Drug Carriers

International Edition: DOI: 10.1002/anie.201412055
German Edition: DOI: 10.1002/ange.201412055

Regioselective Ring-Opening Polymerization of a Polyhydroxycarboxylic Acid for the Synthesis of a Nanoscale Carrier Material with pH-Dependent Stability and Sustained Drug Release**

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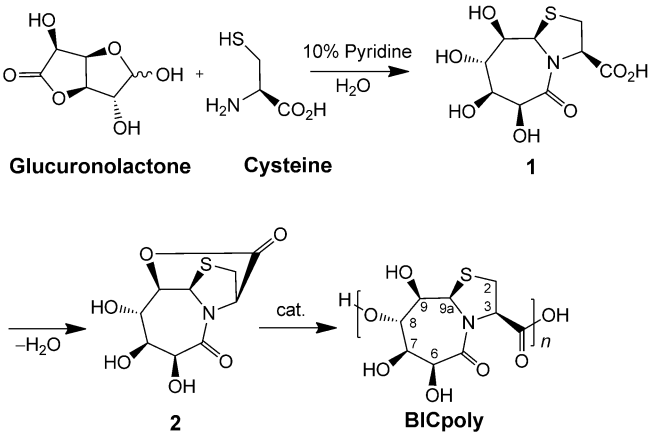
Abstract: *Synthetic polyesters are usually composed of monohydroxycarboxylic acids to avoid the problem of regioselectivity during ring-opening polymerization. In contrast, the linear polyester BICpoly contains four secondary OH groups and is nevertheless esterified regioselectively at only one of these positions. Neither the synthesis of the tricyclic monomers nor the ring-opening polymerization requires protecting groups, making BICpoly an attractive novel and biocompatible polymer. BICpoly nanoparticles can be loaded with low-molecular weight drugs or coated onto surfaces as thin films. The release of loaded compounds makes BICpoly an attractive depot for drug release, as shown herein by loading BICpoly with dyes or the cytostatic drug doxorubicin. BICpoly is distinguishable from other polymers by its characteristic pH-dependent degradation.*

Biocompatible polymers are employed in medicine, for example, for coatings or as depots for drug release.[1] Often used building blocks are amino acids (polyamides)[2], or the monohydroxycarboxylic acids lactic acid (polylactide; PLA),[3] or hydroxybutyric acid (poly-(*R*)-3-hydroxybutyrate; PHB).[4] The challenge posed by oligohydroxycarboxylic acids lies in the regioselective ring-opening polymerization (ROP): Neither activated monomers that can be accessed without laborious protecting-group chemistry nor suitable polymerization catalysts are currently available. Polymers of biological origin, such as polysaccharides (e.g. cellulose) or polyhydroxycarboxylic acids (e.g. alginates), are known for their selective formation of only one regioisomer, even if a multitude of different isomers would be possible. Polymeric uronic acids are generally linked as acetals. Herein we describe a novel polyester that is accessible by ROP just like other synthetic polyesters, but involves the typical polysaccharide number of four OH groups on each building block (Scheme 1).



Scheme 1. Condensation of D-glucuronolactone with L-cysteine yields thiazolidinlactam **1**, which undergoes lactonization with EEDQ to give Monomer **2**. Nucleophilic catalysis of the ROP leads to polyester **BICpoly**.

The condensation of D-glucuronolactone with L-cysteine can be conducted in water on a large scale.[5] Only a low percentage of pyridine is needed to prevent the reaction from stopping at the *S,N*-acetal formation step. Compound **1** and similar thiazolidinlactams were incorporated as dipeptide isosters in bioactive peptides,[6] synthetic proteins,[7] and polyamides,[8] or were starting materials for natural-product syntheses.[9] Thiazolidinlactam **1** cyclizes with EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) to give lactone **2**, which can be stored as a powder. Pyridine is completely removed during the subsequent work-up. It can be replaced by pyridoxal, a catalyst of biological origin, without affecting the course of reaction. EEDQ conveys the regioselective condensation of the established tetrahydroxycarboxylic acid **1** to lactone **2**, which precipitates from the reaction medium without the need for chromatographic purification. A crystal structure (deposition number CCDC 1017210) of **2** was obtained and is shown in Figure 1.

