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Smart surfaces based on thermo-responsive polymer brushes prepared from L-alanine derivatives for cell capture and release

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Two novel thermo-responsive polymer brushes were prepared from L-alanine derivatives using surface-initiated atom transfer radical polymerization (SI-ATRP) technique. The temperature-induced cell capture and release on both polymer brush modified substrates were further explored.

Introduction

Polymer brushes, consisting of a dense array of polymer chains with one of their chain ends immobilized on a surface or interface, have attracted increasing attention for potential applications, including stimuli-responsive surfaces, cell adhesive surfaces, nonbiofouling surfaces.[1] Among them, thermo-responsive polymer brushes are of particular interest as the constituent polymer chains can switch from a hydrophilic, swollen state to a hydrophobic, collapse state at a certain temperature. The ability to adjust the surface wettability simply by changing temperature enables thermo-responsive polymer brushes as potential smart materials in the applications of cell-sheet engineering[2-7] and bioseparation.[8-10] Poly(*N*-isopropylacrylamide) (PNiPAM), the most widely studied thermo-responsive polymer brush, undergoes a phase transition at a lower critical solution temperature (LCST) of around 32 °C in water.[11, 12] Okano et al. successfully harvested confluent cell sheets on PNiPAM-based surfaces by simply reducing temperature.[2-7] Such a cell sheet detachment strategy avoids the use of digestive enzyme and therefore maintains the intact extracellular matrix (ECM). This is extremely attractive in tissue repair. Recently, poly(oligo ethylene glycol methacrylate) (POEGMA)-based thermo-responsive brushes have received a great deal of interest due to their nontoxic and nonimmunogenic properties, as well as the tunable collapse temperature.[13, 14] These materials have been used in controlled cell adhesion and detachment.[15-17]

Unlike PNiPAM and POEGMA, known as synthetic polymers lack of chirality, most biological macromolecules present high chiral preference for monomers (e.g. L-amino acids, D-saccharides, and L-phospholipids). The polymer brushes composed of chiral molecules provide additional factors to regulate substrate properties and represent a step forward to biomimetic surface. α-Amino acids have emerged as an interesting platform to fabricate functional surfaces for their advantages over other natural biomolecules, such as easy availability, diverse functionality as well as the ability to form high ordered structures. Liu et al. developed serine-based zwitterionic polymer brushes and further explored their potential applications as antifouling surface.[18] Sun and co-workers prepared chiral surfaces with a library of amino acids and found that L-type amino acids-based substrates were preferred for cell adhesion and proliferation.[19-21] Our group has previously developed shape-persistent, thermo-responsive polypeptide brushes consisting of poly(L-glutamate) with oligo(ethylene glycol) side chain.[22] In comparison to the vast work based on non-chiral polymer brushes, the research exploring the chiral polymer brushes for the fabrication of functional surfaces, particularly stimuli-responsive surfaces, has rarely been done.

In this paper, we presented the preparation of a novel type of thermo-responsive polymer brush from L-alanine derivatives. Similar to PNiPAM brushes, these polymer brushes exhibit LCST-type thermo-responsive behaviour and their surface wettability is switched by changing temperature. We further explored the polymer brushes as smart surface for temperature-induced cell capture and release.

Experimental section

Materials

Dichloromethane (DCM) was purified by purging with dry nitrogen, followed by passing through columns of activated alumina. Deionized water was obtained from a Millipore Milli-Q purification unit. 2-Bromoisobutyryl bromide, methacryloyl chloride and allylamine hydrochloride were obtained from Aladdin reagent. Triethylamine was obtained from Beijing Chemical Co. L-Alanine (L-Ala) and L-alanine methyl ester hydrochloride (L-Ala-OMeHCl) were obtained from GL Biochem (Shanghai) Ltd. Other reagents were purchased from Aldrich.

