pH-degradable PVA-based nanogels via photo-crosslinking of thermo-preinduced nanoaggregates for controlled drug delivery

Wei Chen *, Yong Hou, Zhaoxu Tu, Lingyan Gao, Rainer Haag *

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Takustrasse 3, Berlin 14195, Germany

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A B S T R A C T
pH-Degradable PVA nanogels, which are prepared by photo-crosslinking thermo-preinduced PVA nanoaggregates in water without any surfactants or toxic organic solvents, are used for intracellular PTX release and anticancer treatment. These nanogels fast degraded at mildly acidic conditions with a pH-triggered PTX release, and the degradation products are only native PVA and poly(hydroxyethyl acrylate) (PHEA) as well as acetaldehyde without any toxic byproducts. The nanogel sizes could be tailored by different temperatures during the crosslinking process. The results of confocal microscopy and flow cytometry revealed that smaller nanogels exhibited enhanced internalization with MCF-7 cells than the ones treated with larger nanogels, by which the smaller PTX-loaded nanogels induced a more significant cytotoxicity against MCF-7 cells.

Graphic abstract: pH-Degradable PVA nanogels can be prepared by photo-crosslinking of thermo-preinduced nanogels with tailored nanogel sizes given their pH-triggered PTX release and fast acid-degradation into native PVA and cell-compatible poly(hydroxyethyl acrylate) (PHEA) as well as acetaldehyde.

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1. Introduction

Nanomedicines have conferred many advantages for cancer diagnosis and therapy [1–4]. The preclinical and clinical studies have demonstrated that advanced drug delivery nanosystems provide prolonged circulation time, efficient tumor-targeted accumulation via the enhanced permeability and retention (EPR) effect, reduced side effects, and improved drug tolerance, that has resulted in better drug bioavailability and therapy [5–9]. To meet the pharmaceutical requirements, biocompatible nanocarriers including liposomes, polymeric nanoparticles, micelles, and nanogels have been developed for delivery of various types of drugs for cancer treatment. Compared with other nanocarriers, nanogels with internally crosslinked three-dimensional (3D) structures show high water content, desirable chemical and mechanical properties, and a large surface area for multivalent bioconjugation [10–14]. They are able to stabilize bioactive compounds such as drugs, peptides/proteins, and DNA/RNA in their polymeric networks, and moreover, nanogels actively participate in the drug delivery process due to their intrinsic properties such as stimuli-responsive behavior, swelling and softness, to achieve a controlled drug release at the target site [15–18].

Poly(vinyl alcohol) (PVA) is a synthetic polymer with OH-hydrophilicity prepared by radical polymerization of vinyl acetate and followed with partial hydrolysis. Due to its unique properties including water solubility, multiple OH-groups for further decoration, and FDA-approved biocompatibility and low toxicity, the use of PVA as a biomaterial has attracted great attention in biomedical applications such as protein/enzyme immobilization, cell encapsulation in the form of micro/hydrogel scaffolds [19–22]. However, PVA nanostructures failed to meet the demand, especially in the field of nanomedicine area, which is mostly due to their inhomogeneous interior, high porosity, low drug affinity, and uncontrollable release behavior. The modification of OH-groups on PVA is largely considered to introduce more convenient sites for conjugation and chain extension using ester, carbamate, ether and acetal linkages [23]. For example: Kupal et al. reported that core–shell PVA-based microgels shielded with hyaluronic acid (HA) were prepared by “click” chemistry and inverse emulsion techniques for targeted local delivery of doxorubicin to adenocarcinoma colon cells (HT-29) [24]. We recently developed charge-conversional reducible PVA nanogels by combining nanoprecipitation and “click” chemistry for an enhanced cellular uptake towards universal tumor cells and efficient tumorous cytotoxicity against human cancer MCF-7 cells [25].

