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Thermo/redox/pH- Triple Sensitive Poly(N-isopropylacrylamide-co-acrylic acid) Nanogels for Anticancer Drug Delivery

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The clinical application of doxorubicin (DOX), like other anticancer drugs, is limited by insufficient cellular uptake and the numerous drug resistance mechanisms existent in cells. The development of smart nanomaterials able of carrying the drugs into the cells and of releasing them under the control of the microenvironment is an interesting approach that may increase the success of the anticancer drugs currently in use. Herein, we report an easy process to prepare biocompatible nanogels (NGs) with thermo/redox/pH- triple sensitivity which are highly effective in the intracellular delivery of DOX. Redox-sensitive/degradable NGs (PNAO-BAC) and nondegradable NGs (PNAO-MBA) were prepared through in situ polymerization of N-isopropylacrylamide (NIPAM) and acrylic acid (AA) in the presence of sodium dodecyl sulfate (SDS) as a surfactant, using N,N'-bis(acryloyl)cystamine (BAC) as a biodegradable crosslinker or N,N'-methylene bisacrylamide (MBA) as a nondegradable crosslinker, respectively. After that, the cationic DOX drug was loaded into the NGs through electrostatic interactions, by simply mixing them in aqueous solution. Compared to nondegradable PNA-MBA NGs, PNA-BAC NGs not only presented a higher DOX drug loading capacity, but also allowed a more sustainable drug release behavior under physiological conditions. More importantly, PNA-BAC NGs displayed thermo-induced drug release properties and an in vitro accelerated release of DOX in conditions that mimic intracellular reductive conditions and acidic tumor microenvironments. The thermo/redox/pH multi-sensitive NGs can quickly be taken up by CAL-72 cells (an osteosarcoma cell line), resulting in a high DOX intracellular accumulation and an improved cytotoxicity when compared with free DOX and DOX-loaded nondegradable PNA-MBA NGs. The developed NGs can be possibly used as an effective platform for the delivery of cationic therapeutic agents for biomedical applications.
INTRODUCTION

Although doxorubicin (DOX) has been widely employed in the treatment of different types of cancer (e.g. breast cancer, ovarian cancer, multiple myeloma and AIDS-related Kaposi's sarcoma), its use has been associated with an ineffective drug influx, and the activation of several biological mechanisms (like increased drug efflux, DNA repair, alterability in drug metabolism, detoxification, etc.) that cause drug resistance. Therefore, to maintain the needed DOX concentration level, a large dosage or an increased number of injections are often used, which may cause serious side effects on normal tissues, especially the heart and the kidneys.

Recently, various kinds of nanocarriers with intelligent responsiveness to internal or external stimuli (such as changes in pH, redox conditions, temperature, electric/magnetic field, light, etc.) have been developed for the delivery of various therapeutic agents. Among them, nanogels (NGs) which are physically or chemically crosslinked three-dimensional hydrophilic polymer networks at the nanoscale or submicron-size scale are receiving more and more attention, due to their unique features. Compared to other rigid nanoparticles (NPs), the flexibility and softness of NGs endow them with a faster responsiveness upon different stimuli, and easier penetration ability into tissues (e.g., human skin). They are also reported to present prolonged circulating lifetime (their flexibility reduces the possibility of their entrapment by macrophages), efficient cell uptake capability, as well as an improved bioavailability and biocompatibility in vivo. These advantages make them promising systems to deliver various therapeutic agents through proper physical encapsulation or chemical conjugation.

Among the stimuli sensitive drug vehicles, thermosensitive nanocarriers have been employed for the hyperthermia therapy of tumors where a local increase of temperature in the tumor area can trigger drug release, resulting in the selective death of tumor cells while the normal tissues are not affected. Poly(N-isopropylacrylamide) (PNIPAM), the most known temperature-sensitive
polymer, has a lower critical solution temperature (LCST) which is about 32 °C in water. The increase of temperature above its LCST induces a disruption of water-polymer hydrogen bonding and the formation of intra- and interchain hydrogen bonding, thus inducing a change in the macromolecular conformation from a hydrophilic random coil state to a desolvated globular state. However, the LCST of PNIPAM is far lower than biological temperature, limiting its potential applications in biomedical field. The introduction of a second hydrophilic monomer, such as acrylic acid (AA), into PNIPAM via free radical polymerization using \(N,N'\)-methylene bisacrylamide (MBA) as crosslinker has been used to develop PNIPAM-AA (PNA-MBA) NGs with adjustable LCST near or above body temperature. This procedure confers PNIPAM NGs more advantages, such as an easier drug loading, and drug release controllability. These NGs can also be functionalized with tumor targeting moieties. However, the nondegradability of the PNA-MBA NGs may not be beneficial for their complete elimination by renal clearance, thus leading to permanent accumulation in normal organs like heart or liver and subsequent adverse effects in the body.

