Degradable Multilayer Films and Hollow Capsules via a 'Click' Strategy

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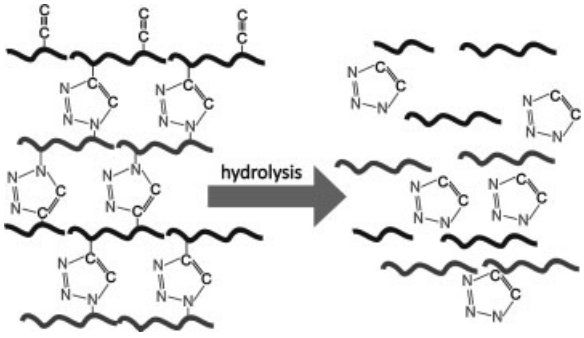
Degradable polymeric multilayer films and microcapsules are fabricated by 'click' chemistry using dextran modified with azide and alkyne moieties. Alternating layers of dextran modified with azide and alkyne groups, respectively, are covalently bonded by virtue of the 1,3-dipolar cycloaddition reaction, known as click chemistry, which leads to the formation of stable triazole cross-links between the successive layers. The dextran is modified in such a way that the azide and alkyne moieties are connected to the dextran backbone by a hydrolyzable carbonate ester. Therefore, the obtained multilayered structures can be degraded by simple hydrolysis. This type of multilayer avoids the use of polyelectrolytes, which are potentially cytotoxic, and could, therefore, be interesting for the fields of drug delivery and tissue engineering.



Introduction

Polymeric multilayer thin films[1,2] and microcapsules[3–6] fabricated by consecutive layer-by-layer (LbL) coating of an oppositely charged surface are undoubtedly an emerging research area. For instance, in the biomedical and pharmaceutical field,[7] for example, polyelectrolyte multilayers on flat substrates have shown feasibility for the functionalization of stents[8–11] to render them more biocompatible[12] or to provide them with drug eluting properties.[13] Polyelectrolyte microcapsules have shown opportunities for stimuli-responsive delivery of therapeutic molecules and for intracellular drug delivery.[14–27] These microcapsules are fabricated by the LbL coating of a sacrificial spherical template followed by its decomposition.[6,15,28,29] The main advantage of this polyelectrolyte approach is the use of the mild aqueous conditions for their synthesis. Moreover, the design can be adjusted to specific requirements by incorporation of various functional groups, nanoparticles, or biomolecules. Several research groups have made important contributions towards the incorporation of release mechanisms within the wall of multilayered microcapsules.[21] In all cases these mechanisms were based on a physico-chemically triggered system such as pH,[30–33] ionic strength,[34,35] glucose,[19] laser light,[22,36–40] ultrasound,[24,41,42] osmotic pressure,[16–18]

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or enzymatic degradation.[43] In contrast to a triggered release mechanism, certain applications require microcapsules that gradually degrade, which results in the release of their content in a sustained way over a relatively long time period. In a way to meet this requirement, the use of a multilayer system based on interactions other than electrostatic ones may be considered.[44,45] Moreover, because polycations, generally speaking, are rather cytotoxic,[46,47] there is a need for novel strategies to design and prepare LBL coatings.[48]

In this paper we report on the fabrication of degradable multilayers films and capsules produced by a 'click' chemistry approach using biocompatible building blocks. The click chemistry used in this paper involves the Cu^{I}-catalyzed triazole formation between azides and alkynes, known as the Huisgen 1,3-dipolar cycloaddition reaction.[49–55] Caruso et al. recently reported on the formation of multilayer films and capsules based on 'click' chemistry using poly(acrylic acid) modified with azide and alkyne moieties, respectively.[56,57] It was shown that multilayers could be constructed as a result of triazole ring formation between the successive layers. However, they were not intended to be degradable. Degradability of polyelectrolyte multilayers was introduced by Picart et al. using polysaccharides and polypeptides as building blocks.[58] These macromolecules are prone to enzymatic degradation and drug release from such multilayers can be considered as 'triggered release'. 'Erodible' polyelectrolyte multilayers that can decompose by chemical hydrolysis were developed by Lynn et al. who introduced hydrolytic 'erodable' multilayers on flat substrates.[59] These multilayers were composed of a polycation, a poly(β-aminoester), that contained an ester group in the polymer backbone which can by cleaved by hydrolysis.[60] Both of these approaches comprised the use of polycations, which are considered to be potentially cytotoxic as mentioned above.[46,47]