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[**] We thank Prof. Dr. M. Ulbricht and Dr. Jackely Aragón Gómez, Department of polymer analytics, Chair for Technical Chemistry II, University Duisburg–Essen, for the execution of GPC measurements. We thank B.Sc. Wolf Mathis Rink for his support in conducting experiments.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201412055.

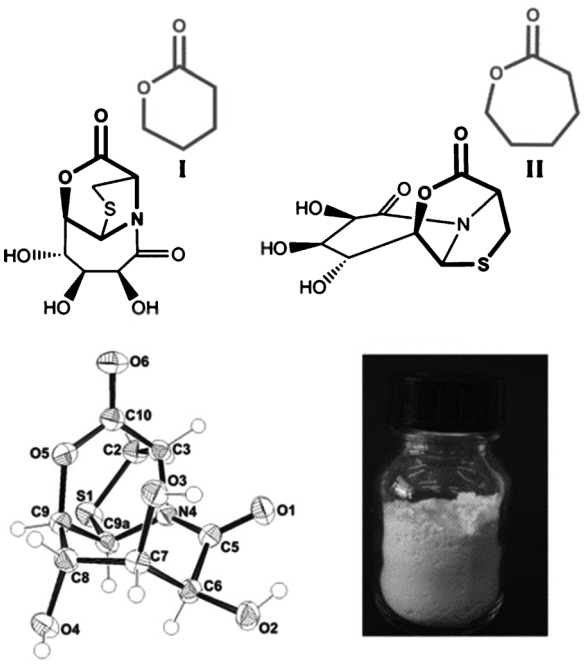

Figure 1. The upper structural formulae show that **2** contains both a δ-valerolactone ring and a ε-caprolactone ring. The crystal structure illustrates the compact shape of the oligocyclic ring systems. A glass vial containing **2** as white powder is shown alongside.

The bicyclic ring geometry gives a fixed orientation of the OH groups in **2**, thus creating a sufficient difference in reactivity for the regioselective synthesis of only one of the possible esters. During the base-catalyzed ROP to the polyester **BICpoly** no other bonds, transesterifications or even branched esters are observed in the ^{1}H NMR spectrum.

Dimethyl sulfoxide (DMSO) proved to be suitable as reaction medium and **BICpoly** can easily be separated from it by precipitation in water. Different bases and catalysts have been investigated for the ROP (see Supporting Information for details). A reaction temperature of about 100 °C is needed to complete the reaction after 30 min. ROP provides the polymer with homogenous regio- and stereochemistry. Hence, **BICpoly** consists only of the structural element that is shown in Scheme 1. The saponification of the polyester by adding small amounts of base (e.g. NaOH) leads to complete hydrolysis of the polymer (> 99 % by ^{1}H NMR) to yield building block **1**. No additional fragments that would require separate toxicological analyses are observed upon hydrolysis (Supporting Information Figure S1).

The degree of polymerization is less than 20 building blocks, as indicated by mass spectrometry (MS; see below; data not shown). On the basis of ESI-MS or MALDI-MS it is not possible to decide whether the terminal building block exists as lactone or a macrocyclic structure is formed. The esterification at O8 is identified by NMR spectroscopy based on the deshielding of C8 in comparison to monomer **1**. The tetramer could be isolated by HPLC, its NMR spectroscopy data are given in the Supporting Information, Figure S1. Although the ^{1}H NMR spectra show the expected microheterogeneity for a polymer of statistical length, only this analytical method is capable of verifying the otherwise not detectable regioselectivity of the esterification at O8 (Figure 2).

Degrees of polymerization up to $n = 9$ have been reliably confirmed by MALDI-TOF MS. In addition, degrees of polymerization up to $n = 14$ are observed despite the poorer signal-to-noise ratio (Supporting Information, Figure S2 A). The resulting molar mass of the polymer of approximately 3500 g mol^{-1} was confirmed by gel permeation chromatography ($M_n D = 3427$ g mol^{-1}; Supporting Information Figure S2 B).

