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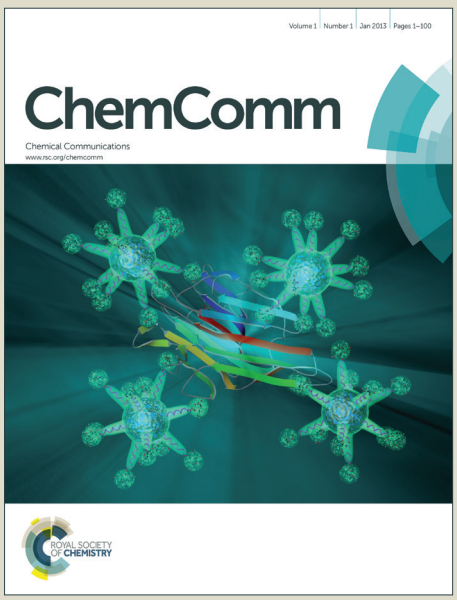

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Amphiphilic Sugar Poly(orthoesters) as pH-Responsive Nanoscopic Assemblies for Acidity-Enhanced Drug Delivery and Cell Killing

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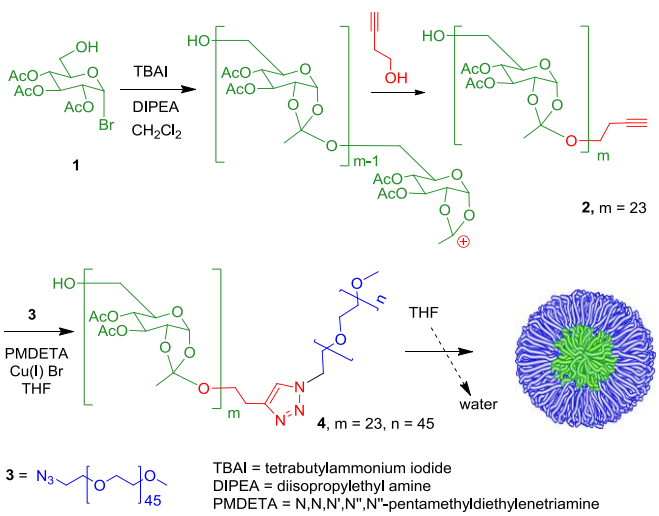
A sugar poly(orthoester)-based drug delivery system was constructed to achieve acidity-enhanced drug delivery and cell killing.

Polymeric nanoparticles (NPs), with the capability and versatility to encapsulate drugs within their structures, have demonstrated great potentials in drug delivery.[1, 2] However, despite much progress, there are still many issues that are limiting their clinical applications. Notably, the pre-loaded drugs within the NP systems cannot be released in an efficient and controlled manner. To address this issue, stimuli-responsive NP delivery systems have been studied,[3-6] which can undergo disassembly or degradation in response to the change or application of internal or external stimuli, such as temperature, ultrasound, enzymes and acidity. Among these stimuli, the use of acidity is particularly attractive because of the many pH discrepancies that exist between normal and malignant tissues/cells.[7] For example, tumour and inflammatory tissues are often exposed to mildly low pH values (pH = 5-6).[8, 9] Presumably, such pH discrepancies may allow for selective or targeted delivery.[10-12]

Current pH-responsive systems often employ acid-degradable linkages such as acetal and ketal.[13-17] For example, Fréchet and co-workers reported the syntheses of acetal-containing amphiphilic block polymers to achieve acidity-enhanced doxorubicin (DOX) release.[15] Murthy and co-workers reported the syntheses of polyketal copolymers to deliver imatinib in the treatment of acute inflammatory disease.[14] Although these delivery systems have shown potential, they were only mildly sensitive to acidolysis. For example, for an acetal-based NP delivery system, it took 42 h to release 50% of the pre-loaded DOX at pH = 5.[15] Other acid-labile linkages such as imine and hydrazone have also been reported but they suffer from similar drawbacks.[18, 19].

