Magneto-responsive Cell Culture Substrates that can be Modulated *in situ*

Federica Crippa[a], Barbara Rothen-Rutishauser[a], and Alke Petri-Fink[ab]*

Abstract: Understanding the interaction between cells and their environment is fundamental for mechanobiology. To mimic the behavior of cells in physiological and pathological conditions, synthetic substrates must have topographical and/or mechanical properties that evolve in time. Dynamic substrates mainly rely on stimuli-responsive materials where an external stimulus induces controlled variations in topography or mechanics. Herein, we describe the development of a dynamic cell culture substrate where mechanical properties are reversibly tuned *in situ* using magnetically-responsive superparamagnetic iron oxide nanoparticles (SPIONs).

Keywords: Magnetic nanoparticles · Mechanobiology · Stimuli-responsive substrates

1. Introduction

Several decades of research in nanotechnology have led to the development and use of magnetic nanoparticles (MNPs). These materials have found applications in different fields such as storage media, jet printing, high gradient magnetic separation, biosensing and in medicine.[1]

Due to their magnetic responsiveness, superparamagnetic iron oxide nanoparticles (SPIONs) have also been used in the field of cell mechanics as nanosized probes to study the mechanical properties of cells and to investigate how cellular behavior depends on the external environment (*i.e.* other cells or the extracellular matrix (ECM)).[2] In particular the role of ECM in influencing cell morphology, migration and differentiation is a topic of relevant interest for the scientific community.[3–5] Previously, various synthetic cell culture substrates able to mimic the mechanical and topographical properties of the ECM have been developed to understand how ECM properties affect cell behavior.[6–9] Many of these substrates are synthesized starting from responsive polymers that vary their conformation upon external stimulation (*e.g.* temperature, light, pH) and imitate the dynamic environment of ECM that changes and evolves continuously. In this context, responsive nanoparticles such as SPIONs offer new possibilities for the development of dynamic cell culture substrates; they can be incorporated in a polymeric matrix and can be excited by a magnetic field to modify the matrix conformation and properties by the induction of heat.[10–12]

2. Superparamagnetic Iron Oxide Nanoparticles

SPIONs are MNPs composed of single crystals of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) with a core diameter of about 4–25 nm. To synthesize these MNPs several chemical routes have been developed[13] and the synthetic approach generally determines size, polydispersity, shape, crystallinity and surface. Bulk magnetite and maghemite display typical ferrimagnetic behavior, whereas the nanosized materials show drastically altered magnetic properties. At the nanoscale, SPIONs are composed of single crystalline domains (mono-domain particles) showing so-called superparamagnetic behavior.[14] The particles are typically used for *e.g.* magnetic hyperthermia applications. This method is fundamentally linked to the nanosize of the magnetic particles, which, when exposed to an alternating magnetic field (AMF),[1,15] dissipate heat through relaxation losses. Typically, the heating potential of MNPs depends on the material itself and its size (distribution) and energy dissipation can occur either through the physical rotation of the particles themselves in the fluid (Brownian relaxation), or rotation of the atomic magnetic moments within each particle (Néel relaxation).

3. Interaction of Cells with ECM

Cell mechanics describes the relationship between force and cell structure and originates from the well-established knowledge that eukaryotic cells are able to generate and withstand mechanical forces in their environment.[16] For example, endothelial cells situated in the interior part of blood vessels are subjected to shear stress,[17] while chondrocytes located in the meniscus cartilage mainly sustain compression.[18] At the same time cells mechanically interact with their surrounding (*i.e.* ECM and neighboring cells), adapt their structure and behavior accordingly, and can modify the ECM.[3–5,19]

The adhesion between the cells and the external environment mainly occurs through a series of cell adhesion molecules called integrins, which are transmembrane receptors.[20] In the presence of high ligand density, integrins migrate and cluster on the cellular membrane. As the extracellular component binds ECM ligands, the intracellular domain undergoes a conformational change and triggers the formation of the focal adhesion complex. These complexes activate intracellular pathways, thus strengthening and orienting