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All commercially obtained reagents were used as received without further purification unless otherwise noted. The silicon wafers were obtained from Beijing Xinxing Braim Technology Co., Ltd. Breast cancer cell line (MCF-7) was purchased from Beijing Xiehe Hospital. Trypsin-EDTA was obtained from Invitrogen. *N*-methacryloyl-L-alanine methyl ester (MA-L-Ala-OMe) and *N*-methacryloyl-L-alanine isopropyl amide (MA-L-Ala-iPA) were synthesized as previously reported.[23] The ATRP initiator 2-bromo-*N*-(3-(chlorodimethylsilyl)propyl)-2-methylpropanamide was synthesized in a similar way as reported except using allylamine hydrochloride instead of allylamine.[24] The AO/PI working solution was prepared by mixing 0.01 mL 670 μmol/L AO (acridine orange) solution and 1mL 750 μmol/L PI (propidium iodide) solution, and then diluted by 10 fold Dulbeccos solution and filtered through a 0.22 μm filter.

Instruments

X-ray photoelectron spectroscopy (XPS) was performed on a Thermo Scientific ESCALab 250Xi using 200 W monochromated Al Kα radiation. The 500 μm X-ray spot was used for XPS analysis. The base pressure in the analysis chamber was about 3×10^{-10} mbar. Typically the hydrocarbon C_{1s} line at 284.8 eV from adventitious carbon is used for energy referencing. The brush thicknesses were measured using an M-2000V, J.A. Woollam spectroscopic ellipsometer at an incidence angle of 70° and a wavelength scan from 370.1 to 999.1 nm. All calculations were done with a Cauchy model, assuming the polymer brushes homogenous and isotropic. Three different locations for each sample were measured and the mean values and the standard deviations were given as results. Quartz crystal microbalance with dissipation (QCM-D) measurements was conducted on a Q-Sense E1 system using SiO_2 coated quartz crystals purchased from Q-Sense. The quartz sensor was mounted in a flow module with one side exposed to Milli-Q water. The temperature was increased with a rate of 0.2 °C/min. Atomic force microscopy (AFM) was performed in tapping mode (Multimode 8, Bruker, Inc.) with silicon cantilever probes. The scanning rate was 1 Hz. Water contact angles were recorded on a Dataphysics OCA25 semiautomatic contact angle measurement instrument with a homemade heater using Milli-Q water.

Initiator immobilization

The silicon wafers were cleaned by sonication in water and then acetone, followed by drying under a stream of nitrogen. The silicon wafers were treated with freshly prepared "piranha" solution (a mixture of concentrated sulfuric acid and 30% hydrogen peroxide (v/v = 7:3)) at 120 °C for 40 min. (*Caution: Piranha solution is extremely corrosive! Please be careful with this solution*.) The silicon wafers were washed with large amount of deionized water and then acetone, followed by drying under a stream of nitrogen. The cleaned substrates were then immersed in a 2% v/v solution of 2-bromo-*N*-(3-(chlorodimethylsilyl)propyl)-2-ethylpropanamide in DCM overnight. After that, the substrates were washed extensively with DCM and dried under a stream of nitrogen. The glass substrates were modified in the same way. Silicon oxide coated quartz crystals were modified in a similar way except using oxygen plasma rather than "piranha" solution to clean the substrates.

Surface-initiated ATRP polymerizaiton

A solution of MA-L-Ala-OMe (3 mmol, 513.6 mg) and *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA) (0.1 mmol, 17.3 mg, 21μL) in a mixture of methanol (1 mL) and deionized water (3 mL) was degassed by three freeze/pump/thaw cycles, and then 7.2 mg copper (Ⅰ) bromide was added under the protection of nitrogen. The solution was degassed by one more freeze/pump/thaw cycle and then transferred via syringe into a nitrogen purged reactor containing ATRP initiator modified silicon wafers. The polymerization was conducted at room temperature. After a desired time, the substrates were washed extensively with water and methanol and then dried under a stream of nitrogen. The poly(MA-L-Ala-iPA) brushes were prepared in a similar way except using a mixture of methanol and deionized water (v/v = 3/2) as solvents.