Much effort has been made towards the development of pH-sensitive nanocarriers for intracellular drug delivery, since there are natural pH gradients in the tumor tissue microenvironment (pH 6.5–7.2) as well as the endosomal/lysosomal compartments of tumor cells (pH 4.0–6.5) [26–32]. It is noteworthy that the strategy of pH-triggered drug release has been exploited to meet the challenges of various extra- and intra-cellular barriers towards successful cancer chemotherapy for drug release nanosystems. The acid-labile acetal linkage has been
widely introduced to fabricate pH-sensitive nanostructures and networks for intracellular drug delivery, in which the acetal groups are relatively stable under physiological conditions, while rapidly hydrolyzed at a mildly acidic pH to release the encapsulated cargo [33–35]. Acetalization reactions were usually used to equip PVA chains with acrylamide groups and conjugated heterocyclic chromophores, as well as photoactive groups [36,37]. In this study, we developed pH-degradable nanogels based on acetal-linked PVA with defined shape and size for encapsulation of PTX and intracellular release (Scheme 1). These functionalized PVA materials could firstly form nanogelaggregates in water by thermo-trigger, which was followed by photo-irradiation to produce nanogels with high stability under physiological conditions. These nanogels could degrade fast at a mildly acidic pH, and more interestingly, the degradation products are only native PVA and cell-compatible poly(hydroxyethyl acrylate) (PHEA) as well as acetaldehyde without any toxic byproducts. We investigated the thermo-transition behavior of the functionalized PVA, the stability and degradation of the nanogels, together with the in vitro drug release, size-dependent cellular uptake and tumorous cytoxicity of PTX-loaded nanogels.

2. Experimental section

2.1. Materials and methods

Ethylene glycol vinyl ether (Aldrich, 97%), acryloyl chloride (Aldrich, 97%), triethylamine (Et3N, Acros, 98%), 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, Aldrich, 98%), paclitaxel (PTX, Sigma, 97%), triethylamine (Et3N, Acros, 99%), 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, Aldrich, 98%), paclitaxel (PTX, Sigma, 97%), p-toluenesulfonic acid monohydrate (PTSA, Sigma-Aldrich, 98%) were used as received. Polyvinyl alcohol (PVA, Mowiol 3-85, Mw = 15,000 g/mol) was provided by Kuraray Europe GmbH (Germany). For cell culture experiments, MCF-7 cells (DSMZ, ACC 107) were cultured in RPMI supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, and 10 μg/mL human insulin. A549 cells (DSMZ, ACC 107) were cultured in DMEM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal calf serum, and 2 mM glutamine.

1H 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 SX C that was equipped with a DTGS detector from 4000–600 cm to. 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. Transmission electron microscopy (TEM) measurement was performed on Philips EM12 and operated at 100 kV with a nanogel concentration of 1 mg/mL. The TEM samples were prepared by dropping 5 μL of nanogel suspensions on the microscopical 200 mesh grids, which were placed on liquid nitrogen. After 5 min, the samples were lyophilized for TEM measurement.

2.2. Synthesis of vinyl ether acrylate (VEA)

Ethylene glycol vinyl ether (20.00 g, 0.227 mol) and Et3N (41.6 mL, 0.30 mol) were dissolved in dichloromethane (CH2Cl2, 300 mL), followed by a drop wise addition of acryloyl chloride (22.5 mL, 0.272 mol) at 0 °C. After 8 h, the reaction solution was extracted with NaCO3 aqueous solution twice, and the organic phase was then dried over MgSO4 and concentrated by rotary evaporation. The final product was collected by distillation under reduced pressure. Yield: 27.72 g (86%). 1H NMR (400 MHz, CDCl3): δ 6.60 (1H, CH2=CH–O–), 5.85–6.45 (3H, CH2=CH–C–O–), 4.40 (2H, –CH2–O–C–O–), 4.05–4.20 (2H, CH2=CH–O–), 3.94 (2H, –CH2–CH2–O–C–O–). 13C NMR (400 MHz, CDCl3): δ 166.10 (CH=CH=O), 151.50 (CH–CH=O), 131.40 (CH=CH–C–O–), 128.12 (CH2=CH–C–O–), 87.75 (CH2=CH–O–O–). 1H NMR of final product was collected by distillation under reduced pressure. Yield: 27.72 g (86%).

