



Title	Modulation and Characterization of the Double Network Hydrogel Surface-Bulk Transition
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1 Modulation and characterization of the double network hydrogel 2 surface-bulk transition

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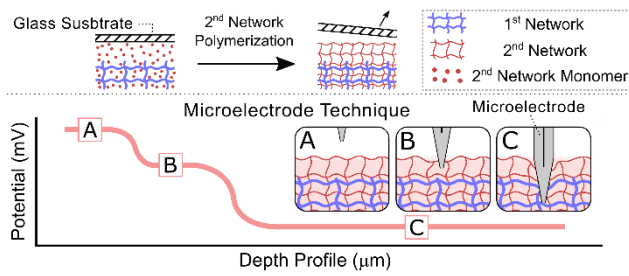
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29

30 Abstract

31 The hydrogel chemical structure at the gel-solution interface is important towards practical use,
32 especially in tough double network (DN) hydrogels that have promising applications as structural
33 biomaterials. In this work, we regulate the surface chemical structure of DN hydrogels and the surface-
34 bulk transition by the molding substrate used for the synthesis of the 2nd network. To characterize the
35 surface and bulk structure, we combined ATR-FTIR and a newly developed microelectrode technique
36 that probes the electric potential distribution within a hydrogel. We found that the polymerization on
37 a repulsive substrate leads to the formation of a thin layer of 2nd network on the surface of DN
38 hydrogels, which makes the surface different from the bulk. By controlling the 2nd network
39 polymerization conditions and molding substrate, the surface-bulk transition region can be regulated,
40 so that either only the 2nd network or both networks are present at the DN hydrogel surface. Through
41 these findings we gained a new insight on the structure formation at the DN hydrogel surface, and this
42 leads to easy regulation of the hydrogel surface structure and properties.

43

44 Introduction

45 The double network (DN) concept gave rise to hydrogels with rubber-like strength and toughness and
46 inspired the development of novel hydrogel materials for potential applications in industrial¹⁻³ and
47 medical fields⁴⁻⁶. DN hydrogels have structural similarities to living soft matter, and they therefore
48 have received attention for use as cell scaffolds^{7,8}, implants^{9,10} and anti-adhesive membranes^{11,12}. For
49 such applications in wet environments, understanding and controlling the surface physicochemical
50 properties of DN hydrogels is crucial.

51 A typical DN hydrogel owes its high strength and toughness to the contrasting architecture of the two
52 interpenetrating networks: a rigid and brittle 1st network, acting as sacrificial bonds, and a soft and
53 stretchable 2nd network as its interpenetrating counterpart, synthesized in a two-step polymerization
54 approach¹³⁻¹⁶. To obtain the contrasting architecture, a DN hydrogel is usually made from a densely
55 crosslinked polyelectrolyte as the 1st network and a sparsely crosslinked neutral polymer as the 2nd
56 network. Driven by the high osmotic pressure of the counter-ions, the polyelectrolyte network
57 substantially swells in the aqueous precursor solution of the 2nd network, reaching a rigid and brittle
58 state, while the 2nd network, formed in the presence of the first network, can hardly swell due to the
59 constrain of the already highly swollen first network, reaching a soft and stretchable state. Hydrogels
60 made from polyanions such as poly(acrylic acid), poly(sodium p-styrene sulfonate), poly(2-acrylamido-
61 2-methyl-propane sulfonic acid) (PAMPS) and PAMPS – sodium salt, have shown many unique
62 properties, including low sliding friction on glass substrates in water, the adsorption of proteins and
63 platelets, known as biofouling, and cell attachment¹⁷⁻¹⁹. However, after introduction of the 2nd neutral
64 network in such polyelectrolyte hydrogels to create a DN hydrogel, these properties change
65 remarkably, so that cells hardly attach to the DN hydrogel²⁰⁻²². Yet, evidence suggests that DN
66 hydrogels from PAMPS and poly(dimethylacrylamide) have therapeutic potential by inducing in vivo
67 articular cartilage regeneration^{23,24}, in which the surface physicochemical properties of the DN
68 hydrogels should play an important role in the initial cell adhesion^{25,26} or morphology^{27,28}.
69 Understanding and regulating cell-hydrogel interactions are among the most challenging problems in

70 bioengineering, and are affected by hydrogel characteristics including charge density²⁹, water
71 content³⁰, hydrophilicity³¹ and mechanical properties³².

72 In the case of DN hydrogels, it could be argued that cells do not adhere, but do so on the corresponding
73 single network (SN) polyelectrolyte hydrogel, because the concentration of the electrically neutral 2nd
74 network, is already high enough to weaken the surface charge sufficiently^{33–35}. Another reason might
75 relate to the two-step synthesis approach and the interactions between the substrate material and the
76 1st network hydrogel³⁶. We consider that the surface of the DN hydrogels are sensitive to the two-step
77 polymerization approach and the interaction between the 1st network and the wall of the molding
78 substrate used to synthesize the 2nd network. In the case of a polyelectrolyte hydrogel, at the hydrogel-
79 solution interface, an electric double layer is formed based on the balance of attraction of mobile
80 counter-ions and repulsion of mobile co-ions to the immobile ions of the polyelectrolyte network. As
81 a result, there is an electric potential difference between the polyelectrolyte hydrogel and the
82 solution^{37,38}. The intensity of the electric potential of the hydrogel in relation to the solution depends
83 on the ionic strength of the solution and the immobile charge density of the hydrogel³⁹. Such electric
84 double layer is the origin of repulsion between two like-charged polyelectrolyte hydrogels. When two
85 like-charged surfaces are forced to approach each other, an osmotic repulsion is generated between
86 them to avoid an overlap of the electric double layers of the two surfaces and a liquid film is formed
87 at the interface⁴⁰. Due to the existence of the liquid film as lubricant, two like-charged polyelectrolyte
88 hydrogels show low sliding friction under water^{41,42}.

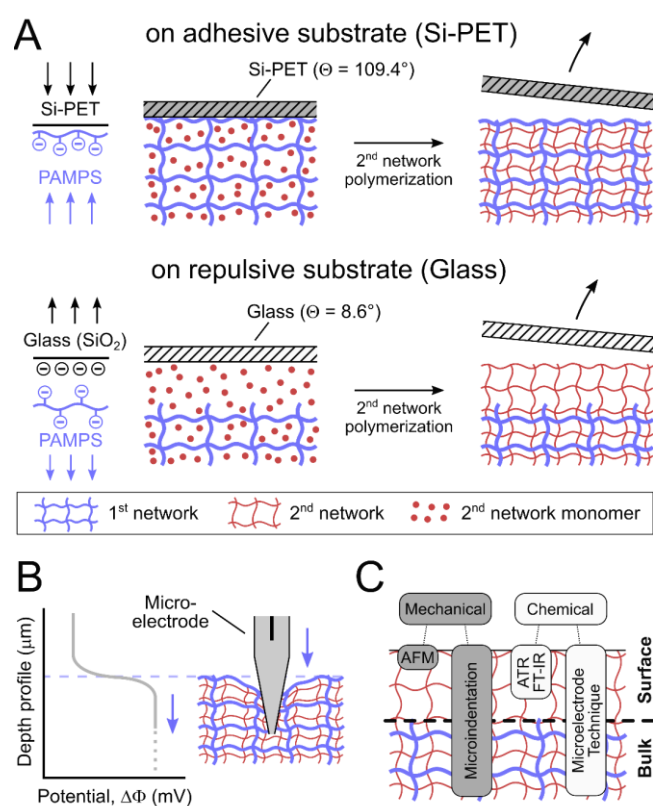
89 The above results suggest that the surface of a DN hydrogel could be regulated by the molding
90 substrate used for synthesizing the 2nd network. When a polyanion is used as the 1st network and a
91 negatively charged substrate is used as mold, a thin hydrogel layer of the 2nd network should be formed
92 on the surface of the DN hydrogel. In contrast, if a hydrophobic mold is used, to which the 1st network
93 is adhesive, no thin hydrogel layer of the 2nd network will be formed on the DN gel surface. In this work,
94 we synthesized DN hydrogels from PAMPS as the 1st network and polyacrylamide (PAAm) as the 2nd
95 network in a modified two-step synthesis approach. To regulate the surface-bulk transition we used

