This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Amphiphilic Poly(esteracetal)s as Dual pH- and Enzyme-Responsive Micellar Immunodrug Delivery Systems†

Leon Bixenmann, a Judith Stickdorn a and Lutz Nuhn a,b,‡

Poly(esteracetal)s are equipped with both acid-labile acetal and a base-labile ester functionalities in their backbone, thus, providing dual pH-responsive degradation profiles. In this study, the cyclic esteracetal monomer 2-methyl-1,3-dioxan-4-one (MDO) was polymerised via cationic ring-opening mechanism onto methoxy poly(ethylene glycol) (mPEG) affording amphiphilic mPEG-b-P(MDO) poly(esteracetal) block copolymers that self-assemble into well-defined monomodal micelles by direct hydration with aqueous buffers. Their pH-responsive behaviour towards rapid particle degradation was confirmed by DLS and 1H-NMR. Upon acidification or basification as well as lipase-mediated enzymatic hydrolysis immediate polymer degradation and cargo release could be monitored. As an example for further suitable applications, an amphiphilic immune stimulatory TLR-7 agonist (Adifectin) was co-formulated into the mPEG-b-P(MDO) block copolymer micelles and its activity was investigated by TLR stimulation of RAW Blue macrophages. The degradable amphiphilic block copolymer was able to solubilize the drug more efficiently and retained its receptor activity. Consequently, mPEG-b-P(MDO) based amphiphilic poly(esteracetal)s can be considered as promising delivery system for further immunodrugs and, thus, may contribute to improve their translatability.

1. Introduction

Delivering pharmaceutically active molecules to their specific therapeutic target by using nanosized carrier systems has attracted major interest to overcome current limitations of drug therapies.1,2 Many hydrophobic drugs hamper from low solubility and unspecific binding which cause non-controllable toxicities.3,4 This includes novel promising anticancer or immune therapeutics which accumulate insufficiently at their target side. As a result, their related off-target effects limit any clinical translatability. In that context, amphiphilic block copolymers may support the drugs when formulated into nanometre-sized micellar delivery systems. They are structurally composed of a hydrophilic corona and a hydrophobic core where the therapeutic compounds can be entrapped physically.5,6,7 Size, surface properties and the micelles’ responsive behaviour can determine the drugs’ accessibility and tissue accumulation.8 Many attempts have been followed to translate this general principle into clinical practice including paclitaxel formulations like Genexol-PM, which was approved in 2007 in South Korea for breast cancer.4,7,9 In parallel, novel therapeutic antitumour concepts have been evolved during the last decade, especially with respect to the role of the immune system in cancer progression. Scientists envision to reinforce immune reactions against cancer cells by delivering immune-stimulating therapeutics to the tumour microenvironment and its draining lymph nodes.6,10 Immunostimulants such as the Toll-like receptor (TLR) 7 agonist Imiquimod are highly potent in inducing innate immune responses and result in an activation of a broad spectrum of antigen-presenting cells and sufficient activation of cytotoxic T-cells. Unfortunately, if administered systemically, uncontrolled inflammations are elicited also in healthy tissues leading to severe side effects.11 To overcome these dose-limiting toxicities, nanocarriers are considered as ideal delivery vehicles especially for such small molecular immunotherapeutics, e.g. to enhance their transport, release and activity in the lymph nodes.12-14 Consequently, a fundamental challenge is to design carriers with sufficient tissue mobility to lymph nodes that show at the same time proper uptake by relevant immune cells. Recently reported findings imply that lymph nodes can exhibit acidic compartments that inhibit T-cell effector functions.15 Therefore, an acidic triggered cleavage of the shielding corona followed by the release of immunotherapeutics might be a beneficial strategy towards lymph node targeted delivery of immune stimulatory drugs. As advanced multi-responsive class of materials, poly(esteracetal)s can provide such acid-cleavable features beyond classical ester hydrolysis (the latter commonly guarantees avoiding long-term particle accumulation by e.g. enzyme-mediated ester cleavage).

Poly(esteracetal)s are accessible by ring-opening polymerisation and gain growing interest especially towards the development of degradable polymers for sustainable plastics.16,17,18 To that respect, Hillmyer and coworkers have recently provided major contributions to a mechanistic understanding of their fabrication.17,18 In one of their seminal

‡ Electronic Supplementary Information (ESI) available under DOI: 10.1039/x000000x
works, Hillmyer et al. applied cationic ring-opening polymerisation conditions to cyclic ester acetal monomers which prevented the expulsion of acetaldehyde. Using the acidic organocatalyst diphenyl phosphoric acid (DPP), they showed successful poly(esteracetal) formation with different end groups based on the applied alcohol initiator. However, to the best of our knowledge, a block copolymerisation with macroinitiators towards amphiphilic diblock copolymers has so far not been reported.

Expanding classical micellar drug carrier systems, we believe that micelles derived from poly(esteracetal) diblock copolymers are a promising platform for lymph node-responsive delivery of immunotherapeutics. The acid-degradable acetal group assures rapid carrier degradation and cargo release in acidic environments like immunosuppressive cancers and their draining lymph node, while the ester functionalities further enable long-term enzymatic carrier clearance from the body. In this context, our work aims at providing first proof-of-concept studies on applying amphiphilic poly(esteracetal) block copolymers and their derived micelles for pH- and lipase-responsive delivery of immunodrugs.

Figure 1: (A) Synthesis and dual pH-responsive (or enzymatic) degradation of amphiphilic poly(esteracetal) block copolymers. (B) Schematic illustration of the concept using amphiphilic poly(esteracetal) block copolymers and their derived micelles for pH- and lipase-responsive delivery of immunodrugs.

2. Experimental Section

2.1 Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich or Fisher Scientific. 1,2-Dichloroethane (DCE) and dichloromethane (DCM) as solvents were dried by heating under reflux over calcium hydride for 3 h, then stirred overnight at room temperature and distilled prior to use. The cyclic esteracetal monomer 2-methyl-1,3-dioxan-4-one (MDO) was synthesized from commercial 2-methyltetrahydrofuran-3-one, as recently reported. Prior to use, MDO was dried over calcium hydride overnight at room temperature (rt) and freshly distilled. Magnesium sulphate was obtained from Acros Organics and diphenylphosphoric acid (DPP) was purchased from Alfa Aesar. DPP was dried under high vacuum (0.05 mbar) overnight. DPP-catalyst stock solutions were prepared from dried DPP in either anhydrous 1,2-dichloroethane or dichloromethane. Polyethylene glycol was purchased from Fluka® Analytical. Prior to use, it was also dried by azeotropic distillation with toluene three times and then left in high vacuum (0.05 mbar) overnight. 120 mM phosphate buffer (10x PB) was prepared from sodium hydrogen phosphate (100 mM) and potassium dihydrogen phosphate (20 mM) diluted with Millipore water. Millipore water was obtained from a Milli-Q Reference A+ System (water was used at a resistivity of 18.2 MΩ cm⁻¹ and total organic carbon of <5 ppm). Deuterated solvents were obtained from Deutero. Deuterated toluene was purchased from Sigma Aldrich. For enzymatic degradation studies, a recombinant lipase immobilized on acrylic
resin beads was used (Novozyme 435 ≥5,000 U/g, recombinant, expressed in Aspergillus niger, from Sigma Aldrich). The solid support enabled enzymatic polymer degradation studies both in organic (toluene) and aqueous environment. Dialysis was performed using Spectra/Por7 dialysis membranes with a molecular weight cut-off of 1000 g mol⁻¹. Sterile Dulbecco’s PBS, cell culture medium and supplements were purchased from Thermofisher. The RAW-Blue reporter cell line, QUANTI-Blue reagent and Adifectin (CL347) were purchased from Invivogen and handled as recommended by the supplier.

