Polymer Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/polymers

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Facile Fabrication of Ultrathin Antibacterial Hydrogel Films via Layer-by-Layer "Click" Chemistry†

Huan Wang,‡[a] Guangyu Zha,‡[b] Hong Du,[a] Lilong Gao,[a] Xiaodong Li,*[b] Zhiquan Shen[a] and Weipu Zhu*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

We report a facile strategy to fabricate ultrathin hydrogel films via layer-by-layer (LbL) technique and "click" chemistry. Poly[oligo (ethylene glycol) fumarate]-*co*-poly[dodecyl bis(2-hydroxyethyl) methylammonium fumarate] (POEGDMAM) containing multi-ene and poly[oligo (ethylene glycol) mercaptosuccinate] (POEGMS) containing multi-thiol were synthesized by polycondensation respectively, which were used as precursors for LbL thiol-ene "click" reaction under ambient conditions without any metal catalyst or light irradiation. Due to the ammonium groups with long alkyl chain from POEGDMAM, the ultrathin hydrogel films exhibited excellent antibacterial activity against both *Staphylococcus aureus* and *Escherichia coli*, which was enhanced by the growing numbers of layers. This kind of biocompatible antibacterial ultrathin hydrogel films is a promising candidate for biomedical applications.

Introduction

Ultrathin hydrogel films are being paid more and more attention owing to their promising applications on sensing, advanced delivery and external functionalization of materials.[1-5] Unlike macro-hydrogels, ultrathin hydrogel films are capable of fast response and show widespread practical value.[6] Usually, ultrathin hydrogel films are fabricated by spin-casting with hydrogel precursors, and subsequent crosslinking.[7, 8] It is a simple method but with serious limitations. For one thing, it can only apply to the specific geometry such as smooth plane. For another, it's difficult to control the thickness of hydrogel films below micron scale.[9]

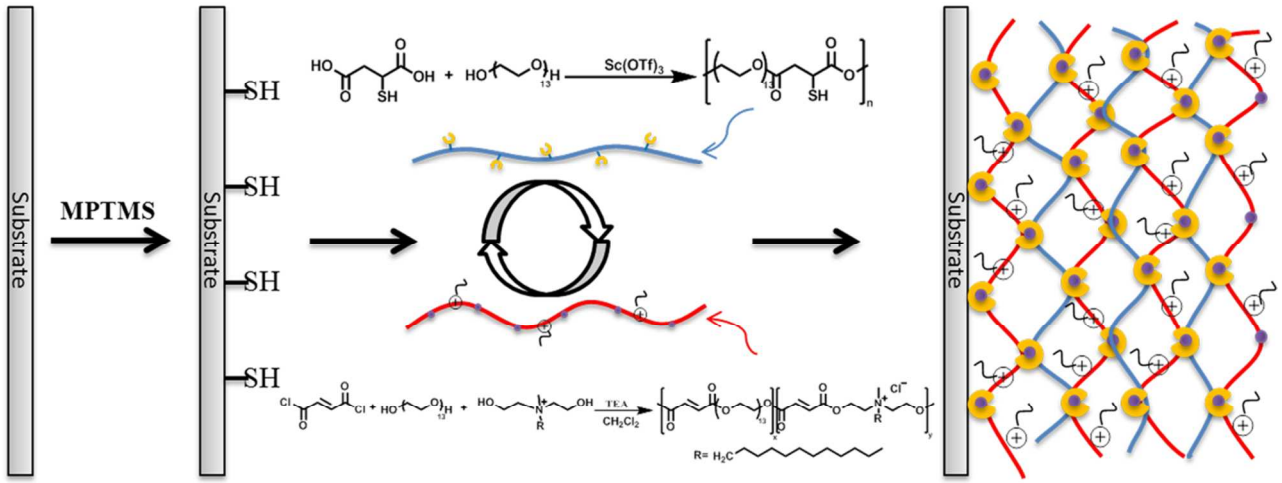
Layer-by-Layer (LbL) technique is a versatile strategy for fabricating ultrathin films,[10-13] with the possibility of controlling and tailoring the interfacial properties in the preparation of advanced materials with various functions on virtually any substrate. However, most of the attention is paid to the assembly of polymers driven by electrostatic interaction,[8, 14] hydrogen bonding,[15, 16] charge-transfer interaction,[17] metal-ligand interaction,[18, 19] which limit the robustness of the films because they are not covalently linked and their stability depends on different factors such as solvent, pH value, polyelectrolyte concentration and so on. "Click" chemistry was defined by Sharpless in 2001 as a set of reactions proceeding under mild reaction conditions with virtually no side reactions or byproducts, which makes the reaction of particular interest in the formation of covalently linked thin film.[20] In 2006, Caruso was the first to fabricate LbL thin films based on a CuAAC strategy. The covalent bonds between poly(acrylic acid) chains allowed a reversible swelling of the films when the pH was changed.[21] Moreover, the obtained films were found to be resistant to a set of organic solvents. However, the biocompatibility of the hydrogel thin films was reduced by the residue copper ions which cannot be removed entirely due to the coordination of copper ions and triazole ring. Thus, the applications of this kind of hydrogel thin films may be greatly limited in many fields such as biology and medicine.

