This is a pre- or post-print of an article published in
Krejci, I., Piana, C., Howitz, S., Wegener, T., Fiedler,
S., Zwanzig, M., Schmitt, D., Daum, N., Meier, K., Lehr,
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(2012) Acta Biomaterialia, 8 (3), pp. 1239-1247.

Process optimization and biocompatibility of cell carriers suitable for automated magnetic manipulation

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Abstract

To date there is an increasing demand for automated cell reprogramming in the fields of cell biology, biotechnology and biomedical sciences. Microfluidic-based platforms that provide unattended manipulation of adherent cells, promise to be an appropriate basis for cell manipulation. In this study we developed a magnetically driven cell carrier to serve as a vehicle within an in-vitro environment. To elucidate the impact of the carrier on the cells, biocompatibility was estimated applying the human adenocarcinoma cell line Caco-2. Besides evaluation of the quality of the magnetic carriers by FE-SEM, the rate of adherence, proliferation, and differentiation of Caco-2 cells grown on the carriers was quantified. Moreover, the morphology of the cells was monitored by immunofluorescent staining. Early generations of the cell carrier suffered from release of cytotoxic nickel from the magnetic cushion. Biocompatibility was achieved by complete encapsulation of the nickel bulk within galvanic gold. The insulation process had to be developed stepwise and was controlled by parallel monitoring of the cell viability. The final carrier generation proved to be a proper support for cell manipulation, providing a proliferative activity of Caco-2 cells equal to glass or polystyrene as a reference for up to 10 days. Functional differentiation was enhanced by more than 30% as compared to the reference. All in all, a flat, ferromagnetic, and fully biocompatible carrier for cell manipulation was developed for application in microfluidic systems. Beyond that, this study offers advice for the development of magnetic cell carriers and the estimation of their biocompatibility.

Keywords: Caco-2, cell carrier, automated manipulation, magnetic manipulation, microfluidic system.

1. Introduction

In nature, the stimuli for the differentiation of cells are recognized at the surface and transmitted to the nucleus via intracellular signalling pathways. Currently, advance towards induced differentiation was made either by addition of soluble factors like growth factors [1-3], determination factors [4-5], and vitamins [6] or by surface mediated cell differentiation via immobilization of adhesion molecules [7] or matrix molecules [8]. Additionally, cells have been manipulated by genetic programming [9].

Recently, in-vitro cell cultivation and differentiation have been recognized as fundamental tools for progress in medicine and pharmaceuticals especially considering the fact that defined differentiation of cells and moreover targeted manipulation of the destiny of an individual cell becomes more and more feasible. However, cell culture requires work-intensive handling restricting its routine applicability. Efforts have been made to automate screening assays in microplate format [10] and to monitor cellular changes by measuring physical values like voltage [11] or impedance [12].

Addressing the increasing demand for miniaturization, microfluidic systems provide a well-established basis to scale down analytical devices for biological applications. Moreover, microfluidics is a promising technology in cell-based screening industry as their benefits include reduced reagent consumption and thus lower cost [13]. Furthermore, its feasibility for real-time monitoring of erythrocytes was demonstrated and applied for nanoparticle-driven manipulation of cells [14]. The techniques to manipulate cells within a microfluidic system include dielectrophoresis, standing-wave ultrasound or magnetism using either particles as vehicles for adherent cells [15-17] or optical tweezers for automatic cell recognition and separation [18]. Amongst all, however, magnetic manipulation offers the distinct advantage that the cells are barely exposed to mechanical stress. Thus, magnetic beads are widely used for separation, isolation, and detection of cells [19]. A major challenge faced with practical application of magnetic beads is to improve the binding efficiency of the cells to the coated beads which often results in a time-consuming optimization process [20-21].

An integrated approach towards automated cell manipulation and defined differentiation within a poly(dimethyl siloxane) (PDMS)-based microfluidic system is described by Gómez-Sjöberg *et al.* [22]. It enables culture and differentiation of cells in different compartments. Here, the defined localisation of stimulating factors as required for cell differentiation is inherent. In fluidic systems mimicking different cell environments in the same fluidic compartment diffusion of dissolved factors is the limiting element and tricky to be controlled by pure microfluidics. Only recently, the same authors reported about modifications to be made to the PDMS to allow a better control on small molecule concentration [23]. Immobilisation of molecules on substrates is an alternative solution. This approach describes convenient magnetic handling in liquids and production of a low price

disposable. Apart from that, biology requires sterilization-resistant materials, transparency for inspection of the cells, and long-time biocompatibility.

