalanine-phenylalanine (GFF), surrounded by a quenching FRET system attached to the ε-amino groups of Fmoc-lysine: 7-methoxycoumarin-4-yl-acetic acid (Mca) as the fluorophore and 2,4-dinitrophenyl (Dnp) as a suitable quencher.[32] A variable number (2 and 6) of lysine residues was introduced at the extremities, resulting in 3 and 7 amine groups per peptide, respectively. The chemical structures of these two peptides are presented in Figure 1a and b. The peptides are named [Mca/3NH_2] and [Mca/7NH_2].

2.2. Capsules Preparation

The nanocapsules were prepared as follows: the peptide (50 mg) and, in case of dye encapsulation, sulforhodamine 101 (SR101, 5 mg), were dissolved in 0.75 g *N*,*N*-dimethyl formamide (DMF) containing 5 g L^{-1} lithium bromide as osmotic pressure agent. Then, the continuous phase, consisting of a 0.5 wt% block copolymer surfactant poly(butylene-*co*-ethylene)-*block*-poly(ethylene oxide)

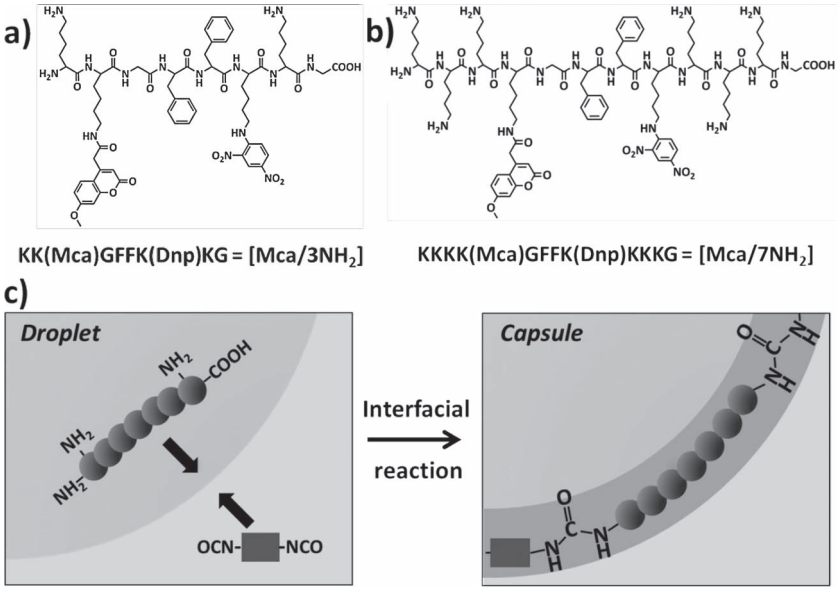

Figure 1. (a) Structure of peptide [Mca/3NH_2]; (b) structure of peptide [Mca/7NH_2]; (c) schematic representation of the interfacial polyaddition in miniemulsion (the spheres represent the amino acids of the peptide while the rectangle represents the TDI molecule).

(P(B/E-*b*-EO)) solution in 6.75 g cyclohexane, was added. After intense stirring and ultrasonication (3 min total, applied in pulses of 10 s with 10 s pause, 70% intensity) on a Branson 450D sonifier (1/4" diameter), a freshly prepared toluene diisocyanate (TDI, amount according to the number of amino functions) in cyclohexane (0.5 g) solution was added drop wise. The mixture was stirred for 15 h. Then, the miniemulsion was redispersed in twice its weight of aqueous 0.3 wt% sodium dodecyl sulfate solution until complete cyclohexane evaporation at 40 °C, followed by mild ultrasonication (3 min total, applied in pulses of 10 s with 10 s pause, 30% intensity). Finally, the sample was dialyzed against deionized water (dialysis tube, cut-off 14 kDa) during at least 1 week to remove surfactants, non-encapsulated dye, and possible unreacted peptide. Photographs of the final dispersions are shown in Supporting Information, Figure S1.

