Title

Ultrastrong and flexible hybrid hydrogels based on solution self-assembly of chitin nanofibers in gelatin methacryloyl (GelMA)

Journal

Journal of Materials Chemistry B, 4(15)

ISSN

2050-750X

Authors

Hassanzadeh, P
Kazemzadeh-Narbat, M
Rosenzweig, R
et al.

Publication Date

2016-04-21

DOI

10.1039/c6tb00021e

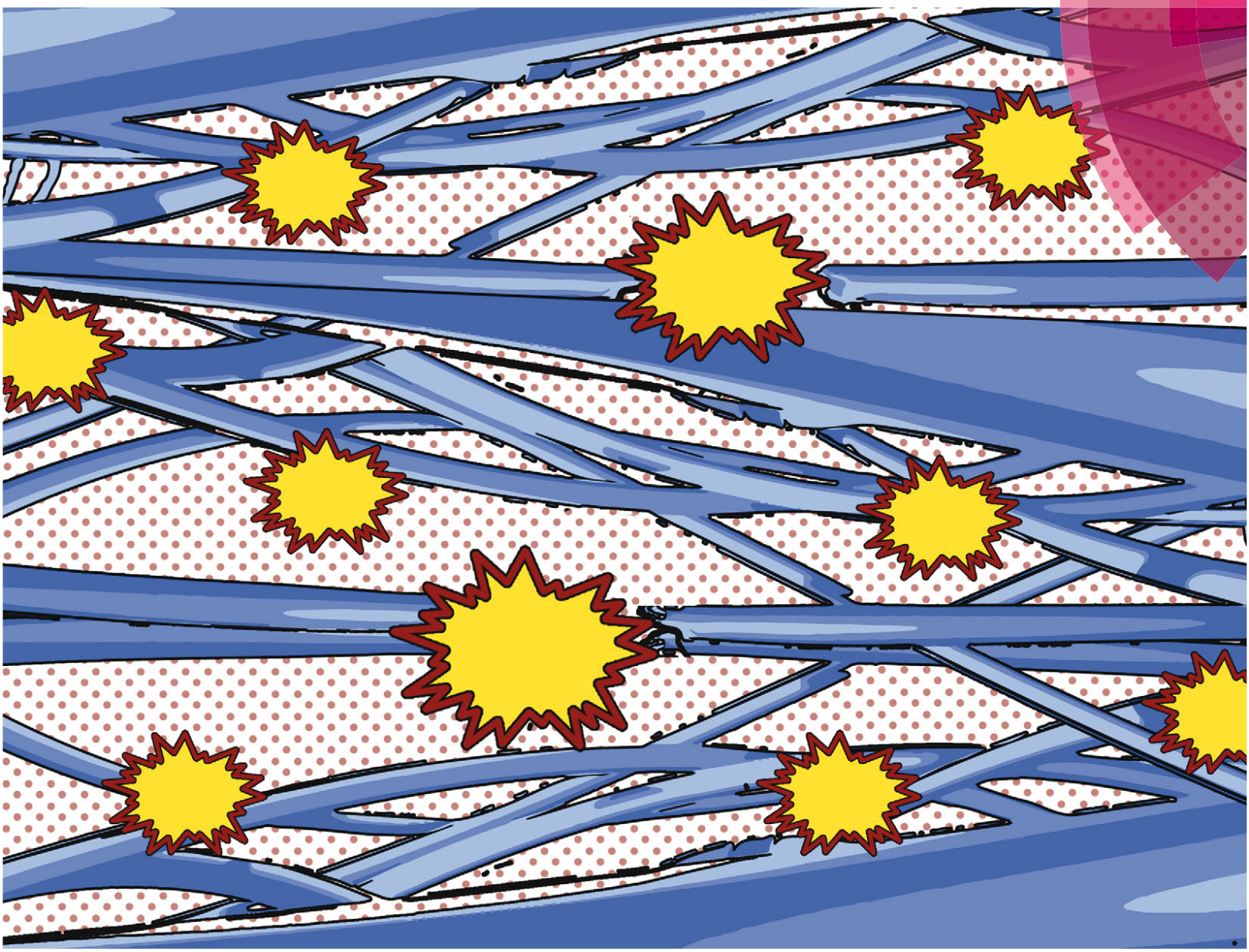
Peer reviewed

Volume 4 | Number 15 | 21 April 2016 | Pages 2529–2742

Journal of Materials Chemistry B

Materials for biology and medicine

www.rsc.org/MaterialsB



ISSN 2050-750X

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

A. Khademhosseini, N. Annabi, M. Rolandi *et al.*

Ultrastrong and flexible hybrid hydrogels based on solution self-assembly of chitin nanofibers in gelatin methacryloyl (GelMA)

