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Author(s)	Frauenlob, Martin
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Study on the control of the double network hydrogel surface-bulk transition
and its effect on mesenchymal stem cells

ダブルネットワークゲル表面-バルク転移の制御およびその間葉系幹細胞への影響に関する研究

Martin FRAUENLOB

北海道大学大学院生命科学院
Graduate School of Life Science, Hokkaido University

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List of Abbreviations:

UV-VIS	Ultra violet and visible spectrum
ATR/FT-IR	Attenuated total reflectance Fourier transformation infrared spectroscopy
AFM	Atomic force microscopy
RT-qPCR	Real-time quantitative polymerase chain reaction
MET	Microelectrode technique
DN	Double network
SN	Single network
PAMPS	Monomer: 2-acrylamido-2-methylpropanesulfonic acid
PAAm	Monomer: polyacrylamide
PDMA	Monomer: N,N-dimethylacrylamide
PCDME	Monomer: N-(Carboxymethyl)-N,N-dimethyl-2-(methacryloyloxy) ethanaminium, inner salt
MBAA	Crosslinker: N,N'-methylenebis(acrylamide)
α -keto	2-oxoglutaric acid
1 st nw	First network
2 nd nw	Second network
Si-PET	Silicone coated polyethylene film
PS	Polystyrene
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MSC	Mesenchymal stem cell
UCB	Umbilical cord blood
BM	Bone marrow
[a]	Soft single network hydrogel corresponds to DN hydrogel surface polymer density
[b]	Stiff single network hydrogel corresponds to DN hydrogels elastic modulus
[c]	Double network hydrogel used to induce stem cells
GSEA	Gene set enrichment analysis
GO	Gene ontology
STD	Standard deviation
SEM	Standard error of the mean

List of symbols and constants:

ΔG_{mix}	Gibbs free energy of mixing
n	number of moles
ν	Volume fraction
χ	Thermodynamic interaction parameter between polymer and solvent
ξ	Mesh size
ϕ_s, ϕ_g	Potential of solvent (s) and gel (g)
A	Absorbance (a.u.)
A_{max}	Absorbance at the peak (a.u.)
$\Delta\Phi$	Electric potential (mV)
$\Delta\Phi_s$	Electric Surface potential (mV)
t	Surface layer thickness (μm)
R	Gas constant (8.314 J/K, mol)
F	Faraday constant (96485 As/mol)
z	Valence of mobile counter ions (... , -2, -1, 0, +1, +2,...)
T	Absolute temperature (K)
Θ	Contact angle ($^\circ$)
E	Elastic modulus (kPa) at the surface ($E_{Surface}$) and of the bulk hydrogel (E_{Bulk})
Q	Volumetric swelling degree (mm^3/mm^3)
Q_r	Relative swelling ratio (mm^3/mm^3)
h	Hydrogel thickness (mm)
m	Hydrogel weight (g)
V_r	Volume ratio (mL/mL)
c_g, c_s	Mobile counter-ion concentration in the gel (g) and solution (s)
C_{M1}, C_{M2}	Molar concentration (M) of first ($M1$) and second ($M2$) network polymer
W_{M1}, W_{M2}	Weight fraction, Surface density (wt%) of first ($M1$) and second ($M2$) network polymer
X_{X1}, X_{X2}	Crosslinking degree (mol%) of first ($X1$) and second ($X2$) network
X_{I1}, X_{I2}	Initiator concentration (mol%) of first ($X1$) and second ($X2$) network
ΔC_q	Relative quantification cycles
$-\Delta\Delta C_q$	Relative mRNA expression

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1 General Introduction

1.1 Overview

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

A typical DN hydrogel owes its high strength and toughness to the contrasting architecture of the two interpenetrating networks: a rigid and brittle 1st network, acting as sacrificial bonds, and a soft and stretchable 2nd network as its interpenetrating counterpart, synthesized in a two-step polymerization approach¹³⁻¹⁶. To obtain the contrasting architecture, a DN hydrogel is usually made from a densely crosslinked polyelectrolyte as the 1st network and a sparsely crosslinked neutral polymer as the 2nd network. Such complex structures are generally challenging to analyze. Further it is problematic to analyze the DN hydrogel structure, because it is not clear if the surface differs from the bulk composition. First, in swollen state, the water molecules and hydrogen bonds absorb a large bandwidth of electromagnetic waves, rendering spectroscopy techniques ineffective¹⁷. Second, common electric potential characterization methods such as ζ -potential measurements, used to determine hydrogel charges, are not capable of analyzing a structure-related charge difference between the surface and bulk of the hydrogel¹⁸.

As the DN hydrogel was introduced investigations on its therapeutic potential and applicability in regenerative medicine were conducted by Yasuda et al. ¹⁹. They found that implanted DN hydrogels induced articular cartilage regeneration in large osteochondral defects by double network hydrogel implantation. Since then, multiple studies on chondrogenic re- and dedifferentiation on hydrogels have supported the assumption that double network hydrogels might be involved in pluripotency

expression and inducing stem cells ^{20–24}. However, a comprehensive characterization of the DN hydrogels surface and bulk properties is not yet established, to proof that mechanical or chemical hydrogel properties are guiding the stem cell induction.

In cell-based therapies and related *in vitro* stem cell expansion a key factor is the maintenance of pluripotency ²⁵. Evidence suggests that cell-substrate and cell-cell interactions are highly influential pluripotency regulation factors and depending on the deployed cultivation strategy either cell-substrate or cell-cell interactions are preferentially favored ^{26,27}. Extensive research has shown that the integrin-based adhesion gives human cells the capability to respond to the scaffolds stiffness over a length scale proportional to their size ^{28,29} but recent studies on membrane-based aggregation cell cultures using chitosan ³⁰, agarose ³¹ and polyacrylamide ^{32,33} presented evidence for substrate-based regulation of pluripotency in the non-adhesive regime as well. However, these results must be interpreted with caution, since limited comparability prohibits valid conclusions, due to the lack of adequate control samples or the inclusion of minimal amount of cell lines and substrates.

It is certainly challenging to design an experimental setup out of highly hydrated synthetic or biological polymeric scaffolds, where physicochemical parameters are individually tunable to ensure reliable comparisons ³⁴. Stiffness (elastic modulus, E) of soft matter, one such parameter, is determined by the density of polymer strands and their chemical structure. Therefore, degree of crosslinking changes polymer density, equilibrium water content and stiffness at once, demonstrating the coupling of the mechanical and chemical scaffold properties on a fundamental level. The cell-scaffold interaction is one of the most complex phenomena to study in biomedical engineering, therefore the establishment of a universal platform to compare adhesive with non-adhesive growth is essential and the DN hydrogel might be the platform that is able to combine these two cultivation types.

1.2 Outline of this Thesis

This study pursues one major goal in three steps. At first, the characterization of the surface and bulk chemical structure of the double network hydrogel to investigate if there is a difference between them. Then based on that, the regulation and quantification of chemical and mechanical hydrogel surface properties to synthesize a series of hydrogels where those properties are decoupled. Third, this decoupled hydrogel series are tested in stem cell expansion experiments to investigate how either chemical or mechanical DN hydrogel properties guide the pluripotency marker gene expression. Finally, this should contribute to the understanding of why double network hydrogels outperform single network hydrogels as stem cell expansion scaffolds.

Chapter 2 is a review on the fundamentals of hydrogels and strategies how to synthesize and characterize the double network hydrogels with the microelectrode technique. An overview on the usage of hydrogels as cell scaffolds is given and how mechanical and chemical hydrogel properties are tuned to affect cellular behavior.

Chapter 3 focused on the impact of the hydrogel synthesis mold on the surface and bulk hydrogel structure of double network hydrogels. Here it is demonstrated how the combination of infrared spectroscopy and the microelectrode technique gives new insights on the chemical structure and spatial distribution of the two polymer networks if the hydrogel is synthesized in a hydrophilic or hydrophobic mold.

Chapter 4 describes how extremely small amounts of monomers, not reacted in the first polymerization step affect the surface potential of the double network hydrogel. The hypothesis here was that this negatively charged residual monomers are incorporated in the second network via the second network polymerization step and therefore decrease the surface potential which might affect the stem cell morphology.

Chapter 5 applies the knowledge gained from the double network hydrogel surface characterization, so that a second network polymer layer is formed on top of the bulk material. It is

demonstrated how the thickness of this surface layer is freely adjustable from the micro- to the macro-scale. Further the effect of the presence of a second network surface layer on the stem cell morphology is investigated.

Chapter 6 investigates the how the double network hydrogel could be used to independently regulate mechanical or chemical hydrogel properties. This demonstrated for three kinds of double network hydrogels, which are deployed in a in vitro stem cell expansion assay. Here it was speculated whether the growth on hydrogel induced the pluripotent stem cell state. The stem cell induction was analyzed by monitoring pluripotency marker genes via RT-qPCR and GSEA analysis.

2 Background on hydrogels and cell scaffolds

2.1 Introduction

Simply, this works topic is to evaluate the interaction between double network hydrogel surfaces and human cells. As there are mechanical and chemical hydrogel surface properties, defined by the hydrogels structure and molecular composition, the introductory chapter focuses at first on the general concept of hydrogels, their fabrication and the double network hydrogels unique mechanical and chemical properties. Second, the use of hydrogels as cell scaffolds and literature is reviewed on the cell-substrate interactions based on the scaffolds surface energy, chemistry and mechanical performance.

2.2 Hydrogel and the double network concept

2.2.1 Hydrogel

A hydrogel is a branched and interconnected material, with a big water storage capacity. The absorption of water is facilitated by the hydrophilic functional groups of the hydrogel polymer strands. Because of the strong affinity of water to the hydrophilic groups the hydrogel swells, and the volume drastically increases. If the polymers are arranged in a three-dimensional mesh the polymer strand crosslinks lead to the formation of a material that cannot dissolve in water (**Figure 1A**). Even under mechanical load the water is kept within the hydrogel because it is attracted by the hydrophilic groups. The amount of water or solvent stored in a hydrogel is dependent on the polymer length and the chemical structure³⁵.

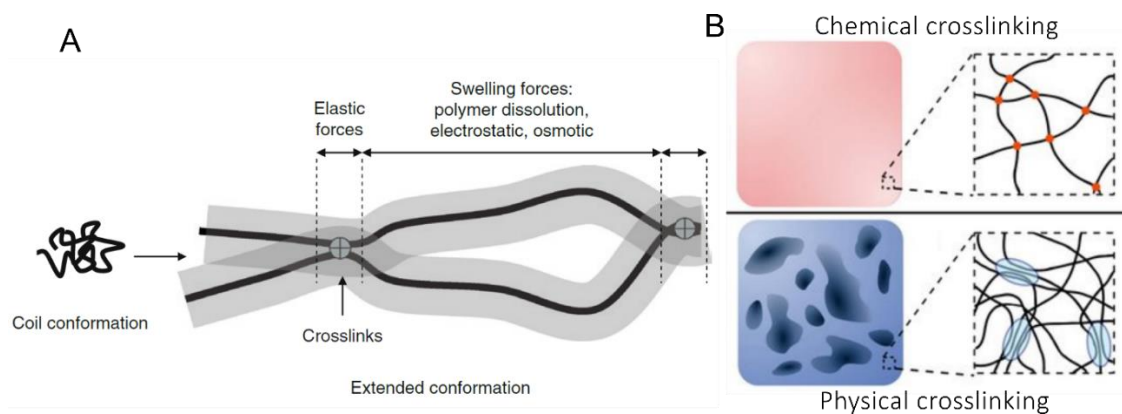


Figure 1: (A) The active forces within the hydrogel mesh that induce swelling and maintain the hydrogel shape (reproduced from literature³⁶). (B) A schematic drawing of chemically crosslinked and physically crosslinked hydrogel (reproduced from literature³⁷).

The hydrogel is synthesized via chemical or physical crosslinking (**Figure 1B**). The chemical crosslink forms irreversible covalent bonds via crosslinking molecules in the reaction mix, that connect two polymer chains in a chemical reaction. For the physical crosslinking a hydrogel is synthesized based on reversible interactions such as hydrogen bonds, ionic bonds, hydrophobic interactions and entanglement. Since hydrogels consist out of a solid and fluid phase, they have unique properties and are stimuli responsive materials. Dependent on the temperature, the solvent ionic strength or the type of solvent the polymer chains of the hydrogel might aggregated leading to phase separation. This collapse of structure changes physical hydrogel properties such as volume, stiffness and transparence (**Figure 2**). The solubility of the polymer in the water is described by the thermodynamic relationship below.

$$\Delta G_{mix} = RT [n_1 \ln v_1 + n_2 \ln v_2 + n_1 v_2 \chi_{12}] \quad (\text{Eq. 1})$$

This relationship established (**Eq. 1**) by Paul J. Flory and Maurice L. Huggins predicts how in polymer solution the Gibbs free energy of mixing changes (ΔG_{mix}) if a certain number of moles of polymers (n_1) of defined volume (v_1) are mixed with a solvent of defined number of moles (n_2) and volume fraction (v_2). The dimensionless parameter χ_{12} describes the difference in interaction energy between the solvent and the polymer compared to the interacting between the solvent molecules themselves. In the Flory-Huggins solution theory this polymer-solvent interaction parameter is empirically defined and describes how well a polymer is soluble in the solvent of choice. This consideration assumes that the polymer chains are of equal length and are much larger than the solvent molecules and that in the mixture the change of volume is constant ($\Delta V_{mix} = 0$). If the change in Gibbs free energy is positive ($\Delta G_{mix} > 0$) the solid and fluid phase separate³⁸. Because of this responsive behavior of hydrogels to solvent properties they find applications in tissue engineering, drug delivery, biosensors, microfluidics, as swelling agents and carrier material used in the biomedical pharmaceutical field and in agriculture and industry³⁶.

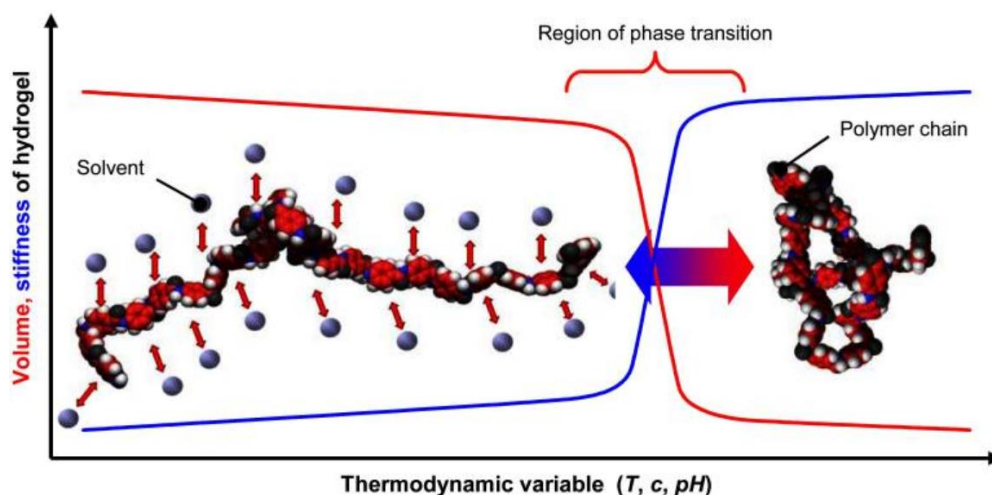


Figure 2: Phase transition behavior of polymer chain based on the thermodynamic equilibrium. How thermodynamic variables such as temperature (T), concentration (c) and pH affects the conformation of the polymer chains, which regulates hydrogel volume and stiffness (reproduced from literature³⁹).

2.2.2 Free radical photopolymerization

The free radical polymerization is used to synthesis hydrogels by binding together monomers through covalent bonds to oligomers and finally polymer chains. The radical polymerization is initiated by radicals, molecules with unpaired electrons which are for this reason very reactive. Precursors of this radicals, known as initiators, are added to the polymerization reaction mix. Such initiator molecules are activated by temperature, redox potential or exposure to light. In the radical photopolymerization the initiator molecule is activated by the exposure to light in the UV-VIS region. The free radical polymer chain growth includes three consecutive steps, the initiation, propagation and termination (**Figure 3**). First, when the photosensitive initiator (PI) is excited by the absorption of radiation energy it decays into two radicals ($R\bullet$). In the initial reaction the formed radical couples with a monomer (M) from solution to form an active monomer ($R(M)_n\bullet$). Originating from the active monomer the covalent bonding of monomers is possible. If a monomer has a terminal double bond like the acrylate group of acrylamides, a polymer chain reaction is initiated. The chain propagation is terminated if the reaction reaches a thermodynamic equilibrium state initiated by the concentration ratio between the reactants and product, or the addition of reaction inhibitors. Since the described three steps in the free radical polymerization process just synthesize single polymer chains the addition of crosslinkers are needed to synthesize a covalent bond hydrogel. These crosslinkers are molecules with e.g. two terminal

acrylate groups such as N,N'-methylenebisacrylamide (MBAAm), added to the reaction mix. MBAAm is a bifunctional co-monomer and, as it applies to the monomer, the free radicals associate with MBAAm, on each side one, to synthesize a polymer strand⁴⁰.

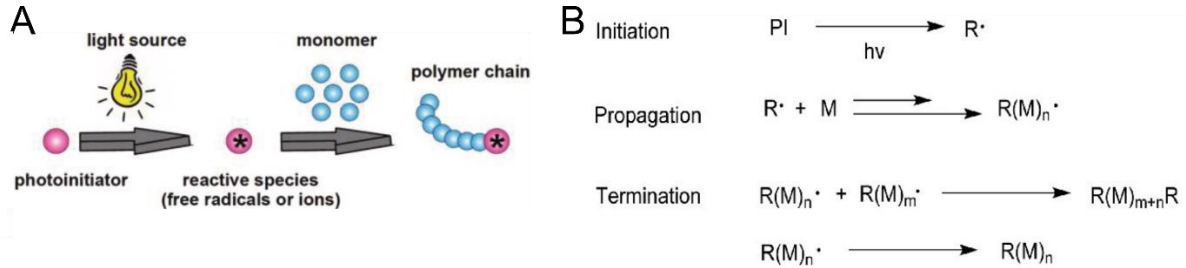


Figure 3: Free radical photopolymerization. (A) Schematic drawing how photoinitiation starts the polymerization of monomers in the reaction mix (reproduced from literature⁴⁰). (B) Scheme of the three consecutive photopolymerization steps the initiation, propagation and termination (reproduced from literature⁴¹).

The photoinitiator are classified by the number of radical pairs per photoinitiator molecule are generated. The bigger the number of radical pairs the faster the polymerization reaction rate is⁴². Initiators such as 4,4'-azobis-4-cyanopentanoic acid and 2,2'-azobisisobutyronitrile generate singlet radical pairs but α -ketoglutaric acid and β -ketoglutaric acid generate triplet radical pairs and are therefore more efficient⁴³. Atmospheric oxygen from the surrounding environment however could initiate or inhibit photopolymerization reactions. Since oxygen appears as O_2 and O_3 (ozone), the ozone is reactive and could start a chain reaction. Further, the initiator is consumed to form less reactive oxygen radicals that inhibit a fast and homogeneous polymerization reaction. For these reasons, the radical photopolymerization is performed in an oxygen free environment filled with inert gas such as argon⁴⁴.

2.2.3 Hydrogel swelling

After the polymerization process is finished a hydrogel in the so called as-prepared state is immersed in a solvent. The space that is occupied by the solvent within the hydrogel after the swelling process reaches equilibrium is determined by the volumetric degree of swelling or gravimetric solvent fraction.

$$Q = \left(\frac{h}{h_0}\right)^3 \quad (\text{Eq. 2})$$

(Eq. 2) The volumetric degree of swelling (Q) is determined by the thickness of the hydrogel in the as-prepared state (h_0) divided by the thickness in the swollen state (h).

$$W = \left(\frac{m_{wet} - m_{dry}}{m_{wet}} \right) \quad (\text{Eq. 3})$$

(Eq. 3) The gravimetric solvent fraction (W) determined by the mass of the hydrogel in wet or swollen state (m_{wet}) and after drying (m_{dry}) that removes the solvent from the hydrogel. The factor that regulates and affects the degree of swelling the most is the crosslinker density. The amount of crosslinker effectively incorporated in the polymerization process defines the average distance between two crosslinking points and therefore the expansion of the mesh in the solvent.

The swelling is facilitated by polymer-solvent interaction forces based on the hydrogel structure and the type of solvent. The structure of hydrogels is separated into five groups: non-ionic, cationic, anionic, ampholytic and hydrogels with hydrophilic backbones containing hydrophobic functional groups (Figure 4). Basically, a hydrogel would swell infinitely in a good solvent but that is prohibited by the elastic forces generated by the chemical and mechanical crosslinks. The hydrogel is fully swollen when the counteracting elastic forces from the crosslinks are equal to the swelling forces including the polymer-solvent interactions (polymer dissolution), electrostatic forces and the osmotic pressure³⁶ (Figure 1A).

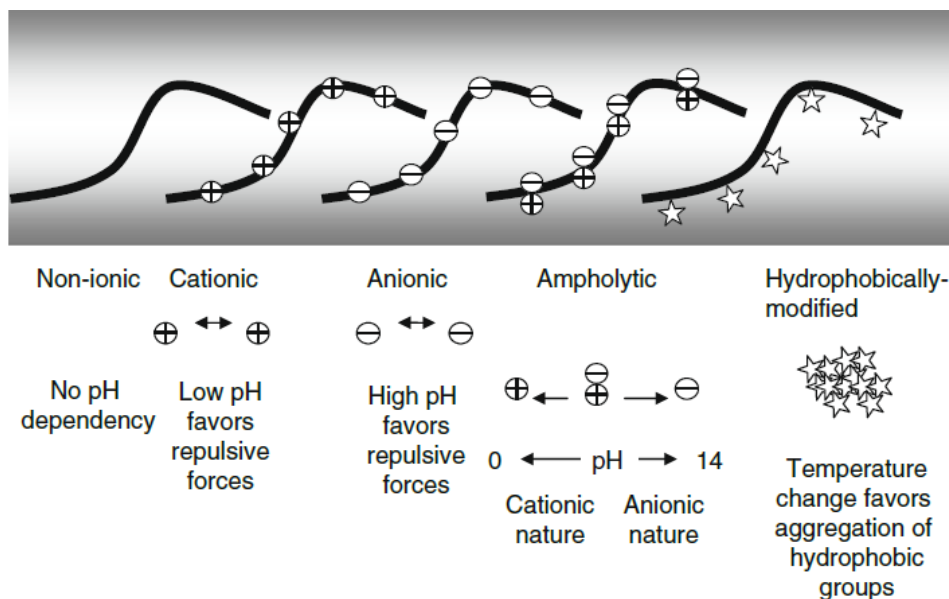


Figure 4: Types of hydrogel sidechains. The composition of the hydrogel side chains determines the swelling behavior (reproduced from literature³⁶).

The cationic or anionic hydrogels having positively or negatively charged functional groups and are known as polyelectrolytes (e.g. 2-acrylamido-2-methylpropanesulfonic acid) (PAMPS). The most dominant force driving swelling is the electrostatic repulsion of the like-charged groups in the hydrogel mesh. Among such charged groups are hydroxy groups (-OH), carboxy groups (-COOH), sulfoxides (-SO₂, -SO₃) and amides (e.g. acrylamide) (PAAm). The swelling properties of such hydrogels are highly affected by the strength of these ionic groups to attract ions from solution and the ions concentration in the solution. The ionic strength of the solution is regulated by the number of dissociated ions in the solution classified as co- and counterions. If the ionic groups on the polymer chain and the dissociated ions are like-charged, these dissociated ions are classified as co-ions. The opposite-charged dissociated ions are the counter-ions. When ionic polymers are immersed in a solution containing dissociated ions, ion pairing appears, the association of counterions from solution to the polymer functional groups, osmosis is generated. The bigger the concentration difference between counterions in the gel, associated through ion pairing, and in solution the bigger the generated osmotic pressure and the degree of swelling. However, if the concentration of counterions in solution is higher than in the gel phase, the hydrogel shrinks. Therefore, the ionic strength in solution can directly guide the degree of swelling and restricts the expansion of the hydrogel mesh⁴⁵.

The Crosslinking degree and type of crosslinker not only defines the equilibrium degree of swelling but also the elasticity of the hydrogel. Basically, if the crosslinking degree increases the elasticity increases as well because the distance between crosslinks is smaller which results in a smaller degree of swelling. Based on this relation, the synthesis of a hydrogel with a high water content and elastic modulus is prohibited. However, to meet such requirements a second polymer network is introduced in a highly swollen hydrogel, to reinforce the structure^{35,36}.

2.2.4 Double network hydrogel

Usually hydrogels are highly swollen and soft and therefore too weak for practical use in biomedical engineering or load-bearing applications. However, there is an exception the double network hydrogel.

Unlike most hydrogels which are brittle in their swollen state, the double network (DN) hydrogel retains high mechanical strength and toughness while having a water content of ~90 wt%.

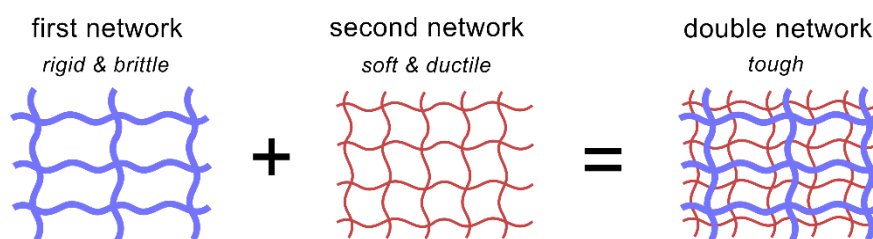


Figure 5: Double network hydrogel structure. The DN hydrogel is synthesized out of a rigid and brittle first network and a soft and ductile second network in a two-step polymerization.

The DN hydrogel has an interpenetrating structure of two separate hydrophilic polymer networks of contrasting mechanical properties and architecture (**Figure 5**). A rigid and brittle polyelectrolyte (e.g. PAMPS) that is highly crosslinked at a low molar concentration functions as the first network (1st nw). The second network (2nd nw) consists out of a soft and ductile, electrically neutral polymer (e.g. PAAm) that is loosely crosslinked, where the molar concentration is 20 – 30 times higher than that of the first network. The DN hydrogel is synthesized via a two-step sequential free-radical polymerization where in the first step the rigid first network is synthesized from monomers in the presence of a crosslinker and a photoinitiator. Second, the as-prepared first gel is immersed in an aqueous precursor solution containing the second network monomers, low amount of crosslinker and a photoinitiator. After the first gel reaches swelling equilibrium, the second network is polymerized in the presence of the first polyelectrolyte network. To fabricate a DN hydrogel the big discrepancy of molar concentration between 1st and 2nd network is achieved via the osmotic pressure driven, large swelling of the polyelectrolyte gel in the 2nd network precursor solution. Therefore, various types of electrolytic and electrically neutral monomers forming the 1st and 2nd network respectively, could be combined to synthesize mechanically strong and tough DN hydrogels.

Among the studied DN hydrogels, the one containing PAMPS and PAAm as 1st and 2nd network respectively shows outstanding mechanical performance. **Figure 6A** shows the compressive stress strain curves for PAMPS/PAAm DN hydrogel and the corresponding SN PAMPS and SN PAAm hydrogel. The single network hydrogels fracture at stresses below 1 MPa with a maximum compressive strain of

41 and 84 % for PAMPS and PAAm respectively. The DN hydrogel however, exceeds in both categories with a fracture stress of 17.2 MPa at a compressive strain of 92 %. As mentioned, for DN hydrogel the molar concentration ratio between the two networks is important, as it affects the fracture stress. At the optimized polymer network composition of PAMPS/PAAm of 1/20 the mechanical performance reaches its peak at 17.2 MPa (**Figure 6B**). However, if the PAAm concentration increases or drops the fracture stress and the elastic modulus decrease (**Figure 6C**).

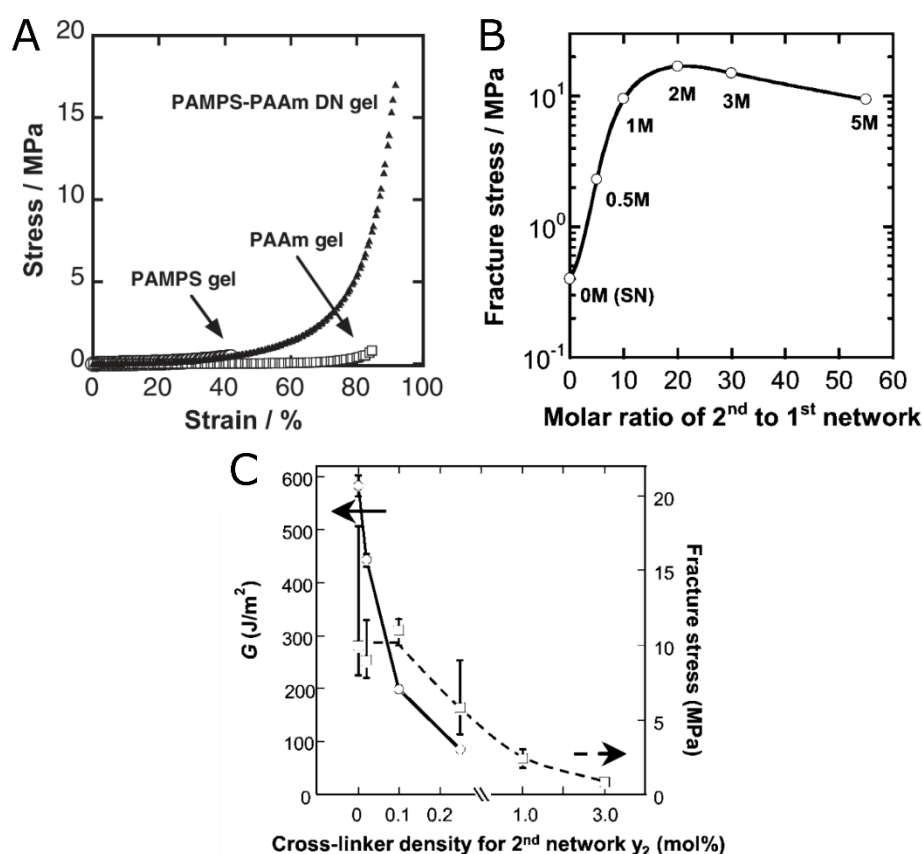


Figure 6: Mechanical performance of DN hydrogel under compression. (A) The stress-strain curve of PAMPS, PAAm and the corresponding DN hydrogel. (B) The relation fracture stress of PAMPS/PAAm DN hydrogel to its 1st to 2nd network ratio. Figure (A) and (B) are from reference Gong et al⁴⁶. (C) The effect of 2nd network crosslinking degree on the fracture energy and the fracture stress of PAMPS/PAAm DN hydrogels (reproduced from literature⁴⁷).

The fracture mechanism of DN hydrogels differ drastically from single network hydrogels. To single network hydrogels applies that under mechanical load, energy is stored elastically until energy is dissipated by breaking the chemical crosslinks of polymer chains only located at the crack tip and fracture appears. In such a case there is no hysteresis is observable in the stress strain curve. However, the fracture behavior of DN hydrogels while uniaxial elongation is much more complex (**Figure 7**). In

the first loading curve a velocity independent necking process is observed, where the stress reaches a plateau while the hydrogel is stretched more and more. This is because of an internal fracture of the first brittle PAMPS network breaks up into small clusters. This sacrificial bonds from the first network dissipate a huge amount of energy that is not found in the second loading curve. As the necking propagates the damage done to the first network structure increases and contributes to the DN hydrogel toughness. As a study on the necking phenomena showed that among other factors such as chain pulling and disentanglement the necking phenomena remains as the dominant factor since the mechanical performance highly tensile speed independent⁴⁸.

