Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

Controlling the orientation of cell-synthesized extracellular matrix by using engineered gelatin-based building-blocks

F A Pennacchio, C Casale, F Urciuolo, G Imparato, R Vecchione and P A Netti

Synthesis of acrylamide-modified gelatin B.

Gelatin type B (Bloom strength of 225 g, MW range 50000-100000 Sigma) isolated from bovine skin, acrylic acid *N*-hydroxysuccinimide ester (Aldrich) and 4-Methoxyphenol (MeHQ) (Sigma-Aldrich) were used as received. Starting from a protocol reported elsewhere, gelatin was chemically modified with acrylic side groups.[1] After dissolution of 1 g of gelatin in phosphate buffer (pH 7.8) at 40 °C, 0.76 mmol of acrylic acid *N*-hydroxysuccinimide (NHS)-ester (128.5 mg) and 46 ppm of MeHQ were added while vigorously stirring. After 1 h, the reaction mixture was diluted and dialyzed for 48 h against distilled water at 40 °C. The reaction product was then freeze-dried leading to a white fluffy solid. The degree of substitution was verified using ^{1}H-NMR spectroscopy at 40 °C (Figure S1). Deuterium oxide (D_2O) was used as solvent for the ^{1}H-NMR analysis. The methyl signal (at 1.1 ppm) of Val, Ile, and Leu can be used as an internal standard in ^{1}H NMR spectroscopy because it can be considered chemically inert. The presence of peaks to the methylene protons H_2C=CH- between 5.8-6.5 ppm indicates the success of the reaction since it is relative to the acrylamide groups. This functionalization has been quantified by integrating and comparing this peak with the peak relative to the methyl signal at 1.1 ppm (we used gelatin functionalized at 85%). In particular, starting from the work of Billiet et al. [1], degree of substitution (DS) has been calculated as follows :

$$DS(\%) = \frac{0.37mol}{100g} * \frac{integration\ at\ 5.8\ ppm}{integration\ at\ 1.1\ ppm} * \frac{100}{0.038mol/100g}.$$

Here 0.37 mol/100 g correspond to the integration of methyl signals of Val, Ile and Leu, while 0.038 mol/100 g is the total amount of free amines present in gelatin B.