It is known that intracellular sites (cytoplasm, mitochondria, or nucleus) present a high concentration of reducing compounds containing sulfhydryl groups (-SH), which are able to reduce disulfide bonds by serving as electron donors. The development of disulfide bond containing nanocarriers that are easily degraded in the reducing intracellular environment while remaining stable in the extracellular space (with lower concentration of reducing compounds) is an interesting approach to enhance the drug delivery efficiency.

In the present study, we developed a new type of PNIPAM-based NGs with redox sensitivity that can trigger the intracellular release of DOX, by following an organic-free synthetic approach. The process involved two steps: first, \(N,N'\)-bis(acryloyl)cystamine (BAC, a reducible crosslinker) was introduced into a solution of NIPAM and AA monomers in the presence of sodium dodecyl
sulfate (SDS) surfactant. The obtained mixture was *in situ* polymerized into disulfide-containing NGs (PNA-BAC). The drug delivery properties were studied by encapsulating the DOX drug into the NGs (PNA-BAC-DOX) via electrostatic interactions between the anionic NGs and the cationic DOX. The results indicated that the PNA-BAC-DOX NGs had an improved DOX encapsulation efficiency and a good ability to release DOX in a sustained manner under physiological conditions. Importantly, PNA-BAC-DOX NGs also presented sensitivity to temperature and pH beyond that to a reducing environment (triple sensitivity). The developed PNA-BAC-DOX NGs enhanced DOX internalization in CAL-72 cells (an osteosarcoma cell line) and presented an higher anticancer activity (cytotoxicity) as compared to free DOX and the corresponding nondegradable DOX-loaded NGs (prepared using \(N,N'\)-methylene bisacrylamide (MBA) instead of BAC). As far as we know, we report here for the first time the preparation and *in vitro* antitumor activity evaluation of thermo, pH and redox –sensitivity of PNIPAM-AA NGs for anticancer drug delivery. This study can provide new enlightenment for development of an effective and safe alternative nanoplatform for therapeutic delivery.

**EXPERIMENTAL SECTION**

**Cells and Materials.** N-isopropylacrylamide (NIPAM) was purchased from Tokyo Chemical Industry Co., Ltd.. \(N,N'\)-methylenbisacrylamide (BIS) was bought from Tianjin Kemiou Chemical Reagent Co., Ltd.. \(N,N'\)-bis(acryloyl)cystamine (BAC) was bought from Sigma. Dithiothreitol (DTT), acrylic acid (AA), potassium persulfate (KPS) and sodium dodecyl sulfate (SDS) were obtained from Sinopharm Chemical Reagent Co., Ltd.. Doxorubicin hydrochloride (DOX) was purchased from Zibo Ocean International Trade Co., Ltd.. NIPAM was recrystallized from \(n\)-hexane, and AA was previously distilled under reduced pressure in nitrogen atmosphere. KPS was recrystallized under room temperature. Formaldehyde was obtained from Lab-scan Analytical Sciences. All the other reagents were purchased from Sigma, unless otherwise stated. CAL-72 cells were purchased from DSMZ, Germany.
Preparation, Characterization and Drug Loading of PNA-BAC-DOX NGs.

NIPAM (1.62 mmol) was dissolved in a BAC aqueous solution (10.4 g, 3.85 mM). Then, the above solution was mixed with an aqueous solution of AA (1.01 g, 177 mM) and an aqueous solution of SDS (surfactant, 1.06 g, 17.36 mM). Finally, additional water was added to ensure that the total quantity of solution was 20 g. This mixture was then degassed by nitrogen purge for 1 h at 70 °C, followed by the addition of KPS aqueous solution (5 g, 10 mM). The polymerization was carried out at 70 °C for at least 7 h under nitrogen atmosphere. The obtained solution was purified through dialysis against distilled water at room temperature using a dialysis bag (MWCO: 8,000-12,000 Da, Biosharp) for 3 days (500 mL x 9 times), followed by lyophilization to get PNA-BAC NGs. The nondegradable NGs (PNA-MBA) were prepared using a similar procedure but using MBA, instead of BAC, as a crosslinker.

