Charge-conversional and reduction-sensitive poly(vinyl alcohol) nanogels for enhanced cell uptake and efficient intracellular doxorubicin release

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Charge-conversional and reduction-sensitive polyvinyl alcohol (PVA) nanogels were developed for efficient cancer treatment by enhanced cell uptake and intracellular triggered doxorubicin (DOX) release. These PVA nanogels were prepared in a straightforward manner by inverse nanoprecipitation via “click” reaction with an average diameter of 118 nm. The introduction of COOH into the PVA nanogels efficiently improved the DOX encapsulation due to the electrostatic interaction. The in vitro release result showed that the decrease of electrostatic interaction between COOH and DOX under a mimicking endosomal pH, in combination with the cleavage of the intervening disulfide bonds in response to a high glutathione (GSH) concentration led to a fast and complete release of DOX. Furthermore, confocal laser scanning microscopy (CLSM) revealed that the ultra pH-sensitive terminal groups allowed nanogels to reverse their surface charge from negative to positive under a tumor extracellular pH (6.5–6.8) which facilitated cell internalization. MTT assays and real time cell analysis (RTCA) showed that these DOX-loaded charge-conversional and reducible PVA nanogels had much better cell toxicity than DOX-loaded non-charge-conversional or reduction-insensitive PVA nanogels following 48 h of incubation. These novel charge-conversional and stimuli-responsive PVA nanogels are highly promising for targeted intracellular anticancer drug release.

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1. Introduction

Drug delivery systems (DDSs) have appeared as a promising and reliable approach for delivering potent drugs to the site of action in a precise and timely fashion. Polymeric nanosystems which include micelles, liposomes, nanoparticles, and nanogels have been the most investigated DDSs, because of their prolonged circulation time, enhanced accumulation in the tumor sites via the enhanced permeability and retention (EPR) effect, decreased adverse effects, and improved cellular internalization versus cellular internalization dilemma has been quite a challenge for targeted tumor chemotherapy. In the past decade, several strategies have been developed to enhance tumor cell uptake of nano-systems, including the decoration of active targeting ligands for receptor-mediated endocytosis and the usage of tumor extracellular microenvironment stimuli, i.e., tumoral acidity and temperature gradients [17–19]. As compared to the strategy of active targeting ligand-decoration, the nanosystems using the tumor pH for enhanced cellular internalization is easy to prepare and can be exploited for the universal tumor cells, since the mildly acidic extracellular pH (in the range of 6.5–6.8) exists in most tumor tissues, which is lower than that of normal tissues and the blood stream (pH 7.2–7.4) [20,21]. Several ultra pH-sensitive polymers including poly(β-amino ester), poly(methacryloyl sulfadimethoxine), polyhistidine, and polymer with 2,3-dimethylmaleic anhydride (DMMA) modified amine moieties, have been developed for the construction of polymeric delivery systems in response to weakly acidic tumor tissue microenvironments. For example, Kataoka et al. first reported the concept of charge-conversional polymer based on citraconic amides, which are stable under neutral conditions while they can be cleaved only under slightly acidic pH conditions, thus leading to the transformation of negatively charged carbonate functionalities into positively charged primary amines [22]. Recently, Wang et al. further developed dual pH-sensitive prodrug nanoparticles using two pH-sensitive linkages with different stabilities towards acidity, DMMA-based amides, and hydrozone linkages, thereby achieving...
improved cellular internalization of nanoparticles under a tumor extracellular pH and effective release of covalently conjugated DOX under endosomal/lysosomal pH [23].

Another considerable challenge for cancer therapy is how to accomplish a rapid drug release after the nanosystems arrive at the pathological site for better therapeutic efficacy. In the past decade, a tremendous effort has been directed to develop intelligent drug nanocarriers that release their payload in response to intrinsic intracellular signals, particularly to endosomal/lysosomal pH and cytoplasmic glutathione (GSH) [24–26]. Polymeric components with pH-sensitive groups (pH-induced protonation/deprotonation or degradation) are used to produce drug-formulation nanosystems that are relatively stable in the circulation but also have the ability to rapidly release the entrapped drugs in the tumor tissue (pH 6.8) as well as in the intracellular compartments, such as endosomes (pH 5.5–6) and lysosomes (pH 4.5–5.0) of cells [27–29]. Following escape from the endosome and by taking advantage of the high redox potential in cytoplasm and nuclei of cancer cells, which have much higher concentration of reducing glutathione (GSH) tripeptide than body fluids and extracellular milieu (0.5–10 mM versus 2–20 μM GSH), reduction-sensitive nanosystems containing S–S bonds have been designed and exploited for active cytoplasmic release of various potent chemotherapeutics [30–32]. It should be noted that polymeric nanosystems with dual and multi-stimuli responses have shown unprecedented control over drug delivery and thus led to superior in vitro and/or in vivo anti-cancer efficacy, whereby these combined responses take place either simultaneously or in a sequential manner at the pathological site [33–35]. Very recently, we developed pH and redox dual-responsive prodrug nanogels using an inverse nanoprecipitation method without any surfactants. After cleavage of the hydrazone linkers and degradation of S–S crosslinked networks under intracellular conditions, conjugated DOX could be efficiently released from the hyperbranched polyglycerol (hPG) nanogel matrix and internalized by the tumor cells to induce cell death [36].