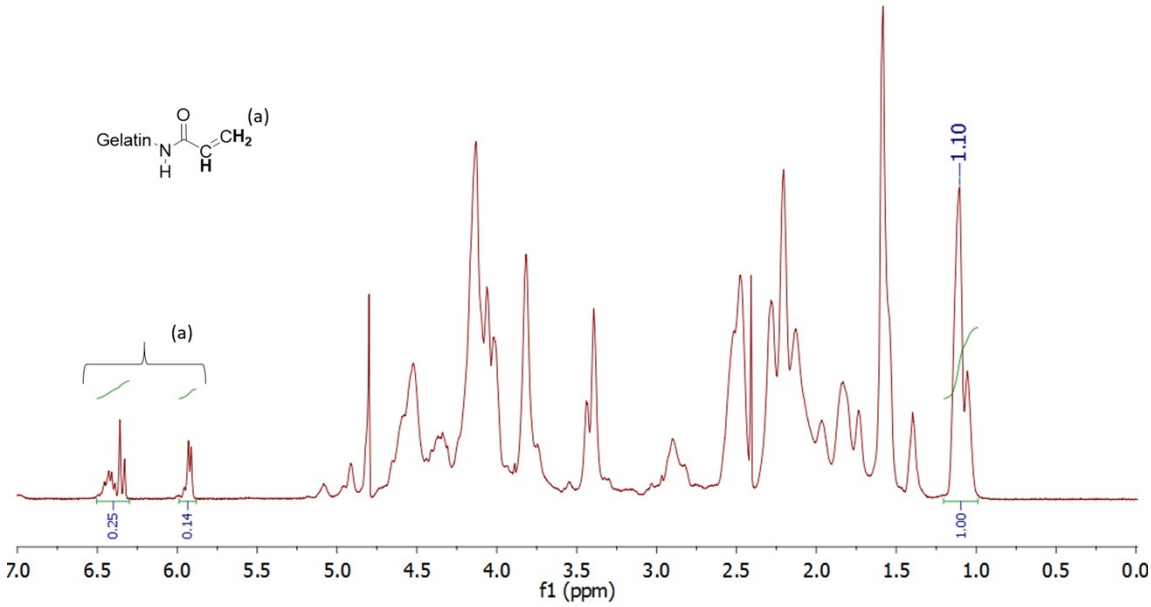
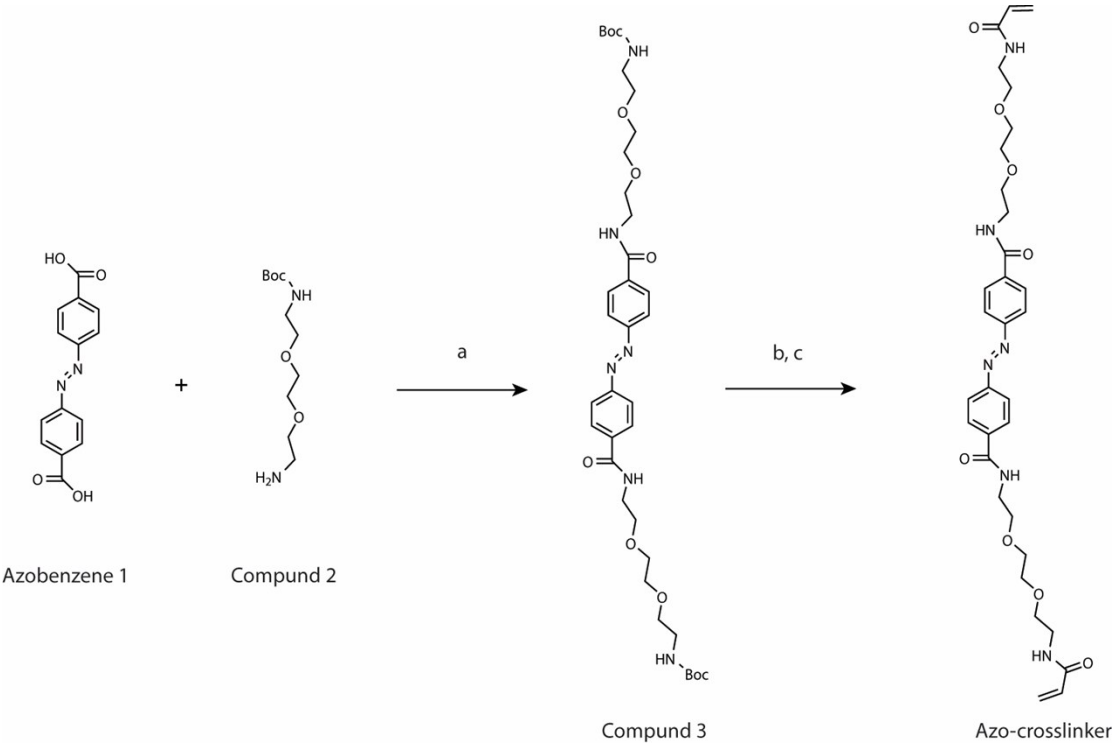


Figure S1 ^{1}H NMR spectrum of modified gelatin B.

Synthesis of azobenzene-based crosslinker (Azo-crosslinker).