Recently, the reaction between thiols and enes is considered as the secondary generation of "click" reactions.[22, 23] Thiol-ene polymer network has significant advantages over traditional polymer networks as they are formed rapidly and quantitatively under ambient conditions and are insensitive to the presence of water and oxygen.[24, 25] What's more, Michael addition type thiol-ene "click" reactions between electron-deficient enes and thiols could be carried out under physiological conditions without metal catalyst or light irradiation, and has been widely used in biomedical applications. Poly(ethylene glycol) (PEG) is an FDA proved hydrophilic polymer possessing excellent biocompatibility which is widely applied to scaffolds,[26] drug delivery,[27-29] cell culturing[30] and other biomedical fields. In our previous work, we synthesized novel PEG derivatives containing multiple "clickable" groups by polycondensation of dihydroxyl oligo(ethylene glycol) (OEG) with maleic anhydride or thiolmalic acid respectively under mild conditions.[31, 32] Fully biodegradable PEG-based hydrogels could be facilely prepared through metal and light free Michael addition type thiol-ene "click" reaction by simply mixing the PBS solutions of the two PEG derivatives under room temperature, which can be used as a physical barrier to lower the extent and the severity of postoperative adhesions.[33]

As bacterial infections are a series of severe threats to public health, the design and development of antibacterial materials are of great significance.[34] Quaternary ammonium salts (QAS) have long been one of the most widely used low-molecular-weight

antibacterial agents due to their excellent cell membrane penetration properties, low toxicity, good environmental stability, lack of skin irritation, low corrosivity, and extended residence time and biological activity.[35] It has also been reported that the polymers with pendant QAS group attached by covalent bonds exhibit strong antibacterial activity.[36] In order to incorporate QAS moieties into hydrogel backbone, we designed and synthesized a novel PEG derivative containing multiple QAS and "clickable" double bonds (POEGDMAM) by the direct polycondensation of OEG, fumaryl chloride (FC) and dodecyl bis(2-hydroxyethyl) methylammonium chloride (DMA) as one of the precursors for the preparation of hydrogels. The injectable "click" hydrogels containing QAS moieties in the backbone possess significant antibacterial ability and low toxicity.[37]

While in this paper, ultrathin antibacterial hydrogel films were facilely fabricated via LbL thiol-ene "click" reaction using POEGDMAM and POEGMS as precursors as shown in Scheme 1. Owning to the QAS in the hydrogel backbone, this kind of biocompatible ultrathin PEG hydrogel films shows satisfactory antibacterial ability against both gram-negative and gram-positive bacteria.[38-40]



Scheme 1 Demonstration of fabricating ultrathin hydrogel films by LbL thiol-ene "click" chemistry.

