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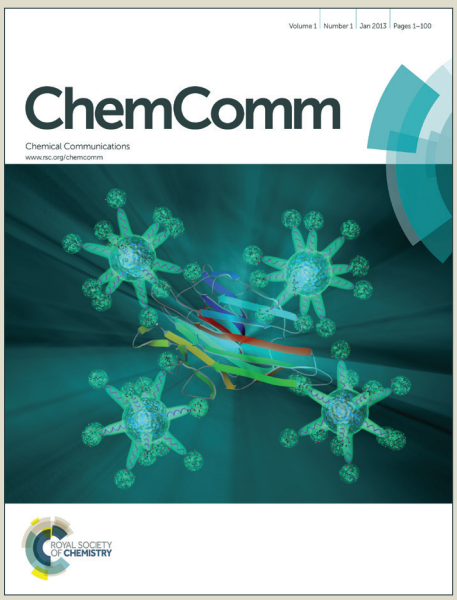

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Saccharide and Temperature Dual-Responsive Hydrogel Layer for Harvesting Cell Sheet

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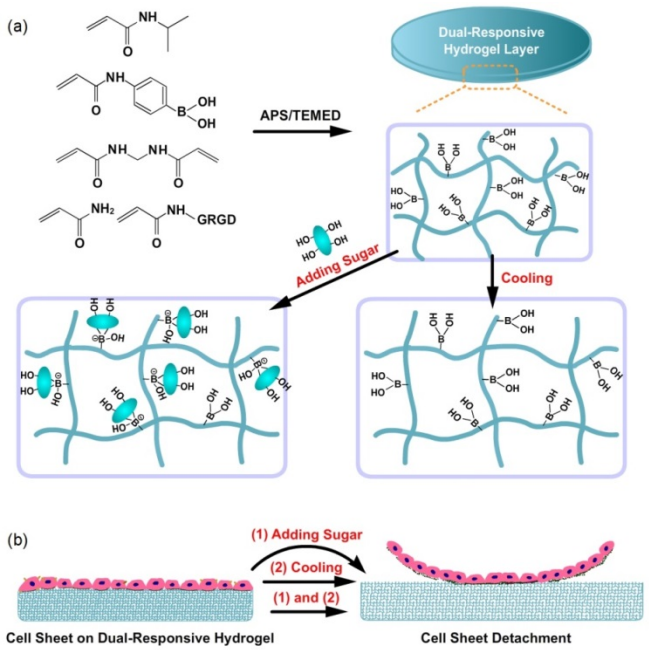
Saccharide and temperature dual-responsive hydrogels have been prepared based on PNIPAAm copolymers containing phenylboronic acid (PBA) groups and used for harvesting cell sheet. The cell sheet could be released from the hydrogel layer at 37 °C simply by increasing sugar concentration, and could be more efficiently released at a lower temperature and elevated sugar concentration.

Stimuli-responsive polymers have shown great potential in biomedical applications due to their "smart" physicochemical properties.[1] One of the most popular examples is the application of poly(*N*-isopropylacrylamide) (PNIPAAm) as thermo-responsive cell culture substrates for cell sheet engineering, which is mainly based on the temperature-induced surface changing from hydrophobic to hydrophilic. Benefiting from the almost non-invasive harvest of an intact monolayer cell sheet along with their deposited extracellular matrix (ECM), this method has shown great promise in cell-based therapies and regenerative medicine, especially scaffold-free tissue engineering.[2] Nevertheless, the temperature-induced cell detachment generally needs to incubate the cells at a low temperature (e.g., 20 °C) or even lower and last for about 30 min or even longer. Evidences showed that the cell metabolic processes were obviously suppressed below 32 °C,[3] which may result in low cell viability of the recovered cell sheet and consequent inefficient clinical therapies. Therefore, it is desirable to achieve rapid cell sheet detachment under warmer temperature (around 37 °C). However, this expectation seems to be impossible puzzle with current temperature-induced cell sheet harvest systems, because enough cooling amplitude is essential for efficient cell sheet harvest with such systems.

