Drug delivery has been considered as the key to the clinical success of numerous drugs.[1–3] In the past decade, with an aim to improve chemotherapy, tremendous effort has been directed to the development of polymer nanoparticles for the controlled delivery of anticancer drugs, including doxorubicin (DOX) and paclitaxel (PTX).[4–12] These nanovehicles offer several unique features, such as enhancing the aqueous solubility and bioavailability of the drug, prolonging the circulation time, preferential accumulation at the tumor sites by the enhanced permeability and retention (EPR) effect, and reducing systemic side effects.[13,14] However, one practical challenge with nanoparticles is their low stability in vivo because of the large dilution volume and/or interactions with cells and biomolecules present in the blood, which often lead to premature drug release, aggregation, and a diminished ability of the drug to reach its target.[15] In the past few years, different cross-linking approaches have been adopted to improve their stability,[16,17] for example, through cross-linking of the hydrophobic shell,[18] within the hydrophobic core,[19–21] or at the core–shell interface.[22] It should be noted, on the other hand, that overly stable nanoparticles are also far from optimal for drug-delivery applications, because drug efficacy is significantly reduced by the prohibited release of drugs from the nanoparticles even though they reach the target sites.[23,24] Last but not least, few of the cross-linked nanoparticles so far reported are biocompatible and degradable,[19,20] which are nevertheless fundamental prerequisites for biomedical applications.

There exists a large difference in the redox potential between the mildly oxidizing extracellular milieu and the reducing intracellular fluids, such as the cytoplasm and the cell nucleus,[25] which renders reduction-sensitive polymers particularly appealing for biomedical applications.[26] For example, reduction-sensitive polymer/DNA complexes,[27] polyeon complex micelles,[28,29] micelles,[30] polymersomes,[31] cross-linked polymersomes,[32] and degradable nanogels[33,34] have been reported to achieve fast intracellular release of DNA, siRNA, or drugs.

Herein, we report on reversibly stabilized multifunctional dextran nanoparticles for the triggered intracellular delivery of DOX, one of the most potent lipophilic anticancer drugs widely used in the clinics. The nanoparticles were prepared from dextran-lipoic acid derivatives (Dex-LAs) and were readily cross-linked using a catalytic amount of dithiothreitol (DTT; Figure 1). Dextran is a natural analogue of polyethylene glycol (PEG), and has been used in a range of biomedical applications because of its excellent aqueous solubility, biocompatibility, and nonfouling properties.[35–38] Lipoic acid is produced naturally in the human body and commonly used as an antioxidant drug for treating diseases such as diabetes and HIV.[39,40] Thus, these nanoparticles are based solely on well-accepted medical materials. The resulting cross-linked dextran nanoparticles (C-DNPs) are stable against dilution and a high salt concentration. Notably, they showed a high drug loading efficiency of 84%. The in vitro release studies showed that the release of DOX was minimal (ca. 10%) even under extensive dilution, while in the presence of 10 mM DTT (which mimics the intracellular reductive environment) over 90% of the DOX was released in 11 h. Confocal laser scanning microscopy (CLSM) studies using HeLa and RAW 264.7 (a mouse leukaemic monocyte macrophage cell line) cells showed a rapid and efficient delivery of DOX into the cell nucleus (Figure 1). MTT assays showed that DOX-loaded C-DNP had a similar drug efficacy as the non-cross-linked counterparts. These dextran nanoparticles, although extremely simple, display multifunctionalities including degradability, biocompatibility, and reversible cross-linking properties, which renders them superb for the tumor-targeted delivery of anticancer drugs.