Poly(vinyl alcohol) (PVA) has an excellent history of biomedical applications, specifically in the form of micro/hydrogel materials that are used for enzyme immobilization, cell encapsulation, and clinical applications as embolic bodies [37]. However, due to PVA’s inhomogeneous interior, high porosity, and lack of polar PVA nanogels, it failed to meet the demands of (nano)biotechnology, especially in the nanomedicine area because of its inferior drug encapsulation capability and uncontrollable release behavior. Therefore we developed a unique charge-conversional and reducible PVA nanogel system for enhanced cell uptake and efficient intracellular DOX release. These PVA nanogels are prepared in a straightforward manner by inverse nanoprecipitation via a “click” reaction with an ultra pH-sensitive terminator which enables nanogels to reverse their surface charge from negative to positive at tumor extracellular pH (6.5–6.8) for enhanced cell internalization (Scheme 1). Subsequently, in vitro drug release and tumor cell killing activity of DOX-loaded nanogels were investigated and the results compared with those obtained using DOX-loaded non-charge-conversional or reduction-insensitive counterparts.

2. Experimental section

2.1. Materials

Cystamine bishydrochloride (Aldrich, 96%), succinic anhydride (Aldrich, 99%), triethylamine (Acros, 99%), propargyl chloroformate (Aldrich, 96%), propargylamine (Aldrich, 98%), 2,3-dimethylmaleic anhydride (DMMA, Acros, 97%), mesyl chloride (MsCl, Acros, 99%), sodium azide (Acros, 99%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Acros, 97%), N-hydroxysuccinimide (NHS, Acros, 98%), doxorubicin hydrochloride (DOX, Sigma, 98%) were used as received. Polyvinyl alcohol (PVA, Mowiol 3-97, $M_w = 16,000$ g/mol) was provided by Kuraray Europe GmbH (Germany). For cell culture experiments, MCF-7 cells (DSMZ no.: 115) were cultured in RPMI supplemented

Scheme 1. Illustration of charge-conversional and reduction-sensitive PVA nanogels for enhanced cell uptake and efficient intracellular DOX release.
with 10% fetal calf serum, MEM nonessential amino acids, 1 mM sodium pyruvate, and 10 g/mL human insulin HeLa cells (DSMZ no.: ACC 57) were cultured in RPMI supplemented with 10% fetal calf serum and 1 mM sodium pyruvate.

### 2.2. Characterization

$^1$H NMR spectra were recorded on a Bruker ECX 400. The chemical shifts were calibrated against residual solvent peaks as the internal standard. IR measurements were carried out on a Nicolet AVATAR 320 FT-IR 5 SXC that was equipped with a DTGS detector from 4000–6000 cm$^{-1}$. The size of nanogels was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser. The morphology of nanogels was observed using cryogenic transmission electron microscopy (Cryo-TEM, Philips CM12). Microscopy was carried out at a 94 kV sample temperature using the low-dose protocol of the microscope at a primary magnification of ×58,300 and an accelerating voltage of 100 kV (LaB6-illumination).

### 2.3. Synthesis of propargyl-cystamine

Cystamine bis-hydrochloride (4.00 g, 17.6 mmol) and triethylamine (7.40 mL, 3 equiv.) were dissolved in methanol (300 mL), followed by drop wise addition of propargyl chloroformate (2.10 g, 17.6 mmol) dissolved in DCM at 0 °C (Scheme S1). After 4 h the solvent was evaporated, and Na$_2$HPO$_4$ aqueous solution was added (100 mL, 1 M, pH 4.2). The aqueous solution was extracted with diethyl ether three times to remove the byproduct, di-propargyl-cystamine. The aqueous solution was basified to pH 9 by NaOH aqueous solution (1 M) and extracted with ethyl acetate three times. The combined organic phases were dried over MgSO$_4$ and evaporated to yield the product as a viscous yellow liquid. Yield: 1.32 g (32%). $^1$H NMR (400 MHz, CD$_3$OD): δ 4.63 (2H, −CH$_2$−C≡CH), 3.40 (2H, −NH−CH$_2$−CH$_2$−), 2.91 (2H, −CH$_2$−NH$_2$), 2.86 (1H, −C≡CH), 2.78 (4H, −CH$_2$−SS−CH$_2$−) (Fig. S1).

