

## **Ultrastrong and Flexible Hybrid Hydrogels based on Solution Self-Assembly of Chitin Nanofibers in Gelatin Methacryloyl (GelMA)**

Pegah Hassanzadeh, Mehdi Kazemzadeh-Narbat, Rachel Rosenzweig, Xiaolin Zhang, Ali Khademhosseini,\* Nasim Annabi,\* Marco Rolandi\*

### **Experimental Procedure**

*Materials:* The materials and suppliers used are as follows: Squid pen chitin flakes (Industrial Research Ltd-New Zealand), hexafluoro-2-propanol (HFIP) (Oakwood Products, Inc.), gelatin type A made from porcine skin and methacrylic anhydride (MA) (Sigma-Aldrich Chemical Co., USA), 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) (Ciba Chemicals, Osaka, Japan), optical grating GE 2550-0875 Echelle Grating, 12.66  $\mu\text{m}$ , 75° blaze angle (THORLABS) and polydimethylsiloxane (PDMS) (SYLGARD 184).

*Preparation of GelMA-Chitin Solutions:* Appropriate amounts of squid pen  $\beta$ -chitin are dissolved in HFIP for four days under vigorous stirring at room temperature to obtain concentration of 0.4 (w/v) %. Then, appropriate amounts of lyophilized highly methacrylated GelMA are dissolved in chitin/HFIP solution for 1 day under mild stirring at room temperature. The GelMA prepolymer is synthesized as described previously.<sup>1</sup> A high degree of methacrylation (~80%) is obtained by adding 8 mL of MA to 10 g of gelatin in 100 mL of Phosphate-Buffered Saline (PBS). The mixture is dialyzed in a 12-14 KDa cutoff membrane in the distilled water for one week at 40 °C and then lyophilized for one week. The GelMA-chitin solutions are GMAC31 (GelMA:chitin, 3 to 1 weight ratio), GMAC11 (1 to 1 weight ratio), and GMAC13 (1 to 3 weight ratio). The photoinitiator (PI) (Irgacure 2959) with the ratio of PI/GelMA=2 is added to each GMAC/HFIP solution and left to dissolve for 6 hrs at room temperature. The chitin/HFIP and GelMA/HFIP (PI/GelMA = 2) solutions are also prepared by dissolving appropriate amount of

each material in HFIP under stirring at room temperature for 4 days and 1 day, respectively. HFIP is a toxic solvent and particular care is required during handling. All the above and following procedures are performed in a fume hood. In order to prepare gelatin-chitin solution, the same procedure as GelMA-chitin is used. GelMA is substituted by porcine gelatin and PI is not used.

*Preparation of GelMA-Chitin and Gelatin-Chitin Hybrid Films:* GelMA-chitin (GMAC), gelatin-chitin (GC), chitin and GelMA films for further measurement and testing are prepared by pouring 10 mL of each solution into a rectangular-shaped (50 × 25 mm) PDMS mold with the volume of 17.5 ml. The molds are covered by a tight parafilm with 5 holes to afford slow evaporation of the HFIP over a period of 5 days. Films obtained after shorter period of times are brittle due to increased number of defects and stress accumulated while drying. After complete drying, GMAC and GelMA films are exposed to UV irradiation (365 nm, 100W, 115V, UVP™ Blak-Ray™ B-100A UV Lamps) for 3 minutes in the distance of 10 cm to the lamp in dry condition to crosslink the methacrylate functional groups of GelMA. The samples are then washed with copious amount of DI water following the same procedure that was followed for our previous work with GelMA.<sup>2-4</sup> While the presence of unreacted PI cannot be completely excluded, unreacted PI would be toxic to cells and we do not observe any cytotoxicity of these samples. Subsequently, they dry in room temperature. The GC, chitin, and gelatin films are used for characterization as they are upon complete drying.

*Preparation of Micropatterned GMAC13:* The GMAC13/HFIP solution is drop cast into the master PDMS mold (replica of GE 2550-0875 Echelle Grating) to create micropatterns on top. The dried film is then exposed to UV for 3 minutes and washed with DI water for 5 min as described before.

