

MORPHOLOGY AND TAXIS OF OSTEOLASTS ADHERED ON SURFACES WITH TAILORED ELECTRIC POTENTIALS

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ABSTRACT

Electrical cell stimulation is a promising technique to manipulate cell functions and thus to improve osseointegration. In this work, electric fields are applied in the vicinity of MG-63 osteoblast-like cells to study their morphology, cell alignment and guided migration mainly via Scanning Ion Conductance Microscopy (SICM) and optical microscopy. As shown for microcontact printed molecules stripes, representing zeta potential landscapes on glass substrates, the cells align along the structures. We attribute this cell response to double-sided cathodotaxis due to local electric fields occurring at the glass-molecule transition site. Concerning the apical membrane morphology, we observe a higher density of membrane protrusions in relation to cells that are not aligned. In contrast to molecule landscapes an electrical stimulation setup via faradaic currents does not result in a significant preferred migration direction, nor do the cells align or elongate.

ZUSAMMENFASSUNG

Die elektrische Zellstimulation ist eine vielversprechende Technik zur Beeinflussung der Zellfunktionen und somit zur Verbesserung der Osseointegration. In dieser Arbeit werden elektrische Felder in der Nähe von MG-63 Osteoblasten-ähnlichen Zellen genutzt, um deren Morphologie, Ausrichtung und gerichtete Migration hauptsächlich mit Hilfe der Rasterionenleitfähigkeitsmikroskopie (SICM) und der optischen Mikroskopie zu untersuchen. Wie für mikrokontaktgedruckte Molekülstreifen, die Zetapotentiallandschaften auf Glassubstraten darstellen, gezeigt wird, richten sich die Zellen entlang der Strukturen aus. Das Zellverhalten führen wir auf eine beidseitige Kathodotaxis aufgrund lokaler elektrischer Felder an der Glas-Molekül-Übergangsstelle zurück. Bezüglich der Morphologie der apikalen Membran können wir eine höhere Dichte von Membranausstülpungen im Vergleich zu den nicht ausgerichteten Zellen feststellen. Im Gegensatz zu den Moleküllandschaften führt eine elektrische Stimulierung durch faradaysche Ströme weder zu einer signifikanten bevorzugten Migrationsrichtung noch zu einer Ausrichtung oder gerichteten Ausdehnung der Zellen.

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ACRONYMS

AC	alternating current
AFM	atomic force microscopy
ALP	alkaline phosphatase
BMP	bone morphogenic protein
BSA	bovine serum albumin
BSP	bone sialoprotein
CAM	cell-adhesion molecule
CNT	carbon nanotubes
COL	collagen
DC	direct current
DCN	decorin
DMEM	dulbeccos modified eagle medium
ECM	extracellular matrix
EDL	electrical double layer
EF	electric field
ES	electrical stimulation
FCS	fetal calf serum
GE	gene expression
GF	growth factor
GFR	growth factor receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB	high mobility group box
IGF	insulin-like growth factor
MSC	mesenchymal stem cell
μCP	microcontact printing
OC	osteocalcin
ON	osteonectin

OPG	osteoprotegerin
OPN	osteopontin
Osx	osterix
PAMAM	polyamidoamine
PBS	phosphate buffered saline
PDADMA	polydiallyldimethylammonium
PDMS	polydimethylsiloxane
PEI	polyethylenimine
PFA	paraformaldehyde
PPAAm	plasma-polymerized allylamine
PSS	polystyrenesulfonate
ROS	reactive oxygen species
Runx	runt-related transcription factor
SACC	stretch activated calcium channel
SAM	self-assembled monolayer
SEM	scanning electron microscopy
SICM	scanning ion conductance microscopy
SPM	scanning probe microscopy
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TMP	transmembrane potential
VEGF	vascular endothelial growth factor
VGCC	voltage-gated calcium channel

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INTRODUCTION

The phenomenon of an induced electric potential of bone was discovered in 1957 [1]. This was attributed to a piezoelectric effect which is only present during mechanical stress. If stress is absent, the electric field (EF) is almost zero. Over the long term, this bone deteriorates, which is particularly evident in jaw bone resorption in cases of missing teeth.

Various diseases such as osteoporosis and osteoarthritis demand new approaches, especially in an increasingly ageing society (from avg. 39.3 years (1990) to 44.6 years (2022)) [2] with higher life expectancy (1990: 72 years (m), 79 years (f); 2023: 78 years (m), 83 years (f)) [3]. Hence, it is hardly surprising that surgeries involving bone implants such as hip and knee arthroplasty are among the most common surgeries and represent the major reason for inpatient stay in hospitals in Germany in 2023 [4]. Successful implantations (survival rates) exceed 95% for dental implants, approx. 92% for total knee replacements, and range from 62% to 98% for total hip replacements [5–7].

The process of osseointegration, i.e., the formation of a structural and functional connection between living bone and the implant surface, is critical for success. Osseointegration comprises three major phases. Initially, blood surrounds the artificial implant and thus blood clotting begins. The first phase is characterized by a non-specific inflammatory response triggered by phagocytic cells that transform the blood clot within the first few days after surgery (see Figure 1a). Over time the inflammatory response becomes more specific due to increasing numbers of T cells, B cells, Killer (K) cells, Natural Killer (NK) cells, and macrophages [8]. During the second (proliferative or reparative) phase, new blood vessels are formed from pre-existing capillaries in the surrounding vital tissue (angiogenesis) to provide nutrients and oxygen to cells. Local mesenchymal stem cells differentiate into fibroblasts, osteoblasts and chondroblasts. These cells form a soft callus, composed of fibrous and cartilaginous tissue, which replaces the blood clot at the implant-bone inter-

face. Simultaneously, a layer of woven bone (random and not stress oriented) is deposited. Eventually, the soft callus develops into harder bone callus (via chondro- and osteoblasts and -clasts). The final phase (after approximately four weeks) involves bone remodeling of the woven bone and bony callus by osteoblasts and osteoclasts. The remodeling is a continuous, lifelong process where compact bone surrounded by lamellar bone is formed.

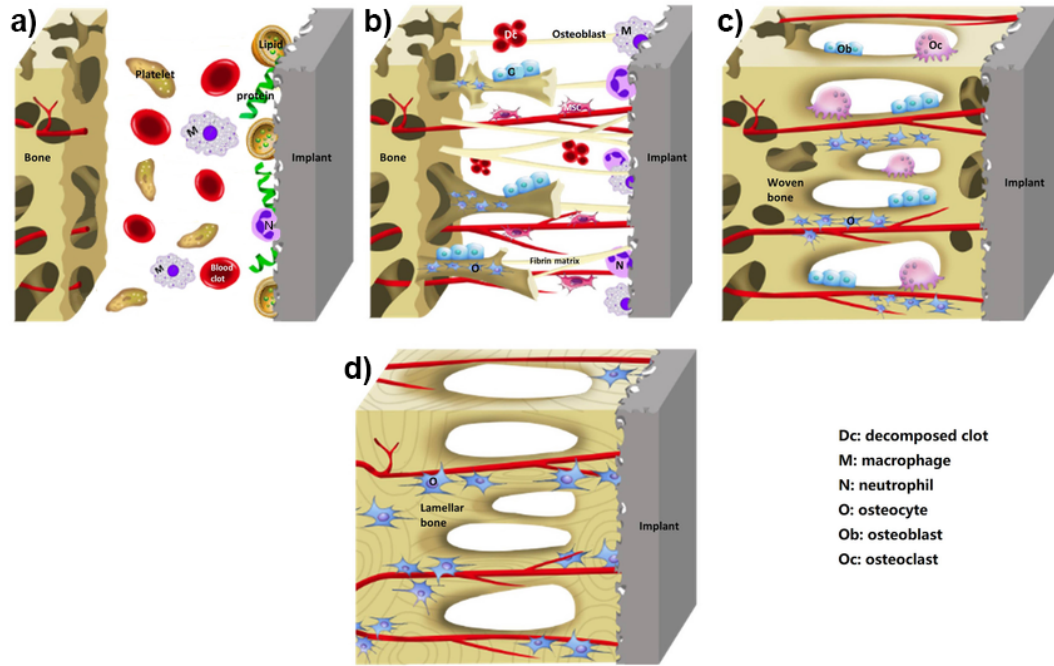


Figure 1: Schematic illustration of osseointegration. a) represents inflammation phase; b) and c) proliferation/repair phase; d) maturation phase. Figure adapted from [9].

The failure of osseointegration has numerous reasons. For example, the insufficient recruitment of mesenchymal stem cells (MSCs) at the implant site or an imbalance of osteoblasts and osteoclasts [10]. Therefore, the targeted recruitment and migration of MSCs as well as the regulation of their differentiation into osteoblasts and their migration to the bone-implant interface is crucial. The development of new implant materials and techniques may help to enhance and support these processes.

Especially electrical stimulation (ES) is a promising technique, which is already successfully used clinically for bone healing [11]. Despite its clinical application, the de-

tailed mechanism underlying ES is poorly understood. Solid fundamental research is therefore required to develop targeted ES therapies.

To address local effects on the cellular level a microscopic approach is advantageous. By applying scanning ion conductance microscopy (SICM) to in vitro osteoblast-like cells, it is possible to observe morphological responses and spatial distributions of local surface potentials and surface charges, respectively, in liquid environment. However, in future the SICM could also be used to locally apply a high EF (10^8 V/m) to cells. Measurements are accompanied by atomic force microscopy (AFM) and fluorescence microscopy to characterize the cell adhesion substrates.

The main concepts and theoretical basics are briefly introduced in Chapter 2. The following Chapter 3 gives an overview of the experimental setups and methods as well as sample preparations. Chapter 4 deals with the apical membrane morphology and its dynamics of osteoblast-like cells in the living state. For comparison and evaluation reasons the cells were also measured after being fixed by paraformaldehyde (PFA). In the following Chapter we report on modified adhesion substrates to achieve local surface potential landscapes. Due to cells responding to a lot of cues, such as surface roughness, step edges, contact guidance, and chemical cues, we employed microcontact printing (μ CP) to deposit various molecules in a monolayer fashion. In the last Chapter we established a planar resistive electrode setup to apply macroscopic EFs for cell stimulation. Particular attention was given to cell migration behavior and cell orientation upon stimulation. This setup could potentially be used in the SICM to investigate the microscopic response.

2.1 CELL ADHESION

Cell adhesion describes the process by which cells bind to each other, to the extracellular matrix (ECM) or to an artificial substrate. It plays a crucial role in diverse processes including cell communication, proliferation, migration, and differentiation [12–16]. Therefore, understanding cell-material interactions is essential for gaining insight into these processes and improving tissue maintenance and development and in the special case of osteoblasts the osseointegration. In vitro, the adhesion process of cells can be structured in 3 phases. The first phase describes the initial contact with the substrate while the second contains cell shape changes and binding of cell-adhesion molecules (CAMs). The last phase represents the final spreading, reorganization of the cytoskeleton and formation of focal adhesions. During the first phase even before cells settle on the substrate due to sedimentation, different proteins in physiological medium (e.g. fetal calf serum (FCS)) adsorb onto the surface. Small proteins adsorb first onto the surface, but over time, they are replaced by larger proteins like fibrinogen (Vroman effect) [17]. The final composition of the formed protein layer is determined by the specific substrate properties, since proteins in solution interact with the substrate by long range van der Waals and Coulomb interaction. Initial cell binding is accompanied by formation of single receptor-ligand pairs, whose number increases over time leading to higher adhesion strength. The connection between adsorbed proteins and cell is mediated by transmembrane proteins, so-called integrins. Once the integrins bind to their ligands they change their conformation, which promotes linking to actin and thus the cytoskeleton. This is crucial to achieve rigid cell-material connections. Especially for osteoblasts the presence of vitronectin, fibronectin and collagen I, which are present in the ECM is crucial for initial adhesion [18]. During the spreading phase, the cell

starts to flatten while forming more bonds to the substrate. In the final phase, focal adhesions are formed and the connections of the respective vinculin-integrin bonds with the cytoskeleton are established.

2.2 ELECTRICAL DOUBLE LAYER

Exposing solids, particles, proteins, molecules etc. to a liquid environment leads to the formation of an electrical double layer (EDL) due to an electrochemical potential difference at the phase boundary. The electric potential of the immersed species develops in different ways:

- 1) the species is charged by dissociation or ionization of chemical groups that are attached (e.g., carboxylic groups ($-\text{COOH} \rightarrow -\text{COO}^- + \text{H}^+$) or amine groups ($-\text{NH}_2 + \text{H}^+ \rightarrow -\text{NH}_3^+$))
- 2) by desorption or adsorption of ions to obtain an electrochemical equilibrium (e.g. release of Ag^+ ions when Ag is immersed in AgNO_3 solution)

Helmholtz already in 1879 postulated that an EDL forms which consists of rigid counter ions that screen the surface charges so that the electric potential drops linearly with distance [19]. However, he did not account for the thermal energy of the ions which leads to a diffusive layer where ions are constantly transferred and reorganized. This model was developed by Gouy and Chapman [20, 21] and later complemented by Stern [22]. Closest to the surface a rigid layer consisting of approximately just an ion (and its hydration shell) monolayer, the Stern or Helmholtz layer. It is followed by a diffuse layer, which begins at the electric potential ζ . This layer becomes more important concerning the capacitance at lower ionic concentrations, which are present in the physiological medium and phosphate buffered saline (PBS) [23]. The screening layers are almost instantly formed at timescales of the order of 10^{-9} s which is orders faster than that of typical processes in cells like enzyme turnover (10^{-6} s), activation of ion channels (10^{-3} s) or action potential duration (10^{-3} s) [24]. However, changes of the potential via chemical reactions (e.g. upon change of the pH value of electrolytes) might be effective on longer timescales. Taking into account the Poisson equation: $\Delta\varphi = -\frac{4\pi\rho}{\epsilon_r}$, where φ is the electrical potential, ϵ_r the relative permittivity, and ρ the space charge density. For the space charge density the Boltzmann equation is valid: $\rho = F \sum_i z_i c_i \exp(z_i F \varphi / RT)$ where

φ is the electrical potential with respect to the bulk of the electrolyte, z_i and c_i the charge number and the concentration of the ions i , respectively. F is the faraday constant, T temperature, and R the gas constant. Combining the equations leads to a homogeneous differential equation of second order. For a planar surface the equation only depends on one spacial coordinate (e.g. x) and it is solved by $\varphi = \varphi_0 \exp(-\kappa x)$. The electric potential therefore drops exponentially with distance x from φ_0 (the surface potential) to the potential of the bulk solution. The parameter $\kappa = (4\pi F^2 / \epsilon_r RT \sum z_i^2 c_i)^{1/2}$ defines the double layer thickness.

As mentioned the ζ potential defines the electric potential at the shear plane, which is present between the stationary layer and the diffuse layer. It is thought to be a critical biomaterial parameter for cell adhesion. It is dependent on several parameters e.g. the ion concentration or pH-value (which is critical for 1)) of the liquid as well as the surface potential of the solid material. Commonly the ζ potential of planar substrates is acquired through a capillary where an applied pressure difference (Δp) results in a liquid flow (and ion current flow at the shear plane) and thus generates a streaming potential (for non-conductive surfaces) or a streaming current (I_{str}). The ζ potential is derived from the equation: $\zeta = \frac{\partial I_{str}}{\partial \Delta p} \frac{\eta}{\epsilon_r \epsilon_0} \frac{L}{A}$, where η and ϵ_r are the viscosity and the relative permittivity of the liquid, respectively and L and A the length and cross section area of the capillary channel.

2.3 ELECTRICAL STIMULATION OF CELLS

The communication of cells with their surrounding, as well as intracellular signaling pathways (e.g., Wnt/GSK3 β , PI3K/Akt), involve electrochemical processes. Consequently, the [ES](#) of cells to modulate such electrochemical cascades has been a focus of research in recent years. Studies have demonstrated that [ES](#) can influence cell functions, including enhanced adhesion, directed migration, increased proliferation, and differentiation of stem cells. However, the underlying mechanisms remain poorly understood, and the variety of [ES](#) setups, along with the cell types being used in the literature do not provide a clear, straightforward picture. This section provides a brief overview of commonly used [ES](#) setups and suggested or reported mechanisms of galvanotaxis.

2.3.1 ELECTRICAL STIMULATION SETUPS

Generally, ES configurations can be divided into three categories: faradaic current, capacitive coupling, and inductive coupling.

The faradaic ES setups are characterized by oxidative or reductive processes at the electrodes. One can distinguish between three commonly used setups in literature. Either the electrodes, which consist of inert materials to prevent corrosion (e.g., platinum, gold or graphite), are in contact with the physiological medium (applies for rod/plate electrodes or substrate-mediated setups, see Figure 2a and b) or in a separate volume (agar salt bridge, see Figure 2c). In substrate mediated setups, the cells are seeded directly onto an electrically conductive substrate, which serves as a current-carrying conductor. In rare cases where another electrode is immersed in the medium, the substrate functions as either an anode or cathode. The major advantage of the faradaic setups is their versatile applicability, such as in direct current (DC) operation for studying galvanotaxis or alternating current (AC) operation to address signaling pathways ([25]). However, chemical reactions at the electrode-medium interface can give rise to pH changes and the creation of reactive oxygen species (ROS) [26] in the cell medium.

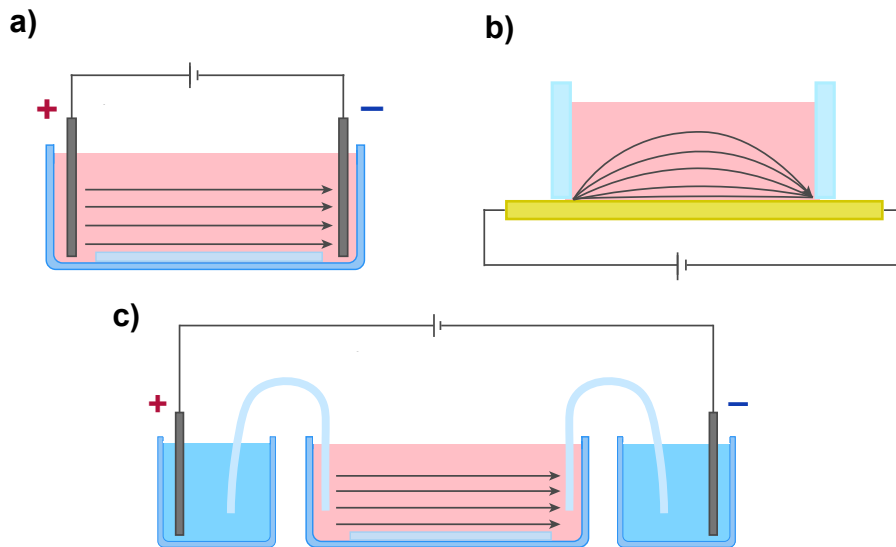


Figure 2: Schematic view of the electric fields in (a) rod/plate-electrodes setup, (b) substrate-mediated setup, and (c) agar salt bridge setup.

To circumvent these issues, agar salt bridges are occasionally utilized. The agar tubes act as an ion-conductive electrical connection between the physiological medium and the bath solution containing the electrodes. As a result, faradaic byproducts are restricted to the electrode-interface and are unable to diffuse through the salt bridges into the cell vicinity.

Capacitive setups typically consist of two metal plates which are placed outside of the culture dishes, either in a horizontal or vertical arrangement (see [Figure 3](#)). Occasionally, one of the electrodes is immersed in the cell medium (known as semi-capacitive). Applying voltage to the plates leads to an electric field between them, leading to a charge shift inside the cell medium. This setup avoids chemical reactions. However, the charge displacement - and thus an effective screening of the electric field in the medium - occurs in the order of 10^{-9} s, neglecting interface effects. It should be noted that this represents an upper limit approximation based on the bulk properties of the physiological medium. The actual threshold is highly dependent on the setup, such as the geometry and the double layer capacitance and is expected to be in the order of 10^{-4} s for common configurations. Consequently, capacitive setups are only applicable in AC mode and may only generate a significant electric field over time in the vicinity of cells at frequencies beyond 10^4 Hz. Nevertheless, effects on cells have also been reported at frequencies in the range of 10^1 Hz (see [Table 1](#)).

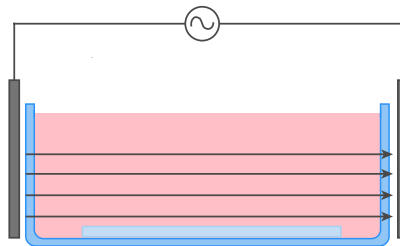


Figure 3: Horizontal capacitive setup of two metal plates, creating a homogeneous EF in cell vicinity.

Inductive setups utilize coils - often in Helmholtz configuration - surrounding the culture dishes (see [Figure 4](#)). Usually, a pulsed voltage is applied to the coils, resulting in a time-varying magnetic field and thus in an induced non-conservative electric field in cell vicinity.

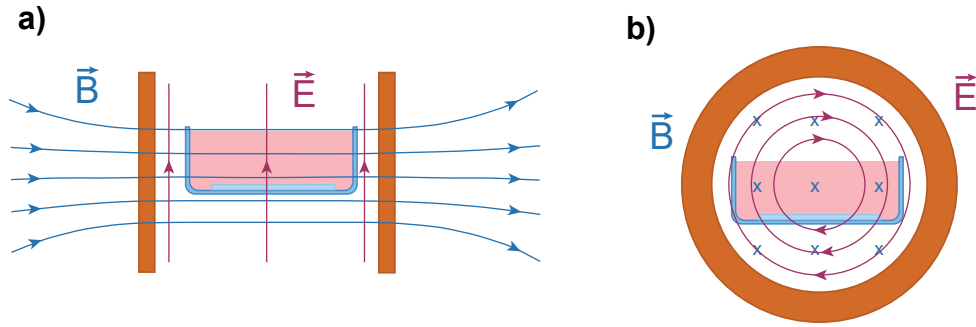


Figure 4: A common inductive setup, consisting of 2 Helmholtz-coils [27]. (a) Schematic view of magnetic and electric field during ES, (b) side view

Various ES setups are widely used in the literature to influence cellular behavior. Since this work focuses on the osteoblast-like cell line MG-63 (see Section 2.4.1), only experiments related to osteoblasts are summarized.

However, comparisons remain demanding due to the wide variety of setups, cell lines, and the amount of stimulation variables, such as ES-duration, EF strength, voltage (V), electric current (I).

Generally, the estimated electric fields applied to cells are mostly in the order of 10^1 V/cm which corresponds to physiological values [28, 29]. It should be noted that most reported electric fields are not explicitly simulated but rather estimated by dividing the applied voltage by the setup dimensions (indicated with *). Thus the reported values may differ from the effective electric field in cell vicinity. Effects on adhesion & proliferation performance, metabolic activity, galvanotaxis and the expression of several genes related to differentiation are reported.

As shown in Table 1, most studies focus on the MC3T3-E1 cell line. Even when similar faradaic setups were used, experimental outcomes varied. While most studies report enhanced proliferation, the metabolic activity and the specific upregulated genes do not yield a consistent picture. Regarding morphological changes of cells exposed to EF, elongation and alignment of various cell types (MG-63, SaOS-2, fetal rat calvaria, bovine osteoblast-like) generally occur perpendicular to the EF direction. However, only a limited number of studies stated morphological changes. It remains unclear whether other studies did not confirm these findings or did not investigate cell morphology.

ES config.	cell type	stimulation parameters	effective EF	cellular effects	Ref.
rods/plates	hFOB 1.19 human osteoblasts	rect. pulses (0.4 ms), $f = 20$ Hz $V = 15$ V, $j = 4.2$ A/m ² ES-duration = 1 h/d	-	increased proliferation and Ca deposition after 3 weeks	[30]
	human primary osteoblast-like cells	sinusoidal voltage, $f = 20$ Hz $V_{rms} = 0.7$ V	1 V/cm 1.50 V/cm	increased metabolic activity, mineralization capacity increased gene expression (<i>Runx2</i> , <i>COL1</i> at 1.5 V/cm) (<i>OPN</i> , <i>OPG</i> at 1 V/cm)	[31]
	human primary osteoblast-like cells	pulses (0.4 ms), $f = 100$ Hz ES-duration = 5 min/12h	0.5-4 V/cm*	increased cell proliferation (0.5-1 V/cm*)	[32]
	MC3T3-E1	rect. pulses (500 μ s), $f = 50$ -100 Hz $I_p = 10$ -20 μ A	-	cell viability & collagen production increased	[33]
	MC3T3-E1	DC, $V = 125$ -500 mV	-	promotion of proliferation & differentiation	[34]
	MC3T3-E1	DC, $V = 2.2$ V $I = 0.07$ mA ES-duration = 1-2 h/d (3 d)	1 V/cm*	reduced metabolic activity upregulation of <i>BMP2</i> & <i>OPN</i> no change in expression of <i>Runx2</i> , <i>VEGFA</i> & <i>HMGB1</i>	[35]
	MC3T3-E1	sinusoidal voltage, $f = 1$ kHz $V_p = 500$ mV	-	increased proliferation & <i>ALP</i> expression cell alignment parallel to field lines	[36]
	MG-63	rect. pulses of ± 1 V or ± 5 V pulse duration 10 ms at 7.9 Hz or 3.6 ms at 20 Hz	0.9 V/cm 4.5 V/cm	enhanced initial adhesion performance higher intracellular calcium increased initial <i>ROS</i> production ($t < 4$ h)	[26]
substrate mediated	MC3T3-E1	DC, $I = 200$ μ A ES-duration = 4 h/d	-	increased metabolic & <i>ALP</i> activity; upregulation of <i>Runx2</i> , <i>OPN</i> , <i>Osx</i> , <i>OC</i>	[37]
	SaOS-2	ES-duration = 2-8 h	1-4 V/cm*	increased <i>ALP</i> (2 V/cm*) & <i>OC</i> production increased proliferation (2 V/cm*), decreased proliferation (> 2 V/cm*)	[38]
	SaOS-2	ES-duration = 3x6 h/6d	2 V/cm*	increased phosphate & Ca deposition higher Gene expression (<i>ALP</i> , <i>BMP2</i> , <i>Runx2</i> , <i>OC</i>)	[39]
	SaOS-2	rect. pulses, $f = 100$ Hz, duty cycle 4% $V_p = 1$ -10 V ES-duration = 2x5 min/24h	0.33-3.33 V/cm*	increased cell proliferation (at 0.66 V/cm*)	[40]
	Sprague-Dawley (SD) rat's osteoblasts	$I = 10$ μ A, $f = 10$ Hz duty cycle 50% ES-time = 6 h/d	-	increased Ca production and proliferation increased gene expression (<i>ON</i> , <i>OC</i> , <i>COL1</i> , <i>OPG</i>) no effect on gene expression (<i>ALP</i> , <i>BSP</i> , <i>OPN</i>)	[41]
	Sprague-Dawley (SD) rat's osteoblasts	$j = 1.5$ μ A/cm ² , $f = 3000$ Hz ES-duration = 24 h/d or 6 h/d	-	increased proliferation (only if continuously stimulated) no upregulation of gene expression (<i>GE</i>) (<i>ALP</i> , <i>OPN</i> , <i>COL1</i>) decreased expression of <i>cbfa1</i> ; no upregulation of <i>GF</i> (<i>BMP-2</i> , <i>-4</i> , <i>IGF-2</i> , <i>TGF-β1</i>)	[42]
	Rat bone marrow stromal cells	DC, $V = 0.1$ -10 V ES-duration = 2-12 h	0.035-3.5 V/cm*	decreased cell viability (for 12 h stim.); increased calcium deposition (4 h stim., 0.35 V/cm*)	[43]
Agar Salt Bridge	RCJ 1.20 and RCB2.2A from fetal rat calvaria	DC, ES-duration = 17.2 h	1-10 V/cm*	galvanotaxis toward cathode; cell orientation perpendicular to EF	[44]
	fetal rat calvaria primary osteoblastic cells; SaOS-2	DC, ES-duration = 5 min-5 h	1-14 V/cm*	elongation and orientation perpendicular to EF; guided migration to anode (SaOS-2) or cathode (calvarial cells); local Ca^{2+} gradient is essential for galvanotaxis	[45]
	SaOS-2	degenerate wave signal, $f = 16$ Hz ES-duration = 8 h (within 28 h)	0.1 V/cm*	reduced proliferation; increased expression of <i>ALP</i> , <i>COL1</i> , <i>OC</i> , <i>OPN</i> , <i>ON</i>	[46]
	hFOB 1.19 human osteoblasts	DC, ES-duration = 30 min	0.5-5 V/cm*	increase in intracellular Ca^{2+} after 20 min of ES; time delay decreased with increasing field strength	[47]
	bovine osteoblast-like cells; MG-63	DC, ES-duration = 5 h	0-20 V/cm*	alignment and elongation perpendicular to EF (≥ 4 V/cm*)	[48]

ES config.	cell type	stimulation parameters	effective EF	cellular effects	Ref.
capacitive	bovine osteoblast-like cells	asymmetric saw-tooth signal repet. rate 16 Hz $V_{p-p} = 100$ V ES-duration = 1-16 days	60 V/cm	increased cell proliferation & ALP activity; enhanced ECM protein synthesis	[49]
	bovine osteoblast-like cells	asymmetric saw-tooth signal repet. rate 16 Hz $V_{p-p} = 100$ V ES-duration = 14 days	60 V/cm	increased calcium content (mineralization)	[50]
	Human osteoblast-like cells	sinusoidal voltage, $f = 60$ kHz $V_{rms} = 0.1$ V or 1 V	< 0.1 V/cm	increased metabolic activity ($0.1 V_{rms}$) decreased COL1 ($0.1 V_{rms}$) & increased ALP ($1 V_{rms}$) levels upregulation of OPG and TIMP1 ($0.1 V_{rms}$)	[51]
	MC3T3-E1	sinusoidal voltage, $f = 14$ Hz ES-duration = 1-7 days	3 V/cm	decreased cell proliferation no significant change in metabolic activity unchanged levels of ON & COL1 increased ALP activity	[52]
	MC3T3-E1	sinusoidal voltage, $f = 60$ kHz ES-duration = 24 h 50% duty cycle	$A_{field} = 0.02$ V/cm	increased BMP-2 and ALP expression; increased mRNA expression for BMP-2, -4, -5, -6, and -7	[53]
	MC3T3-E1	sinusoidal voltage, $f = 60$ Hz ES-duration = 24 h	$A_{field} = 0.02$ V/cm	increased proliferation, Ca^{2+} ion through voltage-gated calcium channel (VGCC)	[54]
	MG-63	$V = 100$ -500 mV (DC), ES-duration = 2 h	-	decreased cell number, lowest at cathode increased OC & OPG production	[55]
	SaOS-2	Degenerate Wave signal, $f = 16$ Hz; $A = 10$ mV/mm ES-duration = 8 h (within 28 h)	-	reduced proliferation; increased expression of ALP, COL1, OC, OPN, ON	[46]
inductive	rat calvarial osteoblasts	rect. pulses (300 μ s), $f = 7.5$ Hz $B_{max} = 6.75$ mT ES-duration = 2 h/d or 8 h/d	up to 8 mV/cm*	increased proliferation ($B_{rms} = 0.13$ mT) decreased proliferation ($B_{rms} \geq 0.32$ mT) increased ALP activity ($B_{rms} \geq 0.24$ mT)	[56]
	human osteoblasts	$H = 400$ Oe, $f = 100$ Hz magnetoelectric coeff. 50 V/(cm Oe) ES-duration = 1 h/d	-	increased proliferation (after 7 days) higher expression of COL1, ALP, ON no change of OC & BSP levels	[57]
	MC3T3-E1	$f = 10$ Hz $B = 0.1$ mT or 0.4 mT	0.4 mV/cm* at 0.1 mT	no change in ALPase activity activation of mTOR pathway after 10 min	[58]
	MC3T3-E1	bursts (5 ms) of rectangular pulses (0.2 ms) $B = 2$ mT, $f = 15$ Hz	-	increased cellular attachment; better developed cytoskeleton	[59]
	MG-63	bursts (5 ms) with 20 pulses $B = 1.8$ mT, $f = 15$ Hz ES-duration = 8 h/d	-	increased ALP, OC, and collagen production decreased proliferation	[60]
	SaOS-2	rect. pulses (1.3 ms), $f = 75$ Hz $B = 2$ mT ES-duration = 24 h/d for 22 days	-	increased levels of DCN, OC, OPN, COL1, COL3	[61]

Table 1: overview of in vitro ES studies related to osteoblasts, * where the effective field was not simulated nor verifiable where the value was derived from

2.3.2 GALVANOTAXIS

In literature, a vast majority of cell types (fibroblasts, keratinocytes, chondrocytes, epithelial cells, osteoblasts and neurons) typically show a directed migration towards the cathode (cathodotaxis) [44, 62–65]. Only few types move to the anode (e.g. corneal endothelial cells and human vascular endothelial cells). This might be related to wound healing processes, where a laterally oriented EF develops pointing towards the wound center [66]. However, independent from the migration direction they generally align perpendicular to the EF and their migration speed increases with field strength. Additionally the substrate where the cells are adhered has a significant influence in migration performance, which is why comparisons within the literature have to be critically questioned.

Two main mechanisms are discussed, however their specific effect on galvanotaxis is unclear and might even change with different cell types. The first one is the inflow and outflow of Ca^{2+} from cells [67]. Due to the EF, cations (Ca^{2+} among others) accumulate at the outer side of the cell membrane facing the anodic side. The concentration gradient leads to passive Ca^{2+} influx into the cell and therefore a higher Ca^{2+} concentration at the anodic side of the cell. Ca^{2+} is responsible for actomyosin contraction and thus higher concentrations could lead to a contraction and therefore retraction of the anodic cell (rear) side. The concentration of Ca^{2+} is decreased on the cathodic side. The free barbed ends of the actin cytoskeleton makes polymerization easier, leading to expansion of the cytoskeleton in direction of the cathode. This mechanism could explain that migration speed increases with increasing field strength. However the EF also leads to a local change of the transmembrane potential (TMP), and therefore to depolarization of the membrane potential (making the potential less negative or even positive) opening voltage-gated calcium channels (VGCCs), which could lead to an influx of Ca^{2+} at the cathodic side and thus reduce or change the sign of the Calcium concentration gradient within the cell. The process might be the reason for galvanotaxis in one direction at low field strength and in the other direction with increasing electric field strength (e.g. for peripheral lens epithelial cells [68]). Overall the theory that calcium concentration gradients are responsible for galvanotaxis is highly dependent on parameters like the membrane conductance for calcium ions [69], the Ca diffusion coefficient, the specific TMP and the presence of VGCCs.

It should be noticed that Stretch activated calcium channels (SACCs) and calcium induced calcium release through the endoplasmic reticulum are also essential processes for cathodotaxis of various cells (e.g. lung cancer cells [70]). Li et al. blocked the SACCs with GdCl_3 and noticed a significant decrease in directed migration speed to the cathode, however VGCCs seemed not to be involved. The second hypothesis includes growth factors (GFs) and their corresponding growth factor receptors (GFRs) that regulate various cellular processes like mitosis, migration and differentiation. For example in case of corneal epithelial cells the GFRs accumulated at the leading edges and thus correlated with the direction of migration under electrostimulation [71]. Nonetheless the directionality did not increase when applying GFs during electrostimulation, only the migration rate changed. Sometimes the two mechanisms that are discussed in this section overlap and GFs are even required during electrostimulation to achieve a directed galvanotaxis of cells e.g. for human keratinocytes [28, 63].

2.4 BONE AND BONEMINERALIZING CELLS

Human bone, especially the diaphysis (see Figure 5), consists of a hard outer layer, called compact bone since it represents the densest part of the skeleton. It is mostly responsible for the mechanical stability as well as for storing and releasing chemical elements like calcium. Trabecular or spongy bone is found in the axial skeleton (skull and trunk) as well as in the metaphyses and epiphyses. It is highly porous and consists of trabeculae structures that surround pores filled with bone marrow. Both types of bone consist of bone tissue, called lamellar bone [72]. It is arranged in small columns, so called osteons or haversian systems. In the center of each column is the haversian canal, which allows blood vessels and nerves to go through the bone to supply the osteocytes (former osteoblasts and osteoclasts), which are located in multiple layers around the center, with minerals. Osteoblasts are found on the surface of osteons. Their function is to form osteoid around themselves, which primarily consists of Type I collagen (98 wt %) and several noncollagenous proteins [73]. Once the osteoblast encapsulated itself completely the osteoid mineralizes to lamellar bone, and the osteoblast becomes an osteocyte. Latter are responsible for bone maintenance. Since the bone underlies constant remodeling it also has to be resorbed by so called osteoclasts before being rebuild.