Because of their slow responsiveness, the above mentioned chemical linkages may not be suitable to construct pH-responsive delivery systems that aim for efficient release. Compared to acetal and ketal linkages, orthoester linkages are orders of magnitude more sensitive towards acid-catalysed hydrolysis.[20] Early syntheses of poly(orthoesters) have been studied by Heller and co-workers.[21, 22] However, the reported poly(orthoesters) were not pH-responsive, due to their high hydrophobicity that limited water and acid penetration. Recently, Du and co-workers reported the synthesis of a sugar-based poly(orthoester), which was highly pH-responsive with a half-life ($t_{1/2}$) = 0.6 h at a mildly acidic environment (pH = 5).[23] We perceived that if such sugar orthoester linkages were employed to construct NP delivery systems, efficient and even targeted release may be achieved. Herein we report the synthesis of amphiphilic sugar poly(orthoesters) to construct highly pH-responsive NPs.

As depicted in Scheme 1, di-functional monomer **1** was subjected to polymerization using tetrabutylammonium iodide (TBAI) as a promoter according to the procedure reported previously.[23] At the end of the polymerization, 3-butyn-1-ol was added as a terminator to give alkyne-functionalized sugar poly(orthoester) **2**.



Scheme 1 The synthesis of sugar poly(orthoester)-*block*-PEG **4**, and the subsequent construction of NPs through self-assembly.

The successful addition of the terminator was confirmed by NMR studies, which showed the $C\equiv C-CH_2$ protons resonating at 2.4 ppm (Figure 1, bottom). A calculation of the molecular weights based on these protons gave M_n^{NMR} = 6.3 kDa, which is close to that

obtained from gel permeation chromatography (GPC) analysis (M_n^{GPC} = 6.1 kDa) (Figure 2A). It should be noted that we have also attempted the use of OH-containing polymers such as poly(ethylene glycol) (PEG, M_n = 750 or M_n = 2000) as a terminator, aiming for a direct formation of an amphiphilic block polymer. Unfortunately, this route was not successful. Possibly, the steric hindrance or the limited accessibility of the chain-end -OH group of the PEG had adversely affected the conjugation.

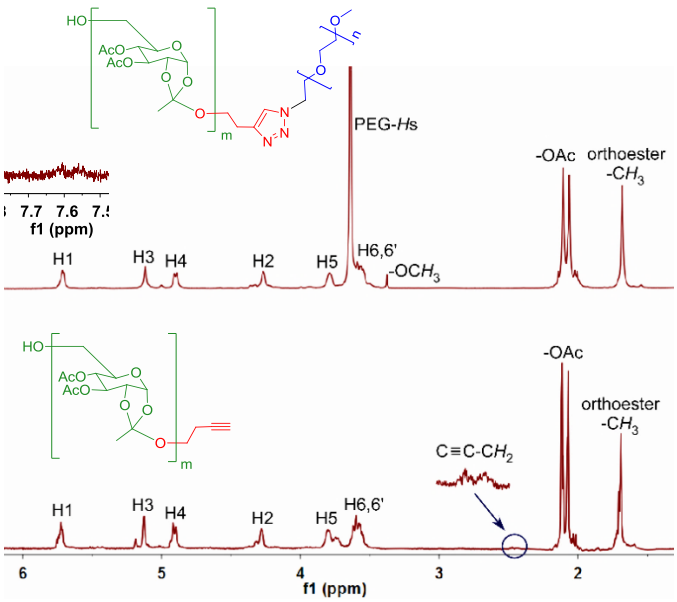


Fig. 1 ^{1}H-NMR spectra of alkyne-terminated sugar poly(orthoester) **2** (bottom) and PEG-conjugated amphiphilic block polymer **4** (top).

Our next step was to perform click-conjugation of **2** with an azide-functionalized hydrophilic polymer. We chose PEG as the hydrophilic polymer because of its favourable biocompatibility.[24] An azide-functionalized PEG (**3**, M_n = 2.0 kDa) was synthesized[25] and the click conjugation was performed according to the reported procedure (Scheme 1).[26, 27] After the click conjugation, an extensive dialysis (mwco = 3.2 kDa) was performed to remove the excess PEG, the catalyst, as well as the chelating agent. As evidenced by the NMR analyses (Figure 1, top), the successful click conjugation was achieved with the formation of triazole rings, for which the protons resonate at 7.6 ppm. The GPC analyses also confirmed the successful conjugation of PEG, with the molecular weights increasing from M_n^{GPC} = 6.2 kDa to M_n^{GPC} = 8.7 kDa (Figure 2A). It should be noted that the GPC analyses gave a clean curve, indicating that most, if not all, of the excess PEG had been successfully removed.

