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Main-chain degradable single-chain cyclized polymers as gene delivery vectors

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ABSTRACT

Single-chain technology (SCT) allows the manipulation of polymeric architectures at an individual polymer chain level, providing a new platform for the fabrication of nanoscale polymeric objects. However, it remains problematic to apply this newborn technology to the biological and medical fields, since synthesis of single-chain polymeric nanoparticles relies heavily on controlled/living radical polymerization of vinyl based monomers, yielding a persistent non-degradable carbon-carbon based backbone. Moreover, the ultrahigh dilution conditions often required for single-chain polymer nanoparticle synthesis limits large-scale applicability. A versatile approach to achieve backbone degradability in single-chain cyclized polymers was developed by combining ring-opening addition polymerization and intramolecular cyclization into a one-pot RAFT copolymerization of cyclic and mono/multi-vinyl monomers system under concentrated conditions. The in situ intramolecular cyclization of individual propagating chains was achieved by kinetic control and statistical manipulation of mono- and multi-vinyl monomer copolymerization. The cyclic allylsulfide monomer 3-methylidene-1,9-dioxo-5,12,13-trithiacyclopentadecane-2,8-dione (MDTD) was copolymerized via the ring-opening pathway to introduce disulfide groups into the vinyl-based backbone without compromising the single chain propagation nature. Backbone degradable single chain polymeric nanoparticles were obtained with molecular weights of 10 kDa and MDTD incorporation ratios of 4.7%. Chemical degradation of the nanoparticles confirmed both their single chain nature, as well as backbone degradability. The single-chain cyclized polymeric nanoparticles were evaluated for their gene transfection capabilities. The backbone degradable nanoparticles displayed high transfection efficiencies and low cytotoxicities in both 3T3 and HeLa cells.

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1. Introduction

The preparation of nano-sized objects consisting of single polymeric chains, recently coined single-chain technology (SCT) [1] brings great promise to different areas in nanotechnology, including materials science, biomedicine and energy [2–8]. Interest in producing macromolecular architectures through manipulation of single polymer chains is rapidly increasing, because of the promise of smart, autonomous and tailor-designed functions at the individual polymeric chain level. Over a decade of development has resulted in single-chain polymer nanoparticles becoming new building blocks for bottom-up nanotechnology [2].

Nanomedicine may greatly benefit from their drug transport properties, multivalent character [2,9,10] and, though still crude, resemblance to natural proteins and enzymes [11,12].

Several techniques have been explored to synthesize single-chain polymeric nanomaterials. In particular, intramolecular crosslinking of individual linear polymer chains in ultra-dilute solution leads to unimolecular nanoparticles – i.e. single-chain polymeric nanoparticles (SCNPs) – have received ample attention [2,8]. Hawker and coworkers reported pioneering work in which random intramolecular dimerization of benzocyclobutene units was employed to form compact polystyrene nanoparticles [13]. This intrachain crosslinking has also been achieved afterwards by other covalent [7,14,15], dynamic covalent [16,17] and noncovalent bonding [18–20]. Interestingly, Meijer et al. have shown that directional self-assembly of supramolecular binding motifs can be used to guide the compaction of single polymer chains [21,22]. However, in order to avoid intermolecular crosslinking, these post-polymerization modification reactions need to be conducted under extremely dilute conditions (typically <0.1 mg/mL) [3],

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practically forbidding large-scale production. Promotion of intra-chain crosslinking is therefore a challenging key aspect in the advancement of the single-chain technology.

Recently, Wang and coworkers expanded the toolbox of the single-chain technology by demonstrating facile manipulation of inter- and intramolecular reactions during the polymerization of multivinyl monomers (MVMs) [23–27]. In contrast to the aforementioned post-polymerization crosslinking strategies, this approach eventually leads to single-chain cyclized/knotted polymeric nanoparticles (SCKNPs) under concentrated reaction conditions. Key to this approach is achieving kinetic control to ensure that same-chain pendant vinyl moieties have the highest probability of reacting with their own propagating active center in the initial linear chain growth stage. The benefits of this internal cyclization in the controlled polymerization of MVMs are two-fold: first, keeping chain propagation within single molecules, which is a prerequisite for the formation of single-chain cyclized polymer nanoparticles; second, consumption of most of the pendant vinyl moieties to minimize intermolecular coupling and hence delay gelation.