175 YEARS

Journal of Materials Chemistry B

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: J. Mater. Chem. B, 2016, 4, 2539

Received 6th January 2016,
Accepted 23rd February 2016

DOI: 10.1039/c6tb00021e

www.rsc.org/MaterialsB

Ultrastrong and flexible hybrid hydrogels based on solution self-assembly of chitin nanofibers in gelatin methacryloyl (GelMA)†

P. Hassanzadeh,[ab] M. Kazemzadeh-Narbat,[cd] R. Rosenzweig,[a] X. Zhang,[ab] A. Khademhosseini,*[cdefg] N. Annabi*[cdh] and M. Rolandi*[ab]

We demonstrate the formation of ultrastrong and flexible hydrogels *via* self-assembly of chitin nanofibers in the presence of gelatin methacryloyl. We tune the mechanical properties of the hydrogel using the chitin nanofiber content and show proof-of-concept applications in engineering vascular tissues.

Hydrogels are biological scaffolds with applications in tissue engineering and regenerative medicine.[1] Hydrogels are prepared from hydrophilic networks of polymers with porous structures that allow efficient transport of oxygen and nutrients for optimal cell growth.[2] Hydrogels closely match the mechanical properties and flexibility of soft tissues for flawless organ integration. However, when hydrogels are too soft, their handling while maintaining tissue integrity becomes challenging.[3] To overcome this challenge, hydrogels are reinforced with more rigid components to create hybrids that are still soft but with improved mechanical robustness.[4] Here, we introduce the one-pot self-assembly of new crosslinked gelatin methacryloyl (GelMA) hydrogels reinforced with chitin nanofibers (GMAC) (Fig. 1). The chitin nanofiber reinforcement increases both stiffness and strain-to-failure of the hydrogels improving their handling and integrity.

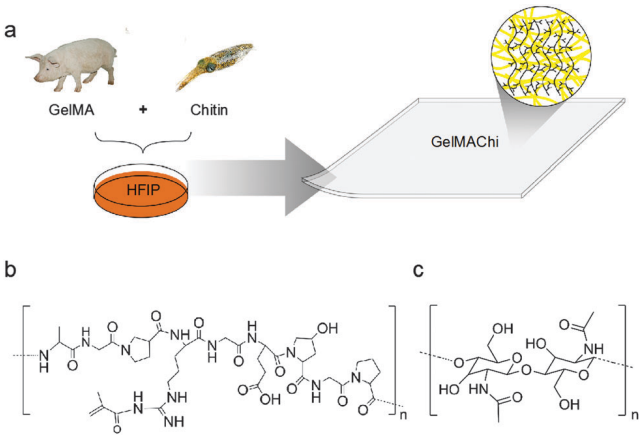


Fig. 1 (a) Schematic illustration of the self-assembly process of chitin nanofibers-gelatin methacryloyl (GMAC) films. Lyophilized porcine gelatin methacryloyl (GelMA) and squid pen β-chitin (Chi) are co-dissolved in hexafluoroisopropanol (HFIP) to yield clear solutions with different GelMA : chi weight ratios. Upon drying, the solution forms transparent films comprised of ultrafine (3 nm) chitin nanofibers self assembled within UV-crosslinkable GelMA, (b) molecular structure of GelMA, (c) molecular structure of chitin.

The simple self-assembly process for these hydrogels is amenable to soft lithography strategies to create microstructures. Human Umbilical Vein Endothelial Cells (HUVECs) co-cultured with Human Mesenchymal Stem Cells (HMSCs) proliferate, and align on the micropatterned hydrogels and express vasculogenic markers indicating cellular differentiation and vascular network formation.

GelMA is a gelatin derivative that is synthesized by the direct reaction of gelatin with methacrylic anhydride (MA) (Fig. 1b).[5] Gelatin is a hydrolytically degraded collagen, one of the most abundant components of the extracellular matrix (ECM).[6,7] Photocrosslinking of GelMA using a water-soluble photoinitiator enhances stability of the water-soluble gelatin in tissue culture for applications in cardiac and vascular tissue engineering, and drug-incorporated hydrogels for bone tissue engineering.[8] GelMA is soft with an elastic modulus of 3.3–110 KPa depending on the degree of methacrylation and concentration of GelMA.[9,25]