Our next aim was to construct the NPs through transitioning polymer **4** from THF into water. During the process, a self-assembly process occurred to give the NPs. The TEM measurements indicated that the NPs are nearly spherical with sizes of 56 ± 8 nm (Figure 2B). The dynamic light scattering (DLS) measurements gave the NP sizes of 96 ± 15 nm (see ESI). The increased DLS sizes (compared to the TEM sizes) are the result of hydration of the NPs in aqueous solution. Furthermore, we have also measured the critical micelle concentration (CMC) to be 0.02 mg/mL (2.3 ×10^{-6} M), indicating the formation of fairly stable NPs in aqueous solution (see ESI).

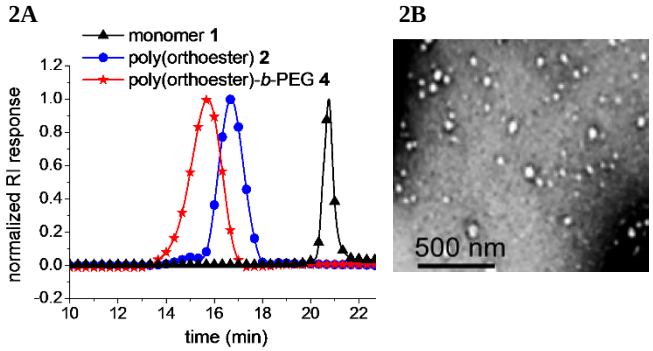


Fig. 2A: The GPC curves of sugar poly(orthoester) **2** and amphiphilic block polymer **4**. The GPC curve of monomer **1** is also included as a reference. **2B:** The TEM image of the NPs derived from polymer **4**.

Because of the embedded sugar orthoester linkages, the derived NPs may be degraded under acidic conditions. To study the acid-catalysed degradation, we employed DLS to monitor the size changes during the course of acidolysis.[15, 28] As shown in Figure 3, under mildly acidic conditions (pH = 5), the degradation of the NPs proceeded with a gradual decrease of the average sizes, consistent with the disintegration of the NPs into soluble sugar monomers. It should be noted that the NPs were very stable at pH = 7.4 with no significant change of the sizes after a 7-day storage at rt (see ESI).

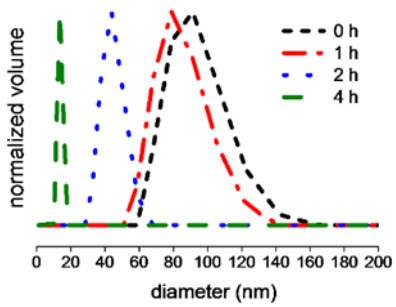


Fig. 3. The progress of acid-catalyzed disintegration of the NPs at pH = 5.0, as monitored by DLS.

The NPs, composed of sugar poly(orthoesters) in the core and PEG on the shell, were expected to exhibit minimal cytotoxicity to cells. We then performed a cell viability assay using HEK293 cells. As shown in Figure 4 (black line, square), the cells were comfortable with the addition of these NPs; there was no decrease of cell viability at a NP concentration of 50 μg/mL.

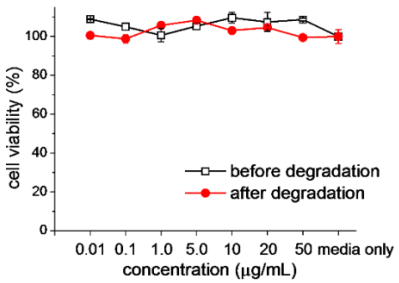


Fig. 4 The cell viability studies of the NPs before (black) and after (red) degradation, using HEK293 cells.