Experimental section

Materials

Diol oligo(ethylene glycol) (OEG_{13}, M_n=600, Sinopharm Chemical Reagent Co., China) was dried in toluene by azeotropic distillation. Triethylamine (TEA) and dichloromethane was dried over CaH_2 and distilled before use. Fumaryl chloride (TCI, Japan), terephthaloyl chloride (Aladdin, China), dodecyl bis(2-hydroxyethyl) methylammonium chloride (Xiamen Pioneer Technology Co., China) and other reagents were used as received. The single-side polished silicon wafer and quartz wafer were purchased from LiJing Co., Ltd and GuangLiang High technology Co. Ltd, respectively. POEGDMAM and POEGMS were synthesized as previously described. [33, 34]

Synthesis of POEGDMAMT

The dried OEG_{13} (7.20 g, 12.0 mmol), dodecyl bis(2-hydroxyethyl) methylammonium chloride (1.94 g, 6.00 mmol), terephthaloyl chloride (0.73 g, 3.60 mmol) and triethylamine (3.64 g, 36.0 mmol) were dissolved in 80 mL of anhydrous methylene chloride and cooled by an ice bath. Fumaryl chloride (2.20 g, 14.4 mmol) was added to the solution dropwise via a funnel over 2 h under vigorous stirring. The reaction was continued under an argon atmosphere for 24 h at room temperature. Afterward, the solvent was removed through rotary evaporation, and the residue was dissolved in ethyl acetate. After removing triethylamine hydrochloride salt by filtration, the crude product was precipitated into cold diethyl ether, washed three times by diethyl ether and dried in vacuum to constant weight (7.93 g, yield: 69.4 %).

Preparation of ultrathin hydrogel films

The silicon or quartz slides were cut into 10 × 10 mm^2 pieces, sonicated in ethanol for 30 min, and rinsed with deionized (DI) water. The clean substrates were hydroxylated by treating in a 30: 70 (v/v) mixture of H_2O_2 and H_2SO_4 at 80 °C for 1 h. The resulting substrates were dried under a nitrogen (N_2) stream. Then 3-mercaptopropyl trimethoxysilane (MPTMS) in toluene at a concentration of 1 % was prepared in a N_2 atmosphere. The substrates were immersed into MPTMS solution at room temperature for 30 min in a N_2 atmosphere, then successively washed with toluene, ethanol and DI water, and finally dried in a N_2 stream. The substrates modified by MPTMS were alternately immersed in phosphate buffer solutions (PBS, pH 7.4) of POEGDMAM (or POEGDMAMT) and POEGMS at the concentration of 1% for 10 min with intermediate water rinsing and N_2 drying. Multilayer films can be formed by repeating these two steps in a cyclic fashion.

Characterization

^1H NMR was measured on Bruker Avance DMX500 NMR spectrometer (500 MHz) at room temperature with $CDCl_3$ as the solvent. Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with Waters 208 apparatus equipped with Waters 2410 RI detector (set at 60 °C). The eluent was DMF at a flow rate of

1.0 mL/min. The number-average (M_n) was calculated using a calibration curve which was obtained from polymethylmethacrylate standards with low polydispersity indices. UV-vis absorption spectra were conducted with UV-vis spectroscopy (UV-2450) at 25 °C. The wettability of the surfaces was characterized by means of a video-based optical contact angle measure system DropMeter A-200 (MAIST). Film thickness measurements were performed using a GES 5E spectroscopic ellipsometer (Semilab) on Si wafers. Measurements were performed between 190 nm to 990 nm at an angle of 60°. The refractive indexes of the films were set to 1.50 for all the films and at least three different measurements were performed for each bilayer. The antibacterial activity of the ultrathin hydrogel films was determined by disc diffusion method. 500 mL of nutrient medium (pH 7.0) containing beef extract 2.50 g, peptone 5.00 g, agar 10.0 g and NaCl 2.50 g was autoclaved at 126 °C for 30 min. 50.0 μL bacteria suspension (10^6 cfu mL^{-1}) was added onto the agar plates containing 15.0 mL nutrient medium. The bacteria suspensions were then homogeneously dispersed by using coated rods. The ultrathin hydrogel sample were finally put on the media for bacteria and followed by culturing in 37 °C for 24 h. The biocompatibility tests including cell adhesion and proliferation behavior was conducted using Hela cells. The cells were maintained using a standard protocol. Different layers' hydrogel films were prepared on quartz slides (10 × 10 mm^2) while a piece of clean quartz slide was chosen as the negative control. Samples were then rinsed thoroughly with ethanol and PBS, placed in a 24-well plate with DMEM media with 10 % FBS, and seeded with Hela cells adjusted to a population of 2.5×10^5 cells per well. Finally, the cell-seeded samples were incubated at 37 °C in 5 % CO_2 for 24 h. The cell adhesion and proliferation behavior were observed by inverted phase contrast microscope (Olympus CKX41). All the samples were magnified 200 times.

