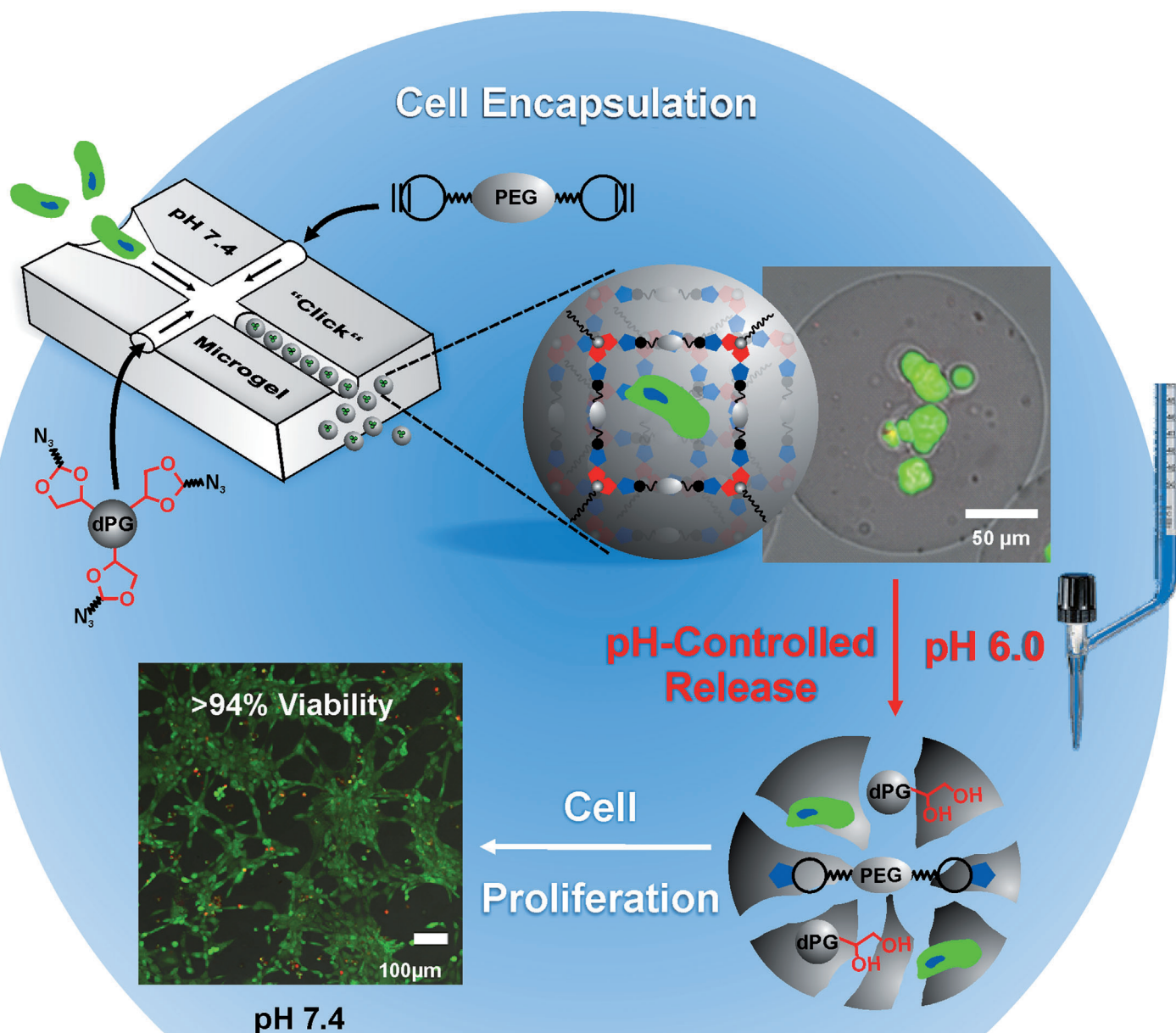


# A Microgel Construction Kit for Bioorthogonal Encapsulation and pH-Controlled Release of Living Cells\*\*

Dirk Steinhilber, Torsten Rossow, Stefanie Wedepohl, Florian Paulus, Sebastian Seiffert, and Rainer Haag\*



