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## Pure hydrophilic block copolymer vesicles with redox- and pH-cleavable crosslinks†

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The self-assembly of a novel double hydrophilic block copolymer consisting of biocompatible blocks, namely pullulan-*b*-poly(*N*-vinylpyrrolidone), is presented. Completely hydrophilic spherical structures with an average apparent radius of 800 nm at increased concentrations in water are observed *via* dynamic light scattering as well as cryo scanning electron microscopy and confocal laser scanning microscopy techniques. Moreover, the pullulan block is converted to present aldehyde groups acting as anchor point for crosslinker attachments. It is demonstrated, that the oxidized self-assembled particles could be cross-linked *via* the bifunctional crosslinker cystamine forming dynamic covalent imine linkages with aldehyde groups. The afforded vesicles with an average diameter of 700 nm are stable upon high dilution and could be observed *via* cryo scanning electron microscopy and transmission electron microscopy. Furthermore, it is possible to cleave the crosslinking bonds with the treatment of acid or the application of a reducing agent. The responsivity is a key feature taking future applications in the biomedical sector into account.

## Introduction

Block copolymer self-assembly has significant impact on polymer science as well as chemical science in general.<sup>1-3</sup> Plenty of utilizations of block copolymer self-assemblies are found in various chemical disciplines, *e.g.* block copolymer templating in mesoporous silica synthesis,<sup>4,5</sup> energy related materials<sup>6</sup> or nano structure engineering.<sup>7,8</sup> One of the most frequently utilized types of block copolymers for self-assembly are amphiphilic block copolymers that can be used for the formation of micelles,<sup>9-11</sup> cylindrical micelles<sup>12,13</sup> or vesicles<sup>14,15</sup> in aqueous environment. Concomitantly colloidal structures from amphiphilic block copolymers have found broad interest with respect to applications, *e.g.* as drug delivery vehicles,<sup>14,15</sup> hybrid materials,<sup>16</sup> photoluminescent materials<sup>17</sup> or membranes.<sup>18</sup>

In contrast to the self-assembly of amphiphilic block copolymers in aqueous solution, pure hydrophilic block copolymers, *e.g.* double hydrophilic block copolymers (DHBCs), can form self-assembled structures in aqueous solution as well. On one hand stimuli-responsive blocks were employed to form such structures. Usually a stimulus like temperature or pH change is utilized to render the solubility of one block in the

DHBC to hydrophobic.<sup>19</sup> Certainly, a triggered self-assembly has several advantages but in this case the self-assembly is due to the hydrophobic effect and the block copolymer not a pure DHBC at the time of self-assembly. On the other hand self-assembly of pure DHBC in water without external triggers is possible as shown by several researchers, *e.g.* giant vesicles of pullulan-*b*-poly(ethylene oxide) (Pull-*b*-PEO),<sup>20</sup> vesicles from Pull-*b*-poly(*N*-ethylacrylamide),<sup>21</sup> particles from poly(2-ethyl-2-oxazoline)-*b*-poly(*N*-vinylpyrrolidone) (PEtOx-*b*-PVP),<sup>22</sup> micelles from poly(2-hydroxyethyl methacrylate)-*b*-poly(2-*O*-(*N*-acetyl- $\beta$ -D-glucosamine)ethyl methacrylate)<sup>23</sup> or lyotropic mesophases from PEO-*b*-poly(2-methyl-2-oxazoline).<sup>24</sup> Certainly, depending on the choice of the blocks DHBCs might feature significant biocompatibility.<sup>25</sup>

The synthesis of DHBCs can be performed *via* reversible deactivation radical polymerization strategies, *e.g.* the reversible addition-chain transfer fragmentation (RAFT)/macromolecular architecture design *via* the interchange of xanthates (MADIX) process,<sup>26</sup> as well as modular conjugation reactions such as the copper catalyzed azide alkyne cycloaddition reaction.<sup>27</sup> While the block copolymer formation is straightforward, the self-assembly process of DHBCs is not completely understood. Although there are theoretical investigations,<sup>28</sup> the microstructure of the formed self-assemblies is still unknown. In general relatively high polymer concentrations are required for the formation of self-assembled structures, which renders them on the other hand unstable in dilute environment. In order to preserve the completely hydrophilic self-assemblies crosslinking in aqueous solution is a valid option as it has been utilized frequently in nano particle<sup>29,30</sup> and hydrogel formation.<sup>31,32</sup> In

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the case of DHBC-based systems crosslinking has been performed with self-assemblies from PEO-*b*-poly(*N*-vinylpyrrolidone) (PEO-*b*-PVP)<sup>33</sup> and PEO-*b*-PEtOx.<sup>34</sup>

A bio-derived building block that has proven to be useful in DHBC self-assembly so far is Pull,<sup>20,21</sup> which is a linear non-ionic poly(saccharide) consisting of  $\alpha$ -1,6-linked maltotriose units.<sup>35</sup> It entails significant biodegradability,<sup>35</sup> biocompatibility<sup>36</sup> and can be end functionalized easily.<sup>20,37</sup> A combination with the likewise biocompatible PVP<sup>38</sup> seems to be an efficient combination with respect to future application in biomedical science. In such a way a novel DHBC is generated that can be investigated regarding self-assembly processes in water (Scheme 1). According to research by Whitesides and co-workers, PVP and dextran homopolymers form multiphase systems in water.<sup>39</sup> Therefore, a dextran-*b*-PVP block copolymer might form self-assemblies in aqueous solution, which is an indication that Pull-*b*-PVP forms self-assemblies as Pull is structurally related to dextran.

As stated above, crosslinking of the formed self-assembled structure is beneficial for stabilization and a significant requirement for utilization in applicable concentration ranges. In that regard oxidation of the Pull backbone for the formation of aldehyde moieties allows crosslinking *via* the formation of pH responsive dynamic covalent imine bonds<sup>40,41</sup> together with a diamine molecule, *e.g.* cystamine. Therefore, pH cleavable crosslinking is reached. Moreover, utilization of the functional diamine cystamine entails the system with another feature of triggered cleavage. As cystamine contains a disulfide bond, cleavage can be induced in reductive environment.<sup>42,43</sup> In such a way DHBC-based crosslinked self-assemblies are obtained that can be cleaved *via* pH or redox triggers and intentionally disassembled at high dilution (Scheme 1).

Here a novel DHBC, namely Pull-*b*-PVP, undergoing self-assembly in aqueous solution is presented. The block copolymer is synthesized *via* a Pull macro chain transfer agent suitable for chain extension with VP *via* RAFT polymerization. Subsequently, the formation of self-assembled structures was

probed *via* dynamic light scattering (DLS), cryo scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and differential interference contrast microscopy (DIC) showing particles in the range of 150 nm to 1  $\mu$ m. Moreover, the Pull block could be oxidized and crosslinked *via* addition of cystamine. Therefore, stable structures could be generated and analyzed *via* DLS, cryo SEM and transmission electron microscopy (TEM). As shown *via* cryo SEM hollow spheres are obtained indicating vesicular structures in solution. Additionally, the formed crosslinks could be cleaved *via* pH due to the dynamic covalent nature of the imine bond or redox triggers due to the incorporated disulfide moiety leading to degraded vesicles at high dilution.

