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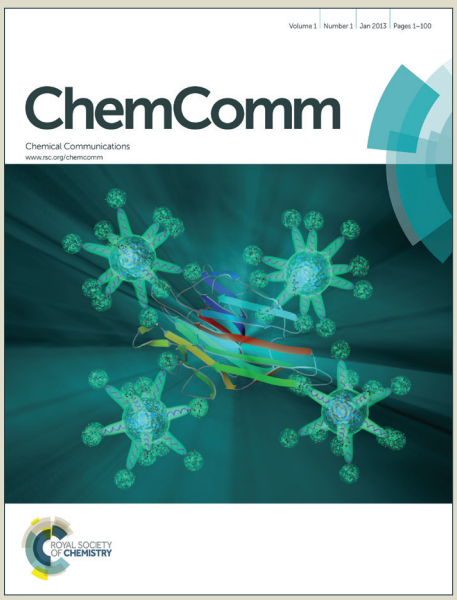

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Directed Self-assembly of Polypeptide-engineered Physical Microgels for Building Porous Cell-laden Hydrogels

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A novel approach to build porous cell-laden hydrogels through the self-assembly of coiled-coil polypeptide on the surface of physical microgels was developed. Both the extracellular microenvironments of pores and physical microgels within assembled constructs could be tailored simultaneously by tuning the polypeptide and morphological features of microgels.

Hydrogels have attracted much scientific interest and have been extensively used as intelligent biomaterials in tissue engineering and regenerative medicine applications due to their unique properties, such as high moisture, softness, acceptable elasticity, and good biocompatibility.[1] Up to date, two major types of hydrogels, which is prepared by chemically cross-linked or physically cross-linked method, have been extensively studied as scaffolds.[2] In recent years, physically cross-linked hydrogels have presented a great potential for biomedical applications.[3] The major reason is that it does not depend on the addition of organic solvents or cross-linking reagents. Compared with synthetic polymer materials, polypeptide-based physical hydrogel can better mimic the complex and dynamic natural extracellular matrix (ECM) because proteins are major players in providing structural support, cell adhesion, and signal regulation in natural ECM.[4]

Microscale hydrogels (microgels) received considerable attention in the past few decades due to their well defined shapes, biocompatibility, controlled microenvironment, and uniform cell density.[5] Assembly of microgels with defined three-dimensional (3-D) structure is a potentially promising approach for bottom-up engineering of tissue construction to mimic the complexity of living tissue because most living tissues and organs are composed of numerous microscale repeating functional units (i.e., neure, muscle fiber, islet, or nephron).[6] Two major types of approaches have been developed for microgel assembly. One is physical association, such as acoustic assembly, magnetic assembly, surface tension driven assembly, and microfluidic assembly,[7] which cannot ensure the mechanical integrity and stability of assembled constructs and limits the final size of assembled constructs. The other kind of assembly is formed through covalent or non-covalent conjugation between microgels. For instance, multicomponent cell-laden constructs could be generated by polyethylene glycol diacrylate (PEGDA) microgels and be stabilized by a secondary UV cross-linking reaction. In our previous study, we have used polypeptide AC_{10}cys as a cross-linker to create large porous constructs with cell-laden PEGDA microgels by a Michael-type addition reaction.[8] Although the extracellular microenvironment of pores within the assembled constructs could be tailored by morphological features of microgels and fusing some bioactive ligands into the polypeptide, encapsulated cells could not spread and migrate in PEGDA microgels. Since cell-cell and cell-extracellular matrix interactions play critical roles in cell and tissue function, control over the cellular microenvironments is a key to create large porous constructs for tissue engineering applications.[7c,9] However, no approaches have been developed so far that can tailor both microenvironments of pores and microgels within assembled constructs.

Herein, we report an approach of modular assembly for building large porous cell-laden hydrogels to address the challenges in 3-D tissue engineering. A photopolymerizable macromer (RGDP-PEG-acrylate) having PEG flanked by a photoreaction acrylate group and a self-assembly polypeptide P was chosen to fabricate physical microgels. The cell-laden physical microgels are able to self-assemble directly forming large constructs in one step (Scheme 1). The extracellular microenvironments of pores and physical microgels within assembled constructs were tailored simultaneously by tuning the polypeptide and morphological features of microgels. The assembly method reported here may provide unique opportunities to create large 3-D tissue engineered products.