The drug was loaded into the NGs by dropping 2 mg of DOX in 1 mL water into 5 mL of ultrapure water containing 50 mg of NGs. The mixture was kept under magnetic stirring, overnight, and subjected to dialysis (MWCO: 8000-12,000 Da) to remove the free drug. The media removed from the dialysis membrane were collected for spectrophotometrical analysis at 490 nm using an ultraviolet-visible (UV-Vis) spectrometer (Lambda 35 CA, Perkin Elmer) for indirect determination of the DOX encapsulation efficiency. The purified samples were lyophilized to obtain the PNA-MBA-DOX and PNA-BAC-DOX NGs, which were kept at 4 °C for further study. All the drug loading process was performed under dark.

The particle size and the zeta potential of the Dox-loaded and Dox-free NGs were measured using a Zetasizer (Nano ZS, Malvern Instruments). The NGs were dispersed in PBS or water (0.5 mg/mL) and sonicated for 15 minutes before measurements.

The morphology of the NGs was examined by transmission electron microscope (TEM) (FEI Tecnai G20, USA) with an accelerating voltage of 200 kV. Before measurement, the samples were dispersed in ultrapure water (0.5 mg/ml) in the absence or presence of 4 mM dithiothreitol (DTT) (for redox degradability study) under sonication for 10 min, and then put in 37 °C water bath. At different time
intervals, a slight amount of the NGs dispersion was taken out for TEM observation. The aqueous suspensions of the samples were dropped onto a 400 mesh copper grid, followed by air-drying before analysis.

**In vitro drug release studies.** For the drug release experiments, 2 mg of PNA-MBA-DOX or PNA-BAC-DOX NGs was dispersed in 1 mL of PBS and introduced in a dialysis membrane (MWCO: 8000-12,000 Da). Dialysis was then performed against 5 mL PBS solution, under different pH values (7.4, 6.5 or 5.0), at a temperature of 25, 37 or 42 ºC, and in the absence or presence of 4 mM DTT. At different time intervals, an aliquot of the PBS solution was taken out for spectrophotometrical analysis at 490 nm and refreshed with 0.5 mL of new PBS solution. The cumulative release \( C_r \) of DOX against time was obtained according to the equation:

\[
C_r = 100 \times \frac{Abs_t}{Abs_{tot}}
\]  

where \( Abs_t \) and \( Abs_{tot} \) are the absorbance value of the solution at time \( t \) and the absorbance value of a solution containing the total amount of drug existent inside the NGs used for drug release, respectively.

**Cell Biological Evaluation.** CAL-72 cells (an osteosarcoma cell line) were cultured in Dulbecco’s Modified Eagle Medium (D-MEM) containing 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) of an antibiotic-antimycotic 100x solution (AA, Gibco, with penicillin, streptomycin, and amphotericin B). The medium was supplemented with 1% (v/v) of L-glutamine 100x solution (Gibco) and 1% (v/v) of insulin-transferrin-selenium 100x solution (ITS, Gibco). The cells were grown at 37 ºC, in a humidified atmosphere with 5% carbon dioxide. Afterwards, the cells were harvested at 70-80% confluence, using trypsin-EDTA solution for the enzymatic detachment of the cells from the plastic substrate.

For the cytotoxicity experiments, CAL-72 cells were first plated in 48-well plates at a seeding density of 10 x 10^3 cells per well. After one day, free DOX, PNA-MBA-DOX, PNA-BAC-DOX nanogel solutions (75 µL, with equivalent DOX concentrations), prepared in PBS solution with pH value of 7.4, were added to the cell culture medium (425 µL) and cells were then incubated for 48 h, at 37 ºC, before the resazurin reduction assay. Solutions of PNA-MBA and PNA-BAC NGs in PBS pH 7.4 (containing
equivalent mass concentrations to those used in the DOX-loaded nanogel solutions) were used as controls.