[a] Department of Materials Science and Engineering, University of Washington, Seattle, WA 98195, USA
[b] Department of Electrical Engineering, University of California-Santa Cruz, Santa Cruz, CA 95064, USA. E-mail: mrolandi@ucsc.edu
[c] Biomaterials Innovation Research Center, Division of Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02139, USA. E-mail: alik@rics.bwh.harvard.edu
[d] Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[e] Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA
[f] Department of Bioindustrial Technologies, College of Animal Bioscience and Technology, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[g] Department of Physics, King Abdulaziz University, Jeddah 21569, Saudi Arabia
[h] Department of Chemical Engineering, Northeastern University, Boston, MA, 02115-5000, USA. E-mail: n.annabi@neu.edu

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6tb00021e

As a result, reinforcement of GelMA is desirable for many applications.[10] Mineralized GelMA hydrogel with either Ca^{2+} binding-carboxyl groups[11] or titanium[12] has improved modulus for tissue repair. The elastic modulus of soft GelMA hydrogels increases from 10 KPa to 24 KPa *via* the incorporation of dextran glycidyl methacrylate upon photocrosslinking,[13] and to 50 KPa upon reinforcement with aligned carbon nanotubes.[3] Also, adding silk fibroin to GelMA hydrogels increases their compressive elastic modulus up to 80 KPa.[2] Following this exciting route, here we develop GelMA with self-assembled chitin nanofibers to make a fiber-reinforced hybrid hydrogel.

Chitin [poly(β-(1,4)-*N*-acetyl-D-glucosamine)] (Fig. 1c) is the second most abundant natural polysaccharide after cellulose.[14] Chitin occurs as ordered crystalline nanofibers and is the major structural component of cell walls in fungi and yeast, the exoskeleton of arthropods and mollusk shells.[15] Chitin is mechanically robust with an elastic modulus of 2 GPa and an ultimate tensile strength of 140 MPa comparable to aluminum.[16] Biomedical applications of chitin include sutures and wound dressings,[17] tissue engineering scaffolds,[14] microneedles for diagnostics,[18] and biocompatible electronic devices.[19] We have previously developed the self-assembly of 3 nm chitin nanofibers from solution[16,20,21] and demonstrated simple solution co-assembly of chitin nanofiber-silk biocomposites.[22] This solution process is amenable to facile soft-lithography strategies to create microstructures[23] for tissue engineering[16,24] and biomedical devices.[18]

Gelatin is water soluble and is not stable in an aqueous environment.[2] To form the chitin reinforced hybrid hydrogel, we first synthesize GelMA by functionalizing the primary amines in gelatin with methacryloyl groups according to previously published procedures (Fig. 2a).[2] To create the GelMA-chitin (GMAC) hydrogels, solutions of squid pen β-chitin and GelMA co-dissolved in HFIP are dried on a polydimethylsiloxane (PDMS) mold to yield GMAC films (Fig. 2b). The same process also yields films of gelatin and chitin nanofibers (GelChi) when pristine gelatin is used instead of GelMA (Fig. S1, ESI†). Exposure of GMAC to UV light (365 nm, 100 W, 115 V, UVP™ Blak-Ray™ B-100A UV lamps) for 3 minutes in the presence of Irgacure 2959 as the photoinitiator results in the crosslinking of the methacryloyl groups in GelMA producing a covalently-bonded matrix intertwined with the chitin nanofibers (Fig. 2b). GMAC films are more stable in an aqueous environment than GelChi films with a polymerization efficiency of approximately 40% (Fig. S2, ESI†). In GelChi films, the entirety of the gelatin washes away after only one day under physiological conditions (Fig. S2, ESI†).

Chitin nanofibers self-assemble within GelMA and yield GMAC hybrid films with a variable chitin nanofiber content (Fig. 3). As the prepared GelMA film is smooth (Fig. 3a), increasing the relative concentration of chitin and GelMA in GMAC31, GMAC11, GMAC13 (GMACXY, where X : Y = GelMA : chitin weight ratio) yields films with an increased fraction of chitin nanofibers as expected (Fig. 3b–d).[20] In GMAC31 (Fig. 3b), the large amount of GelMA may result in the formation of GelMA agglomerates. In GMAC11 and GMAC13 (Fig. 3c and d), the chitin nanofibers in the co-assembled hybrid hydrogel have the same entangled