### 2.4. Synthesis of dimethylmaleic propargylamide

Propargylamine (0.50 g, 9.09 mol) and 2,3-dimethylmaleic anhydride (1.14 g, 9.09 mmol) were sequentially dissolved in anhydrous THF (25 mL), and the solution was stirred for 4 h at room temperature (Scheme S2). The final product was recrystallized in THF. Yield: 1.50 g (91%). $^1$H NMR (400 MHz, CD$_3$OD): δ 3.70 (2H, −CH$_2$−C≡CH = CH), 2.01 (1H, −CH$_2$−C == CH), 1.89 (6H, −C(CH$_3$)$_2$ == C(CH$_2$)$_3$−) (Fig. S2).

### 2.5. Synthesis of carboxyl-alkynyl-functionalized PVA (PVA-COOH-alkynyl)

The synthesis of carboxyl-alkynyl functionalized PVA (PVA-COOH-alkynyl) was carried out in two steps. Briefly, PVA (2.00 g, 36.38 mmol of OH group) was dissolved in anhydrous DMSO (100 mL) at 50 °C and the solution cooled down to room temperature. Succinic anhydride (1.09 g, 10.90 mmol) and a catalytic amount of Et$_3$N were sequentially added to the reaction. After 24 h reaction, a sample was taken for determination of carboxyl functionality. The reaction solution was divided into two aliquots. EDC (494.2 mg, 2.58 mmol) and NHS (296.7 mg, 2.58 mmol) were added to both aliquots (PVA-COOH, 5.17 mmol of COOH group). After 30 min, propargyl-cystamine (604.0 mg, 2.58 mmol) and propargylamine (142.0 mg, 2.58 mmol) were separately added into these two aliquots, and the reaction was allowed to proceed with overnight stirring at room temperature. The polymers were isolated by dialysis, first in ethanol for 8 h, then in water for another 48 h. After that they were freeze-dried.

### 2.6. Synthesis of azido-functionalized PVA (PVA-N$_3$)

The synthesis of azido-functionalized PVA was carried out in a two-step protocol [38]. In the first step, 10 mL pyridine and methylsulfonyl chloride (0.84 mL, 10.90 mmol) were added to a solution of PVA (2.00 g, 36.38 mmol of OH group) in DMSO (100 mL). The reaction mixture was stirred overnight in a cold water bath; Na$_2$N$_3$ (1.42 g, 21.8 mmol) was added into the solution. The reaction was allowed to proceed with stirring overnight at 80 °C. After cooling to room temperature, the polymer was isolated by precipitation in diethyl ether/acetone (1/1), and the solid was further purified by dialysis in water to give a light yellow solid after freeze-drying.

### 2.7. Preparation of PVA nanogels by inverse nanoprecipitation via ‘click’ chemistry

PVA-COOH-alkynyl and PVA-N$_3$ were dissolved separately in phosphate buffer (PB, pH 7.4, 10 mM) with a concentration of 10 mg/mL. THPTA (110 mg/mL, 10 μL), CuSO$_4$ (21 mg/mL, 10 μL), and NaAsc (50 mg/mL, 10 μL) were sequentially added into 800 μL of PVA-alkynyl solution. The solution was kept at 4 °C and mixed with 800 μL of PVA-N$_3$ solution. The mixed solution was quickly added into 20 mL of acetone. After 24 h, the reaction was quenched by the addition of excess dimethylenemal propargylamide (charge-conversional nanogel) dissolved in acetone or propargylalcohol (non-charge-conversional nanogel). After 10 min, 20 mL of PB was added and acetone was evaporated to obtain PVA nanogels dispersed in water. The nanogels were collected by centrifugation (5000 rpm) with a MWCO of 10,000, washing 5 times with Milli-Q-water, and freeze-drying. The nanogel samples were redispersed in PB by sonication and characterized by DLS and cryo-TEM. For the preparation of DOX-loaded nanogels, 160 or 320 μL of DOX-HCl solution in Milli-Q-water (5.0 mg/mL) was added into 800 μL of PVA-alkynyl solution, and the following steps were carried out similar to the preparation of blank NGs except that the procedure was performed in the dark.