In recent studies Caco-2 cells were used to estimate the influence of growth supports on several cell characteristics [24-26]. The Caco-2 cell line was established by Fogh *et al.* in 1974 and derived from a human colon adenocarcinoma of a 72-year-old Caucasian patient [27]. Despite of their colonic origin the cells form monolayers that undergo complete and terminal differentiation forming a columnar absorptive epithelium similar to that of the human small intestine [28]. For this reason Caco-2 cells are widely accepted as model for the human small intestine [29-30]. Furthermore, this cell line is recommended by the FDA to be applied for the Biopharmaceutics Classification System (BCS) [31].

The aim of this work was to develop and optimize millimetre sized flat substrates for cell seeding. These carriers are engineered to be used as cell shuttles in a microfluidic system enabling free positioning based on automated magnetic manipulation. Based on a thin glass, those carriers provide two-sided optical access to the adherently growing cells via a standard microscope. Addressing the above mentioned challenges, the gap between technological and biological demands was bridged by a stepwise development of carriers with concomitant biocompatibility studies. The magnetic carriers are expected to offer the possibility for differentiation of cells by temporary contact with signal factors in differently prepared chambers. Moreover, such carriers should be readily modifiable either physically or biochemically using techniques like nano-imprinting to trigger the differentiation process via direct immobilization of signal factors on the growth area of the cells. Hence, the adhesion, proliferation, and differentiation of cells grown on the individual cell carrier (cc) generations were assessed. The viability data provided the basis for improvement of the carriers. The quality of the carriers was additionally investigated by scanning electron microscopy (SEM) as well as by Graphite Furnace Atomic Absorption Spectrometry. The results of this study may give valuable advice for the development of magnetic carriers offering an alternative to magnetic beads or optical tweezers in the field of automated cell manipulation.

2. Material and methods

2.1 Processing of the magnetic cell carriers

The production of the ccs was optimized stepwise as a 4" wafer process. The final processing technology is given below.

2.1.1. Initial metallization of the glass substrates

On 150 or 175µm thick 4" borosilicate glass wafers, Type D263 T (Schott AG, Mainz, Germany) a Cr (10 nm)/Au (150 nm) plating base was superimposed by lift-off patterning technology to enhance nickel adhesion. The Cr-Au-system was coated without vacuum interruption by magnetron sputtering (sputter system MSB400,

Malz & Schmitt, Meißen, Germany). Subsequently, this side will be the backside of the carrier and obtain a gold-sealed nickel cushion as described in section 2.1.2.

The metallization at the backside has a rhomboidal shape with a round window of 540 µm for later inspection of the cells. To realize this design a positive photoresist of the AZ 1500 series (Clariant, Wiesbaden, Germany) was spin coated applying an RC8system and optical lithography was realized with a 4" mask aligner MA25 (both Karl Süss, Garching, Germany).

On the front side of the carrier a Ti (10 nm)/Pt (100 nm) metallization was superimposed by lift-off patterning technology to label the carriers with a running individual number.

2.1.2. Nickel plating and gold sealing of the magnetic nickel cushion

Initially a negative photoresist (dry resist Ordyl AR200 series/ Elga Europe, Milan, Italy) of 65 to 70 µm thickness was applied onto the backside of the glass substrate with a prestructured plating base. After illumination, development and postbaking the structured substrates were cleaned with an O₂-plasma flash.

To guarantee complete encapsulation of the magnetic Ni cushion, a galvanic sub-layer of Au (3-5 µm) was required followed by galvanic Ni-plating. For an efficient magnetic handling a 50 µm thick Ni-layer had to be plated on the rather thin, brittle glass wafer. Since there is no standard process to plate stressless magnetic nickel layers on thin glass, a special designed wafer holder was developed by Silicet® (Lohfelden, Germany) and Fraunhofer IZM (Berlin, Germany) to provide a sufficient electrical current for homogenous galvanic deposition. Moreover, a sulphamate based nickel-plating bath was created being similar to those bath compositions used for LIGA - applications.

Subsequently, the dry-resist was removed by stripping and the carriers were cleaned by mechanical brushing.

Finally, the Ni-rhomb was covered with a 3-5 µm thick pore free galvanic gold layer for nickel encapsulation. An immersion gold plating step was necessary before to prevent the dissolution of nickel in the final gold plating bath. A schematic illustration of the production process is given in figure 1, the bath chemistry and process parameters of the electroplating are shown in table 1.

Table 1: Electroplating process

Process step	Bath parameter						Additional thickness
	Metal content	complexer	additives	Temp. [°C]	Current density	pH	
resist mask (dry resist, double laminated)							
Soft gold 1	10 g/l	CN ⁻	grain refiner	65	0,1 A/dm²	6,0	2 µm
Nickel	60 g/l	Sulfamate	Cl ⁻ ; SDS; H ₃ BO ₃	55	1 A/dm²	4,0	50 µm
remove resist							
Immersion gold	3,5 g/l	CN ⁻	Ammonium citrate	95	electroless	4,8	0,1µm
Soft gold 2	10 g/l	CN ⁻	grain refiner	65	0,1 A/dm²	6,0	3 µm

Figure 1: Schematic illustration of the final cell carrier production technique. A median cut of a single carrier is shown.