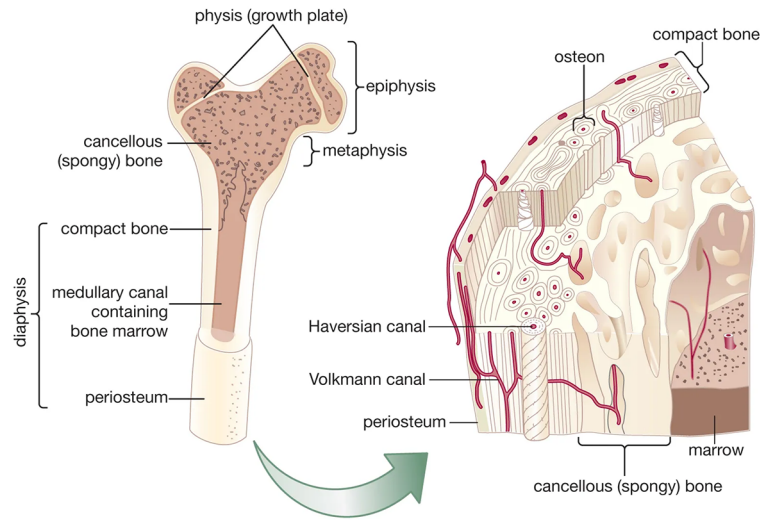


Figure 5: Schematic view of human bone. Figure adapted from [74].

In implantology the osseointegration meaning the direct structural and functional connection between the bone and an implant is of outstanding importance. In case of insufficient integration fibrous tissue forms and the implant loosens. To circumvent these complications the implant surface has to be designed in such way that adhesion and differentiation of osteoblasts are ensured [75]. In this work the osteoblast-like cell system MG-63 was used to investigate the adhesion and migration performance in dependence of different surface properties.

2.4.1 MG-63 CELL SYSTEM

The MG-63 cell line was derived from the osteosarcoma of a 14 year old boy in 1978 [76]. The osteosarcoma cells have been reported to show dose dependent stimulated alkaline phosphatase (ALP) expression upon $1,25(\text{OH})_2\text{D}_3$ exposure, which is characteristic for human osteoblast cells [77]. However the low basal ALP-level as well as a high synthesis of type III collagen indicates that the MG-63 cells are not fully differentiated osteoblasts [77, 78]. The fact that also osteocalcin is being produced when exposed to $1,25(\text{OH})_2\text{D}_3$ further supports the close relation of MG-63 cells to mesenchymal osteoprogenitor or osteoblast-like cells. For cell-material interaction the predominant factor are the integrins at the cell membrane (see Sec-

tion 2.1). MG-63 cells express to human osteoblasts partially similar integrin subunits (α_2 , α_5 , α_v , β_1 and β_3) [77, 79]. As a result the MG-63 are a well established cell system to investigate initial adhesion in context of osseointegration [80–83]. The phenotypical stability, that is common for sarcoma cells also leads to a versatile and widely used cell line [84].

3

EXPERIMENTAL SETUPS AND METHODS

3.1 SCANNING ION CONDUCTANCE MICROSCOPY

The [SICM](#) which is a type of scanning probe microscopy ([SPM](#)) was developed in 1989 by Hansma and coworkers to specifically investigate the topography of soft, non-conductive specimens in the environment of electrolyte solution [85]. Although they only demonstrated the capabilities of the microscope on polymer films, they had in mind that it would be well suited for measuring biological samples in the future. Only years later, in 1997, the [SICM](#) was exploited to image living biological samples, such as murine and human cells, without damaging them [86]. In 2000 they already mapped single active K^+ ion channels of cardiac myocytes by combining the [SICM](#) with a patch-clamp system [87]. Another milestone was achieved in 2002, when Gorelik et al. applied [SICM](#) together with confocal microscopy to simultaneously achieve topographical and fluorescence information of fibroblast-like cell surfaces [88]. However, the reliable scanning of surfaces of living cells was still demanding, because of high slopes and membrane protrusions. The invention of a novel scanning mode, called backstep mode which is nowadays commonly known as hopping mode (see [Section 3.1.1](#)), solved this issue [89].

3.1.1 PRINCIPLE OF SCANNING ION CONDUCTANCE MICROSCOPY

The [SICM](#) employs a nanopipette as a probe, which is immersed in an electrolyte (e.g. physiological medium). An electric potential of a few mV is applied between two electrodes - one situated in the nanopipette the other in bulk solution. As a

result an ion current in the order of a nA runs through the nanopipette opening, acting as a feedback signal. The electrodes usually consist of Ag/AgCl to avoid high polarization at the liquid-electrode interface (see [Section 2.2](#)) and therefore a high voltage drop in the direct vicinity of the electrodes. When the nanopipette opening approaches a surface, the effective cross-section reduces resulting in an increasing resistance and a decreasing ion current, which is referred to as current squeezing [90]. The ion current can therefore be utilized to keep a constant distance between pipette and specimen to measure the topography. The lateral resolution of the [SICM](#) is approximately the diameter of the nanopipette opening (≈ 50 nm in this work, see [Section A.6](#)). Typical measurement modes are the continuous or non-modulated mode in which the pipette is driven laterally over the sample by varying the pipette-sample distance to keep the ion current at a specified value. Due to the fact that the ion current through the nanopipette is mostly vertically sensitive, this measurement mode is only suitable for flat or smooth surfaces. In case of cell topography, which is the main focus point of this work, the hopping mode is essential to avoid collisions of the nanopipette at steep edges of the cell membrane. Although the latter mode requires higher data acquisition time, it is also less susceptible to electrical potential drift. This is due to the fact that at each measurement point the nanopipette retracts from the substrate measuring the reference current and approaches after that until the current drops down to a value relative to this reference. Afterwards the pipette is retracted again, moves to the next measurement spot and repeats the procedure (see [Figure 6](#)). Over the years the [SICM](#) modes were further developed to not only measure the topography but also gather locally resolved functional information, e.g. the surface charge, pH value, ion concentration and stiffness [91].

3.1.2 SICM SETUP

The setup used in this work can be seen in [Figure 7](#). It consists of an [SICM](#) head under which the sample stage and an optical microscope are situated. The [SICM](#)- (or optionally [AFM](#)-) head comprises the probe and a sample cover. It is vertically driven by a step motor for coarse approach of the probe to the sample. For fine approach and height correction during measurement, the probe is moved by a z-piezoactuator

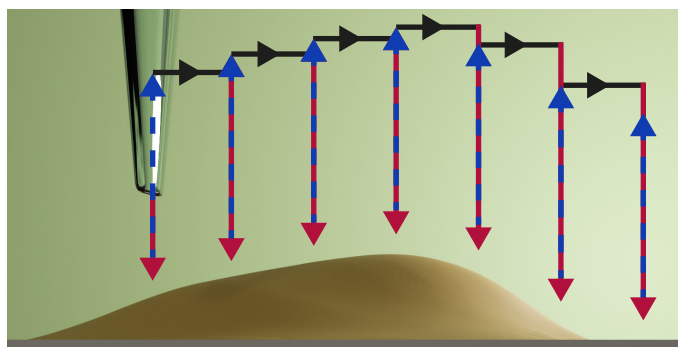


Figure 6: Schematic representation of the hopping mode in SICM. The reference current is measured at the position of the blue arrowheads, far away from the sample. The red arrow indicates the approach distance of the nanopipette until the relative current setpoint is reached. Blue arrow indicates retraction height after the setpoint was reached and before the lateral movement of the nanopipette to the next location.

which has a workspace of 20 μm . The sample is placed on a stage containing an x-y-piezoactuator with a moving distance of 100 μm in both directions. For living biological samples, the stage can be heated locally by a resistive heater to keep the specimen at 37°C. Between the sample and the sample cover an inlet passes a gas mixture (95 % air, 5 % CO_2) to keep the pH level of physiological medium at 7.4. An external humidifier keeps a small water reservoir at 37°C through which a gas mixture is passed to saturate the humidity inside the chamber. A Faraday cage surrounds the probe head and the sample to minimize the pickup of electronic noise through unscreened cables.

The inverted optical microscope (Ti-U) consists of several objectives under the sample stage to precisely position the probe on the sample. The gathered light can either be projected onto a camera (DS-Qi2, Nikon Corporation, Tokyo, Japan) or passed through an ocular. A filter cube that can be positioned in the optical path allows fluorescence microscopy (details in appendix Section A.9). The whole setup is positioned on an active damping table to decouple the system from external vibrations.

In this work, the focus was on the topography of living and fixed MG-63 osteoblast-like cells. Unless not otherwise stated, the hopping mode was used for topography measurements. In case of living cells, the measurements were performed in dulbeccos modified eagle medium (DMEM) with 10% FCS at 37°C and under 5 %

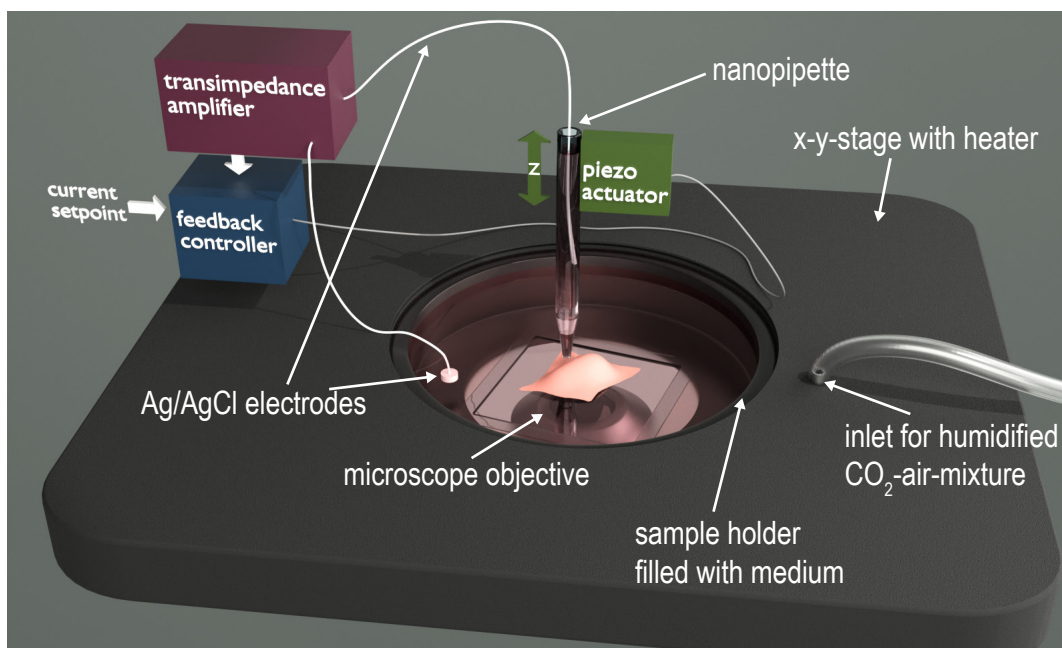


Figure 7: 3d rendered representation of the SICM setup.

CO₂ atmosphere. With 4 % PFA fixed cells (see appendix Section A.8) were measured at room temperature (21°C) in PBS (ROTI Cell PBS, Carl Roth, Karlsruhe, Germany). The nanopipettes were produced with a CO₂ laser-based pipette puller (Sutter P-2000, Sutter Instrument, Novato, CA, USA) from capillary borosilicate-glass (inner diameter 0.58 mm) with the following parameters: Heat: 260, Filament: 4, Velocity: 50, Delay: 225, and Pull: 140. Nanopipette openings of approx. 50 nm in diameter could be achieved.

3.2 AFM

AFM measurements were performed to evaluate the μ CP samples in ambient conditions (21°C, 50 % relative humidity). A commercial AFM (NX-bio, Park Systems, Suwon, South Korea) was operated in non-contact mode (also referred to as dynamic mode) with ACTA cantilevers (nominal spring constant: 37 N/m) (AppNano, Mountain View, CA, USA).

3.3 FLUORESCENCE MICROSCOPY

Fluorescence microscopy (Olympus IX73, Tokyo, Japan) via a UV filter cube (see attachment for details) was performed under atmospheric conditions to ensure the successful molecule transfer upon μ CP as well as the integrity of the molecule landscape on the macroscopic scale. After seeding, the response of the cells to the molecular layers was observed optically in situ (Nikon ECLIPSE Ti-U, Tokio, Japan) via a GFP (green fluorescent protein) filter cube (for details, see attachment). Due to low autofluorescence intensity of the molecule layers (bovine serum albumin (BSA) and polyamidoamine (PAMAM)) the culture medium was replaced by isotonic 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer.

3.4 SAMPLE PREPARATION

3.4.1 MICROCONTACT PRINTING

μ CP is a technique that was invented by Whitesides and coworkers originally in order to create molecular patterns faster than before possible by conventional photolithography methods [92]. Therefore, patterned elastomeric stamps, which often consist of polydimethylsiloxane (PDMS), are immersed in an "ink" solution, containing the molecules to be printed. After that, the stamp is brought into contact with the substrate such that a self-assembled monolayer (SAM) forms in the pattern of the stamp. In the beginning octanethiols were printed onto gold, forming covalently bound SAMs. However over the years the process was further improved for other molecules so even polar solutions could be used on the hydrophobic PDMS stamps.

In this work PDMS stamps were produced by mixing the Sylgard 184 elastomer (Dow Chemical, Midland, Michigan, USA) with the respective curing agent at a weight ratio of 10:1 (see Figure 8). To avoid cavities in the resulting mold the mixture was placed under vacuum for 15 min. After pouring the mixture over the glass master (Center for Microtechnologies, Technical University of Chemnitz, Chemnitz, Germany) it was cured in an oven (Universal Oven UF30, Memmert, Schwabach,

Germany) at 40°C for 24 h to aid polymerization. Subsequently the elastomer was peeled off the glass master and finally cut into molds with $\approx 1 \times 1 \text{ cm}^2$ base area.

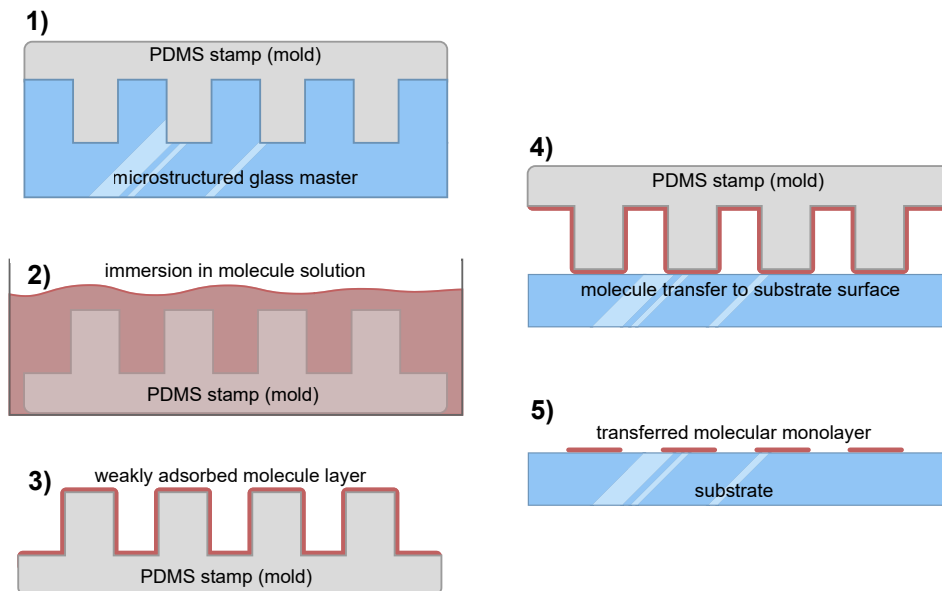


Figure 8: Schematic representation of the μ CP-process. (1) Micro-structured PDMS stamps were produced by pouring Sylgard 184 elastomer on glass masters. (2) & (3) The stamp was immersed in the respective molecule solution for 24 h. Thus, molecules weakly adsorb onto the PDMS stamp. (4) & (5) Molecular stripe patterned monolayers were transferred on the glass surface by pressing the PDMS stamp onto it.

To transfer molecules to the PDMS molds, they were immersed for 24 h in the respective molecule solutions. The composition of the solutions as well as the molecules used are listed below this section in Table 2. The PDMS stamps were rinsed with double-distilled water to remove excessive molecules. The next step included drying the stamps with nitrogen gas, to avoid formation of a closed liquid film on the substrate and therefore mobile molecules that would form unpatterned SAMs. Finally, the mold was brought into contact with the substrate to transfer the respective molecules. Here, the latter were borosilicate glass coverslips with dimensions of $22 \times 22 \text{ mm}^2$ (Menzel cover slips, VWR International, Darmstadt, Germany). Prior to μ CP the glasses were cleaned for 10 min in isopropanol in an ultrasonic bath. To make the samples more hydrophilic they were subsequently exposed to an ozone-UV cleaner (PSD-UV, Novascan Technologies, Inc., Boone, Iowa) for 30 min.

molecule	solvent	molecule concentration
BSA (Carl Roth, Karlsruhe, Germany)	double distilled water	0.15 mM
amine dendrimer (PAMAM gen. 1) (Sigma-Aldrich, St. Louis, MO, USA)	double distilled water	1.4 mM
carboxyl dendrimer (G1-TMP-COOH) (Polymer Factory, Stockholm, Sweden)	double distilled water	1.5 mM
polystyrenesulfonate (PSS) (Polymer Standard Service, Mainz, Germany)	100 mM NaCl	1 mM
polydiallyldimethylammonium (PDADMA) (Polymer Standard Service, Mainz, Germany)	100 mM NaCl	1 mM

Table 2: Listing of the molecule solutions used for [μCP](#). The respective chemical structural formulas are stated in appendix [Section A.3](#)

3.4.2 CULTURE OF MG-63 OSTEOBLASTIC CELLS

Human osteoblast-like cells of the cell line MG-63, were cultured in [DMEM](#) (DMEM, 31966-021, Life Technologies Limited, Paisley, UK), with 10 % [FCS](#) (FCS, Biochrom FCS Superior, Merck, Darmstadt, Germany) and 1 % antibiotics (gentamicin, Ratiopharm, Ulm, Germany) in a humidified atmosphere at 37°C with 5 % CO₂ (incubator New Brunswick Galaxy 14S, Eppendorf SE, Hamburg, Germany). The cells were passaged after a confluent layer in T25 cell culture flask was reached (roughly every third or fourth day). The cell culture medium was removed, and the cells were washed twice with 1 ml [PBS](#) each time. To suspend the adherent cells from the culture flask they were trypsinized (0.05 % trypsin-EDTA, ethylenediaminetetraacetic acid; PAA Laboratories, Pasching, Australia) and incubated for 5 minutes at 37°C. Afterwards 1 ml [DMEM](#) was added to stop the enzymatic reaction. The cell suspension was transferred into a centrifuge tube and centrifuged for 5 min at 120 x g. After removing the excessive liquid, the cells were resuspended in 1 ml [DMEM](#) and the population was measured with hemocytometers. The respective volume for 3×10^5 cells was seeded into a new T25 cell culture flask with 5 ml [DMEM](#) and 1 μl of gentamicin.

3.5 TOPOGRAPHY IMAGES

If not otherwise stated topography images of cell surface membranes were usually prepared by a line-wise height offset subtraction by their median of differences. Also a background plane was calculated and subtracted from the height data in cases where a plain reference layer (like the substrate) was recorded. Only for topography data where the cell membrane was exclusively measured, a 2D-polynomial background was calculated and subtracted to remove the membrane curvature. This enabled us to better visualize the small features occurring on the membrane.

3.6 STATISTICS

Student's t-tests with the software GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, CA, USA) were performed for the two respective groups (unoriented vs. oriented cells and glass- vs. molecule-monolayer-adhered cells) in [Chapter 5](#). Differences on p-values < 0.05 were considered significant. The cell preference results are presented as mean $\pm$ sem (standard error of the mean).

4

APICAL MEMBRANE MORPHOLOGY OF ADHERED OSTEOBLASTS¹

The functionality of living cells is inherently linked to subunits with dimensions ranging from several micrometers down to the nanometer scale. The cell's plasma membrane plays a particularly important role, since it serves as a separation of the inner cell contents and the extracellular space. Several proteins are situated on and in the membrane to exchange substances (via integral proteins e.g., carrier or ion channels), information (via peripheral proteins e.g., signaling proteins) or to aid adhesion and contact of the cell (e.g., integrins).

The membrane surface of cardiomyocytes shows characteristic periodic grooves, which result from highly organized sarcomere structures close to the cell surface to facilitate directed contraction. Also t-tubule invaginations are present that aid a fast calcium-supply for the excitation-contraction mechanism [95, 96].

HeLa cells express specific protrusions at the plasma membrane, called microvilli [97]. They are crucial for nutrient absorption and their number increases when cells are exposed to serum-free medium.

While the macroscopic morphology of MG-63 osteoblasts is well studied on varying substrates via light microscopy or scanning electron microscopy [98, 99], experiments focusing on sub-micrometer features are scarce in literature.

Hence, the apical membrane morphology of MG-63 osteoblasts is studied in this chapter. First, we will focus on living cells to investigate the unaltered morphological features and their dynamics. In the second part, the cells are investigated in a fixed state to evaluate their morphology without dynamics and to identify differences depending on the substrate to which the cells are adhered to.

¹ Parts of this chapter are adopted from the manuscripts [93] and [94].

4.1 MORPHOLOGY OF LIVING CELLS

Living MG-63 cells were cultured (see [Section 3.4.2](#)) and seeded onto borosilicate glass cover slips (Menzel, VWR International, Darmstadt, Germany). Usually, after 24 h of incubation they were investigated by SICM (see [Section 3.1.2](#)).

[Figure 9a](#) exemplarily shows a typical SICM topography of the border region in the live state (see the red square in [Figure 9b](#) for the corresponding bright-field microscopy image). The height for each location respective pixel in the image is displayed in form of a color, where bright spots represent positions of high- and dark spots of low height values. The corresponding value for each color can be identified by the color scale to the left-hand side of the image. The fast scan direction is from left to right, the slow scan from bottom to top.

The profile (red line, [Figure 9c](#)) shows that the border region of the cell is not flat. The cell surface is tilted 30° with respect to the glass substrate, which is represented by the horizontal segment on the right side of the profile. A sudden height change (≈ 500 nm) from the cell to the flat glass surface is visible, which in the following is referred to as step edge. The apical cell membrane surface exhibits features with lateral dimensions of approximately $1\ \mu\text{m} \times 0.8\ \mu\text{m}$ protruding 100–300 nm from the surrounding ([Figure 9d](#)), at a density of 0.3–0.5 features per μm^2 . These sheet-like protrusions seem to be oriented to the right side, since there is a smooth transition in height from left to right (see [Figure 9d](#)). In contrast, an abrupt height difference appears on the right edges of these sheets. Changing the fast scan direction did not change the orientation of these sheets. However, the dynamic rearrangement of the membrane features and possibly migration of the cell lead to artifacts which are visible in the measurements. The streaky appearance corresponds to the fast scan direction. Scanning of the topography took 68 min which only enabled us to perform measurements where the membrane dynamics are highly relevant. This becomes particularly evident when the individual scan lines cannot be aligned smoothly, leading to steps in the height image (see bottom left corner of [Figure 9a](#)).

[Figure 10a](#) shows a typical topography of membrane surfaces in flatter regions of the cell. The exact tilting angle cannot be calculated, since the reference glass surface is missing in the topography image. However, since the cell is spread wider in the scanning region (see [Figure 10b](#)) it is most likely a flat region. The sheet-like pro-

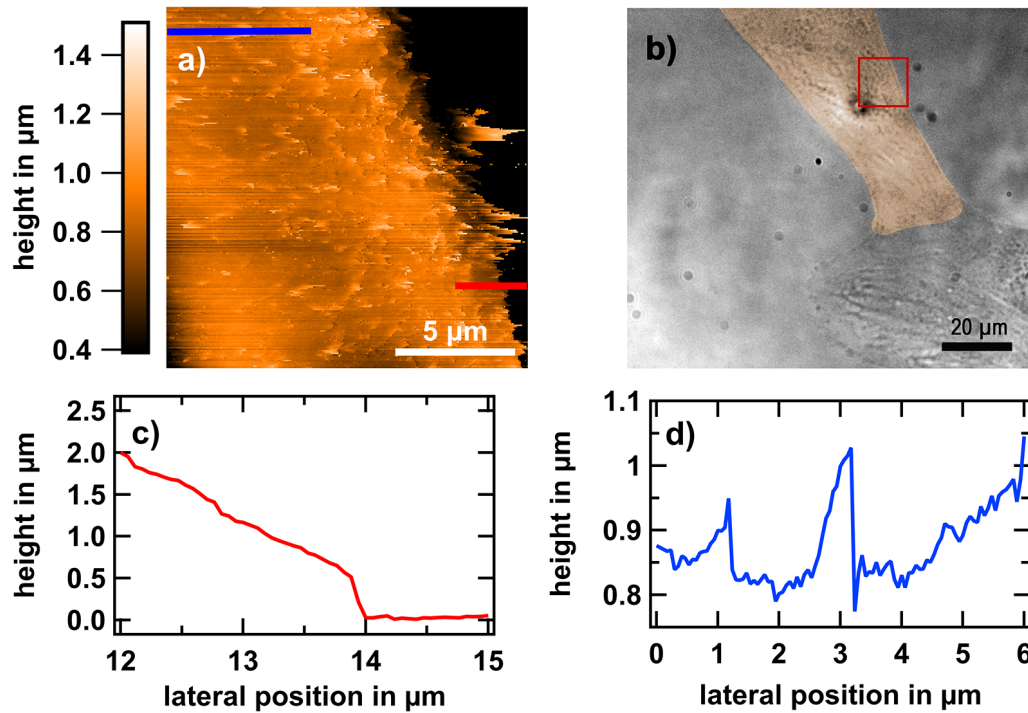


Figure 9: Living osteoblast cell adhered to glass after 24 h of culturing. (a) *SICM* topography image. Acquisition time: 68 min. (b) Corresponding bright-field microscopy image of the living cell taken prior to the *SICM* measurement. The cell has been digitally stained in orange for better visibility. The area marked in red was measured by *SICM*. (a) Sheet-like protrusions that mostly orient to the cell edge (right side in the image) are visible. (c) Line profile at the cell rim, as indicated in (a). A sudden jump from the cell surface to the flat glass surface is visible (≈ 500 nm). (d) Height profile along the blue line indicated in (a). Typical feature heights are several hundred nanometers.

trusions observed earlier are absent. Instead, blurred, bright, almost circular spots are visible in the colored image. They appear in different diameters of up to $1\ \mu\text{m}$ with elevations of 100 - 200 nm. In contrast to the sheet-like protrusions, these spots exhibit no sharp height transition. However, the density is similar. The dark spot on the right edge of the image shows an invagination that we encountered occasionally when measuring live membrane surfaces. The diameter of this depression is roughly $1.5\text{--}2\ \mu\text{m}$ and the depth was determined to be 800 nm.

Similar to the previously discussed topography, temporal undersampling leads to artifacts like the steps due to the scan line misalignment. Also, there is a slight wavi-

ness present in the image, it seems like the features are moving over time to the left and right, respectively.

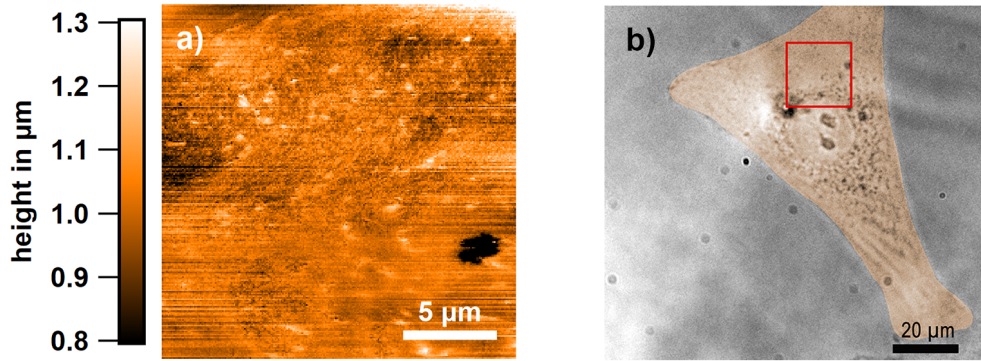


Figure 10: Living osteoblast cell adhered to glass after 24 h of culturing. (a) SICM topography image. The acquisition time was 68 min. Cell membrane protrusions can be seen as bright blurred spots. (b) Corresponding bright-field microscopy image of the living cell taken prior to the SICM measurement. The cell has been digitally stained in orange for better visibility. The area marked in red was measured.

To better assess the dynamics on the cell surfaces smaller frames were subsequently measured at the same position. This enables us to reduce the retraction height of the tip in the hopping mode (see Section 3.1.1), which dramatically enhances the scanning speed. Figure 11 shows SICM topography images, where the acquisition time could be reduced to 60 seconds each. The features remind more of the sheet-like protrusions in Figure 9a than the circular spots discussed before. The lateral dimensions are similar with $0.5\ \mu\text{m} \times 0.8\ \mu\text{m}$ while the maximum elevation is in range of 100 - 200 nm. Striking are the fine filaments that seem to protrude from the sheet-like structures (see red marker in top right or bottom left image, Figure 11). The upper limit of their width is 62.5 nm, which corresponds to the pixel width of the images. The filaments are several 100 nm up to micrometers long and seem to be anchored only on one end of the sheets, which is why they change appearance between measurements (see Figure 11).

Taking a closer look at the dynamics of sheet-like protrusions, one can see that most of them are not migrating over the surface. They seem to be laterally fixed within a moving radius of a few 100 nm. However, their shape changes over time, either because they change orientation or they get absorbed. Over the course of the mea-

surement one can observe the appearance and disappearance (from Figure 11a ff.) of such a protrusion (marked blue). The shape is fully developed after ≈ 4 min and by ≈ 7 min the protrusion disappears.

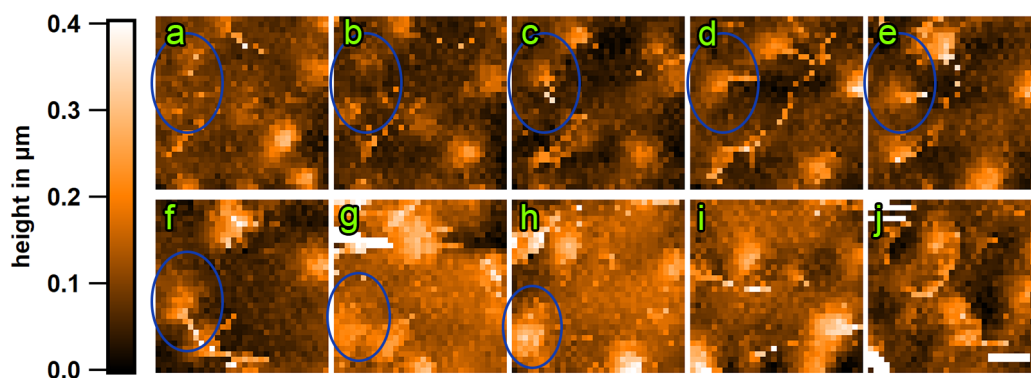


Figure 11: Subsequent images taken of the topography of a MG-63 cell adhered on glass. Data for each image was acquired within 60 s. A 2nd degree 2D polynomial layer was subtracted to pronounce the features of the cell surface better. The white scale bar in figure j represents 500 nm.

The dynamics of circular structures (Figure 10a) are shown in Figure 12. The diameter of the structures ranges from ≈ 120 nm to ≈ 400 nm. Their maximum height is about 250 nm. The graph in Figure 12 shows the maximum height of the feature marked in red over time. The shown values are means of the maximum height measured by forward scan and corresponding backward scan. The structure seems to retract linearly with a speed of (9 ± 3) nm/min.

Similar to the dynamics of the sheetlike protrusions, the circular ones seem laterally fixed and only move a few 100 nm in the presented time frame of 9 min. However, the dynamics are still too fast. We sometimes cannot distinguish whether a feature migrates or it retracts and another feature forms next to it.

Frequently we observe that structures associate together (see yellow marker) if they are within a moving radius of a few 100 nm or they dissociate (see blue marker). These processes appear to be faster than the acquisition time of 60 s.

Figure 13 shows subsequently taken SICM topographies of a living MG-63 cell at the rim. Most likely the rim is a lamellipodium, since the step edge height is only 100 - 200 nm. The contour of the rim is dynamic, while some areas expand laterally

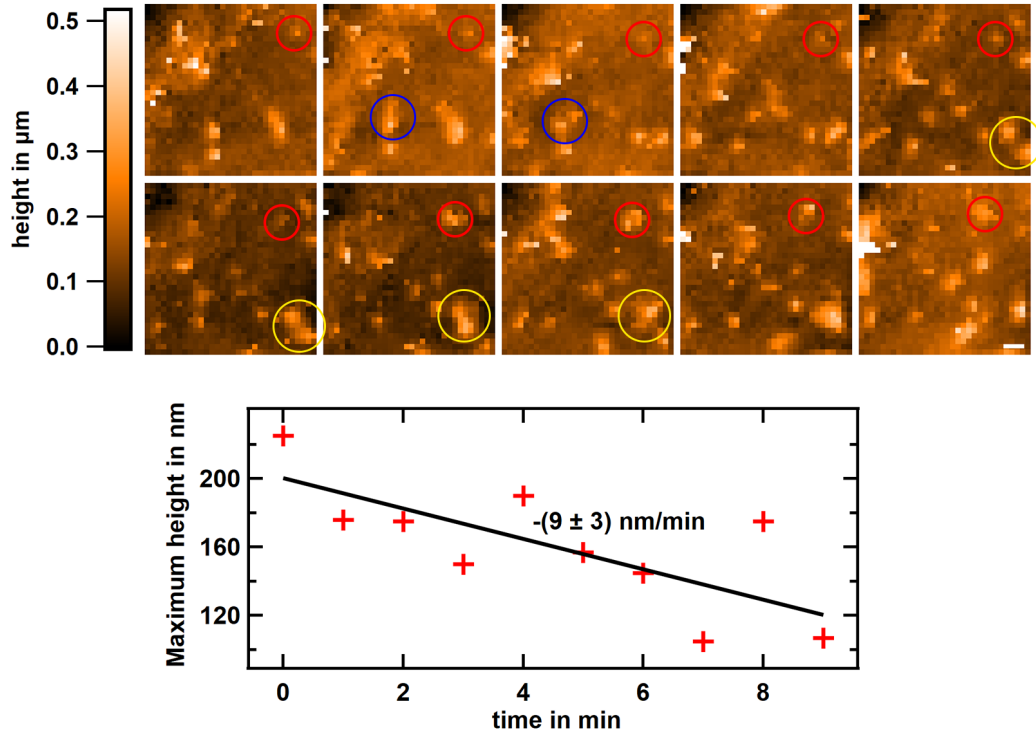


Figure 12: Subsequent images taken of the topography of a MG-63 cell adhered on glass. Data for each image was acquired within 60 s. A 2nd degree 2D polynomial layer was subtracted to pronounce the features of the cell surface better. The white scale bar represents 500 nm. The graph shows the maximum height of the feature marked in red over time. The plotted heights are mean values of the SICM forward- and corresponding backward scan.

a few 100 nm, others shrink within the scanning time of a frame. However, the cell doesn't seem to migrate or expand, since the cell's rim kept staying in the small measurement frame for 24 min. During this, elongated features develop at the cell rim. Interestingly they arise from broader micrometer wide and hundreds of nanometers long protrusions at the cell rim upon partial retraction of these protrusions. The resulting structures measure 100 nm in height and initially a few 100 nm in width but over time they get narrower (only 100 nm, see Figure 13). We observed similar growth and retraction speeds (regarding the length of the protrusion) of $1 \mu\text{m/min}$ (not shown). It should be noted that this is a lower limit since the temporal resolution is one minute.

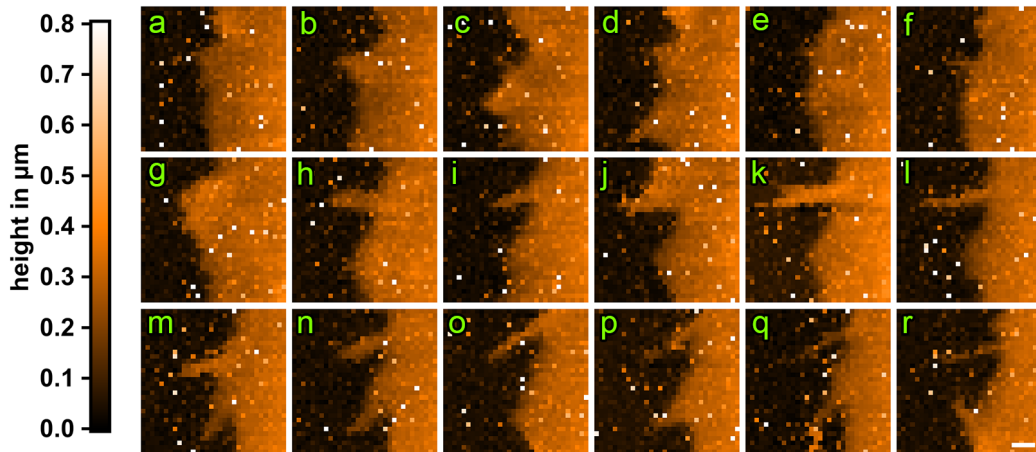


Figure 13: Subsequent images taken of the topography of a MG-63 cell adhered on glass. Data for each image was acquired within 80 s. The white scale bar in figure r represents 500 nm.

