
4 Nanoparticle-loaded bioactive hydrogels

Abstract: Laser-induced sub 100 nm structures were prepared on silicon, thoroughly characterized, and the process of their formation was elucidated. They were used as substrates for a controlled deposition of calcium phosphate nanoparticles by electrophoretic deposition. Calcium phosphate nanoparticles are used as carriers for biomolecules such as nucleic acid for transfection or gene silencing in the context of gene therapy. It is important to apply only a defined quantity of nanoparticles and nucleic acids to a given cell culture or tissue. A defined number of nanoparticles were deposited onto a ripple structured silicon surface by electrophoretic deposition. Alginate beads were then rolled on this surface allowing the nanoparticles to adsorb onto the surface of the alginate bead. This resulted in alginate beads with a defined quantity of particles and biomolecules on their surface. The effect of calcium phosphate nanoparticle-coated alginate beads on cells was studied by the hanging drop method.

4.1 Introduction

There is great interest in the genetic manipulation of living cells by the administration of nucleic acids (DNA or RNA) in experimental medicine, e.g. for the integration of fluorescent reporter genes [1]. It can additionally enable the induction of pluripotency in patient-derived cells using epigenetic reprogramming methods, opening up new possibilities for personalized medicine and disease-specific drug discovery. While these artificially generated cells do not exist in the human body, comparable to embryonic stem cells, human induced pluripotent stem cells (hiPSC) have the ability to differentiate into every cell type of the human body and to renew themselves by mitotic cell division [2].

Due to the fact that nucleic acids alone are not able to enter a cell, suitable carriers are needed. Currently, a retrovirus-mediated gene transduction is often used, but non-viral systems are becoming increasingly important [3]. Nanoparticles can be used for the transport of nucleic acids because they can easily be taken up by cells and no further transport vectors are necessary [4]. In order to standardize this process, the exposure of cells to a defined number of nanoparticles is necessary. Note that with dispersed nanoparticles, it is difficult to clearly define the dose of nanoparticles in direct cell contact. As most cell types, including fibroblasts and hiPSCs, grow in adherent culture, a homogeneous immobilization of nanoparticles on a surface is one possible method of controlling nanoparticle exposure [5]. The cultivation of a defined num-
ber of cells on specified growth areas homogeneously loaded with DNA-containing nanoparticles can lead to a reproducible gene transfection [5].

Different inorganic nanoparticles have been used for the transport of nucleic acids into cells [6, 7]. In comparison to organic particles, inorganic particles are often easier to synthesize, less toxic, not sensitive to microbial degradation and easier to store. In this case, we used calcium phosphate nanoparticles as drug carriers [8, 9]. Calcium phosphate is a biomineral found in mammalian bone and teeth, leading to high biocompatibility [10]. We used the cationic polymer poly(ethyleneimine) (PEI) to stabilize the calcium phosphate nanoparticles. This led to a positive zeta potential and colloidal stability. Note that the particles need to carry a surface charge for electrophoretic deposition. Electrophoretic deposition was used to coat silicon, both smooth and ripple-nanostructured [11, 12]. In general, the coating of implants is an interesting area in medicine to increase their bioactivity [13]. These deposited nanoparticles were then transferred to alginate beads by rolling the beads over the nanoparticle-coated surface. In earlier work we have shown that laser-structured surfaces are ideally suited for controlled deposition of calcium phosphate nanoparticles [12] and that cells show distinct behavior towards such coated surfaces [11].

Alginate, a hydrogel derived from marine brown algae, is a highly suitable matrix for immobilization of nanoparticles. Alginate is often used for cell encapsulation and is easy to modify according to specific cell characteristics and applications [14]. Ultra-pure alginates can be used for cell-based assays, due to the low risk of contamination (in the case of in vitro application), inflammation and immune response (in case of in vivo application). Gel formation from fluid alginates to gelled alginate hydrogels takes place by ionotropic crosslinking with alkaline-earth metal cations (e.g. Ca^{2+}, Ba^{2+}) under gentle conditions (neutral pH, room temperature). This allows the encapsulation of cells into alginate without significantly damaging the cells [15]. Several methods for dispensing [16] and modification have been published in the last decades ranging between coatings [17], physical modifications [18], and covalent coupling of bioactive molecules [19].