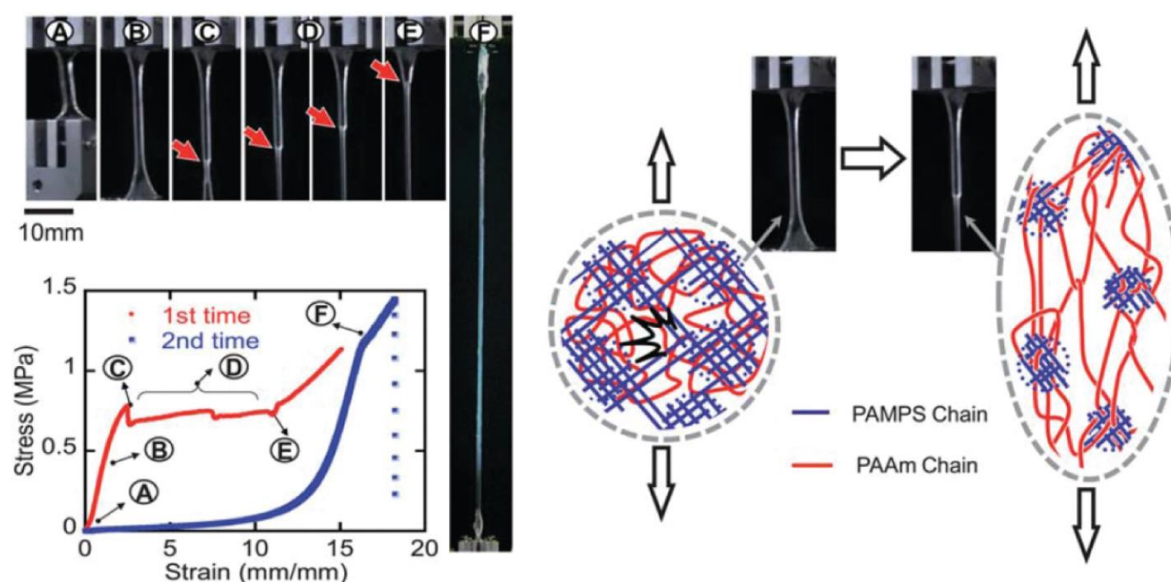


Figure 7: Necking phenomena of DN hydrogel and illustration on structural change. The two-time loading of the PAMPS/PAAm DN hydrogel and macroscopic images visualize the elongation, necking and yielding of DN hydrogels with huge hysteresis observable between the 1st and 2nd loading curve. (red arrows indicate the necking front, Enumeration of pictures correspond to that in the stress–strain curves). The illustration of the DN polymer structure before and after necking, where the 1st network is already fractured (reproduced from literature^{48,49}).

After the toughening mechanism was initially described, follow-up development on the DN hydrogel concept demonstrated the wide applicability of this system. Looking back on DN hydrogel literature of the last decade, remarkable publications demonstrated that the DN hydrogel was successfully deployed to induce cartilage regeneration^{19,20} or used as anti-adhesive membrane in surgery¹¹. Other publications focused on the fabrication of mechanically strong DN hydrogels out of various molecules^{12,50,51}, fabric-hydrogel composites^{52,53} and on self-toughening DN hydrogels¹.

2.2.5 The microelectrode technique (MET)

The microelectrode technique is a lately developed method to analyze the chemical structure of a hydrogel by studying the electric potential in the hydrogel depth profile of polyelectrolytes⁵⁴. The polymer network of a polyelectrolyte carries fixed macro ions surrounded by small ions, mobile in aqueous solution surrounding the polymer strands (**Figure 8**). The mobile ions, co- and counter-ions are present in the hydrogel. Because of the nanoscale distribution of these ions inside the hydrogel, a potential variation along the mesh size is present. However, the observation on a bigger scale, larger than the mesh size, the potential appears to be constant in homogeneous gels. If a heterogeneous hydrogel structure occurs the distribution of fixed macro ions changes and alters the electric potential⁵⁵.

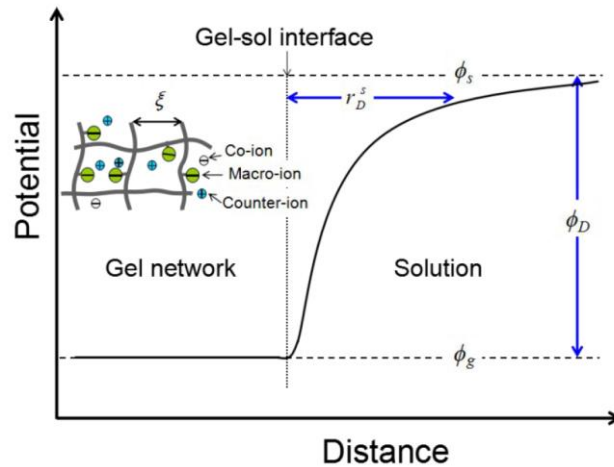


Figure 8: Electric potential at the solution gel interface of a polyelectrolyte. Since small positively charged counter-ions are attracted by the negative macroions the concentration between counter-ions in the gel and the surrounding bath solution is disproportional. Because of that concentration difference, an electric double layer at the solution-gel interface is formed with a certain electric potential difference. The Donnan potential (ϕ_D) is defined as the difference between the gel potential (ϕ_g) and the bath solution (ϕ_s) at thermodynamic equilibrium (reproduced from literature⁵⁴).

The Donnan potential, describes the differences in electric potential between hydrogel and the bath solution. The concentration difference of small mobile ions between gel and solution, a result of the entropy-driven ion migration alongside the gel-solution interface, forms an electric double layer at the interface. This happens because the fixed ions attract and repel co-ions and counter-ions respectively through electrostatic interactions, to keep the hydrogel electrically neutral⁵⁶. The thermodynamic equilibrium state where the electrostatic interactions the diffusion of mobile ions rendered the

hydrogel electrically neutral is also known as the Donnan equilibrium, which generated large osmotic pressure that leads to the swelling polyelectrolyte hydrogels in water and solutions with low ionic strength⁵⁵.

Previously methods were only capable to indirectly detect the Donnan potential via investigations on the osmotic pressure, the electric conductance and the detection of proton concentrations in the hydrogels by fluorescent indicators. Another method the zeta potential method, detects the hydrogel surface charge but is not capable to analyze the hydrogel bulk potential^{57,58}.

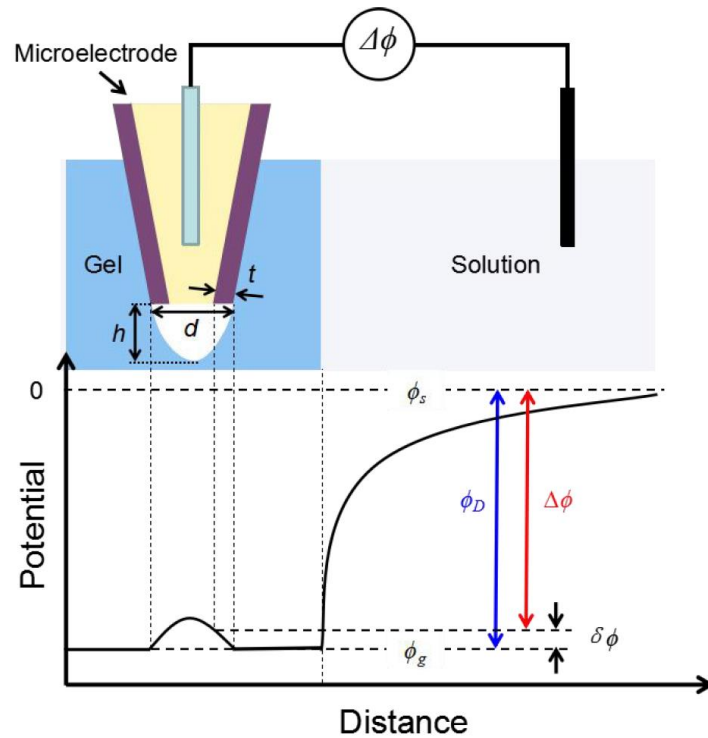


Figure 9: Geometry and potential at the microelectrode tip. The measured potential between working and reference electrode ($\Delta\phi$) is smaller than the theoretical Donnan potential (ϕ_D) because a potential loss is contributed to the wall thickness (t) microelectrode. This is valid if the wall thickness is smaller than the hydrogel fracture length (h), (reproduced from literature⁵⁴).

Using electrodes or microelectrodes is widely applied to evaluate physicochemical properties of solutions such as pH or impedance. The detection of neuronal signal transduction of living cells via electric signals was studied using electrode probes as well⁵⁹. For the microelectrode technique it is important to minimize the probe diameter and the probe wall thickness (**Figure 9**). Both parameters if they are too big, affect the potential drop at the electrode tip. Therefore, the electrode is inserted into a glass capillary that is fabricated via a glass capillary puller. With that it is possible to fabricate glass

capillary tips with a wall thickness and inner diameter down to ~ 20 nm and ~ 60 nm respectively. It was shown that an inner diameter smaller than 200 nm is necessary to measure spatial electric potential distributions inside the hydrogel. Further the nanoscale capillary diameter reduces the amount of reference solution into the bath solution.

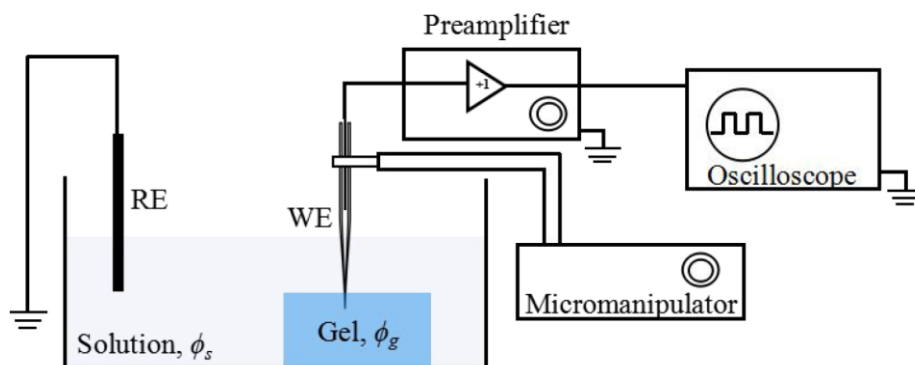


Figure 10: The experimental setup of the microelectrode technique. The movement of the working microelectrode (WE) is controlled by the micromanipulator and the signal transmitted from the reference electrode (RE) is recorded on the oscilloscope after preamplification (reproduced from literature⁵⁴).

The experimental setup of the MET is adopted from a previously described setup to record the electric potential of polymer solutions using a reference (RE) and working electrode (WE), (**Figure 10**)⁶⁰. Here it is possible to adjust the position of the glass capillary containing the working electrode, to penetrate the hydrogel at defined velocity. The signal of electric potential generated between the two electrodes is pre-amplified and record via an oscilloscope. With this method it is possible to measure an electric potential that is very close to the theoretically predicted Donnan potential of polyelectrolyte hydrogels in solutions of weak ionic strength. Since this methods resolution allows the detection of spatial electric potential distributions it should be possible to detect the separation of 1st and 2nd network at the double network hydrogel surface area.

2.3 Hydrogel utilized as cell scaffolds

2.3.1 History and development of cell scaffolds

When investigators firstly figured out a way to culture living cells on glass and later polystyrene dishes, in vitro cell culture was established and by that standardized. Widely accepted as cell culture substrate, polystyrene was deployed and improved the notoriously weak performance and reproducibility of cell

culture studies⁶¹. Decades after however, the same cell culture substrate is still in use while knowledge on cell-substrate interaction has grown. These findings established a new understanding which indicates that dense hydrophobic polymer mesh works such as polystyrene do not properly resemble the living cells physiological environment⁶². Furthermore, in vitro cell culture studies in preclinical trials for drug discovery showed primarily good results and reproducibility but failed in the clinical trial. In such cases, medicinal compounds were declared ineffective when a follow-up in vivo test after in vitro culture failed, showing that cellular phenomena observed on polystyrene dishes are a poor way of understanding biological processes^{63,64}. In parallel the development on soft matrices such as fibronectin⁶⁵, collagen⁶⁶ and Matrigel⁶⁷, an extracellular protein mix, succeeded in the maintenance of human tissue cells which were at that point considered as non-culturable. In the mean while as well agarose and acrylamide based soft matter was deployed in fractioning and isolation of genome, transcriptome and proteome fragments via gel electrophoresis which is the golden standard for cell analytics up until now⁶⁸. As hydrogels were introduced in the biological field their downsides became obvious. The usability was limited based on the weak mechanical performance of highly hydrated polymers and related problems including hydrogel fracture while handling and sterilization⁶⁹. Nonetheless, since the early 90's the scientific community reported on a huge variety of innovative 2D and 3D hydrogel scaffolds are available for mimicking specific physiological environments of human and animal tissue^{62,70,71}. This led to a whole new understanding on how mechanical and chemical signals from the extracellular matrices are processed by living cells and on how the cell scaffolds are applied to guide cell morphology, motility and differentiation which is essential for regenerative medicine, cancer therapy and fundamental cell biological research⁷²⁻⁷⁵. Nowadays, the scope of research is the fabrication of complex biomaterials to mimic interface or gradient structures found in human tissue for the reproducible in vitro investigation of biological processes^{76,77}.

2.3.2 How hydrogel relates to human tissue

Roughly speaking the bulk material of human tissue consists of tissue specific living cells and the extracellular matrix which functions as a supporting grid that facilitates the motility of cells. The compounds of the extracellular matrix are deposited by the cells themselves, allowing the cells to shape their surroundings. However, just to a certain extent. In reverse, the extracellular matrix composition and related physicochemical properties and scaffold geometries of the extracellular matrix grid guides the cells properties⁷⁸. It tends to be a challenge between the feedback loop of the cell shaping the surroundings and the extracellular matrix guiding the cell. In this dynamic balance the stronger feedback loop finally determines the overall properties of this heterogeneous tissue. In reverse, by fabricating scaffolds with predefined rigidity, dimensionality, topology and adhesiveness it is possible to guide cell behavior useful in biomedical applications (**Figure 11**)⁷⁸. Materials mimicking structures found in human tissue are classified as biomaterials. Since hydrogels from synthetic and biopolymers are highly practical to reproduce environmental conditions in human tissue. Similar to human tissue, hydrogels have a high water content.

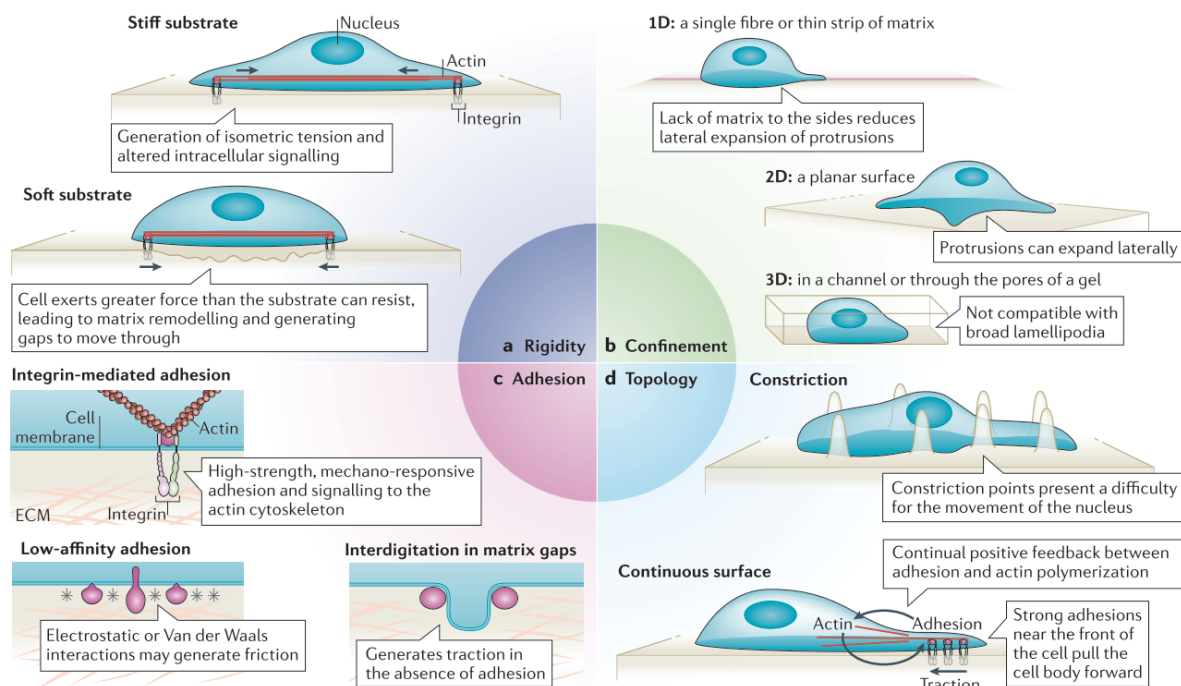


Figure 11: The interaction between cells and scaffolds that guide cellular behavior. A summary on the how the scaffold properties, mimicking the natural, cellular environment, such as rigidity, confinement, adhesion and topology affect the cellular response (reproduced from literature⁷⁸).

By simple modification of the hydrogels side chain structures or crosslinking densities the scaffold elasticity is regulated in the biological relevant elasticity from $\sim 10^3$ Pa to $\sim 10^6$ Pa for neuron and bone tissue respectively⁷⁴. Further, functional groups containing integrin recognition sequences to facilitate cell attachment⁷⁹. Especially, physical gels out of collagen, alginate and cellulose have the potential to form hierarchical fibrous structures found in human tissue such as the tendon^{80,81}.

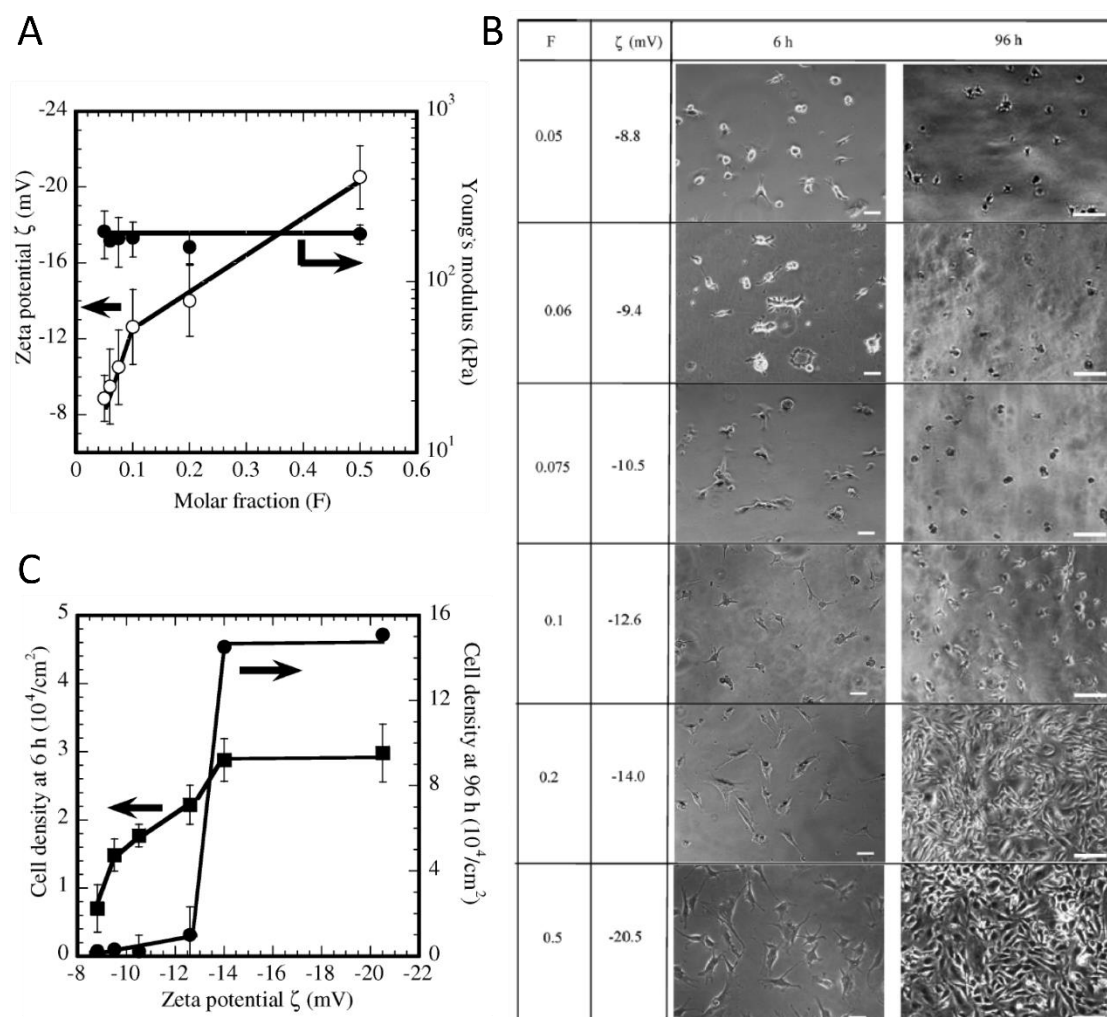


Figure 12: Critical surface charge for long-term cell adhesion. (A) The comparison of the zeta potential and the stiffness (Young's modulus) to the molar fraction of negatively charged NaSS (F) monomer in the reaction mix containing neutral charged DMA. (B) Cell morphology of bovine fetal aorta endothelial cells on NaSS-co-DMAAm polymer obtained after 6 and 96 hours of incubation by phase-contrast microscopy (scale bar represents 50 μ m). (C) The cell density after 6 and 96 hours in relation to the hydrogel zeta potential (reproduced from literature⁸²).

Using hydrogels has the benefit to polymerize the scaffold after the addition of the cells which results in the fabrication of a three-dimensional scaffold. It was previously identified that cellular response of cells to growing in 2D or 3D highly differ in terms of gene expression⁸³. For mimicking human tissue,

the hydrogel chemical structure needs to be modified. However, that limits regulation possibilities as it affects multiple properties at once such as wettability, elasticity and charge⁸⁴. Therefore, methods to decouple these chemical and mechanical hydrogel properties are developed^{34,85,86}. As shown in **Figure 12A**, the addition of small fractions of negative charged monomers to the reaction mix of neutral charged monomer resulted in various hydrogels that have zeta potentials, a parameter related to surface charge, that change from -8 to -20 mV, while the stiffness (kPa) is kept constant⁸². This modification of the hydrogel surface potential leads to the control over the cell attachment. After just 6 hours cells attached to all hydrogels even if the zeta potential changed, however after 4 days of incubation cell attachment only increased on hydrogels with a potential more negative than -12.6 mV (**Figure 12, B-C**). This reveals that for cells there is a critical surface charge that regulates cell adhesion. However, it needs to be noted that this threshold changes between the deployed cell lines⁸⁷.

2.3.3 Application of double network hydrogel in tissue engineering

As society grows older, the wear of tissues such as cartilage, that not spontaneously regenerate, is a problem to overcome in nowadays medical treatment. To find tough biomaterials, that are compatible to this niche environment at the bone-cartilage interface, is of high interest. For such implant the biocompatibility, stability and degradability are important factors to consider⁸⁸. For such application the PAMPS/PDMA DN hydrogel was implanted into subcutaneous tissue of rabbits and it was found that after 1 week after surgery the tissue was mildly inflamed. The follow-up friction and stress testes concluded that from a mechanical point of view this DN hydrogel is applicable for cartilage replacement⁸⁹. When PAMPS/PDMA hydrogel was implanted in a surgically created osteochondral defect as shown in **Figure 13A** the regeneration of the wound was documented in comparison to a defect without DN hydrogel¹⁹. The osteochondral defect cross-section was analyzed weekly and the stain with safranin-O for cartilage tissue and reveal that after 2 weeks the cartilage tissue started to regenerate and that after 4 weeks the whole area of the implant was covered with cartilage like tissue (**Figure 13B**). In comparison, the control developed no cartilage-like tissue after 4 weeks. The RT-qPCR analysis on the newly formed tissue of the control and the DN hydrogel in relation to actual cartilage

tissue was performed. The results showed that the tissue formed on DN hydrogel (T) expresses genes for cartilage-like tissue such as Type 2 Collagen, Aggrecan and SOX9, though the expression is not as high as in regular cartilage tissue (**Figure 13C**). This demonstrates the implantation of DN hydrogel induced spontaneous cartilage regeneration.

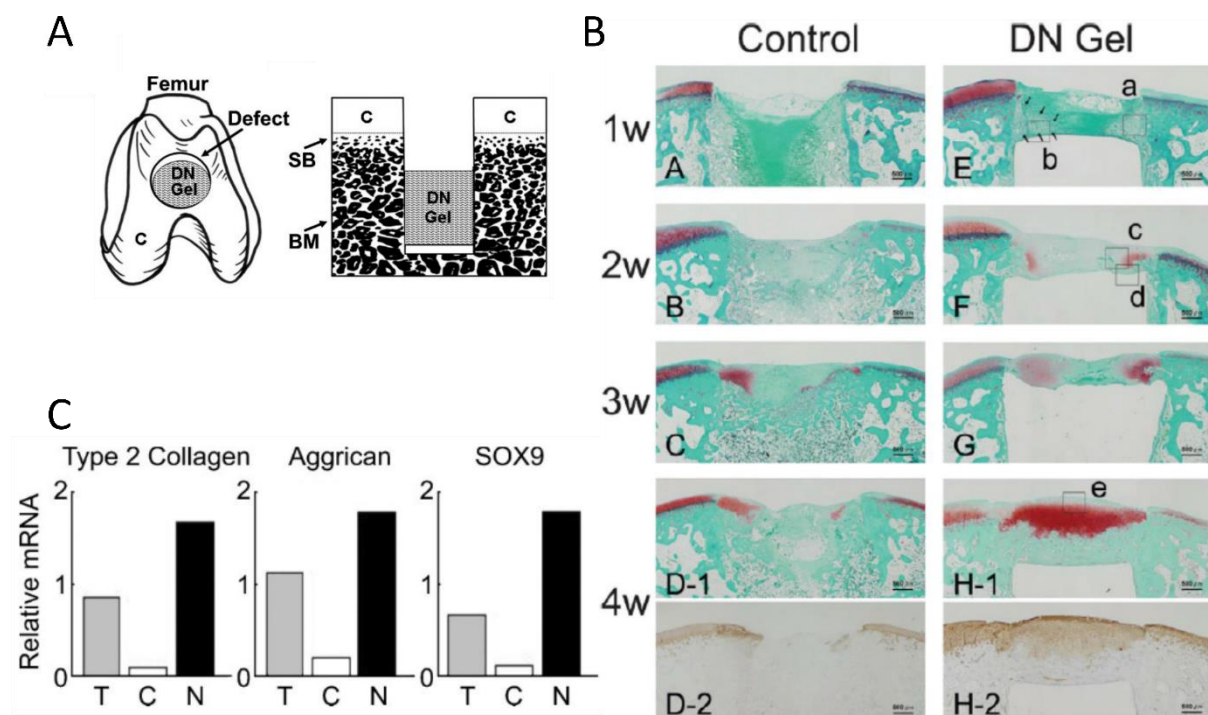


Figure 13: Spontaneous cartilage regeneration on DN hydrogel. (A) Schematic illustration of the implantation of DN hydrogel into the osteochondral defect at the femoral groove (C: Cartilage, SB: subchondral bone, BM: bone marrow). (B) Micro graphs of the defect cross-sections stained with Safranin-O (a - e are highlights on the regeneration zone). (C) The RT-qPCR results on the DN hydrogel (T), Control (C) and regular cartilage (N) tissue after 4 weeks. (reproduced from literature¹⁹).

The bonding between implant and tissue is of importance to increase the stability of region over time. However, the PAMPS/PDMA hydrogel does just weakly bond to the surrounding bone tissue. Therefore, the surface of the DN hydrogel was improved by the growth of hydroxyapatite crystals at its surface⁴ (**Figure 14A**). Hydroxyapatite is the mineral present in bones, that give the bone tissue its characteristic solid structure. After the hydroxyapatite coated PAMPS/PDMA hydrogel (Hap/DN) was implanted in an osteochondral defect of 4.3 mm diameter (**Figure 14B**)¹⁰. After 1 and for weeks the bonding strength between bone and regular DN or Hap/DN hydrogel was tested by a push out test with an indenter (**Figure 14C**). It was shown that after 1 week the DN and Hap/DN hydrogels did not yet bonded to the bone (**Figure 14D**). However, after 4 weeks the bonding strength only for the

Hap/DN increased up to 4 ON. This fosters the assumption that the hydroxyapatite coating led to the diffusion of the minerals and the increased bonding strength. These studies have shown that DN hydrogels might play an important role in tissue regeneration which implies that there must be an interaction present between stem cells that are induced by the hydrogel to form the cartilage tissue. Such pluripotent stem cells present at the bone-cartilage niche are capable to induce the regeneration of various tissues in the body via differentiation^{83,90}. However, an evaluation that relates the growth and induction of pluripotent stem cells to DN hydrogels chemical or mechanical properties has not yet been reported.

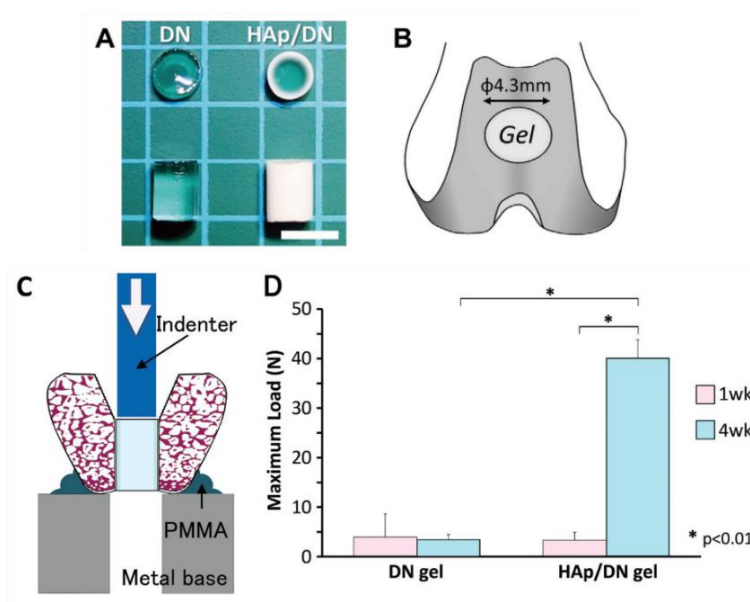


Figure 14: Improved hydroxyapatite DN hydrogels. (A) The cylindrical shape of DN and Hap/DN hydrogel before implantation (the scale bar represents of 5 mm). (B) Illustration of the implantation area at the femoral groove. (A and B are reproduced from literature¹⁰). (C) Illustration of the bonding-strength test via pull-out with an cylindric indenter of diameter 3.7 mm (PMMA: poly(methyl methacrylate)). (D) Measured bonding strength between bone and hydrogel after 1 and 4 weeks (C and D are reproduced from literature⁴).

3 Characterization of the surface-to-bulk transition of double network hydrogels

3.1 Introduction

The first objective of this thesis is to characterize the chemical and physical properties of double network hydrogels. The DN hydrogel is usually made from a densely crosslinked polyelectrolyte as the 1st network and a weakly crosslinked neutral polymer as the contrasting 2nd network. Driven by the high osmotic pressure of the counter-ions, the polyelectrolyte network substantially swells in the aqueous precursor solution of the 2nd network, reaching a rigid and brittle state, while the 2nd network, formed in the presence of the first network, can hardly swell due to the constrain of the already highly swollen first network, reaching a soft and stretchable state. Hydrogels made from polyanions such as poly(acrylic acid), poly(sodium p-styrene sulfonate), poly(2-acrylamido-2-methyl-propane sulfonic acid) (PAMPS) and PAMPS – sodium salt, have shown many unique properties, including low sliding friction on glass substrates in water, the adsorption of proteins and platelets, known as biofouling, and cell attachment^{20,91,92}. However, after introduction of the 2nd neutral network in such polyelectrolyte hydrogels to create a DN hydrogel, these properties change remarkably.

