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Reversibly self-assembled pH-responsive PEG-p(CL-g-TMC) polymersomes

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Abstract
Polymersomes have gained much interest within the biomedical field as drug delivery systems due to their ability to transport and protect cargo from the harsh environment inside the body. For an improved drug efficacy, control over cargo release is however also an important factor to take into account. An often employed method is to incorporate pH sensitive groups in the vesicle membrane, which induce disassembly and content release when the particles have reached a target site in the body with the appropriate pH, such as the acidic microenvironment of tumor tissue or the endosome. In this paper, biodegradable poly(ethylene glycol)-poly(caprolactone-gradient-trimethylene carbonate)-based polymeric vesicles have been developed with disassembly features at mild acidic conditions. Modifying the polymer backbone with imidazole moieties results in vesicle disassembly upon protonation due to the lowered pH. Furthermore, upon increasing the pH efficient re-assembly into vesicles is observed due to the switchable amphiphilic nature of the polymer. When this re-assembly process is conducted in presence of cargo, enhanced encapsulation is achieved. Furthermore, the potency of the polymeric system for future biomedical applications such as adjuvant delivery is demonstrated.

KEYWORDS
biodegradable, encapsulation, pH-responsive, polymersome, reversible

1 | INTRODUCTION
Polymeric nanoparticles have been recognized as an interesting carrier platform in the field of nanomedicine. The macromolecular character of the building blocks provides the particles with robustness, whereas their synthetic nature allows for the incorporation of functional elements contributing to their versatility. Furthermore, they have the capacity to encapsulate, protect and deliver cargo to a target site to enhance drug efficacy.[1]-[10] The use of polymeric vesicles or polymersomes for drug delivery is of particular interest due to their ability to transport both hydrophobic and hydrophilic cargo.[11]-[13] One import issue of all polymeric carrier systems is to incorporate a mechanism for content release. In order to evoke a desired biological response, release of the encapsulated cargo is required at the site of interest and, therefore, responsive nanoparticles have been designed that can disassemble employing a specific trigger. Stimulus-responsiveness has been extensively studied in the field of polymersomes, and
functionalities sensitive to pH, redox conditions or light have been integrated in the polymer membrane.\[14\]-[28] pH sensitivity is of particular interest as it varies in different parts of the body and consequently can lead to targeted delivery. For example, the acidic endosomal environment (pH <6) enables pH responsive platforms to deliver and release their bio-active compounds inside the cell after endocytosis, enhancing the therapeutic effect.\[29\] Many pH-sensitive polymersomes have been prepared. By exploiting the hydrolysis of acid labile linkers, such as acetals, hydrazine and imines, pH sensitive polymersomes have been devised and used for intracellular drug delivery.\[18,19\] More widely studied is the use of ionizable groups for triggered membrane disruption. By incorporating carboxylic or sulfonic acid groups, or basic moieties, such as amines, pH sensitivity can be achieved.\[19,20,30,31\] Up to now pH responsiveness has mainly been used for the triggered disassembly of the particles. The reversed process, particle reassembly, remains underexplored, whereas this could be exploited to ensure cargo loading in an aqueous environment.

Often, responsive systems are composed of non-biodegradable polymers, which could hamper their facile translation to biomedical applications.\[15,24,32-35\] To this end, biodegradable block copolymers were developed to facilitate their implementation in the medical field. Recently, in our group we reported on poly(ethylene glycol)-poly(caprolactone-gradient-trimethylene carbonate) (PEG-p(CL-g-TMC)) amphiphilic block copolymers with biomedical potential. The carbonate group is known to be biodegradable, and the TMC monomer can easily be modified to carry functional side chains.\[36]-[38]\] The incorporation of pH-sensitive imidazole side chains in the hydrophobic domain of this amphiphilic block copolymer allowed the polymers to form multi-lamellar particles at physiological pH, while particle reconfiguration into positively charged micellar structures occurred upon a pH decrease (pH < 6.5), due to protonation of the imidazole groups.\[36\] This change in particle size and charge facilitated enhanced tissue penetration in a 3D tumor model. This class of block copolymers however has not been exploited yet for the construction of pH responsive vesicles.

Herein, we have developed pH-responsive PEG-p(CL-g-TMC) polymersomes which are destabilized at mild acidic conditions, as found in the endosome,\[39\] for future use as intracellular delivery vesicles. In order to achieve polymersome destabilization at endosomal pH, a series of block copolymers was synthesized carrying the imidazole functionality (Figure 1(A)). After establishing appropriate conditions for polymersome assembly the pH dependent behavior of the vesicles was investigated. Remarkably, polymersomes could be fully disassembled in acidic pH and subsequently reassembled in similar sized vesicles upon a pH increase (Figure 1(B)). Taking advantage of the interaction between the imidazole-functionalized polymer and hydrophilic cargo, upon reassembly a higher encapsulation was accomplished as compared to the conventional direct hydration method used previously.