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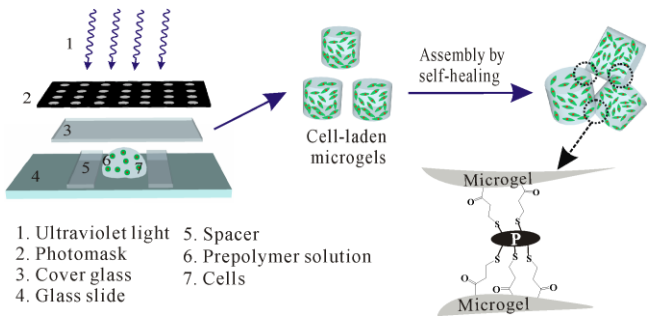

Scheme 1 Schematic illustration of fabrication RGDP-PEG-acrylate microgels by photolithography and directed self-assembly of cell-laden microgels into porous hydrogels.

To prepare cell-laden physical microgel, coiled-coil polypeptide P which can self-assemble into pentamer was chosen to act as the physical junctions in the microgels.[10] The design of multi-functional mocromer and amino acid sequence of the polypeptide RGDPcys are shown in Fig. S1. Photopolymerizable macromer RGDP-PEG-acrylate was synthesized via the Michael-type addition reaction between the cysteine residue of coiled-coil polypeptide RGDPcys and acrylate of PEGDA (molecular weight: 6000) and verified by using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Large excess of PEGDA (molar ratio RGDPcys:PEGDA = 1:10) was used to prevent the production of conjugations in which both acrylate groups of PEGDA were modified (Fig. S2). Far-UV circular dichroism (CD) was employed to determine the secondary structure of polypeptide RGDPcys and RGDP-PEG-acrylate (Fig. S3). The result confirmed that modification of RGDPcys with PEGDA did not alter the coiled-coil structure of the polypeptide. Photo-cross-linkable multi-branched macromer formed from RGDP-PEG-acrylate having PEG flanked by a photoreaction acrylate group and a self-assembly polypeptide P. All microgels including cell-laden ones were fabricated by using photolithography (Scheme 1). The height of the microgels was controlled using microscope cover glass slides as spacers. Microgels of desired shapes (cube, cylinder, star) and sizes were tuned by changing the photomasks (Fig. 1). Our study shows that porosity, permeability, and pore interconnectivity of assembled constructs are strongly dependent on the morphological features of microgels.[8] RGDP-PEG-acrylate microgels disassembled when it was immersed in an 8 M urea buffer. In contrast, RGDP-PEG-acrylate microgels immersing in PBS retained its integrity. These results suggested that this kind of microgels was physical gel, which the physical bonds formed from self-assembly of P domain in the macromer as the junctions.

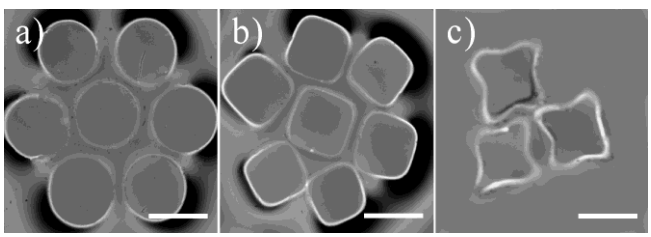

Fig. 1 Optical microscope image of photolithographically fabricated RGDP-PEG-acrylate microgels with different shapes, a) cylinder, b) cube, c) star. Scale bars: 500 μm.

