RSC Advances

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

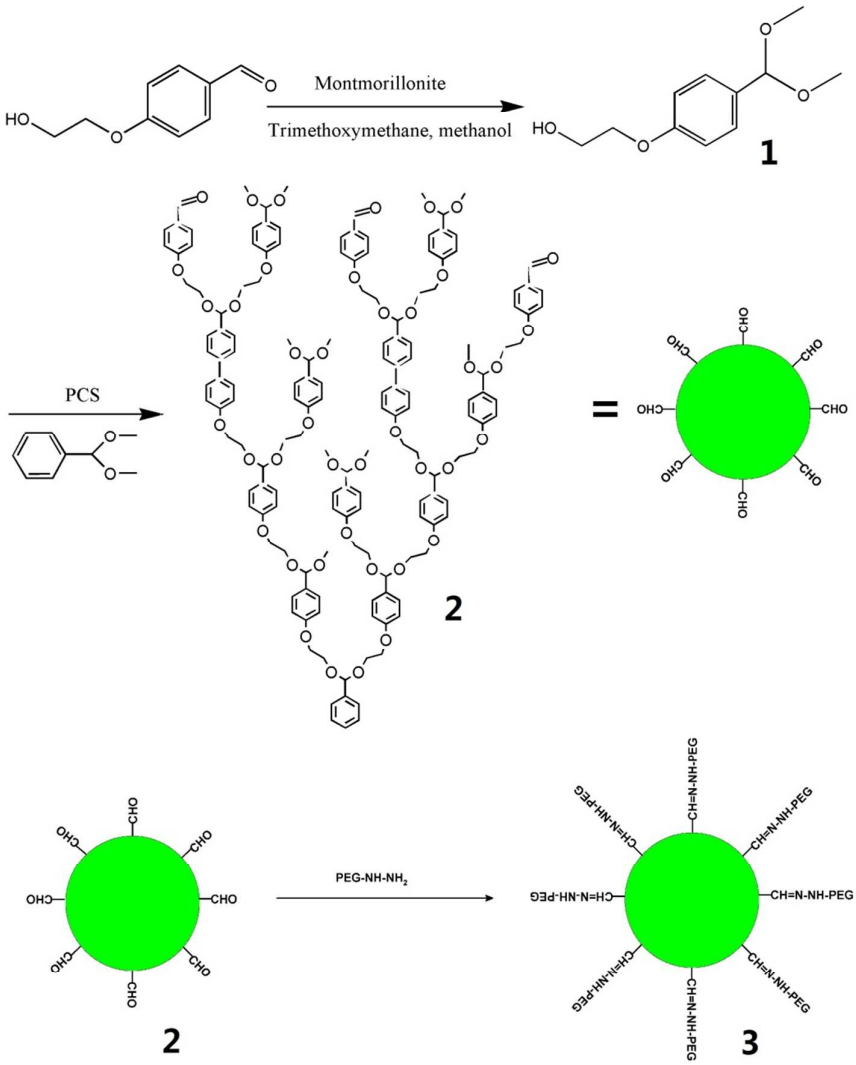
Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Abstract:

Amphiphilic hyperbranched polymer containing quantitative of pH sensitive bonds-acetal and hydrazone bonds-was synthesized in our group. The hydrophobic chains consist of hyperbranched polyacetals and the hydrophilic chains are PEG. PEG are attached to the hyperbranched polyacetals by the hydrazone bonds. The amphiphilic hyperbranched polymer could assembled into micelles easily by dialysis method. The micelles containing quantitative of pH sensitive bonds were quite fragile in pH 5.0 buffer solution but very stable in pH 7.4 buffer solution. DOX-loaded micelles were also prepared by the dialysis method. The size of blanked micelle and DOX-loaded micelle were 30nm and 35nm respectively. The increase of the diameter confirmed that DOX was loaded into the micelle successfully. Drug loading and drug loading efficiency was 2.34% and 23.4% respectively which was detected by the UV-VIS at the wavelength of 482nm. The drug release behavior demonstrated that DOX was released faster in pH 5.0 buffer solution than in pH 7.4 buffer solution.