2.1.3. Preparation of the single magnetic cell carriers

In order to yield single carriers, the last step of the production process is to cut the magnetic carriers from the 4'' wafer. For cutting the wafers to yield cell shuttles with 1140 μm x 2100 μm x 230 μm in size a standard semiconductor wafer saw DAD 320 (Disco Ltd., Tokyo, Japan) was used. At this, the critical step was the clamping of the small ccs. To prevent cracks a special gluing technology using the hotmelt stepwax no.1 (Nikka Seiko Co., Tokyo, Japan) and a silicon support wafer were developed instead of applying the standard adhesive coated blue-tape.

After cutting the wafers, the single carriers were cleaned stepwise by washing the chips three times with acetone followed by deionized water. Afterwards the chips were treated with Piranha solution and rinsed again with deionized water. Finally, the quality of the carriers was controlled by optical inspection using a standard stereo magnifying glass.

2.1.4. Quality assurance of the magnetic cell carriers

2.1.4.1. Microscopic inspections

Throughout the process the quality of cc-samples was evaluated by conventional light microscopy and field emitting scanning electron microscopy (FE-SEM).

A FIB/FE-SEM Crossbeam (Zeiss/Leo 1540XB, Oberkochen, Germany) was used to analyze the surface quality of the electroplated carrier. The focused gallium ion beam (FIB) has been used to prepare cross-sections of the gold encapsulated nickel cushions. The FIB-cut was examined with the FE-SEM under an angle of 36°.

2.1.4.2. Determination of the nickel release

The nickel release from the different cc-generations was quantified by determination of the dissolved nickel with Graphite Furnace Atomic Absorption Spectrometry (GF-AAS). After cleaning the carriers with 0.5 ml RPMI-1640 cell culture medium (GIBCO Intvitrogen, Karlsruhe, Germany) in Eppendorf vessels they were washed twice with 1 ml deionized water. Then the carriers were stored for two days in 500 μl RPMI-1640 medium at room temperature. A 90 μl aliquot of this medium was mixed with 20 μl 14M HNO_3 suprapure and 90 μl deionized water to prepare the final sample solution. Released nickel was quantified in 10 μl of the sample solution using a polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi Z-8000, Hitachi, Tokyo, Japan) at a wavelength of 232 nm and a slit width of 0.2 nm. For calibration, a stock solution containing 10 μg nickel in 1ml 1M HNO_3 was diluted with RPMI-1640 medium, 14M HNO_3 and deionized water (9:2:9, v:v:v) to yield standard solutions containing 0.1 $\mu\text{g}/\text{ml}$, 0.2 $\mu\text{g}/\text{ml}$ or 0.5 $\mu\text{g}/\text{ml}$ nickel. If necessary, the sample solution was diluted with 1M HNO_3 . The detection limit ranged between 17 and 30 ng.

2.2. Cell culture

Caco-2 cells (ATCC, American Type Culture Collection, Rockville, MD, USA) of passages 25 to 68 were maintained in RPMI-1640 – High Glucose with L-glutamine (RPMI, GIBCO Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (GIBCO) in a humidified 5% CO₂/ 95% air atmosphere at 37°C. Cells were subcultured once a week by animal origin free Tryple Select (GIBCO). Biocompatibility studies were performed on 2x3 carrier arrays. Unless otherwise indicated, Caco-2 cells were seeded on these 2x3 cc-arrays placed in the wells of a 96 well microplate at a density of 62,500 cells per cm² and cultured for up to 14 days. As a reference cells were seeded at the same density into the wells of a conventional 96 well polystyrene microplate or into wells containing D263 glass platelets of the same dimension as the 2x3 cc-arrays. For disinfection the cc-arrays as well as the platelets were treated with 70% ethanol.

2.3. Adhesion studies

To determine the number of cells adhering to the magnetic cc, the DNA content of the cells was estimated by the CyQuantTM test kit (Molecular Probes, Eugene, OR, USA) pursuing a modified protocol. After removal of the supernatant cells were lysed by incubation with a mixture of 25 µl 20mM Hepes/NaOH buffer pH 7.0 and 25 µl CellLyticTM-M reagent (Molecular Probes) for 15 min at room temperature. The DNA of the lysed cells was stained by addition of 150 µl dye solution consisting of 0.5 µl fluorescent dye, 7.5 µl buffer, and 142.5 µl water. After storage for 20 min in the dark the fluorescence intensity was determined at 485/535nm in a fluorescence microplate reader. All assays were performed at least in triplicate to assure statistical relevance.