Our previous studies have shown that macroscopic RGDP-PEG-acrylate hydrogel has self-healing property,[11] which may be contributed to the dynamic exchange of polypeptide P at the interface of hydrogels. We hypothesized that the RGDP-PEG-acrylate microgels also have self-healing characteristic. Therefore, RGDP-PEG-acrylate microgels were expected to build large porous 3-D tissue construct through self-healing of microgels. After centrifugation (225 × g) for 5 min, microgels were assembled into a stable construct which retained certain mechanical integrity (Fig. 2a). Low speed centrifugation only made microgels contact with each other. Microgels remained their original morphology in the assembled construct and high stability. This result is ascribed to their high mechanical strength (Fig. S4). Microgels failed to assemble into mechanically integrated construct when 10 mg mL^{-1} polypeptide P was added during assembly. High concentration of polypeptide P could prevent assembly of the microgels because high concentrations of polypeptide P in solution competed with the polypeptide P on the surface of micorgels.[8] This result indicates that porous constructs were formed through self-assembly of polypeptide P on the surface of mcirogels. Compared with previously reported assembly approaches, extra reagents and operations, such as cross-linker and second UV exposure, were avoided in the RGDP-PEG-acrylate microgels assembly system.[5a,8] Since the polypeptide P uniformly distributed on the surface of microgel, large constructs could be assembled within one step, and they would not have limitations in size. In addition, because the polypeptide P is biosynthesized by gene engineering, sequences of interest can be incorporated into engineering polypeptides through the flexibility of recombinant DNA technology. By incorporating bioactive ligands of interest into polypeptide molecules we are not only able to tailor the microenvironment of microgels for cells, but also tune the parameters of pores in the assembled constructs. Compared with preformed porous scaffolds,[12] bioactive ligands incorporated into the photo-cross-linkable macromer could be remained when it was cultured with perfusion flow.

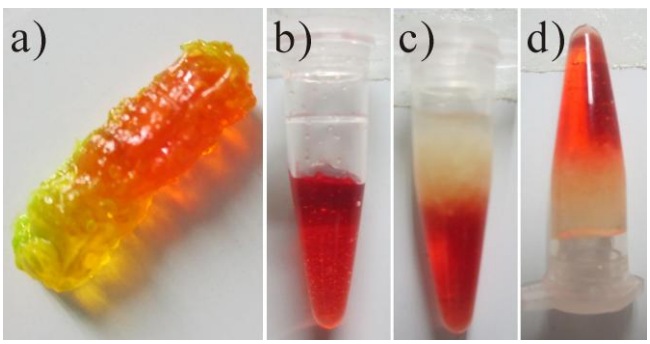

Fig. 2 Directly assembled porous constructs. a) Optical microscope image of a construct assembled from circle-shaped microgels. b) A construct assembled from circle-shaped microgels was stained with a red hydrophobic dye 1,5-diphenylthiocarbazone. c) Microgels stained with a yellow hydrophobic dye 2-amino-4,6-bis-[(4-N,N'-diphenylamino)styryl] pyrimidine were sequentially assembled in a centrifuge tube. d) Microgels maintained their mechanical integrity and original positions when the tube was inverted.

Bottom-up fabrication technique for building large or complex constructs has been explored as approaches to address the key of challenges in 3-D tissue engineering because much of native tissues are often made from repeating functional units.[13] To further evaluate the ability to prepare complex 3-D construct, sequential assembly of different microgels was examined. Two batches of microgels were prepared by photolithography and stained with a yellow hydrophobic dye 2-amino-4,6-bis-[(4-*N*,*N*'-diphenylamino)styryl] pyrimidine[14] and a red hydrophobic dye 1,5-diphenylthiocarbazone, respectively. The microgels containing different dyes were sequentially gathered into a tube to create complex constructs. The mechanical integrity of the assembled construct and the original positions of the distinct

layer were retained after the sample was inverted (Fig. 2b-2d). This observation suggests that different types of cells encapsulated in distinct microgels may create a complex 3-D construct to mimic natural tissue using layer-by-layer assembly.

Cytotoxicity of the photo-cross-linked RGDP-PEG-acrylate hydrogels was examined (Fig. S5). After 48 h culture, only a few dead cells were observed, suggesting that hydrogels containing P domains and PEG are not toxic to mammalian cells *in vitro*. *In vitro* cytotoxicity of the fabrication process of photolithography and assembly process was also tested. Viability of the NIH 3T3 fibroblasts encapsulated in the microgels was measured before and after assembly. Most cells remained viable in the microgels after fabrication, indicating that the photolithographic fabrication step did not result in a significant amount of cell death (Fig. 3a). Almost no change of viability of encapsulated cells was observed when the cell-laden microgels were assembled through self-healing of the microgels (Fig. 3b). Compared with previously reported assembly approaches, such as second UV exposure and acoustic waves,[5a,7a] assembly process through self-healing of the microgels reported here is unaffected with the viability of cells encapsulated in the microgels. Additionally, cells were uniform throughout the microgels and the assembled constructs. Microengineered *in situ* forming hydrogels expected to address the challenges of cell seeding in the preformed porous scaffolds, which typically have a high cell density in the periphery of the scaffolds.[7c,8,13a,15]