A method of studying the interaction of cells and dispersed particles is the hanging drop cultivation method [20]. Here, cells are cultivated in a small drop (20–40 μL) of culture medium which is placed in the inner lid of a cell culture dish. Due to gravitation, all cells in the droplet are forced to form one homogeneous aggregate in the lower part of the droplet. In combination with alginate microcarriers loaded with nanoparticles containing nucleic acids, this method is highly suitable for the transfection of cells due to the enforced cell-surface contact within the drop. The defined and homogeneous spatial arrangement of nanoparticles with transcription factors for cell reprogramming on the growth surface of cells will increase reproducibility and standardization. Using the advantages of automation, this cell culture method additionally permits almost complete control of the microenvironment as well as a significantly increased reproducibility of the results by volume miniaturization and parallelization.
4.2 Material and methods

4.2.1 Laser-induced sub 100 nm structures

Periodic pattern formation on surfaces under laser irradiation in a certain fluency range has long been known and is termed laser-induced periodic surface structures (LIPSS) [21, 22]. Depending on the illumination parameters and the material properties, a variety of topologies and scales can be realized. A basic classification of these structures is made by the spatial frequency of the pattern in relation to the wavelength of the generating laser light: patterns with structural dimensions comparable to the laser wavelength are called low spatial frequency LIPSS (LSFL) [23, 24], those with sub-diffractive structural dimensions are accordingly called high spatial frequency LIPSS (HSFL) [23, 25, 26].

By the application of appropriate irradiation and material parameters, HSFLs can have periodicities of around 100 nm and below. This is of the order of typical diameters of nanoparticles for biomedical and pharmaceutic use [27–29]. Hence it is promising to study the potential of HSFL-patterned surfaces for biomedical nanoparticle arrangement and transfer. To this end, extended areas of HSFL patterns are needed in contrast to the single focal spot structures widely produced for mechanistic elucidation of the structure generation [27]. This was achieved by scanning the laser focus along the substrate surface. In order to have better process control during patterning, low energy ultra-short pulses (nJ) were utilized at a high repetition rate (MHz) in contrast to the usual high energy pulses at low repetition rate (Hz–kHz) used by amplified laser systems. A compact ultra-fast Ti:sapphire tunable laser system (80 MHz, \( E_{\text{max}} \approx 40 \text{nJ/pulse} \), \( \tau = 140 \text{fs (FWHM)} \), \( \lambda = 800 \text{nm} \)) coupled to a laser workstation specially designed and developed for accurate micro- and nanoprocessing was used to generate these periodical ripples on large areas. The surface patterns were created by pulse trains of some 150 000 pulses in a quasi-continuous fashion in contrast to mostly single pulse evoked structures known from the literature [23, 26]. Since a quasi-continuous evolution of HSFL in spatial contact to already formed HSFL (due to scanning) may have very specific mechanisms of formation, this was the subject of extensive studies [30–33]. The results of these studies now help to understand, control and predict periodicities and orientation of our large area HSFL upon irradiation and material parameters.

In order to achieve a defined orientation and surface density, the calcium phosphate nanoparticles were deposited onto reusable stamps, consisting of silicon wafers patterned with HSFL. These high spatial frequency ripples were generated in air on standard one-side-polished, boron-doped p-type 525 μm thick Si wafers with a crystallographic orientation of 100. The low energy femtosecond laser pulses were focused by a 20× focusing objective with a relatively high numerical aperture (NA) of 0.75 to reach the fluency threshold of HSFL generation. The focus diameter was about 1.3 μm at the surface of the sample. More details on the experimental setup can be found...
in [34]. A peculiarity of laterally extended, pulse train-generated HSFLs is the necessity of removing expelled material via an adapted etching step. For silicon, this was accomplished by treatment with aqueous ammonium fluoride solution.

4.2.2 Production of alginate beads and hydrogels

Alginate beads were produced using an air-jet droplet generator (Fig 4.1) as described in [15], [35] and [14]. Liquid Na-alginate solution or Na-alginate/cell suspensions were supplied in 1mL syringes. The solution was transported with constant feed through a disposable nozzle (Fig. 4.1(b) and (c)), producing homogeneous droplets using an
The geometry of the disposable nozzle was optimized for the production of alginate beads with a diameter of 500 μm. The droplet generator allowed adjustment of the bead size by increasing air flow. After droplet break-off and during freefall, alginate droplets were internally gelled by bombardment with BaCl₂ crystals. External gelling of alginate usually takes place with a 20 mM BaCl₂ iso-osmolar solution, producing perfectly round alginate beads (Fig. 4.1(d)). Alginate beads were usually gelled for 15 min at room temperature and intensively washed in iso-osmolar NaCl solution to remove excess Ba²⁺ ions. Due to low mechanical stress and physiological conditions of all media used, excellent cell viability was achieved [36].

4.2.3 Synthesis of calcium phosphate nanoparticles

4.2.3.1 Calcium phosphate nanoparticles, dispersed in ethanol

The calcium phosphate nanoparticles in ethanol were prepared by precipitation in the presence of poly(ethylenimine) (PEI) [5]. Aqueous solutions of calcium-L-lactate (c = 18 mmol L⁻¹) and di-ammonium hydrogen phosphate (c = 10.8 mmol L⁻¹) were pumped into a beaker by a peristaltic pump. A PEI solution (c = 2 g L⁻¹) was added by a second peristaltic pump. The volume flow rate was adjusted to a ratio of 1:1:2 (Ca²⁺ : PO₄⁻ : PEI). Labelled PEI was used for the fluorescently labelled particles, fluorescein or tetramethylrhodamine isothiocyanate (FITC or TRITC, respectively). The calcium phosphate nanoparticles were isolated by centrifugation for 1 h at 1500 g, washed with water and freeze-dried. After freeze-drying, the particles were redispersed in ethanol by ultrasonication to achieve a stable colloidal dispersion.