*Correspondence: Prof. A. Petri-Fink
E-mail: alke.fink@unifr.ch
[a]Adolphe Merkle Institute, Faculty of Science and Medicine, University of Fribourg
Chemin des Verdiers 4, CH-1700 Fribourg
[b]Chemistry Department, University of Fribourg

the cytoskeleton which anchors the cell mechanically to the ECM through the focal adhesion. This allows the cytoskeleton to contract and transmit micro-forces inside the cell.[21] Moreover, contractile activity influences cell shape and elasticity and determines activation or inhibition of biochemical signalling through a complex series of pathways regulating diverse cell functions.[4] The process of conversion of mechanical stimuli into biochemical and molecular activity is defined as mechanotransduction and influences cells functions such as migration, proliferation, apoptosis and differentiation.[22,23]

4. Synthetic Responsive Substrates to Mimic ECM–Cell Mechanical Interaction

Cell differentiation, migration and morphology depend on the rigidity, viscosity and topography of the ECM which changes *in vivo* during tissue development, remodelling and disease.[3] Currently, the majority of the studies that aim to elucidate how cellular phenomena are dependent upon ECM geometry and mechanics focus on static synthetic substrates where cells interact with a defined topographical feature or material.[3,4,6,9,24–26] Although these studies are crucial, passive substrates are not sufficient to mimic the natural environment of cells, which undergoes remodelling in physiological or pathological conditions.[27]

To overcome this limitation, different approaches have been developed, including the use of stimuli-responsive polymers as cell culture substrates. To be more specific, varieties of substrates were fabricated which alter their stiffness and topography due to changes in temperature,[7,28,29] light[30] or pH.[8] These responsive cell culture substrates present advantages over static ones. First, their mechanical properties and topography can be altered *in situ* (*e.g.* in presence of the cells) and it is therefore possible to study changes in cell morphology, adhesion and migration in real time. Stimulation is often reversible, *i.e.* substrates can change between two different conformations several times (*i.e.* flat/grooved surface, soft/stiff substrate). This kind of dynamic stimulation can be interesting for cell mechanics investigations as it gives more insights into dynamic cellular behavior. The following subsections offer a schematic overview of the state of the art where responsive substrates are used to study cells mechanics.

4.1 *Temperature-responsive Substrates*

Temperature-responsive polymers with a customized lower critical solution temperature (LCST) near physiological range (*i.e.* 37 °C) can be useful to develop dynamic cell substrates, since the stimulation (*i.e.* change in stiffness, viscosity or topography) can be easily delivered by varying the temperature of the cell culture medium. So far temperature-responsive substrates have been employed to investigate the cellular response to mechanical stretching,[31] surface roughness[29] and to provide reversible encapsulation of adherent cells.[7]

Reconfigurable micro- and nano-topographical substrates have been implemented starting from shape memory polymers that change their topography near physiological temperature.[28,32–38] Even if the topographical transition is a singular event and cannot be repeated cyclically, shape memory systems have been a fundamental tool to assess the role of nanotopography and strain in cell alignment,[28,34,35] to investigate the role of actin in the regulation of cell morphology[33] and to direct stem cells differentiation.[35]

Regarding temperature-responsive substrates, it is important to note that the influence of the temperature itself on cellular behavior has not been fully clarified yet; although several authors have not noticed a significant effect on cells,[28,35] Le *et al.* have obtained discrepant results.[32]

4.2 *Light-responsive Substrates*

One of the most explored approaches to provide dynamic stimulation is based on photo-responsive substrates where UV light is tuned to be non-invasive and biocompatible.[30,39] 2D and 3D substrates containing photo-cleavable agents can change rigidity, topography and crosslinking ratio upon UV irradiation and have been used in the presence of cells to assess cell morphology,[39–41] motility,[30,41] migration[42,43] and to trigger phenotypic changes.[39,40] Despite the extensive use of photo-cleavable substrates, only few works where the substrate stiffness is increased have been reported.[44–47] Nevertheless these works are elegant solutions to study the cellular response to stiffening.

In general, light-responsive substrates are closer to the natural ECM when compared to temperature-based responsive ones. However, in almost all the mentioned cases stimulation is not reversible since the proposed substrates undergo irreversible degradation or stiffening.