### 2.8. Reduction-sensitivity and surface charge-conversion of PVA nanogels

PVA nanogel suspension (1.0 mg/mL) was divided into two aliquots of 1 mL, and 10 μL of GSH solution (1.0 M) was added to one of the two aliquots with a final GSH concentration of 10 mM. The samples were slowly stirred at 37 °C under a N$_2$ atmosphere and the change in the nanogel size was monitored over time by DLS. To monitor the surface charge conversion, PVA nanogel suspension (1.0 mg/mL) was similarly divided into six aliquots of 1 mL whereby 25 μL of acetic buffer (pH 6.8, 4 M) was added to one of the two aliquots with a final pH of 6.8. The surface charge of the nanogels was followed by zeta potential measurements.

### 2.9. In vitro release of DOX from PVA nanogels

The in vitro release of DOX from PVA nanogels was investigated at 37 °C under four different conditions: (i) PB (10 mM, pH 7.4), (ii) acetate buffer (10 mM, pH 5.5), (iii) PB (10 mM, pH 7.4) containing 10 mM GSH, and (iv) acetate buffer (100 mM, pH 5.5) containing 10 mM GSH. DOX-loaded micelle suspension was divided into six aliquots and immediately transferred to a dialysis tube with a MWCO of 12,000-14,000. The dialysis tube was immersed into 20 mL of appropriate buffer and shaken at 37 °C. At set time intervals, 5.0 mL of the release medium was taken out and replenished with an equal volume of fresh medium. To avoid oxidation of GSH, the release media were perfused with argon gas. The concentration of DOX was determined by fluorescence measurements (excitation at 480 nm). To determine the drug loading content, DOX-loaded nanogel suspensions were freeze-dried, dissolved in DMSO, and analyzed with UV spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations.
To determine the amount of released DOX, calibration curves were run with DOX/phosphate buffer solutions with different DOX concentrations at pH 7.4. The emission at 480 nm was recorded. Release experiments were conducted in triplicate. The results are presented as the average ± standard deviation.

Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

\[
\text{DLC (wt. %)} = \left( \frac{\text{weight of loaded drug}}{\text{total weight of polymer and loaded drug}} \right) \times 100%
\]

\[
\text{DLE (\%)} = \left( \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \right) \times 100%
\]

2.10. Confocal laser scanning microscopy

MCF-7 cells were plated on microscope slides in a 24-well plate (5 × 10^3 cells/well) using 1640 culture medium containing 10% FBS. After 24 h incubation, the medium was replaced by 450 μL of fresh culture medium and 50 μL of prescribed amounts of DOX-loaded nanogels or free DOX. After incubation for 4 h and 8 h, respectively, the culture medium was removed and the cells were washed twice with PBS. The cells were fixed with 4% paraformaldehyde and the nuclei were stained with DAPI. Fluorescence images of cells were obtained with a Confocal Laser Scanning Microscope (Leica, Germany) and analyzed by Leica 2.6.0 software.

To study the cellular uptake of charge-conversional nanogels at different pHs, DOX was linked to PVA-COOH-(SS-alkynyl) using a carbodiimide chemistry via the amino group of DOX and the carboxyl group of the polymer. DOX-conjugated nanogels were prepared as mentioned above using dimethylmaleic propargylamide (charge-conversional nanogel) or propargyl alcohol (non-charge-conversional nanogel) as the terminators. Similarly, MCF-7 cells were plated on microscope slides in a 24-well plate (5 × 10^3 cells/well) using 1640 culture medium containing 10% FBS. After 24 h incubation, the medium was replaced by 450 μL of fresh culture medium with two pHs (pH 6.8 or 7.4) and 50 μL of prescribed amounts of DOX-loaded nanogels or free DOX. After incubation for 1 h and 2 h, respectively, the culture medium was removed and the cells were washed twice with PBS. The cells were fixed with 4% paraformaldehyde for 20 min, incubated with early endosome antibody-FITC (Invitrogen, Germany) at 37 °C for 1 h, and stained with DAPI. Fluorescence images of cells were obtained with CLSM.

2.11. Real time cell analysis (RTCA) of DOX-loaded PVA nanogels

RTCA was used for dynamically monitoring the cell proliferation and viability in real time, based on the Real-Time Cell Electronic Sensor (RT-CES) system. 50 μL of culture medium was added to each well of the E-plate 96 (Roche, Mannheim, Germany) for the background measurement, followed by adding 40 μL of MCF-7 cell suspension into the sensor wells (1 × 10^4 cells/well). After 24 h incubation, 10 μL of nanogel samples at different concentrations in PB were added into each well. Four replicates for each sample were measured and the results were reported as the mean value. The E-plate was incubated with 5% CO2 at 37 °C and monitored on the RTCA SP system (Roche, Mannheim, Germany) with time intervals of at least 15 min for 48 h after treatment. Analysis was performed using the RTCA software version 2.0.

