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# ROS-Sensitive Polymer Micelles for Selective Degradation in Primary Human Monocytes from Patients with Active IBD

Elena Gardey, Fabian H. Sobotta, Stefanie Quickert, Tony Bruns, Johannes C. Brendel,\* and Andreas Stallmach\*

Inflammatory bowel disease (IBD) is characterized by increased levels of reactive oxygen species (ROS) in inflamed areas of the gastrointestinal tract and in circulating immune cells, providing novel opportunities for targeted drug delivery. In the recent experiments, oxidation-responsive polymeric nanostructures selectively degrade in the presence of H<sub>2</sub>O<sub>2</sub>. Based on these results, it is hypothesized that such degradation process can be triggered in a similar way by the incubation with stimulated monocytes isolated from patients with IBD. A first indication is given by a significant correlation between excessive ROS and degradation of micelles in monocytes isolated from healthy individuals after phorbol 12-myristate 13-acetate (PMA) stimulation. But even if the ROS-sensitive micelles are incubated with nonstimulated monocytes from patients with active IBD, a spontaneous degradation is observed in contrast to micelles incubated with monocytes from healthy donors. The findings indicate that the thioether-based micelles are indeed promising for selective drug release in the presence of activated immune cells.

many inflammatory diseases.<sup>[1–6]</sup> Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory diseases associated with severe oxidative stress.<sup>[6,7]</sup> It has been shown that the production of free radicals dramatically increases in the mucosa<sup>[8]</sup> and in monocytes of patients with IBD.<sup>[9,10]</sup> However, excessive ROS in IBD provides an opportunity for selective drug delivery. Correspondingly, ROS-sensitive nanoparticles can be disintegrated and used for controlled drug release in inflamed areas with excessive ROS production. This approach can help to reduce drug-related side effects and improve efficacy of anti-inflammatory drugs in targeted inflamed areas.<sup>[11–13]</sup> ROS-sensitive nanoparticles showed their potential in cancer models,<sup>[14]</sup> and recent studies have used polymer nanoparticles for targeting inflamed intestine in IBD models.<sup>[15–18]</sup>

However, this promising approach has some limitations and issues in the design and fabrication of appropriate and selective carrier system.<sup>[19]</sup> The polymers themselves, nanoparticles, and their oxidized products have to be biocompatible<sup>[20]</sup> and demonstrate noninflammatory properties.<sup>[21]</sup> Recently we have reported a straightforward process to create novel ROS-sensitive polymeric micelles comprising thioether moieties, which can

## 1. Introduction


Reactive oxygen species (ROS) play a fundamental role in several physiological processes in the body. Immune cells release ROS in inflamed areas to effectively protect the organism from the pathogenic infections. However, increased release of ROS is now known to be a principal component in the development of

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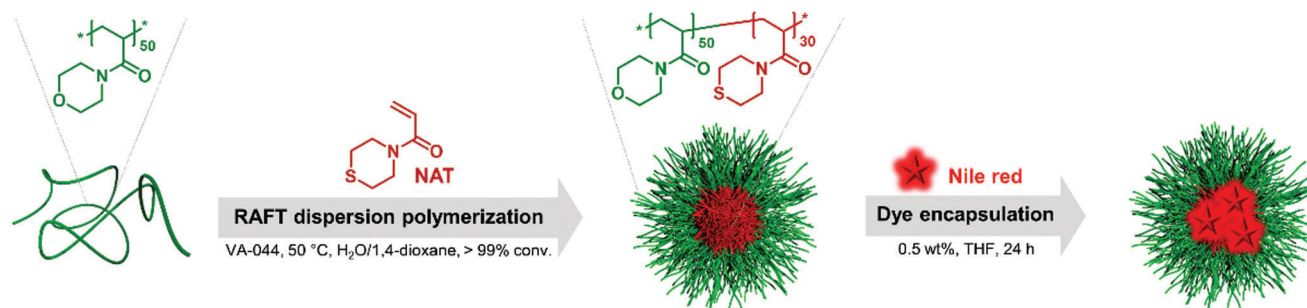
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**Scheme 1.** Schematic illustration of the one-pot synthesis of block copolymer-based micelles via aqueous reversible addition fragmentation chain transfer (RAFT) dispersion polymerization and subsequent dye loading.