96 substrates of low (silicone coated polyethylene, Si-PET) and high (borosilicate glass substrate) surface
97 energy in the 2nd network polymerization step (Scheme 1A). On the low surface energy substrate, we
98 predict the adhesion of the 1st network to the substrate through hydrophobic interactions resulting in
99 a DN hydrogel (DN/Si-PET) with 1st and 2nd network present at the surface. On the borosilicate glass
100 mold that also carries negative charges on its surface, however, we assume that a PAAm layer forms
101 (DN/Glass) because of the electrostatic repulsion between the PAMPS and the glass. As the thickness
102 of the liquid layer at the repulsive interface is determined by the balance of the ionic osmotic pressure
103 and the normal pressure applied, we tried to regulate the surface layer thickness through applying
104 normal compression to the glass and PAMPS hydrogel in the 2nd network polymerization (DN/Glass-C,
105 Figure S5A).

106 It is challenging to analyze the heterogeneous DN hydrogel structure, especially if it differs between
107 the surface and the bulk. First, in swollen state, the water molecules and hydrogen bonds absorb a
108 large bandwidth of electromagnetic waves, rendering spectroscopy techniques ineffective⁴³. Second,
109 common electric potential characterization methods such as ζ -potential measurements, used to
110 determine hydrogel charges, are not capable of analyzing a structure-related charge difference
111 between the surface and bulk of the hydrogel⁴⁴. To overcome these difficulties, we adopted a recently
112 developed method that provides a new perspective on the charge distributions in hydrogels, called the
113 microelectrode technique (Scheme 1B)⁴⁵. By inserting a microelectrode into the hydrogel and
114 recording the electric signal in accordance to the electrode penetration velocity, an electric potential-
115 depth profile of the hydrogel is generated, which reveals the charge density distribution inside of the
116 hydrogel. Here in this study we deployed attenuated total reflectance Fourier-transform infrared
117 spectroscopy (ATR FT-IR) and the microelectrode technique to characterize the surface and bulk
118 structure of the DN hydrogels. ATR FT-IR provides surface structure information while the
119 microelectrode technique is a highly sensitive method giving a depth profile with a low spatial
120 resolution of the microelectrode diameter (~200 nm). Using these two complementary methods, we

121 can characterize the surface layer structure and the surface-bulk transition of the hydrogels (Scheme
 122 1C).

123 Ultimately, we identified that modulating the 2nd network polymerization condition regulates the
 124 surface-bulk transition and allows us to switch-on or switch-off the cell adhesive properties on DN
 125 hydrogels. By modifying the two-step DN hydrogel synthesis approach we provide an easily accessible
 126 opportunity to design tough hydrogels with functional surface charge transitions, which are useful in
 127 cell scaffolding and in vivo applications.



128 **Scheme 1: Strategy to regulate and to analyze the surface-bulk transition of DN hydrogels by using different**
 129 **molds for second network synthesis.** (A) DN hydrogels synthesized on the hydrophobic substrate (Si-PET)
 130 have surfaces containing both polymer networks due to adhesion of the 1st network on the substrate.
 131 DN hydrogels synthesized on the glass substrate are covered with a thin layer of 2nd network polymer
 132 due to the osmotic repulsion between the negatively charged glass and the 1st network. (B) The
 133 microelectrode technique measures the electric potential of the hydrogel depth profile (blue dotted
 134 line represents the hydrogel-solution interface). (C) Summary of the deployed mechanical and
 135 chemical characterization methods used to analyze the surface-bulk transition of DN hydrogels.
 136

137

138 Experimental Section

139 Materials

140 The 1st network monomer 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Toagosei Co., Ltd.,
141 Japan) was used as received. The 2nd network monomer acrylamide (AAM), crosslinker N,N'-
142 methylenebis(acrylamide) (MBAA) and initiator 2-oxoglutaric acid were used as obtained from Wako
143 Pure Chemical Industries, Ltd., Japan. The molding surfaces for the 2nd network polymerization, such
144 as borosilicate glass substrate (Asahi Glass Co., Ltd., Japan), were heat treated at 500 °C for 2 hours
145 prior to use and silicone coated polyethylene film (Si-PET), SP-PET-03-38-Bu (Mitsui Chemicals
146 Tohcello. Inc., Japan) was used as purchased. Unless otherwise noticed, all chemicals and solvents used
147 in cell culture were purchased from Sigma-Aldrich Inc, United States. The human mesenchymal stem
148 cell (MSC) line derived from umbilical cord blood (UCB) (JCRB1110 UCB408E6E7TERT-33) was obtained
149 from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Japan).

150 Surface energy of molding substrates

151 To characterize the surface energy of the molding substrates used in the 2nd network polymerization
152 step, the static water contact angles (Θ) of the glass substrate and the Si-PET film were measured with
153 double distilled water (ddH₂O, droplet volume 2 μ L) on a Drop Master 300 (Kyowa Interface Science
154 Co., Ltd.). The contact angle images are found in the supplementary information in Figure S1.

155 Single and double network hydrogel synthesis

156 The DN hydrogel synthesis was modified from Gong et.al⁴⁶ and sample codes and formulations are
157 found in Table 1. Briefly, in the first step, aqueous precursor solutions of AMPS monomer (C_{M1}),
158 crosslinker MBAA (X_{X1}) and initiator 2-oxoglutaric acid (X_{I1}) were poured into glass molds with a spacer
159 thickness of 0.5 to 2 mm (h_0) and free-radical polymerization was initiated by irradiated of 365 nm UV
160 light in argon atmosphere (2.5 mbar) for 8 hours to obtain 1st network PAMPS hydrogels. This first

161 polymerization process was followed by 3-day immersion of the freshly synthesized hydrogels in the
 162 2nd network precursor solution composed of AAm monomer (C_{M2}), crosslinker MBAA (X_{X2}) and initiator
 163 2-oxoglutaric acid (X_{I2}). In the second step, the PAMPS hydrogels soaked in the 2nd network precursor
 164 solution, were sandwiched between glass plates (hereafter referred as DN/Glass) or Si-PET film
 165 (referred as DN/Si-PET) and compressed by 0.1 – 0.3 kPa. For the DN sample group DN/Glass-C, the
 166 PAMPS hydrogels were sandwiched between glass plates and compressed by 50 kPa (Figure S5A). For
 167 the thickness gradient DN hydrogels, the PAMPS hydrogel was sandwiched between glass plates in a
 168 mold with a gradual increase in spacer thicknesses (Scheme S1). The hydrogel-mold assemblies were
 169 incubation in the argon atmosphere for 1 hour, and the 2nd network was polymerized within the PAMPS
 170 network by UV irradiation for 8 hours.

171 Single network (SN) hydrogels corresponding to the 1st or 2nd network were synthesized in a similar
 172 manner to the 1st network polymerization of DN hydrogels from aqueous solutions of either AMPS or
 173 AAm monomer with various crosslinker densities of MBAA. Prior to hydrogel characterization, the
 174 synthesized SN and DN hydrogels were equilibrated in double distilled water (ddH₂O) for at least for 7
 175 days.