4.3 *DNA-crosslinked Substrates*

Another strategy to provide mechanical dynamic stimulation on cells has been proposed by Langrana's group and is based on DNA-crosslinked hydrogels.[48,49] Taking advantage of the high affinity of DNA with complementary sequences, paired oligonucleotides can be selectively forced or removed by electrophoresis inside the hydrogel. This leads to changes in the crosslinking ratio and therefore in the overall stiffness of the substrate.[50]

In general DNA-based stiffening processes are relatively slow and the transitions are not sharp, which limits the flexibility of these substrates in terms of dynamic stimulation.

4.4 *Physical-chemical Responsive Substrates (pH, Ions, Chemical Cues)*

In addition, other solutions have been proposed. For example, pH-responsive substrates capable of increasing their stiffness up to 40-fold were developed by Yoshikawa *et al.*[8] and ion-responsive 3D scaffolds made of a collagen-alginate matrix[51] were used to investigate cells morphology upon dynamic stimulation. Other solutions are based on the incorporation of chemical cues that can trigger Michael-type addition reactions,[52] in the substrates or in the presence of supramolecular hydrogels.[53]

Even though physical and chemical responsive substrates provide biocompatible changes in substrate mechanical properties, to date they have not found a broad application in biomechanics since large pH and ions variations may interfere with cells and limit the overall biocompatibility of the substrates.

5. Magneto-responsive Substrates

Magnetic micro- and NPs can be remotely controlled using a magnetic field and therefore offer the possibility to create magneto-responsive cell culture substrates that change topography and mechanical properties on demand.[10–12,54] Mayer *et al.*[12] developed magneto-active elastomers (PDMS-based) that tuned stiffness and topography when stimulated by static and oscillating magnetic field, respectively.

In our recent work we have synthesized thermo-responsive magnetic substrates starting from SPIONs and *N*-isopropylacrylamide (NIPAM) (Fig. 1).[54] The obtained nanocomposite substrates were able to cyclically change phase when exposed to an alternating magnetic field since SPIONs act as hotspots which heat up the surrounding thermo-responsive matrix. In general, these substrates presented some interesting features that made them appealing for cell culture, since they were biocompatible for fibroblast cells and the stimulation occurred close to physiological temperature (37 °C). Moreover phase transition was tuned down to a narrow temperature range (2–3 °C) and was reversible.[54]

Here we report on the characterization of thermo-responsive magnetic substrates building up on our previous work[54] and we propose preliminary experiments that analyze the effects of magneto-thermal stimulation on cells.

As SPIONs were physically trapped in the poly-NIPAM matrix, composition analyses on the substrates were performed to

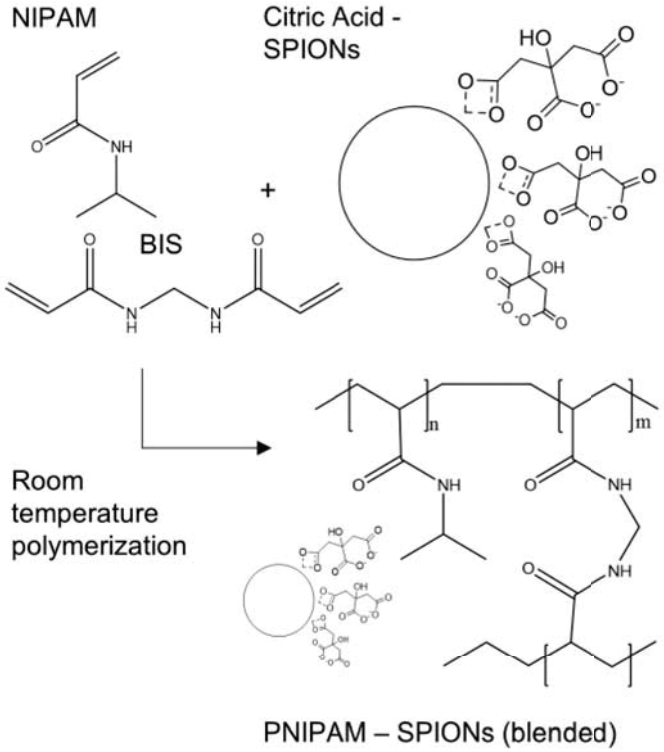

Fig. 1. PNIPAM-SPIONs substrates are obtained by polymerization of *N*-isopropylacrylamide (NIPAM) and *N,N'*-methylenebis(acrylamide) (BIS) in presence of citric acid coated SPIONs following a previously reported method.[54]