Scanning electron micrographs (SEM) were taken on a Gemini 1530 (Carl Zeiss AG) with an In-Lens detector and acceleration voltages between 0.2–0.3 kV. Samples were prepared by drop-casting 5 μL of diluted dispersions in cyclohexane on a silicon wafer. Transmission electron micrographs (TEM) were obtained on a FEI Tecnai F20 microscope under an acceleration voltage of 200 kV. The samples were prepared with 5 μL of diluted dispersions placed on a 300 mesh carbon-coated copper grid. The particle size was determined by photon cross correlation spectroscopy (PCCS) using a Nanophox device (Sympatec GmbH). Measurements were performed with 100 μL of dispersion diluted in 2.5 mL cyclohexane or water, with the detector set to 90° from the incident beam. The size given is the average of the mean values obtained from three measurements.

2.3. Enzymatic Cleavage/Degradation

The cleavage experiments were carried out by diluting the dispersion five times in water and adding trypsin to a final concentration of 2.5 g L^{-1}. In each case, a control experiment was prepared from the same sample under the same conditions but without the addition of enzyme. Fluorescence intensities (Mca, λ_{em} = 405 nm, λ_{exc} = 340 nm, and SR101, λ_{em} = 605 nm, λ_{exc} = 580 nm) were monitored using a Tecan M1000 microplate reader at 37 °C. Measurements were conducted every minute and the plate was briefly shaken (5 s) before each recording. Each data point corresponds to the average value of eight replicates.

3. Results and Discussion

3.1. Peptides

The peptide sequences were prepared in such a way that they contained several features: the recognition sequence (GFF) for the peptidase is surrounded by a FRET system consisting of a fluorophore (Mca) and a suitable quencher (Dnp) (Figure 1a and b). FRET systems are of common use for live monitoring of enzymatic cleavage in solution.[32,33] Concerning an intact peptide, the fluorescence is quenched by energy transfer from Mca to Dnp. Once the recognition sequence has been cleaved, the distance between the fluorophore and its quencher is too large for the quenching to happen efficiently and fluorescence recovery from the dye is observed. At both ends of the peptide, lysine amino acids were added to introduce various amounts of amino groups (3 and 7) in the peptide and to allow the interfacial addition reaction with TDI in miniemulsion. A higher number of NH_2-groups is expected to lead to a denser polymeric network, with higher encapsulation efficiency and less leakage.

3.2. Degradation of the Peptide

In order to confirm that the FRET pair and the lysine amino acids did not interfere with protease action, the peptide sequence [Mca/7NH_2] was subjected to trypsin solution (Figure S2, Supporting Information). When trypsin was added, a strong increase of fluorescence was observed within only a few minutes. This confirmed that the recognition sequence could be successfully cleaved by trypsin and the Mca/Dnp pair is an efficient internally quenched fluorescent system to monitor the degradation in real time.

3.3. Structures of the Capsules

The miniemulsion technique is based on stabilized nanodroplets, which act as independent nanoreactors because of the presence of an osmotic agent that prevents Ostwald ripening.[34–36] The polyaddition reaction between the peptide dissolved in the dispersed phase and the TDI introduced via the continuous phase occurs at the interface (Figure 1c). In an ideal case, the generated polymer is insoluble in both phases: phase separation takes place and liquid core capsules are obtained. An example of this is shown in Figure 2a and b, using a DMF/water mixture (50/50 w/w) as dispersed phase. When pure DMF is used as dispersed phase, the polymer remains soluble in the droplets and particle-like structures are obtained (Figure 2c and d).

3.4. Unloaded Nanocapsules

Peptide-based nanocapsules were initially prepared without encapsulated dye. After the polyaddition in cyclohexane, a fraction of each sample was freeze-dried and analyzed by size-exclusion chromatography (SEC) in DMF with UV detection at the wavelength of 325 nm (absorption maximum of the peptide) to allow specific detection of the products. In Figure 3a, the chromatograms of both peptides and their respective polymers obtained after the interfacial polyaddition are shown. For each polymer, one can observe a clear shift toward a lower elution volume (i.e., higher molecular weight) compared with the corresponding peptide. This evidences that the interfacial polyaddition has proceeded successfully. Surprisingly,

