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Amylose-Coated Biohybrid Microgels by Phosphorylase-Catalyzed Grafting-From Polymerization

Elisabeth Gau, Franziska Flecken, Thomke Belthle, Masyitha Ambarwati, Katja Loos, and Andrij Pich*

Herein, the synthesis of amylose-coated, temperature-responsive poly(*N*-vinylcaprolactam) (VCL)-based copolymer microgels by enzyme-catalyzed grafting-from polymerization with phosphorylase b from rabbit muscle is reported. The phosphorylase is able to recognize the oligosaccharide maltoheptaose as primer and attach glucose units from the monomer glucose-1-phosphate to it, thereby forming amylose chains while releasing inorganic phosphate. Therefore, to enable the phosphorylase-catalyzed grafting-from polymerization of glucose-1-phosphate from the PVCL-based microgels, the maltoheptaose primer is covalently attached to the microgel in the first synthesis step. This is realized by adding *N*-(2-aminoethyl) methacrylamide (AEMAA) as a comonomer to the PVCL microgel to integrate primary amino groups and subsequent coupling of maltoheptaonolactone. Both the PVCL/AEMAA microgel as well as the obtained microgel–maltoheptaose construct are characterized in detail by dynamic light scattering, electrophoretic mobility measurements, IR spectroscopy, and atomic force microscopy. From the microgel–maltoheptaose construct, the grafting-from polymerization of glucose-1-phosphate is performed by the addition of phosphorylase b. Atomic force microscopy images clearly demonstrate the formation of an amylose shell around the microgels. The developed amylose-coated microgels open up promising application possibilities, for example, as colloidal scavengers, since amylose helices can serve as host molecules for inclusion of hydrophobic guest molecules.

Aqueous microgels are crosslinked porous polymer networks with a spherical shape which form colloiddally stable dispersions in water.^[1–3] Special attention has been paid to microgels based on *N*-vinylcaprolactam (VCL) during the last years, since these exhibit outstanding properties like a high biocompatibility and temperature-responsive behavior.^[4–6] PVCL microgels show a volume phase transition at a temperature of 32 °C,^[7,8] being swollen at temperatures below while losing water and thus collapsing at temperatures above. Due to the unique properties of PVCL microgels, numerous applications in the biomedical field were developed in the last years, for example, the use of PVCL-based microgels for drug release^[6,9,10] or as contrast agent in tumor magnetic resonance (MR) imaging after gadolinium loading.^[5]

The combination of different types of microgels and biomacromolecules has always been an interesting challenge to enhance the application possibilities such constructs are offering not only in the biomedical field, but also in other application areas.^[11] Regarding the literature, different types of biomacromolecules have been already attached to microgels, mainly

peptides, proteins, and enzymes.^[12] Covalent attachment of enzymes to microgels is often reached by the active ester method (*N*-hydroxysuccinimide, NHS) and by the carbodiimide coupling strategy (*N,N'*-dicyclohexylcarbodiimide, DCC or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDC).^[12,13] In our group, recently a method for selective surface-modification of microgels with biomacromolecules was developed, the so called sortase-mediated ligation (SML). After having covalently attached the peptide sequence LPETG for recognition by the transpeptidase enzyme sortase A (SrtA) to PVCL microgels, SrtA is added to enzymatically attach an oligoglycin-tagged protein or enzyme.^[14] Besides, also the conjugation of DNA oligonucleotides to microgels was already reported. Ali et al. described the covalent coupling of 5'-amine modified DNA oligonucleotides to carboxyl-containing PNIPAm microgels via EDC/*N*-hydroxysulfosuccinimide (NHSS) chemistry.^[15,16] However, polysaccharides and also shorter oligo- and monosaccharides have not been combined with microgels very often so far, probably because it is challenging to attach these molecules bearing most of the times no functional and reactive groups except hydroxyl groups to the polymer network.