**Abstract:** *pH-Cleavable cell-laden microgels with excellent long-term viabilities were fabricated by combining bioorthogonal strain-promoted azide–alkyne cycloaddition (SPAAC) and droplet-based microfluidics. Poly(ethylene glycol)-dicyclooctyne and dendritic poly(glycerol azide) served as bioinert hydrogel precursors. Azide conjugation was performed using different substituted acid-labile benzacetal linkers that allowed precise control of the microgel degradation kinetics in the interesting pH range between 4.5 and 7.4. By this means, a pH-controlled release of the encapsulated cells was achieved upon demand with no effect on cell viability and spreading. As a result, the microgel particles can be used for temporary cell encapsulation, allowing the cells to be studied and manipulated during the encapsulation and then be isolated and harvested by decomposition of the microgel scaffolds.*

**H**ydrogels represent an important class of biomaterials for applications in regenerative medicine<sup>[1]</sup> and controlled delivery of therapeutic biomolecules,<sup>[2]</sup> because their mechanical properties are similar to those of many objects in living systems. This makes them ideal scaffolds to encapsulate, stabilize, culture, and deliver living cells,<sup>[2a]</sup> proteins,<sup>[3]</sup> and oligonucleotides.<sup>[4]</sup> A crucial challenge in these applications is to release the biological objects with precise control over the location and release rate.<sup>[5]</sup> To achieve this goal, the degradation of hydrogels by the cleavage of chemical bonds in the constituent polymer network, followed by dissolution of the gel and release of encapsulated guests is a promising strategy. Among the various hydrogel mechanisms of hydrogel degradation, including photolytic,<sup>[6]</sup> hydrolytic,<sup>[7]</sup> and enzymatic degradation,<sup>[8]</sup> hydrolysis at acidic pH has great potential for applications relying on controlled release.<sup>[9]</sup> Because protons are known to play a major role in cellular communication,<sup>[10]</sup> encapsulated cells can actively degrade their hydrogel matrix, which has direct impact on cell differentiation,<sup>[8]</sup> proliferation,<sup>[11]</sup> and migration.<sup>[12]</sup> In contrast to enzymatic degradation, which is diffusion controlled,<sup>[13]</sup> acidic hydrolysis shows

no diffusion limitation because of the small size of the proton catalyst. Thus, the degradation kinetics depend only on the chemical bond cleavage and therefore can be precisely controlled for the release at the given target site.

Recently, acid-cleavable hydrogels have been prepared by free radical cross-linking<sup>[14]</sup> and copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC).<sup>[15]</sup> However, these procedures are not suitable for the encapsulation of sensitive biomolecules, because the generation of free radicals and metal ions can damage them.<sup>[16]</sup> To achieve homogenous and efficient encapsulation, however, cells must be encapsulated during hydrogel formation. Hence, mild cross-linking reactions are required that are 1) orthogonal to the functional groups of the biomolecules, 2) non-cytotoxic, and 3) fast at 37 °C.<sup>[5]</sup> The strain-promoted azide–alkyne cycloaddition (SPAAC) fulfills all these requirements<sup>[17]</sup> and has recently been applied for the encapsulation of living cells into bulk hydrogels.<sup>[18]</sup> Although these are promising approaches, cell release under physiological conditions has not been achieved; however, this is essential for cell therapy and cellular biophysics.<sup>[19]</sup> Additionally, hydrogel particles with micrometer-scale dimensions (microgels) are much more useful for these applications than bulk hydrogels, because they can be used to control the cellular microenvironment in the culture very precisely<sup>[20]</sup> and can be handled by micropipettes and microsyringes.<sup>[21]</sup>

Herein we present a new microgel construction kit for the bioorthogonal encapsulation by SPAAC and the programmed release of cells triggered by benzacetal hydrolysis. We combined this chemistry with droplet-based microfluidics<sup>[19a,22]</sup> to generate stimuli-responsive mammalian-cell-laden microgels (Figure 1). Since we used different substituted benzacetals as pH-cleavable linkers on the dendritic building block, we could control the release kinetics in the interesting pH range between 4.5 and 7.4 without changing the initial mechanical matrix properties; both parameters are known to mainly influence cellular fate.<sup>[23]</sup> This makes our approach superior to conventionally used enzymatic degradation, in which the kinetics can only be varied by changing the initial degree of cross-linking.<sup>[8]</sup>