*Preparation of Hybrid Hydrogels:* After washing the crosslinked GelMA, GMAC films and patterned GMAC13 with DI water, the films are immersed in DPBS at 37 °C for 24 hrs. The hydrophilic films absorb water and swell.

*Hydrogels Weight Stability Analysis:* The extent of stability of GelMA and gelatin in the GMAC and GelChi hydrogels in an aqueous environment is analyzed by comparing the weight of dry hybrid films before ( $m_{\text{before}}$ ) and after immersion in DPBS at 37 °C for 24 hrs (physiological conditions) ( $m_{\text{after}}$ ) and drying for the three weight ratios of hydrogels. Chitin and GelMA/gelatin content of the final dry films are estimated from the initial amounts considering chitin is stable and insoluble in DPBS at 37 °C for 24 hrs. We use this measurement to assess the crosslinking reaction efficiency of GelMA because in the FTIR spectra the unreacted C=C bond appears around  $1640\text{cm}^{-1}$ ,<sup>5</sup> which is too close to the amide I (C=O stretching) at  $1657\text{cm}^{-1}$  for chitin<sup>6</sup> for a precise estimate. Additionally, the C=O peak for GelMA is at  $1636\text{cm}^{-1}$ .<sup>7</sup> We define the reaction efficiency = (GelMA mass after immersion)/(total GelMA mass in the sample). Thus the reaction efficiency is 40% and is consistent through GMAC31, GMAC11, and GMAC13.

*Characterization:* Tapping mode Atomic Force Microscopy (AFM) is performed on a Veeco Multimode V with a Nanoscope IV controller. Veecoprobes Sb-doped Si cantilevers ( $\rho = 0.01\text{-}0.025 \text{ }\Omega\text{-cm}$ ,  $k = 40 \text{ N/m}$ ,  $\nu \sim 300 \text{ kHz}$ ) are used on dry samples. Fourier Transform Infrared (FTIR) spectra are recorded with a Bruker vectex 70 FTIR spectrophotometer ( $4000 \text{ to } 400 \text{ cm}^{-1}$ ,  $4 \text{ cm}^{-1}$  resolution) working in ATR mode. Stress vs. strain data is recorded on three rectangular samples for each material measuring  $3 \times 30 \text{ mm}$  with thickness of approximately  $300 \text{ }\mu\text{m}$  for hydrogels. After cutting the samples with razor blade, they are examined under optical microscope to assure they do not contain any notches and major microcracks formed on the edges during cutting process. All tests are conducted according to ASTM D882-12 with a Shimadzu AGS-X ( $0.1 \text{ mm/min}$  rate)

equipped with a 100 N load cell and 50 N pneumatic grips. The reported values are average from three measurements and ASTM D882-12 does not include reporting standard deviation due to the reduced number of data points. In the case of hydrogels, a vaporizer with dual output to cover both sides of the hydrogel with some water splash on the surface is used in order to keep the humidity high (~70%) and prevent the hydrogel from drying out.

*Cell Culture and Materials:* Endothelial growth medium (EGM-2 BulletKit) is used to culture HUVECs. hMSCs (Lonza) are cultured in alpha-MEM medium supplemented with 10% FBS and 1% penicillin/streptomycin, 2mM L-glutamine, 1 ng/ml basic fibroblast growth factor (bFGF, Life Technologies) and 0.2 M ascorbic acid (AA, Sigma). Cells are cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The media are changed every two days. After the confluence is reached, cells are harvested using trypsin/EDTA and counted on a hemocytometer. Subsequently, an equal cell quantity of HUVECs and hMSCs (10<sup>5</sup> cells 1:1) are mixed in EGM-2, before seeding the cells on GMAC13 substrates. Sample is then placed in a multi-well plate, cultured in EGM-2 media and incubated. All experiments are performed at passages 4-8.