4.2.3.2 Calcium phosphate nanoparticles, dispersed in water

The water-dispersed calcium phosphate nanoparticles were prepared by precipitation. Aqueous solutions of calcium-L-lactate (c = 6 mmol L⁻¹) and di-ammonium hydrogen phosphate (c = 3.6 mmol L⁻¹) were pumped together into a 5 cm tube with a volume flow rate of 25 mL min⁻¹. After nucleation in the tube, PEI (c = 2 g L⁻¹) was added with a flow rate of 12.5 mL min⁻¹. After stabilization the particles were isolated by centrifugation (20 min, 1100 g) and redispersed in water by ultrasonication (50 % amplitude, 0.8 pulse). Different amounts of EGFP-DNA (50 μg mL⁻¹, 25 μg mL⁻¹ and 15 μg mL⁻¹) were added to the positively charged calcium phosphate-PEI nanoparticles [37]. A stable colloidal dispersion was obtained.

4.2.3.3 Calcium phosphate nanorods, dispersed in water

PEI-functionalized calcium phosphate nanorods were synthesized by adding a 6 % Ostim®-suspension to 6 mL water and 3 mL PEI-solution (c = 10 g L⁻¹). Ostim® is a commercially available bone substitution material and consists of water-dispersed
nanoscopic hydroxyapatite, \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \) [38]. These calcium phosphate nano-rods can be functionalized by nucleic acids and used for transfection and gene silencing [38]. The mixture was dispersed by ultrasonic treatment. The particles were then isolated by centrifugation for 30 min at 4700 g and redispersed in water by ultrasonication. A stable colloidal dispersion of calcium phosphate nanorods was obtained with a concentration of 6 mg mL\(^{-1}\) calcium phosphate. For electrophoretic deposition the dispersion was diluted to 1 mg mL\(^{-1}\) calcium phosphate.

All nanoparticles were characterized by dynamic light scattering (DLS), zeta potential measurements and scanning electron microscopy (SEM). Dynamic light scattering and zeta potential measurements were carried out with the Zetasizer Nano ZS instrument from Malvern. The SEM images were recorded with an ESEM Quanta 400 FEG instrument from FEI.

4.2.4 Electrophoretic deposition

Electrophoretic deposition was carried out with ethanol and water as dispersion media. The medium is important for the choice of voltage and deposition time. The calcium phosphate nanoparticles in ethanol were deposited at 50 V DC. The deposition time varied between 30 s and 2 min. The water-dispersed calcium phosphate nanoparticles and nanorods were deposited at 2 V DC. The deposition time was between 30 min and 5 h. Voltage is limited in water to avoid the electrolysis of water. All electrophoretic depositions were carried out at room temperature. Silicon, indium-doped tin oxide (ITO) and silicon with ripple structures were used as substrates. All substrates were dried in air after deposition. The apparatus for electrophoretic deposition is shown in Fig. 4.2.

4.3 Results

4.3.1 Characterization of laser-induced sub 100 nm structures

Investigations were performed to characterize the HSFL structures in terms of morphology, periodicity, depth profile, aspect ratio and the influence of varying parameters such as energy per pulse, number of applied pulses (scan speed), wavelength, polarization, NA of the focusing optics and incident angle of the laser beam. For these purposes, the resulting irradiated surface morphologies were examined with scanning electronic microscopy (SEM). Fine cross-sectional cuts of structured fields recovered with HSFL were performed by focused ion beam (FIB) milling to analyse the depth profiles. An example of HSFL generated in Si is shown in Fig. 4.3. The periodicity of the ripples was measured in two ways: Classically by directly measuring the distances between two rims at several locations on the SEM image with the help of image analysis
Fig. 4.2: Setup for the electrophoretic deposition (EPD) of nanoparticles on structured surfaces.

Fig. 4.3: (a) SEM image of HSFL generated in Si at 700 nm at an energy per pulse of 3 nJ and at a scan speed of 1 mm s$^{-1}$. (b) Details of the HSFL with direct measurements. (c) 2D-FT of image (b). The double arrow represents the polarization of the laser irradiation.
software as seen in the magnified image of Fig. 4.3 (b). The average periodicity based on limited measurements was $\Lambda_{\text{average}} \approx 111 \text{ nm} \pm 10 \text{ nm}$. This method is relatively accurate but limited to a very small area. The other method which is not limited to the size of the area and directly gives the quantitative averaged result consists of taking the discrete two-dimensional Fourier transformation (2D-FT) of the whole SEM image of Fig. 4.3 (b), as shown in Fig. 4.3 (c). The quantitative periodicity obtained by 2D-FT was $\Lambda \approx 119 \text{ nm}$.