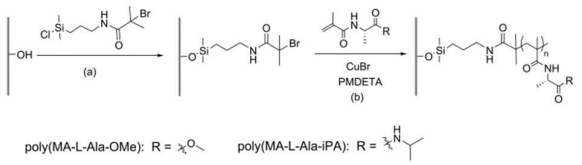
Cell culture

Human breast cancer cell line MCF-7 was cultured according to ATCC recommendation. MCF-7 cells were cultured in RPMI 1640 growth media supplemented with 10% fetal bovine serum (FBS) in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ºC and 5% CO_2 in cell incubator (Thermo Forma Series II, Thermo Scientific), and sub-cultured every three day using 0.5% Trypsin-EDTA.

Cell capture and release

Firstly, the polymer brush grafted substrates were placed into a well of a six-well cell culture plate. Then, 3 mL MCF-7 cell suspension (density = 1×10^4 mL^{-1}) was loaded onto the polymer brush grafted substrates. After 24 h culture at 37 °C, the substrates were gently rinsed with PBS at least three times to wash away the non-adhered cells. According to the previous description, the adhered cells were stained with AO/PI, immediately imaged and counted by a fluorescence microscope (Nikon Ti-E, Japan).[25] To explore the effect of temperature change, cells after culture at 37 ºC for 24 h were moved to a 20 ºC incubator and cultured for another 2 h, rinsed with PBS for three times, and stained with AO/PI for imaging and counting. The adhered cell numbers at 37 ºC and 20 ºC for each substrate were averaged from at least 5 different areas and reported as mean value and standard deviation.

Results and discussion



Scheme 1. Illustration of the synthesis of polymer brushes via SI-ATRP: (a) immobilization of ATRP initiator on silicon wafers; (b) SI-ATRP of MA-L-Ala-OMe or MA-L-Ala-iPA to form polymer brushes.

Two polymer brushes with different pendent groups, *i.e.*, poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA), were synthesized by SI-ATRP in this study (**Scheme 1**). The substrates were firstly modified with the silane-functionalized initiator, followed by SI-ATRP of methacrylamide derivatives of L-alanine. Using the SI-ATRP protocol, a poly(MA-L-Ala-OMe) brush with thickness up to 35 nm was obtained in 24 hours. **Figure 1A** illustrates the growth of poly(MA-L-Ala-OMe) brush as a function of polymerization time at 25 °C. The brush thickness increases rapidly in the first 5 hours and then gradually reaches a plateau. In contrast to the SI-ATRP of (meth)acrylate monomers, which normally presents a linear growth of brush thickness with time, the polymerization of MA-L-Ala-OMe from substrates is less-controlled. Such deviation from "living"/controlled polymerization can be inferred from the non-controlled solution ATRP of (meth)acrylamide monomers. A few reasons were proposed for the non-controlled polymerization behavior of (meth)acrylamide monomers, including the competitive complexation of poly(meth)acrylamide with copper, the strong C-Br bond between the terminal (meth)acrylamide unit in the polymer and the bromine atom as well as cyclization side reaction induced bromine lost.[26, 27] The polymerization of poly(MA-L-Ala-iPA) brush shows a similar growth profile but with a smaller film thickness compared to poly(MA-L-Ala-OMe) **(Figure 1B)**, which indicates the lower reactivity of the MA-L-Ala-iPA monomer. This may be attributed to the presence of one more amide group in the side chain of the MA-L-Ala-iPA monomer. The atomic force microscopy (AFM) topography scans as well as cross sectional profiles of poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes indicate both polymer brushes are homogenous and form smooth films in the scanning range (**Figure S1**). The average root mean square (RMS) roughness calculated for poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes is 2.0 ± 1.3 nm and 0.76 ± 0.02 nm, respectively (5 images were counted for each).