2.2 Instrumentation

All ¹H-Nuclear Magnetic Resonance (NMR) spectra were recorded at room temperature on a Bruker 250 MHz, 300 MHz, 400 MHz or 600 MHz FT NMR spectrometer (Bruker Avance III 250, Bruker Avance III HD 300, Bruker Avance II 400, Bruker Avance III 600). Chemical shifts (δ) are provided in parts per million relative to TMS. NMR spectra were processed with the MestReNova 11.0.4 from Mestrelab Research. Samples were prepared in respective deuterated solvent signal. Integrals of proton signals of the block copolymer were normalized to the integral of the ethylene glycol matrix.

Diffusion Ordered Spectroscopy DOSY spectra were recorded at room temperature on a Bruker 400 MHz FT NMR spectrometer (Bruker Avance III HD 400) and processed by Bayesian DOSY Transformation (minimum 1.00e-08; maximum 1.00e-04; resolution 1.00e-04) from Mestrelab Research. 1H; -C(O)-C(H), δ [ppm] = 5.39 (q, J=5.1, 1H; -O-C(CH₂⁻)-O-), 4.17 (ddd, δ = 11.5, 8.2, 2.3, 1H; -C(O)-CH₂⁻-O-), 3.91 (ddd, δ = 11.4, 10.7, 5.4, 1H; -C(O)-CH(CH₂⁻)O-), 2.75 (ddd, δ = 17.8, 10.7, 8.2, 1H; -C(O)-CH₂⁻-O-), 2.56 (ddd, δ = 17.9, 5.4, 2.3, 1H; -C(O)-CH₂⁻-O-), 1.45 (d, δ = 5.2, 3H; -O-CH(CH₃)O-).

2.3 Synthesis of 2-methyl-1,3-dioxan-4-one (MDO) monomer

According to literature, the cyclic esteracetal-monomer 2-methyl-1,3-dioxan-4-one (MDO) was synthesized from racemic 2-methylidihydropyran-3-one via Baeyer-Villiger Oxidation. 77% m-CPBA (69.17 g; 309 mmol; 1.2 eq) was dissolved in 600 mL methylene chloride (DCM), dried over MgSO₄ and filtered. With 100 mL of DCM, residual MgSO₄ was rinsed and filtered. The combined filtrates were transferred into a 2000 mL round bottom flask under nitrogen atmosphere and cooled to 0°C. 2-methyltetrahydropyran-3-one (25 mL; 257.2 mmol; 1 eq) was added stepwise via syringe over a time range of 30 minutes. The solution was then brought to room temperature and stirred for further 21.5 h. The precipitated meta-chlorobenzoic acid was removed by repeated filtration at -78°C. The aqueous fractions were extracted three times with DCM. The organic layers were washed with 10 mL of deionized water and subsequently dried over MgSO₄. Dichloromethane was removed by distillation. The crude oil was transferred into a 50 mL round bottom flask, dried over calcium hydride (100 mL) until no further carbon dioxide emission was evident. The aforesaid fractions were transferred into a 2000 mL round bottom flask under nitrogen atmosphere and cooled to 0°C.

The filtrate was washed with saturated sodium bicarbonate (8x 20 mL) until no further carbon dioxide emission was evident. The aqueous fractions were extracted three times with DCM. The organic layers were washed with 10 mL of deionized water and subsequently dried over MgSO₄. Dichloromethane was removed by distillation. The crude oil was transferred into a 50 mL round bottom flask, dried over calcium hydride for 12 h at room temperature and subsequently distilled via a short path distillation apparatus (56°C, 5·10⁻² mbar). The product was obtained as colourless liquid (17.58 g; 151.40 mmol; 59%) and stored at -17°C. The purified product was characterised by ¹H, ¹³C, COSY- and HSQC-NMR spectroscopy. All spectra are provided in Figure S1 - S4.

2.4 Homopolymerisation of MDO

One day before polymerisation, pyrene butanol (35.15 mg; 0.13 mmol; 1/80 eq.) was weighed into a dried 5 mL Schlenk flask, dried by azeotropic distillation with toluene for three times and further kept under high vacuum overnight. The polymerisation of MDO was carried out in a nitrogen atmosphere using dried glassware and a Teflon stir bar. MDO (1.0 mL; 10.25 mmol; 1 eq) was added to pyrene butanol (volumetrically by using a 1 mL syringe). 0.27 mL of dichloroethane (DCE) was added as solvent. After complete solvation of all compounds, DPP was added from a prepared stock solution using a 1 mL syringe (200 µL of a 24.635mM stock solution in DCE; 4.93 µmol; amount corresponds to a ratio of 1/2080 DPP/MDO). Conversion of MDO was determined by ¹H-NMR. After 24 h
polymisation was quenched by triethylamine (3.4 µL dissolved in 1 mL of DCM; 5 eq.) and stirred for 10 minutes. The crude product was subsequently purified by repeating precipitation three times in 40 mL of a THF/n-Hexane solution (1:9). The precipitate was isolated by centrifugation (4500 rpm, 20 min, 4°C) and removal of the supernatant. The remaining pellet was dissolved in 1 mL of DCM and precipitated again. After drying in vacuum at room temperature 237 mg of poly(pyrene butanol-PMD) was obtained as slightly yellowish, highly viscous liquid (19% yield relative to 100% of monomer). The homopolymer was analysed by SEC, MALDI and 1H-NMR as well as 13C-NMR, COSY, HSQC and HMBC spectroscopy. All data are provided in Figure S5 - S12.

2.5 Block copolymerisation of MDO onto mPEG

As an example, the synthesis of mPEGm-b-P(MDO13) is described. One day before polymerisation, 129.36 mg (64.9 µmol; 1/158 eq.) mPEG was weighed into a 10 mL Schlenk tube and equipped with a stir bar. For drying, 1 mL of toluene was added and the suspension was stirred for 10 min. Afterwards, the solution was frozen in liquid nitrogen and dried under reduced pressure (0.05 mbar). This procedure was repeated three times and the dried polymer was then kept at 0.05 mbar overnight. Into the prepared Schlenk tube equipped with a stir bar and dried mPEG, MDO was added volumetrically via a purged syringe (1 mL; 10.25 mmol 1 eq.). All compounds were dissolved without adding additional solvent. Subsequently, 150 µL catalyst stock solution was added volumetrically (concentration: 32.846 mM stock solution in dichloromethane; 4.93 µmol; amount corresponds to a ratio of 1/2080 DPP:MDO). Then, polymerisation was conducted at room temperature. Conversion of MDO was tracked by UV (m, 2H; -C(O)-C(CH3)-). Then, polymerisation was conducted at room temperature. Conversion of MDO was tracked by UV (m, 2H; -C(O)-C(CH3)-).

2.6 Self-assembly process of mPEG-b-poly(MDO)

a) Solvent switch method:

Approximately 10 mg of the diblock copolymer was dissolved in an appropriate volume of DMSO to target a concentration of 5 mg/mL. The solution was dialyzed against 1 L of phosphate buffered-saline solution at a pH of 7.4. In the first attempt, dialysis was carried out over 4 days (the pH was checked to be neutral by using pH indicator strips (MERCK) before and after dialysis). The solvent was exchanged every 24 hours. To prove preliminary degradation, the dialyzed solution was exchanged with DCM and analysed via 1H-NMR in CDCl3 where only PEG as degradation product could be found (acetaldheyde and 3-hydroxypropionic acid are water-soluble and could not be exchanged). Therefore, for the subsequent micellar preparation dialysis was reduced to 8 h and the solvent exchanged in 2 h intervals. Dialysis usually yielded particle solution of approximately 6 mL. Particles were subsequently measured by DLS (Figure S32 and Table S1).

b) Direct hydration technique:

Approx. 4 mg of polymer was weighed in a 5 mL Eppendorf tube, and an appropriate volume of 120 mM phosphate buffer (adjusted to a pH between 7.2 and 7.4) was added to target a concentration of 4 mg/mL. After ten minutes of shaking, the dissolution process was assisted by sonication for 15 min at 0°C. Directly after preparation, micellar size distribution was analysed by DLS measurement at 25°C (Figure 3 and Figure S33).
in vacuo within approximately 2 h affording a polymer pyrene film. All following subsequent steps were done as fast as possible to minimize possible hydrolysis. 354 µL of 10x PB buffer was added to each polymer pyrene film. The samples were sonicated for 30 min and repeatedly vortexed. Finally, 300 µL of each sample were pipetted into black 96 well plates and measured immediately after being measured with a microplate reader. Fluorescence emission was recorded at room temperature with an excitation wavelength of 333 nm and monitored from 360–450 nm (step size 1 nm).