Alternatively, several approaches have been developed recently and provided more flexible options for the harvest of cell sheet by using other stimuli-responsive surface materials.[4] These newly emerged developments, such as UV light-induced,[4a] pH-induced,[4b] electricity-induced,[4c] chemical-induced,[4d,4e] and magnetism-induced methods,[4f] however, still have problems. For example, in the UV- and pH-induced methods, ultraviolet irradiation and the deviations from the physiological pH will definitely harm the recovered cells to a certain extent. For other mentioned methods, besides the potential harm caused by chemical agents, electrochemical process or magnetic force, these kinds of recovered cell sheets all inevitably contain residual materials on the bottom due to their detachable surface layer, thus also probably leading to unpredictable side-effects on clinical therapies. Therefore, the exploitation of novel substrate for harvesting cell sheet under a mild operating condition and with desirable non-toxic is of great importance to cell sheet engineering.

Recent studies reported that PNIPAAm copolymers containing phenylboronic acid (PBA) groups had dual responsiveness to temperature and saccharides, especially exhibiting a gradual rising of the lower critical solution temperature (LCST) as the addition of *cis*-diol compounds (e.g., saccharide biomolecules).[5] This is because the hydrophobic PBA can fast complex with some saccharides (e.g., glucose and fructose) to form stable hydrophilic boronate esters, finally improving the hydrophilicity of the PNIPAAm-based copolymers.[6] Taking advantage of this phenomenon, it might be possible to harvest cell sheet using the saccharide-induced surface hydrophilicity change if the cells are cultured on a PBA-containing substrate. Importantly, this method would be non-harmful to the harvested cell sheet because the saccharides, such as glucose and fructose, are essential nutrients for cell culture. Intuitively, it can be speculated that the cell sheet might be detached from a PBA-containing substrate at a faster rate or at a relatively warm temperature (around 37 °C) with the assistance of glucose- or fructose-induced hydration. Such an approach may ingeniously overcome the abovementioned drawbacks in traditional cell sheet technology using thermo-responsive substrates.

In this work, we developed, for the first time, a saccharide and temperature dual-responsive cell sheet technology. The dual-responsive cell culture substrate is based on a saccharide and temperature dual-responsive hydrogel layer, which was composed of both thermo-sensitive PNIPAAm and saccharide-sensitive poly(3-acrylamidophenylboronic acid) (PAPBA). Here, saccharide-responsive monomer 3-acrylamidophenylboronic acid (APBA) was chosen because of its suitable equilibrium association constant with glucose (pK_a = 8.2). Previous studies have shown that, PAPBA are insensitive to a low-glucose concentration (e.g. 1 ~ 2 g/L) at pH of 7.4, however, remarkable responsiveness happens at high concentration of glucose or fructose (> 5 g/L).[7] Thus, the resultant dual-responsive substrate in our system could be tactfully used for cell culture in a low-sugar culture medium (e.g., 1 g /L) and then used for cell detachment in a high-sugar culture medium (i.e., 5 ~ 10 g /L). To gain insights into our novel system, we studied the cell



Scheme 1. (a) Schematic illustration of the saccharide and temperature dual-responsive hydrogel layer. (b) Harvesting of cell sheet from the dual-responsive hydrogel layer by adding the medium with sugar, i.e., glucose or fructose (1), reducing temperature (2), or both.

sheet detachment behaviours by reduction of temperature, changing the medium with a high-sugar medium, or both of the two above means.

The saccharide and temperature dual-responsive hydrogel layer was prepared via a redox initiated polymerization at 37 ^{o}C in phosphate buffer saline (PBS, pH 7.4). In reaction mixture, NIPAAm, APBA, acrylamide (AAm), methylene bisacrylamide (MBAAm), were used as the thermo-responsive monomer, saccharide-responsive monomer, auxiliary monomer, and the crosslinker, respectively (Scheme 1, see details in ESI†). Also, cell adhesive peptide RGD (Arg-Gly-Asp) capped with polymerizable acryloyl group (Acry-RGD) was incorporated into the hydrogel to improve cell adhesion. Polymerization was performed between two glass plates and resulted in a hydrogel thin layer with a thickness of 0.75 mm (according to our previous method).[2d] Following that, the adsorbed oligomers and unreacted monomers on the hydrogel layer were removed by alternately washing with ultrapure water and PBS solution. For comparison, control hydrogels without the saccharide-responsive monomer AAPBA were also prepared.