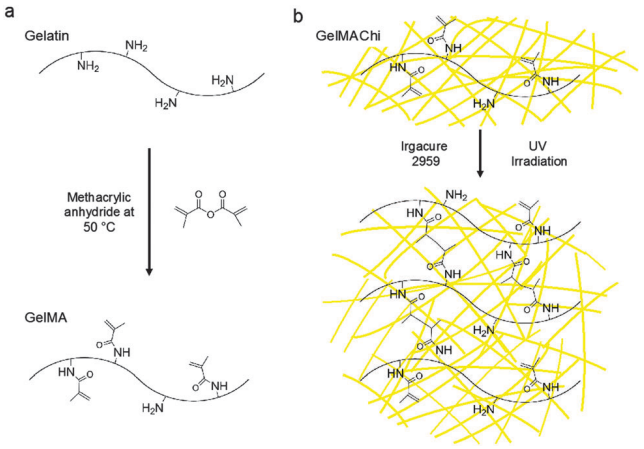


Fig. 2 Synthesis of (a) gelatin methacryloyl (GelMA) through functionalizing gelatin's primary amine groups by methacrylic anhydride (MA) at 50 °C, (b) crosslinked GelMA-chitin (GMAC) films by exposure of dry GMAC films to UV irradiation in the presence of a photoinitiator (PI), Irgacure 2959, for 3 minutes. This process crosslinks the methacryloyl groups producing a stable covalently-bonded GelMA structure intertwined with chitin nanofibers.

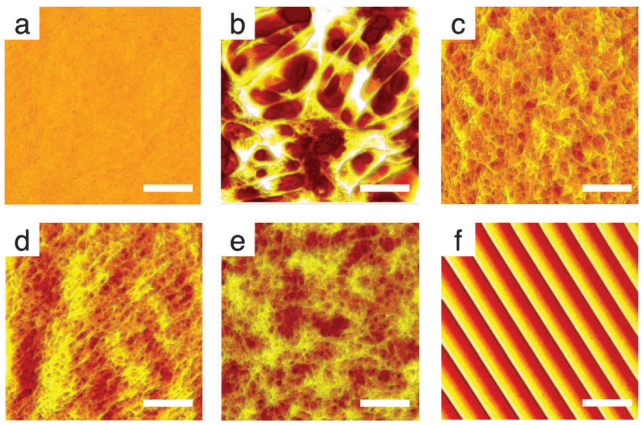


Fig. 3 (a–e) Topographic atomic force microscopy (AFM) images of GelMA, GMAC films with different GelMA : chitin weight ratios, and chitin. GMACXY are GMAC films with XY = GelMA : chitin weight ratio. (a) GelMA, (b) GMAC31, (c) GMAC11, (d) GMAC13, and (e) chitin, respectively (scale bars are 500 nm). (f) Topographic AFM image of micro-patterned GMAC13 with a pitch of 12.15 μm and a height of 450 nm (scale bar is 20 μm).

structure as the chitin nanofibers self-assembled from a chitin-only HFIP solution (Fig. 3e) indicating the robustness of the chitin nanofiber self-assembly in the presence of GelMA. This microstructure control affords a simple strategy to fine-tune the mechanical properties of GMAC hydrogels.

The GMAC films are solution-processable and amenable to soft-lithography strategies that we have previously developed for chitin[16] and chitin-silk.[22] Micropatterns with the pitch of 12.15 μm and height of 450 nm are fabricated on GMAC13 using solution-based replica molding (Fig. 3f).

GMAC hydrogels are overall more robust than their GelMA counterparts without chitin nanofiber reinforcement (Fig. 4). GelMA hydrogels have elastic moduli in the range of 3.3–110 kPa depending on the GelMA concentration and degree of methacrylation.[25] Chitin nanofibers in the matrix of GMAC hydrogels

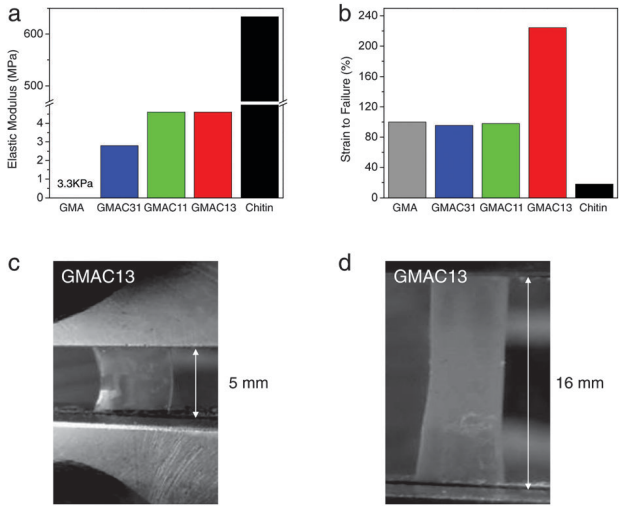


Fig. 4 Mechanical properties of hybrid GMAC hydrogels from tensile test analysis, (a) elastic moduli obtained from slopes of linear region in engineering stress–strain curves (Fig. S4, ESI†) of the hydrogels with different GelMA : chitin weight ratios. The elastic modulus for all GMAC hydrogels are higher than what has been reported for GelMA[25] and they are lower than chitin alone, (b) values of engineering strain to failure for hybrid hydrogels with different GelMA : chitin weight ratios. GMAC13 is >100% more extensible than GelMA[25] and >200% more extensible than chitin alone, (c) optical image of GMAC13 at the initial state before load application in mechanical tensile tester, (d) optical image of GMAC13 showing 224% extensibility under tensile load, showing the final length of 16 mm.