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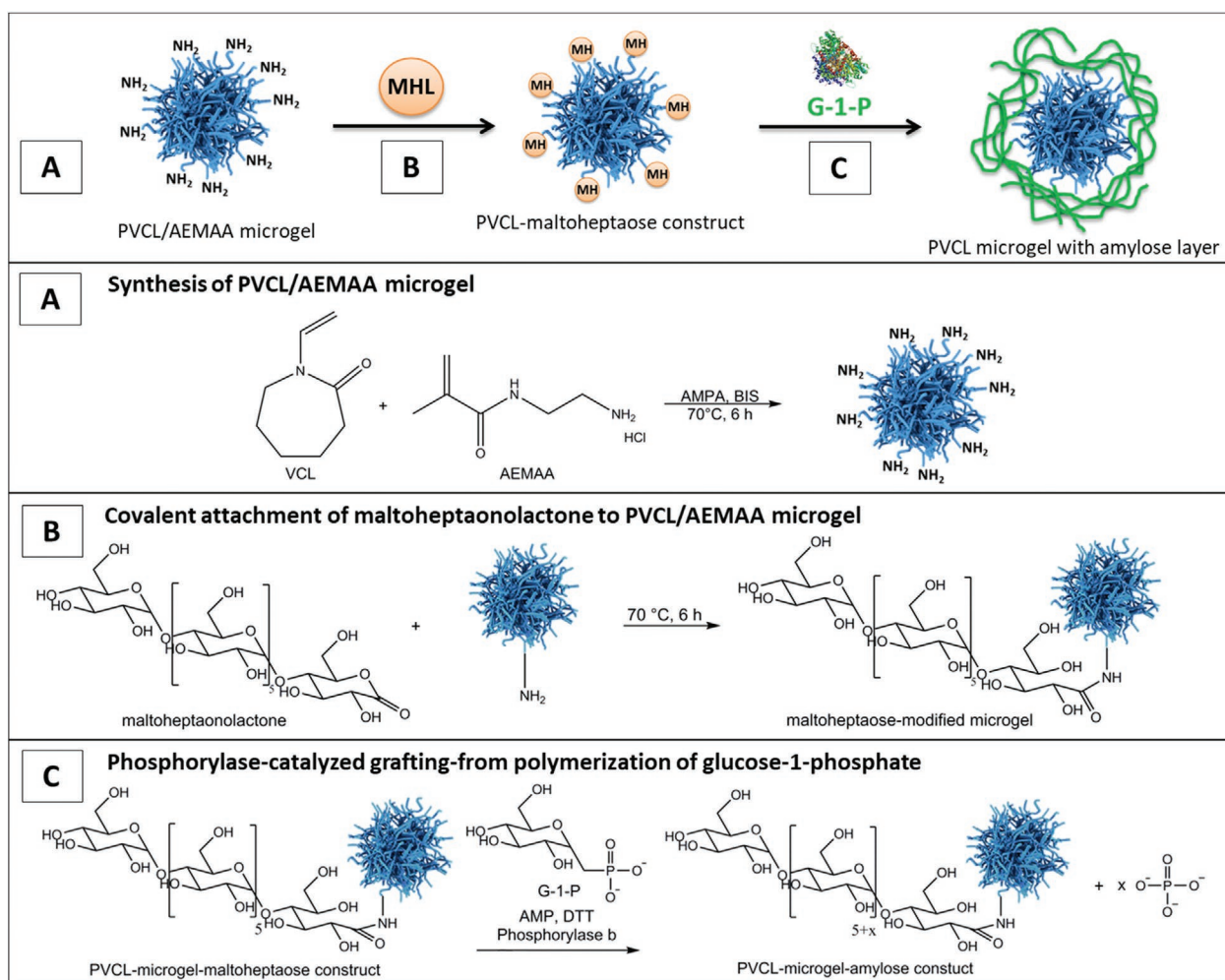
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Since simple and complex carbohydrates are highly important in biological systems regarding metabolic, structural, and physical roles,^[17] the binding of such substances to microgels would lead to the design of interesting materials combining the promising properties of the polymer network with the biological functions of the saccharide. Recently reported examples for the incorporation of glycans into microgels are the synthesis of polyethylene glycol (PEG)-based microgels containing *N*-acetylglucosamine (GlcNAc) and *N*-acetyllactosamine (LacNAc) using microfluidics^[18] and the synthesis of poly(*N*-isopropylacrylamide) (PNIPAm)-based microgels containing trehalose in the crosslinks for application as soft matrices in 3D cell culture.^[19]