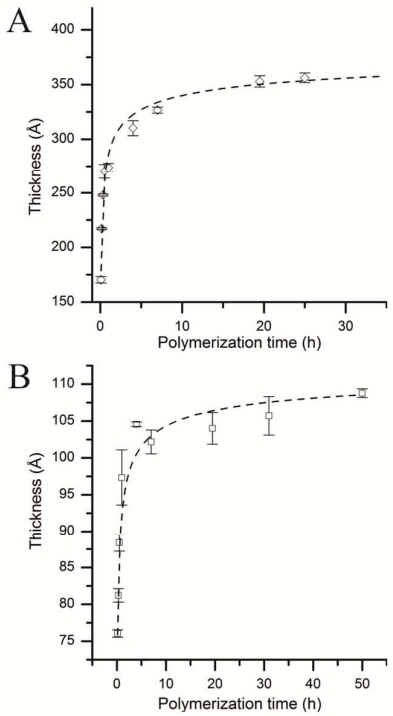


Figure 1. Evolution of the thickness of the poly(MA-L-Ala-OMe) brush (A) and poly(MA-L-Ala-iPA) brush (B) as a function of polymerization time, measured by ellipsometry. The dotted lines were used to guide the eyes.

The poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes were characterized by X-ray photoelectron spectroscopy (XPS). **Figure 2** shows the XPS survey spectra as well as the high resolution C_{1s} scans of the ATRP initiator, poly(MA-L-Ala-OMe) brush and poly(MA-L-Ala-iPA) brush modified substrates. In **Figure 2A**, the initiator survey spectra show the presence of O_{1s}, C_{1s}, N_{1s} and Br_{3d} signals, which is consistent with the composition of the initiator. Compared to the initiator modified substrate, poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes modified substrates show much higher intensity of N_{1s} and C_{1s} signals. Furthermore, the high resolution C_{1s} scans of poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes can be deconvoluted into 5 signals with expected area ratio and electron binding energy, which demonstrate the successful grafting of polymer brushes from the substrates. The C_{1s} scans of poly(MA-L-Ala-OMe) brush are characterized by an ester C=O peak at 289.0 eV (signal e) and an amide C=O peak at 284.7 eV (signal c) while poly(MA-L-Ala-iPA) brush C_{1s} scans merely show the amide C=O peak. The elemental compositions of ATRP initiator, poly(MA-L-Ala-OMe) brush and poly(MA-L-Ala-iPA) brush modified substrates are given in Table S1

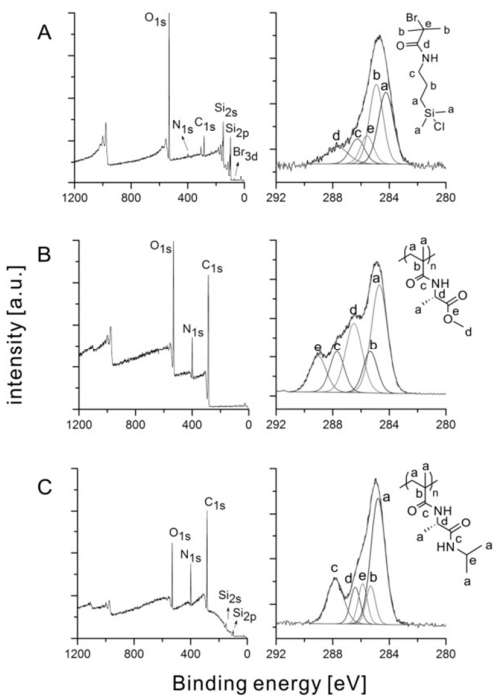


Figure 2. XPS survey spectra and C_{1s} high resolution scans of (A) an initiator modified substrate; (B) a poly(MA-L-Ala-OMe) brush modified substrate and (C) a poly(MA-L-Ala-iPA) brush modified substrate.