RSC Advances Accepted Manuscript

RSC advances RSCPublishing

ARTICLE

Synthesis of an amphiphilic hyperbranched polymer as a novel pH-sensitive drug carrier

Cite this: DOI: 10.1039/xoxxoooooox

Chen Rui[a,c], Wang Liqun[a,b]*

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

Amphiphilic hyperbranched polymer containing quantitative of pH sensitive bonds-acetal and hydrazone bonds-was synthesized in our group. The hydrophobic chains consist of hyperbranched polyacetals and the hydrophilic chains are PEG. PEG are attached to the hyperbranched polyacetals by the hydrazone bonds. The amphiphilic hyperbranched polymer could assembled into micelles easily by dialysis method. The micelles containing quantitative of pH sensitive bonds were quite fragile in pH 5.0 buffer solution but very stable in pH 7.4 buffer solution. DOX-loaded micelles were also prepared by the dialysis method. The size of blanked micelle and DOX-loaded micelle were 30nm and 35nm respectively. The increase of the diameter confirmed that DOX was loaded into the micelle successfully. Drug loading and drug loading efficiency was 2.34% and 23.4% respectively which was detected by the UV-VIS at the wavelength of 482nm. The drug release behavior demonstrated that DOX was released faster in pH 5.0 buffer solution than in pH 7.4 buffer solution.

RSC Advances Accepted Manuscript

Introduction

Hyperbranched polymers are highly branched macromolecules with three-dimensional dentritic architectures. Due to their unique physical and chemical properties and potential applications in various fields from drug delivery to coatings, interest in hyperbranched polymers is growing rapidly [1-5].

Compared to linear polymers, they have a number of beneficial attributes for biomedical applications, including the following: 1) biodistribution and pharmacokinetic properties that can be tuned by controlling dendrimer size and conformation; 2) high structural and chemical homogeneity; 3) ability to be functionalized with multiple copies of drugs, chromophores orligands either at their peripheries and/or their interiors; 4) high ligand density; 5) controlled degradation[6]. Due to the above advantages, many hyperbranched polymers are designed as drug delivery system for the cancer therapy, such as PAMAM[7-9], Polyether[10], Polyester[11] and Polypeptide[12-14].

However, as the cancer therapy, it is better to bestow the carrier with some response characteristics, such as pH, redox, and temperature responsiveness. Jean M.J. Frechet and cowokers[10] synthesized hyperbranched polyesters which was used as drug carrier. In this system, DOX was covalently bound via a hydrazine linkage to a high molecular weight 3-arm poly (ethylene oxide)-dendrimer hybrid. Drug release was a function of pH, and the release rate was more rapid at pH<6. Doron shabat and coworkers[15] designed a dendritic pro-drug that is activated through a single catalytic reaction by a specific enzyme. It could offer significant advantages in the inhibition of tumor growth, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. Jayachandran N. Kizhakkedathu and co-workers[16] synthesized a new class of biodegradable polymers, main chain biodegradable hyperbranched polyglycerols with randomly distributed acid-labile ketal groups in the medium high molecular weight range. In vitro degradation studies showed that this polymer was relatively stable at physiological pH, but underwent pH dependent dydrolysis at acidic pH values.

Among all of the hyperbranched polymers, we have not heard of a hyperbranched polymer containing both acetal and hydrazine bonds in the backbone of the macromelecular. In our group, we synthesized the amphiphilic hyperbranched polymer which contains dual pH responsive bonds: acetal bond and hydrazine bond. The hyperbranched core which is composed of acetal linkages is

surrounded by the PEG shell, and the PEG was linked to the core by the acid sensitive linkage hydrazine bond. This new type of hyperbranched polymers are quite pH sensitive which can be used as drug carrier in the future.