Amylose is a natural occurring linear polysaccharide consisting of glucose units linked by α -(1 \rightarrow 4)-glycosidic linkages and exhibiting a helical conformation.^[20] Amylose is one of the two main components of starch, making up about 20% of it while the remaining 80% are amylopectin.^[21] Separation of amylose from amylopectin inside starch is difficult, preventing the availability of pure amylose from this source. This is why pure amylose is until now only accessible by an enzymatic pathway which is the

phosphorylase-catalyzed polymerization of glucose-1-phosphate (G-1-P).^[22] The limited availability of pure amylose is probably the reason why only combinations of microgels with starch have been reported in the literature so far,^[23] but to the best of our knowledge, pure amylose has not been integrated into microgels until now, even though they would offer interesting application possibilities. Due to the helical conformation of amylose creating a hydrophobic cavity in its interior since the hydroxyl groups are located on the outside, amylose can act as a host to include low molecular weight guest molecules exhibiting a hydrophobic character.^[22,24] Therefore, it would be possible, for example, to use amylose-coated microgels as scavengers for the removal of hydrophobic compounds from solutions, since the microgels would enable a facile separation from the solution by centrifugation.

In this work, we report for the first time the enzyme-catalyzed amylose shell formation around temperature-sensitive PVCL microgels by grafting-from polymerization (**Scheme 1**). Phosphorylase b from *rabbit muscle* is able to catalyze amylose-formation by grafting glucose units from the monomer glucose-1-phosphate to a maltoheptaose primer while inorganic



Scheme 1. The amylose-coating of microgels by phosphorylase-catalyzed grafting-from polymerization consists of three steps: A) synthesis of poly(*N*-vinylcaprolactam-*co*-*N*-(2-aminoethyl) methacrylamide) (PVCL/AEMAA) microgels, B) covalent attachment of maltoheptaonolactone to the primary amino groups of the PVCL/AEMAA microgel, and C) phosphorylase-catalyzed grafting-from polymerization of glucose-1-phosphate (G-1-P) to PVCL-based maltoheptaose-modified microgels leading to the formation of amylose-covered PVCL microgels.

phosphate is released. This principle has been already used to attach amylose to silica beads^[25,26] and is now transferred from hard particles to soft microgel networks.

To realize an amylose–microgel construct, a way to synthesize a microgel containing the covalently attached maltoheptaose primer for the enzyme-catalyzed polymerization was needed. Since the maltoheptaose does not contain reactive groups which can be used for covalent attachment to the microgel, the first step was the oxidation of the oligosaccharide to introduce a carbonyl group into the molecule. According to the literature, iodine and potassium peroxide were used to synthesize the maltoheptaonolactone from maltoheptaose (Figure S1, Supporting Information).^[27–29] The successful lactone synthesis was confirmed by IR- and ¹³C-NMR-spectroscopy (Figure S3, Supporting Information).