The thermo-responsive properties of the polymer brushes were studied with quartz crystal microbalance with dissipation (QCM-D). Both poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes were exposed to water and heated at a rate of 0.2 °C/min in the temperature range of 10 to 40 °C (**Figure 3**). The frequency shift (Δf) for both brushes increases with increasing temperature, which indicates the brushes dehydrate and collapse at elevated temperature. In contrast, the dissipation shift (Δ*D*) for both brushes decreases with increasing temperature, which can be attributed to the increased rigidity of the brushes as they collapse. The curves of Δf versus temperature show different slopes in the experimental temperature range. A collapse temperature for both polymer brushes, $T_c^{heating}$, can be estimated from the inflection point of the Δf curves. The collapse temperature is estimated to be ~ 25 °C for poly(MA-L-Ala-OMe) brush, which is lower than the reported cloud point (35 °C) of poly(MA-L-Ala-OMe)$_{89}$ at 1 mg/mL aqueous solution.[23] In contrast, the collapse temperature for poly(MA-L-Ala-iPA) brush is estimated to be ~ 22.5 °C, which is slightly lower than that of poly(MA-L-Ala-OMe) brush. This result is in good agreement with the fact that poly(MA-L-Ala-OMe) has higher solution cloud point than poly(MA-L-Ala-iPA) with comparable molecular weight under the same condition. The dehydration of the polymer brushes upon heating were further investigated by water contact angle measurements at different temperatures. **Table S2** summarizes the water contact angles of poly(MA-L-Ala-OMe) and poly(MA-L-Ala-iPA) brushes at room temperature (R.T.) and 50 °C. In the case of poly(MA-L-Ala-OMe) brush, the average value of water contact angle increases at elevated temperature, confirming the dehydration of the polymer brush. The small variation of the water contact angle for poly(MA-L-Ala-iPA) brush between R.T. and 50 °C is probably because R.T. is very close to the collapse region, as indicated by QCM-D experiments.

Figure S2 further shows Δf curves as a function of temperature for both polymer brushes under a heating and cooling cycle. Both polymer brushes present reversible thermo-responsive behaviour in the experimental temperature range, while only the poly(MA-L-Ala-iPA) brush shows obvious hysteresis in the cooling ramp. A collapse temperature for both polymer brushes, $T_c^{cooling}$, can be estimated from the inflection point of the Δf curves in the cooling ramp. The $T_c^{cooling}$ of the poly(MA-L-Ala-OMe) brush is estimated to be ~ 22.5 °C while the poly(MA-L-Ala-iPA) brush shows the $T_c^{cooling}$ of ~ 17.5 °C. The $T_c^{cooling}$ of the poly(MA-L-Ala-iPA) brush measured in the cooling ramp is 5 °C lower than that in the heating process. In contrast, the $T_c^{cooling}$ of the poly(MA-L-Ala-OMe) brush only shows 2.5 °C difference between the heating and cooling ramps. This difference is probably due to the presence of one more amide bond in the side chain of poly(MA-L-Ala-iPA) brush. The significant hysteresis in the heating and cooling cycle of the poly(MA-L-Ala-iPA) brush indicates that stronger intra- and inter-chain hydrogen bonding interactions are formed in the poly(MA-L-Ala-iPA) brush during the heating ramp. Therefore more energy is required to break up the hydrogen bonds during the cooling process.

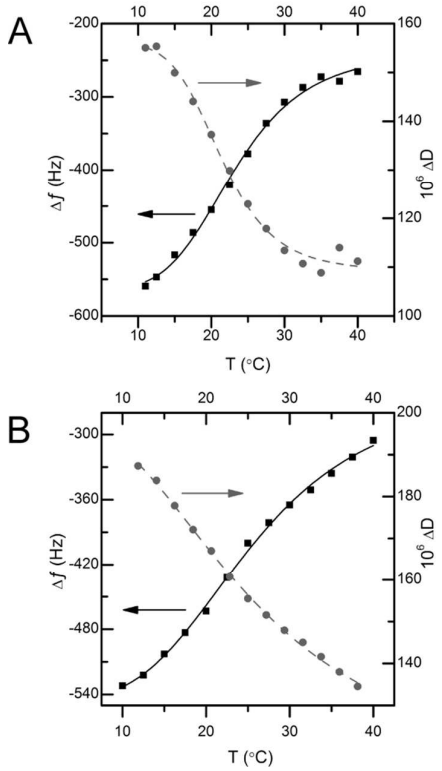


Figure 3. Temperature dependence of the third overtone of the frequency shift (Δf, ■) and of the dissipation shift (ΔD, ●) for (A): a poly(MA-L-Ala-OMe) brush

(thickness = 22 nm) and (B) a poly(MA-L-Ala-iPA) brush (thickness = 10 nm) as measured by QCM-D in water. The brush thickness was measured by ellipsometry.