Photon correlation spectroscopy (PCS) and phase analysis light scattering (PALS) show that upon precipitation of DMSO-dissolved **BICpoly,** nanoparticles are formed. The nanoparticle size was dependent on the buffer conditions

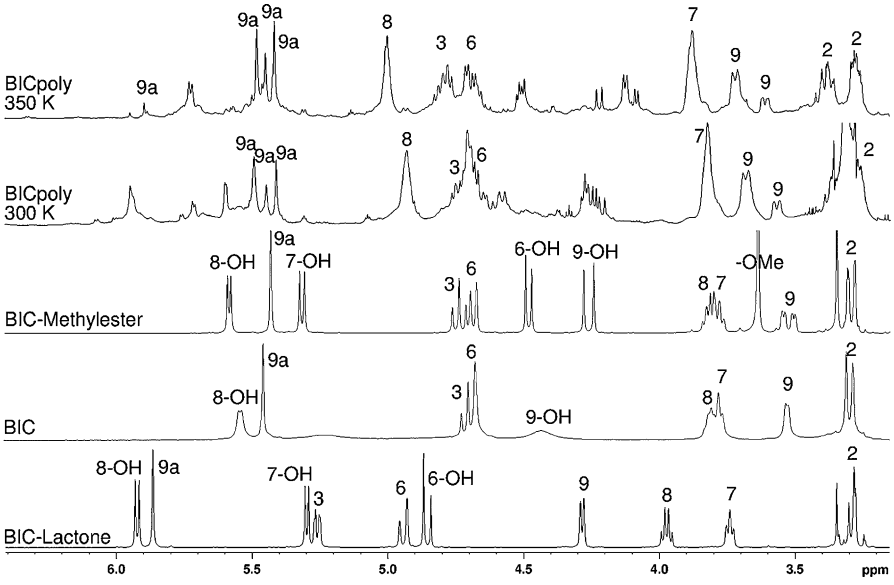

Figure 2. ^{1}H NMR spectra of **BICpoly** (500 MHz, [D$_6$]DMSO, upper two spectra at 300 K and 350 K) and of the three monomeric BIC derivatives (300 MHz, 300 K, [D$_6$]DMSO). The bridgehead protons (H9a) are typically situated at around δ = 5.5 ppm in the upper four spectra, while H9a in **2** (lowermost spectrum) resonates at δ = 5.85 ppm, just like the terminal group of **BICpoly** at 350 K (uppermost spectrum). The selective esterification at O8 leads to a deshielding of H8, which resonates at δ = 4 ppm in all the monomers and is noticeably deshielded to δ = 5 ppm in **BICpoly** as the only one of all three possible linkage positions (O6, O7, and O8). Different degrees of polymerization are the reason for the heterogeneity of the side signal sets in the ^{1}H NMR spectra of **BICpoly**. Chemical exchange with the proton of the carboxylic acid leads to broadening of the OH signals. BIC methyl ester shows the best comparability with **BICpoly** in regard to the 6-OH and 9-OH groups in the region of δ = 4.0–4.6 ppm.

during precipitation and could be adjusted to a range from around 200 nm to over 1000 nm. Thus nanoparticles with a size of approximately 200 nm (PCS, measured with Zetasizer Nano ZS, Malvern Instruments) and a surface charge of −25 to −30 mV (zeta potential, measured with ZetaPALS, Brookhaven) are obtained in pure water (see Supporting Information, Figure S3 A,B), while salt solutions (NaCl) with increasing ionic strength lead to microparticles that sediment quickly (Supporting Information, Figure S3 C). No difference in size is found when comparing loaded (see below) and unloaded nanoparticles in water (data not shown). However, treatment of the nanoparticles with ultrasound (ultrasonic finger, ultrasonic bath) affects the size and results in smaller nanoparticles. Size measurements by nanoparticle tracking (NTA)—which always leads to higher values than PCS measurements—show a decrease from 385 nm to 294 nm when applying ultrasound (Supporting Information, Figure S3 D).