The FIB cross-section procedure includes firstly the deposition of a protective platinum layer on the surface, and secondly the successive removal of the silicon layer in the depth, using gallium ions with a continuously decreasing energy down to 30 keV for ultimate fine removal and polishing. An example of a cross-section ($15 \times 5 \mu\text{m}^2$) performed on the silicon-surface covered with HSFL generated at 800 nm, at an energy of 4.5 nJ/pulse and a scan speed of 1 mm s$^{-1}$ is shown in Fig. 4.4. Comparatively homogeneous ripples in morphology, periodicity and depth with a repeatable sinusoidal-like profile can be observed in Fig. 4.4 (b). The depth of the crater ($\sim 135 \text{ nm}$) is close to the distance between two rims ($\sim 125 \text{ nm}$). This leads to an aspect ratio of about $1:1$ which was deduced from the magnified image of a ripple in Fig. 4.4 (c).

![Fig. 4.4: SEM images of the crosssectional profile of HSFL in Si. (a) FIB section overview. (b) High magnification of HSFL profile. (c) Crosssectional profile overview of the HSFL.](image-url)
The influence of the various parameters listed above was systematically studied by SEM and FIB. It was shown that HSFL initiation, formation, and arrangement in silicon under ultra-short pulses with low energy at high repetition rates combine the structural modification of the surface, initiated by heat accumulation of successive pulses with second harmonic generation (SHG). The periodicity of HSFL is wavelength-dependent and increases with a wavelength between 700 nm and 950 nm [25, 26]. At a first approximation, the periodicity of the HSFL tends to follow the equation \( \Lambda = \lambda / 2n_\lambda \) satisfactorily [39]. A revisited and refined model of the SHG ripple spacing theory taking the modified femtosecond laser excited silicon refractive index \( n^*_\lambda \) as a function of the wavelength for Si into account was proposed. It matched the experimental data very well [32, 39]. These results are summarized in Fig. 4.5.

![Fig. 4.5: HSFL periodicity as a function of the wavelength. Comparison of experimental data and theoretical models of HSFL spacing.](image)

The orientation of the HSFL is dependent on the direction of polarization and perpendicular to this, but no influence of the direction of polarization was observed on periodicity, profile and aspect ratio of the crosssections of HSFL [24, 30]. The periodicity, morphology, depth and aspect ratio of the HSFL are quite independent from the energy, the number of pulses applied, the focusing geometry and the incidence angle of the laser beam [34, 40].
4.3.2 Electrophoretic deposition of nanoparticles

Unstructured silicon wafers were coated with TRITC- or FITC-PEI-labelled fluorescing spherical cationic calcium phosphate nanoparticles. The nanoparticles were redispersed in ethanol, and electrophoretic deposition was carried out at 50 V DC for 1 min. After deposition, the surface showed a multilayer of spherical nanoparticles (Fig. 4.6(a)). These coated wafers were subsequently used to coat the alginate beads. Anionic DNA-functionalized spherical nanoparticles in water were also used for electrophoretic deposition. The surface showed a nanoparticle monolayer after electrophoretic deposition at 2 V for 30 min (Fig. 4.6(b)). The electrically conducting glass indium-doped tin oxide (ITO) was coated with cationic PEI-stabilized calcium phosphate nanorods in water. After deposition for 5 h at 2 V, the surface showed a nanorod multilayer with about 17 layers (Fig. 4.6(c)).

The dose of nanoparticles both on the silicon substrate and on the alginate beads is important to judge the biological effect. A length of 159 nm and a diameter of 35 nm of the calcium phosphate nanorods were assumed for the calculation of the number of nanorod layers on the substrate [37]. The volume was calculated assuming a cylindrical shape of the particles with $1.33 \times 10^{-22} \text{ m}^3$ per particle. The mass of one nanorod was calculated with the density of hydroxyapatite (3160 kg m$^{-3}$), giving $4.20 \times 10^{-19} \text{ kg}$ per particle.

The amount of calcium was measured by elemental analysis (dissolution of the calcium phosphate nanorod layer with nitric acid, followed by atomic absorption spectroscopic determination of the calcium concentration in the acid). A mass of 0.0565 mg calcium per cm$^2$ was determined. This corresponds to a mass of 0.14 mg hydroxyapatite, assuming the stoichiometry of hydroxyapatite, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$. Consequently, $3.33 \times 10^{11}$ nanorods were deposited on 1 cm$^2$ of the substrate.

The layer thickness of the calcium phosphate nanorods was 599 nm assuming the spheres were closepacked (74 vol%) as approximation. It was assumed that the nanorods were lying on the long edge of the substrate on calculation of the number of layers.