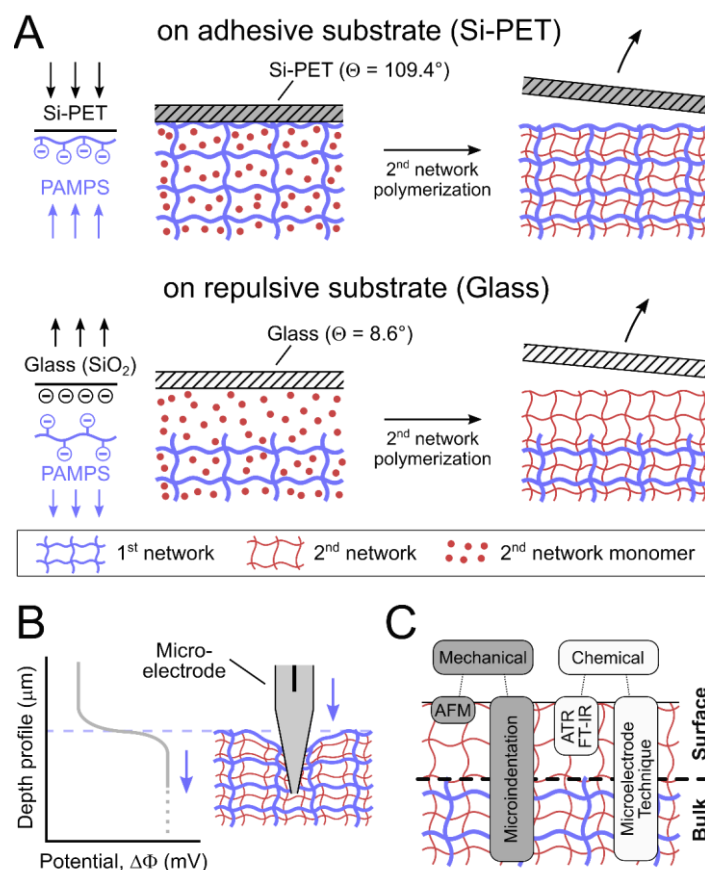
In the case of DN hydrogels, it could be argued that, compared to the corresponding single network (SN) polyelectrolyte hydrogel, the concentration of the electrically neutral 2nd network, is already high enough to weaken the surface charge and alter the surface properties^{87,93,94}. Another reason might relate to the two-step synthesis approach and the interactions between the substrate material and the 1st network hydrogel⁹⁵. I consider that the surface of the DN hydrogels are sensitive to the two-step polymerization approach and the interaction between the 1st network and the wall of the molding substrate used to synthesize the 2nd network. In the case of a polyelectrolyte hydrogel, at the hydrogel-solution interface, an electric double layer is formed based on the balance of attraction of mobile counter-ions and repulsion of mobile co-ions to the immobile ions of the polyelectrolyte network. As a result, there is an electric potential difference between the polyelectrolyte hydrogel and the solution^{96,97}. The intensity of the electric potential of the hydrogel in relation to the solution depends on the ionic strength of the solution and the immobile charge density of the hydrogel⁹⁸. Such an

electric double layer is the origin of repulsion between two like-charged polyelectrolyte hydrogels. When two like-charged surfaces are forced to approach each other, an osmotic repulsion is generated between them to avoid an overlap of the electric double layers of the two surfaces and a liquid film is formed at the interface⁹⁹. Due to the existence of the liquid film as lubricant, two like-charged polyelectrolyte hydrogels show low sliding friction under water^{100,101}.

The above results suggest that the surface of a DN hydrogel could be regulated by the molding substrate used for synthesizing the 2nd network. When a polyanion is used as the 1st network and a negatively charged substrate is used as mold, a thin hydrogel layer of the 2nd network should be formed on the surface of the DN hydrogel. In contrast, if a hydrophobic mold is used, to which the 1st network is adhesive, no thin hydrogel layer of the 2nd network will be formed on the DN gel surface. In this work, I synthesized DN hydrogels from PAMPS as the 1st network and polyacrylamide (PAAm) as the 2nd network in a modified two-step synthesis approach. To regulate the surface-bulk transition I used substrates of low (silicone coated polyethylene, Si-PET) and high (borosilicate glass substrate) surface energy in the 2nd network polymerization step (**Scheme 1A**). On the low surface energy substrate, I predict the adhesion of the 1st network to the substrate through hydrophobic interactions resulting in a DN hydrogel (DN/Si-PET) with 1st and 2nd network present at the surface. On the borosilicate glass mold that also carries negative charges on its surface, however, I assume that a PAAm layer forms (DN/Glass) because of the electrostatic repulsion between the PAMPS and the glass. As the thickness of the liquid layer at the repulsive interface is determined by the balance of the ionic osmotic pressure and the normal pressure applied, I tried to regulate the surface layer thickness through applying normal compression to the glass and PAMPS hydrogel in the 2nd network polymerization (DN/Glass-C, **Figure 18A**).

Here in this chapter I deployed attenuated total reflectance Fourier-transform infrared spectroscopy (ATR FT-IR) and the microelectrode technique (**Scheme 1A**) to characterize the surface and bulk structure of the DN hydrogels. ATR FT-IR provides surface structure information while the microelectrode technique is a highly sensitive method giving a depth profile with a low spatial

resolution of the microelectrode diameter (~ 200 nm). Using these two complementary methods, the characterization of the surface layer structure and the hydrogel surface-bulk transition should be investigated (**Scheme 1C**).



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

3.2 Experimental section

3.2.1 Materials

The 1st network monomer 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Toagosei Co., Ltd., Japan) was used as received. The 2nd network monomer acrylamide (AAm), crosslinker N,N'-methylenebis(acrylamide) (MBAA) and initiator 2-oxoglutaric acid were used as obtained from Wako Pure Chemical Industries, Ltd., Japan. The molding surfaces for the 2nd network polymerization, such

as borosilicate glass substrate (Asahi Glass Co., Ltd., Japan), were heat treated at 500 °C for 2 hours prior to use and silicone coated polyethylene film (Si-PET), SP-PET-03-38-Bu (Mitsui Chemicals Tohcello. Inc., Japan) was used as purchased.

3.2.2 Surface energy of molding substrates

To characterize the surface energy of the molding substrates used in the 2nd network polymerization step, the static water contact angles (Θ) of the glass substrate and the Si-PET film were measured with double distilled water (ddH₂O, droplet volume 2 μ L) on a Drop Master 300 (Kyowa Interface Science Co., Ltd.). The contact angle images are found in **Figure 16**.

3.2.3 Single and double network hydrogel synthesis

The DN hydrogel synthesis was modified from Gong et.al⁴⁶ and sample codes and formulations are found in **Table 1**. Briefly, in the first step, aqueous precursor solutions of AMPS monomer (C_{M1}), crosslinker MBAA (X_{X1}) and initiator 2-oxoglutaric acid (X_{I1}) were poured into glass molds with a spacer thickness of 0.5 to 2 mm (h_0) and free-radical polymerization was initiated by irradiated of 365 nm UV light in argon atmosphere (2.5 mbar) for 8 hours to obtain 1st network PAMPS hydrogels. This first polymerization process was followed by 3-day immersion of the freshly synthesized hydrogels in the 2nd network precursor solution composed of AAm monomer (C_{M2}), crosslinker MBAA (X_{X2}) and initiator 2-oxoglutaric acid (X_{I2}). In the second step, the PAMPS hydrogels soaked in the 2nd network precursor solution, were sandwiched between glass plates (hereafter referred as DN/Glass) or Si-PET film (referred as DN/Si-PET) and compressed by 0.1 – 0.3 kPa. For the DN sample group DN/Glass-C, the PAMPS hydrogels were sandwiched between glass plates and compressed by 50 kPa (**Figure 18A**). The hydrogel-mold assemblies were incubation in the argon atmosphere for 1 hour, and the 2nd network was polymerized within the PAMPS network by UV irradiation for 8 hours.

Single network (SN) hydrogels corresponding to the 1st or 2nd network were synthesized in a similar manner to the 1st network polymerization of DN hydrogels from aqueous solutions of either AMPS or AAm monomer with various crosslinker densities of MBAA. Prior to hydrogel characterization, the

synthesized SN and DN hydrogels were equilibrated in double distilled water (ddH₂O) for at least for 7 days.

Table 1: Hydrogel sample codes and formulations Chapter 3 and 4

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

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

3.2.4 Hydrogel swelling and polymer weight fraction

The bulk volumetric swelling degree of PAMPS hydrogels, $Q = (h/h_0)^3$, was determined from the thickness of PAMPS hydrogels swollen in double distilled water (ddH₂O) or 2nd network precursor solution (h), and that in the as-prepared-state (h_0) after polymerization. The polymer weight fraction, W_{M1} and W_{M2} (wt%) of SN hydrogel was determined by the gravimetric wet (m)/dry (m_0) ratio ($\times 100$) of hydrogel equilibrated in ddH₂O (m), and after vacuum oven drying procedure at 75°C and reduced pressure of 1 bar for at least 2 days (m_0).

3.2.5 Polymer surface density via ATR/FT-IR

To analyze polymer surface density of the 1st and 2nd network at the SN and DN hydrogel surface, attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR) on a FT-IR 6600 spectrometer (Jasco, Japan) with diamond prism was performed. Prior to analysis, hydrogel discs were immersed in D₂O for 12 h and hydrogel spectra were measured in full contact with the prism for wavenumbers of 1800 – 800 cm⁻¹, at 16x accumulation and resolution of 2 cm⁻¹. Absorbance maxima¹⁰², (A_{max}) of the characteristic sulfonic group peak ($\nu_{S=O}$) of PAMPS at 1042 cm⁻¹, and carbonyl group peak ($\nu_{C=O}$) for PAAm at 1640 cm⁻¹ were determined using the manufacturers software. Since infrared absorption peak height resembles polymer surface density, a calibration curve that relates IR absorption peak height and polymer weight fraction of SN hydrogel allows the detection of 1st and 2nd network at the DN hydrogel surface (**Figure 20**). Since the gels slightly swelled in deuterium oxide compared to water, the relative swelling ratio (Q_r) was used as a correction factor for the polymer surface density calibration. The $Q_r = (h_{H2O}/h_{D2O})^3$ was determined by the hydrogel thickness in ddH₂O

(h_{H_2O}) relative to thickness in deuterium oxide (h_{D_2O}), (**Figure 20**). Polymer surface densities were expressed as weight fractions and as PAMPS to PAAm molar concentration ratio, C_{M1}/C_{M2} (M/M).

3.2.6 Depth profiling via microelectrode technique

The surface layer thickness (t) at which the hydrogel structure transitions from the surface to bulk was evaluated by a microelectrode insertion technique previously described⁵⁴. Here the thickness is based on the displacement of the microelectrode moving at a constant speed and the shift in electric potential ($\Delta\Phi$) due to the relative change in the local small ion concentration. Equilibrated in 10^{-4} M NaCl solution, hydrogels were probed with a microelectrode made from a glass capillary with a fiber filament (outer diameter = 180 nm, inner diameter = 128 nm) fabricated with a P-1000 capillary puller (Sutter Instrument Co., U.S.), at an insertion speed of $0.5 - 2 \mu\text{m/s}$. Firstly the surface electric potentials ($\Delta\Phi_s$) were estimated from the 1st derivative of the electric potential curves and secondly the peak distance measured from the first valley to the last hill of the 2nd derivative was used to estimate the surface layer thickness (t) of hydrogels (**Figure 17**). The 1st and 2nd derivative curves were processed via smoothing and y-axis scaling in Origin (OriginLab Corp.) software. The Debye length of the bath solution (10^{-4} M NaCl) is 30.4 nm ⁵⁴.

3.2.7 Mechanical characterization of hydrogels

The surface elastic modulus (E_{Surface}) was tested on an atomic force microscope (AFM, Nanowizard 4, Bruker, U.S.) mounted to an inverted microscope (Nikon Eclipse TE 300, Japan) in aqueous conditions at 25°C . The spring constants of $0.079 - 0.083 \text{ N/m}$ were obtained by thermal tuning prior to each 4×4 force-distance curve array in force mapping-mode on the AFM (QITM - mode) for the used pyramid shaped cantilever (A triangular, MLCT series, Bruker, U.S.). The peak force setpoint was normalized to 0.2 nN . The bulk elastic modulus (E_{Bulk}) was determined by indentation method using a tensile-compressive mechanical tester (Autograph AG-X, Shimadzu Co., Japan) in air at 25°C . A sphere-shaped micro-indenter ($r = 250 \mu\text{m}$, material: stainless steel) probed the hydrogel at a deformation velocity of $250 \mu\text{m/min}$. By applying the Hertzian model¹⁰³ for rigid sphere indenters, with an assumed Poisson's

ratio for the hydrogels of 0.5 the elastic modulus (E) was estimated from the AFM and micro-indentation loading curves.

3.3 Results and discussion

3.3.1 Chemical properties of the surface-bulk transition region

Here the surface chemical compositions of DN hydrogels prepared on different substrates is characterized using ATR/FT-IR which reveals the chemistry of the topmost surface of the hydrogels. PAMPS has a sulfonic group and PAAm has an amide carbonyl group (**Figure 21A**) that can be characterized by ATR/FT-IR. On the absorption spectra (**Figure 21B**), a sharp peak at 1042 cm^{-1} , characteristic for the sulfonic group ($\nu_{\text{S=O}}$) of the PAMPS network, and a strong sharp absorption peak around 1640 cm^{-1} , from the primary amide carbonyl group ($\nu_{\text{C=O}}$) of PAAm, were observed on the spectra for the SN PAMPS (1-3-1) gel and PAAm (2-0.1-0.1) gel, respectively.

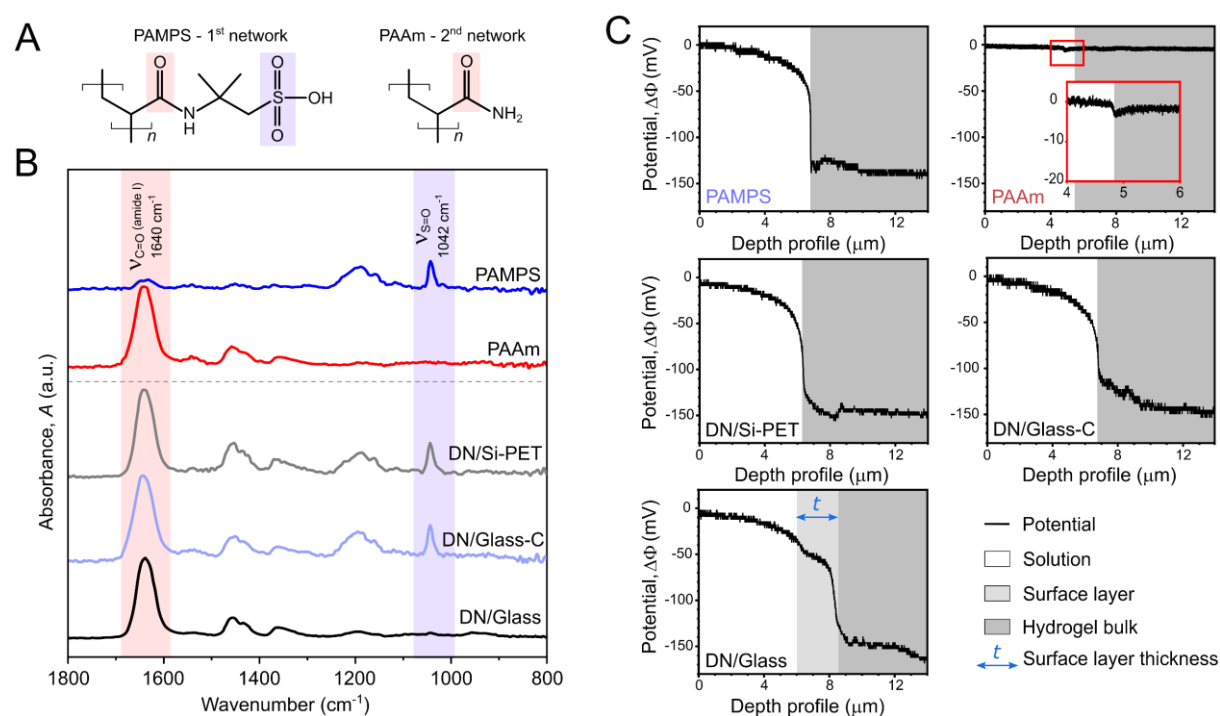


Figure 15: Analysis of the surface structure of DN hydrogels where the 2nd network was synthesized on different substrates. (A) The molecular structures of the 1st and 2nd network polymers. The red and blue background represent the IR bond absorptions, used to characterize the hydrogels. (B) The ATR/FT-IR absorption spectra and (C) the electric potential depth profiles ($\Delta\Phi$) of SN PAMPS (1-3-1), 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-PET) and glass substrates (DN/Glass), and on glass under compression (DN/Glass-C, **Figure 18A**).

On the PAMPS hydrogel, a peak at 1640 cm^{-1} is also observable since the molecular structure of PAMPS contains a primary amide carbonyl group ($\nu_{\text{C=O}}$). However, the signal is much weaker compared to PAAm and therefore it is neglectable. The spectrum of the DN/Si-PET, exhibits both strong absorption peaks at 1042 cm^{-1} for the sulfonic group and at 1640 cm^{-1} for the primary amide carbonyl group, while the DN/Glass hydrogel only exhibits absorption at 1640 cm^{-1} due to the amide carbonyl group. These ATR/FT-IR results indicate that both PAMPS and PAAm are present on the surface of the DN/Si-PET hydrogel while only the 2nd network PAAm is present on the surface of the DN/Glass hydrogel. Based on this analysis, I infer that there is a surface layer of PAAm on the DN/Glass hydrogel.

To characterize the thickness of the surface layer on the DN/Glass hydrogel, I probed the depth profile of the electric potential near the surface of the hydrogels by the microelectrode technique. A charged hydrogel generates an electric potential ($\Delta\Phi$) relative to the bath aqueous solution due to the Donnan effect⁵⁵.

$$\Delta\Phi = - \frac{2.3RT}{zF} \log \left(\frac{c_g}{c_s} \right) \quad (\text{Eq. 4})$$

Here (Eq. 4), c_g and c_s are the mobile counter-ion concentrations in the hydrogel and in the bath solution, respectively. z is the valence of the mobile counter-ion, F the Faraday constant, R the gas constant, and T the absolute temperature. The covalent bound ions on the polymer network guide the mobile counter-ion concentration to keep neutrality in a thermodynamic equilibrium state. As a result, by probing the local electric potential, I can detect the local structure of the hydrogel. For the PAMPS hydrogel, that carries immobile negative charges on the polymer network, the potential shifts to a negative value relative to the bath solution since the hydrogel traps high numbers of positively charged mobile ions (z is positive), while the PAAm hydrogel has almost zero potential since its polymer network carries a negligible amount of charge. Accordingly, I can probe the surface layer thickness of the DN/Glass hydrogels via the electric potential depth profiling.

As shown in **Figure 21C**, the microelectrode, inserted into a SN PAMPS hydrogel, measures an initial gradual drop of electric potential until the displacement of $6.8\text{ }\mu\text{m}$, which is followed by an abrupt drop

to a large negative value of -150 mV. The initial potential drop is due to the deformation before the penetration of the electrode into the hydrogel. Like the piezoelectric effect, the compression at the probe tip increases the local polymer charge density and decreases the electric potential. The large potential shift ($\Delta\Phi$) to -150 mV indicates that a high amount of Na^+ ions ($z = +1$) are deployed as counterions to neutralize the fixed negative charges of the sulfonic groups of PAMPS. In contrast, only a very small potential shift of -4 mV is measured for the neutral SN PAAm hydrogel. The potential depth profile of the DN/Si-PET hydrogel, where the 2nd network was synthesized on adhesive Si-PET (contact angle to ddH₂O $\Theta = 109.4 \pm 0.8^\circ$, **Figure 16**), shows an immediate drop of the potential to -150 mV after the initial drop coming from the compression before electrode penetration, indicating the absence of the surface layer, in good agreement with the ATR/FT-IR result.

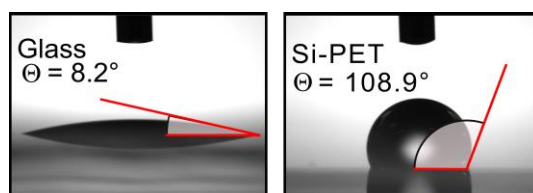


Figure 16: Molding substrate surface energy. The water contact angles of $8.5 \pm 1.5^\circ$ and $109.4 \pm 0.8^\circ$ (mean $\pm$ SD, $n = 10$) demonstrate the high and low surface energy of borosilicate glass and Si-PET used as molding substrates in the 2nd network synthesis, respectively.

In contrast, the depth profiles of the DN/Glass hydrogel (1-3-1/2-0.1-0.1), where the 2nd network was synthesized on repulsive glass (contact angle to ddH₂O, $\Theta = 8.5 \pm 1.5^\circ$, **Figure 16**), also shows an initial drop of electric potential to a probe displacement of 6.1 μm , like the SN PAMPS hydrogel before the probe penetration to the hydrogel. Different from the profile of the SN PAMPS hydrogel, a small hump 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 presence of a weakly charged surface layer. At a displacement larger than 8.5 μm , the potential shifts drastically to -150 mV, indicating the transition from the surface layer to the bulk material composition of PAMPS/PAAm because the shift intensity is like that of the corresponding PAMPS SN hydrogel. From the 2nd derivative of the depth profile, a surface layer of thickness $t = 2.23 \mu\text{m}$ on the DN/Glass hydrogel was determined (**Figure 17**).

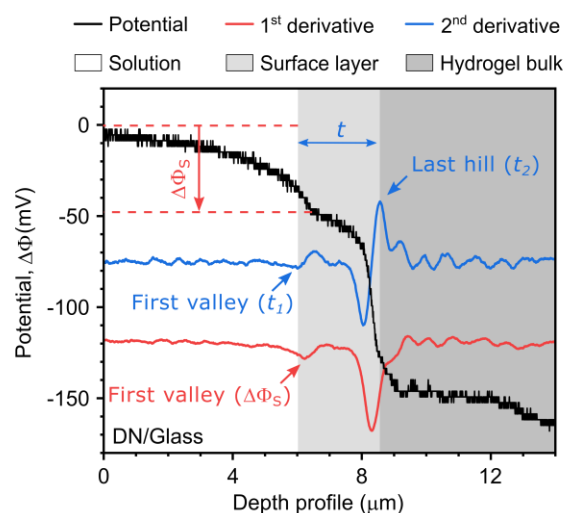


Figure 17: Analysis of electric potential depth profiles. The electric surface potential ($\Delta\Phi_s$) is defined as the difference in potential between the initial relative potential (0 mV) and the electric potential at the first valley of the 1st derivative at the hydrogel surface. The surface layer thickness (t) corresponds to the distance between the first valley (t_1) and the last hill (t_2) of the 2nd derivative in the hydrogel depth profile (Sample: DN/Glass 1-3-1/2-0.1-0.1).

The microelectrode results reveal the presence of a surface layer on the DN/Glass hydrogel while no surface layer is formed on the DN/Si-PET hydrogel. These results are consistent with the ATR/FT-IR results. However, the microelectrode method reveals that the surface layer on the DN/Glass hydrogel has a larger electric potential (-50 mV) than that of the corresponding PAAm hydrogel (-4 mV), indicating that the surface layer of the DN/Glass hydrogel is not pure PAAm, which is not consistent with the ATR/FT-IR measurement that shows no signal from PAMPS. This discrepancy is due to the relatively poor sensitivity of the ATR/FT-IR. From the potential shift of -50 mV in relation to the bath solution containing 10^{-4} M NaCl, the concentration of mobile Na^+ ion in the surface layer of the DN/Glass gel is estimated as 7×10^{-4} M by using Equation 1. The presence of this excess number of mobile ions in the surface layer of the DN/Glass hydrogel suggests that a very small amount of AMPS monomers, as residue from the 1st network synthesis, of 7×10^{-4} M in concentration, is copolymerized in the 2nd PAAm polymerization to form a copolymer. Such a small amount of AMPS in the surface layer of the DN/Glass could not be detected by the ATR/FT-IR method that has a lower detection limit for PAMPS of 9.4×10^{-4} M (**Figure 19**).

The PAAm surface layer formation on the DN/Glass hydrogel is presumably caused by the counter ions osmotic repulsion between negatively charged PAMPS hydrogel and the glass substrate during the 2nd

network polymerization. To verify the hypothesis, I tried to eliminate the surface layer by applying compression (50 kPa) to the 1st network hydrogel during the synthesis of the 2nd network to counteract the osmotic repulsion (**Figure 18A**). To choose a proper compressive pressure, the compressive stress-strain behavior of PAMPS hydrogels, immersed in AAm solution, was studied for various crosslinking degrees (X_{X1}) of the PAMPS hydrogels. The compressive stress and fracture stress increased but the fracture strain decreased with increasing 1st network crosslinking (**Figure 18B**). The weakest PAMPS hydrogel, with 1.5 mol% crosslinker, fractured at around 120 kPa.

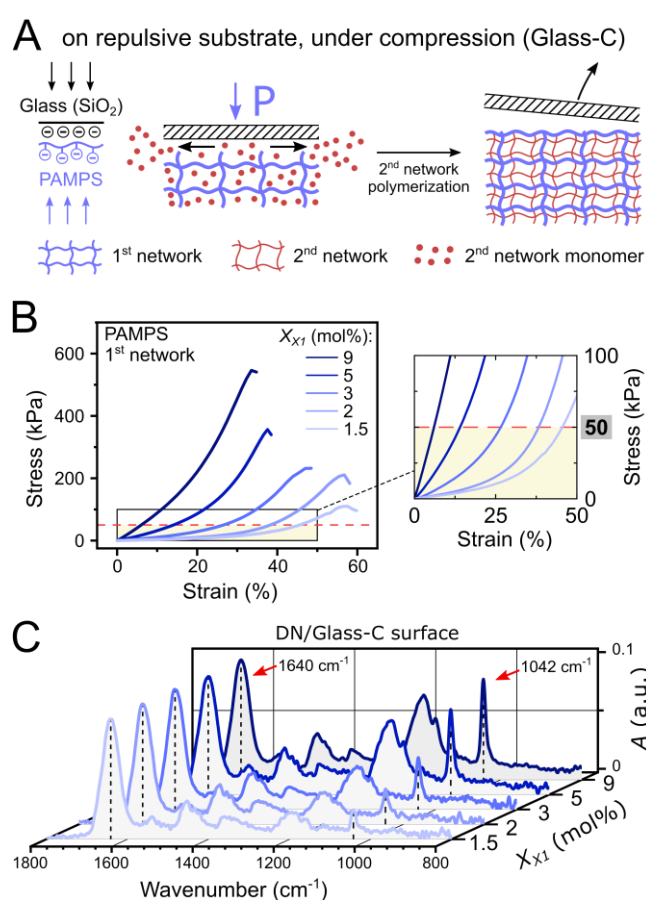


Figure 18: Applying compression to the PAMPS hydrogel ($P = 50$ kPa) during synthesis of the 2nd network to prevent the formation of a surface layer. (A) Applying normal pressure to counteract the osmotic repulsion at the hydrogel-glass interface during the 2nd network synthesis (DN/Glass-C). (B) The compressive stress-strain curves of the PAMPS hydrogel (1- X_{X1} -1) immersed in the AAm solution (2-0.1-0.1) to determine the normal pressure. The compressive stress-strain curves of the 1st network hydrogel swollen in 2nd network precursor solution was performed on hydrogel discs of 1 cm in diameter and 2 – 3 mm in thickness at a loading strain of 10 %/min using a tensile-compressive mechanical tester (Tensilon RTC-1310A, Orientic Co.). (C) The ATR/FT-IR spectra of the DN/Glass-C hydrogels (1- X_{X1} -1/2-0.1-0.1) with varied 1st network crosslinker concentration X_{X1} .

To make sure that the 1st network is compressed but does not fracture during the 2nd network polymerization, I applied to each differently crosslinked PAMPS hydrogel a compression of 50 kPa. This overall compression of 50 kPa is low compared to the bulk modulus of these highly crosslinked SN hydrogels which is in the range of 100 ~ 1000 kPa but in each ATR/FT-IR spectrum both peaks at 1042 and 1640 cm⁻¹, representing the 1st and the 2nd network respectively, are observable (**Figure 18C**).

3.3.2 Construction of a calibration curve for the surface polymer density

Here, I characterized the single network hydrogels surface via ATR/FT-IR spectroscopy to investigate the polymer surface density of the 1st and 2nd network polymers PAMPS (W_{M1}) and PAAm (W_{M2}) respectively. In **Figure 19**, the infrared absorption spectra reveal that the peak intensities of the SN hydrogels PAMPS (X_{X1} : 1.5 – 9 mol%) and PAAm hydrogels (X_{X2} : 0.1 – 4 mol%) increase with the crosslinking degree. Since this relationship exists, it is possible to determine the surface polymer density from the peak heights.

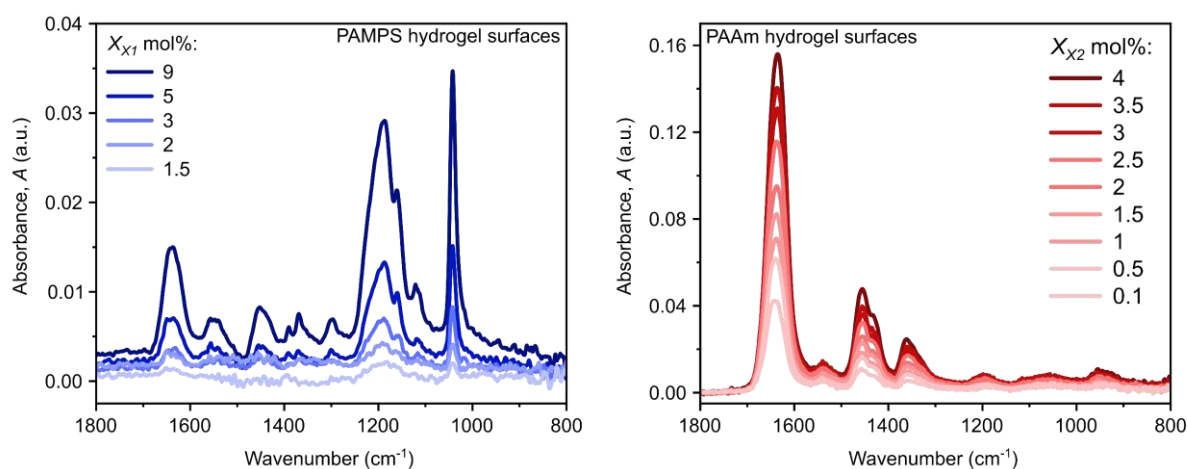


Figure 19: Infrared absorption spectra and signal intensity of SN hydrogel surfaces. Differently crosslinked hydrogels, PAMPS (X_{X1} : 1.5 – 9 mol%) and PAAm hydrogels (X_{X2} : 0.1 – 4 mol%), swollen in D₂O, demonstrate the relation between polymer density at the surface and X_{X1} and X_{X2} .

To construct the calibration curves from SN PAMPS and PAAm hydrogel for the detection of the polymer surface density on DN hydrogel, the gravimetric polymer weight fractions of hydrogels swollen in water (W_{M1} , W_{M2}), the absorption peak heights of hydrogels immersed in D₂O (A_{max}) and the relative swelling ratio, (Q_r) were related to the crosslinking degree (X_{X1} , X_{X2}), (mean $\pm$ SD, $n = 3$ hydrogels per symbol), (**Figure 20**). To correlate the polymer weight fractions with the absorption peak height, the ATR/FT-IR data need to be corrected by Q_r because of the swelling discrepancy between water and

D₂O. The relative swelling ratio, $Q_r = (h_{H_2O}/h_{D_2O})^3$ was determined by the hydrogel thickness after swelling in ddH₂O (h_{H_2O}) relative to swelling thickness in deuterium oxide (h_{D_2O}). The resulting calibration curves for PAMPS and PAAm surface density based on the relation of the corrected absorbance maxima ($x = A_{max} \times Q_r$) to the corresponding polymer weight fraction ($y = W_{M1}, W_{M2}$) were used to quantify the polymer surface density of DN hydrogels in the following chapters.

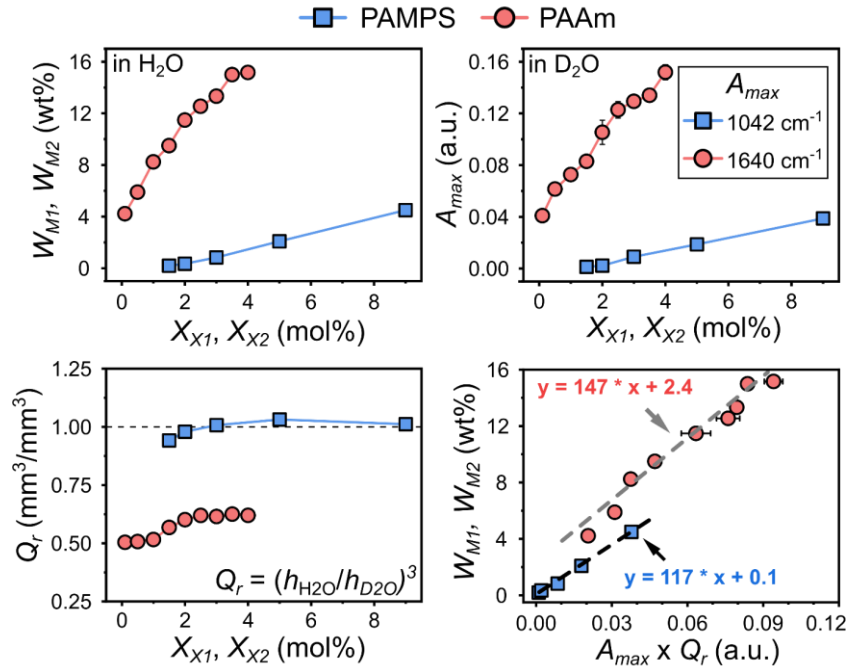


Figure 20: Calibration curves to analyze PAMPS and PAAm densities at the DN hydrogel surface. The calibration curve is determined by the infrared spectroscopy peak heights corrected by the swelling ratio ($A_{max} \times Q_r$) and the weight fraction (W_{M2}) of PAMPS and PAAm (wt%), determined by gravimetry.