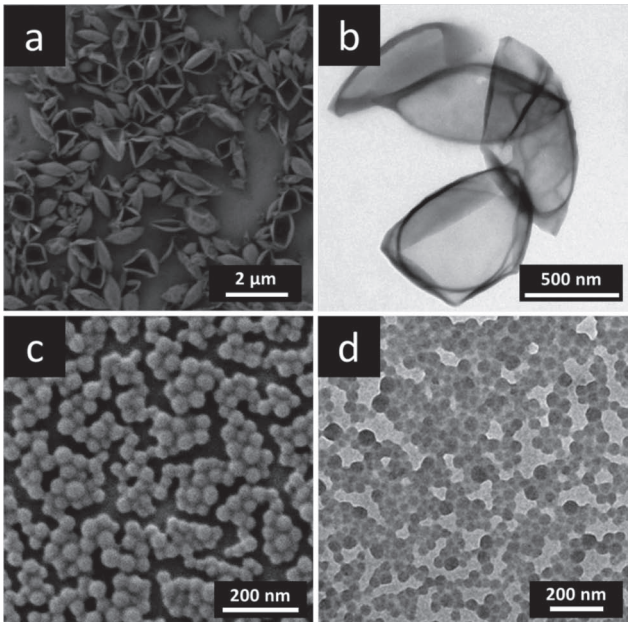

Figure 2. Scanning electron micrographs (SEM) and transmission electron micrographs (TEM) of capsules prepared from [Mca/7NH_2] with 2 equivalent TDI molecules compared to the number of amino groups on the peptide: (a) and (b) SEM and corresponding TEM of the sample with 50% w/w DMF/H_2O as dispersed phase; (c) and (d) SEM and corresponding TEM with DMF as dispersed phase.

the elution volume is not lower for the polymer generated from peptide [Mca/7NH_2] than from [Mca/3NH_2]. This suggests that increasing the functionality of the peptide does not significantly increase the size of the peptide-based polyurea. However, after interfacial polyaddition of peptide [Mca/7NH_2], the chromatogram shows a monomodal distribution, whereas the polymer made from peptide [Mca/3NH_2] has a bimodal distribution with a shoulder corresponding to unreacted peptide. Therefore, the increase of peptide functionality has the advantage of almost quantitative inclusion of the peptide into the polymer, resulting in less free, unreacted peptide.

The size of the capsules was measured with PCCS (Table 1). Taking the example of the capsules prepared from peptide [Mca/7NH_2] with 2 equivalents of TDI, a narrow monomodal distribution centered on 155 nm was observed. After redispersion in water and subsequent dialysis, a particle size of 294 nm was measured. Ultrasonication at 30% intensity allows breaking the aggregates without damaging the capsules. The addition of trypsin led to enzymatic cleavage as detected by a strong fluorescence increase (factor 45) at the wavelength of the Mca group within a few hours, whereas the fluorescence signal remains constant without the addition of enzyme (Figure 3b). Moreover, the fluorescence still increases after 12 h, whereas the equilibrium state was reached within a few minutes when cleaving the peptide in solution. As the time of this fluorescence increase is significantly longer than for the free peptide, it is concluded that peptide sequences incorporated in the hybrid nanoparticles were cleaved during the experiment.

3.5. Encapsulation and Release of Dye

Successful degradation of the model capsules containing the FRET pair has shown that the sequences can be cleaved as heterophase substrates and are thus suitable for dye release experiments. A series of samples with encapsulated sulforhodamine (SR101) was prepared. Synthetic parameters and the results observed are summarized in Table 1.

The influence of the amount of TDI equivalents on the polymer chain length was investigated (Figure 3c). When more TDI was added, the peak of the SEC chromatogram shifted toward lower elution volumes, that is, the molecular weight of the polymer chain increases. The average size of the capsules in cyclohexane is approximately 150 nm for all samples, which is similar to the size of sample without encapsulated SR101. Neither the nature of the peptide nor the amount of TDI has a significant influence on the size of the capsules. With the exception of capsules prepared from [Mca/7NH_2] with 5 TDI equivalents, which started flocculating, all samples were nicely redispersed in water. The number of reactive groups of the peptide (functionality) appears to be a key parameter for efficient encapsulation, for example, samples with the same amount of 2 TDI equivalents, the encapsulation efficiency increases from 75% for capsules made from peptide [Mca/3NH_2] to 93% for capsules made from peptide [Mca/7NH_2]. An increasing amount of TDI from 1 to 5 TDI equivalents slightly enhances the (already high) encapsulation rate in the capsules made of peptide [Mca/7NH_2] from 91% to 98%. This is confirmed by fluorescence microscopy, where both Mca and SR101 fluorescence signals were observed with the same spatial distribution, confirming an efficient and homogeneous entrapment of SR101 in the peptide-based nanocapsules. (Figure S3, Supporting Information). Moreover, it was verified by time-correlated single photon counting (TCSPC) experiments that the fluorescence of the encapsulated SR101 was indeed efficiently quenched inside the capsules, due to its high local concentration. The free dye dissolved in water showed a monoexponential decay curve with a fluorescence lifetime of $\tau = 4.18$ ns. After encapsulation, two lifetimes can be extracted from the curve ($\tau_1 = 180$ ps and $\tau_2 = 3.60$ ns). Additionally, the steady state fluorescence intensity is about 40 times lower after encapsulation. (Figure S4, Supporting Information).

