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A new pathway towards polymer modified cellulose nanocrystals *via* a "grafting onto" process for drug delivery

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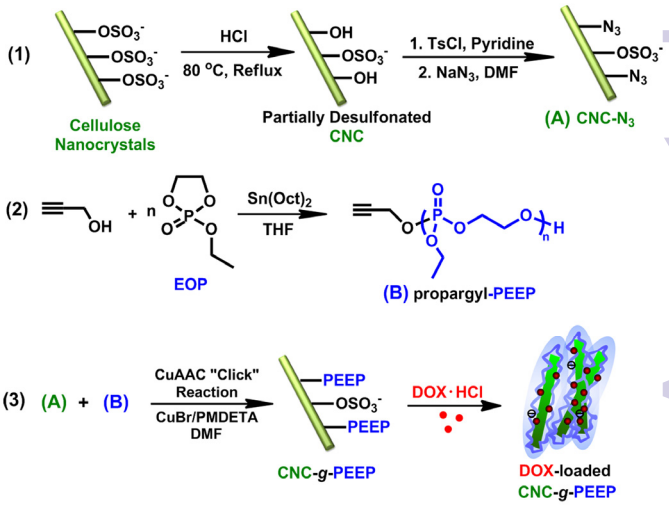
Poly(ethyl ethylene phosphate) (PEEP) modified cellulose nanocrystals (CNCs) (CNC-*g*-PEEP) were synthesized through a "grafting onto" process, in which a combination of ring-opening polymerization (ROP) and Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) "click" chemistry were utilized. The resultant suspension of negatively-charged CNC-*g*-PEEP nanocrystals could be used to encapsulate doxorubicin (DOX) by electrostatic interaction and release the drug in the tumor cell environment.

Cancer therapy has become one of the main challenges in the biomedical field worldwide. During the past decades, as a sustainable material gained from renewable sources, cellulose nanocrystals (CNCs) have been extensively studied due to their desirable properties, such as uniform nanorod-like shape, high specific surface area, biocompatibility, biodegradability, sustainability and good mechanical strength.[1-4] CNCs, consisting of the hydrogen-bonded linear β-D-glucopyranose chains, can form the nanorods with width of 10-20 nm and length of 200-400 nm.[5,6] The negatively-charged surface and hydroxyl groups on CNCs provide a good platform to combine them with various positively-charged materials, for example, metal nanoparticles or some drugs.[1,7] Both of the physical and chemical properties of CNCs can be adjusted by grafting polymers onto their surface.[8,9]

Polyphosphoesters (PPEs) are a class of promising biomaterials with good biocompatibility and biodegradability because of their similar structures with teichoic acid and nucleic acid.[10,11] PPEs are prone to degradation through the scission of ester bonds under hydrolytic or enzymatic conditions.[12-14] Up to now, several kinds of polymer modified CNCs have been synthesized *via* the "grafting from" process.[8,15] However, it is difficult to purify and clearly characterize these grafted polymers. To the best of our knowledge, there are no reports working on how to introduce PPEs to the CNCs *via* the "grafting onto" method, which may extend the applications of CNCs in biomedical fields.



Scheme 1 Schematic illustration of the synthesis pathway of CNC-*g*-PEEP *via* CuAAC "click" reaction and the formation of DOX-loaded nanocrystals.

Herein, we report on a new strategy to modify CNC with hydrophilic PPE through a "grafting onto" process. As shown in Scheme 1, the propargyl-terminated poly(ethyl ethylene phosphate) (propargyl-PEEP) was synthesized by the ROP reaction of 2-ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP) monomer, which was then used for the highly efficient CuAAC "click" reaction with the azide-modified CNC (CNC-N_3) to yield the PEEP-grafted and negatively-charged nanocrystals (CNC-*g*-PEEP). This kind of material possesses the nanorod-like morphology and can be used to load antitumor drug doxorubicin (DOX) by means of electrostatic interaction because the protonization of amine group endows DOX with positive charges. The hydrophilic PEEP chains will provide favorable stability to the DOX-loaded nanocrystals in blood circulation.[16,17] Besides, the diameter of CNC-*g*-PEEP nanocrystals is around 30-40

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Electronic Supplementary Information (ESI) available: Materials, synthesis and characterization of CNC-N_3, propargyl-$PEEP_n$, CNC-*g*-$PEEP_n$, ^{1}H NMR spectrum, GPC results, elementary data and zeta-potential results. See DOI:10.1039/x0xx00000x

nm, which allowed them to be internalized by tumor cells. In the tumor acidic environment, the electrostatic interaction will be destroyed by the attack from ions (e.g., H^+ or Cl^-) in cytoplasm[18] and PEEP segments can be degraded in the presence of phosphodiesterase I or under the acidic condition, thus leading to a pH-triggered release of DOX.