Experimental

Materials

Mono-methoxy polyethylene glycol(Mw:1900), 4-(2-hydroxyethoxy)-benzaldehyde were purchased from Tokyo Chemical Industry Co.,Ltd. Trimethyl orthoformate, (±)-camphor-10-sulfonic acid, Montmorillonite K10 and Benzaldehyde dimethyl acetal were purchased from Alfa Aesar Co.,Ltd. 4-Nitrophenyl carbonochloridate(p-NPC) was purchased from Adamas Reagent Co., Ltd. Hydrazine monohydrate was purchased from Aladdin Reagent Co., Ltd. Triethylamine, methylene chloride, methanol and n-hexane were purchased from Sinopharm Chemical Reagent Co., Ltd. The all reagents were used as received.

Synthesis and preparation

(1) The synthesis of HBPAs-Hydrazone-PEG [17, 18]

a. The synthesis of the monomer 4-(2-Hydroxyethoxy)-benzaldehyde dimethyl acetal

5g of 4-(2-Hydroxyethoxy)-benzaldehyde, 50mg of Montmorillonite K10 (catalyst), 30ml of Trimethyl orthoformate and 100ml of methanol were added into a 250 ml of flask and refluxed at 100℃ with magnetic stirring for 24h. After the reaction, the catalyst was removed by vacuum filtration and the solvents (methanol and trimethylorthoformate) were removed by rotary evaporation. And then, the monomer 4-(2-Hydroxyethoxy)-benzaldehyde dimethyl acetal was acquired. (The H^1-NMR characterization was in supporting information Fig 2).

b. The synthsis of hyperbranched polyacetals (HBPAs)

1.7g of 4-(2-Hydroxyethoxy)-benzaldehyde dimethyl acetal, 0.08g of PCS [19]and 20μl of Benzaldehyde dimethyl acetal were added in a 10ml of reaction tube. The mixture was stirred for 15min under Ar atmosphere at room temperature and kept stirring at 70 ℃ for another 15min. And then, the temperature was heated to 110℃ slowly and continued stirring for 1 hour under Ar atmosphere. 1 hour later, the reaction was cooled to room temperature and then heated to 110℃ again under vacuum (< -0.1MPa) for another 1h. After the reaction cooled to room temperature, 5ml of THF was added to dissolve the crude product and then the catalyst PCS was removed by vacuum filtration. THF was removed by rotary evaporation and HBPAs was acquired.

c. The synthesis of HBPAs-hydrazone-PEG

180mg of HBPAs and 2.75g of pre-synthesized Mono-methoxy PEG-$NHNH_2$ [20, 21](see supporting information) were dissolved in 10ml of DMF. 20μl of triethylamine was added into the solution. The reaction was kept at room temperature and stirred for 24 hours. 24 hours later, the solution was dialyzed against deionized water for another 24 hours (molecular weight cut off: 3500). Finally, HBPAs-Hydrazine-PEG was obtained by freeze-drying.

(2) The degradation of HBPAs and micelles

a. The degradation of HBPAs

5mg of HBPAs was dissolved into 5ml of DMF, and dialyzed against 50 ml of buffer solution (molecular cutoff: 100-500). At the predetermined time, 4 ml of buffer solution was withdrawn and 4 ml of fresh buffer solution was then added. 20μl of collected buffer solution was injected into the HPLC system to detect the hydrolyzed product 4-(2-Hydroxyethoxy)-benzaldehyde. And then, calculated the accumulation of the hydrolyzed 4-(2-Hydroxyethoxy)-benzaldehyde as a function of the time.

b. The degradation of the HBPAs-hydrazone-PEG micelle

1mg/ml HBPAs-hydrazone-PEG micelle was diluted by the same volume of buffer solution. 30min later, the diluted micelle was detected by the DLS instrument for size analysis.

(3) Preparation of the HBPAs-hydrazine-PEG micelle and DOX-loaded micelles

The HBPAs-hydrazone-PEG micelle and DOX-loaded micelle was prepared by the dialysis method: 3mg of HBPAs-hydrazine-PEG (or 3mg of HBPAs-hydrazone-PEG and 300μg of DOX) was dissolved into 3ml of DMF and dialyzed against deionized water for 24 hours. And then, the micelle (or DOX-loaded micelle) was condensed into 3ml and kept at 4℃ for use.