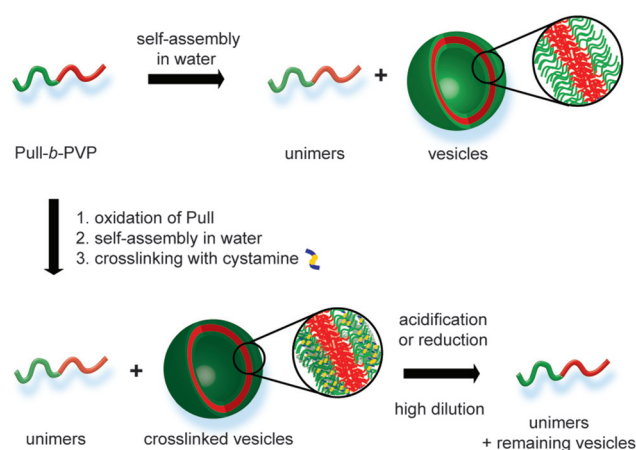
## Experimental

### Chemicals

*N*-Vinylpyrrolidone (VP, 99%, Sigma Aldrich) was dried over anhydrous magnesium sulfate and purified by distillation under reduced pressure. Acetone (analytical grade, J.T. Baker) and dichloromethane (DCM, analytical grade, Acros Organics) were stored over molecular sieves (3 Å) prior to use. Millipore water was obtained from an Integra UV plus pure water system by SG Water (Germany). Ammonium chloride (99%, Roth KG), 2-bromopropionyl bromide (97%, Sigma Aldrich), *t*-butyl peroxide (70% solution in water, Acros Organics), cystamine dihydrochloride (96%, Sigma Aldrich), diethylene glycol (99%, Fischer Chemical), diethyl ether (ACS reagent, Sigma Aldrich), *N,N*-dimethylformamide (DMF, analytical grade, Sigma Aldrich), dimethylsulfoxide (DMSO, analytical grade, VWR Chemicals), ethyl acetate (EtOAc, analytical grade, Chem Solute), hexamethylene diamine (96%, Sigma Aldrich), hexane (analytical grade, Fluka), hydrochloric acid (HCl, fuming, Roth KG), *N*-hydroxy succinimide (NHS, 98%, Sigma Aldrich), magnesium sulfate (dried, Fisher Scientific), methanol (MeOH, analytical grade, Fisher Scientific), potassium-*O*-ethyl xanthate (98% Alfa Aesar), pyridine (99% extra dry, Acros Organics), Rhodamine B (Sigma Aldrich), sodium bicarbonate (>99%, Fluka), sodium cyanoborohydride (NaCNBH<sub>3</sub>, 95%, Sigma Aldrich), sodium (*meta*)periodate (NaIO<sub>4</sub>, pure, VWR Chemicals), sodium sulfite (97%, Acros Organics), triethylamine (Sigma Aldrich) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, >98%, Roth KG) were used as received. Spectra/Por dialysis tubes with MWCOs of 10 000 and 1 000 000 were purchased from Spectrum Labs. 2,5-Dioxypyrrolidin-1-yl-2-bromopropanoate was synthesized according to the literature (see ESI† for details).<sup>44</sup> Pullulan was depolymerized and conjugated with an amine according to the literature (see ESI† for details).<sup>37,45</sup>

### Analytical techniques

<sup>1</sup>H- and <sup>13</sup>C-NMR spectra were recorded at ambient temperature at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C with a Bruker Ascend400. Size exclusion chromatography (SEC) was conducted in *N*-methyl-2-pyrrolidone (NMP, Fluka, GC grade) with



**Scheme 1** Overview for self-assembly of Pull-*b*-PVP, crosslinking *via* cystamine and pH or redox induced cleavage.





**Xanthate functionalized pullulan (Pull-X).** In a dry argon purged 50 mL round bottom Schlenk flask pullulan-NH<sub>2</sub> (2.4 g, 0.150 mmol, 1 eq.) was dissolved in dry DMSO (25 mL).

1.9  $\mu\text{L}$  (3.8  $\mu\text{L}$  for the 10% oxidized block copolymer, respectively) of the stock solution was added to the Pull<sub>124</sub>-*b*-PVP<sub>263</sub> solution and the reaction mixture was shaken for 2 days. The reaction mixture was diluted to 0.1 wt% (100  $\mu\text{L}$  of crosslinked solution was added to 5 mL Millipore water) and dialyzed against Millipore water with a 1 000 000 MWCO dialysis tube for 3 days. The resulting solution was analyzed *via* DLS at 25 °C.



**Acid induced disassembly of oxidized Pull<sub>124</sub>-b-PVP<sub>263</sub> self-assemblies.** Hydrochloric acid (150  $\mu$ L, 0.1 mol L<sup>-1</sup>) was added to 2 mL of a 0.1 wt% solution of crosslinked and dialyzed Pull<sub>124</sub>-b-PVP<sub>263</sub> oxidized to 5%. The vial was sealed and immersed in a water bath at 40 °C for 24 h. The pH induced disassembly was monitored *via* DLS at 25 °C.

**Redox induced disassembly of oxidized Pull<sub>124</sub>-b-PVP<sub>263</sub> self-assemblies.** A 0.1 wt% solution of crosslinked and dialyzed Pull<sub>124</sub>-b-PVP<sub>263</sub> oxidized to 5% was degassed with argon for 10 minutes. TCEP (20.0 mg, 0.08 mmol) was added and the sealed vial was immersed in a water bath at 40 °C for 24 h. The redox induced disassembly was monitored *via* DLS at 25 °C.

## Results and discussion

### Synthesis of $\omega$ -functionalized pullulan-xanthate

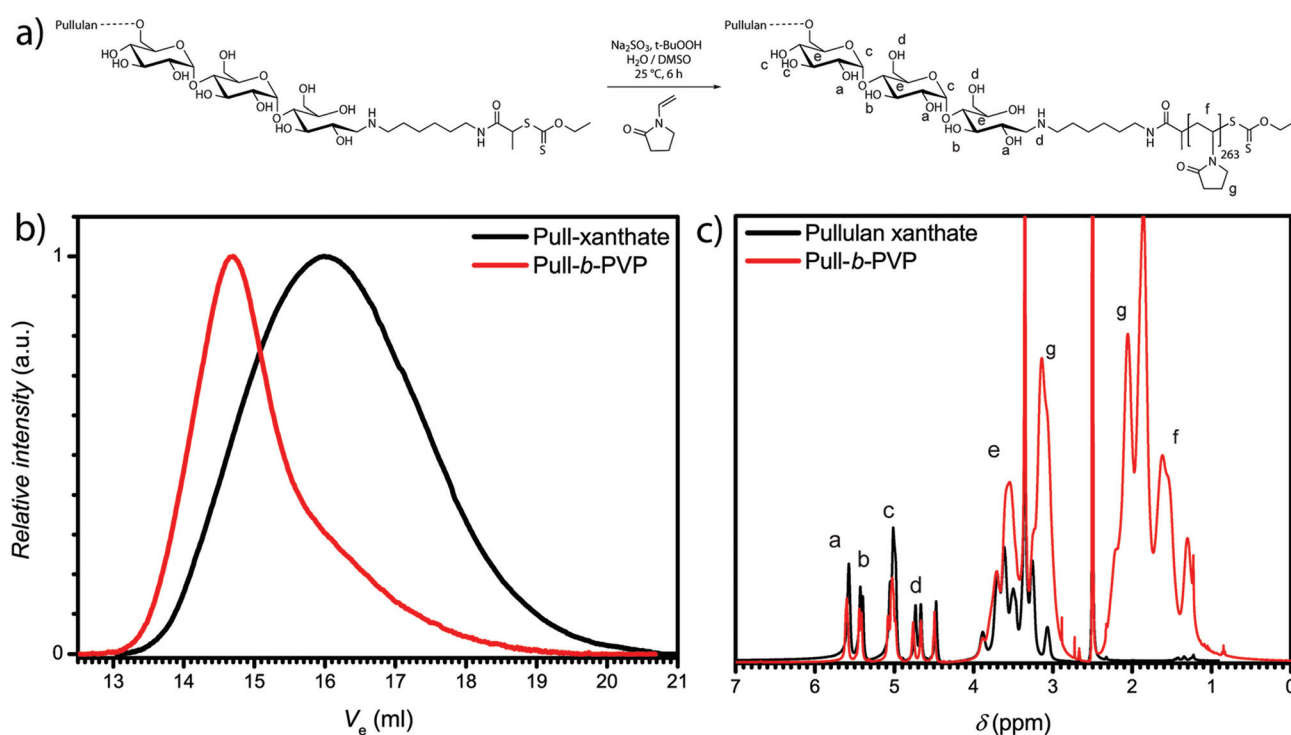
In order to synthesize a Pull macroinitiator for the block copolymer synthesis of Pull-*b*-PVP, natural Pull precursor was depolymerized according to the literature (see ESI for details, Fig. S1 and Table S1†). In a subsequent step the depolymerized Pull was end functionalized with hexamethylene diamine to introduce an amine functionalization (see ESI for details, Fig. S2 and S3†).