Azobenzene 1 was synthesized following an already reported protocol.[2] Azobenzene 1 (30 mg, 0.111 mmol) was dissolved in dichloromethane (CH_2Cl_2) and 74 µl of triehtylamine (TEA) at room temperature. Then 2.4 equiv. of 1-Hydroxybenzotriazole hydrate (HOBt·H_2O), 2.4 equiv. of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) and 2.4 equiv. of compound 2 (N-Boc- 2,2-(ethylenedioxy)diethylamine, Aldrich) were added and left reacting overnight. The reaction was followed by thin layer chromatography (TLC) and the product was extracted in dichloromethane. Compound 2 was purified by silica column chromatography (Biotage ZIP 5g, flow rate 18 mL/min) and eluted with 30% of MeOH in CH_2Cl_2. The product formation was confirmed by mass spectrometry. MS (ESI): m/z calculated for $C_{36}H_{54}N_6O_{10}$: 731.39 $[M+H]^+$; found: 731.40. After that, compound 2 was treated for 2 hours with a solution of 50/50 v/v trifluoroacetic acid (TFA) in CH_2Cl_2 to remove the Boc protecting group, then co-evaporated with toluene and treated with TEA. Finally, compound 3 was reacted with 2.4 equiv. of acrylic acid (197 mmol, 14.2 mg), 2.4 equiv. of HOBt·H_2O, 2.4 equiv. of EDC·HCl and 4.8 equiv. of TEA. The reaction was followed by thin layer chromatography (TLC). The product Azo-crosslinker was purified by silica column chromatography (Biotage KP-

SIL 10g, flow rate 20 ml/min) and eluted with 10% of MeOH in CH_2Cl_2. The product formation was confirmed by mass spectrometry and characterized by UV/Vis spectrophotometry (Figure S2). MS (ESI): m/z calculated for $C_{32}H_{42}N_6O_8$: 639.39 $[M+H]^+$; found: 639.31. The product was also characterized by ^1H NMR (600 MHz, [D4] CD_3OD, 25 °C, TMS): δ (ppm) = 8.0 (s, 8 H; Ar-CH), 6.3-6.2 ppm (m, 4 H; $H_bC=CH_c$), 5.6 (dd, $J(H_a,H_b)$ = 2.4 Hz, $J(H_a,H_c)$ = 9.6 Hz, 2 H; $=CH_a$), 3.7-3.5 (m, 20 H; -CH_2- PEO), 3.4 (t, 4H; J= 6 Hz, -CH_2- PEO).



Azobenzene 1 Compund 2 Compund 3 Azo-crosslinker

Scheme S1. Synthetic scheme of Azo-crosslinker. Reaction conditions: a) 0.111 mmol of azobenzene 1, 4.8 equiv. of TEA, 2.4 equiv. of HOBt·H_2O, 2.4 equiv. EDC·HCl and 2.4 equiv. of compound 2, overnight at room temperature. b) TFA/CH_2Cl_2 50/50 v/v for 2 hours to remove the Boc protecting group. After the reaction, the product was treated with TEA and co-evaporated with toluene. c) Boc-deprotected compound 3 was reacted with 2.4 equiv. of acrylic acid, 2.4 equiv. of HOBt·H_2O, 2.4 equiv. of EDC·HCl and 4.8 equiv. of TEA overnight at room temperature.

