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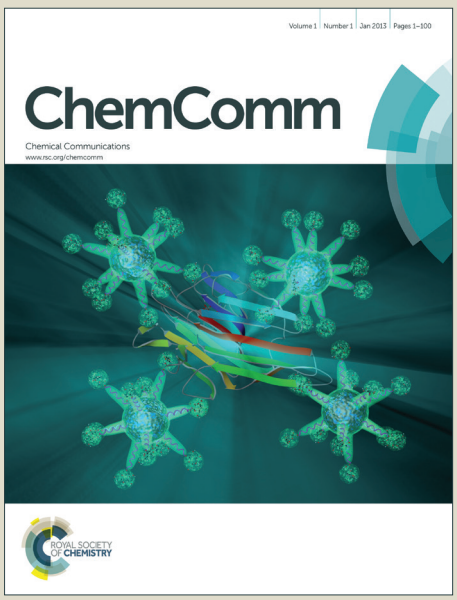

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ARTICLE TYPE

Dual pH-triggered multistage drug delivery system based on host-guest interaction-associated polymeric nanogels

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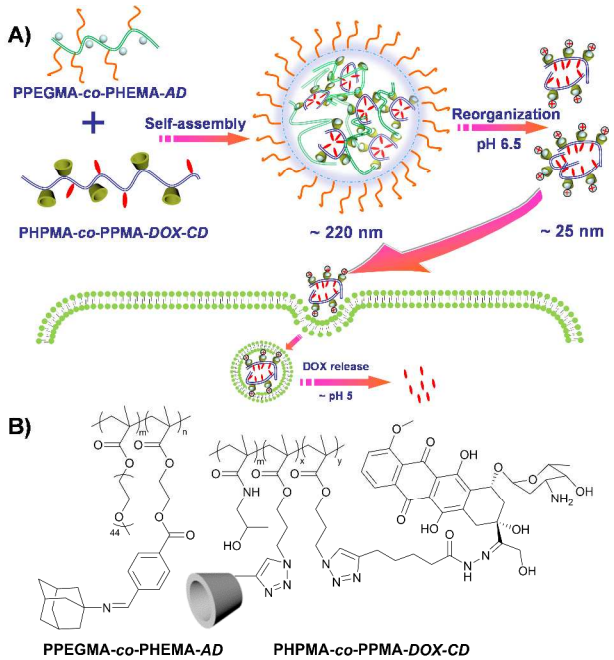
The polymeric nanogels were constructed via host-guest interactions for dual pH-triggered multistage drug delivery, which showed tumor acidity-triggered nanogel reorganization into smaller nanoparticles for deep tissue penetration, high-efficiency cellular uptake, and intracellular endo-lysosomal pH-responsive drug release.

Polymeric nanocarriers have been emerged as widely investigated anticancer drug delivery platforms due to many advantages mainly including optimization of pharmaceutical and pharmacological features of drugs, enhanced permeation and retention (EPR) within tumor tissues, targeted delivery to specific tissues or cells, facile structural fixation for enhanced stability, tunable nanoparticle sizes, and possible incorporation of on-demand release or deeper penetration properties.[1] Various systems addressing varying extracellular and intracellular barriers have been designed and constructed for more efficient delivery and enhanced therapeutic efficacy.[1] However, the complicated microenvironments in tumor tissue or cells result in modest therapeutic efficacy utilizing the current clinically used systems (e.g. Doxil and Abraxane).[2] One of the major challenges is the difficulty of delivering the drugs throughout entire tumor tissue, especially the centre regions consisting of dense collagen matrix.[3] The optimum particles sizes for tumor tissue accumulation and deep penetration are different.[4] To overcome this issue, various size-alterable multistage nanocarriers for on-demand drug delivery in response to specific stimuli have been constructed.[5] For instance, the small-sized quantum dots-encapsulated gelatin multistage nanoparticles showed size change under degradation of proteases, which was demonstrated to be deep penetration in tumor tissue.[5a] On the other hand, the UV light-triggered volume shrunk micelles were also designed based on spiropyran-containing PEGylated lipid.[5b] However, further endeavours should be made for practical and clinical applications using nontoxic polymers under mild stimuli.