Experimental Part

Materials

Polyacrylic acid (PAA; 50 kDa) was purchased from Polysciences. Propargyl alcohol (PA-OH), fluorescein isothiocyanate dextran (FITC-dextran; $\overline{M}_w \approx 150$ and 2 000 kDa), 1,1′-carbonyldiimidazole (CDI), dextran ($\overline{M}_w \approx 19$ kDa), dimethyl sulfoxide (DMSO), ethyl acetate, polyethylene imine (PEI; 22 kDa), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and propargylamine were purchased from Sigma–Aldrich and Fluka. Sodium ascorbate and tetrabutylammonium hydrogensulfate were purchased from Acros. Dichloromethane, ether, copper sulfate, magnesium sulfate, and sodium sulfate were purchased from Merck. 3-Azidopropanol was synthesized according to the literature.[61]

Synthesis of Propargyl Carbonylimidazole (PA-CI) (1)

A dry round-bottomed flask was charged with 29.19 g (180 mmol) of CDI and 200 mL of dichloromethane to yield a turbid suspension. Propargyl alcohol (5.82 mL, 100 mmol) was added under vigorous stirring to yield a clear solution upon dissolution of the propargyl alcohol. After 1 h reaction at room temperature the mixture was extracted three times with 35 mL of water. The organic layer was dried over magnesium sulfate. After filtering off the magnesium sulfate, the liquid was evaporated by rotary evaporation and propargyl carbonylimidazole (PA-CI, 11.86 g, 79% yield) was obtained as a dry powder. ^{1}H NMR spectra were recorded with a Bruker AVANCE 500 MHz spectrometer. ^{1}H NMR ($CDCl_3$): $\delta = 2.6$ (t, 1H, $HC{\equiv}C$), 5.0 (d, 2H, $CH_2{-}O$), 7.0 (s, 1H, $C{=}CH{-}N$), 7.4 (s, 1H, $N{-}CH{=}C$), 8.1 (s, 1H, $N{-}CH{=}N$).

Synthesis of Dextran-propargylcarbonate (dex-C≡C) (3)

In a dry round-bottomed flask, 1 g of dextran (which corresponded to 6.167 mmol of glucopyranose repeating units) was dissolved in 20 mL of anhydrous DMSO. To this mixture, 280 mg (1.86 mmol) of PA-CI was added and the reaction was stirred overnight at 50 °C under a nitrogen atmosphere. Subsequently, the reaction mixture was put in dialysis bags ($\overline{M}_w$ cut off 3.5 kDa, Spectra Por) and dialyzed against pure water for 5 d. After lyophilization dextran-propargylcarbonate was obtained as a white fluffy powder. ^{1}H NMR (D_2O) $\delta = 5.01$ ($1H_{dextran}$, $O{-}C(CH){-}O$), 4.88 (2H, $C{\equiv}C{-}CH_2$), 3.5–4.3 (6H, dextran).

Synthesis of 3-Azidopropyl Carbonylimidazole (AP-CI) (2)

A dry round-bottomed flask was charged with 18.24 g (112.5 mmol) of CDI and 200 mL of ethyl acetate to yield a turbid suspension. Ethyl acetate was used as a solvent instead of dichloromethane to avoid the formation of diazomethane, which is prone to detonation. 3-Azidopropanol (6.96 mL, 75 mmol, 7.58 g) was added dropwise under vigorous stirring while the reaction mixture turned into a clear solution. After 2 h reaction at room temperature the solution was extracted three times with 200 mL of water. The organic layer was dried over magnesium sulfate. After filtering off the magnesium sulfate the solvent was evaporated by rotary evaporation and 3-azidopropyl carbonylimidazole (AP-CI) was obtained as a liquid. Yield was 52%. ^{1}H NMR ($CDCl_3$) $\delta = 2.03$ (m, 2H, $CH_2{-}CH_2{-}CH_2$), 3.45 (t, 2H, $N_3{-}CH_2$), 4.47 (t, 2H, $CH_2{-}O$), 7.0 (s, 1H, $C{=}CH{-}N$), 7.4 (s, 1H, $N{-}CH{=}C$), 8.1 (s, 1H, $N{-}CH{=}N$).