2 | EXPERIMENTAL SECTION

2.1 | Materials

All chemicals were used as received unless otherwise stated. Monomethoxy-polyethylene glycol, 1 kDa (≥95%), was obtained from JenKem Technology USA. Monomer trimethylene carbonate was purchased from TCI Europe (>98%). Monomer ε-caprolactone (99%) and reagent bis(pentafluorophenyl) carbonate (97%) were purchased from Fluorochem. CpG-ODN 2006 was purchased from Biomers.net. Sulfo-Cyanine5 NHS ester (95%) was purchased from Lumiprobe. Amicon Ultra 0.5 ml (MWCO = 100 kDa) spin-filters were purchased from Sigma Aldrich. All other chemicals and reagents, including ovalbumin and 2,2-bis(hydroxymethyl) propionic acid (98%), methanesulfonic acid (≥99%), 1-(3-aminopropyl)imidazole (≥97%), cesium fluoride (99%), triethylamine (≥99.5%) were supplied by Sigma–Aldrich. Ultra-pure water was obtained from Merck.
Millipore Q-Pod system with a 0.22 μm Millipore Express 40 filter (18.2 MΩ).

2.1.1 | Experimental note

All reactions are performed under argon atmosphere using dried glassware unless stated otherwise.

2.2 | Measurements

2.2.1 | Nuclear magnetic resonance

$^1$H-nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 MHz spectrometer with CDCl$_3$ as a solvent and TMS as internal standard.

2.2.2 | Gel permeation chromatography

Gel permeation chromatography (GPC) was conducted using a Shimadzu Prominence-i GPC system equipped with a PL gel 5 μm mixed D and C column (Polymer Laboratories) and a Shimadzu RID-20A differential refractive index detector; THF was used as an eluent with a flow rate of 1 ml min$^{-1}$; polystyrene standards.

2.2.3 | Protein concentration measurements

Protein concentrations were determined by the Pierce™ BCA Protein Assay according to the manufacturer’s instructions at 562 nm or by fluorescence spectroscopy, Ex/Em = 598/664, on a Tecan Safire II UV–Vis fluorescence and absorbance plate-reader in a Greiner Flat Transparent (absorbance) or Black (fluorescence) 96-well plate.

2.2.4 | CpG ODN concentration measurements

CpG-ODN concentrations were determined on a Thermo Scientific NanoDrop™ 1000 (absorbance 280 nm).

2.2.5 | Dynamic light scattering and zeta-potential measurements

Dynamic light scattering(DLS) and zeta-potential measurements were executed on a Malvern Zetasizer Nano ZSP and the supplied software, Zetasizer Software v7.13 was used for processing and analyzing the data.

2.2.6 | Cryogenic transmission electron microscopy

Experiments were performed using the TU/e CryoTitan (Thermo Fisher Scientific) equipped with a field emission gun and autoloader and operated at 300 kV acceleration voltage in low-dose bright-field TEM mode. Samples for cryogenic transmission electron microscopy (cryo-TEM) were prepared by glow-discharging the grids (Lacey carbon coated, R2/2, Cu, 200 mesh, EM sciences) in a Cressington 208 carbon coater for 40 s. Then, 4 μl of the polymersome solution was pipetted on the grid and blotted in a Vitrobot MARK III at room temperature and 100% humidity. The grid was blotted for 3 s (offset −3) and directly plunged and frozen in liquid ethane. Cryo-TEM images were acquired with zero loss energy filtering mode (Gatan GIF 2002, 20 eV energy slit) on a CCD camera (Gatan model 794). Image analysis and processing was done using the software ImageJ (version 1.53c), provided for free by the National Institute of Health, USA. Membrane width was determined by the Line function followed by the Measure option.

2.3 | Preparation of poly(ethylene glycol)$_{22}$-block-poly[(e-caprolactone)$_{38}$-gradient-(trimethylene carbonate)$_{37}$]

The block copolymer was synthesized according to a previously reported literature procedure aiming for a composition of poly(ethylene glycol)$_{22}$-block-poly[(e-caprolactone)$_{38}$-gradient-(trimethylene carbonate)$_{37}$] (PEG$_{22}$p(CL$_{38}$g-TMC$_{37}$))$_{12,41}$ Briefly, a 250 ml single necked round bottom flask, equipped with a magnetic stir bar and argon inlet, was charged with macrorinitiator monomethoxy-poly (ethylene glycol) (Mn ~1000 g/mol; 705.2 mg; 0.71 mmol), e-caprolactone (εCL) (2.74 ml; 35 eq.; 24.7 mmol) and trimethylene carbonate (TMC) (2.52 g; 35 eq.; 24.7 mmol). Traces of water were removed by dissolving the solids in anhydrous toluene followed by concentration in vacuo; this was done twice. Under argon, the dried reagents were redissolved in dry dichloromethane (DCM) (150 ml, [εCL] = 0.17 M) while stirring, and methane sulfonic acid (MSA) was added (0.1 eq. with respect to εCL). The reaction was stored at room temperature for approximately 24 h until there was no evidence of residual monomer from the $^1$H-NMR spectra. After completion of the reaction, confirmed by $^1$H-NMR spectroscopy, the reaction mixture was diluted using DCM. The organic phase was washed with
saturated NaHCO₃ (aq.) followed by water and finally a brine wash. The remaining water from the organic phase was removed using Na₂SO₄ (5–10 min) and the mixture was then filtered and concentrated in vacuo. An oily colorless product was collected. The remaining oil was lyophilized from 1,4-dioxane for 2 days to yield a waxy oil (92% yield, D > 1.20, Figure S6).