Since the polymer was acid-degradable, we also wanted to evaluate the toxicity that resulted from the degraded products. The degradation was performed at pH = 5 using trifluoroacetic acid (TFA) to adjust the pH values. After incubation of the NPs at rt for 24 h, the pH value was adjusted back to pH = 7.4 (using PBS buffer) to study the cytotoxicity. As shown in Figure 4 (red line, circle), the degraded NPs also exhibited very low toxicity We further performed the ^{1}H-NMR studies to elucidate the structure of the degraded products, which were a mixture of acetylated glucose (see ESI), resulting from the acid-catalysed cleavage of the orthoester linkages, as well as the rearrangements and the migration of the acetyl groups during the acidolysis.[29]

Compared to polyacetal and polyketal systems, which release potentially toxic aldehydes or ketones upon acidolysis, the sugar poly(orthoesters) system reported here may have advantages in terms of long term cell toxicity from the degraded products.

Because of the low toxicities, these NPs may find applications in drug delivery. We then performed the drug loading/release studies using DOX as a model drug, due to its unique UV absorbance that allows for convenient detection and quantification.[30-32] The drug was incorporated into the NPs using an emulsion-evaporation method reported previously,[31] which gave a drug loading efficiency (DLE) of 73% and drug loading content (DLC) of 20 wt%.

The release of DOX was conducted according to the reported dialysis protocol[31] in PBS of varying pH values (pH = 5.0 and pH = 7.4), as well as in fetal bovine serum (FBS, pH = 7.4). As illustrated in Figure 5, there was a small amount (19%) of DOX leakage in PBS buffer at pH = 7.4 after dialysis for 6 h (Figure 5, square). This pre-mature release may translate to a loss of DOX during circulation in plasma and therefore a decrease of treatment efficacy. Fortunately, the stability of the DOX-loaded NPs in FBS was excellent with minimal pre-mature release after a 6-h dialysis (Figure 5, triangle). The improved stability of the DOX-loaded NPs in FBS is possibly due to the higher hydrophilicity of the FBS media which may slow the passive release of DOX.

On the other hand, at pH = 5, the release was very rapid with 72% of the drug released within 1 h, and nearly 100% released within 4 h (Figure 5). The release rate at this pH value is orders of magnitude faster than those based on ketal/acetal delivery systems.[14, 15] Such high pH-responsiveness may be clinically significant; in the treatment of many diseases, such as acute liver infection, a fast release of drugs, *e.g.* within a few hours, is critical, due to the fast deterioration of tissue and organ functions.[33]

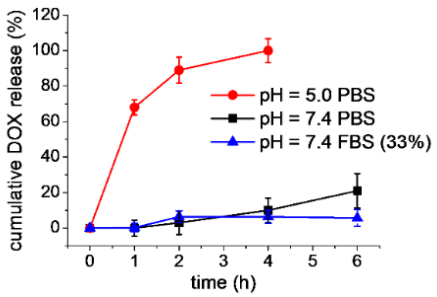


Fig. 5 The release profile of DOX, encapsulated in the NPs at pH = 7.4 and 5.0, respectively. The release profile in FBS was also included to demonstrate the stability of DOX-loaded NPs in FBS.

Having this exciting data, we then evaluated the cell killing effects under mildly low pH values, such as pH = 5. With the consideration that the acidic environment may complicate the cell viability, we then identified cells that were healthy at pH = 5.0. We found that HEK293 cells were very comfortable at this condition; the cell viability at pH = 5.0 was virtually the same as that at pH = 7.4 (Figure 6, media only).

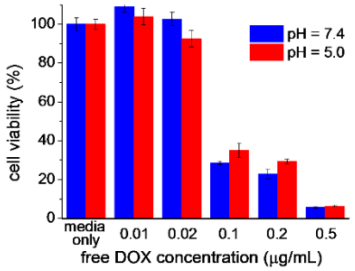


Fig. 6 The cell viability studies of HEK293 cells using free DOX of varying concentrations at pH = 5.0 and pH = 7.4, respectively.