2.4. Proliferation studies

The proliferative activity was determined using the BrDU cell proliferation ELISA test kit (Roche, Mannheim, Germany) adhering to the manufacturer's instructions. Prior to quantification of the reaction product in a microplate reader (Anthos ELISA Reader 2001, Krefeld, Germany) at 450nm 1M H₂SO₄ was added to stop enzyme activity.

2.5. Differentiation studies

Functional differentiation of the cells was estimated from the activity of the brush border hydrolase Aminopeptidase N (APN). After removal of the culture medium, the enzyme activity was assessed by incubating the cell layer with 150 µl substrate solution containing 1.5 mM L-alanine-7-amido-4-methyl-coumarine trifluoroacetate salt in isotone 20 mM Hepes/NaOH buffer pH 7.4. Following incubation at 37°C for 1 h in the dark, 100 µl of the supernatant were transferred into a 96 well microplate and the converted substrate was quantified in a fluorescence microplate reader at 360 nm / 465 nm using substrate without cells as a blank. The activity of APN was calculated from a standard curve of 7-amido-4-methyl-coumarine in 20 mM Hepes/NaOH

buffer pH 7.4. One unit is defined as the amount of protein that hydrolyses 1.0 μmol L-alanine-7-amido-4-methyl-trifluoroacetate salt per minute.

2.6. Definition of the nickel tolerance limit

To estimate the concentration of nickel that affects the viability of cells in culture, Caco-2 cells were cultured in RPMI-1640 medium supplemented with 50, 100, 250, 500, 1000 or 5000 ng nickel per well (160 μl) using water-soluble NiCl_2 . After 2, 7, and 16 days in culture adhesion studies (section 2.3.) were performed to evaluate the influence of the individual nickel concentration on the vitality of the cells.

2.7. Biocompatibility of the individual cell carrier generations

Biocompatibility of the particular cc-generations was scrutinized by performing adhesion and proliferation studies as described in sections 2.3 and 2.4. Moreover, the activity of the enzyme Aminopeptidase N was quantified to elucidate the effect on the functional differentiation of Caco-2 cells. These biocompatibility studies were carried out in different labs to confirm the results. For the final screening the same passage of Caco-2 cells was applied in order to exclude any day-to-day variation and to verify the biocompatibility of all cc-generations.

To confirm the quality of the final generation cc5 the BrDU cell proliferation ELISA was modified by extending the incubation time of the BrDU labelling reagent from 1h to 24 h

2.8. Morphology studies

2.8.1. Immunofluorescent staining of ZO-1 and villin

On day 7 postseeding cells cultivated on 2x3 cc5 arrays were fixed for 20 min with ice-cold methanol at -20°C . After washing the cells were rehydrated with PBS at room temperature. The cell layer was stained with either purified mouse anti-ZO-1 or anti-Villin antibody (BD Biosciences, San Jose, CA, USA) as a primary antibody and the secondary antibody for 1 hour at 37°C each. The secondary antibody solution containing goat-anti-mouse Immunoglobulin/FITC (DAKO, Vienna, Austria) was supplemented with 1 $\mu\text{g}/\text{ml}$ propidium iodide (SIGMA, St. Louis, MO, USA) in order to counter-stain the cell nuclei. Finally the cell layer was washed three times and mounted for microscopic inspection using Fluor SaveTM Reagent (Calbiochem, Darmstadt, Germany). Immunofluorescence images of labelled cells were acquired using a Nikon Eclipse 50i microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. Excitation and emission filter blocks were at 465-495/515-555 nm for green fluorescence and at 510-560/590 nm for red fluorescence. All pictures were acquired at 20x magnification using Lucia Gv5.0 software for image evaluation.

3. Results

3.1 Stepwise optimization of the cell carrier production

3.1.1. Main modifications of the individual cell carrier generations

The final carrier was developed by stepwise improvement of the processing technique. The main modifications are given in table 2. The first prototype, cc0, had a Ti/Pt plating base and a rectangular nickel bulk of about 40 μm covered with about 1400 μm Si_3N_4 on its backside. The distinct nickel release of this carrier was reduced on cc1-R1 by removing Ni from the cutting lines. Moreover, the shape of the nickel bulk was changed to a rhombus. The next generation, cc2-R3, had for the first time a Ti/Pt front side metallization and a front side label. In addition, the thickness of the nickel core of cc2-R3 was reduced to 30 μm . In addition to the 200-300 nm Si_3N_4 coating a PMMA layer of 1 μm was superimposed to establish a barrier reducing nickel release. With the cc2-R4 generation the plating base on the backside was changed to Cr/Au instead of Ti/Pt to decrease the resistance and thus enhance the nickel electroforming. Additionally, instead of PMMA a 1 μm galvanic Au layer was superimposed to improve the nickel coverage. Major improvements were achieved with the generation cc4-R2. On these carriers, a running individual number was realized as front side label, the plating base on the backside was further improved and the nickel cushion of 50 – 65 μm was covered with spin coated SU8 (Micro Resist Technology, Berlin, Germany). Moreover, a galvanic Au sublayer of 3 μm was added to reduce interfacial tension. On the final carrier cc5 (fig. 2) the nickel cushion was completely isolated by an additional galvanic Au top layer instead of SU8 to minimize Ni leakage.