After the successful transformation of the  $\omega$ -aldehyde to a primary amine,<sup>37</sup> the attachment of a RAFT/MADIX chain transfer agent was performed in the next step (Scheme S1†). Therefore, a *N*-succinimidyl xanthate, namely 2,5-dioxopyrroli-

din-1-yl 2-((ethoxycarbonothioyl)thio)propanoate was synthesized *via* literature known procedures (Fig. S4 and S5†).<sup>44,46</sup> The active ester route was chosen to avoid side reactions, *e.g.* aminolysis of the RAFT agent as well as multiple attachments. The synthesis of the pullulan chain transfer agent was carried out in DMSO at ambient temperature to ensure complete dissolution of pullulan amine and the xanthate transfer agent. The xanthate functionalized pullulan was dialyzed and lyophilized after the reaction to remove DMSO and unreacted xanthate transfer agent from the solution (Scheme S2†). An apparent average molecular weight of 26 500 g mol<sup>-1</sup> with *D* of 1.7 was obtained (Table S1 and Fig. S6†). <sup>1</sup>H-NMR displays the successful attachment of the xanthate group to pullulan amine. The proton signals corresponding to the xanthate and the hexamethylene group could be assigned *via* the magnification of the area with a low chemical shift (Fig. S7†). Subsequently, block copolymer formation *via* chain extension with VP was performed.

### Block copolymer formation of Pull-*b*-PVP

Block copolymer formation *via* RAFT polymerization of VP in aqueous solution according to our previous method<sup>33</sup> led to unsatisfying results. Therefore, the system was diluted with another polar but aprotic solvent, namely DMSO (Fig. 1a). DMSO is a good solvent for monomer and macro RAFT/MADIX chain transfer agent. The polymerization was performed at 25 °C with the redox couple sodium sulfite/*t*-butyl hydroperoxide as initiator. A Pull<sub>124</sub>-b-PVP<sub>263</sub> block copolymer with



**Fig. 1** (a) Polymerization scheme for Pull-*b*-PVP block copolymer formation in water/DMSO mixture, (b) SEC traces of pullulan xanthate and Pull<sub>124</sub>-b-PVP<sub>263</sub> block copolymer synthesized in water/DMSO mixtures recorded in NMP at 70 °C and (c) <sup>1</sup>H-NMR of pullulan xanthate and Pull<sub>124</sub>-b-PVP<sub>263</sub> recorded at 400 MHz in DMSO-*d*<sub>6</sub>.





an apparent number weighted molecular mass of 58 800 g mol<sup>-1</sup> and *D* of 1.8 was obtained.

Nevertheless, the SEC traces (Fig. 1b) display slight tailing, which can be explained with the broad molecular weight distribution of pullulan. Additionally, increased interactions with the SEC column as well as the occurrence of dead chains during the polymerization can play a role for the observed tailing. The <sup>1</sup>H-NMR spectrum (Fig. 1c) further displays the successful block copolymerization with the proton signals of both polymer blocks between 4.4 ppm and 5.7 ppm for pullulan and from 1.2 ppm to 2.1 ppm for PVP, being present in the spectrum. According to the integration of the protons corresponding to the a-OH group of pullulan (Fig. 1c) and the relation to the integral of the protons corresponding to the PVP backbone, indexed as f (Fig. 1c) in the <sup>1</sup>H-NMR spectrum, a VP incorporation of 263 repeating units was calculated. The

block copolymer composition was therefore assessed to be Pull<sub>124</sub>-*b*-PVP<sub>263</sub>. In the next step the self-assembly behavior of Pull-*b*-PVP was investigated in diluted aqueous solutions.

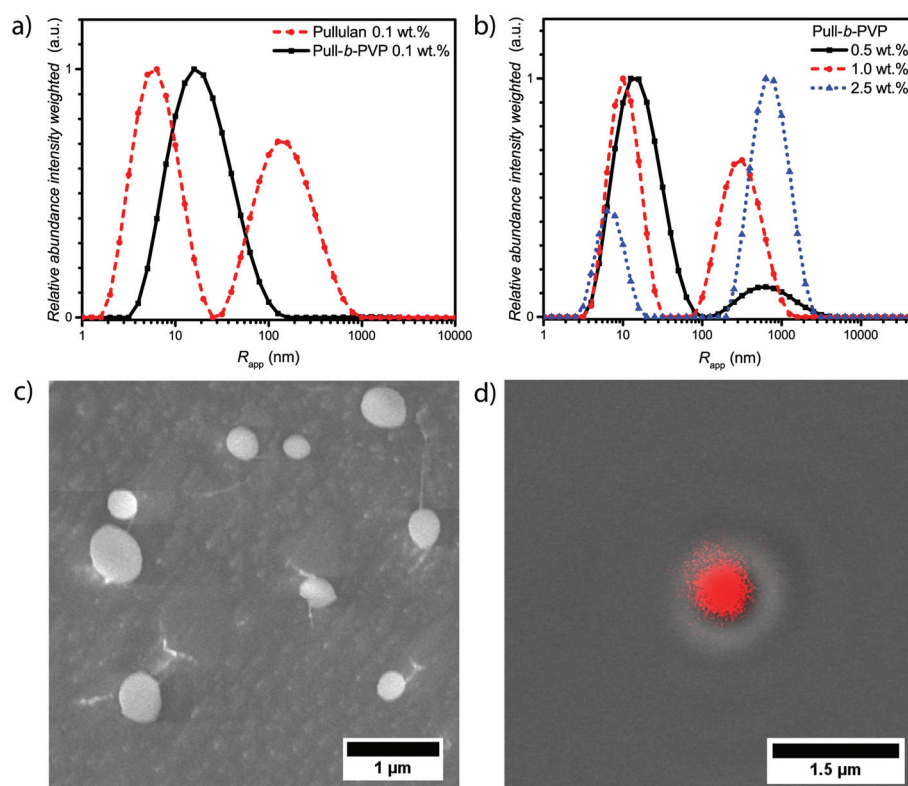
### Self-assembly of Pull-*b*-PVP block copolymers in aqueous solution

After the successful synthesis of Pull<sub>124</sub>-*b*-PVP<sub>263</sub>, the self-assembly behavior was investigated. Therefore, solutions containing 0.1, 0.5, 1.0, and 2.5 wt% of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> in Millipore water were prepared, filtered with 0.45 μm CA syringe filters, and analyzed *via* DLS. In order to compare the block copolymer with the pullulan homopolymer, a pullulan solution containing 0.1 wt% in Millipore water was prepared as well and investigated *via* DLS (Table 1).

The intensity weighted size distributions of pullulan and Pull<sub>124</sub>-*b*-PVP<sub>263</sub> at 0.1 wt% in Fig. 2a (Fig. S8†) display a strong

**Table 1** Summary of apparent average hydrodynamic radii of pullulan and Pull<sub>124</sub>-*b*-PVP<sub>263</sub> determined *via* DLS at 25 °C

Polymer	Concentration (wt%)	Peak 1 <i>R</i> <sub>h,app</sub> (nm)	Rel. abund.	Peak 2 <i>R</i> <sub>h,app</sub> (nm)	Rel. abund.
Pullulan	0.1	7	1.0	180	0.7
Pull <sub>124</sub> - <i>b</i> -PVP <sub>263</sub>	0.1	25	1.0	—	—
	0.5	16	1.0	750	0.12
	1.0	11	1.0	350	0.66
	2.5	7	0.42	800	1.0



**Fig. 2** (a) Intensity weighted size distributions of pullulan and Pull-*b*-PVP at 0.1 wt%; (b) intensity weighted size distributions of Pull-*b*-PVP at higher concentrations in Millipore water measured *via* DLS at 25 °C; (c) cryo SEM micrographs of a 0.5 wt% Pull<sub>124</sub>-*b*-PVP<sub>263</sub> solution and (d) differential interference contrast (DIC) image with confocal laser scanning microscopy (CLSM) overlay displaying the particle structure of Pull-*b*-PVP at 2.5 wt% stained with 0.08 mM Rhodamine B.