We used cytocompatible dendritic polyglycerol (dPG) and linear poly(ethylene glycol) (PEG) as bioinert hydrogel building blocks.<sup>[24]</sup> With these materials, nonspecific hydrogel–cell interactions are avoided.<sup>[15,25]</sup> Thus, we prepared homo-bifunctional PEG-dicyclooctyne (PEG-DIC) and dPG-polyazide and used them as macromonomers for the in situ cross-linking by SPAAC. To achieve microgel degradability at low pH, the azide was conjugated to dPG through an acid-labile benzacetal linker. First, hetero-bifunctional linkers were prepared that bore a terminal azide and dimethylacetal group (Scheme 1). By using aromatic core segments that carry either an electron-donating methoxy group in *ortho* position to the aldehyde (**1a**) or a hydrogen atom (**1b**), it was possible to fine-tune the kinetics of the acetal degradation, which is known to strongly depend on the electron density of the acetal.<sup>[26]</sup> Our synthesis started from *para*-hydroxybenzaldehydes, which are ideal building blocks because phenolates are excellent nucleophiles for coupling reactions with electrophilic azides **2**. Since the reactions are high yielding and the

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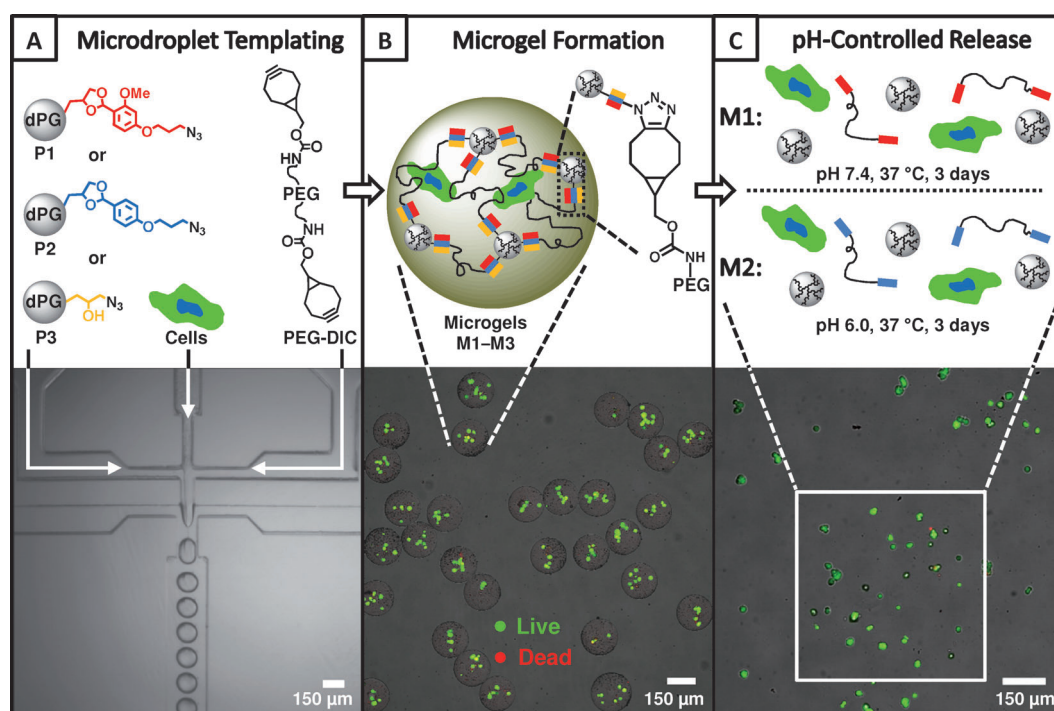
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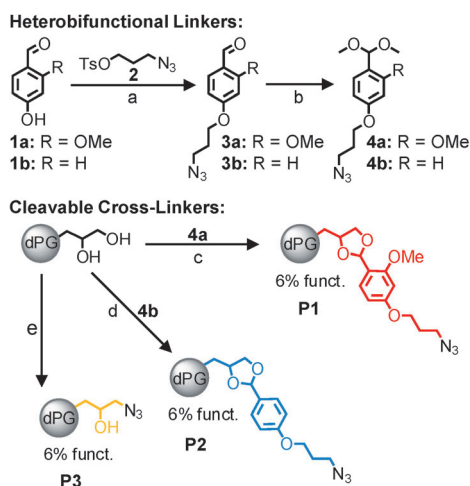
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**Figure 1.** Cell encapsulation and release: A) A set of dPG-azide precursors **P1–P3** with acid-cleavable linkers that had different hydrolysis kinetics and PEG-dicyclooctyne were prepared. One of the dPG macromonomers, the PEG-dicyclooctyne cross-linker, and NIH 3T3 cells were injected into a microfluidic device that served to form monodisperse, micrometer-sized precursor droplets. Subsequent mixing of the three liquids inside the monodisperse droplets led to droplet gelation by SPAAC. B) Thereby, micrometer-sized, cell-laden hydrogel particles **M1–M3** were formed. The viability of cells encapsulated into microgel particles was at least 94 %, as determined by fluorescence-based live–dead assays, in which living cells were stained green and dead cells were stained red. C) Incubation of the microgels **M1–M3** at pH 7.4, 37 °C, and 5 % CO<sub>2</sub> led to complete particle degradation of **M1** after three days and release of the cells with unchanged residual viability of 96 %. Under these incubation conditions microgel **M2** showed incomplete degradation and no release of cells for an observation period of two weeks. Incubation of microgel **M2** at pH 6.0, 37 °C, and 5 % CO<sub>2</sub> led to complete particle degradation after three days and release of the cells with an unchanged residual viability of 94 %. Microgel **M3** showed no degradation between pH 6.0–7.4.