Reversible addition-fragmentation chain transfer polymerization (RAFT) polymerization is a particularly promising way to synthesize kinetically controlled polymers. The absence of possibly toxic metal ions in the polymerization, combined with the wide variety of compatible vinyl monomers bode well for future biomedical application. Moreover, intrachain cyclization is promoted by the inherently high deactivation rates [26] characteristic for RAFT polymerization. Typical constants of chain transfer (k_{tr}) are 10^2 to 10^3 times higher than for vinyl propagation (k_p) [28], largely preventing intermolecular crosslinking. As such, SCKNPs are readily obtained when the polymerization is quenched in time, before the primary polymer chains starts to combine. SCKNPs architecturally resemble SCNPs given the unimolecular carbon-carbon based backbone with numerous cyclized loops bridged by 'crosslinkers', whose degradation results in linear polymer chains [23,25,26,29]. However, in contrast to SCNPs, the synthesis of SCKNPs features a one-pot in situ intramolecular reaction, resulting in self-cyclization of the propagating polymer chains in concentrated solution, without the need for additional post-polymerization steps.

Synthesis of single-chain polymer nanoparticles relies heavily on controlled/living radical polymerization (CRP) techniques, owing to the high fidelity of the resulting vinyl polymers and the wide variety of easily accessible functional monomers. However, degradability of vinyl polymers remains a critical issue for their biomedical application, as the carbon-carbon backbone does not easily undergo hydrolysis or enzymatic degradation. As a result, unwanted immune responses and toxicity hamper their translation to the clinic [30]. Several different design strategies have been developed to introduce labile units into the backbone of vinyl polymers [30]. Bifunctional CRP initiators containing cleavable units such as redox-responsive [31], thermally degradable [32], acid-labile [33] and photocleavable groups [34] have been reported, from which polymers containing a central degradable junction can be grown [35]. For instance, Matyjaszewski and coworkers demonstrated the synthesis of backbone degradable polymers employing disulfide containing initiators for atom-transfer polymerization (ATRP) [31,36]. Multisegmented degradable polymers have been synthesized by combining difunctional macromonomers, which are generated for example by nucleophilic substitution of the halide endgroups of ATRP-derived polymers [37] or aminolysis of RAFT-derived polymers [38–40]. This step-growth polymerization approach generally results in broad molecular weight distributions, while the additional synthetic steps limit the applicability of this approach.

Radical ring opening polymerization of cyclic vinyl monomers provides for an elegant, broadly applicable way of incorporating degradable and responsive linkages [41,42]. The most studied cyclic monomers for this purpose are cyclic ketene acetals (CKAs), initially investigated by Bailey et al. [43], and later used to synthesize different copolymer structures [44–47]. A particularly versatile class of cyclic monomers is based on the allyl sulfide moiety [48–50]. Paulusse et al. demonstrated the

facile incorporation of ester, thioester and disulfide functionalities into the vinyl polymer backbone under CRP conditions [51]. The introduction of multiple orthogonally degradable units into the vinyl backbone resulted in tunable, selective and stepwise degradation with the ability for further functionalization of the polymer backbone [51].

Combining backbone degradability with single-chain technology would enable its safe application in nanomedicine. However, the stringent requirements to achieve intramolecular chain collapse and prevent unwanted side reactions, such as intermolecular crosslinking, have hampered the development of degradable single chain polymer nanoparticles. Herein, we report the synthesis of backbone degradable single-chain cyclized polymer nanoparticles via one-pot RAFT copolymerization of *N,N*-dimethylaminoethyl methacrylate (DMAEMA) with tri(ethyleneglycol) diacrylate (TEGDA) and a 15-membered cyclic allyl sulfide monomer, 3-methylidene-1,9-dioxo-5,12,13-trithiacyclopentadecane-2,8-dione (MDTD). Incorporation of the cyclic monomer MDTD is monitored on the basis of copolymer composition and degradation behavior. Degradation profiles of the polymer nanoparticles are evaluated in vitro and the gene trafficking capabilities and cytotoxicity of the polymer nanoparticles are evaluated on 3T3 and HeLa cells.

2. Materials and methods

2.1. Materials

2,2'-Azobis(2-methylpropionitrile) (AIBN, 98%, Sigma-Aldrich), 2,2'-(dimethyl amino)ethyl methacrylate (DMAEMA, 98%, Sigma-Aldrich), poly(ethylene glycol) diacrylate (TEGDA, $M_n = 250$ g/mol, Sigma-Aldrich), toluene (>99.5%, Sigma-Aldrich), acryloyl chloride ($\geq 97\%$, Sigma-Aldrich) 2-hydroxyethyl disulfide (90%, Sigma-Aldrich), triethylamine (TEA, 99%, Sigma-Aldrich), and hydrazine hydrate (reagent grade, Sigma-Aldrich) were used as received. The disulfide containing cyclic 3-methylidene-1,9-dioxo-5,12,13-trithiacyclopentadecane-2,8-dione monomer was synthesized following a literature procedure [51]. The RAFT agent 2-cyano-2-propyl dithiobenzoate (CPBDT) was kindly provided by Dr. Tai, University of Bangor, United Kingdom. Solvents were purchased from Fisher Scientific and used as received.