The linear fit parameters for the PAMPS and PAAm calibration curves, $y = kx + d$ (slope - k , intercept - d), are displayed in **Table 2**. Using this calibration curves, it is estimated that at a PAMPS weight fraction (W_{M1} , **Figure 19**) of ~0.2 wt% (corresponding to a molar concentration C_{M1} of 9.4×10^{-4} M), the detection limit for PAMPS is almost reached for the weakly crosslinked PAMPS hydrogel (1-1.5-1), since the peaks have intensities similar to the background signal.

Table 2: Linear fit parameters of the PAMPS and PAAm surface density calibration curves

Linear fit parameters	1 st network	2 nd network
($y = d + kx$)	PAMPS	PAAm
Intercept (d)	0.084 ± 0.014	2.366 ± 1.046
Slope (k)	116.8 ± 4.5	146.5 ± 13.9
r^2	0.9956	0.9401

y: W_{M1} or W_{M2} , x: $A_{max} \times Q_r$

3.3.3 Quantitative analysis of chemical and mechanical properties of the surface-bulk transition region

We further analyzed the surface structure and modulus, in comparison with the bulk quantities for DN/Glass, DN/Si-PET and DN/Glass-C hydrogels synthesized by different formulations, and the results are summarized in **Figure 21** and **Figure 22**. The **Figure 21A** shows that the polymer surface density of SN PAMPS and SN PAAm hydrogels increases with the crosslinking degree (X_{X1} , X_{X2}), due to the decrease of the volumetric swelling degree (Q) that comes along with the increased crosslinking degree. The polymer densities can be tuned 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 adjusting the crosslinking degree. Due to its polyelectrolyte nature, the swelling degree of PAMPS hydrogels is much higher than that of the neutral PAAm hydrogels even at a high crosslinking degree. The swelling degree of PAMPS in 2nd network precursor solution is almost the same to that of PAMPS in water because the AAm monomer is neutral⁹⁷.

The changes in surface polymer density and electric potential of DN gels in accordance to the 1st network crosslinking degree (X_{X1}) is shown in **Figure 21B**. On DN hydrogels, the PAMPS surface densities (W_{M1}) between DN/Glass, DN/Si-PET and DN/Glass-C is different because of the presence or absence of the surface layer (**Figure 21B**, upper left side). As the 1st network crosslinking (X_{X1}) increases, the PAMPS surface densities of DN/Si-PET and DN/Glass-C hydrogels increased, in agreement with that of the SN PAMPS hydrogel. However, on the DN/Glass hydrogel surface, the PAMPS density is below a weight fraction of 1 wt% regardless of the change in the X_{X1} for the whole crosslinking range. The PAAm surface density on DN/Si-PET and DN/Glass-C hydrogels slightly increases with increasing PAMPS network crosslinking (X_{X1}), though the amount of crosslinker for the PAAm network for all DN hydrogels was kept constant (X_{X2} : 0.1 mol%). However, on the DN/Glass hydrogel, the PAAm surface density is higher than that of the DN/Si-PET and DN/Glass-C hydrogels, showing almost a constant value, independent of the change in X_{X1} . The increase in the PAAm density with X_{X1} on DN/Si-PET and DN/Glass-C gels is because the swelling of the PAAm is constrained by the presence of PAMPS. The higher the X_{X1} of the 1st network, the more the PAAm network is constrained¹⁵. The surface molar ratio of PAMPS to PAAm (C_{M1}/C_{M2}) increases with the increase in the 1st network crosslinking degree for

DN/Si-PET and DN/Glass-C gels while it keeps a low but constant value for DN/Glass gels (**Figure 21B**, lower left side). By cutting the surface hydrogel I exposed the bulk material so that a comparison between the surface and the bulk molar ratio C_{M1}/C_{M2} is possible (**Figure 21B**, upper right side). While the bulk values were in good agreement with the theoretical values, the surface molar ratio C_{M1}/C_{M2} is about 2 times higher than 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} are the feed monomer concentrations of the 1st network and the 2nd network, respectively, and Q is volumetric swelling degree of PAMPS hydrogels in AAm solution. The high molar ratio on the surface compared to the bulk probably originates from the faster polymerization of the PAMPS hydrogels near the glass substrates in relation to the central region due to the higher UV intensity at the surface regions.

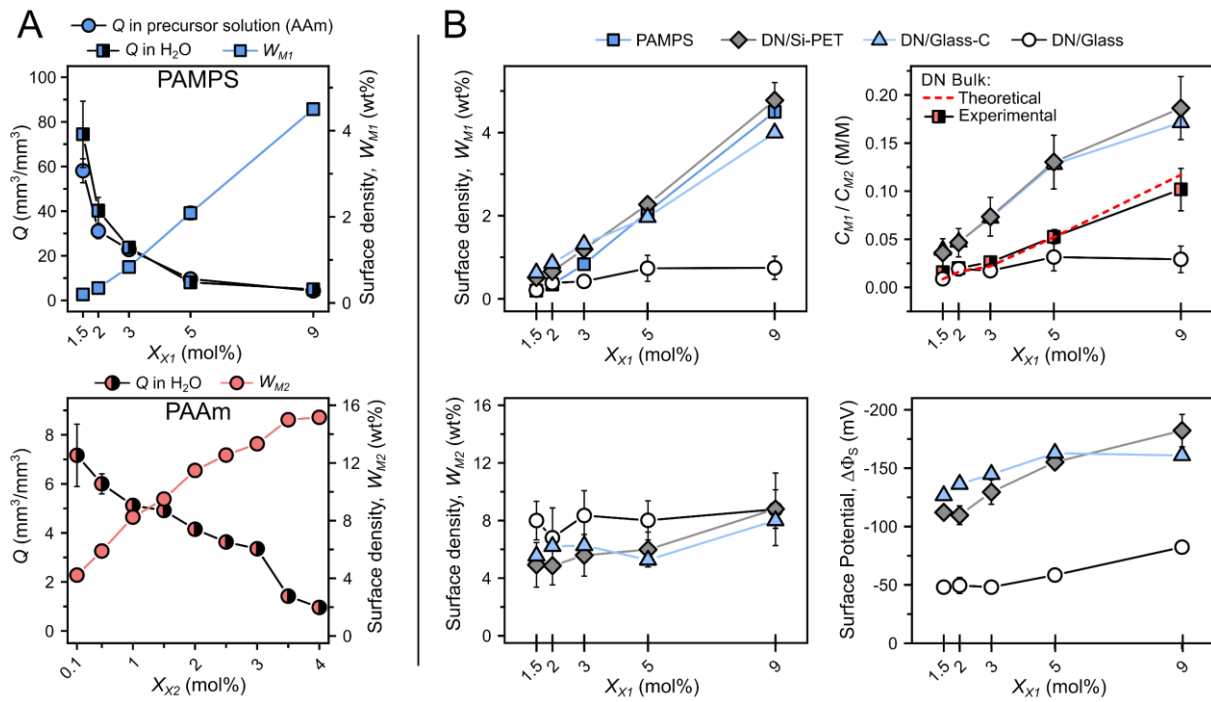


Figure 21: Regulation of polymer surface density (W_{M1} , W_{M2}) by crosslinking degree (X_{X1} , X_{X2}) of the first network. (A) The volumetric swelling degree (Q) and the corresponding surface polymer density (W_{M1} , W_{M2}) of SN PAMPS and PAAm hydrogels (mean $\pm$ SD, $n = 3$ hydrogels per symbol) for varied crosslinking degree (X_{X1} , X_{X2}). (B) DN hydrogel surface polymer density (W_{M1} , W_{M2}), the corresponding molar concentration ratio (C_{M1}/C_{M2}), and the surface potential ($\Delta\Phi_s$) for DN gels prepared with various 1st network crosslinking degree (X_{X1}) (mean $\pm$ SD, sample groups SN PAMPS, DN/Si-PET, DN/Glass contained sample sizes of $n = 3 - 6$ hydrogels per symbol, and for DN/Glass-C $n = 1$ hydrogel per symbol). The standard deviations (SD) of the PAAm density values tend to be high because the low accuracy of the ATR/FT-IR data used for the quantitative analysis resulted in a poorly fitted calibration curve (**Figure 19**, **Figure 20**).

The increase in the amount of crosslinker (X_{X1}) reduces the swelling of the PAMPS hydrogel in the 2nd network precursor solution and consequently increases the number of negative charges through the PAMPS density. Therefore, the electric surface potential ($\Delta\Phi_s$), defined as the initial drastic potential drop at the hydrogel surface (**Figure 17**), of the DN/Si-PET and DN/Glass-C hydrogels 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 21B**, lower right side). Similar increase in surface electric potential was published previously, when crosslinker density increased in SN PAMPS hydrogels⁵⁴. The DN/glass hydrogel reaches a value of -75 mV at 9 mol% crosslinker (X_{X1}) and is arguably related to the increased incorporation of unreacted AMPS because of a low conversion rate in the polymerization of highly crosslinked PAMPS.

As **Figure 22A** demonstrates, in the case of DN/Glass hydrogels the surface layer thickness is between 3 - 4 μm for different X_{X1} . Because of the presence of the surface layer, the elastic modulus at the surface is lower than the bulk in the case of the DN/Glass hydrogel, and only weakly increased with X_{X1} . However, the bulk modulus of the DN/Glass hydrogel increases with increasing 1st network crosslinking (X_{X1}), showing the same values as the bulk elastic modulus of DN/Si-PET hydrogels. For SN PAMPS and DN/Si-PET hydrogels, the surface and the bulk elastic modulus both increase with X_{X1} .

From the above results, I can tune the bulk modulus while keeping the same surface chemistry. As shown in **Figure 22B**, when the bulk elastic modulus of DN/Glass and DN/Si-PET was changed through the 1st network crosslinking degree, the polymer surface densities evolved differently, depending on the presence or absence of the surface layer. When no surface layer is present, the chemical structure at the surface is controlled through the PAMPS/PAAm ratio and the swelling in the 2nd network precursor solution. However, when there is a surface layer, the surface chemical structure depends on the formulation of the 2nd network precursor solution and is independent of the bulk elastic modulus. As shown for the SN PAAm hydrogels, polymer surface density and modulus increase with crosslinking density X_{X2} . By varying X_{X1} and X_{X2} of the DN/Glass hydrogel, it is possible to individually tune the surface layer polymer density (or modulus) and the bulk polymer density (or modulus).

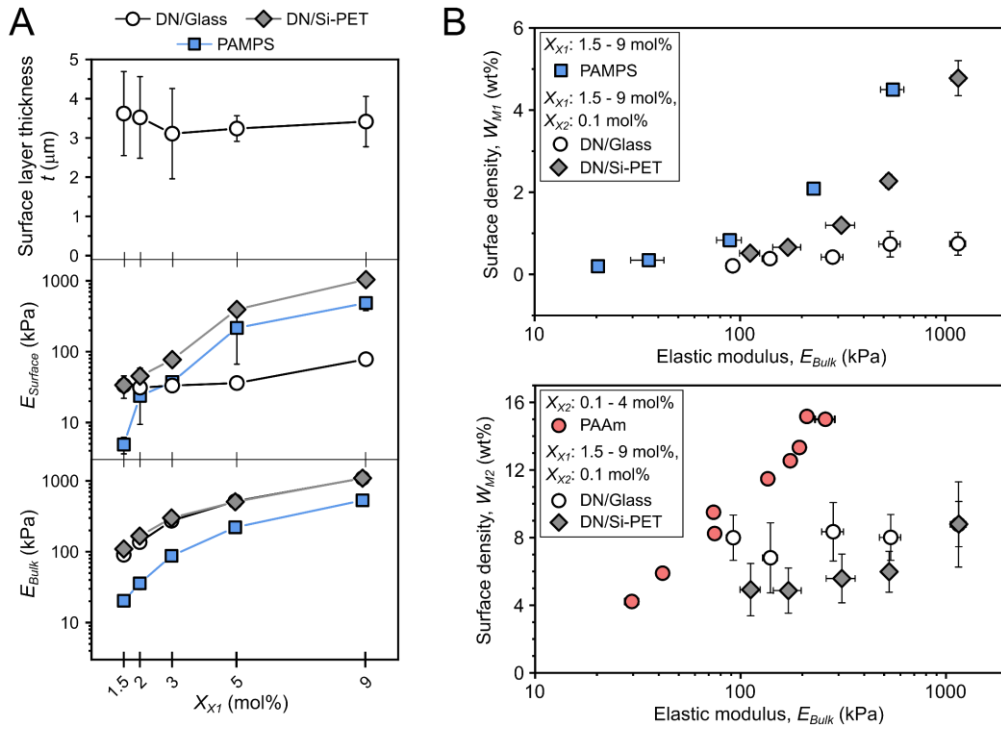


Figure 22: Effect of Surface layer thickness on elastic modulus. (A) The surface layer of 3 – 4 μm thickness (t) on the DN/Glass hydrogel affects the elastic modulus at the surface (E_{Surface}), (mean $\pm$ SD, $n = 15 - 48$ technical replicates per symbol) but not the bulk (E_{Bulk}). The sample groups SN PAMPS, SN PAAm, DN/Glass and DN/Si-PET contained sample sizes of $n = 3 - 6$ hydrogels per symbol (mean $\pm$ SD). (B) Independent tuning of bulk modulus and polymer surface densities (W_{M1} , W_{M2}) by DN hydrogel synthesis.

3.4 Conclusion

The surface energy and charge of the substrates used in the 2nd network synthesis play a crucial role in determining DN hydrogel surface chemistry and surface stiffness. While the use of a low energy molding substrate facilitates the synthesis of DN hydrogels with both networks present at the surface, the electrostatic interactions between glass and like-charged polyelectrolyte hydrogels are responsible for the formation of a 2nd network polymer surface layer. The regulation of the DN hydrogel surface potential is mainly related to the 2nd network surface layer thickness that is modifiable through molding substrate surface energy or normal compression during synthesis. If no surface layer is present, a secondary regulation of the surface potential is possible via the molar ratio between the polyelectrolyte and neutral polymer. The combination of the ATR/FT-IR and the microelectrode technique has proven to be a powerful approach for characterizing hydrogel surfaces since each technique makes up for the disadvantage of the other technique. The microelectrode technique, which is highly sensitive in detecting electric potentials but has a poor spatial resolution in the depth profile,

complements the ATR/FT-IR technique, which gives structural information on the topmost hydrogel layer with lower sensitivity. In the following chapters this surface modification via the molding substrate is deployed to regulate cell adhesion on DN hydrogels.

4 The effect of residual monomers on the double network hydrogel surface potential

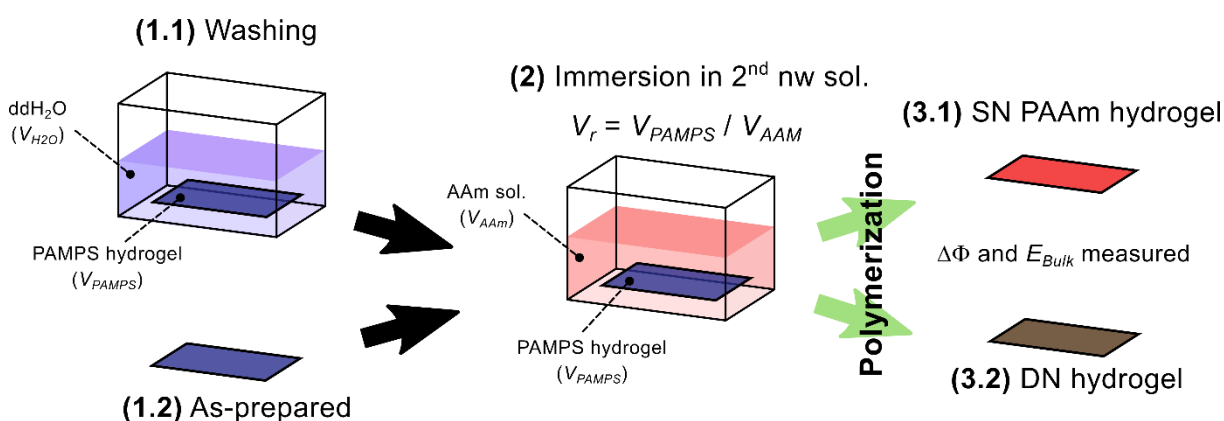
4.1 Introduction

As demonstrated in chapter 3, a surface layer of 2nd network polymer (PAAm) based on the repulsion between glass substrate and 1st network hydrogel (PAMPS) formed on top of the DN hydrogel bulk. There, it was surprising that the DN/Glass hydrogel surface potentials increased slightly with the crosslinking degree (X_{XL}) shown in **Figure 21**. Based on the ATR-FT/IR observation on DN/Glass hydrogels no peak characteristic for the 1st network polyelectrolyte, that could affect the surface potential, was observed in the infrared spectrum (**Figure 15**). Therefore, the surface potential of DN/Glass hydrogels should not have increased. Although, one hypothesis for the observed surface potential would be, that the PAAm hydrogel at the surface cannot swell freely, since it is entangled with the bulk material. Because of that swelling mismatch the mesh expansion to reach thermodynamic equilibrium, in solutions with weak ionic strength, is hindered and therefore the surface potential is more negative than that of the corresponding freely swollen PAAm hydrogel. Another explanation was already proposed in chapter 3, which is related to unreacted AMPS monomers from the 1st network polymerization step. In this case the surface potential could be still increased because residual AMPS monomers are incorporated into the 2nd network polymer through the second polymerization step. The observation via infrared spectroscopy, where no characteristic peak for PAMPS was observed, most likely appeared because the amount of AMPS to sufficiently change the PAAm surface potential is beyond the spectroscopies detection limit. However, via the microelectrode technique a potential difference is still detectable.

To test the mentioned explanation, where the residual AMPS monomers are the cause for the increased surface potential, I synthesized DN hydrogels where the residual 1st network monomers were removed and analyzed the surface potential. To accomplish the removal, 1st network hydrogels were washed with water multiple times (**Scheme 2**). To monitor the concentration of AMPS molecules in solution the conductivity of the washing water was analyzed since even small numbers of AMPS molecules in water increase the conductivity of a solution. After the washing procedure, the PAMPS

hydrogels were immersed in 2nd network precursor solution containing AAm monomers and the DN hydrogel was synthesized as performed in the previous chapters. The addition of a control group, where the unwashed as-prepared PAMPS hydrogel was used to synthesize DN hydrogel, is used to compare hydrogel surface potentials analyzed with the microelectrode technique. Further a calibration group out of PAMPS-co-PAAm hydrogels, where the fraction of PAMPS was regulated in the range of $2 \times 10^{-2} - 2 \times 10^{-5}$ M (**Table 4**), was synthesized to demonstrate how small PAMPS concentration in the DN hydrogel surface layer could impact the electric potential.

The results in this section ultimately demonstrate that only the removal of AMPS from the 1st network hydrogel allows the fabrication of DN/Glass hydrogels with electric potentials comparable to the corresponding SN PAAm hydrogels.



Scheme 2: Setup to investigate the effect of residual 1st network monomers on the DN/Glass hydrogel surface potential. removal of residua. The washed (1.1) and as-prepared (1.2) PAMPS hydrogel was immersed in 2nd network precursor solution (2). After equilibration DN/Glass hydrogel and SN PAAm hydrogel from the precursor solution in the box was synthesized. The washing and immersion process were monitored via the washing water conductivity and the hydrogel surface potentials were analyzed via the microelectrode technique.

4.2 Experimental section

Unless otherwise noticed the hydrogel synthesis, the microelectrode technique and the elastic modulus characterization were performed as described in chapter 3.2.

4.2.1 Materials

The 1st network monomer 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Toagosei Co., Ltd., Japan) was used as received. The 2nd network monomer acrylamide (AAM), N,N-dimethylacrylamide,

the crosslinker N,N'-methylenebis(acrylamide) (MBAA) and initiator 2-oxoglutaric acid were used as obtained from Wako Pure Chemical Industries, Ltd., Japan.

4.2.2 Single and double network hydrogel synthesis

The hydrogel synthesis was performed as described in chapter 3.2.3 and hydrogel formulations are found in **Table 3**. The DN hydrogels were synthesized using borosilicate glass as molding substrate to form a 2nd network polymer surface layer.

Table 3: Hydrogel sample codes and formulations Chapter 5

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	3	1	-	-	-
PAAm (C_{M2} - X_{X2} - X_{I2})	-	-	-	2	0.1	0.1
DN/Glass (C_{M1} - X_{X1} - X_{I1} / C_{M2} - X_{X2} - X_{I2})	1	3	1	2	0.1	0.1
PAMPS-co-PAAm	$0.02 - 2 \times 10^{-5}$	-	-	~2	0.1	0.1

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

To synthesize the PAMPS-co-PAAm hydrogels, the aqueous AMPS and AAm precursor solutions were mixed by the ratios shown in **Table 4**. To fabricate weakly charged co-polymers only small fractions of AMPS were added to the AAm solution.

Table 4: PAMPS-co-PAAm formulation

Total volume (mL)	AMPS precursor sol. 1-0.1-0.1 (μL)	AAM precursor sol. 2-0.1-0.1 (mL)	AMPS conc. (M)	AMPS/AAm ratio (M/M)
50	1000	49	2×10^{-2}	1×10^{-2}
50	500	49.5	1×10^{-2}	0.5×10^{-2}
50	100	49.9	2×10^{-3}	1×10^{-3}
50	10	50	2×10^{-4}	1×10^{-4}
50	1	50	2×10^{-5}	1×10^{-5}

4.2.3 Conductivity of the hydrogel bath solution

The residual AMPS monomers in the hydrogel washing water (ddH₂O) were monitored on a conductivity meter (FE30 KIT, Mettler-Toledo) to evaluate the concentration of unreacted monomers in solution. Briefly, the evaluation included the correlation between ionic conductivity and the molecular concentration of the dissolved AMPS monomers via a dilution series ranging from 10⁻¹ to 10⁻⁶ M, using water or 2nd network precursor solution (AAm with a formulation of: 2-0.1-0.1) as diluting

agent. Since the volume ratio between hydrogel and bath solution is affecting the amount of ion exchange between hydrogel and bath solution hence the conductivity, the volume ratio (V_r) was defined by $V_r = V_{PAMPS}/V_{H_2O}$. To monitor the PAMPS hydrogel washing procedure the as-prepared hydrogel of volume 2 cm^3 (V_{PAMPS}), with a geometry ($l \times b \times h$) of $4 \times 2.5 \times 0.2\text{ cm}$, was immersed in 100 mL ddH₂O (V_{H_2O}). After each 24 hours of incubation the conductivity of the washing water was detected, and the old washing water was disposed and exchanged by new 100mL ddH₂O. To demonstrate that unreacted 1st network monomers diffuse into solution, the conductivity change over time was monitored of the washed and as-prepared PAMPS hydrogels (V_{PAMPS}) immersed in 2nd network precursor solution (V_{AAM}) at volume ratios in the range of 0.02 - 0.001 ($V_r = V_{PAMPS}/V_{AAM}$). Hydrogels were stored at 25 °C in the dark on a horizontal shaker plate at 60 rpm. It needs to be noted that the conductivity of the AAm bath solutions was corrected by the daily decrease in conductivity of the control AAM bath solution since the UV/VIS reactive 2-oxoglutaric acid is consumed slightly over time.

4.2.4 Depth profiling via microelectrode technique

To investigate the relationship between the 2nd network precursor solution conductivity and the DN hydrogel surface charge the electric potential was analyzed via the microelectrode technique. Prior to the sampling hydrogels were washed in water (ddH₂O) until the washing water reached a conductivity $\geq 2.0\text{ }\mu\text{S/cm}$. Then the hydrogel was immersed in the bath solution of NaCl $1 \times 10^{-4}\text{ M}$, until it reached equilibrium at a conductivity of $\sim 15\text{ }\mu\text{S/cm}$ at 25 °C. The depth profiling method and the analysis of the potential curve were described above in chapter 3.2.6.

4.3 Results and discussion

4.3.1 Calibration and PAMPS washing water conductivity

The **Figure 23A** demonstrates that the ionic conductivity correlates with the AMPS concentration changes in aqueous solution and solutions with weak ionic strength such as 2nd network precursor solution containing AAm. Since monomers of polyelectrolytes are highly soluble in water the change in ionic conductivity of hydrogel washing water over time gives a hint on how well charged molecules diffuse from the hydrogels into the bathing solution. Because there were no other stable and highly

charged molecules in the hydrogel reaction mix the ionic conductivity is mostly guided by the AMPS concentration. With this calibration curve the AMPS concentration can be detected down to values of 2×10^{-6} M. However, in the AAm solution the lower detection limit for AMPS molecules lies at a concentration of 1×10^{-4} M, as the conductivity approaches a constant value of $340 \mu\text{S}/\text{cm}$ which is characteristic for the pure 2nd network precursor solution of AAm. The hydrogel washing procedure of the 1st network PAMPS hydrogel samples (2cm^3) in 100 mL ddH₂O was monitored by measuring washing water conductivity daily after 24 hours of incubation. **Figure 23B** shows that, after 5 - 7 days the AMPS concentration in the sample with daily water exchange stayed constant below 5×10^{-5} M, corresponding to the ionic conductivity of $\geq 2\mu\text{S}/\text{cm}$. For samples without water exchange conductivity wasn't changed over time, indicating the necessity to wash hydrogels for at least 5 - 7 days to reduce the concentration of residual AMPS molecules.

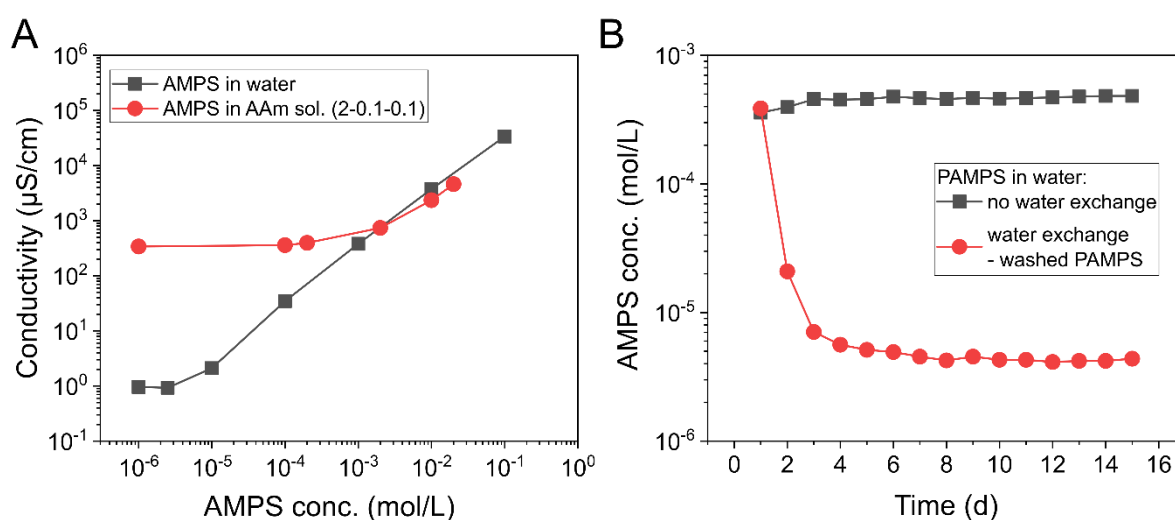


Figure 23: Hydrogel washing procedure validation. (A) The ionic conductivity correlates with the AMPS concentration in water and AAm solution ($n = 1$ per point, blue dashed line indicates the ionic conductivity of ddH₂O). (B) The PAMPS hydrogel washing procedure of 2 cm^3 (geometry: $l \times b \times h$, $4 \times 2.5 \times 0.2 \text{ cm}$, $n = 1$ hydrogel) hydrogel samples in 100 mL ddH₂O was monitored by measuring washing water conductivity daily after 24 hours of incubation.

4.3.2 The impact of PAMPS hydrogel washing on DN hydrogel surface potential

To investigate if the residual AMPS molecules affect conductivity of the AAm 2nd network precursor solution, the washed and the as-prepared PAMPS hydrogels were immersed in the solution and the ionic conductivity was monitored for 3 days. **Figure 24 A** and **B** visualize the change of conductivity at

different time scales. In the case where the as-prepared PAMPS hydrogels are immersed in the precursor solution it is shown that, as the volume ratio (V_r) approaches 1, where V_{PAMPS} and V_{AAm} are equal, the increase in conductivity is the highest at $V_r = 0.02$. This demonstrates that residual AMPS monomers diffuse into the AAm solution. At the tested volume ratio of 0.02 to 0.001 the conductivity changes after 3 days of hydrogel immersion reaches a conductivity of 510 and 370 $\mu\text{S}/\text{cm}$ respectively, which is associated with AMPS concentrations of 1.6×10^{-4} M to 2.3×10^{-3} M. In contrast, when the washed PAMPS hydrogel is immersed in AAm solution, at any of the tested volume ratios, the conductivity stayed stable and showed no deviation from the control sample which was freshly prepared AAm solution containing no PAMPS hydrogel. This shows that the PAMPS washing procedure effectively removed residual AMPS monomers from the 2nd network precursor solution to an undetectable degree which is below 1×10^{-4} M shown in **Figure 23A**.

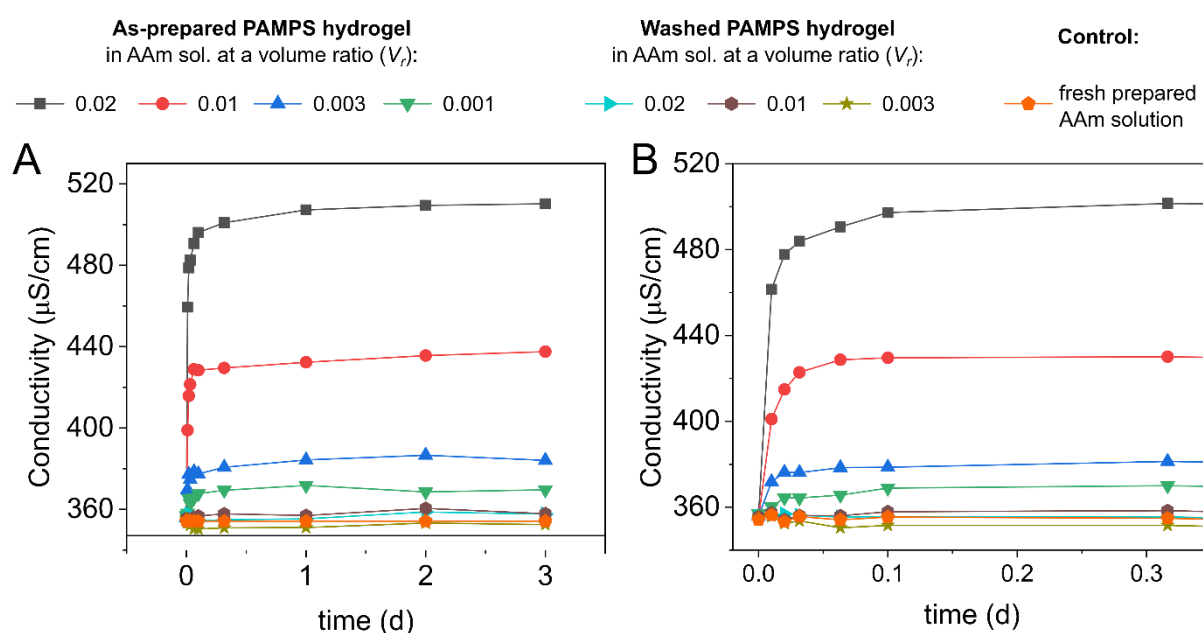


Figure 24: Time-lapse analysis on the conductivity of 2nd network precursor solution. The conductivity of AAm solutions (2-0.1-0.1) was measured over the time course of (A) 0 - 3 days, (B) with zoom-in on 0.0 – 0.3 days, when as-prepared or washed PAMPS hydrogels (1-3-1) were immersed in the solution at volume ratios (V_r) ranging from 0.02 to 0.001 ($n = 1$ hydrogel per condition).