Synthesis of Dextran-Azidopropylcarbonate (dex-N_3) (4)

In a dry round-bottomed flask 1 g of dextran (which corresponded to 6.167 mmol of glucopyranose repeating units) was dissolved in 20 mL of anhydrous DMSO. To this mixture 0.301 g (1.54 mmol) or 1.204 g (6.16 mmol) of AP-CI was added and the reaction was stirred overnight at 50 °C under a nitrogen atmosphere.

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Subsequently, the reaction mixture was put in dialysis bags ($\overline{M}_w$ cut off 3.5 kDa, Spectra Por) and dialyzed against pure water for 5 d. After lyophilization, dextran-azidopropylcarbonate was obtained as a white fluffy powder. ^{1}H NMR (D_2O): $\delta = 2.02$ (2H, C≡C–CH_2), 3.5–4.3 (6H, dextran), 4.36 (2H, CH_2–CH_2–O), 5.02 (1$H_{dextran}$, O–C(CH)–O).

Synthesis of Polyacrylic Acid-propargylamine (PAA-C≡C)

Polyacrylic acid (1 g of dry product) was dissolved in 50 mL of water and the pH was adjusted to 5 using 1 M HCl and 1 M NaOH solutions. Propargylamine (237 μL, 3.46 mmol, aiming at 25% derivatization of the carboxy groups) was added under vigorous stirring and the pH was adjusted to 5. EDC (1.33 g, 2 equivalents to the propargylamine) was dissolved in the mixture and the reaction proceeded under vigorous stirring while the pH was continuously adjusted to 5 for the first hour of the reaction. After reaction overnight, the mixture was put in dialysis bags ($\overline{M}_w$ cut off 25 kDa, Spectra Por) and dialyzed for 5 d against distilled water followed by freeze-drying. The degree of substitution was determined to be 25% by ^{1}H NMR in D_2O.

LbL Coating of Planar Surfaces

Quartz slides were cleaned and hydrophylized by treatment with freshly prepared piranha solution (H_2O_2 (35%)/H_2SO_4 (98%) 1:1 v/v), and then abundantly rinsed with distilled water. The treated quartz slides were precoated with a PEI layer by immersion in a 2 mg · mL^{-1} PEI solution that contained 0.5 M NaCl followed by immersion in 2 mg · mL^{-1} PAA-C≡C solution that contained 0.5 M NaCl. Multilayers of dex-N_3/dex-C≡C were deposited on the quartz slides at room temperature by alternate immersion in dex-$N_3$and dex-C≡C solutions, respectively, starting with dex-N_3. Both these solutions contained 1 mg · mL^{-1} of dex-N_3 or dex-C≡C, respectively, 0.5 mg · mL^{-1} of $CuSO_4$ and 1 mg · mL^{-1} of sodium ascorbate in distilled water. The pH of each solution was adjusted to 4.5 with 0.1 M solutions of HCl and NaOH to minimize the hydrolysis of the carbonate esters, which link the azide and alkyne moieties, respectively, to the dextran backbone. The solutions were freshly prepared before each deposition step in the following order: dex-C≡C/dex-N_3/$CuSO_4$/sodium ascorbate/pH equilibration. Each deposition step took 10 min and afterwards the quartz slides were rinsed with distilled water and dried under a nitrogen stream. This was repeated until the desired amount of bilayers was deposited. After each deposition of a bilayer the absorbance of the film was measured with a Pharmacia Biochrom 4060 UV-VIS spectrophotometer. Degradation of the multilayer was performed by incubating the quartz slide in a 0.1 M carbonate buffer (pH 9) at 37 °C.

Fabrication of Microcapsules

$CaCO_3$ microparticles (with an average diameter of 3 μm) were used as a sacrificial template for microcapsule fabrication and were fabricated according to Volodkin et al.[62,63] Briefly, $CaCl_2$ and Na_2CO_3 solutions (0.33 M) were mixed under vigorous stirring for 30 s, which led to the precipitation of $CaCO_3$ particles. Subsequently, four centrifugation and washing steps with water were performed in order to remove the unreacted species. In a last step the particles were washed with acetone and subsequently air-dried.