The samples were finally investigated in the presence of enzyme; one example of fluorescence evolution is displayed in Figure 3d. The time-dependent increase of Mca

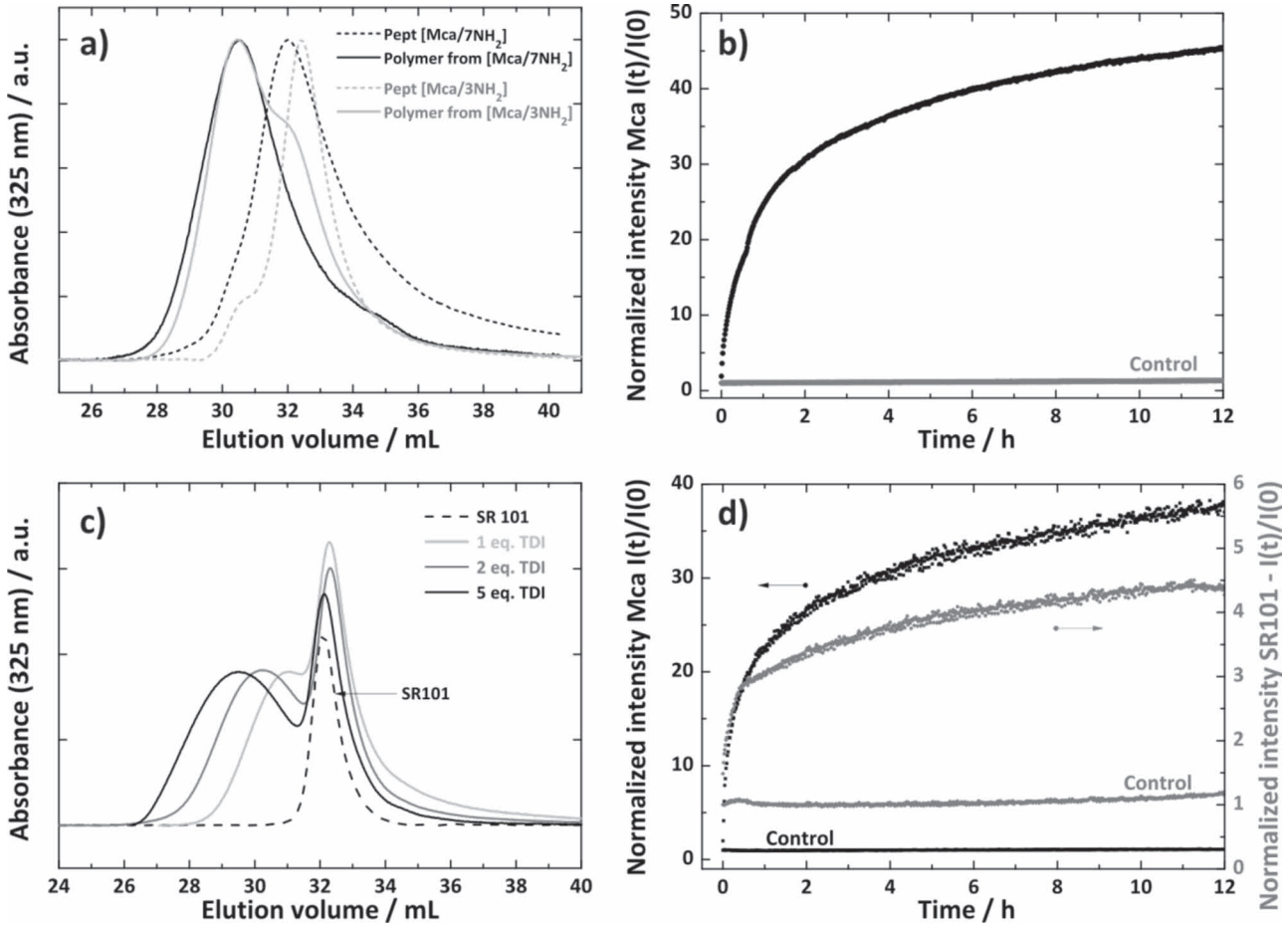

Figure 3. (a) Normalized SEC chromatograms of peptide [Mca/3NH_2] (grey, dashed line) and its polymer (solid line); peptide [Mca/7NH_2] and its polymer are in black; (b) fluorescence recovery from Mca dye (λ_{em} = 405 nm, λ_{exc} = 340 nm) on the peptide observed during enzymatic cleavage (trypsin) of the recognition site in the nanocapsules (black). The control experiment is plotted in gray. As control, the same sample was monitored under the same experimental conditions, but without the addition of enzyme; (c) SEC chromatograms of polymer obtained from peptide [Mca/7NH_2] with different amounts of TDI. All chromatograms from peptides were normalized to the same total area. The second eluting peak is mainly attributed to the absorbance of entrapped SR101 (d) Fluorescence recovery of the Mca dye on the peptide (black) and of released SR101 (grey, λ_{em} = 605 nm, λ_{exc} = 580 nm) during enzymatic cleavage of the nanocapsules and their respective control sample without addition of enzyme.

fluorescence is quite similar to the unloaded sample; thus, peptide degradation occurs in the same time range. In addition, SR101 fluorescence increased simultaneously only when enzyme was added. This demonstrates that SR101 is released from the capsules, and that its fluorescence is no longer quenched because of encapsulation.

Table 1. Synthetic parameters, size of the capsules, and encapsulation efficiency of SR101.

Peptide	TDI amount[a)] [eq]	Size in cyclohexane [nm]	Size in water [nm]	Encapsulation efficiency[b)] [%]
Mca/3 NH_2	2	153 ± 2	468 ± 28[c)]	–[d)]
Mca/3 NH_2	2	140 ± 3	308 ± 11	75
Mca/7 NH_2	1	166 ± 7	211 ± 7	91
Mca/7 NH_2	2	154 ± 8	249 ±14	–[d)]
Mca/7 NH_2	2	155 ± 7	294 ± 6	93
