Electronic Supplementary Material (ESI)

Intestine-penetrating, pH-sensitive and double-layered nanoparticles for oral delivery of doxorubicin with reduced toxicity

Min Sun, Dapeng Li, Xin Wang, Le He, Xiaodong Lv, Yong Xu, Rupei Tang*

Engineering Research Center for Biomedical Materials, School of Life Science, Anhui Key Laboratory of Modern Biomanufacturing, Anhui University, 111 Jiu long Road, Hefei, Anhui Province, 230601, P. R. China

*Corresponding author.

E-mail address: tangrp99@iccas.ac.cn

Tel.: +86 55163861493

Fax: +86 55163861863

1. Characterization of Poly (ortho ester urethane)

Molecular weight and polydispersity index (PDI) of pure Poly (ortho ester urethane) (POEU) were detected by a Waters 1515 gel permeation chromatography (GPC), with DMF as the mobile phase at a flow rate of 1 mL/min. Then, all GPC results and yield were listed in **Table. S1**.

Table. S1 Diameter index and degree of substitution (DS) of prepared nanogels

Polymer	Yield (%)	Mn [a] ($\times 10^4$)	Mw [b] ($\times 10^4$)	PDI
POEU	85.6	1.81	2.91	1.61

[a] represented the number-average molecular weight.
[a] represented the weight-average molecular weight.

The yield of POEU still hold a high level (85.6%) after three settlement of ice ethyl ether, indicating that this two-step polymerization is a simple and economical reaction. When HMDI-OE-HMDI-PCL was considered as a repeating structural unit, we could calculate that the structural elements were 13.1 by analyzing the Mn of POEU.[1,2]

2. Preparation method of CMC-coated nanoparticles

CMC-coated POEU nanoparticles (NP2) were prepared by similar single emulsion technology. Briefly, 1 mL of DCM that dissolved 20 mg of POEU was mixed intensively with 5 wt% CMC/PBS (pH 8.0) in different volumes, and then sonicated for five times (10 s each time) on ice. Subsequently, the intermixture was injected into 30 mL of 0.3 wt% PVA/PBS (pH 8.0). Immediately, different amounts of glutaraldehyde solution were fed and the system was stirred stirring for 2 hours. Finally, pure NP2 were acquired by centrifugation and then suspended in PBS to determine corresponding hydrated size and PDI by DLS. Additionally, all fed ratios and results was displayed in **Table. S2.**

Table. S2 Diameter index and degree of substitution (DS) of prepared nanogels

Name	POEU (mg)	CMC (mg)	Glutaraldehyde (μg)	Size (nm)	PDI
Formulation 1	20	0.1	2.5	Solid [b]	
Formulation 2	20	0.1	5	872 ± 14	0.43 ± 0.06
Formulation 3 (NP2)	**20**	**0.2**	**2.5**	**359 ± 8**	**0.22 ± 0.02**
Formulation 4	20	0.2	5	Colloid [c]	
Formulation 5	20	0.2	0.125	1613 ± 42	0.77 ± 0.08
Formulation 6	20	0.4	2.5	561 ± 27	0.51 ± 0.11
Formulation 7	20	0.4	5	Colloid [c]	

[b] represented a large amount of precipitation was generated in solution.

[c] represented solution had been converted to colloidal form.

As CMC was used as a cross-linkable emulsifier to prepare the W/O double-layered nanoparticle, its usage must be explored. From **Table. S2,** POEU tend to precipitate more when CMC was less, which was attributed to the inadequate emulsifying capacity of CMC. Besides, when the amount of CMC or glutaraldehyde was high, relative system change to hydrogels due to the over-crosslinking.[3] In general, we

choice formulation 3 to fibrate the oral dual-layered nanoparticles (NP2) because its uniform and suitable size for drug delivery.[4]