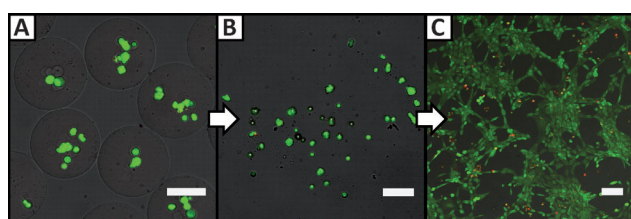


**Scheme 1.** General reaction scheme for the preparation of acid-cleavable dPG-polyazides (**P1** and **P2**) and stable dPG-polyazide (**P3**) with a degree of functionalization of 6 %. The following reaction conditions were used: a) acetone, K<sub>2</sub>CO<sub>3</sub>, reflux, 12 h, 81 %; b) methanol, trimethyl orthoformate, PTSA, reflux, 1 h, full conversion; c) DMF, PTSA, RT 3 h 80 %; d) DMF, PTSA, RT 3 h 83 %; e) synthesized according to a published procedure.<sup>[28]</sup> PTSA = *para*-toluenesulfonic acid, Ts = tosyl.

starting materials are commercially available, these compounds can be prepared on a multigram scale. Then, the aldehydes **3a** and **3b** were activated by dimethylacetal formation to obtain **4a** and **4b** and the linkers were attached to dPG by an acid-catalyzed transacetalization reaction using conditions that were recently reported by our group and others.<sup>[15,27]</sup> Therefore, we obtained two azide-functionalized dPGs with different degradation kinetics (**P1** and **P2**) and additionally we synthesized nondegradable dPG-azide<sup>[28]</sup> (**P3**). Since azides are used in many chemical reactions and can be converted into other useful reactive groups,<sup>[29]</sup> this approach to acid-cleavable dPG polyazides might be of broad use for applications that require stimuli-responsive bond cleavage. For the synthesis of PEG-DIC, we followed a procedure reported by van Delft et al.<sup>[30]</sup> and prepared carbonate-functionalized cyclooctynes. PEG-DIC cross-linkers were formed by carbamate formation between a PEG diamine and the carbonate-activated cyclooctyne.