be oxidized in the presence of H<sub>2</sub>O<sub>2</sub> resulting in a disintegration of the micelle.<sup>[22]</sup> These polymeric nanoparticles are directly prepared in aqueous solution by polymerization induced self-assembly (PISA), which represents a straightforward way to prepare polymer micelles circumventing tedious assembly steps. A biocompatible water-soluble block based on the monomer *N*-acryloylmorpholine (NAM) is formed first,<sup>[21,23–25]</sup> and after full conversion of the monomer, the chain is extended by a second, hydrophobic block using *N*-acryloylthiomorpholine (NAT). In addition, we have demonstrated cellular uptake of these micelles without signs of cytotoxicity.<sup>[22]</sup> A key advantage of these micelles is the ability to degrade in the presence of high levels of ROS. Therefore, they have the capacity to release their cargo only in restricted and target areas characterized by an oxidative environment.

In recent years, a wide variety of oxidation-sensitive nanomaterials have been reported.<sup>[17,26]</sup> An important factor concerning IBD is that ROS-sensitive nanoparticles should be stable at normal physiological concentrations of ROS but selectively degrade under oxidative stress during inflammation.<sup>[27,28]</sup> Many reported in vitro studies relate the efficacy of oxidation-responsive nanomaterials to their response in cultivated cell lines or artificial tissue systems, which are often further treated with additional H<sub>2</sub>O<sub>2</sub> to induce the degradation.<sup>[5,15,29,30]</sup> A more sophisticated approach for a more reliable investigation of nanoparticle efficacy considers the use of primary immune cells which are selectively activated by added stimuli.<sup>[5,16,31]</sup>

It remains debatable, whether the aggravation of the immune cells by stimulation reflects realistic conditions within the disease.<sup>[32,33]</sup> A direct evaluation of the response of ROS sensitive particles to primary, already activated cells isolated directly from patients with inflammation would be highly desirable to circumvent any stimulation. In comparison to cells or tissue from healthy donors, a significant effect should be observed to prove a selective degradation. In this study, we demonstrate that such an approach is indeed suitable to evaluate the effectiveness of ROS-sensitive nanoparticles. Based on our previous findings,<sup>[22]</sup> we hypothesized that our ROS-sensitive polymeric micelles can degrade in the presence of high levels of ROS presented in activated monocytes which are isolated from patients with active IBD. We compared the results with monocytes from healthy donors and patients in clinical remission to investigate the selectivity and stability of the micelles in the presence of nonactivated cells. As a positive control, we further tested monocytes treated

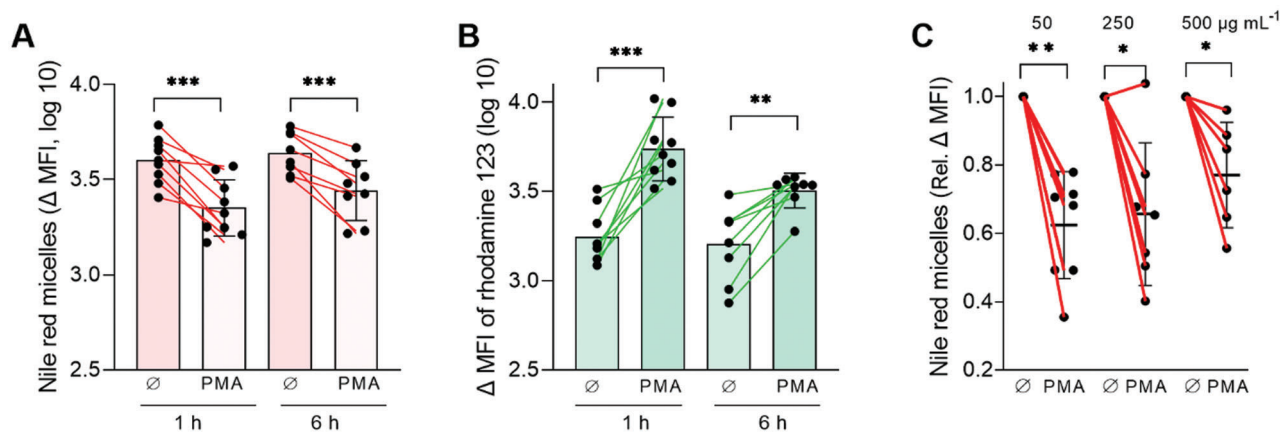
with the protein kinase C agonist phorbol 12-myristate 13-acetate (PMA), that activates pathways leading to the generation of superoxide anions.<sup>[34,35]</sup> The obtained results corroborate that the thioether based ROS-responsive polymeric micelles can indeed be degraded in the presence of activated monocytes isolated from patients with active form of chronic inflammatory disease while they remain unaffected in case of healthy individuals.