Scheme 1. N-isopropylacrylamide (NIPAM), acrylic acid (AA) were in situ polymerized into redox and thermo-sensitive NGs (PNA-BAC) upon initiation of potassium persulfate (KPS) in the presence of sodium dodecyl sulfate (SDS), using N,N’-bis(acryloyl)cystamine (BAC) as a biodegradable crosslinker. The formed NGs can load cationic doxorubicin (DOX) drug by mixing it with the NGs in aqueous solution. The drug-loaded NGs (PNA-BAC-DOX) are expected to have accelerated DOX release ability upon both heating and redox stimuli.

For the cell uptake study, cells were plated for 24 h before incubation with the test solutions, to allow cell attachment. In these experiments, 75 µL of the solutions of free DOX, PNA-MBA-DOX, PNA-BAC-DOX NGs were used at the same DOX concentration (the final concentrations in the wells were 2.0). Cells were then incubated with the test solutions (425 µL) at 37 ºC for 2, 4, 6, 8 or 48 h. Subsequently, the cultures were washed with sterilized PBS buffer, stained with DAPI for 30 min, fixed with 3.7% formaldehyde for 20 min, and visualized using a fluorescence microscope (Nikon Eclipse TE 2000E).
ImageJ (Wayne Rasband), an image analysis software was used to merge and adjust the contrast of the pictures (all images were treated in the same way).

RESULTS AND DISCUSSION

Preparation and Physical Characterization of DOX-loaded NGs (PNA-BAC-DOX).

![Graph showing encapsulation efficiency vs crosslinker concentration](image)

**Figure 1.** The effect of crosslinker concentration on DOX encapsulation efficiency (EE) of PNA-MBA and PNA-BAC NGs.

PNA-BAC-DOX NGs were prepared by a radical polymerization method as shown in Scheme 1. Firstly, an aqueous solution of NIPAM and BAC was mixed with an aqueous solution of AA in the presence of SDS as surfactant. After that, the mixture was degassed under nitrogen bubbling at 70 °C, followed by the addition of KPS to initiate the polymerization process. After dialysis purification and lyophilization, PNA-BAC NGs were obtained. Then, DOX was loaded into the NGs through the establishment of electrostatic interactions between both. The special properties of PNIPAM will endow the NGs with thermo-sensitivity, and the presence of AA in PNIPAM is used to adjust the LCST of PNIPAM and for the improvement of the electrostatic interactions of the NGs with cationic drugs. The introduction of a disulfide-containing crosslinker (BAC) will confer redox sensitivity to the NGs, and the presence of a surfactant is beneficial for the preparation of nanoparticles in a monodispersed state.26 The
encapsulation efficiency (EE) was firstly investigated as a function of crosslinker concentration. As shown in Figure 1, with the increase of crosslinker concentration from 0.16 to 1.6 mM, the EE of PNA-BAC NGs continuously increased from 54 to 64%, while the EE of PNA-MBA NGs firstly increased and then decreased (48, 50 and 40% at 0.16, 0.8 and 1.6 mM, respectively). PNA-BAC NGs had higher EEs than PNA-MBA NGs for all studied concentrations. Since BAC as crosslinker has a longer and flexible molecular structure than MBA due to the existence of a disulfide bond in the former, PNA-BAC NGs possibly allow an easier internal DOX diffusion. As a too high crosslinking density may not be beneficial to drug release, the NGs at a crosslinker concentration of 1.6 mM were chosen for further study. The corresponding DOX-free and -loaded PNA-MBA and PNA-BAC NGs were designated as PNA-MBA_1.6, PNA-BAC_1.6, PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6, respectively.