*Cell Alignment:* The alignment of cells on patterned and non-patterned GMAC13 substrates is investigated using fluorescence images based on angle of orientation with respect to the direction of the actin filaments. After culturing the HUVECs/hMSCs on the GMAC13 substrates for 3 and 5 days, the specimens are rinsed 3 times with prewarmed DPBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Subsequently, the cells are washed with DPBS 3 times and permeabilized by incubating in 0.1% (w/v) Triton X-100 in DPBS for 15 min and rinsed 3 times with DPBS for 5 min. The cells are then blocked in 1% (w/v) Bovine Serum Albumin (BSA) in DPBS for 1 h, followed by staining with 1/40 dilution of Alexa Fluor 488<sup>®</sup> phalloidin (Invitrogen) in 0.1% BSA blocking solution for 2 h at room temperature to visualize F-actin. Cell nuclei are stained

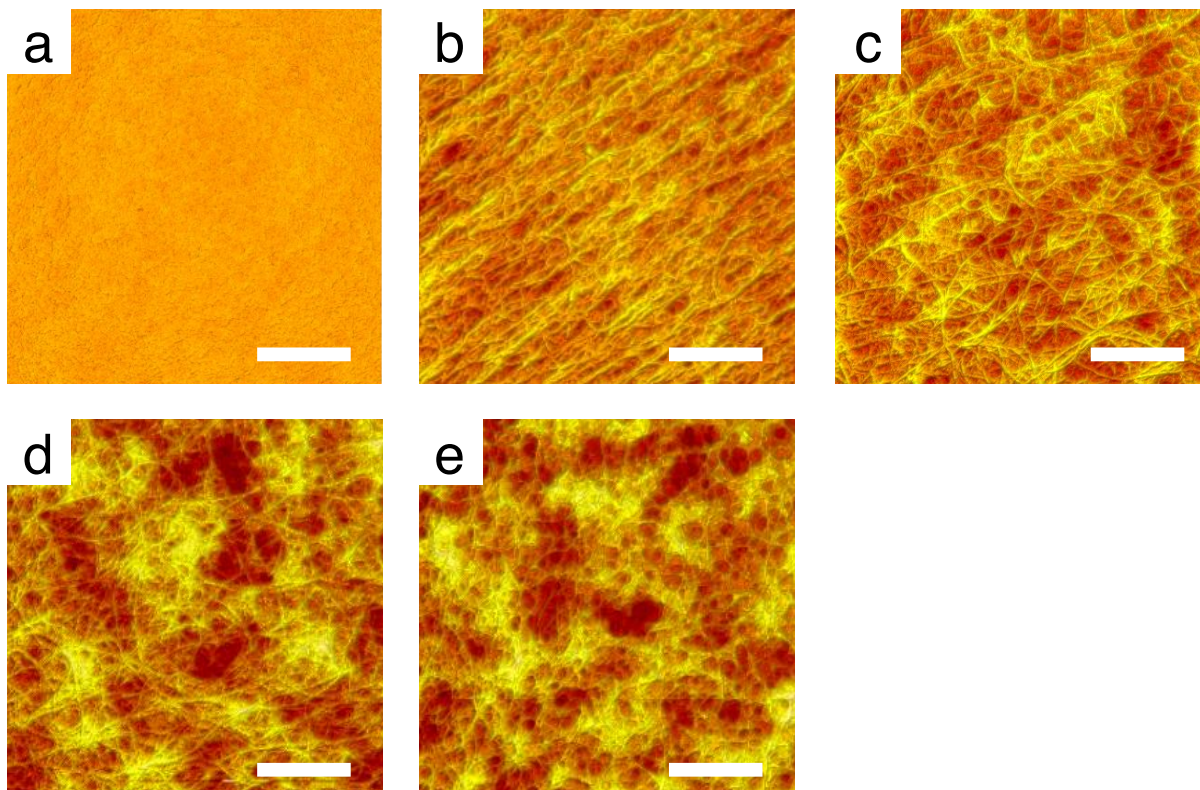
with 1/1000 dilution of 4'-6-Diamidino-2-phenylindole (DAPI, Invitrogen) in DPBS for 5 min. Alignment analysis is performed by measuring the deviation of actin filaments with respect to the grooves' axis using fluorescence images (6 random images for each sample). The normalized alignment angles are grouped in 10 degrees increments to compare the orientation of cells on patterned and un-patterned samples. The analysis is performed in triplicate for each condition.