3. **FT-IR of nanoparticles**

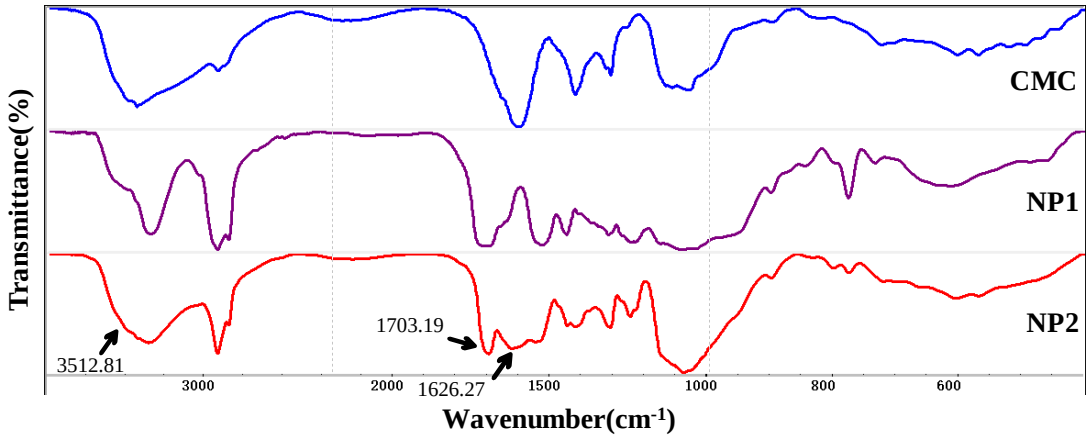

Fig. S1 FT-IR spectra of CMC, NP1 and NP2.

The chemical composition of NP1 and NP2 were analyzed by ATR-Fourier transform infrared spectroscopy (ATR-FTIR, NEXUS-870, Nicolet Instrument Co., USA). Powered carboxymethyl chitosan (CMC) was set as control groups. Then, the results were exhibited in **Fig. S1**.

4. NMR spectrum of HO-OE-OH

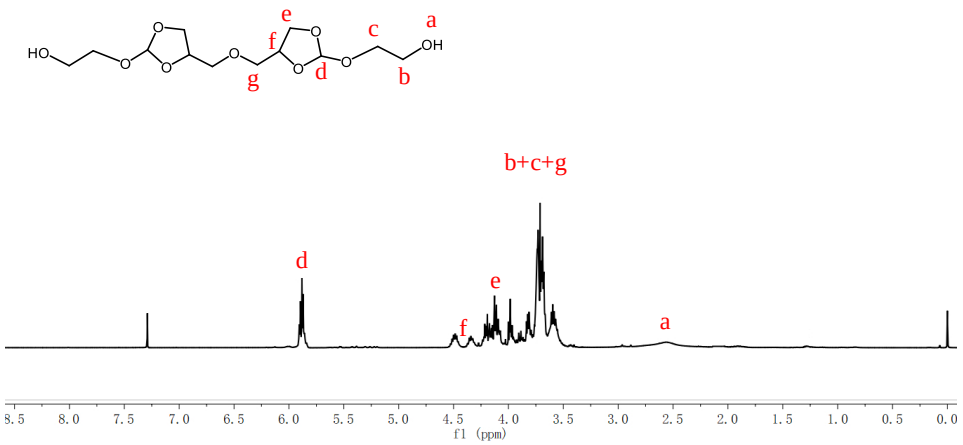

Fig. S2 ^{1}H NMR spectra of HO-OE-OH in $CDCl_3$.

2,2′-((4,4′-(oxybis(methylene)))bis(1,3-dioxolane-4,2-diyl))bis(oxy))diethanol (HO-OE-OH) was synthesized according to pervious works[2] and detail process was showed in manuscript. Then, this compound was dissolved in $CDCl_3$ to obtain corresponding NMR spectrum (Fig. S2). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 2.47 (s, 2H, OH), 3.46–3.84 (m, 12H, CH_2), 3.86–4.22 (m, 4H, CH-O-CH_2), 4.27–4.54 (m, 2H, CH_2-CH-CH_2), 5.81–5.92 (d, 2H, $CH(O)_3$). The integral area ratio of typical peak d and f was 1:1, which is consisted with theoretical proportion.

