Drug Delivery

DOI: 10.1002/anie.200605254

Targeted Charge-Reversal Nanoparticles for Nuclear Drug Delivery**

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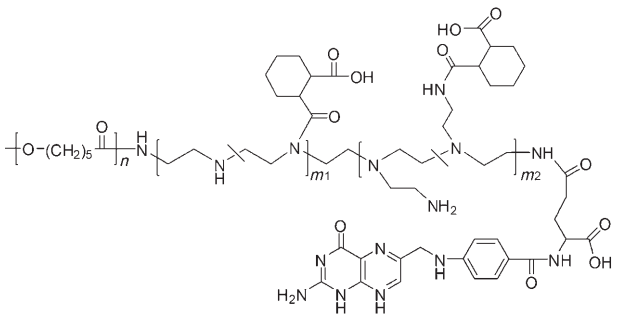
Most cancer chemotherapy drugs, such as anthracyclines and cisplatin, target nuclear DNA to cause DNA damage and/or topoisomerase inhibition to induce cell death (apoptosis).[1,2] In addition to the overexpressed multidrug-resistance mechanism in the cell membrane,[3,4] drug-resistant cancer cells have many intracellular drug-resistance mechanisms to limit the access of cytosolic drugs to the nucleus.[5,6] Consequently, only a small percentage of drugs delivered into the cytosol finally reach the nucleus. For example, less than 1% of the cisplatin molecules that enter the cell actually bind the nuclear DNA.[2] Thus, a drug carrier capable of localizing and directly releasing drugs into the nucleus would circumvent the multidrug-resistance and intracellular drug-resistance mechanisms to effectively deliver drugs to the vicinity of DNA, leading to a high therapeutic efficacy.

Polymer nanoparticles[7,8] can carry drugs preferentially to cancerous tissues by means of the enhanced permeation and retention (EPR) effect[9,10] and bypass the multidrug resistance in the cell membrane,[11] but the nanoparticles developed to date were found retained in cytoplasmic organelles including lysosomes rather than the nucleus.[12] Nuclear localization peptides (NLPs)—short highly positively charged peptides that actively transport large proteins across the nuclear membrane—have been used to localize drug molecules from the cytosol to the nucleus.[13,14] A cationic polymer, polyethyleneimine (PEI), has been used extensively in nonviral gene delivery. It can carry DNA across the cell membrane, harness the molecular motors to actively move along the microtubule network, and finally enter the nucleus.[15–17] NLPs and PEI, however, are highly positively charged at physiological pH. Positively charged polymers or colloidal particles can cause severe serum inhibition and are rapidly cleared from the plasma compartment,[18,19] and thus cannot be used in vivo.

An ideal regime would be to activate the cationic charges only in cancerous tissues or their intracellular compartments. Herein, we report nanoparticles with a negative-to-positive charge-reversal PEI outer layer triggered by the solid tumor extracellular acidity ($pH < 7$,[10,20]) or lysosomal (pH 4–5,[21]) for nuclear drug delivery. Negatively charged polymers have little interaction with the blood components and have been used extensively in vivo.[22,23]

Amides with neighboring carboxylic acid groups exhibit pH-dependent hydrolysis.[24] The hydrolysis of model amides of primary and secondary amines made from *cis*-1,2-cyclohexanedicarboxylic anhydride was tested at different pH values (Figure S1 in the Supporting Information). The amide of the secondary amine almost instantly hydrolyzed at pH 5, slightly slower at pH 6, but only 50% even after 60 h at pH 7.4. The amide of the primary amine hydrolyzed more slowly at pH 5 and 6 than that of the secondary amine amide, and did not hydrolyze at pH 7.4. Thus, we used this type of amides to preserve the primary and secondary amines of PEI: At neutral pH, the amides are stable and negatively charged because of the β-carboxylic acid groups, while at a low pH, the amides hydrolyze to regenerate the amine groups to carry cationic charges.