determine the exact concentration of SPIONs and to quantify potential SPIONs leakage from the substrate. To do so, the substrates were digested in a lab microwave and the iron was quantified by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). From these analyses we observed that approximately 30% of the total SPIONs used during synthesis leaked from the substrates during the post-synthesis cleaning process. Therefore, further experiments should attempt to chemically cross-link the SPIONs to the polymeric matrix. To do so, synthetic strategies may involve the use of SPIONs functionalized with specific polymers that present acrylic acid or acryl amide groups able to crosslink with NIPAM[55] or the SPIONs surfaces could be used to directly start the polymerization of poly-NIPAM.[56]

The mechanical properties of the resulting substrates were tested at the macro- and the micro-scale at temperatures below and above the phase transition. Uniaxial stress-strain tensile tests (Fig. 2A) and quasi-static atomic force microscopy (AFM) surface indentations (Fig. 2B) highlighted the effect of temperature on the bulk and on the surface elastic moduli, respectively. The bulk elastic modulus increased upon phase transition by a factor of 1.5 (from 31 °C to 36 °C) and the obtained values were in the same order of magnitude as reported for similar poly-NIPAM-based soft materials.[57,58] At the micro-scale the surface elastic modulus depended on the environmental temperature and increased upon phase transition. High variability in the measures of E^* could be correlated with inhomogeneity of the surface mechanics or with the intrinsic limitations of the quasi-static AFM method.[59]

To investigate cellular behavior, a series of experiments were performed by stimulating the substrates with an AMF. In these experiments NIH 3T3 fibroblasts were seeded on sterilized thermo-responsive magnetic substrates and cultured for 2 hours at 31 °C. Then they were cultured in the presence of an AMF, which showed no adverse effects on the cells (magnetic field: 14.7 kA/m, frequency: 523.5 kHz) or at 31 °C for 1 hour. Investigation of cell areas by laser scanning microscopy (LSM) and statistical analysis revealed that AMF-mediated stiffening of the substrates resulted

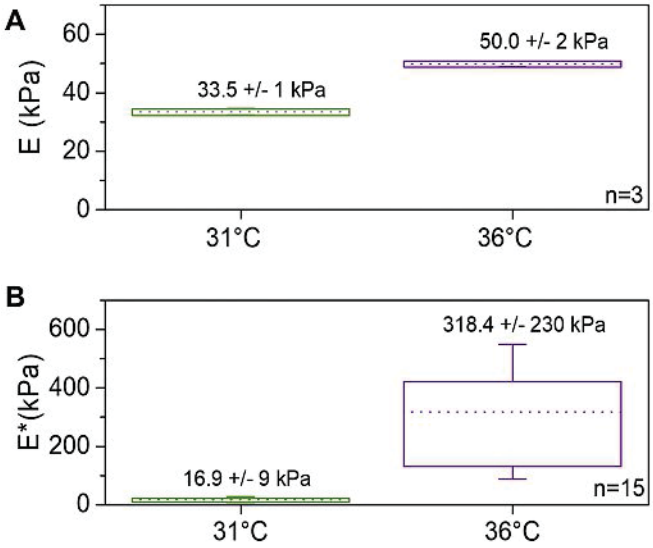

Fig. 2. Macro- (A) and micro-scale (B) mechanical characterization of substrates below (31 °C) and above (36 °C) phase transition. The bulk elastic moduli *E* and the surface ones *E** were calculated using uniaxial stress-strain tensile tests (A) and quasi-static AFM surface indentations (B).