Nanoparticles with a size of 300 nm and a charge of −25 mV adopt a size of approximately 400 nm and a charge of −30 mV when stored for several weeks in distilled water (pH 5.5 to 6.0) at 4 °C. This indicates that these nanoparticles can be kept in water without aggregation or other changes, unlike many other polymeric nanoparticles which then require extensive modifications (e.g. see Ref. [10]). When stored under physiological conditions (HEPES buffered physiological saline solution), the formation of a distinct second population of particles with a size of around 1.5 μm is observed in addition to the simultaneous detection of the 400 nm, −30 mV nanoparticles. This could be important for anticipated applications in biological systems where no degradation occurs (see below), although our studies on the biological functionality of loaded drugs (see below) suggest that the particle size is not crucially important. Subsequently, **BICpoly** nanoparticles can also be loaded as thin layer onto a surface. For this purpose, the suspension is applied onto the surface and air-dried. The type of film formed turned out to be dependent on the number of washing steps after precipitation, which can be attributed to varying particle sizes or the presence of residual DMSO. The film is initially transparent while turning white after several washing steps.

The low solubility of the polymer and its residue-free hydrolysis to BIC (structure **1**) or its two monomers make it suitable as depot for sustained drug release. The low molecular weight compound loading and release properties of **BICpoly** were investigated by using the dyes methylene blue and rhodamine as well as the cytostatic drug doxorubicin as a model substance for possible medical applications (see e.g. Ref. [11,12]). We were able to show that the nanoparticles, in the form of the precipitate in suspension or as film on a surface, can be loaded with drugs. This is possible independent of whether the drug is dissolved in DMSO (= initial solvent of polymer) or in water (= medium that is added to precipitate the polymer from DMSO). As expected, the efficiency of the loading was dependent on the initial drug concentration; an excess is beneficial. When utilizing methylene blue, a maximum loading efficiency of 10 μg mg^{-1} **BICpoly** was measured (Supporting Information, Fig-