The $CaCO_3$ microparticles were pre-coated (in order to provide the $CaCO_3$ microparticles with a surface reactive towards click chemistry) through electrostatic interaction with a layer of PAA-C≡C by 10 min incubation of the $CaCO_3$ microparticles in a solution of PAA-C≡C (1 mg · mL^{-1} that contained 0.5 M NaCl, equilibrated at pH 7 with 0.1 M HCl and 0.1 M NaOH solutions) followed by two centrifugation/washing steps with water. The LbL coating was started by incubating the pre-coated microparticles at room temperature in a 1 mg · mL^{-1} solution of dex-N_3 (that contained 0.5 mg · mL^{-1} of $CuSO_4$ and 1 mg · mL^{-1} of sodium ascorbate, in distilled water with the pH equilibrated at 4.5) for 10 min followed by two washing/centrifugation steps with water. The second dextran layer was reacted on the previous one by incubation of the microparticles at room temperature in a 1 mg · mL^{-1} solution of dex-C≡C (that contained 0.5 mg · mL^{-1} of $CuSO_4$ and 1 mg · mL^{-1} of sodium ascorbate, in distilled water with the pH equilibrated at 4.5) for 10 min followed by two washing/centrifugation steps with water. The solutions were freshly prepared before each deposition step in the following order: dex-C≡C/dex-N_3/$CuSO_4$/sodium ascorbate/pH equilibration. These steps were repeated until four bilayers of dex-N_3/dex- C≡C were deposited onto the microparticles. Hollow capsules were obtained by dissolving the $CaCO_3$ microparticles in ethylenediaminetetraacetate (EDTA, 0.2 M; pH adjusted to 5.2 using 1 M HCl and 1 M NaOH solutions).

Microscopic Characterization

Scanning electron microscopy (SEM) images were recorded with a Quanta 200 FEG FEI scanning electron microscope operated at an acceleration voltage of 5 kV. Optical microscopy images were recorded with a EZCI-si confocal laser scanning microscope equipped with a 60× water immersion objective.

Results and Discussion

Very recently we have introduced hydrolyzable click cross-links for the synthesis of hydrogel microspheres.[64] Dextran (40 kDa) was modified with alkyne and azide moieties, respectively, by hydrolyzable carbonate esters according to the reaction scheme in Figure 1. Dextran-propargylcarbonate (dex-C≡C) was synthesized by coupling carbonylimidazole-activated propargylalcohol to dextran while dextran-azidopropylcarbonate (dex-N_3) was synthesized by coupling carbonylimidazole-activated 3-azidopropylalcohol to dextran. In this study, we have synthesized dex-C≡C and dex-N_3 with a degree of substitution of 20 (20 pending azide/alkyne moieties per 100 glucopyranose units). Covalent binding of the dex-C≡C and dex-N_3 layers is performed by triazole ring formation