5. DLS determine

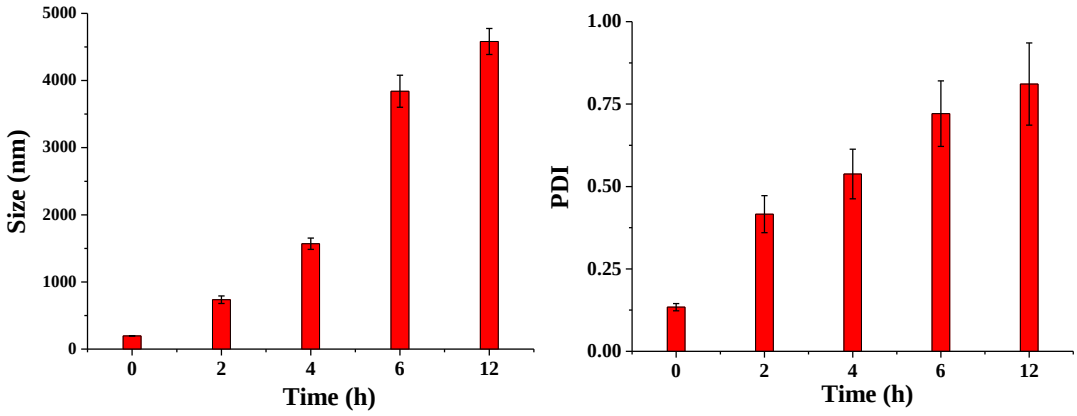

Fig. S3 Average size and polydispersity index change of NP1 over time at pH 1.0.

For investigation of NP1 degradation performance in PBS with pH 1.0, the changes in hydrate diameter and polydisperse index were monitored by DLS and all results were showed in **Fig. S3**. The size and PDI showed a significant increase, represent the quick degradation and aggregation. Besides, the cuvette image at pH 1.0 in **Fig. 3C** showed a remarkable increase in turbidity. To sum up, all phenomenon can be the quick degradation of orthoester and aggregation of hydrophobic POEU.[5,6]

6. Acid stability study of NP1

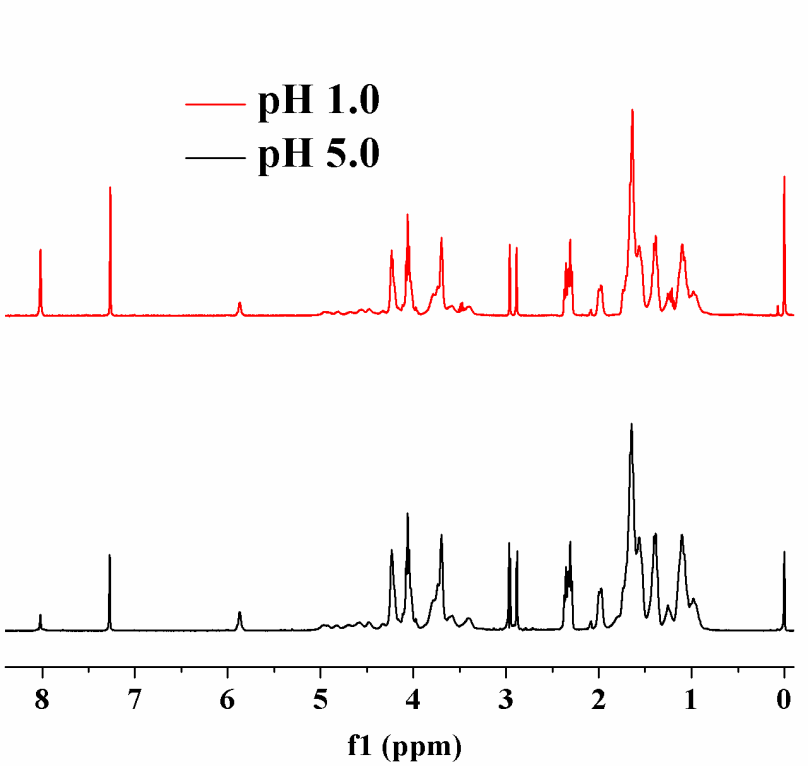

Fig. S4 NMR spectra of NP1 under different acid conditions.