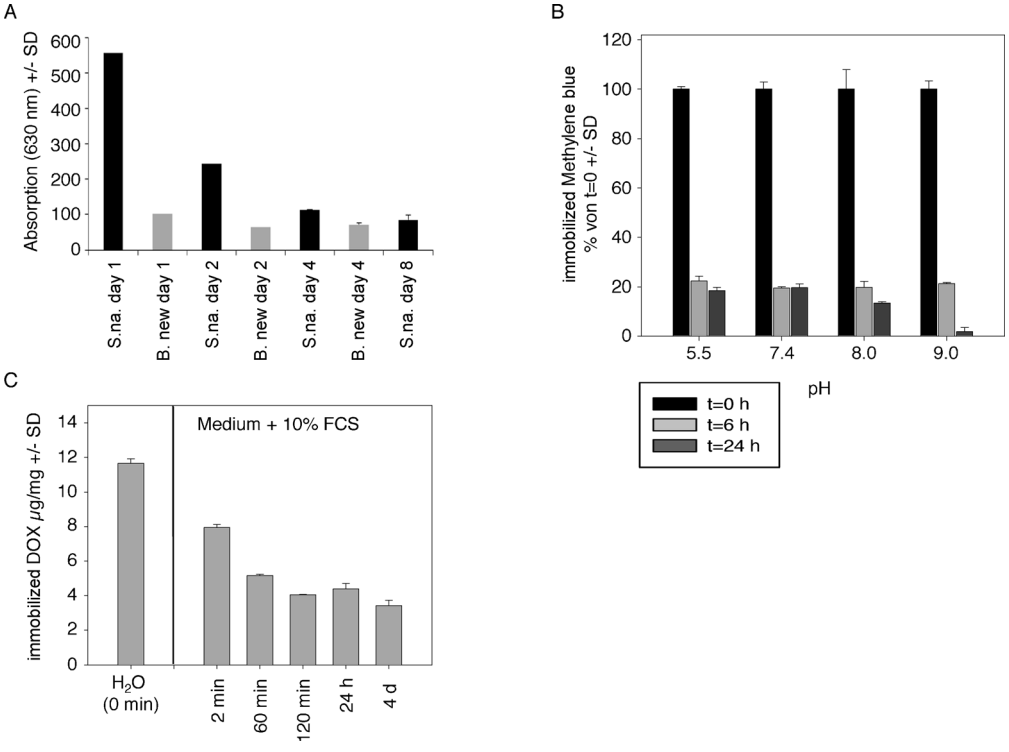

Figure 3. Sustained release of methylene blue from precipitated **BICpoly** nanoparticles. A) 50 μL of the nanoparticle sediment, which was blue after dye loading, was, after removal of non-loaded dye, resuspended in 0.1 M Tris buffer (pH 7.4). The release of methylene blue over time was determined by absorption measurements of the centrifuged sample supernatants ("S.na.", black bars) at 630 nm. "B. (buffer) new" represents absorption measurements directly after a buffer change (= background), and the difference between the gray and the black bars of the subsequent time point indicates the release of methylene blue. B) Time dependence of the amount of methylene blue loaded onto **BICpoly** in relation to the pH value. **BICpoly** loaded with methylene blue (time $t = 0$) was incubated under shaking at 37 °C in 50 mM phosphate buffer at the pH values indicated(). After 6 h or 24 h, the samples were centrifuged and the supernatant discarded, prior to washing of the sediment twice with water and the determination of the residual amount of methylene blue still loaded. For this purpose the **BICpoly** sediment was lysed as described in the Supporting Information; the absorption of the lysate is depicted, each normalized to $t = 0$. C) Release of DOX under physiological conditions in the presence of serum. 1 mg **BICpoly** was loaded with 20 μg DOX and incubated in cell-culture medium. The residual amounts of still immobilized DOX were determined at the time points indicated.

ure S4A). The time-dependent analysis of the loading revealed a rapid process that is completed within a few minutes; additional incubation time leads to no further improvement (Supporting Information, Figure S4B). No desorption of the loaded nanoparticles was observed in pure water, even in the presence of proteins that could interfere with the interactions (Supporting Information, Figure S4C). In contrast, a fast desorption reaching a plateau of approximately 2 $\mu g\,mg^{-1}$ **BICpoly** was detected in physiological NaCl solution. This result correlates with the value found for the loading of methylene blue under the same saline conditions (data not shown) and indicates electrostatic and ionic-strength dependent interactions. A slightly higher loading giving values of approximately 12 $\mu g\,mg^{-1}$ **BICpoly** was achieved in the case of doxorubicin (DOX). This process again turned out to be fast and was independent of DOX being added during the precipitation ("pre-loading") or afterwards to the prefabricated nanoparticles ("post-loading"; Supporting Information, Figure S4D). While again no desorption occurred in pure water even in the presence of proteins, a decrease of the loading capacity was observed under physiological saline conditions, albeit loaded DOX amounts remained higher (ca. 6 $\mu g\,mL^{-1}$ **BICpoly**) than with methylene blue (data not shown).

At pH 7.4, the drug loaded on **BICpoly** was released slowly over a few days. This process was first shown for methylene blue release from **BICpoly** nanoparticles into the supernatant (Figure 3A; see the difference between the gray and corresponding black bars).

When applying multiple buffer changes ("B. (buffer) new"), the **BICpoly** sediment was completely decolorized after 4 days, without observing any optical evidence for polymer decomposition under these buffer conditions. At low pH conditions, a fast, ionic strength dependent partial desorption of methylene blue loaded onto **BICpoly** was observed, after 6 h reaching a plateau where no further desorption occurred (Figure 3B). In contrast, a subsequent partial or complete methylene blue release was observed at pH 8 or pH 9, respectively, that is based on the partial (pH 8) or complete (pH 9) hydrolysis of **BICpoly** (see below). For analysing DOX loaded **BICpoly**, the decrease of the immobilized drug was monitored during incubation in cell-culture medium. An initially fast decrease slowed down over longer time periods (Figure 3C). Values were reached that proved to be smaller than the loading capacity achieved under physiological saline conditions, again indicating a slow degradation of the carrier material.

Indeed, a simultaneous degradation of the polymer can occur parallel to the release of loaded substances, as indicated by a decrease of the visible, insoluble precipitate. Interestingly, this process is highly pH-dependent within a narrow pH range. More specifically, we determined that the degradation at pH 8–9 and 37 °C takes place within a few hours (up to 90 % in the first 5 h), while the precipitate remained completely stable at pH 3–7 for days (measured up to 6 days; Figure 4). Normally, an inverse behavior of nanoparticles regarding their pH-dependent degradation is observed, namely their decomposition at low pH values (see e.g. Ref. [12,13]). Our finding of stability at acidic pH values has rarely been described and opens up completely different application possibilities. For example, an oral ingestion (safe transit through the stomach and subsequent degradation in the