Result and discussion

POEGMS and POEGDMAM were synthesized via "one pot" polycondensation and characterized by 1H NMR. The molar ratio of OEG segments to thiol groups in POEGMS is 1: 0.98, calculated by the integral areas of Hb and Hd (Fig. S1). Similarly, based on the integral areas of Hg, Hb and Hi from Fig. S2, the molar ratio of OEG segments, DMA units and fumaryl units in POEGDMAM was 1: 0.55: 1.51, which is very close to the theoretic value.

Scheme 1 illustrates the multilayer deposition of POEGDMAM and POEGMS onto thiolated quartz substrates via "click" chemistry. POEGDMAM with multi-ene first reacted with the thiols on the surface to form a single layer of polymer. Subsequently POEGMS with multi-thiol reacted with the immobilized ene groups of the polymer surface to give the second layer. Repeated deposition of the polymers gave the corresponding multilayer films. UV-vis spectroscopy was used to monitor the multilayer fabrication process. Considering neither POEGDMAM nor POEGMS exhibits an obvious UV absorbance between 190 nm and 800 nm after "click" reaction. POEGDMAMT with aromatic rings in the backbone was synthesized similarly by direct polycondensation of OEG_{13}, FC, DMA and terephthaloyl chloride, to replace the POEGDMAM when conducted the UV-vis spectroscopy. Figure 1 shows the UV-vis absorption spectra of 1, 2, 3, 4 and 5 bilayers of POEGDMAMT and POEGMS reacted on a quartz slide. The linear increase in absorbance at 256 nm with the number of layers indicates a progressive reaction with almost an equal amount of the copolymers in each cycle.

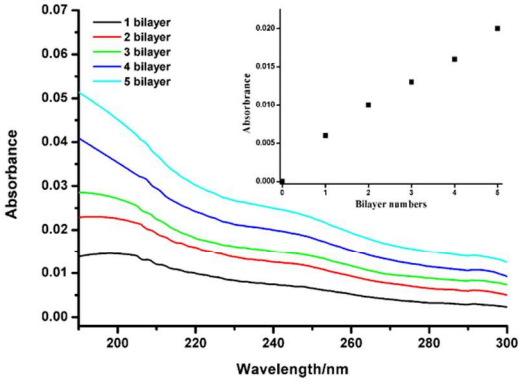


Fig. 1 UV- vis absorption spectra for multilayer films assembled on quartz with increasing bilayer numbers. Inset: absorbance as a function of bilayer number monitored at 256 nm.

The thickness of the ultrathin hydrogel films was further monitored by ellipsometry. Fig. 2 plots the film thickness as a function of bilayer number. Again a linear relationship was observed, confirming a linear growth for the hydrogel film build-up. As can be seen in the graph, the hydrogel films grow with a regular thickness increase averaging 2.8 nm per bilayer which demonstrates "click" chemistry allows excellent control over film thickness.

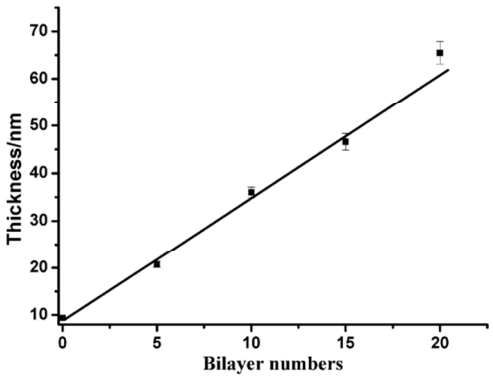


Fig. 2 Relationship between film thickness as determined by ellipsometry and number of bilayers for the ultrathin hydrogel film.

Contact angles of the ultrathin hydrogel films were measured to quantify the change of surface hydrophilicity, as presented in Figure 3. The hydroxylated silicon wafers were quite hydrophilic after washing with the Piranha solution owing to the covering with hydroxyl group. We chose the widely used silane coupling agent MPTMS to anchor the thiol on the silicon wafers through the hydrolysis reaction of the methoxysilyl group with hydroxyls. The surfaces turned out to be much less hydrophilic after being thiolated as the contact angles were about 60° (Fig. 3b). Then the

hydrophilicity was increased with the bilayer numbers of the hydrogel ultrathin films since the hydrogels were quite readily absorbing water. When the bilayer number reached 15 (Fig. 3e), the contact angle decreased to 0°, which indicated the surfaces were thoroughly hydrophilic owing to the formation of hydrogel with a certain thickness. The change of the hydrophilicity was in conformity with the results of UV and the ellipsometry.