increase the elastic modulus significantly (from 3.3 KPa to 2.8 MPa for GMAC31 and to 4.6 MPa for GMAC11 and GMAC13) (Fig. 4a). The increase in the elastic modulus for GMAC is due to physical reinforcement of the soft matrix of GelMA with entangled chitin nanofibers. This increase in elastic modulus is more pronounced for GMAC13, which has a higher content of chitin nanofibers. While we have previously observed strong hydrogen bonding between chitin and silk,[22] GelMA and chitin do not show any substantial hydrogen bonding between the two molecules as indicated by the Fourier Transform Infrared (FTIR) spectra (Fig. S3, ESI†).[22] This low degree of hydrogen bonding between GelMA and chitin might be due to a small number of available amine groups in the highly methacryloyl-modified (>80%) GelMA. These amine groups are required for hydrogen bonding with the C=O in chitin (Fig. 2). It is likely that this reduced degree of hydrogen bonding in the GMAC composites causes a decreased elastic modulus from 633.6 MPa for chitin to 4.6 MPa for GMAC with only 10% GelMA (Fig. 4a). It is conceivable that GelMA positions itself between the chitin nanofibers, reducing the intrafiber hydrogen bonding, and acts as a lubricant to yield a material with a lower elastic modulus than what is expected from the rule of mixtures (Fig. 4a). This lubrication effect affords, on the other hand, a very high strain-to-failure or extensibility of 224% for GMAC hydrogels. This strain-to-failure is >100% improvement with respect to GelMA hydrogels and >200% improvement with respect to chitin alone (Fig. 4b–d). The GMAC11 and GMAC31 hydrogels also stretch 80% more than chitin (Fig. 4b). We select GMAC13 for cell culture because of its higher elastic modulus and superior extensibility (Fig. 4c and d).

As a proof-of-concept we co-culture HUVECs/HMSCs on micropatterned GMAC13 hydrogels (Fig. 5a–d). We quantify the alignment of HUVECs/HMSCs on day 3 according to the direction of the actin filaments on both patterned and non-patterned hydrogels and grouped in 10° increments (Fig. 5e and f). For patterned hydrogels, the cells mostly align and elongate along the axis of the micropatterns with the pitch size of 12.15 μm and a height of 450 nm. There is no cell alignment after 3 and 5 days on non-patterned hydrogels used as control. For micropatterned hydrogels, over 50% of the cells align within the 0–10° increment angle and approximately 95% of cells are aligned with an angle <40° with respect to the direction of the micropatterns (Fig. 5e). Unlike the patterned hydrogels, the cells randomly orient on the non-patterned control hydrogels (Fig. 5f). Fluorescence images of cells on day 5 on either patterned hydrogels or hydrogels without any patterns demonstrate the formed organized networks with neighboring cells or random interconnected networks of neighboring cells (Fig. 5c and d). In our previous study, higher cellular alignment was observed on patterned chitin scaffolds compared to non-patterned ones. We found that cell alignment, within the 0–10° increment angle, was around 55% in the patterned scaffold which was significantly higher than in the non-patterned chitin scaffold (15%).[16] Here, we observe a higher degree of alignment for the micropatterned GMAC13 substrate as compared to patterned chitin in our previous work (95% *vs.* 55%). This might be due to