2.7 Dual pH-responsive degradation studies

a) NMR degradation kinetics (Figure S43 and S44):
Micelles were prepared via dissolution method as described above. 8 mg mPEG<sub>b</sub>-b-P(MDO)<sub>15</sub> was weighed in a 5 mL Eppendorf tube, and 1 mL of 120 mM phosphate buffer prepared in D<sub>2</sub>O was added. The pH value was measured with a glass electrode standardized by aqueous buffer solutions at pH 4, 7 and 10. The pH-value can be approximated by:

\[ pH_{(buffer \text{ of } D_2O)} = pH_{measured} - 0.41 \]

Accordingly, the measured pH value of 7.5 correlates to a buffer with a pH of 7.1. After degradation, the pH dropped to a value of 7.0 which corresponds to a pH value of approx. 6.6.

After ten minutes of shaking, the dissolution process was assisted by sonication at 0°C for 15 min. Subsequently, NMR analysis was conducted. The degradation was calculated by subtracting the relative amount of 3-hydroxypropanoic acid (determined by its proton signal referenced to the proton signal of the HO-CH<sub>2</sub>- group) from the degree of polymerisation determined in CDC<sub>3</sub>. 

b) NMR analysis of micellar degradation products after exposure to acidic and basic conditions:
For acidic degradation micelles were prepared at a concentration of 8 mg/mL in D<sub>2</sub>O. To 1 mL of micellar solution, 50 µL of 1 M HCl or NaOH was added respectively and 1H-NMR spectra were 4 h after addition.

c) SEC analysis of micelles after exposure to acidic and basic conditions:
The degraded NMR samples from 2.7.b) were analysed by THF-SEC. Before measurement, the pH was neutralized. The solution was diluted by THF to approx. 4 mg/mL, filtered and analysed.

d) DLS analysis of micelles after exposure to acidic and basic conditions (Figure 4A, Figure S38 and S39):
To investigate the micellar response to poly(esteracetal) degradation, micelles were prepared via the direct dissolution method (as described in 2.6.b) and again analysed via DLS. For that purpose, micelles were prepared in 120 mM phosphate buffer at pH 7.4. After preparation micelles were observed for approximately 2 h confirming normal particle formation and stability, and subsequently divided into three new cuvettes. To each of them either 10 vol% 1 M HCl, phosphate buffer or 1 M NaOH was added. Subsequently, all probes were characterised by DLS overtime at given time points of approximately 2 h (addition of 1M HCl afforded pH 5.7, phosphate buffer remained neutral at pH 7.2 and addition of 1 M NaOH resulted in pH 10.8).

e) Zeta potential analysis of micelles immediately after exposure to neutral, acidic and basic conditions (Figure S40 and Figure S54):
To verify the proposed mechanism of micelle behaviour in response to change in pH, micelles were prepared (and loaded, respectively) as described via direct hydration technique at a concentration of 4 mg/mL in 5 mM HEPES buffer (at pH 7.14) and subsequently used for zeta potential analysis. Micelles were diluted at a mass concentration of 0.25 mg/mL in deionized water (0 mL of buffer), further acidified by 2 µL 1M HCl (affording a final pH = 4) and basified respectively by 3.5 µL of 1M NaOH (affording a final pH = 10) and measured again. Data is given as mean ± standard deviation of three repeated measurements per sample.

2.8 Enzymatic degradation studies

a) Enzymatic NMR degradation kinetics in toluene (Figure S45-S47):
The non-self-assembled block copolymers could be degraded enzymatically by solid-supported lipase in organic solvent. For that purpose, 19.55 mg of mPEG<sub>b</sub>-b-P(MDO)<sub>71</sub> was dissolved in 0.7 mL toluene-d8 and 25 mg of Novozyme 435 was added. A sample without enzyme served as negative control. The degradation was determined based on the formation of acetalddehyde referenced to the aromatic resonance signal of residual toluene. The signal ratio of intact polymer to toluene could be determined by NMR-analysis before enzyme addition.

b) Enzymatic NMR degradation kinetics in D<sub>2</sub>O phosphate buffer (Figure S5 and Figure S48):
The self-assembled block copolymer micelles could also be degraded enzymatically in aqueous solvent by solid-supported lipase. For that purpose, mPEG<sub>b</sub>-b-P(MDO)<sub>71</sub> micelles were prepared at a concentration of approx. 15 mg/mL in 10x PB buffer (prepared from D<sub>2</sub>O with 1% dimethyl sulfoxide (DMS) as internal standard; dimethyl sulfoxide was chosen as reference, because the chemical shift is apart from any relevant resonance signal; furthermore, it is highly hydrophilic, which minimizes potential interactions with the enzyme or micelles during degradation). 20 mg of Novozyme 435 per 1 mg of polymer was added into the NMR tube. The resin floated on top of the sample and therefore, did not interfere with the NMR measurement. In periodic intervals NMR measurements were recorded and analysed (compare Figure S48).

c) Enzymatic DLS degradation kinetics (Figure S5 and Figure S49):
Enzymatic micelle degradation could also be followed by DLS, as solid-supported lipase beads float on top of the aqueous medium inside the DLS cuvette. For that purpose, micelles were prepared as described by direct hydration technique at a concentration of 4 mg/mL. Subsequently, 93.70 mg of Novozyme 435 were added to 900 µL of micellar solution inside the cuvette and degradation monitored via DLS. A sample without lipase served as reference. Between each measuring time point, sealed samples were shaken on an orbital lab shaker.

2.9 Encapsulation and release of Nile Red into mPEG-b-poly(MDO) block copolymer micelles

a) Nile Red formulation:
In order to target a theoretical loading of 2.5 wt.% of Nile Red, approx. 4 mg polymer was weighed in a 5 mL Eppendorf tube and dissolved in an appropriate volume of a Nile Red stock solution (c = 0.111 mg/mL in acetone). Subsequently, the solvent was removed in vacuum for 2.5 h, resulting in a thin layer of Nile Red and mPEG-b-poly(MDO) covering the Eppendorf tube which enables a fast dissolution afterwards. 120 mM phosphate buffer was added targeting a polymer concentration of 4 mg/mL. Self-assembly and dye loading was assisted by sonication at 0°C for 30 minutes. Upon

This journal is © The Royal Society of Chemistry 20xx

J. Name., 2013, 00, 1-3 | 5

Please do not adjust margins
sonication, the particles were filtered via a GHP-filter with a pore size of 0.45 μm. The filtration step removed precipitated dye, which was not encapsulated. After filtration, a pink solution without any visible precipitations was obtained. In analogy, reference samples were prepared that contained only polymer or only Nile Red and served as blank probes (Figure S50). Directly after preparation micelles were analysed by DLS and UV/Vis spectroscopy.

2.12 Raw Blue macrophage cell culturing

RAW-Blue macrophages were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 0.02% Normocin and 0.01% Zeocin at 37 °C with 5% CO₂ saturation.