176 **Table 1: Hydrogel sample codes and formulations**

Sample code	PAMPS - 1 st network			PAAm - 2 nd network		
	Monomer	Crosslinker*	Initiator*	Monomer	Crosslinker*	Initiator*
	C_{M1} (M)	X_{X1} (mol%)	X_{I1} (mol%)	C_{M2} (M)	X_{X2} (mol%)	X_{I2} (mol%)
PAMPS (C_{M1} - X_{X1} - X_{I1})	1	1.5, 2, 3, 5, 9	1	-	-	-
PAAm (C_{M2} - X_{X2} - X_{I2})	-	-	-	2	0.1 – 4	0.1
DN(C_{M1} - X_{X1} - X_{I1} / C_{M2} - X_{X2} - X_{I2})	1	1.5, 2, 3, 5, 9	1	2	0.1	0.1

177 *mol% of X_{X1} , X_{I1} and X_{X2} , X_{I2} are relative to the amount of C_{M1} and C_{M2} , respectively.

178 Hydrogel swelling and polymer weight fraction

179 The bulk volumetric swelling degree of PAMPS hydrogels, $Q = (h/h_0)^3$, was determined from the
 180 thickness of PAMPS hydrogels swollen in double distilled water (ddH₂O) or 2nd network precursor
 181 solution (h), and that in the as-prepared-state (h_0) after polymerization. The polymer weight fraction,
 182 W_{M1} and W_{M2} (wt%) of SN hydrogel was determined by the gravimetric wet (m)/dry (m_0) ratio ($\times 100$)

183 of hydrogel equilibrated in ddH₂O (*m*), and after vacuum oven drying procedure at 75°C and reduced
184 pressure of 1 bar for at least 2 days (*m*₀).

185 Polymer surface density via ATR/FT-IR

186 To analyze polymer surface density of the 1st and 2nd network at the SN and DN hydrogel surface,
187 attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR) on a FT-IR 6600
188 spectrometer (Jasco, Japan) with diamond prism was performed. Prior to analysis, hydrogel discs were
189 immersed in D₂O for 12 h and hydrogel spectra were measured in full contact with the prism for
190 wavenumbers of 1800 – 800 cm⁻¹, at 16x accumulation and resolution of 2 cm⁻¹. Absorbance maxima⁴⁷,
191 (*A*_{max}) of the characteristic sulfonic group peak (*v*_{S=O}) of PAMPS at 1042 cm⁻¹, and carbonyl group peak
192 (*v*_{C=O}) for PAAm at 1640 cm⁻¹ were determined using the manufacturers software. Since infrared
193 absorption peak height resembles polymer surface density, a calibration curve that relates IR
194 absorption peak height and polymer weight fraction of SN hydrogel allows the detection of 1st and 2nd
195 network at the DN hydrogel surface (Table S1, Figure S3, Figure S4). Since the gels slightly swelled in
196 deuterium oxide compared to water, the relative swelling ratio (*Q*_r) was used as a correction factor for
197 the polymer surface density calibration. The $Q_r = (h_{H_2O}/h_{D_2O})^3$ was determined by the hydrogel thickness
198 in ddH₂O (*h*_{H₂O}) relative to thickness in deuterium oxide (*h*_{D₂O}), (Figure S4). Polymer surface densities
199 were expressed as weight fractions and as PAMPS to PAAm molar concentration ratio, *C*_{M1}/*C*_{M2} (M/M).

200 Depth profiling via microelectrode technique

201 The surface layer thickness (*t*) at which the hydrogel structure transitions from the surface to bulk was
202 evaluated by a microelectrode insertion technique previously described⁴⁵. Here the thickness is based
203 on the displacement of the microelectrode moving at a constant speed and the shift in electric
204 potential ($\Delta\Phi$) due to the relative change in the local small ion concentration. Equilibrated in 10⁻⁴ M
205 NaCl solution, hydrogels were probed with a microelectrode made from a glass capillary with a fiber
206 filament (outer diameter = 180 nm, inner diameter = 128 nm) fabricated with a P-1000 capillary puller
207 (Sutter Instrument Co., U.S.), at an insertion speed of 0.5 – 2 μm/s. Firstly the surface electric potentials

208 ($\Delta\Phi_s$) were estimated from the 1st derivative of the electric potential curves and secondly the peak
209 distance measured from the first valley to the last hill of the 2nd derivative was used to estimate the
210 surface layer thickness (t) of hydrogels (Figure S2). The 1st and 2nd derivative curves were processed via
211 smoothing and y-axis scaling in Origin (OriginLab Corp.) software. The Debye length of the bath solution
212 (10^{-4} M NaCl) is 30.4 nm⁴⁵.

213 Mechanical characterization of hydrogels

214 The surface elastic modulus ($E_{Surface}$) was tested on an atomic force microscope (AFM, Nanowizard 4,
215 Bruker, U.S.) mounted to an inverted microscope (Nikon Eclipse TE 300, Japan) in aqueous conditions
216 at 25°C. The spring constants of 0.079 - 0.083 N/m were obtained by thermal tuning prior to each 4 x
217 4 force-distance curve array in force mapping-mode on the SFM (QITM - mode) for the used pyramid
218 shaped cantilever (A triangular, MLCT series, Bruker, U.S.). The peak force setpoint was normalized to
219 0.2 nN. The bulk elastic modulus (E_{Bulk}) was determined by indentation method using a tensile-
220 compressive mechanical tester (Autograph AG-X, Shimadzu Co., Japan) in air at 25°C. A sphere-shaped
221 micro-indenter ($r = 250 \mu\text{m}$, material: stainless steel) probed the hydrogel at a deformation velocity of
222 250 $\mu\text{m}/\text{min}$. By applying the Hertzian model⁴⁸ for rigid sphere indenters, with an assumed Poisson's
223 ratio for the hydrogels of 0.5 the elastic modulus (E) was estimated from the AFM and micro-
224 indentation loading curves.

225 Hydrogel cross-section structure observation

226 The swollen DN hydrogel sample with a surface layer thickness gradient was frozen in liquid N₂ and
227 fractured to expose the cross section and freeze-dried (Advantage XL-70, VirTis freeze dryer). After
228 gold sputtering (E-1010, Hitachi, Japan), the structure of the hydrogel cross-section was analyzed on a
229 scanning electron microscope (SEM, JSM-6010LA, JEOL Ltd., Japan).