Table 2: Technological modifications of the individual cell carrier generations.

generation	D263 glass	front side	backside
cc0	150 μm		<ul style="list-style-type: none"> plating base: Ti (10 nm)/Pt (100 nm) Ni-bulk: rectangular, 40 $\mu\text{m} \pm 15 \mu\text{m}$ Si_3N_4: 1400 - 1450 nm
cc1-R1			<ul style="list-style-type: none"> Ni bulk: rhomboidal Si_3N_4: 400 – 500 nm
cc2-R3		<ul style="list-style-type: none"> metallization: Ti (10 nm)/Pt (100 nm) label: "FS" 	<ul style="list-style-type: none"> Ni bulk: 30 $\mu\text{m} \pm 15 \mu\text{m}$ Si_3N_4: 200 – 300 nm 1 μm PMMA
cc2-R4		<ul style="list-style-type: none"> metallization: Ti (10 nm)/Pt (100 nm) label: „CC2“ 	<ul style="list-style-type: none"> plating base: Cr (10 nm)/Au (100 nm) 1 μm galvanic Au Si_3N_4: 500 nm
cc4-R2	175 μm	<ul style="list-style-type: none"> metallization: Ti (10 nm)/Pt (100 nm) running individual number as label 	<ul style="list-style-type: none"> plating base: Cr (10 nm)/Au (150 nm) sublayer: 3 μm galvanic Au Ni bulk: 50 – 65 μm 10 – 15 μm spin coated SU8
cc5	175 μm and 150 μm		<ul style="list-style-type: none"> toplayer: 3 – 5 μm galvanic Au

Figure 2: Backside (left) and front side (right, with running number) of the final cell carrier cc5.

Chip size: 2100 μm x 1400 μm x 230 μm ; round window for microscopic

inspection of cells with 540 μm in diameter.

3.1.2. Realization of the final nickel encapsulation by gold

To ensure a tight gold-sealing of the thick nickel layer on a thin glass substrate the commonly used galvanic plating techniques had to be adapted and optimized. Initially, the masks for the metal adhesion layers and the thick resist were applied with a lateral overlap resulting in a narrow rim of free plating base around the later plated nickel cushion. After resist removal this rim served as additional cathode for the deposition of the final Au-layer. Further, a thin golden sub-layer was superimposed to achieve inherent cementation of the nickel to be deposited in the next step. Finally, tight sealing was accomplished by subsequent galvanic plating of gold on the top of the nickel cushion and the plating base rim.

For galvanic deposition of the nickel-rhomb and the gold-sealing layers, some major modifications of the processing technique were necessary. Since the electrical contact of the initially metalized glass substrates requires even distribution of current densities the wafer layout was modified by producing separate contact spots at each wafer quarter. Moreover, a specially designed wafer holder was developed to contact all four quadrants of the cc-wafer by galvanic bath resistant sealed pins. As compared to earlier generations, this even distribution of current densities improved stress-inducing uneven layer-plating significantly. Finally, a sulphamate-based plating bath composition was chosen to achieve strain and stress compensation.

3.2. Quality assurance of the magnetic cell carrier

3.2.1. Quality defects monitored by microscopy

To evaluate the appearance of the different layers, to measure their thickness, and to inspect the random sample carriers at different process stages, conventional light microscopy and FE-SEM analysis were applied.

Light microscopy revealed that the combination of a thick metal layer and a thin glass substrate induces wafer warping (fig. 3A). Moreover, using FE-SEM, the rupture of the whole nickel cushion from the initial metalized glass substrate became apparent (fig. 3B).

Figure 3: Light microscopical view of nickel stress induced glass cracks in the cc4-R2 generation (A).

SEM image of a single dismantled Ni-cushion of the carrier generations cc4-R2, showing the glass remainder (B).

For high resolution analysis of the metal layers FE-SEM was employed to assess the assembling of the Au-Ni-Au bulk and to elucidate the origin of nickel leakage. Figure 4 shows the gold-sealed nickel cushion and its assembly.