After incubation with acid solution, a new NMR peak (8.05) belonged to the specific degradation peaks of ortho ester[1,2] was obviously observed in corresponding NMR spectra. Moreover, the area of ortho ester NMR peak at 5.81 ppm become smaller compare to NP1 NMR spectrum showed in Fig. 2B. These phenomena indicated that ortho ester in NP1 could be broken under acid environment. Besides, the area of degradation peak in pH 1.0 was bigger than that in pH 5.0, which proved that strong acid could trigger a faster degradation.

7. Acid stability study of NP2

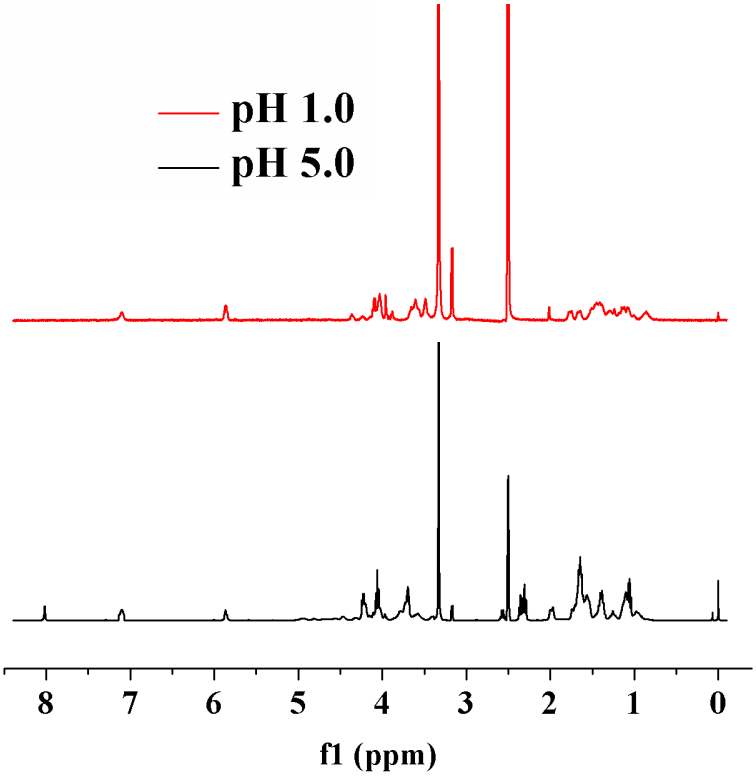

Fig. S5 NMR spectra of NP2 under different acid conditions.

Although the degradation peak of ortho ester in NP2 could be observed after incubation with pH 5.0 for 6 h, the NMR spectrum of NP2 after treatment with strong acid was little different form that in physiological pH (shown in Fig. 2B). All results demonstrated that NP2 possessed great strong-acid stability, which was consisted with its drug release performance. Additionally, these phenomena laid the foundation for NP2 as an oral drug carrier.

8. Cell uptake analysis

We analyze cellular drug concentration by measuring the fluorescence intensity in NP1/DOX and NP2/DOX groups at an excitation wavelength of 480 nm and an emission wavelength of 590 nm[7]. Briefly, 0.2 mL of NP1/DOX or NP2/DOX at same DOX concentration of 8 μg/mL were fed into 1.8 mL of medium cultured with H22 cells. After incubation for pre-set time point (2, 4, 8, 12 h), unbroken cell deposition was collected by centrifugation and washed twice with fresh PBS. All H22 cells were disrupted by cell lysis buffer at 37 °C for 15 min, corresponding cytoplasmic matrix was obtained and detected using SpectraMax M2e Molecular devices. H22 cells treated with free DOX was set as control group and the intracellular drug concentration was calculated by standard curve. In addition, these results were showed as following:

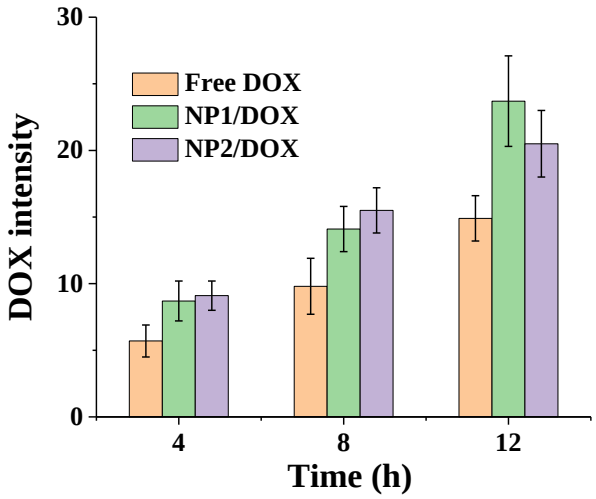

Fig. S6 DOX fluorescence intensity after treatment with virous DOX formulations.

9. Cytotoxicity analysis of DOX-loading nanoparticles

We tested the anticancer activity of drug-loading NP by CCK8 assay. Briefly, 20 μL of NP1/DOX or NP2/DOX at a series of DOX concentration (2, 4, 8, 16 μg/mL) were fed into 180 μL of medium cultured with H22 cells. After culture for 24 h, 10 μL of CCK8 solution were added for further 2 h of incubation. The absorbance of samples was measured by SpectraMax M2e Molecular at 450 nm. In addition, all tests at least were repeated three times and H22 cells treated with PBS was rated as 100% cell viability.

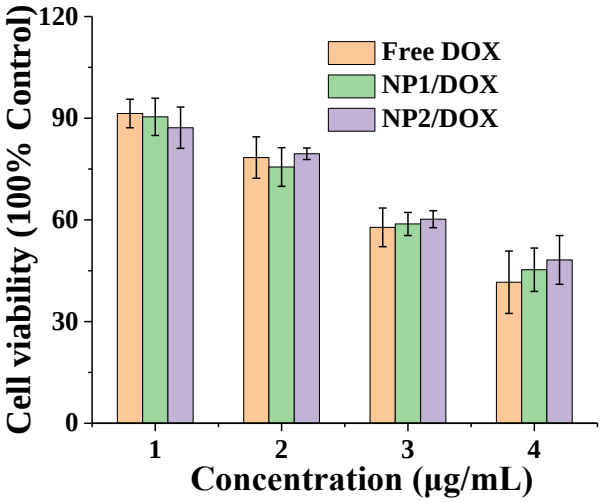

Fig. S7 CCK8 assay against H22 cells with nanoparticles at various concentration.

Reference

1. S. Fu, G. Yang, J. Wang, X. Wang, X. Cheng, R Tang, *Polymer,* 2017, **114**, 1–14.

2. S. Fu, G. Yang, J. Wang, X. Wang, X. Cheng, Q. Zha, R. *Tang, Eur. Polym. J.,* 2017, **95**, 275–288.

3. M. N. R. Kumar, *React. Funct. Polym.,* 2000, **46**, 1-27.

4. K. Y. Win, S. S. Feng, *Biomaterials*, 2005, ***26*****,** 2713-2722.

5. J. Heller, J. Barr, S. Y. Ng, K. S. Abdellauoi, R. Gurny, *Adv. Drug Deliver. Rev.,* 2002, **54**, 1015-1039.

6. Z. Qiao, R. Zhang, F. Du, D. Liang, Z. Li, *J. Control. Release,* 2011, **152**, 57-66.

7. M. Sun, X. Wang, X. Cheng, L. He, G. Yan, R. Tang, *Carbohyd. Polym.,* 2018, **198**, 142-154.