## 2. Results and Discussion

The investigated polymers and micelles were prepared by polymerization-induced self-assembly (PISA) following a previously established procedure (Scheme 1).<sup>[22]</sup> Starting from a PNAM<sub>50</sub> macrochain transfer agent (CTA), micelles based on PNAM<sub>50</sub>-*b*-PNAT<sub>30</sub> block copolymers could be directly created in situ during the block extension step in a one-pot process in aqueous solution (characteristics of the polymers are given in Table S1 in the Supporting Information). Compared to the common post-polymerization formulation techniques, PISA allows the reproducible synthesis of micelles of different sizes in large quantities and at high concentrations in short time scales.<sup>[36]</sup> The resulting spherical micelles (see Figure S1 in the Supporting Information for additional cryo-TEM images) contain a hydrophilic shell, based on PNAM, while the hydrophobic PNAT induces the aggregation. The core of the micelles is prone to oxidation at the thioether moieties, which results in the formation of hydrophilic sulfoxide units and, in consequence, to the disintegration of the micelles. It is important to note, that both the polymeric micelles as well as the oxidized and water-soluble polymer revealed no signs of cytotoxicity, which was previously investigated by our group.<sup>[22]</sup>

While the previous studies focused on solutions in water, we first investigated the degradation under physiological conditions. To monitor the disintegration of the micelles they were loaded with fluorescent dye Nile red (0.5 wt%), which acts as sensor for a changing environment.<sup>[23]</sup> The fluorescence intensity of the Nile red dye decreases drastically in an hydrophilic environment and therefore the oxidation of micelles should induce a fluorescence quenching of the Nile red dye.<sup>[22,23,37]</sup>

In a first experiment investigating the degradation of Nile red loaded polymeric micelles by activated immune cells, we examined monocytes of self-declared healthy donors which were actively stimulated by PMA to induce a strong release of ROS. Therefore, the micelles were incubated for 1 h with the isolated



**Figure 1.** A) Fluorescence intensity of Nile red micelles ( $50 \mu\text{g mL}^{-1}$ ) incubated with monocytes from healthy donors; B) ROS production after phorbol-12-myristat-13-acetat (PMA) stimulation determined as intracellular oxidation of dihydrorhodamine 123; C) disintegration of ROS-sensitive micelles after 6 h at different concentrations ( $50\text{--}500 \mu\text{g mL}^{-1}$ ) in monocytes isolated from healthy donors after treatment with PMA. Monocytes were stimulated with PMA for 10 min. Untreated cells were used as a control ( $\emptyset$ ). MFI: median fluorescence intensity. The results are presented as  $M \pm \text{SD}$ ; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ,  $n = 6\text{--}8$  (healthy donors).

monocytes and one sample was treated with PMA, while the control remained untreated. The observed fluorescence intensity decreased significantly ( $p = 0.0004$ , paired  $t$ -test) for the activated sample compared to the nonstimulated control (Figure 1A). As mentioned above, the quenching of Nile red fluorescence helps to evaluate the solubility switch of the core polymer, which is directly related to micelles oxidation.<sup>[23]</sup> In addition, the production of ROS was monitored by the oxidation of dihydrorhodamine 123 to rhodamine, which corroborated an enhanced oxidative environment in the PMA treated monocytes ( $p = 0.0004$ , paired  $t$ -test) (Figure 1B).