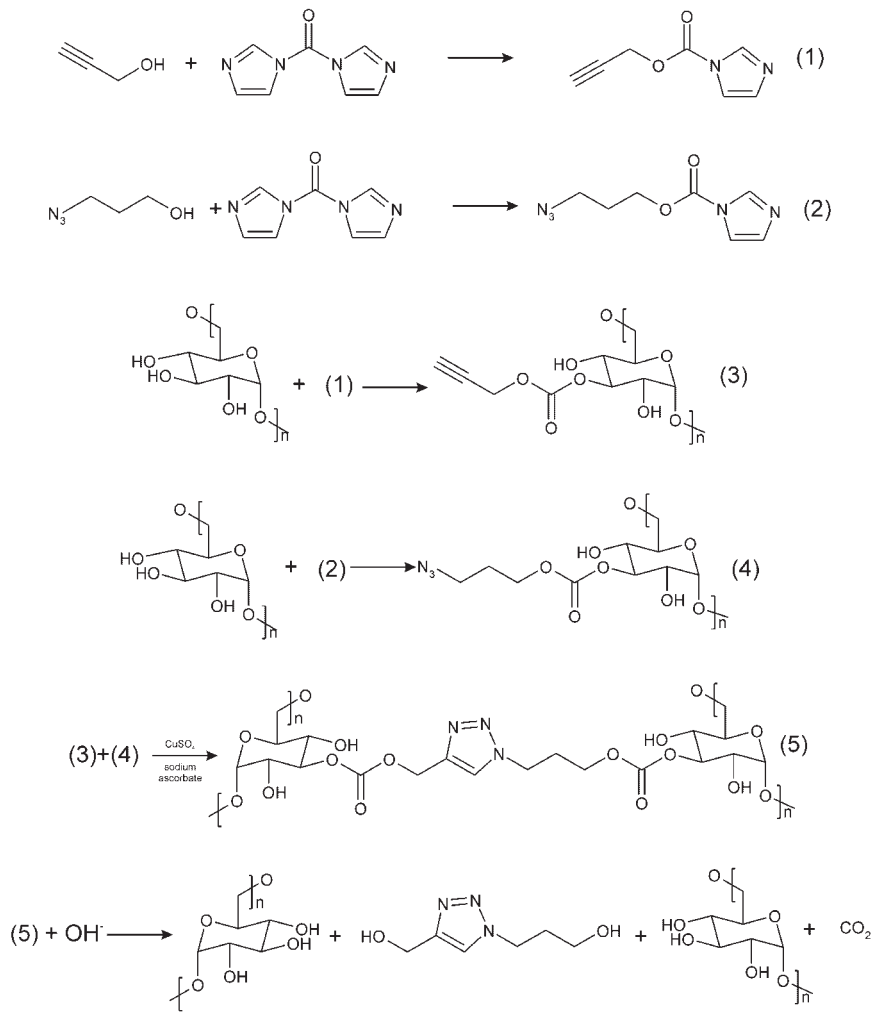

Figure 1. Reaction scheme of the synthesis of dextran-propargyl carbonate (**3**) and dextran-azidopropylcarbonate (**4**). Propargyl alcohol and 3-azidopropanol are activated with CDI to yield **1** and **2**. Dextran is grafted with the activated compounds **1** and **2** to yield alkyne **3** and azide **4** modified dextran. 'Click' reaction between **3** and **4** crosslinks the dextran chains by formation of a triazole ring (**5**). Hydrolysis of the carbonate esters degrades the dextran network with the formation of dextran chains, CO_2, and a low-molecular-weight triazole compound as degradation products.

between the alkyne and azide groups by the Cu^{I}-catalyzed Huisgen click reaction. Cu^{I} is generated in situ by reduction of $Cu^{II}SO_4$ to Cu^{I} in the presence of sodium ascorbate. Degradation of the resulting multilayer films is able to occur under physiological conditions through hydrolysis of the carbonate esters[65] that connect the triazole ring with two glucopyranose units of the dextran chains, which disintegrate into the original dextran, CO_2, and a low molecular weight triazole compound (see Figure 1).

To assess the possibility to construct degradable multilayer films by 'clicking' azide (dex-N_3) and alkyne (dex-C≡C) modified dextran chains, we first investigated the multilayer build-up on a planar surface, in this case quartz slides. The process is schematically illustrated in Figure 2. To obtain a surface that is reactive towards click chemistry, i.e., functionalized with alkyne or azide moieties, the quartz slides were first coated with a layer of polyethylene imine (PEI, 22 kDa), which is a polycation that is generally used as an adhesion promoter prior to thin film deposition. Subsequently, to provide the substrate with click reactive alkyne moieties, a layer of polyacrylic acid-propargylamine (PAA-C≡C; synthesized by substituting 25% of the carboxylic acid groups with propargylamine using carbodiimide coupling) was adsorbed on the PEI layer through electrostatic interaction. In a next step, the pre-coated quartz slides were coated by click coupling alternating dex-N_3 layers and dex-C≡C layers in the presence of $CuSO_4$ and sodium ascorbate, to result in the