The cryo SEM measurements of a 0.5 wt% solution of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> display an increased amount of spherical particles with average diameters between 150 nm and almost 1  $\mu\text{m}$  (Fig. 2c). Moreover, tubular structures containing free dissolved block copolymer can be observed (Fig. S9†). The tubular structures form due to the growth of ice crystals during the freezing process in liquid nitrogen pushing the free dissolved block copolymers to the crystal border, where they concentrate and form tubular alignments.<sup>48</sup> Furthermore, string-like connections between a significant amount of smaller particles can be observed, *e.g.* the particles are aligned in the fashion of pearls that are lined up on a string composed of block copolymer (Fig. S9†). The occurrence of these inter-particle connections is not completely understood yet and is possibly an artifact of the cryo SEM process. Nonetheless, a high amount of spherical particles could be confirmed *via* cryo SEM measurements. In agreement to the intensity weighed

A comparison of the obtained average diameters from CLSM/DIC and cryo SEM with the average apparent radii obtained from DLS shows significant differences at the corresponding concentrations. The average apparent diameters calculated from DLS exceed the diameters determined *via* microscopy about a factor of 30–50%, which might be due to the intensity weighted particle size distribution. As larger particle are overexpressed in contrast to smaller ones in the distribution curve. Nevertheless, particle formation of completely hydrophilic block copolymers in aqueous solution is evident. In order to stabilize the particles against dilution and open up opportunities for future applications, crosslinking of the particles was studied subsequently.

The confirmation of spherical particles self-assembled from Pull-*b*-PVP block copolymers already displays the potential of this system. In order to preserve the structures and generate more applicable systems for future utilization in drug delivery, a crosslinking strategy has to be developed. Since the application of polysaccharides in biomedical and pharmaceutical field as hydrogel components is well studied, several techniques to crosslink polysaccharides with or without the application of reversible crosslinking agents were reported.<sup>47,49–52</sup> The reversibility of the crosslinking was usually enabled *via* crosslinkers bearing redox responsive groups such as dithiols or a pH sensitive linkage with the polysaccharide, such as imines.<sup>51</sup> To enable imine formation with polysaccharides such as dextran or pullulan, the cyclic hexoses need to be oxidized to alde-



hydres. The most frequently reported pathway to accomplish a mild oxidation of polysaccharides without the destruction of the backbone is carried out with sodium (*meta*)periodate in aqueous solutions (see ESI† for details).<sup>49,50</sup> Maia *et al.* reported a facile oxidation and crosslinking method for dextran.<sup>47</sup> In order to investigate the possibility of selective pullulan oxidation to a pullulan dialdehyde, test oxidations with depolymerized pullulan with different percentages of NaIO<sub>4</sub> as oxidizing agent were conducted (Table S2†). The state of oxidation was characterized *via* <sup>1</sup>H-NMR spectroscopy (Fig. S11 and Table S3†). Furthermore, SEC measurements should indicate depolymerization or degradation of oxidized pullulan (Fig. S12†). In addition to the formation of aldehyde units, a decrease in pullulan molecular weight was observed for high NaIO<sub>4</sub> equivalents that might be due to fractured polymer chains. For that reason, oxidations exceeding 10% of NaIO<sub>4</sub> were not conducted with the Pull<sub>124</sub>-*b*-PVP<sub>263</sub> block copolymers.

In order to oxidize the pullulan moieties of a Pull-*b*-PVP block copolymer, the DHBC was dissolved in Millipore water and the corresponding amount of NaIO<sub>4</sub> was added to oxidize 5% and 10% of the pullulan units to dialdehyde (Fig. 3a). As visible from the <sup>1</sup>H-NMR spectra in Fig. 3b, the oxidation of glucose units of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> can be regarded as successful. The appearance of the anomeric aldehyde protons in the area between 6.1 ppm and 7.5 ppm confirms a successful oxidation procedure. The integrals normalized to the internal standard DMF display an increase from the control sample with an integral of 0.04 to 0.67 and 1.35 for the 5% and 10% oxidation, respectively. However, absolute values of oxidized units cannot be determined *via* the previous method because the exact molecular mass of the block copolymer is unknown and only the apparent average number weighted molecular masses were determined. Therefore, no clear statement on the actual oxidation state can be given, but from the comparison of the integrals corresponding to 5% oxidation and 10% oxidation a