Both purity and copolymer composition were confirmed using ¹H-NMR, Figure S1 (400 MHz, CDCl₃). Composition was calculated by using the protons of 1 kDa poly(ethylene glycol) (3.62–3.68 ppm), terminal methyl unit (s, 3.38 ppm), TMC-CH₂ (m, 1.89–2.11 ppm), ε-CL CH₂ (m, 1.33–1.48 ppm), ε-CL 2× CH₂ (m, 1.54–1.76 ppm), ε-CL 2× CH₂ (m, 2.25–2.40 ppm) and 2× CH₂ TMC + CH₂ ε-CL (4.01–4.36 ppm).

2.4 | Synthesis of pentafluorophenyl 5-methyl-2-oxo-1,3-dioxane-5-carboxylate (TMC-OPhF₅)

The compound was synthesized according to a previously reported procedure.³⁶,⁴⁰ A 100 ml three necked round bottom flask, equipped with a magnetic stirrer, was charged with 2,2-bis(hydroxymethyl)propionic acid (Bis-MPA; 5 g; 37.3 mmol), bis(pentafluorophenyl) carbonate (36.7 g; 2.5 eq.; 93.2 mmol) and CsF (1.7 g; 0.3 eq.; 11.2 mmol). 70 ml anhydrous THF was added and the mixture was stirred overnight under argon atmosphere. First a heterogeneous solution was obtained, which became homogenous after an hour. After ~24 h the mixture was concentrated in vacuo and reddissolved in DCM. The precipitated by-product, pentafluorophenol, was removed via filtration. The organic phase was collected and washed with NaHCO₃ (aq.) and water. The organic phase was collected and dried over MgSO₄. After filtration, DCM was removed in vacuo, yielding a solid/oily product. The compound was recrystallized by dissolving in hot ethyl acetate and, subsequently, n-hexane was added till cloudy. Hereafter, the mixture was heated until the solution became clear. The pure compound crystallized out, resulting in a white crystalline powder. Yield: 7.75 g (64%).

¹H-NMR, Figure S2, (400 MHz, CDCl₃), δ ppm: 4.85 (d, J = 10.8 Hz, 2H), 4.36 (d, J = 10.8 Hz, 2H), 1.55 (s, 3H).

¹⁹F-NMR, Figure S5A, (376 MHz, CDCl₃), δ ppm: 152.70–154.93 (m, 2F), 156.13 (t, 1F), 161.15–162.38 (m, 2F).

2.5 | Preparation of PEG₂₂-p(CL₃₆-g-TMC) (OPhF₅)₁₁

The same protocol was used as described for (PEG-p(CL-g-TMC)). Monomethoxy-poly(ethylene glycol) (Mn ~1000 g/mol), ε-caprolactone (35 eq.) and TMC-OPhF₅ (11 eq.) were used, resulting in PEG₂₂-p(CL₃₆-g-TMC(OPhF₅)₁₁) as a waxy oil (92% yield, D = 1.15, Figure S6). Both purity and copolymer composition were confirmed using ¹H-NMR, Figure S3 (400 MHz, CDCl₃).

Composition was calculated by using the protons of the 1 kDa poly(ethylene glycol) (3.62–3.68 ppm), terminal methyl unit (s, 3.38 ppm), ε-CL CH₂ + TMC-OPhF₅ CH₃ (m, 1.33–1.48 ppm), ε-CL 2× CH₂ (m, 1.54–1.76 ppm), ε-CL 2× CH₂ (m, 2.25–2.40 ppm), CH₂ ε-CL (4.01–4.19 ppm) and TMC-OPhF₅ 2× CH₂ (m, 4.30–4.60 ppm). ¹⁹F-NMR, Figure SSB, (376 MHz, CDCl₃), δ ppm: 152.70–153.08, (m, 2F), 157.07 (b, 1F), 161.66–162 (m, 2F).

2.6 | Preparation of PEG₂₂-p(CL₄₆-g-TMC) (OPhF₅)₆

The same protocol was used as described for (PEG-p(CL-g-TMC)). Monomethoxy-poly(ethylene glycol) (Mn ~1000 g/mol), ε-CL (45 eq.) and TMC-OPhF₅ (5 eq.) were used, resulting in PEG₂₂-p(CL₄₆-g-TMC(OPhF₅)₆) as a sticky solid (83% yield, D = 1.21, Figure S6). Both purity and copolymer composition were confirmed using ¹H-NMR, Figure S3, (400 MHz, CDCl₃).