The resultant hydrogels were first characterized using Fourier transform infrared spectroscopy (FT-IR) (see ESI†, Fig.S1). Besides the characteristic peaks of PNIPAAm such as the amide I band (1656 cm^{-1}, C=O stretching) and amide II band (1543 cm^{-1}, N-H stretching), there is also a characteristic peak of PAPBA (1335 cm^{-1}, B-O stretching) in the spectrum of the resultant hydrogel but not in the control hydrogels. These results confirmed the successful incorporation of both NIPAAm and APBA in the hydrogel.

We then investigated the temperature and saccharide-responsive properties of the hydrogels. Changes in their swelling ratios in PBS containing different amount of sugar (from 0 to 20 g/L) and at different temperature (i.e., 20 or 37 ^{o}C) were measured after swelling equilibria were achieved (in 20 min). As shown in Fig.1 and Fig.S2 in ESI†, the resultant hydrogel exhibited not only great volume

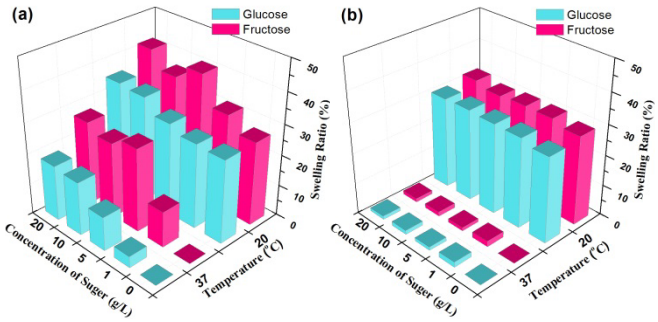


Figure 1. Changes in the swelling ratios of (a) the saccharide and temperature dual-responsive hydrogel and (b) the control temperature-responsive hydrogel in PBS (pH 7.4) containing different amount of sugars at 37 or 20 ^{o}C. The measurements were carried out after immersion of the hydrogels in different PBS and different temperature for 2 h. The swelling ratio at 37 ^{o}C and in PBS without glucose or fructose was defined as 0%.

changes at different temperature (37 vs. 20 ^{o}C) but also marked swelling behaviours in the presence of glucose or fructose, clearly indicating the saccharide and temperature dual-responsiveness of the PNIPAAm- and PAPBA- containing hydrogels. In contrast, the control hydrogel only exhibited temperature-responsive swelling behaviour due to lack of PBA groups in the polymeric network.

Considering that the surface hydrophilicity/hydrophobicity of the hydrogel is a key factor for cell adhesion, we also checked the surface wettability using air bubble contact angle measurement technique (Fig.2). For subaqueous air bubble contact angle test, a more spreading air bubble under the surface indicates a more hydrophobic surface property.[8] Thus, the results in Fig.2 clearly show that the hydrophilicity of the hydrogels increased gradually if the hydrogels were incubated in PBS containing increased amount of glucose or fructose. This is mainly due to the formation of more hydrophilic boronate esters with sugar molecules compared to the original phenylboronic acid groups. It is worth noting that, because of the higher association constant of PBA/fructose complexes,[5c] the hydrophilicity of the dual-responsive hydrogel in the presence of fructose was obviously more than that of glucose, which was also similar with the changes of swelling ratios showed in Fig.1. More importantly, the dual-responsive hydrogel all showed similar swelling ratio and similar surface wettability at 37 ^{o}C and when the

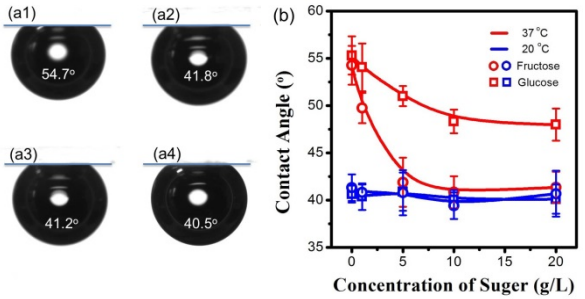


Figure 2. (a) The representative air bubble profiles below the dual-responsive hydrogel in (a1) pure PBS at 37 ^{o}C, (a2) PBS containing 10g/L fructose at 37 ^{o}C, (a3) pure PBS at 20 ^{o}C and (a4) PBS containing 10 g/L fructose at 20 ^{o}C. (b) Changes in the contact angle of the dual-responsive hydrogel in PBS (pH 7.4) containing different amount of sugar at 37 and 20 ^{o}C, respectively. All the data were tested by air bubble contact angle measurement. Before bubble tests, the measurements were carried out after immersing the hydrogels in PBS containing different concentrations of sugar and at different temperature for 30 min.