The next step was the synthesis of a suitable microgel exhibiting reactive groups for the attachment of the primer. Since the terminal carbonyl group of the synthesized lactone is able to react with primary amino groups, poly(*N*-vinylcaprolactam-*co*-*N*-(2-aminoethyl) methacrylamide) (PVCL/AEMAA) microgels were synthesized by free radical precipitation polymerization in water (1A). *N,N'*-methylenebis(acrylamide) (BIS) was used as crosslinker and 2,2'-azobis(2-methylpropionamide) dihydrochloride (AMPA) as initiator for the precipitation polymerization (see Supporting Information for experimental procedure). The PVCL/AEMAA microgel was obtained in the form of a stable, turbid dispersion with a microgel yield of 60.2% determined gravimetrically after the purification by dialysis and lyophilization. The hydrodynamic radii and the polydispersity indices (PDI) of the microgel were analyzed by dynamic light scattering at different temperatures to study the temperature-responsive behavior. At a temperature of 20 °C, so below the VPTT, the hydrodynamic radius of the microgels was determined to be 96.0 ± 5.4 nm (PDI = 0.489 ± 0.070), while at 50 °C, above the VPTT, the hydrodynamic radius is with 46.4 ± 1.5 nm significantly lower (PDI = 0.357 ± 0.009). These results display the expected temperature-sensitive behavior of the microgels due to the incorporation of VCL as the main monomer. However, the PDIs of the microgel are quite high at both temperatures, indicating that the microgels are slightly polydisperse. The incorporation of the primary amino groups bearing comonomer into the microgel network was investigated by IR-spectroscopy. The characteristic bands for both the additional amide bond as well as the primary amino group appear at the same wavenumbers and can be observed at 1024 cm⁻¹ (C–N stretching vibration), at 1530 cm⁻¹ (N–H bending vibration), and at 3050–3300 cm⁻¹ (N–H stretching vibration) (Figure 1; see Figure S2, Supporting Information for comparison with PVCL reference). Besides, the electrophoretic mobility of the microgels was measured and turned out to be positive (2.33 ± 0.13 μm·cm (Vs)⁻¹ in H₂O), due to the presence of primary amino groups from the comonomer being positively charged.

Next, the covalent attachment of the maltoheptaonolactone to the PVCL/AEMAA microgels was performed according to a procedure described in the literature for the coupling of lactones to primary amino groups (1B).^[29] The lactone was directly solved in the microgel dispersion and the mixture was heated up to 70 °C (see Supporting Information for experimental procedure). Through the attachment of the oligosaccharide, the hydrodynamic radius of the microgel increased to 242.5 ± 11.4 nm (PDI = 0.280 ± 0.014) (before: 96.0 ± 5.4 nm

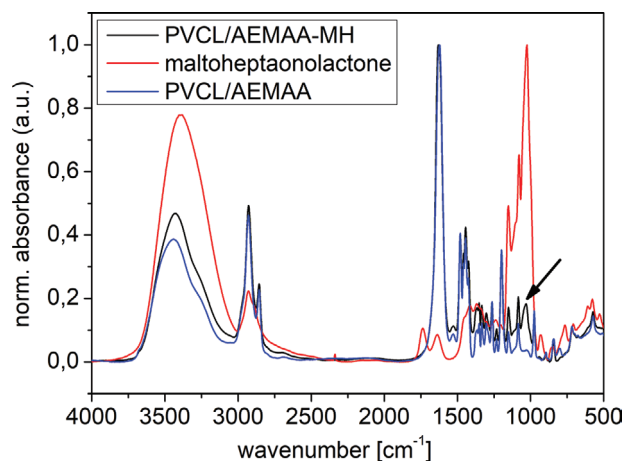


Figure 1. FTIR-spectra of the PVCL/AEMAA microgel and the maltoheptaonolactone before the coupling reaction and of the PVCL/AEMAA-maltoheptaose microgel–primer construct.