*Cell Viability:* Fluorescence-based LIVE/DEAD<sup>®</sup> viability/cytotoxicity assay kit (Invitrogen) consisting intracellular green-fluorescent calcein AM and red-fluorescent ethidium homodimer-1 stains is used to determine the viable and non-viable cells. The percentage of viable HUVECs/hMSCs ( $10^5$  cells 1:1) co-cultured on the GMAC13 substrates is quantified after 1, 3, and 5 days. First a solution containing two components at 0.5  $\mu$ l/ml (calcein) and 2  $\mu$ l/ml (ethidium homodimer-1) are dissolved in DPBS, respectively. At each time point, the media are removed and the hydrogels covered with cells are rinsed with DPBS and subsequently 1 mL of the solution is added to each sample. Afterwards, the samples are incubated for 30 min at room temperature and then imaged with 10X magnifications using an inverted fluorescence microscope (Nikon TE 2000-U, Nikon instruments Inc., USA). The total number of cells (red and green) and number of live cells (green) are counted using ImageJ software (NIH). Finally, the cell viability is calculated and quantified by dividing live cells by total number of cells. The calculations are based on three independent samples and reported based on the mean  $\pm$  standard deviation.

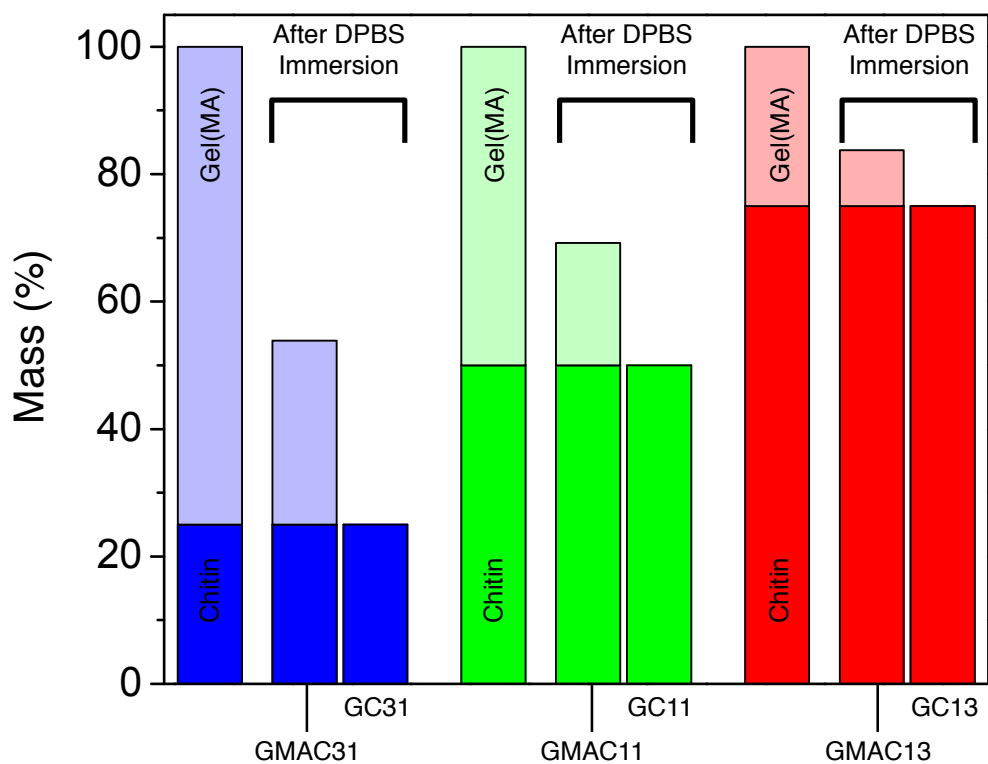
*Cell Proliferation:* Proliferation of cells is assessed using the resazurin-based PrestoBlue<sup>®</sup> assay (Invitrogen, CA), a non-toxic metabolic indicator for viable cells. Briefly, HUVECs/hMSCs co-culture ( $10^5$  cells 1:1) are seeded on each GMAC13 substrate and incubated for 1, 3, and 5 days. After each time point the culture medium is removed and the samples are rinsed with DPBS. Subsequently, the DMEM containing 10% PrestoBlue<sup>®</sup> reagent is added to each

well and incubated at 37 °C for 1 h, and the well with reagent with no cells served as the blank control. The fluorescence of the reduced PrestoBlue<sup>®</sup> dye is read at 570 (excitation) and 600 nm (emission) with a microplate reader (Biotek, USA), and all values are corrected based on blank control. Three replicates are analyzed continuously for 1, 3 and 5 days and growth were plotted based on the mean ± standard deviation.