2.2. Synthesis of disulfide diacrylate (DSDA)

THF (600 mL) was dried over sodium sulfate for 24 h. 2-Hydroxyethyl disulfide (15.38 g, 12.2 mL, 100 mmol) and TEA (40.66 g, 402 mmol) were mixed with THF (450 mL) in a 1 L round-bottom flask. The solution was flushed with argon for 10 min and kept under argon environment. Acryloyl chloride (32 mL, 35.65 g, 394 mmol) diluted in THF (50 mL) was added dropwise. The reaction was left stirring for 24 h in an ice bath. The product was filtered by vacuum filtration to remove undesired triethylammonium chloride salts. The solution was washed 5 times by extraction using a 0.2 M sodium carbonate solution, followed by 5 times distilled water washing. After drying with sodium sulfate, the solvent was removed by rotary evaporation. The obtained product (42% yield) was characterized by ^1H NMR spectroscopy in CDCl_3 : 2.84 ppm (t, 4H), 4.45 ppm (t, 4H), 5.80 ppm (d, 2H), 6.05 ppm (q, 2H), 6.43 ppm (d, 2H).

2.3. Polymer synthesis

2.3.1. Synthesis of poly(DMAEMA-co-TEGDA) (P1)

DMAEMA (320 equiv.; 5.30 g), TEGDA (80 equiv.; 2.11 g), RAFT agent CPBDT (1 equiv.; 0.0239 g), AIBN (1 equiv.; 0.0174 g) and toluene (20 mL) were added into a 50 mL round bottom flask. The solution was bubbled for 15 min with argon and put into an oil bath at 60 °C. The reaction was sampled manually at desired time points. The obtained polymer mixture was purified by dialysis using a Spectra/Por® dialysis

membrane (pretreated RC tubing, MWCO of 2 kDa) in acetone with a solvent change every 12 h.

2.3.2. Synthesis of poly(DMAEMA-co-DSDA) (**P2**)

DMAEMA (320 equiv., 5.33 g), DSDA (80 equiv., 2.19 g) RAFT agent CPBDT (1 equiv.; 0.0230 g), AIBN (1 equiv.; 0.0170 g) were mixed in toluene (20 mL). The solution was bubbled for 15 min with argon and put into an oil bath at 60 °C. The reaction was sampled manually at desired time points. The purification by dialysis was performed under the same protocol as previously described.

2.3.3. Synthesis of poly(DMAEMA-co-MDTD-co-TEGDA) (**P3**)

DMAEMA (300 equiv., 4.77 g), TEGDA (80 equiv., 2.02 g), MDTD (20 equiv. 0.624 g), CPBDT (1 equiv.; 0.022 g) and AIBN (1 equiv.; 0.0166 g) were mixed with toluene (20 mL). The solution was bubbled for 15 min with argon and put into an oil bath at 60 °C. The reaction was sampled manually at desired time points. The purification by dialysis was performed under the same protocol as previously described.

2.4. Degradation experiments

P1–3 (5.0 mg) was dissolved in DMF (2.00 mL) or chloroform-d (0.80 mL). Hydrazine hydrate (50–60%, 5 μ L) or glutathione/H₂O solution (20 mM) were added to the solutions and left for 1 h or up to 16 h. The DMF mixture was purified using an aluminium oxide column and monitored by GPC. The chloroform-d solution was analyzed by ¹H NMR spectroscopy.

2.5. End capping of polymers **P1–3**

P1–3 was redissolved in THF to make a concentration of 100 mg/mL. A 10-fold excess of 3-morpholinopropylamine (MPA) with respect to vinyl groups of the polymers was dissolved in same volume of THF. The two solutions were mixed together and stirred for 24 h. MPA was removed by precipitation (3 $\times$) against a large excess of cold hexane/diethyl ether (10:7 v/v). The mixture was subsequently dried under vacuum.

2.6. Gel permeation chromatography (GPC) characterization

Weight averaged molecular weight (M_w), number average molecular weight (M_n) and polydispersity ($\bar{D}$) were obtained by GPC (Agilent 1260) equipped with RI detector. The columns (30 cm PLgel Mixed-C, two in series) were eluted using DMF (containing 0.1% LiBr) and calibrated using a series of 12 near-monodisperse PMMA standards (M_p from 690 to 1,944,000 g/mol). The polymers were analyzed at a concentration of 5.0 mg/mL in DMF by RI detector. All calibrations and analyses were performed at 60 °C and a flow rate of 1 mL/min.