4.2 MORPHOLOGY OF FIXED CELLS

In order to suppress the live-cell dynamics, adhered osteoblasts were fixed with 4 % PFA (see appendix [Section A.8](#)) and subsequently investigated by SICM. A corresponding SICM topography is shown in [Figure 14a](#). Here, the sheet-like protrusions resemble those in the living state ([Figure 11](#)) during fast scans. Their lateral and vertical dimensions are similar. However, their shape is more pronounced and the features are stable over hours of measurements. The protrusions frequently show twisting, even in the fixed state. Similarly to the living state, we did not observe flapping when reversing the scan direction.

Usually the error current, that is, the deviation between setpoint and actual current at the time the approach is stopped is higher on hard than on soft materials (see, e.g., the white area on the right side of [Figure 14b](#), corresponding to glass). The reason is the different slope of the current–distance curves [100]. Because of this, the delay between current sensing and motion termination results in a larger error signal on hard materials than on soft ones. The fixed ruffles exhibit an extremely low ion current error ([Figure 14b](#)), lower than that of the surrounding membrane. In case of living cells such a contrast is absent as can be seen in [Figure 14c](#) and [Figure 14d](#).

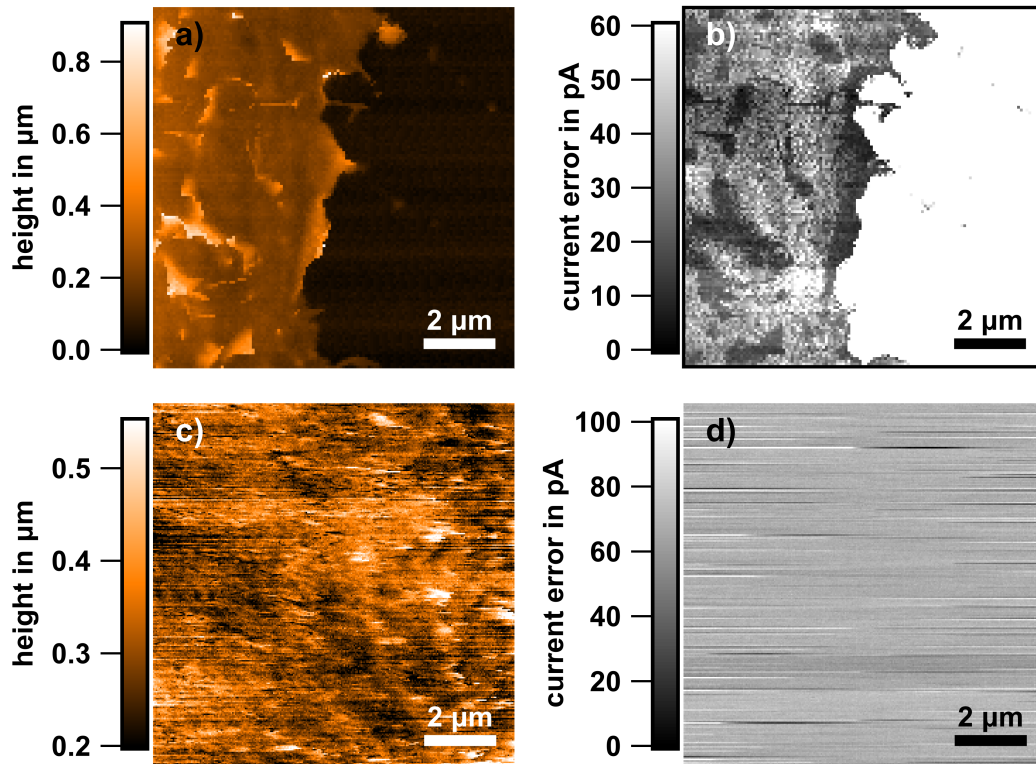


Figure 14: Comparison of living and fixed MG-63 cells by SICM. (a) Osteoblast adhered to glass after fixation (3 h). SICM topography image of the edge of the cell. The ruffles are clearly visible. (b) The corresponding current error image. Note that the ruffles appear to lead to smaller error values, resulting in a contrast in the error map. (c) SICM topography of a living osteoblast adhered to glass (24 h). (d) The corresponding current error map shows that there is no error contrast in the live state.

Interestingly, the sheet-like protrusions occur even directly at the cell rim (see [Figure 14a](#)). We observed such protrusions more pronounced and to a high extent. The location at the cellular periphery seemed arbitrary. [Figure 15a](#) shows typical protrusions. Frequently we observed that they are present numerously, so one cannot distinguish between separate sheets. They overlap, are twisted, and show maximum elevations of more than $1\text{ }\mu\text{m}$. The upper limit of the width is 180 nm , assuming that it forms an angle of 90° with respect to the glass surface ([Figure 15c](#)).

Though the sheet-like protrusions are the most prominent feature we observe on the osteoblastic cells, further membrane features, such as holes and circular protrusions, have been encountered. Circular tail-like protrusions were sometimes ob-

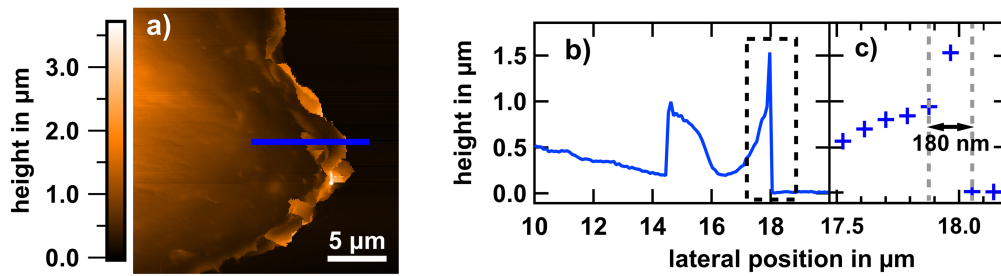


Figure 15: SICM analysis of a fixed cell adhered for 24 h to a 10 nm Au layer. (a) SICM topography of the peripheral protrusions of the cell. Such sheets can exhibit extensions of more than 1 μm as shown by the blue line in (a), the height profile of which is shown in (b). (c) Expansion of the section highlighted by the dashed box in (b).

served along with the sheets (Figure 16a), especially in the vicinity of the cell nucleus. They measure around 100 nm in length and their lateral scale is ca. 1 μm . They are similar to the structures that were measured in the living state (see Figure 10a).

Occasionally, we also observed depression or hole features in the membrane when cells were fixed (Figure 16b). They are between a few hundred of nanometers and one micrometer deep and mostly occur in close vicinity to extensive protrusions. Since these holes show lateral dimensions of several hundred nanometers, we can expect the measured depths to be close to the actual value. This is due to the fact that the used nanopipettes have a very high aspect ratio (the cone half angle is ca. 4°) with a small opening diameter (below 80 nm). Rarely, pancake-shaped structures with diameters of 0.5 - 1 μm are seen on the membrane, such as those shown in Figure 16c. They are characterized by their plateau-like shape that is elevated by approximately 200 nm.

Membrane topography in SEM

Since SICM only enables us to see projections of the topography in the xy-plane, we investigated the topography also by scanning electron microscopy (SEM). Therefore the cells were fixed with glutardialdehyde and subsequently critical point dried. To reduce the invasiveness, we omitted the typical Au coating of the specimen.

Similar to SICM, we could recognize the sheet-like protrusions, especially at the cell edge (Figure 17). However, the features at the top right of the image look more distorted. They seem to point vertically with respect to the cell membrane and remind

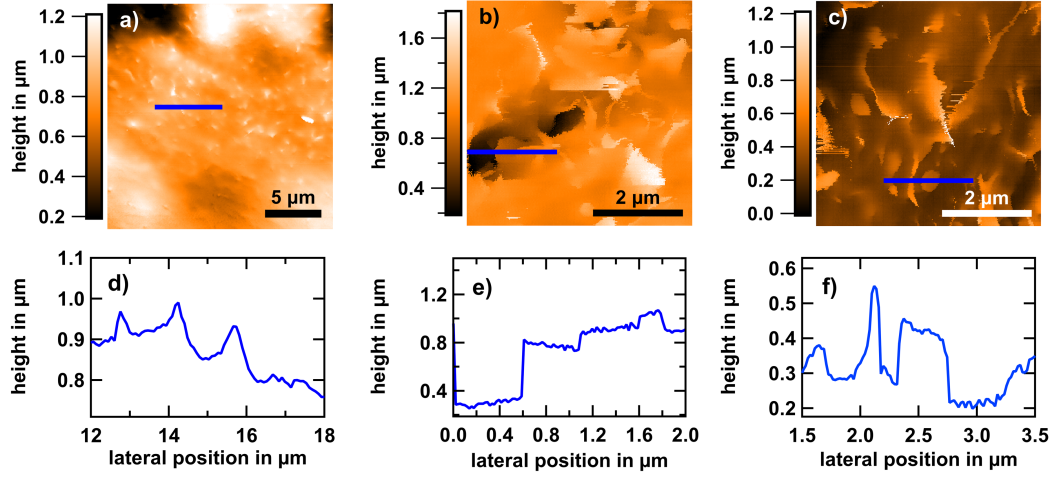


Figure 16: Other membrane features observed by SICM on fixed osteoblastic cells on glass (24 h). (a) Circular tail-like protrusions in the vicinity of the cell nucleus. These tail-like features are characterized by heights of less than 100 nm and seem to show no flapping, which results in almost orthogonal orientation with respect to the cell membrane. (b) Depressions or holes in the cell membrane of ca. 500 nm depth. (c) Pancake-like structures, which were rarely observed, show plateau-like characteristics. The heights observed are similar to those of dorsal ruffles. (d–f) The corresponding line profiles

more of the circular protrusions observed with SICM.

A few elongated protrusions, several micrometers long and connected to the cell rim, are visible. The observations are in agreement with those by SICM (Figure 13).

Excess membrane

To quantify the excess membrane of the cells, which results from the previously observed protrusions, we fixed the cells just 3 h after adhesion. The reason is that most of the spreading happens in the early phase after initial adhesion. In order to quantify the excess membrane associated with sheet-like protrusions, we focused on local frames in the vicinity of the cell rim, not on the large-scale cell morphology. Overall, four different cell passages were investigated. The cells were seeded on pristine glass substrates and plasma-polymerized allylamine (PPAAm) coated glass. On each cell, three edge locations were chosen randomly and the corresponding excess surface was determined. We define the excess surface A_{exc} as the difference between the effective and projected surface area ($A_{\text{eff}} - A_{\text{proj}}$). The effective surface is

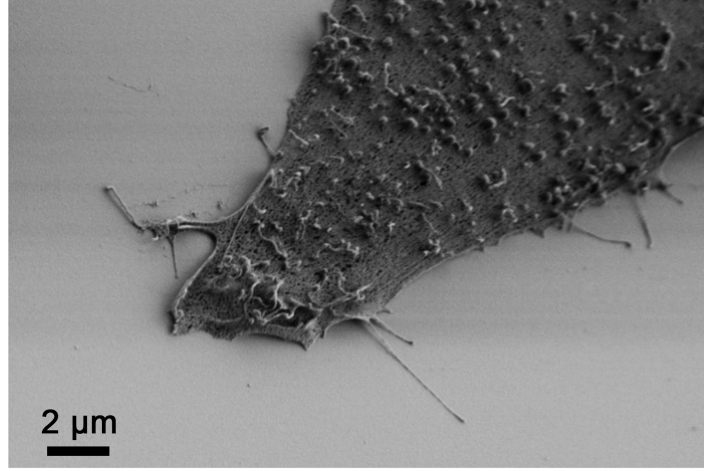


Figure 17: SEM of an MG-63 osteoblast after 3 h of adhesion to titanium. The ruffles on the cell membrane are visible. (FE-SEM SUPRA25, 1 kV, 30° angle, 100 nm Ti on Si wafer, fixation by 2.5 % glutardialdehyde (GA), acetone series, critical point drying).

the undulated surface area of the three-dimensional function $z(x, y)$ as determined from SICM topography images,

$$A_{\text{eff}} = \iint \left[\left(\frac{\partial z(x, y)}{\partial x} \right)^2 + \left(\frac{\partial z(x, y)}{\partial y} \right)^2 + 1 \right] dx dy ,$$

and the projected area is the frame or base area $A_{\text{proj}} = \iint dx dy$ (Figure 18a).

The relative excess surface is then

$$A_{\text{rel,exc}} = \frac{A_{\text{eff}} - A_{\text{proj}}}{A_{\text{proj}}} .$$

Figure 18b shows the relative excess surface as a function of the adhesion area of the whole cell, $A_{\text{rel,exc}}(A_{\text{adh}})$. The total adhesion area A_{adh} was determined via bright-field microscopy images. Relative excess surfaces between 5 % and 60 % were found assessing ca. 40 cells. Although the data is substantially scattered, we can observe a clear anticorrelation, namely $A_{\text{rel,exc}} \propto A_{\text{adh}}^{\alpha}$ with $\alpha = -1.13 \pm 0.25$, obtained by least-square fit and indicated by the red curve in Figure 18b. This means that a larger adhesion interface area is associated with a smaller excess surface (i.e., a lower number of protrusions or less extended ones).

Looking at the substrate-specific data, cells with extremely large adhesion interface areas (more than $2600 \mu\text{m}^2$) are only found on PPAAm layers. Nevertheless, the span of measured adhesion areas is large. On average (analyzing 44 cells and taking three samples on each cell) the excess surface on glass (0.25 ± 0.03) is larger than on PPAAm (0.17 ± 0.04).

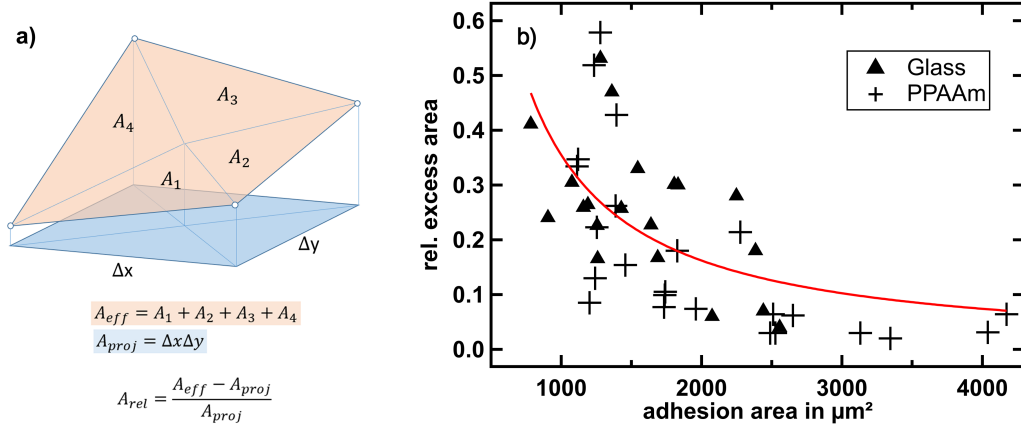


Figure 18: (a) Illustration of the effective surface area (orange) and the corresponding projected surface area (blue). The white dots represent height data points. (b) Correlation plot of the relative excess surface (ordinate) and the total adhesion interface area (abscissa) 3 h after seeding. The values depicted in the graph are the arithmetic mean of the corresponding relative excess areas determined for each cell. An anticorrelation becomes evident (red graph).

Rim edge heights

In Figure 19a, we show a typical example of the rim step edge height of an osteoblastic cell. Other MG-63 cells exhibit similar edge heights, though the weighting of the different height regimes measured varies with the overall shape of the cell. The step edge heights were systematically measured at the rim of the lamellipodia as well as at ordinary edges of the cells. Note that the step edge height not only incorporates the gap between the cell and the surface of the material but also the local height of the cell itself (Figure 19b). Both parameters may vary independently, and from the three-dimensional cell morphology we can only determine the sum of gap and cell height. At the higher edges (above $1 \mu\text{m}$) the step edge slope is greater than 89° and there is hardly any curvature detectable on the cell before its height falls to the substrate level. This could mean that the cell has a pronounced undermined configuration, compatible with the gap distance increasing at the rim of cells (see

discussion below). The flatter cell edges show slight curvatures on top and at the ledge side, and slope angles measure down to 45° . The smallest heights at homogeneous regions measured about 100 nm (Figure 19a), which is in agreement with the step edge height measured on living cells (Figure 13). Sometimes we observe rugged traces with only 60 nm in height or less. Apart from that, we find relatively high step edges of approximately $1\ \mu\text{m}$ and above, which we attribute to ordinary or trailing edges of osteoblastic cells (Figure 19a).

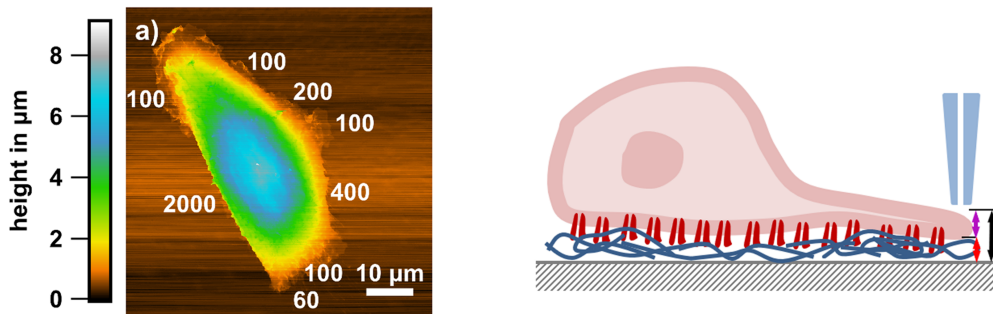


Figure 19: Edge height analysis. (a) Topography of a fixed osteoblast on a 10 nm Au layer on glass with indicated results (in nm) of step edge height analyses in the rim region. Cells show vastly differing edge heights, depending on the macroscopic location. However, the transition from regions of large edge heights to smaller ones is rather smooth. (b) Scheme of the measuring principle of the step edge heights. We determine the overall height of the edges (black arrow), which contains the cell thickness at the rim (purple arrow) as well as the gap between substrate and basal cell membrane (red arrow).

The edge height at the border of cells can be helpful to judge adhesion performance. At the edge of the cell, the height inheres in strongly curved or folded membrane regions and adjustment of the cleft distance between the basal membrane and the material's surface. An interesting parameter is the cell edge height, which is directly accessible by SICM. As pointed out earlier, the cell edge height is a strongly varying quantity (between 100 nm and a few micrometers) when measuring around the whole perimeter of the cell. Therefore, 3 topography images ($10 \times 10\ \mu\text{m}^2$, 128×128 pixels) at randomly chosen spots at the cell edges for each cell ($n = 6$ cells per surface after 1 h, $n = 18$ cells per surface after 3 h) were taken. Cell edge heights were measured exemplarily at 5 representative locations for each topography image and the respective values for each cell averaged. For osteoblastic cells after only 1 h culturing on the substrate, the average cell edge heights were

227 nm $\pm$ 18 nm on glass and 164 nm $\pm$ 7 nm on PPAAm, i.e., the cell edge is significantly $\approx 30\%$ smaller on PPAAm, indicating a higher cell–substrate contact (Figure 20). After culturing for 3 h on the surfaces, this difference has already vanished (on glass 171 nm $\pm$ 11 nm, on PPAAm 150 nm $\pm$ 8 nm). However, if we consider regions of peripheral ruffles, we find again a significant difference between 246 nm $\pm$ 17 nm on glass and 174 nm $\pm$ 10 nm on PPAAm. Note: This type of sheet-like protrusion is a loosened and folded-back membrane and it can originate from membrane excess, which was more pronounced on glass (see Figure 20 and Figure 18b). The protrusions need to be excluded for this analysis, because they would lead to an overestimation of the edge height in the case of glass.

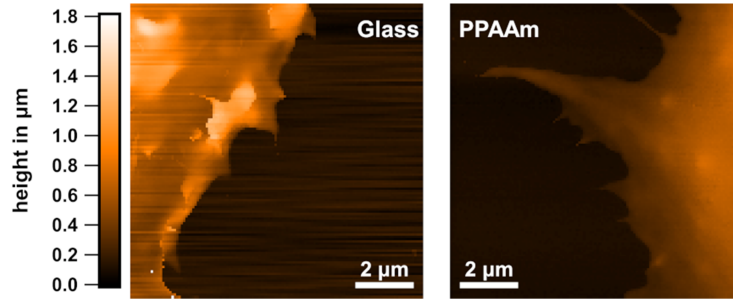


Figure 20: SICM topography images ($10 \times 10 \mu\text{m}^2$) of fixed MG-63 osteoblast peripheries after 1 h adhesion on glass (left) and plasma polymer PPAAm (right). The color scale represents the height information. Note that the cells exhibit more and higher peripheral ruffles (bright, leaf-like structures on the cell rim) when cultured on glass compared with flat cell edges on PPAAm.

4.3 DISCUSSION

Circular protrusions

Circular protrusions were found on the apical membrane of MG-63 osteoblastic cells, not only in the living state but also in the fixed state (see Figure 12 and Figure 16a). We can therefore expect that the shape and dimensions of these protrusions are not an artifact due to cellular dynamics or the fixation process.

The features' shape (circular pillars, standing out perpendicular to the surface) are similar to those of microvilli reported in literature [101]. Microvilli have been found on a wide variety of cell types including epithelial cells, dendritic cells, and

neurons. They are known to consist of a core of bundled F-actin filaments [102].

However, we observed that our pillar-like protrusions are up to 250 nm in height, while Gorelik et al. described microvilli heights (in case of living A6 cells) of up to ≈ 500 nm. Those of lymphocytes show mean heights of 0.3–0.4 μm and maximum heights well beyond 500 nm in SEM [103]. Even though our (chosen) lateral resolution might not have been sufficient enough to resolve single microvilli, it is unlikely that we missed those high structures, especially when scanning in forward and backward direction over a timeframe of 9 min (see Figure 12).

The width or diameter of microvilli is extensively studied on lymphocytes. Jung et al. describe 70–100 nm [104], while Polliack et al. observed 70–230 nm (and rarely up to 350 nm) [105]. In contrast, the smallest diameters on MG-63 found in this work are 400 nm. This could be explained by the fact that we did not observe single microvilli. However, Gorelik et al. observed no clumps, meaning that microvilli only attached along specific axis, forming ridges. If this was the case in our observations, we would measure the widths of single microvilli. Since SICM only measures the surface from the top, it is not excluded that we did observe angled, associated microvilli (like those of lymphocytes [103]) and thus did not observe widths of 200 nm and below.

The retraction speed of a circular feature (9 ± 3) nm/min is not compatible with that of single microvilli (1.2 nm/s) [101]. Since we expect to observe associated microvilli this might falsify the retraction speed, microvilli ridges have been reported to be more stable than single microvilli [101].

Elongated features at the cell rim

The elongated structures at the lamellipodial rim (see Figure 13) that were also observed by SEM (Figure 17) are suggested to be filopodia. Figure 13g shows a lamellipodial extension (advanced in < 60 sec) prior to the development of the filopodium. We expect that during this phase the Λ -shaped precursor is built within the lamellipodial actin network as reported e.g. for B16-F1 fibroblast-like cells [106]. In agreement with their observations, we could see that this extension, after building the precursor, retracted in less than 60 s leaving behind a filopodium. Filopodia fusion was also observed (not shown), which is a frequent process. For that, the filopodia moved sideways to each other, while the lamellipodia expanded around and between the fused filopodia just to retract again after fusion. The process takes no longer than 120 s.

We observe retraction speeds of $1\text{ }\mu\text{m}/\text{min}$ (or $17\text{ nm}/\text{s}$) which is in agreement with those measured for growth cones ($17\text{ nm} - 170\text{ nm}$) [107] or HeLa cells ($10\text{ nm}/\text{s}$) [108]. This retraction speed is faster than the depolymerization speed of F-actin ($\approx 2\text{ nm}/\text{s}$) [109, 110], which is the main constituent of filopodia. Therefore, we suspect a significant share of the filopodial retraction to result from the actin network retrograde flow in the lamellipodium, which is connected to the filopodium. It is driven mainly by actin polymerization (which is, with hundreds of nm/s , significantly faster than depolymerization) which pushes against the lamellipodial edge and contractile forces in the cell [107].

Even though, in general, the growth of filopodia was reported to be faster than the retraction (e.g. $100\text{ nm}/\text{s}$ for HeLa) there are filopodia that grow with a speed of our measured $17\text{ nm}/\text{s}$, e.g. growth cones and NG108-15 neuroblastoma cells ($1 - 2\text{ }\mu\text{m}/\text{min}$) [111].

Sheet-like protrusions

Similar sheet-like structures that we observed (see Figure 9a, Figure 11, Figure 14a) have been reported for dendritic cells. It has been shown that these structures, called ruffles, serve as catchers for T cells [112]. They have also been described on osteoblasts upon exposure to parathyroid extract [113] and after internalization of polymer or metal particles [114], on fibroblastoid cells [115, 116], on breast cancer cells [117], and on keratinocytes [118]. They appear in various sizes (laterally and vertically) and shapes. In general, these features are associated with migration, receptor internalization, and macropinocytosis [117, 119, 120]. Membrane ruffling is regulated by a distinct signaling pathway [121] and the supporting actin is denser and more cross-linked [118] compared to flat membrane regions. Three types of ruffles can be distinguished, namely (linear) dorsal, peripheral, and circular dorsal ruffles [119]. Colocalization with hyaluronan synthase has been found in a breast cancer cell line (MCF-7) [117].

Figure 15a shows an example of a peripheral ruffle. Peripheral ruffles are sometimes attributed to loosened lamellipodia. Such loosening and the subsequent retraction of lamellipodia towards the cell body has been shown for keratinocytes [118]. Either the attachment of the lamellipodium via integrin was not successful or such processes may serve the cell to attain structural flexibility and thereby responsiveness. Dynamic remodeling of structures, which requires a continuous balance between assembly and disassembly, is well known for cytoskeletal fibers.

The comparison between ion current error images of living and fixed cells can provide insight into the protein content in the ruffle volume, because fixation leads to cross-linking of proteins resulting in a higher Young's modulus. The fixed ruffles exhibit an extremely low ion current error (Figure 14b), lower than that of the surrounding membrane. PFA usually increases the Young's modulus of cell surfaces, for instance, from 3.5 to 18 kPa on fibroblasts [122]. Therefore, the lower ion current error appears counterintuitive regarding the material properties. However, considering the fin or springboard morphology of ruffles, the elasticity may not result from changes in material properties only, but also from the flexible shape. Thus, the extremely low error probably points towards lower stiffness of the ruffle structure, even when a harder (fixed) protein content is present.

Comparison with SEM

Osteoblastic cells have been studied extensively by scanning electron microscopy (SEM) [113, 114, 123]. Electron microscopy can yield nanoscopic resolution but requires invasive preparation, such as critical point drying and deposition of thin Au layers. This enables the observation of cell surfaces containing membrane protrusion features; however, apart from [113], no clear ruffles, as observed by SICM, are recognizable. When no Au coating was applied on the cells of the same osteoblastic cell line (MG-63) that were fixed after 3 h of adhesion on titanium and afterwards critical point dried, the ruffles remain clearer to see (Figure 17).

There are two issues regarding the contrast discrepancy between SEM and SICM that cannot be fully excluded to play a role. By means of critical point drying, volume changes of the cell throughout the preparation procedure for SEM are believed to be largely avoided. This might not fully hold at the nanoscale and some membrane protrusion features could get altered during cell death and the drying procedure. Thus, an option to explain the difference between SEM and SICM images is that the ruffles might be too fragile and are retracted or collapse upon critical point drying. However, especially attractive van der Waals forces might lead to the attachment of ruffles to the membrane surface and, hence, their deformation under vacuum conditions. In contrast, the attractive surface forces are screened in physiological medium by ions, leading to undistorted ruffles. Indeed, numerous of worm-like structures are typically observed on osteoblastic cells in SEM images, which could be bent microvilli or residual ruffles. In summary, plasma membranes in the rim region tend to appear slightly smoother (less ruffled) on cells that have

formed large adhesion interface areas. This may indicate that membrane supply is an issue during faster spreading.

Excess membrane

We observed that the relative excess membrane decreased with increasing adhesion area of the cells, independent of the substrate (Figure 18b). This could mean that excess membrane was “consumed” in more anisotropic or spread cell shapes to compensate for the increased surface-to-volume ratio in the course of spreading. Ruffles could serve as a membrane reserve prior to formation of large adhesion interfaces. This would be compatible with the observation that there are fewer ruffles found on lamellipodia or rim regions than in more central regions of the cell. For keratinocytes an anticorrelation between ruffling and lamellipodia persistence has been reported [118], supporting this scenario. Another option is that ruffles and excess membrane are signs of former unsuccessful spreading or deliberate lamellipodia loosening, for instance, for the purpose of resuming migration [124].

A slightly positive zeta potential, such as measured for PPAAm on titanium, has been shown to increase the spreading speed [125, 126]; our tentative observation of larger maximum adhesion areas on PPAAm than on glass substrates is compatible with these earlier findings.

The fact that the span of measured adhesion areas for cells on PPAAm is quite large (1100–4200 μm^2) may indicate that either the PPAAm layers on our glass surfaces are heterogeneous or that cells do not always respond to it. Another origin of the large spreading may be that, depending on the stage in the cell cycle, the adhesion program may get more or less priority. It has been shown that isolated cells do not synchronize their cell cycle in contrast to cells in groups [127] or in tissues [128, 129]. Hence, each cell may be in a different stage. This may contribute to different spreading speeds as observed on PPAAm. According to Pu et al. [130], MG-63 osteoblast-like cells are to 62 %, 18.6 %, and 19.4 % in the G1, S, and G2 phases, respectively. Our results are not compatible with [123], where ruffles have been predominantly observed on primary rat osteoblasts cultured on bioglass, which has a less negative zeta potential compared to quartz glass.

Rim edge height

The smallest heights at homogeneous regions measured about 100 nm (Figure 19 and Figure 12). Taking into account that the ECM already takes up about 60 nm [131],

the curvature radius of the membrane would correspond to 20 nm. Values in this regime are in line with protein-aided cellular membrane curvatures. For example, intracellular membrane tubes exhibit diameters of a few tens of nanometers, a maximum curvature is assumed to be generated by the Bin/ampiphysin/Rvs (BAR) protein and corresponds to less than $1/(10 \text{ nm})$ [132, 133]. Sometimes we find rugged traces with only 60 nm height. We suspect that those may originate from protein secretions that the extracellular matrix left behind, material that was formerly present within the gap between cell and substrate. Either this protein becomes exposed because the lamellipodium has folded away from the surface (to become a peripheral ruffle) or parts of the cell have been retracted from that region in the course of migration.

We observed that the cell edge height is lower on moderately positively charged PPAAm compared with negatively charged borosilicate glass after 1 h. Since we measured the sum of both cleft and cell height at the cell edge, we cannot directly compare with work addressing the cleft only. However, our results are compatible with reported cleft thicknesses of about 60 nm, as measured by metal-induced energy transfer [134] and about 40 nm by interferometric photo-activated localization microscopy [135].

Interestingly, after 3 h, there is no significant difference in cell edge height on PPAAm and glass. However, taking into account regions of peripheral ruffles, we observe a higher average cell edge height on glass. This indicates that either the region of peripheral ruffles is higher on glass i.e., specifically at the edges, the cell is not well attached to the substrate, or that, if the edges fold back, they fold back further, leading to longer peripheral ruffles and therefore higher edge heights.

Invaginations

We occasionally observed invaginations with SICM (see Figure 10a). We expect those features neither to be artifacts nor caused by poration due to nanopipette crashes. Otherwise, the invagination would appear suddenly from one scanned line to the next. In addition, the diameter of 1.5–2 μm does not match the width of our nanopipettes with $\approx 350 \text{ nm}$ at the height of 800 nm (see Appendix Section A.6).

Therefore we expect the invaginations to be part of a macropinocytosis mechanism, where the cell takes up extracellular fluid [136].

4.4 CONCLUSION AND OUTLOOK

SICM is a suitable method to resolve apical membrane features of living cells. It is largely non-invasive and thus cell responses due to the method can be neglected. Our high-resolution **SICM** topographies of living and fixed MG-63 osteoblast cells reveal the three-dimensional morphology and dynamics of their cell membranes. The most striking feature observed is the large amount of sheet-like plasma membrane protrusions, that is, dorsal and peripheral ruffles. Dorsal ruffles vanish to a large extent at cell rim regions when spreading is promoted and the cells exhibit large adhesion areas (above $2500\text{ }\mu\text{m}^2$). This was the case on amine-covered substrates, leading to smoother regions towards the lamellipodia. This suggests that ruffles act as membrane storage. However, we cannot discriminate between the scenario in which the cell anticipates rapid adhesion and thus ruffled membrane is brought toward the cell rim in order to establish lamellipodia and the scenario in which the ruffles remain behind after unsuccessful lamellipodia formation. Yet, the former scenario is rather unlikely since cells in the early stages of adhesion (low adhesion area) on **PPAAm** coating did not show a higher ruffle density than those on glass. Heights at the rim edges of the osteoblastic cells vary between 100 nm and more than $2\text{ }\mu\text{m}$ along the circumference. The smallest heights of around 60 nm may be due to **ECM** material left behind after the cell has retracted from such positions. Small heights of a few hundreds of nanometers may refer to lamellipodia regions, while the larger heights of a few micrometers and less curvature are typical for an ordinary configuration at cell rims.

During initial adhesion (1 h), the cells show significantly lower cell edge heights on **PPAAm** than on glass. This might indicate a higher adhesion performance of **PPAAm**. The speed of scanning with the hopping mode is sufficient to enable time-lapses of dynamics in membrane protrusions, like ruffles, which frequently change the shape but are laterally immobile. Smaller circular protrusions with diameters of 400 nm and heights up to 250 nm (unlikely microvilli), that might be subunits of ruffles, show similar immobility. Filopodia were measured at lamellipodia with widths of $\approx 100\text{ nm}$ and several μm in length. We observed growth and retraction speeds of $1\text{ }\mu\text{m/s}$ (lower limit).

However, in the future the scanning speed would still need to be increased to properly resolve processes of membrane protrusions with high dynamics on the cell surface. The limiting factor are oscillations (560 Hz), which occur at higher scanning speeds in the current signal. They probably stem from vibrations of the nanopipette tip. They are induced by higher accelerations upon rapid upward and downward movement of the nanopipette during scanning (see appendix [Figure 43](#)). Therefore a reduction in tip length by adapting the pulling parameters (see [Section 3.1.2](#)) could increase the resonance frequency and thus avoid inducing oscillations as quickly.

Another option would be the modification of the scan mode. Currently, movement of the nanopipette is abrupt, which leads to high acceleration peaks at points of reversal. Once the ion current setpoint is reached, the pipette gets pulled back instantaneously which can be seen by the zig-zag signal of the capacitive z-distance sensor over time (see [Figure 43](#)). The abrupt change in movement direction or stopping of the nanopipette after retraction induces the previously described oscillations. By changing the movement pattern to a smoother shape, e.g. close to sinusoidal, the peak acceleration could be reduced, and thus oscillations may be avoided, enabling higher scan speeds. However, the parameters have to be adjusted carefully since the tip may be more susceptible to crashes.

To further investigate the circular protrusions and to evaluate whether those are microvilli, simultaneous fluorescence microscopy and [SICM](#) are suitable methods. However, since the inner structure of microvilli and thus the proteins involved are very similar to the cell's cytoskeleton, it may be difficult to just stain and identify microvilli. Villin e.g., is an actin-bundling protein which seems to specifically occur in microvilli of epithelial cell brush borders [[137](#)]. However, proteomics of microvilli of SaOS-2 osteoblast-like cells did not show the presence of villin [[138](#)]. In literature we did not find a method to specifically target microvilli of MG-63.

5

CELLULAR RESPONSE TO MOLECULAR LANDSCAPES¹

In the previous chapter we studied the apical membrane morphology of cells, its dynamics, and changes in dependence of the substrate. In the following, we further investigate cellular behavior on the macroscopic scale. For this, molecular landscapes were printed on glass substrates via [μCP](#) which were characterized by fluorescence microscopy, [AFM](#) and [SICM](#). Furthermore, the cell adhesion sites were evaluated optically.

Regarding osteoblasts, during the initial phase upon insertion of joint prostheses, the speed of covering the material surface is a critical factor in avoiding inflammation. This is a complex process which comprehends directed migration towards the prosthesis interface, adhesion performance, differentiation and proliferation. There are a variety of factors that modify each of these aspects. While cell adhesion is largely affected by surface properties, which include surface roughness (on different scales), anisotropy, stiffness and chemical composition [[140](#)], the migration direction depends on gradients of e.g. chemical, electrical and magnetic nature.