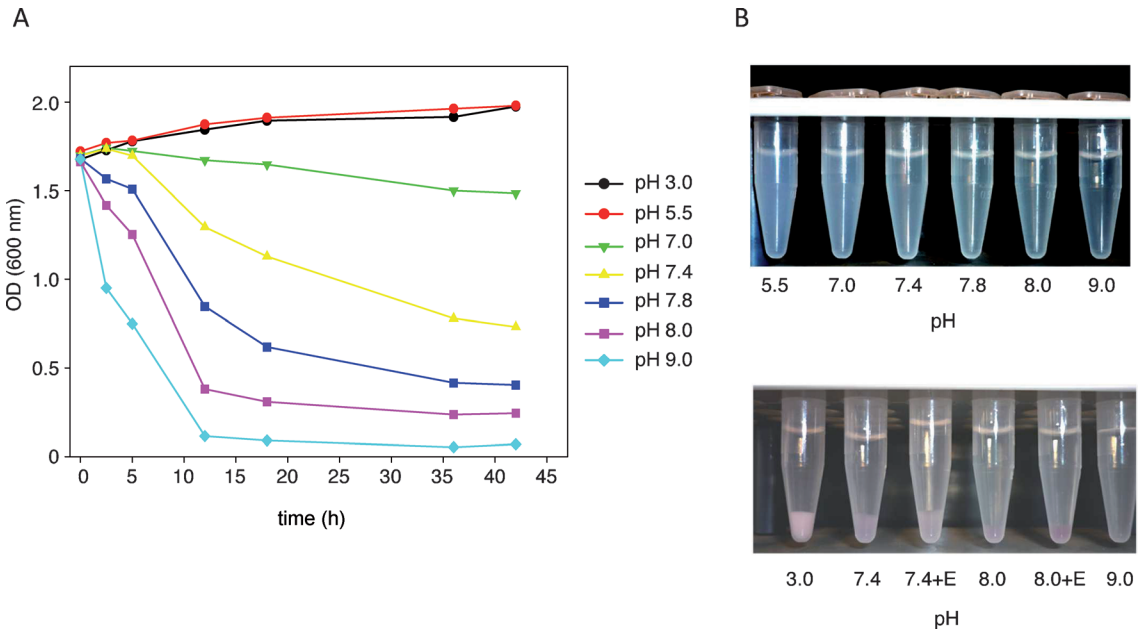

Figure 4. Degradation of **BICpoly** nanoparticles, dependent on the pH value of a 0.5 M Tris buffer used for suspension. The nanoparticles are loaded with doxorubicin for staining. A) Measurement of the optical densities of the polymer suspensions over a period of 6 days at the different pH values. B) Residual turbidity of the solutions from (A) after 42 h (top). Centrifugation of the suspensions after incubation at the indicated pH values reveal partial degradation at pH 7.4 as well as the largely (pH 8.0) or complete (pH 9.0) degradation. The addition of esterase ("+E") has no influence (bottom).

intestine upon pH increase) should be possible, as shown recently for poly(ester amides) for oral insulin application.[14]

At the particularly important physiological pH value of 7.4, a degradation of around 40% occurs within the first two days, after which the degradation becomes slower. This fact is extremely important in terms of the applicability to biological systems.

Different biological assays demonstrated that **BICpoly** as well as its precursors **1** and **2** are highly biocompatible in vitro (cell culture). In a cell viability assay, no decrease in the number of viable cells was observed over a wide range of **BICpoly** concentrations (Figure 5A). This result was confirmed in a lactate dehydrogenase (LDH) release assay, where no induction of LDH release into the medium was detected, thus verifying the absence of cell damage (Figure 5B). Only at the very high concentration of 20 mg mL^{-1} an impairment of the cell growth is observed. However, this effect can be explained by the formation of nanoparticle deposits as precipitates on the cells (Supporting Information, Figure S5A). In the case of the (non-precipitating) intermediate products BIC or **2**, no cytotoxicity is monitored (Supporting Information, Figure S5B), even at higher concentrations (100 mM; ca. 28 mg mL^{-1}).

After coating of a **BICpoly**-layer onto a cell-culture support, this film allowed even the attachment and cultivation of adherent cells (Figure 5C). Consequently, interactions between the cells and the **BICpoly** support take place without negatively affecting the cells. Accordingly, particles that were formed during degradation of the precipitate attached to the cells.