Table 1. Characterization of DOX-free and loaded NGs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>25 °C</th>
<th>37 °C</th>
<th>EE, %</th>
<th>LC, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size,nm</td>
<td>Zeta, mV</td>
<td>Size,nm</td>
<td>Zeta, mV</td>
</tr>
<tr>
<td>PNA-MBA_1.6</td>
<td>237 ± 32</td>
<td>-10.7 ± 0.3</td>
<td>204 ± 1</td>
<td>-9.5 ± 0.6</td>
</tr>
<tr>
<td>PNA-MBA-DOX_1.6</td>
<td>300 ± 20</td>
<td>-7.2 ± 0.4</td>
<td>272 ± 25</td>
<td>-7.7 ± 0.9</td>
</tr>
<tr>
<td>PNA-BAC_1.6</td>
<td>300 ± 3</td>
<td>-10.4 ± 0.3</td>
<td>274 ± 3</td>
<td>-10.6 ± 0.5</td>
</tr>
<tr>
<td>PNA-BAC-DOX_1.6</td>
<td>385 ± 4</td>
<td>-9.8 ± 0.2</td>
<td>341 ± 3</td>
<td>-9.8 ± 0.1</td>
</tr>
</tbody>
</table>

\[ a \] Size and Zeta potential were measured in PBS with a pH value of 7.4 at 25 or 37 °C.

\[ b \] Encapsulation efficiency = \(100 \times \frac{W_t}{W_0}\), where \(W_0\) and \(W_t\) are the total DOX weight used for encapsulation and the weight of encapsulated DOX, respectively.

\[ c \] Loading capacity = \(100 \times \frac{W_t}{W}\), where \(W_t\) and \(W\) are the weight of encapsulated DOX and the weight of DOX-loaded nanogels, respectively.

Dynamic light scattering (DLS) was used to analyze the hydrodynamic size of the formed NGs in PBS buffer (Table 1). PNA-BAC_1.6 NGs had a larger size (274 ± 3 nm) than that of PNA-MBA_1.6.
NGs (204 ± 1 nm) under physiological condition, which may be one reason why the former had higher therapeutic loading capacity than the latter. Furthermore, all the NGs had a ζ-potential of around -10 mV, indicating that the drug loading exerted a negligible effect on the surface charge of the NGs. This negative-charged surface (although moderate) may be beneficial for the improvement of the circulation lifetime of the NGs in the blood, by reducing the possibility of interaction with the negatively charged proteins in plasma. Both PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6 NGs had sizes larger than the drug-free ones, again suggesting the successful loading of the DOX drug which resulted in a more swollen state in PBS solution. PNA-BAC-DOX_1.6 had higher drug encapsulation efficiency and loading capacity than PNA-MBA-DOX_1.6 (as shown in Table 1).

Figure 2. Transmission electron microscope (TEM) images of the PNA-BAC_1.6 NGs in ultrapure water in the absence (a, b) and presence (c, d) of 4.0 mM DTT for a period of 0.5 h (a, c) and 10 h (b, d).
TEM was further employed for investigation of the morphology of PNA-BAC_1.6 NGs. As shown in Figure 2, PNA-BAC_1.6 NGs are present as monodispersed nanoparticles, with smaller sizes (about 100-150 nm) than those measured by DLS (Table 1). Since TEM micrographs reflect the dry state of materials, the larger DLS sizes of the PNA-BAC_1.6 NGs may be ascribed to a higher swollen state in PBS solution. To check if the PNA-BAC NGs are redox sensitive, their sizes were also investigated via treatment by DTT. The results indicated that the treatment of the NGs for 0.5 h in the presence of 4.0 mM DTT resulted in an obvious increase of the size to 200 nm (Figure 2c). When the treatment time increased to 10 h, the PNA-BAC_1.6 samples in the presence of 4.0 mM DTT lost their original 3D shape (Figure 2d), suggesting the disintegration of the NGs through cleavage under reducing conditions.

Differential scanning calorimetry (DSC) was employed to study the thermosensitivity of the NGs in solid state. As shown in Figure 3a, PNA-MBA_1.6 had a lower critical solution temperature (LCST) at 36.1 °C, similar to the previous report.\textsuperscript{28,29} Compared to PNA-MBA_1.6, PNA-BAC_1.6 indicated a LSCST at 35.5 °C with a broader shape, probably associated with their bigger size. The hydrodynamic diameter of the NGs as a function of temperature was further studied to evaluate their thermosensitivity in aqueous solution (Figure 3b). Since DOX is a hydrophilic molecule, its loading into the NGs (by electrostatic interactions with the carboxylic groups in the NGs) make the size of the loaded NGs higher than the non-loaded ones for all the temperature range. On the other hand, it is clear that the size of the PNA-BAC_1.6 NGs gradually decreased with a temperature increase from 25 to 45 °C. This size decrease is probably due to the increase of the hydrophobicity of PNIPAM induced by its thermosensitivity, which results in the loss of bounded water and shrinkage of the NGs. However, this behavior is different when compared to the PNIPAM itself that suffers an abrupt size decrease around 32 °C which corresponds to its LCST.\textsuperscript{19} For the drug delivery approach, this result is interesting due to the fact that offers a sustainable release instead of a sudden one. As such, by increasing the temperature until
or near above the physiological temperature, the NGs will slowly shrink, lose water and probably release
the drug if they are loaded.