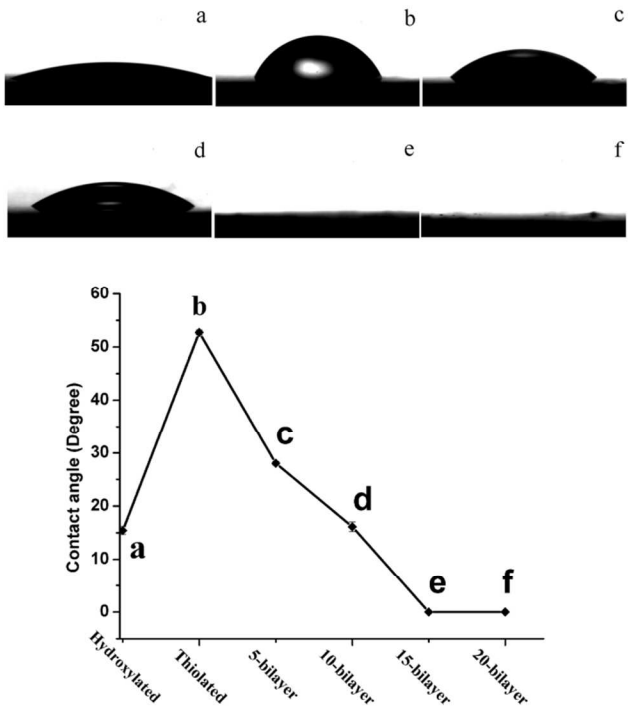


Fig. 3 The contact angle test of hydroxylated and thiolated silicon wafers, as well as ultrathin hydrogel films with different bilayers.

The antibacterial activity of the ultrathin hydrogel films on silicon wafers was determined by disc diffusion method against gram-negative (*Escherichia coli*) and gram-positive (*Staphyloccocus aureus*) bacteria. The tests were carried out according to a standard operation procedure. After each kind of bacteria was inoculated on LB agar evenly, the silicon samples were put on it. The samples were cultured under 37 °C for 24 h. As shown in Figure 4, the control groups without any hydrogel films generated no inhibition zones, while the others exhibited inhibition zones to both of these two kinds of bacteria. The zones were quite obvious considering the thickness of the films was just below 100 nm. Moreover, the inhibition zones increased significantly with the numbers of bilayer which confirmed the LbL reaction on the substrates.

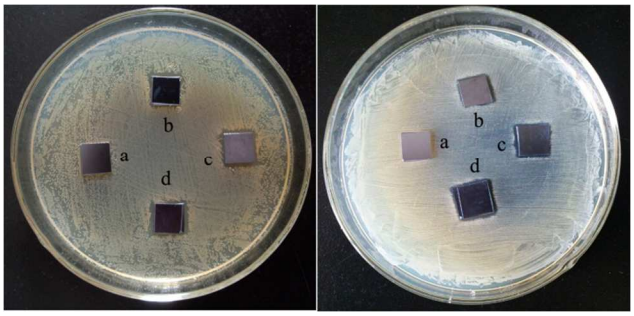


Fig. 4 The antibacterial activity of ultrathin hydrogel films with different layers against *Staphyloccocus aureus* (left) and *Escherichia coli* (right) (a to d: bilayer 0, 5, 10 and 20).

The biocompatibility tests of these ultrathin hydrogel films were conducted with Hela cells and observed directly by inverted phase contrast microscope. The cells were cultivated on quartz slides, on which the hydrogel films were prepared. The operation was conformed to a standard protocol. Cells are able to keep their integrate structures and readily to stick to the surfaces of quartz slides to proliferate in favorable conditions. As showed in Figure 5, a great majority of cells retained the complete structures, which were well attached to each surface of the fabricated samples. Only the 20-bilayer hydrogel film exhibited a slight decline of the attached cells. The antibacterial ultrathin hydrogel films own considerable biocompatibility, which is expected to be applied to the biological and medical field.