(4) Doxorubicin Release

3ml of the prepared DOX-loaded micelle was dialyzed against 50ml of buffer solution. At the predetermined time, 4 ml of buffer solution was withdrawn and 4 ml of fresh buffer solution was then added (molecular weight cutoff: 3500). Doxorubicin was measured using a UV-VIS at a wavelength of 482nm**.**

Methods

^{1}H-NMR spectroscopy (ADVANCE2B/400MHZ) was used with DMSO-d_6 and 1, 2-Dichlorobenzen d_4 as the solvent. Transmission electron microscopy (JEM-1200EX, 80KV) was performed to investigate the morphology of the HBPAs-Hydrazine-PEG micelles and drug-loaded micelles. Size distribution analysis was studied using dynamic light scattering (Malven). Two instruments were used to crosscheck the reliability of the obtained data. The amount of released DOX was measured by a UV detector (UV-1800, SHIMADZU) at a wavelength of 482nm. Molecular weight was detected by the GPC (Waters 1515)..

Result and discussion

The synthesis of the amphiphilic hyperbranched polymers

Fig 1 gives the synthesis route of HBPAs-Hydrazone-PEG. The monomer 4-(2-hydroxyethoxyl) -benzaldehyde dimethyl acetal was firstly synthesized by aldol condensation. The transfer efficiency from 4-(2-hydroxyethoxyl) benzaldehyde to 4-(2-hydroxyethoxyl) benzaldehyde dimethyl acetal is closed to 100% which was confirmed by the H^1-NMR spectrum (supporting information Fig 2). Hyperbranched polyacetals were then synthesized from the monomer at the ratio of monomer to initiator (benzaldehyde

RSC Advances Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

dimethyl acetal) 60:1. By changing the monomer to initiator ratio, we could synthesize HBPAs with different molecular weights.

The proton NMR spectra of the polymer HBPAs along with their peak assignments are shown in Figure 2 (upper). Compared to the monomer spectrum (supporting information Fig 2), the peaks in the polymer spectrum become broad, and a substantial reduction in the relative intensity of the methoxy protons (peak g), because of the expected loss of methanol. Interestingly, the methane proton of the acetal unit (peak a), which is a singlet in the monomer, splits into three well-resolved peaks in the polymer. On the basis of the relative positions of these three peaks, the peaks can be assigned into three well-resolved peaks. The peaks can be assigned to dendritic (D), linear (L) and terminal (T) units, as shown in Figure 3. From the relative intensities of these three peaks, the degree of branching (DB) of the polymer was estimated to be around 0.52, which is roughly the expected value for a statistically random growth process[22]. The DB was calculated by the following equation (1):

$$DB = \frac{D+T}{D+L+T} \qquad (1)$$

Another interesting aspect of the spectrum is the presence of an aldehyde proton peak at around 10.2 ppm. This is presumably is due to inadvertent hydrolysis of some of the acetal groups. The relative intensities of the three acetal methane peaks (peaks a) appear to suggest that preferential hydrolysis of the terminal dimethylacetal (T) units may have occurred; this is reflected by the decrease in the intensity of the terminal methane peak with increase in the intensity of the aldehyde peak (the H^1-NMR spectrum was in supporting information Fig 3) [17]. Of course the containing of the aldehyde group could be adjusted by adding 4-(2-Hydroxyethoxy)-benzaldehyde into the monomer in the synthesis procedure of HBPAs (supporting information). Compared to acetal bonds (5.5-6.2 ppm), the content of the aldehyde group (10.2 ppm) was about 17% which was estimated by the H^1-NMR spectrum.