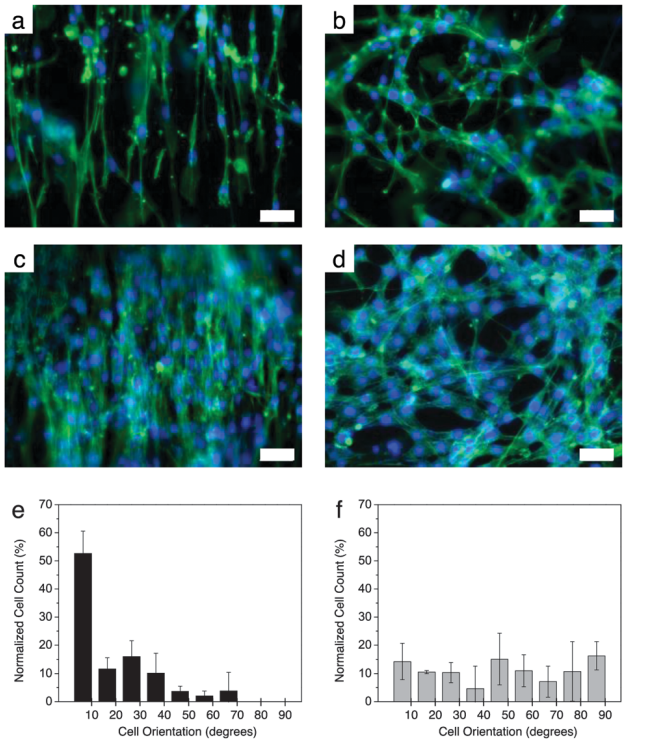


Fig. 5 (a–d) Representative fluorescence images demonstrate Actin/Dapi stained cell orientation after 3 (a and b) and 5 days (c and d) on the patterned (a and c) and non-patterned (b and d) GMAC13 hydrogels (Scale bars = 50 μm), (e and f) normalized cell count of cellular alignment measured with respect to the longitudinal direction of the actin filaments on micropatterned GMAC13 hydrogels (e) and non-patterned GMAC13 hydrogels as the control (f).

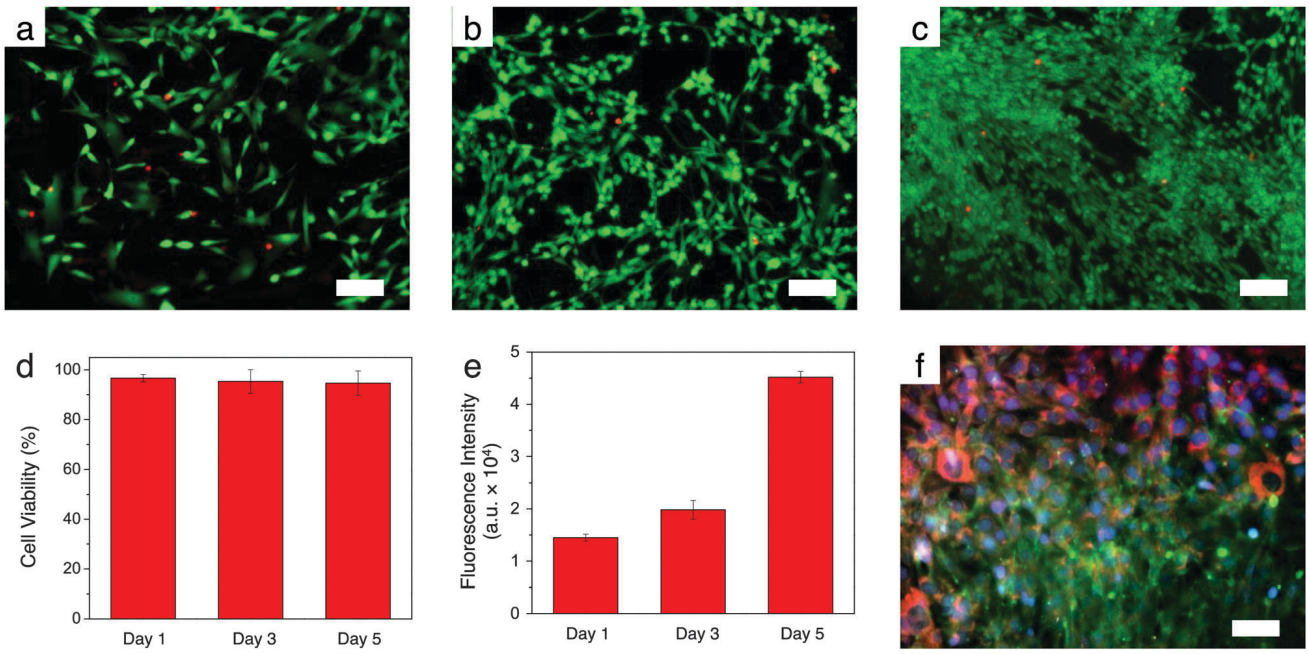


Fig. 6 (a–c) Fluorescence microscopy images showing the HUVECs/HMSCs co-culture growing on GMAC13 hydrogels after 1, 3 and 5 days, stained with Live (green) and Dead (red) viability assay kit (scale bars represent 100 μm), (d) quantification of cell viability on GMAC13 hydrogels after 1, 3 and 5 days, (e) PrestoBlue® assay showing the proliferation of the HUVECs/HMSCs co-culture on the GMAC13 surface after 1, 3 and 5 days, (f) immunostaining of vascular markers for HUVECs/HMSCs grown on GMAC13 after 5 days (green: CD31, red: alpha-SMA, blue: DAPI) (scale bar represents 50 μm).

the higher flexibility of the GMAC13 substrate, facilitating surface patterning.