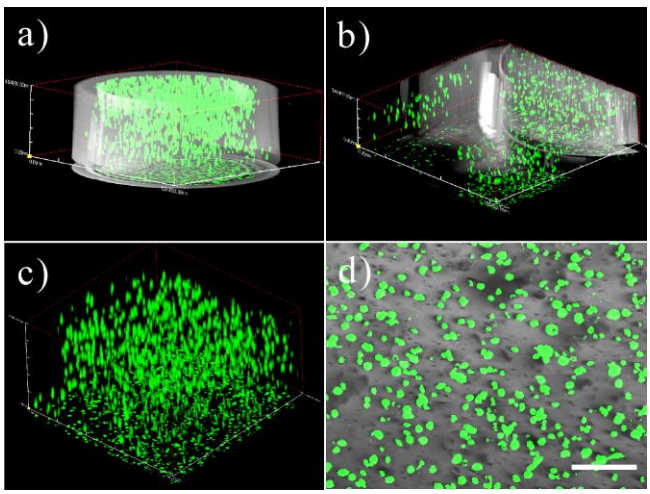

Fig. 3 3-D encapsulation of fibroblast in microgels and directly assembled constructs. All images were acquired after the samples were stained with calcein AM and ethidium homodimer-1. 3-D confocal images of NIH 3T3 fibroblasts encapsulation in microgels before (a) and after (b) self-assembly. 3-D confocal images (c) and 2-D confocal fluorescence images (d) of assembled cell-laden construct after 24 h culture. A 10× objective (0.40 numerical apertures) was used. The scale bar is 200 μm.

Spreading, migration, and proliferation of cells in biomaterials are a key for engineering 3-D artificial tissues. Because RGDP-PEG-acrylate microgel is a physical hydrogel containing a dynamic pentamer formed from the P domain and an RGD cell-binding domain, cells encapsulated in the microgels are expected to spread and migrate freely.[16] After culture for 24 h, the confocal images of encapsulated cells in the assembled constructs are shown in Fig. 3c. NIH 3T3 fibroblasts encapsulated in the RGDP-PEG-acrylate physical microgels spread and migrate freely. This may be due to the dynamic polypeptide P in the RGDP-PEG-acrylate physical microgels to provide the necessary paths for the spreading and migration of cells. Cell fast spreading and migration indicate that cells can migrate and spread well through dynamic paths formed by the polypeptide P, and that the dynamism of pentamer is continuous and fleet. The mechanical strength of the microgels may remain unaffected by spreading and migration of cells because the assembly of coiled-coil polypeptide P is reversible.[10] In comparison, cells in the proteolytically degradable hydrogel started to spread and migrate through the hydrogel matrix after three days, and the mechanical strength of the proteolytically degradable hydrogels gradually decreased with culturing. In addition, the linkages between the microgels are identical composition of the physical microgel. Thus, the physical, dynamic self-assembly of polypeptide P may allow cells, such as endothelial cells, to adhere and migrate freely in the pores of the assembled construct, where this assembled approach through self-healing of the microgels is beneficial to form vascular system in the assembled construct.