2.12. Cytotoxicity of DOX-loaded PVA nanogels

The cytotoxicity of blank and DOX-loaded PVA nanogels was studied by MTT assay using MCF-7 and HeLa cells. Cells were seeded into a 96-well plate at a density of 1 × 10^4 cells per well in 90 μL of 1640 culture medium containing 10% FBS and incubated at 37 °C with 5% CO2. After 24 h, 10 μL of nanogel samples at different concentrations in PB (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, and then 10 μL of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and the medium was replaced by 150 μL of DMSO to dissolve the resulting purple crystals. The optical densities were measured by a microplate reader at 570 nm. The experiments were conducted in triplicate and the results were presented as the average ± standard deviation.

To evaluate whether the high drug efficacy was specially caused by tumoral pH-activated endocytosis, MCF-7 cells seeded in a 96-well plate at a density of 1 × 10^4 cells per well for 24 h were incubated at pH 6.8 or 7.4 for 4 h with DOX-loaded nanogels or free DOX. After that, the medium was replaced by 100 μL of fresh medium and the cells were cultured for another 48 h. The same procedure was carried out as mentioned above for the cytotoxicity measurement.

3. Results and discussion

3.1. Synthesis of functional PVA derivatives

Carboxyl/alkynyl-functionalized PVA was prepared by two steps: (i) carboxylation of PVA with succinic anhydride; (ii) conjugation of propargyl-cystamine or propargylamine via carbodiimide chemistry (Scheme 2). The 1H NMR displayed new signals at 2.42 that corresponded to methylene protons in the vicinity of the carboxyl group, whereby the carboxyl was calculated at 28.5% according to the integral ratio of δ 1.94 (methyl group on PVA side chain) and δ 2.42 (Fig. S3B). The conjugation of propargyl-cystamine or propargylamine to carboxyl-functionalized PVA efficiently proceeded with 9.5% functionality as expected (Fig. S3C). PVA-N3 was accordingly synthesized with a functionality of 23.4% and characterized by FT-IR (Fig. S4A) and elemental analysis [38].

3.2. Formation of reduction-sensitive crosslinked nanogels

Copper-catalyzed Huisgen [2 + 3] cycloaddition was selected as a crosslinking reaction due to its high conversion, fast reaction kinetics, and good bioorthogonality [39]. The aqueous solutions of PVA-COOH-(SS-alkynyl) and PVA-N3 were separately prepared with a concentration of 10 mg/mL. After cooling to 4 °C, a catalytic amount of THPA/ CuSO4/NaAsc was added, and PVA nanoaggregates were formed by precipitation into acetone. The polymer concentration drastically increased during this process, which induced gelation reactions and PVA network formation. After gelation, excessive propargyl-cystamine was added to quench the remaining reactive azido groups. Finally, the charge-conversional reducible PVA nanogels (CC-SS-NGs) were collected by evaporating acetone, washing with PB, and freeze-drying. Interestingly, CC-SS-NGs with an average size of 118 nm were determined by dynamic laser scattering (DLS) (Fig. 1A), which is larger when dispersed in acetone (98 nm) due to their swelling property in water. The cryo-TEM micrograph showed that these nanogels had a spherical morphology with particle sizes in accord with those determined by DLS (Fig. 1B). Meanwhile, the charge-conversional nanogels without S-S bonds (CC-NSS-NGs) were prepared with an average size of 88 nm as a reduction-insensitive control (Fig. 1A). To confirm whether the “click” reaction went well during the nanogel formation process, nanogels diluted with DMSO were measured by DLS. The result showed that both of the chemical crosslinked CC-SS-NGs and CC-NSS-NGs were only swollen in DMSO, in contrast to the hydrogen-bonding induced physically crosslinked PVA nanogels, which dissolved into unimer in DMSO (Fig. 1A). FT-IR also showed that during the nanogels’ formation the peak at 2000 cm−1 attributed to azido group decreased after crosslinking and disappeared with the addition of a terminator (Fig. S4). It is confirmed that combining this inverse nanoprecipitation technique with a click reaction is a very facile and efficient method for the preparation of hydrophilic nanogel particles.
### 3.3. Surface charge-conversion and reduction-sensitivity