Correlating both experiments, we found a significant negative correlation ( $r = -0.7214$ ,  $p = 0.0007$ ) between oxidation of dihydrorhodamine 123 and the fluorescence intensity of Nile red micelles after incubation with the monocytes. We therefore conclude that the increased generation of ROS is directly related to the decreased fluorescence intensity of micelles (Figure S2A, Supporting Information). Similar results were observed if the incubation period is extended to 6 h ( $p = 0.0006$ , paired  $t$ -test) (Figure 1A,B). However, it has to be kept in mind that further increases in incubation time may lead to falsified results due to an unintended diffusion of Nile red dye out of the micelles. The Nile red dye might consequently integrate into the cell membrane or intracellular lipid droplets.<sup>[38,39]</sup>

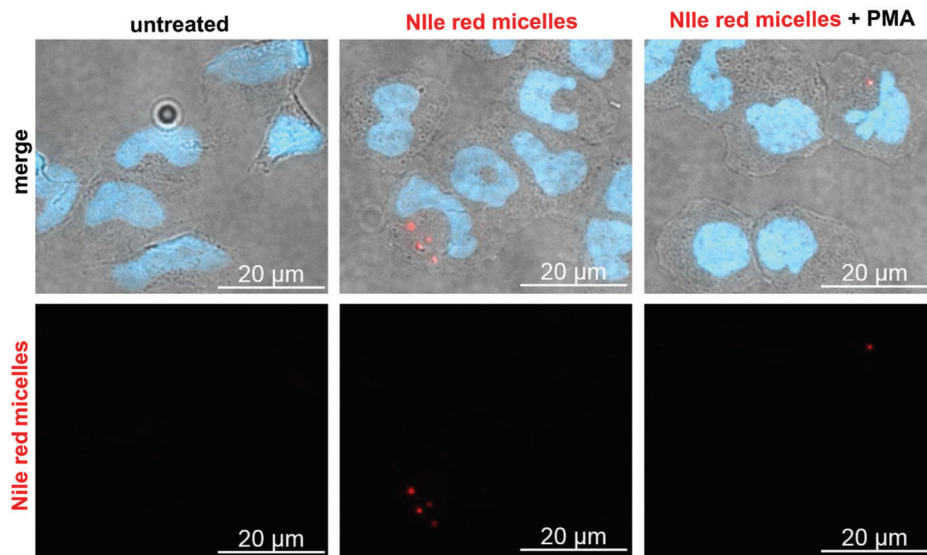
Following these first experiments, we further tested the influence of micelle concentration on the degradation behavior in stimulated monocytes. Equal number of monocytes were incubated with  $50\text{--}500 \mu\text{g mL}^{-1}$  ROS-sensitive micelles for 6 h. After PMA activation, fluorescence intensity of Nile red was significantly decreased in all groups (Figure 1C,  $p = 0.0078$ ,  $p = 0.0313$ ,  $p = 0.0313$ , Wilcoxon test). Due to the most significant difference, the concentration of ROS-responsive polymeric micelles was kept at  $50 \mu\text{g mL}^{-1}$  for further experiments.

Our experiments demonstrated that Nile red fluorescence was not completely quenched after incubating micelles with stimulated human monocytes in contrast to the previous oxidation experiments with  $\text{H}_2\text{O}_2$  in water.<sup>[22]</sup> This effect could either be related to a partial diffusion of the released dye into the membrane

or an interaction with serum proteins present in the solutions. To investigate the impact of this serum in the medium on the fluorescence intensity during the oxidation of the polymeric micelles, we incubated them in 10% fetal bovine serum (FBS) medium containing  $\text{H}_2\text{O}_2$ . Previously, we observed that almost all micelles were disintegrated after 1 h incubation in PBS with 1 mol  $\text{H}_2\text{O}_2$ .<sup>[22]</sup> However, it has to be mentioned that the fluorescence decay in case of Nile red loaded micelles was also delayed compared to measurements based on dynamic light scattering (DLS), which was related to the interaction of the dye molecules with remaining hydrophobic thioether groups on the polymers. The presence of thioether groups after disintegration have also been confirmed in a subsequent study on the polymer oxidation.<sup>[40]</sup> A further delay is now observed when the micelles were incubated in 10% FBS medium with 1 mol  $\text{H}_2\text{O}_2$ , as the fluorescence intensity was only reduced by around 65% after 2 h (Figure S2B, Supporting Information). It is interesting to note that no change is observed, if the loaded micelles are kept in the medium without  $\text{H}_2\text{O}_2$ . We assume that the released sensor dyes are not only interacting with the remaining hydrophobic groups but also with serum proteins partially conserving its fluoresces and reducing the sensitivity of the approach. Nevertheless, the approach allows still monitoring of the degradation, since no change occurs in absence of the oxidant. It can further not be excluded that serum-contained medium induces some protection of the ROS-sensitive micelles due to formation of protein corona, which was however beyond the scope of this study.