**Figure 3.** a) DSC curves of PNA-MBA_1.6 and PNA-BAC_1.6 samples; b) Hydrodynamic size of the
PNA-BAC_1.6 and PNA-BAC-DOX_1.6 NGs in UP water as a function of temperature.

*In vitro drug release of DOX-loaded NGs.* An ideal drug delivery platform should release the
encapsulated drug in a controllable and sustainable way to exert long time effects. Under physiological
conditions (pH 7.4), the DOX drug was released from both PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6 NGs in a more sustainable mode as compared to free DOX drug (Figure 4a). Interestingly and
in accordance with the previous results, the increase of temperature from 25 to 42 °C obviously
accelerated the drug release process (Figure 4b). Indeed, as explained before, PNIPAM tends to shrink
due to the increase in its hydrophobicity at high temperature,\(^{30}\) which probably promotes the diffusion of
the hydrophilic doxorubicin hydrochloride from the PNA-BAC-DOX_1.6 NGs, thereby accelerating the
release process. This thermo-accelerated drug release behavior of the NGs can be useful for thermo-
guided antitumor treatment.\(^{31}\)

It is reported that some tumor tissues and intracellular sites present a reducible microenvironment due
to the existence of a high concentration of reducing compounds containing sulphydryl groups (-SH).\(^{24,32}\)
Therefore, the release of DOX was also investigated under a non-reducing and an intracellular-mimicking reducing environment (4 mM DTT in PBS buffer at pH 7.4). As it can be seen from Figure 4c, the cumulative DOX release after 9 h (81 ± 13%) for PNA-BAC-DOX_1.6 was boosted in the presence of 4 mM DTT, while the samples under normal conditions led to a lower extension of drug release (63 ± 2%). As comparison, there is no significant difference for the PNA-MBA-DOX_1.6 system under similar conditions. This redox responsiveness revealed by the PNA-BAC-DOX_1.6 NGs is very important since it can conduct to higher values of drug release after cellular internalization. In fact, it is expected that the high intracellular concentrations of compounds containing –SH groups, such as glutathione (the concentration of glutathione is about 1000 times higher inside cells than in the extracellular environment), will result in the cleavage of the disulfide crosslink bonds present in the nanogel (Figure 2c and d).

Considering the quite acidic environment that can exist in solid tumor sites (pH 6.5) and endo/lysosomal compartments (pH 5.0-6.0), we also compared the release behavior of DOX under both physiological (pH 7.4) and acidic (pH 6.5 and 5.0) conditions. The DOX release efficiency was accelerated under acid condition when compared with the physiological pH, revealing that the NGs were pH sensitive (Figure 4e). For instance, DOX cumulative release at 10 h was 63 ± 2%, 72 ± 3%, and 81 ± 7% at pH 7.4, 6.5 and 5.0, respectively. This pH sensitivity in DOX release means that even if DOX release is limited under physiological conditions (less toxicity to normal tissues), the NGs will release more of their drug cargo in the solid tumor extracellular environment (pH 6.5), and in the endo-lysosomal vesicles (pH 5.0), resulting in enhanced anticancer activity. The redox-induced degradability and the thermo- and pH-mediated drug release properties will synergistically improve the delivery efficiency of DOX from the NGs and help overcoming the DOX resistance commonly found in cancer cells.
Figure 4. \textit{In vitro} DOX cumulative release in PBS buffer from a) free DOX, PNA-MBA-DOX \_1.6 and PNA-BAC-DOX \_1.6 NGs at 37 °C, b) PNA-BAC-DOX \_1.6 NGs at different temperatures (25, 37 and 42 °C), c) PNA-BAC-DOX \_1.6 NGs and d) PNA-MBA-DOX \_1.6 in the presence and absence of DTT (4.0 mM) at 37 °C, e) PNA-BAC-DOX \_1.6 at different pH values (7.4, 6.5 and 5.0).
**Figure 5.** CAL-72 cell viability exposed to free DOX, PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6 NGs (with equivalent DOX concentration), and PNA-MBA_1.6 and PNA-BAC_1.6 (with equivalent weight concentration of the corresponding PNA-BAC-DOX_1.6 NGs) after 48 h of cell culture (± standard deviation, n = 3).