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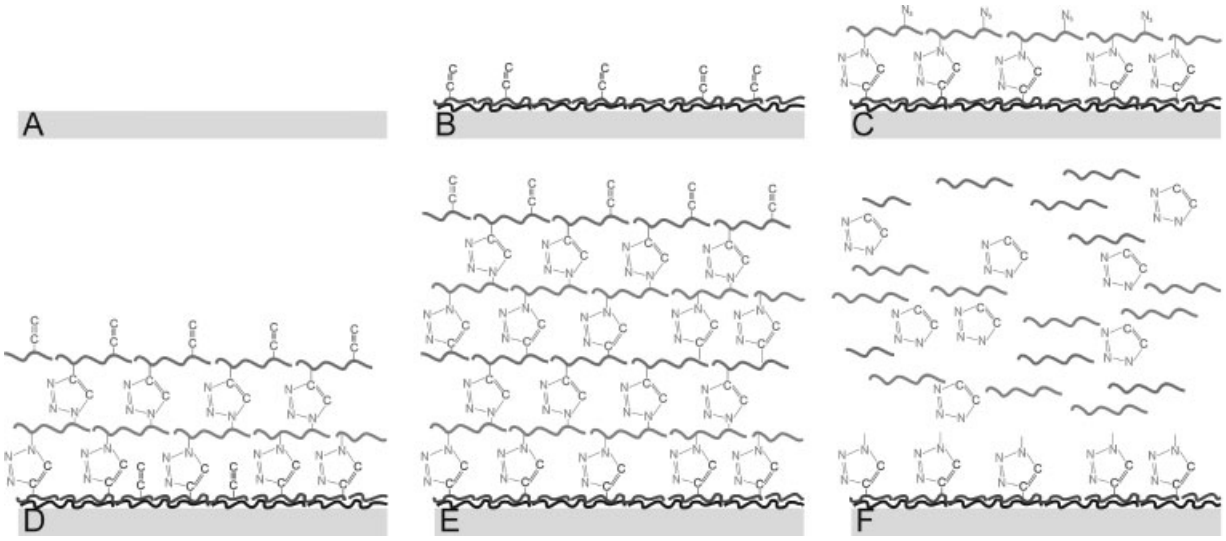

Figure 2. Schematic representation of the build-up of a degradable 'clicked' multilayer. A bare substrate (A) is pre-coated with a PEI/PAA–C≡C (i.e., polycation/polyanion) layer (B) to induce the surface reactive towards click chemistry. A dex-N_3 layer (C) is clicked to the PAA–C≡C layer followed by the clicked deposition of consecutive layers (D and E). Hydrolysis of the carbonate esters, which link the triazole bonds to the dextran backbones, leads to the disassembly of the clicked multilayer film.