We also evaluate the viability, proliferation, and vascularization of HUVECs/HMSCs co-cultured on the GMAC13 hydrogels after 1, 3 and 5 days of culture (Fig. 6). HMSCs are ideal cell sources for cardiovascular tissue engineering because they can differentiate into smooth muscle cells and endothelial cells *in vitro*[26,27] and *in vivo*.[28,29] Typically, the co-culture of HUVECs and HMSCs results in the formation of robust functional vascular networks within the first week.[30] On the GMAC13 hydrogels, live cells (green) attach well and exhibit a normal morphology with uniform distribution and very few dead cells (red) (Fig. 6a–c). Live/dead fluorescent staining indicates that over 90% of the cells are viable on the GMAC13 hydrogels after 1, 3 and 5 days (Fig. 6d). HUVECs/HMSCs cells are metabolically active (PrestoBlue®) and continue to proliferate with an enhanced proliferation activity on day 5 (Fig. 6e). To investigate the vasculogenic capacity of the HUVECs/HMSCs co-culture on GMAC13 hydrogels, we study early vasculogenesis by immunostaining the expressions of the endothelial cell specific marker CD31 and the early marker of smooth muscle cell differentiation, α-SMA. GMAC13 is conducive to HMSCs differentiation into smooth muscle cells (Fig. 6f). Relative expression of CD31 by HUVECs and α-SMA by HMSCs suggests a close association of HMSCs with HUVECs inducing an ongoing process of perivascular activity of cells. Consequently, the HUVECs/HMSCs co-culture on GMAC13 is a novel platform for studying vasculogenesis that is easier to source than Matrigel[31] and more mechanically robust than collagen-based hydrogels.[32]

In conclusion, we report the one-pot self-assembly of new crosslinked GelMA hydrogels reinforced with 3 nm chitin nanofibers (GMAC). The chitin nanofiber reinforcement increases the hydrogel elastic modulus by one-thousand fold and strain-to-failure by >200% improving handling and integrity for tissue engineering applications. The simple self-assembly process for these hydrogels is amenable to soft lithography strategies to create microstructures. HUVECs co-cultured with HMSCs grow, proliferate, and align on the micropatterned hydrogels, and express vasculogenic markers indicating cell differentiation and the formation of stable vasculature on these substrates.

Acknowledgements

This work is supported by the Office of Naval Research Award N000141410724 (MR) and AK acknowledges funding from the National Science Foundation (EFRI-1240443), IMMODGEL (602694), ONR PECASE Award, and the National Institutes of Health (EB012597, AR057837, DE021468, HL099073, AI105024, AR063745).

References

1 A. Khademhosseini and R. Langer, *Biomaterials*, 2007, **28**, 5087–5092.
2 W. Xiao, J. He, J. W. Nichol, L. Wang, C. B. Hutson, B. Wang, Y. Du, H. Fan and A. Khademhosseini, *Acta Biomater.*, 2011, **7**, 2384–2393.
3 S. Ahadian, J. Ramon-Azcon, M. Estili, X. Liang, S. Ostrovidov, H. Shiku, M. Ramalingam, K. Nakajima, Y. Sakka, H. Bae, T. Matsue and A. Khademhosseini, *Sci. Rep.*, 2014, **4**, 4271.
4 J. Kopeček, *Biomaterials*, 2007, **28**, 5185–5192.