2.3. Synthesis of VEA-functionalized PVA (PVA-VEA)

PVA (1.00 g, 17 mmol of OH group) was dissolved in anhydrous DMSO (100 mL), and then VEA (16, 20, and 40 mol% of OH group in PVA) and a catalytic amount of PTSA were sequentially added to the reaction to prepare PVA-VEA with different functionalities. After 6 h, the reaction was quenched by addition of Et3N. The solvent was removed by dialysis against methanol, and then the polymer solution was concentrated by rotary evaporation. Finally, the polymer was isolated by precipitation in diethyl ether, and re-dissolved in water and freeze-dried.

2.4. Preparation of pH-degradable nanogels

PVA-VEA polymer dissolved in water with a concentration of 1.0 mg/mL containing 0.05 mg/mL of I2959 photo-initiator was kept

Scheme 1. Illustration of PTX-loaded PVA nanogels via photo-crosslinking of thermo-preinduced nanoaggregates, and pH-triggered degradation of nanogels to native PVA and poly(hydroxyethyl acrylate) (PHEA) as well as acetaldehyde.

in ice-water bath, and then slowly warmed with argon perfusion. The polymer solution was stirred under UV exposure for 10 min. The nanogels were purified by dialysis in water with a molecular weight cut off (MWCO) of 2000 and collected by freeze-drying. For the preparation of PTX-loaded nanogels, PTX dissolved in ethanol (5.0 mg/mL) was added into PVA-VEA solution (1.0 mg/mL containing 0.05 mg/mL of I2959) with a feeding ratio at 10 wt% of PVA-VEA polymer, and the following steps were performed similarly to the preparation of the blank nanogel. The free drug was removed by centrifugation with a MWCO of 10,000, and PTX-loaded nanogels were collected by freeze-drying.

2.5. pH-degradation of PVA-VEA polymer and nanogels

PVA-VEA dissolved in acetic acid (pH 5.0) with a concentration of 0.5 mg/mL was stirred at room temperature. To monitor the degradation of VEA group from PVA backbone, 10 mL of polymer solution was taken out at the desired time points and freeze-dried. The degradation degree of PVA-VEA was characterized by $^1$H NMR. PVA-VEA nanogels were separately suspended in phosphate buffer (pH 7.4) and acetic acid buffer (pH 5.0) with a concentration of 1.0 mg/mL. The samples were slowly stirred at 37 °C, and the nanogel size was monitored over time by DLS.

2.6. In vitro release of PTX

The in vitro release of PTX from PTX-VEA nanogels was investigated at 37 °C in acetic buffer (pH 5.0) and PB (pH 7.4). PTX-loaded nanogels were divided into two aliquots of 1 mL, and immediately transferred to a dialysis tube with a MWCO of 12,000–14,000. The dialysis tubes were immersed into 20 mL of appropriate buffers and shaken at 37 °C. At set time intervals, 5.0 mL of the release medium was taken out from each experimental group and replenished with an equal volume of fresh appropriate medium. The release medium was freeze-dried and the amount of PTX was determined by HPLC (Knauer, Advanced Scientific Instruments), with UV detection at 227 nm using a 60/40 (v/v) mixture of acetonitrile and water as a mobile phase. Release experiments were conducted in triplicate. The results are presented as the average ± standard deviation.