2.7. ¹H NMR characterization

¹H NMR spectroscopic analysis was carried out on a Varian NMR system 400 MHz spectrometer with MestReNova 6.1 processing software and reported in parts per million (ppm) relative to the response of the CDCl₃ (7.26 ppm) and tetramethylsilane (0.00 ppm).

2.8. Polyplex preparation

The expansion, isolation and purification of cell secreted Gaussia-luciferase plasmid (pCMV-GLuc, obtained from New England Biolabs UK) was performed using the Giga-Prep (Qiagen) kit as per protocol and its purity was confirmed by UV spectroscopy (NanoDrop™ ND1000 Spectrophotometer, Thermo Scientific) and gel electrophoresis. The polyplex formed via the electrostatic attraction between the DNA and cationic polymer. **P1–3** were firstly diluted in 25 mM aqueous NaOAc (pH = 5.2) and then mixed with DNA (60 μ g/mL in 25 mM aqueous NaOAc)

at a 1:1 volume ratio with polymer/DNA w/w ratio ranged from 15:1, 30:1 to 45:1. For PEI, N/P ratio 10:1 was used. To prepare the polyplexes, PEI and DNA were dissolved in 40 μ L of Opti-MEM media, respectively, and then vortexed for 15 s and allowed to stand for 10 min. SuperFect was used according to manufacturer's instructions, briefly, DNA was diluted in serum free cell culture media, SuperFect was then added, mixed by vortexing for 10 s and incubated for 10 min. DNA binding efficiencies were confirmed by PicoGreen.

2.9. Cell culture

Rat embryonic 3T3 fibroblasts (3T3) and human cervical cancer cells (HeLa) were grown in Dulbecco modified Eagle's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) at 37 °C with 5% CO₂ using standard cell culturing techniques.

2.10. Luciferase transfection

Under usual cell culture sterile conditions cells were seeded in 96 well plates at a density of 10,000 cells per well 24 h prior to the addition of the polyplexes (formed with 0.25 μ g of plasmid DNA per well). After incubation at 37 °C and 5% CO₂, the cell culture media was replaced with polyplex solutions of varying weight ratios made up as described above, in serum containing media. A positive control of cells was subjected to the same treatment, but received commercially available transfection agents/plasmid according to the standard protocol. After 48 h of incubation, analysis of the luciferase activity was performed as per the provided protocol, which, for this secreted luciferase means analysis of the cell supernatant, and subsequent plotting of luciferase activity directly in terms of relative light units (RLU).

2.11. AlamarBlue® reduction

The biocompatibility of **P1–3** was evaluated by alamarBlue™ assay (Thermo scientific), which assessed metabolic activity of transfected cells. 48-h post-transfection, cell viability was assessed by following the alamarBlue™ assay manufacturers' protocol. After 3 h incubation with 100 μ L alamarBlue reagent, the fluorescence intensity was measured using standard protocol on a multi-plate reader and alamarBlue reduction percentage was calculated.

2.12. Green fluorescent protein (GFP) expression

Expression of pCMV-GFP green fluorescent protein plasmid (New England Biolabs UK, also obtained as described earlier) was used to assess polymer transfection. The transfection was conducted as aforementioned. 48 h post transfection, the cells were visualized with an Olympus fluorescence microscope.

2.13. Statistical analyses

G-luciferase activity and cell viability after transfection with **P1–3**, DNA polyplexes underwent one-way ANOVA compared to controls. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Design philosophy

The synthesis of single-chain cyclized polymeric nanoparticles from RAFT homopolymerization of MVMs is extended here to the copolymerization system to prepare well-defined degradable single-chain cyclized polymer nanoparticles for gene delivery. The monovinyl monomer DMAEMA was chosen because of the promising gene carrier properties of poly(DMAEMA); though these polymers suffer from relatively high