These new macromonomers were used to prepare NIH3T3-cell-laden microgels **M1–M3** with different degradation kinetics by applying droplet-based microfluidics (Figure 1A; see the Supporting Information for details). The microgels swelled to uniform sizes of 170  $\mu$ m in cell medium and contained homogeneously distributed cells (Figure 1B).

To keep the degree of swelling and thus the mechanical properties of all microgels constant, dPG-polyazide macromonomers **P1–P3** were used with the same molecular weights and same degrees of functionalization. In addition, all microgels **M1–M3** had the same polymer content ( $217\text{ g L}^{-1}$ ). By using model microgels<sup>[22a]</sup> with the same composition as the actual microgels, but without any cell load, we determined gravimetrically the mass-based degree of swelling,  $Q = w_{\text{wet}}/w_{\text{dry}}$  with  $w_{\text{wet}}$  the weight of the swollen and  $w_{\text{dry}}$  the weight of the dry microgels. For degradable and nondegradable microgels almost identical  $Q$  values in the range of 20 to 26 were obtained. Hence, we were able to study cell viability in the three different microgel batches **M1–M3** depending on their degradation kinetics but independent of their initial degree of swelling. After one day of encapsulation, excellent cell viabilities of more than 94 % in all microgel batches were obtained (Figure 2A and Figure S3 in the Supporting Infor-



**Figure 2.** Confocal micrographs recorded after staining of the cells using a live–dead assay. Green staining denotes living cells, whereas red staining marks dead cells. A) Cell-laden microgels **M1** at day 1. B) Just released cells from **M1** at day 3. C) Cells from **M1** after release and subsequent 11 days of incubation in a polystyrene cell culture dish. All scale bars denote 100  $\mu\text{m}$ .

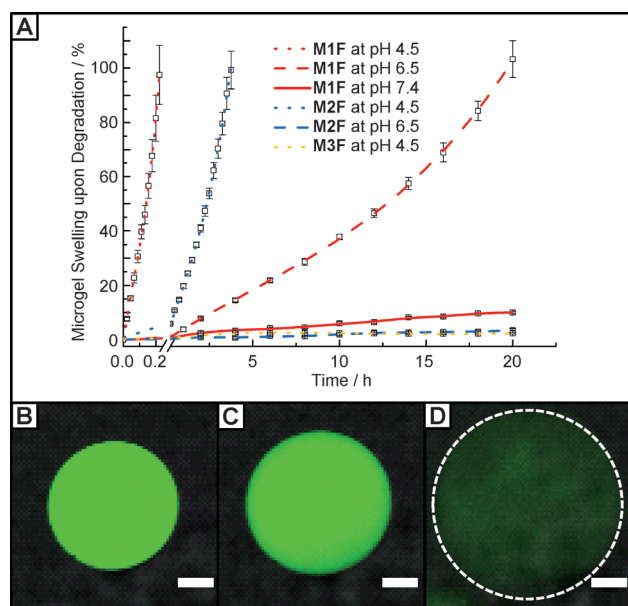
mation). Even one week after cell encapsulation and cultivation at pH 7.4, 37°C, and 5 %  $\text{CO}_2$ , there was no decrease in cell viability for microgels **M2** and **M3**. For microgel **M1**, we found a complete degradation of the matrix and liberation of the encapsulated cells on day 3, with excellent viabilities of 96 % (Figure 2B). In contrast, microgels **M2** and **M3** showed no degradation under these incubation conditions for an observation period of two weeks. Interestingly, incubation of microgel **M2** at pH 6.0, 37°C, and 5 %  $\text{CO}_2$  led to complete degradation and release of the cells after three days, again with excellent viabilities of 94 % (Figure S4). After their release the cells exhibited a spherical morphology, because they had been encapsulated in a bioinert material to which they could not adhere and spread. After subsequent 11 days of incubation in a polystyrene cell culture dish, the released cells were investigated again and highly proliferating and spreading cells were found (Figure 2C). Hence, the chemistry of encapsulation and subsequent degradation of the microgels into polyglycerol and benzaldehyde-terminated PEG had no impact on the viability and behavior of the cells in accordance to *in vivo* studies from the literature.<sup>[24a,31]</sup> This makes our new microgel kit an excellent candidate for the encapsulation and implantation of therapeutic stem cells in tumor tissues (pH 6.5–7.0).<sup>[32]</sup> After several days, during which the immune system is known to be most harmful, the cells

could be released from the pH-responsive microgels and enabled to differentiate and reconstruct diseased tissues and eradicate residual tumor cells after tumor section.<sup>[33]</sup>