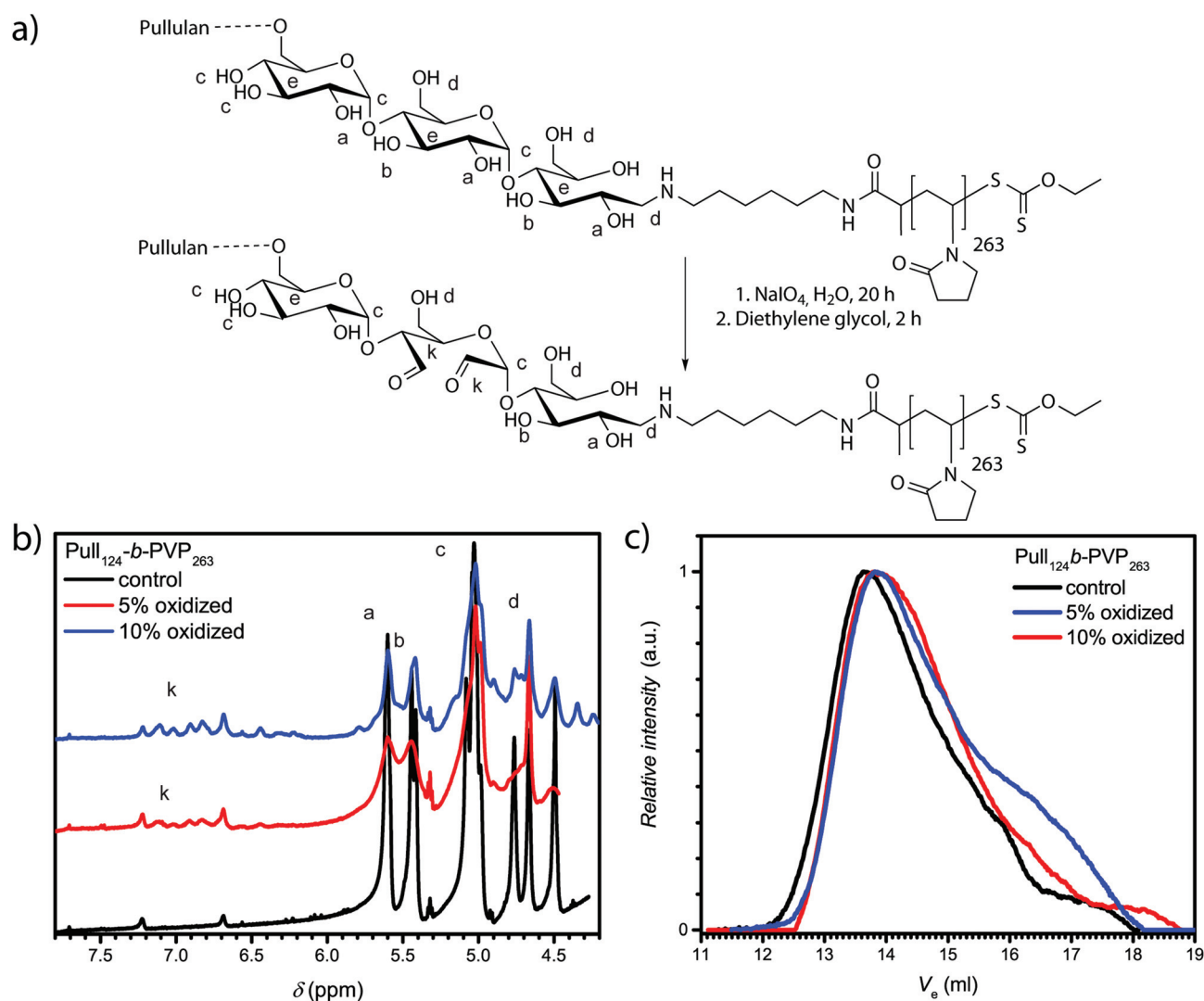


Fig. 3 (a) Schematic oxidation of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> with NaIO<sub>4</sub>; (b) <sup>1</sup>H-NMR spectra of oxidized Pull<sub>124</sub>-*b*-PVP<sub>263</sub> emphasizing on the anomeric proton peaks of the oxidized glucose units recorded at 400 MHz in DMSO-*d*<sub>6</sub>; (c) corresponding SEC elution curves recorded in NMP at 70 °C.





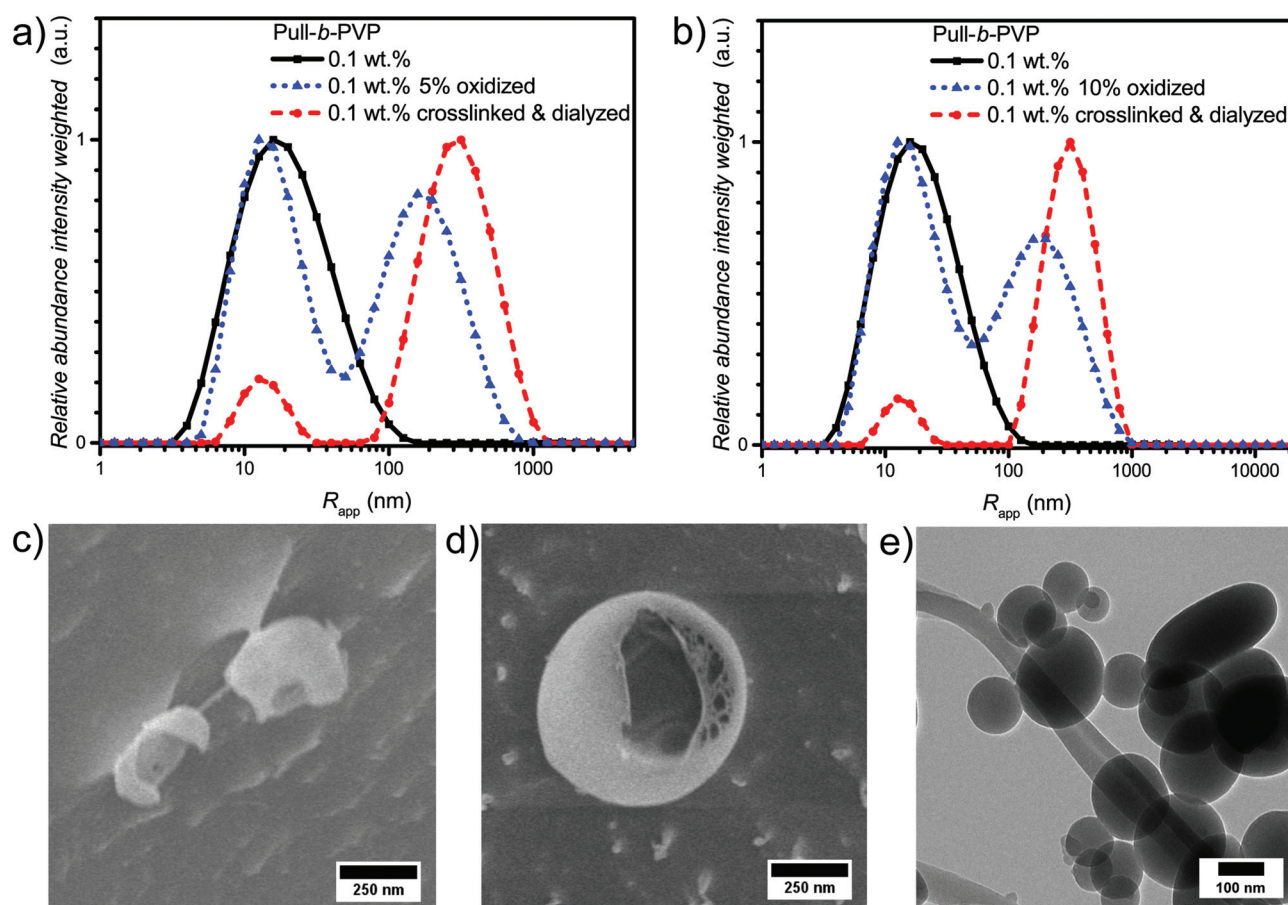
two fold increase in the absolute value of anomeric aldehyde protons can be stated.

The SEC elution curves in Fig. 3c display a decent change of the elution curves upon oxidation with  $\text{NaIO}_4$ . The oxidized block copolymers elute with a significantly broader curve compared to the initial block copolymer. The drastic change in the appearance of the elugrams can be attributed to a different interaction of the oxidized block copolymer with the solvent NMP resulting in a peak broadening towards higher elution volumes and a slightly more pronounced tailing. Another reason for tailing towards longer retention times might be chain degradation after the oxidation as species of lower molecular weight are present. Nevertheless, polymer chain fracture or depolymerization occurs only to a minor extent as seen from the elugrams and a successful oxidation of pullulan is indicated. Therefore, the appearance of the anomeric proton signals in the  $^1\text{H-NMR}$  spectrum and the SEC results both point towards a successful oxidation.

As already indicated by SEC measurements a change in the chemical behavior of the block copolymer is induced by oxidation of the Pull backbone, which is also indicated by DLS measurements of aqueous solutions of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> and its

oxidized derivatives at 0.1 wt%. As visible from the particle size distribution in Fig. 4 the unimodal average apparent particle size distribution of Pull-*b*-PVP at 0.1 wt% turned to a bimodal distribution for both oxidized species. The apparent average particle size distributions of the oxidized block copolymers show an increased tendency for self-assembly already at lower concentrations in contrast to non-oxidized Pull-*b*-PVP. The origin of the increased tendency can be referred to a change in the hydrophilicity of pullulan. As oxidation of alcohols to aldehydes decreases hydrophilicity and increases the probability of hydrogen bonding of pullulan, microphase separation is encouraged. An enhanced self-assembly is very beneficial for the attempted crosslinking step. As shown for higher concentrated solutions of Pull-*b*-PVP (Fig. 2b), the tendency to form structures such as particles increases with raising concentration. Conclusively, a higher amount of self-assembled structures should be present for the oxidized species at more concentrated solutions than it was the case for general Pull-*b*-PVP.

Due to the enhanced amount of self-assembled structures at higher block copolymer concentrations, the crosslinking procedure was conducted at a highly concentrated state of



**Fig. 4** Intensity weighted particle size distributions of 0.1 wt% solutions of initial Pull<sub>124</sub>-*b*-PVP<sub>263</sub>, oxidized block copolymer before and after crosslinking and dialysis with (a) 5% of oxidized pullulan and (b) 10% of oxidized pullulan in Millipore water measured via DLS at 25 °C. Cryo SEM micrographs of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> particles at a concentration of 0.1 wt% after crosslinking and dialysis with (c) 5% of oxidized pullulan and (d) 10% of oxidized pullulan. (e) TEM image of 10% oxidized Pull<sub>124</sub>-*b*-PVP<sub>263</sub> after crosslinking and freeze drying on a copper grid.