230 *In vitro* cell culture and morphology

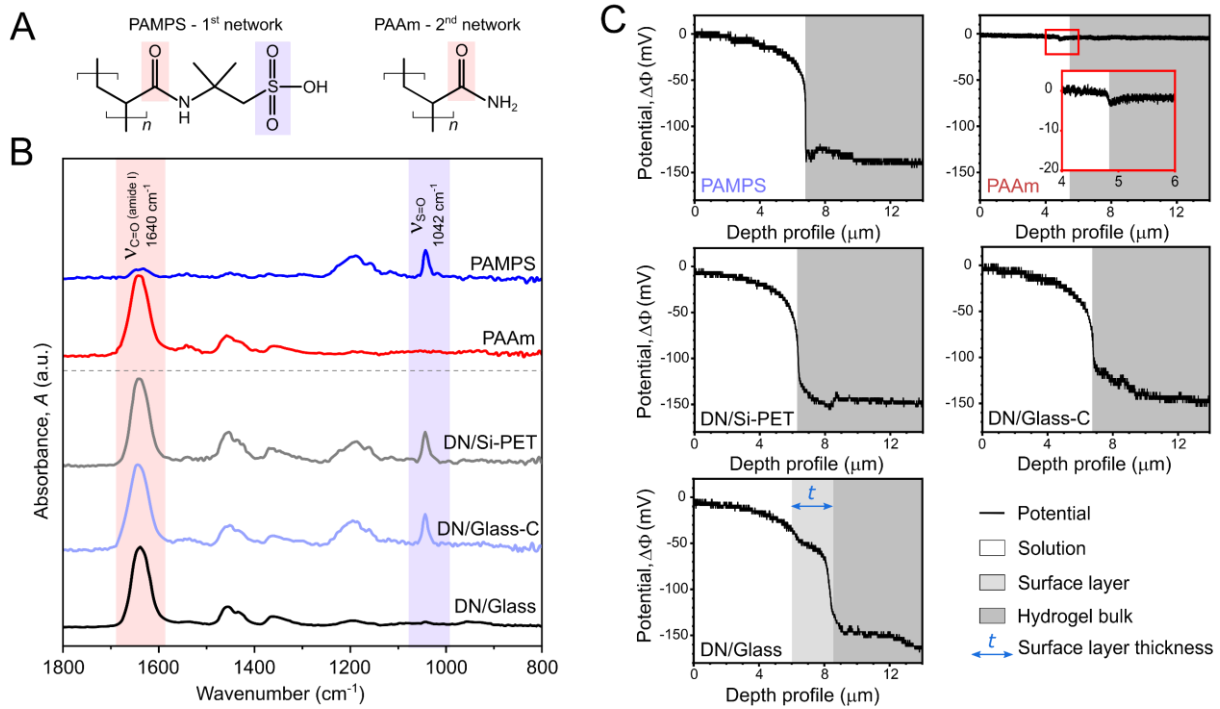
231 Hydrogel membranes were equilibrated and sterilized in HEPES-buffer and then incubated in culture
232 medium overnight. The Dulbecco's modified Eagle's culture media was supplemented with 10% fetal
233 bovine serum (Thermo Fisher Sci.), 1% Penicillin/Streptomycin and 1% L-glutamine. The mesenchymal
234 stem cells were expanded on polystyrene dishes prior to cell seeding on hydrogel membranes. On day
235 0, trypsinized single-cell suspensions of stem cells were tested by trypan blue dye live/dead exclusion.
236 Cell cultures with a viability over 95% were seeded onto hydrogel membranes at a concentration of 1
237 $\times 10^5$ cells/mL and incubated for 3 days at 37°C and 5% CO₂ in humidified atmosphere. Cell cultivation
238 was stopped on day 3 and cell morphologies were imaged through phase-contrast microscopy
239 (OLYMPUS - IX73, Japan).

240

241 Results and Discussion

242 Chemical and mechanical properties of the surface-bulk transition region

243 First, we characterized the surface chemical compositions of DN hydrogels prepared on different
244 substrates using ATR/FT-IR which reveals the chemistry of the topmost surface of the hydrogels.
245 PAMPS has a sulfonic group and PAAm has an amide carbonyl group (Figure 1A) that can be
246 characterized by ATR/FT-IR. On the absorption spectra (Figure 1B), a sharp peak at 1042 cm^{-1} ,
247 characteristic for the sulfonic group ($\nu_{\text{S=O}}$) of the PAMPS network, and a strong sharp absorption peak
248 around 1640 cm^{-1} , from the primary amide carbonyl group ($\nu_{\text{C=O}}$) of PAAm, were observed on the
249 spectra for the SN PAMPS (1-3-1) gel and PAAm (2-0.1-0.1) gel, respectively. On the PAMPS hydrogel,
250 a peak at 1640 cm^{-1} is also observable since the molecular structure of PAMPS contains a primary amide
251 carbonyl group ($\nu_{\text{C=O}}$). However, the signal is much weaker compared to PAAm and therefore it is
252 neglectable. The spectrum of the DN/Si-PET, exhibits both strong absorption peaks at 1042 cm^{-1} for
253 the sulfonic group and at 1640 cm^{-1} for the primary amide carbonyl group, while the DN/Glass hydrogel
254 only exhibits absorption at 1640 cm^{-1} due to the amide carbonyl group. These ATR/FT-IR results
255 indicate that both PAMPS and PAAm are present on the surface of the DN/Si-PET hydrogel while only
256 the 2nd network PAAm is present on the surface of the DN/Glass hydrogel. Based on this analysis, we
257 infer that there is a surface layer of PAAm on the DN/Glass hydrogel.



258
 259 **Figure 1: Analysis of the surface structure of DN hydrogels where the 2nd network was synthesized**
 260 **on different substrates.** (A) The molecular structures of the 1st and 2nd network polymers. The red and
 261 blue background represent the IR bond absorptions, used to characterize the hydrogels. (B) The
 262 ATR/FT-IR absorption spectra and (C) the electric potential depth profiles ($\Delta\Phi$) of SN PAMPS (1-3-1),
 263 SN PAAm (2-0.1-0.1) and corresponding DN hydrogels (1-3-1/2-0.1-0.1) synthesized on Si-PET (DN/Si-
 264 PET) and glass substrates (DN/Glass), and on glass under compression (DN/Glass-C, Figure S5).

265 To characterize the thickness of the surface layer on the DN/Glass hydrogel, we probed the depth
 266 profile of the electric potential near the surface of the hydrogels by the microelectrode technique. A
 267 charged hydrogel generates an electric potential ($\Delta\Phi$) relative to the bath aqueous solution due to the
 268 Donnan effect⁴⁹.

269

$$\Delta\Phi = -\frac{2.3RT}{zF} \log\left(\frac{C_g}{C_s}\right) \quad (1)$$

270 Here, C_g and C_s are the mobile counter-ion concentrations in the hydrogel and in the bath solution,
 271 respectively. z is the valence of the mobile counter-ion, F the Faraday constant, R the gas constant,
 272 and T the absolute temperature. The covalent bound ions on the polymer network guide the mobile
 273 counter-ion concentration to keep neutrality in a thermodynamic equilibrium state. As a result, by
 274 probing the local electric potential, we can detect the local structure of the hydrogel. For the PAMPS
 275 hydrogel, that carries immobile negative charges on the polymer network, the potential shifts to a
 276 negative value relative to the bath solution since the hydrogel traps high numbers of positively charged

277 mobile ions (z is positive), while the PAAm hydrogel has almost zero potential since its polymer
278 network carries a negligible amount of charge. Accordingly, we can probe the surface layer thickness
279 of the DN/Glass hydrogels via the electric potential depth profiling.

280 As shown in Figure 1C, the microelectrode, inserted into a SN PAMPS hydrogel, measures an initial
281 gradual drop of electric potential until the displacement of $6.8 \mu\text{m}$, which is followed by an abrupt drop
282 to a large negative value of -150 mV . The initial potential drop is due to the deformation before the
283 penetration of the electrode into the hydrogel. Like the piezoelectric effect, the compression at the
284 probe tip increases the local polymer charge density and decreases the electric potential. The large
285 potential shift ($\Delta\Phi$) to -150 mV indicates that a high amount of Na^+ ions ($z = +1$) are deployed as
286 counterions to neutralize the fixed negative charges of the sulfonic groups of PAMPS. In contrast, only
287 a very small potential shift of -4 mV is measured for the neutral SN PAAm hydrogel. The potential depth
288 profile of the DN/Si-PET hydrogel, where the 2nd network was synthesized on adhesive Si-PET (contact
289 angle to ddH_2O $\Theta = 109.4 \pm 0.8^\circ$, Figure S1), shows an immediate drop of the potential to -150 mV after
290 the initial drop coming from the compression before electrode penetration, indicating the absence of
291 the surface layer, in good agreement with the ATR/FT-IR result.