The uptake and potential localization of the micelles was subsequently investigated. The later was analyzed by fluorescence microscopy for which the micelles were incubated for 1 h with monocytes from healthy donors without and with PMA stimulation. The monocytes were subsequently washed by centrifugation to remove any remaining micelles from the surface and deposited on slides by Cytospin.

The microscopy images clearly demonstrate an uptake into the untreated monocytes and the punctuate pattern indicates entrapment in phagolysosomes (Figure 2). In contrast to the flow cytometry data, which indicate that  $98.9 \pm 0.7\%$  of the cells take

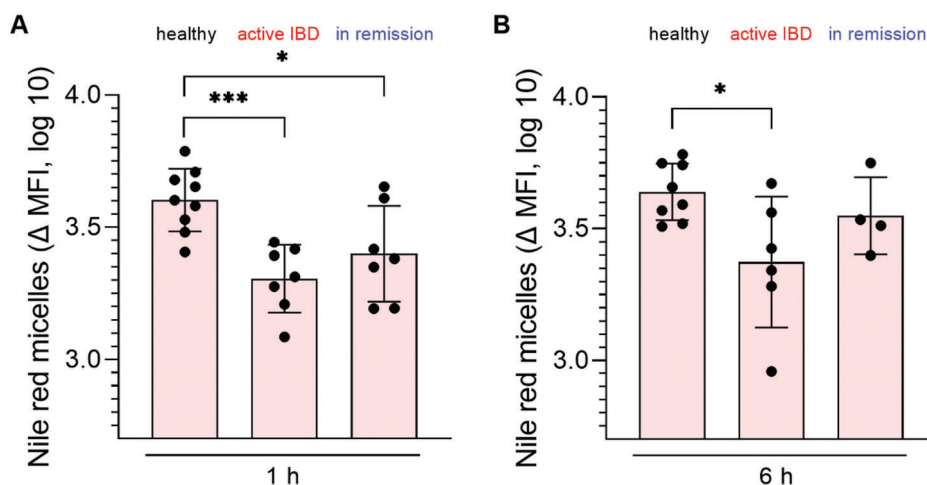


**Figure 2.** Disintegration of ROS-sensitive polymeric micelles in monocytes isolated from a healthy donor after treatment with phorbol-12-myristat-13-acetat (PMA). The cells were treated with Nile red micelles (red) for 1 h. Cells were stimulated with PMA for 10 min. The cell nuclei were stained with DAPI (blue). Bar is 20  $\mu\text{m}$ .

up micelles after 1 h incubation (Figures S3 and S4, Supporting Information), not all cells display the respective intense signal for the Nile red. We relate this discrepancy to the limited optical resolution and sensitivity of the microscope which cannot detect individual Nile red micelles but mostly accumulations in the cells.<sup>[41,42]</sup> Nevertheless, the images of the treated cells display only very few spots indicating the disintegration of the present micelles, which corroborates the change in intensity observed in flow cytometry experiments (Figures S3 and S4, Supporting Information).

Encouraged by these initial results on actively stimulated immune cells, we further examined monocytes isolated from peripheral blood of patients with IBD to verify our initial hypothesis. While those immune cells are known for an enhanced ROS

production compared with immune cells of healthy persons,<sup>[9,10]</sup> it has yet to be demonstrated, if such a disease related activation is sufficient to induce a degradation of ROS-sensitive nanoparticles. Like the previous experiments, we incubated our micelles again for 1 h and 6 h with the isolated monocytes and performed control experiments with monocytes from healthy donors. In the case of 1 h incubation, the fluorescence intensity of the Nile red loaded polymeric micelles was indeed found to be significantly decreased for monocytes isolated from patients with active IBD ( $p = 0.0003$ , unpaired  $t$ -test) and in clinical remission ( $p = 0.0169$ , unpaired  $t$ -test) compared with the cells from healthy donors (Figure 3A). After 6 h incubation the effect is decreased, but still monocytes from patients with active IBD patients revealed a decreased fluorescence intensity compared with unstimulated



**Figure 3.** Fluorescence intensity of Nile red micelles incubated with monocytes from healthy donors and patients with inflammatory bowel disease (IBD) after 1 h A) and 6 h B). MFI: median fluorescence intensity. The results are presented as  $M \pm \text{SD}$ ; \* $p < 0.05$ , \*\*\* $p < 0.001$ ,  $n = 4-9$  (patients or healthy donors).

monocytes isolated from healthy donors (Figure 3B,  $p = 0.0183$ , unpaired  $t$ -test). This reversal might again point at secondary effects due to a potential integration of the dye into hydrophobic compartments of the cells with time causing a renewed increase in fluorescence intensity.