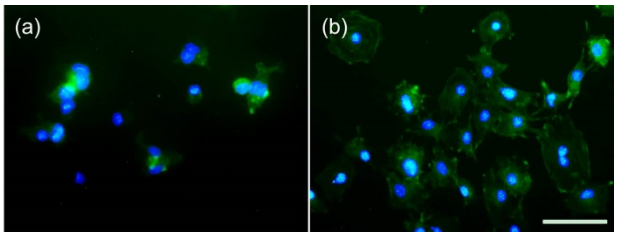


Figure 3. Representative fluorescence micrographs of MC 3T3-E1 cells adhered on the dual-responsive hydrogel without (a) and with AcryRGD as the monomer during polymerization. The molar ratio of the AcryRGD monomer was 5%. Scale bar 100 μm.

concentration of fructose was over 5 g/L, as compared to that at 20 °C and without any sugar in the solution. It clearly showed that the volume phase transition temperature (VPTT) of the dual-responsive hydrogel could be greatly increased in PBS with sugar molecules, which is similar to previous reported increase of LCST in a linear PBA-*co*-PNIPAAm copolymer.[5] More importantly, it also implies that, in our dual-responsive system, increasing sugar concentration in the cell culture medium could dramatically improve the surface hydrophilicity and facilitate cell detachment. However, such an effect could only be achieved by reducing the temperature in traditional thermo-responsive cell sheet harvest systems.

With the dual-responsive hydrogel layer in hand, the detachment of cell sheet from the hydrogels was then checked to determine whether the cell release was dual-responsive toward saccharide and temperature. As mentioned before, to obtain improved cell adhesive properties (because a PBA containing hydrogel is unfavourable to cell adhesion as compared to a hydrogel without PBA in our system), cell adhesive monomer AcryRGD was used during polymerization. MC3T3-E1 cells were then seeded on the hydrogels and cultured at 37 °C in a low-glucose medium (DMEM with 1 g /L of glucose). We first checked the ability of cells to adhere on the hydrogels after 6 h of culture. DAPI and phalloidin reagents were applied for staining of nuclei and F-actin cytoskeleton of adhered cells, respectively (Fig.3). Clearly, the cells showed significant increases in cell adhesion on the hydrogels containing RGD peptide compared to the hydrogel without RGD, indicating the improved cell adhesion property of our dual-responsive hydrogels. We also found that, with RGD peptide copolymerized in the hydrogel, there was no significant difference in cell proliferation between a PBA containing hydrogel and a no PBA one.

After 2 days of culture, the adhered cells proliferated to form a confluent cell monolayer (or cell sheet). The saccharide-responsive cell detachment was first tested by adding the medium with 10 g/L of glucose or fructose at 37 °C. Here, 10 g/L of glucose or fructose were chosen according to the obvious changes in swelling ratio and surface wettability of the hydrogels at 37 °C (Fig.1 and 2). Typically, when medium was added with 10 g/L of fructose, the adhered cell sheet was continuously peeled off from the hydrogel (usually started from the edge of hydrogel) and detached completely within 42 min (Fig.4). Apparently, the saccharide-responsiveness in our system is beneficial for the detachment of an intact cell sheet in which the cell-cell connections are well maintained. Furthermore, the harvested cell sheet could re-adhere on a new substrate in low-glucose DMEM (see Fig.5), implying that the cellular functions as well as ECM components are not impaired during the detachment of cell sheet and therefore the saccharide-triggered cell detachment in this work is in a non-invasive manner and will minimize the adverse impact in therapy efficiency. It should be noted that, when the same concentration of glucose was added in the medium at 37 °C, the cell sheet cannot be completely detached even after 1 h (the blue curve in Fig.6a, see also Fig.S3 in ESI†). This is mainly due to the relatively low association constant of PBA/glucose complexes[5c] at pH 7.4, which results in very limited changes in swelling ratio and surface wettability upon glucose concentration change (Fig 1a and 2a).

