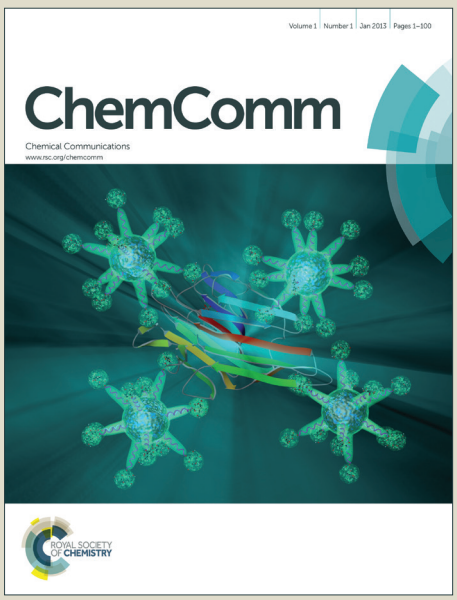
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Injectable drug-loaded hydrogel based on supramolecular polymeric prodrug†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Lu Xiong,‡[a] Qiaojie Luo,‡[b] Ying, Wang,[a] Xiaodong Li,*[b] Zhiquan Shen,[a] and Weipu Zhu*[a]

We reported a novel injectable doxorubicin-loaded hydrogel based on host-guest interaction and Schiff's base reaction. A supramolecular polymeric prodrug was prepared through the inclusion of adamantane-modified doxorubicin into the β-cyclodextrin cavity on the polyaldehyde dextran chain, which was *in situ* crosslinked by carboxymethyl chitosan.

Cancer is one of the leading causes of death in the world today and has seriously threatened human health.[1, 2] Although surgical resection is the preferred therapy for the early stage solid tumors, the recurrence rate of tumors is high after surgery.[3] Chemotherapy is the main treatment for tumor recurrence, especially for the middle and late stage tumors.[4] However, chemotherapeutic drugs are difficult to reach the tumor tissues selectively and cause undesirable side effects on human normal tissues during therapy.[5, 6]

To overcome these drawbacks, nanocarriers for drug delivery, including liposomes,[7-10] microspheres,[11-13] micelles,[14-19] have been widely investigated and developed. Nevertheless, these nanocarriers are transported along the blood circulatory to tissues and penetrate from blood vessel walls into targeted tumor sites.[20] As a result, only a fraction of drugs can reach the tumor tissues, thus reducing therapeutic efficacy and increasing toxicity to normal tissues.[21] Compared to the traditional chemotherapy, the localized drug delivery can not only control the steady load and sustained release of anticancer drugs, but also avoid the systemic circulation of drugs and reduce toxicity to normal tissues. To achieve the best therapeutic efficacy of anticancer drugs, numerous attempts have been made to develop localized drug delivery systems.[22, 23]

Hydrogels are a class of materials which present a three dimensional structure and can maintain a significant amount of water, which have showed promising potential for biomedical applications.[24-27] Injectable hydrogels, which can *in situ* form gel in a minimally invasive way, maintain the drugs within the target tumor tissues, and perform sustained drug release, have caused widespread concern for their potential applications for intratumor drug delivery.[28, 29] Direct drug embedding into the hydrogel can allow the hydrophilic drugs to homogeneously diffuse into the pores of the hydrogel. However, the release of the loaded drugs is not well regulated and shows a burst release.[30] One the other hand, some hydrophobic drug could also be diffused into the hydrogels employing amphiphilic block polymers as precursors.[31, 32] Nevertheless, because of the weak interaction between drug and hydrophobic segment, the stability and drug loading capacities of this kind of hydrogels are limited.