*In vitro cytotoxicity and cellular internalization of DOX-loaded NGs.* In order to check if the PNA-BAC-DOX_1.6 NGs could improve the therapeutic efficacy of the DOX drug, the cytotoxicity of DOX-loaded NGs was quantitatively evaluated using CAL-72 cells (an osteosarcoma cell line). As it can be seen in Figure 5, both PNA-MBA-DOX_1.6 (IC50: 0.75 µM) and PNA-BAC-DOX_1.6 NGs (IC50: 0.50 µM) displayed an improved cytotoxicity toward CAL-72 cells, while free DOX alone had a higher IC50 value (3.0 µM), probably related with drug resistance processes. Since the blank NGs did not display any cytotoxicity, the therapeutic efficacy comes only from the effect of the loaded drug. Although PNA-MBA-DOX_1.6 NGs also exhibited an improved cytotoxicity compared to free DOX, their nondegradability features may pose some risks associated with accumulation in human organs, which is a
limiting factor for their *in vivo* application. The degradability, excellent cytocompatibility of the PNA-BAC_1.6 NGs, as well as their better sustainable drug release behavior, pH/thermo/redox-sensitivity, and high therapeutic efficacy make them more appropriate to be used as an effective platform for the intracellular delivery of DOX and, potentially, of other anticancer drugs.

**Figure 6.** Bright field and fluorescence microscopy images of CAL-72 cells after 4 h culture. Results are presented for the control, DOX (2.0 µM), and PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6 NGs with an equivalent amount of DOX.

The cellular internalization of the nanocarriers is an important factor to achieve the required therapeutic efficacy.\(^{35-37}\) Since DOX is fluorescent, fluorescence microscopy was used to study its internalization by CAL-72 cells. As can be seen in Figure 6, 7 and 8, an obvious higher reddish
intensity was observed inside cells after they were treated from 2 to 48 h with the PNA-BAC-DOX_1.6, compared to those treated with free DOX drug and PNA-MBA-DOX_1.6 NGs. The feeble intracellular drug accumulation of free DOX may be associated with an ineffective drug influx due to protonated DOX trapping as well as increased drug efflux that results in multidrug resistance.\textsuperscript{2-6} The higher drug accumulation inside cells and the enhanced cell death activity may be attributed to the higher cell uptake of PNA-BAC-DOX_1.6 NGs, as well as to their thermo/redox/pH- triple sensitivity which facilitated the DOX release.\textsuperscript{38-40}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Merged fluorescence microscopy images (blue:stained nucleus, red:DOX) of CAL-72 cells after culture for different period (2 - 8 h). Results are presented for the control, DOX (2.0 µM), and PNA-MBA-DOX_1.6 and PNA-BAC-DOX_1.6 NGs with an equivalent amount of DOX.}
\end{figure}
**CONCLUSIONS**

In summary, we present a simple strategy to develop poly(N-isopropylacrylamide-co-acrylic acid) NGs which have good cytocompatibility, high drug encapsulation efficiency, and thermo, pH and redox-sensitivity in terms of drug release. Upon cellular internalization, the drug in the NGs can be effectively released and exert its therapeutic bioactivity. The good cytocompatibility of the NGs, as well as their intelligent drug release controllability (thermo/pH/redox- sensitivity) make them a promising alternative platform for the delivery of various cationic anticancer therapeutic agents, beyond DOX drug. In addition,
the preparation of the NGs is not involved in organic solvents, which is not only a green approach as it is also interesting from the pharmaceutical application point of view, providing new insights for the rational design of optimal platforms for the intracellular delivery of therapeutic agents.

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NOTES AND REFERENCES

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Doxorubicin is effectively loaded into disulfide-crosslinked poly(N-isopropylacrylamide-co-acrylic acid) nanogels, which can be triggerably released under heating or reducing/acidic tumor microenvironment.