cytotoxicity due to the persistent nondegradable carbon-carbon chain [52]. Two divinyl monomers, i.e. TEGDA and disulfide diacrylate (DSDA), were used as 'crosslinkers' to intramolecularly bridge the chain-propagating radical with the same-chain pendant vinyls, enabling the formation of the single-chain cyclized architecture during the polymerization process. The cyclic monomer MDTD was copolymerized to incorporate multiple disulfide and ester groups into the single vinyl based polymer backbone, endowing the single-chain cyclized polymers with degradable moieties. Fig. 1 outlines the three types of targeted single-chain cyclized polymers prepared: non-degradable single-chain poly(DMAEMA-co-TEGDA) (**P1**), crosslinker-degradable single-chain poly(DMAEMA-co-DSDA) (**P2**) and backbone degradable single-chain poly(DMAEMA-co-MDTD-co-TEGDA) (**P3**). It should be noted that the copolymerization systems for **P1–2** employed here are similar to the controlled radical cross-linking copolymerization (cRCC, a controlled version of the "Strathclyde route"), in which monovinyl monomers are copolymerized with multivinyl crosslinkers by controlled/living methods normally leading to nano-/microgels or branched polymers [53,54]. One distinct difference however, is the ratio of MVMs to initiator being much higher than 1, in contrast to the traditional cRCC system, wherein the amount of cross-linker is controlled to be lower than that of

the initiator. The widely accepted Flory-Stockmayer theory predicts the critical gelation occurring when the average molar ratio of crosslinks to primary chains exceeds unity [55,56]. However, as previously mentioned, with kinetic control and statistical manipulation, the intramolecular cyclization contributes significantly to the consumption of pendant vinyls during the linear chain propagation, thus providing a highly intramolecularly cyclized single-chain, provided the polymerization is quenched timely.

3.2. Polymerizations

The polymerizations were monitored by manually sampling at desired time intervals followed by the analysis through gel permeation chromatography (GPC) (Table 1). As outlined in Fig. 2, the GPC traces for these three polymerization reactions show similar trends with respect to polymer growth. In the early stage, the highly symmetrical and unimodal peaks observed in GPC traces indicate a controlled linear chain growth without intermolecular crosslinking reactions [52]. The controlled living nature of the linear chain growth is further manifested by the parallel shift of the corresponding GPC traces as the polymerization proceeds during this early stage. For instance the symmetrical peak

Fig. 1. Synthesis of three different single-chain cyclized polymers via RAFT copolymerization: non-degradable poly(DMAEMA-co-TEGDA) (**P1**), crosslinker-degradable poly(DMAEMA-co-DSDA) (**P2**) and backbone-degradable poly(DMAEMA-co-MDTD-co-TEGDA) (**P3**).

Table 1
Polymerization conditions and molecular weight characteristics of **P1–3**.

Polymers ^a	Time [h]	M _n ^b [kDa]	PDI ^b	PVC ^c [%]	IP ^c [%]
Poly(DMAEMA-co-TEGDA) (P1)	1	4.9	1.7	33	–
	2	11.2	1.7	39	–
	3	17.6	2.0	41	–
	4	27.2	3.2	46	–
Poly(DMAEMA-co-DSDA) (P2)	6	4.0	1.8	14	–
	8.5	9.0	1.8	17	–
	10	16.5	1.9	–	–
	12	32.0	8.0	23	–
Poly(DMAEMA-co-MDPA-co-TEGDA) (P3)	2	5.7	1.8	23	3.6
	4	9.8	2.1	38	4.1
	5	10.0	2.5	40	4.7
	8	17.4	3.6	44	3.7

^a Azobisisobutyronitrile (AIBN) and 2-cyano-2-propyl dithiobenzoate (CPBDT) were used as initiator and RAFT agent respectively. All reactions were conducted in toluene at 60 °C with a vinyl monomer concentration of 2.5 M. **P1**: AIBN/CPBDT/DMAEMA/TEGDA = 0.1:0.1:34:8, **P2**: AIBN/CPBDT/DMAEMA/DSDA = 0.1:0.1:34:8, **P3**: AIBN/CPBDT/DMAEMA/TEGDA/MDTD = 0.1:0.1:32:8:2.

^b Number-averaged molecular weights (M_n) and polydispersity indices (PDI) were determined by GPC with a refractive-index detector.

^c Pendant vinyl conversions (PVC) and incorporation percentages (IP) were calculated from ¹H NMR spectroscopic data.

of the **P1** polymerization at 1 h is practically parallel with the trace at 2 h, a typical feature of controlled/living linear chain polymerization [57]. This initial linear chain growth results in the formation of predominantly linear chains with minimal interchain crosslinking events, an important prerequisite for the formation of single-chain polymers. Combination of these polymer chains only occurred after the critical overlap concentration is reached, manifested by the occurrence of the multimodal peaks and high polydispersity as observed in the GPC traces at 4 h, 12 h and 8 h for **P1**, **P2** and **P3**. The targeted single-chain polymers **P1**, **P2** and **P3** were obtained by quenching the three polymerization reactions timely, just before intermolecular crosslinking reaction occurred, which is at 2 h, 8.5 h and 5 h respectively herein.