2.7. Size-dependent cellular uptake of PVA nanogels

PVA nanogels with different sizes were prepared by UV-crosslinking of PVA-VEA with the functionality of 6% (PVA-VEA6%) under the thermo-trigger ranging from 18 to 30 °C. After that, FITC was linked to the nanogels using an ester condensation reaction between the isothiocyanate group (NCS) of FITC and the hydroxyl group of PVA nanogels. Free FITC was removed by centrifugation with a MWCO of 10,000. MCF-7 cells were plated on microscope slides in a 24-well plate ($1.0 \times 10^4$ cells/well) using 1640 culture medium containing 10% PBS. After 24 h incubation, the medium was replaced by 450 μL of fresh culture medium and 50 μL of prescribed amounts of FITC-labeled nanogel samples. After incubation for 3 h, the culture medium was removed and the cells were washed twice with phosphate buffered saline (PBS). The cells were fixed with 4% paraformaldehyde for 20 min, incubated with Alexa Fluor 594 (ThermoFisher, Germany) to label the cell membrane at 37 °C for 1 h, and the cell nuclei were stained with DAPI. Fluorescence images of cells were obtained with confocal laser scanning microscope (CLSM, Leica, Germany) and analyzed by Leica 2.6.0 software.

Cellular uptake of FITC-labeled PVA nanogels was also quantified by flow cytometry analysis. MCF-7 cells were cultured in a 24-well plate ($1.0 \times 10^4$ cells/well) for 24 h, and then the medium was replaced by 450 μL of fresh culture medium and 50 μL of prescribed amounts of FITC-labeled nanogel samples. After 6 h incubation, the culture medium was removed, and the cells were rinsed thrice with PBS and treated with trypsin. The cell suspensions were washed twice with PBS and re-suspended in PBS. The quantification of fluorescence was performed by a FACScalibur (BD Accuri C6).

2.8. Cytotoxicity of PTX-loaded PVA nanogels

The cytotoxicity of blank PVA nanogels was studied by the MTT assay using A549 and MCF-7 cells. Cells were seeded into a 96-well plate at a density of $1 \times 10^4$ cells per well in 90 μL of culture medium and incubated at 37 °C with 5% CO2. After 24 h, 10 μL of blank nanogel samples at different concentrations in PB (10 mM, pH 7.4) were added and the cells were incubated for another 48 h. To study the cytotoxicity of PTX-loaded PVA-VEA6% nanogels, 10 μL of prescribed amounts of PTX-loaded nanogel samples were added. The cells were incubated with PTX-loaded samples for another 48 h incubation. After that, 10 μL of MTT solution (5 mg/mL) was added and the cells were incubated for 4 h. 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. To further study the cytotoxicity of PTX-loaded nanogels induced by different sizes, MCF-7 cells were incubated with 10 μL of prescribed amounts of PTX-loaded nanogel samples for 6 h, and then the medium was replaced by fresh medium for another 48 h incubation. After that, the cell treatment procedure was carried out as above-mentioned to test the cytotoxicity value. The experiments were conducted in triplicate. The results are presented as the average ± standard deviation.

3. Results and discussion

3.1. Synthesis of PVA-VEA polymer

Vinyl ether acrylate (VEA) was synthesized by acrylation of ethylene glycol and vinyl ether with a yield over 85% (Fig. S1). It has been demonstrated that the acetalization between vinyl ether and hydroxyl or thiol is quite efficient and the formed acetal linkers are labile to mildly acidic conditions [38]. VEA-functionalized PVA polymers with the VEA functionalities of 6%, 3.5%, and 2% (denoted as PVA-VEA6%, PVA-VEA3.5%, and PVA-VEA2%, respectively) were synthesized by acetalization between vinyl ether group and hydroxyl in the presence of PTSA (Scheme 2, and Table 1). The degree of VEA functionalities was determined by $^1$H NMR according to the integral ratio of the signals at δ 5.88–6.42 and 2.01, which are attributed to the double bond of VEA and the methyl group on PVA, respectively (Fig. S2A). It is interesting to note that the VEA functional domain is highly flexible for crosslinking or modification via UV-irradiation or Michael-type addition reaction. Furthermore, the acetal linker between VEA and PVA backbone is pH-degradable, and the UV-crosslinked structure based on PVA-VEA can degrade to native PVA, PHEA, and acetaldehyde without any toxic byproduct.