In addition, temperature-induced cell sheet detachment in the PNIPAAm- and PAPBA- containing hydrogels was also carried out. Similar to fructose-induced cell detachment, an intact cell sheet could be harvested within 35 min when the temperature was dropped from 37 to 20 °C (the pink curve in Fig.6, see also Fig.S4 in ESI†). This result, together with the abovementioned saccharide-responsive cell detachment, clearly demonstrates that the saccharide and temperature dual-responsive hydrogels can be used for harvesting cell sheet by reducing temperature or adding sugar in the medium, or both. Moreover, in the case of simultaneously reducing the

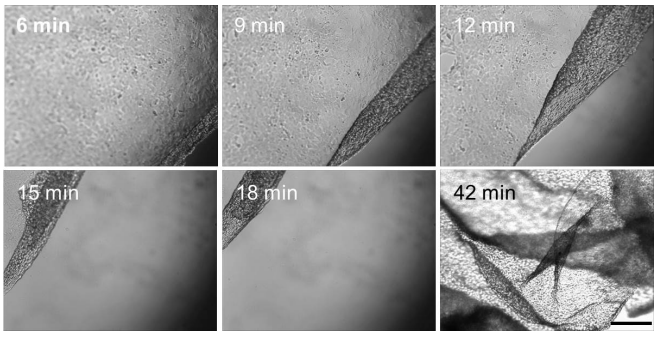


Figure 4. Detachment of a cell sheet from the dual-responsive hydrogel after incubated in 10 g/L of fructose-containing DMED at 6, 9, 12, 15, 18 and 42 min at 37 °C, respectively. Note that the cell sheet migrated out of the view of the microscope after 18 min. However, a harvested cell sheet was found in the medium after 42 min. Scale bar 400 μm.

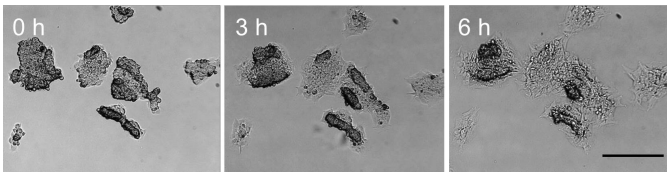


Figure 5. The saccharide-triggered harvested cell sheet re-adhered on a cell culture plate in low-glucose DMEM after 0, 3 and 6 h of culture at 37 °C. To facilitate the cell adhesion, the harvested cell sheet was divided into small pieces. Scale bar 400 μm.

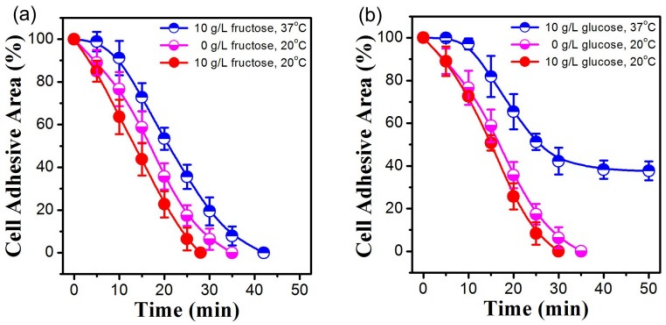


Figure 6. Detachment profiles of cell sheet from the dual-responsive hydrogel after incubated in a low-glucose medium DMEM without or with 10 g/L of glucose (a) or 10 g/L of fructose (b) at different temperature. In both figures, initial area of the cell sheet was defined as 100%.

temperature and increasing the sugar concentration in one system, more efficient cell sheet detachment could be achieved (the red curve in Fig.6a and Fig.6b, see also Fig.S5 and Fig.S6 in ESI†). For example, the cell sheet could be completely detached in 10 g/L of fructose at 20 ^{o}C within 30 min, which is markedly shorter than temperature-induced or sugar-induced cell sheet detachment. These results clearly demonstrate that the dual-responsiveness in our PNIPAAm- and PAPBA- containing hydrogels can be used to effectively accelerate cell sheet detachment as a result of the synergistic effect of reducing temperature and adding sugar.

In summary, we demonstrated here a saccharide and temperature dual-responsive hydrogel layer composed of PNIPAAm and PAPBA copolymers for efficiently harvesting cell sheet. Besides reducing the temperature, an intact cell sheet could, for the first time, be obtained by supplementing nontoxic saccharides (e.g., glucose or fructose) to the culture medium. In addition, with the assistance of saccharide-induced surface hydration, the efficiency of cell sheet detachment at lower temperature could be accelerated. Therefore, our hydrogel system provides a novel strategy for rapid and non-invasive harvest of cell sheet and will hold great promise in cell-based tissue engineering and regenerative medicine.