CNC-*g*-PEEP was prepared by the following steps as shown in Scheme 1. First of all, the partial desulfation treatment was conducted to obtain more active hydroxyl groups on the surface,[19] followed by the chlorination with *p*-toluenesulfonyl chloride (TsCl) to yield the CNC-Cl. Sodium azide (NaN_3) was then used to react with CNC-Cl to yield the azide-modified CNC (CNC-N_3);[9,20] Secondly, propargyl-PEEP was prepared by the ROP reaction of EOP monomer according to our previous report;[21] Finally, CNC-*g*-PEEP was obtained by the CuAAC "click" reaction between CNC-N_3 and propargyl-PEEP. The ^{1}H NMR spectrum and GPC results of propargyl-PEEP samples with different molecular weights (see Fig. S1 and Table S1 in ESI†) demonstrate the successful synthesis of propargyl-PEEP with controlled molecular weights.

FT-IR analysis was used to monitor the changes of functional groups during the reactions and the results are shown in Fig. 1. Compared to the original CNC shown in Fig. 1(A), a new absorption peak at 2105 cm^{-1} corresponding to the stretching vibration of azide group is clearly detected in Fig. 1(B), demonstrating the successful introduction of azide group onto CNC. Moreover, the results from the elementary analysis (Table S2 in ESI†) also indicate the introduction of nitrogen atom in the CNC-N_3. In addition, the mole number of grafted PEEP can be calculated based on the N content from elemental analysis because the molar ratio of azide group and PEEP chains grafted on CNC-N_3 is close to 1:1. The amount of grafted $PEEP_{29}$ chains is about 81.7 mg per 100 mg CNC-N_3 (See ESI† for the detailed equations). In addition, the absorption peak of azide group disappeared after CuAAC "click" reaction, while the peaks at 2890 cm^{-1} and 1273 cm^{-1} respectively ascribed to the stretching vibrations of CNC and –P=O from propargyl-$PEEP_{29}$ maintained, indicating the successful preparation of CNC-*g*-$PEEP_{29}$.

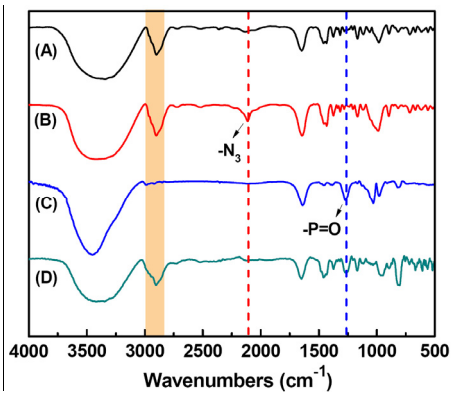

Fig. 1 FT-IR spectra of (A) CNC, (B) CNC-N_3, (C) propargyl-$PEEP_{29}$ and (D) CNC-*g*-$PEEP_{29}$.

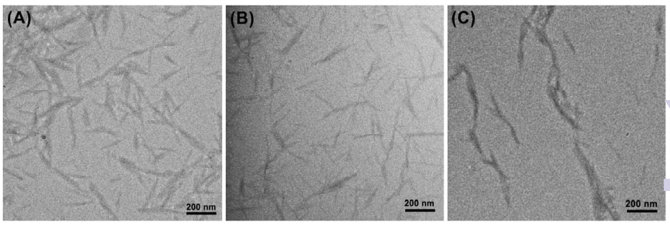

Fig. 2 TEM images of (A) CNC, (B) CNC-N_3 and (C) CNC-*g*-$PEEP_{29}$. The scale bars correspond to 200 nm in all images.

It was interesting to find that the zeta potential values of CNCs changed after each modification and the results are shown in Fig. S2 in ESI†. One can find that the zeta potential value increased from -56.2 mV to -35.9 mV after the desulfation treatment, while the value of CNC-*g*-$PEEP_{29}$ was -24.6 mV, illustrating that this kind of negatively-charged CNC nanocrystals own a possibility to bind some positively-charged molecules. After loading DOX, the zeta potential value changed to -10 mV, demonstrating that CNC-*g*-$PEEP_{29}$ has been successfully combined with DOX. TEM was employed to observe the morphologies of various CNC nanocrystals. As shown in Fig. 2, both CNC (Fig. 2A) and CNC-N_3 (Fig. 2B) samples possess nanorod-like structures with a diameter about 10-20 nm, while the CNC-*g*-$PEEP_{29}$ (Fig. 2C) exhibits a similar nanorod-like structures but with larger sizes.