Especially electric fields are known to influence numerous of the previously mentioned cellular processes. To understand how fields act on cell adhesion behavior, in vitro experiments are conducted using electrostimulation chambers (see [Chapter 6](#)). To check the effect of [DC](#) electric fields, it is also possible to use intrinsic surface potentials, directly. Due to advances in lithography, even localized surface potential landscapes can be created reliably and cost-efficiently. Since surfaces in vivo and in vitro are located in a liquid environment (often blood or culture medium, respectively) the relevant parameter is the ζ potential (see [Section 2.2](#)). It is defined as the residual unscreened surface potential at the hydrodynamic dis-

¹ Parts of this chapter are adopted from the manuscript [[139](#)].

tance or shear plane. The ζ potentials of surfaces are usually determined in a fluid channel via the streaming current or potential, depending on whether the substrate is electrically conductive or not [141, 142].

Most material surfaces, due to electron spill-out, exhibit more or less negative ζ potentials. The surface charge can be rendered towards the positive direction by attachment of molecular groups, which bind protons or by cationic dissociable groups. More difficult is the shift of the charge to more negative values than typical materials surfaces. In an aqueous medium, such a proton-binding molecular group is an amine. Amine groups will increase the ζ potential, i.e., the residual surface potential at the hydrodynamic distance. At the relevant pH of 7, the ζ potential is raised by more than ≈ 50 mV upon application of a polyallylamine layer [126, 143]. Laterally, a ζ potential difference of 30 mV at the transition between a molecular monolayer and glass, adapting over a distance of two protein diameters (≈ 10 nm) will already give rise to local electric fields of more than 10^6 V/m. This field is quite localized, but at least three orders of magnitude higher than the fields typically applied or operative in electrotaxis or electrostimulation chambers [48]. It should be noted that in the literature, the externally applied field is most often given, while the field actually operative in the chamber may be more than 10 times weaker, depending on geometry, ion current, electrode material and medium permittivity [144]. Since the ζ potentials on surfaces are already obtained at the shear plane the respective fields do indeed represent the actual fields.

We used molecular species such as amine- and carboxyl-terminated dendrimers (PAMAM, generation 1 and trimethylolpropane, generation 1, respectively), BSA and polyelectrolytes (PSS and PDADMA) to create surface potential landscapes. Even though we tried to utilize "innocent" molecules, it cannot be fully excluded that these molecular species provide biochemical cues. Albumin mainly occurs in the blood where it has the task of aiding thromboresistance by passivating blood vessel surfaces and suppressing platelet adhesion and fibrinogen adsorption. In non-native environments, it can also promote cell adhesion or be innocent [145–148]. For osteoblast-like cells, enhanced adhesion on BSA is observed compared to heat-denatured BSA [149], which can be further increased if it is treated with calcium. The dendrimers are synthetic, and in principle, they can damage the cell membrane or even enter the cell. However, this is unlikely at dendrimer diameters as small as chosen here [150]. Studies with MG-63 osteoblasts on homogeneous amine-terminated molecular layers revealed superior adhesion performance, i.e., the largest adhesion

areas and the most vital intracellular Ca^{2+} mobilization on plasma-polymerized polyallylamine and (3-Aminopropyl)triethoxysilane (APTES), in comparison to natively oxidized Ti surfaces [126].

Most studies use [ECM](#) species for stripe patterns, where the cell adhesion behavior is governed by the strong chemical affinity to bind (e.g. fibronectin), even at large topographic heights. Though the situation is distinct from our study, where we aim to explore other types of mechanisms and use “innocent molecular species” for stripe patterns, we provide a brief summary of findings on stripe patterns with [ECM](#) species [151–158]: Spindle shapes and alignment of fibroblasts in between fibronectin island rows have been observed [153]. The islands appear to be quite high, since only the template underneath has been prepared by μCP . Similarly, 2 μm fibronectin stripes with varying spacing gave rise to elongation and directional migration of fibroblasts in terms of contact guidance [155]. REF52 fibroblasts prefer adhesion on fibronectin stripes, in between non-adhesive polyethylene glycol (PEG) stripes, particularly if the PEG stripe width exceeds 8 μm [159]. Since the fibronectin is adsorbed to photolithographically prepared Au structures, these regions may protrude. Extremely high structures (pillars) lead to reduced adhesion area, in terms of formation of bridges and traction forces [157]. Muscle cells and murine myoblasts adhered to microprinted, assumingly flat, stripes of fibronectin or laminin took the characteristic spindle shape and aligned [152, 158]. Human vena saphena cells, at large stripe spacings, also adhere to fibronectin stripes and adopt a spindle shape [151]. Non-adhesive (hydrophobic) and adhesive stripes have been constituted by methyl termination and untreated regions of fused quartz, respectively, fabricated photolithographically [154]. The height differences should be in the region of the dimension of one methyl group, thus below one nanometer. Beyond a 24 μm stripe period a spindle shape and orientation have been observed with hamster (BHK) and canine (MDCK) kidney cells [154]. An interesting option is backfilling in between [ECM](#) stripes by other materials such as poly-L-lysine (PLL) or PLL-polyethyleneglycol [156]. PLL has positive charges, and the observation is that cells adhere to ECM stripes with the typical spindle shape and orientation. [ECM](#) protein species, such as collagen or fibronectin allow biochemical adhesion, i.e., expression of focal adhesion points, so that one may expect, that with these species, any electric field effects or other physical cues are overruled. In electric [DC](#) fields live cells, with their negative ζ potential, often migrate toward the cathode, thus in the direction of the electric field [160, 161]. Dead systems, such as lipid vesicles

with negative ζ potential, will drift opposite to the electric field $\vec{E}$ (thus towards the anode). The speed of electrophoresis [162] is $\vec{v} = \epsilon_0 \epsilon \zeta \vec{E} / \eta$ with ϵ_0 denoting the permittivity of vacuum, ϵ the relative permittivity of the medium, and η its viscosity. Thus, live cells most often move opposite to electrophoresis. Electrophoresis is a passive process, while cell migration is active. The directions of electrotaxis may be related to the wound potential, which is negative. In the case of bone tissue, the migration behavior of osteoblasts and osteoclasts appears compatible with the aim of driving bone remodeling at wounds toward excess mineralization [161].

5.1 CHARACTERIZATION OF MOLECULAR STRIPE PATTERNS

Molecular stripe patterns were printed on glass cover slips by μ CP and subsequently rinsed with distilled water (see Section 3.4.1). The mesoscopic characterization was performed by fluorescence microscopy (using UV filter cube, see appendix Section A.9) since we noticed a rather faint luminescence of the various molecules. We attribute this effect to aggregation-induced emission [163]. An example of the amine-dendrimer can be seen in Figure 21a. For these amine-terminated dendrimers as well as the other molecule types we noticed a homogeneous fluorescence intensity that only occasionally showed defects.

The topography of the various μ CP samples on cover slips was characterized by AFM. The height of these stripes is particularly important because high structures influence cell alignment, known as contact guidance [164]. A representative AFM image of amine-dendrimer stripes is shown in Figure 21c. The topographic height of the stripes was found to be 2 nm from the averaged line profile (see Figure 21e). Compared to the apparent root mean square roughness of the topographically lower stripe areas ($R_{\text{rms}} \approx 10$ nm, which we attribute to the glass substrate) the roughness of the molecular stripes is lower (≈ 9 nm). This, in addition to the homogeneous distribution of the high (white) spots in Figure 21c, indicates that these result from the bare glass substrate and that we achieved homogeneous monolayer stripe patterns of amine dendrimers by μ CP.

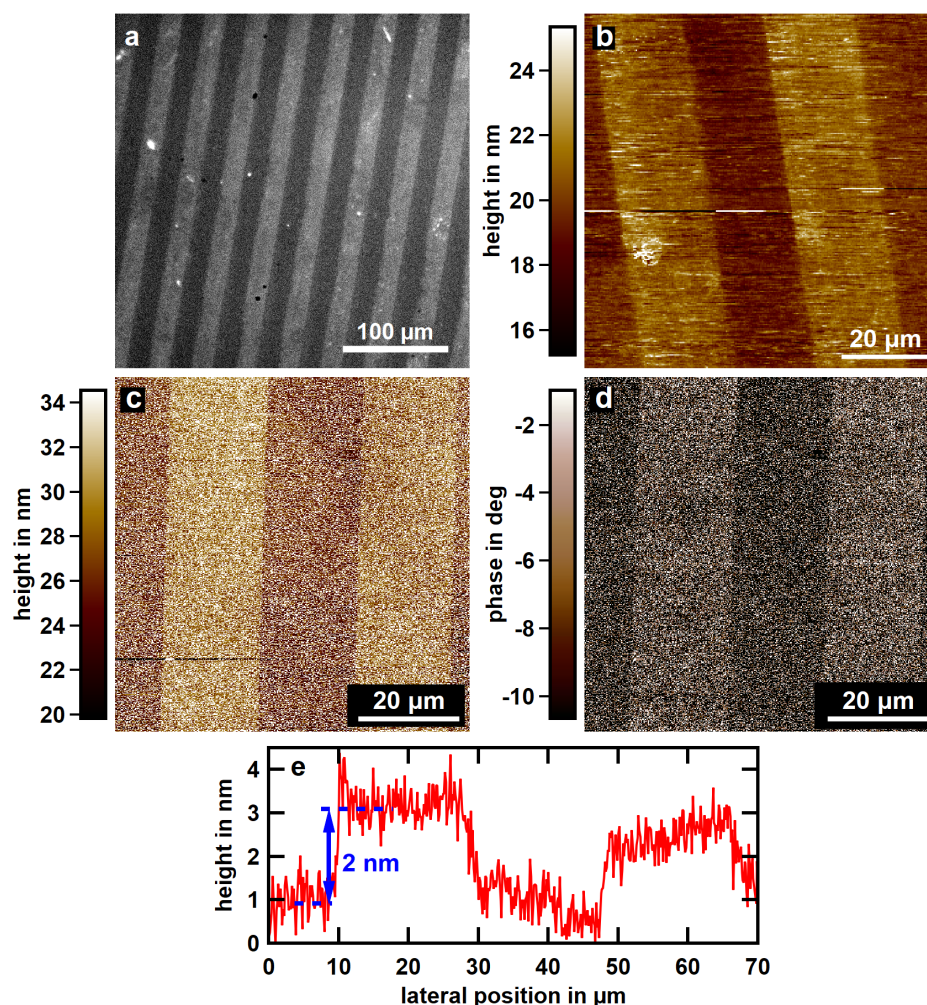


Figure 21: Characterization of molecular landscapes (in this case NH_2 -terminated (PAMAM) dendrimer monolayers) on glass upon μCP . (a) Fluorescence image, obtained with an UV filter cube in ambient. The molecule stripe patterns, which are bright, are macroscopically intact and only occasionally show defects. (b) SICM-topography image of an NH_2 dendrimer/glass sample in PBS. Bright colors show higher areas. The molecule stripes (high areas) remain intact in liquid environment. (c) AFM-topography image of $20\text{ }\mu\text{m}$ -wide molecule stripes with $20\text{ }\mu\text{m}$ spacing between them under ambient conditions (air). Bright colors indicate topographically high locations. The white spots partly originate from oscillations. (d) Corresponding phase image. One can see that topographically higher areas show lower absolute phase values than the lower areas. This indicates different mechanical properties between those areas and, therefore, points to different materials being present, namely NH_2 -terminated dendrimers and glass. (e) Horizontal line profile of the AFM-topography image (c) averaged over the whole area. The height of the molecular layer (2 nm) is clearly visible.

The corresponding AFM phase image (Figure 21d) shows a lower absolute phase value at topographically high positions, revealing that the molecules indeed cover only 20 μm -wide stripes, and that in between these stripes the bare glass substrate is still visible. To validate the integrity of the molecular layers in liquid (PBS) we performed SICM measurements. The stripe-patterned molecular layers stayed intact and, in case of the amine dendrimer, heights of 2 nm (see Figure 21b) were measured which is similar in ambient conditions. It should be noted that the sample appears to have a lower roughness ($R_{\text{rms}} \approx 2$ nm) in liquid. This is due to the lower lateral resolution of the SICM (≈ 50 nm) compared to AFM (a few nanometers) [165, 166]. Height analysis was conducted for the various molecule types. The results are shown in Table 3.

molecule species	AFM [nm]	SICM [nm]
BSA	4	2
NH ₂ -terminated dendrimer (PAMAM gen. 1)	2	2
COOH-terminated dendrimer (G1-TMP-COOH)	1	2 - 3
PDADMA	1 - 3	0.5 - 1
PSS	1	2

Table 3: In ambient conditions (AFM) and in liquid (SICM) measured molecule layer thicknesses.

5.2 CELL RESPONSE TO MOLECULAR LANDSCAPES

MG-63 osteoblastic cells were seeded on BSA and NH₂-terminated dendrimer-printed glass samples and incubated for 24 h, allowing them to adhere and migrate to preferred locations. Subsequently, the cell culture medium was exchanged by HEPES buffer to localize the molecular stripes by luminescence (GFP filter cube). In areas where molecular stripe patterns were present, a significant amount of single cells ($(89 \pm 3)\%$ for BSA and $(78 \pm 6)\%$ for NH₂ dendrimer samples) adopted a distinct spindle shape and aligned parallel to the stripes (Figure 22). This observation remained true only when the cells were separated from each other. In case of connected cells (e.g., via filopodia) the response to the molecular patterns seemed to be

largely absent (see blue marker in Figure 22d). They were often randomly oriented and did not necessarily maintain a spindle shape.

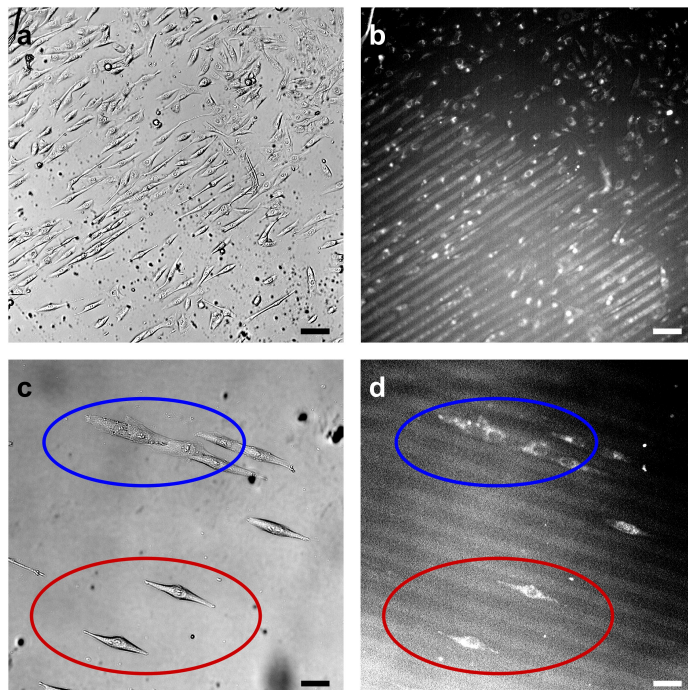


Figure 22: Optical microscopy images of MG-63 cells after 24h adhesion time on glass with molecule stripe landscapes. The scale bars in the bottom right corner show 100 μm or 50 μm for images (a,b) and (c,d), respectively. (a) Brightfield image of adhered cells. Most cells orient diagonally and adopt a spindle shape. (b) Corresponding auto-fluorescence image (GFP-filtercube). The bright stripes (indicating NH_2 -terminated dendrimer layers) are oriented in the same direction as the cells. At spots where the stripe pattern is absent (top right) the cells orient randomly and do not necessarily take on a spindle shape. (c) Brightfield image of adhered MG-63 cells on BSA μCP glass. The cell clusters (marked in blue) are oriented in the same way as the separated cells (marked in red). However, it is visible that the adhesion area is not so well defined for the cell-clusters. (d) Corresponding auto-fluorescence image. The separated cells orient along the bright BSA stripes, but adhere on the dark glass-strips. In case of the cell clusters (marked in blue), orientation and confinement onto the glass site is largely absent.

Taking only separated cells into account, we noticed a significant adhesion preference onto glass sites (see Figure 23). While for the BSA-samples ($87 \pm 5\%$) of single cells residing on glass, the preference seemed to be slightly lower in case of PAMAM

samples with $(70 \pm 5)\%$. We can exclude that this observation is due to different adhesion probabilities on glass or flat molecule layers. When we offered a flat unstructured layer of BSA or NH_2 -terminated dendrimer, the preference for glass vanished. A similar amount of cells were then found on the glass and molecule sites after 24 h incubation (see Figure 24). This may also serve to dispel doubts regarding the fact that albumin is not fully innocent in terms of adhesion [145–148].

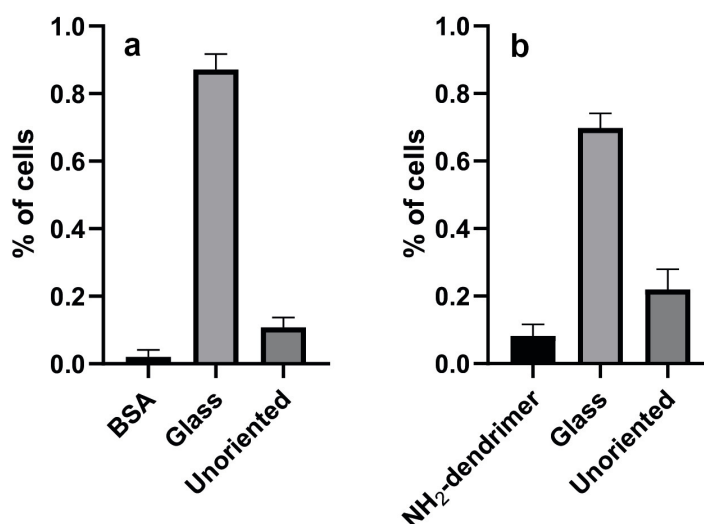


Figure 23: Percentage of separated cells on glass samples with structured molecular landscapes after 24 h adhesion time. Cells either oriented along the structures (adhered on molecule layer or glass) or they did not respond to the landscape and remained unoriented. At least 4 independent experiments were performed, all graphs show significance (mean $\pm$ sem) between oriented (glass and molecule layer) vs. unoriented cells and molecule layer vs. glass (Student's t-test, $p < 0.05$). (a) Cell-distribution on BSA μCP structured glass. (b) Cell-distribution on NH_2 -terminated dendrimer μCP structured glass.

The luminescence intensity of the polyelectrolytes (PSS and PDADMA) and COOH -terminated dendrimers was not sufficient enough when exposed to HEPES buffer to discriminate between the glass and molecule sites. Thus, we decided to check random samples using SICM. Three cells for each molecule type were chosen and their topography as well as location were investigated. The cell topography at the poles featured membrane protrusions, so-called ruffles (as described in the previous chapter, see Figure 25). Strikingly, the amount of ruffles is higher than at the rim of

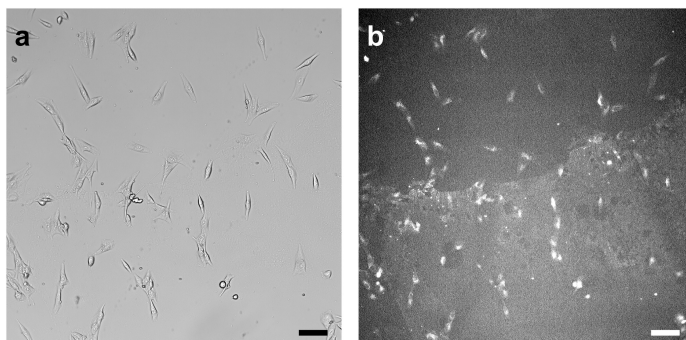


Figure 24: MG-63 cells 24 h after seeding onto a BSA μ CP glass surface. The scale bars indicate 100 μ m. (a) Brightfield image of a representative spot at the transition from glass to BSA-surface. A homogeneous cell distribution is present. (b) Corresponding auto-fluorescence image. The bright area (bottom half) shows the presence of a BSA-layer. A total of 36 cells are adhered on glass while 43 reside on the BSA-layer. Considering the area of the molecule layer vs. glass (0.74 mm² and 0.65 mm², respectively) the cell density is similar.

cells which did not adopt the spindle shape. In all cases, the cell alignment was along the stripe-patterns and the cells chose to adhere at the glass site.

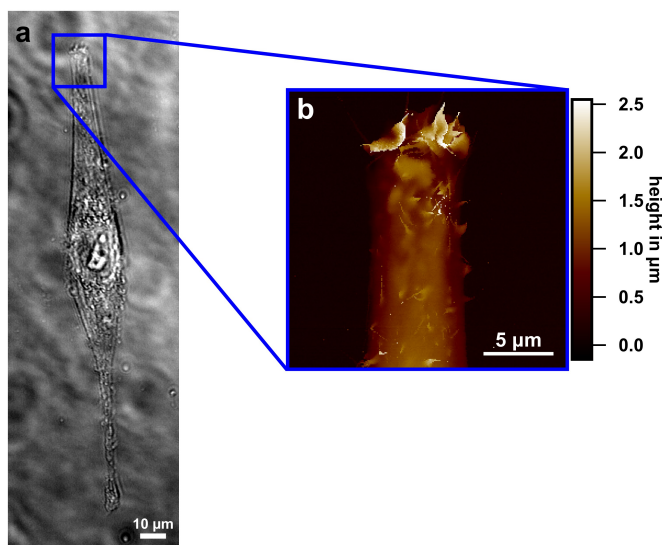


Figure 25: Fixed MG-63 cell after 24 h adhesion time on BSA μ CP glass in PBS. (a) Bright-field image of a spindle-shaped cell oriented parallel to the molecule stripes. The blue square at the cell pole shows the area of investigation by SICM. (b) SICM-topography image of the cell pole. Bright colors mark high spots. Extensive membrane ruffling is visible. The ruffles (peripheral) at the edges are several μm higher compared to the surrounding cell membrane, indicating that the cell either folded back its membrane or that the cell attempted to spread further and, therefore, provided membrane at the pole.

5.3 DISCUSSION

Stripe characterization

The AFM data correspond well to the respective molecule monolayer heights [167–169]. All measured heights fall in the regime between 0.5 and 4 nm. In general, under ambient conditions (air), which were present for AFM measurements it is well known that a thin film of water adsorbs onto the surface. The film is usually not measured by AFM but might lead to a swelling behavior of the molecular films. In contrast, SICM is performed in liquid (PBS) containing various dissolved salt species.

In case of BSA we expect a laying configuration with the long molecule axis ($\approx 14\text{ nm}$) parallel and the short axis ($\approx 4.2\text{ nm}$) perpendicular to the surface [167]. The protein structure (tertiary and quaternary) of BSA may be the reason why e.g., the Stokes radius is only 3.48 nm [170]. This would imply a high ion-conductivity

and could explain why an artificially low height of 2 nm was measured by SICM. The NH₂-dendrimers exhibit a higher mass density than BSA (50 %) taking into account the Stokes volume and the molecular weight. Therefore the ion-conductivity may be negligible which results in similar heights measured with AFM and SICM (2 nm). Even though, no data on the Stokes radius is available in literature, we expect a similar mass density of the COOH-terminated dendrimer. However, in contrast to the NH₂-dendrimer the height in SICM was measured up to a 3-fold higher than in AFM. Taking into account the ζ -potential, which is negative for both the glass surface and the COOH-dendrimer, we can expect a repulsive effect. However, this effect should be lower in a high ionic environment due to the screening and thus should lead to an opposite effect of what has been measured.

The swelling in liquid environment is especially known for polyelectrolytes and results in height changes of a few nanometers in case of a few multilayers [171]. However, the swelling is usually $\approx 30\%$ and the effect observed here is larger. More likely there are only weak binding sites formed between the negatively charged PSS and glass, so polymer fragments protrude into the liquid (loops and tails). This would explain the increase in layer thickness in liquid. In contrast, layers of positively charged molecules (e.g., PDADMA) are equally thin or thinner in liquid compared to ambient atmosphere because the positive monomers are electrostatically attracted to the glass.

The rectifying behavior of the ion current through a nanopipette due to the different ζ potentials of the molecular layer and glass can be neglected here, since the Debye length is ≈ 0.7 nm in PBS [91]. The edge sharpness of the stripes is in the regime of 25 nm according to AFM measurements at the transition between glass and stripe.

Adhesion site preference

The apparent glass preference when molecular layers are offered in the direct vicinity of cells is not explainable by means of ζ potentials of the different species: the potential of glass is strongly negative (≈ -80 mV), BSA films on glass raise its potential by a few 10 mV. The amine groups of the dendrimer exposed to aqueous medium tend to bind a proton and become positively charged, resulting in a positive ζ potential of 22 mV [172]. This would give rise to a preference to the molecular layers since ζ potentials in the regime around 0 mV are favorable for MG-63 activity [126]. Especially on larger, covalent bound NH₂-terminated dendrimers (gen. 5) the os-

teoblasts exhibited tight cell attachment with well-organized actin stress fibers and well-spread morphology [172].

However, if a chemical gradient would be operative, the cells could sense and mistake or regard the amine as the presence of cells or extracellular matrix. In terms of (pseudo-)tissue formation priority, i.e., swiftly covering a surface, it would make sense, that cells fill voids, i.e., the space in between the molecule stripes. The observation of mini-tissue, i.e., the accumulation of two or more cells that did not reside on the glass site supports this scenario. The UV-ozone pre-treatment of the glass slides as a reason for the cell response could be excluded since experiments without treatment yielded similar results. Contact guidance occurs beyond a threshold of ≈ 33 nm deep grooves for osteoblasts [173, 174]. For fibroblasts, the threshold for contact guidance was reported to be 35 nm with groove structures with a 1:1 pitch ratio [173]. Therefore, we regard contact guidance as an extremely unlikely process in our experiment since our structures are less than 4 nm in height.

Instead, we suggest that the different ζ potentials between glass and the respective molecular layer give rise to a horizontal electric field localized at the molecule-glass transition lines. Considering the low distance over which the electric potential drops (e.g., roughly assuming twice the molecular diameter), the electric field already amounts to the order of 10^6 V/m. Such high field strengths are usually not present in conventional electric chambers [44, 48]. In case of the BSA and NH_2 -terminated dendrimer landscapes, the electric field vectors would point in-plane, perpendicularly from the stripe edges toward the glass site, which is the retraction (or deformation) direction of osteoblastic cells in our experiments (see Figure 26).

Often, mammalian cells appear to show cathodic taxis [160, 161], including keratinocytes [175], neuronal growth cones [176], meniscus cells [177], and SaOS-2 osteoblast-like cells [44, 45]. Some cell types show anodic taxis, e.g., osteoclasts [44] and dermal fibroblasts [175]. Furthermore, primary patient-derived osteoblasts [178] and calvarial osteosarcoma osteoblasts [45] show anodic taxis. A paucity of literature exists on the behavior of the osteosarcoma cell line MG-63 [48, 144]. This type of osteoblastic cell appears not to migrate to one or the other electrode, however, MG-63 adopts a crescent shape with the convex side toward the cathode [48], as has also been observed for migrating RCJ.1.20 osteoblastic cells [44]. This may possibly represent an attempt to minimize electric potential differences along the cell body.

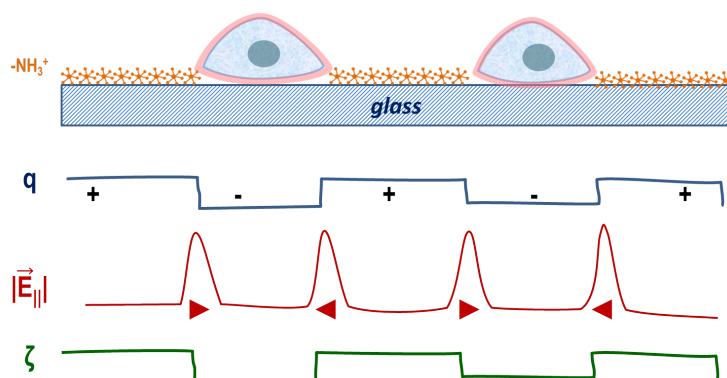


Figure 26: Depicted is the cross section of an NH_2 -terminated dendrimer printed on a glass sample where MG-63 osteoblastic cells are adhered. The difference in ζ potential between the molecules and glass (illustrated in green) lead to an alternating lateral electric field (red) at the glass–molecule interface. We put forward a double-sided cathodic taxis ansatz to explain the apparent cell preference on glass.

This is in agreement with our observation, since the cells adhered along the glass and thus avoided the horizontal electric field at the glass–molecule interface. To corroborate that the glass preference is due to electro-deformation, we assessed the cell response to COOH-terminated dendrimers, [PDADMA](#), and [PSS](#) landscapes. [PSS](#) shows a slightly lower ζ potential (-88 mV) than glass (assuming -80 mV for glass), which may allow us to alter the electric field vectors in comparison to [BSA](#) and NH_2 -terminated dendrimer-printed glass samples [126]. COOH-terminated dendrimers and [PDADMA](#) were chosen to vary the electric field strength due to their ζ potential (-47 mV and 51 mV, respectively) [126, 172]. Through bright-field microscopy, we noticed an alignment and orientation of single cells, similar to previous observations with positively charged stripes. Unfortunately, the autofluorescence of the three molecule types was too faint to observe under liquid conditions, which is why we could only conduct spot checks to investigate the cell’s preference with [SICM](#). Of the three cells chosen on each molecular species sample (COOH-dendrimer, [PSS](#), and [PDADMA](#)), none of them were located on the molecule stripe. Thus, the orientation of the electric field vector at the glass–molecule interface (COOH and [PSS](#)) either might not be the dominating factor that controls the adhesion preference or the lowering of the ζ potential was not successful. Since the ζ potentials of glass and [PSS](#) are very similar, reversal of the electric field vector at the transition lines might not have taken place.

In general, the error of ζ potentials with molecule coating is $\approx \pm 10$ mV. It is much more difficult to increase the negativity of already negative surfaces compared to the charging of negative surfaces toward the positive direction. This is due to the electron spill-out, i.e., the fact that electrons are less localized in atoms than the positive nuclei.

SICM-topography at the cell poles

Significant amounts of ruffles were present at the cell poles (see Figure 25). Since ruffles act, among other functions, as membrane storage, the spindle-shaped cells apparently provide additional membrane at the poles. This shows that the apical membrane morphology indicates the spreading direction (along the poles).

If we were to assume cathodic taxis of the osteoblasts, the movement could be explained by the push-pull model [67] or the directional sensing model [179]. Both suggest the Ca^{2+} -flow being essential for the mechanism. A transcellular Ca^{2+} current may pass from the rear to the leading edge, resulting in a Ca^{2+} concentration gradient along the cell. The low Ca^{2+} concentration toward the anode leads to depolymerization of actin while an increased Ca^{2+} level at the cathodic side leads to a promotion of polymerization. The concentration gradient also comes along with actomyosin exertion and thus to a migration towards the cathode [67]. Since, in our experiment, there is no uniform cathodic side due to alternating electric field directions, we suggest a double-sided local galvanotaxis of cells in the direction of the electric field vectors being responsible for the observed behavior.

5.4 CONCLUSION AND OUTLOOK

Monolayer-striped patterns with $40\text{ }\mu\text{m}$ periodicity and $20\text{ }\mu\text{m}$ stripe width have been prepared by μCP and characterized by AFM and SICM. The heights were found to range between 0.5 and 4 nm with the largest apparent height observed with BSA stripes in ambient (air) and the smallest with COOH-terminated dendrimers G1 in physiological medium. Taking into account the sharpness of the molecular stripe pattern edges and the ζ potential difference between molecules and glass, the electric field lines at the interface should amount to 10^6 V/m. Indeed, osteoblasts preferentially adhere to the glass (with an abundance of $87 \pm 5\%$ and $70 \pm 5\%$) and orient along the molecular stripes, adopting a spindle shape. The spindle poles exhibit

excessive ruffling, pointing towards a membrane reservoir for the purpose of longitudinal spreading. The deformation is compatible with a cathodic taxis mechanism, where Ca^{2+} ions flow into the cell at the anodic side, leading to retraction.

Since we could not discriminate whether we achieved an alternation of the direction of the EF (by applying molecules with a lower ζ potential than glass) it would be helpful to change the substrate (in this case, glass). However, as stated previously the ζ potential of most substrates is highly negative. Therefore, we advise creating alternating molecular stripe patterns with 2 molecule species that cover the whole glass surface. A variety of different lithographic methods are common, but they are often operable at too low lateral scales for cell applications (e.g., nanografting via AFM or dip-pen nanolithography [180]) or require chemical agents (e.g. photolithography) which often conflicts with biocompatibility.

We consider μCP to be the most efficient and "non-invasive" method to achieve alternating molecule stripe patterns. The ordinary μCP , which we have performed, has already been further developed by other workgroups [181, 182].

One approach is subsequent μCP of different molecule species [181]. However, substantial effort has to be made aligning substrate and PDMS stamp during the second printing step (e.g. via micrometer drive stages). Misalignment as well as the case that the two molecular species share the same solvent, could lead to the transition between the stripes being soft and thus to a diffuse EF distribution.

Microdisplacement printing could solve this issue, but the method is highly dependent on the specific molecules being used [183]. It requires a "labile" SAM that is adhered to the substrate (e.g., by immersing the substrate). The second molecule species is printed (via μCP) on the SAM, which results in displacement of the SAM at the printed locations via competitive adsorption.

Another possibility is the combination of μCP and degas-driven flow-guided patterning [182]. In contrast to the previously mentioned techniques, it is easily applicable and not reliant on specific molecules. The working principle was demonstrated by Lee and coworkers with FITS-BSA that was applied to the μCP stamp in the ordinary way (see Figure 27). The molecule was stamped onto glass under vacuum condition, which leads to air-release in the highly air-soluble PDMS. After restoring the atmospheric pressure, the PDMS reabsorbed air over time. During this phase the second ink (in this case, PEG-silane in ethanol) is introduced, which is pulled into

the stamp cavities due to reduced local air pressure.

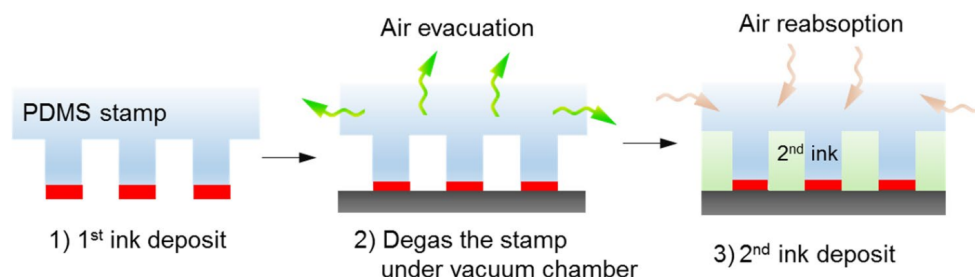


Figure 27: Illustration of μ CP with degas-driven flow guided patterning. Figure adapted from [182].

The concept of ζ potential of monolayers on surfaces has always been addressed by means of a surface averaged value. In the future it may be eligible to resolve the ζ potential locally to achieve a better understanding of the transition between the molecule stripes. SICM is a suitable method to evaluate surface charge densities due to the electric double layer formed in liquid environment [91]. Although the ζ potential can only be accessed at the shear plane, we can draw conclusions about its relative value by SICM (see Section A.7).

Figure 28 shows ion current (I) - distance (d) curves on glass, measured with SICM. The measurement medium was a mixture of PBS and double distilled water (ratio 1 : 2). The effective reduction of the salt ions leads to an extension of the Debye length such that the SICM sensitivity is sufficient to detect the EDL. The EDL leads to an effective increase in ion current when approaching the sample with the nanopipette, before decreasing again due to current squeezing (reduction in cross-section of the current paths). Depending on the charge species of the EDL this current increase occurs either when the nanopipette is at positive or negative potential (relative to bath electrode).