292 In contrast, the depth profiles of the DN/Glass hydrogel (1-3-1/2-0.1-0.1), where the 2nd network was
293 synthesized on repulsive glass (contact angle to ddH_2O , $\Theta = 8.5 \pm 1.5^\circ$, Figure S1), also shows an initial
294 drop of electric potential to a probe displacement of $6.1 \mu\text{m}$, like the SN PAMPS hydrogel before the
295 probe penetration to the hydrogel. Different from the profile of the SN PAMPS hydrogel, a small hump
296 with a change in potential of -50 mV appears at the displacement of $6.1 \mu\text{m} \sim 8.5 \mu\text{m}$, indicating the
297 presence of a weakly charged surface layer. At a displacement larger than $8.5 \mu\text{m}$, the potential shifts
298 drastically to -150 mV , indicating the transition from the surface layer to the bulk material composition
299 of PAMPS/PAAm because the shift intensity is like that of the corresponding PAMPS SN hydrogel. From
300 the 2nd derivative of the depth profile, a surface layer of thickness $t = 2.23 \mu\text{m}$ on the DN/Glass hydrogel
301 was determined (Figure S2).

302 The microelectrode results reveal the presence of a surface layer on the DN/Glass hydrogel while no
303 surface layer is formed on the DN/Si-PET hydrogel. These results are consistent with the ATR/FT-IR
304 results. However, the microelectrode method reveals that the surface layer on the DN/Glass hydrogel
305 has a larger electric potential (-50 mV) than that of the corresponding PAAm hydrogel (-4 mV),
306 indicating that the surface layer of the DN/Glass hydrogel is not pure PAAm, which is not consistent
307 with the ATR/FT-IR measurement that shows no signal from PAMPS. This discrepancy is due to the
308 relatively poor sensitivity of the ATR/FT-IR. From the potential shift of -50 mV in relation to the bath
309 solution containing 10^{-4} M NaCl, the concentration of mobile Na^+ ion in the surface layer of the
310 DN/Glass gel is estimated as 7×10^{-4} M by using Equation 1. The presence of this excess number of
311 mobile ions in the surface layer of the DN/Glass hydrogel suggests that a very small amount of AMPS
312 monomers, as residue from the 1st network synthesis, of 7×10^{-4} M in concentration, is copolymerized
313 in the 2nd PAAm polymerization to form a copolymer. Such a small amount of AMPS in the surface layer
314 of the DN/Glass could not be detected by the ATR/FT-IR method that has a lower detection limit for
315 PAMPS of 9.4×10^{-4} M (Figure S3).

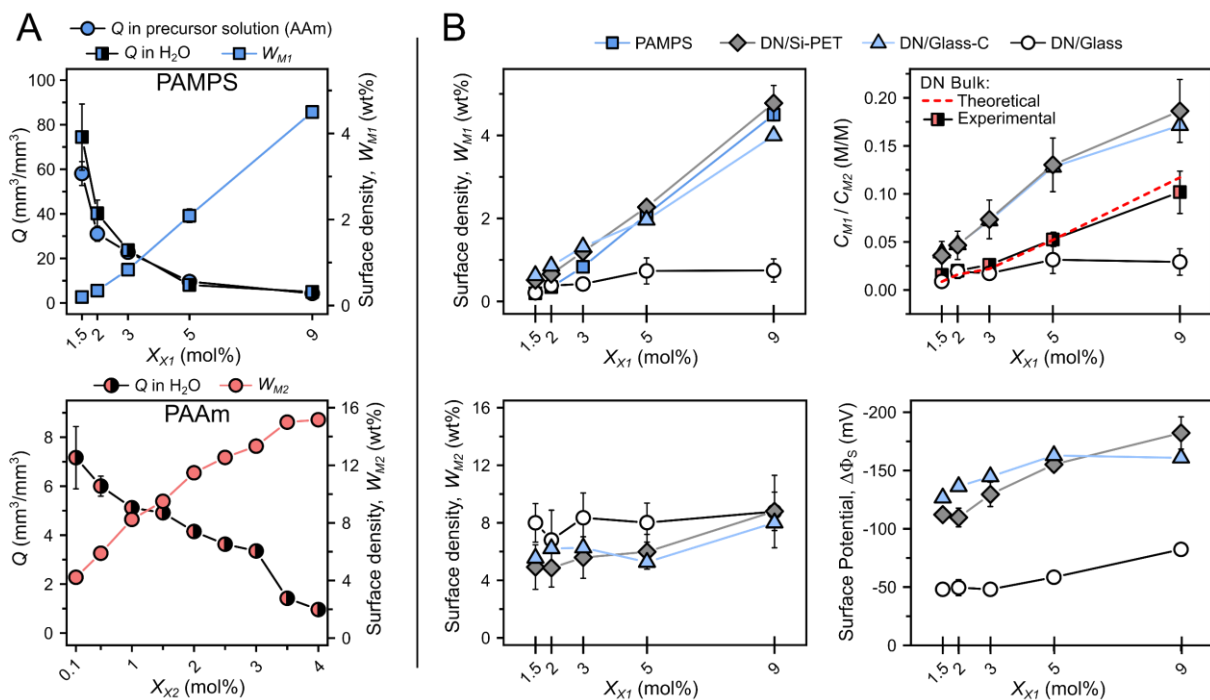
316 The PAAm surface layer formation on the DN/Glass hydrogel is presumably caused by the counter ions
317 osmotic repulsion between negatively charged PAMPS hydrogel and the glass substrate during the 2nd
318 network polymerization. To verify the hypothesis, we tried to eliminate the surface layer by applying
319 compression (50 kPa) to the 1st network hydrogel during the synthesis of the 2nd network to counteract
320 the osmotic repulsion (Figure S5A). The ATR/FT-IR spectra show both peaks from PAAm (1640 cm^{-1})
321 and PAMPS (1042 cm^{-1}) (Figure 1B), indicating no surface layer formation on the DN/Glass-C hydrogel.
322 The electric potential depth profile also confirms no surface layer formation. These results
323 demonstrate that we can switch on or off the surface layer of DN gels by either substrate chemistry or
324 compression during the 2nd network polymerization.

325 We further analyzed the surface structure and modulus, in comparison with the bulk quantities for
326 DN/Glass, DN/Si-PET and DN/Glass-C hydrogels synthesized by different formulations, and the results
327 are summarized in Figure 2 and 3. Figure 2A shows that the polymer surface density of SN PAMPS and

328 SN PAAm hydrogels increases with the crosslinking degree (X_{X1} , X_{X2}), due to the decrease of the
329 volumetric swelling degree (Q) at increased crosslinking degree. The polymer densities can be tuned
330 in a range of 0.2 ~ 4.5 wt% and 4.2 ~15.2 wt% for PAMPS (W_{M1}) and PAAm (W_{M2}), respectively, by
331 adjusting the crosslinking degree. Due to its polyelectrolyte nature, the swelling degree of PAMPS
332 hydrogels is much higher than that of the neutral PAAm hydrogels even at a high crosslinking degree.
333 The swelling degree of PAMPS in 2nd network precursor solution is almost the same to that of PAMPS
334 in water because the AAm monomer is neutral³⁸.