Figure 4: Detailed view of the gold encapsulated nickel cushion. SEM images of a FIB-cut in the edge of an Au encapsulated Ni cushion. Viewing angle of the cut is 36°.

Further critical areas are shown in figure 5. Dark parts (5A, B) of the gold layer were found to be polymer residues that can cause electrolyte uptake followed by corrosion and nickel leakage. Moreover, corrosion effects on ccs (5C, D) were observed.

Figure 5: Image 5A shows dark edges of the gold layer. The detailed image 5B reveals dry-resist residues obviously causing electrolyte uptake followed by corrosion and nickel release. Moreover, a typical defect of the cell carriers by corrosion (5C) and the corresponding corrosion spot (5D) are displayed.

3.2.2. Estimation of the nickel release

The nickel leakage of cc4-R2 and cc5 was quantified by GF-AAS. In case of cc4-R2 nickel release was detectable for 66.6% of all tested carriers. For cc5 two thirds of the examined carriers had a nickel release below the lower limit of detection.

On average the nickel release amounted to 72.00 ± 35.36 ng per cc4-R2 carrier. In case of cc5, a reduction of about 40% could be observed with leakage rates of 28.70 ± 13.70 ng nickel per carrier.

3.2.3. Nickel tolerance limit of Caco-2 cells

In order to identify the maximum amount of nickel present in the culture medium that exerts no negative influence on viability cells were cultured in presence of different concentrations of nickel for up to 16 days.

Adhesion studies (fig. 6) revealed that concentrations between 50 ng and 250 ng nickel per well had no major impact on cell growth up to day 16 postseeding as compared to the reference ($124,700 \pm 2496$ cells per well on day 16 postseeding). In contrast, exposition to higher nickel concentrations resulted in reduced adhesion rates on day 7 and 16 postseeding. In case of 500 ng nickel per well cell adhesion was reduced to 87% and in case of 1000 ng nickel per well to 74% as compared to cells cultured in nickel-free medium. Obviously, addition of 5000 ng nickel per well induced cell death, since adherent cells were detectable neither on day 7 nor on day 16 postseeding.

Figure 6: Cell count per well (160µl) 2, 7 and 16 days postseeding. Cells were cultured in RPMI-1640 medium supplemented with different concentrations of NiCl_2 (mean $\pm$ SD; n=3).

3.3. Biocompatibility of the individual cell carrier generations

The applicability of magnetic ccs to serve as growth supports for Caco-2 cells was elucidated by adhesion and proliferation studies as well as by determining the activity of the brush border hydrolase Aminopeptidase N.

Proliferation studies revealed that the design of the magnetic cc has a tremendous impact on the proliferative activity of Caco-2 cells. As shown in figure 7 the proliferation rate of cells cultured on the carriers reflects the

stepwise production improvement. The first generations cc0 and cc1-R1 reduced cellular proliferation to about 50% as compared to the reference 3 days postseeding. However, 10 days postseeding the proliferative activity was even decreased to 11% (cc0) and 4% (cc1-R1) as compared to the control. The PMMA-coated generation cc2-R3 seemed to serve as a proper support, but further experiments revealed a non-acceptable standard deviation (0.489 ± 0.378 AU (absorbance units)). Three days postseeding the proliferative activity on cc2-R4 was similar to that of cc2-R3, whereas at day 10 postseeding proliferation on cc2-R4 was even lowest yielding only 10% of the reference (0.061 ± 0.069 AU). The proliferative activity of cells grown on the next carrier generation, cc4-R2, reached about 60% of the reference (0.399 ± 0.092 AU) 3 days postseeding and even 80% of the reference (0.714 ± 0.043 AU) at day 10 postseeding. cc5 was finally equal to polystyrene or glass, with a proliferative activity of 0.633 ± 0.112 AU on day 3 postseeding and 0.863 ± 0.095 AU on day 10 postseeding, respectively.

Figure 7: Proliferative activity of Caco-2 cells cultivated on different cell carrier generations as compared to D263 glass and polystyrene as growth support (mean $\pm$ SD; n = 6).

The quality of the final generation cc5 was confirmed by extending the incubation time of the BrDU labeling reagent to 24 hours. This prolonged incubation with BrDU revealed that cells cultured on the magnetic carrier cc5 had the same proliferative activity as compared to the conventional tissue culture material polystyrene. Two days postseeding the proliferation of cells reached a value 0.75 ± 0.02 AU. Furthermore, the same result was observed on day 10 postseeding (cc5 0.73 ± 0.07 AU; polystyrene 0.75 ± 0.12 AU).

Evaluation of the functional differentiation of Caco-2 cells on the individual generations of the magnetic ccs revealed results similar to the proliferation studies.