We performed similar I - d curves along a line roughly perpendicular to PAMAM stripes printed on glass (stripe width: $6\ \mu\text{m}$, periodicity: $12\ \mu\text{m}$). The process was repeated 3 times at the same location. Averaged values of the maximum ion current measured at negative bias are plotted in Figure 29. All measured current values are higher than the reference current ($0.95\ \text{nA}$, far away from the sample). Thus we can

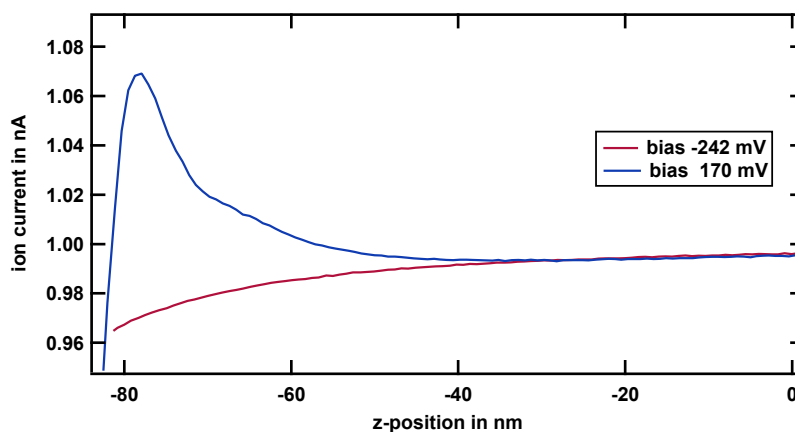


Figure 28: Ion current as a function of nanopipette-height at different electric potentials of the nanopipette electrode (relative to the bath electrode). Lower height-values mean a closer distance between glass surface and nanopipette.

conclude that the ζ potential along the measured position is always negative. However, a slight difference is noticeable. The highest values (around 1.1 nA) are found at a plateau with a distance of 5 μm (blue background), which is in close agreement with the stripe width. We expect those values to result from the pristine glass surface.

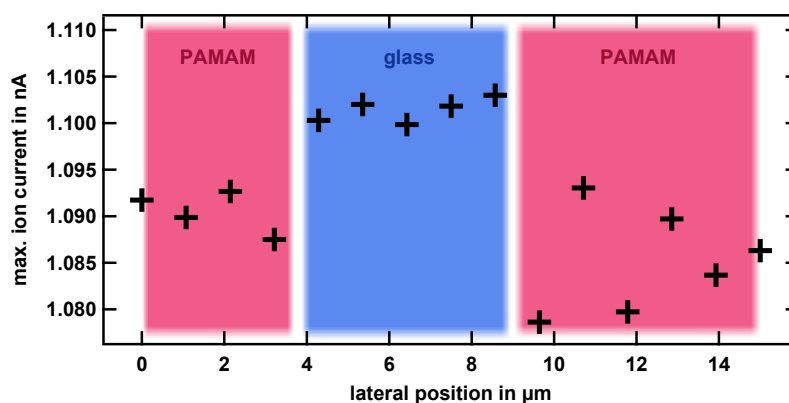


Figure 29: Maximum ion current as a function of the position along a straight line perpendicular to molecular stripes.

Where we expect the PAMAM stripes to be (red background), lower maximum ion currents are measured (around 0.9-0.8 nA). Therefore, the PAMAM molecules indeed shift the ζ potential towards the positive, but do not reverse the ζ potential to positive values. It should be noted that these measurements were only spot checks and may not represent the actual ζ potential at the location of PAMAM stripes.

6

SURFACE-MEDIATED ELECTRICAL STIMULATION OF ADHERED OSTEOBLASTS

In the previous chapter we established localized EFs via molecular landscapes to investigate the cellular response. The cells aligned parallel to the molecule stripes and thus avoided the glass-molecule interface, which we attribute to a localized form of the directed migration of cells due to the EF. However, to further investigate the cellular response to EFs we need to vary the setup, since DC EFs resulting from varying surface potentials are almost instantly screened by the ions in liquid environment. As an alternative approach to the classical capacitor-like electrode configuration, we employed conductive substrates, acting as planar resistive electrodes to achieve a homogeneous EF in cell vicinity. The aim is to achieve a more predictable field distribution, especially at the cell-substrate interface, and to minimize electrochemical reactions in the liquid container.

6.1 INTRODUCTION

Endogenous EFs occur in various biological systems and across different scales. Starting from the embryonic morphogenesis of various species where EFs are suspected to lead to the development of extremities [184, 185]. Even on a single-cell scale, endogenous EFs are responsible for guidance of the axis of cellular division [186]. EFs occur at cell membranes where the TMP, which usually ranges from -40 mV to -90 mV, is kept constant by an active ion transport via molecular pumps [187]. Neuronal communication through axons would not be possible without endogenous dynamic EFs, which are also known to play a crucial role in wound healing. The

disrupted epithelial barrier is more negative than the underlying intact epidermis, which results in an EF that acts as a guiding cue for cells [188, 189].

Therefore, the ES of cells to influence such electrochemical cascades has been of great interest in research in recent years. Indeed, researchers have been able to influence cell function such as improved adhesion, directed migration, increased proliferation, and differentiation of stem cells. However, the underlying mechanisms are not yet well understood and the variety of ES setups, as well as the specific cell types being used in literature, don't provide a clear, straightforward picture. Generally, the ES configurations can be divided into three groups: faradaic current, capacitively coupled, and inductively coupled chambers (see Section 2.3.1).

In capacitive setups, mostly capacitor plates outside the culture dish are being used. Since dissolved ions in physiological medium lead to an effective screening of the EF, it is not possible to establish a constant electric potential gradient in cell vicinity. However, if a time-dependent voltage is applied, the electrochemical layer of the physiological medium is charged and discharged, causing an ion current flow. In this case an AC EF in cell periphery is accomplished. In contrast, chambers with Faradaic currents being present can provide constant EFs. Usually two electrodes are immersed in the medium and placed next to cells. Similar setups, often referred to as (conductive) substrate-mediated, consist of a single electrode on which the cells are directly seeded [190–192]. Materials being used for such electrodes are metals, metal oxides, polymers and graphite. Due to a constant EF (in DC operation) the directional migration of cells (known as electrotaxis or galvanotaxis) can be investigated with adhered cells.

Moreover, DC setups imply electrochemical reactions at the liquid-electrode interface, known as electrolysis. It has been suggested that the reactions lead to a pH shift in the physiological medium (particularly in the vicinity of electrode surfaces) or even result in more or less toxic species such as hydrogen peroxide or free radicals [26], and therefore in unintentional cell responses to stress. To prevent these issues, agar salt bridges were introduced in some studies to physically separate the physiological medium including the cells, and the one containing the electrodes, where the reactions are taking place [193].

6.2 CONCEPT OF THE PLANAR RESISTIVE ELECTRODE

The planar resistive electrode configuration consists of a substrate which is electrically conductive and contacted at the edges. Between those contacts, a silicone reservoir filled with physiological medium and the respective cells is positioned. A 3D representation and the cross-section of the setup as well as the corresponding simplified model of an equivalent circuit can be seen in Figure 30. Applying a voltage at the substrate leads to an electric current through it. The liquid which consists of various types of ions acts as a resistor connected in parallel. At the liquid-electrode interface redox reactions result in transfer of charges into and out of the substrate. However, since an EDL forms at the liquid-electrode interface a significant

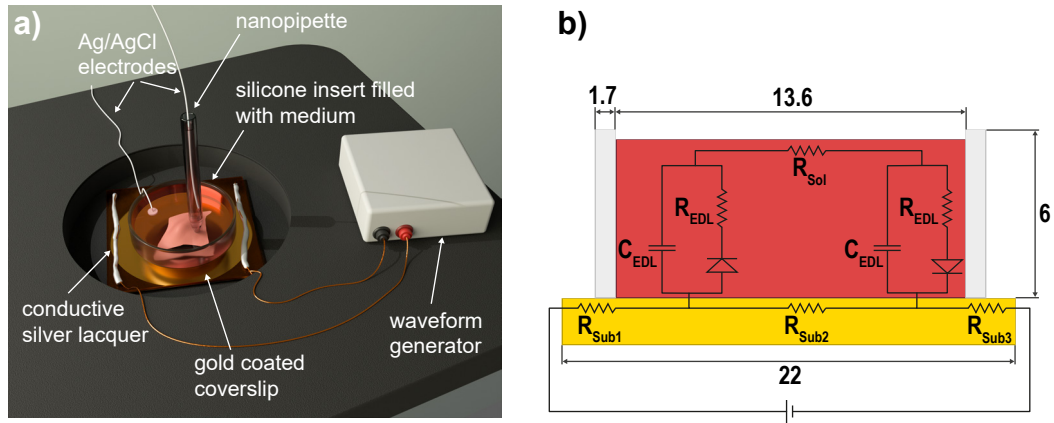


Figure 30: (a) 3D representation of the ES chamber within the SICM-stage (not to scale). (b) Cross section of the stimulation chamber (dimensions are given in mm) and the corresponding simplified equivalent circuit. The substrate (with resistance $R_{Sub} = R_{Sub1} + R_{Sub2} + R_{Sub3}$) is connected to a voltage supply. Parallel to the substrate is the medium (DMEM) which has a certain resistance R_{Sol} . The liquid-electrode interface leads to a formation of an electric double layer, that can be further charged or discharged (capacitance: C_{EDL}). If the potential drop at the electrode-liquid interface is high enough (depending on the reacting ion species) due to a charged EDL a faradaic current will flow. This threshold behavior is accounted for by diodes in forward-direction.

portion of the electric potential already drops within a few nanometers. The EDL can be simulated by a capacitance that can be charged and discharged, depending on the voltage applied to the electrode. For the reduction or oxidation of ions at the

liquid-electrode interface, which is required to achieve a Faradaic current through the medium and thus an [EF](#), a minimal potential difference across the [EDL](#) is needed. The specific value is dependent on the reacting species. This is accounted for by diodes in forward direction in the equivalent circuit that only pass current when a threshold voltage is reached. The required minimal potential drop at the interface causes the origin of ion current through the medium being mainly at the edges of the silicone cylinder.

Simulations were performed by Julius Zimmermann in his time in the research group "Electromagnetic Field Theory" (U. van Rienen) that are based on the finite element method. For every element Maxwell's equations in the electroquasistatic limit were computed via COMSOL Multiphysics. The respective material properties used can be found in the Appendix [Section A.4](#). The simulation shows indeed a homogeneous [EF](#) in the center of the silicone ring. The [EF](#) penetrates the medium only in cell vicinity, close to the electrode (see [Figure 31](#)). However, it should be noted that the [EDL](#) is not included in the simulation, which potentially leads to an overestimation of the magnitude of the [EF](#). With [DC](#) stimulation there are the typical disadvantages such as electrolysis (mainly water splitting becoming effective at about 1.8 V) and electron-transfer reactions at the electrode. Since an ion current passes the inter-cellular medium and charges are being transferred by ions, material will be deposited at the liquid-electrode interface and thus a local concentration gradient of ionic species and potentially a pH gradient will develop which could be stressful or cytotoxic [194]. The issue could be potentially avoided by [AC](#) stimulation in combination with charge compensation, which means that the ion current should equal zero on average over time. Depending on the specific configuration one needs to consider ohmic heat due to the electric current passing through the substrate.

For our setup the resistive electrode sample is required to be partially transparent due to an inverted microscope (see [Figure 30a](#)). Since in vitro cell experiments are performed the electrode should be biocompatible and non-degradable over time. Gold exhibits all these properties, it is widely used in the field of nanomedicine e.g., in form of nanoparticles [195, 196] or multi-electrode arrays in field potential measurements of neurons. Among the typical inert metals (e.g. silver, gold, platinum) gold is most suitable because it is not cytotoxic in the form of an ion (in contrast

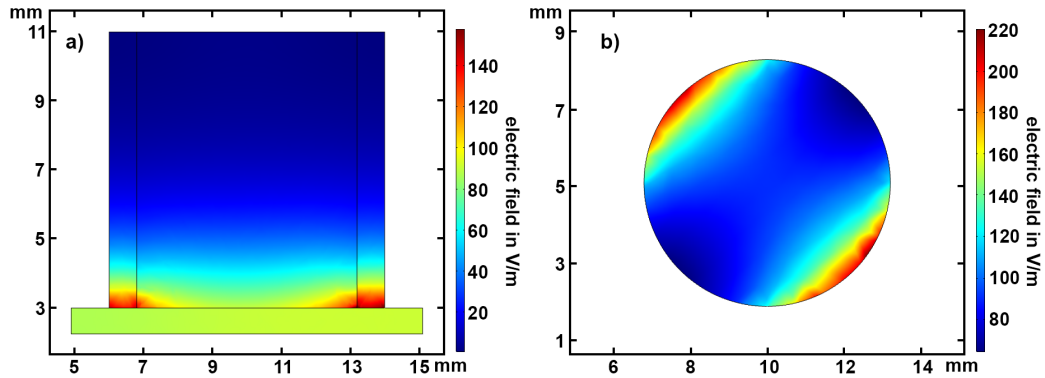


Figure 31: EF distribution calculated for a planar resistive electrode under a cylindrical polystyrene ring. Note that the dimensions of the setup as well as the electrode and cylinder material used in our experiments differs slightly from the simulation. The EF is homogeneous in the center and mostly affecting medium in close vicinity of the electrode. For further information about the simulation, which is kindly provided by Julius Zimmermann, see text above and appendix [Section A.4](#).

to silver, which leads to the production of reactive oxygen species and thus oxidative stress in cells, which damages membranes and organelles [197]) and the electric double layer formed is smaller than that of Pt regarding the capacitance at low frequencies in NaCl aqueous solutions [198]. One disadvantage is the high electrical conductivity, which leads to significant ohmic heating during stimulation. This is why we had to check the resistance of the sputtered gold layer at a given voltage in dependence of layer thickness and the ohmic heat development during electrostimulation.

6.3 RESULTS

6.3.1 CHARACTERIZATION OF THE RESISTIVE ELECTRODE LAYERS

The gold layers were deposited with a sputter coater (Cressington 108auto/SE, TESCAN GmbH, Dortmund, Germany) and their corresponding resistances were mea-

sured. Afterwards the layer thickness was measured by partially scratching off the gold layer and measuring the resulting steps by AFM. The maximum resistance that could be achieved was around 190 Ohm (see Figure 32) at a layer thickness of 10 nm. The upper limit of the resistance is due to the requirement for a closed or percolated gold layer.

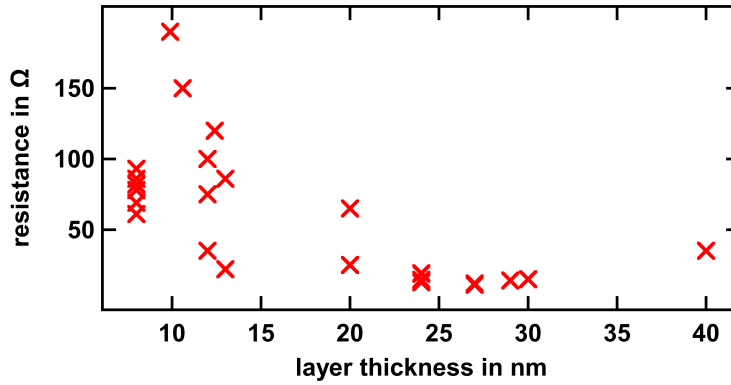


Figure 32: Resistance of sputtered gold layers on glass cover slips ($22 \times 22 \text{ mm}^2$) in dependence of layer thickness, according to AFM measurements. Maximum resistances of 190Ω could be achieved while maintaining a closed gold layer at thicknesses of $\approx 10 \text{ nm}$. For layers of 20 nm and more values of $\approx 15 \Omega$ could be obtained reliably. Measured by Regina Lange.

To evaluate the temperature development of the setup due to ohmic heating the planar resistive electrode configuration (see Figure 30) was placed in an incubator which was set to 37°C . A silicone ring was placed on the substrate and filled with DMEM. A constant voltage was applied to the gold electrode while the corresponding electric current was monitored. The temperature was measured by using an encapsulated miniature thermistor, which was placed inside the medium. Therefore the thermistor's resistance was read out once a constant value, and thus thermal equilibrium, was established. The temperature and the electric power in dependence of the applied voltage can be seen in Figure 33. The diagram shows that a power of 30 mW already leads to $\approx 38^\circ\text{C}$. This ohmic power was chosen as an upper limit, since cell viability is assumed not to be affected and only minor effects on cellular physiology, like alkaline phosphatase, proliferation and mineralization are to be expected [199, 200].

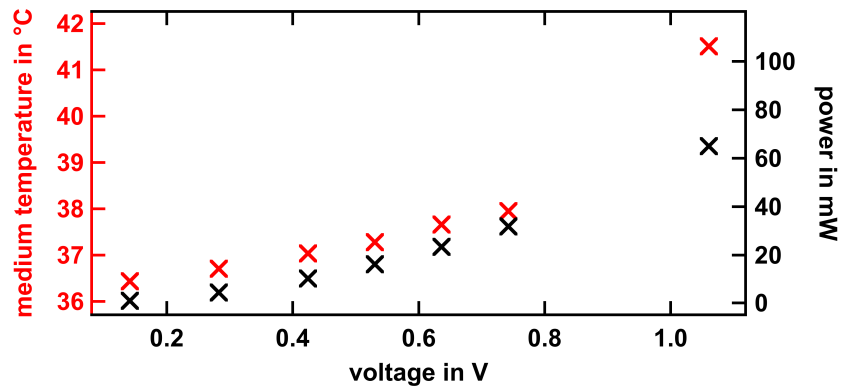


Figure 33: Temperature of the physiological medium and power of heating within the substrate-mediated [ES](#) chamber in dependence of applied voltage at a ≈ 20 nm thick gold layer.

This leads to the need of other substrates when the [EF](#) in the liquid should be in the physiological window of several volts/cm. In the ideal case where the full electric potential drops linearly across the liquid we would need substrates with a resistance of $4\text{ k}\Omega$ to achieve 5 V/cm . This is regarded as the physiological window for beneficial electrostimulation of bone. Such fields lead to galvanotaxis of human osteosarcoma cells (SaOS-2) [45]. Correspondingly, at the substrate 11 V would need to be applied taking into account the lateral dimensions of the gold layer ($22 \times 22\text{ mm}^2$). This is a lower limit due to formation of an [EDL](#) where a significant voltage drop occurs. Since for an $100\text{ }\Omega$ gold layer, only $\approx 1.7\text{ V}$ can be applied until the limit of 30 mW is reached we decided to use polymers mixed with carbon nanotubes ([CNT](#)), which were kindly provided by the group "Soft Matter and Biophysics" (C. Helm, University of Greifswald) [201]. The specific sequence of layers used is: PEI/PSS/PDADMA/([CNT](#)/PDADMA) $_n$ ([CNT](#)/PEI) (further information in Appendix [Section A.2](#)). The number of bilayers ([CNT](#) & Polymer) can be varied. In the scope of this work, 6 bilayers were sufficient (i.e., $n=5$). The polymers polyethylenimine ([PEI](#)), [PSS](#), and [PDADMA](#) are polyelectrolytes, which become charged in a liquid environment. Polymers in combination with single wall carbon nanotubes are already commonly used in literature for [ES](#) purposes [202–204]. The advantage of such a configuration is the variable resistance that can easily be tuned by varying the number of layers adsorbed onto the surface (see [Figure 34](#)) [201].

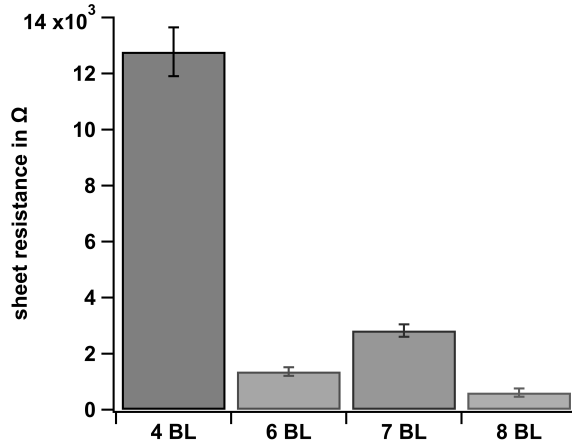


Figure 34: Average sheet resistances of substrates (& the corresponding standard error of mean) with different numbers of polyelectrolyte-CNT bilayers.

6.3.2 CELL MIGRATION DURING DC ELECTRICAL STIMULATION

The cell culture was performed as previously described in [Section 3.4.2](#). MG-63 osteoblastic cells were seeded on the respective electrode material at a density of 3500 cm^{-2} . 24 h after cell seeding, the setup was put under an optical microscope and cells were stimulated by applying a constant voltage to the conductive substrate. The temperature of the medium was measured by a Pt-100 resistor to ensure that 38°C was not exceeded. Optical images were acquired and analyzed before and after stimulation. To overcome drift during stimulation, inhomogeneities in the substrate were used as fixed reference markers.

Gold Electrodes

During the first experiment the cells on the sputtered gold layer were continuously stimulated for 2 h at 1 V. The distance the centroid of the respective cells moved is plotted in a polar coordinate system ([Figure 35a](#)). The cathode (negative potential or ground) and the anode are positioned at 270° and 90° direction, respectively. On average the 12 cells investigated migrated $(0.6 \pm 0.3) \mu\text{m}$ in x- and $(-0.3 \pm 0.3) \mu\text{m}$ in y-direction. Disregarding the migration direction, i.e. only considering the average migration distance from the respective starting point, the average migration speed is $(0.7 \pm 0.2) \mu\text{m/h}$. The cells' movement is randomly oriented, i.e. no galvanotaxis could be discerned.

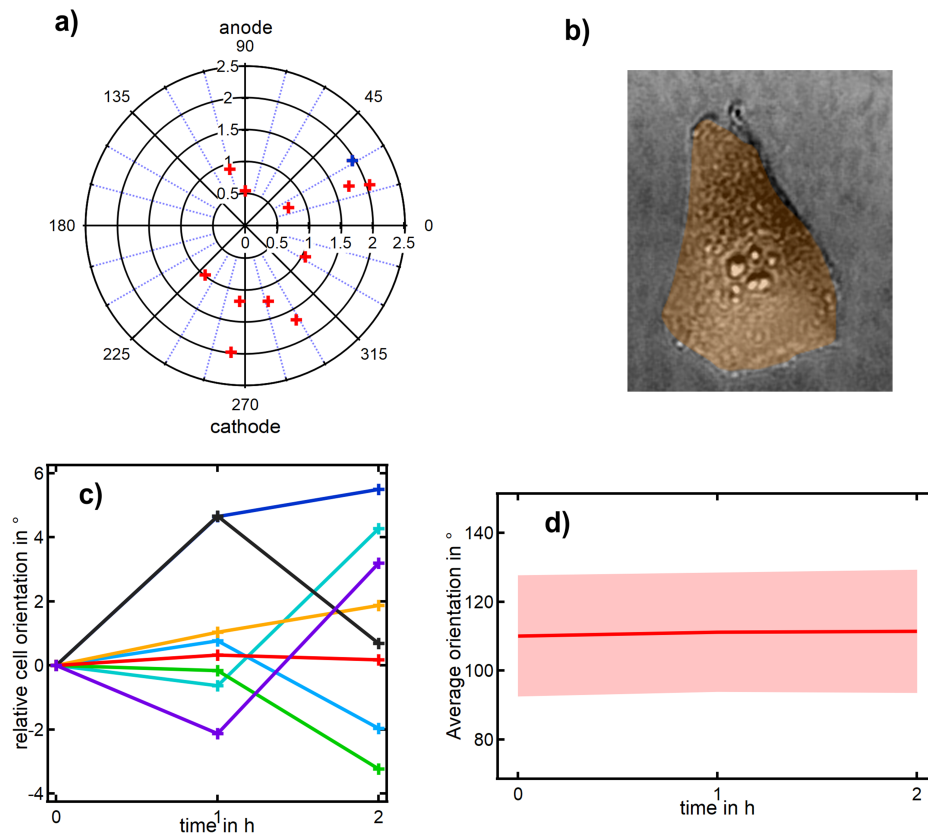


Figure 35: ES of MG-63 cells for 2 h with 1 V applied to a resistive gold layer on glass. (a) Centroid movement of single cells in polar coordinate system. Radial units: μm , 90° and 270° refers to the anode and cathode direction, respectively. (b) Brightfield image of a cell before (orange area) and after stimulation. The cell's movement is marked blue in (a). (c) Relative orientation of selected cells over stimulation time. (d) Average orientation of the cells' long axis over time. The light red shading shows the standard error of the mean. See the text above for details.

To evaluate changes in the orientation of the cells, ellipses were fitted and the angle between the primary axis and the horizontal axis calculated (ImageJ). Cells in which a distinct orientation could be recognized, meaning that the length of the primary axis of the ellipse is at least 1.5 times the length of the minor axis were selected and their orientation change over time presented in Figure 35c. The average absolute change of orientation of $(2.6 \pm 0.7)^\circ$ is not significant. The orientation of the cells does not seem to converge to a specific angle regarding the standard error of the mean (Figure 35d). Therefore, the EF seems to have no effect on cellu-

lar orientation. Since it is not clear whether, and if so, to which parameters of ES the cells could respond, the second experiment was performed by stimulating for 1 h at 1 V, followed by 2.5 h of resting, meaning the stimulation was turned off. The cells responded in a similar way (see Figure 36). Migration was not directed since the 12 cells were displaced on average $(0.4 \pm 0.5) \mu\text{m}$ in x- and $(0.2 \pm 0.6) \mu\text{m}$ in y-direction. However the mean migration speed of $(0.7 \pm 0.09) \mu\text{m/h}$ was similar to the experiment where the stimulation duration was longer. Note that a comparison of the mean migration speed between experiments with different observation times is only valid if the cells perform a uniform rectilinear motion. In the other limiting case of non-directed migration of cells (i.e., random walk) analyzing the mean-squared displacement would be beneficial. A directed reorientation of cells could also not be observed.

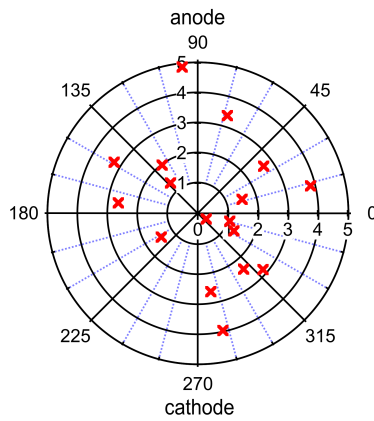


Figure 36: Centroid movement of MG-63 cells within a timeframe of 3,5h. In this experiment the cells rested 2.5h (no stimulation) after 1 h of stimulation at 1 V. Cells were seeded on sputtered gold layer on glass.

Polyelectrolyte-CNT Layers

Since no directionality of migration or of the shape of the cells could be observed the substrate was changed to layers of polyelectrolytes and CNTs, where higher voltages could be applied without extensively heating the medium.

In experiments similar to those of gold layers the cells were stimulated two times at 2 V for 15 minutes and a 1 h break after each stimulation (see Figure 37a). The cells' movement is represented by red crosses. The cells' migration directions seems uniformly distributed. The mean net migration distance in x is $(-1.3 \pm 2.9) \mu\text{m}$ and in y $(0.7 \pm 1.2) \mu\text{m}$. The average migration speed $(3.5 \pm 0.9) \mu\text{m/h}$ is substantially increased in comparison to the previous gold layer experiments.

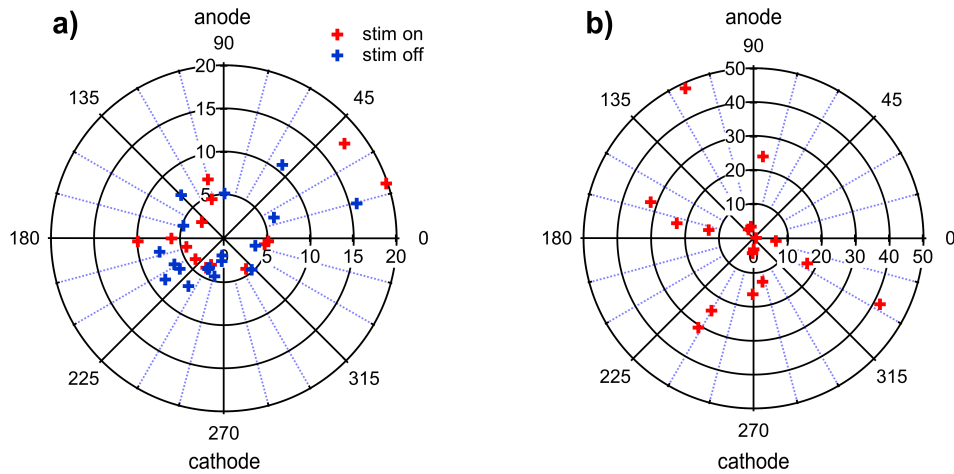


Figure 37: Centroid movement of MG-63 cells. Cells were seeded on Polymer-CNT-bilayers (6) on glass. (a) Blue crosses represent single cells where the stimulation was turned off, red crosses where the stimulation was turned on at 2 V (for 2×15 min and 1 h break after each). (b) The cells were stimulated with a voltage starting at 1 V for 15 min afterwards a 1 h stimulation break and subsequently repeated the procedure with increments of 1 V. Timeframe: 3.75 h

The control experiment on the same kind of substrate without stimulation shows similar isotropic migration of the cells (blue crosses in Figure 37a). The mean migration in x-direction was $(-0.6 \pm 1.3) \mu\text{m}$ and in y-direction $(-0.8 \pm 1.0) \mu\text{m}$. The average migration speed was $(2.6 \pm 0.4) \mu\text{m/h}$. Concerning even higher EFs in cell vicinity the MG-63 osteoblasts seem not to change their isotropic migration. Stimulation for 15 minutes each at 1–3 V with increments of 1 V and stimulation break for 1 h in between shows migration of $(-3.1 \pm 3.9) \mu\text{m}$ and $(-1.2 \pm 4.2) \mu\text{m}$ in x- and y-direction, respectively within an overall timeframe of 3.75 h (see Figure 37b). Applying 5 V for 15 minutes led to blebbing which started already a few minutes after the stimulation was turned off (see Figure 38). Blebbing occurred on several cells. The blebs stayed intact for the hour observed after stimulation with 5 V. They had dimensions of several μm in diameter and occurred on the cells directionally independent (isotropic appearance of blebs).

To ensure that the substrate-mediated electrostimulation setup is a valuable approach, we used two simple rod-shaped Ag/AgCl electrodes in the reservoir to stimulate cells. This setup has the advantage that no additional heating due to a current flow through the substrate occurs and all of the electric current flowing re-

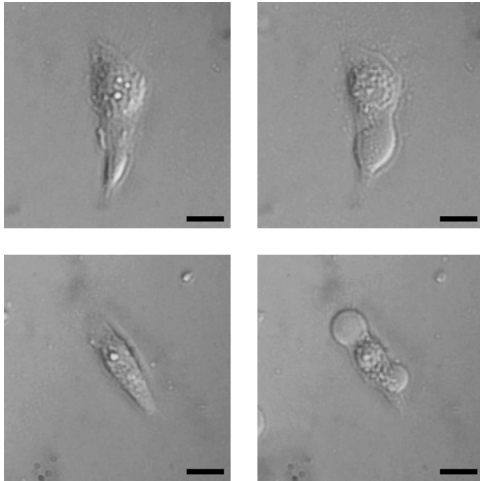


Figure 38: Two selected MG-63 cells seeded on Polymer-CNT layers before (left) and after (right) 3.75 h of ES (see text for details). Blebs of several μm diameter on the cellular edges are visible after stimulation. Scale bars correspond to 20 μm .

sults from ion movement inside the medium, which is the only effective way to establish an EF in cell vicinity. 3 V was applied continuously between them for 15 h in total. Despite having a large voltage compared with earlier experiments, only after ≈ 13 h, blebbing started. However, after giving the cells several hours to migrate and rearrange their cytoskeleton accordingly, the locomotion was isotropic with an average movement of $(0.2 \pm 1.1) \mu\text{m}$ in x- and $(-0.4 \pm 1.3) \mu\text{m}$ in y-direction. The cells also were quite immobile considering the average migration speed was $(0.32 \pm 0.06) \mu\text{m/h}$. The change in orientation of cells is negligible, the mean change in orientation angle (not considering the rotation direction) is $(3.3 \pm 1.2)^\circ$. Regarding single cell orientations over time, there is no change of the direction of the cell orientation visible. The angle of orientation seems stationary with minor deviations over time (see Figure 39b). The time evolution of the average orientation angle of the observed cells can be seen in Figure 39c. The different orientation angles seem not to converge to a direction parallel or perpendicular to the EF, as indicated by the high standard error of the mean shown as the light red shading.

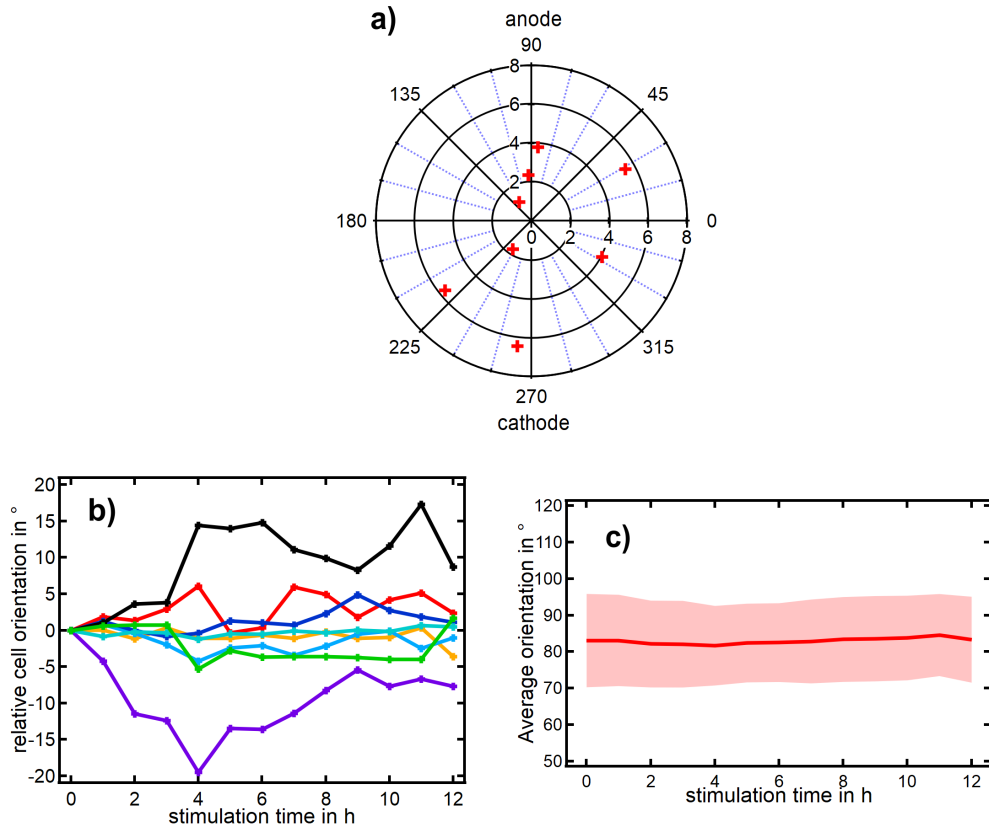


Figure 39: [ES](#) of MG-63 cells for 12 h with 3 V applied to rod-shaped Ag/AgCl electrodes. (a) Centroid movement of single cells in polar coordinates. Radial Unit: μm , 270° points to cathode, 90° to anode (b) Relative orientation of selected cells over stimulation time. (c) Average orientation of the cells long axis over time. The light red shading shows the standard error of mean.

6.4 DISCUSSION

Galvanotactic Behavior

Modulation of spatial and temporal Ca^{2+} concentration is thought to be one of the main driving forces of galvanotaxis [45]. In [DC-EFs](#) Ca^{2+} ions move to the cathode. At the membrane towards the anode, Ca^{2+} ions pass Calcium permeable ion channels due to diffusion. This leads to a higher Ca concentration at the anodal side. Calcium ions in combination with the protein calmodulin are responsible for phospho-

rylating the myosin light-chain kinase (MLCK). This stimulates the myosin ATPase causing cell contraction. Also actin depolymerization is promoted by increased Ca concentration. In contrast to that the calcium concentration decreases at the cathodal side, leading to higher actin polymerization and thus a net movement of the cell in the cathodal direction. Although most cell types show galvanotaxis in the direction of the cathode, a few move in opposite direction. One reason for the difference could be the cell-dependent ion channel density. Active ion channels e.g., VGCCs, that are voltage-sensitive Ca-permeable channels located at the plasma membrane of cells, need to be considered. They get activated and thus open up when the TMP depolarizes. One generally differentiates between two main types of VGCCs - the low-voltage-activated (LVA) and high-voltage-activated (HVA) channels. Since relatively small TMP changes (e.g., at 2 V applied, we expect a potential difference of 2 mV over the whole cell) are to be expected during the ES we focus in the following on LVA (also called t-type) channels. T-type channels consist of a primary protein unit called alpha1. It constitutes the pore and the voltage sensitive part. Three different groups of t-type channels are known containing alpha1G, alpha1H, or alpha1I (Cav3.1, Cav3.2, and Cav3.3, respectively). The cell line MG-63 expresses all these channels [205]. They seem to have an activation threshold of ≈ -70 mV [206], which is why the membrane of MG-63 should be partly Ca^{2+} permeable at their resting TMP of -60 mV [207]. During the DC ES, the TMP changes. The part facing the cathode should depolarize, which could lead to further opening of the T-type channels. The resulting Ca^{2+} influx into the cell could depolarize the membrane further until HVA channels open which increases the ion current further. Thus the Ca influx from VGCCs increases the Ca concentration at the cathodal side, which counteracts the previously described Ca gradient resulting from passive ion channels. However it has been reported that the Ca conductance increases by further depolarization until it converges around -20 mV [208]. Since no galvanotactic behavior on the MG-63 cells was observed in our experiments, it is conceivable that the passive as well as stimulated influx of Ca^{2+} lead to an insufficient Ca^{2+} concentration gradient within the cells. In human osteoblasts it was also shown that a high endogenous Ca^{2+} concentration led to a reduction in directional migration speed [178]. It is therefore also possible, that a high concentration of ion channels of MG-63 cells resulted in a high Ca^{2+} concentration, inhibiting migration in our ES experiments. Another widely discussed mechanism refers to GFRs being responsible for galvanotaxis. The receptors are expected to be uniformly distributed over the cell's plasma membrane. If an

EF is applied, electrophoresis will act onto the mostly charged receptors. Therefore positively charged receptors will accumulate at the cathodal membrane site and vice versa. However, since the TMP will lead to an accumulation of cations at the interface of membrane and medium, electroosmosis will occur. The ion flow along the membrane will therefore create a shear force onto the receptors. Since the ions accumulated at the membrane are mostly positive, the electroosmosis will support the movement of positively charged receptors and inhibit the movement of negatively charged receptors toward the anode, or even lead to a movement in the direction of the cathode. The zeta potential of the receptors and the zeta potential of the cell surface is crucial to estimate the moving direction [209].