It has been demonstrated that the amide bond neighboring to the dimethylmaleic group was pH-labile even under such slightly acidic conditions as pH 6.8 [40,41]. The CC-SS-NGs were therefore expected to show a similar charge-conversional behavior in the tumoral extracellular pH environment, i.e., at pH 6.8. Initially, the CC-SS-NGs were negatively charged at pH 7.4. When the pH value of the medium was adjusted to pH 6.8 and kept in equilibrium for 2 min, however, the zeta potential of these nanogels significantly increased and became positive within 10 min because the cleavage of the amide bond in the ultra pH-sensitive terminator transformed the carboxyl groups to amino groups (Fig. 2A). Despite the slow increase of the nanogels’ zeta potential upon incubation at pH 7.4, the charge remained negative for 3 h. Interestingly, we observed that the zeta potential of non-charge-conversional reduction-sensitive nanogels (NCC-SS-NGs, using propargyl alcohol as the pH-insensitive terminal group) did not significantly change at both pH 6.8 and 7.4 and stayed negative for the whole monitoring process (Fig. 2A). Since cell membranes are generally negatively charged, the charge-conversion behavior of CC-SS-NGs in the tumoral extracellular pH environment improved their internalization by tumor cells. It should be noted that, although the zeta potential of CC-SS-NGs quickly increased at pH 6.8, the NG size did not change after 18 h (Fig. S5), which indicates that the nanogels kept their spherical structure before cellular uptake and subsequent restrictions from drug leaching.

The reduction-sensitivity of PVA nanogels was studied by monitoring the nanogels’ size over time in response to 10 mM GSH using DLS. The results showed that CC-SS-NGs quickly dissociated in the presence of 10 mM GSH, whereby a large population was observed that had reduced sizes of about 60 nm and 30 nm at 1 h and 6 h, respectively (Fig. 2B). Unexpectedly, the nanogels did not degrade into soluble unimers. This was probably due to the presence of the hydrogen bonding and propargyl-cystamine hydrophobic modification, which resulted in small PVA aggregations in the aqueous solution. In contrast, little size change was detected for CC-NSS-NGs within 24 h under the same reducing conditions (Fig. 2B), as well as for the CC-SS-NGs within 24 h in the absence of GSH. It is therefore evident that CC-SS-NGs can be rapidly disrupted under intracellular-mimicking reducing conditions.

### 3.4. Loading and triggered release of DOX

DOX can be readily encapsulated into nanogels by inverse nanocoprecipitation in acetone, followed by a “click” reaction to form the DOX-loaded nanogels. These nanogels can be quenched by dimethylmaleic propargylamide (charge-conversional nanogels) or
propargyl alcohol (non-charge-conversional nanogels), and, the
unloaded DOX can be removed by centrifugation. The results showed that both CC-SS-NGs and CC-NSS-NGs had a higher loading capability for DOX than the physically crosslinked PVA nanogels (Fig. S6). This can be attributed to the following factors: (1) the presence of carboxyl groups in the nanogels that significantly improved the DOX-loading content due to electrostatic interactions [42] and (2) the propargyl-cystamine hydrophobic modification and the chemically crosslinked networks in the nanogels, which provided the hydrophobic interaction with DOX. Due to the similar degrees of functionality (DF) of the same PVA, CC-SS-NGs and CC-NSS-NGs exhibited similar DOX loading levels with the drug loading efficiency (DLE) ranging from 67.4 to 79.2% at theoretical drug loading contents (DLC) of 5 and 10 wt.% (Table 1). The DLE decreased as the theoretical DLC increased. The average size of CC-SS-NGs expanded from 118 to 154 nm after loading 4.0 wt.% DOX and then rose to 177 nm upon a further increase in the DLC to 7.1 wt.%. All the DOX-loaded nanogels were shown to be sufficiently stable with no size change after 24 h in the presence of 10% FBS.

The in vitro release of DOX from nanogels was investigated at 37 °C in the following four different media: (i) pH 7.4, (ii) pH 5.5, (iii) pH 7.4 and 10 mM GSH, and (iv) pH 5.5 and 10 mM GSH. The DOX release at physiological pH (pH 7.4) was highly restricted with a released amount of ca. 23% after 48 h (Fig. 3A). The release of DOX was significantly accelerated at pH 5.5 with 63% of DOX released after 48 h under otherwise the same conditions and was likely due to the decrease of the electrostatic interaction between nanogels and DOX. A similar pH-dependent DOX release behavior could be also observed from the poly(acrylic acid)-DOX complex [43]. The DOX release rate accelerated under the reducing environment containing 10 mM GSH at pH 7.4, in which 58% of DOX was released in 10 h (Fig. 3A). CC-SS-NGs were rapidly destabilized and the electrostatic interaction with DOX became weaker under the dual stimuli. In contrast, DOX release from CC-NSS-NGs was not influenced so much by the presence of GSH, both at pH 5.0 and pH 7.4 (Fig. 3B), in which only 8% difference in cumulative DOX release was observed most likely due to the diffusion of GSH into the nanogels. Li et al. also reported that the anticancer drug paclitaxel (PTX) release from the (ortho ester)-containing and disulfide-crosslinked nanogels was greatly accelerated by a cooperative effect of both acid-triggered hydrolysis and DTT-induced degradation [44]. These results clearly indicate that the DOX released from CC-SS-NGs proceeds in a controlled manner and can be activated by a synergistic trigger of low pH and reductive environments.