In order to avoid the burst release and enhance drug loading capacity, antitumor drugs have been covalently bonded onto the hydrophilic polymeric carriers to form polymeric prodrugs, so called polymer-drug conjugates,[33-35] which can release the drugs when triggered by unique biological stimuli such as pH,[36] redox potential[37] or enzyme[38] for anticancer chemotherapy. For example, the obvious difference in pH between normal tissues (pH ~ 7.4) and acidic tumor tissues (pH < 6.8) has motivated researchers to design pH-responsive polymeric prodrugs via acid-labile covalent linkages like benzoic-imine bond,[39] Schiff's base bond,[40] and hydrazone bond [41] for tumor targeted delivery. Kataoka et al[42] have reported a pH-sensitive polymeric prodrugs by which doxorubicin (DOX) was conjugated to the poly(ethylene glycol)-*b*-poly(β-benzyl-L-aspartate) through acid-sensitive hydrazone linkages, which showed a highly sensitive drug release under an acidic microenvironment. This kind of covalent conjugations has improved the stability and loading capacity of drugs. However, because all polymers have polydispersity, these covalently bonded polymeric prodrugs are not pure chemical compounds, but mixtures, which makes them difficult to be approved by the drug administration of most countries.

In this study, based on host-gust interaction, we designed and synthesized a novel supramolecular polymeric drug as hydrogel precursor to solve this problem. First, β-cyclodextrin-modified polyaldehyde dextran (PAD-CD), was synthesized through the Schiff's base reaction between amino β-cyclodextrin and polyaldehyde dextran. Meanwhile, a small molecular prodrug, adamantane-modified doxorubicin (AD-DOX), was synthesized via an acid-labile benzoic–imine linkage between adamantane benzaldehyde and doxorubicin. The supramolecular polymeric prodrug could be facilely prepared form PAD-CD and AD-DOX through the strong host-guest interaction between CD and AD moieties, which could combine the advantages of polymeric prodrug and purity of small molecular prodrug. Then supramolecular polymeric prodrug was *in situ* crosslinked by carboxymethyl chitosan, resulting in an injectable DOX-loaded hydrogel (Scheme

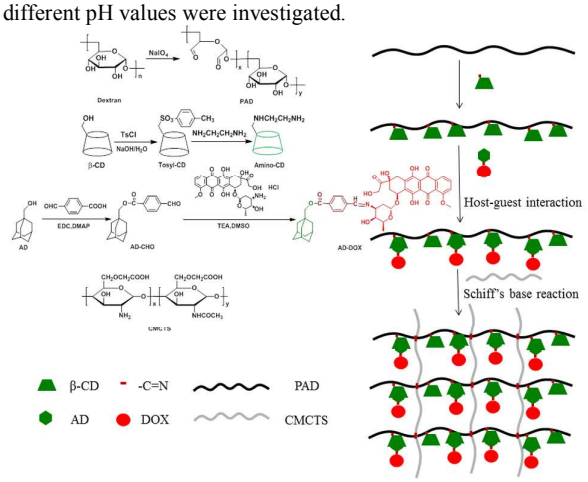
[a] *MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, People's Republic of China. Fax: +86 571 87953727; Tel: +86 571 87953739; E-mail: zhuwp@zju.edu.cn.*

[b] *Affiliated Stomatology Hospital, School of Medicine, Zhejiang University, Hangzhou 310006, China. Fax: +86 571 87217430; Tel: +86 571 87217430; E-mail: cisarli@zju.edu.cn.*

† Electronic Supplementary Information (ESI) available: Details of synthesis and characterizations. See DOI: 10.1039/x0xx00000x

‡ L. Xiong and Q. J. Luo contributed equally to this work.

1). The degradation behavior and *in vitro* DOX release upon different pH values were investigated.



Scheme 1 Preparation of the injectable DOX-loaded hydrogel.