To determine whether the encapsulated cells could actively degrade **M1**, microgels with the same composition but without cell load were prepared and cultured under the same conditions as those that contained cells. A prolonged degradation time of four days was observed, which indicates that the cells acidified the microgels from the inside due to their metabolic activity.<sup>[10]</sup>

For a detailed study of the microgel degradation kinetics, the PEG cross-linker was labeled with an azide-functionalized green fluorescent probe, and fluorescent microgels **M1F–M3F** (without cell loading but with the same macromonomer content as in **M1–M3**) were prepared. After workup, these microgels were stored under basic conditions in order to reach their equilibrium degree of swelling without degradation. The microgels were then incubated with buffer solutions of pH 4.5, 6.5, and 7.4, and their swelling was observed by fluorescence microscopy at 25°C. The swelling of microgels at acidic pH resulted from acetal hydrolysis and reduced degree of cross-linking because no swelling was observed for the stable microgel **M3** as a control. Particle swelling was then plotted as a function of time (Figure 3A). A typical course of the degradation experiments is depicted in Figure 3B–D. Microgel **M1F** had the fastest degradation kinetics at all pH values tested. At pH 4.5, **M1F** completely degraded after 13 min. Such rapid particle dissolution is ideal for the burst release of active agents. **M1F** degraded quickly at pH 6.5 after 20 h, rendering this microgel suitable for cell therapy in tumor and inflamed tissues.<sup>[33,34]</sup> For the same incubation time at pH 7.4, **M1F** showed no significant degradation. Thus, our system is excellent for encapsulating and stabilizing cells under physiological conditions and releasing them after injection at the target site. Microgel **M2F** showed a slower dissolution with a complete degradation after 3.75 h at pH 4.5 with no significant degradation at pH 6.5. This type of microgel is ideal for applications in which cells need to be stabilized and released over a long time. The microgels **M1F** and **M2F** had remarkably different degradations at physiologically relevant pH, which makes them interesting transporters for a variety of biological applications.

In conclusion, we have designed a new macromonomer construction kit that allows the bioorthogonal encapsulation of living cells into microgels and, for the first time, their programmed release under acidic conditions. The encapsulated cells could be cultured inside the microgels with full retention of their viability. Microgel degradation was precisely controlled by different substituted benzacetals as pH-cleavable linkers on the dendritic building block and had no detrimental effect on the encapsulated and released cells. As a result, the microgel particles can be used for temporary cell encapsulation, allowing the cells to be studied and manipulated during encapsulation and then isolated and harvested on demand by decomposition of the microgel scaffolds. Thus, our approach will advance the understanding of cellular survival in artificial 3D matrix environments. Additionally, our construction kit has potential for the stabilization and controlled



**Figure 3.** Tracking of microgel particle swelling as an indicator of degradation at different pH. A) Fluorescently labeled microgels **M1F–M3F** with the same composition as microgels **M1–M3** but without any cell load were incubated at various pH at 25°C and observed by fluorescence microscopy. Their diameter growth can be regarded as a measure of their swelling upon degradation. The maximal diameter growth had the same values (24–26%, within experimental certainty) for all microgel samples at the point of maximal swelling. Hence, this maximal diameter growth was normalized to 100%. The lines were drawn manually to guide the eye. B–D) Fluorescence micrographs of a particle from batch **M1F** at pH 4.5: B) at the beginning of incubation, C) when degradation is halfway complete, and D) when the degradation is complete. All scale bars denote 50 μm.

release of many other therapeutic relevant biological systems such as proteins, genes, and even bacteria.<sup>[35]</sup>

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