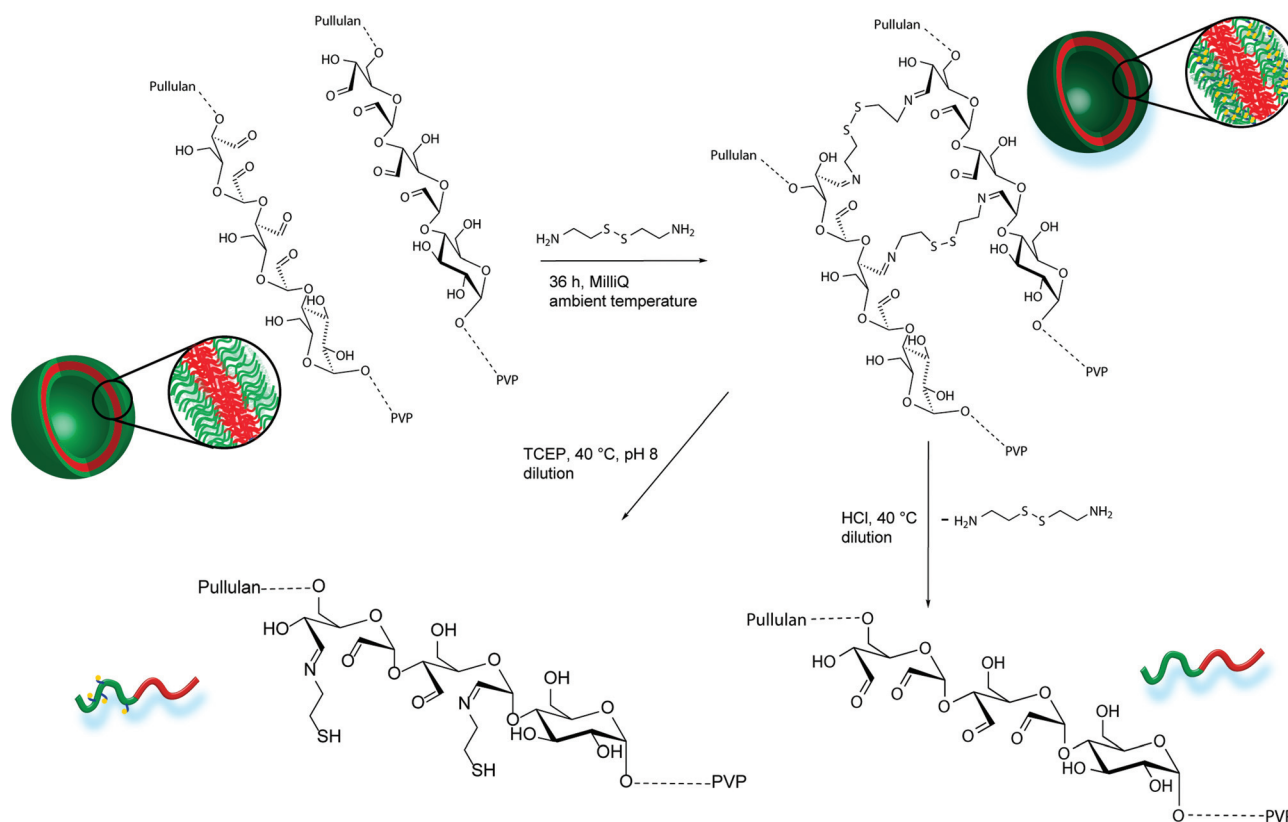




5.0 wt%. Cystamine dihydrochloride was selected as cross-linking agent. The crosslinker can reversibly attach to the oxidized glucose units of the pullulan block *via* imine formation with aldehyde groups. Furthermore, the disulfide bridge can be cleaved redox chemically by the application of suitable reducing agents, such as tricarboxyethyl phosphine (TCEP). Thus, two different triggers, pH and redox, can be applied for disassembly and probably release applications. To conduct the crosslinking, an aqueous solution containing 5 wt% of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> was prepared and a 0.044 mmol mL<sup>-1</sup> cystamine dihydrochloride solution was added. The mixture was shaken for 36 hours in order to achieve a high crosslinking density (Scheme 2). The DLS distributions do not show significant differences before and after crosslinking (Fig. S13†). In order to remove free dissolved block copolymer as well as dimers and lower aggregates, the crosslinked solution was diluted to 0.1 wt% and dialyzed for three days with an 1000 000 MWCO dialysis tube against Millipore water. The high MWCO of the dialysis membrane and the long dialysis time should ensure a complete removal of smaller species, but trap the larger crosslinked aggregates inside the tube.

As visible from the intensity weighted particle size distributions of the oxidized Pull-*b*-PVP block copolymer before, after crosslinking and dialysis (Fig. 4a), crosslinking and dialysis drastically shifted the average apparent particle size distribution towards the large species. At the same concentration of

0.1 wt% the amount of unimers would be significantly increased without crosslinking. Thus, crosslinking preserves larger particles under diluted conditions. The relative abundance of the free dissolved 5% oxidized block copolymer species decreased by 80% from 1.0 to 0.2 while maintaining the average apparent radius of 14 nm. Nevertheless, complete removal of unimer species was not possible *via* dialysis. Moreover, the average apparent radius of the large particle species increased to 340 nm being the most abundant species by intensity weighting. However, free dissolved block copolymer is still present in the solution. Due to the intensity weighted size distribution, the abundance of the free dissolved block copolymer species appears to be significantly lower than it is the case in reality. Therefore, it can be stated that dialysis was not able to completely remove all free dissolved species. One explanation for this inability could be the reversibility of the imine formation due to concentration and entropic reasons leading to slight disassembly with time which may result in free dissolved block copolymer still being abundant in the solution of crosslinked structures. The crosslinked and dialyzed sample of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> oxidized to 10% displays a similar bimodal intensity weighted particle size distribution with average apparent radii of 14 nm for the free dissolved block copolymer and 340 nm for the crosslinked structures (Fig. 4b and Table S4†). Again, complete removal of small species was not possible *via* dialysis.



**Scheme 2** Schematic crosslinking procedure of oxidized Pull-*b*-PVP self-assemblies with cystamine in Millipore water, pH or redox induced disassembly of crosslinked Pull-*b*-PVP vesicles.





In addition, the cryo SEM micrographs of the crosslinked and dialyzed sample of Pull<sub>124</sub>-*b*-PVP<sub>263</sub> oxidized to 5% display spherical particles and a small amount of vesicular structures (Fig. 4c and S14†). Moreover, no tubular alignments of free dissolved block copolymer are visible as before (Fig. S14†). The crosslinked and dialyzed particles possess average diameters between 250 nm and 1.2  $\mu$ m. Larger spherical structures with diameters between 700 nm and 1.2  $\mu$ m often show a ruptured morphology with a hollow interior indicating vesicle formation. Moreover, some ruptured smaller particles with diameters around 250 nm could be observed (Fig. S14†) which indicate a hollow structure as well. The presence of vesicular structures increases with a higher oxidation state of pullulan at 10%. The cryo SEM micrographs (Fig. 4d and S15†) display a high amount of vesicular structures with average diameters ranging between 250 nm and 1.2  $\mu$ m. Only a small amount of small structures without vesicular shape could be observed (Fig. S15†). An even more impressive observation can be seen in Fig. S15† with the presence of anisotropic structures, which could possibly origin from two structures that merged during the crosslinking process. Such a merging of vesicles is not unlikely due to the high concentration of 5.0 wt% during the crosslinking procedure, which results in close proximity of the spherical structures and entanglement of block copolymer chain is possible. The magnification of a crosslinked Pull-*b*-PVP vesicle (Fig. 4d) gives some more insight into its interior displaying an interpenetrating network of crosslinked block copolymer which increases in density towards the outer border to form a closed shell. Despite the insight into the vesicular structure, the cryo SEM technique prevents a clear postulation about the origin of the observations. These network structures can either be an intrinsic feature of the vesicles or be artifacts of the sample preparation, especially during the freezing and sputtering process.