A supramolecular polymeric prodrug was prepared *via* the host-guest interaction between adamantane-modified doxorubicin (AD-DOX) and β-Cyclodextrin-modified polyaldehyde dextran (PAD-CD). AD-DOX was synthesized through the reaction between the aldehyde group of 1-adamantanemethyl 4-formylbenzoate (AD-CHO) and the amino group of DOX, which was characterized by ^{1}H NMR. The signals of corresponding protons were well assigned with the chemical structure. Notably, the absence of the signal of aldehyde proton (H^g, 10.1 ppm, Fig. S1A) and the presence of the imine proton ($H^{g'}$, 8.0 ppm, Fig. S1B) clearly confirmed the formation of the imine bond. PAD-CD was synthesized by Schiff's base reaction between amino-CD (Fig. S2) and PAD, which was characterized by ^{1}H NMR (Fig. S3) and Fourier transform infrared spectroscopy (FT-IR) (Fig. S4). The new absorption peaks at 1650 cm^{-1} and 850 cm^{-1} in FT-IR spectrum corresponding to azomethine (CH=N) group[40] suggested the acquisition of PAD-CD. The supramolecular polymeric prodrug (PAD-CD/AD-DOX) was characterized by proton nuclear magnetic resonance (^{1}H NMR) with D_2O as the solvent (Fig. S5). The signals of AD-DOX that was insoluble in water were clearly observed, which indicated the AD-DOX had been successfully attached onto PAD-CD backbone *via* the host-guest interaction. The actually loaded DOX in supramolecular polymeric prodrug is 2.06 wt-%, which was determined by UV-vis spectrometry after treating the supramolecular polymeric prodrug with citrate buffer solution (pH 3.0) for 48 h.

An injectable DOX-loaded hydrogel was prepared via Schiff's base reaction between supramolecular polymeric prodrug and carboxymethyl chitosan. Fig. 1A clearly displayed the *in situ* formation of DOX-loaded hydrogel. Briefly, after mixing supramolecular polymeric prodrug and carboxymethyl chitosan solutions at a concentration of 4 wt-%, the DOX-loaded hydrogel was formed rapidly within 1 minute. The formation of injectable hydrogels was further confirmed by FT-IR spectra as shown in Fig. S6. The disappearance of the peak at 1730 cm^{-1} and the enhanced absorbance at 850 cm^{-1} clearly demonstrated the complete reaction between the aldehyde groups of PAD-CD and the amino groups of CMCTS. The mechanical properties of DOX-loaded hydrogel and blank hydrogel were evaluated by monitoring the variations of storage modulus (G') and loss modulus (G'') as a function of frequency at 37 °C. As shown in Fig. 1B, G' values of both DOX-loaded hydrogel and blank hydrogel were independent of frequencies, indicating the formation and stability of covalently crosslinked hydrogel networks. Moreover, G' values (2.4 kPa) were significantly higher than the corresponding G'' values, demonstrating the high elasticity of hydrogels. Notably, the storage modulus of DOX-loaded hydrogel was slightly higher than that of blank one, which may be attributed to hydrophobic interactions of DOX moieties leading to a denser network structure in DOX-loaded hydrogel than that in blank hydrogel. SEM images were used to characterize the morphologies of DOX-loaded hydrogel and blank hydrogel (Fig. 1C). Both hydrogels displayed macroporous and regular networks, indicating a homogeneous reaction during the gelation. Importantly, the pore diameter of DOX-loaded hydrogel was slightly smaller than that of blank hydrogel, which was consistent with the results of rheological experiments. Swelling curves of the lyophilized blank hydrogel in different phosphate buffer saline (PBS) solutions were presented in Fig. S7. All hydrogels in PBS solutions basically reached equilibrium swelling within 72 h with high swelling ratios ranged from 3,500 % to 4,000 %, depending on the pH values of PBS solutions.

