Scanning force microscopy on spatially and temporally varying magnetic substrates for cell cultivation

Juliane Issle and Uwe Hartmann
Institute of Experimental Physics, University of Saarland, P.O. Box 151 150, 66041 Saarbruecken, Germany
j.issle@mx.uni-saarland.de

Abstract. The general intention of this work is to analyze spatially and temporally variable nanostructured surfaces generated by functionalized magnetic beads on a biocompatible carrier substrate to induce cell differentiation. Magnetic thin films with particular domain structures are used to align magnetic nanobeads of around 250nm diameter (Fe₃O₄ core) by means of magnetic interaction. The beads’ properties are investigated by atomic force microscopy (AFM) and magnetic force microscopy (MFM), respectively. As the magnetic substrates as well as the beads are biocompatible the set up is used as a substrate for cell growth. AFM gives information about the cells’ behavior on the artificial structures. By using external magnetic fields in the range of 10mT the magnetic structure of the thin films can easily be modified in vivo which gives the opportunity to study the influence of structured substrates not only with respect to topographical but also to dynamical changes.

1. Introduction
As it becomes of more and more interest to influence cells, especially stem cells, in a controlled manner, this work describes a new approach of structuring biocompatible substrates to induce cell differentiation. Up to now many techniques have been established to provide the possibility to structure a substrate in terms of chemical patterning on the one hand and topographical on the other. There are some problems which must be overcome. The substrates have to be nontoxic and the patterning must extend over a large area of several square millimeters. The latter can be achieved by soft lithography like microcontact printing or nanoimprint lithography [1]. These methods are widely-used to obtain certain protein patterns [2] or topographical structures [3]. It was already proven that cells react to structures in the micrometer range [3]. So far the shape of the structures used was fixed in advance. It could not be changed during cell cultivation.

We developed a set up which can be easily varied in vivo. It consists in a magnetic carrier substrate, more precisely in a garnet film onto which magnetic nanobeads with a diameter of around 250nm are aligned by means of magnetic interaction between the domains of the garnet film and the magnetic core of the beads. To the beads’ surface, different kinds of proteins and growth factors can be bound covalently, depending on what is required for a certain cell type. The magnetic properties of the garnet film change with external magnetic fields in the range of some mT. The saturation field is about 10 mT. It is possible to vary the whole structure as the beads follow those changes as long as they are kept in liquid environment. That gives the opportunity to change the shape and geometrical structure during cell cultivation in vivo.
The garnet films are not toxic. They do not contain heavy metals like nickel or chromium as it is often the case for magnetic thin layers. The beads consist mainly of magnetite (Fe₃O₄) which is not toxic either. Actually it is widely found in biology and is supposed to play an important role in the navigation in the terrestrial magnetic field. Examples are magnetotactic bacteria [4] or pigeons [5].

As the diameter of the beads is below the resolution of light microscopes other methods must be used to study the behavior of the beads under different magnetic treatments. Atomic force microscopy (AFM) is a powerful method to gain information about the topography of the beads and about their behavior during or after a structural change of the magnetic thin layer. Magnetic force microscopy (MFM) is used to study the magnetic properties of the described set up. Electron microscopy shows details of the cells and the magnetic beads.

2. Sample Preparation

2.1 Magnetic nanobeads
The beads used in our experiments (fluidMAG-ARA, chemicell, Berlin, Germany) consist in a core of approximately 80% (v/v) magnetite particles, each of a diameter of 10 nm to 20 nm (see fig. 1). They are embedded in a polysaccharide matrix, which can be modified to carry reactive groups like carboxylic ones. Due to amino groups in most of the proteins, binding of the proteins to the beads is possible via an activation step with carbodiimid. Each bead is of a maximum diameter of 250 nm and is superparamagnetic. The beads can be separated from solution by means of a permanent magnet, which is necessary for the functionalization and the application in cell culture.

2.2 Garnet films
The garnet films (YSmBiGaFe) are of a thickness of 4 to 7 µm. The domain structure can vary from linear to triangular and mazes, and to bubble-shaped domains [6]. Those structures are metastable (stable without external magnetic field) once they are generated, see fig. 2. To gain linear structures the garnet film must be exposed to temperatures above the Curie temperature which is around 150°C and be cooled down slowly. Mixtures between the above mentioned structures can be achieved by external magnetic fields with varying the orientation to the surface (see fig. 2 b,c). The width of a domain is around 5 µm. The domain walls between two antiparallel magnetized domains provide the possibility to deposit magnetic nanobeads [7] as the magnetic gradient field causes strong magnetic forces between the beads and the surface. The garnet films are biocompatible and stable under culturing conditions (see fig. 3).

2.3 Experimental set up
The garnet films with the original domain structure are kept in liquid environment during bead deposition and afterwards. A home made incubator controls the environmental conditions for cell culturing. It is combined with a microscope in order to have the possibility for cell imaging in vivo. Magnetic coils are built around to obtain certain magnetic conditions in vivo, concerning the field strength and orientation. Feed back loops for temperature and field control keep a steady environment. For testing of the whole setup L929 fibroblasts (fig.3) and MAPCs (multipotent adult progenitor cells, stem cells) have been cultured (see fig. 4).