TEM imaging of 10% oxidized Pull<sub>124</sub>-*b*-PVP<sub>263</sub> after crosslinking and freeze drying on a copper grid (Fig. 4e and S16†) shows spherical particles with diameters between 100 and 500 nm. The particles might appear smaller than expected as drying of the samples certainly leads to collapsed structures. Moreover, some particles seem to have ruptured shells (Fig. S16b†), which is another indication for vesicular structures being present in solution.

Despite the uncertainties regarding the interior vesicle structure, the self-assembled and crosslinked vesicles already mark a significant step towards a model drug delivery cargo system. The vesicular structures encourage an encapsulation of target molecules. Therefore, a triggered cleavage of the crosslinked structures would be beneficial, *e.g.* *via* a pH or redox trigger (Scheme 2).

In order to assess pH triggered disassembly of the self-assembled and crosslinked Pull-*b*-PVP vesicles, 2.0 mL of 0.1 wt% solutions of the 5% oxidized and crosslinked samples were treated with HCl. The pH of the solution was adjusted to 3 (150  $\mu$ L of a 0.1 mol L<sup>-1</sup> HCl solution) and the solution was heated to 40 °C overnight. Reduction triggered disassembly was performed *via* TCEP addition to self-assembled and crosslinked Pull-*b*-PVP vesicles, 2.0 mL of 0.1 wt% solutions of the 10% oxidized and crosslinked samples were treated with 20 mg TCEP for the cleavage of the disulfide bridges. Therefore, the solution containing the crosslinked vesicles was placed in a DLS vial and argon was bubbled through to remove the oxygen inside the solution. Subsequently, TCEP was added and the sealed vial was heated in a water bath at 40 °C overnight. In both cases heat treatment was necessary to generate a certain activation energy which is required to accelerate the desired reaction pathway and encourage the disassembly process. After the addition of the cleavage agent and heat treatment, the two samples were investigated *via* DLS and com-

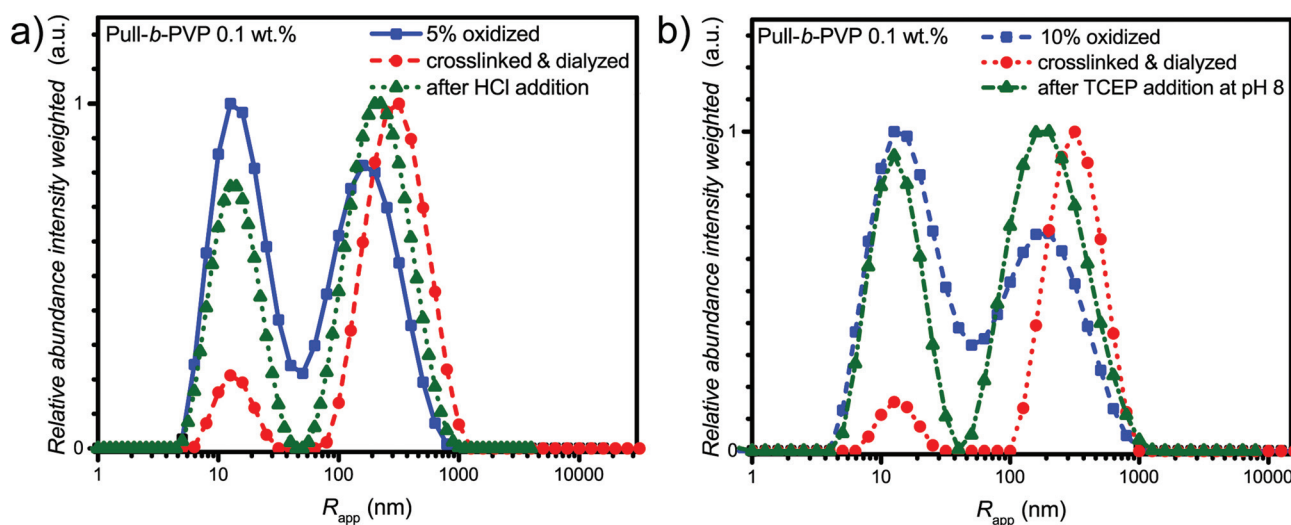


Fig. 5 Comparison of intensity weighted particle size distributions of 0.1 wt% solutions in Millipore water measured *via* DLS at 25 °C of (a) 5% oxidized Pull<sub>124</sub>-*b*-PVP<sub>263</sub>, oxidized block copolymer after crosslinking and dialysis and after treatment with HCl; (b) 10% oxidized Pull<sub>124</sub>-*b*-PVP<sub>263</sub>, oxidized block copolymer after crosslinking and dialysis and after treatment with TCEP.





pared with the particle size distribution of the oxidized and the crosslinked samples (Fig. 5).

The DLS results of the acid treatment of the crosslinked Pull-*b*-PVP vesicles (Fig. 5a) display a significant disassembly of the crosslinked structures. The relative abundance of the free dissolved species increased by four-fold to 0.77 and the average apparent radius decreased by 27% from 340 nm to 250 nm (Table S4†). Nevertheless, complete disassembly was not observed but the observed distribution is close to the initial distribution after oxidation. Moreover, in the case of TCEP treatment as visible from the intensity weighted average particle size distribution (Fig. 5b) the addition of TCEP to the crosslinked vesicle solution resulted in disassembly of the vesicular structures as well. The relative abundance of the peak corresponding to the free dissolved block copolymer increased by four fold from 0.2 to 0.92. Taking the intensity weighing into account, the increase in relative abundance is already very significant and a decent success in disassembly can be stated. Nevertheless, quantitative disassembly was not the case, yet the apparent size distribution is close to the initial size distribution after oxidation. Furthermore, the average apparent radius decreased by 30% from 340 nm to 240 nm (Table S4†). When comparing the average apparent particle size distribution of the oxidized species with the crosslinked one after TCEP addition, a complete disappearance of the aggregated species can probably not be achieved because the oxidized species displays a certain tendency to form aggregates as well. Overall, acid and TCEP addition display a facile pathway towards the disassembly of the crosslinked DHBC vesicles.

## Conclusions

Herein, a novel DHBC Pull-*b*-PVP was synthesized *via* RAFT/MADIX techniques starting from the biomacromolecule pullulan and the related macro RAFT/MADIX chain transfer agent. The block copolymer self-assembled to spherical structures with an average apparent radius of 800 nm at increased concentrations in water. Furthermore, spherical structures could be observed with cryo SEM and CLSM techniques. The pullulan block could be successfully converted to present aldehyde groups acting as anchor point for crosslinker attachments. It was demonstrated, that the oxidized self-assembled particles could be crosslinked *via* the bifunctional crosslinker cystamine forming dynamic covalent imine linkages with aldehyde groups. The afforded vesicles with an average diameter of 700 nm were stable upon high dilution and could be observed *via* cryo SEM and TEM. Furthermore, it was possible to cleave the crosslinking bonds with the treatment of acid or the application of the reducing agent TCEP. The triggered cleavage is a key feature taking future applications in the biomedical sector into account.

## Conflicts of interest

There are no conflicts to declare.