The cell capture and release experiments were performed on polymer brush modified glass substrates by changing temperature between 37 °C and 20 °C. **Figure 4** shows the captured cell number on both polymer brush modified substrates at 37 °C and the remained cell number after cell release at 20 °C. In the case of poly(MA-L-Ala-iPA) brush modified substrate, the cell density on the substrate at 37 °C is significantly larger than that at 20 °C. The calculated cell density of poly(MA-L-Ala-iPA) brush modified substrate is (1.31 ± 0.99) $\times 10^4$ cm^{-2} at 37 °C. (96.5 ± 3.5) % of captured cells is released as the temperature is reduced to 20 °C. Note that the adhered cells on the substrates are inhomogenous as indicated by the relative big statistic deviations. One possible reason is that the grafted polymers on substrates are polydispersed due to the less-controlled ATRP polymerization. The statistical results demonstrate that poly(MA-L-Ala-iPA) brush can be used to fabricate thermo-responsive substrate for cell capture and release. In comparison, the poly(MA-L-Ala-OMe) brush modified substrate presents lower cell capture and release efficiency, although the statistic analysis of the data indicates the cell density at 37 °C is still larger than that at 20 °C ($P < 0.01$ for the t-test). Given the similar collapse temperature and comparable thickness for poly(MA-L-Ala-OMe) brush and poly(MA-L-Ala-iPA) brush, the difference in cell capture and release efficiency is probably due to the different side chain functionality.[2, 28, 29]

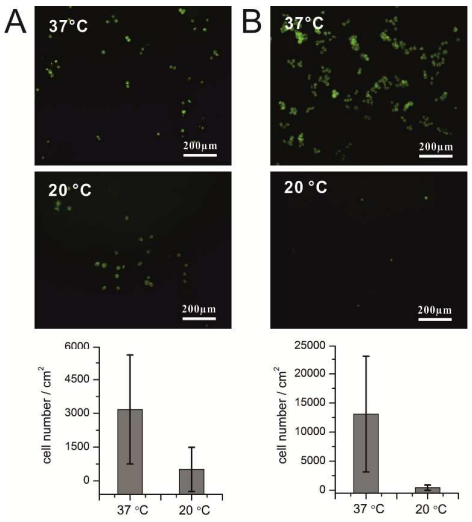


Figure 4. The cell capture and release on (A) a poly(MA-L-Ala-OMe) brush (thickness = 25 nm) and (B) a poly(MA-L-Ala-iPA) brush (thickness = 17 nm) modified substrates at 37 °C and 20 °C, respectively. The histograms indicate the cell density on polymer brush modified substrates at different temperatures. The brush thickness was measured by ellipsometry.

Conclusions

We prepared two novel polymer brushes from methacrylamide derivatives of L-alanine. Both polymer brushes exhibit reversible LCST-type thermo-responsive behavior. The poly(MA-L-Ala-iPA) brush has similar collapse temperature with poly(MA-L-Ala-OMe) brush but shows obvious hysteresis. As a proof of concept, the poly(MA-L-Ala-iPA) brush can be used to fabricate thermo-responsive substrates for cell capture and release, which may have potential applications in cell-sheet engineering.