3.2. Nanogel formation and stability

The PVA-VEA polymer was readily dissolved in water (1.0 mg/mL) with an average size of 6–10 nm at a low temperature, indicating that PVA-VEA exists as a unimer (Fig. 1A). However, the clear solution turned turbid upon increasing the temperature, and dynamic light scattering (DLS) showed a lower critical solution temperature (LCST) of 16, 22 and 40 °C for PVA-VEA6%, PVA-VEA3.5%, and PVA-VEA2%, respectively (Table 1). The hydrophobic modification with VEA units on PVA could weaken the intra- and intermolecular hydrogen bonds of adjoining OH-groups, which endows PVA material with thermo-transition ability. It is interesting to note that the transition temperature of PVA-VEA solution increased as the decrease of VEA functionality, in which the respective sizes of thermo-preinduced nanoaggregates were 220, 201, and 170 nm for PVA-VEA6%, PVA-VEA3.5%, and PVA-VEA2%. As the VEA functionality was further increased to 8.2%, the polymer couldn’t be directly dissolved in water even at low temperature, and formed...
nanoaggregates with the size of 90 nm in water at 5 °C by the assistance of methanol in suspending. The nanoaggregates were sequentially exposed under UV-light to form nanogels with decreased sizes varying from 125 to 180 nm (Scheme 2), and remarkably PVA-VEA<sub>6</sub>% nanogels showed 180 nm with a particularly narrow size distribution of 0.05 (Fig. 1B). The crosslinking reaction of PVA-VEA was confirmed by 1H NMR in deuterated methanol using PVA-VEA<sub>6</sub>%, in which proton resonances at δ 5.88–6.42 attributed to the double bond clearly disappeared after UV-exposure (Fig. S2A). FT-IR showed that the peak at 1645 cm<sup>−1</sup> attributed to C–C group also disappeared after crosslinking (Fig. S2B). To further confirm the stability of the nanogels, PVA-VEA<sub>6</sub>% nanogels were measured by DLS at 10 and 25 °C, and the results showed that there was no size difference for the crosslinked PVA-VEA<sub>6</sub>% at both temperatures, while the non-crosslinked PVA-VEA<sub>6</sub>% still presented reversible thermo-response (Fig. 1C). The DLS results performed at 25 °C also showed that there was just a little swelling (30–50 nm increase) for PVA-VEA<sub>6</sub>% nanogels by 1000-fold dilution in water or using methanol for a suspension, in contrast to the non-crosslinked PVA-VEA<sub>6</sub>%, which dissociated by high dilution or dissolved in methanol (Fig. 1D). This indicates that these thermo-preinduced nanoaggregates under UV-irradiation are good process for preparing degradable PVA nanogels.

### 3.3. pH-dependent degradation

The pH-dependent acetal hydrolysis of PVA-VEA<sub>6</sub>% was characterized by 1H NMR according to the disappearance of acrylate proton resonance at 5.88–6.42 ppm (Fig. S3). By calculating the integral ratio of δ 5.88–6.42 and 2.01 (proton resonance of acrylic and methyl group on PVA, respectively), the half-life of acetal hydrolysis at pH 5.0 was about 6 h. By contrast, a negligible acetal hydrolysis of PVA-VEA<sub>6</sub>% was observed at pH 7.4 over 24 h. This hydrolysis rate of acetal at pHs was similar to those reported by Fréchet, Zhong, and our group for PEG copolymers with (tri)methoxybenzylidene acetals attached at the side or periphery [39–42]. The size change of nanogels in response to acetal hydrolysis was followed by DLS. The placement of nanogels into pH 5.0 acetate buffer (100 mM) resulted in rapid and remarkable swelling, in which the size increased from 180 nm to about 520 nm in 2 h that reached over 1000 nm after 6 h. It is interesting to notice that after 24 h the size of nanogels further decreased to 7–10 nm, which is similar to the size of non-functionalized PVA dissolved in water (Fig. 2A). In contrast, no change of nanogel size was observed over 24 h at pH 7.4. The degradation of the nanogels remained consistent with degradation of PVA-VEA<sub>6</sub>% at pH 5.0 and 7.4. The degradation of PVA-VEA<sub>6</sub>% nanogels was also checked by 1H NMR (400 MHz, CD3OD), and the spectra showed that the nanogels after the treatment at the acidic conditions presented the new signal at δ 3.60 attributed to the methylene protons neighboring to the OH-group in the degradation product of PHEA, and the new signals at δ 8.07 and 2.46 attributed to the acetaldehyde protons (Fig. S4), indicating that the degradation product of PVA-VEA nanogels are PVA, PHEA as well as acetaldehyde, without any toxic side product.