Composition was calculated by using the protons of the 1 kDa poly(ethylene glycol) (3.62–3.68 ppm), terminal methyl unit (s, 3.38 ppm), ε-CL CH₂ + TMC-OPhF₅ CH₃ (m, 1.33–1.48 ppm), ε-CL 2× CH₂ (m, 1.54–1.76 ppm), ε-CL 2× CH₂ (m, 2.25–2.40 ppm), CH₂ ε-CL (4.01–4.19 ppm) and TMC-OPhF₅ 2× CH₂ (m, 4.30–4.60 ppm). ¹⁹F-NMR, Figure SSB, (376 MHz, CDCl₃), δ ppm: 152.70–153.08, (m, 2F), 157.07 (b, 1F), 161.66–162 (m, 2F).

2.7 | Preparation of PEG₂₂-p(CL₃₆-g-TMC) (TMCI₁₁) (TMCI₁₁)

According to a literature procedure PEG₂₂-p(CL₃₆-g-TMC) was synthesized.³⁶ A 10 ml single necked round bottom flask, equipped with a magnetic stirrer, was charged with PEG₂₂-p(CL₃₆-g-TMC(OPhF₅)₁₁) (407.96 mg; 0.0454 mmol) which was dissolved in 4 ml dry THF. The reaction mixture was cooled to 0°C using an ice bath. Hereafter, TEA (63.8 µl; 0.5 mmol; 1.1 eq. with respect to the pentafluoro phenyl ester) and 1-(3-aminopropyl)imidazole (74 µl; 0.52 mmol, 1.05 eq. with respect to the pentafluoro phenyl ester) were dissolved in 1,4-dioxane for 2 days to yield a white crystalline powder. Yield: 7.75 g (64%).
removed, and the reaction mixture was allowed to react for approximately 1 h. The reaction mixture was precipitated in diethyl ether (liq. N2 cooled to almost freezing). The supernatant was removed and the remaining solid was precipitated again from THF. Hereafter, the supernatant was removed and the solid was lyophilized from 1,4-dioxane for 1–2 days resulting in an oil (50% yield).

Both purity and copolymer composition were confirmed using $^1$H-NMR, Figure S4, (400 MHz, CDCl₃). Degree of functionalization was calculated by using the protons of the 1 kDa poly(ethylene glycol) (3.51 ppm), TMCI CH₂ (m, 1.76–1.85 ppm), TMCI CH₂ (t, 2.96–3.07 ppm) and TMCI CH₂ (t, 3.86–3.93 ppm). $^{19}$F-NMR, Figure S5D, (376 MHz, CDCl₃); no signal.

2.8 | Preparation of PEG₂₂-p(CL₄₆-g-TMCI₆) (TMCI₆)

The same protocol was used as described for TMCI₁₁ using PEG₂₂-p(CL₄₆-g-TMC(OPhF₅)₆) as starting compound and resulting in PEG₂₂-p(CL₄₆-g-TMCI₆) as a sticky solid (81% yield).

Both purity and copolymer composition were confirmed using $^1$H-NMR, Figure S4, (400 MHz, CDCl₃). Degree of functionalization was calculated by using the protons of the 1 kDa poly(ethylene glycol) (3.51 ppm), TMCI CH₂ (m, 1.76–1.85 ppm), TMCI CH₂ (t, 2.96–3.07 ppm) and TMCI CH₂ (t, 3.86–3.93 ppm). $^{19}$F-NMR, Figure S5D, (376 MHz, CDCl₃); no signal.

2.9 | Labeling ovalbumin with Cy5-sulfo-NHS (Cy5-ovalbumin)

Ovalbumin (OVA) was dissolved in PBS (0.5 mg/ml) and Cy3-sulfo-NHS (5 eq.) was added. After complete dissolution the mixture was shaken at 37°C for 1 h. Hereafter, the unreacted dye was washed away by spin-filtration (MWCO = 10 kDa) and the protein was washed 3x with PBS (1x) followed by a wash with MilliQ. The remaining solution was lyophilized overnight resulting in a dark blue powder. Successful labeling was confirmed by fluorescence spectroscopy.

2.10 | Polymersome formation by direct hydration

PEG-p(CL-g-TMC[I]) polymer was dissolved in PEG350 at 10 wt%. A volume of 20 μl of the mixture was added to a 5 ml flat-bottom glass vial, equipped with a magnetic stir bar (length stir bar $\approx$ inner diameter vial), by placing the solution directly next to the stir bar in the middle of the vial. 80 μl PBS (1x) at room temperature was added rapidly to the mixture while stirring at 250 rpm. An opaque mixture was formed immediately. After 5 min of stirring PBS (1x) was slowly added in order to dilute the sample to the concentration needed. The sample was stored at 4°C.

2.11 | DLS and zeta-potential measurements

DLS and zeta-potential samples were prepared by diluting the polymersome samples 10 times in medium of choice, e.g. PBS, McIlvaine buffer or MilliQ at a specific pH.