We have also studied the pH effects on the potency of free DOX, which was virtually unaffected by the change in the pH values. At low DOX concentrations, *e.g.* below 0.02 μg/mL, DOX did not exhibit cell killing effects (Figure 6). At higher concentrations, for example, at 0.1 and 0.2 μg/mL, the DOX potency was only slightly higher at pH = 7.4 than that at pH = 5.0, possibly due to the increased solubility of DOX at higher pH values. When the DOX concentration reach a high value (0.5 μg/mL), there is no discrepancy in the potency.

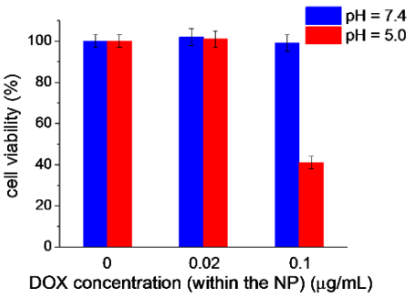


Fig. 7 The cell viability studies of DOX (encapsulated within the NPs) of varying concentrations at pH = 5 and pH = 7.4, respectively.

Using this data as a reference, we then performed the cell killing studies at an encapsulated DOX concentration of 0.02 μg/mL, which indicated a NP concentration of 0.1 μg/mL (Figure 7). After incubating the cells with the DOX-loaded NPs at pH = 7.4 for 4 h, there was no cell killing (cell viability = 102 ± 4%). When the pH value was dropped to pH = 5, there was no cell killing, either (cell viability = 101 ± 4%, Figure 7), possibly, the amount of encapsulated DOX was insignificant. Therefore, we increased the DOX concentration to 0.1 μg/mL (NP concentration = 0.5 μg/mL). At this concentration, there was virtually no cell killing at pH = 7.4 (cell viability = 99 ± 4%, Figure 7), indicating a minimal DOX release. However, when the pH value was decreased to pH = 5, the cell viability dropped to 41%. In comparison to the cell viability at pH = 7.4, there is obviously an acidity-enhanced cell killing, which resulted from the degradation of the orthoester linkages embedded inside the NPs. It should be noted that at this concentration, the potency of encapsulated DOX was only slightly decreased than that from free DOX (cell viability = 35%, Figure 6). The lower potency may result from the gradual release of DOX from the NPs.[15] However, such a drawback may be compensated by the accumulation of the NPs in tumor tissues through enhanced permeation and retention (EPR) effect.[34]

The degradation of the NPs may occur extracellularly, or inside the cells' lysosomes or endosomes after the cell uptake.[11, 12] However, our studies showed that the cell viability was almost 99%

at pH =7.4, suggesting insignificant degradation occurred inside the cells. The extracellular degradation could be a very useful tool for selective drug delivery to the tumour and inflammatory tissues, for which the extracellular pH values are mildly acidic in the range of 5-6. [8, 9]

Conclusions

In conclusion, we reported the synthesis of a novel pH-responsive drug delivery system to achieve acidity-enhanced drug release and cell killing. The system, built upon sugar poly(orthoesters), exhibited minimal cytotoxicity to the HEK293 cells. It should be noted that the degraded products, which are acetylated glucose, also had low cytotoxicity to these cells. Due to the low toxicity, the reported sugar-based NP delivery system may be able to find clinical applications.

Because of the embedded acid-labile orthoester linkage, the reported NP delivery system allows for selective drug release and cell-killing under mildly acidic conditions. This is clinically attractive in the treatment of many diseases, such as cancer and acute inflammatory diseases, for which the cells and the tissues are often exposed to acidic environment.

However, it should be noted that the results reported here can only serve as a proof-of-concept to demonstrate the potential of these sugar poly(orthoester)-based NPs. The parameters for the cell killing studies, including the concentration, the incubation time, as well as the degradability of the NPs, must be carefully studied in order to achieve acid-enhanced cell killings. With the promising data obtained in this study, we are currently working on the synthesis of sugar poly(orthoesters) with varying chemistry and structures, aiming to achieve tuneable degradability of the NPs. With the successful anchoring of an alkyne terminator, the chemistry demonstrate herein may provide a robust and versatile tool to achieve a variety of sugar-based polymers and NP delivery systems.

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Notes and references

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