Nanogels characteristic of nanoscale networks via physically or chemically associated cross-linkers were considered as promising drug delivery carriers due to high stability even in highly diluted medium.[6] Stimuli-responsiveness can be incorporated into nanogels by using stimuli-responsive polymers or cleavable cross-linkers to achieve structural adjustment or on-demand drug release.[6a-6c] Herein, we proposed a novel nanogel drug delivery system possessing tumor acidity-triggered size change and endo-lysosomal pH-responsive drug release (Scheme 1). The random copolymers, adamantly (AD)-benzoic imine-conjugated poly[poly(ethylene glycol) monomethyl ether methacylate]-*co*-poly(2-Hydroxyethyl methacrylate) (PPEGMA-*co*-PHEMA), PPEGMA-*co*-PHEMA-*AD*, as well as doxorubicin (DOX)-hydrazone and *β*-cyclodextrin (*β*-CD)-conjugated poly[*N*-(2-hydroxypropyl) methacrylamide]-*co*-poly(3-azidopropyl methacrylate) (PHPMA-*co*-PAzPMA), PHPMA-*co*-PPMA-*DOX-CD*, were synthesized and used to prepare nanogels via self-assembly in aqueous solution. In virtue of benzoic imine linker between AD and PPEGMA-*co*-PHEMA backbones, the nanogels can be triggered by tumor acidity to reorganize into nanoparticles of polymer-DOX conjugates with smaller size (~ 25 nm) and slightly positively charged surface.[5d,7] Further investigation demonstrated that the nanoparticles can penetrate deeply in collagen hydrogels mimicking dense matrix of tumor tissue. Moreover, the nanoparticles without PEG coverage facilitated cellular uptake by HeLa cells and showed enhanced cytotoxicity as DOX can be released quickly in intracellular endo-lysosomes at ~ pH 5 due to existence of pH-cleavable hydrazone linkage.[7b,8]



Scheme 1 A) Schematic illustration of construction of polymeric nanogels with dual pH-triggered multistage drug delivery. B) Structural formulas of AD-benzoic imine-linked PPEGMA-*co*-PHEMA-*AD* and DOX-hydrazone-linked PHPMA-*co*-PPMA-*DOX-CD*.

The synthetic routes employed to prepare PPEGMA-*co*-PHEMA-*AD*, and PHPMA-*co*-PPMA-*DOX*-*CD*, were shown in Scheme S2. Firstly, the random copolymer, PPEGMA-*co*-PHEMA, was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization of PEGMA (M_n, ~ 2000) and HEMA using 4-cyanopentanoic acid dithiobenzoate (CTP) as the chain transfer agent (CTA) at the molar ratio of 20:40:1. 4-Formylbenzoic acid (FBA) was attached onto the hydroxyl groups via condensation reaction, followed by AD conjugation via formation of benzoic imine. The degrees of polymerization (DPs) of PPEGMA and PHEMA were determined to be 13 and 21, respectively, and the functionality of hydroxyl groups is 90.5% according to ^{1}H NMR analysis (Fig. S1). After reaction with 1-adamantylamine, the disappearance of aldehyde signals in ^{1}H NMR spectrum was indicative of quantitative transformation of aldehyde groups into benzoic imine. Thus, the polymer was denoted as PPEGMA$_{13}$-*co*-PHEMA$_{21}$-*AD*$_{19}$. On the other hand, *alkynyl*-functionalized *β*-CD and DOX were conjugated to the backbone of the polymer, PHPMA-*co*-PAzPMA, via click reaction to afford PHPMA-*co*-PPMA-*DOX*-*CD*. Notably, *β*-CD and DOX were first modified with alkynyl groups to obtain *alkynyl*-CD[9] and *alkynyl*-DOX (Scheme S1). Azido groups-containing polymer, PHPMA-*co*-PAzPMA, was synthesized through RAFT polymerization.[10] The DPs of PHPMA and PAzPMA can be determined be 78 and 13, respectively, based on ^{1}H NMR analysis (Fig. S2). FT-IR spectra bear out quantitative azido groups transformation evidenced by the complete disappearance of the characteristic peak of azido moieties at ~ 2100 cm^{-1} (Fig. S3). The amount of DOX can be determined to be 7 on each polymer chain with the conjugation efficiency of 86.4% by UV-vis analysis using DOX as the calibration standard. Thus, the copolymer can be termed PHPMA$_{78}$-*co*-PPMA$_{13}$-*DOX*$_7$-*CD*$_6$.