Having demonstrated the ability to create large 3-D tissue products by the assembly approach reported here, we subsequently used NIH 3T3 cells as a model to evaluate the cell viability after the assembly process steps. We observed that cell viabilities in constructs assembled from circle-shaped microgels over 4 days, and in controls of nonporous hydrogels formed from 8% (w/v) RGDP-PEG-acrylate, were comparable (Fig. 4). At 0.5 cm below the top surface, the live/dead assay revealed that the cell viabilities in both assembled constructs and in controls were above 90%. The cell viabilities at 1.5 cm and 0.5 cm below the top surface of the assembled constructs under perfusion culture have no obvious difference. This result suggests that the construct formed from the RGDP-PEG-acrylate microgels is suitable to create large complex tissue products under perfusion culture. In contrast, for nonporous hydrogels, many dead cells were observed at 1.5 cm below the top surface under statically culture. The difference of cell viability was mainly determined by the diverse permeability of nutrients and oxygen in different gel type or gel depth. In the top layer, both cell viabilities of assembled porous hydrogels and nonporous hydrogels are similar and relatively high, indicating that nutrients and oxygen could diffuse into this layer in both assembled constructs and nonporous hydrogels. A possible reason for this is that the pore sizes of 8% (w/v) nonporous hydrogel are relatively large (about 20 μm) (Fig. S6).[11] However, in the bottom layers, the cell viabilities of assembled constructs were higher than those in nonporous hydrogels. These results indicated that large 3-D constructs modularly assembled from RGDP-PEG-acrylate microgels have well interconnected channels which can be used to perform the function of vessels to transport enough nutrients and oxygen to cells and maintain cell viability throughout the whole assembled constructs during 3-D culture.

In conclusion, we have developed a modular assembly approach to create large porous cell-laden constructs through self-healing of photolithographically fabricated RGDP-PEG-acrylate microgels. Different microgels could be assembled to form complex constructs through a sequential bottom-up self-assembly. The physical bonds formed from the self-assembly of the polypeptide P in the microgels provide reversible opening and closing paths for the spreading and migration of encapsulated cells. Both microenvironments of the microgels and the pores in the assembled constructs could be tailored by modification of the identical polypeptide in the microgels and linkages. Furthermore, the constructs assembled from circle-shaped microgels presented a better permeability and pore interconnectivity than isopyknic nonporous hydrogels. Therefore, this assembly approach is expected to address the challenges in 3-D tissue engineering and provide unique opportunities to generate biological tissues.

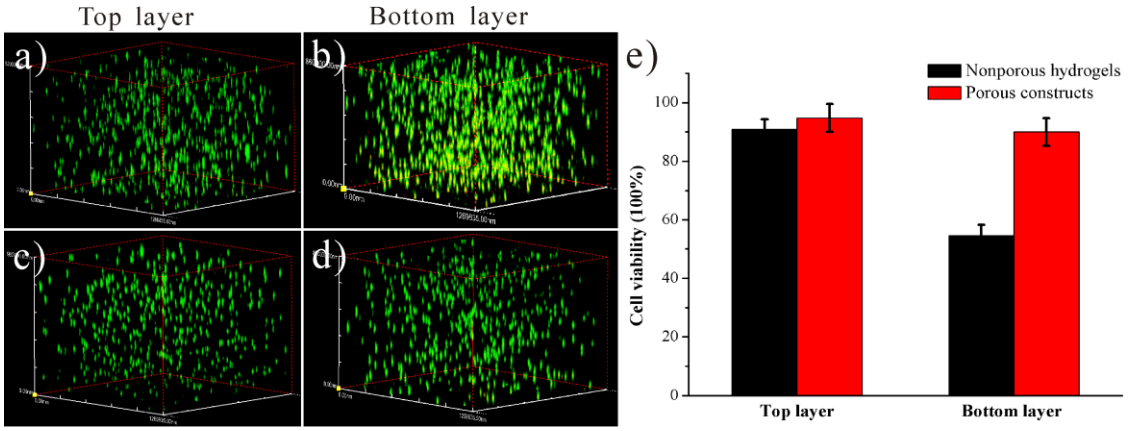

Fig. 4 Viability of fibroblasts cultured in assembled construct. All samples were stained with calcein AM and ethidium homodimer-1. 3-D confocal fluorescence images of nonporous RGDP-PEG-acrylate hydrogels after 4 days of statically culture as revealed by the samples at 0.5 cm (a) and 1.5 cm (b) below the top surface of the constructs. 3-D confocal fluorescence images of porous constructs assembled from circle-shaped RGDP-PEG-acrylate microgels after 4 days of perfusion culture as revealed by the samples at 0.5 cm (c) and 1.5 cm (d) below the top surface of the constructs. e) Quantifying viability of fibroblasts cultured in nonporous and porous constructs after 4 days culture. A 10× objective (0.40 numerical apertures) was used.