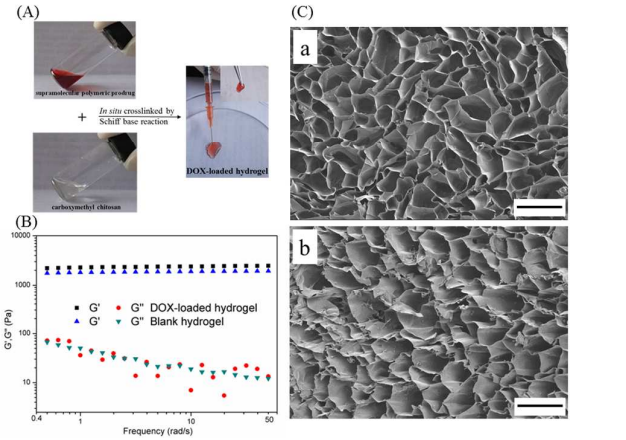


Fig. 1 (A) Photographs of *in situ* formation of DOX-loaded hydrogel; (B) Frequency dependency of storage modulus (G') and loss modulus (G'') of DOX-loaded hydrogel and blank hydrogel; (C) SEM images of DOX-loaded hydrogel (a) and blank hydrogel (b), scale bars are 100 μm for both images.

In vitro DOX release behavior from DOX-loaded hydrogels was investigated at pH 5.8, 6.8 and 7.4. As shown in Fig. 2A, the cumulative release of DOX was suppressed at pH 7.4, and only about 20 % of DOX was released from the hydrogels within 17 days. However, under acidic conditions (pH 6.8 and 5.8), the DOX was released much faster than at a physiological pH. For instance, about 45 % of DOX was eleased from the hydrogels at pH 6.8 was in 17 days. Moreover, the DOX release was markedly accelerated by decreasing the pH of release media to 5.8. All of the loaded DOX was released from the hydrogels in 17 days. This pH-responsive release behavior may be ascribed to the acid-labile benzoic-imine linkage of the small molecular prodrug, which is stable at physiological pH and labile at acidic pH. Based on the cumulative release of DOX from the hydrogel in pH 5.8 PBS solution, the actually loaded DOX in hydrogel was calculated to be 318 μg/mL, which is in great accord with the feeding value.

When DOX was recovered due to the cleavage of benzoic-imine bond at low pH, it may remain in the 3D network of the hydrogels for a certain period before diffusing into the release medium. So the degradation rate of hydrogel may also influence the DOX release behavior. To confirm this speculation, the *in vitro* degradation behaviors of the blank hydrogels were also investigated at pH 5.8, 6.8 and 7.4. As presented in Fig. 2B, the degradation of the blank hydrogels was greatly accelerated in the acidic environment (pH 5.8) with respect to the physiological pH condition (pH 7.4), due to the acid-sensitive imine crosslinkages. That is to say, the hydrogels exhibited a pH-responsive degradation behavior, similar to the DOX

release behavior from DOX-loaded hydrogels. Notably, an apparent increase of weight loss of the blank hydrogels was observed from the twelfth day at pH 5.8 (in Fig. 2B), and a significant enhancement of cumulative DOX release from DOX-loaded hydrogels also appeared at corresponding time (in Fig. 2A). This phenomenon verified that the pH-responsive degradation of the hydrogel made it easy for DOX diffusing from the hydrogel to the release medium, especially in the latter period of release. As we know, the pH of tumor tissues is acidic (pH < 6.8). The benzoic-imine bonds incorporated to the hydrogels could be cleaved under this condition. Moreover, the hydrogel destructed under weak acidic environment, leading to rapid drug release from the DOX-loaded hydrogel due to the breakage of the pH cleavable Schiff's base bonds.