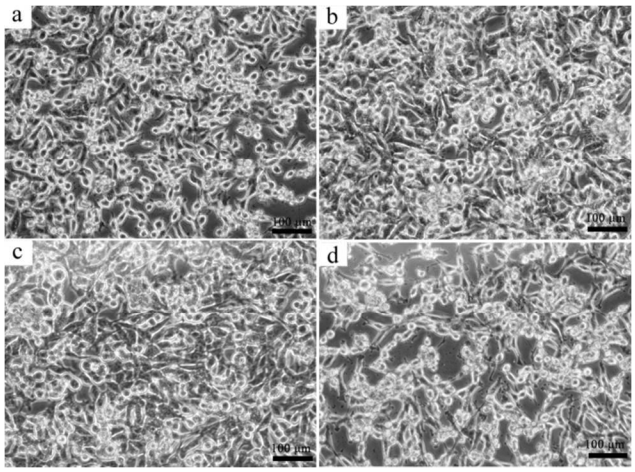


Fig. 5 Cell viability tests of ultrathin hydrogel films with different layers. (The cells were cultivated on hydrogel films with a certain bilayer number, a to d: 0, 5, 10 and 20)

Conclusions

We have demonstrated a facile approach to fabricate ultrathin PEG hydrogel films via a combination of LbL technique and thiol-ene "click" chemistry using multifunctional PEG derivatives as precursors. Due to the quaternary ammonium groups in the hydrogel backbone, these ultrathin hydrogels show significant antibacterial activity against both gram-negative and gram-positive bacteria, enhanced by the increase of hydrogel thickness. Furthermore, this kind of antibacterial ultrathin hydrogels exhibits satisfactory biocompatibility, which makes it a potential candidate for biomedical applications.

Acknowledgements

The authors are grateful for the National Natural Science Foundation of China (21274121 and 51173163), the Major State Basic Research Project (2011CB606001), the National Science-technology Support Plan Project of China (2012BAI07B01) and the Fundamental Research Funds for the Central Universities (2012QNA7043).

Notes and references

[a] *MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China. E-mail: zhuwp@zju.edu.cn*

[b] *Department of Oral and Maxillofacial Surgery, Affiliated Stomatology Hospital, School of Medicine, Zhejiang University, Hangzhou 310006, China. E-mail: cisarli@zju.edu.cn*

† Electronic Supplementary Information (ESI) available: ESI includes the 1H NMR characterization of POEGMS, POEGDMAM POEGDMAMT, and GPC traces of POEGMS, POEGDMAM. See DOI: 10.1039/b000000x/