It is the presence of the aldehyde group that makes the reaction between HBPAs and PEG-$NHNH_2$ come true. The addition amount of PEG-$NHNH_2$ was 10 fold to aldehyde group of HBPAs by mole ratio so that the aldehyde group was transferred into hydrazone bond completely. The proton NMR spectra of the polymer HBPAs-hydrazone-PEG along with their peak assignments are shown in Figure 2 (bottom). HBPAs-hydrazone-PEG could not dissolve into DMSO very well, but could dissolve into 1, 2-Dichlorobenzen. This is the reason why we use 1, 2-Dichlorobenzen d_4 as the deuterated solvent. PEG was successfully attached to the HBPAs, which was confirmed by the disappearance of the peak at 10.2 ppm (aldehyde proton peak) and the appearance of the peak at 8.9 ppm (the hydrazone proton peak). The number average molecular weight of HBPAs and HBPAs-hydrazone-PEG were 3600 and 8600, respectively (GPC). The molecular weight can be controlled by the ratio of monomer to initiator.

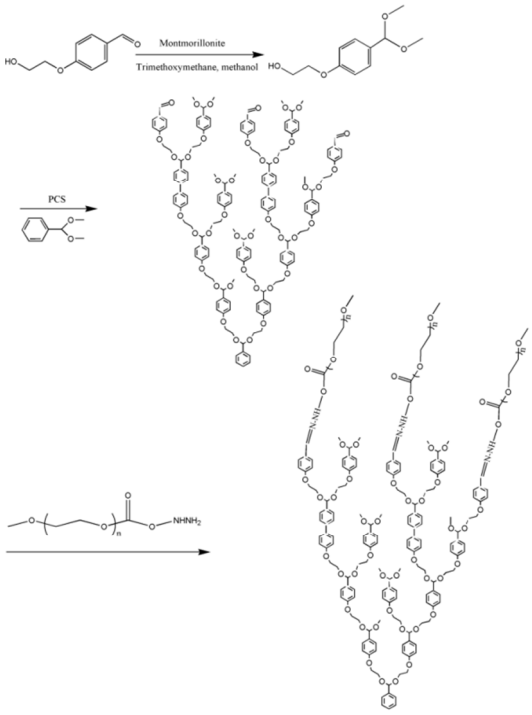


Figure 1 the synthesis process of the HBPAs-Hydrazone-PEG

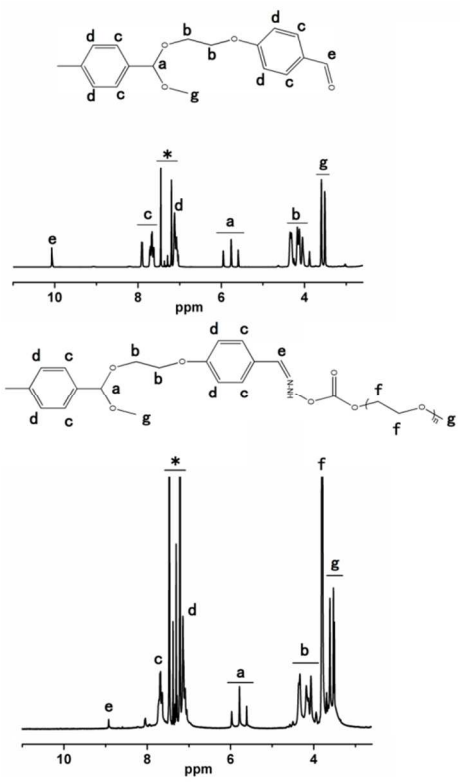


Figure 2 ^{1}H-NMR spectra of HBPAs and HBPAs-Hydrazone-PEG

RSC Advances Accepted Manuscript

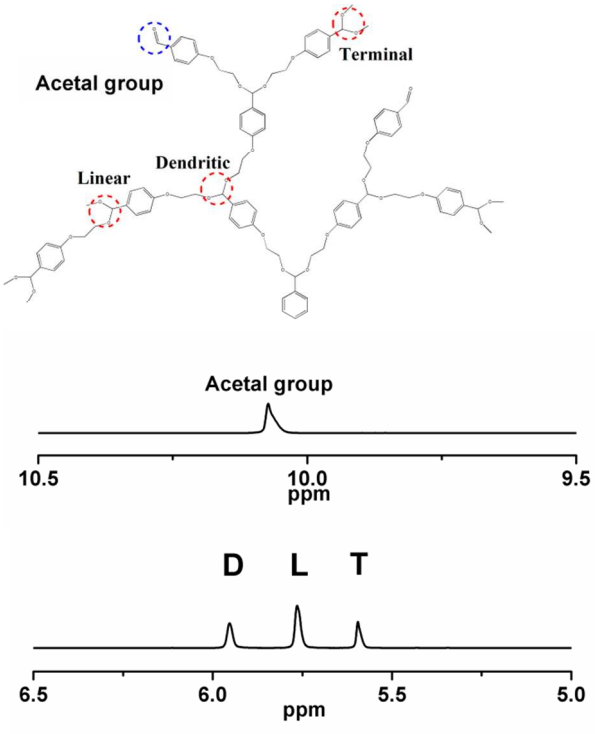


Fig 3 the internal structure of HBPAs

Fig 4 gives the hydrolysis behavior of HBPAs by the dialysis method. A certain amount HBPAs was firstly dissolved into DMF solvent, and then dialyzed against to the buffer solution with different pH. At the pre-determined time, 4ml of buffer solution was withdrawn and 4 ml fresh buffer solution was added into the dialysis system. 