2.13 TLR 7 stimulation assay

Adifectin TLR receptor stimulation followed by NF-κB/AP-1 activation (detected by secretion of embryonic alkaline phosphatase) was performed on RAW-Blue cells as recommended by the manufacturer (InvivoGen). RAW-Blue cells were seeded into 96-well plates at a density of 90,000 cells/well in 180 μL culture medium. Cells were adhered overnight and then treated in each well was treated with 20 μL of sample at given Adifectin concentrations (listed in Table S2). After stimulation for 16 h, 50 μL of the supernatant from each well was collected and probed for secreted embryonic alkaline phosphatase (SEAP) using the QUANTI-Blue assay (InvivoGen). 150 μL QUANTI-Blue was added to each sample and incubated at 37 °C. Secretion of embryonic alkaline phosphatase levels were determined by measuring optical density at 650 nm using a microplate reader. Activity was determined by an increase in optical density relative to the negative control treated with PBS. The TLR 7/8 agonist 1-(4-(aminomethyl)benzyl)-2-butyl-1H-imidazo[4,5-c]quinolin-4-amine (IMDQ) served as positive control, as used in previous experiments. 12-14 A minimal interference between the 120 mM phosphate buffer and the SEAP assay was observed, due to an increased phosphate concentration. Therefore, the obtained results were corrected based on the contribution of the 120 mM phosphate buffer blank and the PBS blank. All experiments were conducted in quadruplicate (n = 4).

2.14 Cell viability assay

50 μL of 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL in PBS) was added to the RAW-Blue macrophages that were treated with the corresponding samples at given Adifectin/polymer concentrations. After MTT incubation for 3 h, the formed formazan crystals were dissolved by addition of 100 μL 10% SDS/0.01 M HCl and incubated overnight at 37 °C. Quantification was done by measuring the absorbance at 570 nm using a microplate reader. Cell viability was calculated in relation to positive (blank PBS, referred to as 100% viability) and negative (10% DMSO, referred to as 0% viability) control samples. All experiments were conducted in quadruplicate (n = 4).

3. Results and Discussion

Among different classes of amphiphilics polymers used for drug delivery, only a few guarantee full fragmentation into small molecules upon exposure to external stimuli. Poly(esteracetal)s can provide such degradation features towards variation in pH both below and above physiological values. While the ester group in the backbone is prone to basic hydrolysis or enzymatic degradation, the additional acetal group renders acidic triggered depolymerisation occurring in tumour microenvironments and their draining lymph nodes, as well as intracellularly inside endolysosomes. To the best of our knowledge, polymerising hydrophobic poly(esteracetal)s onto hydrophilic polymers providing amphiphilic poly(esteracetal)s has not been reported before and will be examined in this work with respect to applications in immunodrug delivery.
3.1 MDO Monomer Synthesis

According to literature, the cyclic esteracetal-monomer 2-methyl-1,3-dioxan-4-one (MDO) was synthesized from racemic 2-methylidihydrofuran-3-one via Baeyer-Villiger oxidation. The purified product was dried over calcium hydride and distilled in vacuum. The compound was obtained as colourless liquid with a yield of 59 %. The product’s structure could be validated by $^1$H-NMR, $^{13}$C-NMR spectroscopy, COSY- and HSQC-NMR spectroscopy shown in Figure S1 - S4. As we observed MDO to slowly degrade over time into 3-hydroxypropanoic acid and acetaldehyde, it was stored at $-18^\circ$C and always distilled freshly prior to use.

3.2 Homopolymerisation of poly(MDO)

Hillmyer et al. have previously evaluated the ring-opening polymerisation (DPP) as organic catalyst.

Hillmyer et al. performed homopolymerisations in bulk. These conditions seem to be primarily difficult for later block copolymerisations. Accordingly, MDO polymerisation conditions are required which take into account both solubility and dilution by a macro-initiator.

To address this question, we initially tested polymerisation at 7 M monomer dilution using pyrene butanol as initiator (compared to ~10 M in bulk). Polymer purification was performed as described by Hillmyer et al. via precipitation in hexane THF (9:1) and subsequent drying under reduced pressure at room temperature. Poly(pyrene butanol-P(MDO)) was characterised by NMR, SEC and MALDI-ToF MS (Figure S5 - S12). $^1$H-NMR analysis confirmed successful ring-opening of the cyclic monomer. The characteristic quartet resonance signal at 5.96 ppm verified incorporation of poly(esteracetal)s into the polymer backbone. By size exclusion chromatography (SEC) we observed reasonable narrow dispersities and in the elugrams UV absorption emerged nearly simultaneously with the refractive index (Figure S11), which confirmed that pyrene butanol was present as an end group on the poly(esteracetal)s (note that poly(esteracetal)s themselves are not UV active). In accordance with that, we could find intact MDO monomer repeating units (116 g/mol) by MALDI ToF mass spectrometry.

A variety of amphiphilic block copolymers were synthesized by polymerising MDO onto mPEG (2000 g mol$^{-1}$). mPEG is commonly used as hydrophilic, non-toxic coating for nanomedicines, because its stealth-like properties prevent opsonisation and plasma protein binding, increase colloidal stability and prolong circulation half-life. For polymerising MDO onto it, we applied the previous conditions of polymerisation. Alternatively, reduced temperatures would thermodynamically favour higher conversions. We, therefore, performed also polymerisations at 0°C, and indeed higher conversions could be found at longer reaction times, yet, we also observed broader molecular weight distributions (PDI increased from 1.31 to 1.79). We attribute this to lower viscosities at these temperatures that might further be affected by the presence of polymers. The latter would be more relevant for block copolymerisations using mPEG macroinitiators.

3.3 Block copolymerisation of mPEG-b-poly(MDO)

To conclude, polymerisations at room temperature and monomer dilutions at 7 M seem to provide acceptable product formation and were, therefore, applied in the following block copolymerisation reactions.
We carefully applied additional characterisation methods first MDO monomer unit by mPEG. S14 - S29, the degree of polymerisation of the MDO block was copolymer formation, as displayed by Figure 2B-D. By size groups referenced to the ethylene glycol units of mPEG). C: MALDI-ToF mass spectra of mPEG-\(\text{b-P(MDO)}\) block copolymer (red) and the mPEG macroinitiator (blue). D: \(\text{H-derived DOSY spectra (400 MHz)}\) of the mPEG-\(\text{b-P(MDO)}\) block copolymer (red) and the mPEG macroinitiator (blue).

solubility experiments with pyrene butanol initiated homopolymers indicated that they are slightly soluble in diethyl ether, whereas mPEG does precipitate nicely. Therefore, the crude product was subsequently precipitated in Et\(\text{O}\) for three times at \(-17^\circ\text{C}\). We obtained different PMDO block lengths at polymerisation degrees between 15 to 71 relative to the mPEG units. The obtained results are summarized in Table 1. \(\text{H-}^{1},\text{C-}^{13}\text{- and 2D-NMR analyses confirmed successful formation of mPEG-poly(esteracetal) block copolymers in all cases and the absence of major impurities. Extraordinary, the }^{1}\text{H-NMR spectrum of mPEG-\(\text{b-P(MDO)}\) is shown in Figure 2A while all remaining spectra can be found in the Supplementary Information (Figure S14 - S29). The degree of polymerisation of the MDO block was determined based on the integral of the poly(esteracetal) groups referenced to the ethylene glycol units of mPEG. Notably, the presence of exactly one single acetal proton signal can further be observed derived from the ring-opening of the first MDO monomer unit by mPEG.}

We carefully applied additional characterisation methods beyond \(\text{H-NMR spectroscopy to prove successful block copolymer formation, as displayed by Figure 2B-D. By size exclusion chromatography (SEC) as well as by DOSY NMR and MALDI-ToF MS analyses a distinct shift to higher molecular}

Table 1: Result of MDO block copolymerisation onto mPEG.