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Notes and references

1 a) K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869-1879; b) J. Kopeček, *Biomaterials*, 2007, **28**, 5185-5192.

2 a) D. Seliktar, *Science*, 2012, **336**, 1124-1128; b) S. Himmelein, V. Lewe, M. A. Stuart and B. J. Ravoo, *Chem. Sci*., 2014, **5**, 1054-1058.

3 a) M. Guvendiren, H. D. Lu and J. A. Burdick, *Soft Matter*, 2012, **8**, 260-272; b) Y. Cao and H. Li, *Chem. Commun*., 2008, 4144-4146.

4 W. Shen, in *Biomaterials for Tissue Engineering Applications*, ed. A. B. Jason and L. M. Robert, Springer, New York, 2011, pp. 244-269.

5 a) Y. Du, E. Lo, S. Ali and A. Khademhosseini, *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 9522-9527; b) J. A. Hanson, C. B. Chang, S. M. Graves, Z. Li, T. G. Mason and T. J. Deming, *Nature*, 2008, **455**, 85-89.

6 a) U. A. Gurkan, S. Tasoglu, D. Kavaz, M. C. Demirel and U. Demirci, *Adv. Healthcare Mater.*, 2012, **1**, 149-158; b) R. Gauvin and A. Khademhosseini, *ACS Nano*, 2011, **5**, 4258-4264.

7 a) F. Xu, T. D. Finley, M. Turkaydin, Y. Sung, U. A. Gurkan, A. S. Yavuz, R. O. Guldiken and U. Demirci, *Biomaterials*, 2011, **32**, 7847-7855; b) Y. Li, G. Huang, X. Zhang, B. Li, Y. Chen, T. Lu, T. J. Lu and F. Xu, *Adv. Funct. Mater.*, 2013, **23**, 660-672; c) B. Zamanian, M. Masaeli, J. W. Nichol, M. Khabiry, M. J. Hancock, H. Bae and A. Khademhosseini, *Small*, 2010, **6**, 937-944; d) L. E. Euliss, S. G. Grancharov, S. O'Brien, T. J. Deming, G. D. Stucky, C. B. Murray and G. A. Held, *Nano Lett.*, 2003, **3**, 1489-1493.

8 B. Liu, Y. Liu, A. K. Lewis and W. Shen, *Biomaterials*, 2010, **31**, 4918-4925.

9 S. Levenberg, J. Rouwkema, M. Macdonald, E. S. Garfein, D. S. Kohane, D. C. Darland, R. Marini, C. A. van Blitterswijk, R. C. Mulligan, P. A. D'Amore and R. Langer, *Nat. Biotechnol.*, 2005, **23**, 879-884.

10 a) S. K. Gunasekar, M. Asnani, C. Limbad, J. S. Haghpanah, W. Hom, H. Barra, S. Nanda, M. Lu and J. K. Montclare, *Biochemistry*, 2009, **48**, 8559-8567; b) W. Shen, K. Zhang, J.A. Kornfield and D. A. Tirrell, *Nat. Mater.*, 2006, **5**, 153-158.

11 M. H. Yao, J. Yang, M. S. Du, J. T. Song, Y. Yu, W. Chen, Y. D. Zhao and B. Liu, *J. Mater. Chem. B*, 2014, **2**, 3123-3132.

12 S. H. Cartmell, B. D. Porter, A. J. Garcia and R. E. Guldberg, *Tissue Eng.*, 2003, **9**, 1197-1203.

13 a) A. Khademhosseini and R. Langer, *Biomaterials*, 2007, **28**, 5087-5092; b) A. R. Bausch and K. Kroy, *Nat. Phys.*, 2006, **2**, 231-238.

14 B. Liu, X. L. Hu, J. Liu, Y. D. Zhao and Z. L. Huang, *Tetrahedron Lett.*, 2007, **48**, 5958-5962.

15 M. Radisic, M. Euloth, L. Yang, R. Langer, L. E. Freed, and G. Vunjak-Novakovic, *Biotechnol. Bioeng.*, 2003, **82**, 403-414.

16 B. D. Olsen, J. A. Kornfield and D. A. Tirrell, *Macromolecules*, 2010, **43**, 9094-9099.