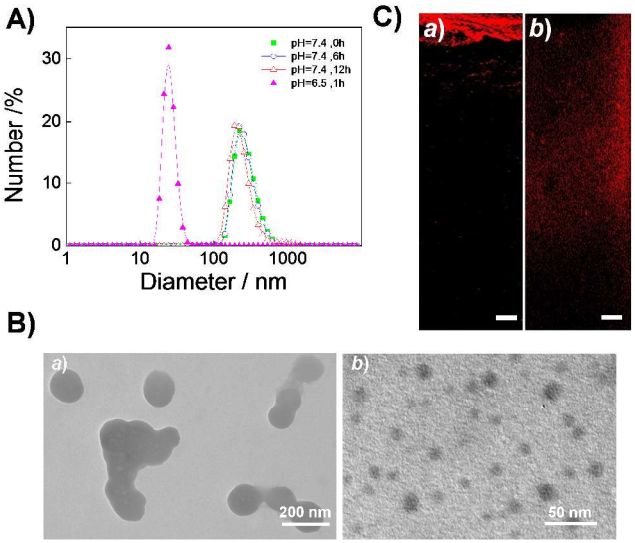


Fig. 1 Tumor acidity-triggered reorganization of PPEGMA-*co*-PHEMA-*AD*/PHPMA-*co*-PPMA-*DOX*-*CD* nanogels undergoing size reduction. A) Size distribution after incubation at pH 7.4 or pH 6.5 for the desired time; B) TEM morphologies before (a) and after (b) incubation at pH 6.5 for 1 h; and C) Penetration profiles of the nanogels before (a) and after (b) size change in the collagen hydrogels mimicking dense matrix of tumor tissue. Scale bars represent 150 μm.

It is well-established that AD and *β*-CD can form inclusion complexes via host-guest interaction in aqueous solution, hence the cross-linkers can be formed via inclusion complexation between AD and *β*-CD moieties-containing polymers.[11] The preparation of nanogels was performed by dialysis against PBS buffer (pH 7.4) from dimethyl sulfoxide (DMSO) solution of PPEGMA-*co*-PHEMA-*AD* and PHPMA-*co*-PPMA-*DOX*-*CD* at AD/CD molar ratio of 1:1. The formed nanogels were characterized by dynamic laser light scattering (DLS) and transmission electron microscopy (TEM) (Fig. 1). DLS characterization revealed that average particle size of nanogels was ~ 220 nm, while the TEM images exhibited ~ 150 nm presumably due to shrinkage in the dry sate during TEM sample preparation. Moreover, PEG shells can not be observed owing to low contrast in TEM images.

Notably, PPEGMA-*co*-PHEMA-*AD* polymer can be dispersed in water (pH 7.4) forming nanoparticles evidenced by strong laser light scattering intensity. Upon addition of equivalent free *β*-CD relative to AD moieties, the solution turned out transparent indicative of good solubility of PPEGMA-*co*-PHEMA-*AD* in the presence of *β*-CD. With pH values decreasing to 6.5, the solution maintained transparent and ^{1}H NMR analysis showed the appearance of aldehyde group signals revealing the cleavage of benzoic imine (Fig. S5). For the nanogels, the size remained constant even after incubation for 12 h at pH 7.4, whereas the size underwent remarkable decrease from ~ 220 nm to ~ 25 nm with slight zeta potential increase after 1 h incubation as the incubation pH was adjusted from 7.4 to 6.5 (Fig. 1A and Fig. S6). The TEM images also showed smaller size (< 20 nm) after preincubation at pH 6.5 for 1 h (Fig. 1B). These results suggested that the nanogels dissociated at pH 6.5 forming CD/AD inclusion complexes and DOX-conjugated PHPMA-*co*-PPMA-*DOX*-*CD/AD* nanoparticles with smaller size due to cleavage of benzoic imine bonds. Moreover, the size of the nanogels can preserve structural integrity upon large-volume dilution. On the other hand, the nanogels can also transformed into smaller nanoparticles with average size of 29 nm after incubation at pH 6.5 for 2 h in the presence of 10% serum, which is similar to that in PBS buffer (Fig. S7).