As to generations cc0 and cc1-R1 the activity of APN (fig. 8) was about 50% (cc0 106.44 ± 10.57 ; cc1-R1 102.67 ± 113.58 nU (nanoUnits) APN/10,000 cells) of the references on day 3 postseeding and even lower than 10% 10 days postseeding (cc0 47.64 ± 40.83 ; cc1-R1 0.00 ± 0.00 nU APN/10,000 cells). Functional differentiation of Caco-2 cells on cc2-R3 and cc2-R4 was highly variable resulting in high standard deviations, both 3 and 10 days postseeding. The magnetic carrier cc4-R2 was found to be a suitable support to yield properly differentiated cells as indicated by an enzyme expression of 221.07 ± 98.95 nU APN/10,000 cells 3 days postseeding and 379.00 ± 92.91 nU APN/10,000 cells 10 days postseeding. The final carrier cc5 revealed even an enhanced APN activity ranging from 150% (day 3 postseeding) to 130% (day 10 postseeding) as compared to D263 glass.

Figure 8: Activity of the brush border hydrolase aminopeptidase N (nanoUnits per 10,000 cells) after cultivating the cells on different magnetic cell carriers (mean $\pm$ SD; n = 6).

3.4. Morphology of cells cultured on cc5

To confirm the formation of a continuous cell layer on the magnetic carrier the tight junctions of Caco-2 cells were monitored by staining the ZO-1 protein as a protein contributing to cell-cell bonds of epithelial cells. At this, a dense network of tight junctions was assessed on cc5 7 days postseeding (fig. 9, A). Hence, the final generation of the magnetic carriers supported the formation of a tight Caco-2 cell layer. Furthermore, morphological differentiation of Caco-2 cells was evaluated by staining villin, a protein found in the microvilli of human intestinal cells. The dots observed on cells cultured on cc5 for 7 days provide evidence for the

formation of microvilli which are buttressed by bundles of actin filaments (fig. 9, B). Consequently cells grown on cc5 revealed also proper morphological differentiation.

Figure 9: Immunofluorescent images of Caco-2 cells stained with the anti-ZO-1-antibody and propidium iodide (A) or with anti-villin-antibody (B).

4. Discussion

Recent advances in knowledge from cell biology, biotechnology as well as biomedical sciences revealed that each single cell is influenced by the surrounding environment. Not only in research and biotechnology but also in cell therapy there is an increasing demand for constructing molecular environments to trigger cells according to their inherent but variable determination. Since signals for differentiation are collected at the cell surface and transmitted to the nucleus via intracellular pathways, an approach towards a miniaturized system for manipulating the cell surface opens up wide areas of application in biotechnology, medicine and pharmaceuticals. First approaches towards automated cell reprogramming were proposed by Gómez-Sjöberg *et. al.* [22] by designing a fully automated microfluidics-based platform which provides unattended stimulation of adherent cells.

The main objective of this study was to develop flat, ferromagnetic carriers that provide a suitable support for adherent cells and that can easily be modified by established methods to trigger the differentiation process via direct immobilization of signal factors. Moreover, these microcarriers should be movable by external magnetic forces to serve as supports for contact-free handling of cells within a microfluidic system. The major technological challenge of the carrier development was the serious demand for long-term biocompatibility.

Glass wafers were considered to be a suitable basic material for these carriers due to their transparency as necessary for microscopic inspection of cells and their well known biocompatibility. Additionally, glass can be

easily modified chemically by conventional silane coupling techniques that allow efficient covalent immobilization of biomolecules [32].

The magnetically addressable structure of the carrier was chosen very carefully. Among all elements there are only a few metals, e.g. iron or nickel, with ferromagnetic properties at room temperature. Both, iron and nickel are used for the production of magnetic beads [33-34] and both are also known to exert toxic effects [35] depending on the concentration and exposition time.

Suitable production technology was an important aspect to be considered selecting the best magnetic compound for the ccs. In order to guarantee efficient magnetic handling of the microcarriers within rather narrow channels of the microfluidic system a thick ferromagnetic metal layer on a thin, brittle glass substrate was an inevitable prerequisite. However, electroforming of thick metal layers on thin glass substrates is a skilful process considering the mechanical stress ratio resulting from the mismatching coefficients of expansion of glass and metal. To date LIGA is the preferable technology which allows on-demand manufacturing of high-aspect-ratio structures with lateral precision below one micrometer. This technique is also used for metal batch fabrication of microsystems in biomedical sciences [36-37]. However, the combination of metal thick-films and thin glass wafers is by far not simple. Well-established processing technologies had to be combined in a particular manner and the individual steps had to be adapted considering the requirements of a biocompatible magnetic carrier. Further, since electroforming of iron is a complex process leading to release of hydrogen as a result of the required excess voltage, nickel was chosen to be the magnetically active material of the carriers.