20 μl of the collected buffer solution was injected into the HPLC system. 4-(2-hydroxyethoxyl) benzaldehyde which was water soluble was created from the hydrolysis of HBPAs and was detected by the UV-VIS system. The accumulated amount of the 4-(2-hydroxyethoxyl) benzaldehyde was calculated and functioned to the time shown in Fig 4. HBPAs were hydrolyzed faster in pH 5.0 buffer solution than in pH 7.4 buffer solution. This demonstrated that HBPAs were pH sensitive.

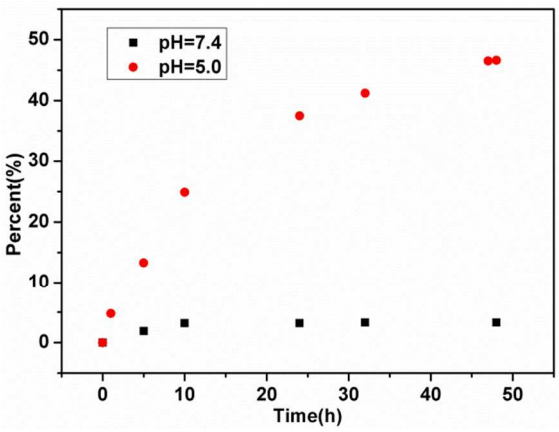


Fig 4 degradation curve of HBPAs at different pH values

The micelle formation of HBPAs-Hydrazone-PEG

Micelle was prepared by dissolving HBPAs-hydrazone-PEG in DMF and dialyzed in the deionized water for 24h. Fig 5 gives the DLS curve and TEM of the prepared HBPAs-hydrazone-PEG micelle. The diameter of the micelle is about 30nm and the PDI is 0.391. The micelle was quite stable in neutral environment but fragile in acidic solution as was illustrated in Fig 6. A certain volume of micelle solution (1.0mg/ml in deionized water) was diluted by same volume of buffer solution with different pH. When diluted by the pH 7.4 buffer solution (0.1M), the diameter of the micelle was unchanged. However, when diluted by the pH 5.0 buffer solution (0.1M), it hydrolyzed quickly. The critical micelle concentration was studied using pyrene as fluorescent agent. The value of the CMC is about 2.0 μg (supporting information Fig 5).