### 3.4. PTX encapsulation and pH-triggered release

Paclitaxel (PTX) was encapsulated into PVA-VEA nanogels to study the drug loading capability. Before UV-irradiation, PTX dissolved in ethanol was added into PVA-VEA aqueous solution with a theoretical drug loading content (DLC: weight of loaded drug/total weight of polymer and loaded drug × 100%); of 10 wt%. The results showed that DLC and drug loading efficiency (DLE: weight of loaded drug/weight of drug in feed × 100%) of PVA-VEA<sub>6</sub>% for paclitaxel were approximately 6.2 wt% and 59.5%, respectively (Table 1). The drug loading capabilities of PVA-VEA<sub>3.5</sub>% and PVA-VEA<sub>2</sub>% were very poor, with PTX DLE of only 18.4% and 10.9%, respectively, which were mainly due to the insufficient hydrophobic domains inside the nanogels for PTX interaction. In the following, PVA-VEA<sub>6</sub>% nanogels were further used for an in vitro PTX release study. The in vitro release profile of PTX from nanogels was investigated at pH 7.4 and pH 5.0 at 37 °C. PTX release at physiological pH (pH 7.4) was highly restricted with a released amount of only 21.2% after 30 h (Fig. 2B). However, the PTX release was enhanced under an endosomal/lysosomal pH condition (pH 5.0) due to the cleavage of acid-labile acetal linker, in which 77.0% of PTX was released in 6 h, and release amount reached 90% in 30 h. PTX release from PVA nanogels clearly proceeds in a controlled manner and can be triggered by a low pH.

### 3.5. Size-dependent cellular uptake

It is interesting to note that the size of PVA-VEA<sub>6</sub>% aggregates before UV-crosslinking can be tailored by different thermo-triggers ranging from 160 nm to 450 nm, followed by UV-crosslinking to produce pH-degradable nanogels with different sizes. The average size of the polymer nanoaggregates became larger as the temperature increased from 18 to 25 °C.
30 °C, which is probably due to Oswald ripening. For example: nanoaggregates were crosslinked at 18–20 °C to form nanogels with an average size of 180 nm (NG-180) determined by DLS and TEM, and as the temperature increased during the UV-crosslinking, the nanogel sizes could reach 324 (NG-324) and 486 nm (NG-486) crosslinked at 22–25 °C and 28–30 °C, respectively (Fig. 3). The nanogel size evidently affects the efficiency of cellular uptake and subsequent cellular internalization [43–45]. We investigated the cellular uptake behavior of FITC-labeled nanogels with different sizes using MCF-7 cells. By CLSM images, it was found that FITC-labeled NG-180 well distributed in the MCF-7 cells after 6 h incubation, while NG-324 displayed a weaker FITC intensity than that of NG-180, and there was only a little FITC fluorescence for the cells incubated with NG-486 under otherwise the same conditions (Fig. 4). Cellular uptake of FITC-labeled nanogels was further quantified by flow cytometry analysis. As expected, the flow cytometry results showed that MCF-7 cells following 6 h treatment with NG-180 displayed enhanced FITC fluorescence, 1.5–3.5-fold higher than that of cells incubated with NG-324 and NG-486 (Fig. 5A). Hence, the cellular uptake is highly size-dependent in the order: NG-180 < NG-324 < NG-486, indicating that the smaller nanogels endow the cellular uptake
more effectively [46]. The mechanism is supposed to be caused by a combination of factors, i.e. the surface ratio of adhesion, membrane stretching, and the bending energy of cell membrane with different sizes of nanogels, as well as polydispersity of nanogels particles.