To demonstrate this concept, a model polymer, polycaprolactone (M_n=3800)-*block*-PEI (M_n=1800) (PCL-PEI) was synthesized (Scheme S1 in the Supporting Information). Its PEI block reacted with 1,2-cyclohexanedicarboxylic anhydride to convert the primary and secondary amines into their amides (PCL-PEI/amide). The degree of amidization was optimized. The PEI block with 20% of its primary and secondary amines converted into their amides was found optimal in terms of the charge-reversal kinetics of the resulting nanoparticles (Scheme 1). Folic acid (FA) moieties



Scheme 1. The structure of folic acid functionalized poly(ε-caprolactone)-*block*-PEI with the amines converted into their amides (PCL-b-PEI/amide-FA).

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[**] We thank the National Science Foundation (BES-0401982) and the American Cancer Society (RSG-06-118-01-CDD) for financial support.

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were also conjugated to the PEI block to form PCL-b-PEI/amide-FA for folate-receptor targeting.[25] It was estimated from the NMR spectra that there were 0.79 molecules of folic acid per PCL-PEI chain on average. The PCL-PEI/amide-FA formed nanoparticles of about 210 nm in diameter in water. The nanoparticles were about 120 nm in diameter if loaded with 14.6 wt% doxorubicin (DOX). Transmission electron microscopy (TEM) showed that these nanoparticles were spherical (Figure S2 in the Supporting Information).

The hydrolysis kinetics of the amides in the PCL-PEI/amide was determined by dispersing the nanoparticles in solution at pH 7.4, 6.0, or 5.0. The amides hydrolyzed to an extent of about 70% at pH 5.0 and 40% at pH 6.0 in 2 h. At pH 7.4, only about 25% of the amides hydrolyzed even after 24 h. The amides were hydrolyzed to more than 75% and 50% at pH values of 5.0 and 6.0, respectively, after 24 h (Figure S3 in the Supporting Information).

Accordingly, the charge reversal of the PCL-PEI/amide micelles was determined by measuring their ζ potentials at different acidities (Figure 1). The micelles of PCL-PEI/amide

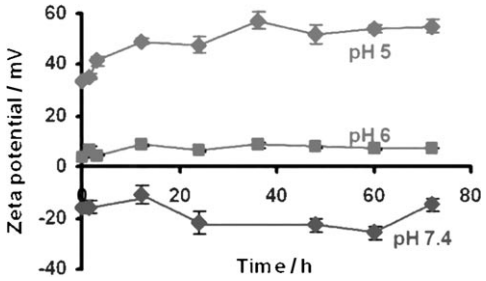
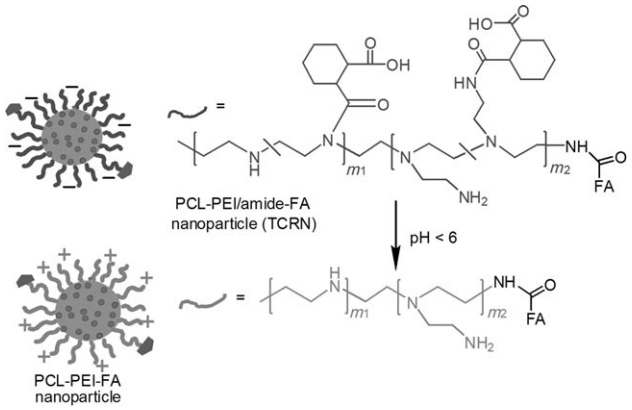


Figure 1. The ζ potential of the PCL-PEI/amide nanoparticles at 37°C as a function of time at different pH values.

revealed a ζ potential of about -20 mV at pH 7.4 even after more than 60 h, indicating that they were always negatively charged as a result of the presence of COOH groups. At pH 5, they immediately became highly positively charged and gradually reached a ζ potential of about $+50$ mV in about 10 h. At pH 6, the ζ potential was about $+8$ mV. For comparison, the micelles of PCL-PEI were always positively charged. Their ζ potential was $+36.1$ mV at pH 5, $+18.4$ mV at pH 6, and $+17.5$ mV at pH 7.4. Thus, the PCL-PEI/amide micelles were indeed charge-reversal nanoparticles: they were negatively charged at physiological pH and thus are suitable for in vivo applications. Once localized in solid tumors or lysosomes, the PEI/amides hydrolyze and recover the PEI and the micelles become positively charged. With the folic acid targeting groups, the micelles are named targeted charge-reversal nanoparticles (TCRNs; Scheme 2).