| \(\chi_{15}^{\text{H}}\) | con. [%] | \(\chi_{nprod}^{\text{H}}\) | \(M_{MDO}^{\text{prod}}\) [g/mol] | \(M_{nMDO}^{\text{prod}}\) [g/mol] |
|----------------|----------|----------------|-------------------------------|-------------------------------|
| mPEG-\(\text{b-P(MDO)}\) | 95 | 44 | 15 | 3700 | 5000 | 1.46 |
| mPEG-\(\text{b-P(MDO)}\) | 126 | 44 | 23 | 4700 | 6000 | 1.57 |
| mPEG-\(\text{b-P(MDO)}\) | 158 | 55 | 50 | 7800 | 8800 | 1.65 |
| mPEG-\(\text{b-P(MDO)}\) | 190 | 54 | 71 | 10200 | 10300 | 1.48 |

\(\chi_{15}^{\text{H}},\text{ initial monomer to initiator (= mPEG) ratio; con.: monomer conversion during polymerisation determined by }^{1}\text{H-NMR prior to polymer purification, }\chi_{nprod}^{\text{H}},\text{ monomer to initiator (= mPEG) composition of the purified polymer determined by }^{1}\text{H-NMR using the resonance signals of the poly(esteracetal) proton and referencing them to the proton signals of the ethylene glycol units of PEG. }M_{MDO}^{\text{prod}},\text{ calculated molecular weight based on the }\chi_{nprod}^{\text{H}}\text{ of the monomer and adding the molecular weight of the initiator (= 2kDa mPEG). }M_{nMDO}^{\text{prod}}\text{ and }\chi_{nMDO}^{\text{H}},\text{ number average molecular weight and distribution determined by THF SEC using mPEG calibration.}

weights/slower diffusion species was obtained for all block copolymers, while signals of remaining unmodified mPEG macroinitiator were generally absent (Figure S16, S19, S22 and S25).

In the MALDI-ToF mass spectra repetitive MDO (116 g mol\(^{-1}\)) and ethylene glycol (44 g mol\(^{-1}\)) units can be found (Figure S15, S18, S21 and S24). By detailed analysis and comparison with simulated mass signals (Figure S30) several mass peaks can be annotated to block copolymer species and not be explained by non-modified mPEG or self-initiated P(MDO) homopolymers. Taken together, these results clearly demonstrate that poly(MDO) can be attached with varying molecular weights onto mPEG and the resulting amphiphilic poly(esteracetal) block copolymers are further investigated for their self-assembly and drug encapsulation performance.

3.4 Block copolymer self-assembly

Generally, nanoparticles within a size range between 10 nm to 100 nm are considered as ideal drug carrier systems for several purposes including lymph node targeting after subcutaneous injection,\(^{13,23,24}\) For the latter, such particles would be large enough to prevent fast clearance by the bloodstream, but still small enough to provide sufficient diffusion through the water channels of the interstitium into the lymphatic system.

Therefore, we investigated the influence of the hydrophobic chain length of the mPEG-\(\text{b-poly(MDO)}\) block copolymers on their self-assembly behaviour in aqueous media. Note that poly(MDO) homopolymers alone could not be solubilised in water after extensive mixing (Figure S31). Thus, it was at first intended to prepare block copolymer micelles via a simple dialysis-derived solvent switch method, which is most commonly used for micelle preparations.\(^{25,26}\) For that purpose, the block copolymers were dissolved in DMSO and dialyzed against water. Subsequent DLS measurement revealed aggregates with a size of approximately 100 nm (Figure S32). However, an unusually low scattering intensity implied
poly(MDO) homopolymers seem to provide such low $T_g$ as the isolated polymer behaved like a highly viscous liquid (according to literature, the $T_g$ is around -30°C). A direct hydration without assistance of further organic solvents is a fast preparation method and would reduce preliminary hydrolysis as the hydrophobic domains are immediately forced into self-assembled and shielded structures in the aqueous environment. Thus, they would instantaneously be protected from hydrolysis compared to solvent exchange methods, where mixtures of organic and aqueous solvents are present during dialysis and cannot shield the hydrophobic block from preliminary degradation during the self-assembly process. In fact, when dialysis was performed for several days, no particles could be detected anymore and $^1$H-NMR proved polymer degradation to mPEG solely (Figure S32).

To that respect, an alternative preparation method had to be applied to access block copolymer micelles rapidly. It has been reported that for amphiphilic block copolymers with relatively low glass transition temperatures ($T_g$) for their hydrophobic block, micelles can be prepared via direct hydration. Interestingly, poly(MDO) homopolymers seem to provide such low $T_g$ as the isolated polymer behaved like a highly viscous liquid (according to literature, the $T_g$ is around -30°C). A direct hydration without assistance of further organic solvents is a fast preparation method and would reduce preliminary hydrolysis as the hydrophobic domains are immediately forced into self-assembled and shielded structures in the aqueous environment. Thus, they would instantaneously be protected from hydrolysis compared to solvent exchange methods, where mixtures of organic and aqueous solvents are present during dialysis and cannot shield the hydrophobic block from degradation immediately.

For the direct hydration formulation, 120 mM phosphate buffer was directly added to the block copolymers and the subsequent dissolution and self-assembly process was assisted by ultrasonication at 0°C. Indeed, DLS measurements proved the formation of micelles at different sizes depending on the length of the hydrophobic block. As shown in Figure 3, micellar sizes could be varied from 26 nm to 141 nm depending on the molecular weight of the P(MDO) block. Moreover, reproducibility for the direct hydration formulation method could further be validated by preparing micelles from mPEG-b-poly(MDO)$_{30}$ independently for three times. Subsequent DLS measurement always indicated almost identical micelles with similar sizes and distributions (Figure S33). In addition, critical micelle concentration (CMC) was accessed for mPEG$_{48}$-b-poly(MDO)$_{15}$ and mPEG$_{48}$-b-poly(MDO)$_{30}$ via pyrene fluorescence spectroscopy in order to compare the influence of the hydrophobic P(MDO) block on the self-assembly behavior. The intensities of pyrene’s first and third vibronic band maxima $I_1$ and $I_2$ at 375 nm and 387 nm varied depending on the molar block copolymer concentration (Figure S34 and S35). The critical micellar concentration (CMC) was then estimated by plotting the ratio $I_2/I_1$ versus logarithmic molar polymer concentration (Figure S36 and S37). Thereby, for the mPEG$_{48}$-b-poly(MDO)$_{30}$ block copolymer with the 3.3-fold larger P(MDO) block, a 3.3-fold lower CMC could be determined, which confirms that block copolymer self-assembly is triggered by the P(MDO) block in aqueous media.

Altogether, the synthesized amphiphilic mPEG-b-poly(MDO) block copolymers cover an attractive self-assembling behaviour and their resulting micellar size ranges are ideal for drug delivery purposes including passive lymph node delivery.$^{13,23}$

### 3.5 Dual pH-responsive degradation

To generally verify the dual pH-responsive degradation profile of mPEG-b-poly(MDO) block copolymers, micelles were exposed to acidic and basic conditions in aqueous media and analysed via $^1$H-NMR. For that purpose, micelles were prepared from mPEG$_{48}$-b-poly(MDO)$_{15}$ in D$_2$O. Under these conditions, the poly(MDO) block is not observable (Figure 4B). However, already 30 min after addition of 5 vol% 1 M HCl or NaOH, more than 80% of the degradation products appeared in the $^1$H-NMR spectra. A measurement after 4 hours proved complete degradation, as shown in Figure 4B. Micelles degrade into acetaldehyde, 3-hydroxypropanoic acid and mPEG$_{48}$. Note that acetaldehyde can afterwards either form a hydrate under acidic conditions, or it isomerizes to its enol as well as react further by aldol condensation under basic conditions (Figure 4B). All of these degradation products could be found in the $^1$H-NMR spectra. In analogy to these observations, also the elugram of subsequent SEC measurements proved complete poly(MDO) block degradation to the remaining hydrophilic mPEG-block, as shown by Figure S42. To investigate the degradation of the block copolymers on the micellar self-assembly behaviour, intact micelles were prepared in 120 mM phosphate buffer and monitored by DLS under acidic, neutral and basic conditions. After the integrity of the micelles was monitored at neutral conditions for up to two hours, they were treated with either 10 vol% 1 M HCl, phosphate buffer or 1 M NaOH, respectively. Thereby, the pH either dropped to 5.7, remained constant at 7.2 or increased to 10.8. For the varied pH values a significant drop in the scattering intensity was found after 2 hours indicating that under both acidic and basic conditions micelle degradation was tremendously accelerated (Figure 4A).