A typical DLS measurement was executed as follows: 100 μl polymersome solution (~ 5 mg/ml) was diluted in 900 μl PBS or McIlvaine buffer of pH of choice. Hereafter, the pH was monitored, and the sample measured on DLS for zeta-potential and hydrodynamic radius.

2.12 | Typical encapsulation experiment

PEG-p(CL-g-TMC[I]) polymer was dissolved in PEG350 at 10 wt%. A volume of 20 μl of the mixture was added to a 5 ml flat-bottom glass vial, equipped with a magnetic stir bar (length stir bar $\approx$ inner diameter vial), by placing the solution directly next to the stir bar in the middle of the vial. A volume of 80 μl of a solution of OVA(-Cy5) or CpG (at various concentrations, see Table 2), was added rapidly to the mixture while stirring at 250 rpm. An opaque mixture was formed immediately. After 5 min buffer was slowly added in order to dilute the sample to the concentration needed. Hereafter, the sample was purified. Typically, a 500 μl spin-filter (MWCO = 100 kDa) was filled with 100 μl polymersome-cargo mixture and cooled to 4°C. The sample was filtered at 3500 rcf at 4°C for 30 min. This was repeated three times adding approx. 400 μl buffer each time. After the last run the sample volume was diluted to the original volume in order to measure the cargo concentration and encapsulation efficiency. This was done by absorption and/or fluorescence spectroscopy (see procedure concentration and encapsulation efficiency determination, Section 2.14).

2.13 | Encapsulation by re-assembly

PEG-p(CL-g-TMCI) polymer was dissolved in PEG350 at 10 wt%. A volume of 20 μl of the mixture was added to a
5 ml flat-bottom glass vial, equipped with a magnetic stir bar (length stir bar ≈ inner diameter vial), by placing the solution directly next to the stir bar in the middle of the vial. A volume of 80 μl PBS (1x) at room temperature was added rapidly to the mixture while stirring at 250 rpm. An opaque mixture was formed immediately. After 5 min of stirring 40 μl of 0.1 M HCl was added to disassemble the polymersome sample, obtaining a clear solution. Then, 80 μl CpG (916 μg/ml) was added, followed by the addition of 300 μl PBS (1x) inducing polymersome formation. If needed, pH was adjusted to >7.4 using 0.25 M NaOH while stirring. Hereafter, the sample was purified. Typically, a 500 μl spin-filter (MWCO = 100 kDa) was filled with 480 μl polymersome-cargo mixture and cooled to 4°C. The sample was filtered at 3500 rcf at 4°C for 30 min. This was repeated three times adding approx. 400 μl PBS (1x) each time. After the last run the sample volume was diluted to the original volume, 100 μl, in order to measure the cargo concentration and encapsulation efficiency. This was done by absorbance spectroscopy see procedure concentration and encapsulation efficiency determination, Section 2.14.

2.14 | Concentration and encapsulation efficiency determination

OVA concentration and encapsulation efficiency were determined by the colorimetric Pierce™ BCA Protein Assay using a plate-reader. In short, a stock solution of OVA in PBS was made, diluted and subsequently loaded in a transparent 96-well plate to create a calibration curve (562 nm, 0–2000 μg/ml, R² = 0.99) according to the Pierce™ BCA Protein Assay kit. Hereafter, empty polymersomes were loaded, as blank, as well as the protein filled polymersomes. Coloring reagent was added and after an incubation period of 30 min at 37°C, allowing the reagent to color the protein, the absorbance values were measured from each well (562 nm). The measured absorbance value of the blank polymersome sample was subtracted from the OVA filled polymersome sample in order to remove the background signal caused by the opaque polymersome solution. Hereafter, concentration was determined by comparing the measured signal to the calibration curve.

CpG concentration and encapsulation efficiency were determined by first creating a dilution series of CpG dissolved in a mixture of DMSO and water (3:1) followed by measuring the absorbance of the dissolved molecule on a Nanodrop creating a calibration curve (280 nm, 0–1000 μg/ml, R² = 0.99). Hereafter, empty and CpG loaded polymersome samples were dissolved in DMSO (3:1; DMSO:sample), resulting in dissolution of the polymer, and measured using the Nanodrop. The absorbance value of the empty polymersome sample was subtracted from the CpG loaded polymersome sample in order to remove the background signal of the dissolved polymer. Hereafter, concentration was determined by comparing the measured signal to the calibration curve.

Encapsulation efficiency was calculated as follows:

\[
\text{Encapsulation efficiency} \, (\%) = \frac{[\text{Encapsulated cargo}] \times 100}{[\text{Total cargo added}]}
\]

Measurements were carried out in duplicate and the concentration, standard deviation as well as the encapsulation efficiencies are given in Table 2.