Mca/7 NH_2	5	146 ± 4	780 ± 88	98

[a)]Number of TDI equivalent calculated regarding the total amount of amino group on the peptide; [b)]Calculated from the amount of SR101 removed during dialysis; [c)]The sample was not ultrasonicated after redispersion and is more liable to contains aggregates; [d)]Sample without SR101.

Comparable results were observed for all samples. Within 12 h, Mca fluorescence increases by at least a factor of 25, while SR101 fluorescence is increased about fivefold. Comparing fluorescence signals of the different samples and quantifying the cleavage efficiency is, however, not straightforward. Firstly, the amount of peptide present is not the same because of different types of peptides and amounts dialyzed out. Secondly, the quenching efficiency of the FRET system varies, either by the proximity to another FRET pair in the nanocapsules, or due to the rigidity of the polymer chains compared with free peptide.[37,38]

4. Conclusion

The inverse miniemulsion process was used to prepare peptide-based hybrid nanocapsules via interfacial polyaddition. A hydrophilic dye was efficiently encapsulated and the influence of the synthetic parameters on the size of the polymer chains, the morphology of the capsules and the encapsulation efficiency, was studied. The intrinsic property of the peptide sequence—its cleavability by trypsin—was used to degrade the nanocapsules and release the loaded dye. Cleavage and release were monitored in real time via fluorescence recovery, using two different methods, (1) due to the quenching FRET system enclosing the recognition site and, (2) due to the release of the quenched encapsulated dye. This new approach opens interesting perspectives for specific enzyme-triggered drug delivery from nanostructured materials.

Supporting Information

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

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