Interestingly, further analyses revealed that under acidic conditions micelles first formed micrometre-sized aggregates, as shown by an increase in scattering intensity (Figure 4A). Corresponding DLS-derived size distribution curves can be found in Figure S38. This responsive aggregation was also visible by eye due to the massive increase in turbidity, yet, it disappeared after 2 h completely. We hypothesize that this aggregation might be a consequence of preliminary hydrolysis of the stabilising PEG units at the interface between the hydrophobic cores and their hydrophilic coronas. Consequently, the hydrophobic domains are not sterically shielded anymore.
Figure 4: Dual pH-responsive degradation profile of mPEG-b-P(MDO) derived block copolymer micelles. (A) Observed scattering intensity by DLS (derived count rate) overtime at acidic (pH = 5.7), neutral (pH = 7.2), and basic conditions (pH = 10.8) with photographs of the corresponding samples inside the cuvette and a proposed mechanism explaining the degradation and self-assembly behaviour. (B) $^1$H-NMR analysis (400 MHz) of micelles at neutral conditions as well as after acidic and basic degradation. Due to the suppressed molecular motion of the P(MDO) block inside the micellar core, corresponding proton signals are diminished. Under acidic as well as in basic conditions micelles degrade into 3-hydroxypropionic acid and acetaldehyde (note acetaldehyde can form hydrates under acidic conditions or further isomerizes to enols as well as form aldol adducts at basic pH.

and, therefore, aggregate into large particles. To support this hypothesis, we performed zeta potential measurements immediately after lowering the pH and found an increase from slightly negative (-13 ± 6 mV) to neutral (-3 ± 7 mV) zeta potential values corresponding to a charge neutralization by protonation of the formed carboxylate groups (Figure S40). We schematically depicted this behaviour in Figure 4A.

On the contrary, under basic conditions micelles did not aggregate but gradually fragmentized into smaller particles, as found by a gradual decrease in count rate (Figure 4A) as well as by the monitored size distribution plots (Figure S35). Presumably, under basic conditions particles are slightly charged by the deprotonated carboxylic end groups, which prevent an aggregation due to charge repulsion. This could again be confirmed by zeta potential measurements directly after increasing the pH, where slightly negative zeta potential values (-13 ± 6 mV) further dropped to more negative (-25 ±
6 mV) which contribute to the colloidal stability of the formed degradation fragments in aqueous media (Figure S40).

According to preliminary published data suggesting acidic compartments to be present in compartments of lymph nodes,\textsuperscript{15} we want to pinpoint that such pH-responsive aggregation behaviour might be a promising strategy for tissue-selective particle trapping.

Micelle stability was also monitored over time under neutral conditions. In Figure 4A a gradual decrease in light scattering count rate was found for mPEG-b-P(MDO) block copolymers, too, although in a slower fashion. Retained micellar breakdown is desirable because degradation into hydrophilic small molecules with reasonable low molecular weight is expected to enable easy renal carrier clearance from the body, preventing long-term accumulation.\textsuperscript{37}

Comparison of micelles derived from different P(MDO) block lengths showed that those with the shortest P(MDO) content were already degraded in a few hours (Figure S41). We were able to track their degradation kinetics via \textsuperscript{1}H-NMR in phosphate-buffered D\textsubscript{2}O, too, as illustrated by Figure S43 and S44. Interestingly, the kinetics perfectly matched to the timeframes of disassembly observed by DLS from Figure S41. An increased particle stability, however, was confirmed for block copolymers with increasing hydrophobic block lengths (Figure S41). Micelles from mPEG\textsubscript{48}-b-P(MDO)\textsubscript{48} exhibited a reasonable stability and combined with their size below 100 nm they were considered as most promising for further delivery purposes.

### 3.6 Enzymatic degradation

Acidic hydrolysis conditions can physiologically occur either intracellularly (e.g. in endolysosomes at pH levels of up to 5) or extracellularly (e.g. in immunosuppressive tumour microenvironments or lymph nodes). Basic pH levels, however, are generally not reached under physiological conditions, yet, many hydrolytic esterase enzymes apply base-catalysed mechanisms to cleave ester bonds. Consequently, we studied poly(MDO) degradation behaviour in presence of esterases and chose lipase as an enzyme with high ester-cleaving activity at hydrophilic-hydrophobic interfaces.

In first trials, the enzymatic degradation was tested on nonself-assembling single mPEG-b-P(MDO) poly(esteracetal) block copolymers. Yet, this could only be conducted in presence of organic solvents (Figure S45). For that purpose, a recombinant lipase immobilized on acrylic resin beads was used (Novozyme 435), which facilitates ester cleavage in both organic (toluene) and aqueous environment according to the manufacturer. Block copolymer degradation experiments were performed in toluene-D\textsubscript{8} in order to monitor the formation of small molecular degradation products by \textsuperscript{1}H-NMR overtime (Figure S46). As control samples, mPEG-b-P(MDO) block copolymers were analysed in toluene-D\textsubscript{8} without enzyme, too, and they proved high polymer stability in organic media over 1 week (Figure S47). In the presence of lipase, however, gradual P(MDO) block degradation was observed by an increase in the formation of acetaldehyde coupled with a decrease of the polymeric esteracetal resonance signal (Figure S46). According to the low amount of water present in the toluene reaction mixture, polymer degradation was not complete and reached around 50% after 90 h (Figure S47). These results motivated us to perform further lipase degradation experiments also in buffered aqueous media (Figure 5).

For that purpose, mPEG-b-P(MDO)\textsubscript{48} micelles were prepared in H\textsubscript{2}O or D\textsubscript{2}O (supplemented with 120 mM phosphate at pH 7) by the direct hydration method (Figure 5A). After addition of lipase Novozyme 435, accelerated release of 3-hydroxypropionic acid as well as acetaldehyde (and its hydrate form, respectively) could be detected by \textsuperscript{1}H-NMR (Figure 5B). Kinetic monitoring of the \textsuperscript{1}H-NMR samples over time revealed that already within the first 1.5 h almost all P(MDO) was hydrolysed into its small molecular degradation products (Figure 5C), while in control sample without enzyme those hydrolysis products could be found gradually (after 90 h the expected amount of degradation products could be observed, Figure S48).

These findings could nicely be correlated to the scattering intensities observed during DLS kinetic experiments in presence of lipase. The
count rates of the formulated mPEG-b-P(MDO) micelles dropped within 2 h dramatically (Figure 5D), while a gradual decrease was found for the control samples only over longer time frame (Figure S49). Interestingly, in analogy to the DLS kinetic experiments in presence of NaOH, no increase in turbidity or scattering intensity was found during lipase-mediated degradation in contrast to the acid-catalysed degradation process at lower pH (Figure 4A). Consequently, lipase-mediated P(MDO) degradation probably follows a similar base-catalysed hydrolysis process, as hypothesized in Figure 4A (right), and confirms the unique dual pH-responsive versatility of mPEG-b-P(MDO)-based micelles for drug delivery purposes.