3 | RESULTS AND DISCUSSION

3.1 | Polymersome preparation

In order to attain pH sensitive amphiphilic block copolymers capable of forming polymersomes which could be disassembled at low pH, a series of monomethoxy-PEG-p(CL-g-TMCI) polymers was synthesized. Earlier work in our group showed that similar polymers can self-assemble in multilamellar structures that reassembled into cationic micelles when exposed to low pH.[36] To develop a system that provided well-defined vesicles which could quickly disassemble upon lowering the pH to levels found in the endosome, different amounts of a functional TMC monomer, carrying the pH responsive imidazole group, were incorporated in the hydrophobic chain of the amphiphilic copolymer. The imidazole moiety was introduced via a post-polymerization modification strategy. First, cationic ring opening polymerization was performed of trimethylene carbonate pen-tafluorophenyl ester (TMC-OPhF₅) with εCL using macroinitiator monomethyl PEG and MSA as catalyst (Scheme S1).[37] After co-polymerization, the pH sensitive intensity of the blank polymersome sample was subtracted from the Cy5-OVA filled polymersome sample in order to remove the background signal caused by the opaque polymersome solution. Hereafter, concentration was determined by comparing the measured signal to the calibration curve.
imidazole functionality was introduced by reacting the primary amine of 1-(3-aminopropyl)imidazole with the pentafluorophenyl ester incorporated in the backbone of the polymer (Scheme S2).\[36,38,40\] To allow robust formation of polymersomes the hydrophilic volume of the block copolymers was set to 10–14 wt\%.[12,41] To this end, PEG\(_{22}\)-p(CL\(_{36}\)-g-TMC\(_{11}\)) (TMCI11) and PEG\(_{22}\)-p(CL\(_{46}\)-g-TMC\(_{6}\)) (TMCI6) were synthesized. Non-pH responsive polymer PEG-p(CL-g-TMC) was synthesized according to literature procedures by cationic ring opening polymerization using MSA as catalyst.[12,36] The degree of polymerization was determined by \(^1\)H-NMR spectroscopy and dispersity of all polymers was measured by SEC. An overview of the obtained polymers is shown in Table 1.

Polymersome formation for PEG-p(CL-g-TMC), TMCI11 and TMCI6 was induced by the direct hydration method.[12,41] In short, the polymer was dissolved in low molecular weight poly(ethylene glycol) and stirred in a vial equipped with a stir bar while rapidly adding PBS (pH 7.4). Hereafter, size measurements were performed by DLS and polymersome formation was confirmed by cryo-TEM analysis (Figure 2). Polymersomes with a hydrodynamic size of 130, 160 and 150 nm and with a membrane thickness of 17.1 (±2.0, \(n = 13\), \(n = \) number of vesicles, see experimental section Cryogenic transmission electron microscopy [cryo-TEM] for more information, Section 2.2.6), 11.6 (±2.2, \(n = 15\)) and 12.7 nm (±1.6, \(n = 16\)) were obtained for PEG-p(CL-g-TMC), TMCI11 and TMCI6, respectively. These values, size and membrane thickness, are typical for polymersomes and are in line with previous research with regards to PEG-p (CL-g-TMC) vesicles.[12,42,43,44]

### 3.2 pH triggered polymersome destabilization

To investigate the effect of the imidazole group on polymersome stability they were studied at different pH conditions. First, non-pH responsive PEG-p(CL-g-TMC) polymersomes were kept for 10 days at pH 7.4 at 37°C (-Figure S7). No size changes were observed, demonstrating

### Table 1 Overview of obtained polymers including yield and dispersity

| Polymer \(^{b}\) | Abbreviation | Yield (%) | Dispersity (Đ) \(^{b}\) |
|-----------------|--------------|-----------|-------------------|
| PEG\(_{22}\)-p(CL\(_{36}\)-g-TMC\(_{35}\)) | PEG-p(CL-g-TMC) | 92 | 1.22 |
| PEG\(_{22}\)-p(CL\(_{36}\)-g-TMC(OF\(_{5}\))\(_{11}\)) | — | 92 | 1.15 |
| PEG\(_{22}\)-p(CL\(_{46}\)-g-TMC(OF\(_{5}\))\(_{6}\)) | — | 83 | 1.20 |
| PEG\(_{22}\)-p(CL\(_{36}\)-g-TMCI\(_{11}\)) | TMCI11 | 84 | — |
| PEG\(_{22}\)-p(CL\(_{46}\)-g-TMCI\(_{6}\)) | TMCI6 | 81 | — |

**Abbreviations:** PEG-p(CL-g-TMC), poly(ethylene glycol)-poly(caprolactone-gradient-trimethylene carbonate).

\(^{b}\)DP determined by \(^1\)H-NMR spectroscopy (Polymer composition determination is presented in the experimental section, Section 2).

\(^{b}\)Determined by SEC (eluent:THF; standard:polystyrene; details are presented in the experimental section; Section 2).