*Immunofluorescence for Vasculogenic Markers:* The vasculogenic activity of HUVECs/hMSCs co-culture (10<sup>5</sup> cells 1:1) grown on GMAC13 substrates is investigated by immunostaining for CD31 (Abcam, USA), and anti-Smooth Muscle Actin (α-SMA; Abcam, USA) expressions. The GMAC13 covered with cells were rinsed in DPBS and fixed in 4% paraformaldehyde solution in DPBS for 20 min. Subsequently, the cell membranes are permeabilized in 0.1% Triton X-100 in DPBS for 15 min and washed with DPBS for 3 times. The samples are then blocked with 1% (w/v) BSA in DPBS for 1 h, followed by primary antibody staining with 1/40 dilution of rabbit monoclonal anti-CD31 antibody (Abcam) and 1/100 dilution of mouse monoclonal anti-alpha smooth muscle actin antibody (Abcam) in 0.1% BSA blocking solution overnight at 4 °C. The samples are washed in DPBS three times with 1 h intervals in between the washing steps. After primary antibody staining, the samples are incubated in 1/200 dilution of Alexa Fluor-488 conjugated goat anti-rabbit (Abcam) and 1/200 dilution of Alexa Fluor-594 conjugated goat anti-mouse secondary antibodies (Abcam) in 0.1% BSA in DPBS for 2 h at room temperature. Subsequently, the samples are washed again with the same procedure as above followed by 1/1000 dilution DAPI staining for 5 min. After rinsing the samples with DPBS, confocal images are taken.

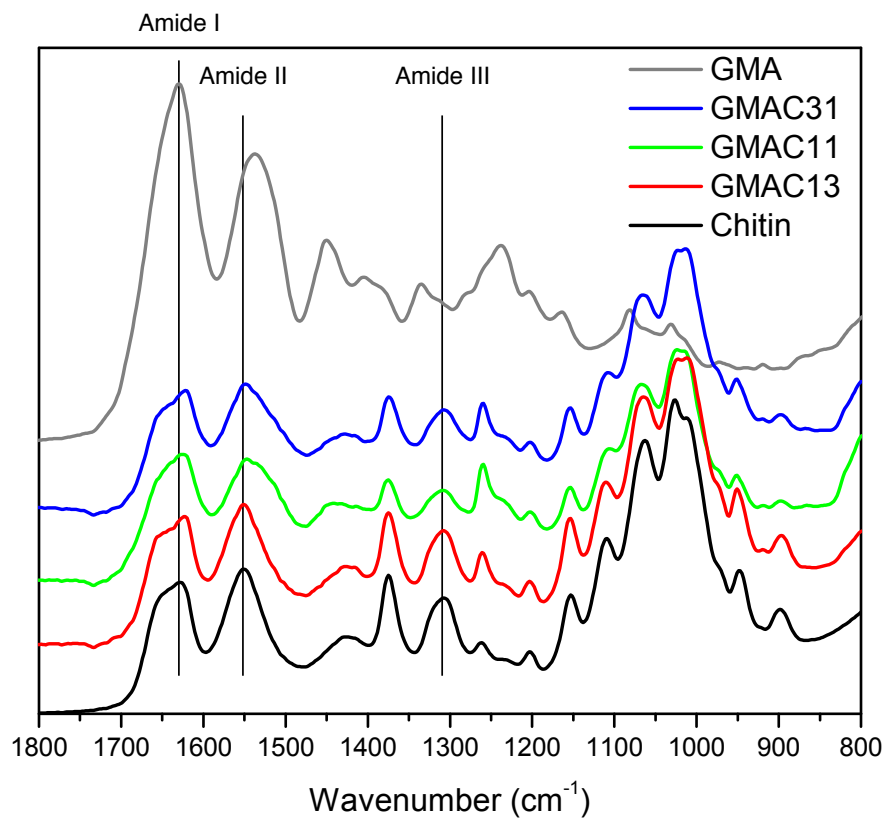


**Fig. S1** (a-e) Topographic atomic force microscope (AFM) images of Gelatin, GelChi films with different gelatin:chitin ratios, and Chitin. GCXY are GelChi films with  $XY = \text{gelatin:chitin weight ratio}$ . (a) Gelatin, (b) GC31, (c) GC11, (d) GC13, and (e) Chitin, respectively (scale bars are 500 nm).