The cellular internalization of TCRNs loaded with DOX (TCRNs/DOX) was measured using flow cytometry (Figure 2). The percentage of DOX-positive cells cultured with TCRNs/DOX was significantly higher than that cultured with free DOX under the same conditions at pH 7.4. This is a significant improvement compared with reported results in which the cellular uptake of DOX in drug carriers was generally slower than that of free DOX; free DOX enters cell by a rapid diffusion process, while drug carriers enter cells by



Scheme 2. The structure of the targeted charge-reversal nanoparticle (TCRN) and its pH-triggered charge reversal.

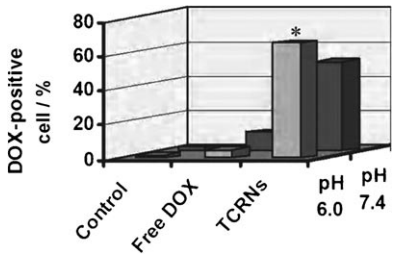


Figure 2. SKOV-3 cellular uptake of TCRNs/DOX at different pH values. DOX dose: 1 $\mu g\,mL^{-1}$; 1 h incubation at 37°C. Results are presented as the mean of three experiments. Statistical significance * $P < 0.05$ with respect to all others.

the slower endocytosis process.[26] Figure 2 also shows that TCRNs/DOX entered cells faster at pH 6 than at pH 7.4. This observation agrees with the result in Figure 1, which shows that some positive charges were regenerated on the TCRNs at pH 6. Positive charges promote the cellular internalization through electrostatically adsorptive endocytosis.[27,28]

The effectiveness of the targeting group folic acid on the TCRNs in binding folate receptors and promoting the cellular uptake was evaluated using SKOV-3 ovarian cancer cells, which are known to overexpress folate receptors.[25] TCRNs were internalized much faster into the cancer cells than the charge-reversal nanoparticles without the folic acid moieties (CRNs) (Figure 3). This indicates that TCRNs indeed effectively target the folate-receptor-overexpressing cancer cells.

After internalized, the TCRNs must localize in lysosomes to regenerate the PEI layer. The intracellular trafficking of the nanoparticles was analyzed using confocal scanning laser fluorescent microscopy (Figure 4). Clearly, most internalized TCRNs were localized in lysosomes (yellow spots in Figure 4D). Some TCRNs were not associated with lysosomes (red spots in Figure 4D), suggesting that these TCRNs might have already escaped from lysosomes within 2 h incubation. The ability of the TCRNs to escape from lysosomes was evaluated by a hemolysis assay. Hemolysis of red blood cells (RBCs) has been used as a measure of the ability of a drug carrier to rupture lysosomes.[29] The hemolysis of TCRNs was evaluated at pH 6 rather than at the lysosomal pH value (pH 4–5) because at this low pH value a significant fraction of

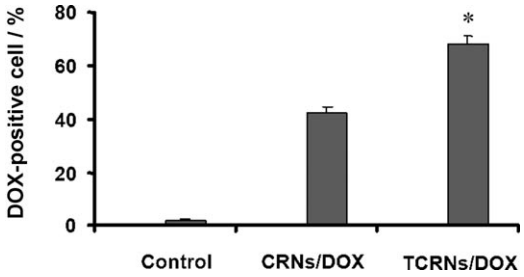


Figure 3. SKOV-3 cellular uptake of DOX-loaded TCRNs and charge-reversal nanoparticles (CRNs) made from PCL-PEI/amide as shown in Scheme 2 but without the folic acid moieties. DOX dose: 0.5 μg mL^{-1}; pH 7.4; 2 h incubation at 37 °C. Results are presented as the mean of three experiments with standard deviations. * $P < 0.05$ with respect to CRNs/DOX.