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\delta_{\text{layers}} = \frac{N \cdot V_{\text{NR}}}{A \cdot 0.74} = \frac{3.33 \times 10^{11} \cdot 1.33 \times 10^{-22} \text{ m}^3}{10^{-4} \text{ m}^2 \cdot 0.74} = 5.99 \times 10^{-7} \text{ m} = 599 \text{ nm}
\]

\[
N_{\text{layers}} = \frac{\delta_{\text{layers}}}{d} = \frac{599 \text{ nm}}{35 \text{ nm}} = 17
\]

$N$: number of nanoparticles on the substrate

$A$: area of the substrate ($10^{-4}$ m$^2$)

$\delta_{\text{layers}}$: layer thickness assuming close-packed spheres

$d$: one nanorod thick

$N_{\text{layers}}$: number of layers on the substrate

This gives 17 nanorod layers, with an estimated error of ± 20%.
4.3.3 Combination of bead production and coating of dispersed calcium phosphate nanoparticles

Nanoparticle-coated alginate beads can be realized in two different ways. On the one hand, the gelled alginate beads can be coated with nanoparticles by incubation in a nanoparticle dispersion. On the other hand, alginate bead production and coating with nanoparticles can be combined during bead production as described above.

The nanoparticles for deposition were supplied in the crosslinking bath. The contact of liquid alginate drops with the crosslinking bath containing nanoparticles affected the accumulation of calcium phosphate nanoparticles on the bead surface and, at the same time, gelation of alginate resulted in stable alginate beads. Bead preparation using different crosslinking baths containing 20 mM BaCl$_2$, 10 mM BaCl$_2$ and 0.15 mg mL$^{-1}$ calcium phosphate nanoparticles as well as a pure calcium phosphate...
nanoparticle dispersion with 0.1 mg mL\(^{-1}\), 0.2 mg mL\(^{-1}\) and 0.3 mg mL\(^{-1}\) (mass refers to calcium phosphate) showed that nanoparticle dispersions alone are not sufficient for the production of stable alginate beads, underscoring the need for other procedures.

PEI-TRITC-functionalized calcium phosphate nanoparticles were used for better analysis of the loading procedure from dispersion. A pure nanoparticle dispersion with 0.1 mg mL\(^{-1}\) and 0.2 mg mL\(^{-1}\) alginate tended to form large agglomerates on the bead surface (Fig 4.7 (a) and (b)). At a concentration of 0.3 mg mL\(^{-1}\), partial bead formation, and large agglomerates of alginate beads and unshaped hydrogel were observed (Fig 4.7 (c) and (d)). In contrast, a mixture of calcium phosphate nanoparticle dispersion with BaCl\(_2\) crosslinking solution (final concentration 0.15 mg mL\(^{-1}\) calcium phosphate nanoparticles in 10 mM BaCl\(_2\)) resulted in stable alginate beads (Fig. 4.7 (e)), comparable to beads produced with standard crosslinking solution (Fig. 4.7 (f)). Calcium phosphate nanoparticle-loaded alginate beads can be distinguished by increased surface contrast and are also generally darker in comparison to standard alginate beads.

The stability of standard and calcium phosphate nanoparticle-loaded alginate beads was monitored over 4 days and analyzed with respect to their diameter. Standard alginate beads at day 0 and day 4 and calcium phosphate-loaded beads at day 0 showed comparable equivalent diameters of about 600 μm (Fig. 4.7 (g)). Alginate beads loaded with calcium phosphate nanoparticles showed a slight decrease in diameter of approximately 50 μm after storage for 4 days. Differences were significant between standard alginate beads and calcium phosphate-loaded beads on day 0 as well as between calcium phosphate-loaded alginate beads on day 0 and day 4. The differences between standard and calcium phosphate-loaded beads can be explained by additional particles in the crosslinking solution, influencing bead shape, whereas differences of calcium phosphate-loaded beads on day 0 and day 4 are probably caused by further crosslinking by calcium and thus shrinking of alginate beads over time. Nevertheless, calcium phosphate nanoparticle-loaded alginate beads were intact after 4 days and showed no signs of disintegration. The reproducible shape, as well as the stability over several days, gave the basis for integrating these beads into the cell-based hanging drop cultivation assay. Future studies must also focus on long-term stability with additional mechanical stress to evaluate the applicability of calcium phosphate nanoparticle-loaded alginate beads in stirred bioreactor systems.