Pendant vinyl conversion, calculated comparison of the fully reacted divinyl monomer units with those with only a single reacted vinyl moiety, (Fig. S1 and Eq. S1) is as high as 33% at the first time point of the **P1** polymerization system, indicating that approximately one third of the pendant groups has reacted during that linear chain growth stage. Given the unimodal and symmetrical GPC peaks, these pendant vinyl are most likely consumed intramolecularly. Such high conversions of the pendant vinyl groups at this stage indicate high percentages of divinyl monomers incorporated in the primary chain through intrachain ring-closing reactions, resulting in the formation of cyclic

subunits. Relatively high polydispersity indices are observed for the different polymers as compared with those obtained for RAFT homopolymerization of DMAEMA (PDI ~ 1.4 by You et al. [58] and PDI ~ 1.1 by Chieffari et al. [59]). Most likely, the copolymerization of methacrylate-based DMAEMA with acrylate-based TEGDA or DSDA are the cause of these increases. In addition, the choice of the RAFT-agent may not be optimal for the polymerization of acrylates. Although polydispersities observed here are higher than those of traditional SCNPs, these increases are not due to interchain combination as demonstrated by the degradation results in the following section, indicating that the single chain nature of the polymers is maintained.

As shown in Fig. 2c the different sampling time points show comparable traces for co-polymerization **P3** as those observed for **P1** and **P2**. The parallel shift of the symmetrical and unimodal GPC traces manifest characteristics of a controlled/living linear chain growth. The level of pendant vinyl conversion (23–40%) during the linear chain propagation period is consistent with the observations for **P1**, confirming substantial intramolecular cyclization. In comparison to **P1** slower chain propagation is observed for **P3**. This lower reaction rate is attributed to the formation of thiyl-radicals during ring opening of MDTD [51]. It is reported that higher bond dissociation energies (BDE) are required to form thiyl-radicals, than for carbon-radical in vinyl monomers [60,61], in this case DMAEMA and TEGDA. The difference in BDEs, however, has only limited influence on the incorporation ratios of the cyclic monomer, as determined from the incorporation percentages (IP) of MDTD (Table 1, Fig. S1 and Eq. S2). The incorporation percentages determined throughout the polymerization match feed ratios closely.

3.3. Degradation experiments

Disulfides were chosen as the degradable functionality, because of their ability to undergo degradation in the cellular reducing environment [62], allowing for targeted intracellular delivery of a drug or gene cargo. Incorporation of disulfide bonds in the cyclized polymers was evaluated by exposure of the polymers to the strong reducing agent hydrazine hydrate or the biological antioxidant glutathione (GSH). The degradation reaction was carried out by mixing polymers **P1–3** with hydrazine hydrate solution and monitoring for changes in molecular weight by GPC at 0 h (before degradation), 0.5 h after addition of hydrazine hydrate and after 18 h. Even upon extended exposure to reducing agents, the molecular weight of **P1** remained unaltered, with fully overlapping GPC traces before and after reaction (see Table 2 and Fig. S2a, b). Hence, no degradation occurred. In contrast, the degradation of **P2** shows a slight decrease in molecular weight as the peak shifted toward a higher retention time (see Table 2 and Fig. 3a). The degradation reaction in this case represents cleavage of the disulfide

Fig. 2. Molecular weight evolution during the polymerizations to give **P1** (a), **P2** (b) and **P3** (c), as monitored by GPC (refractive-index). Traces show highly symmetrical and unimodal peaks at the beginning of the reaction indicating controlled linear chain growth with minimal intermolecular crosslinking reactions and bimodal and multimodal peaks in the later stages. The single-chain polymers **P1**, **P2** and **P3** were obtained by quenching the three polymerization reactions at 2 h, 8.5 h and 5 h respectively (encircled).

Table 2
Molecular weight distribution of degraded polymers **P1–3** at different time points.

Polymer	Time	M_n [kDa]	PDI
Poly(DMAEMA-co-TEGDA) (P1b)	0 h	10.2	1.8
	0.5 h	8.8	2.0
	overnight	9.0	2.0
Poly(DMAEMA-co-DSDA) (P2)	0 h	10.9	1.7
	0.5 h	8.1	1.7
	overnight	7.9	1.8
Poly(DMAEMA-co-MDTD-co-TEGDA) (P3)	0 h	9.9	2.5
	0.5 h	3.1	2.4
	overnight	2.8	2.1

Note: the different polymers were degraded with hydrazine hydrate and measured by GPC before and 0.5 h after the addition of hydrazine hydrate; mixtures were stored overnight to examine molecular weights of the final degradation products.