It is further interesting to note that additional PMA-stimulation of monocytes isolated from active IBD patients resulted again in a significant decrease in fluorescence intensity compared to the untreated cells ( $p = 0.0123$ , paired  $t$ -test; Figures S5 and S6, Supporting Information).

These results are further corroborated by microscopy analysis of the corresponding cells, which still shows some intact micelles for the nonstimulated cells from active IBD patients while nearly all fluorescence is quenched for the PMA stimulated cells (Figure S7, Supporting Information). This result underlines that active stimulation induces additional effects in terms of ROS production, which needs to be considered in the evaluation of such degradation studies. It has to be kept in mind, that all patients underwent immunosuppression therapy (Table S2, Supporting Information) and 30% of the patients with IBD use 5-aminosalicylic acid (5-ASA) to scavenge reactive species.<sup>[6]</sup> Nevertheless, our experiments verify that monocytes isolated from patients with active IBD induce a sufficient ROS production for degradation of the presented ROS-sensitive micelles.

### 3. Conclusion

In summary, we demonstrated the degradation of ROS-sensitive nanoparticles by activated monocytes isolated from patients with IBD compared to cells from healthy individuals. The chosen PNAM-*b*-PNAT micelles comprise thioether units in the hydrophobic core, which can be oxidized to sulfoxide units inducing a hydrophobic to hydrophilic transition and a disintegration of the micelles. Nile red was loaded into the micelles as sensor dye to monitor the oxidation process. In first experiments on PMA stimulated monocytes from healthy donors, a significant degradation of the polymeric micelles was observed when compared to untreated cells and the result correlated well with the enhanced ROS production in stimulated monocytes. More interesting, the direct incubated with monocytes isolated from IBD patients similarly resulted in a significant decrease in fluorescence compared to monocytes from healthy donors. Therefore, our work demonstrates in a first proof of concept that the disintegration of thioether based micelles can be selectively triggered by monocytes isolated from patients with IBD, which renders these materials promising carriers for drug delivery. Furthermore, our approach to monitor ROS-sensitive polymeric micelles in the presence of monocytes isolated from patients suffering from IBD appears a promising strategy for direct evaluation of comparable drug delivery systems, since extremely short half-lives of ROS render it challenging to design suitable in vitro experiments with representative concentration of ROS as in blood and intestine of patients with IBD.<sup>[6]</sup>

### 4. Experimental Section

**Micelle Preparation and Characterization:** Micelle preparation and characterization were published previously.<sup>[22]</sup> Briefly, amphiphilic

block copolymers P(NAM-*b*-NAT) were synthesized in a sequential polymerization starting from the chain transfer agent 2-(butyl)-thiocarbonylthiopropionic acid and using first *N*-acryloylmorpholine (NAM) and after full conversion adding *N*-acryloylthiomorpholine (NAT). In both steps VA-044 was used as initiator. The resulting hydrodynamic diameter of the micelles is 50.41 nm. The micelles were further loaded with Nile red according to a previously published procedure.<sup>[43]</sup>

**Isolation of Mononuclear Cells:** Peripheral blood was collected from 16 (median age: 31, range: 29–66) patients with IBD and 9 self-declared healthy donors (median age: 29.5, range: 26–59) at the Jena University Hospital. Informed consent was obtained from all subjects involved in the study. The study was approved by local internal review board (Ethics committee, no. 3285-10/11). The diagnosis of IBD was made by clinical data, symptoms, and histological evaluation. Disease activity status was divided into two groups (active IBD and IBD in remission). Disease activity status was assessed by two independent clinicians considering patient's symptoms and clinical examinations. All patients were obtained medical treatment (Table S2, Supporting Information). Monocytes were isolated from blood by density gradient centrifugation using Lympholyte-H (Tebu-bio, CL5020 Human, Canada). Human monocytes were isolated from peripheral blood mononuclear cells with purities greater than 97% using CD14 MicroBeads (Miltenyi Biotec, Germany) for the positive selection according to manufacturer's instructions.