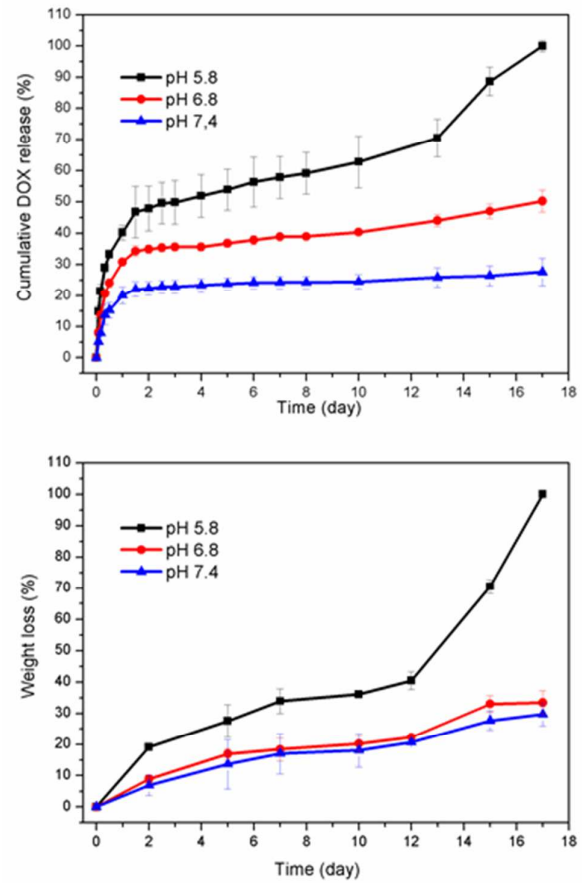


Fig. 2 (A) *In vitro* DOX release profiles of DOX-loaded hydrogels at pH 5.8, 6.8 and 7.4; (B) *In vitro* degradation profiles of blank hydrogels at pH 5.8, 6.8 and 7.4.

To evaluate the cytotoxicity of the hydrogels, HeLa cells were exposed to blank hydrogels, DOX-loaded hydrogels and free DOX for a series of time ranging from 24 h to 72 h by the MTT assay. The dose of DOX was 300 μg/mL. As shown in Fig. 3, the blank hydrogels showed no significant cytotoxicity on the growth inhibition of HeLa cells, while a clear inhibition of the growth of HeLa cells was observed when the cells were treated with either DOX-loaded hydrogel or free DOX in PBS solution with time increasing. In addition, it should be noticed that the DOX-loaded hydrogel at the equivalent DOX dosage exhibited lower cytotoxicity compared to the free DOX, which may result from the controlled sustained release behavior of DOX from the hydrogel.

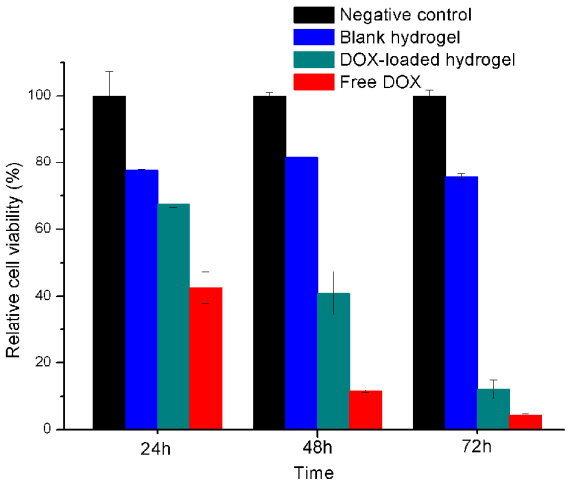


Fig. 3 *In vitro* cytotoxicities of blank hydrogel and DOX-loaded hydrogel on HeLa cells determined by MTT assay.

In conclusion, a supramolecular polymeric prodrug was prepared based on host-guest interaction from β-cyclodextrin-modified polyaldehyde dextran and adamantane-modified doxorubicin, which could be *in situ* crosslinked by carboxymethyl chitosan under physiological conditions, to give injectable DOX-loaded hydrogel. Acid-responsive release of DOX from the hydrogel was observed due to the acid-labile benzoic-imine bond between DOX and adamantane moieties, as well as the imine crosslinkages of the hydrogels. Furthermore, as a small molecular prodrug with high chemical purity, AD-DOX was non-covalently conjugated to the framework of hydrogel, which makes this drug-loaded hydrogel a promising candidate as drug carrier for intratumor drug delivery in clinical applications.