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## Notes and references

- 1 C. J. Hawker and K. L. Wooley, *Science*, 2005, **309**, 1200–1205.
- 2 Y. Mai and A. Eisenberg, *Chem. Soc. Rev.*, 2012, **41**, 5969–5985.
- 3 T. H. Epps III and R. K. O'Reilly, *Chem. Sci.*, 2016, **7**, 1674–1689.
- 4 D. Zhao, J. Feng, Q. Huo, N. Melosh, G. H. Fredrickson, B. F. Chmelka and G. D. Stucky, *Science*, 1998, **279**, 548–552.
- 5 E. Krämer, S. Förster, C. Göltner and M. Antonietti, *Langmuir*, 1998, **14**, 2027–2031.
- 6 M. C. Orilall and U. Wiesner, *Chem. Soc. Rev.*, 2011, **40**, 520–535.
- 7 J. Y. Cheng, A. M. Mayes and C. A. Ross, *Nat. Mater.*, 2004, **3**, 823–828.
- 8 M. Inam, G. Cambridge, A. Pitto-Barry, Z. P. L. Laker, N. R. Wilson, R. T. Mathers, A. P. Dove and R. K. O'Reilly, *Chem. Sci.*, 2017, **8**, 4223–4230.
- 9 G. Riess, *Prog. Polym. Sci.*, 2003, **28**, 1107–1170.
- 10 J.-F. Gohy, in *Block Copolymers II*, ed. V. Abetz, Springer, Berlin, Heidelberg, 2005, pp. 65–136.
- 11 E. Blasco, B. V. K. J. Schmidt, C. Barner-Kowollik, M. Pinol and L. Oriol, *Polym. Chem.*, 2013, **4**, 4506–4514.
- 12 N. Petzetakis, A. P. Dove and R. K. O'Reilly, *Chem. Sci.*, 2011, **2**, 955–960.
- 13 J. B. Gilroy, T. Gädt, G. R. Whittell, L. Chabanne, J. M. Mitchels, R. M. Richardson, M. A. Winnik and I. Manners, *Nat. Chem.*, 2010, **2**, 566–570.
- 14 B. Jeong, Y. H. Bae, D. S. Lee and S. W. Kim, *Nature*, 1997, **388**, 860–862.
- 15 A. Rösler, G. W. M. Vandermeulen and H.-A. Klok, *Adv. Drug Delivery Rev.*, 2012, **64**, 270–279.
- 16 S. Förster and M. Antonietti, *Adv. Mater.*, 1998, **10**, 195–217.
- 17 S.-J. Park, S.-G. Kang, M. Fryd, J. G. Saven and S.-J. Park, *J. Am. Chem. Soc.*, 2010, **132**, 9931–9933.
- 18 C. Hörenz, C. Pietsch, A. S. Goldmann, C. Barner-Kowollik and F. H. Schacher, *Adv. Mater. Interfaces*, 2015, **2**, 1500042.
- 19 B. V. K. J. Schmidt and C. Barner-Kowollik, *Polym. Chem.*, 2014, **5**, 2461–2472.
- 20 S. M. Brosnan, H. Schlaad and M. Antonietti, *Angew. Chem., Int. Ed.*, 2015, **54**, 9715–9718.
- 21 J. Willersinn, A. Bogomolova, M. Brunet Cabré and B. V. K. J. Schmidt, *Polym. Chem.*, 2017, **8**, 1244–1254.





- 22 J. Willersinn and B. V. K. J. Schmidt, *Polymers*, 2017, **9**, 293.
- 23 H. Park, S. Walta, R. R. Rosencrantz, A. Korner, C. Schulte, L. Elling, W. Richtering and A. Boker, *Polym. Chem.*, 2016, **7**, 878–886.
- 24 A. Taubert, E. Furrer and W. Meier, *Chem. Commun.*, 2004, 2170–2171.
- 25 K. Knop, R. Hoogenboom, D. Fischer and U. S. Schubert, *Angew. Chem., Int. Ed.*, 2010, **49**, 6288–6308.
- 26 A. B. Lowe and C. L. McCormick, *Prog. Polym. Sci.*, 2007, **32**, 283–351.
- 27 J. F. Lutz, *Angew. Chem., Int. Ed.*, 2007, **46**, 1018–1025.
- 28 J. Wu, Z. Wang, Y. Yin, R. Jiang, B. Li and A.-C. Shi, *Macromolecules*, 2015, **48**, 8897–8906.
- 29 A. F. Hirschbiel, B. V. K. J. Schmidt, P. Krolla-Sidenstein, J. P. Blinco and C. Barner-Kowollik, *Macromolecules*, 2015, **48**, 4410–4420.
- 30 W. Zhang, Z. Kochovski, B. V. K. J. Schmidt, M. Antonietti and J. Yuan, *Polymer*, 2016, **107**, 509–516.
- 31 A. M. Kloxin, A. M. Kasko, C. N. Salinas and K. S. Anseth, *Science*, 2009, **324**, 59–63.
- 32 B. Kumru, M. Shalom, M. Antonietti and B. V. K. J. Schmidt, *Macromolecules*, 2017, **50**, 1862–1869.
- 33 J. Willersinn, M. Drechsler, M. Antonietti and B. V. K. J. Schmidt, *Macromolecules*, 2016, **49**, 5331–5341.
- 34 V. Ďordovič, M. Uchman, K. Procházka, A. Zhigunov, J. Pleštil, A. Nykänen, J. Ruokolainen and P. Matějček, *Macromolecules*, 2013, **46**, 6881–6890.
- 35 R. S. Singh, N. Kaur and J. F. Kennedy, *Carbohydr. Polym.*, 2015, **123**, 190–207.
- 36 L. Chen, X. Wang, F. Ji, Y. Bao, J. Wang, X. Wang, L. Guo and Y. Li, *RSC Adv.*, 2015, **5**, 94719–94731.
- 37 C. Schatz, S. Louguet, J.-F. Le Meins and S. Lecommandoux, *Angew. Chem., Int. Ed.*, 2009, **48**, 2572–2575.
- 38 M. Teodorescu and M. Bercea, *Polym.-Plast. Technol. Eng.*, 2015, **54**, 923–943.
- 39 C. R. Mace, O. Akbulut, A. A. Kumar, N. D. Shapiro, R. Derda, M. R. Patton and G. M. Whitesides, *J. Am. Chem. Soc.*, 2012, **134**, 9094–9097.
- 40 Y. Jin, C. Yu, R. J. Denman and W. Zhang, *Chem. Soc. Rev.*, 2013, **42**, 6634–6654.
- 41 Y. Azuma, T. Terashima and M. Sawamoto, *Macromolecules*, 2017, **50**, 587–596.
- 42 J. Xu, K. Jung, N. A. Corrigan and C. Boyer, *Chem. Sci.*, 2014, **5**, 3568–3575.
- 43 W. C. de Vries, D. Grill, M. Tesch, A. Ricker, H. Nüsse, J. Klingauf, A. Studer, V. Gerke and B. J. Ravoo, *Angew. Chem., Int. Ed.*, 2017, **56**, 9603–9607.
- 44 F. Lecolley, L. Tao, G. Mantovani, I. Durkin, S. Lautru and D. M. Haddleton, *Chem. Commun.*, 2004, 2026–2027.
- 45 L. Ilić, K. Jeremić and S. Jovanović, *Eur. Polym. J.*, 1991, **27**, 1227–1229.
- 46 G. Pound, F. Aguesse, J. B. McLeary, R. F. M. Lange and B. Klumperman, *Macromolecules*, 2007, **40**, 8861–8871.
- 47 J. Maia, L. Ferreira, R. Carvalho, M. A. Ramos and M. H. Gil, *Polymer*, 2005, **46**, 9604–9614.
- 48 B. Wicklein, A. Kocjan, G. Salazar-Alvarez, F. Carosio, G. Camino, M. Antonietti and L. Bergstrom, *Nat. Nanotechnol.*, 2015, **10**, 277–283.
- 49 K. H. Bouhadir, D. S. Hausman and D. J. Mooney, *Polymer*, 1999, **40**, 3575–3584.
- 50 J. P. Draye, B. Delaey, A. Van de Voorde, A. Van Den Bulcke, B. Bogdanov and E. Schacht, *Biomaterials*, 1998, **19**, 99–107.
- 51 J. McCann, J. M. Behrendt, J. F. Yan, S. Halacheva and B. R. Saunders, *J. Colloid Interface Sci.*, 2015, **449**, 21–30.
- 52 S. K. Tripathi, R. Goyal and K. C. Gupta, *Soft Matter*, 2011, **7**, 11360–11371.