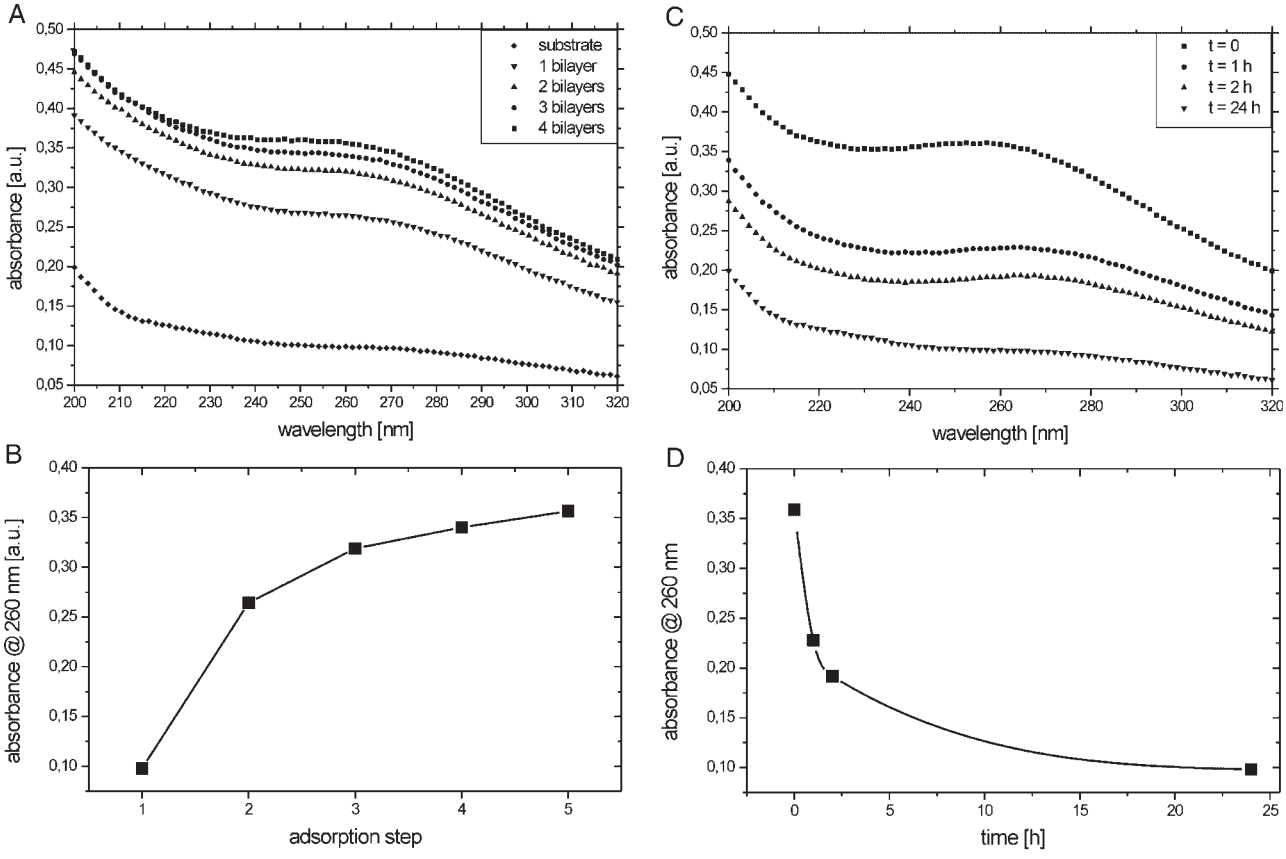

Figure 3. UV-vis absorption spectrum showing the increase in absorbance per deposited bilayer on a quartz slide (A) and the corresponding increase in absorbance at 260 nm as a function of adsorption step (B). UV-Vis absorption spectrum showing (C) the decrease in absorbance as a function of degradation time when the coated quartz slides are placed in a pH 9 buffer at 37 °C. (D) shows the corresponding decrease in absorbance at 260 nm as a function of time.

formation of a chemically stable triazole ring between the successive dextran layers. After the deposition of each dex-N_3/dex-C≡C bilayer, the UV-vis spectrum was measured. Figure 3A shows the increase in absorbance as a function of the layer number, which indeed indicates the deposition of a thin film on the quartz slides. In total four dex-N_3/dex-C≡C bilayers were deposited as this agrees with the number of polymer layers usually applied for the fabrication of hollow capsules,[16,21–23,43] which was the final aim in this study. Figure 3B shows the increase in absorbance at 260 nm as a function of deposition step. The graph clearly shows a non-linear growth, which indicates that saturation occurs after the deposition of several 'clicked' layers. The exact reason for this phenomenon is not clear at this moment. To prove that the constructed multilayer film can be degraded by hydrolysis, we immersed the quartz slides in a solution buffered at pH 9 and heated at 37 °C. At several time intervals, the film was dried and the UV-vis absorption spectrum was recorded. As shown in Figure 3C,D, the absorbance gradually decreases and after 24 h it is reduced to the absorbance of a bare quartz slide coated with PEI/PAA-C≡C. These observations clearly prove that multilayer films can be constructed and broken down based on alternating dex-N3/dex-C≡C layers that are connected through degradable 'click' linkages. It is worth noting that the rate of hydrolysis of carbonate esters is minimal at pH values between 4 and 5 (i.e., the conditions used for building-up the multilayer) and increases exponentially at alkaline pH (i.e. the conditions used to assess multilayer degradation).[66]

The final aim of this research is the fabrication of degradable hollow capsules based on the above described 'click' reactions. Therefore, $CaCO_3$ microparticles, fabricated by mixing aqueous solutions with equimolar amounts of calcium chloride and sodium carbonate, were used as sacrificial templates.[62,63] These $CaCO_3$ microparticles, having a mean diameter of 3 μm, were first pre-coated with a layer of PAA-C≡C to render the microparticles' surface reactive towards click chemistry. Because of their high porosity,[62,63,67] the $CaCO_3$ microparticles efficiently adsorb the PAA-C≡C and no PEI layer was required in this case. Thereafter, four bilayers of dex-N_3/dex-C≡C were deposited on the surface of the $CaCO_3$ microparticles, followed by the dissolution of the $CaCO_3$ in an EDTA solution. To remove the dissolved ions, the obtained capsule dispersion was dialyzed against water for 2 d (at 4 °C to avoid capsule degradation) as the capsules appeared to be too light to be able to sediment through centrifugation. Figure 4A and 4B show scanning electron microscopy images of hollow capsules. Whereas capsules prepared from oppositely charged polyelectrolytes templated on $CaCO_3$ microparticles can have thicknesses up to several hundreds of nanometers,[24,68] the clicked capsules are clearly extremely thin.