335 The changes in surface polymer density and electric potential of DN gels in accordance to the 1st
336 network crosslinking degree (X_{X1}) is shown in Figure 2B. On DN hydrogels, the PAMPS surface densities
337 (W_{M1}) between DN/Glass, DN/Si-PET and DN/Glass-C is different because of the presence or absence
338 of the surface layer (upper left side). As the 1st network crosslinking (X_{X1}) increases, the PAMPS surface
339 densities of DN/Si-PET and DN/Glass-C hydrogels increased, in agreement with that of the SN PAMPS
340 hydrogel. However, on the DN/Glass hydrogel surface, the PAMPS density is below a weight fraction
341 of 1 wt% regardless of the change in the X_{X1} for the whole crosslinking range. The PAAm surface density
342 on DN/Si-PET and DN/Glass-C hydrogels slightly increases with increasing PAMPS network crosslinking
343 (X_{X1}), though the amount of crosslinker for the PAAm network for all DN hydrogels was kept constant
344 (X_{X2} : 0.1 mol%). However, on the DN/Glass hydrogel, the PAAm surface density is higher than that of
345 the DN/Si-PET and DN/Glass-C hydrogels, showing almost a constant value, independent of the change
346 in X_{X1} . The increase in the PAAm density with X_{X1} on DN/Si-PET and DN/Glass-C gels is because the
347 swelling of the PAAm is constrained by the presence of PAMPS. The higher the X_{X1} of the 1st network,
348 the more the PAAm network is constrained¹⁵. The surface molar ratio of PAMPS to PAAm (C_{M1}/C_{M2})
349 increases with the increase in the 1st network crosslinking degree for DN/Si-PET and DN/Glass-C gels
350 while it keeps a low but constant value for DN/Glass gels (Figure 2B, lower left side). By cutting the
351 surface hydrogel we exposed the bulk material so that a comparison between the surface and the bulk
352 molar ratio C_{M1}/C_{M2} is possible (Figure 2B, upper right side). While the bulk values were in good
353 agreement with the theoretical values, the surface molar ratio C_{M1}/C_{M2} is about 2 times higher than

354 the bulk values. The theoretical values were estimated from, $C_{M1}/C_{M2} = Q^{-1}C_{M1}/C_{M2}$, where C_{M1} and C_{M2}
 355 are the feed monomer concentrations of the 1st network and the 2nd network, respectively, and Q is
 356 volumetric swelling degree of PAMPS hydrogels in AAm solution. The high molar ratio on the surface
 357 compared to the bulk probably originates from the faster polymerization of the PAMPS hydrogels near
 358 the glass substrates in relation to the central region due to the higher UV intensity at the surface
 359 regions. The increase in the amount of crosslinker (X_{X1}) reduces the swelling of the PAMPS hydrogel in
 360 the 2nd network precursor solution and consequently increases the number of negative charges
 361 through the PAMPS density. Therefore, the electric surface potential ($\Delta\Phi_s$), defined as the initial
 362 drastic potential drop at the hydrogel surface (Figure S2), of the DN/Si-PET and DN/Glass-C hydrogels
 363 increased up to a value of -180 mV at 9 mol% crosslinker (X_{X1}), with a molar ratio C_{M1}/C_{M2} of 0.2 (Figure
 364 2B, lower right side). Similar increase in surface electric potential was published previously, when
 365 crosslinker density increased in SN PAMPS hydrogels⁴⁵. The DN/glass hydrogel reaches a value of -75
 366 mV at 9 mol% crosslinker (X_{X1}) and is arguably related to the increased incorporation of unreacted
 367 AMPS because of a low conversion rate in the polymerization of highly crosslinked PAMPS.

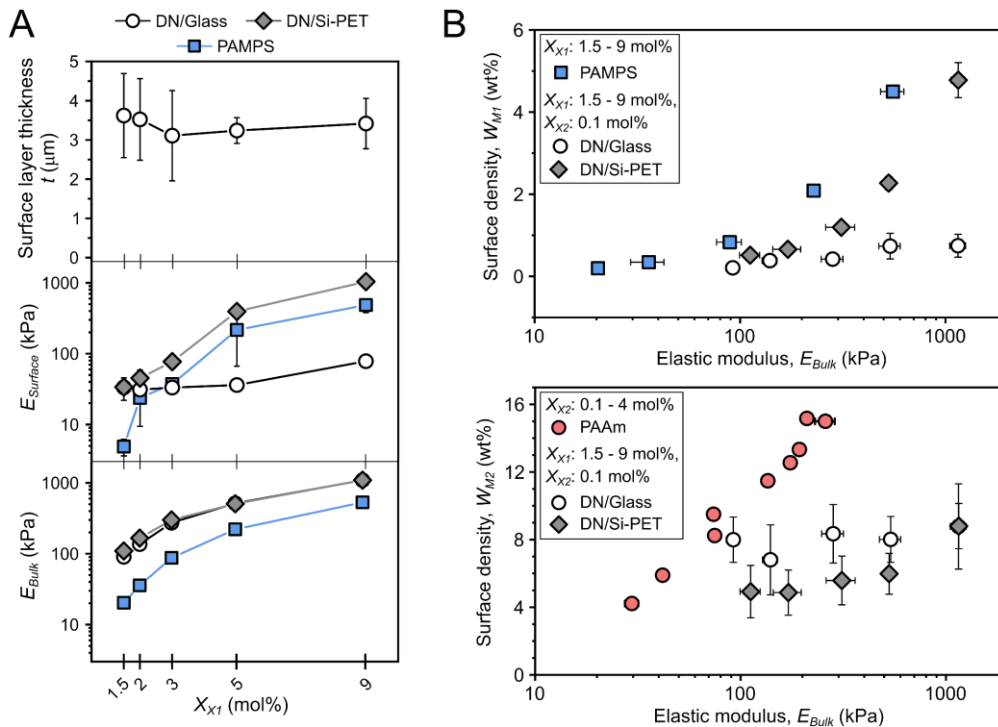


368
 369 **Figure 2: Regulation of polymer surface density (W_{M1} , W_{M2}) by crosslinking degree (X_{X1} , X_{X2}) of the**
 370 **first network.** (A) The volumetric swelling degree (Q) and the corresponding surface polymer density
 371 (W_{M1} , W_{M2}) of SN PAMPS and PAAM hydrogels (mean \pm SD, $n = 3$ hydrogels per symbol) for varied
 372 crosslinking degree (X_{X1} , X_{X2}). (B) DN hydrogel surface polymer density (W_{M1} , W_{M2}), the corresponding

373 molar concentration ratio (C_{M1}/C_{M2}), and the surface potential ($\Delta\Phi_s$) for DN gels prepared with various
374 1st network crosslinking degree (X_{X1}) (mean \pm SD, sample groups SN PAMPS, DN/Si-PET, DN/Glass
375 contained sample sizes of $n = 3 - 6$ hydrogels per symbol, and for DN/Glass-C $n = 1$ hydrogel per
376 symbol). The standard deviations (SD) of the PAAm density values tend to be high because the low
377 accuracy of the ATR/FT-IR data used for the quantitative analysis resulted in a poorly fitted calibration
378 curve (Figure S3, Figures S4).

379 As Figure 3A demonstrates, in the case of DN/Glass hydrogels the surface layer thickness is between 3
380 - 4 μm for different X_{X1} . Because of the presence of the surface layer, the elastic modulus at the surface
381 is lower than the bulk in the case of the DN/Glass hydrogel, and only weakly increased with X_{X1} .
382 However, the bulk modulus of the DN/Glass hydrogel increases with increasing 1st network crosslinking
383 (X_{X1}), showing the same values as the bulk elastic modulus of DN/Si-PET hydrogels. For SN PAMPS and
384 DN/Si-PET hydrogels, the surface and the bulk elastic modulus both increase with X_{X1} .