Acknowledgements

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Notes and references

1. R. Barbey, L. Lavanant, D. Paripovic, N. Schuwer, C. Sugnaux, S. Tugulu and H. A. Klok, *Chem. Rev.*, 2009, **109**, 5437-5527.
2. H. Takahashi, N. Matsuzaka, M. Nakayama, A. Kikuchi, M. Yamato and T. Okano, *Biomacromolecules*, 2012, **13**, 253-260.
3. H. Takahashi, M. Nakayama, K. Itoga, M. Yamato and T. Okano, *Biomacromolecules*, 2011, **12**, 1414-1418.
4. H. Takahashi, M. Nakayama, M. Yamato and T. Okano, *Biomacromolecules*, 2010, **11**, 1991-1999.
5. A. Mizutani, A. Kikuchi, M. Yamato, H. Kanazawa and T. Okano, *Biomaterials*, 2008, **29**, 2073-2081.
6. M. Yamato, M. Utsumi, A. Kushida, C. Konno, A. Kikuchi and T. Okano, *Tissue Eng.*, 2001, **7**, 473-480.
7. N. Matsuzaka, M. Nakayama, H. Takahashi, M. Yamato, A. Kikuchi and T. Okano, *Biomacromolecules*, 2013, **14**, 3164-3171.
8. K. Nagase, J. Kobayashi, A. Kikuchi, Y. Akiyama, H. Kanazawa and T. Okano, *Langmuir*, 2007, **24**, 511-517.
9. R. Xie, S. B. Zhang, H. D. Wang, M. Yang, P. F. Li, X. L. Zhu and L. Y. Chu, *J. Membr. Sci.*, 2009, **326**, 618-626.
10. H. Kanazawa, E. Ayano, C. Sakamoto, R. Yoda, A. Kikuchi and T. Okano, *J. Chromatogr. A*, 2006, **1106**, 152-158.
11. S. Fujishige, K. Kubota and I. Ando, *J. Phys. Chem.*, 1989, **93**, 3311-3313.
12. H. G. Schild, *Prog. Polym. Sci.*, 1992, **17**, 163-249.
13. S. Han, M. Hagiwara and T. Ishizone, *Macromolecules*, 2003, **36**, 8312-8319.
14. J.-F. Lutz, Ö. Akdemir and A. Hoth, *J. Am. Chem. Soc.*, 2006, **128**, 13046-13047.
15. S. Desseaux and H. A. Klok, *Biomacromolecules*, 2014, **15**, 3859-3865.
16. E. Wischerhoff, K. Uhlig, A. Lankenau, H. G. Börner, A. Laschewsky, C. Duschl and J.-F. Lutz, *Angew. Chem. Int. Ed.*, 2008, **47**, 5666-5668.
17. A. Dworak, A. Utrata-Wesolek, D. Szweda, A. Kowalczuk, B. Trzebicka, J. Aniol, A. L. Sieron, A. Klama-Baryla and M. Kawecki, *ACS Appl. Mater. Interfaces*, 2013, **5**, 2197-2207.
18. Q. Liu, A. Singh and L. Liu, *Biomacromolecules*, 2013, **14**, 226-231.
19. X. Wang, H. Gan, M. Zhang and T. Sun, *Langmuir*, 2012, **28**, 2791-2798.
20. X. Wang, H. Gan, T. Sun, B. Su, H. Fuchs, D. Vestweber and S. Butz, *Soft Matter*, 2010, **6**, 3851-3855.

21. T. Sun, D. Han, K. Rhemann, L. Chi and H. Fuchs, *J. Am. Chem. Soc.*, 2007, **129**, 1496-1497.
22. Y. Shen, S. Desseaux, B. Aden, B. S. Lokitz, S. M. Kilbey, Z. Li and H.-A. Klok, *Macromolecules*, 2015, **48**, 2399-2406.
23. D. Yu, C. Luo, W. Fu and Z. Li, *Polym. Chem.*, 2014, **5**, 4561-4568.
24. D. Paripovic and H. A. Klok, *Macromol. Chem. Phys.*, 2011, **212**, 950-958.
25. S. Wang, H. Wang, J. Jiao, K.-J. Chen, G. E. Owens, K.-i. Kamei, J. Sun, D. J. Sherman, C. P. Behrenbruch, H. Wu and H.-R. Tseng, *Angew. Chem.*, 2009, **121**, 9132-9135.
26. J. T. Rademacher, M. Baum, M. E. Pallack, W. J. Brittain and W. J. Simonsick, *Macromolecules*, 2000, **33**, 284-288.
27. M. Teodorescu and K. Matyjaszewski, *Macromolecules*, 1999, **32**, 4826-4831.
28. Y. Arima and H. Iwata, *Biomaterials*, 2007, **28**, 3074-3082.
29. E. Cooper, L. Parker, C. A. Scotchford, S. Downes, G. J. Leggett and T. L. Parker, *J. Mater. Chem.*, 2000, **10**, 133-139.