Since the conductivity in the AAm solutions increased the impact of this increase was analyzed in relation to the hydrogels surface potential. Therefore, an aliquot of the precursor solution was removed from the bath solution to synthesize a PAAm hydrogel. These hydrogels were analyzed via the microelectrode technique and it was found, shown in **Figure 25A**; that the electric surface

potentials of SN PAAm hydrogels are inversely correlated with the volume ratio (V_r). However, for the SN PAAm hydrogel where the washed PAMPS hydrogel was immersed in the precursor solution the conductivity and the surface potential ($\Delta\Phi_s$) like that of the SN PAAm hydrogel from the control group where now PAMPS hydrogel was immersed in. These results show that the conductivity of the AAm precursor solution s are alters the potential of the PAAm hydrogels in a range of -20 mV to -55 mV. However, from these results so far it is not clear if this increase in surface charge is just related to the AMPS monomers or some other unknown factor. Therefore, the electric surface potentials of the PAMPS-co-PAAm hydrogels where investigated, shown in **Figure 25B**. The comparison of the surface potentials and stiffnesses of the SN PAAm hydrogels and PAMPS-co-PAAm hydrogels in **Figure 25C**, show how well these two sample groups overlap.

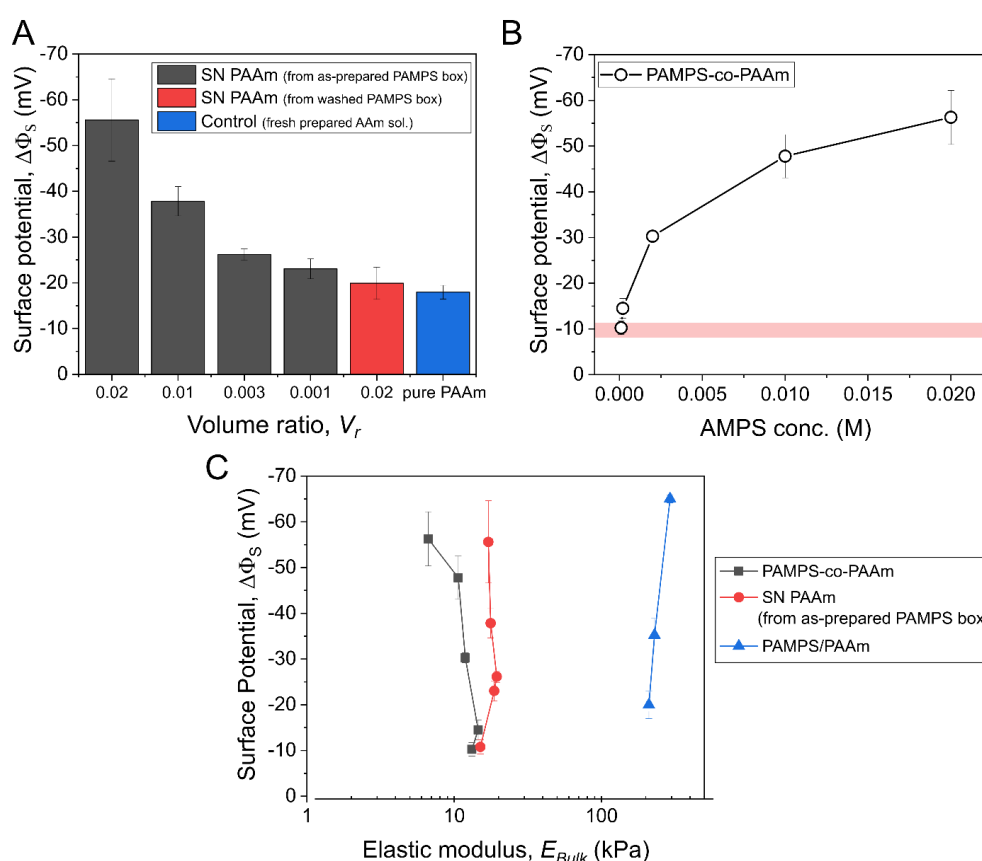


Figure 25: Electric surface potentials of SN and DN hydrogels based on the PAMPS hydrogel washing procedure. (A) The relation of volume ratio (V_r) on the potential of SN PAAm hydrogels (2-0.1-0.1) out of the 2nd network precursor solutions where either the as-prepared or the washed PAMPS hydrogel (1-3-1) was immersed for 3 days. (B) The impact of AMPS concentration (M) in the reaction mix on the hydrogel surface potential of PAMPS-co-PAAm polymers (formulations are found in **Table 4**). (C) Relationship between electric surface potential ($\Delta\Phi_s$) and hydrogel bulk stiffness (E_{Bulk}) for the tested hydrogels (Mean $\pm$ STD, $n = 3$ measurements per hydrogel).

This comparison fosters the idea that this surface potential changes are related just to the concentration of AMPS in the reaction mix. To see if the DN hydrogels show similar behavior, the as-prepared PAMPS hydrogels immersed in AAm solution at volume ratios (V_r) = 0.02, 0.02 and 0.03 were used to synthesis DN hydrogels. The mold substrate, borosilicate glass was used to synthesize DN hydrogels with a 2nd network surface layer. **Figure 25C** shows that the results for the electric surface potentials between SN PAAm and DN hydrogels are similar. As expected, the bulk elastic moduli differ between SN PAAm and DN hydrogels two groups. With these observations it is possible to reject the hypothesis that, the DN hydrogels surface potential is mainly related to the swelling discrepancy between bulk and surface because in this scenario the DN hydrogels immersed at different volume ratios (V_r) should have had the same surface potentials which was not the case.

4.4 Conclusion

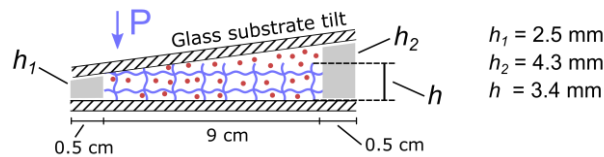
Basically, the surface potential between solution and gel is regulated by the ionic osmotic pressure, which is controlled by the hydrogels chain energy and chain density as well as the ionic strength of the solvent. Which means that for the PAAm single network hydrogel and the PAAm surface layers on DN hydrogels, both having a formulation of 2-0.1-0.1 (Monomer-crosslinker-initiator), the potential should be the same. Here however, it is shown why the previously observations in chapter 3 detected a difference between their electric potentials. It was shown here that unreacted monomers from the 1st network polymerization diffuse into the 2nd network precursor solution. In the 2nd network polymerization step this leads to the polymerization of PAMPS-co-PAAm polymer at the surface of the DN hydrogel with a PAMPS concentration of 10^{-3} to 10^{-4} M. These concentrations at first seem negligible, however it was shown that the DN hydrogels surface potential is drastically changed. With two methods it is possible to maintain the surface potential of the DN/Glass hydrogels. First, the PAMPS hydrogel needs to be washed after synthesis for 5 - 7 days with the proposed method to remove most of the residual AMPS monomers. Second, when the PAMPS hydrogel was immersed in the 2nd network precursor solution, the volume ratio (V_r) must be extremely small which would waste a lot of reagent solution. Since these results were obtained after the observations on cell growth and

gene expression of stem cells on DN hydrogels described in chapter 5 and chapter 6, the DN hydrogel surface potentials in these chapters varied in the range of -30 to -70 mV.

5 Application of the modified surface-bulk transition of double network hydrogels

5.1 Introduction

Hydrogels made from polyelectrolytes show many unique properties, such as low sliding friction on glass substrates in water and cell attachment^{20,91,92}. However, after introduction of the 2nd neutral network in such polyelectrolyte hydrogels to create a DN hydrogel, these properties change, so that cells hardly attach to the DN hydrogel^{23,24,104}. Yet, evidence suggests that DN hydrogels from PAMPS and poly(dimethylacrylamide) have therapeutic potential by inducing in vivo articular cartilage regeneration^{19,105}, in which the surface physicochemical properties of the DN hydrogels should play an important role in the initial cell adhesion^{74,106} or morphology^{107,108}. Understanding and regulating cell-hydrogel interactions are among the most challenging problems in bioengineering, and are affected by hydrogel characteristics including charge density¹⁰⁹, water content⁸⁴, hydrophilicity¹¹⁰ and mechanical properties⁷⁸. Therefore, investigating the applicability of DN hydrogels in bioengineering and cell cultivation is of interest.



Scheme 3: Polymerization mold assembly to synthesize a DN hydrogel with surface layer thickness gradient. This cross-sectional view demonstrates how the 1st network PAMPS hydrogel (blue lines, PAMPS 1-3-1) soaked in 2nd network precursor solution (red dots, AAm 2-0.1-0.1) is sandwiched between two glass plates (length: 10 cm, width: 10 cm).

With the described method in chapter 3 it is possible to adjust the DN hydrogels surface potential and elastic modulus, by the choice of the synthesis mold and the concentration ratio of the 1st and 2nd network. By specifically controlling the spatial distribution of the two networks, a complex structure is achieved where the second network preferentially organizes at the surface, and the bulk consists of a mixture of both networks. To demonstrate that the thickness of this surface layer is adjustable, the PAMPS hydrogel was sandwiched between glass plates in a mold with a gradual increase in spacer thicknesses to form as surface layer gradient DN hydrogel (**Scheme 3**). Therefore, the hydrogel is

surrounded by a 3D printed spacer (grey) with a height starting from 2.5 mm (h_1) on the left and gradually increasing up to 4.3 mm (h_2) on the right, to form a surface layer thickness gradient out of the 2nd network precursor solution containing AAm.

During the 2nd network synthesis, the 1st network hydrogel was partially compressed on one side, through the spacer of height h_1 , thinner than the hydrogel thickness h , reaching a maximum compression of ($P = 50\text{kPa}$). On the other side, a spacer of height h_2 , thicker than the hydrogel was used, where the gap between the PAMPS network and the glass substrate was filled with excess AAm solution. The maximum gap distance creates a surface layer thickness of 0.9 mm ($t \sim h_2 - h_1$) in the as-prepared state. Because of the spacer height difference, a structural change at the surface-bulk transition zone through sample preparation should be induced. The formed surface layer gradient should drastically change from a negative potential related to the PAMPS/PAAm concentration ratio to the neutral potential of the PAAm surface layer. This change of surface electric potential ($\Delta\Phi_s$) might have a drastic impact on cell adhesion.

First the surface potential as well as the evaluation of the thickness gradient were analyzed by the microelectrode technique introduced in chapter 3. Second, the cell adhesive properties of DN hydrogels synthesized and characterized in chapter 3, as they have surface potentials ranging from -50 to -170 mV (**Figure 21**) related to that of the gradient DN hydrogel. Because the gradient DN hydrogel has a tilted surface it is not applicable to cell culture, since the cells would rather start rolling and fall off the hydrogel surface and land in the niche between the petri dish and the hydrogel. To investigate if cell morphology is not just affected by the surface potential it was related to the DN hydrogels and the corresponding PAMPS SN hydrogels stiffness and polymer surface density.

5.2 Experimental section

5.2.1 Materials

In this section the hydrogels synthesized and characterized in chapter 3 were deployed in the following experiments. Unless otherwise noticed, all chemicals and solvents used in cell culture were purchased from Sigma-Aldrich Inc, United States. The human mesenchymal stem cell (MSC) line derived from

umbilical cord blood (UCB) (JCRB1110 UCB408E6E7TERT-33) was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Japan).

5.2.2 Hydrogel cross-section structure observation

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

5.2.3 *In vitro* cell culture and morphology

Hydrogel membranes were equilibrated and sterilized in HEPES-buffer and then incubated in culture medium overnight. The Dulbecco's modified Eagle's culture media was supplemented with 10% fetal bovine serum (Thermo Fisher Sci.), 1% Penicillin/Streptomycin and 1% L-glutamine. The mesenchymal stem cells were expanded on polystyrene dishes prior to cell seeding on hydrogel membranes. On day 0, trypsinized single-cell suspensions of stem cells were tested by trypan blue dye live/dead exclusion. Cell cultures with a viability over 95% were seeded onto hydrogel membranes at a concentration of 1×10^5 cells/mL and incubated for 3 days at 37°C and 5% CO₂ in humidified atmosphere. Cell cultivation was stopped on day 3 and cell morphologies were imaged through phase-contrast microscopy (OLYMPUS - IX73, Japan). Obtained images were converted to binary and the extended particle analyzer tool (BioVoxel Toolbox) in FIJI ¹¹¹ was used to measure ferret diameters of multicellular aggregates and cell spreading area of adherent cells.

5.3 Results and discussion

5.3.1 Control of the surface layer thickness

To develop DN hydrogels with spatially modulated surface properties, the DN hydrogel was synthesized in a polymerization mold where the glass substrate was tilted which allows the regulation of the surface layer thickness, and through that, the control of the surface electric potential (**Figure 26A**). The cross-section view of the DN hydrogel shows that there is a clear difference in structure of the surface layer and the bulk region, as shown by the microscopic observations at position 8 cm

(**Figure 26A**). The position is determined as the distance of the sampling position to the edge of the compressed hydrogel side. While the surface layer of $t \sim 800 \mu\text{m}$ thick (**Figure 26A**, 1) shows a big porous structure, in the bulk material (**Figure 26A**, 3) a hierarchical structure of big pores filled with smaller ones is observed. The transition between the surface layer and the bulk is a clear cut (**Figure 26A**, 2, white dotted line), with a transition zone thinner than $20 \mu\text{m}$. The electric potential depth profiles measured at different positions reveal the gradual increase of the surface layer thickness with increasing distance to the compressed side of the DN hydrogel. Three typical depth profiles are shown in **Figure 26B**. The electric potential between the gel and the bath solution on the compressed side is above -100 mV (Distance: 0 cm), related to the DN bulk potential. At positions of 2 cm and 6 cm , the surface layer thickness increased so that the surface potential is weakened to a value of -20 mV , related to the PAAm surface layer. The increase in surface thickness along the distance from the compressed side, analyzed by the electric potential depth profiles, is summarized in **Figure 26C**.

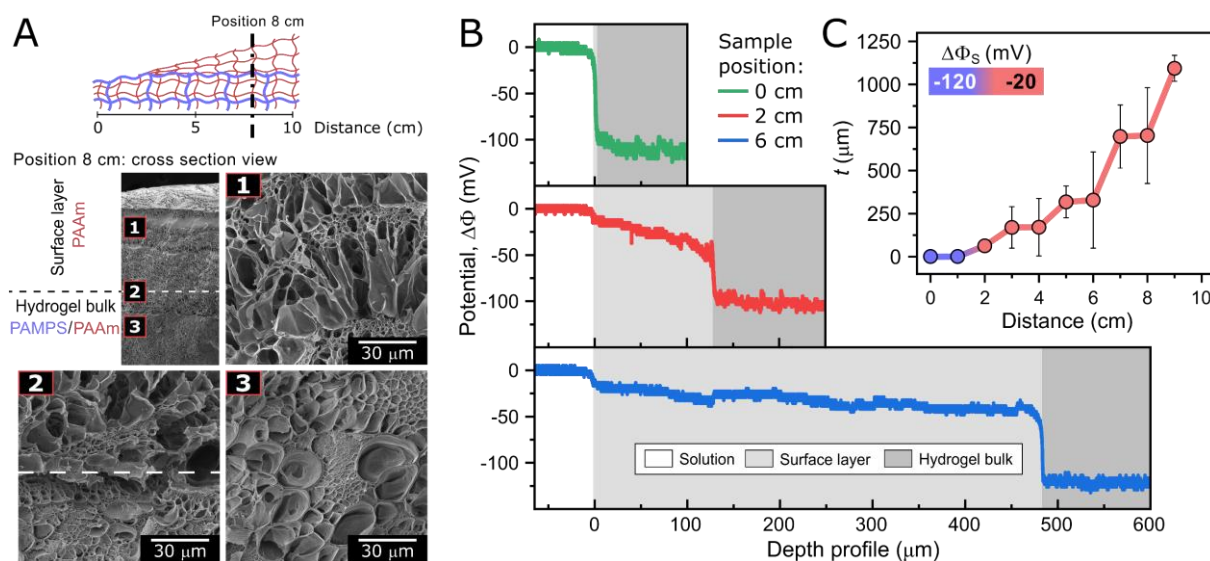


Figure 26: Control of PAAm layer thickness on the DN/Glass surface from micro-to-macro scale. (A) The 2nd network polymerization setup where the spacer heights (**Scheme 3**) and the compressive stress regulate the surface layer thickness of the DN hydrogel (PAMPS/PAAm, 1-3-1/2-0.1-0.1). The DN hydrogel SEM cross-sectional view reveals a surface layer with thickness $t \sim 800 \mu\text{m}$, with structural details on the PAAm surface layer (1), the transition zone (2, white dotted line represents the surface-bulk transition) and the DN bulk structure (3). (B) Selected electric potential depth profiles of the DN hydrogel at specific positions showing the surface layer thickness gradient. (C) Position profile of surface layer thickness and the corresponding surface potential ($\Delta\Phi_s$) visualized by a blue (strong charged) and red (weak charged) color map ($n = 2 - 5$ measurements per position).

On the compressed side (Position: 0 cm), no surface layer is formed, and the surface potential of the gel is the same as the bulk, showing a value of -120 mV. The surface potential abruptly changes from -120 mV to -20 mV between positions of 1 and 2 cm, due to the formation of surface layer. With the increase of the distance to the compressed side, the surface layer thickness gradually increases and reaches values higher than 1 mm at the position of 9 cm, while the surface potential remains constant at -20 mV. Consequently, I obtained a hydrogel surface with a strong negatively charged region ($\Delta\Phi_s = -120$ mV, blue) that switches within 1 cm to an almost neutral ($\Delta\Phi_s = -20$ mV, red) region. Since the surface layer thickness increases with the distance from the compressed side, the surface modulus integrated from the surface layer and the bulk might change with the distance as well¹¹². Such a surface might be used for studying the modulus effect on cells with a constant surface chemistry.

5.3.2 Cell adhesion to DN hydrogels with varied surface potential

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

The **Figure 27** shows the morphology of the mesenchymal stem cells after 3 days of incubation on various hydrogels with different surface potentials. When the hydrogel surface potential was weaker than -60 mV, as seen on PAAm and DN/Glass hydrogels, cells did not adhere to the surface, instead they aggregated to multicellular spheroids. On hydrogels with surface potential stronger than -100 mV, as seen on PAMPS, DN/Glass-C, and DN/Si-PET hydrogels, cells started to adhere. On the SN PAMPS hydrogel the individual cells were circularly spread, while on the DN/Glass-C and DN/Si-PET hydrogels the cell shapes are more irregular and spindle-like (shown by white arrows in **Figure 27**). In case of DN/Si-PET hydrogels, on the low crosslinked sample (X_{X1} 1.5 mol%) dome-shaped adherent cell aggregates were observed, but as the crosslinking degree increased the cells gradually become slightly more spread and the dome-shaped aggregates were not observable anymore. The cell-substrate

adhesion is usually guided by protein adsorption^{113,114}. However, cell adhesion is a highly complex process and it was found that cell morphological changes are influenced by substrate roughness¹¹⁵ and mechanical properties^{74,106,112} as well. The results shown in **Figure 27** suggest that the surface electric potential is a major factor regulating cell morphology and it should be noted that the critical surface potential for cell adhesion varies from cell line to cell line⁸⁷.

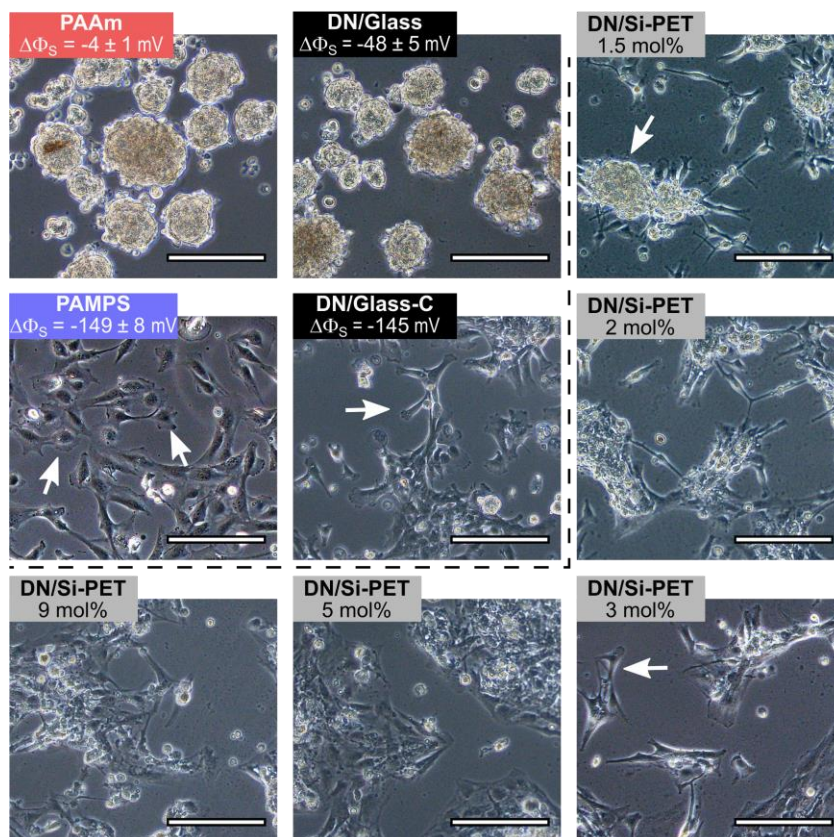


Figure 27: Increasing hydrogel surface potential induces cell attachment. Phase-contrast microscopic images (10 x magnification) of umbilical cord blood derived mesenchymal stem cells cultured on hydrogels for 3 days. Low surface electric potential facilitates stem cell aggregation (SN PAAm and DN/Glass) while high surface electric potential results in cell-hydrogel adhesion (SN PAMPS, DN/Glass-C, and DN/Si-PET). The scale bars represent 180 μm . The surface electric potentials for the DN/Si-PET hydrogels are shown in **Figure 21C**.

The detailed analysis on the cellular aggregates in **Figure 28A** demonstrates that the spheroid diameters on the hydrogels DN/Glass and the corresponding PAAm gel (2-0.1-0.1) are not significantly different. The diameters are constant for the whole range from 1.5 to 9 mol% of 1st network crosslinking (X_{X1}) in the bulk DN gel and show standard deviations of $\sim 100\mu\text{m}$. This shows that even the DN/Glass hydrogel with 9 mol% crosslinker, where the surface potential is the highest, the cells do not adhere or show interactions with the hydrogel surface. If the cells start to interact with the surface,

I assumed that the spheroid diameter becomes smaller as the interaction between cells is weakened. Differently, in **Figure 28B** on the adhesive SN PAMPS hydrogel the cell spreading area of the adherent cells increases with the crosslinking degree. This correlation between cell spreading area and crosslinker degree also happens in the DN/Si-PET sampling group, however in comparison to the corresponding SN PAMPS hydrogels to a smaller degree.

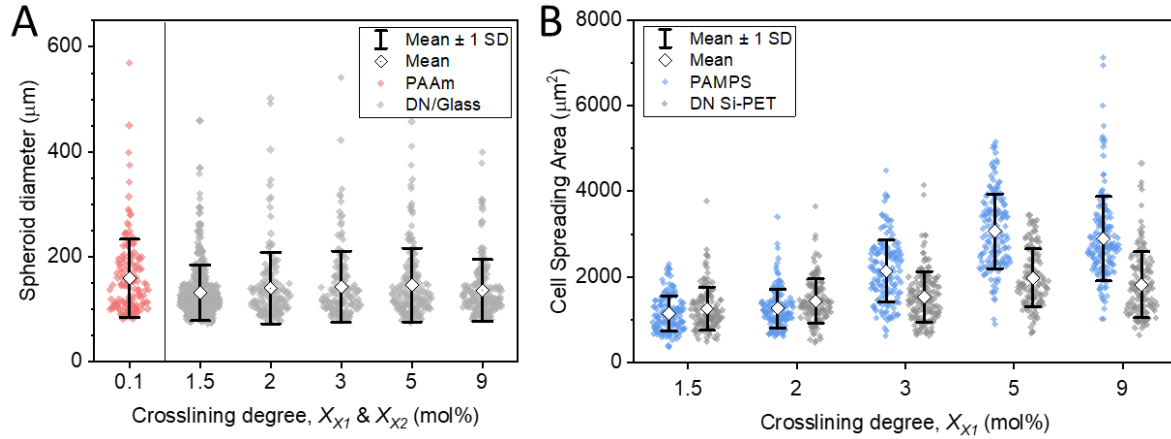


Figure 28: Quantification of the cell morphology on SN and DN hydrogels. The analysis of the cell spheroid diameter of UCB derived MSCs on SN PAAm (2-0.1-0.1) and DN/Glass (1- X_{X1} -1/2-0.1-0.1) and the cell spreading area of adherent cells on SN PAMPS (1- X_{X1} -1) and DN/Si-PET were quantified using the phase-contrast microscopic images (10 x magnification) as shown in **Figure 27** ($n \geq 127$ cells per sample group).

To investigate if cell morphology of adherent cells is affected by mechanical or chemical hydrogel properties the cell spreading area was related to the PAMPS density at the surface (mol/mm^2) and the hydrogel stiffness (E_{Bulk}). The relation in figure XX shows that cell spreading areas on SN PAMPS and DN/Si-PET increase with the PAMPS density. In the high-density region, above $0.015 \text{ mol}/\text{mm}^2$ PAMPS density, the cells spread much more on SN PAMPS than they did on the DN/Si-PET hydrogels. When the cell spreading is compared to the elastic modulus of the hydrogel, in **Figure 29B**, both spreading area increases with the elastic modulus. However, there is parallel shift between the PAMPS and DN/Si-PET data sets. Since the PAMPS and DN/Si-PET data sets overlap in the region up to $0.01 \text{ mol}/\text{mm}^2$ PAMPS, I assume that the charge density at the surface, related to the polyelectrolyte concentration, is a more dominant driving force in cell morphology than the hydrogel stiffness is, which shows no data set overlap (**Figure 29A**). In the proposed case the elastic modulus region is on the upper edge of the biologically significant range of elasticity which could be a reason for the small impact on

cell spreading⁷⁴. Further, it could be argued that on the DN/Si-PET cell spreading is slightly inhibited because the PAMPS attachment points are entangled with the 2nd network mesh and therefore not as moveable as on SN PAMPS hydrogels.

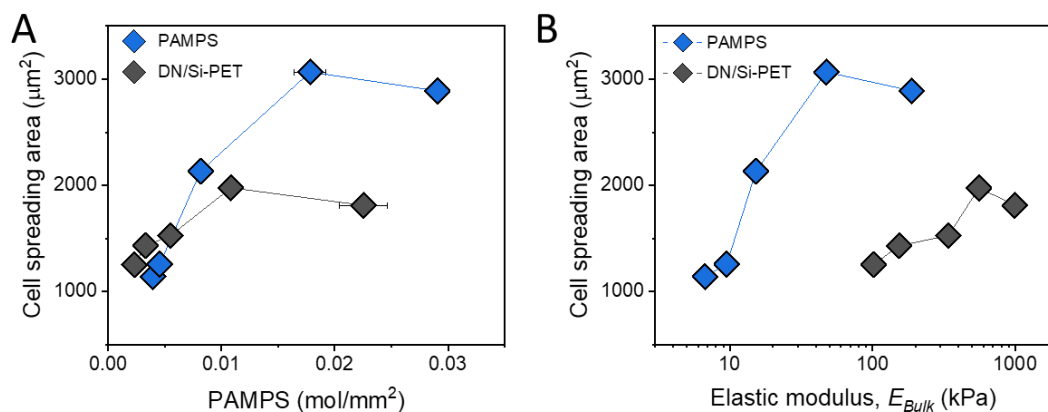


Figure 29: Relation of hydrogel properties to cell morphology. The cell spreading areas (Mean $\pm$ SEM) of UCB derived MSCs are related to the PAMPS surface density (mol/mm², Mean $\pm$ STD) and elastic modulus (kPa, Mean $\pm$ STD) of SN PAMPS and DN/Si-PET hydrogels. The data for the PAMPS surface density and elastic modulus were adopted from the hydrogel characterization in chapter 3 (**Figure 21** and **Figure 22**).

5.4 Conclusion

Here I demonstrated the applicability of the DN hydrogel surface modifications. Usually, DN hydrogels made from two chemically crosslinked networks are synthesized in a two-step approach, which is time consuming and work intensive. However, the second synthesis step is an easily accessible technique that fabricates not just a tough hydrogel but simultaneously functionalizes the hydrogel surface, which will be useful in controlling surface friction, cell scaffolding, and in vivo applications. It is shown that the surface layer thickness is precisely tunable from the micro and macro scale. The cell experiment proves that the previously observed phenomena, where cells do not adhere to DN hydrogels, is not valid for DN hydrogels in general, but is a consequence of the synthesis condition. Above a critical value, the surface PAMPS/PAAm ratios, applying to a wide range of DN hydrogel compositions, have just a minor impact on the surface electric potential and the cell morphology, since no big difference in cell spreading diameters were observed. This makes clear that publications investigating the interactions between cells and DN hydrogels must explicitly describe the utilized polymerization substrate and the related surface potentials, since it drastically changes the hydrogels cell-adhesive

properties. Further the relation to the elastic modulus shows that there is a slight impact on the morphology as it was described in various ways in the last decade. Recently, there is evidence that the induced stem cell state, of interest to cell therapies, is enhanced by cell aggregation. Therefore, the detailed hydrogel characterization and physicochemical decoupling based on the regulation of the surface-bulk transition described here is applied in the following chapter 6, to investigate how the DN/Glass hydrogels regulate gene expression in mesenchymal stem cells.

6 The effect of chemical and mechanical properties of hydrogels on mesenchymal stem cells

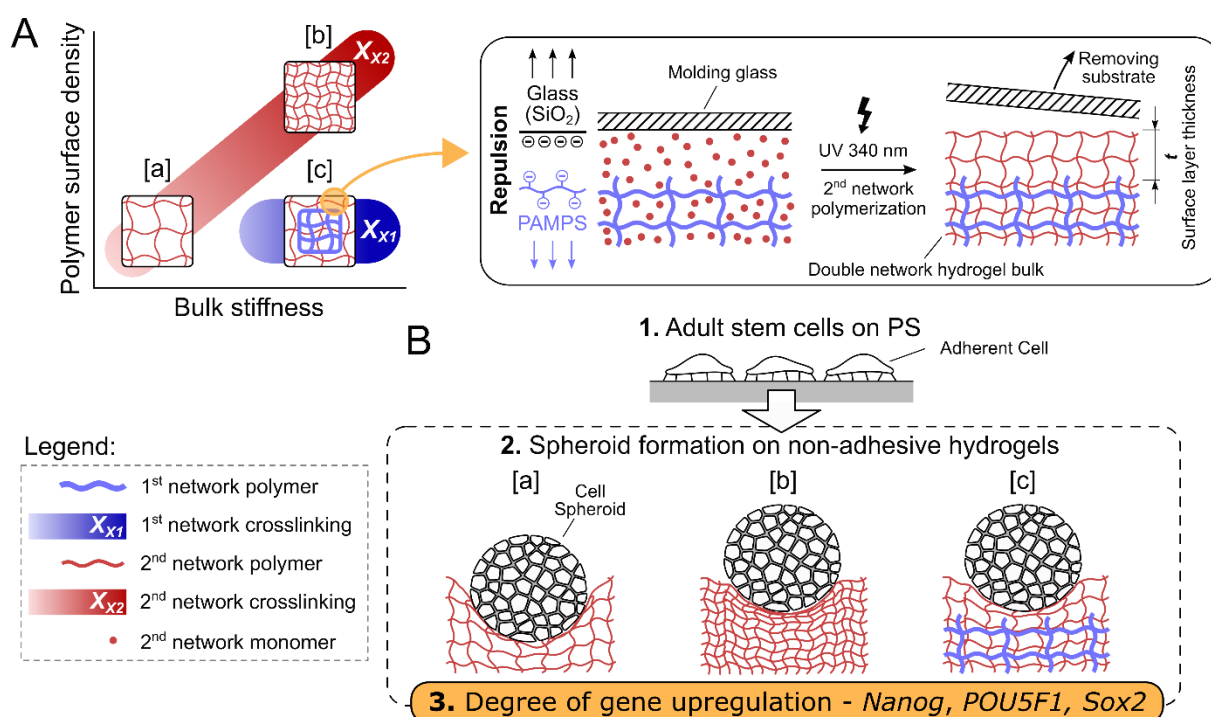
6.1 Introduction

A method commonly used for inducing and maintaining a pluripotent state is in vitro multicellular aggregation on low attachment substrates ^{116,117}, such as Poly (2-hydroxyethyl methacrylate) (PHEMA) and commercially available ultra-low attachment culture plates. Although this cultivation strategy has its clear advantages based on the high expression levels of angiogenesis ¹¹⁸, anti-inflammatory ¹¹⁹ and stemness ¹²⁰ proteins, little is known about the seemingly complex spheroid-surface interactions where cell to substrate adhesion is not clearly observable. Moreover, there is reasonable concern if physicochemical properties of scaffolds influence stem cell behavior since mostly cell-cell interactions are present in the spheroid growth cultivation strategy. Recent studies on membrane-based aggregation cell cultures using chitosan ³⁰, agarose ³¹ and polyacrylamide ^{32,33} presented evidence for substrate-based regulation of pluripotency. These results are not conclusive and should be interpreted with caution, since limited comparability prohibits valid conclusions, due to the lack of adequate control samples or inclusion of minimal amount of cell lines and substrates.