To assess the biodegradability of the microcapsules, we have visualized the degradation process by confocal microscopy. A drop of sodium hydroxide was added to

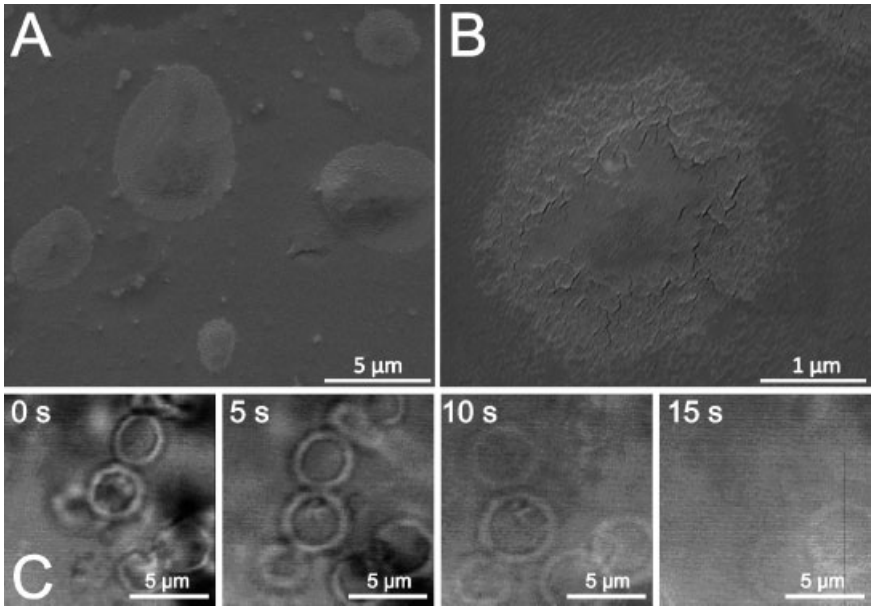

Figure 4. A,B) SEM images at different magnification of a microcapsule that consists of four clicked dex-N3/dex–C≡C bilayers. C) Confocal transmission microscopy snapshots of degrading clicked multilayer capsules taken at different time intervals after the addition of sodium hydroxide.

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accelerate the degradation of the carbonate esters, which link the triazole rings to the dextran chains. Figure 4C shows the behavior of the capsules at consecutive time intervals. After the addition of sodium hydroxide (0 s), the capsules start to swell and gradually dissolve in the surrounding solution, showing the degradability of the capsules. As Jiang et al. previously showed that click-linked carbonate esters can also hydrolyze under physiological conditions (i.e., pH 7.4 and 37 °C),[69] a similar behavior can be expected from the capsules reported in this paper. Indeed when the capsules are incubated at physiological conditions for one week no trace of the capsules is observed by optical microscopy.

Conclusion

In conclusion, we have shown that hydrolytically degradable multilayers and capsules can be fabricated by layer-by-layer build-up using the Cu^{I}-catalyzed Huisgen 1,3 dipolar click cycloaddition reaction, to result in triazole ring linkages between alkyne and azide modified dextrans. By the introduction of carbonate esters, which link the alkyne and azide groups to the dextran chains, we have made the resulting clicked multilayers and capsules degradable. To the best of our knowledge, hydrolytically degradable multilayers based on non-electrostatic interactions have not yet been reported. The mild, non-harmful reaction conditions for click chemistry are of great importance when labile biological compounds have to be incorporated. Thanks to the introduction of hydrolytically degradable carbonate ester linkages, it is believed that this type of coating technique will become an important strategy for the design of, e.g., drug delivery systems, degradable drug formulations, tissue engineering scaffolds, and others. Moreover, as the wall of the hollow capsules was observed to be extremely thin they could be of interest for intracellular delivery as upon cellular internalization, they are expected to be largely deformed,[70] which could facilitate the release of their payload.

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