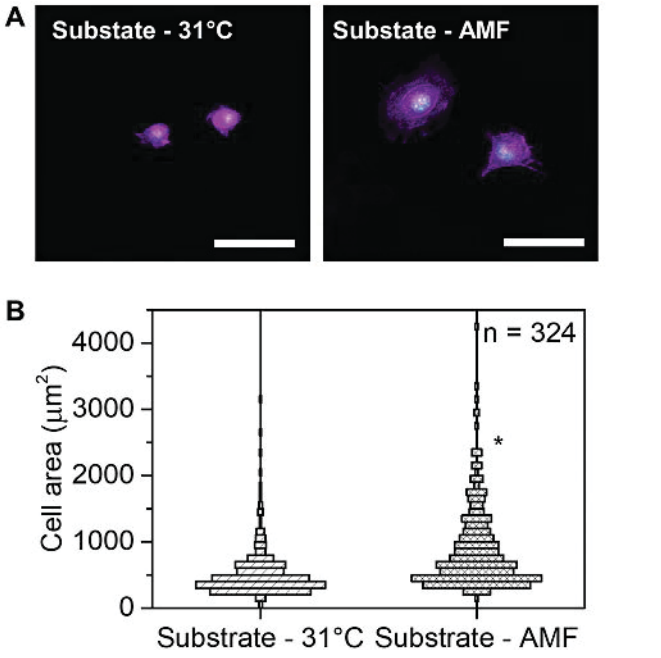

Fig. 3. Cellular adhesion experiments on substrates at 31 °C and stimulated by an AMF. LSM images (A) and the analysis on the cell adhesion area (B) revealed the effect of the AMF stimulation on cell morphology. Upon AMF-mediated phase transition the NIH 3T3 fibroblasts were more prone to adhere and spread on the substrates in comparison to the cells cultivated at 31 °C. Statistical significance was determined *via* a one-way ANOVA comparison of geometric means and Tukey's honestly significant difference test (p-value = 0.05). *Indicates a statistically significant difference from the sample at 31 °C. Scale bar: 50 μm. Fuchsia: stain for F-actin cytoskeleton, light blue: stain for the nucleus.

in a significant increase in the adhesion of the cells to the surface (Fig. 3A and Fig. 3B).

In conclusion, for the proposed magnetic thermo-responsive substrates, the adhesion area of NIH 3T3 fibroblasts was influenced by the substrate mechanical properties. Our result is in agreement with several scientific publications.[29–31]

6. Challenges and Open Questions

Presently, there are few studies reporting magneto-responsive substrates available in literature. Despite this, substrates which

apply SPIONs as actuators could present advantages: SPIONs are biocompatible, the magnetic fields can be compatible with cell survival, and the fields can be localized very specifically on a few cells. Furthermore, the substrates can change phase reversibly and they could be used to deliver dynamic mechanical stimulation to the cells and study the effect of repeated mechanical variations on the cell adhesion behavior.

However, there are some possible challenges that limit the development of magneto-responsive substrates. Technical challenges are mainly related to the stimulation itself, *i.e.* the AMF. Operating a microscope or a mechanical testing device in the proximity of an AMF is challenging as it induces eddy currents in the metallic parts of instruments, potentially resulting in overheating of the instrument and possible damage.[60] Moreover, commercially available AMF generators will often dissipate heat after some time limiting the possibility to perform long-term magnetic stimulation experiments on the substrates (*i.e.* 12 or 24 hours). Use of an AMF limits the possibility to visualize the behavior of SPIONs in the substrate (*i.e.* colloidal stability, aggregation, Brownian motion).

Possible solutions have been recently proposed. To image cells during AMF stimulation, Connord *et al*.[61] have developed a new set-up where the magnetic coil was miniaturized and was placed in close proximity to a confocal microscope. Moreover, a possible method to characterize the mechanical properties in the presence of an AMF would involve optical measurements of the substrate curvature during the de-swelling process.[62] These experiments, proposed by Yoon *et al*.[62] were performed on PNIPAM hydrogels swelling in water and could be potentially repeated in the AMF as they do not necessitate a microscope or a mechanical testing device. However, these are complex custom-made platforms and these analyses can be demanding in terms of methodology and instrumentation.

7. Outlook and Conclusion

Overall, the ability of SPIONs to produce heat upon AMF stimulation renders these nanoparticles extremely appealing for the study of cell mechanics. This field is increasingly relying on responsive cell culture substrates that allow *in situ* manipulation of the mechanical and topographical cues of the substrates. Herein, we have presented several types of responsive cell culture substrates and have shown how cell mechanics studies can potentially benefit from the implementation of SPIONs in responsive cell culture substrates. To conclude, we have discussed some challenging aspects related to the use of AMF stimulation that will need to be overcome to promote future investigations on magneto-responsive cell culture substrates.

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