bonds in the divinyl crosslinker DSDA. Scission of the intrachain crosslinkers results in opening of the cyclic subunits. Full cleavage of the crosslinkers (as confirmed by the ^1H NMR spectroscopy, Fig. S3) results in complete removal of the cyclic units, and hence ‘untying’ of the single cyclized chain to a single linear polymer chain. The slight decrease in molecular weight, as reported earlier for the degradation of MVM-derived homopolymers [23,25–27,29], is apparent because of two opposite effects occurring during degradation: cleavage of the crosslinks results in the release of small fragments (side chains and small branches) and a concomitant decrease in hydrodynamic volume; untying of the cyclized polymer chains on the other hand will increase the hydrodynamic volume. These synergistic effects determine the final change in molecular weight. The substantial differences in degradation profiles of **P2** shown here with those observed for the crosslinked degradable hyperbranched polymers reported by Zhao et al. [27] and Armes et al. [63] further confirm the single cyclized chain nature.

Degradation of **P3** shows a dramatic decrease in molecular weight (Table 2 and Fig. 3b), although only 5% MDTD was copolymerized. M_n decreased from 9.9 kDa to 2.8 kDa. It should be noted that after degradation the GPC peak is still unimodal, indicating a relatively statistical distribution of disulfide units along the polymer backbone. Considering the incorporation percentage of MDTD, the molecular weight after

degradation should be approximately 2.6 kDa. The obtained number-averaged molecular weight observed by GPC decreased to 3.1 kDa with a broad molecular weight distribution ($\text{PDI} = 4.4$) within 0.5 h, but after prolonged degradation M_n was lowered down to 2.8 kDa with a narrow molecular weight distribution, close to the theoretical value.

To fully verify their degradability, more detailed degradation studies on single-chain polymers **P1–3** at different time points were performed following the same procedure (Fig. S2). This revealed that only the molecular weight of **P3** decreased significantly (Table S1). Finally, degradation of **P3** was also carried out with the antioxidant glutathione (GSH) to ensure degradability of **P3** in the intracellular redox environment. The concentration of GSH (20 mM) employed here is in the millimolar range comparable to intracellular GSH [64]. Purified polymer **P3** with M_n of 9.8 kDa is degraded into small fragments ($M_n = 2.3$ kDa) as shown in Fig. S4, demonstrating the backbone biodegradability of **P3**.

3.4. Gene delivery

It is widely accepted that successful DNA delivery requires the vectors to protect the DNA from enzymatic degradation during its journey to the cell nucleus, but also release DNA for efficient transcription of mRNA [65]. Polymer degradation may enhance the release of genetic cargos from polyplexes and reducing cytotoxicity [66]. As such, the degradation is a critical issue for biomedical application of single-chain polymeric nanoparticles. Although the ester groups can be (slowly) hydrolyzed in the extracellular environment, the disulfide bonds are not reduced until these are exposed to GSH in intracellular cytoplasm, where the GSH concentration is 100–1000 times higher than that in extracellular environment and cleavage occurs rapidly [64]. Newland et al. reported the promising transfection efficiencies of nanoscale single-chain cyclized poly(DMAEMA-co-EGDMA-co-PEGEMA) [24] and poly(DSDMA-co-DMAEMA-co-PEGEMA) [29], owing to the strong interactions between the multiple cycle polymeric structure with plasmid DNA. However, both copolymers decreased cell viabilities by up to 20%, which was attributed to the long non-degradable vinyl backbone. A short vinyl backbone achieved by an oligomer combination strategy showed much higher cell viabilities [52], but high initiator percentages

Fig. 3. Schematic depiction of degradation process and molecular weight distributions corresponding to the degradation study on crosslink-degradable **P2** and backbone-degradable **P3** with hydrazine hydrate. The degradation with hydrazine hydrate of the crosslink-degradable poly(DMAEMA-co-DSDA) results in an untied linear polymer. This untying of the single-chain cyclized polymer results in a slight change in molecular weight. The degradation of backbone-reducible poly(DMAEMA-co-MDTD-co-TEGDA) results in a significant decrease in molecular weight owing to the cleavage of the vinyl based polymer backbone at randomly distributed disulfide units.

limit biocompatibility of these nanostructures. Therefore, incorporation of MDTD into single-chain polymeric nanoscale objects is expected to further reduce cytotoxicity of the polyplexes, without compromising transfection efficiencies.

The transfection capabilities of the three polymers **P1–3**, all end-capped with 3-morpholinopropylamine (MPA), was assessed by examining the Gaussia (G)-luciferase activity and green fluorescence protein (GFP) expression in transfected 3T3 and HeLa cell lines. In each study, three polymer/plasmid weight ratios of 15:1, 30:1 and 45:1 were evaluated. The commercial transfection agents PEI (branched, 25 kDa) and SuperFect were used as positive controls, following standard protocols of the manufacturers. Subsequent cell viabilities post-transfection were analyzed using the alamarBlue reduction method.