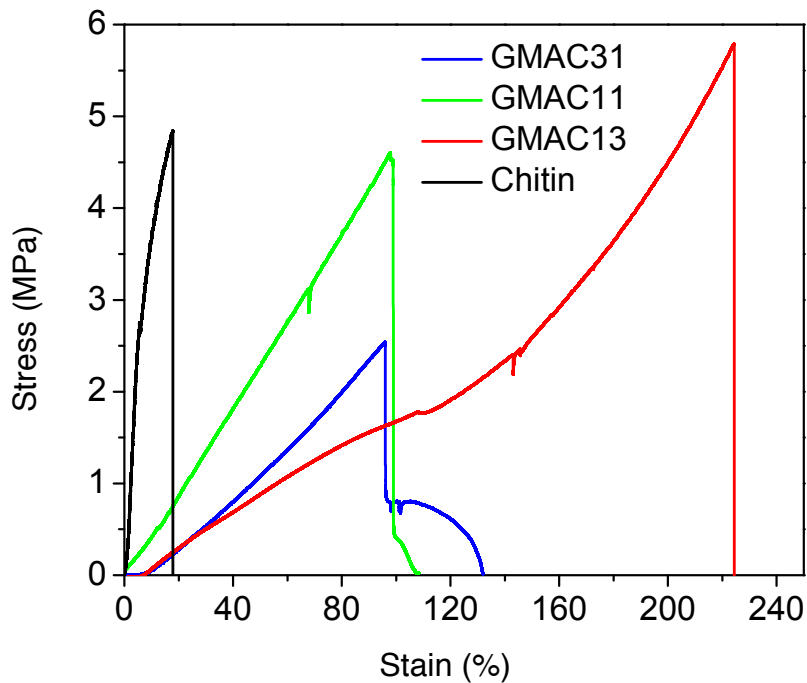


**Fig. S2** Values of mass percent showing stability of GelMA in comparison with gelatin in hybrid cross-linked GMAC (GMAC) hydrogels and gelatin-chitin (GC) hydrogels after immersion into DPBS for 24 hrs at 37 °C. Crosslinking GelMA will make it stable and less soluble in DPBS in comparison with fully soluble gelatin.





**Fig. S3** FTIR spectra of corss-linked GeIMA, GMAC31, GMAC11, GMAC31, and Chitin. The spectra do not show any indication of chemical bonding between GeIMA and chitin molecules. No significant change of frequency and/or intensity in major peaks of chitin (Amide I (C=O) and Amide II and III (CN-NH)) is observed.



**Fig. S4** Engineering stress-strain curves of chitin and GMAC hydrogels with different GelMA:chitin weight ratios.

## Reference

1. A. I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E. H. Schacht, M. Cornelissen and H. Berghmans, *Biomacromolecules*, 2000, **1**, 31-38.
2. K. M. Tsang, N. Annabi, F. Ercole, K. Zhou, D. Karst, F. Li, J. M. Haynes, R. A. Evans, H. Thissen, A. Khademhosseini and J. S. Forsythe, *Adv Funct Mater*, 2015, **25**, 977-986.
3. 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.
4. G. Camci-Unal, D. Cuttica and N. Annabi, *Biomacromolecules*, 2013, **14**, 1085-1092.
5. H. Shin, B. D. Olsen and A. Khademhosseini, *Biomaterials*, 2012, **33**, 3143-3152.
6. M. K. Jang, B. G. Kong, Y. I. Jeong, C. Lee and J. W. Nah, *Journal of Polymer Science Part A: Polymer Chemistry*, 2004, **42**, 3423-3432.
7. C. B. de Vidal and M. L. S. Mello, *Micron*, 2011, **42**, 283-289.