**Incubation of Cells with Micelles:** Monocytes ( $4 \times 10^5$  cells) were incubated (37 °C, 5% CO<sub>2</sub>) in 200  $\mu$ L phenol-red free RPMI 1640 medium (Biochrom, Germany) with 1% L-glutamine, Penicillin, Streptomycin and 10% heat-deactivated fetal bovine serum (FBS). Cells were incubated with ROS-sensitive micelles (50  $\mu$ g mL<sup>-1</sup>) for 1 or 6 h and subsequently left untreated or treated with either phorbol 12-myristate 13-acetate (PMA, 25  $\mu$ g mL<sup>-1</sup>, Abcam, UK) and/or 1.25  $\mu$ g mL<sup>-1</sup> dihydrorhodamine 123 (GlycoTope Biotechnology, Phagoburst, Germany) for the final 10 min of incubation. Unstimulated cells served as a background control. ROS generation was monitored by oxidation of dihydrorhodamine 123 (DHR) to rhodamine after PMA stimulation. Monocytes isolated from healthy donors were additionally incubated with 50, 250, and 500  $\mu$ g mL<sup>-1</sup> of ROS-sensitive micelles for 6h. After incubation cells were washed twice by centrifugation (250  $\times$  g, 5 min, Rotanta 460R, Hettich, Germany) with buffer (phosphate-buffered saline (PBS), 2% FBS, 2mmol EDTA, pH 7.2). Cells were placed on ice and analyzed by flow cytometry (CytoFlex, Beckman Coulter, Germany).

**Cellular Uptake (Flow Cytometry):** Flow cytometry was performed on a CytoFlex Flow Cytometer (Beckman Coulter, Germany) with a 488 nm laser and ECD channel (610/20 nm) for Nile red and FITC channel (525/40 nm) for rhodamine. 20 000 cells were counted per sample. Cellular debris were excluded according to forward and side scatter characteristics. Micelles from the surface of the cells were removed by washing as described above.

**Cellular Uptake (Microscopy):** Monocytes from patients with active IBD and self-declared healthy donors were incubated with ROS-sensitive micelles (50  $\mu$ g mL<sup>-1</sup>) in phenol-red free RPMI 1640 medium for 1h (37 °C, 5% CO<sub>2</sub>). PMA (25  $\mu$ g mL<sup>-1</sup>) was added for the last 10 min. After incubation cells were washed twice by centrifugation. CytoSpin 3 Cell Preparation System (1000  $\times$  g, 5 min, Thermo Shandon, UK) was used to deposit monocytes on slides for microscopy images (Axio Observer Z.1, Zeiss, Germany). DAPI (4',6-diamidino-2-phenylindole) Fluoromount-G (SouthernBiotech, USA) was used to stain the nuclei of the cells. All images were acquired using the same exposure time of 5s on the red channel.

**Time-Dependent Disintegration of ROS-Sensitive Micelles without Cells:** 100  $\mu$ L of ROS-sensitive micelles loaded with Nile red (1 mg mL<sup>-1</sup>) were incubated in 100  $\mu$ L of medium (10% FBS) with 1 mol H<sub>2</sub>O<sub>2</sub>, 0.1 mol H<sub>2</sub>O<sub>2</sub>, and 0.02 mol H<sub>2</sub>O<sub>2</sub> at 37 °C. Fluorescence intensity of micelles was measured every 2 h with a microplate reader Tecan Infinite M200 Pro (Ex./Em.: 535 nm/612 nm).

**Statistical Analysis:** Results are presented as mean values  $\pm$  standard deviation ( $M \pm SD$ ). Log-transformed data were used. A two-tailed paired and unpaired Student's  $t$ -test, Wilcoxon tests were applied to detect significant differences, carried out by GraphPad Prism 9.0.0 (GraphPad Software, San Diego, California USA).

## Supporting Information

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

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## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Keywords

controlled release, PISA, RAFT polymerization, reactive oxygen species, smart delivery

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