### 4.3.4 Coating of alginate beads from solution

The alginate beads were coated with PEI-TRITC-functionalized calcium phosphate nanoparticles. Nanoparticle dispersion was used after precipitation without purification. 1.5 mg alginate beads were added to 30 mL nanoparticle dispersion and the dispersion was stirred for 1 h. The beads were isolated from the dispersion by sedi-
Fig. 4.7: Alginate beads which were modified with PEI-functionalized calcium phosphate nanoparticles (labelled TRITC) from solution. (a)–(d) Alginate beads were modified with pure calcium phosphate nanoparticle dispersion with concentrations of (a) 0.1 mg mL\(^{-1}\), (b) 0.2 mg mL\(^{-1}\), (c) and (d) 0.3 mg mL\(^{-1}\). (e) Alginate beads with 0.15 mg mL\(^{-1}\) nanoparticles in 10 mM BaCl\(_2\). Stable modified alginate beads could only be produced reproducibly with a mixture of calcium phosphate and BaCl\(_2\). (f) Unmodified native alginate beads. (g) Dimensional properties of alginate beads quantified by equivalent diameter after day 0 and day 4. Data are presented as mean ± standard deviation (SD, \(n = 3\)), (*) indicates statistical significance (\(p < 0.05\), t-test). Scale bar: 500 μm.

mentation and subsequent washing with water. The coated alginate beads are shown in Fig. 4.8(a). The calcium phosphate nanoparticles (red) formed a homogeneous coating on the beads. However, the alginate beads lost their round shape after coating. This may be due to free calcium ions in the dispersion.
Experiments were carried out with the purified nanoparticle dispersion to check whether the calcium ions have an effect on bead shape. The experiments were performed as described above. The beads were washed with water after 1 h of stirring. Figure 4.8 (b) shows the coated beads. Notably, the beads did not lose their shape during coating.

The calcium concentration of the beads was measured with elemental analysis (atomic absorption spectroscopy of the calcium concentration; computation according to the example given above). With these results, it was possible to estimate the number of calcium phosphate nanoparticles from $10^{10}$ to $10^{11}$ on each alginate bead.

![Fluorescence microscopic images of alginate beads](image)

**Fig. 4.8:** Fluorescence microscopic images of alginate beads (a) with a coating of PEI-TRITC-functionalized calcium phosphate nanoparticles with free Ca$^{2+}$ in dispersion; (b) with a coating of calcium phosphate nanoparticles without free Ca$^{2+}$ in dispersion; (c), (d) with a coating of calcium phosphate nanoparticles. (c) For this coating, the alginate beads were rolled over an electrophoretically coated surface with PEI-TRITC-functionalized calcium phosphate nanoparticles. (d) For this coating, the alginate beads were sequentially rolled over electrophoretically coated surfaces with PEI-FITC- and PEI-TRITC-functionalized calcium phosphate nanoparticles.
4.3.5 Coating from substrates

Unstructured silicon substrates were coated with calcium phosphate nanoparticles by electrophoretic deposition. A multilayer with fluorescent calcium phosphate nanoparticles was used for the coating. The first coating was carried out with PEI-FITC-functionalized alginate beads and PEI-TRITC-functionalized calcium phosphate nanoparticles. PEI-TRITC-functionalized calcium phosphate nanoparticles were deposited on a silicon surface with a layer thickness of about 400 nm. The alginate beads were then rolled over the surface. The coated alginate beads are shown in Fig. 4.8 (c). The surface coating was clearly inhomogeneous and patchy. The particles were agglomerated (red) on the surface and there were gaps with no particles. However, the particles adhered to the bead surface very well.

The second coating procedure was carried out with two different colored fluorescent calcium phosphate nanoparticles. For this coating, two different silicon wafers were coated, each with one kind of fluorescent nanoparticles (PEI-TRITC- or PEI-FITC-functionalized calcium phosphate nanoparticles). The alginate beads were rolled over the first surface and then over the second surface. The first layer on the alginate beads consisted of PEI-TRITC-functionalized calcium phosphate nanoparticles (red). Some free patches were then coated with PEI-FITC-functionalized calcium phosphate nanoparticles (green). Figure 4.8 (d) shows alginate beads which were coated with two kinds of fluorescent calcium phosphate nanoparticles in the way described.

4.3.6 Cell reactions to calcium phosphate nanoparticle-coated alginate membranes

Alginate membranes coated with calcium phosphate nanoparticles were used to study the effects of the physicochemical surface properties and thus the changed cellular behaviour. Native and calcium phosphate-coated alginate membranes and a tissue culture-treated polystyrene surface were inoculated with L929 fibroblasts and monitored over 24 hours in automated microscopes (Biostation IM, Nikon). As an indicator of the changed surface properties of alginate introduced by coating with calcium phosphate nanoparticles, the attachment of fibroblasts was quantified after 24 h.