The Dex-LA conjugates were conveniently prepared by treating dextran with lipoic acid anhydride in DMSO (see Scheme SI in the Supporting Information). 'H NMR spectroscopic analysis showed that the lipoic acid was successfully grafted on to the dextran (see Figure S1 in the Supporting Information). The degree of substitution (DS, defined as the number of LA units per 100 anhydroglucosidic (AHG) units), as determined by comparing the signals at δ = 1.86–1.91 ppm.
and δ = 4.68 ppm (anomeric proton in dextran), revealed that good control over the DS could be achieved by careful choice of the LA/AHG ratios: DS values of 34, 67, and 80 were obtained for LA/AHG mole feeding ratios of 0.5:1, 0.8:1, and 1:1, respectively (see Table S1 in the Supporting Information).

These Dex-LA conjugates readily formed nanosized particles in water. The hydrodynamic sizes, as determined by dynamic light scattering (DLS) measurements, of the nanoparticles decreased from 221 to 145 nm as the DS increased from 34 to 80 (see Table S1 in the Supporting Information). Zeta-potential analyses demonstrated that these nanoparticles had negative surface charges (−19.9 to −10.2 mV). The critical aggregation concentrations (CACs) determined using pyrene as a fluorescence probe were 16.0, 13.5, and 11.2 mg L⁻¹ for Dex-LA conjugates with DS values of 34, 67, and 80, respectively (see Table S1 in the Supporting Information). It is evident, therefore, that both the particle size and CAC could be elegantly controlled by the DS.

These nanoparticles were subsequently cross-linked in phosphate buffer (pH 7.4, 10 mM) by introducing 10 mol% DTT relative to the lipoyl units. DLS studies revealed that the nanoparticles shrunk by 20–35 nm after cross-linking (see Table S1 in the Supporting Information). The decrease in the particle size upon cross-linking was further verified by transmission electron microscopy (TEM) measurements (see Figure S2 in the Supporting Information). The smaller particle size of C-DNP is advantageous, since it may be taken up more efficiently by cells. Lipoyl groups have previously been exploited to obtain cross-linked liposomes[41,42] and to generate a reversibly polymerized lipid for gene delivery.[43] The cross-linking/polymerization mechanism is based on thiol–disulfide exchange under catalysis by DTT, wherein lipoyl rings are opened to form preferentially linear disulfide bonds between different lipoyl units.[41] The ultraviolet (UV) spectra of the nanoparticles in DMSO showed that the absorbance at 330 nm, which is characteristic of lipoyl rings, completely disappeared for the C-DNP (see Figure S3 in the Supporting Information), thus confirming that the DTT had successfully promoted the cross-linking of the dextran nanoparticles. Colloidal stability studies with DLS revealed that C-DNP was sufficiently stable against high salt concentrations (up to 2 M) as well as extensive dilution: the size distribution remained narrow even though the particle sizes increased from about 110 to 200 nm (see Figure S4 in the Supporting Information). In contrast, large aggregates (1000 nm and above) were observed for the non-cross-linked dextran nanoparticles (NC-DNP) at 0.2 M NaCl as well as with 1000-fold dilution (see Figure S4 in the Supporting Information).

The change in the particle size in response to 10 mM DTT was monitored over time in water at an ultralow concentration to investigate whether C-DNP can be un-cross-linked in a reductive environment analogous to that of the cytoplasm and cell nucleus. Interestingly, the results showed that the size of the C-DNP (DS 67) increased from about 190 nm to over 500 nm in 4 h, and to 1000 nm after 21.5 h (Figure 2). In contrast, the particle sizes varied only slightly in the absence of DTT. It should be noted that lipoic acid is conjugated to dextran through an ester bond, which is degraded over several weeks to give the nontoxic products lipoic acid and dextran. We have examined the stability of C-DNP in 20 mM phosphate buffer (PB) at 37 °C. In line with our expectation,
substantial aggregation occurred after 15 days as a result of hydrolytic degradation (see Figure S5 in the Supporting Information). $^1$H NMR spectroscopic analysis showed that approximately 5 and 22% of the lipoyl esters were degraded after 8 and 15 days, respectively (see Figure S6 in the Supporting Information). The biocompatibility studies using HeLa and RAW 264.7 cells revealed that the Dex-LA nanoparticles are nontoxic up to the highest testing concentration of 2.5 mgmL$^{-1}$ (Figure 3).