Both the crosslinker degradable **P2** and backbone degradable **P3** exhibited higher transfection efficiencies than the non-degradable **P1** (Fig. 4a), demonstrating the essential role of the disulfide bond for high gene transfection efficacy in 3T3 cells [67]. The high gene transfection with **P3** as a vector was likely achieved because after endosomal escape, the high concentration of GSH in the cytoplasm prompts rapid and complete backbone degradation of the polyplexes releasing complexed DNA for effective transfection [66]. The higher transfection efficiencies were further validated by the expression of GFP, as observed by fluorescence microscopy imaging (Fig. 4e–h). GFP expression in 3T3 cells after transfection correlates well with the activities of G-Luciferase. As shown in Fig. 4g, more cells were transfected as evidenced by widespread GFP expression when **P3** was used as transfection agent.

In terms of the 3T3 cell viability, non-degradable polymer vector **P1** showed higher cytotoxicity within 48 h, according to a cell metabolic activity study. Decreased cytotoxicity was observed for the crosslinker degradable vector **P2** (Fig. 4b). However, when the polymer/plasmid weight ratios are higher than 30:1, cytotoxicities of **P1** and **P2** have reached similar levels. A possible explanation is that both of these polymers have long non-degradable vinyl backbones with high inherent cytotoxicity [30,68]. Much lower cytotoxicity was achieved when the backbone degradable polymer **P3** was employed, with cell viabilities as high as 90% even at 45:1 weight ratios, demonstrating its excellent biocompatibility.

Similar trends were observed for the transfection efficiencies of **P1–3** in HeLa cells (Fig. 4c). Backbone degradable **P3** exhibited much higher transfection efficiencies (by around 40 fold) than the non-degradable **P1** and crosslinker degradable **P2**, which further confirmed that essential role of the rapid and complete backbone degradation of the polyplexes releasing complexed DNA for effective transfection. Cytotoxicities tested on the HeLa cell line showed similar results, with over 90% cell viability preserved by polymer vector **P3** (see Fig. 4d). Remarkably, both transfection efficiencies and cell viabilities of the synthesized degradable **P3** are superior to the commercial agent PEI and are comparable to SuperFect.

4. Conclusions

Backbone degradable single-chain cyclized polymer (**P3**) was successfully and efficiently synthesized through RAFT copolymerization of DMAEMA, TEGDA and cyclic monomer MDTD under concentrated conditions in a one-step procedure. Similar to the synthesis of non-degradable **P1** and crosslinker degradable **P2**, the polymerization process for **P3** displays an initial linear chain growth period with high pendant vinyl conversion (~30%). Chemical degradation of **P2** resulted in untying of the single-chain cyclized polymers with only slight decreases in the molecular weight, while degradation of **P3** into much smaller fragments demonstrated backbone degradability. Moreover, molecular weights of the degradation products are consistent with the incorporation percentage of MDTD, providing a predictable and tailorable degradation behavior. The gene transfection results demonstrate that **P3** is an efficient in vitro gene delivery agent: high transfection efficiency and low cytotoxicity, proving the significance of degradability of single chain polymers for biomedical application. These first attempts toward backbone degradable single-chain polymeric nano-objects demonstrate the ease with which degradability can be incorporated in complex macromolecular architectures via radical ring opening polymerization. These promising results essentially represent the missing link in the design of advanced single-chain polymeric materials and their safe use in biomedical applications, and should facilitate the move of the SCT research beyond its current proof of concept stage.

Fig. 4. G-luciferase activity (a) and cell viability (b) of 3T3 cells after transfection; G-luciferase activity (c) and cell viability (d) of HeLa cells after transfection; One-way ANOVA, mean $\pm$ SD, $n = 4$, * $p < 0.05$ superior luciferase activity and cell viability compared with **P1**/DNA polyplex; # $p < 0.05$ superior luciferase activity and cell viability compared with **P2**/DNA polyplex. GFP expression of 3T3 cells after transfection with **P1**/DNA (e), **P2**/DNA (f), **P3**/DNA (g) and SuperFect/DNA (h). Scale bars are 200 μm.

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Appendix A. Supplementary data

Detailed materials and experimental methods, ¹H NMR spectra, calculation of pendant vinyl conversion and incorporation percentage, degradation studies, transfection in HeLa cell lines. Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.jconrel.2016.07.046>.

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