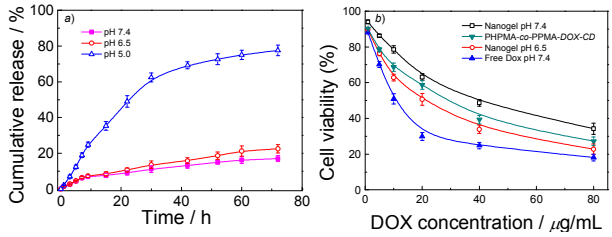


Fig. 2 a) Drug release profiles of the DOX-loading nanogels in aqueous solution at various pH values (pH 7.4, 6.5 and 5.0) (mean ± SEM, n = 3). b) Cytotoxicity of the nanogels after preincubation at varying pH values (pH 7.4 and 6.5), free DOX, and PHPMA-*co*-PPMA-*DOX*-*CD* polymer against HeLa cells (mean ± SEM, n = 4).

As the large nanogels reorganized into smaller nanoparticles, the nanocarriers were expected to penetrate deeply in tumor tissues with dense matrix. To verify this hypothesis, the diffusive transport of the nanogels after 2 h preincubation at pH 6.5 was evaluated using a collagen hydrogel with the concentration of 7.54 mg/mL mimicking dense matrix of tumor tissue. A small amount of nanogels solutions after preincubation at pH 7.4 or pH 6.5 was injected in contact with collagen hydrogels and incubated

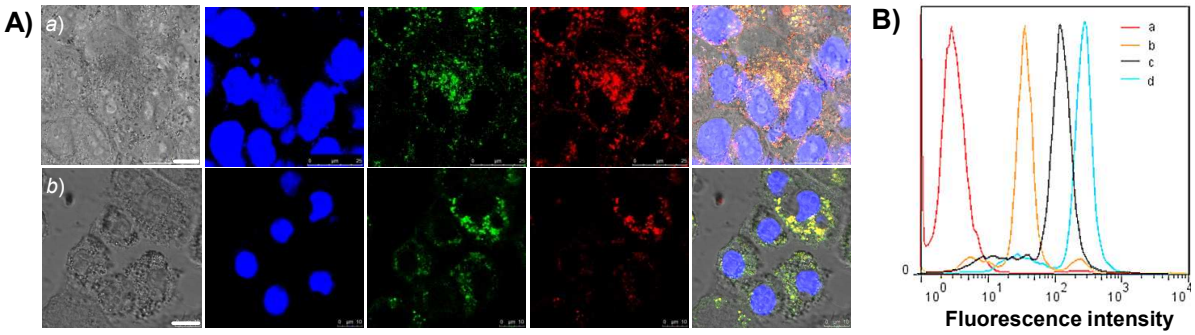


Fig. 3 A) CLSM observation of the intracellular distribution of DOX (red) from the nanogels with endo-/lysosomes (green) and nuclei (blue) in HeLa cells after preincubation in PBS at pH 6.5 (a) and pH 7.4 (b) for 2 h and incubation with cells for 4 h. Bars represent 10 μm. B) Cellular uptake of DOX from the nanogels carriers against HeLa cells examined by flow cytometry (a: control samples without addition of nanogels; b: nanogels after preincubation at pH 7.4; c: PHPMA-*co*-PPMA-*DOX*-*CD* polymer; d: nanogels after preincubation at pH 6.5).

for 4 h. Confocal laser scanning microscopy (CLSM) was used to observe the distribution of DOX by fluorescence intensity at the wavelength of 560-590 nm. Fig. 1C shows that the nanogels after preincubation at pH 7.4 exhibited very limited penetration mainly on the top of the collagen hydrogels. On the other hand, after preincubation at pH 6.5, the DOX-containing nanoparticles showed > 2 mm penetration into the hydrogel within 4 h as a result of small size.