3.5. Cellular uptake and intracellular release of DOX

To further demonstrate that nanogels can be more efficiently internalized by cancer cells at extracellular pH, we investigated the cellular uptake behaviors of DOX-conjugated nanogels at pH 7.4 and 6.8. MCF-7 cells were incubated with DOX-conjugated CC-SS-NGs and NCC-SS-NGs at each pH for 1 h and 2 h, and their cellular distribution was observed with confocal laser scanning microscopy (CLSM). It was demonstrated that charge-conversional CC-SS-NGs were internalized at pH 6.8 after 1 h incubation and intensively distributed in the cytoplasm after 2 h overlaying with the endosomes (Fig. 4A and B), while it was rarely observed in cells incubated with CC-SS-NGs at pH 7.4 after 2 h (Fig. 4C). The CLSM images also showed that the DOX-conjugated, non-charge-conversional NCC-SS-NGs, as a negative control, had a very low cellular uptake at both pH 6.8 and 7.4 for 2 h, similar to that of CC-SS-NGs at pH 7.4 (Fig. S7). It was concluded that the charge-conversional CC-SS-NGs indeed exhibited significantly enhanced cellular internalization at the tumoral extracellular pH.

To demonstrate that DOX could be efficiently released from the nanogels following cellular uptake and be further internalized with the cell nucleus, we investigated the intracellular DOX release from the DOX-loaded nanogels in MCF-7 cells using CLSM. Interestingly, significant DOX fluorescence was observed in the cytoplasm and nuclei of MCF-7 cells following 4 h incubation with CC-SS-NGs (Fig. 5A). The DOX fluorescence became even stronger in the cell nuclei at a longer incubation time of 8 h (Fig. S8), which was notably similar for MCF-7 cells following 4 h incubation with free DOX (positive control, Fig. S5). In contrast, very weak DOX fluorescence was observed in the peri-nuclei region of cells incubated for 4 h with CC-NS-SS-NGs (reduction-insensitive control) under otherwise the same conditions (Fig. 5C), and this released DOX was found in the cell nuclei after 8 h incubation (Fig. 5D). The different intracellular drug release behaviors of CC-SS-NGs and CC-NSS-NGs were most likely due to the fast and complete DOX release from CC-SS-NGs following their escape from the endosomes as a result

![Fig. 2. Changes in the zeta potential of PVA nanogels at pH 6.8 and 7.4 (A), and in the nanogel sizes in response to 10 mM GSH (B).](image-url)
of reduction-triggered nanogel dissociation in the cytosol, which is in line with the in vitro release under dual stimuli. Although the DOX release from CC-NSS-NGs could be accelerated under endosomal pH, it would have been significantly restricted in the cytosol (reducing environment and neutral pH), as indicated in Fig 3B. These results demonstrate that pH and reduction dual-responsive CC-SS-NGs mediate a more efficient intracellular anticancer drug release than reduction-insensitive nanogels.

3.6. Tumor cytotoxicity of DOX-loaded nanogels

MTT assays in MCF-7 and HeLa cells revealed that the blank PVA nanogels were practically non-toxic (cell viabilities ≥ 80%) up to a tested concentration of 1.0 mg/mL (Fig. 6A and S8A), which confirms that these degradable nanogels have good biocompatibility. DOX-loaded nanogels, however, displayed significant cell killing activity towards MCF-7 and HeLa cells following 48 h incubation (Fig. 6B and S8B). It
should be noted that DOX-loaded CC-SS-NGs had low half inhibitory concentration (IC₅₀) values of 0.32 and 0.45 μg DOX equiv./mL for MCF-7 and HeLa cells, respectively, which was lower than those obtained with the reduction-insensitive CC-NSS-NGs under otherwise the same conditions (IC₅₀ = 2.04 and 1.42 μg DOX equiv./mL for MCF-7 and HeLa cells, respectively). This higher cell killing activity of CC-SS-NGs is in accordance with the CLSM observations that CC-SS-NGs mediated a faster and better intracellular drug release than the corresponding reduction-insensitive CC-NSS-NGs. Interestingly, it was found that both DOX-loaded charge-conversional CC-SS-NGs and CC-NSS-NGs presented superior cell killing activity towards the cancer cells compared to the corresponding non-charge converted NCC-SS-NGs and NCC-NSS-NGs, which was attributed to enhanced cellular uptake via the cleavage of pH-labile amide bonds.