### Table 2 - Overview of cargo concentration and encapsulation efficiency

| Polymer \(^{a}\) | (Polymer) mg/ml | Encapsulation method | Cargo | (Maximum amount of cargo) μg/ml | (Encapsulated cargo) μg/ml | SD (μg/ml) | Encapsulation efficiency (%) | SD (%) |
|-----------------|-----------------|----------------------|-------|-------------------------------|--------------------------|----------|-----------------------------|--------|
| PEG-p (CL-g-TMC) | 5 Direct hydration | OVA | 8000 | 400 | 2.67 | 5.0 | 0.04 |
| TMCI11 | 5 Direct hydration | OVA | 8000 | 1750 | 55.5 | 21.9 | 0.42 |
| TMCI6 | 5 Direct hydration | OVA | 1000 | 184 | 3.08 | 18.4 | 0.25 |
| TMCI11 | 24 Direct hydration | CpG | 733 | 39 | 2.59 | 5.3 | 0.35 |
| TMCI11 | 24 Re-assembly | CpG | 733 | 188 | 2.53 | 25.6 | 0.35 |

**Note:** The encapsulation of CpG in PEG-p(CL-g-TMC) polymersomes did not result in a detectable value and is therefore omitted from the table.
the stability of this polymersome system. Next, both imidazole functionalized systems were exposed to 37°C at physiological pH, by incubating the samples in phosphate-buffered saline (PBS 1X, pH 7.4, without calcium and magnesium chloride), while shaking and the change in hydrodynamic diameter was monitored to assess the particle’s stability (Figure 3). As the pKa of imidazole is approximately 6.5,[36,45,46] partial protonation could affect the vesicle’s stability. Indeed, polymersomes composed of TMCI6, with fewer imidazole groups, showed improved stability compared to vesicles made of TMCI11. Within a day the TMCI11 polymersomes destabilized, which was visually observed by the decrease in turbidity of the sample (opaque to a clear solution) and confirmed by DLS.

**Figure 2**  Dynamic light scaterring (DLS) spectra and cryogenic transmission electron microscopy (cryo-TEM) pictures of poly(ethylene glycol)- poly(caprolactone-gradient-trimethylene carbonate) (PEG-p(CL-g-TMC)) (A),(D); TMCI11 (B),(E); and TMCI6 polymersomes (C),(F) at pH 7.4. Scale bar represents 100 nm [Color figure can be viewed at wileyonlinelibrary.com]

**Figure 3**  The hydrodynamic size (A) and the PDI (B) of TMCI6 (red) and TMCI11 (black) polymersomes as a function of time at pH 7.4. After the 1-day time point TMCI11 polymersomes were destabilized [Color figure can be viewed at wileyonlinelibrary.com]
measurements (Figure S8). The stability of the TMCI6 polymersomes can possibly be accounted for by the larger semi-crystalline pCL block (67 vs. 51 wt%), stabilizing the membrane, and a decrease in imidazole content (20 vs. 37 wt%) as compared to the TMCI11 sample. This demonstrates the ability to tune the timeframe of disintegration of the vesicle by changing the chemical composition and, consequently, control the release of cargo.

In order to simulate endosomal conditions, the pH of the polymersome formulations was adjusted to pH 6.0 which increased the degree of protonation of the imidazole group and, consequently, destabilized the vesicle. As expected, the non-pH responsive polymersomes, PEG-p(CL-g-TMC), remained stable after decreasing the pH (- Figure S9). Direct destabilization of both imidazole containing polymersomes, upon acidification, was observed as shown by DLS and cryo-TEM analysis (Figure 4). DLS measurements revealed large fluctuation in the corregogram, as opposed to a standard S-curve obtained by well-defined particles, indicating destabilization of the vesicle (Figure 4(A)). Furthermore, a steep drop in signal intensity, as indicated by a ~50-fold decrease in derived count rate, signifies the change in morphology. Cryo-TEM of the TMCI11 solution revealed the appearance of small nanoparticles (10–20 nm, Figure 4(B)). In case of the TMCI6 sample, a signal drop of ~20-fold and fluctuations in the corregogram were measured by DLS and large aggregates were observed after adjusting the pH, possibly due to the higher pCL content, which resulted in stronger hydrophobic interactions (Figure 4(C),(D)).

Subsequently, it was investigated what would happen with the assembly state of the polymers when the pH was re-adjusted to ≥7.4. Interestingly, re-assembly was observed for the TMCI11 sample resulting in 120 nm sized polymersomes and the S-curve of the corregogram was restored to the original shape and quality as observed by DLS; the polymersome morphology was confirmed by cryo-TEM with a membrane thickness of 9.9 nm (±1.6, n = 21) (Figure 5(A),(B), Figure S10). Increasing the pH after disassembly of the TMCI6 polymersomes resulted in clustered fibrous aggregates (PDI ~ 0.5) (Figure 5(C), (D)). The higher pCL content presumably inhibits re-assembly into polymersomes, resulting in aggregated structures.