5 J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar and A. Khademhosseini, *Biomaterials*, 2010, **31**, 5536–5544.
6 B. B. A. I. Van Den Bulcke, N. De Rooze, E. H. Schacht, M. Cornelissen and H. Berghmans, *Biomacromolecules*, 2000, **1**, 31–38.
7 A. Abi-Dargham, N. Miyake, M. Skinbjerg, X. Xiaoyan, R. R. Girgis and M. Slifstein, *Schizophrenia Bulletin*, 2011, **37**, 121.
8 O. Jeon, D. W. Wolfson and E. Alsberg, *Adv. Mater.*, 2015, **27**, 2216–2223.
9 X. Zhao, Q. Lang, L. Yildirimer, Z. Y. Lin, W. Cui, N. Annabi, K. W. Ng, M. R. Dokmeci, A. M. Ghaemmaghami and A. Khademhosseini, *Adv. Healthcare Mater.*, 2016, **5**, 108–118.
10 K. Yue, G. Trujillo-de Santiago, M. M. Alvarez, A. Tamayol, N. Annabi and A. Khademhosseini, *Biomaterials*, 2015, **73**, 254–271.
11 L. Zhou, G. Tan, Y. Tan, H. Wang, J. Liao and C. Ning, *RSC Adv.*, 2014, **4**, 21997.
12 G. Tan, L. Zhou, C. Ning, Y. Tan, G. Ni, J. Liao, P. Yu and X. Chen, *Appl. Surf. Sci.*, 2013, **279**, 293–299.
13 H. Wang, L. Zhou, J. Liao, Y. Tan, K. Ouyang, C. Ning, G. Ni and G. Tan, *J. Mater. Sci.: Mater. Med.*, 2014, **25**, 2173–2183.
14 M. Rinaudo, *Prog. Polym. Sci.*, 2006, **31**, 603–632.
15 C. K. S. Pillai, W. Paul and C. P. Sharma, *Prog. Polym. Sci.*, 2009, **34**, 641–678.
16 P. Hassanzadeh, M. Kharaziha, M. Nikkhah, S. R. Shin, J. Jin, S. He, W. Sun, C. Zhong, M. R. Dokmeci, A. Khademhosseini and M. Rolandi, *J. Mater. Chem. B*, 2013, **1**, 4217–4224.
17 R. Jayakumar, M. Prabaharan, S. V. Nair and H. Tamura, *Biotechnol. Adv.*, 2010, **28**, 142–150.
18 J. Jin, V. Reese, R. Coler, D. Carter and M. Rolandi, *Adv. Healthcare Mater.*, 2014, **3**, 349–353.
19 C. Zhong, Y. Deng, A. F. Roudsari, A. Kapetanovic, M. P. Anantram and M. Rolandi, *Nat. Commun.*, 2011, **2**, 476.
20 C. Zhong, A. Cooper, A. Kapetanovic, Z. Fang, M. Zhang and M. Rolandi, *Soft Matter*, 2010, **6**, 5298.
21 M. Rolandi and R. Rolandi, *Adv. Colloid Interface Sci.*, 2014, **207**, 216–222.
22 J. Jin, P. Hassanzadeh, G. Perotto, W. Sun, M. A. Brenckle, D. Kaplan, F. G. Omenetto and M. Rolandi, *Adv. Mater.*, 2013, **25**, 4482–4487.
23 C. Zhong, A. Kapetanovic, Y. Deng and M. Rolandi, *Adv. Mater.*, 2011, **23**, 4776–4781.
24 A. Cooper, C. Zhong, Y. Kinoshita, R. S. Morrison, M. Rolandi and M. Zhang, *J. Mater. Chem.*, 2012, **22**, 3105.
25 N. Annabi, S. M. Mithieux, P. Zorlutuna, G. Camci-Unal, A. S. Weiss and A. Khademhosseini, *Biomaterials*, 2013, **34**, 5496–5505.
26 S. B. J. Oswald, B. Jorgensen, S. Feldmann, G. Ehninger, M. Bornhauser and C. Werner, *Stem Cells*, 2004, **22**, 377–384.
27 M. Abdel-Hamid, M. R. Hussein, A. F. Ahmad and E. M. Elgezawi, *Int. J. Exp. Pathol.*, 2005, **86**, 117–123.
28 F. W. Sutherland, T. E. Perry, Y. Yu, M. C. Sherwood, E. Rabkin, Y. Masuda, G. A. Garcia, D. L. McLellan, G. C. Engelmayr, Jr., M. S. Sacks, F. J. Schoen and J. E. Mayer, Jr., *Circulation*, 2005, **111**, 2783–2791.
29 G. Matsumura, S. Miyagawa-Tomita, T. Shin'oka, Y. Ikada and H. Kurosawa, *Circulation*, 2003, **108**, 1729–1734.
30 S. B. J. S. Jeon, M. Gilardi, G. Dubini, J. L. Charest, M. Moretti and R. D. Kamm, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 214–219.
31 H. K. Kleinman and G. R. Martin, *Semin. Cancer Biol.*, 2005, **15**, 378–386.
32 C. Helary, I. Bataille, A. Abed, C. Illoul, A. Anglo, L. Louedec, D. Letourneur, A. Meddahi-Pelle and M. M. Giraud-Guille, *Biomaterials*, 2010, **31**, 481–490.