Visual inspection of the cells showed cells attached to standard tissue culture polystyrene surface (TCPS; Fig. 4.9 (a)) and no fibroblasts attached to native alginate hydrogel surfaces (Fig. 4.9 (b)). Cells on native alginate hydrogels tended to agglomerate due to the absence of integrin binding sites. In contrast, fibroblasts were able to attach and spread on calcium phosphate nanoparticle-loaded alginate hydrogel surfaces (Fig. 4.9 (c)). Compared to cells attached to rigid TCPS, cells on nanoparticle-coated alginate hydrogel surfaces appeared to be less flat and spread due to different substrate stiffness. Overall, 94 % ± 2 % cells were attached to the TCPS surface after 24 h, whereas 76 % ± 21 % cells were attached to calcium phosphate nanoparticle-coated alginate hydrogels. Only 2 % ± 2 % of the cells were attached to native alginate
hydrogel surfaces (Fig. 4.9 (d)). These data lead to the conclusion that calcium phosphate nanoparticles, stabilized with TRITC-PEI, trigger an integrin-mediated adhesion of anchorage-dependent cells like fibroblasts. Positive surface charges introduced by PEI as well as probably an increased roughness of the hydrogel surface by deposited nanoparticles further enhanced the cytocompatibility for cells like fibroblasts. Future studies should focus on the modification of nanoparticles with extracellular matrix (ECM) proteins like collagen or laminin to engineer more specific cellular environments for multipotent or pluripotent stem cells.

**Fig. 4.9:** Reactions of L929 fibroblasts, cultured on different surfaces. (a) Fibroblasts attached to rigid tissue culture-treated polystyrene surface. (b) Fibroblasts attached to native Ba-alginate membranes forming multicellular agglomerates indicating low cell-matrix interactions. (c) Fibroblasts attached to a nanoparticle-coated Ba-alginate membrane. (d) Overall attachment rate of L929 fibroblasts on the surfaces shown in (a)–(c). The modification of native Ba-alginate significantly increased the attachment rate, presented as average with SD ($n = 3$ independent experiments). The highest attachment rate was observed on the TCPS surface. The scale bars in (a), (b) and (c) indicate 200 μm.
4.3.7 Robotic coating of alginate beads with calcium phosphate nanoparticles from substrates

The coating from nanostructured, two-dimensional templates requires an adjustable robotic stage for moving and loading alginate beads at the same time. On the basis of a commercially available xyz-table (Nano-Plotter, GeSiM mbH, Grosserkmannsdorf, Germany) and its passive tip holder, we mounted an aluminum endplate for collection and moving of alginate beads to be coated (Fig. 4.10 (a)). Alginate beads interacted with the aluminum endplate due to a thin layer of liquid around the beads (Fig. 4.10 (b)). To load the alginate beads with calcium phosphate nanoparticles, the endplate with adhered beads was contacted with the surface and moved in the x- and y-directions in order to roll the beads over the deposited nanoparticles (Fig. 4.10 (c)). A rectangular moving pattern was implemented where the head was moved for 3 mm in the x- and y-directions. The moving pattern was repeated four times. Unloaded native alginate beads (Fig. 4.10 (d)) were coated with nanoparticles by this procedure. Figure 4.10 (e) shows calcium phosphate nanoparticle-coated alginate beads after the robotic loading procedure was performed, as indicated by the red fluorescence of TRITC. Figure 4.10 (f) illustrates alginate beads being loaded with calcium phosphate nanoparticles by intensity histograms of fluorescence channels before and after the loading procedure. Small deformations observed on alginate beads after the loading procedure had to be overcome, e.g. by spring-loaded surfaces. The successful transfer of nanoparticles using this prototype gave the basis for further studies with the focus on electrophoretically deposited nanoparticles on structured surfaces as shown in Fig. 4.3 (a).

4.3.8 Hanging drop technology with calcium phosphate nanoparticle-coated alginate beads

After alginate beads had been successfully loaded with nanoparticles as shown in Fig. 4.7 and the first promising results with respect to the increased cytocompatibility of alginate hydrogels (Fig. 4.9) were achieved, calcium phosphate nanoparticle-coated alginate beads were used as microcarriers for human mesenchymal stem cells (hMSCs) in the hanging drop cultivation technique. hMSCs (PromoCell GmbH, Heidelberg, Germany) were cultivated in hanging drop cultivation substrates in volumes of 40 μL. A schematic drawing of the hanging drop principle is shown in Fig. 4.10 (g). Microcarriers and cells cultivated in hanging drops accumulated in the drop meniscus and were allowed to interact by cell-cell or cell-matrix interactions. In our experiments, microcarriers, native alginate beads, calcium phosphate nanoparticle-coated alginate beads (both produced as described in Section 4.3) were cultivated with 4000 cells per droplet (40 μL) in commercially available hanging drop plates (GravityPLUS™, InSphero AG, Schlieren, Switzerland). Cells and microcarriers were harvested after
Fig. 4.10: (a)–(f) Prototype for the coating of alginate beads with nanoparticles from surfaces. (a) Holder for the coating of alginate beads from two-dimensional surfaces. (b) Tool with sticky alginate beads before (inset) and after (large image) coating. (c) Two-dimensional coating template, loaded with calcium phosphate nanoparticles, after the coating procedure. The coating area is clearly identifiable (red arrows indicate moving direction of xy robot). (d) Native alginate beads before the coating procedure: bright field image (inset) and fluorescence image (large image). (e) Modified alginate beads after coating procedure: bright field image (inset) and fluorescence image (large image). (f) Histograms of the red channel from fluorescence images in (d) and (e). The increased intensity of the red channel shows the loading of alginate beads with calcium phosphate nanoparticles (TRITC modified). Scale bars in (d) and (e): 200 μm. (g)–(i) Hanging drop technology. (g) Schematic drawing of the hanging drop principle. Small volumes of medium with cells and microcarrier are placed on compatible surfaces for droplet formation and cell cultivation. Cells and microcarrier were located in the droplet’s meniscus. (h) hMSCs after cultivation in HD with native alginate beads. (i) hMSCs after cultivation in HD with calcium phosphate nanoparticle modified alginate beads. (h), (i): Image 1: bright field microscopic; Image 2: fluorescence image (triple band filter). Scale bars: 500 μm.
5 days, stained with fluorescein diacetate and ethidium bromide and analyzed by bright field and fluorescence microscopy.