The use of DN hydrogels in cell culture has led in previous cases only to cellular aggregation. Here in this thesis, it was shown that the cell morphology highly depends on how the chemical structure at the DN hydrogels surface-bulk transition is modified. The application of DN hydrogel of earlier studies brought up evidence for the therapeutic potential of double network hydrogels in regenerative medicine, through inducing articular cartilage regeneration in large osteochondral defects by double network hydrogel implantation¹⁹. Since then, multiple studies on chondrogenic differentiation on hydrogels have supported the assumption that double network hydrogels have a potential in guiding cell differentiation and therefore might be involved in pluripotency expression, which still requires experimental evaluation ^{20–24}.

This section evaluates if mechanical or chemical hydrogel properties of DN hydrogels affect the gene expression of pluripotency genes in stem cells, that support the regeneration of tissue. Here it is

described how the development of a polymer-independent experimental technique allows the decoupling of hydrogel stiffness and polymer surface density based on the double network hydrogel fabrication method using glass as synthesis mold substrate (DN/Glass). I produced SN hydrogel controls that match either the polymer surface density (forth on referred as hydrogel [a]) or bulk elastic modulus (forth on referred as hydrogel [b]) or polymer surface density of the DN hydrogels synthesized on glass molding substrate (forth on referred as hydrogel [c]), (**Scheme 3A**). The DN and SN hydrogels were utilized in mesenchymal stem cell (MSC) cultures, where pluripotency marker gene expressions and the gene set enrichment were investigated (**Scheme 3B**).



Scheme 4: Setup to independently observe the effect of mechanical or chemical hydrogel properties on stem cell gene expression. (A) The fabrication of DN/Glass hydrogels facilitates the independent tuning of bulk stiffness and polymer surface density outside of the region that can be obtained with the modification of crosslinker density (X_{X2}) of SN hydrogels such as [a] and [b]. (B) The cell experiment includes the cultivation of stem cells on polystyrene (PS), the transfer of these cells to hydrogels [a] to [c] and the analysis of pluripotency gene expression of the obtain cell aggregates after the incubation of 3 days.

The growth of cells on the SN PAAm and the DN/Glass hydrogels, which have low surface potentials, should lead to the formation of cellular aggregates. To examine the role of the mechanical or chemical hydrogel properties on stem cell pluripotency on a broader scale, I deployed three 2nd network polymer types including poly(acrylamide) (PAAm), poly(dimethylacrylamide) (PDMA) and poly(N-(car-

boxymethyl)-N,N-dimethyl-2-(methacryloyloxy) ethanaminium, inner salt) (PCDME) in cell culture and used umbilical cord blood (UCB) and bone marrow (BM) derived mesenchymal stem cells as cell models. In relation to previous studies, this technique allows to draw a more precise conclusion since compares only cell aggregation cultures on hydrogels synthesized out of the same monomer. Ultimately, this result demonstrate that the gene expression patterns correlate with the hydrogels bulk elastic moduli, independent of polymer surface density. This finding contributes towards the understanding of substrate-based regulation on the pluripotent stem cell state in non-adhesive growth.

6.2 Experimental section

6.2.1 Materials

Chemicals for hydrogel synthesis were used as obtained from Wako Pure Chemical Industries, Ltd., Japan. The 1st network monomer 2-acrylamido-2-methylpropanesulfonic acid (AMPS) (Toagosei Co., Ltd., Japan) was used as received. The 2nd network monomer acrylamide (AAm), N,N-dimethylacrylamide (DMAAm) and N-(Carboxymethyl)-N,N-dimethyl-2-(methacryloyloxy) ethanaminium, inner salt (CDME) (Osaka Organic Chemical Co., Ltd., Japan) were used as received and molecular structures are found in **Figure 30**. The crosslinker N,N'-methylenebis(acrylamide) (MBAA) and initiator 2-oxoglutaric acid were used as obtained from Wako Pure Chemical Industries, Ltd., Japan. Unless otherwise noticed, all chemicals and solvents used in cell culture and cell analytics were purchased from Sigma-Aldrich Inc, United States. Human mesenchymal stem cell (MSC) lines derived from umbilical cord blood (UCB) (JCRB1110 UCB408E6E7TERT-33) and bone marrow (BM) (JCRB1140 UBE6T-6), were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Japan).

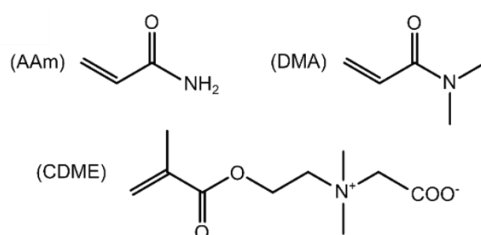


Figure 30: Chemical structure. Structure of monomers used in the 2nd network polymerization of DN hydrogels deployed in stem cell culture.

6.2.2 Hydrogel characterization and formulation

The hydrogel characterization of the chemical composition was performed via ATR/FT-IR and the microelectrode technique described above in chapters 3.2.5 and 3.2.6 respectively. The mechanical performance of the hydrogels was tested by microindentation and AFM measurement described in chapter 3.2.7. The formulations of DN hydrogels composed of PAMPS/PAAm, PAMPS/PCDME and PAMPS/PDMAAm are shown in **Table 5**. The hydrogels were washed with water to remove cytotoxic unreacted, acrylate monomers ^{121,122}, which was detected by conductivity measurements described above (chapter 4.2.3).

Table 5: Hydrogel sample codes and formulations Chapter 6

Sample code	PAMPS - 1 st network			PAAm - 2 nd network		
	Monomer C_{M1} (M)	Crosslinker* X_{X1} (mol%)	Initiator* X_{I1} (mol%)	Monomer C_{M1} (M)	Crosslinker* X_{X1} (mol%)	Initiator* X_{I1} (mol%)
PAMPS	1	1.5, 2, 3, 5, 9	1	-	-	-
DN/Glass (PAAm)	1	1.5, 2, 3, 5, 9	1	2	0.1	0.1
PAAm	-	-	-	2	0.1 - 4	0.1
PDMA DN/Glass	1	3, 5, 9	1	2	0.1	0.1
PDMA	-	-	-	2	0.1 - 8	0.1
PCDME DN/Glass	1	3, 5, 9	1	2	0.1	0.1
PCDME	-	-	-	2	0.1 - 8	0.1

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

6.2.3 Hydrogel surface roughness measurements

The surface roughness of hydrogels equilibrated in HEPES-buffer solution (pH 7.4) was measured on a Keyence VK 9710 (Keyence, Japan) laser scanning microscope. Hydrogel surface scans obtained with a Z-axis resolution of 0.05 μm and Z measurement distance of 5 μm at 50x magnification on an area of 50 x 50 μm were analyzed with the manufacturers software to determine the arithmetical mean deviation-based surface roughness (R_a) as previously described ¹²³.

6.2.4 *In vitro* cell culture and morphology

Mesenchymal stem cells were expanded on polystyrene dishes (PS) prior to cell seeding on hydrogel membranes. Hydrogel membranes were equilibrated and sterilized in HEPES-buffer and then incubated in culture medium overnight. On day 0, trypsinized single-cell suspensions from mesenchymal stem cell passages 1 – 7 were tested by trypan blue dye live/dead exclusion. Cells with viabilities more than 95% were seeded onto hydrogel membranes (*Table 8*) and PS controls at a

concentration of 1×10^5 cells/mL and incubated for 3 days. Cells on hydrogels and PS were incubated in DMEM supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Sci.), 1% Penicillin/Streptomycin and 1% L-glutamine at 37°C and 5% CO₂ in humidified atmosphere. Cell cultivation was stopped on day 3 and cell morphologies were imaged through bright field microscopy (OLYMPUS - IX73, Japan). Obtained images were converted to binary and the extended particle analyzer tool (BioVoxxel Toolbox) in FIJI ¹¹¹ was used to measure ferret diameters of multicellular aggregates.

6.2.5 RT-qPCR gene expression

After the cells were collected on day 3, total RNA isolation was performed with an RNeasy® Mini Kit (QIAGEN N. V., Netherlands) according to the manufacturers isolation protocol. Of the obtained mRNA samples with acceptable quality (wavelength absorbance ratio, 260 nm/280 nm >1.8), tested on a Nanodrop 2000C (Life Technologies, Thermo Fisher Sci.), cDNA was synthesized by using a SuperScript™ VILO™ cDNA Synthesis Kit (Life Technologies, Thermo Fisher Sci.). RT-qPCR (reverse transcriptase -, quantitative - PCR) was performed with 5 – 10 ng cDNA, 900 nM forward- and 900 nM reverse primer and 10 µL Power SYBR™ Green PCR Master Mix 2x (Applied Biosystems, Life Technologies) per well on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Life Technologies), using the comparative $\Delta\Delta C_q$ method. Firstly, the target gene expression was normalized to a cell differentiation- and cell source - independent reference gene triplicate ¹²⁴, encoding *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *TATA-binding protein (TBP)* and *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)* and secondly normalized to the control group on PS dishes. Standardization ¹²⁵ of biological replicates $n \geq 3$ was performed in accordance to MIQE guidelines ¹²⁶. In all figures, relative gene expression is reported as relative fold change ($-\Delta\Delta C_q$). The primer sequences for reference genes and genes of interest were designed (**Table 6**), using Primer3 software v.4 ^{127,128} and Primer-BLAST (NCBI website, National Center for Biotechnology Information). All RT-qPCR runs were performed with a hot start (95 °C, 10 minutes), 45 amplification cycles (95° C, 15 seconds and 61 °C, 1 minute) and step-and-hold

melting curves from 60 °C to 95 °C in 0.3 °C steps. Primer and RT-qPCR run efficiencies were analyzed via dilution series on PCR 96-well plates and LinRegPCR software v.11^{129,130}.

Table 6: RT-qPCR primer design

Accession no.	Gene		Primer sequence (5' - 3')	Amplicon length [bp]
NM_002046	<i>GAPDH</i>	Forward	AGCCACATCGCTCAGACAC	66
		Reverse	GCCCAATACGACCAAATCC	
NM_003406	<i>YWHAZ</i>	Forward	TCATACAAAGACAGCAGCT	105
		Reverse	TTCCCTCCTTCTCCTGCTT	
NM_003194	<i>TBP</i>	Forward	AGTTCTGGGATTGTACCGCA	116
		Reverse	CGCTTGGGATTATATTCGGCG	
NM_024865	<i>NANOG</i>	Forward	GAAGTGTGTCTCTTCCACCC	118
		Reverse	TGTTTGTAGCTGAGGTTCAAGGA	
NM_002701	<i>POU5F1</i>	Forward	CCAGGACATCAAAGCTCTGC	135
		Reverse	TGGCTGAATACCTTCCCAAAT	
NM_003106	<i>SOX2</i>	Forward	CACATGAACGGCTGGAGCAA	205
		Reverse	GCTGCGAGTAGGACATGCTG	

6.2.6 Protein expression by Western blotting

Cells were washed with PBS and lysed in RIPA lysis buffer for 10 minutes on ice. After centrifuging the cell lysates at 15.000 rpm and 4 °C for 10 minutes, the total protein in the extracted supernatant was quantified by a Bradford assay¹³¹ based protein kit (Bio-Rad, Life Technologies). Then, 15 µg of the total protein per sample were fractionated in a 13% SDS-PAGE¹³², transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked for 1 hour in TBS-T buffer containing 5% skim milk. PVDF membranes were probed with primary antibodies for anti-AKT (rabbit, #9272 Cell Signaling Technologies, U.S.) as a control protein and anti-NANOG (rabbit, #4903 Cell Signaling Technologies) by overnight incubation at 4°C. Immune complexes were then exposed to peroxidase-coupled rabbit secondary antibodies for 1 hour. Proteins were detected through chemiluminescence by Amersham ECL Western Blotting Detection Reagent (GE Healthcare, U. K.) and the signal was acquired via an Image Quant LAS 4000 mini (GE Healthcare) system.

6.2.7 Microarray transcriptome profiling and gene set enrichment analysis (GSEA)

Cell harvest and isolation was performed on day 3 with a RNeasy® Mini Kit. RNA integrity number (RIN) was evaluated on a 2100 Bioanalyzer and 2200 TapeStation system (Agilent, CA, U.S.). For each sample, 0.1 µg of total RNA with RIN > 9, were labeled with Cyanine-3 (Cy3) based on a Low Input Quick Amp Labeling Kit (one color, Agilent) protocol. Cy-3 labeled cRNA was fragmented and hybridized to a

SurePrint G3 Human GE 8x60K Microarray Ver3.0 (Agilent) chip using a Gene Expression Hybridization Kit (Agilent) and Gene Expression Wash Pack (Agilent). At a scan resolution of 3 μm , the dye signal was detected on a G2600D SureScan (Agilent) Microarray Scanner. Data processing such as background subtraction, signal intensity processing (Lowest and highest 2% of signal data were removed) and signal normalization via the global scaling method was performed individually for each array by Feature Extraction Software 12.0.3.1 (Agilent). Lists containing genes with differences in expression levels of more than 4-fold between SN and DN hydrogels were compiled (**Table S 1**, **Table S 2**). Gene set enrichment analysis with modified Fisher's exact test ($p \leq 0.05$) was performed via utilizing DAVID bioinformatics resources^{133,134} to identify significantly enriched biological processes that are related to gene ontology (GO) terms (**Table S 3**, **Table S 4**).

6.2.8 Statistical analysis

Unless otherwise noticed results are displayed as mean $\pm$ SD. Statistical analysis was performed with Origin (OriginLab Corp.) software, by one-way analysis of variance (ANOVA) with a follow-up mean comparison via Tukey test. Results were considered as suggestive evidence, if $P < 0.05$ (*) and significant evidence if $P < 0.005$ (**).

6.3 Results and discussion

6.3.1 Analysis of the surface bulk-transition region of DN hydrogels deployed in cell culture

To investigate the polymer surface density of DN/Glass hydrogels containing PDMA and PCDME as 2nd network, a calibration curve out of the polymer weight fraction (WM2) obtained by gravimetric analysis and the peak height of polymer specific peak of the infrared spectra obtained by ATR/FT-IR spectroscopy. The method to obtain the calibration curve for PDMA and PCDME like that demonstrated for PAMPS and PAAm shown chapter 3.3.2 in **Figure 22**. The **Figure 31** shows that no swelling discrepancy (Q_r) between the volume in H₂O and in D₂O occurred ($Q_r = (h_{\text{H}_2\text{O}}/h_{\text{D}_2\text{O}})^3$). Using the maximum absorbance of the peak at 1608 cm⁻¹ and 1627 cm⁻¹ for PDMA and PCDME respectively, a linear relation between the polymer weight fraction and the corrected absorbance ($A_{\text{max}} \times Q_r$).

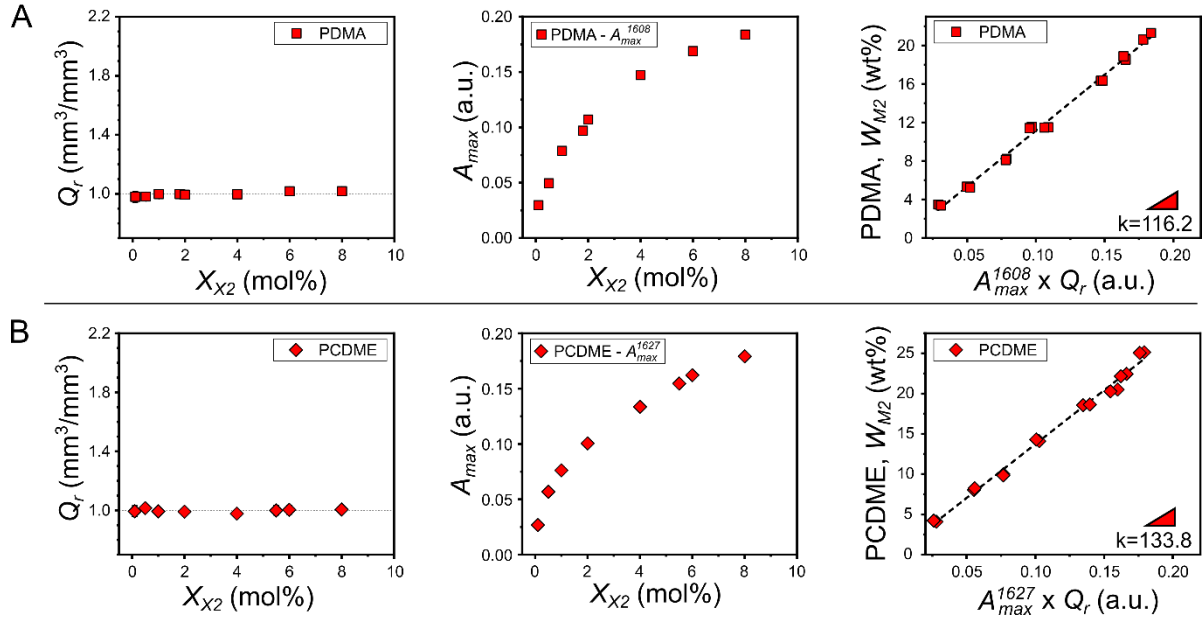


Figure 31: Calibration curve to investigate surface polymer density of DN/Glass hydrogels containing PDMA and PCDME. The calibration curve is determined by the infrared spectroscopy peak heights corrected by the swelling ratio ($A_{max} \times Q_r$); ($Q_r = (h_{H2O}/h_{D2O})^3$) and the weight fraction (W_{M2}) of PDMA and PCDME (wt%), determined by gravimetry.

The summary of the linear fit for the polymer surface density calibration curves for PAMPS, PAAm, PDMA and PCDME was summarized in **Table 7**. In comparison with the previous observation PAMPS and PAAm the new calibration curves as well have good a good fit and an r^2 -value above 0.99.

Table 7: Calibration curve fit parameters for 1st and 2nd network surface density

Linear fit parameters ($y = d + kx$)	1 st network	2 nd network		
	PAMPS*	PAAm*	PDMA	PCDME
Intercept (d)	0.084 ± 0.014	2.366 ± 1.046	-0.456 ± 0.297	0.356 ± 0.388
Slope (k)	116.8 ± 4.5	146.5 ± 13.9	116.19 ± 2.51	133.79 ± 3.14
r^2	0.9956	0.9401	0.9935	0.9923

y: W_{M1} or W_{M2} , x: $A_{max} \times Q_r$

*PAMPS and PAAm values were introduced in Chapter 3

For the cell cultivation DN/Glass hydrogels and corresponding SN hydrogels of PAAm and PDMA and PCDME were synthesized with the formulations found in **Table 5**. The evaluation on polymer surface density, surface-bulk transition depth, bulk and surface elastic modulus of PAAm based SN and DN hydrogels was extensively described in chapter 3. The surface characterization of SN and DN hydrogels with PDMA as 2nd network in **Figure 32A** demonstrate that the IR absorption spectrum of the DN/Glass gel (PAMPS/PDMA 1-3-1/2-0.1-0.1) shows that only PDMA network is present at the hydrogel surface since the absorption peak at 1042 cm^{-1} for PAMPS 1st network is not quantifiable at the DN hydrogel

surface. Since borosilicate glass was used as synthesis mold the thickness of the surface-bulk transition phase (t) on the DN/Glass gels investigated and resulted in a value of $7.3 \pm 1.5 \mu\text{m}$ (**Figure 32B**) and has an electric potential like PAAm based DN/Glass hydrogel. **Figure 32C** displays the polymer surface density independent tuning of the bulk elastic modulus capability of the PDMA based DN/Glass hydrogels.

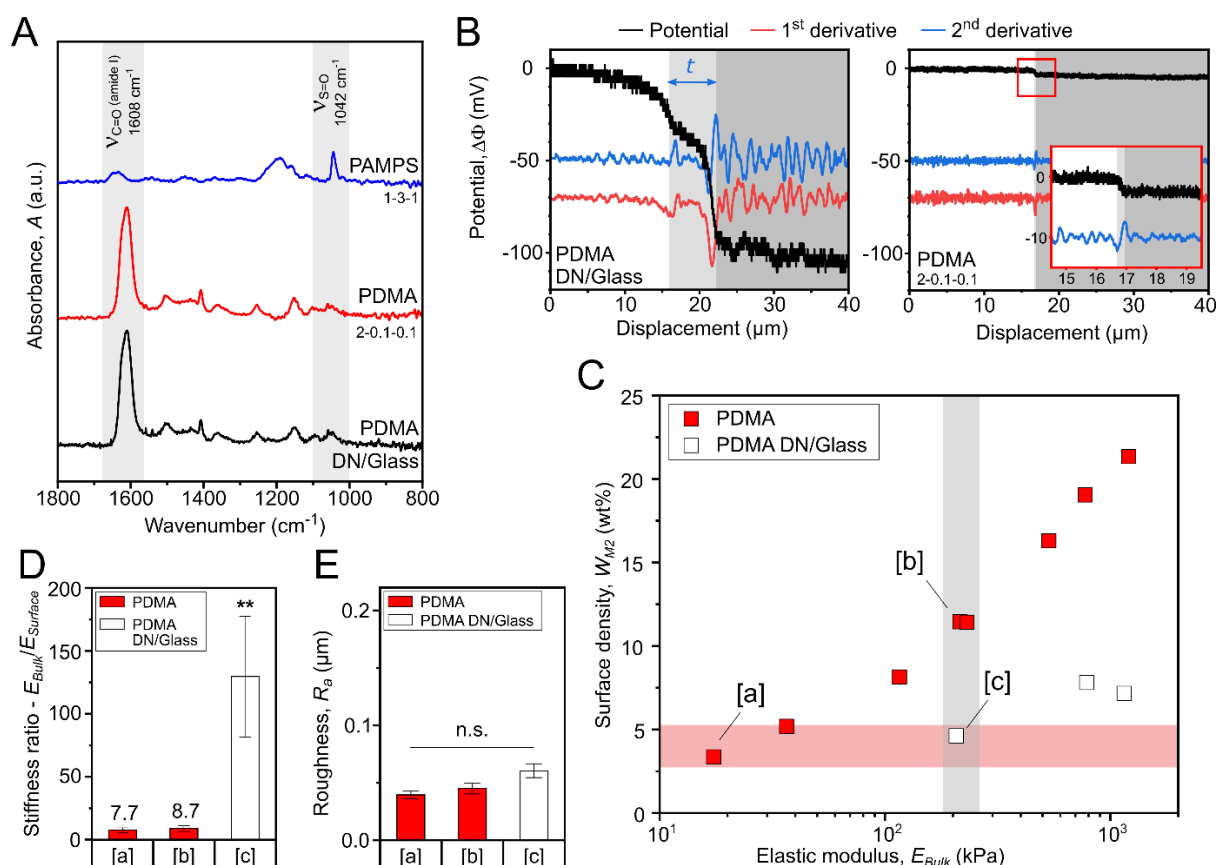


Figure 32: Surface characterization of SN and DN hydrogels with PDMA as 2nd network. (A) The IR absorption spectrum of SN PAMPS (1-3-1), SN PDMA (2-0.1-0.1) and the corresponding DN/Glass gel. (B) Electric potential profiling on PDMA DN/Glass (1-3-1/2-0.1-0.1) and the corresponding SN PDMA hydrogel in 10^{-4} M NaCl bath solution ($n = 3$ hydrogels, mean $\pm$ SD, white band: solvent, light grey band: T, dark grey band: hydrogel bulk). (C) The relation between PDMA surface density (W_{M2}) and bulk elastic modulus of the SN and DN hydrogels ($n = 1$ hydrogel per point). (D) The stiffness ratio obtained from the hydrogel bulk (E_{Bulk} , $n = 3$ hydrogels with 2 measurements, mean $\pm$ SD) and the surface (E_{Surface} , $n = 1$ hydrogel with 21 – 44 measurements, mean $\pm$ SD) via microindentation and AFM measurements for SN samples [a] and [b] and the corresponding DN sample [c]. (E) The hydrogel surface roughness of samples used in the cell culture experiment ($n = 2$ with 2 measurements, mean $\pm$ SD).

The letters [a], [b] and [c] the hydrogels deployed for the follow-up cell culture experiments. The stiffness ratio in **Figure 32D**, of the hydrogel bulk surface elastic modulus shows a significant difference between DN/Glass hydrogel [c] and SN hydrogels [a] and [b], because the bulk consists out of a stiff double network structure, whereas the surface contains weak crosslinked PDMA. **Figure 32E** shows that there is no difference in hydrogel surface roughness between samples [a] to [c].

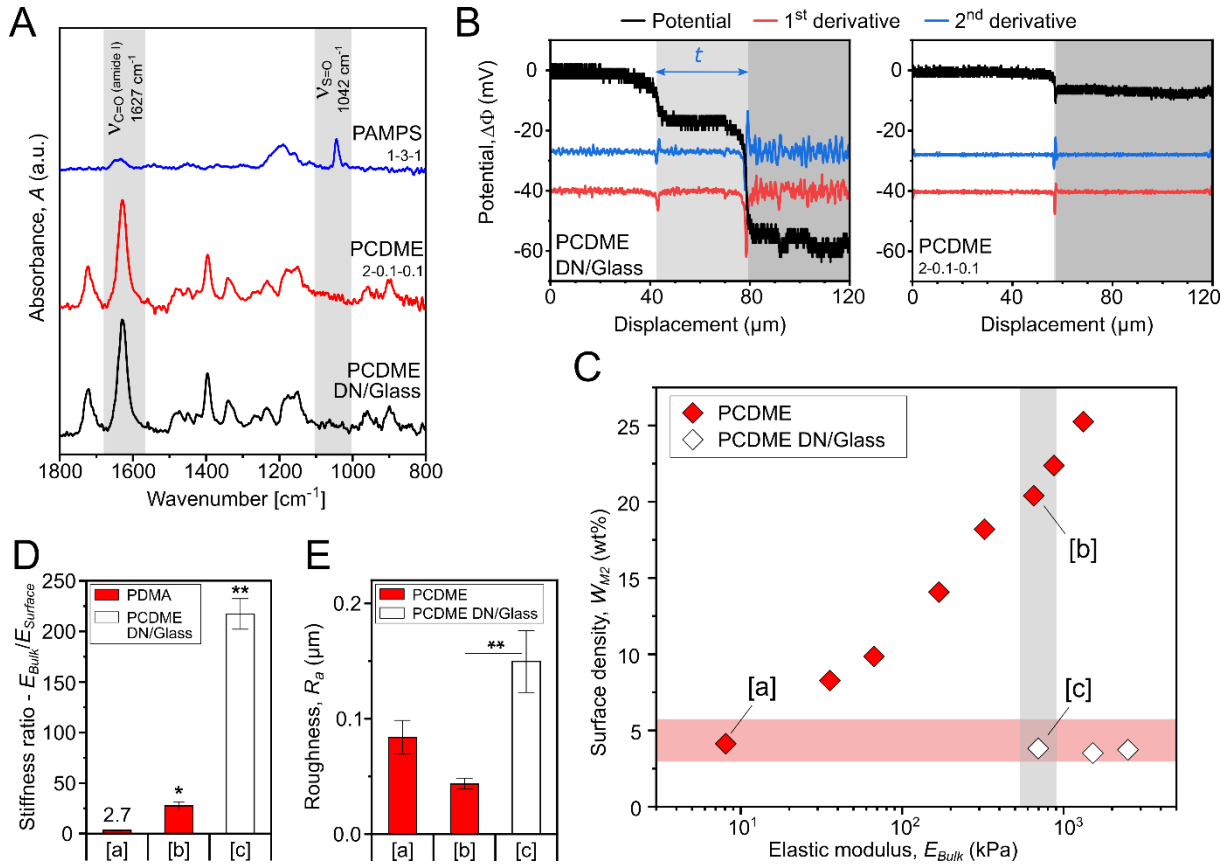


Figure 33: Surface characterization of SN and DN hydrogels with PCDME as 2nd network. (A) The IR absorption spectrum of SN PAMPS (1-3-1), SN PCDME (2-0.1-0.1) and the corresponding DN/Glass gel. (B) Electric potential profiling on PCDME DN/Glass (1-3-1/2-0.1-0.1) and the corresponding SN PCDME hydrogel in 10⁻⁴ M NaCl bath solution (n = 3 hydrogels, mean ± SD, white band: solvent, light grey band: T, dark grey band: hydrogel bulk). (C) The relation between PCDME surface density (W_{M2}) and bulk elastic modulus of the SN and DN hydrogels (n = 1 hydrogel per point). (D) The stiffness ratio obtained from the hydrogel bulk (E_{Bulk} , n = 3 hydrogels with 2 measurements, mean ± SD) and the surface ($E_{Surface}$, n = 1 hydrogel with 21 – 44 measurements, mean ± SD) via miroindentation and AFM measurements for SN samples [a] and [b] and the corresponding DN sample [c]. (E) The hydrogel surface roughness of samples used in the cell culture experiment (n = 2 with 2 measurements, mean ± SD).

Analog to the results of the PDMA hydrogel is the PCDME hydrogel characterization. However, differences are that the IR absorption spectra for PCDME have the characteristic peak for the carboxy group at 1627 cm^{-1} wavenumber (**Figure 33A**). The thickness of the surface-bulk transition phase (t) on the DN/Glass gels of $27.9 \pm 10.7\text{ }\mu\text{m}$ is significantly thicker than on the PAAm and PDMA DN/Glass hydrogels (**Figure 33B**) of similar formulation (1-3-1/2-0.1-0.1). The display of the decoupling capability of PCDME based hydrogels in **Figure 33C** demonstrates which hydrogels (SN hydrogels [a], [b] and DN hydrogel [c]) were picked for the follow-up cell culture experiment. The stiffness ratio from hydrogel bulk (E_{Bulk}) to surface ($E_{Surface}$), shows a significant difference between DN/Glass hydrogel [c] and SN hydrogels [a] and [b], because the bulk consists out of a stiff double network structure whereas the surface contains weak crosslinked PCDME (**Figure 33D**). This difference in material composition near the surface leads to swelling discrepancies and a rougher surface on the PCDME DN/glass hydrogel (**Figure 33E**).

6.3.2 Cell aggregation on hydrogel scaffolds

Here, the cell aggregation capability of umbilical cord blood (UCB) and bone marrow - derived mesenchymal stem cells (MSCs) cultured on SN PAAm, SN PDMA, SN PCDME and the corresponding DN/Glass hydrogels was evaluated. Therefore, hydrogel scaffolds listed in **Table 8** were deployed in cell culture.

Table 8: Physicochemical properties of hydrogel scaffolds used in cell culture

Hydrogel properties	Single network		Double network
	[a]	[b]	[c] DN/Glass
Formulation: PAAm	<i>PAAm 2-0.1-0.1</i>	<i>PAAm 2-3.5-0.1</i>	<i>PAMPS/PAAm 1-3-1/2-0.1-0.1</i>
Polymer density, W_{M2} (wt%)	$4.22 \pm 0.06^{\alpha}$	$14.91 \pm 0.16^{\alpha, \beta}$	$5.58 \pm 1.44^{\beta}$
$E_{Surface}$ (kPa)	$3.52 \pm 0.29^{\gamma}$	$13.55 \pm 2.47^{\gamma, \delta}$	$5.07 \pm 0.54^{\delta}$
E_{Bulk} (kPa)	$29.6 \pm 2.2^{\epsilon, \zeta}$	$258.9 \pm 28.5^{\epsilon}$	$245.7 \pm 30.3^{\zeta}$
Formulation: PDMA	<i>PDMA 2-0.1-0.1</i>	<i>PDMA 2-1.8-0.1</i>	<i>PAMPS/PDMA 1-3-1/2-0.1-0.1</i>
Polymer density, W_{M2} (wt%)	$3.36 \pm 0.15^{\alpha}$	$11.42 \pm 0.16^{\alpha, \beta}$	$4.62 \pm 0.35^{\beta}$
$E_{Surface}$ (kPa)	$2.26 \pm 0.14^{\gamma}$	$24.29 \pm 2.76^{\gamma, \delta}$	$1.54 \pm 0.52^{\delta}$
E_{Bulk} (kPa)	$17.4 \pm 0.6^{\epsilon, \zeta}$	$215.3 \pm 6.9^{\epsilon}$	$198.9 \pm 30.2^{\zeta}$
Formulation: PCDME	<i>PCDME 2-0.1-0.1</i>	<i>PCDME 2-5.5-0.1</i>	<i>PAMPS/PCDME 1-3-1/2-0.1-0.1</i>
Polymer density, W_{M2} (wt%)	$4.12 \pm 0.19^{\alpha}$	$20.4 \pm 0.68^{\alpha, \beta}$	$3.83 \pm 0.55^{\beta}$
$E_{Surface}$ (kPa)	$3.29 \pm 0.24^{\gamma}$	$24.34 \pm 2.16^{\gamma, \delta}$	$3.23 \pm 0.17^{\delta}$
E_{Bulk} (kPa)	$8.8 \pm 0.1^{\epsilon, \zeta}$	$654.2 \pm 21.9^{\epsilon}$	$697.5 \pm 27.2^{\zeta}$

$\alpha, \beta, \gamma, \delta, \epsilon, \zeta$ represent $P < 0.005$

The cell morphologies were analyzed after 3 days of incubation and show that in all cases cell aggregate to spheroids, whereas on polystyrene they grow adherent (**Figure 34A**).