3.6. Cytotoxicity of PTX-loaded nanogels

MTT assays using MCF-7 and A549 cells revealed that PVA-based nanogels were practically non-toxic (cell viabilities ≥ 90%) up to a tested concentration of 1.0 mg/mL (Fig. S5A), confirming that these nanogels had a good cell-compatibility. Notably, PTX-loaded NG-180 displayed significant tumorous cytotoxicity towards MCF-7 and A549 cells following 48 h incubation (Fig. S5B). For example, the half maximal inhibitory concentrations (IC\textsubscript{50}) of PTX-loaded nanogels towards MCF-7 and A549 cells were determined to be 0.79 and 1.50 μg PTX equiv./mL, respectively, which were close to the values of free PTX (MCF-7 cell: 0.58 μg PTX equiv./mL, and A549 cell: 1.07 μg PTX equiv./mL). To further study size-dependent tumorous cytotoxicity of PVA-VEA\textsubscript{6%} nanogels, MCF-7 cells were incubated only for 6 h with PTX-loaded nanogels with different sizes. The culture medium was removed and replenished with fresh

Fig. 3. Size distribution of PVA-VEA\textsubscript{6%} nanogels prepared under different thermo-trigger measured by DLS and TEM.

Fig. 4. CLSM images of MCF-7 cells incubated with FITC-labeled PVA-VEA\textsubscript{6%} nanogels with different sizes for 6 h. The images show for each panel from left to right cell nuclei stained by DAPI (blue), cell membrane labeled by Alexa Fluor 594 (red), FITC-labeled nanogels in cells (green), overlays of three fluorescent images, and bright field image. The scale bars correspond to 25 μm in all the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
culture medium and the cells were cultured for another 48 h. Interestingly, MCF-7 cells treated with PTX-loaded NG-180 displayed cell viabilities of 52.9% and 43.0% with the PTX feeding concentrations of 5 and 10 μg/mL, respectively, which was much lower than those incubated with PTX-loaded NG-324 (5 μg/mL: 69.6%, and 10 μg/mL: 44.5%) and NG-486 (5 μg/mL: 83.8%, and 10 μg/mL: 70.7%) (Fig. 5B). The results demonstrated that smaller nanogels exhibited enhanced cellular uptake and more significant tumorous cytotoxicity. The in vitro therapeutic effects of nanogels were determined by complicated reasons, but at least this provides an insight that the interactions between particle sizes and biological effects should be definitely considered in investigating therapeutic applications.

4. Conclusion

We have successfully developed biocompatible and pH-degradable PVA-based nanogels for intracellular PTX release and anticancer treatment, in which thermo-preinduced PVA-VEA nanopreaggregates were formed and UV-irradiated to prepare nanogels without any surfactants or toxic organic solvents. These nanogels possess well-defined structure and size with excellent colloid stability but rapid degradation and a pH-triggered drug release under intracellular acidic conditions. Furthermore, the size of PVA-VEA aggregates can be tailored by different thermal triggers, followed by UV-crosslinking to fabricate pH-degradable nanogels with different sizes. The smaller nanogels exhibited enhanced internalization with MCF-7 cells, inducing a higher tumorous cytotoxicity. As a promising template, this nanogel system can be further developed for targeting ligands for tumor tissue or cell targeting binding, providing a highly potential platform for delivery of various chemotherapeutics and proteins to actively treat different malignant tumors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.10.032.

Fig. 5. (A) Flow cytometry profiles of MCF-7 cells after 6 h incubation with FITC-labeled PVA-VEA nanogels with different sizes (The cells without any treatment were used as a blank control), and (B) cytotoxicity of PTX-loaded PVA-VEA nanogels determined by MTT assay using MCF-7 cells (The cells were treated with PTX-loaded nanogels or free PTX for 6 h, then the medium was removed and replenished with fresh culture medium for another 48 h. The data are presented as the average ± standard deviation (n = 3, student's t-test: *p < 0.05, **p < 0.01).

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