[PDI = 0.489]) at 20 °C. If the temperature is raised to 50 °C, the hydrodynamic radius of the microgel–primer construct is 134.5 ± 11.1 nm (PDI = 0.256 ± 0.021) (before: 46.4 ± 1.5 nm [PDI = 0.357]) indicating that the temperature-responsive behavior of the microgels is preserved after the oligosaccharide coupling. The electrophoretic mobility increased slightly to 2.73 ± 0.21 μm·cm (Vs)⁻¹ (before: 2.33 ± 0.13 μm·cm (Vs)⁻¹) through the attachment of the maltoheptaonolactone. Since the maltoheptaonolactone itself does not carry any charge, the positive value indicates that probably there are still some residual, non-reacted primary amino groups present, which are positively charged. To monitor the different chemical compositions of the microgels after the primer coupling, IR-spectroscopy was used and the resulting spectrum was compared to the spectrum of the native PVCL/AEMAA microgel (Figure 1). The band at 1028 cm⁻¹ resulting from the C–O stretching vibration of the numerous C–O bonds in the oligosaccharide, which is the band with the highest intensity in the spectrum of the lactone, is also visible inside the microgel–primer construct after the coupling reaction, indicating successful attachment.

After having realized this important preliminary step of covalently attaching the maltoheptaose in form of its corresponding lactone to the PVCL-based microgel, in the next step the phosphorylase-catalyzed grafting-from polymerization of glucose-1-phosphate (G-1-P) was performed (1C). Therefore, phosphorylase b from rabbit muscle was added to the microgel–primer construct as well as adenosine 3',5'-cyclic monophosphate (AMP) and DL-dithiothreitol (DTT) to ensure its activity. The addition of AMP as an allosteric activator in excess is required to shift the equilibrium from the inactive T-form to the active R-form of the phosphorylase.^[30–33] The DTT is necessary to protect the thiol groups inside the enzymatic structure from oxygen induced oxidation and thereby preserving the activity of the enzyme.^[34,35] Besides, the monomer G-1-P was added to the reaction mixture (see Supporting Information for experimental procedure).

The formation of an amylose coating around the microgels resulting from enzyme-mediated grafting-from polymerization was analyzed by different characterization methods (Table S1, Supporting Information). As a first indicator, dynamic light

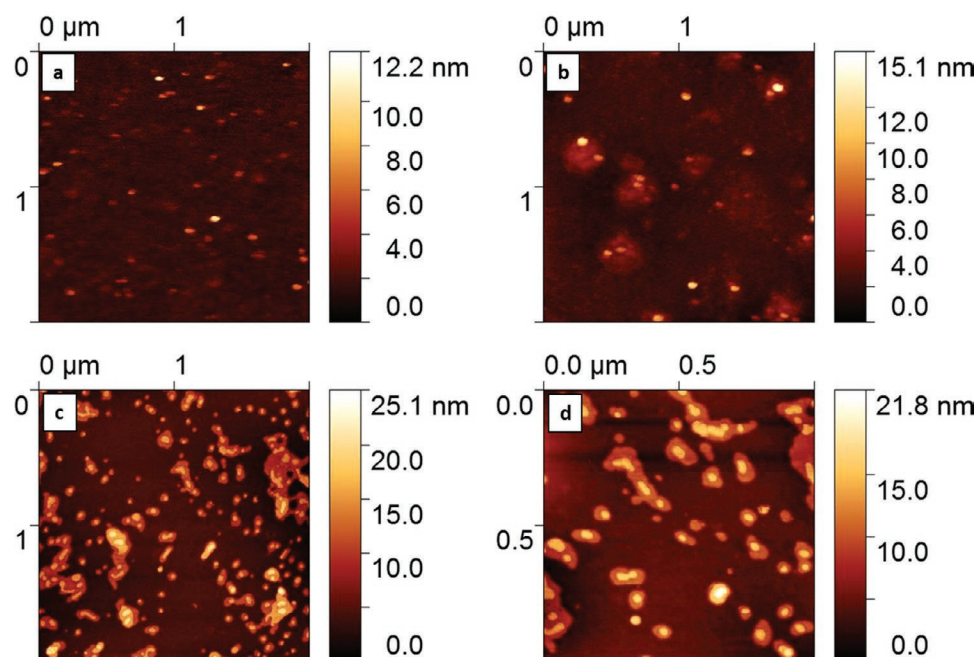


Figure 2. AFM images of a) the PVCL/AEMAA microgel, b) the PVCL-maltoheptaose microgel–primer construct, and c,d) the amylose-coated microgels after the phosphorylase-catalyzed grafting-from polymerization of G-1-P in two different magnifications.