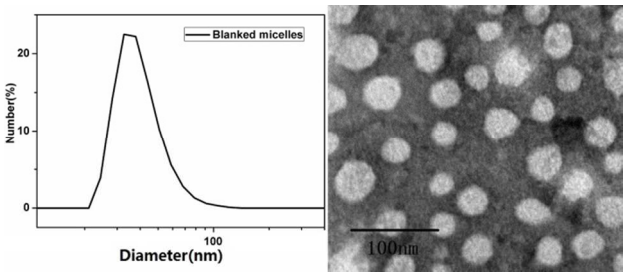


Fig 5 DLS and TEM of blanked micelle

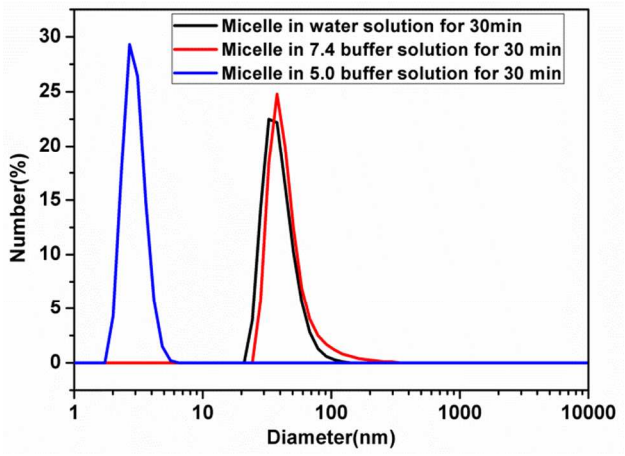


Fig 6 the changes of the micelle size at different pH values

The DOX-loaded micelles and drug release behavior

DOX-loaded HBPAs-hydrazone-PEG micelles were prepared by the dialysis method. Before drug loading, doxorubicin hydrochloride (DOX·HCl) was pre-treated with excess triethylamine in DMC overnight. The weight ratio of the DOX to micelle is 10%, and the drug loading content (DLC) and drug loading efficiency (DLE) is 2.34% and 23.4%, respectively. DOX was detected by UV-VIS at predetermined wavelength of 482nm. DLC and DLE were calculated by the following equation (2):

$$DLC = \frac{\text{weight of loaded DOX}}{\text{weight of drug loaded micelle}};\ DLE = \frac{\text{weight of loaded DOX}}{\text{weight of devoted DOX}} \quad (2)$$

RSC Advances Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

Compared to the blanked micelles, the particle size of drug-loaded micelles increased from 30nm to 35nm (Fig 7). The increase of the diameter is mainly due to the encapsulation of the DOX.

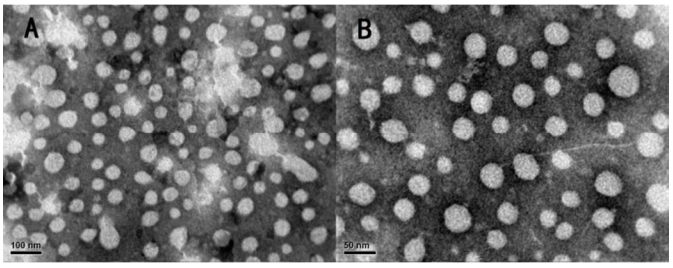


Fig 7 TEM of micelle before and after DOX loaded

Fig 8 gives the drug release curve at different pH buffer solutions. DOX was released faster in pH 5.0 buffer solution than in pH 7.4 buffer solution. This is due to the carrier of HBPAs-hydrazone-PEG is pH sensentive. More than 50% of DOX was released from the carrier in less than 10 hours at pH 5.0 buffer solution, while less than 10% of DOX was released from the carrier at pH 7.4 buffer solution. The leakage of the DOX at neutral solution is due to the hydrophilicity of HBPAs. In fact, HBPAs is different from the traditional hydrophobic materials such as PLA and PCL. There are plenty of ethenyl-oxy group which is hydrophilic similar to PEG in the backbone of HBPAs, but the benzene ring bestowed HBPAs with hydrophobicity. So, more or less, there is a little hydrophilicity for HBPAs. Water can penetrate into the core of the micelle and swell the micelle. This is the reason why DOX could also leak out from the carrier at neutral buffer solution.