‡ These authors contributed equally

1 D. Matsukuma, K. Yamamoto and T. Aoyagi, *Langmuir.*, 2006, **22**, 5911-5915.

2 X. Zhang, Y. Guan and Y. Zhang, *Biomacromolecules*, 2011, **13**, 92-97.

3 L. Zhai, *Chem. Soc. Rev.*, 2013, **42**, 7148-7160.

4 H. Shih, A. K. Fraser and C.-C. Lin, *ACS Appl. Mater. Interfaces*, 2013, **5**, 1673-1680.

5 S. Pavlukhina and S. Sukhishvili, *Adv. Drug Deliver. Rev*, 2011, **63**, 822-836.

6 X. Liang, V. Kozlovskaya, Y. Chen, O. Zavgorodnya and E. Kharlampieva, *Chem. Mater.*, 2012, **24**, 3707-3719.

7 V. Gopishetty, I. Tokarev and S. Minko, *J. Mater. Chem.*, 2012, **22**, 19482-19487.

8 K. M. Beckwith and P. Sikorski, *Biofabrication*, 2013, **5**.

9 Y. Wang, V. Kozlovskaya, I. G. Arcibal, D. M. Cropek and E. Kharlampieva, *Soft Matter*, 2013, **9**, 9420-9429.

10 G. Decher, *Science*, 1997, **277**, 1232-1237.

11 R. Zhou and M. P. Srinivasan, *J. Colloid Interf. Sci*, 2013, **392**, 158-166.

12 A. Krawicz, J. Palazzo, G.-C. Wang and P. H. Dinolfo, *RSC Adv.*, 2012, **2**, 7513-7522.

13 V. Kozlovskaya, O. Zavgorodnya, Y. Wang, J. F. Ankner and E. Kharlampieva, *ACS Macro Lett.*, 2013, **2**, 226-229.

14 S. Park, S. H. Bhang, W. G. La, J. Seo, B. S. Kim and K. Char, *Biomaterials.*, 2012, **33**, 5468-5477.

15 I. Erel-Unal and S. A. Sukhishvili, *Macromolecules.*, 2008, **41**, 8737-8744.

16 S. W. Lee, K. E. Tettey, I. L. Kim, J. A. Burdick and D. Lee, *Macromolecules.*, 2012, **45**, 6120-6126.

17 Y. Zhang and W. Cao, *Langmuir.*, 2001, **17**, 5021-5024.

18 F. Zhang and M. P. Srinivasan, *Langmuir.*, 2007, **23**, 10102-10108.

19 X. Y. Huang, A. B. Schubert, J. D. Chrisman and N. S. Zacharia, *Langmuir.*, 2013, **29**, 12959-12968.

20 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angewandte Chemie International Edition*, 2001, **40**, 2004-2021.

21 G. K. Such, J. F. Quinn, A. Quinn, E. Tjipto and F. Caruso, *J. Am. Chem. Soc.*, 2006, **128**, 9318-9319.

22 H. Shih, A. K. Fraser and C. C. Lin, *ACS Appl. Mater. Interfaces*, 2013, **5**, 1673-1680.

23 X. Sui, L. van Ingen, M. A. Hempenius and G. J. Vancso, *Macromol. Rapid. Comm.*, 2010, **31**, 2059-2063.

24 C. E. Hoyle and C. N. Bowman, *Angew. Chem. Int. Edit.*, 2010, **49**, 1540-1573.

25 C. A. DeForest, B. D. Polizzotti and K. S. Anseth, *Nat. Mater.*, 2009, **8**, 659-664.

26 J. Zhu, *Biomaterials.*, 2010, **31**, 4639-4656.

27 J. Liu, C. Detrembleur, M.-C. De Pauw-Gillet, S. Mornet, E. Duguet and C. Jerome, *Polym. Chem.*, 2014, **5**, 799-813.

28 P. F. Gou, W. P. Zhu and Z. Q. Shen, *Biomacromolecules*, 2010, **11**, 934-943.

29 P. F. Gou, W. P. Zhu and Z. Q. Shen, *Polym. Chem.*, 2010, **1**, 1205-1214.

30 Y. Hao, H. Shih, Z. Muňoz, A. Kemp and C.-C. Lin, *Acta Biomaterialia*, 2014, **10**, 104-114.

31 L. L. Gao, Q. J. Luo, Y. Wang, H. Du, X. D. Li, Z. Q. Shen and W. P. Zhu, *RSC Adv.*, 2014, **4**, 4177-4180.

32 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, DOI: 10.1039/C1034PY00577E.

33 W. P. Zhu, L. L. Gao, Q. J. Luo, C. Gao, G. Y. Zha, Z. Q. Shen and X. D. Li, *Polym. Chem.*, 2014, **5**, 2018-2026.

34 W. E. Stamm, *Ann. Intern. Med.*, 1978, **89**, 764-769.

35 B. Dizman, M. O. Elasri and L. J. Mathias, *J. Polym. Sci. Pol. Chem.*, 2006, **44**, 5965-5973.

36 X. Liu, H. Zhang, Z. Tian, A. Sen and H. R. Allcock, *Polym. Chem.*, 2012, **3**, 2082-2091.

37 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.

38 M. R. J. Salton, J. H. Freer and D. J. Ellar, *Biochem. Bioph. Res. Co*, 1968, **33**, 909-915.

39 W. P. Zhu, H. Du, Y. Huang, S. Sun, N. Xu, H. G. Ni, X. Cai, X. D. Li and Z. Q. Shen, *J. Polym. Sci. Pol. Chem.*, 2013, **51**, 3667-3673.

40 W. P. Zhu, Y. Wang, S. Sun, Q. J. Zhang, X. D. Li and Z. Q. Shen, *J. Mater. Chem.*, 2012, **22**, 11785-11791.