It is well-known that both CNC and PPEs possess good biocompatibility.[1,4,10,11] MTT assay was used to investigate the *in vitro* cytotoxicity of CNC, propargyl-$PEEP_{29}$ and CNC-*g*-$PEEP_{29}$ against HeLa cells and L929 cells, respectively. As shown in Fig. 3, all the samples show negligible cytotoxicity against both cells at different concentrations, and almost the average cell viabilities are above 90% even at high concentrations, indicating the good biocompatibility of CNC and PEEP-modified CNC. In addition, the *in vitro* cytotoxicity of DOX-loaded CNC-*g*-$PEEP_{29}$ nanocrystals against

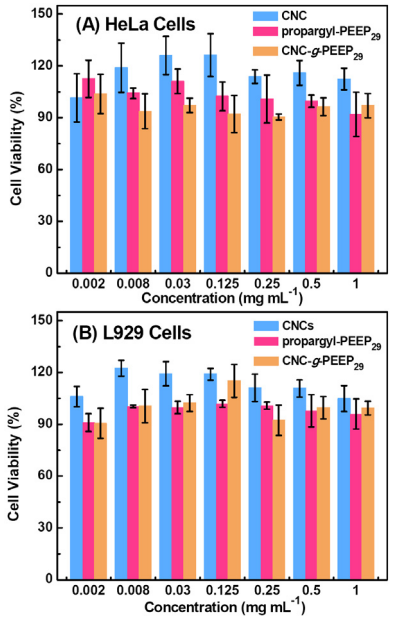

Fig. 3 Cell viability of (A) HeLa cells and (B) L929 cells incubated with CNC, propargyl-$PEEP_{29}$ and CNC-*g*-$PEEP_{29}$ at different concentrations for 48 h, respectively.

HeLa cells was also investigated by the MTT assay using free DOX as a control. The DOX-loaded nanocrystals possessed a slightly lower cytotoxicity against HeLa cells compared with free DOX at the same dosage, as shown in Fig. 4. This result may be caused by the prolonged DOX release from the dissociation of electrostatic interaction. The IC_{50} values (inhibitory concentration to produce 50% cell death) of DOX-loaded CNC-*g*-$PEEP_{29}$ is 9.95 mg DOX equiv L^{-1}, which is higher than that of free DOX (6.38 mg DOX equiv L^{-1}).

The *in vitro* pH-triggered DOX release from DOX-loaded nanocrystals was studied at pH 5.0 and 7.4, respectively. As shown in Fig. 5, the DOX-loaded nanocrystals exhibit a noticeably slow and controlled release process at pH 7.4 compared with the burst release of free DOX as we reported before.[22] Moreover, the release rates at pH 5.0 is much faster than that at pH 7.4, indicating the highly pH-dependent release behavior of DOX-loaded CNC-*g*-$PEEP_{29}$ nanocrystals. This slow release at pH 7.4 is beneficial to a drug carrier during circulation since most of DOX would not be released, thus avoiding some side effects to normal tissues. The fluorescent measurement showed that the drug loading content (DLC) and drug loading efficiency (DLE) values are 13% and 27%, respectively.

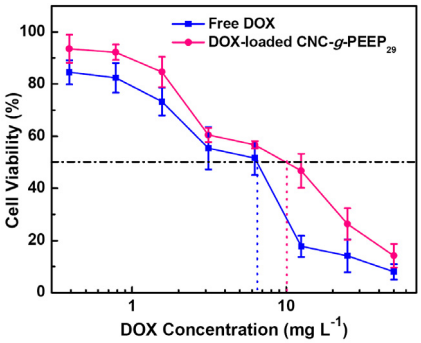

Fig. 4 Cell viability of HeLa cells treated with the DOX-loaded CNC-*g*-$PEEP_{29}$ nanocrystals and free DOX with different DOX concentrations for 48 h.

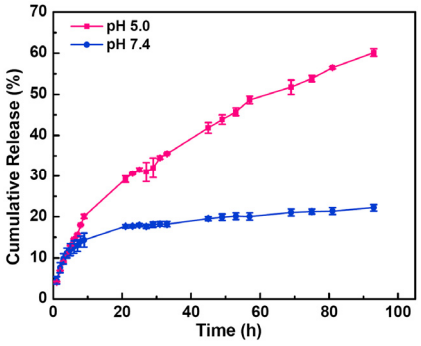

Fig. 5 *In vitro* drug release of the DOX-loaded CNC-*g*-$PEEP_{29}$ nanocrystals at pH 5.0 and 7.4.