The observation that the MG-63 cells do not perform galvanotaxis is in agreement with literature [48]. However, Curtze et al. recognized significant reorientation and elongation perpendicular to the EF within a timeframe of 2 - 3 h. The reason could be that they claim to have an EF of 10 V/m, which is quite high compared to other galvanotactic experiments. The threshold of galvanotactic behavior is e.g. 1.5 V/cm for keratinocytes, 2 V/cm for fibroblasts and 1 V/cm in case of neural crest cells [210–212]. Regarding elongation perpendicular to the EF, thresholds of, e.g., 1.5 V/cm for embryonic fibroblasts, 6 V/cm for retinal pigment epithelial cells or 1.5 V/cm for corneal epithelial cells were determined [213–215]. For osteosarcoma cells (SaOS-2) 2 V/cm seem to be sufficient to induce a significant reorientation [45]. However, those EF strengths have to be questioned. We expect that a lower EF is present, since they did not mention simulations of their stimulation chambers. In the study of MG-63 osteoblasts platinum wires were used which are physically separated from the cells' medium by agar bridges. Already in the agar bridges a significant part of the voltage should drop and thus is not available in cell vicinity [178]. Depending on the specific setup that was used by Curtze et al., it is possible that the EF strength in their experiments was close to that in our experiments. In this case we should have observed a reorientation of the MG-63 cells as well.

However, we did not observe such a reorientation of cells. One reason could be the different medium the cells were cultured in. Curtze et al. used RPMI 1640 instead of DMEM, and the experiment was not performed in cell culture medium due to fluorescence measurements [48]. The stimulation was performed in 10 mM HEPES solution, without FCS. In similar studies the absence of FCS had significant impact on galvanotaxis [215].

The discrepancy between our results and the ones of Curtze et al. could also be

caused by a lower EF being present in our experiments. If we consider the ideal case of a linear voltage drop across the length of the stimulation chamber the upper limit of 1.5 V/cm can be expected. However raising it by increasing the voltage further led to blebbing, thereby limiting the applicable field strength.

Blebbing

Blebbing is a mechanism that is associated with various cellular functions like e.g., motility [216], mitosis [217] and apoptosis [218]. Blebs are protrusions of the cell's plasma membrane. Usually the membrane is bound to the cell cortex. However, due to pressure, this binding can break or the cortex ruptures, enabling the membrane to form blebs.

Not every cell type is capable of utilizing blebs for motility. While some cells exclusively form blebs for migration purposes (e.g. zebrafish primordial germ cells [219]), others use protrusions like lamellipodia. It is known that some cells have the ability to switch between those types of migration. However, motility-related blebbing occurs at the leading edge [220] and in our experiments the blebs did not appear directional. There is also no evidence in literature that MG-63 form blebs for motility purposes. The dynamics are also different. During our ES the blebs stayed intact for hours, while bleb formation and retraction during migration occurs within a few seconds [221]. Bleb formation is performed by cytosol that flows between the cortex and the membrane due to a pressure difference. This means that the volume of the cell stays constant [222]. When the MG-63 expressed blebs it seemed like the cell's volume changed which would mean that extracellular medium entered the cell.

A reason for the influx could be the build-up of osmotic pressure. Since there is an ion current passing through the extracellular medium, ions undergo oxidation or reduction at the electrodes. This reduces the ion concentration inside the liquid and thus increases the concentration gradient between the intra- and extracellular media. The osteoblastic cells could reduce this gradient with their passive ion pumps, which can transport roughly 10^7 ions per second [223] and active sodium-potassium-pumps that are significantly slower with only 400 - 500 ions per second [224]. However this will probably not be sufficient to completely counterbalance the ions that transition into the solid state. That's why water could enter the cell, reducing the osmotic pressure and thus leading to swelling or blebbing of the cells. Such behavior has already been observed when HeLa cells were exposed to hypoosmotic condition by only using a 10 % PBS-solution [225].

The physiological medium used in our experiments (DMEM, see appendix [Section A.1](#)) has an ion concentration of roughly 2×150 mM if all molecular constituents are completely dissociated. Regarding the electric current measured (e.g. 200 μ A) and the volume of liquid, no ions would be available anymore after 60 h (in case all ions are monovalent). It should be noted that the 200 μ A result partly from the electric current through the planar resistive substrate and the ions present are not exclusively monovalent. Besides that, the possibility of ions provided by the electrode is disregarded to contribute to the electrical current. Thus, 60 h is a lower limit. Therefore the osmotic pressure would probably lead to a negligible inflow of liquid into the cells. What also points towards a different reason is that blebbing occurred immediately after the stimulation with 5 V was turned off. The build-up of an osmotic pressure would take several hours. The same argumentation applies for changes in the pH value of the medium that could have led to blebbing [226].

Another reason for blebbing could be the activation of voltage-gated ion channels by [ES](#). Changes in the [TMP](#) lead to conformational changes of ion channels. Voltage-gated sodium channels open upon membrane depolarization (on the cathodal side), while voltage-gated potassium channels will open at the anodal side (hyperpolarization). This gives rise to an action potential firing in MG-63 cells [207]. However it means that there is a self-regulatory mechanism which would inhibit the uncontrolled influx of sodium and potassium. This way ions would effectively diffuse from locations of high concentration to those of low concentration. Since the blebs occurred at random spots on the cells the hyperpolarization and depolarization of the membrane seems not to be the reason.

The bleb formation could also be a necrotic behavior of MG-63 cells, which could be caused by local heating of the cells. Our temperature measurements only regarded the temperature in the bulk physiological medium. If one assumes a homogeneous, isotropic specific electrical resistance of the electrode, which should be the case for the gold layers, the Joule heat during stimulation dissipates over the whole electrode surface area. The heat transfer to the liquid via heat conduction and convection is sufficient to not let the electrode heat up compared to the bulk liquid. This can be estimated as follows: It is assumed that the power limit of 30 mW (see [Section 6.3.1](#)) dissipates only via the upper surface area of the electrode (i.e. the glass substrate beneath the gold electrode does not act as a heat sink). This way in equilibrium the heat transferred per unit time and surface area is: $30 \text{ mW} / 22 \times 22 \text{ mm}^2 \approx 22.6 \text{ W/m}^2$. Generally one can approximate the heat being transferred at the gold-

liquid interface by: $\frac{Q}{A} = c\Delta T$, where Q is the heat transfer per unit time, A is the interface area, c is the heat transfer coefficient and ΔT temperature difference between the liquid and the gold electrode [227, p. 5]. The temperature difference is approximately 0.019 - 0.226 K in case the liquid is water ($c = 100\text{-}1200 \text{ W}/(\text{m}^2\text{K})$) [228]. This indicates that the temperature difference between the gold layer (electrode) and the bulk physiological medium is negligible. However, this approximation might not hold for the polymer/CNT electrodes. In this case, only the CNTs are responsible for the electrical conductivity. If we assume only discrete current paths, local heating would occur beneath the cells, where the current density is high due to local accumulation of CNT at the interface. This results in a smaller localized heat transfer (due to the surface area A for heat dissipation is orders of magnitude smaller) and thus the electrode surface might heat up significantly (locally). Such heating is capable of causing cell necrosis or apoptosis [229]. In contrast to apoptosis necrosis results in an increased cell volume [230].

6.5 CONCLUSION AND OUTLOOK

We established a stimulation chamber that applies homogeneous EFs in cell vicinity and is applicable within the SICM setup, where the access from top with a nanopipette and the presence of a bath electrode are crucial. Optical investigations with an inverted microscope are also possible due to the electrode being partially transparent. Due to the construction of the ES chamber, the EF is established quite effectively, meaning that the highest current density (and thus EF) occurs in cell vicinity at the electrode-liquid interface. In contrast to vertically inserted plate electrodes, which lead to field lines parallel to the substrate even in areas where no cells are present, the electrolysis is expected to be reduced while maintaining the same effect on cells. However the magnitude of the EF is hard to quantify and due to the mechanisms acting on largely different geometric scales (e.g. the development of an electric double layer at the electrode-liquid interface, and the electric current pathways being on the mm-lengthscale) the numerical simulation is demanding. Therefore, an experimental way of determining the local EF is highly desirable. One possibility is the SICM with its nanopipette. By introducing two electrodes into the system the location dependent electric potential can be measured. One of these electrodes should be the reference electrode and thus stationary. The second electrode

(in the nanopipette) would be scanned over the planar resistive electrode and thus the potential with respect to the reference electrode could be mapped.

However, even in case the **EF** is fully known, it remains open how the **TMP** of the cells is affected, since they can vary their intracellular electric potential through ion channels. The **TMP** change upon **ES** could be investigated by voltage-sensitive dyes. These dyes bind to the cellular membrane and change their fluorescence intensity depending on the local **TMP**. However the voltage resolution is critical. Most dyes show a 2-6 % change in intensity at a potential difference of 100 mV. They are often used for action potential monitoring of e.g., cardiomyocytes or axons [231, 232]. During **ES**, we expect a change of only a few mV. Since the voltage-sensitive dyes have a short response time upon potential change it might be advantageous to stimulate the cells with **AC** and record the fluorescence intensity over time, to increase the signal to noise ratio.

Noteworthy galvanotactic behavior of MG-63 cells could not be observed with the setup. At various voltages, the cells showed neither directed migration nor reorientation. As stated previously the exact mechanism of galvanotaxis is unknown and might differ between specific cell types. Therefore, the discussed mechanisms should be examined to determine whether they are applicable to the MG-63 cell type. Ca^{2+} imaging with specifically binding dyes (e.g., fluo-3) offers the opportunity to investigate the hypothesized calcium influx from extracellular space as well as the calcium release from the endoplasmic reticulum [126]. This might provide insight into whether a calcium gradient can be achieved across the cells. Another option would be to stimulate the cells while the T-type **VGCCs** are selectively blocked using Nickel ions or other substances [233–235]. Therefore Ca influx takes only place at the anodal side by passive, Ca permeable ion channels which could lead to a calcium gradient and thus galvanotaxis. To avoid the possibility of hypothetical electrolysis and pH changes being responsible for inhibiting galvanotaxis of MG-63 cells, the physiological medium could either be continuously exchanged during stimulation or agar bridges could be introduced to separate electrolytic byproducts from the cellular medium.

In the future, not only the galvanotactic behavior could be observed, but also other crucial cellular parameters such as cell stiffness, which is closely related to cell motility and gives insight in differentiation. **SICM** enables local cell-mechanical mea-

surements, but the number of analyzable cells is highly limited. In contrast, cell cytometry offers high throughput and thus allows for statistical analysis of cell stiffness [236].

The occurrence of blebs during ES points towards an uncontrolled influx of extracellular medium into the cells. To exclude osmotic pressure as the cause of bleb formation, the volume of physiological medium inside the chamber could be varied. This should not influence the EF but the osmotic pressure when stimulating with the same parameters.

Another approach is the continuous exchange of the medium via pumps, which would exclude osmotic pressure effects and allow for longer stimulations without risking pH changes.

Regarding the clinical application of the substrate-mediated DC setup, it has the advantage of locally stimulating cells and is therefore more efficient. Yonemori and colleagues observed a significantly higher bone formation in the bone marrow of rabbits when stimulating with a DC-EF instead of pulsed electromagnetic fields [237]. Concerning osseointegration, which is the process of permanently attaching the implant to the bone, the planar resistive electrode could be applied locally at the implant-bone interface. This would ensure the ES of cells over the whole implant surface and thus lead to potentially higher proliferation and migration speeds of osteoblasts resulting in an improved osteoconduction [238]. In case of small, single electrodes in the vicinity of the implant, an insulating tissue layer that might form around the electrodes due to inflammation could lead to an insufficient EF at the interface. A conductive layer that contacts the liquid environment also leads to "pinning" of the electric potential inside the liquid to some extent. If a sufficiently high potential gradient exists locally between the liquid and the electrode, charge transfer and thus an electric current would flow from the liquid to the electrode or substrate, and vice versa.

A

APPENDIX

A.1 CONTENTS OF DMEM

DMEM is available in various formulations. For clarity, the specific inorganic salts contained in DMEM (used for cell culture in [Section 3.4.2](#)) are listed in the following table.

inorganic salts	molecular weight	concentration (mM)
Calcium Chloride (CaCl_2) (anhyd.)	111.0	1.8
Ferric Nitrate ($\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$)	404.0	2.5×10^{-4}
Magnesium Sulfate (MgSO_4) (anhyd.)	120.0	0.8
Potassium Chloride (KCl)	75.0	5.3
Sodium Bicarbonate (NaHCO_3)	84.0	44.0
Sodium Chloride (NaCl)	58.0	110.3
Sodium Phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)	138.0	0.9

Table 4: For further contents see [\[239\]](#).

A.2 PREPARATION OF CNT-POLYELECTROLYTES-MULTIFILMS

The multilayers consisting of CNT and polyelectrolytes enabled us to tune the sheet resistance of the substrates used for ES (see [Section 6.3.1](#)) and thus higher voltages could be applied for ES. The samples were kindly provided by the group "Soft Mat-

ter and Biophysics" (C. Helm, University of Greifswald).

The following protocol was largely taken verbatim from [201].

CNTs (512 mg, single-walled/double-walled mixture; CNT ratio > 90%, diameter 1 to 2 nm, length 5 to 30 μm , abcr GmbH, Karlsruhe, Germany) were treated with 50 ml of a 1:3 volume ratio of a HNO_3 (60-68%)/ H_2SO_4 (98%) acid mixture at 80°C for 3.5 h according to López-Oyama [240]. The obtained dispersion was filtered under vacuum and washed with deionized water until the pH of the rinsing water became neutral. The solvent was removed with the use of a liquid nitrogen rotary evaporator and modified CNTs were dried overnight via lyophilization. After the chemical treatment, 65 mg of dry CNTs were obtained (at ambient conditions), due to addition of some negative charges. This allowed the CNTs to be used as polyanions. For a fine dispersion in water, raw CNTs were put in a solution containing 2 wt% sodium dodecyl sulfate (SDS), purchased from Carl Roth GmbH+Co. KG, Karlsruhe, Germany.

The glass slides ($22 \times 22 \text{ cm}^2$) were cleaned according to a RCA treatment protocol [241] and used as substrate. The polyelectrolyte multilayers in combination with freshly modified CNTs were prepared by the Layer by Layer-method [242], sequential adsorption of oppositely charged PEs and CNTs from solution with the help of a dipping robot (Riegler & Kirstein GmbH, Potsdam, Germany) and a refrigerated circulation thermostat (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 20°C . As polycations poly(ethylenimine) (PEI, branched, $M_w = 750 \text{ kDa}$, $\text{PDI} = 12.5$) and PDADMA ($M_w = 44.3 \text{ kDa}$ and 322 kDa , $\text{PDI} = 2.19$) were used. As polyanions we used PSS ($M_w = 666 \text{ kDa}$, $\text{PDI} < 1.2$) and modified CNTs. PEI was purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany), PDADMA and PSS from Polymer Standards Service (Mainz, Germany). For the polyelectrolyte solutions, the composition of the adsorption solution was 1 mM PE with respect to the monomer concentration solved in 100 mM NaCl (Merck KGaA, Darmstadt, Germany). The modified CNT concentration was 0.15 mg/ml and 0.25 mg/ml suspended in ultrapure water. The adsorption time for each deposition step was 30 min with three subsequent washing steps (each step 1 min) with ultrapure water (nominal conductivity of $0.054 \mu\text{S/cm}$, Sartorius arium advance, Göttingen, Germany, followed by Millipore purification system, Molsheim, France) to remove the loosely bond polycations or polyanions. For all films the first adsorbed layer was PEI followed by a PSS layer. Then the PDADMA and the CNTs were absorbed in an alternating se-

quence. The final layers where CNT followed by PSS. Therefore, the overall film structure is PEI/PSS/PDADMA/(CNT/PDADMA)_N/(CNT/PSS).

A.3 CHEMICAL STRUCTURAL FORMULAS

The following figures illustrate the structural formulas of the specific molecules used for μ CP in Section 3.4.1.

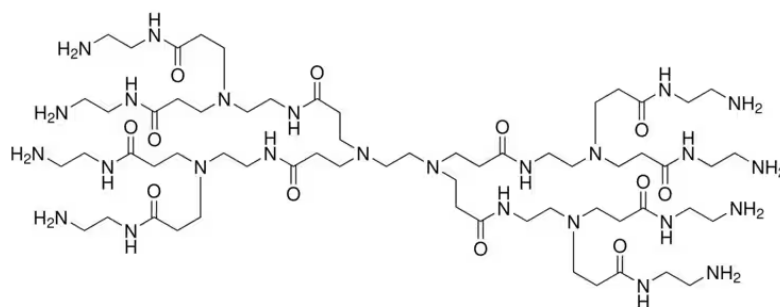


Figure 40: Structural formula of PAMAM (gen. 1) with ethylenediamine core. Adapted from [243].

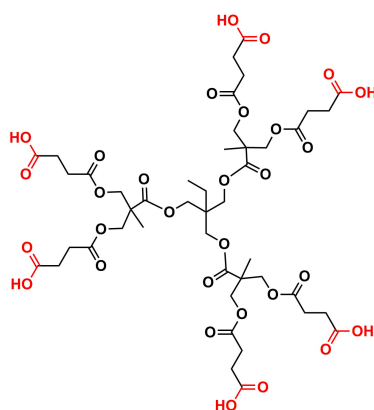


Figure 41: Structural formula of G1-TMP-COOH. Adapted from [244].

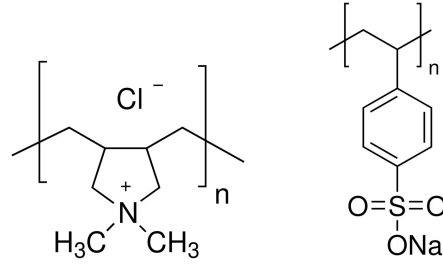


Figure 42: Structural formulas of [PDADMA](#) (left) and [PSS](#) (right). Adapted from [245, 246].

A.4 SIMULATION PARAMETERS

The specific simulation parameters significantly influence the observables (in this case the electric field strength, in [Section 6.2](#)). They are listed in the following table.

material	conductivity σ [S/m]	rel. permittivity ϵ_r
Titanium (Substrate)	2.6×10^6	1
Polystyrene Cylinder	10^{-14}	2.5
DMEM (medium)	1.5	80

Table 5: Used material properties for simulation in [Section 6.2](#).

A.5 ION CURRENT OSCILLATIONS DURING SICM SCAN

The graph depicts the ion current oscillations that arise due to the high accelerations of the nanopipette in the hopping-mode. These oscillations significantly limit the scan speed of the [SICM](#) making it challenging to observe dynamic processes such as changes in the membrane morphology of living cells (see [Section 4.4](#)).

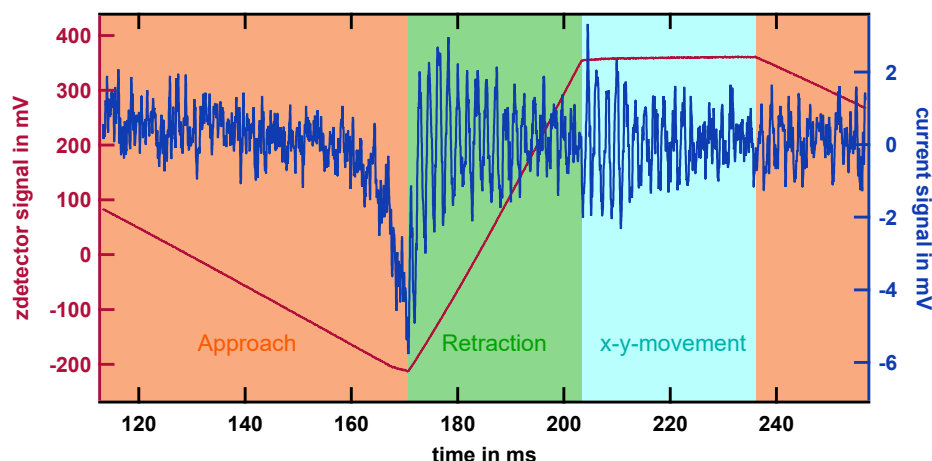


Figure 43: *Z*-detector and ion current signal vs. time during a SICM topography measurement on glass in hopping mode. The ion current signal starts oscillating at a frequency of ≈ 560 Hz when the approach as well as the retraction of the nanopipette is stopped.

A.6 NANOPIPETTE DIMENSIONS

The nanopipette dimensions, particularly the opening diameter, are critical parameters that determine the lateral resolution of the SICM. The high aspect ratio of the measured nanopipette ensured that the observed deep invaginations are not caused by a collision of the pipette (see Section 4.1).

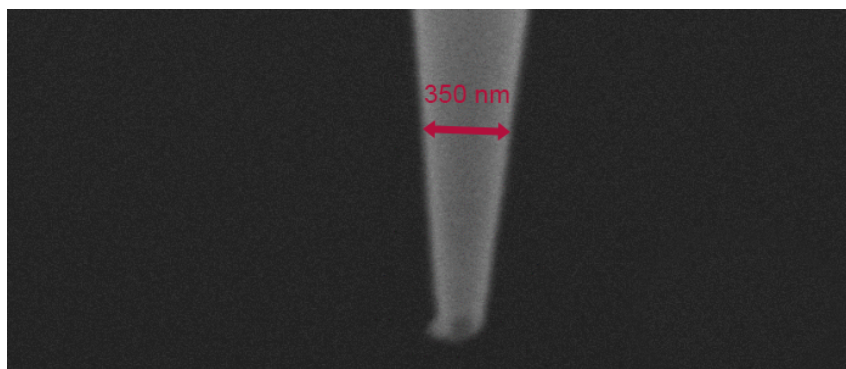


Figure 44: SEM-image of a typical borosilicate glass nanopipette used for SICM. The width was measured to be ≈ 350 nm at a distance of 800 nm from the opening.

A.7 RECTIFYING BEHAVIOR OF NANOPIPETTES

In this section, the mechanism behind the surface charge sensitivity (see [Section 5.4](#)) of a [SICM](#) is elucidated.

If the nanopipette orifice is sufficiently small, or the ion concentration of the medium is low such that the orifice and the (cationic) [EDL](#) at the glass-medium interface are of similar dimension, the ion current through the nanopipette becomes rectified. This occurs because the orifice is preferentially permeable to cations due to the cationic [EDL](#). As a result, the transference number of cations at the nanopipette tip is very high, while the that of anions is low [\[247\]](#). Further away from the tip, the difference in transference numbers vanishes, since the [EDL](#) is small compared to the cross-section of the nanopipette. When the applied voltage is such that a negative potential is present in the pipette, the cations flow from the tip region of high transference number into a region of lower transference numbers. This gradient leads to an accumulation of ions in the tip region of the nanopipette and, consequently, to an increased conductivity. If the polarity is reversed, the cation flux moves from a region of low to the region of high transference numbers, resulting in ion depletion in the nanopipette and thus decreased conductivity.

When a charged surface is in close proximity to the nanopipette orifice, the rectifying behavior is either enhanced (in case of negative surface charges) or diminished. This is because the ion flux must pass the region between the nanopipette orifice and the surface, which can further increase ion-selective permeability (if the polarity of the [EDL](#) matches that of the nanopipette) or reduce it (see [Figure 45](#)) [\[91\]](#).

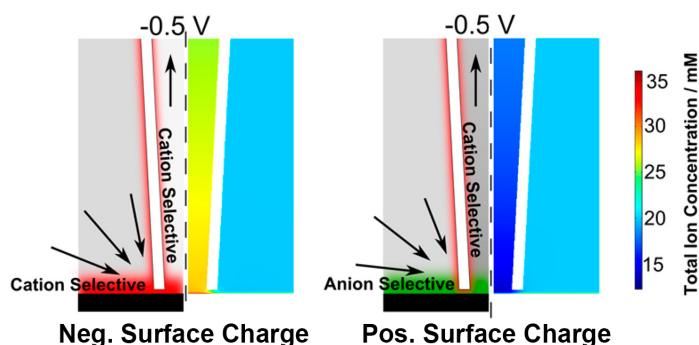


Figure 45: Schematic view of a nanopipette orifice in proximity to a charged surface. The ion concentration inside the nanopipette changes significantly depending on the surface charge. Adapted from [\[91\]](#).

A.8 CELL FIXATION PROTOCOL

Cell fixation is a widely used procedure to preserve the cell structure and morphology at a specific point in time. It allows for the measurement of the apical morphology of MG-63 cells without artifacts resulting from the membrane dynamics of living cells. Additionally, it enables the comparable assessment of membrane morphology in dependence of parameters such as cell adhesion area. (see [Section 4.2](#)).

1. Wash cells briefly with [PBS](#) (2 x)
2. Fix cells with 4 % [PFA](#) in [PBS](#) for 10 min at room temperature.
3. Remove the fixation buffer.
4. Wash cells 3 x with [PBS](#)
5. Leave fixed cells in [PBS](#)

A.9 FLUORESCENCE FILTERCUBE SPECTRA

The specific spectra of the fluorescence filtercubes used (see [Chapter 5](#)) are provided below.

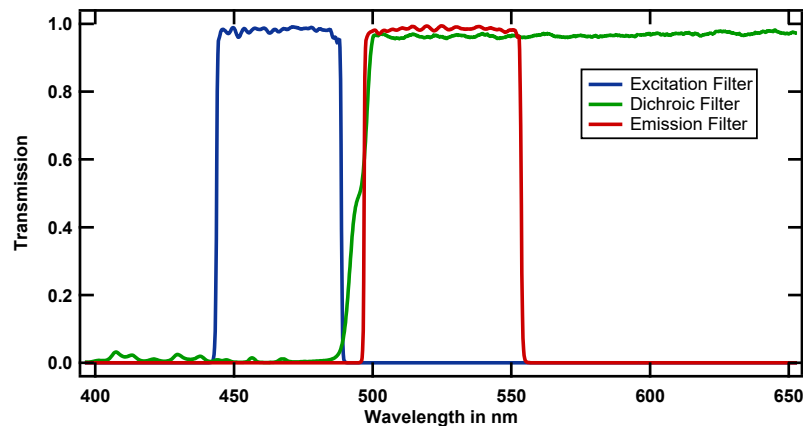


Figure 46: Transmission spectrum of the GFP-Filterset. Figure adapted from [\[248\]](#).

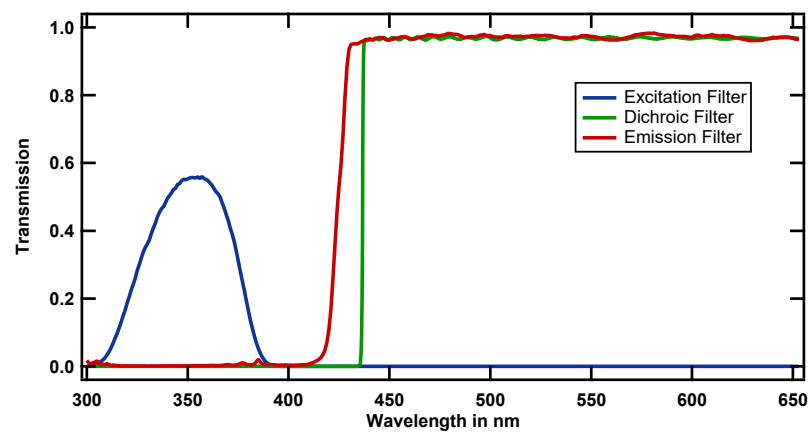


Figure 47: Transmission spectrum of the UV-Filterset.

BIBLIOGRAPHY

- [1] Eiichi Fukada and Iwao Yasuda. "On the Piezoelectric Effect of Bone." *Journal of the Physical Society of Japan* **12** (1957), pp. 1158–1162. DOI: [10.1143/JPSJ.12.1158](https://doi.org/10.1143/JPSJ.12.1158).
- [2] *Average Age Germany*. 2024-11-03. URL: <https://www.bib.bund.de/DE/Fakten/Fakt/B19-Durchschnittsalter-Bevoelkerung-ab-1871.html>.
- [3] *Life Expectancy Germany*. 2024-11-03. URL: <https://www.destatis.de/DE/Themen/Gesellschaft-Umwelt/Bevoelkerung/Sterbefaell-e-Lebenserwartung/Publikationen/Downloads-Sterbefaell-e-statistischer-bericht-sterbetafeln-5126207237005.html?nn=209016>.
- [4] *Operations Germany*. 2024-11-03. URL: <https://www.destatis.de/DE/Themen/Gesellschaft-Umwelt/Gesundheit/Krankenhaeuser/Ta-bellen/drg-operationen-insgesamt.html>.
- [5] Mark-Steven Howe, William Keys, and Derek Richards. "Long-term (10-year) dental implant survival: A systematic review and sensitivity meta-analysis." *Journal of dentistry* **84** (2019), pp. 9–21. DOI: [10.1016/j.jdent.2019.03.008](https://doi.org/10.1016/j.jdent.2019.03.008).
- [6] J-N Argenson, S. Boisdard, S. Parratte, S. Descamps, et al. "Survival analysis of total knee arthroplasty at a minimum 10 years' follow-up: a multicenter French nationwide study including 846 cases." *Orthopaedics & traumatology, surgery & research : OTSR* **99** (2013), pp. 385–390. DOI: [10.1016/j.otsr.2013.03.014](https://doi.org/10.1016/j.otsr.2013.03.014).
- [7] Ahmed M. Negm, Lauren A. Beaupre, C. Michael Goplen, Colleen Weeks, et al. "A Scoping Review of Total Hip Arthroplasty Survival and Reoperation Rates in Patients of 55 Years or Younger: Health Services Implications for Revision Surgeries." *Arthroplasty today* **16** (2022), 247–258.e6. DOI: [10.1016/j.artd.2022.05.012](https://doi.org/10.1016/j.artd.2022.05.012).
- [8] Prutha Vaidya, Swapna Mahale, Sunila Kale, and Agraja Patil. "Osseointegration- A Review." *IOSR Journal of Dental and Medical Sciences* **16** (2017), pp. 45–48. DOI: [10.9790/0853-1601014548](https://doi.org/10.9790/0853-1601014548).
- [9] Yingying Zhang, Zhenmin Fan, Yanghui Xing, Shaowei Jia, et al. "Effect of microtopography on osseointegration of implantable biomaterials and its modification strategies." *Frontiers in bioengineering and biotechnology* **10** (2022), p. 981062. DOI: [10.3389/fbioe.2022.981062](https://doi.org/10.3389/fbioe.2022.981062).

- [10] Weiping Lin, Liangliang Xu, Stefan Zwillingenberger, Emmanuel Gibon, et al. "Mesenchymal stem cells homing to improve bone healing." *Journal of orthopaedic translation* **9** (2017), pp. 19–27. DOI: [10.1016/j.jot.2017.03.002](https://doi.org/10.1016/j.jot.2017.03.002).
- [11] Mit Balvantray Bhavsar, Zhihua Han, Thomas DeCoster, Liudmila Leppik, et al. "Electrical stimulation-based bone fracture treatment, if it works so well why do not more surgeons use it?" *European journal of trauma and emergency surgery : official publication of the European Trauma Society* **46** (2020), pp. 245–264. DOI: [10.1007/s00068-019-01127-z](https://doi.org/10.1007/s00068-019-01127-z).
- [12] Barry M. Gumbiner. "Cell Adhesion: The Molecular Basis of Tissue Architecture and Morphogenesis." *Cell* **84** (1996), pp. 345–357. DOI: [10.1016/S0092-8674\(00\)81279-9](https://doi.org/10.1016/S0092-8674(00)81279-9).
- [13] Amelia Ahmad Khalili and Mohd Ridzuan Ahmad. "A Review of Cell Adhesion Studies for Biomedical and Biological Applications." *International journal of molecular sciences* **16** (2015), pp. 18149–18184. DOI: [10.3390/ijms160818149](https://doi.org/10.3390/ijms160818149).
- [14] Matthew C. Jones, Junzhe Zha, and Martin J. Humphries. "Connections between the cell cycle, cell adhesion and the cytoskeleton." *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **374** (2019), p. 20180227. DOI: [10.1098/rstb.2018.0227](https://doi.org/10.1098/rstb.2018.0227).
- [15] Wenxiang Meng and Masatoshi Takeichi. "Adherens junction: molecular architecture and regulation." *Cold Spring Harbor perspectives in biology* **1** (2009), a002899. DOI: [10.1101/cshperspect.a002899](https://doi.org/10.1101/cshperspect.a002899).
- [16] Anna Huttenlocher and Alan Rick Horwitz. "Integrins in cell migration." *Cold Spring Harbor perspectives in biology* **3** (2011), a005074. DOI: [10.1101/cshperspect.a005074](https://doi.org/10.1101/cshperspect.a005074).
- [17] Lia Stanciu and Susana Diaz-Amaya. "Tissue-biomaterials interactions." In: *Introductory biomaterials*. Ed. by L. Stanciu and Susana Diaz-Amaya. Biomedical engineering. Amsterdam: Academic Press, 2022, pp. 171–200. DOI: [10.1016/B978-0-12-809263-7.00008-1](https://doi.org/10.1016/B978-0-12-809263-7.00008-1).
- [18] C.Rolfe Howlett, Margaret D.M. Evans, William R. Walsh, Graham Johnson, et al. "Mechanism of initial attachment of cells derived from human bone to commonly used prosthetic materials during cell culture." *Biomaterials* **15** (1994), pp. 213–222. DOI: [10.1016/0142-9612\(94\)90070-1](https://doi.org/10.1016/0142-9612(94)90070-1).
- [19] H. Helmholtz. "Studien über electrische Grenzschichten." *Annalen der Physik und Chemie* **243** (1879), pp. 337–382. DOI: [10.1002/andp.18792430702](https://doi.org/10.1002/andp.18792430702).
- [20] M. Gouy. "Sur la constitution de la charge électrique à la surface d'un électrolyte." *Journal de Physique Théorique et Appliquée* **9** (1910), pp. 457–468. DOI: [10.1051/jphystap:019100090045700](https://doi.org/10.1051/jphystap:019100090045700).
- [21] David Leonard Chapman. "LI. A contribution to the theory of electrocapilarity." *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science* **25** (1913), pp. 475–481. DOI: [10.1080/14786440408634187](https://doi.org/10.1080/14786440408634187).