The pH-responsive cumulative drug release was evaluated at various pH values. Anticancer drug, DOX, was conjugated on the polymer backbone of PHPMA-*co*-PPMA-*DOX*-*CD* through hydrazone bonds, which have been demonstrated to be endo-lysosomal pH-sensitive.[7b,8] As shown in Fig. 2a, at endo-lysosomal acidity of pH 5, 76.9% DOX was released after 72 h incubation due to cleavage of hydrazone linkage. In contrast, only 22.4% and 17.1% DOX were released at pH 6.5 and pH 7.4, respectively. These results were consistent with previous reports.[8] Moreover, we further estimated the cytotoxicity of the nanogels against HeLa cells after preincubation at pH 7.4 or pH 6.5. Fig. 2b shows the nanogels exhibited significantly higher cytotoxicity after preincubation at pH 6.5 than that at pH 7.4 and PHPMA-*co*-PPMA-*DOX*-*CD* polymer, which was closer to that of free DOX. In view of the similar DOX release rates of the nanogels at pH 6.5 and 7.4 as well as negligible cytotoxicity of the polymers (PPEG-*co*-PHEMA-AD and PHPPMA-*co*-PAzPMA + β-CD) (Fig. S8), the cytotoxicity differences should be attributed to varying cellular uptake efficiencies.

To further illuminate influences of nanogels reorganization on their association with cells, we observed the cellular internalization and intracellular localization using CLSM and flow cytometry, respectively (Fig. 3). The nanogels were added into the HeLa cell culture medium after preincubation at pH 6.5 for 2 h, where the nanogels transformed into small nanoparticles with slightly positively charged surfaces. Notably, after 4 h cellular internalization, the CLSM images showed stronger red fluorescence for the nanogels pre-incubated at pH 6.5 reflecting larger amount of internalized nanoparticle than those pre-incubated at pH 7.4. The colocalization of red and green colour demonstrated that DOX release occurred in the endo-lysosomes. The quantitative results further confirmed markedly higher cellular uptake activity of the nanoparticles after the nanogel reorganization as compared with the nanogels preincubated at pH 7.4 and PHPMA-*co*-PPMA-*DOX*-*CD* polymers by flow cytometry (Fig 3B). Moreover, after another 24 h incubation, majority of released DOX was localized in the nucleus (Fig. S9). Therefore, the higher cytotoxicity of the nanogels after preincubation at pH 6.5 can be attributed to higher cellular uptake due to the nanogels reorganization into small nanoparticles without PEG protection.

In summary, a nanogel drug delivery system with unique dual-pH-responsive multistage release behaviors was successfully constructed from copolymers of AD-benzoic imine-linked PPEGMA-*co*-PHEMA-*AD* and DOX-hydrazone-linked PHPMA-*co*-PPMA-*DOX*-*CD* via host-guest interaction between AD and β-CD moieties. This nanogel exhibited tumor pH-triggered reorganization into smaller nanoparticles due to cleavage of benzoic imine linkage, which can facilitate deep penetration in the tumor tissue-mimicking collagen hydrogels and promote cellular uptake. Moreover, the reorganized nanocarriers can release DOX quickly in late endosomal or lysosomal acidity, which showed closer cytotoxicity to that of free DOX. This novel nanogel drug delivery system augurs well for applications as smart nanocarriers for deep tumor tissue penetration and promoted therapeutic efficacy.

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† Electronic Supplementary Information (ESI) available: Experimantal section, Scheme S1-S2, and Fig. S1-S5. See DOI: 10.1039/b000000x/

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