385 From the above results, we can tune the bulk modulus while keeping the same surface chemistry. As
386 shown in Figure 3B, when the bulk elastic modulus of DN/Glass and DN/Si-PET was changed through
387 the 1st network crosslinking degree, the polymer surface densities evolved differently, depending on
388 the presence or absence of the surface layer. When no surface layer is present, the chemical structure
389 at the surface is controlled through the PAMPS/PAAm ratio and the swelling in the 2nd network
390 precursor solution. However, when there is a surface layer, the surface chemical structure depends on
391 the formulation of the 2nd network precursor solution and is independent of the bulk elastic modulus.
392 As shown for the SN PAAm hydrogels, polymer surface density and modulus increase with crosslinking
393 density X_{X2} . By varying X_{X1} and X_{X2} of the DN/Glass hydrogel, it is possible to individually tune the surface
394 layer polymer density (or modulus) and the bulk polymer density (or modulus).

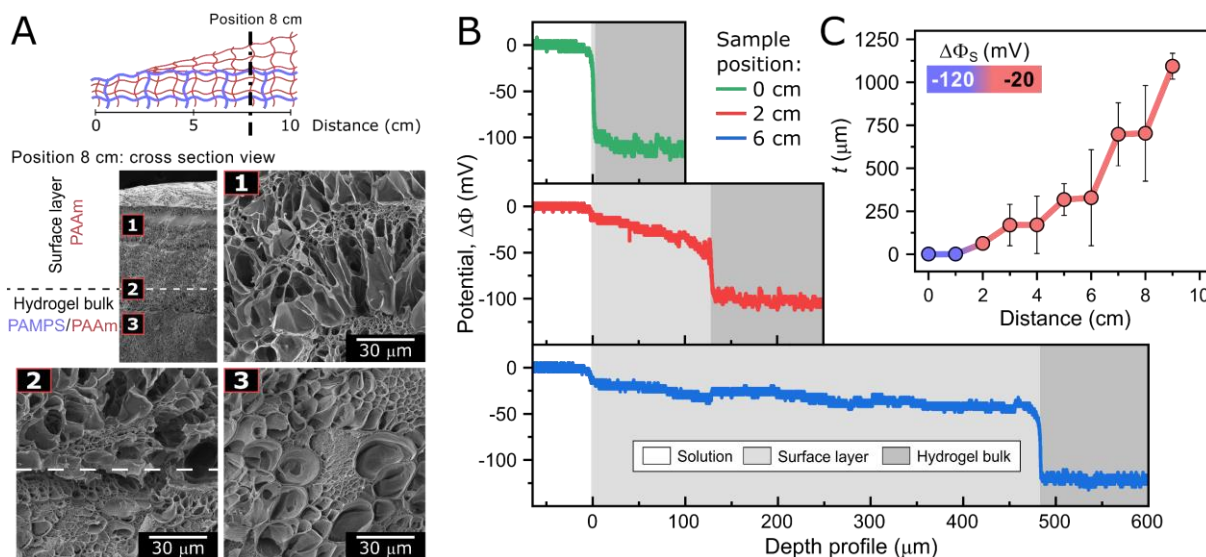


395
 396 **Figure 3: Effect of Surface layer thickness on elastic modulus.** (A) The surface layer of 3 – 4 μm
 397 thickness (t) on the DN/Glass hydrogel affects the elastic modulus at the surface (E_{Surface}), (mean \pm SD,
 398 $n = 15 - 48$ technical replicates per symbol) but not the bulk (E_{Bulk}). The sample groups SN PAMPS, SN
 399 PAAm, DN/Glass and DN/Si-PET contained sample sizes of $n = 3 - 6$ hydrogels per symbol (mean \pm SD).
 400 (B) Independent tuning of bulk modulus and polymer surface densities (W_{M1} , W_{M2}) by DN hydrogel
 401 synthesis.

402 Control of the surface layer thickness

403 To develop DN hydrogels with spatially modulated surface properties, the DN hydrogel was
 404 synthesized in a polymerization mold where the glass substrate was tilted which allows the regulation
 405 of the surface layer thickness, and through that, the control of the surface electric potential (Figure
 406 4A). During the 2nd network synthesis, the 1st network hydrogel was partially compressed on one side,
 407 through a spacer of height h_1 , thinner than the hydrogel thickness h , to reach a maximum compression
 408 of 50 kPa (Scheme S1). On the other side, a spacer of height h_2 , thicker than the hydrogel was used,
 409 where the gap between the PAMPS network and the glass substrate was filled with excess AAm
 410 solution. The maximum gap distance creates a surface layer thickness of 0.9 mm ($t \sim h_2 - h_1$) in the as-
 411 prepared state. Because of the spacer height difference, a structural change at the surface-bulk
 412 transition zone through sample preparation should be induced. The cross-section view of the DN
 413 hydrogel shows that there is a clear difference in structure of the surface layer and the bulk region, as

414 shown by the microscopic observations at position 8 cm (Figure 4A). The position is determined as the
415 distance of the sampling position to the edge of the compressed hydrogel side. While the surface layer
416 of $t \sim 800 \mu\text{m}$ thick (Figure 4A, 1) shows a big porous structure, in the bulk material (Figure 4A, 3) a
417 hierarchical structure of big pores filled with smaller ones is observed. The transition between the
418 surface layer and the bulk is a clear cut (Figure 4A, 2, white dotted line), with a transition zone thinner
419 than $20 \mu\text{m}$. Even if there is a structural difference between the surface layer and the bulk observable,
420 it must be noted that the observed porous structure is not resembling the native hydrogel structure
421 because it is obtained through a freeze-drying process. The electric potential depth profiles measured
422 at different positions reveal the gradual increase of the surface layer thickness with increasing distance
423 to the compressed side of the DN hydrogel. Three typical depth profiles are shown in Figure 4B. The
424 electric potential between the gel and the bath solution on the compressed side is above -100 mV
425 (Distance: 0 cm), related to the DN bulk potential. At positions of 2 cm and 6 cm , the surface layer
426 thickness increased so that the surface potential is weakened to a value of -20 mV , related to the PAAm
427 surface layer. The increase in surface thickness along the distance from the compressed side, analyzed
428 by the electric potential depth profiles, is summarized in Figure 4C. On the compressed side (Position:
429 0 cm), no surface layer is formed, and the surface potential of the gel is the same as the bulk, showing
430 a value of -120 mV . The surface potential abruptly changes from -120 mV to -20 mV between positions
431 of 1 and 2 cm , due to the formation of surface layer. With the increase of the distance to the
432 compressed side, the surface layer thickness gradually increases and reaches values higher than 1 mm
433 at the position of 9 cm , while the surface potential remains constant at -20 mV . Consequently, we
434 obtained a hydrogel surface with a strong negatively charged region ($\Delta\Phi_s = -120 \text{ mV}$, blue) that
435 switches within 1 cm to an almost neutral ($\Delta\Phi_s = -20 \text{ mV}$, red) region. Since the surface layer thickness
436 increases with the distance from the compressed side, the surface modulus integrated from the
437 surface layer and the bulk might change with the distance as well⁵⁰. Such a surface might be used for
438 studying the modulus effect on cells with a constant surface chemistry.