The main technological challenge was the even distribution of a thick low-stressed nickel layer over the whole surface area. Otherwise, stress-induced forces at the metal-glass interface may lead to wafer warping and peeling of the complete nickel cushion from the glass substrate, as observed by FE-SEM. Therefore, the primary Ti-Pt metallization (generations cc0 - cc2-R3) at the backside of the carrier was replaced by a Cr-Au plating base that was subsequently thickened from 10 nm Cr/100 nm Au to 10 nm Cr/150 nm Au to lower the tension at the metal-glass interface. An additional layer of 3-5 μm galvanic gold at the plating base further permitted stress-less electroforming of the nickel-rhomb on cc4-R2 and cc5. Moreover, the plating process itself was improved by an optimized sulphamate based bath and a specifically developed wafer holder. Stress-inducing uneven layer plating of earlier generations was improved considerably by the custom-designed wafer holder with four point electrodes to connect all wafer quadrants, which was applied for the production of cc4-R2 and cc5. That way major differences in Ni-layer thickness between peripheral and central wafer regions could be smoothed. To achieve the required thickness of the nickel-cushion dry-resist lithography was found to be suitable in order to superimpose a sufficiently high solid resist.

To avoid any corrosion of nickel within an electrolytic solution such as culture medium, a reliable insulation of the magnetic cushion is a further technological demand for application in cell culture, since the corrosion of nickel is associated with the release of cytotoxic nickel ions.

First efforts to insulate the nickel bulk were made by PMMA-coating (polymethyl methacrylate) of the backside of cc2-R3. However, proliferation as well as the activity of the brush border hydrolase aminopeptidase N varied significantly with the individual cc2-R3 carriers. These results were attributed to sterilization of the ccs with 70% EtOH, since alcohol in general leads to stress corrosion cracking of PMMA [38]. Thus, PMMA was inadequate for nickel insulation. For the next cc-generation a galvanic gold layer of 3 μm additionally coated with 500 nm Si_3N_4 was prepared to cover the nickel cushion. Biocompatibility studies revealed proper proliferation of cells on cc2-R4 3 days postseeding but 10 days postseeding the proliferative activity was notably decreased as compared to plain glass or polystyrene. Differentiation studies confirmed these observations. As elucidated by FE-SEM the poor biocompatibility of these carriers resulted from an insufficient cover by the gold layer at the metal-glass interface. For cc4-R2 further improvements were accomplished by a 3 μm galvanic gold sub-layer and an additional spin coated SU8 layer sealing the magnetic nickel cushion. On these carriers proliferation of Caco-2 cells was only slightly reduced and functional differentiation was actually similar to those of the references. This probably pointed to local dewetting of the SU8 layer which caused some nickel release. Furthermore, the optical quality of the round windows for cell inspection was decreased by SU8 filling. For the ultimate cc-generation cc5 the nickel cushion was completely encapsulated with gold. Cell culture studies revealed that their biocompatibility was equal to glass and tissue culture treated polystyrene. The enzyme activity was even slightly enhanced. Finally, the morphology of Caco-2 cells grown on cc5 carriers was monitored by immunofluorescent staining techniques. The formation of a tight cell layer with microvilli again confirmed biocompatibility of these carriers.

Despite proper resist removal FE-SEM of cc4-R2 carriers revealed that polymeric residues can remain on the carrier after electroforming of the nickel-rhomb. However, in general these residues should get encased by the finally deposited gold layer. Consequently, defects are possible after plating and a careful inspection of the carriers is mandatory prior to cell cultivation. An additional mechanical brushing after resist removal improved the final generation cc5. Nevertheless, 30% of the investigated carriers still released nickel. Since Caco-2 cells tolerate up to 3125 ng nickel per ml cell culture medium, a mean release of 28.7 ± 13.7 ng per single cc5 carrier is far below the tolerance limit of Caco-2 cells and there is no major impact on cell adhesion.

5. Conclusions

All in all, the results of this study prove the importance to optimize well-established techniques in order to realize magnetic carriers for cell manipulation within a microfluidic system. Hence, careful characterization of the biocompatibility of substrates intended for use in cell culture should go along with the development of processing technology. Finally, our study presents for the first time flat, magnetic ccs appropriate for microfluidic systems as a basis for a miniaturized automated cell programming system.

Acknowledgements

This paper was generated in the context of the CellPROM project, funded by the European Community, contract No. NMP4-CT-2004-500039 under the 6th Framework Programme for Research and Technological Development in the thematic area of “Nanotechnologies and nano-sciences, knowledge-based multifunctional materials and new production processes and devices”. The work and results reported reflect the author’s views and the Community is not liable for any use that may be made of the information contained therein.

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