Acknowledgements

The work was financially supported by the National Natural Science Foundation of China (21274121 and 51173163), the Major State Basic Research Project (2011CB606001) and the Fundamental Research Funds for the Central Universities (2015QNA4036).

Notes and references

1 P. Jha, *Nat. Rev. Cancer*, 2009, **9**, 655-664.
2 D. Wirtz, K. Konstantopoulos and P. C. Searson, *Nat. Rev. Cancer*, 2011, **11**, 512-522.
3 C. R. Kelsey, L. B. Marks, D. Hollis, J. L. Hubbs, N. E. Ready, T. A. D'Amico and J. A. Boyd, *Cancer*, 2009, **115**, 5218-5227.
4 H. Gelderblom, J. Verweij, K. Nooter and A. Sparreboom, *Eur. J. Cancer*, 2001, **37**, 1590-1598.
5 D. Peer, J. M. Karp, S. Hong, O. C. FaroKhzad, R. Margalit and R. Langer, *Nat. Nanotechnol.*, 2007, **2**, 751-760.
6 M. Verbrugghe, S. Verhaeghe, K. Lauwaert, D. Beeckman and A. Van Hecke, *Cancer Treat. Rev.*, 2013, **39**, 610-621.
7 P. Crosasso, M. Ceruti, P. Brusa, S. Arpicco, F. Dosio and L. Cattel, *J. Control. Release*, 2000, **63**, 19-30.
8 G. Wu, A. Milkhailovsky, H. A. Khant, C. Fu, W. Chiu and J. A. Zasadzinski, *J. Am. Chem. Soc.*, 2008, **130**, 8175-8177.
9 Y. Malam, M. Loizidou and A. M. Seifalian, *Trends Pharmacol. Sci*, 2009, **30**, 592-599.
10 H. J. Chen, X. R. Huang, X. B. Zhou, B. Y. Zheng and J. D. Huang, *Chem. Commun.*, 2015, **51**, 4681-4684.
11 G. Ruan and S. S. Feng, *Biomaterials.*, 2003, **24**, 5037-5044.
12 J. You, R. P. Shao, X. Wei, S. Gupta and C. Li, *Small*, 2010, **6**, 1022-1031.