- [22] Otto Stern. "Zur Theorie der elektrolytischen Doppelschicht." *Zeitschrift für Elektrochemie und angewandte physikalische Chemie* **30** (1924), pp. 508–516. DOI: [10.1002/bbpc.192400182](https://doi.org/10.1002/bbpc.192400182).
- [23] Helmut Baumgärtel. *Electrochemistry: A guide for newcomers*. Berlin and Boston: De Gruyter, 2019. DOI: [10.1515/9783110437393](https://doi.org/10.1515/9783110437393).
- [24] Maya Shamir, Yinon Bar-On, Rob Phillips, and Ron Milo. "SnapShot: Timescales in Cell Biology." *Cell* **164** (2016), 1302–1302.e1. DOI: [10.1016/j.cell.2016.02.058](https://doi.org/10.1016/j.cell.2016.02.058).
- [25] Kazuo Katoh. "Effects of Electrical Stimulation on the Signal Transduction-Related Proteins, c-Src and Focal Adhesion Kinase, in Fibroblasts." *Life (Basel, Switzerland)* **12** (2022). DOI: [10.3390/life12040531](https://doi.org/10.3390/life12040531).
- [26] Susanne Staehlke, Meike Bielfeldt, Julius Zimmermann, Martina Gruening, et al. "Pulsed Electrical Stimulation Affects Osteoblast Adhesion and Calcium Ion Signaling." *Cells* **11** (2022). DOI: [10.3390/cells11172650](https://doi.org/10.3390/cells11172650).
- [27] Richard Balint, Nigel J. Cassidy, and Sarah H. Cartmell. "Electrical stimulation: a novel tool for tissue engineering." *Tissue engineering. Part B, Reviews* **19** (2013), pp. 48–57. DOI: [10.1089/ten.TEB.2012.0183](https://doi.org/10.1089/ten.TEB.2012.0183).
- [28] Richard Nuccitelli. "A Role for Endogenous Electric Fields in Wound Healing." In: *Current Topics in Developmental Biology*. Ed. by Gerald P. Schatten. Vol. 58. Current Topics in Developmental Biology. s.l.: Elsevier textbooks, 2003, pp. 1–26. DOI: [10.1016/S0070-2153\(03\)58001-2](https://doi.org/10.1016/S0070-2153(03)58001-2).
- [29] Flavio Fröhlich and David A. McCormick. "Endogenous electric fields may guide neocortical network activity." *Neuron* **67** (2010), pp. 129–143. DOI: [10.1016/j.neuron.2010.06.005](https://doi.org/10.1016/j.neuron.2010.06.005).
- [30] Batur Ercan and Thomas J. Webster. "The effect of biphasic electrical stimulation on osteoblast function at anodized nanotubular titanium surfaces." *Biomaterials* **31** (2010), pp. 3684–3693. DOI: [10.1016/j.biomaterials.2010.01.078](https://doi.org/10.1016/j.biomaterials.2010.01.078).
- [31] Franziska Sahm, Vivica Freiin Grote, Julius Zimmermann, Fiete Haack, et al. "Long-term stimulation with alternating electric fields modulates the differentiation and mineralization of human pre-osteoblasts." *Frontiers in physiology* **13** (2022), p. 965181. DOI: [10.3389/fphys.2022.965181](https://doi.org/10.3389/fphys.2022.965181).
- [32] Ashutosh K. Dubey and Bikramjit Basu. "Pulsed Electrical Stimulation and Surface Charge Induced Cell Growth on Multistage Spark Plasma Sintered Hydroxyapatite–Barium Titanate Piezobiocomposite." *Journal of the American Ceramic Society* **97** (2014), pp. 481–489. DOI: [10.1111/jace.12647](https://doi.org/10.1111/jace.12647).
- [33] Emily Pettersen, Furqan A. Shah, and Max Ortiz-Catalan. "Enhancing osteoblast survival through pulsed electrical stimulation and implications for osseointegration." *Scientific reports* **11** (2021), p. 22416. DOI: [10.1038/s41598-021-01901-3](https://doi.org/10.1038/s41598-021-01901-3).

- [34] Daniel Martín, J. Bocio-Nuñez, Santiago F. Scagliusi, Pablo Pérez, et al. "DC electrical stimulation enhances proliferation and differentiation on N2a and MC3T3 cell lines." *Journal of biological engineering* **16** (2022), p. 27. DOI: [10.1186/s13036-022-00306-8](https://doi.org/10.1186/s13036-022-00306-8).
- [35] Kasama Srirussamee, Sahba Mobini, Nigel J. Cassidy, and Sarah H. Cartmell. "Direct electrical stimulation enhances osteogenesis by inducing Bmp2 and Spp1 expressions from macrophages and preosteoblasts." *Biotechnology and bioengineering* **116** (2019), pp. 3421–3432. DOI: [10.1002/bit.27142](https://doi.org/10.1002/bit.27142).
- [36] Yong Min, Yidong Liu, Yadagiri Poojari, Jen-Chieh Wu, et al. "Self-doped polyaniline-based interdigitated electrodes for electrical stimulation of osteoblast cell lines." *Synthetic Metals* **198** (2014), pp. 308–313. DOI: [10.1016/j.synthmet.2014.10.035](https://doi.org/10.1016/j.synthmet.2014.10.035).
- [37] Jieyu Zhang, Koon Gee Neoh, Xuefeng Hu, En-Tang Kang, et al. "Combined effects of direct current stimulation and immobilized BMP-2 for enhancement of osteogenesis." *Biotechnology and bioengineering* **110** (2013), pp. 1466–1475. DOI: [10.1002/bit.24796](https://doi.org/10.1002/bit.24796).
- [38] Shiyun Meng, Mahmoud Rouabhia, and Ze Zhang. "Electrical stimulation modulates osteoblast proliferation and bone protein production through heparin-bioactivated conductive scaffolds." *Bioelectromagnetics* **34** (2013), pp. 189–199. DOI: [10.1002/bem.21766](https://doi.org/10.1002/bem.21766).
- [39] Shiyun Meng, Ze Zhang, and Mahmoud Rouabhia. "Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation." *Journal of bone and mineral metabolism* **29** (2011), pp. 535–544. DOI: [10.1007/s00774-010-0257-1](https://doi.org/10.1007/s00774-010-0257-1).
- [40] Ashutosh Kumar Dubey, Parnika Agrawal, R. Devesh Kumar Misra, and Bikramjit Basu. "Pulsed electric field mediated in vitro cellular response of fibroblast and osteoblast-like cells on conducting austenitic stainless steel substrate." *Journal of materials science. Materials in medicine* **24** (2013), pp. 1789–1798. DOI: [10.1007/s10856-013-4921-6](https://doi.org/10.1007/s10856-013-4921-6).
- [41] P. R. Supronowicz, P. M. Ajayan, K. R. Ullmann, B. P. Arulanandam, et al. "Novel current-conducting composite substrates for exposing osteoblasts to alternating current stimulation." *Journal of biomedical materials research* **59** (2002), pp. 499–506. DOI: [10.1002/jbm.10015](https://doi.org/10.1002/jbm.10015).
- [42] In Sook Kim, Jong Keun Song, Yu Lian Zhang, Tae Hyung Lee, et al. "Biphasic electric current stimulates proliferation and induces VEGF production in osteoblasts." *Biochimica et biophysica acta* **1763** (2006), pp. 907–916. DOI: [10.1016/j.bbamcr.2006.06.007](https://doi.org/10.1016/j.bbamcr.2006.06.007).
- [43] Wei-Wen Hu, Yi-Ting Hsu, Yu-Che Cheng, Chuan Li, et al. "Electrical stimulation to promote osteogenesis using conductive polypyrrole films." *Materials science & engineering. C, Materials for biological applications* **37** (2014), pp. 28–36. DOI: [10.1016/j.msec.2013.12.019](https://doi.org/10.1016/j.msec.2013.12.019).

- [44] J. Ferrier, S. M. Ross, J. Kanehisa, and J. E. Aubin. "Osteoclasts and osteoblasts migrate in opposite directions in response to a constant electrical field." *Journal of cellular physiology* **129** (1986), pp. 283–288. DOI: [10.1002/jcp.1041290303](https://doi.org/10.1002/jcp.1041290303).
- [45] Nurdan Ozkucur, Thomas K. Monsees, Srikanth Perike, Hoa Quynh Do, et al. "Local calcium elevation and cell elongation initiate guided motility in electrically stimulated osteoblast-like cells." *PloS one* **4** (2009), e6131. DOI: [10.1371/journal.pone.0006131](https://doi.org/10.1371/journal.pone.0006131).
- [46] Michelle Griffin, Anil Sebastian, James Colthurst, and Ardeshir Bayat. "Enhancement of differentiation and mineralisation of osteoblast-like cells by degenerate electrical waveform in an in vitro electrical stimulation model compared to capacitive coupling." *PloS one* **8** (2013), e72978. DOI: [10.1371/journal.pone.0072978](https://doi.org/10.1371/journal.pone.0072978).
- [47] Layla Khatib, David E. Golan, and Michael Cho. "Physiologic electrical stimulation provokes intracellular calcium increase mediated by phospholipase C activation in human osteoblasts." *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18** (2004), pp. 1903–1905. DOI: [10.1096/fj.04-1814fje](https://doi.org/10.1096/fj.04-1814fje).
- [48] Sami Curtze, Micah Dembo, Miguel Miron, and David B. Jones. "Dynamic changes in traction forces with DC electric field in osteoblast-like cells." *Journal of cell science* **117** (2004), pp. 2721–2729. DOI: [10.1242/jcs.01119](https://doi.org/10.1242/jcs.01119).
- [49] M. Hartig, U. Joos, and H. P. Wiesmann. "Capacitively coupled electric fields accelerate proliferation of osteoblast-like primary cells and increase bone extracellular matrix formation in vitro." *European biophysics journal : EBJ* **29** (2000), pp. 499–506. DOI: [10.1007/s002490000100](https://doi.org/10.1007/s002490000100).
- [50] H. Wiesmann, M. Hartig, U. Stratmann, U. Meyer, et al. "Electrical stimulation influences mineral formation of osteoblast-like cells in vitro." *Biochimica et biophysica acta* **1538** (2001), pp. 28–37. DOI: [10.1016/S0167-4889\(00\)00135-X](https://doi.org/10.1016/S0167-4889(00)00135-X).
- [51] Martin Stephan, Julius Zimmermann, Annett Klinder, Franziska Sahm, et al. "Establishment and Evaluation of an In Vitro System for Biophysical Stimulation of Human Osteoblasts." *Cells* **9** (2020). DOI: [10.3390/cells9091995](https://doi.org/10.3390/cells9091995).
- [52] Marco P. Soares Dos Santos, J. Coutinho, Ana Marote, Bárbara Sousa, et al. "Capacitive technologies for highly controlled and personalized electrical stimulation by implantable biomedical systems." *Scientific reports* **9** (2019), p. 5001. DOI: [10.1038/s41598-019-41540-3](https://doi.org/10.1038/s41598-019-41540-3).
- [53] Zhenyu Wang, Charles C. Clark, and Carl T. Brighton. "Up-regulation of bone morphogenetic proteins in cultured murine bone cells with use of specific electric fields." *The Journal of bone and joint surgery. American volume* **88** (2006), pp. 1053–1065. DOI: [10.2106/JBJS.E.00443](https://doi.org/10.2106/JBJS.E.00443).

- [54] C. T. Brighton, W. Wang, R. Seldes, G. Zhang, et al. "Signal transduction in electrically stimulated bone cells." *The Journal of bone and joint surgery. American volume* **83** (2001), pp. 1514–1523. DOI: [10.2106/00004623-200110000-00009](https://doi.org/10.2106/00004623-200110000-00009).
- [55] Rolando A. Gittens, Rene Olivares-Navarrete, Robert Rettew, Robert J. Butera, et al. "Electrical polarization of titanium surfaces for the enhancement of osteoblast differentiation." *Bioelectromagnetics* **34** (2013), pp. 599–612. DOI: [10.1002/bem.21810](https://doi.org/10.1002/bem.21810).
- [56] Ming-Tzu Tsai, Walter Hong-Shong Chang, Kyle Chang, Ru-Jyuan Hou, et al. "Pulsed electromagnetic fields affect osteoblast proliferation and differentiation in bone tissue engineering." *Bioelectromagnetics* **28** (2007), pp. 519–528. DOI: [10.1002/bem.20336](https://doi.org/10.1002/bem.20336).
- [57] Oriol Careta, Aliona Nicolenco, Filippas Perdikos, Andreu Blanquer, et al. "Enhanced Proliferation and Differentiation of Human Osteoblasts by Remotely Controlled Magnetic-Field-Induced Electric Stimulation Using Flexible Substrates." *ACS applied materials & interfaces* **15** (2023), pp. 58054–58066. DOI: [10.1021/acsmi.3c09428](https://doi.org/10.1021/acsmi.3c09428).
- [58] Hironori Miyamoto, Yasunobu Sawaji, Takahiro Iwaki, Toshinori Masaoka, et al. "Intermittent pulsed electromagnetic field stimulation activates the mTOR pathway and stimulates the proliferation of osteoblast-like cells." *Bioelectromagnetics* **40** (2019), pp. 412–421. DOI: [10.1002/bem.22207](https://doi.org/10.1002/bem.22207).
- [59] Da Jing, Mingming Zhai, Shichao Tong, Fei Xu, et al. "Pulsed electromagnetic fields promote osteogenesis and osseointegration of porous titanium implants in bone defect repair through a Wnt/b-catenin signaling-associated mechanism." *Scientific reports* **6** (2016), p. 32045. DOI: [10.1038/srep32045](https://doi.org/10.1038/srep32045).
- [60] C. H. Lohmann, Z. Schwartz, Y. Liu, H. Guerkov, et al. "Pulsed electromagnetic field stimulation of MG63 osteoblast-like cells affects differentiation and local factor production." *Journal of Orthopaedic Research* **18** (2000), pp. 637–646. DOI: [10.1002/jor.1100180417](https://doi.org/10.1002/jor.1100180417).
- [61] Lorenzo Fassina, Enrica Saino, Maria Sonia Sbarra, Livia Visai, et al. "In vitro electromagnetically stimulated SAOS-2 osteoblasts inside porous hydroxyapatite." *Journal of biomedical materials research. Part A* **93** (2010), pp. 1272–1279. DOI: [10.1002/jbm.a.32620](https://doi.org/10.1002/jbm.a.32620).
- [62] Pen-Hsiu Grace Chao, Helen H. Lu, Clark T. Hung, Steven B. Nicoll, et al. "Effects of applied DC electric field on ligament fibroblast migration and wound healing." *Connective tissue research* **48** (2007), pp. 188–197. DOI: [10.1080/03008200701424451](https://doi.org/10.1080/03008200701424451).
- [63] K. S. Fang, B. Farboud, R. Nuccitelli, and R. R. Isseroff. "Migration of human keratinocytes in electric fields requires growth factors and extracellular calcium." *The Journal of investigative dermatology* **111** (1998), pp. 751–756. DOI: [10.1046/j.1523-1747.1998.00366.x](https://doi.org/10.1046/j.1523-1747.1998.00366.x).

- [64] P. H. Chao, R. Roy, R. L. Mauck, W. Liu, et al. "Chondrocyte translocation response to direct current electric fields." *Journal of biomechanical engineering* **122** (2000), pp. 261–267. DOI: [10.1115/1.429661](https://doi.org/10.1115/1.429661).
- [65] Li Yao, Lynne Shanley, Colin McCaig, and Min Zhao. "Small applied electric fields guide migration of hippocampal neurons." *Journal of cellular physiology* **216** (2008), pp. 527–535. DOI: [10.1002/jcp.21431](https://doi.org/10.1002/jcp.21431).
- [66] Bing Song, Min Zhao, John V. Forrester, and Colin D. McCaig. "Electrical cues regulate the orientation and frequency of cell division and the rate of wound healing in vivo." *Proceedings of the National Academy of Sciences of the United States of America* **99** (2002), pp. 13577–13582. DOI: [10.1073/pnas.202235299](https://doi.org/10.1073/pnas.202235299).
- [67] Maria E. Mycielska and Mustafa B. A. Djamgoz. "Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease." *Journal of cell science* **117** (2004), pp. 1631–1639. DOI: [10.1242/jcs.01125](https://doi.org/10.1242/jcs.01125).
- [68] Entong Wang, Min Zhao, John V. Forrester, and Colin D. McCaig. "Bi-directional migration of lens epithelial cells in a physiological electrical field." *Experimental Eye Research* **76** (2003), pp. 29–37. DOI: [10.1016/S0014-4835\(02\)00257-9](https://doi.org/10.1016/S0014-4835(02)00257-9).
- [69] Przemysław Borys. "The role of passive calcium influx through the cell membrane in galvanotaxis." *Cellular & molecular biology letters* **18** (2013), pp. 187–199. DOI: [10.2478/s11658-013-0082-3](https://doi.org/10.2478/s11658-013-0082-3).
- [70] Yaping Li, Tao Xu, Xiaomei Chen, Shin Lin, et al. "Effects of direct current electric fields on lung cancer cell electrotaxis in a PMMA-based microfluidic device." *Analytical and bioanalytical chemistry* **409** (2017), pp. 2163–2178. DOI: [10.1007/s00216-016-0162-0](https://doi.org/10.1007/s00216-016-0162-0).
- [71] Vikki A. McBain, John V. Forrester, and Colin D. McCaig. "HGF, MAPK, and a small physiological electric field interact during corneal epithelial cell migration." *Investigative ophthalmology & visual science* **44** (2003), pp. 540–547. DOI: [10.1167/iovs.02-0570](https://doi.org/10.1167/iovs.02-0570).
- [72] *Principles of Bone Biology*. Elsevier, 2002. DOI: [10.1016/B978-0-12-098652-1.X5100-2](https://doi.org/10.1016/B978-0-12-098652-1.X5100-2).
- [73] *Osteoporosis*. Elsevier, 2013. DOI: [10.1016/C2011-1-04566-6](https://doi.org/10.1016/C2011-1-04566-6).
- [74] *Bone Structure*. URL: <https://www.britannica.com/science/cancer/cancer-bone> (visited on 06/04/2024).
- [75] Andrzej Hudecki, Gerard Kiryczyński, and Marek J. Łos. "Biomaterials, Definition, Overview." In: *Stem Cells and Biomaterials for Regenerative Medicine*. Ed. by Marek J. Łos, Andrzej Hudecki, and Emilia Wiecheć. San Diego: Elsevier Science & Technology, 2018, pp. 85–98. DOI: [10.1016/B978-0-12-812258-7.00007-1](https://doi.org/10.1016/B978-0-12-812258-7.00007-1).
- [76] H. Heremans, A. Billiau, J. J. Cassiman, J. C. Mulier, et al. "In vitro cultivation of human tumor tissues. II. Morphological and virological characterization of three cell lines." *Oncology* **35** (1978), pp. 246–252. DOI: [10.1159/000225298](https://doi.org/10.1159/000225298).

- [77] J. Clover and M. Gowen. "Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype?" *Bone* **15** (1994), pp. 585–591. DOI: [10.1016/8756-3282\(94\)90305-0](https://doi.org/10.1016/8756-3282(94)90305-0).
- [78] R. T. Franceschi, P. R. Romano, and K. Y. Park. "Regulation of type I collagen synthesis by 1,25-dihydroxyvitamin D3 in human osteosarcoma cells." *Journal of Biological Chemistry* **263** (1988), pp. 18938–18945. DOI: [10.1016/S0021-9258\(18\)37372-1](https://doi.org/10.1016/S0021-9258(18)37372-1).
- [79] J. Clover, R. A. Dodds, and M. Gowen. "Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture." *Journal of cell science* **103 (Pt 1)** (1992), pp. 267–271. DOI: [10.1242/jcs.103.1.267](https://doi.org/10.1242/jcs.103.1.267).
- [80] Shu-Fen Chu, Min-Tsan Huang, Keng-Liang Ou, Erwan Sugiatno, et al. "Enhanced biocompatible and hemocompatible nano/micro porous surface as a biological scaffold for functionalizational and biointegrated implants." *Journal of Alloys and Compounds* **684** (2016), pp. 726–732. DOI: [10.1016/j.jallcom.2016.05.134](https://doi.org/10.1016/j.jallcom.2016.05.134).
- [81] Zheng ZHOU, Yao DAI, Bin-bin LIU, Lei-lei XIA, et al. "Surface modification of titanium plate enhanced fibronectin-mediated adhesion and proliferation of MG-63 cells." *Transactions of Nonferrous Metals Society of China* **24** (2014), pp. 1065–1071. DOI: [10.1016/S1003-6326\(14\)63163-0](https://doi.org/10.1016/S1003-6326(14)63163-0).
- [82] Xumeng Pan, Yada Li, Adil O. Abdullah, Weiqiang Wang, et al. "Micro/nano-hierarchical structured TiO2 coating on titanium by micro-arc oxidation enhances osteoblast adhesion and differentiation." *Royal Society Open Science* **6** (2019), p. 182031. DOI: [10.1098/rsos.182031](https://doi.org/10.1098/rsos.182031).
- [83] Hae-Won Kim, Hyoun-Ee Kim, and Vehid Salih. "Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin-hydroxyapatite for tissue engineering scaffolds." *Biomaterials* **26** (2005), pp. 5221–5230. DOI: [10.1016/j.biomaterials.2005.01.047](https://doi.org/10.1016/j.biomaterials.2005.01.047).
- [84] Susanne Staehlke, Henrike Rebl, and Barbara Nebe. "Phenotypic stability of the human MG-63 osteoblastic cell line at different passages." *Cell biology international* **43** (2019), pp. 22–32. DOI: [10.1002/cbin.11073](https://doi.org/10.1002/cbin.11073).
- [85] P. K. Hansma, B. Drake, O. Marti, S. A. Gould, et al. "The scanning ion-conductance microscope." *Science (New York, N.Y.)* **243** (1989), pp. 641–643. DOI: [10.1126/science.2464851](https://doi.org/10.1126/science.2464851).
- [86] Y. E. Korchev, C. L. Bashford, M. Milovanovic, I. Vodyanoy, et al. "Scanning ion conductance microscopy of living cells." *Biophysical journal* **73** (1997), pp. 653–658. DOI: [10.1016/S0006-3495\(97\)78100-1](https://doi.org/10.1016/S0006-3495(97)78100-1).
- [87] Y. E. Korchev, Y. A. Negulyaev, C. R. Edwards, I. Vodyanoy, et al. "Functional localization of single active ion channels on the surface of a living cell." *Nature cell biology* **2** (2000), pp. 616–619. DOI: [10.1038/35023563](https://doi.org/10.1038/35023563).

- [88] J. Gorelik, A. Shevchuk, M. Ramalho, M. Elliott, et al. "Scanning surface confocal microscopy for simultaneous topographical and fluorescence imaging: application to single virus-like particle entry into a cell." *Proceedings of the National Academy of Sciences of the United States of America* **99** (2002), pp. 16018–16023. DOI: [10.1073/pnas.252458399](https://doi.org/10.1073/pnas.252458399).
- [89] S. A. Mann, G. Hoffmann, A. Hengstenberg, W. Schuhmann, et al. "Pulse-mode scanning ion conductance microscopy—a method to investigate cultured hippocampal cells." *Journal of neuroscience methods* **116** (2002), pp. 113–117. DOI: [10.1016/s0165-0270\(02\)00023-7](https://doi.org/10.1016/s0165-0270(02)00023-7).
- [90] Johannes Rheinlaender and Tilman E. Schäffer. "Image formation, resolution, and height measurement in scanning ion conductance microscopy." *Journal of Applied Physics* **105** (2009). DOI: [10.1063/1.3122007](https://doi.org/10.1063/1.3122007).
- [91] Kim McKelvey, Sophie L. Kinnear, David Perry, Dmitry Momotenko, et al. "Surface charge mapping with a nanopipette." *Journal of the American Chemical Society* **136** (2014), pp. 13735–13744. DOI: [10.1021/ja506139u](https://doi.org/10.1021/ja506139u).
- [92] Amit Kumar, Hans A. Biebuyck, and George M. Whitesides. "Patterning Self-Assembled Monolayers: Applications in Materials Science." *Langmuir* **10** (1994), pp. 1498–1511. DOI: [10.1021/la00017a030](https://doi.org/10.1021/la00017a030).
- [93] Christian Voelkner, Mirco Wendt, Regina Lange, Max Ulbrich, et al. "The nanomorphology of cell surfaces of adhered osteoblasts." *Beilstein journal of nanotechnology* **12** (2021), pp. 242–256. DOI: [10.3762/bjnano.12.20](https://doi.org/10.3762/bjnano.12.20).
- [94] Martina Gruening, Jonathan E. Dawson, Christian Voelkner, Sven Neuber, et al. "Automatic Actin Filament Quantification and Cell Shape Modeling of Osteoblasts on Charged Ti Surfaces." *Applied Sciences* **11** (2021), p. 5689. DOI: [10.3390/app11125689](https://doi.org/10.3390/app11125689).
- [95] Julia Gorelik, Lin Q. Yang, Yanjun Zhang, Max Lab, et al. "A novel Z-groove index characterizing myocardial surface structure." *Cardiovascular research* **72** (2006), pp. 422–429. DOI: [10.1016/j.cardiores.2006.09.009](https://doi.org/10.1016/j.cardiores.2006.09.009).
- [96] Alberto Sesena-Rubfiaro, Navin J. Prajapati, Lia Paolino, Lihua Lou, et al. "Membrane Remodeling of Human-Engineered Cardiac Tissue by Chronic Electric Stimulation." *ACS biomaterials science & engineering* **9** (2023), pp. 1644–1655. DOI: [10.1021/acsbiomaterials.2c01370](https://doi.org/10.1021/acsbiomaterials.2c01370).
- [97] Lin Liu, Kaizhe Wang, Jingyuan Liu, Yuhui Wei, et al. "Starvation effect on the morphology of microvilli in HeLa cells." *Biochemical and biophysical research communications* **514** (2019), pp. 1238–1243. DOI: [10.1016/j.bbrc.2019.05.073](https://doi.org/10.1016/j.bbrc.2019.05.073).
- [98] Shailin Zhang, Junying Sun, Ying Xu, Shi Qian, et al. "Biological behavior of osteoblast-like cells on titania and zirconia films deposited by cathodic arc deposition." *Biointerphases* **7** (2012), p. 60. DOI: [10.1007/s13758-012-0060-8](https://doi.org/10.1007/s13758-012-0060-8).

- [99] Guowen Qian, Lemin Zhang, Guoyong Wang, Zhengyu Zhao, et al. "3D Printed Zn-doped Mesoporous Silica-incorporated Poly-L-lactic Acid Scaffolds for Bone Repair." *International journal of bioprinting* **7** (2021), p. 346. DOI: [10.18063/ijb.v7i2.346](https://doi.org/10.18063/ijb.v7i2.346).
- [100] Johannes Rheinlaender and Tilman E. Schäffer. "Mapping the mechanical stiffness of live cells with the scanning ion conductance microscope." *Soft Matter* **9** (2013), p. 3230. DOI: [10.1039/c2sm27412d](https://doi.org/10.1039/c2sm27412d).
- [101] Julia Gorelik, Andrew I. Shevchuk, Gregory I. Frolenkov, Ivan A. Diakonov, et al. "Dynamic assembly of surface structures in living cells." *Proceedings of the National Academy of Sciences of the United States of America* **100** (2003), pp. 5819–5822. DOI: [10.1073/pnas.1030502100](https://doi.org/10.1073/pnas.1030502100).
- [102] Cécile Sauvanet, Jessica Wayt, Taher Pelaseyed, and Anthony Bretscher. "Structure, regulation, and functional diversity of microvilli on the apical domain of epithelial cells." *Annual review of cell and developmental biology* **31** (2015), pp. 593–621. DOI: [10.1146/annurev-cellbio-100814-125234](https://doi.org/10.1146/annurev-cellbio-100814-125234).
- [103] Sonja Majstoravich, Jinyi Zhang, Susan Nicholson-Dykstra, Stefan Linder, et al. "Lymphocyte microvilli are dynamic, actin-dependent structures that do not require Wiskott-Aldrich syndrome protein (WASp) for their morphology." *Blood* **104** (2004), pp. 1396–1403. DOI: [10.1182/blood-2004-02-0437](https://doi.org/10.1182/blood-2004-02-0437).
- [104] Yunmin Jung, Inbal Riven, Sara W. Feigelson, Elena Kartvelishvily, et al. "Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies." *Proceedings of the National Academy of Sciences of the United States of America* **113** (2016), E5916–E5924. DOI: [10.1073/pnas.1605399113](https://doi.org/10.1073/pnas.1605399113).
- [105] A. Polliack, N. Lampen, B. D. Clarkson, E. de Harven, et al. "Identification of human B and T lymphocytes by scanning electron microscopy." *The Journal of experimental medicine* **138** (1973), pp. 607–624. DOI: [10.1084/jem.138.3.607](https://doi.org/10.1084/jem.138.3.607).
- [106] Tatyana M. Svitkina, Elena A. Bulanova, Oleg Y. Chaga, Danijela M. Vignjevic, et al. "Mechanism of filopodia initiation by reorganization of a dendritic network." *The Journal of cell biology* **160** (2003), pp. 409–421. DOI: [10.1083/jcb.200210174](https://doi.org/10.1083/jcb.200210174).
- [107] Thomas Bornschlöggl. "How filopodia pull: what we know about the mechanics and dynamics of filopodia." *Cytoskeleton (Hoboken, N.J.)* **70** (2013), pp. 590–603. DOI: [10.1002/cm.21130](https://doi.org/10.1002/cm.21130).
- [108] Jonathan S. Berg and Richard E. Cheney. "Myosin-X is an unconventional myosin that undergoes intrafilopodial motility." *Nature cell biology* **4** (2002), pp. 246–250. DOI: [10.1038/ncb762](https://doi.org/10.1038/ncb762).
- [109] T. D. Pollard, L. Blanchoin, and R. D. Mullins. "Molecular mechanisms controlling actin filament dynamics in nonmuscle cells." *Annual review of biophysics and biomolecular structure* **29** (2000), pp. 545–576. DOI: [10.1146/annurev.biophys.29.1.545](https://doi.org/10.1146/annurev.biophys.29.1.545).

- [110] Alex Mogilner and George Oster. "Force generation by actin polymerization II: the elastic ratchet and tethered filaments." *Biophysical journal* **84** (2003), pp. 1591–1605. DOI: [10.1016/S0006-3495\(03\)74969-8](https://doi.org/10.1016/S0006-3495(03)74969-8).
- [111] A. Mallavarapu and T. Mitchison. "Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction." *The Journal of cell biology* **146** (1999), pp. 1097–1106. DOI: [10.1083/jcb.146.5.1097](https://doi.org/10.1083/jcb.146.5.1097).
- [112] Phyllis J. Fisher, Peggy A. Bulur, Stanimir Vuk-Pavlovic, Franklyn G. Prendergast, et al. "Dendritic cell microvilli: a novel membrane structure associated with the multifocal synapse and T-cell clustering." *Blood* **112** (2008), pp. 5037–5045. DOI: [10.1182/blood-2008-04-149526](https://doi.org/10.1182/blood-2008-04-149526).
- [113] S. J. Jones and A. Boyde. "Morphological changes of osteoblasts in vitro." *Cell and Tissue Research* **166** (1976), pp. 101–107. DOI: [10.1007/BF00215129](https://doi.org/10.1007/BF00215129).
- [114] C. H. Lohmann, Z. Schwartz, G. Köster, U. Jahn, et al. "Phagocytosis of wear debris by osteoblasts affects differentiation and local factor production in a manner dependent on particle composition." *Biomaterials* **21** (2000), pp. 551–561. DOI: [10.1016/S0142-9612\(99\)00211-2](https://doi.org/10.1016/S0142-9612(99)00211-2).
- [115] A. Boyde and A. Bailey. "Observations on the marginal ruffles of an established fibroblast-like cell line." *Cell and Tissue Research* **179** (1977), pp. 225–234. DOI: [10.1007/BF00219798](https://doi.org/10.1007/BF00219798).
- [116] A. J. Ridley. "Membrane ruffling and signal transduction." *BioEssays : news and reviews in molecular, cellular and developmental biology* **16** (1994), pp. 321–327. DOI: [10.1002/bies.950160506](https://doi.org/10.1002/bies.950160506).
- [117] Kirsi Rilla and Arto Koistinen. "Correlative Light and Electron Microscopy Reveals the HAS3-Induced Dorsal Plasma Membrane Ruffles." *International journal of cell biology* **2015** (2015), p. 769163. DOI: [10.1155/2015/769163](https://doi.org/10.1155/2015/769163).
- [118] Bodo Borm, Robert P. Requardt, Volker Herzog, and Gregor Kirfel. "Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization." *Experimental cell research* **302** (2005), pp. 83–95. DOI: [10.1016/j.yexcr.2004.08.034](https://doi.org/10.1016/j.yexcr.2004.08.034).
- [119] Jing-Ling Hoon, Wai-Keung Wong, and Cheng-Gee Koh. "Functions and regulation of circular dorsal ruffles." *Molecular and cellular biology* **32** (2012), pp. 4246–4257. DOI: [10.1128/MCB.00551-12](https://doi.org/10.1128/MCB.00551-12).
- [120] Erik Bernitt, Hans-Günther Döbereiner, Nir S. Gov, and Arik Yochelis. "Fronts and waves of actin polymerization in a bistability-based mechanism of circular dorsal ruffles." *Nature communications* **8** (2017), p. 15863. DOI: [10.1038/ncomms15863](https://doi.org/10.1038/ncomms15863).
- [121] D. A. Cheres, J. Leng, and R. L. Klemke. "Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells." *The Journal of cell biology* **146** (1999), pp. 1107–1116. DOI: [10.1083/jcb.146.5.1107](https://doi.org/10.1083/jcb.146.5.1107).
- [122] Seong-Oh Kim, Joonhui Kim, Takaharu Okajima, and Nam-Joon Cho. "Mechanical properties of paraformaldehyde-treated individual cells investigated by atomic force microscopy and scanning ion conductance microscopy." *Nano convergence* **4** (2017), p. 5. DOI: [10.1186/s40580-017-0099-9](https://doi.org/10.1186/s40580-017-0099-9).

- [123] W. C. Vrouwenvelder, C. G. Groot, and K. de Groot. "Behaviour of fetal rat osteoblasts cultured in vitro on bioactive glass and nonreactive glasses." *Bio-materials* **13** (1992), pp. 382–392. DOI: [10.1016/0142-9612\(92\)90044-O](https://doi.org/10.1016/0142-9612(92)90044-O).
- [124] Kris A. DeMali and Keith Burridge. "Coupling membrane protrusion and cell adhesion." *Journal of cell science* **116** (2003), pp. 2389–2397. DOI: [10.1242/jcs.00605](https://doi.org/10.1242/jcs.00605).
- [125] Birgit Finke, Frank Luethen, Karsten Schroeder, Petra D. Mueller, et al. "The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces." *Biomaterials* **28** (2007), pp. 4521–4534. DOI: [10.1016/j.biomaterials.2007.06.028](https://doi.org/10.1016/j.biomaterials.2007.06.028).
- [126] Martina Gruening, Sven Neuber, Peter Nestler, Jutta Lehnfeld, et al. "Enhancement of Intracellular Calcium Ion Mobilization by Moderately but Not Highly Positive Material Surface Charges." *Frontiers in bioengineering and biotechnology* **8** (2020), p. 1016. DOI: [10.3389/fbioe.2020.01016](https://doi.org/10.3389/fbioe.2020.01016).
- [127] Ute Abraham, Adrián E. Granada, Pål O. Westermarck, Markus Heine, et al. "Coupling governs entrainment range of circadian clocks." *Molecular systems biology* **6** (2010), p. 438. DOI: [10.1038/msb.2010.92](https://doi.org/10.1038/msb.2010.92).
- [128] Loning Fu, Millan S. Patel, Allan Bradley, Erwin F. Wagner, et al. "The molecular clock mediates leptin-regulated bone formation." *Cell* **122** (2005), pp. 803–815. DOI: [10.1016/j.cell.2005.06.028](https://doi.org/10.1016/j.cell.2005.06.028).
- [129] Takeshi Takarada, Cheng Xu, Hiroki Ochi, Ryota Nakazato, et al. "Bone Resorption Is Regulated by Circadian Clock in Osteoblasts." *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **32** (2017), pp. 872–881. DOI: [10.1002/jbmr.3053](https://doi.org/10.1002/jbmr.3053).
- [130] Feifei Pu, Fengxia Chen, Song Lin, Songfeng Chen, et al. "The synergistic anticancer effect of cisplatin combined with *Oldenlandia diffusa* in osteosarcoma MG-63 cell line in vitro." *OncoTargets and therapy* **9** (2016), pp. 255–263. DOI: [10.2147/OTT.S90707](https://doi.org/10.2147/OTT.S90707).
- [131] Alexey I. Chizhik, Jan Rother, Ingo Gregor, Andreas Janshoff, et al. "Metal-induced energy transfer for live cell nanoscopy." *Nature Photonics* **8** (2014), pp. 124–127. DOI: [10.1038/nphoton.2013.345](https://doi.org/10.1038/nphoton.2013.345).
- [132] Joshua Zimmerberg and Stuart McLaughlin. "Membrane curvature: how BAR domains bend bilayers." *Current biology : CB* **14** (2004), R250–2. DOI: [10.1016/j.cub.2004.02.060](https://doi.org/10.1016/j.cub.2004.02.060).
- [133] Harvey T. McMahon and Emmanuel Boucrot. "Membrane curvature at a glance." *Journal of cell science* **128** (2015), pp. 1065–1070. DOI: [10.1242/jcs.114454](https://doi.org/10.1242/jcs.114454).
- [134] Ingo Gregor, Alexey Chizhik, Narain Karedla, and Jörg Enderlein. "Metal-induced energy transfer." *Nanophotonics* **8** (2019), pp. 1689–1699. DOI: [10.1515/nanoph-2019-0201](https://doi.org/10.1515/nanoph-2019-0201).
- [135] Pakorn Kanchanawong, Gleb Shtengel, Ana M. Pasapera, Ericka B. Ramko, et al. "Nanoscale architecture of integrin-based cell adhesions." *Nature* **468** (2010), pp. 580–584. DOI: [10.1038/nature09621](https://doi.org/10.1038/nature09621).