The cytotoxicity of MCF-7 cells incubated with DOX-loaded nanogels was also monitored by a RTCA assay that could reflect the real-time cell number, cell morphology, and degree of cell adhesion. Adherent cells attached to the bottom of the well were able to cause an increase in impedance and displayed an increase in the cell index (CI) value. After 24 h undisturbed cell growth, the substances were supplied with nanogel samples at a DOX concentration of 5 μg/mL. Cytotoxicity effects of DOX-loaded nanogels were recorded in a time-dependent decrease of the CI value. As predicted, DOX-loaded CC-SS-NGs efficiently inhibited cell growth, and the CI value started to decrease after 14 h treatment (Fig. 7A), which seemed longer than the detected time for intracellular release from CLSM, which might be due to the fact the released DOX needed more time to intercalate with the DNA in cell nuclei in order to induce cell death after cellular uptake [45]. It should be noted that the inhibition of cell growth treated with both DOX-loaded CC-NSS-NGs and NCC-NSS-NGs was much lower than with DOX-loaded CC-SS-NGs, which was mainly due to insufficient intracellular drug release from the reduction-insensitive nanogel carriers. Notably, as the same reduction-sensitive carriers, DOX-loaded non-charge-conversional NCC-SS-NGs showed a characteristic delay in the onset of cytotoxicity (approximately 26 h) as compared to the charge-conversional CC-SS-NGs. To further study the pH-induced cellular uptake and improved cell killing activity of the DOX-loaded nanogels, MCF-7 cells were incubated at pH 6.8 or 7.4 for only 4 h with DOX-loaded nanogels. The
culture medium was removed and replenished with fresh culture medium and the cells were cultured for another 48 h. Interestingly, MCF-7 cells treated with DOX-loaded CC-SS-NGs at pH 6.8 displayed a lower cell viability of 43.6% than those incubated with DOX-loaded CC-SS-NGs at pH 7.4 as well as with DOX-loaded non-charge converted NCC-SS-NGs at pH 6.8 (cell viability: 71.0%) under otherwise the same conditions (Fig. 7B). It should be noted that, although CC-SS-NGs and CC-NSS-NGs are both charge-conversional nanogels, DOX-loaded reduction-sensitive CC-SS-NGs displayed better cell killing activity than the reduction-insensitive CC-SS-NGs (cell viability: 58.9%), with a cell killing activity very close to that of free DOX (cell viability: 47.5%). These results demonstrate that CC-SS-NGs can facilitate efficient cellular uptake and drug delivery to achieve remarkable triggered cell killing activity.

4. Conclusions

We have demonstrated that charge-conversional and reducible PVA nanogels efficiently deliver and release DOX into cancer cells and result in significant tumor cell killing activity. This is a new design and study of degradable PVA nanogel systems that respond to tumoral extracellular pH, intracellular endosomal pH, as well as cytoplasmic glutathione for enhanced cellular uptake and a more efficient intracellular release of anticancer drugs. These intelligent blank nanogels have little cytotoxicity and promote a fast and maximum drug release inside the cancer cells in which the drug release is being activated during the whole extracellular/intracellular trafficking process. These charge-conversional and dual-responsive degradable nanogel systems provide a promising platform for a targeted and controlled intracellular release of potent chemotherapeutics.

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Fig. 6. Cytotoxicity of blank (A) and DOX-loaded (B) PVA nanogels using MCF-7 cells. The cells were incubated with nanogels for 48 h. The data are presented as the average ± standard deviation (n = 4).

Fig. 7. (A) Cytotoxicity profiles determined online with an xCelligence RTCA device. Cell proliferation is given as cell index recorded over time. The nanogel samples or free DOX were added into the MCF-7 cells after 24 h cell seeding. DOX dosage was 5 μg/mL. (B) Tumor cell killing activity of DOX-loaded nanogels in MCF-7 cells using MTT assays. The cells were cultured with DOX-loaded nanogels at pH 6.8 or 7.4. DOX dosage was 10 μg/mL. After 4 h incubation, the medium was replaced by 100 μL of fresh culture medium, and the cells were cultured for another 48 h. Cells without treatment were used as a blank control. Data are presented as the average ± standard deviation (n = 4, Student’s t-test, **p < 0.01, ***p < 0.001).
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Appendix A. Supplementary data

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References