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**FIGURE 4** – Corregogram and cryogenic transmission electron microscopy (cryo-TEM) image of TMCI11 (A, B) and TMCI6 (C, D) at pH 7.4 (black) and 6.0 (red). Cryo-TEM: pH 6, scale bar represents 100 nm [Color figure can be viewed at wileyonlinelibrary.com]
3.3 Cargo encapsulation

Since the polymersome systems are ultimately designed as pH responsive delivery vesicles they were tested on their ability to encapsulate cargo. OVA was used as a model antigen macromolecule with a molecular weight of 45 kDa. OVA was dissolved in PBS (1X) and added during the direct hydration method to allow cargo encapsulation. Hereafter the non-encapsulated cargo was removed by spin-filtration (MWCO = 100 kDa). Compared to the non-pH responsive PEG-p(CL-g-TMC) polymersomes, which showed a statistically determined encapsulation efficiency (E.E., see experimental section Concentration and encapsulation efficiency determination for more information, Section 2.14) of ~5%, the E.E. of both imidazole-functionalized systems was increased to 18–22% (Table 2). This is most likely due to the imidazole groups interacting with the protein. The zeta-potentials of both loaded polymersomes, −5 mV for TMC11 and −7.5 mV for TMC16, were slightly less negative at physiological conditions as compared to the zeta-potential of the non-pH responsive polymersome (−12 mV, Figure 6). At pH 6.0 the protonation of the

**FIGURE 5** Dynamic light scattering (DLS) intensity plot of TMC11 (A) and TMC16 (C) at pH 7.4 (black), and at pH ≥7.4 after pH adjustment from pH 6.0 (blue); cryogenic transmission electron microscopy (cryo-TEM) image of TMC11 (B) and TMC16 (D) after pH adjustment from pH 6.0 to pH 9.0. Scale bar represents 100 nm [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 6** Zeta-potential of TMC11 (blue), TMC16 (green) and poly(ethylene glycol)-poly(caprolactone-gradient-trimethylene carbonate) (PEG-p(CL-g-TMC)) (red) polymersomes at pH 7.0 and pH 6.0 [Color figure can be viewed at wileyonlinelibrary.com]
imidazole group was expected to strongly affect the zeta-potential. After the pH of the samples was adjusted in situ, immediately zeta-potential measurements were performed, before particle disassembly occurred. Indeed, a significant change in zeta-potential was observed, that is $+5 \text{ mV}$ and $+10$ for the TMCI11 and TMCI6 polymersomes respectively, whereas the non-pH responsive PEG-p(CL-g-TMC) remained negative at $-8.5 \text{ mV}$.

Since TMCI11 polymersomes could be reversibly assembled and disassembled upon a pH change, it was studied how reassembly could affect cargo encapsulation. Because the polymers are in a protonated state at low pH, a cargo was selected with affinity for the polymer. CpG ODN is a highly negatively charged short single-stranded synthetic DNA macromolecule ($\sim7.7 \text{ kDa}$) and used as a potent adjuvant to increase vaccine efficacy, targeting amongst others TLR9 receptors.\(^{[48-52]}\) On account of the location of these TLR9 receptors inside the endosome, intracellular delivery of this cargo is necessary, which makes this a suitable system for delivery via pH sensitive polymersomes.\(^{[52]}\) Due to the strong negative charge of this molecule\(^{[53]}\) encapsulation in polymersomes during the regular assembly process is troublesome due to electrostatic repulsion. Indeed, CpG could not be encapsulated in PEG-p(CL-g-TMC) polymersomes, that is E.E. of 0% by utilizing the direct hydration method for encapsulation. By using the imidazole functionalized polymers, an E.E. of $\sim5\%$ was achieved by conventional direct hydration. Next, encapsulation via re-assembly was investigated. TMCI11 polymersomes were disassembled at pH 6.0, followed by the addition of CpG ODN and, subsequently, re-assembly was induced by adjusting the pH to 7.4 resulting in vesicles with as size of 130 nm (PDI $\sim 0.14$). After purification, gratifyingly, an E.E. of $\sim26\%$ was obtained which is approximately 5 times as high as compared to the conventional direct hydration method. The imidazole functionality of the polymer is positively charged at acidic conditions and, therefore, the negative CpG will likely coordinate to the polymers. This coordination results in higher local CpG concentration in the vicinity of the polymers, which also leads to enhanced encapsulation. TMCI11 polymersomes are therefore not only useful for pH controlled cargo release, but their unique re-assembly features also allow a much higher loading efficiency of cargo with affinity for the protonated polymers.

4 | Conclusion

In this research we have demonstrated the reversible, pH-triggered assembly and disassembly of biodegradable PEG-p(CL-g-TMC) based polymersomes. By incorporating pH sensitive functionality in the backbone of the polymer, control over disassembly was obtained. Introduction of the acid sensitive imidazole group resulted in polymersome disassembly at mild acidic conditions comparable to the endosomal environment. Re-assembly of the polymersomes could be achieved by adjustment of the pH, potentially eliminating the need of organic solvent and, thus, creating a more biocompatible system. Furthermore, disassembly of the vesicles at physiological conditions could be altered by changing the polymer composition, underlining the tunability and the application of these vesicles as a sustained release system. Enhanced encapsulation of macromolecules was shown by the imidazole modified system and was further optimized by means of the re-assembly encapsulation method. Although improvements still are required, with regard to the long term stability of the polymersomes, we think this system is a conceptually useful platform with potential applicability as drug delivery vesicle towards the endosome.

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