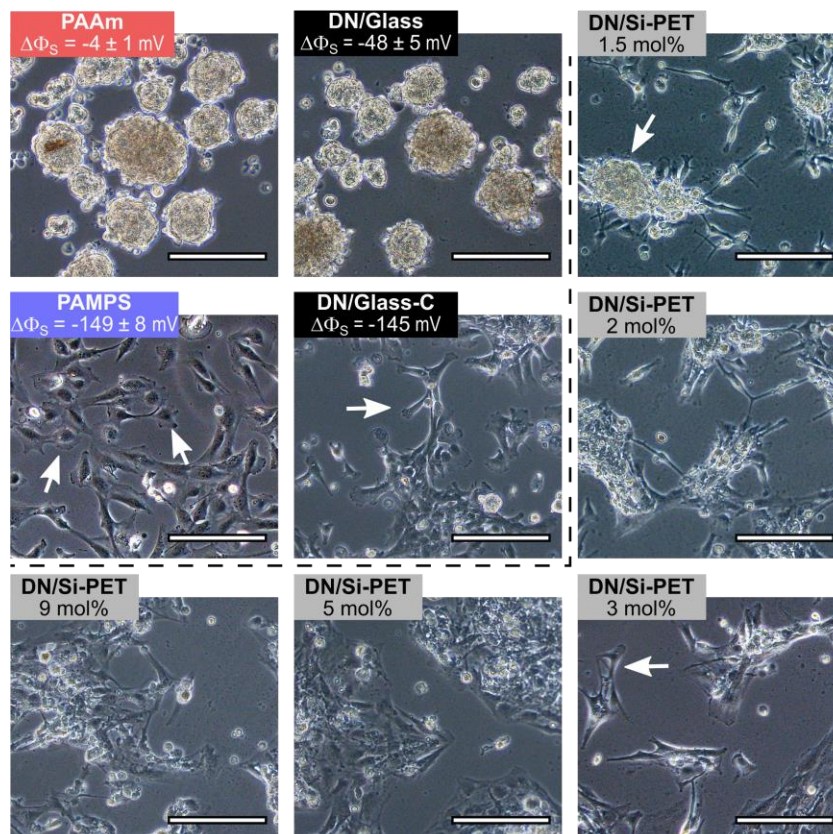
439 **Figure 4: Control of PAAm layer thickness on the DN/Glass surface from micro-to-macro scale.** (A)
 440 The 2nd network polymerization setup where the spacer heights (Scheme S1) and the compressive
 441 stress regulate the surface layer thickness of the DN hydrogel (PAMPS/PAAm, 1-3-1/2-0.1-0.1). The DN
 442 hydrogel SEM cross-sectional view reveals a surface layer with thickness $t \sim 800 \mu\text{m}$, with structural
 443 details on the PAAm surface layer (1), the transition zone (2, white dotted line represents the surface-
 444 bulk transition) and the DN bulk structure (3). (B) Selected electric potential depth profiles of the DN
 445 hydrogel at specific positions showing the surface layer thickness gradient. (C) Position profile of
 446 surface layer thickness and the corresponding surface potential ($\Delta\Phi_s$) visualized by a blue (strong
 447 charged) and red (weak charged) color map ($n = 2 - 5$ measurements per position).

449 Cell adhesion to DN hydrogels with varied surface potential

450 The independent tuning of the surface and bulk properties of DN hydrogels by the surface-bulk
 451 structure transition allows us to control the surface potential of DN gels. Here, we investigate how the
 452 surface potentials of the hydrogels influence the cell attachment. As an example, mesenchymal stem
 453 cell attachment on various types of synthesized DN hydrogels was monitored in a cell culture
 454 experiment.

455 Figure 5 shows the morphology of the mesenchymal stem cells after 3 days of incubation on various
 456 hydrogels with different surface potentials. When the hydrogel surface potential was weaker than -60
 457 mV, as seen on PAAm and DN/Glass hydrogels, cells did not adhere to the surface, instead they
 458 aggregated to multicellular spheroids. On hydrogels with surface potential stronger than -100 mV, as
 459 seen on PAMPS, DN/Glass-C, and DN/Si-PET hydrogels, cells started to adhere. On the SN PAMPS
 460 hydrogel the individual cells were circularly spread, while on the DN/Glass-C and DN/Si-PET hydrogels

461 the cell shapes are more irregular and spindle-like (shown by white arrows in Figure 5). In case of
 462 DN/Si-PET hydrogels, on the low crosslinked sample (X_{XL} 1.5 mol%) dome-shaped adherent cell
 463 aggregates were observed, but as the crosslinking degree increased the cells gradually become slightly
 464 more spread and the dome-shaped aggregates were not observable anymore. The cell-substrate
 465 adhesion is usually guided by protein adsorption^{51,52}. However, cell adhesion is a highly complex
 466 process and it was found that cell morphological changes are influenced by substrate roughness⁵³ and
 467 mechanical properties^{25,26,50} as well. The results shown in Figure 5 suggests that the surface electric
 468 potential is a major factor regulating cell morphology and it should be noted that the critical surface
 469 potential for cell adhesion varies from cell line to cell line³³.



470
 471 **Figure 5: Increasing hydrogel surface potential induces cell attachment.** Phase-contrast microscopic
 472 images (10 x magnification) of mesenchymal stem cells cultured on hydrogels for 3 days. Low surface
 473 electric potential facilitates stem cell aggregation (SN PAAm and DN/Glass) while high surface electric
 474 potential results in cell-hydrogel adhesion (SN PAMPS, DN/Glass-C, and DN/Si-PET). The scale bars
 475 represent 180 μ m. The surface electric potentials for the DN/Si-PET hydrogels are shown in Figure 2B.

476 Summary and Conclusion

477 The surface energy and charge of substrates used in 2nd network synthesis play a crucial role in
478 determining DN hydrogel surface chemistry and surface stiffness. While the use of a low energy
479 molding substrate facilitates the synthesis of DN hydrogels with both networks present at the surface,
480 the electrostatic interactions between glass and like-charged polyelectrolyte hydrogels are responsible
481 for the formation of a 2nd network polymer surface layer. The regulation of the DN hydrogel surface
482 potential is mainly related to the 2nd network surface layer thickness that is modifiable through molding
483 substrate surface energy or normal compression during synthesis. If no surface layer is present, a
484 secondary regulation of the surface potential is possible via the molar ratio between the
485 polyelectrolyte and neutral polymer. Usually, DN hydrogels made from two chemically crosslinked
486 networks are synthesized in a two-step approach, which is time consuming and work intensive.
487 However, with the here-proposed modifications, the second synthesis step is an easily accessible
488 technique that fabricates not just a tough hydrogel but simultaneously functionalizes the hydrogel
489 surface, which will be useful in controllable surface friction, cell scaffolding, and in vivo applications.

490 The combination of the ATR/FT-IR and the microelectrode technique has proven to be a powerful
491 approach for characterizing hydrogel surfaces since each technique makes up for the disadvantage of
492 the other technique. The microelectrode technique, which is highly sensitive in detecting electric
493 potentials but has a poor spatial resolution in the depth profile, complements the ATR/FT-IR technique,
494 which gives structural information on the topmost hydrogel layer with lower sensitivity.

495 The cell experiment proves that the previously observed phenomena, where cells do not adhere to DN
496 hydrogels, is not valid for DN hydrogels in general, but is a consequence of the synthesis condition.
497 Above a critical value, the surface PAMPS/PAAm ratios, applying to a wide range of DN hydrogel
498 compositions, have just a minor impact on the surface electric potential and the cell morphology. This
499 makes clear that publications investigating the interactions between cells and DN hydrogels must
500 explicitly describe the utilized polymerization substrate and the related surface potentials, since it

501 drastically changes the hydrogels cell-adhesive properties. Recently, there is evidence that the induced
502 stem cell state, of interest to cell therapies, is enhanced by cell aggregation. Therefore, the detailed
503 hydrogel characterization and physicochemical decoupling based on the regulation of the surface-bulk
504 transition described here could be applied to investigation how the morphological stem cell changes
505 alter gene expression. Regarding such studies, additional evaluation of hydrogel-protein adsorption
506 and cell viabilities on the synthesized hydrogels is necessary in the future.

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513 Supporting information

514 The supporting information contains Scheme S1, Table S1 and Figure S1 – 5 related to this article.

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