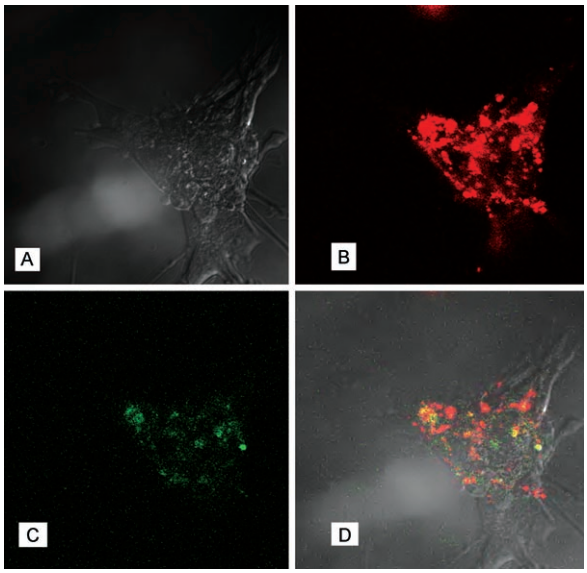


Figure 4. Subcellular localization of TCRNs/DOX observed by confocal scanning laser fluorescent microscopy. A) Transmittance channel; B) DOX channel; C) Lysotracker channel; D) overlap of three channels. Original magnification: 63×; 2 h incubation at 37 °C with TCRNs/DOX at DOX-equivalent dose of 1 μg mL^{-1}.

RBCs lysed. Figure 5 shows that at pH 6 TCRNs lysed RBCs even at very low concentrations. This is in agreement with the results shown in Figure 1. The hydrolysis of PEI/amide at pH 6 produced amine groups carrying positive charges, which caused TCRNs to adsorb on the RBCs and rupture them. One thus can expect that TCRNs would more efficiently rupture lysosomes, where the pH value is 4–5 and TCRNs quickly become fully positively charged. This explains why some of TCRNs were not associated with lysosomes (Figure 4D).

Figure 5 also shows that at neutral pH essentially no RBC hemolysis occurred at TCRN concentrations less than 10 μg mL^{-1}, indicating that TCRNs had little interaction with RBCs. Thus, the nanoparticles are suitable for in vivo applications.[30]

The nuclear localization of TCRNs was monitored by observing the SKOV-3 cells cultured with TCRNs loaded with DOX or PKH26 dye using confocal microscopy. After 8 h incubation with SKOV-3 cells, TCRNs/DOX were found very close to or even associated with the nuclear membrane (Figure S4 in the Supporting Information). To further probe the nuclear localization of TCRNs at longer times, the nanoparticles were loaded with PKH26 (TCRNs/PKH26) instead of DOX because DOX released from the TCRNs could enter the nucleus and might produce misleading results. In addition, the cells that had DOX in their nuclei died very quickly. PKH26 is a cell-membrane dye, that is, it preferentially binds the cell membrane. Thus, it can only be delivered to the nucleus by the TCRNs. It shows no apparent toxic effect to cells. At 12 h, TCRNs/PKH26 localized in some nuclei (arrows in Figure 6A) but mostly associated with

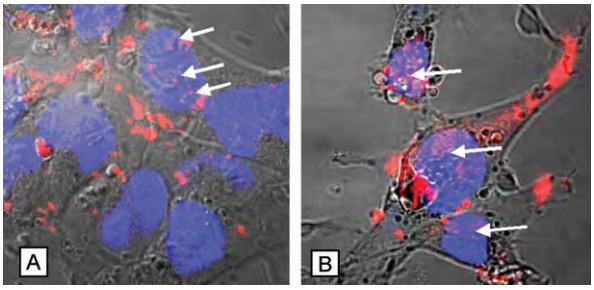


Figure 6. Nuclear localization of TCRNs/PKH26 observed by confocal scanning laser microscopy after being cultured with SKOV-3 cells for 12 h (A) or 24 h (B) at 37 °C. The nuclei were stained with DRAQ5 (blue). PKH26 were assigned to red. Pink spots shown by the arrows correspond to TCRNs/PKH26 colocalized in the nuclei. Original magnification: 63×; the nuclear localizing efficiency was estimated by counting the cells with nuclear TCRNs/PKH26, of which 44% contained the TCRNs in their nuclei.