3.7 Loading and release studies

Ideal micellar-drug delivery systems should be able to encapsulate drugs inside their cores and release them upon desired stimuli that arise at their targeted side of action. To investigate the encapsulation and release performance of mPEG₄₈-b-P(MDO)₅₀ derived block copolymer micelles, Nile Red as hydrophobic and water-insoluble dye was encapsulated to mimic hydrophobic drug model compounds (Figure 6). Interestingly, the fraction of encapsulated Nile Red can be quantified by UV/Vis spectroscopy, while non-encapsulated Nile Red precipitates in water (solubility < 1 µg/mL) and can be filtered off easily (Figure S50 and S51). 28–31

Micelles formulated with Nile Red were prepared via an adapted direct hydration method targeting a theoretical loading of 2.5 wt.% of Nile Red. For that purpose, a polymer film containing Nile Red was first prepared from organic solvent and evaporated prior to direct hydration of the mixture. After sonication particles were filtered in order to remove non-encapsulated, precipitated dye (a schematic representation of the preparation is shown in Figure S50). After filtration, pink solutions without any visible precipitation were obtained. In comparison, applying the same procedure to pure Nile Red but lacking the amphiphilic polymer led to a clear colourless solution which also showed no characteristic absorbance band for Nile Red (compare Figure 6B and Figure S50). Loaded micelles were subsequently analysed by DLS and an increase in size confirmed successful dye encapsulation (Figure 6C). Moreover, Nile Red loading could be quantified by UV/Vis spectroscopy applying an external Nile Red standard curve determined in ethanol (Figure S51 and S52). Pure polymer micelles served as reference for absorbance due to particle scattering (Figure 6D). Based on the observed findings, a dye loading efficiency of 19 % was achieved for the mPEG₄₈-b-P(MDO)₅₀ derived block copolymer micelles, which corresponds to a dye loading of 0.5 wt%. Depending on the length of the P(MDO) block, drug loading and encapsulation efficiency could also be varied (Figure S53). For mPEG₄₈-b-P(MDO)₇₁ only 3.8% of the dye could be encapsulated affording a 0.1 wt.% dye load,

Figure 6: Nile Red formulated into mPEG₄₈-b-P(MDO)₅₀ micelles. (A) Schematic representation of Nile Red loading and release. Under acidic and basic conditions, micellar degradation is highly accelerated. Upon degradation, Nile Red is expected to be released and precipitate. (B) Nile Red formulated with and without polymer. Pictures are shown before and after filtration (450µm). Whereas Nile Red formulated with polymer is stabilized in solution, Nile Red was solely removed by filtration leading to a clear colourless solution. (C) DLS measurement and (D) UV/Vis absorbance spectra of micelles formulated with Nile Red and without. (E) Photograph of Nile Red loaded particles before and after degradation. (F) Lipase-mediated enzymatic degradation of Nile Red loaded mPEG₄₈-b-P(MDO)₇₁ micelles followed by light scattering count rate. (G) Lipase-mediated Nile Red release from mPEG₄₈-b-P(MDO)₇₁ micelles followed by UV-vis absorbance at 549 nm.
while for the larger mPEG$_{48}$-b-P(MDO)$_{71}$ polymers with higher hydrophobic to hydrophilic ratio 36% of dye were formulated into the corresponding micelles affording a 0.9 wt.% dye load, as determined by UV/Vis spectroscopy (Figure S53).

In addition, zeta potential measurements were conducted showing that dye loading hardly affected the zeta potential of the Nile Red loaded mPEG$_{48}$-b-P(MDO) micelles. Unloaded micelles provided a slightly negative zeta potential of -13 ±6 mV that barely increased upon dye loading to -10 ±10 mV, which is not significantly different from unloaded micelles. This behaviour can be explained by the rather low Nile Red loading below 1 wt. %.

Subsequent drug release measurements were conducted and they correspond to previous particle degradation (compare Figure 4A and Figure 5A). After decreasing the pH by addition of HCl, Nile Red loaded micelles started again to aggregate instantaneously, whereas under basic conditions aggregation was not observed (compare Figure S55). Consequently, under acidic conditions, DLS studies cannot be performed properly to correlate them to Nile Red release. We, therefore, followed the process visually by eye (Figure S56) and observed a massive increase in turbidity within the first 6 hours. Simultaneously, released Nile Red starts to adsorb to the bottom of the DLS cuvette. After one day, all drug-loaded micelles were fully degraded and solutions became colourless with precipitated dye on the bottom (Figure 6E and Figure S56).

As the UV absorbance of Nile Red is influenced by its solvation,28–31 UV/Vis spectroscopy can also be used as a tool to monitor its release from the drug carrier over time. However, as the decrease in pH significantly influenced particle aggregation, UV/Vis spectroscopy could not be applied to those formulations to monitor dye release at acidic pH. Alternatively, enzymatic degradation in the presence of lipase at pH 7 did not afford particle aggregation (compare Figure S55) and was, therefore, applied to study dye release kinetics by both UV/Vis spectroscopy and DLS. Similarly to findings of Figure 5D, a rapid decrease in light scattering count rate was found for the samples in the presence of lipase confirming again similar degradation behaviour for both empty and dye loaded micelles (Figure 6F).

In parallel, UV/Vis spectroscopy confirmed what was observed visually by eye (Figure S57). A rapid decrease in Nile Read absorbance maximum around 549 nm (compare Figure 6D) is found for the dye loaded micelles in the presence of lipase (Figure 6G) corresponding to the enzyme-mediated release of Nile Red from the micelles. Without lipase only a sustained degradation of mPEG$_{48}$-b-P(MDO)$_{70}$ derived block copolymer micelles (Figure 6F) corresponding to a gradual release of encapsulated Nile Red over time (Figure 6G) was detected at neutral pH.

3.8 Application as immune drug delivery system

Prior to further investigations as suitable drug carriers, the in vitro cytotoxicity of the mPEG$_{48}$-b-P(MDO)$_{70}$ micelles was studied by MTT-assay on Raw Blue Macrophages. Figure 7D indicates that concentrations up of 400 µg/mL did not influence the viability (compared to PBS buffer as negative control and 10% DMSO as positive control). Encouraged by these results, an immunostimulating TLR agonist could be formulated into mPEG$_{48}$-b-P(MDO)$_{70}$ in analogy to Nile Red.

For that purpose, the amphiphilic TLR 7 agonist Adifectin was selected. Its molecular structure is composed of a hydrophilic TLR 7 interacting head group, a basic spermine linker (which might favour additional interaction with the negatively charged carboxylate end groups of the mPEG$_{48}$-b-P(MDO)$_{70}$ block copolymers) and two hydrophobic phytanyl groups that guarantee interaction with the hydrophobic cores of the mPEG$_{48}$-b-P(MDO)$_{70}$ derived micelles (compare Figure S58). It was fabricated into a polymer film with mPEG$_{48}$-b-P(MDO)$_{70}$ and then rehydrated with 120 mM phosphate buffer. Adifectin itself can also be formulated into water according to the manufacturer’s guideline assisted by ethanol. In both cases with and without mPEG$_{48}$-b-P(MDO)$_{70}$ all samples were sonicated and filtered through GHP-filters of 0.45 µm pore size prior to use.

Subsequent DLS measurement showed only a slight increase in micellar size for the loaded micelles, which can be seen as a first indication for successful immunodrug loading (Figure 7B – note that Adifectin is an amphiphile and might probably be more incorporated into the particle interface than into the core, thus only slightly contributing to an increase in size). In addition, the UV/Vis spectrum in Figure 7C further proved successful formulation of Adifectin into the micelles. The drug load could be quantified by applying an external calibration curve (Figure S61), where micelles prepared without Adifectin served as blank sample.