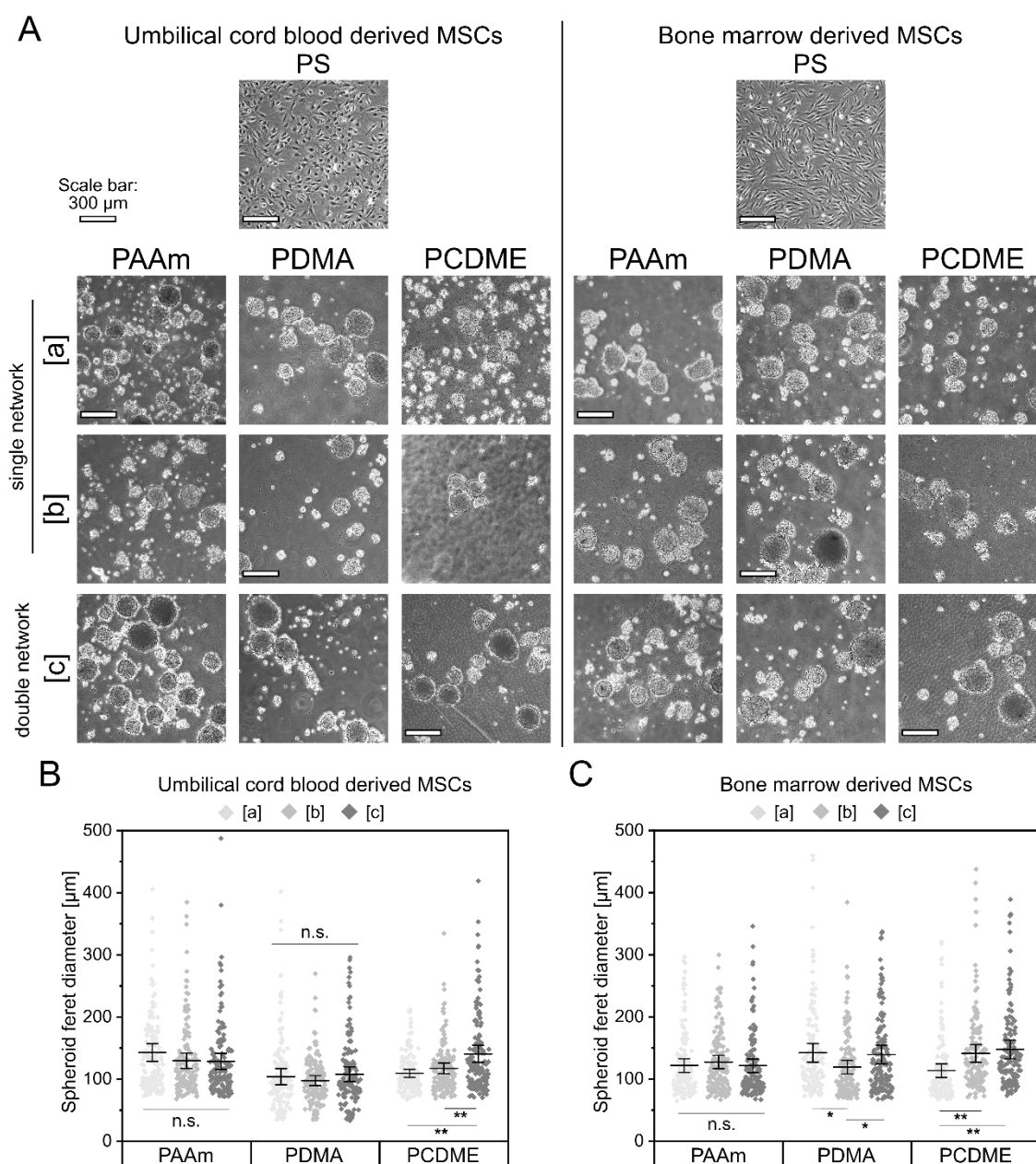


Figure 34: Morphology of UCB and BM derived MSCs on utilized cell substrates. (A) The display of adherent MSCs on PS and MSC aggregates on scaffolds [a], [b] and [c] after 3 days of incubation (bright field microscopy, scale bar represents 300 μ m), deployed in cell culture to investigate the induction of pluripotency. The display of spheroid feret diameters of UCB (B) and BM (C) derived stem cells ($n = 3$ biological replicates containing altogether 150 spheroids, mean $\pm$ 99% CI, n.s. represents no significance) after the incubation for 3 days.

The analysis on spheroid diameters in **Figure 34B** and **Figure 34C**, respectively for UCB and BM derived mesenchymal stem cells, reveals mean diameters between 100 and 200 μ m which is below the diameter limit that causes hypoxia¹³⁵ of cells which would lead to cell death because of a low O_2

concentration in the spheroid core. On PAAm hydrogels no significant difference between spheroid diameters was observed. However, on PDMA and PCDME based hydrogels spheroid diameters vary significantly which could lead to a difference in gene expression or to a low reproducibility rate of these experiments.

6.3.3 Impact of stem cell passage number on pluripotency gene expression

Prior to the gene expression experiment it needs to be evaluated whether the designed primer pairs for the reference and target genes are specific for their sequences which was analyzed by the RT-qPCR melting curve method. Since the DNA sequences, amplified by the polymerase chain reaction, denaturation at a temperature that is related to the sequence length and the chemical structure of the base pairs the loss of fluorescent signal based on the denaturation can be monitored. As **Figure 35** shows, for the reference genes *GAPDH*, *YWHAZ* and *TBP* as well as for the target genes *NANOG*, *POU5F1* (formerly *Oct3/4*) and *SOX2* only one peak per spectrum is observable. This result of the melting curves shows, that only one specific sequence was amplified proofing the specificity of the primer pairs to one sequence each.

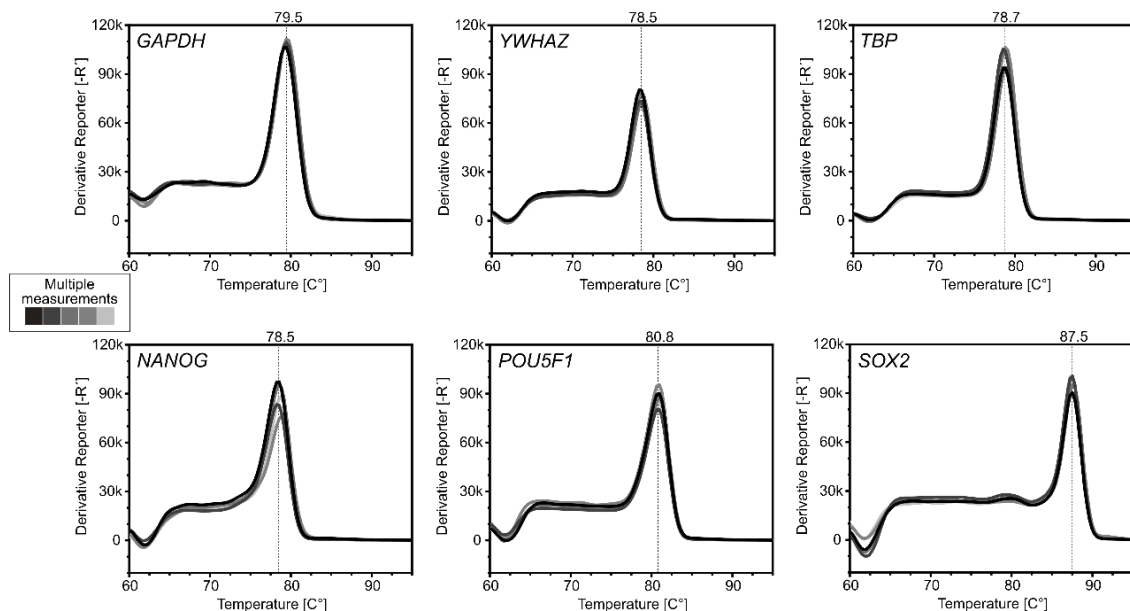


Figure 35: RT-qPCR primer validation. The melting curves after RT-qPCR, of each reference gene (GAPDH, YWHAZ and TBP) and target gene (NANOG, POU5F1 and SOX2) (lines from black to light grey represent, n = 5 technical replicates).

Next, the ΔC_q values of the control group of mesenchymal stem cells on polystyrene dishes were investigated to test, how the gene expression of the cell line changes over time, from passage to passage. A cell passage number is defined by how often cells must be removed from a cultivation chamber to keep them growing, since in in vitro culture, cells are contained in vessels of restricted growth areas and cultivation media volumes such as polystyrene dishes. The ΔC_q value defines the difference in threshold cycles between one target gene and the mean of the reference genes. If this value drastically changes over time, the gene expression results between earlier and later passages cannot be compared since the control group has changed. For stem cell line the gene expression of cell passages needs to be analyzed as the gene expression changes over time^{120,136}. In **Figure 36** the ΔC_q values for the target genes are displayed and reveal that in the early passages from 1 to 4 ΔC_q for a specific gene does change within an acceptable range (± 0.5). However, after passage 4 the ΔC_q value drastically changed. Therefore, in the follow up gene expression experiments only stem cells from passages ≤ 4 are used.

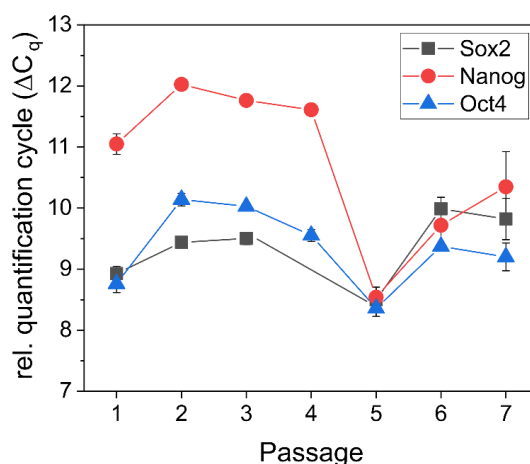


Figure 36: Relation of the ΔC_q value to the passage number. The gene expression of UCB derived MSCs cultured on polystyrene is displayed for the target genes *NANOG*, *POU5F1* and *SOX2* from passage 1 to 7 (n = 3 technical replicates).

6.3.4 Effect of decoupling on UCB derived MSCs gene expression and transcriptome profiles

Here, UCB - derived MSCs were exposed to hydrogel scaffolds [a], [b] and [c] (**Table 8**) for 3 days to investigate how the chemical and mechanical properties of hydrogels containing PAAm as 2nd network affect the pluripotent state of stem cells. On all three hydrogel scaffolds, UCB - derived MSCs tend to

aggregate and form multicellular spheres (**Figure 37A**) with no significant differences between cell aggregates on SN and DN hydrogels (**Figure 37B**), even if the roughness, R_a (**Figure 37C**) of the DN hydrogel sample [c] is significantly different. Therefore, there is no evidence that roughness, over an R_a range of 0.03 – 0.12 μm , affects multicellular aggregation of this cell line on hydrogel scaffolds with PAAm surfaces.

Transitioning from the morphological observation to mRNA gene expression in **Figure 37F**, a significant increase in the relative gene expression of pluripotency marker genes *NANOG*, *POU5F1* (also known as *OCT4*) and *SOX2* on SN hydrogel [b] related to SN hydrogel [a] was detected. Yet, comparison of SN hydrogel [b] to DN hydrogel [c] shows no significant difference. This expression pattern including scaffolds [a], [b] and [c], illustrates that in the UCB - derived MSCs, the pluripotent state is regulated by the hydrogel bulk stiffness and is independent of PAAm surface density, because the rigid SN hydrogel [b] and DN/Glass hydrogel [c] share similar bulk stiffness but have different 2nd network surface density as well as surface stiffness. On the other hand, bulk stiffness and pluripotent gene expression between the soft SN hydrogel [a] and rigid DN/Glass hydrogel [c] are different, even with a 2nd network surface density below 6 wt% for both hydrogels.

To confirm the results of the RT-qPCR, the protein expression levels of NANOG were analyzed by Western blotting. NANOG was selected because it was found that the relative mRNA gene expression levels of *NANOG* tend to be higher than those for *POU5F1* and *SOX2* (**Figure 37F**). As the band intensity in **Figure 37D** demonstrates, the loading reference protein AKT is expressed in almost equal amounts on scaffolds [a], [b], [c] and the PS control. The expression of NANOG protein however correlates with the mRNA expression levels detected by RT-qPCR, since the expression of NANOG protein was low in sample [a], high in samples [b] and [c]. On the PS control NANOG is almost not quantifiable which indicates replicative senescence on PS ¹³⁷.

To investigate if genes other than pluripotent markers follow the elastic modulus related expression pattern, a transcriptome profiling via microarray analysis was performed. I extract 215 genes following

the bulk elastic modulus pattern including 169 genes upregulated in low - and 46 genes upregulated in high – elastic modulus region (**Figure 37E**). For a comprehensive transcriptome profiling output, it was examined if genes could also be identified, following the 2nd network surface density pattern which was not yet investigated with RT-qPCR. This expression pattern involved genes equally expressed in sample [a] and [c] with a 2-fold expression difference to sample [b], which includes 12 genes upregulated in low and 174 genes upregulated in high 2nd network surface density conditions (**Figure 37E**).

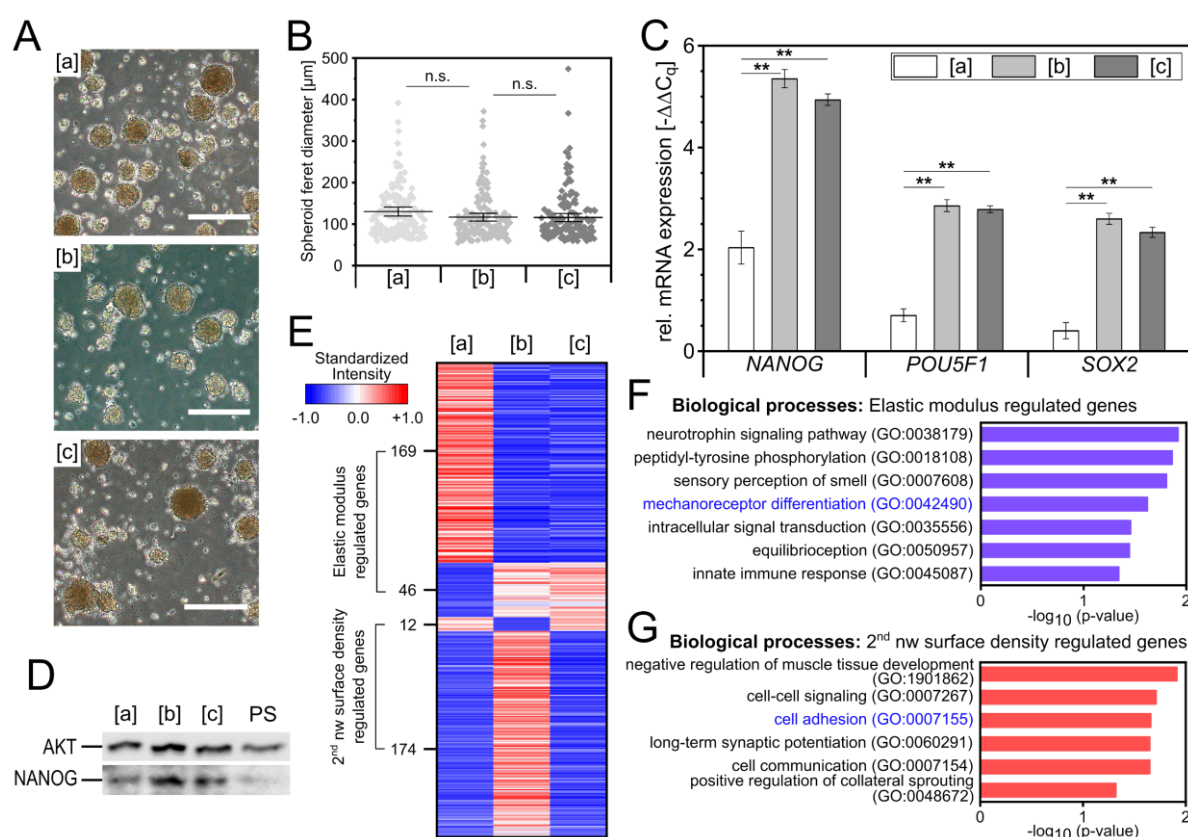


Figure 37: Cell culture of UCB - derived MSCs on single and double network PAAM scaffolds. (A) Morphology (Scale bars represent 360 µm) and (D) spheroid ferret diameter (n = 3 biological replicates containing altogether 150 spheroids, mean ± 99% CI, n.s. represents no significance) of UCB - derived MSCs after 3 days incubation and corresponding (C) hydrogel roughness. (F) The relative mRNA gene expression of pluripotent markers, namely NANOG, POU5F1 and SOX2 (RT-qPCR, relative to PS, n = 3 biological replicates from passages 2 - 4) and (B) Western blotting on AKT and NANOG proteins show a relation of pluripotency to bulk stiffness (n = 1). (E) Microarray analysis displayed as row-scaled Z-scores of gene expressions (n = 1, Fold change > 4) identified 401 genes following either elastic modulus or 2nd network surface density related expression patterns and related biological processes (G,H) identified by GSEA Hydrogel sample codes and properties of scaffolds [a], [b] and [c] are listed in **Table 7**.

To investigate how the stiffness signal is transduced and connects the scaffolds signal with pluripotent marker genes expression, which translate to proteins mainly located in the nucleus ¹³⁸, a GSEA transcriptome profiling was conducted. The analysis on the 215 elastic modulus regulated genes showed that genes *NRTK2* and *NRTK3* are part of significantly enriched gene sets described as the neurotrophin signaling pathway and mechanoreceptor differentiation (**Figure 37G, Table S 1, Table S 3, Table S 4**). Differing compared to the list on 186 2nd network surface density regulated genes, that includes integrin subunit *ITGAL*, gap junction protein *GJB1* and protocadherin *PCDHA2* which are associated with the biological processes of cell adhesion, cell-cell signaling and cell communication (**Figure 37H, Table S 2, Table S 3, Table S 4**). Overall these results provide evidence that the pluripotent state of UCB - derived MSCs cell aggregates is related to mechanoreceptor differentiation and affected by elastic modulus independent of PAAm 2nd network surface density.

6.3.5 Effect of 2nd network polymer type and stem cell line on pluripotency gene expression. Here the cell culture experiment from the previous section was conducted on hydrogels using 2nd network polymers out of DMA and CDME and an additional MSC cell line derived from bone marrow (BM) to observe if pluripotency expression shows similar outcome to confirm the pluripotency regulation via elastic modulus on a broader scale. While DMA was chosen because of its structural similarity to AAm (**Figure 30**), the zwitterionic CDME was deployed because of its exceptional anti-fouling properties and the resistance to temperature, pH and ionic strength induced swelling¹². Both monomers show high swelling ratios in water and are considered neutrally charged which guaranteed multicellular aggregation of MSCs.

Firstly, the detected mRNA expression patterns on PAAm scaffolds are similar between UCB and BM derived MSCs from scaffolds [a], [b] to [c] after 3 days of incubation (**Figure 38**). However absolute gene expression values of UCB - derived MSCs are higher in relation to BM - derived MSCs on the same hydrogel scaffold. Secondly, the choice of 2nd network polymer type changes the gene expression pattern. On scaffolds with PDMA as the 2nd network, the pluripotency marker gene expression is significantly different from scaffold [a] with the lowest expression, over hydrogel [b] to [c] with highest

gene expression. Similar gene expression trends are observed in MSCs on PCDME hydrogels but not as distinct as demonstrated on PAAm or PDMA because expression on PCDME SN hydrogel [a] tends to be higher than expression on corresponding PAAm and PDMA scaffolds [a]. The trend from previous section continues, that on each scaffold the absolute $-\Delta\Delta C_q$ values of *NANOG* are higher than the values of *POU5F1* and *SOX2* independent of cell line or 2nd network polymer type.

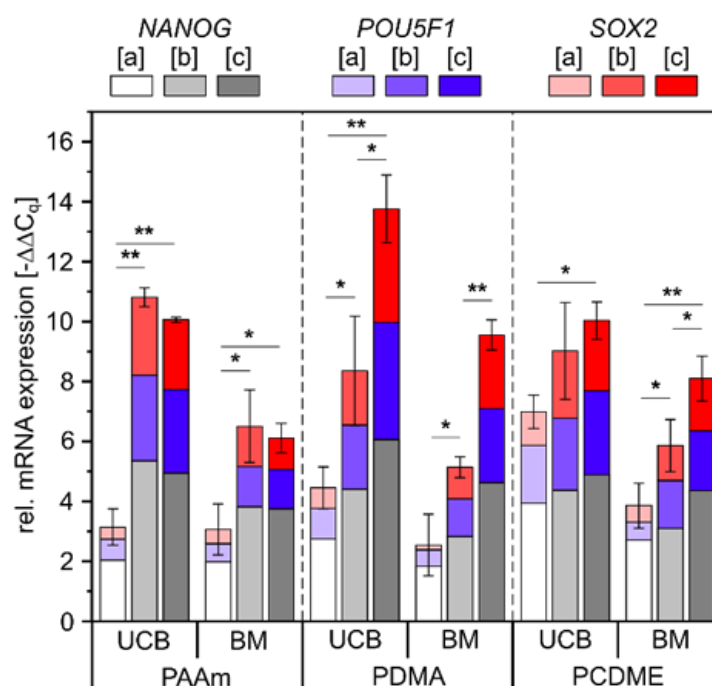


Figure 38: Pluripotent marker gene expression of UCB - and BM - derived MSCs on various hydrogels. (A) Molecular structure of monomers used to synthesize the second network of DN/Glass [c] and corresponding SN hydrogels [a], [b]. Variation of (B) NANOG, POU5F1 and SOX2 in MSC mRNA gene expression on PAAm, PDMA and PCDME hydrogels after 3 days of incubation (RT-qPCR, relative to PS, n = 3 biological replicates from passages 2 - 4). Hydrogel formulations and properties are listed in **Table 8**.

After decoupling the hydrogel stiffness from polymer surface density, it is possible to separately discuss the two properties and reveal the following about their role in stem cell pluripotency. The gene expression analysis on the interaction between UCB - derived MSCs and PAAm scaffolds (**Figure 37**) clearly showed that the bulk elastic modulus affects the stem cell pluripotency. As a consequence, the pluripotent gene expression is not related to the DN hydrogels surface stiffness, since surface stiffness correlates with the polymer surface density. It can be speculated that the down regulation of neurogenic markers such as *NRTK2* and *NRTK3* governs the upregulation of pluripotency on stiff hydrogels since they are involved in mechanoreceptor differentiation. The down regulation of these

neurogenic markers is even more beneficial because their expression would lead to resistance to chemotherapy in breast cancers ¹³⁹. These results might indicate that within 3 days, rapidly a pluripotent state is induced by transferring cells from PS, where NANOG expression was largely decreased ¹⁴⁰, onto stiff PAAm hydrogel scaffolds. The secondary cell experiment on the additional MSC cell line and other polymer types supports the hypothesis that stiffness regulates pluripotency since gene expression on the stiff SN or DN scaffolds, [b] and [c] respectively, is significantly higher compared to the corresponding soft scaffold [a], except for UCB - derived MSCs on the stiff SN PCDME scaffold [b] (**Figure 38**). Interestingly, with PDMA scaffolds, the DN hydrogels are superior at inducing pluripotency even compared to the stiff PDMA SN scaffold [b]. A reason for that could be the variation of marker gene expression in the early passages of the control stem cell culture which were used in the gene expression experiments. It must be noted, that since the gene expression differ slightly between passage 1 to 4 and heave between passage 4 and 7, that experiments conducted on this UCB derived cell line after passage 4 should be interpreted with high caution.

Regarding the hydrogel chemical properties for UCB - derived MSCs on PAAm scaffolds, there was no correlation between pluripotent marker gene expression and 2nd network surface density. However, the GSEA showed that 2nd network surface density regulates biological processes such as cell-adhesion and cell-cell signaling, but it is uncertain if this upregulation is just localized in the outermost cells of the cell aggregate since these are the cells which guide the spheroid-substrate interaction¹⁴¹. The polymer surface density and polymer type likely are involved in cell spheroid formation since charge density and the related swelling degree affect interactions between cell membrane proteins and the polymer surface ^{82,142}. While most multi cellular aggregate diameters are consistent with previous findings ²⁷ and below the diameter limit that causes hypoxia ¹³⁵, cell spheroid diameter of BM derived MSCs on PDMA SN hydrogel [b] and both cell lines on PCDME DN/Glass [c] were significantly different (**Figure 34**). The decreased cell spheroid diameters on the PDMA scaffolds might be explained by a high polymer surface density that results in hydrophobicity-induced protein denaturation at the surface and therefore hinders the cell aggregation process. The sphere diameters on PCDME DN/Glass

hydrogels are increased but inconsistent with hydrogel roughness. However, an impact of polymer surface density cannot be excluded. Even with this limitation, pluripotency gene expression on PCDME scaffolds still follows the trends of PDMA scaffolds but to a lesser extent.

6.4 Conclusion

Previously, it was speculated whether there are scaffold induced interactions in the non-adhesive limit^{30,143} because proper control samples were lacking. For this purpose, I utilized DN hydrogels to decouple the mechanical from the chemical scaffold properties for use in membrane-based cell aggregation cultures. I succeeded in separately investigating the stiffness of the hydrogels through AFM and microindentation at the surface and the bulk respectively, as well as the chemical structure at the surface-bulk transition via ATR/FT-IR and electric potential depth profiles. I conclude that there is a surface polymer density independent relation between bulk scaffold stiffness and pluripotency of mesenchymal stem cells of cell aggregates. Further, stem cell expansion on DN hydrogels exceed the limitations observed on SN hydrogels such as low elasticities, low wettability and hampered cell aggregation capabilities as seen on high crosslinked PDMA hydrogels. These discoveries on scaffold induced pluripotent stem cells in multicellular aggregates, point out new material engineering possibilities in stem cell expansion applicable to stem cell therapy as well as cancer stem cell research. However, still there is the concern elaborated in chapter 4, describing the relation of residual AMPS concentration in the 2nd network polymer and the DN hydrogel surface potential, that small number of AMPS molecules at the DN/Glass hydrogel surface are governing the stem cell response, which would need evaluation in the future.

7 Conclusion

This study focused on the characterization of the surface-bulk transition of DN hydrogels and how the regulation of this region affects stem cells. Since it was not yet clear why cells do not adhere to DN hydrogels as they should, because of the negative charge of the first network, I was motivated to investigate in this direction. It is challenging however, to even investigate the hydrogel surface due to the highly hydrated state. But deploying a combination of ATR/FT-IR spectroscopy and the microelectrode technique has proven highly practical in that regard and let to reveal the following points.

In **chapter 3** it was revealed that in the surface-bulk transition region of DN hydrogel a microscale surface layer of the second network polymer is formed on top of the bulk gel. Because of this surface layer, a model was proposed, based on the surface characterization of double network hydrogel synthesized between glass plates, which assumed that the surface energy of the synthesis mold affects the surface composition on the gel. This model was confirmed by the altering the synthesis conditions of DN hydrogels so that the surface layer is removed, by using a low energy synthesis mold or by compression of the glass synthesis mold while polymerization.

Chapter 4 tried to investigate the question of, why the electric surface potential of the 2nd network surface layer on double network hydrogels is more negative than that of the corresponding single network hydrogel. Here it was shown that extremely small amounts of monomers, not reacted in the 1st polymerization step affect the surface potential of the double network hydrogel surface layer and that of the corresponding single network hydrogel. However, by the implementation of a hydrogel washing step before the immersion of the 1st network hydrogel in the 2nd network precursor solution the unreacted monomers were removed so that the surface potential of the DN hydrogel surface layer was regulated.

Chapter 5 showed that the characterization and modification have application in the control of surface friction and cell scaffolding. A DN hydrogel was synthesized, with a surface layer thickness gradient from the micro- to the macro-scale. Based on the orientation of thickness gradient it was

possible to regulate the spatial distribution of surface charge on the DN hydrogel. Previously, one major issue was that cell didn't adhere to DN hydrogel scaffolds. However, with the surface modifications proposed in this thesis I managed to switch-on or switch-off the cell adhesive properties of DN hydrogel surfaces by choice of the synthesis mold substrate, which expands the applicability of DN hydrogel as biomaterial.

Chapter 6 describes a method to prepare an experiment in which the individual tuning of mechanical and chemical hydrogel properties leads to answering if gene expression of cell spheroid cultures is affected by elastic modulus or polymer surface density of hydrogels. Here I showed that DN hydrogels are uniquely applicable for the decoupling of mechanical and chemical properties, as their surface structure is defined by the 2nd network formulation but the elastic modulus in the bulk is defined by the 1st to 2nd network ratio regulated by the 1st network crosslinking degree. I found that the gene expression of pluripotency genes in stem cells were upregulated on DN hydrogels because of the bulk stiffness and independent of polymer surface density. However, uncertainty factor in the evaluation of the pluripotency gene expression were the not removed unreacted monomers after 1st network polymerization and the expression variation between the cell passages

8 Bibliography

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9 Accomplishments

Original paper:

(1) Title: Modulation and characterization of the double network hydrogel surface-bulk transition

Martin Frauenlob, Daniel R. King, Honglei Guo, Seiichiro Ishihara, Masumi Tsuda, Takayuki Kurokawa, Hisashi Haga, Shinya Tanaka and Jian Ping Gong (DOI: 10.1021/acs.macromol.9b01399), *Macromolecules* 2019, 52, 6704.

(2) Title: Tough and Self-Recoverable Thin Hydrogel Membranes for Biological Applications

Ya Nan Ye, **Martin Frauenlob**, Lei Wang, Masumi Tsuda, Tao Lin Sun, Kunpeng Cui, Riku Takahashi, Hui Jie Zhang, Tasuku Nakajima, Takayuki Nonoyama, Takayuki Kurokawa, Shinya Tanaka, and Jian Ping Gong (DOI: 10.1002/adfm.201801489), *Adv. Funct. Mater.* 2018, 28 (31), 1801489

Papers under revision:

(1) Title: Rapid induction of reprogramming towards cancer stem cells on double-network hydrogels

Jun Suzuka, Masumi Tsuda, Lei Wang, Shinji Kohsaka, Karin Kishida, Shingo Semba, Sachiyo Aburatani, **Martin Frauenlob**, Takayuki Kurokawa, Shinya Kojima, Toshihide Ueno, Yoshihiro Ohmiya, Hiroyuki Mano, Kazunori Yasuda, Jian Ping Gong and Shinya Tanaka (under revision in *nBME*)

Conference:

(1) Title: Regulation of pluripotency through decoupling surface chemistry and polymer physics using double network hydrogels, Martin Frauenlob, Daniel R. King, Masumi Tsuda, Takayuki Kurokawa, Shinya Tanaka and Jian P. Gong, 2018/06, 67th SPSJ Annual Meeting - Nagoya, Japan, Oral

(2) Title: Regulation of pluripotency through decoupling surface and bulk effects using double network hydrogels, Martin Frauenlob, Daniel R. King, Masumi Tsuda, Honglei Guo, Seiichiro Ichihara, Takayuki Kurokawa, Hisashi Haga, Shinya Tanaka and Jian P. Gong, 2018/11, 6th ILSS International Life Science Symposium - Sapporo, Japan, Oral

(3) Title: Decoupling surface and bulk effects of double network hydrogels and their role in stem cell pluripotency, Martin Frauenlob, Daniel R. King, Masumi Tsuda, Honglei Guo, Seiichiro Ichihara, Takayuki Kurokawa, Hisashi Haga, Shinya Tanaka and Jian P. Gong, 2018/12, International Polymer Conference (ICP) 2018 – Hiroshima, Japan, Oral

Summer schools:

(1) Summer school in association with the doctoral dissertation: Advanced course on Cell Mechanobiology: Theory and experiments on the mechanics of life, 2018/09 in Udine, Italy

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Finally, I deeply thank my family, who without a adopt, always supported me in these three years far away from home.