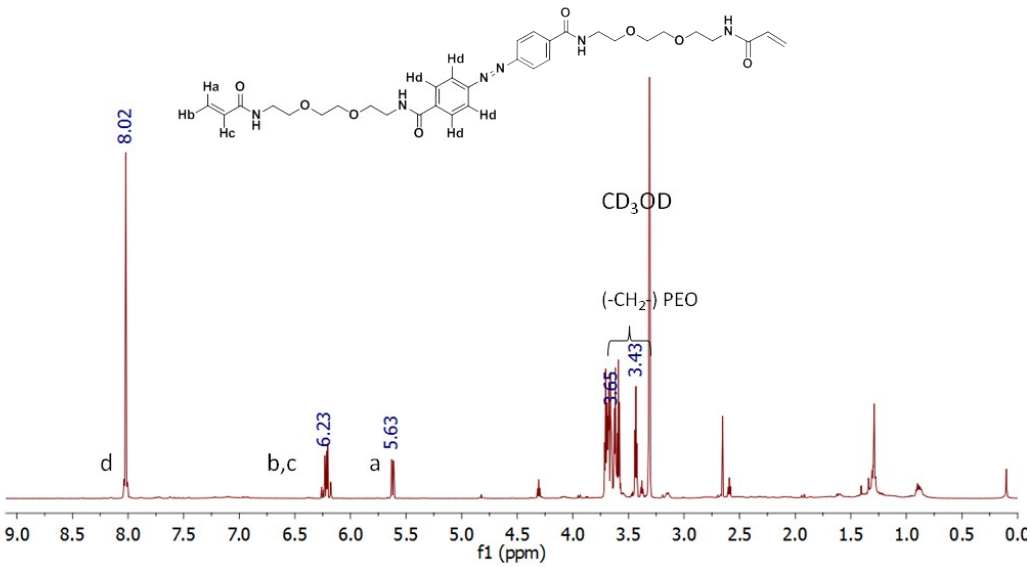


Figure S2. ^{1}H NMR spectrum of Azo-crosslinker.

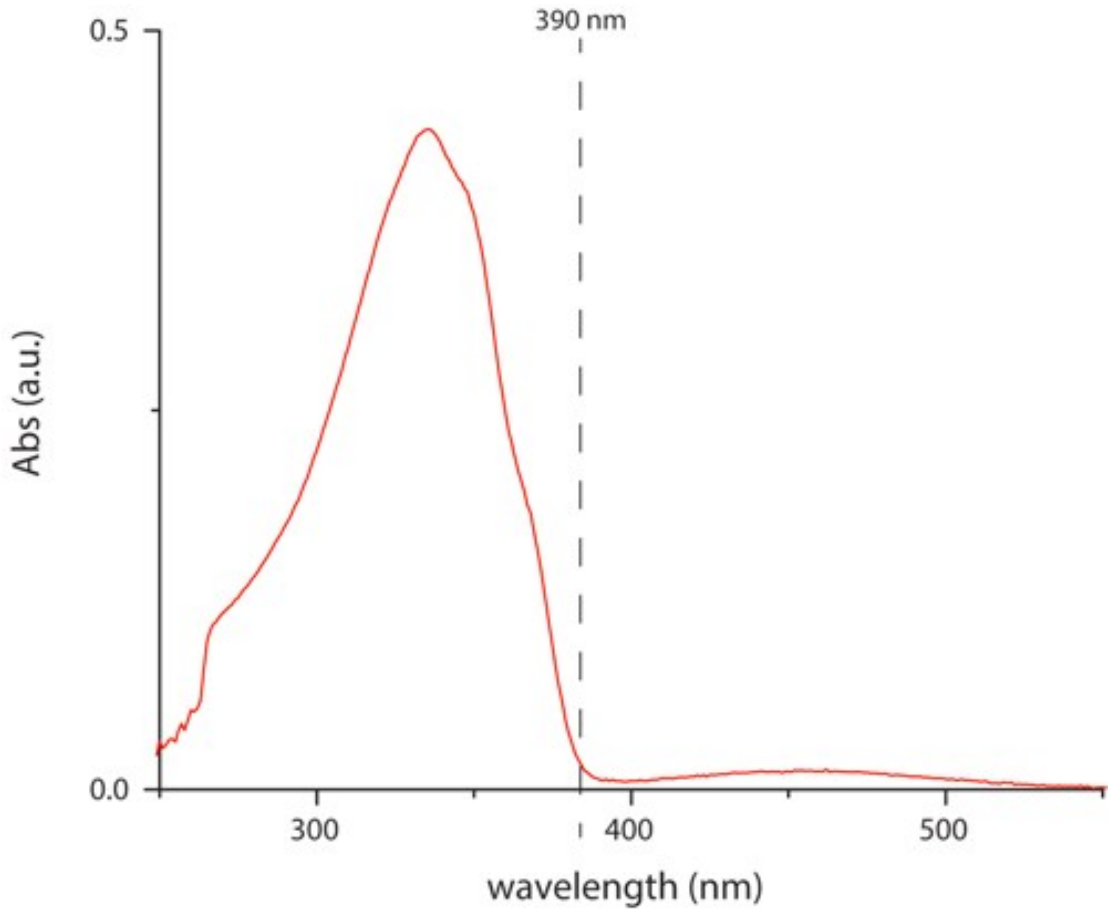


Figure S3. UV/Vis absorption spectra of Azo-crosslinker in DMF using different illumination conditions. The dashed light gray line indicates minimum valley of the

molecule absorption at 390 nm, which corresponds to the wavelenght (□□ of the focal volume of the two-photon polymerization system. In the legend, “UV” indicates the diffused light of a laboratory lamp at 365 nm, while “UV 10x” stays for the light of a mercury lamp focused by a 10x objective and filtered in the UV region. Finally, “blue 10x” represents the same light source filtered in the blue region.