2.4 AFM and MFM measurements
For tapping and contact mode cantilevers with a resonant frequency of 70 kHz and 13 kHz, respectively, have been used together with the Nanoscope IV (Veeco). For the MFM investigations the tips were covered with 30nm CoCr by a sputtering process and magnetized afterwards. In order to study the behavior of the beads during domain changes some of the measurements have been done in liquid environment. For AFM and MFM measurements the cells were fixed with ethanol.
3. Results and discussion
To study the behavior of the magnetic particles under different conditions AFM and MFM were applied. The superparamagnetic particles do not agglomerate. They form layers when they are deposited by spin coating (see fig. 5 a). MFM measurements could be done with a lift height of 50 nm (see fig. 5b). The individual beads do not influence each other. TEM shows that the beads are not spherically shaped (see fig. 1). But a higher resolution which shows more details than the nearly spherical shape could not be obtained with the AFM due to the rather soft polysaccharide matrix.

After bead deposition onto a garnet film the set up was kept in liquid environment (water and culturing medium) for seven days with several washing steps and was investigated afterwards with AFM. Different bead concentrations, functionalizations and sizes (less than 250 nm in diameter) have been tested. Figure 6 shows a 3d view of a garnet film with domain width of 5µm and magnetic beads deposited onto the domain walls. The beads are still bound to the surface in a well defined structure. They were not washed away and did not pile up. No change of the properties of the setup could be verified. Even after applying external magnetic fields the beads still followed the domain changes completely. The beads were not damaged or even destroyed as no AFM investigation showed fragments of particles. The system is very stable over a long period, which makes cell culture possible. Figure 6 shows that the beads are not piling up. As they are of maximum height of 250 nm they do not represent a topographical structure which is relevant for the cells. That leads to the possibility to distinguish between influences based on topography on the one hand [3] and on chemical exposure on the other.

First experiments with beads in cell culture yielded endocytosis. The beads were adsorbed to a normal culturing substrate, like object slide or culture flask. After two to four days the cells did not undergo cell division and finally showed apoptosis. They took up the beads and collected them around the cell core. To overcome this problem experiments with deposition of magnetic beads onto magnetic thin films were done in order to enhance the interaction forces. As many magnetic thin films contain toxic components like heavy metals (Ni, Cr etc.), garnets are a good alternative for cell culture. Additionally they represent the possibility for easily changing the magnetic structure just by using external magnetic fields. Figure 4 shows MAPCs grown on a garnet film. 250 nm beads were deposited on the domain walls. It is clearly visible that the beads remain on the domain walls during cell culture and the cells are not able to take them up anymore. The outer parts of cells are investigated in order to check if the cells are healthy. As they are widely spread across the structure of the beads no changes can be seen in comparison to cells grown on garnets without beads. This shows that the magnetic interaction between the beads and the surface due to the gradient field of the domain walls is strong enough to prevent endocytosis. The set up thus offers an opportunity for the immobilization of proteins without losing the possibility of dynamical structural changes.

The functionalization of the magnetic nanobeads is successful with several proteins (biotin and streptavidin, TGF β as growth factor for fibroblasts), although it was not tested in cell culture yet. AFM measurements showed no visible changes of the beads during the functionalization steps.

4. Summary
A highly variable set up for a straightforward change of the chemical and topographical properties of a substrate for inducing cell differentiation is presented. The usage of functionalized magnetic beads allows a rapid change of the chemical (biomolecules) conditions which are necessary for induction of cell differentiation. AFM measurements show that the magnetic nanobeads do not pile up on the domain walls of the garnet films. The substrate can thus be considered as flat with respect to cell reaction. Further investigations, which will combine stamping techniques with the bead deposition on garnets, will show if there is a biochemical influence of the bead patterns on the cells.

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References
[1] Y. Xia and G. M. Whitesides, Angew. Chem. Int. Ed. 27 (1998) 550-575
[2] J. D. Hoff, L. Cheng, E. Meyhöfer, L. J. Guo and A. J. Hunt, Nano Letters 4 No5 (2004) 853-857
[3] C. A. Mills, J. Escarré, E. Engel, E. Martínez, A. Errachid, J. Bertomeu, J. Andreu, J. A. Planell and J. Samitier, Nanotechnology 16 (2005) 369-375
[4] R. Blakemore, Science 190 (1975) 377
[5] W. Wiltschko und R. Wiltschko, Science 176 (1972) 62-64
[6] A. Hubert and R. Schäfer, Magnetic Domains, Springer (1998) Heidelberg, Germany
[7] L. E. Helseth, T. Backus, T. H. Johansen and T. M. Fischer, Langmuir 21 (2005) 7518-752

Fig. 1: TEM image of a magnetic nanobead. Single magnetite crystals are visible. They are held together by means of a polysaccharide matrix, which can be functionalized.

Fig. 2: Faraday images of a garnet film. Black and white areas are antiparallel magnetized. a) Linear and triangular structures after heating above $T_{\text{Curie}}$ and cooling down again. b) and c) mixed states of bubbles and mazes.
Fig 3: Fibroblasts on beads deposited on a garnet film after 5d of culture. The whole set up is biocompatible. A high concentration of nanobeads in the medium causes piling up. The beads are visible as lines.

Fig 4: Topographical AFM image of MAPCs grown on garnet films with deposited beads. The beads are not taken up by cells as the structure is still visible below the cell membrane and is undamaged.

Fig 5: a) AFM of a layer of magnetic nanobeads of a diameter of less than 100 nm. b) MFM of single magnetic nanobeads. Apart from some topographical artefacts, mainly the stray field is visible.

Fig. 6: 3d topographical image (AFM, tapping mode) of beads deposited onto a garnet film. Beads do not pile up on domain walls.