Initial drug-loading experiments were performed using DOX at a theoretical drug loading content of 9.1 wt% and a polymer concentration of 200 mgL$^{-1}$. Interestingly, cross-linked Dex-LA nanoparticles yielded a high drug-loading efficiency (DLE) of up to 84% (see Table S1 in the Supporting Information). The in vitro release studies were carried out at pH 7.4 and 37°C at a low polymer concentration of 13 mgL$^{-1}$, which is close to its CAC (11 mgL$^{-1}$). These conditions mimicked intravenous administration. The results revealed minimal (ca. 10%) release of DOX from the C-DNP (DS 80) within 11 h (Figure 4), likely because of their particularly sturdy core structure. In the presence of 10 mM DTT, however, sustained and virtually quantitative release of DOX was observed under otherwise identical conditions, and gave a release profile similar to that for DOX-loaded NC-DNP (Figure 4). These results indicate that DTT-triggered de-cross-linking is rapid and complete.

The intracellular trafficking of DOX was studied in HeLa and RAW 264.7 cells by using confocal laser scanning microscopy. Remarkably, DOX-loaded C-DNP efficiently delivered and released the DOX into the cell nucleus. DOX fluorescence was clearly observed in the nucleus of each HeLa cell after incubation for just 0.5 h, thus corroborating fast de-cross-linking of the nanoparticles inside the cells (Figure 5). A longer incubation time (2 h) resulted in stronger DOX fluorescence. In contrast, DOX fluorescence was not detected in cells treated for 0.5 h with the same concentration of free DOX, and after 2 h the DOX was distributed in the surroundings of the cell nucleus (Figure 5). The comparably faster delivery of DOX into the cell nucleus using C-DNP compared to free DOX is unusual, given that inefficient
release of DOX into the cell nucleus was repeatedly reported to be a key challenge encountered with nanoparticles. It should be noted, however, that after 6 h incubation, stronger DOX fluorescence was observed in the cell nucleus of cells treated with free DOX than those with DOX-loaded C-DNP (see Figure S7 in the Supporting Information). In comparison, DOX was mainly distributed in the cytosol of cells incubated with DOX-loaded NC-DNP for 6 h (see Figure S8 in the Supporting Information), which is similar to other particulate delivery systems.[6] Park and co-workers reported that drug is released from polymer micelles into cell membranes rather than inside cells.[44] The fast accumulation of DOX in the presence of C-DNP into the nucleus is likely because C-DNP can overcome this premature drug release as a result of its improved stability, with DOX released rapidly in to the cytoplasm and cell nucleus through fast de-cross-linking.

Notably, cytotoxicity studies using both HeLa and K562 cells demonstrated that DOX-loaded C-DNP had a similar drug efficacy as the non-cross-linked counterpart, with approximately 70% cell death achieved in two days (Figure 6). DOX-loaded DNP revealed, nevertheless, lower cytotoxicity than free DOX (Figure 6). This finding is in line with the CLSM results that more DOX was accumulated in the cell nucleus of cells treated with free DOX than those treated with DOX-loaded C-DNP after a prolonged incubation time (≥ 6 h). However, for in vivo applications, it is unlikely that such a high concentration of free DOX would be present for such a long treatment time.

In conclusion, we have developed simple, biocompatible, and degradable multifunctional cross-linked dextran nanoparticles that are stable under extracellular conditions but are rapidly destabilized under reductive environments that mimic those of the intracellular compartments, such as the cytoplasm and the cell nucleus. These nanoparticles show high drug-loading efficiency and reduction-triggered release of DOX in vitro as well as inside tumor cells, thus resulting in particularly efficient delivery and release of DOX to the cell nucleus. We are convinced that these smart nanoparticles have tremendous potential for tumor-targeted chemotherapy.

Keywords: antitumor agents · cross-linking · drug delivery · nanoparticles · polymers

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