Subsequently, the ability of in vitro immune cell TLR 7 activation was determined by incubating RAW-Blue macrophages as TLR 7 reporter cell line with different poly(esteracetal)/Adifectin concentrations (Table S2). Interestingly, the formulation containing the amphiphilic polymer was more potent compared to the free drug, although the polymer solely did not show any immune stimulation (Figure 7E). Additional quantification of the sample without block copolymer by UV/Vis spectroscopy, as shown in Figure S61, revealed that the increased TLR stimulation activity results from an enhanced ability to solubilise Adifectin during preparation. In fact, the presence of the amphiphilic mPEG$_{48}$-b-P(MDO)$_{70}$ block copolymer promotes the drug’s solubility into water and, thus, its accessibility to the TLR.
Retrospective correction of the determined Adifectin concentration according to Figure S61 showed that formulations with mPEG₄₈-b-P(MDO)₅₀ micelles did not lose Adifectin’s receptor activity and remained almost as potent as the free drug alone (Figure S60 and Table S3).

Furthermore, there was no indication of decreased cell viability on macrophages after treatment with the polymer formulation, as determined by MTT assay (Figure 7D). Taken together, these findings suggest that amphiphilic TLR agonists were safely and efficiently formulated by the mPEG-b-P(MDO)₅₀ block copolymers and retained their drug activity on the TLR receptor in a micellar form. Consequently, the dual pH-responsive behaviour towards rapid particle degradation was confirmed by DLS and ¹H-NMR. While micelles remained stable at pH 7.2, HCI or NaOH addition, as well as exposure to lipase led to immediate degradation.

In subsequent drug loading studies Nile Red as hydrophobic guest molecule could be loaded and released from the degradable amphiphilic block copolymers efficiently. Finally, in vitro studies were conducted where no cellular toxicities were found on macrophages. An amphiphilic TLR-7 agonist (Adifectin) was co-formulated into the mPEG-b-P(MDO) block copolymer micelles and its activity was investigated by TLR stimulation of RAW Blue macrophages. The degradable amphiphilic block copolymer was able to solubilize Adifectin more efficiently and retained the drug’s activity on the TLR receptor.

These findings suggest that mPEG-b-P(MDO) can be utilized for providing optimized delivery opportunities for promising immunodrug molecules, also towards possible lymph node focused delivery. In our ongoing research endeavours, we will focus on enhancing drug loading and micellar stability at neutral pH to prevent premature drug release before reaching its target side.

Conflicts of interest
There are no conflicts to declare.
Acknowledgements

The authors thank Monika Schmelzer for SEC measurements, Hans Joachim Räder and StefanTürk for MALDI-ToF MS measurements, and Petra Kindervater for NMR measurements. Moreover, the authors want to gratefully acknowledge financial support by the DFG through the Emmy-Noether program and the SFB 1066 Project B04.

References

1 a) A. P. Singh, A. Biswas, A. Shukla and P. Maiti, Signal transduction and targeted therapy, 2019, 4, 33; b) S. Senapati, A. K. Mahanta, S. Kumar and P. Maiti, Signal transduction and targeted therapy, 2018, 3, 7; c) Y. Zhang, Y. Huang and S. Li, AAPS PharmSciTech, 2014, 15, 862–871; d) A. Z. Wang, R. Langer and O. C. Farokhzad, Annual review of medicine, 2012, 63, 185–198; e) Rachel S. Riley, Carl H. June, Robert Langer and Michael J. Mitchell; f) E. H. Chang, J. B. Harford, M. A. W. Eaton, P. M. Boisseau, A. Dube, R. Hayeshi, H. Swai and D. S. Lee, Anal. Chem. and biophysical research communications, 2015, 468, 511–517; g) S. Mitragotri, P. A. Burke and R. Langer, Nature reviews. Drug discovery, 2014, 13, 655–672;
2 M. Talelli, M. Barz, C. J. Rijcken, F. Kiessling, W. E. Hennink and T. Lammers, Nano Today, 2015, 10, 93–117;
3 a) H. Chen, C. Khemtong, X. Yang, X. Chang and J. Gao, Drug discovery today, 2011, 16, 354–360; b) A. Tarscay and G. M. Keserü, Journal of medicinal chemistry, 2013, 56, 1789–1795;
4 W. J. Gradishar, S. Tjulandin, N. Davidson, H. Shaw, N. Desai, P. Bhar, M. Hawkins and J. O’Shaughnessy, Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005, 23, 7794–7803.
5 a) K. S. Lee, H. C. Chung, S. A. Im, Y. H. Park, C. S. Kim, S.-B. Kim, S. Y. Rha, M. Y. Lee and J. Ro, Breast cancer research and treatment, 2008, 108, 241–250; b) T. Chida, Y. Miura, H. Cabral, T. Nomoto, K. Kataoka and N. Nishiyama, Journal of controlled release : official journal of the Controlled Release Society, 2018, 292, 130–140; c) K. Kataoka, A. Harada and Y. Nagasaki, Advanced drug delivery reviews, 2012, 64, 37–48; d) N. Nishiyama, Y. Matsumura and K. Kataoka, Cancer science, 2016, 107, 867–874;
6 G. M. Lynn, R. Laga and C. M. Jewell, Cancer letters, 2019, 459, 192–203.
7 H. Cabral and K. Kataoka, Journal of controlled release : official journal of the Controlled Release Society, 2014, 190, 465–476.
8 a) H. Jiang, Q. Wang and X. Sun, Journal of controlled release : official journal of the Controlled Release Society, 2017, 267, 47–56; b) H. Cabral, Y. Matsumoto, K. Mizuno, Q. Chen, M. Murakami, M. Kimura, Y. Terada, M. R. Kano, K. Miyazono, M. Uesaka, N. Nishiyama and K. Kataoka, Nature nanotechnology, 2011, 6, 815–823; c) N. J. Butter, G. M. Mortimer and R. F. Minchin, Nature nanotechnology, 2016, 11, 310–311; d) Z. Zhao, A. Ukidve, V. Krishnan and S. Mitragotri, Advanced drug delivery reviews, 2019; e) K. Miyata, R. J. Christie and K. Kataoka, Reactive and Functional Polymers, 2011, 71, 227–234;
23 A. Schudel, D. M. Francis and S. N. Thomas, Nat Rev Mater, 2019, 4, 415–428.
24 C. Oussoren, J. Zuidema, D.J.A. Crommelin and G. Storm, Biochimica et Biophysica Acta (BBA) - Biomembranes, 1997, 1328, 261–272.
25 Y. Mai and A. Eisenberg, Chemical Society reviews, 2012, 41, 5969–5985.
26 Y. Zhu, B. Yang, S. Chen and J. Du, Progress in Polymer Science, 2017, 64, 1–22.
27 H. S. Choi, W. Liu, P. Misra, E. Tanaka, J. P. Zimmer, B. Ippy, M. G. Bawendi and J. V. Frangioni, Nature Biotechnology, 2007, 25, 1165–1170.
28 J. Jiang, X. Tong and Y. Zhao, Journal of the American Chemical Society, 2005, 127, 8290–8291.
29 S. Binauld and M. H. Stenzel, Chemical communications (Cambridge, England), 2013, 49, 2082–2102.
30 P. Greenspan and S. D. Fowler, Journal of lipid research, 1985, 26, 781–789.
31 J. Bigot, B. Charleux, G. Cooke, F. Delattre, D. Fournier, J. Lyskawa, L. Sambe, F. Stoffelbach and P. Woisel, Journal of the American Chemical Society, 2010, 132, 10796–10801.
Amphiphilic poly(esteracetal) micelles encapsulate potent immune modulatory drugs, but fall apart and release them upon dual pH or enzymatic stimuli.
Amphiphilic poly(esteracetal) micelles encapsulate potent immune modulatory drugs, but fall apart and release them upon dual pH or enzymatic stimuli.