Besides the biocompability of the carrier, the preservation of the loaded drug is an important property and was analysed using DOX. After treatment of cultivated tumor cells with free DOX, a characteristic dose–response curve was obtained (Figure 5D). This curve did not differ, especially regarding the IC50 value (concentration of half maximal inhibition), from the one obtained for the same amount of DOX loaded onto **BICpoly**, indicating the complete preservation of DOX activity upon loading and subsequent release.

In summary, we succeeded in synthesizing a polyester that is composed of alternating D-glucoronic acid and L-cysteine. Remarkably, no protecting groups were necessary for a completely regioselective polymerization. The properties of **BICpoly** as a carrier material for low-molecular-weight drugs open up several very interesting possibilities for biomedical or pharmaceutical applications. Notably, sufficiently high loading capacities are achieved, as shown for the cytostatic drug doxorubicin as one example. The highly pH-dependent degradation of the polymer to its natural and completely biocompatible starting compounds is another noteworthy fact. At pH 7.4, which is particularly relevant with regard to biological systems, degradation kinetics are observed that allow the loading and subsequent sustained release of drugs, while only slight changes in the pH value lead to a considerable stabilization of the nanoparticle precipitate or its highly accelerated degradation. In comparison to other systems, an interesting inverse pH-dependence of stability is observed.

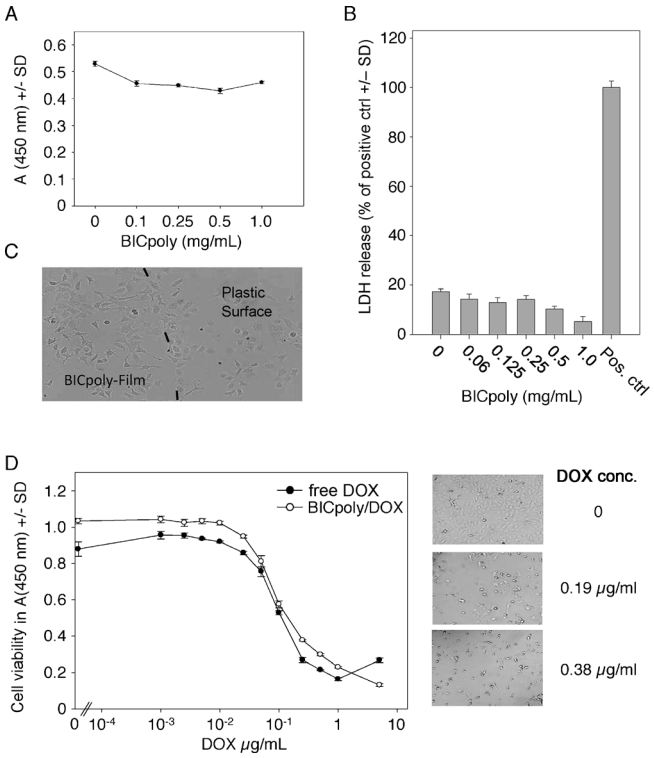

Figure 5. The addition of increasing amounts of **BICpoly** to the cell-culture medium does not lead to reduced cell viability (A) or induce damage (B) of SKOV-3 ovarian carcinoma cells, as determined in WST-1 assay and LDH release assay. The conversion of WST-1, measured as *A*(450 nm), is proportional to the number of viable cells. The release of intracellular lactate dehydrogenase (LDH) indicates the degree of cell damage. C) Attachment and growth of adherent SKOV-3 ovarian carcinoma cells onto the **BICpoly** layer; for comparison, sections without **BICpoly** film are shown where cells grow on the plastic surface. The cells, with apparently the same density and viability, show slightly different morphologies, probably caused by subtle differences in the extracellular matrix. D) Dose-dependent cytotoxic effect of free and **BICpoly**-bound doxorubicin. 72 h after the addition of DOX at the amounts specified on the *x*-axis, either free or loaded onto **BICpoly**, the number of viable cells was determined by a WST-1 assay.

Keywords: cysteine · drug transport · nanoparticles · polyesters · polyhydroxycarboxylic acids

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 6364–6369
Angew. Chem. **2015**, *127*, 6462–6467

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Received: December 16, 2014
Revised: January 23, 2015
Published online: April 13, 2015