- [136] Robert R. Kay. "Macropinocytosis: Biology and mechanisms." *Cells & development* **168** (2021), p. 203713. DOI: [10.1016/j.cdev.2021.203713](https://doi.org/10.1016/j.cdev.2021.203713).
- [137] A. Bretscher. "Molecular architecture of the microvillus cytoskeleton." *Ciba Foundation symposium* **95** (1983), pp. 164–179. DOI: [10.1002/9780470720769.ch10](https://doi.org/10.1002/9780470720769.ch10).
- [138] Cyril Thouverey, Agata Malinowska, Marcin Balcerzak, Agnieszka Strzelecka-Kiliszek, et al. "Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells." *Journal of proteomics* **74** (2011), pp. 1123–1134. DOI: [10.1016/j.jprot.2011.04.005](https://doi.org/10.1016/j.jprot.2011.04.005).
- [139] Christian Voelkner, Issam Assi, Willi Karberg, Regina Lange, et al. "Response of Osteoblasts to Electric Field Line Patterns Emerging from Molecule Stripe Landscapes." *Applied Sciences* **12** (2022), p. 7329. DOI: [10.3390/app12147329](https://doi.org/10.3390/app12147329).
- [140] Kerstin Rabel, Ralf-Joachim Kohal, Thorsten Steinberg, Pascal Tomakidi, et al. "Controlling osteoblast morphology and proliferation via surface microtopographies of implant biomaterials." *Scientific reports* **10** (2020), p. 12810. DOI: [10.1038/s41598-020-69685-6](https://doi.org/10.1038/s41598-020-69685-6).
- [141] S. Roessler, R. Zimmermann, D. Scharnweber, C. Werner, et al. "Characterization of oxide layers on Ti6Al4V and titanium by streaming potential and streaming current measurements." *Colloids and Surfaces B: Biointerfaces* **26** (2002), pp. 387–395. DOI: [10.1016/S0927-7765\(02\)00025-5](https://doi.org/10.1016/S0927-7765(02)00025-5).
- [142] Thomas Luxbacher. *The Zeta potential for solid surface analysis : a practical guide to streaming potential measurement*. 1st. Anton Paar GmbH, 2014.
- [143] Christine C., Cristele J., Yasmine Adriaensen, and Paul G. "Fabrication of Surfaces with Bimodal Roughness Through Polyelectrolyte/Colloid Assembly." In: *Advances in Unconventional Lithography*. Ed. by Gorgi Kostovski. Erscheinungsort nicht ermittelbar: IntechOpen, 2011. DOI: [10.5772/24415](https://doi.org/10.5772/24415).
- [144] Bachir Delenda. *Using reaction-diffusion equations to model and simulate the interaction of bone cells with electrical stimulation*. 2020. DOI: [10.18453/ROSDOK_ID00002860](https://doi.org/10.18453/ROSDOK_ID00002860).
- [145] Jennifer E. Koblinski, Michael Wu, Borries Demeler, Karin Jacob, et al. "Matrix cell adhesion activation by non-adhesion proteins." *Journal of cell science* **118** (2005), pp. 2965–2974. DOI: [10.1242/jcs.02411](https://doi.org/10.1242/jcs.02411).
- [146] S. R. Sousa, M. Lamghari, P. Sampaio, P. Moradas-Ferreira, et al. "Osteoblast adhesion and morphology on TiO₂ depends on the competitive preadsorption of albumin and fibronectin." *Journal of biomedical materials research. Part A* **84** (2008), pp. 281–290. DOI: [10.1002/jbm.a.31201](https://doi.org/10.1002/jbm.a.31201).
- [147] Yunzhi Yang, Renee Cavin, and Joo L. Ong. "Protein adsorption on titanium surfaces and their effect on osteoblast attachment." *Journal of biomedical materials research. Part A* **67** (2003), pp. 344–349. DOI: [10.1002/jbm.a.10578](https://doi.org/10.1002/jbm.a.10578).

- [148] Mathilde Hindié, Emeline Camand, Rémy Agniel, Franck Carreiras, et al. "Effects of human fibronectin and human serum albumin sequential adsorption on preosteoblastic cell adhesion." *Biointerphases* **9** (2014), p. 029008. DOI: [10.1116/1.4867598](https://doi.org/10.1116/1.4867598).
- [149] Stephanie L. Haag, Nathan R. Schiele, and Matthew T. Bernards. "Enhancement and mechanisms of MC3T3-E1 osteoblast-like cell adhesion to albumin through calcium exposure." *Biotechnology and applied biochemistry* **69** (2022), pp. 492–502. DOI: [10.1002/bab.2126](https://doi.org/10.1002/bab.2126).
- [150] Laura J. Fox, Robert M. Richardson, and Wuge H. Briscoe. "PAMAM dendrimer - cell membrane interactions." *Advances in colloid and interface science* **257** (2018), pp. 1–18. DOI: [10.1016/j.cis.2018.06.005](https://doi.org/10.1016/j.cis.2018.06.005).
- [151] Antonetta B. C. Buskermolen, Tommaso Ristori, Dylan Mostert, Mark C. van Turnhout, et al. "Cellular Contact Guidance Emerges from Gap Avoidance." *Cell reports. Physical science* **1** (2020), p. 100055. DOI: [10.1016/j.xcrp.2020.100055](https://doi.org/10.1016/j.xcrp.2020.100055).
- [152] Patrick W. Alford, Alexander P. Nesmith, Johannes N. Seywerd, Anna Grosberg, et al. "Vascular smooth muscle contractility depends on cell shape." *Integrative biology : quantitative biosciences from nano to macro* **3** (2011), pp. 1063–1070. DOI: [10.1039/c1ib00061f](https://doi.org/10.1039/c1ib00061f).
- [153] Nan Xia, Charles K. Thodeti, Tom P. Hunt, Qiaobing Xu, et al. "Directional control of cell motility through focal adhesion positioning and spatial control of Rac activation." *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **22** (2008), pp. 1649–1659. DOI: [10.1096/fj.07-090571](https://doi.org/10.1096/fj.07-090571).
- [154] P. Clark, P. Connolly, and G. R. Moores. "Cell guidance by micropatterned adhesiveness in vitro." *Journal of cell science* **103 (Pt 1)** (1992), pp. 287–292. DOI: [10.1242/jcs.103.1.287](https://doi.org/10.1242/jcs.103.1.287).
- [155] G. R. Ramirez-San Juan, P. W. Oakes, and M. L. Gardel. "Contact guidance requires spatial control of leading-edge protrusion." *Molecular biology of the cell* **28** (2017), pp. 1043–1053. DOI: [10.1091/mbc.E16-11-0769](https://doi.org/10.1091/mbc.E16-11-0769).
- [156] Nicholas R. Romsey, Yue Hou, Laura Lara Rodriguez, and Ian C. Schneider. "The Number of Lines a Cell Contacts and Cell Contractility Drive the Efficiency of Contact Guidance." *Cellular and Molecular Bioengineering* **7** (2014), pp. 122–135. DOI: [10.1007/s12195-013-0299-2](https://doi.org/10.1007/s12195-013-0299-2).
- [157] Olivier M. Rossier, Nils Gauthier, Nicolas Biais, Wynn Vonnegut, et al. "Force generated by actomyosin contraction builds bridges between adhesive contacts." *The EMBO journal* **29** (2010), pp. 1055–1068. DOI: [10.1038/emboj.2010.2](https://doi.org/10.1038/emboj.2010.2).
- [158] Lina Altomare, Mathis Riehle, Nikolaj Gadegaard, Maria Cristina Tanzi, et al. "Microcontact printing of fibronectin on a biodegradable polymeric surface for skeletal muscle cell orientation." *The International journal of artificial organs* **33** (2010), pp. 535–543. DOI: [10.1177/039139881003300804](https://doi.org/10.1177/039139881003300804).

- [159] B. Zimerman, M. Arnold, J. Ulmer, J. Blümmel, et al. "Formation of focal adhesion-stress fibre complexes coordinated by adhesive and non-adhesive surface domains." *IEE proceedings. Nanobiotechnology* **151** (2004), pp. 62–66. DOI: [10.1049/ip-nbt:20040474](https://doi.org/10.1049/ip-nbt:20040474).
- [160] Richard H. W. Funk and Thomas K. Monsees. "Effects of electromagnetic fields on cells: physiological and therapeutical approaches and molecular mechanisms of interaction. A review." *Cells, tissues, organs* **182** (2006), pp. 59–78. DOI: [10.1159/000093061](https://doi.org/10.1159/000093061).
- [161] Roland Glaser. *Biophysics: An Introduction*. 2. 2nd ed. 2012. Berlin, Heidelberg: Springer Berlin Heidelberg, 2012.
- [162] Hans-Jürgen Butt, Karlheinz Graf, and Michael Kappl. *Physics and chemistry of interfaces*. Third, revised and enlarged edition. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 2013.
- [163] Qian Wang, Xueyu Dou, Xiaohong Chen, Zihao Zhao, et al. "Reevaluating Protein Photoluminescence: Remarkable Visible Luminescence upon Concentration and Insight into the Emission Mechanism." *Angewandte Chemie (International ed. in English)* **58** (2019), pp. 12667–12673. DOI: [10.1002/anie.201906226](https://doi.org/10.1002/anie.201906226).
- [164] P. WEISS. "Experiments on cell and axon orientation in vitro; the role of colloidal exudates in tissue organization." *The Journal of experimental zoology* **100** (1945), pp. 353–386. DOI: [10.1002/jez.1401000305](https://doi.org/10.1002/jez.1401000305).
- [165] Johannes Rheinlaender and Tilman E. Schäffer. "Lateral Resolution and Image Formation in Scanning Ion Conductance Microscopy." *Analytical chemistry* **87** (2015), pp. 7117–7124. DOI: [10.1021/acs.analchem.5b00900](https://doi.org/10.1021/acs.analchem.5b00900).
- [166] Yang Gan. "Atomic and subnanometer resolution in ambient conditions by atomic force microscopy." *Surface Science Reports* **64** (2009), pp. 99–121. DOI: [10.1016/j.surfrep.2008.12.001](https://doi.org/10.1016/j.surfrep.2008.12.001).
- [167] A. K. Wright and M. R. Thompson. "Hydrodynamic structure of bovine serum albumin determined by transient electric birefringence." *Biophysical journal* **15** (1975), pp. 137–141. DOI: [10.1016/s0006-3495\(75\)85797-3](https://doi.org/10.1016/s0006-3495(75)85797-3).
- [168] Donald A. Tomalia. "Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry." *Progress in Polymer Science* **30** (2005), pp. 294–324. DOI: [10.1016/j.progpolymsci.2005.01.007](https://doi.org/10.1016/j.progpolymsci.2005.01.007).
- [169] Neus Feliu, Marie V. Walter, Maria I. Montañez, Andrea Kunzmann, et al. "Stability and biocompatibility of a library of polyester dendrimers in comparison to polyamidoamine dendrimers." *Biomaterials* **33** (2012), pp. 1970–1981. DOI: [10.1016/j.biomaterials.2011.11.054](https://doi.org/10.1016/j.biomaterials.2011.11.054).
- [170] Inge Axelsson. "Characterization of proteins and other macromolecules by agarose gel chromatography." *Journal of Chromatography A* **152** (1978), pp. 21–32. DOI: [10.1016/S0021-9673\(00\)85330-3](https://doi.org/10.1016/S0021-9673(00)85330-3).

- [171] John E. Wong, Florian Rehfeldt, Peter Hänni, Motomu Tanaka, et al. "Swelling Behavior of Polyelectrolyte Multilayers in Saturated Water Vapor." *Macromolecules* **37** (2004), pp. 7285–7289. DOI: [10.1021/ma0351930](https://doi.org/10.1021/ma0351930).
- [172] Susanne Staehlke, Jutta Lehnfeld, Andreas Schneider, J. Barbara Nebe, et al. "Terminal chemical functions of polyamidoamine dendrimer surfaces and its impact on bone cell growth." *Materials science & engineering. C, Materials for biological applications* **101** (2019), pp. 190–203. DOI: [10.1016/j.msec.2019.03.073](https://doi.org/10.1016/j.msec.2019.03.073).
- [173] W. A. Loesberg, J. te Riet, F. C. M. J. M. van Delft, P. Schön, et al. "The threshold at which substrate nanogroove dimensions may influence fibroblast alignment and adhesion." *Biomaterials* **28** (2007), pp. 3944–3951. DOI: [10.1016/j.biomaterials.2007.05.030](https://doi.org/10.1016/j.biomaterials.2007.05.030).
- [174] Edwin Lamers, X. Frank Walboomers, Maciej Domanski, Joost te Riet, et al. "The influence of nanoscale grooved substrates on osteoblast behavior and extracellular matrix deposition." *Biomaterials* **31** (2010), pp. 3307–3316. DOI: [10.1016/j.biomaterials.2010.01.034](https://doi.org/10.1016/j.biomaterials.2010.01.034).
- [175] Aihua Guo, Bing Song, Brian Reid, Yu Gu, et al. "Effects of physiological electric fields on migration of human dermal fibroblasts." *The Journal of investigative dermatology* **130** (2010), pp. 2320–2327. DOI: [10.1038/jid.2010.96](https://doi.org/10.1038/jid.2010.96).
- [176] Colin D. McCaig, Ann M. Rajnicek, Bing Song, and Min Zhao. "Controlling cell behavior electrically: current views and future potential." *Physiological reviews* **85** (2005), pp. 943–978. DOI: [10.1152/physrev.00020.2004](https://doi.org/10.1152/physrev.00020.2004).
- [177] Najmuddin J. Gunja, Divya Dujari, Andrew Chen, Alba Luengo, et al. "Migration responses of outer and inner meniscus cells to applied direct current electric fields." *Journal of Orthopaedic Research* **30** (2012), pp. 103–111. DOI: [10.1002/jor.21489](https://doi.org/10.1002/jor.21489).
- [178] Marco Rohde, Josefin Ziebart, Timo Kirschstein, Tina Sellmann, et al. "Human Osteoblast Migration in DC Electrical Fields Depends on Store Operated Ca²⁺-Release and Is Correlated to Upregulation of Stretch-Activated TRPM7 Channels." *Frontiers in bioengineering and biotechnology* **7** (2019), p. 422. DOI: [10.3389/fbioe.2019.00422](https://doi.org/10.3389/fbioe.2019.00422).
- [179] Greg M. Allen, Alex Mogilner, and Julie A. Theriot. "Electrophoresis of cellular membrane components creates the directional cue guiding keratocyte galvanotaxis." *Current biology : CB* **23** (2013), pp. 560–568. DOI: [10.1016/j.cub.2013.02.047](https://doi.org/10.1016/j.cub.2013.02.047).
- [180] R. D. Piner, J. Zhu, F. Xu, S. Hong, et al. "'Dip-Pen' nanolithography." *Science (New York, N.Y.)* **283** (1999), pp. 661–663. DOI: [10.1126/science.283.5402.661](https://doi.org/10.1126/science.283.5402.661).
- [181] J. Park, L. D. Fouché, and P. T. Hammond. "Multicomponent Patterning of Layer-by-Layer Assembled Polyelectrolyte/Nanoparticle Composite Thin Films with Controlled Alignment." *Advanced Materials* **17** (2005), pp. 2575–2579. DOI: [10.1002/adma.200501075](https://doi.org/10.1002/adma.200501075).

- [182] Sang Hun Lee, Won-Yeop Rho, Seon Joo Park, Jinyeong Kim, et al. "Multi-functional self-assembled monolayers via microcontact printing and degas-driven flow guided patterning." *Scientific reports* **8** (2018), p. 16763. DOI: [10.1038/s41598-018-35195-9](https://doi.org/10.1038/s41598-018-35195-9).
- [183] A. A. Dameron, J. R. Hampton, R. K. Smith, T. J. Mullen, et al. "Microdisplacement printing." *Nano letters* **5** (2005), pp. 1834–1837. DOI: [10.1021/nl050981j](https://doi.org/10.1021/nl050981j).
- [184] R. Nuccitelli. "Endogenous electric fields in embryos during development, regeneration and wound healing." *Radiation protection dosimetry* **106** (2003), pp. 375–383. DOI: [10.1093/oxfordjournals.rpd.a006375](https://doi.org/10.1093/oxfordjournals.rpd.a006375).
- [185] A. M. Altizer, L. J. Moriarty, S. M. Bell, C. M. Schreiner, et al. "Endogenous electric current is associated with normal development of the vertebrate limb." *Developmental dynamics : an official publication of the American Association of Anatomists* **221** (2001), pp. 391–401. DOI: [10.1002/dvdy.1158](https://doi.org/10.1002/dvdy.1158).
- [186] M. Zhao, J. V. Forrester, and C. D. McCaig. "A small, physiological electric field orients cell division." *Proceedings of the National Academy of Sciences of the United States of America* **96** (1999), pp. 4942–4946. DOI: [10.1073/pnas.96.9.4942](https://doi.org/10.1073/pnas.96.9.4942).
- [187] Richard H. W. Funk. "Endogenous electric fields as guiding cue for cell migration." *Frontiers in physiology* **6** (2015), p. 143. DOI: [10.3389/fphys.2015.00143](https://doi.org/10.3389/fphys.2015.00143).
- [188] Min Zhao. "Electrical fields in wound healing-An overriding signal that directs cell migration." *Seminars in cell & developmental biology* **20** (2009), pp. 674–682. DOI: [10.1016/j.semcd.2008.12.009](https://doi.org/10.1016/j.semcd.2008.12.009).
- [189] Richard Nuccitelli, Pamela Nuccitelli, Samdeo Ramlatchan, Richard Sanger, et al. "Imaging the electric field associated with mouse and human skin wounds." *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* **16** (2008), pp. 432–441. DOI: [10.1111/j.1524-475X.2008.00389.x](https://doi.org/10.1111/j.1524-475X.2008.00389.x).
- [190] Nadine Wong Shi Kam, Edward Jan, and Nicholas A. Kotov. "Electrical stimulation of neural stem cells mediated by humanized carbon nanotube composite made with extracellular matrix protein." *Nano letters* **9** (2009), pp. 273–278. DOI: [10.1021/nl802859a](https://doi.org/10.1021/nl802859a).
- [191] Wei-Wen Hu, Tun-Chi Chen, Chia-Wen Tsao, and Yu-Che Cheng. "The effects of substrate-mediated electrical stimulation on the promotion of osteogenic differentiation and its optimization." *Journal of biomedical materials research. Part B, Applied biomaterials* **107** (2019), pp. 1607–1619. DOI: [10.1002/jbm.b.34253](https://doi.org/10.1002/jbm.b.34253).
- [192] Shiyun Meng, Mahmoud Rouabhia, and Ze Zhang. "Electrical Stimulation and Cellular Behaviors in Electric Field in Biomedical Research." *Materials (Basel, Switzerland)* **15** (2021). DOI: [10.3390/ma15010165](https://doi.org/10.3390/ma15010165).

- [193] A. K. Harris, N. K. Pryer, and D. Paydarfar. "Effects of electric fields on fibroblast contractility and cytoskeleton." *The Journal of experimental zoology* **253** (1990), pp. 163–176. DOI: [10.1002/jez.1402530206](https://doi.org/10.1002/jez.1402530206).
- [194] Mei Li, Ling Ren, LiHua Li, Peng He, et al. "Cytotoxic Effect on Osteosarcoma MG-63 Cells by Degradation of Magnesium." *Journal of Materials Science & Technology* **30** (2014), pp. 888–893. DOI: [10.1016/j.jmst.2014.04.010](https://doi.org/10.1016/j.jmst.2014.04.010).
- [195] Harshita Sharma, Pawan K. Mishra, Sushama Talegaonkar, and Bhuvaneshwar Vaidya. "Metal nanoparticles: a theranostic nanotool against cancer." *Drug discovery today* **20** (2015), pp. 1143–1151. DOI: [10.1016/j.drudis.2015.05.009](https://doi.org/10.1016/j.drudis.2015.05.009).
- [196] Xingjie Wu, Yanqin Gao, and Chang-Ming Dong. "Polymer/gold hybrid nanoparticles: from synthesis to cancer theranostic applications." *RSC Advances* **5** (2015), pp. 13787–13796. DOI: [10.1039/C4RA16454G](https://doi.org/10.1039/C4RA16454G).
- [197] Tianlu Zhang, Liming Wang, Qiang Chen, and Chunying Chen. "Cytotoxic potential of silver nanoparticles." *Yonsei medical journal* **55** (2014), pp. 283–291. DOI: [10.3349/ymj.2014.55.2.283](https://doi.org/10.3349/ymj.2014.55.2.283).
- [198] Peyman Mirtaheri, Sverre Grimnes, and Orjan G. Martinsen. "Electrode polarization impedance in weak NaCl aqueous solutions." *IEEE transactions on bio-medical engineering* **52** (2005), pp. 2093–2099. DOI: [10.1109/TBME.2005.857639](https://doi.org/10.1109/TBME.2005.857639).
- [199] Zhenwei Ji, Yunlei Ma, Haien Zhao, Wei Li, et al. "The effect of temperature-control microwave on HELA and MG-63 cells." *Journal of cancer research and therapeutics* **14** (2018), S152–S158. DOI: [10.4103/0973-1482.165868](https://doi.org/10.4103/0973-1482.165868).
- [200] C. Shui and A. Scutt. "Mild heat shock induces proliferation, alkaline phosphatase activity, and mineralization in human bone marrow stromal cells and Mg-63 cells in vitro." *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **16** (2001), pp. 731–741. DOI: [10.1359/jbmr.2001.16.4.731](https://doi.org/10.1359/jbmr.2001.16.4.731).
- [201] Sven Neuber, Annekatrin Sill, Ilias Efthimiopoulos, Peter Nestler, et al. "Influence of molecular weight of polycation polydimethyldiallylammonium and carbon nanotube content on electric conductivity of layer-by-layer films." *Thin Solid Films* **745** (2022), p. 139103. DOI: [10.1016/j.tsf.2022.139103](https://doi.org/10.1016/j.tsf.2022.139103).
- [202] Zifei Zhou, Xifeng Liu, Wei Wu, Sungjo Park, et al. "Effective nerve cell modulation by electrical stimulation of carbon nanotube embedded conductive polymeric scaffolds." *Biomaterials science* **6** (2018), pp. 2375–2385. DOI: [10.1039/c8bm00553b](https://doi.org/10.1039/c8bm00553b).
- [203] Aleksandr Markov, Roger Wördenweber, Levan Ichkitidze, Alexander Gerasimenko, et al. "Biocompatible SWCNT Conductive Composites for Biomedical Applications." *Nanomaterials (Basel, Switzerland)* **10** (2020). DOI: [10.3390/nano10122492](https://doi.org/10.3390/nano10122492).

- [204] R. Samba, T. Herrmann, and G. Zeck. "PEDOT-CNT coated electrodes stimulate retinal neurons at low voltage amplitudes and low charge densities." *Journal of neural engineering* **12** (2015), p. 016014. DOI: [10.1088/1741-2560/12/1/016014](https://doi.org/10.1088/1741-2560/12/1/016014).
- [205] Susanne Staehlke, Friederike Kunz, Ronny Loeffler, Claudia Matschegewski, et al. "Regulation of T-Type Calcium Channels in Osteoblasts on Micro-Structured Surface Topography." *Advanced Materials Research* **647** (2013), pp. 476–481. DOI: [10.4028/www.scientific.net/AMR.647.476](https://doi.org/10.4028/www.scientific.net/AMR.647.476).
- [206] Edward Perez-Reyes. "Molecular physiology of low-voltage-activated t-type calcium channels." *Physiological reviews* **83** (2003), pp. 117–161. DOI: [10.1152/physrev.00018.2002](https://doi.org/10.1152/physrev.00018.2002).
- [207] Maria Pangalos, Willem Bintig, Barbara Schlingmann, Frank Feyerabend, et al. "Action potentials in primary osteoblasts and in the MG-63 osteoblast-like cell line." *Journal of bioenergetics and biomembranes* **43** (2011), pp. 311–322. DOI: [10.1007/s10863-011-9354-7](https://doi.org/10.1007/s10863-011-9354-7).
- [208] J. H. Lee, A. N. Daud, L. L. Cribbs, A. E. Lacerda, et al. "Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family." *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19** (1999), pp. 1912–1921. DOI: [10.1523/JNEUROSCI.19-06-01912.1999](https://doi.org/10.1523/JNEUROSCI.19-06-01912.1999).
- [209] S. McLaughlin and M. M. Poo. "The role of electro-osmosis in the electric-field-induced movement of charged macromolecules on the surfaces of cells." *Biophysical journal* **34** (1981), pp. 85–93. DOI: [10.1016/S0006-3495\(81\)84838-2](https://doi.org/10.1016/S0006-3495(81)84838-2).
- [210] Xi Ren, Huanbo Sun, Jie Liu, Xiaowei Guo, et al. "Keratinocyte electrotaxis induced by physiological pulsed direct current electric fields." *Bioelectrochemistry (Amsterdam, Netherlands)* **127** (2019), pp. 113–124. DOI: [10.1016/j.bioelechem.2019.02.001](https://doi.org/10.1016/j.bioelechem.2019.02.001).
- [211] Erik Finkelstein, Winston Chang, P-H Grace Chao, Dorota Gruber, et al. "Roles of microtubules, cell polarity and adhesion in electric-field-mediated motility of 3T3 fibroblasts." *Journal of cell science* **117** (2004), pp. 1533–1545. DOI: [10.1242/jcs.00986](https://doi.org/10.1242/jcs.00986).
- [212] Abijeet Singh Mehta, Pin Ha, Kan Zhu, ShiYu Li, et al. "Physiological electric fields induce directional migration of mammalian cranial neural crest cells." *Developmental biology* **471** (2021), pp. 97–105. DOI: [10.1016/j.ydbio.2020.12.011](https://doi.org/10.1016/j.ydbio.2020.12.011).
- [213] C. A. Erickson and R. Nuccitelli. "Embryonic fibroblast motility and orientation can be influenced by physiological electric fields." *The Journal of cell biology* **98** (1984), pp. 296–307. DOI: [10.1083/jcb.98.1.296](https://doi.org/10.1083/jcb.98.1.296).
- [214] G. L. Sulik, H. K. Soong, P. C. Chang, W. C. Parkinson, et al. "Effects of steady electric fields on human retinal pigment epithelial cell orientation and migration in culture." *Acta ophthalmologica* **70** (1992), pp. 115–122. DOI: [10.1111/j.1755-3768.1992.tb02102.x](https://doi.org/10.1111/j.1755-3768.1992.tb02102.x).

- [215] M. Zhao, C. D. McCaig, A. Agius-Fernandez, J. V. Forrester, et al. "Human corneal epithelial cells reorient and migrate cathodally in a small applied electric field." *Current eye research* **16** (1997), pp. 973–984. DOI: [10.1076/ceyr.16.10.973.9014](https://doi.org/10.1076/ceyr.16.10.973.9014).
- [216] Guillaume Charras and Ewa Paluch. "Blebs lead the way: how to migrate without lamellipodia." *Nature reviews. Molecular cell biology* **9** (2008), pp. 730–736. DOI: [10.1038/nrm2453](https://doi.org/10.1038/nrm2453).
- [217] D. J. Fishkind, L. G. Cao, and Y. L. Wang. "Microinjection of the catalytic fragment of myosin light chain kinase into dividing cells: effects on mitosis and cytokinesis." *The Journal of cell biology* **114** (1991), pp. 967–975. DOI: [10.1083/jcb.114.5.967](https://doi.org/10.1083/jcb.114.5.967).
- [218] M. L. Coleman, E. A. Sahai, M. Yeo, M. Bosch, et al. "Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I." *Nature cell biology* **3** (2001), pp. 339–345. DOI: [10.1038/35070009](https://doi.org/10.1038/35070009).
- [219] Heiko Blaser, Michal Reichman-Fried, Irinka Castanon, Karin Dumstrei, et al. "Migration of zebrafish primordial germ cells: a role for myosin contraction and cytoplasmic flow." *Developmental cell* **11** (2006), pp. 613–627. DOI: [10.1016/j.devcel.2006.09.023](https://doi.org/10.1016/j.devcel.2006.09.023).
- [220] Jan Schick and Erez Raz. "Blebs-Formation, Regulation, Positioning, and Role in Amoeboid Cell Migration." *Frontiers in cell and developmental biology* **10** (2022), p. 926394. DOI: [10.3389/fcell.2022.926394](https://doi.org/10.3389/fcell.2022.926394).
- [221] Benoît Maugis, Jan Brugués, Pierre Nassoy, Nancy Guillen, et al. "Dynamic instability of the intracellular pressure drives bleb-based motility." *Journal of cell science* **123** (2010), pp. 3884–3892. DOI: [10.1242/jcs.065672](https://doi.org/10.1242/jcs.065672).
- [222] Mohammad Goudarzi, Aleix Boquet-Pujadas, Jean-Christophe Olivo-Marin, and Erez Raz. "Fluid dynamics during bleb formation in migrating cells in vivo." *PloS one* **14** (2019), e0212699. DOI: [10.1371/journal.pone.0212699](https://doi.org/10.1371/journal.pone.0212699).
- [223] J. A. Sanchez, J. A. Dani, D. Siemen, and B. Hille. "Slow permeation of organic cations in acetylcholine receptor channels." *The Journal of general physiology* **87** (1986), pp. 985–1001. DOI: [10.1085/jgp.87.6.985](https://doi.org/10.1085/jgp.87.6.985).
- [224] Man Liang, Jiang Tian, Lijun Liu, Sandrine Pierre, et al. "Identification of a pool of non-pumping Na/K-ATPase." *Journal of Biological Chemistry* **282** (2007), pp. 10585–10593. DOI: [10.1074/jbc.M609181200](https://doi.org/10.1074/jbc.M609181200).
- [225] Renquan Ruan, Lili Zou, Sijie Sun, Jing Liu, et al. "Cell blebbing upon addition of cryoprotectants: a self-protection mechanism." *PloS one* **10** (2015), e0125746. DOI: [10.1371/journal.pone.0125746](https://doi.org/10.1371/journal.pone.0125746).
- [226] Maitham A. Khajah, Princy M. Mathew, Nada S. Alam-Eldin, and Yunus A. Luqmani. "Bleb formation is induced by alkaline but not acidic pH in estrogen receptor silenced breast cancer cells." *International journal of oncology* **46** (2015), pp. 1685–1698. DOI: [10.3892/ijo.2015.2884](https://doi.org/10.3892/ijo.2015.2884).

- [227] Yves Jannot, Christian Moyne, and Alain Degiovanni. *Heat transfer 1: Conduction and convection*. Energy series. London and Hoboken, NJ: ISTE and Wiley, 2023. DOI: [10.1002/9781394228102](https://doi.org/10.1002/9781394228102).
- [228] V. A. Kurganov. "HEAT TRANSFER COEFFICIENT." In: *A-to-Z Guide to Thermodynamics, Heat and Mass Transfer, and Fluids Engineering*. Vol. H. Begell-house, 2006. DOI: [10.1615/AtoZ.h.heat_transfer_coefficient](https://doi.org/10.1615/AtoZ.h.heat_transfer_coefficient).
- [229] Joseph L. Roti Roti. "Cellular responses to hyperthermia (40-46 degrees C): cell killing and molecular events." *International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group* **24** (2008), pp. 3–15. DOI: [10.1080/02656730701769841](https://doi.org/10.1080/02656730701769841).
- [230] Carl D. Bortner and John A. Cidlowski. "Ions, the Movement of Water and the Apoptotic Volume Decrease." *Frontiers in cell and developmental biology* **8** (2020), p. 611211. DOI: [10.3389/fcell.2020.611211](https://doi.org/10.3389/fcell.2020.611211).
- [231] Sanaya N. Shroff, Shoshana L. Das, Hua-An Tseng, Jad Noueihed, et al. "Voltage Imaging of Cardiac Cells and Tissue Using the Genetically Encoded Voltage Sensor Archon1." *iScience* **23** (2020), p. 100974. DOI: [10.1016/j.isci.2020.100974](https://doi.org/10.1016/j.isci.2020.100974).
- [232] K. Michel, M. Michaelis, G. Mazzuoli, K. Mueller, et al. "Fast calcium and voltage-sensitive dye imaging in enteric neurones reveal calcium peaks associated with single action potential discharge." *The Journal of physiology* **589** (2011), pp. 5941–5947. DOI: [10.1113/jphysiol.2011.219550](https://doi.org/10.1113/jphysiol.2011.219550).
- [233] J. H. Lee, J. C. Gomora, L. L. Cribbs, and E. Perez-Reyes. "Nickel block of three cloned T-type calcium channels: low concentrations selectively block $\alpha 1H$." *Biophysical journal* **77** (1999), pp. 3034–3042. DOI: [10.1016/S0006-3495\(99\)77134-1](https://doi.org/10.1016/S0006-3495(99)77134-1).
- [234] Wonjoo Choe, Richard B. Messinger, Emily Leach, Veit-Simon Eckle, et al. "TTA-P2 is a potent and selective blocker of T-type calcium channels in rat sensory neurons and a novel antinociceptive agent." *Molecular pharmacology* **80** (2011), pp. 900–910. DOI: [10.1124/mol.111.073205](https://doi.org/10.1124/mol.111.073205).
- [235] Janelle R. Latham, Sriyani Pathirathna, Miljen M. Jagodic, Won Joo Choe, et al. "Selective T-type calcium channel blockade alleviates hyperalgesia in ob/ob mice." *Diabetes* **58** (2009), pp. 2656–2665. DOI: [10.2337/db08-1763](https://doi.org/10.2337/db08-1763).
- [236] Alexander Mietke, Oliver Otto, Salvatore Girardo, Philipp Rosendahl, et al. "Extracting Cell Stiffness from Real-Time Deformability Cytometry: Theory and Experiment." *Biophysical journal* **109** (2015), pp. 2023–2036. DOI: [10.1016/j.bpj.2015.09.006](https://doi.org/10.1016/j.bpj.2015.09.006).
- [237] K. Yonemori, S. Matsunaga, Y. Ishidou, S. Maeda, et al. "Early effects of electrical stimulation on osteogenesis." *Bone* **19** (1996), pp. 173–180. DOI: [10.1016/8756-3282\(96\)00169-X](https://doi.org/10.1016/8756-3282(96)00169-X).

- [238] T. Albrektsson and C. Johansson. "Osteoinduction, osteoconduction and osseointegration." *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society* **10 Suppl 2** (2001), S96–101. DOI: [10.1007/s005860100282](https://doi.org/10.1007/s005860100282).
- [239] *DMEM formulation*. URL: <https://www.thermofisher.com/de/de/home/technical-resources/media-formulation.46.html> (visited on 04/18/2024).
- [240] A. B. López-Oyama, R. A. Silva-Molina, J. Ruíz-García, R. Gámez-Corrales, et al. "Structure, electronic properties, and aggregation behavior of hydroxylated carbon nanotubes." *The Journal of chemical physics* **141** (2014), p. 174703. DOI: [10.1063/1.4900546](https://doi.org/10.1063/1.4900546).
- [241] Werner Kern. "The Evolution of Silicon Wafer Cleaning Technology." *Journal of The Electrochemical Society* **137** (1990), pp. 1887–1892. DOI: [10.1149/1.2086825](https://doi.org/10.1149/1.2086825).
- [242] Gero Decher, Michel Eckle, Johannes Schmitt, and Bernd Struth. "Layer-by-layer assembled multicomposite films." *Current Opinion in Colloid & Interface Science* **3** (1998), pp. 32–39. DOI: [10.1016/S1359-0294\(98\)80039-3](https://doi.org/10.1016/S1359-0294(98)80039-3).
- [243] *PAMAM gen.1 structural formula*. URL: <https://www.sigmaaldrich.com/DE/de/product/aldrich/412384> (visited on 05/10/2023).
- [244] *G1-TMP-COOH structural formula*. URL: <https://www.polymerfactory.com/product-page/bis-mpa-carboxyl-dendrimer-generation-1-tmp-core> (visited on 05/10/2023).
- [245] *PDADMA structural formula*. URL: <https://www.sigmaaldrich.com/DE/de/product/aldrich/409022> (visited on 05/10/2023).
- [246] *PSS structural formula*. URL: <https://www.sigmaaldrich.com/DE/de/product/aldrich/243051> (visited on 05/10/2023).
- [247] D. Woermann. "Electrochemical transport properties of a cone-shaped nanopore: high and low electrical conductivity states depending on the sign of an applied electrical potential difference." *Physical Chemistry Chemical Physics* **5** (2003), pp. 1853–1858. DOI: [10.1039/b301021j](https://doi.org/10.1039/b301021j).
- [248] *Transmission GFP-Filterset*. URL: <https://ahf.de/produkte/spektralanalytik-photonik/optische-filter/filtersets-nach-anwendung/epi-fluoreszenz-weisslicht-led/egfp-hc-filterset-breitband/F36-527> (visited on 10/11/2023).