Cell number

To assess if different value of collagen fraction could be associated to a different number of cells present into the two distinct types of structures, cells were counted from the z stack collected with the confocal microscopy imaging.

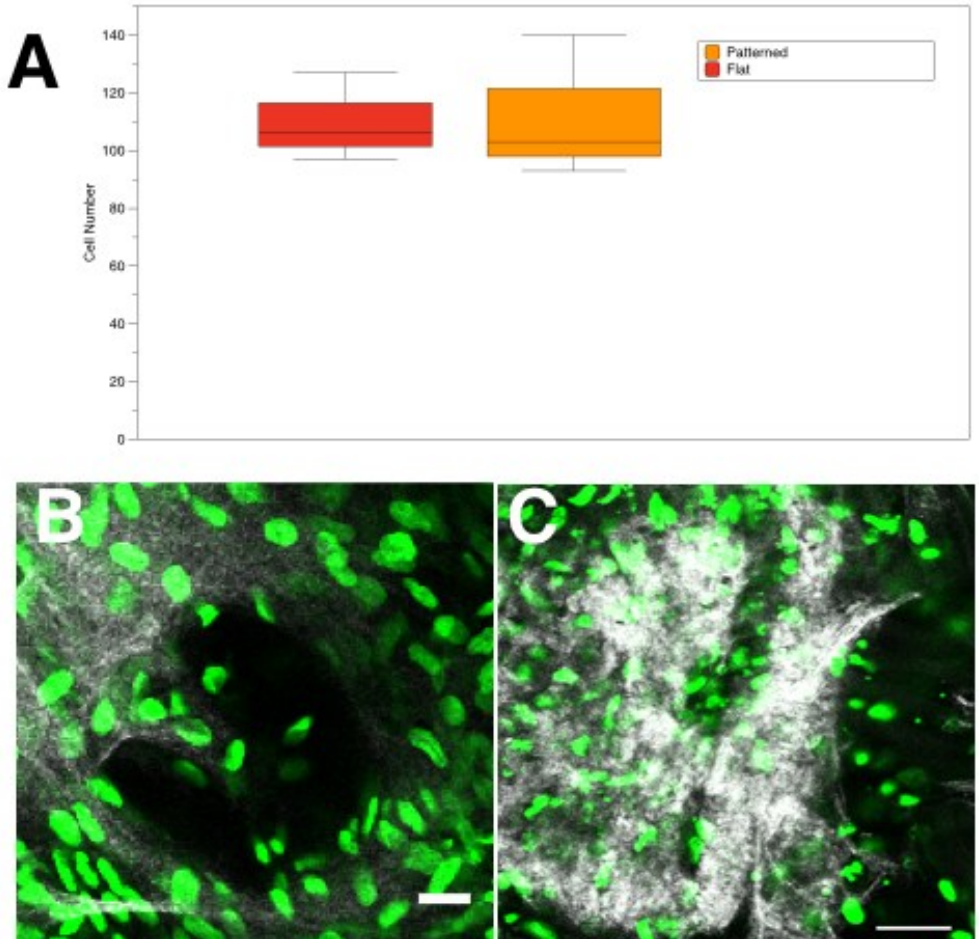


Figure S4. A) Box representation of the number of fixed cells adhered on the surfaces of flat (red box) and of patterned (orange box) plugs. Confocal images of

cells used to quantify cells number on patterned (B) and flat (C) plugs scale bars are 50 µm.

[1] T. Billiet; B. V. Gasse; E. Gevaert; M. Cornelissen; J. C. Martins; P. Dubruel, *Macromol. Biosci.* **2013,** *13*, 1531.