13 X. J. Kang, Y. L. Dai, P. A. Ma, D. M. Yang, C. X. Li, Z. Y. Hou, Z. Y. Cheng and J. Lin, *Chem-Eur. J.*, 2012, **18**, 15676-15682.
14 K. Kataoka, A. Harada and Y. Nagasaki, *Adv. Drug Deliver. Rev.*, 2001, **47**, 113-131.
15 K. Zhang, Y. Wang, W. Zhu, X. Li and Z. Shen, *J. Polym. Sci. Part A: Polym. Chem.*, 2012, **50**, 2045-2052.
16 Y. Wang, H. Du, L. L. Gao, H. G. Ni, X. D. Li, W. P. Zhu and Z. Q. Shen, *Polym. Chem.*, 2013, **4**, 1657-1663.
17 W. P. Zhu, Y. Wang, X. Cai, G. Y. Zha, Q. J. Luo, R. Sun, X. D. Li and Z. Q. Shen, *J. Mater. Chem. B*, 2015, **3**, 3024-3031.
18 J. X. Ding, L. H. Chen, C. S. Xiao, L. Chen, X. L. Zhuang and X. S. Chen, *Chem. Commun.*, 2014, **50**, 11274-11290.
19 R. Sun, Q. J. Luo, C. Gao, Y. Wang, L. L. Gao, H. Du, Y. Huang, X. D. Li, Z. Q. Shen and W. P. Zhu, *Polym. Chem.*, 2014, **5**, 4879-4883.
20 J. B. Wolinsky, Y. L. Colson and M. W. Grinstaff, *J. Control. Release*, 2012, **159**, 14-26.
21 H. L. Wong, R. Bendayan, A. M. Rauth, Y. Li and X. Y. Wu, *Adv. Drug Deliver. Rev.*, 2007, **59**, 491-504.
22 A. C. Anselmo and S. Mitragotri, *J. Control. Release*, 2014, **190**, 15-28.
23 V. P. Torchilin, *Nat. Rev. Drug Discov.*, 2014, **13**, 813-827.
24 C. Gong, C. Wang, Y. Wang, Q. Wu, D. Zhang, F. Luo and Z. Qian, *Nanoscale*, 2012, **4**, 3095-3104.
25 Y. L. Li, J. Rodrigues and H. Tomas, *Chem. Soc. Rev.*, 2012, **41**, 2193-2221.
26 H. Du, G. Y. Zha, L. L. Gao, H. Wang, X. D. Li, Z. Q. Shen and W. P. Zhu, *Polym. Chem.*, 2014, **5**, 4002-4008.
27 H. Wang, G. Y. Zha, H. Du, L. L. Gao, X. D. Li, Z. Q. Shen and W. P. Zhu, *Polym. Chem.*, 2014, **5**, 6489-6494.
28 H. T. Ta, C. R. Dass and D. E. Dunstan, *J. Control. Release*, 2008, **126**, 205-216.
29 L. Zhao, L. Zhu, F. Liu, C. Liu, D. Shan, Q. Wang, C. Zhang, J. Li, J. Liu, X. Qu and Z. Yang, *Int. J. Pharm.*, 2011, **410**, 83-91.
30 X. Huang and C. S. Brazel, *J. Control. Release*, 2001, **73**, 121-136.
31 N. K. Singh and D. S. Lee, *J. Control. Release*, 2014, **193**, 214-227.
32 W. Zhang, X. Zhou, T. Liu, D. Ma and W. Xue, *J. Mater. Chem. B*, 2015, **3**, 2127-2136.
33 R. Duncan, *Nat. Rev. Cancer*, 2006, **6**, 688-701.
34 P. F. Gou, W. P. Zhu and Z. Q. Shen, *Biomacromolecules*, 2010, **11**, 934-943.
35 Y. Wang, Q. Luo, L. Gao, C. Gao, H. Du, G. Zha, X. Li, Z. Shen and W. Zhu, *Biomater. Sci.*, 2014, **2**, 1367-1376.
36 Y. Wang, Q. Luo, R. Sun, G. Zha, X. Li, Z. Shen and W. Zhu, *J. Mater. Chem. B*, 2014, **2**, 7612-7619.
37 L. Bai, X. H. Wang, F. Song, X. L. Wang and Y. Z. Wang, *Chem. Commun.*, 2015, **51**, 93-96.
38 A. Tanaka, Y. Fukuoka, Y. Morimoto, T. Honjo, D. Koda, M. Goto and T. Maruyama, *J. Am. Chem. Soc.*, 2015, **137**, 770-775.
39 H. Deng, Y. Zhang, X. Wang, Jianhuazhang, Y. Cao, J. Liu, J. Liu, L. Deng and A. Dong, *Acta Biomater.*, 2015, **11**, 126-136.
40 J. Shi, W. Guobao, H. Chen, W. Zhong, X. Qiu and M. M. Q. Xing, *Polym. Chem.*, 2014, **5**, 6180-6189.
41 M. Prabaharan, J. J. Grailer, S. Pilla, D. A. Steeber and S. Gong, *Biomaterials.*, 2009, **30**, 5757-5766.
42 Y. Bae, N. Nishiyama, S. Fukushima, H. Koyama, M. Yasuhiro and K. Kataoka, *Bioconjugate Chem.*, 2005, **16**, 122-130.