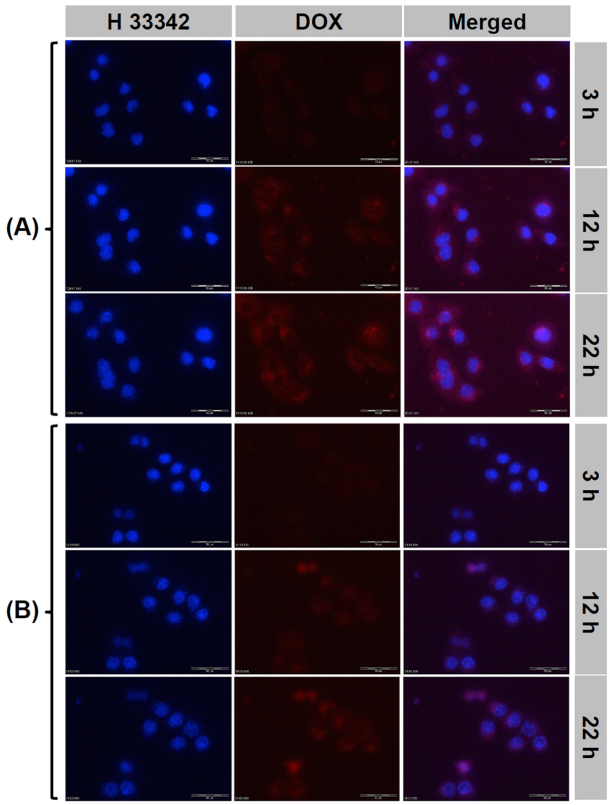

Fig. 6 Fluorescence images of HeLa cells incubated with (A) the DOX-loaded CNC-*g*-$PEEP_{29}$ nanocrystals and (B) free DOX at different incubation time. The DOX dosage was 2 mg L^{-1}. From left to right: Hoechst 33342 (blue), DOX (red), and overlays of the two images. The scale bars correspond to 50 μm in all images.

It has been reported that cellulose has excellent compaction properties when blended with other pharmaceutical excipients so that drug-loaded tablets form dense matrices suitable for the oral administration of drugs. In this case, the key issue is whether CNCs could be internalized by cancer cells or get to the action site.[23,24] This inspired us to speculate that the DOX-loaded CNC-*g*-PEEP may have the ability to deliver DOX into tumor cells. To investigate the intracellular drug release of the DOX-loaded nanocrystals, the live cell imaging system was used to observe the fluorescence intensity of DOX to estimate the release of DOX.

Fig. 6 shows the fluorescence images of HeLa cells incubated with DOX-loaded CNC-*g*-$PEEP_{29}$ using free DOX as a control. From these images, one can find that the red fluorescence intensity of DOX became stronger with the increasing incubation time, indicating the gradual release of DOX from DOX-loaded nanocrystals. Furthermore, we can observe the red fluorescence from DOX in the cytoplasm after 3 h of incubation as shown in Fig. 6(A), while the DOX was dispersed into cytoplasm after 22 h of incubation and several blue fluorescence parts were overlapped by red fluorescence, indicating the successful delivery of DOX into the nuclei. Besides, HeLa cells incubated with free DOX showed the similar fluorescence intensity in the cytoplasm after the same incubation time as shown in Fig. 6(B). Since the cellular uptake of free DOX is a diffusion process, depending on the concentration gradient across the cell membrane of tumor cells, this process

would lead to a slow accumulation of DOX in the nuclei at the low concentration condition.[22,25] The possible mechanism of the interaction between cellulose and cell membrane is still conjectural, as separately reported by Vincent[26] and Brown,[27] that is, some microtubes inside cells may act as the tracks to guide the orientation of cellulose to deposit under specific conditions.

Conclusions

In summary, we have developed a novel polyphosphoester-grafted cellulose nanocrystal (CNC) through the "grafting onto" process. The well-defined propargyl-terminated PEEP was conjugated onto the surface of azide-modified CNC via the CuAAC "click" reaction. The CNC-*g*-PEEP possessed a negatively charged surface which can be used to bind DOX and deliver the drug into the HeLa cells. The results of MTT assay indicated that this CNC-based material showed good biocompatibility to both HeLa cells and L929 cells, while the DOX-loaded CNC-*g*-PEEP nanocrystals exhibited a desirable anticancer activity against HeLa cells. Furthermore, the intracellular drug release observed by a live cell imaging system demonstrated that these DOX-loaded nanocrystals could be internalized into HeLa cells through endocytosis and DOX could be released because of the dissociation of the electrostatic interaction under the acidic environment inside the tumor cells. This research provides a new strategy to prepare the tailor-made polymer-modified CNCs with well-controlled drug release behavior and good pH-response, which can extend the applications of CNCs in biomedical field.

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