11 Appendix

11.1 Appendix A: Supplementary Microarray tables

Table S 1: GSEA analysis related to elastic modulus.

Low Elastic Modulus upregulated: [a] > [b], [c]

Fold change > 4		Normalized signal intensity			
Gene count	169	Hydrogel samples - PAAm			
PrimaryAccession	GeneSymbol	EntrezGeneID	[a]	[b]	[c]
NM_001300778	ZNF140	7699	83.93	10.10	14.12
AK124504	NRG2	9542	254.62	19.95	12.87
NM_024980	GPR157	80045	131.64	23.42	13.53
NM_052995	CLRN1	7401	239.39	11.47	17.07
NR_040710	SNAP25-AS1	100131208	158.56	9.69	16.86
NM_001257357	RAB44	401258	146.94	33.51	9.68
ENST00000613959	ACTR3C	653857	248.55	19.55	19.88
NR_120621	LOC101927419	101927419	164.75	9.73	9.66
NM_212559	XKRX	402415	395.65	54.16	77.79
XR_171099	LOC283028	283028	162.30	16.58	11.42
ENST00000366579	HEATR1	55127	247.71	9.82	20.61
AK094921	LOC100131763	100131763	167.38	10.16	12.57
NR_038980	LOC100506207	100506207	327.94	22.81	75.25
NM_145651	SCGB1C1	147199	166.07	11.43	11.38
NR_046102	LOC646736	646736	465.20	10.56	10.50
NM_002009	FGF7	2252	426.35	84.66	31.22
NM_003874	CD84	8832	113.37	9.80	9.73
ENST00000361625	ZFYVE9	9372	257.40	28.91	11.40
NR_120368	LINC01584	101929701	77.72	9.66	9.60
XR_241571	LOC100507210	100507210	303.62	10.52	10.51
XR_425268	LOC102724630	102724630	172.51	9.90	11.39
NM_080827	WFDC6	140870	408.39	11.62	11.43
NM_177551	HCAR2	338442	299.95	50.47	62.29
NM_153209	KIF19	124602	470.71	28.28	31.49
AL831947	OVOS2	144203	416.19	39.86	27.59
NM_001170633	C12orf56	115749	267.05	28.79	28.34
XR_426854	LOC101928264	101928264	97.14	11.09	11.08
ENST00000494761	LOC101927346	101927346	392.06	42.17	93.74
NM_001080395	AATK	9625	780.21	10.91	10.89
NM_213621	HTR3A	3359	348.55	70.37	83.18
NM_001123228	TMEM14E	645843	196.69	9.68	16.59
NM_001145271	ADAMDEC1	27299	547.73	11.14	11.12
NR_024593	POM121L10P	646074	157.41	34.24	37.37
ENST00000431918	CLDN4	1364	68.09	10.08	10.43
NM_001007253	ERV3-1	2086	222.08	20.56	24.94
AK128253	LOC100132071	100132071	117.34	9.68	9.64
NM_003862	FGF18	8817	89.06	11.62	19.44
NR_015394	LINC00636	285205	91.60	15.83	11.32
NM_001080518	LIPK	643414	955.22	11.31	11.10
NM_138939	CD200R1	131450	284.90	17.74	10.89
NM_002262	KLRD1	3824	539.99	26.34	10.59

NM_145044	ZNF501	115560	143.51	27.94	32.08
NM_014256	B3GNT3	10331	508.71	116.34	113.13
NM_001291937	NTRK2	4915	239.67	9.87	9.85
NR_110433	LOC100505739	100505739	132.21	10.35	10.33
NM_018724	IL20	50604	560.78	15.29	10.29
ENST00000557418	FOXA1	3169	370.76	9.66	23.93
NM_001102562	MARCH11	441061	150.76	10.07	10.05
NM_014460	CSDC2	27254	123.57	10.23	10.20
NR_034018	LOC100130700	100130700	117.74	11.66	18.18
NM_022124	CDH23	64072	652.89	36.30	49.55
NM_002963	S100A7	6278	178.36	20.60	32.99
AB128931	LOC100128226	100128226	274.64	10.13	11.57
NR_105001	LOC101928769	101928769	229.42	10.21	10.18
NR_026992	LOC340017	340017	93.95	11.68	11.59
NM_144984	VSTM4	196740	167.95	29.17	9.62
NM_001278392	CEACAM18	729767	82.35	10.68	10.69
NM_001037329	CNGA4	1262	419.65	14.72	10.09
NM_198492	CLEC4G	339390	127.03	10.50	17.92
NM_001480	GALR1	2587	459.14	11.80	11.67
AK128528	LOC100130057	100130057	164.00	12.80	35.83
NM_001232	CASQ2	845	112.96	11.82	11.63
NM_173853	KRTCAP3	200634	296.56	30.99	13.07
ENST00000417609	LOC100507562	100507562	108.16	11.81	17.28
NM_001001346	CLDN20	49861	164.52	9.56	15.33
AK128478	LOC100129363	100129363	690.21	15.86	11.68
ENST00000624675	LOC100132249	100132249	133.24	11.80	17.28
ENST00000549993	LOC101928207	101928207	155.80	11.57	28.21
ENST00000479783	IQCA1	79781	85.95	9.55	9.51
NR_038873	LOC286059	286059	254.52	9.55	9.51
NM_006271	S100A1	6271	338.60	9.54	11.79
NM_001005288	OR51I1	390063	88.49	9.53	9.50
NR_109923	LAMA5-AS1	101928158	116.75	9.54	14.01
ENST00000567718	LOC100996478	100996478	80.32	11.55	11.49
NM_001012338	NTRK3	4916	447.40	37.93	72.32
XR_429170	LOC102724811	102724811	338.54	15.58	28.41
NR_105010	LINC01333	101929082	70.61	10.01	10.00
NM_024603	BEND5	79656	509.06	10.01	10.01
NR_110951	LINC01258	101928776	301.15	9.49	9.47
NR_109811	ATP11A-AS1	100874205	90.34	9.45	9.42
NM_004709	TMEM257	9142	72.70	9.48	9.45
NM_001145312	ETV3	2117	602.41	33.65	16.10
NM_001243168	PTCRA	171558	117.31	9.82	20.56
NM_207371	SKIDA1	387640	88.55	9.43	9.40
XR_426728	LOC102723542	102723542	345.38	9.42	9.40
NM_004801	NRXN1	9378	174.85	11.43	15.86
NM_032037	TSSK6	83983	164.32	9.51	27.62
NM_133261	GIPC3	126326	203.11	39.66	47.08
NM_003836	DLK1	8788	103.58	9.40	17.11
ENST00000316296	ANO1	55107	257.09	9.43	9.41
ENST00000360835	SCUBE1	80274	269.28	9.43	17.91
AK295906	LOC100507516	100507516	851.26	48.22	25.05

NR_033369	LINC01056	100144597	281.24	10.22	10.21
ENST00000457193	LOC101929374	101929374	457.10	9.47	9.45
NM_001289115	SHBG	6462	184.70	9.37	24.47
NM_001015038	PAGE2B	389860	265.27	40.77	44.42
NM_001164685	THEMIS	387357	471.68	9.78	9.80
NR_026870	LOC100129935	100129935	151.35	9.81	9.83
NM_030576	LIMD2	80774	461.99	106.26	69.01
XR_241812	LOC101928885	101928885	342.57	33.67	30.39
NM_001044369	FAM69C	125704	274.32	9.48	9.48
ENST00000515380	C5orf42	65250	239.47	34.65	31.40
ENST00000328264	DSCR4	10281	225.85	9.33	9.31
ENST00000457853	LINC01270	284751	107.35	10.44	10.52
NM_021015	SSX5	6758	500.61	62.47	46.11
NM_001017978	CT83	203413	300.14	11.58	11.51
NM_175053	KRT74	121391	366.68	39.33	48.60
ENST00000602950	TPD52	7163	334.18	31.76	24.26
NM_001031854	ACCSL	390110	88.71	10.10	10.16
ENST00000554862	LOC101929241	101929241	246.35	15.40	10.80
NR_120565	LOC101928894	101928894	95.07	10.38	10.48
NM_033427	CTTNBP2	83992	254.92	9.08	9.06
NR_033843	LINC01330	646168	123.46	18.12	9.75
NR_105064	LOC101927835	101927835	100.95	9.02	9.68
NR_038438	LOC146513	146513	89.14	10.83	11.02
NR_038972	LOC339593	339593	89.63	9.00	8.99
NM_001024603	LOC154872	154872	97.29	8.99	8.97
XR_432334	OTUD6B-AS1	100506365	506.10	107.75	62.72
XR_247642	LOC101930264	101930264	127.51	9.73	9.72
NM_001001958	OR7G3	390883	63.18	9.06	9.05
NM_001007188	DEPDC5	9681	446.45	63.62	103.41
NM_001242521	C1orf234	729059	550.30	10.80	14.71
NR_027257	FLJ26850	400710	853.85	10.49	22.18
NM_153234	LIX1	167410	685.14	10.45	10.45
NM_000686	AGTR2	186	94.10	11.49	11.43
NM_004319	ASTN1	460	66.05	10.09	10.09
AK127847	FLJ45950	399975	576.30	20.01	27.42
NR_027030	LINC01312	154089	578.80	10.43	10.85
NR_033263	C20orf166-AS1	253868	59.34	9.31	9.34
NM_001009997	C10orf62	414157	253.38	10.51	16.06
NM_181617	KRTAP21-2	337978	122.10	12.46	10.15
NM_080658	ACY3	91703	146.38	10.47	10.46
NM_022479	WBSCR17	64409	465.40	8.84	20.99
NM_018965	TREM2	54209	388.49	8.82	8.81
ENST00000518834	LOC102724453	102724453	108.47	10.88	11.10
XR_252552	LOC101928413	101928413	334.12	10.89	40.27
NM_013289	KIR3DL1	3811	234.16	34.71	42.77
NM_001101391	LINGO3	645191	321.97	10.89	11.11
NR_120463	LOC101928162	101928162	144.56	10.89	11.11
NM_000506	F2	2147	123.05	16.13	27.93
NM_001172501	SLC6A2	6530	471.91	47.02	12.44
NM_203408	FAM47A	158724	147.20	10.52	19.86
NM_001165899	PDE4D	5144	528.33	94.05	120.43

NM_080546	SLC44A1	23446	287.13	41.24	36.30
NR_044996	HCG23	414764	318.04	20.74	16.27
NR_033664	NPSR1-AS1	404744	87.75	10.98	20.41
NM_002022	FMO4	2329	284.48	62.55	69.04
XR_242733	LOC101928701	101928701	321.03	16.03	11.19
NM_182519	BPIFB4	149954	364.08	16.09	11.28
NR_027310	MGC27382	149047	315.81	11.15	11.28
NM_001270805	SYT6	148281	109.97	10.62	10.65
ENST00000354366	NRDE2	55051	109.48	20.82	14.41
XR_241967	LOC101928004	101928004	240.05	9.12	18.17
NM_030904	OR2T1	26696	148.31	9.12	9.10
NR_038235	LOC100507651	100507651	180.20	32.64	41.22
NM_001001956	OR13C9	286362	418.13	10.39	10.42
NM_198229	RGS12	6002	648.43	73.81	99.06
NR_051983	LINC00462	100129597	430.43	26.19	17.69
NM_018849	ABCB4	5244	1060.66	10.40	11.66
NM_006794	GPR75	10936	468.04	11.22	10.42
NM_012314	KIR2DS4	3809	149.26	28.40	29.32
NM_207325	DPY19L3	147991	507.23	76.26	85.40
BC130650	LOC100287160	100287160	279.79	41.75	34.30
NM_001161498	PLEKHD1	400224	592.70	11.48	11.48
NM_001242415	WDR20	91833	568.71	11.49	13.64
NM_004413	DPEP1	1800	92.10	9.92	9.91
NM_054112	DEFB118	117285	166.55	10.17	10.19
NR_104628	LOC101929681	101929681	739.05	9.94	10.06
NM_001010979	C1orf189	388701	148.86	9.98	21.96

High Elastic Modulus upregulated: [b], [c] > [a]

Fold change > 4

Gene count

46

Normalized signal intensity

Hydrogel samples - PAAm

PrimaryAccession	GeneSymbol	EntrezGeneID	[a]	[b]	[c]
NM_053283	DCD	117159	15.33	65.24	61.44
NR_120430	LOC101928100	101928100	12.32	68.18	80.67
AK096041	LOC100129572	100129572	14.39	62.46	58.50
NR_033824	RXRG	6258	18.49	195.10	78.48
NR_026794	LINC00202-2	731789	14.51	134.60	295.96
NM_015320	ARHGEF4	50649	14.45	149.68	132.83
NM_002565	P2RY4	5030	16.63	536.56	390.76
NM_001144995	CCDC85C	317762	14.56	244.39	208.38
NM_152688	KHDRBS2	202559	12.91	91.47	314.10
NM_000833	GRIN2A	2903	12.93	74.84	219.46
ENST00000394457	AHSA2	130872	28.02	149.07	241.33
NM_022162	NOD2	64127	14.38	127.63	175.29
NM_001074	UGT2B7	7364	18.16	78.12	147.20
NR_110036	LOC100996671	100996671	12.19	229.32	134.14
NR_026956	LINC00663	284440	17.84	83.31	85.52
NM_007029	STMN2	11075	14.41	59.46	83.59
NM_080723	NRSN1	140767	14.51	548.07	278.01
NM_016233	PADI3	51702	14.54	480.70	309.32
NM_021201	MS4A7	58475	35.76	145.37	218.86
XR_424240	LOC102723765	102723765	11.49	69.41	104.08

NM_001164443	ANKRD31	256006	11.61	47.00	109.95
XR_246983	LOC101927345	101927345	13.31	62.68	101.79
ENST00000538294	LOC100507065	100507065	11.85	65.57	128.09
NR_047671	BANCR	100885775	11.59	53.83	122.08
XM_006709961	MYRFL	196446	31.17	193.12	184.19
NM_001024457	RGPD1	400966	14.02	75.57	285.37
NM_001029875	RGS7BP	401190	14.93	77.46	131.05
ENST00000423518	GRM8	2918	11.32	49.76	56.58
NR_033987	FLJ37505	400087	10.93	75.15	64.83
NM_001105521	JAKMIP3	282973	320.32	1672.42	2696.97
NM_006188	OCM2	4951	13.05	142.43	55.04
NR_110505	LOC100508046	100508046	12.15	73.55	123.18
NR_033893	LINC01299	286186	10.82	47.52	46.18
NM_001257995	LMO7DN	729420	438.78	2186.89	2973.76
NR_040093	STARD4-AS1	100505678	201.41	838.85	1307.22
NM_139284	LGI4	163175	225.88	1129.22	1468.79
AK093577	MKL2	57496	302.64	1347.39	2313.10
NR_003948	HCG22	285834	10.70	58.18	119.06
NR_125989	LOC101929771	101929771	10.97	87.49	176.97
NM_173625	C17orf78	284099	16.06	92.60	76.46
NM_145893	RBFOX1	54715	14.37	67.06	68.05
NR_046612	HTR2A-AS1	100874082	15.46	76.14	132.69
NM_006781	C6orf10	10665	13.81	120.44	177.10
NM_182522	FAM19A4	151647	16.54	80.09	119.51
NM_032968	PCDH11X	27328	12.99	57.49	126.10
NR_040105	FLJ46066	401103	13.77	82.96	88.52

Table S 2: GSEA analysis related to 2nd network surface density.

High 2nd Network Surface Density upregulated: [b] > [a], [c]

Fold change > 4		Normalized signal intensity				
Gene count	174	Hydrogel samples - PAAm				
PrimaryAccession	GeneSymbol	EntrezGeneID	[a]	[b]	[c]	
NM_033119	NKD1	85407	23.09	261.16	12.31	
ENST00000530102	LINC01493	101928536	13.15	79.16	12.64	
ENST00000538098	WDR74	54663	62.27	258.42	35.53	
NM_006648	WNK2	65268	21.86	104.07	16.59	
NM_001004477	OR10X1	128367	14.48	250.14	21.51	
NR_123723	MMP25-AS1	100507419	17.03	207.01	18.47	
NM_175737	KLB	152831	14.40	66.60	11.33	
NM_001001552	LEMD1	93273	13.52	216.41	10.70	
NR_110391	LOC101928259	101928259	12.57	123.01	9.97	
NM_000166	GJB1	2705	17.61	165.46	9.69	
NM_152701	ABCA13	154664	13.10	211.11	22.57	
NM_001282558	HHLA2	11148	14.13	240.40	27.94	
AK027833	FRAS1	80144	12.76	153.00	10.12	
NM_001871	CPB1	1360	34.40	349.24	39.94	
NR_024378	LINC00051	619434	12.21	85.53	9.67	
NM_005408	CCL13	6357	13.26	107.49	10.48	
ENST00000262676	TTC39A	22996	14.49	456.92	22.07	
NM_001004690	OR2M5	127059	13.30	127.00	10.53	
NM_014364	GAPDHS	26330	12.24	69.01	9.71	
NM_002838	PTPRC	5788	12.21	230.48	9.67	
XR_242019	LOC100505711	100505711	12.13	120.43	9.61	
NM_198253	TERT	7015	132.44	539.71	109.07	
NM_001048	SST	6750	14.59	693.72	11.47	
NM_139167	SGCZ	137868	14.60	193.62	11.48	
NM_014693	ECE2	9718	12.21	571.34	9.67	
NM_213605	ZNF517	340385	12.13	181.48	9.61	
NM_020526	EPHA8	2046	13.02	84.98	10.29	
NR_033883	LOC339529	339529	14.65	533.75	11.51	
NR_109952	LOC101927051	101927051	15.80	645.81	11.40	
NM_004829	NCR1	9437	51.99	265.17	16.06	
NM_001286361	PPIL6	285755	25.53	119.33	25.28	
ENST00000598304	LGALS17A	400696	12.27	140.85	9.72	
NM_002209	ITGAL	3683	13.14	125.47	10.38	
NM_000537	REN	5972	27.45	299.62	11.86	
XR_424296	LOC102725260	102725260	13.17	178.30	16.20	
ENST00000579781	LINC00683	400660	12.10	218.94	26.40	
NR_047479	LINC00854	100874261	12.10	290.63	9.59	
NM_001029870	SOWAHB	345079	13.86	199.53	10.41	
NM_032095	PCDHGB1	56104	12.91	69.33	10.21	
NR_002948	KLKP1	606293	13.18	165.90	10.42	
BC069735	RARB	5915	12.09	183.63	9.58	
NM_000277	PAH	5053	14.80	88.28	11.60	
NR_033753	LRRC37A11P	342666	41.47	388.11	42.03	
NM_032445	MEGF11	84465	12.16	97.70	10.14	

NR_040041	DACT3-AS1	100506068	14.03	92.84	11.05
NR_125815	LOC101929450	101929450	38.14	236.90	41.63
NM_003965	CCRL2	9034	70.48	562.96	106.59
NM_001204118	CLEC17A	388512	25.85	562.51	11.40
ENST00000564041	LINC00922	283867	12.40	329.36	9.82
NM_033122	CABS1	85438	12.01	57.67	9.52
NM_005598	NHLH1	4807	17.06	476.05	9.82
NR_024620	FOXN3-AS2	29018	12.00	66.50	9.51
NR_104190	DISC1FP1	101929222	14.76	518.30	11.57
NR_110017	LOC101929694	101929694	12.17	126.39	9.64
ENST00000507837	MTRR	4552	12.20	386.15	47.14
NR_034012	WDR86-AS1	100131176	13.61	281.52	9.64
NM_014246	CELSR1	9620	14.79	396.15	17.94
NR_110094	MEOX2-AS1	101927524	12.14	49.63	9.61
NM_014430	CIDEB	27141	64.24	370.00	53.04
NM_001012421	ANKRD20A2	441430	13.60	211.27	11.70
NM_153708	RTP1	132112	21.27	204.11	21.77
NR_125806	LINC01411	101928176	12.30	120.83	11.00
NR_121672	LINC00824	101927774	14.29	289.32	11.21
NM_000326	RLBP1	6017	20.83	200.07	40.57
ENST00000448431	LOC101928031	101928031	12.57	362.04	9.95
NR_037890	DNAJB8-AS1	285224	13.28	75.61	10.52
NM_181654	CPLX4	339302	13.56	101.84	10.78
AK098403	LOC100131170	100131170	13.61	139.58	10.70
NM_001042573	ENGASE	64772	59.04	502.85	35.41
XR_429997	LOC102724732	102724732	13.24	113.68	21.29
NM_152891	PRSS33	260429	13.55	362.95	32.47
NM_000599	IGFBP5	3488	26.59	190.79	32.22
NR_104672	LOC102467214	102467214	12.58	269.18	9.96
ENST00000523387	CSMD1	64478	14.66	102.38	11.47
NR_110308	LINC00238	440184	13.75	243.28	14.62
NM_001282301	LOC729159	729159	12.81	123.21	10.14
ENST00000422548	LOC100505918	100505918	13.51	327.97	10.63
ENST00000439237	LOC102725407	102725407	13.09	72.12	13.74
NM_015063	SLC8A2	6543	14.26	396.72	44.87
AK095617	LOC283728	283728	13.80	92.84	22.63
ENST00000374663	CELA3A	10136	15.01	67.79	11.69
XR_242419	LOC101927413	101927413	15.05	65.54	11.72
ENST00000555725	LOC100507277	100507277	13.72	259.77	10.77
NR_026833	LOC400940	400940	11.94	80.50	9.46
ENST00000369743	CHIAP2	149620	11.99	63.75	9.50
NM_152685	SLC23A1	9963	13.01	310.56	10.31
NM_001146336	TMEM114	283953	13.68	116.66	15.44
NM_001142287	SEMA4D	10507	11.94	214.33	9.46
NM_178839	LRRTM1	347730	12.62	66.38	10.00
NR_002216	NXF4	55999	12.99	74.35	14.82
ENST00000415686	LINC01360	101927295	13.08	90.24	10.36
NR_120580	LOC101929497	101929497	24.40	408.69	11.72
NR_125998	LOC102724539	102724539	25.93	146.38	24.25
NM_007125	UTY	7404	24.94	317.61	36.64
NM_001135844	SIRPB1	10326	38.76	163.30	29.98

NR_033946	LOC646268	646268	15.09	131.18	12.56
XR_242026	LOC101929770	101929770	11.87	110.39	9.40
NM_021081	GHRH	2691	24.11	555.40	11.24
NR_110386	LOC101927476	101927476	82.06	559.71	65.02
ENST00000583578	LINC00908	284276	13.55	416.92	13.59
NM_001185149	CLDN24	100132463	21.60	361.76	50.07
NM_178566	ZDHHC21	340481	34.71	292.16	65.74
NM_153488	MAGEA2B	266740	14.06	175.25	20.67
NM_004784	NDST3	9348	12.58	173.10	9.91
NM_033171	B3GALT5	10317	14.02	100.43	10.98
XR_425634	LOC102723759	102723759	15.05	470.35	20.02
NR_046108	CELF2-AS2	439950	15.01	114.04	11.65
NM_004658	RASAL1	8437	99.08	680.69	86.54
XR_427513	LOC102723415	102723415	11.89	119.76	9.40
NM_001145399	MPPED2	744	11.90	56.24	9.40
XR_424068	LOC102723630	102723630	15.11	68.68	11.72
NR_073430	ANKRD20A19P	400110	13.73	298.33	10.95
ENST00000532065	LOC101928132	101928132	13.72	303.56	11.98
NM_031495	PCDHA2	56146	13.56	130.18	10.77
NM_198722	AMIGO3	386724	27.04	249.55	46.65
NM_032054	PCDHGA5	56110	17.30	221.55	25.51
NM_020389	TRPC7	57113	13.54	334.61	21.75
NM_024552	CERS4	79603	200.46	934.84	186.70
NM_152538	IGSF11	152404	12.82	293.97	15.71
NM_020387	RAB25	57111	13.72	80.55	10.95
NM_144614	MBD3L2	125997	13.57	414.56	10.79
NR_038380	SLC7A11-AS1	641364	12.75	109.26	10.02
NR_003255	TSIX	9383	13.34	68.00	10.60
NR_037902	LOC100507537	100507537	11.53	220.22	9.14
NR_024582	JPX	554203	73.21	390.91	83.60
BC132887	LOC642947	642947	42.70	424.28	69.18
NM_145270	PRR35	146325	13.33	361.73	10.59
NM_022893	BCL11A	53335	11.49	160.18	30.63
AK126260	LOC100129275	100129275	26.71	322.80	19.80
NM_001290030	ERICH2	285141	13.35	63.94	10.61
NR_126404	LINC01320	104355288	11.47	136.87	9.26
NR_038232	LOC285889	285889	11.27	49.70	11.75
NM_014621	HOXD4	3233	11.74	61.68	9.24
NM_207341	ZP1	22917	262.33	2218.54	219.07
NM_007308	SNCA	6622	11.13	362.74	8.83
NM_198565	NRROS	375387	96.01	422.73	85.48
NM_001128076	SPDYE4	388333	15.43	302.63	37.24
AK127285	TMBIM4	51643	11.35	126.39	8.94
NM_198173	GRHL3	57822	18.02	332.70	16.82
NR_104620	LOC100505887	100505887	14.11	401.61	19.61
NM_178466	BPIFA3	128861	11.40	223.52	8.97
NR_038968	LINC01281	286442	11.36	406.76	8.95
NM_199047	TBPL2	387332	11.34	135.90	14.30
NM_004925	AQP3	360	46.14	190.64	25.01
NR_034087	LRRC37A5P	652972	11.08	55.42	8.79
NM_001370	DNAH6	1768	11.59	68.57	9.21

NM_001287339	CAB39L	81617	14.22	60.73	10.99
XR_430888	LOC102725022	102725022	11.58	456.28	9.20
NM_014385	SIGLEC7	27036	11.62	85.03	11.57
NM_003301	TRHR	7201	13.80	56.61	10.66
AK095105	FLJ37786	642691	14.85	209.68	28.39
NM_006841	SLC38A3	10991	13.99	60.85	11.19
NM_001122961	C1orf194	127003	13.60	71.16	10.47
NM_005564	LCN2	3934	11.07	56.01	13.50
ENST00000293829	FGF11	2256	185.34	994.55	192.40
NR_033967	SLC2A1-AS1	440584	15.16	62.80	11.35
NM_000189	HK2	3099	11.90	85.22	9.43
ENST00000295706	PPFIA4	8497	74.49	404.59	43.44
NM_001011878	DEFB121	245934	10.90	44.64	8.58
NM_052862	RCSD1	92241	18.71	76.08	8.51
NM_016835	MAPT	4137	11.32	58.56	13.95
NR_040079	LOC399715	399715	29.47	210.10	33.83
NM_003039	SLC2A5	6518	91.16	506.48	105.22
NM_001284260	WDR86	349136	80.92	565.60	49.12
NM_173642	RIMKLA	284716	23.62	137.73	8.90
NM_134260	RORA	6095	22.77	323.15	69.97
NM_016354	SLCO4A1	28231	42.00	428.29	74.07
NM_013332	HILPDA	29923	10998.43	60384.35	7403.91
NM_001782	CD72	971	12.24	53.34	9.42
NM_000160	GCGR	2642	104.29	457.01	98.13
NM_004861	GAL3ST1	9514	13.52	59.89	13.87
NM_006096	NDRG1	10397	22250.93	129705.79	16606.26
AY742823	TSRM	100287664	14.27	57.47	10.84
NM_001114357	C4orf47	441054	106.40	628.71	120.21

Low 2nd Network Surface Density upregulated: [a], [c] > [b]

Fold change > 4		Normalized signal intensity			
Gene count		Hydrogel samples - PAAM			
PrimaryAccession	GeneSymbol	EntrezGeneID	[a]	[b]	[c]
NM_033124	CCDC65	85478	92.46	17.48	85.47
ENST00000466317	FLCN	201163	153.90	13.02	130.84
ENST00000533866	SIGLEC14	100049587	72.09	10.48	84.74
NM_012472	LRRC6	23639	46.05	10.67	76.30
ENST00000553381	YLPM1	56252	47.28	11.31	70.46
NR_024562	HPN-AS1	100128675	66.75	13.05	116.64
NM_001261820	TCP11	6954	69.82	9.23	39.35
NM_000085	CLCNKB	1188	59.13	10.84	79.08
NR_110802	RUNDC3A-AS1	101926996	42.54	10.09	80.85
NM_001168214	C3orf80	401097	74.66	10.52	65.62
NM_198541	IGFL1	374918	117.91	10.00	88.22
NM_133445	GRIN3A	116443	99.83	18.61	75.73

Table S 3: GO-terms related to elastic modulus and 2nd network surface density.

Category		Count	P-Value
Elastic modulus			
GO:0038179	neurotrophin signaling pathway	2	0.0120
GO:0018108	peptidyl-tyrosine phosphorylation	5	0.0137
GO:0007608	sensory perception of smell	5	0.0156
GO:0042490	mechanoreceptor differentiation	2	0.0238
GO:0035556	intracellular signal transduction	7	0.0347
GO:0050957	equilibrioception	2	0.0356
GO:0045087	innate immune response	7	0.0453
2nd Network Surface Density			
GO:1901862	negative regulation of muscle tissue development	2	0.0121
GO:0007267	cell-cell signaling	6	0.0194
GO:0007155	cell adhesion	8	0.0218
GO:0060291	long-term synaptic potentiation	3	0.0223
GO:0007154	cell communication	3	0.0223
GO:0048672	positive regulation of collateral sprouting	2	0.0476

Table S 4: GO genes related to elastic modulus and 2nd network surface density.

Elastic modulus		
ENTREZ_GENE_ID	Name	Symbol
4916	neurotrophic receptor tyrosine kinase 3	NTRK3
3824	killer cell lectin like receptor D1	KLRD1
50649	Rho guanine nucleotide exchange factor 4	ARHGEF4
64127	nucleotide binding oligomerization domain containing 2	NOD2
9681	DEP domain containing 5	DEPDC5
2918	glutamate metabotropic receptor 8	GRM8
4915	neurotrophic receptor tyrosine kinase 2	NTRK2
390063	olfactory receptor family 51 subfamily I member 1	OR51I1
6271	S100 calcium binding protein A1	S100A1
1262	cyclic nucleotide gated channel alpha 4	CNGA4
83983	testis specific serine kinase 6	TSSK6
117285	defensin beta 118	DEFB118
286362	olfactory receptor family 13 subfamily C member 9	OR13C9
8832	CD84 molecule	CD84
8817	fibroblast growth factor 18	FGF18
186	angiotensin II receptor type 2	AGTR2
2252	fibroblast growth factor 7	FGF7
3809	killer cell immunoglobulin like receptor	KIR2DS4
64072	cadherin related 23	CDH23
54209	triggering receptor expressed on myeloid cells 2	TREM2
26696	olfactory receptor family 2 subfamily T member 1	OR2T1
9542	neuregulin 2	NRG2
6278	S100 calcium binding protein A7	S100A7
7401	clarin 1	CLRN1
2nd Network Surface Density		
ENTREZ_GENE_ID	Name	Symbol
9718	endothelin converting enzyme 2	ECE2
152404	immunoglobulin superfamily member 11	IGSF11
3488	insulin like growth factor binding protein 5	IGFBP5
80144	Fraser extracellular matrix complex subunit 1	FRAS1
100049587	sialic acid binding Ig like lectin 14	SIGLEC14
6543	solute carrier family 8 member A2	SLC8A2
201163	folliculin	FLCN
6357	C-C motif chemokine ligand 13	CCL13
10507	semaphorin 4D	SEMA4D
2256	fibroblast growth factor 11	FGF11
2705	gap junction protein beta 1	GJB1
2046	EPH receptor A8	EPHA8
6750	somatostatin	SST
27036	sialic acid binding Ig like lectin 7	SIGLEC7
971	CD72 molecule	CD72
6622	synuclein alpha	SNCA
3683	integrin subunit alpha L	ITGAL
347730	leucine rich repeat transmembrane neuronal 1	LRRTM1
56146	protocadherin alpha 2	PCDHA2
2691	growth hormone releasing hormone	GHRH
53335	B-cell CLL/lymphoma 11A	BCL11A

Study on the control of the double network
hydrogel surface-bulk transition
and its effect on mesenchymal stem cells

Martin Frauenlob