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Notes and references

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†Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

1. *(a)* C. d. l. H. Alarcon, S. Pennadam and C. Alexander, *Chem. Soc. Rev.*, 2005, **34**, 276-285; *(b)* M. A. Cole, N. H. Voelcker, H. Thissen and H. J. Griesser, *Biomaterials*, 2009, **30**, 1827-1850; *(c)* P. M. Mendes, *Chem. Soc. Rev.*, 2008, **37**, 2512-2529; *(d)* C. Li and S. Liu, *Chem. Comm.*, 2012, **48**, 3262-3278.
2 *(a)* Y. Miyahara, N. Nagaya, M. Kataoka, B. Yanagawa, K. Tanaka, H. Hao, K. Ishino, H. Ishida, T. Shimizu, K. Kangawa, S. Sano, T. Okano, S. Kitamura and H. Mori, *Nat. Med.*, 2006, **12**, 459-465; *(b)* A. Nakamura, M. Akahane, H. Shigematsu, M. Tadokoro, Y. Morita, H. Ohgushi, Y. Dohi, T. Imamura and Y. Tanaka, *Bone*, 2010, **46**, 418-424; *(c)* M. Yamato and T. Okano, *Mater. Today*, 2004, **7**, 42-47; *(d)* G. Pan, Q. Guo, Y. Ma, H. Yang and B. Li, *Angew. Chem. Int. Ed.*, 2013, **52**, 6907-6911.
3 *(a)* J. Fujita, *J. Mol. Microbiol. Biotechnol.*, 1999, **1**, 243-255; *(b)* F. Rico, C. Chu, M. H. Abdulreda, Y. Qin and V. T. Moy, *Biophys. J.*, 2010, **99**, 1387-1396.
4 *(a)* Y. Hong, M. Yu, W. Weng, K. Cheng, H. Wang and J. Lin, *Biomaterials*, 2013, **34**, 11-18; *(b)* O. Guillaume-Gentil, O. V. Semenov, A. H. Zisch, R. Zimmermann, J. Vörös and M. Ehrbar, *Biomaterials*, 2011, **32**, 4376-4384; *(c)* O. Guillaume-Gentil, M. Gabi, M. Zenobi-Wong and J. Vörös, *Biomed. Microdevices*, 2011, **13**, 221-230; *(d)* R. Zahn, E. Thomasson, O. Guillaume-Gentil, J. Vörös and T. Zambelli, *Biomaterials*, 2012, **33**, 3421-3427; *(e)* A. Chassepot, L. Gao, I. Nguyen, A. Dochter, F. Fioretti, P. Menu, H. Kerdjoudj, C. Baehr, P. Schaaf and J.-C. Voegel, *Chem. Mater.*, 2012, **24**, 930-937; *(f)* A. Ito, K. Ino, T. Kobayashi and H. Honda, *Biomaterials*, 2005, **26**, 6185-6193.
5 *(a)* G. Qing, X. Wang, L. Jiang, H. Fuchs and T. Sun, *Soft Matter*, 2009, **5**, 2759-2765; *(b)* Z. Guo, I. Shin and J. Yoon, *Chem. Comm.*, 2012, **48**, 5956-5967; *(c)* J. Yan, G. Springsteen, S. Deeter and B. Wang, *Tetrahedron*, 2004, **60**, 11205-11209; *(d)* T. Hoare and R. Pelton, *Macromolecules*, 2007, **40**, 670-678.
6 *(a)* D. Roy, J. N. Cambre and B. S. Sumerlin, *Chem. Comm.*, 2008, **21**, 2477-2479; *(b)* G. Pan, B. Guo, Y. Ma, W. Cui, F. He, B. Li, H. Yang and K. J. Shea, *J. Am. Chem.* Soc., 2014, **136**, 6203–6206.
7 A. Matsumoto, T. Ishii, J. Nishida, H. Matsumoto, K. Kataoka and Y. Miyahara, *Angew. Chem. Int. Ed.*, 2012, **21**, 2124-2128.
8 M. Kobayashi, T. Matsugi, J. Saito, J.-i. Imuta, N. Kashiwa and A. Takahara, *Polym. Chem.*, 2013, **4**, 731-739.