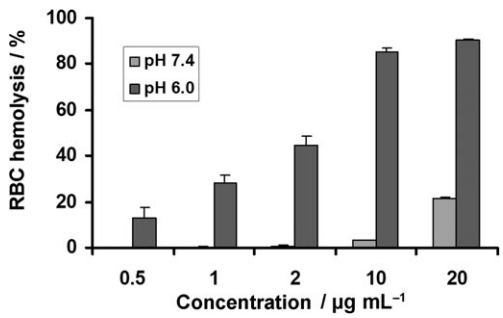


Figure 5. The hemolytic activity of TCRNs on RBCs at pH 6 and 7.4 as a function of TCRN concentration (1 h incubation at 37 °C).

nuclear membranes. Figure 6B shows that after 24 h incubation with the SKOV-3 cells, many TCRNs/PKH26 appeared in the nuclei. z-Serial images of the cell further confirmed the nuclear localization of the TCRNs/PKH26 (Figure S5 in the Supporting Information). This result proves that in contrast to nanoparticles with a PEG corona, which are retained in the lysosomes and other subcellular compartments,[12] TCRNs could indeed enter the nuclei of the cancer cells.

The in vitro cytotoxicity of DOX encapsulated in TCRNs (TCRNs/DOX) was evaluated by measuring the IC_{50} using the MTT assay (Figure 7; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). TCRNs alone showed no detectable cytotoxicity even at high doses. The IC_{50} of free

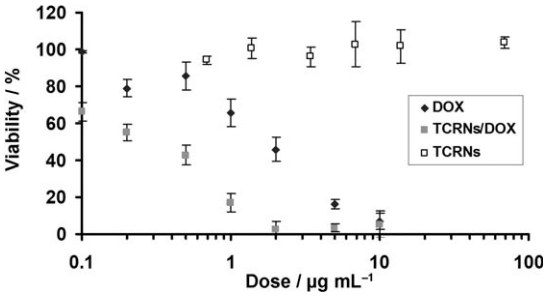


Figure 7. The cytotoxicity of DOX, TCRNs/DOX, and blank TCRNs to SKOV-3 ovarian cancer cells as a function of the DOX or TCRN dose. The results are presented as the mean of four experiments with standard deviations.

DOX was about 1.5 μg mL^{-1}, and it decreased to 0.23 μg mL^{-1} when it was encapsulated in TCRNs (Figure 7). This is different from most other reported nanoparticles as DOX carriers, in which DOX in the nanoparticles showed a lower cytotoxicity than free DOX.[31,32] This comparison suggests that the TCRNs could efficiently cross the cell membrane, escape from the lysosomes, and localize and deliver DOX in the nucleus to result in a greater cytotoxicity.

In summary, we have demonstrated a negative-to-positive charge-reversal technique for preserving primary and secondary amines for in vivo nuclear drug delivery. TCRNs composed of folic acid functionalized PCL-PEI/amide are negatively charged in neutral solution but quickly become positively charged at pH 6 and highly positively charged at pH 5. The hydrolysis kinetics indicate that amides with β-carboxylic acids can hydrolyze in acidic conditions to regenerate the amines, giving rise to a negative-to-positive charge reversal. These recovered amines carry positive charges, which can effectively enhance the cellular uptake of the nanoparticles, and thereafter direct the TCRNs to localize in the nucleus. In vitro experiment shows that TCRNs/DOX are more effective in killing SKOV-3 cancer cells than free doxorubicin is.

Experimental Section

Fabrication of nanoparticles loaded with DOX (TCRNs/DOX): Doxorubicin (DOX) hydrochloride salt (0.5 mg) was dissolved in DMSO (2 mL) and stirred for 10 min. Triethylamine (10 μL) was added and stirred for another 1 h. PCL-PEI/amide-FA (2.5 mg) was dissolved in DMSO (5 mL). The two DMSO solutions were mixed together and stirred for 1 h. The mixture was loaded into a dialysis bag (Spectra Por-7, MWCO 3500) and dialyzed with 2 L phosphate-buffered saline (PBS) solution. The free DOX was removed by filtering the solution through a 0.45 μm filter. The DOX loading was analyzed by measuring the UV absorbance at 486 nm in DMSO/$CHCl_3$. The encapsulation efficiency was 88%, and the loading content was 14.6%. TCRNs without DOX were fabricated similarly except without adding DOX.

Received: December 30, 2006
Revised: March 9, 2007
Published online: May 25, 2007

Keywords: amides · block copolymers · drug delivery · hydrolysis · nanostructures

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