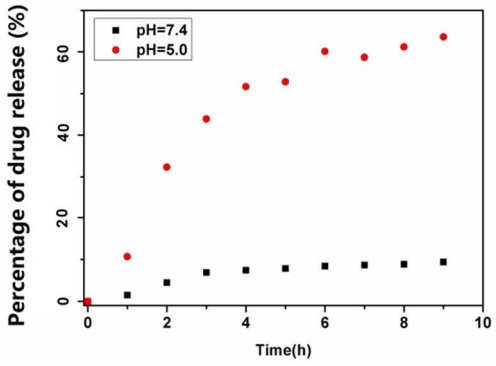


Fig 8 the DOX release curve of DOX-loaded micelle at different pH values

Conclusions

Amphiphilic hyperbranched polyacetals containing dual pH sensitive bonds-acetal and hydrazone bonds-was synthesized in our group. Hydrolysis experiments demonstrated that it hydrolyzed quickly in acidic environment, while it was quite stable at neutral environment. Micelle of HBPAs-hydrazone-PEG could be easily formed by dialysis method. The micelle was stable at neutral buffer solution but hydrolyzed at acidic buffer solution. After DOX was encapsulated into the micelle, the size of the micelle increased to 35nm from 30nm. Drug released faster in pH 5.0 than in pH 7.4, more than 50% of DOX was released from the micelle, but there is also less than 10% of DOX leaked out from the micelle, this is mainly due to the hydrophilicity of HBPAs.

Acknowledgements

This work was supported by the national nature science foundation.

Notes and references

[a] Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China

[b] MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Hangzhou 310027, China.

[c] School of Chemistry and Chemical Engineering, State Key Laboratory of Metal Composites, Shanghai Jiao Tong University,800 Dongchuan Road, Shang hai 200240, People's Republic of China

1. Gao, C. and D. Yan, *Progress In Polymer Science,* 2004, **29**, 183.
2. Hult, A., M. Johansson, and E. Malmstrom, *Advances in Polymer Science: Branched polymers II*, 1999, **143**, 1.
3. Inoue, K., *Progress In Polymer Science*, 2000, **25,** 453.
4. Jikei, M. and M. Kakimoto, *Progress In Polymer Science*, 2001, **26**, 1233.
5. Voit, B., *Journal Of Polymer Science Part A-Polymer Chemistry*, 2000, **38,** 2505.
6. Lee, C.C., *Nature Biotechnology*, 2005, **23**, 1517.
7. Kojima, C., *Bioconjugate Chemistry*, 2000, **11**, 910.
8. Malik, N., E.G. Evagorou, and R. Duncan, *Anti-Cancer Drugs*, 1999, **10**, 767.
9. Kukowska-Latallo, J.F., *Cancer Research*, 2005, **65**, 5317.
10. Liu, M.J., K. Kono, and J.M.J. Frechet, *Journal Of Controlled Release*, 2000, **65**, 121.
11. Morgan, M.T., et al., *Journal Of the American Chemical Society*, 2003, **125**, 15485.
12. King, H.D., *Journal Of Medicinal Chemistry*, 2002, **45**, 4336.
13. Choe, Y.H., *Journal Of Controlled Release*, 2002, **79**, 55.
14. Pasut, G., *Journal Of Bioactive And Compatible Polymers*, 2005, **20**, 213.
15. Haba, K., *Angewandte Chemie-International Edition*, 2005. **44**, 716.
16. Shenoi, R.A., Biomaterials, 2013, **34**, 6068.
17. Chatterjee, S. and S. Ramakrishnan, *Macromolecules*, 2011, **44**, 4658.
18. Lu, Y., *Acta Polymerica Sinica*, 2013, **3**, 391.
19. Behera, G.C. and S. Ramakrishnan, *Journal Of Polymer Science Part A-Polymer Chemistry*, 2004, **42**, 102.
20. Lee, Y., *Bioconjugate Chemistry*, 2008, **19**, 525.
21. Lee, S., *Biomacromolecules*, 2012, **13**, 1190.
22. Holter, D., A. Burgath, and H. Frey, *Acta Polymerica*, 1997. **48**, 30.

RSC Advances Accepted Manuscript