Human mesenchymal stem cells showed no cell-matrix interaction with native alginate as expected. The cells interacted mainly with other cells and built agglomerates (Fig. 4.10 (h)). An increased cell-matrix interaction was observed on calcium phosphate nanoparticle-coated alginate beads. Cells tended to adhere and spread or to build smaller agglomerates on modified alginate beads (Fig. 4.10 (i)). These results indicate that cells were able to interact with nanoparticles on the alginate surface, providing the basis for future gene transfer and drug delivery studies.

4.4 Conclusions

Characterization of the laser-induced sub 100 nm structures was performed by SEM and FIB measurements. Here, the HSFL structures were investigated in terms of morphology, periodicity, depth profile, aspect ratio and how various parameters such as energy per pulse, number of applied pulses (scan speed), wavelength, polarization, NA of the focusing optics and incident angle of the laser beam, influence them. The results show that the periodicity of HSFL is wavelength-dependent and increases with wavelengths between 700 and 950 nm. The resulting structures were relatively homogeneous ripples with a crater depth of 135 nm. This is close to the distance between two rims (∼ 125 nm) which gives an aspect ratio of about 1 : 1.

Silicon wafers with and without ripple structures were used for electrophoretic deposition. Calcium phosphate nanoparticles redispersing in ethanol were deposited and gave a particle multilayer. The electrophoretic deposition with biofunctionalized calcium phosphate nanoparticles from water led to a monolayer. Calcium phosphate nanorods were deposited from water, forming a multilayer with about 17 layers.

Different ways of loading alginate beads with calcium phosphate nanoparticles were investigated. One of these was incubation in a nanoparticle dispersion. The second was loading from the crosslinking bath. The nanoparticle dispersion alone was not sufficient to produce stable alginate beads. Calcium phosphate nanoparticle-coated alginate beads were distinguished by increased surface contrast and were generally darker in comparison to standard alginate beads. The calcium phosphate-coated alginate beads remained intact for at least 4 days and showed no signs of disintegration. Coating with solution gave the best results with a calcium-free dispersion. Nanoparticle coating on the alginate beads was very efficient, and the beads did not lose their shape. The coating of the nanoparticles on the beads was homogeneous in this case.

Coating with a nanoparticle-loaded substrate was also elaborated. The final coating was applied with fluorescent PEI-functionalized calcium phosphate nanoparticles. Fluorescence microscopy pictures showed an inhomogeneous coating. The nanoparticles were agglomerated on the alginate surface. However, it was possible to
coat the surface of the beads with different kinds of fluorescent calcium phosphate nanoparticles.

The best method of coating alginate beads with calcium phosphate nanoparticles from a substrate was using a robot. Electrophoretically coated structured surfaces carrying nanoparticles were used as substrates.

After loading the alginate with calcium phosphate nanoparticles, the effects on the surface properties and the subsequent cellular behavior were studied. Three different samples were examined: native alginate, calcium phosphate-coated alginate and tissue culture-treated polystyrene. The surfaces were colonized with L929 fibroblasts. The nanoparticle-coated alginate surfaces triggered the integrin-mediated adhesion of anchorage-dependent cells like fibroblasts. Finally, calcium phosphate nanoparticle-coated alginate beads were used as microcarriers for human mesenchymal stem cells in the hanging drop cultivation technique. hMSCs showed no cell-matrix interaction on native alginate, whereas the cell-matrix interaction was significantly increased on calcium phosphate-loaded alginate beads.

Overall, the electrophoretic deposition of calcium phosphate nanoparticles on laser-structured surfaces, followed by transfer to alginate beads is a promising method of preparing microcarriers to influence the behavior of cells.

Acknowledgments

This work was supported by the German National Science Foundation (DFG, priority programme SPP 1327). The FIB profiles were prepared by M. Menzel, S. Henning and A. Heilmann at the Fraunhofer Institute for Mechanics of Materials (IWM) in Halle (Saale), Germany.

References

4 Nanoparticle-loaded bioactive hydrogels