scattering was measured to detect any occurring change in the microgel size, showing an increase in the hydrodynamic radius from 242.5 ± 11.4 nm to 326.0 ± 43.4 nm at 20 °C ($PDI = 0.310 \pm 0.233$) after the phosphorylase-catalyzed grafting-from polymerization, indicating the formation of the amylose shell. In addition, also the electrophoretic mobility changed through the formation of the amylose chains from 2.73 ± 0.21 $\mu\text{m}\cdot\text{cm}$ $(\text{Vs})^{-1}$ to a smaller value of -0.90 ± 0.09 $\mu\text{m}\cdot\text{cm}$ $(\text{Vs})^{-1}$. This was not expected since the polymerized amylose chains should not have a different charge than the maltoheptaose on the microgel before the polymerization. One explanation might be the encapsulation of the enzyme phosphorylase into the amylose shell during the polymerization. Since phosphorylase b exhibits an isoelectric point of 6.5,^[36] it is negatively charged above and could therefore decrease, in case of its presence, the electrophoretic mobility of the microgel–amylose constructs.

To visualize the formation of amylose shells around the microgels, atomic force microscopy was performed to monitor the microgels before and after the polymerization (Figure 2).

It is indeed clearly visible, that the thick amylose shell was formed around the microgels. However, not only single-coated microgels can be observed, but also larger aggregates in which several microgels are connected by the amylose shell. These aggregates might be the result of drying effects due to the high forces occurring during spin-coating for AFM sample preparation.

Additionally, IR-spectroscopy was performed to proof the formation of the amylose shell around the microgels (Figure 3). The IR-spectrum of the amylose-coated microgels show very clearly the presence of a large amount of sugar, since the bands being visible before in the spectrum of the maltoheptaose carrying microgel resulting from the saccharide moieties increased significantly.

In conclusion, within our work, it could be shown that phosphorylase-catalyzed grafting-from polymerization is a useful tool for the decoration of microgel surfaces with polysaccharide shells.

Through the covalent attachment of the primer maltoheptaose in form of its corresponding lactone to primary amino groups of a PVCL/AEMAA microgel, the microgels can serve as reactive colloidal templates for the grafting-from polymerization of G-1-P catalyzed by phosphorylase. The helical structure of the amylose chains in obtained microgel–amylose colloidal constructs can serve as depot for hydrophobic guest molecules, thus leading to the development of new scavengers or drug delivery carriers.

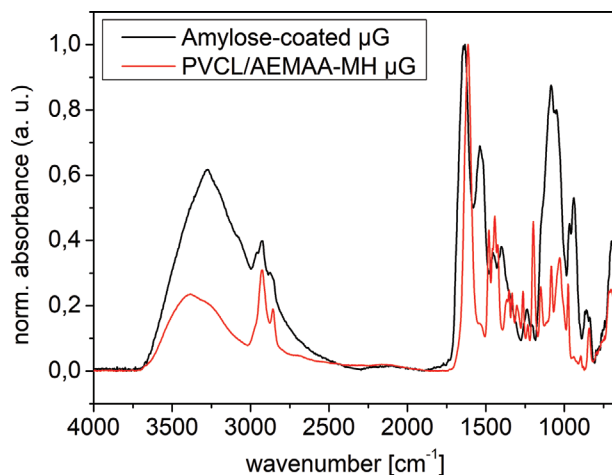


Figure 3. FTIR-spectra of the amylose-coated PVCL microgel and of the PVCL/AEMAA-maltoheptaose microgel–primer construct used as base for the phosphorylase-catalyzed growth of the amylose shell.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

amylose, enzymatic polymerization, microgel modification, phosphorylase

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