

Technical note

Rapid localized cell trapping on biodegradable polymers using cell surface derivatization and microfluidic networking

Jason Sinclair, Aliasger K. Salem*

Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, 52242, USA

Received 23 August 2005; accepted 31 October 2005

Available online 22 November 2005

Abstract

Spatial control over cell attachment is essential for controlling cell behavior and engineering cell-based sensor arrays. Here we report on a patterning procedure that can be utilized on a wide range of adherent and non-adherent cell types without the need to identify the exact peptide sequence or extracellular matrix (ECM) necessary for optimal cell attachment. This is achieved by converting native sialic residues present on the surface of most cells into non-native aldehydes using a mild sodium periodate treatment. The aldehyde groups are then reacted with biotin hydrazide to produce biotinylated cells. Avidin is patterned onto the surface of a biotinylated biodegradable block copolymer, polylactide–poly(ethylene glycol)–biotin (PLA–PEG–biotin) by microfluidic networking using a PDMS stamp. The biotinylated cells then bind specifically to the patterned avidin regions. The PEG that is presented from the PLA–PEG–biotin copolymer in the regions without avidin immobilization minimizes cell binding in the non-patterned regions.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Cell surface engineering; Polymer synthesis; Microfluidics; Soft lithography; Biotin–avidin

1. Introduction

Spatial control over cell binding is essential for modulating cell behavior and engineering cell-based sensor arrays [1–4]. Examples of the need for such geometric control over cell binding include neurons that follow adhesion cues to form contacts with muscle and the patterning of fibroblasts and hepatocytes to maximize the cell-to-cell contact between the two cell types for enhanced viability and functionality over single-cell cultures of hepatocytes [3,5,6]. Soft lithography techniques have shown significant potential in facilitating control over the organization of cells [7,8]. Typically, in this approach, stamps prepared by curing poly-dimethylsiloxane (PDMS) over a photolithographic template are used to transfer extracellular matrix (ECM) or cell adhesive molecules directly onto the substrate either by printing or microfluidic networking [4,6–12]. To elucidate the properties

necessary for optimum cell–substrate interactions, this approach was first used for the transfer of self-assembling molecules onto gold substrates [13]. More recently, micropatterning of ECM and cell-adhesive molecules derived from the ECM (such as RGD from fibronectin) has been carried out on biodegradable substrates [2,4,10,14,15]. Examples of ECMs that are commonly patterned for spatial control over cell attachment include collagen and fibronectin [6]. A limitation to this approach is the need to identify and transfer cell-specific adhesive sequences or ECMs depending on the cell type that is being bound. These methods also remain inapplicable to typically non-adherent cells or cells that do not have sufficient concentrations of functional cell-membrane receptors. In addition, cell (i.e. endothelial) exposure to pulsatile flows after less than a few hours of binding to ECM proteins can result in less than 42% of the cells remaining adhered [16–20]. In this report, we described a biotin–avidin-based patterning procedure that can be utilized for facile spatially controlled rapid attachment of a wide range of cell types. This is achieved by converting native sialic residues on the

*Corresponding author.

E-mail address: aliasger-salem@uiowa.edu (A.K. Salem).

surface of cells into non-native aldehydes using a mild sodium periodate treatment. Sialic acids are a common terminal cell surface monosaccharide group with increased expression in many cancers [21,22]. Cell surface modification has been utilized to engineer cell–cell interactions between myoblasts and to bind endothelial cells, mouse ascite carcinoma cells and chondrocytes to glass or tissue culture plastic substrates with high attachment efficiencies [17–19,23]. The periodate reaction produces a significant proportion of reactive aldehyde groups [23]. The aldehyde groups are then reacted with biotin hydrazide to produce biotinylated cells. Avidin is patterned onto the surface of a biotinylated biodegradable block copolymer, polylactide–poly(ethylene glycol)–biotin (PLA–PEG–biotin) [24] by microfluidic networking using a PDMS stamp [25]. The biotinylated cells then bind specifically to the patterned avidin regions. The PEG that is presented from the PLA–PEG–biotin copolymer without avidin immobilization minimizes cell binding in the non-patterned regions [24,26].

2. Materials and methods

2.1. PLA–PEG–biotin synthesis

α -hydroxy- ω -amine PEG (1 g) was dissolved into acetonitrile (2 mL, Aldrich), methylene chloride (1 mL, Aldrich) and Et_3N (80 mL, Aldrich). After addition of NHS–Biotin (0.250 g, Sigma), the reactants were stirred overnight under argon. The reaction was worked up by the slow addition of diethyl ether (40 mL, Aldrich) to precipitate the polymer. The polymer was re-precipitated from hot isopropanol (70 °C, Aldrich). The polymer (350 mg) was dried azeotropically and left under vacuum. Lactide (2 g, Purac Biochem BV) was added to biotin–PEG–OH (0.35 g) and diluted with 10 mL toluene, $\text{Sn}(\text{Oct})_2/\text{toluene}$ (0.1 g in 1 mL). The reaction was then brought to reflux at 110 °C for 4 h under argon. The product was precipitated from a dichloromethane solution into a cold stirring solution of diethyl ether and isolated by vacuum filtration. Final product was assessed by gel permeation chromatography (GPC) and $^1\text{H-NMR}$ spectroscopy.

2.2. PDMS stamp preparation

To fabricate the mold, a 9:1 ratio of siloxane monomer (Sylgard Silicone Elastomer 184, Dow Corning) to curing agent was cured overnight at 50 °C on a patterned master. The master was prepared by spin coating 250 μl photoresist (SU8) onto a silicone wafer for 55 s at 2500 rpm, solvent baked at 100 °C for 100 s, and then exposed to UV light (11 mJcm^{-2}) from a mercury vapor lamp. The exposed resist was developed in a 4:1 mixture of deionized water and dried with nitrogen. The patterned master was then hard baked for 25 min at 125 °C. The elastomeric mold with the negative imprint on it was peeled off and washed several times with ethanol, hexane, and deionized water.

2.3. Cell culture

Human Dermal Fibroblasts (HDF, Cambrex) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% bovine calf serum (BCS), 0.5% penicillin, 0.5% streptomycin and 1% L-glutamine in a humidified incubator at 37 °C and 5% CO_2 . Cells were passaged 1:4 every 5 days before reaching confluence. Fresh media was added every 2–3 days.

2.4. Fluorescence spectroscopy studies using the [2-(4-hydroxyazobenzene)benzoic acid] (HABA)/avidin reagent

Spectroscopy studies were completed on a SPECTRAMax PLUS (Molecular Devices) at a fixed wavelength of 500 nm at 37 °C. The average read time was 0.5 s and the read mode [Abs]. Three blanks were read followed by three readings of the HABA/avidin in 1 mL cuvettes. Each sample was recorded three times over a subset of four repeats. The assay was completed by measuring the absorbance of the avidin–HABA complex at 500 nm before and after (10 mins) it had been placed over a monolayer of biotinylated cells cultured in 6-well plates. The absorption decreases proportionately to the biotin present on the surface because biotin displaces HABA due to its higher affinity for avidin. The change in absorbance can then be used to calculate the amount of biotin present. A series of biotin solutions of varying concentration were prepared as a calibration curve for determining the quantity of biotin molecules present on the cell surface.

3. Results and discussion

Fig. 1 shows schematically our approach for patterning of cells using the biotin–avidin interaction. First, a biotinylated polylactic acid–polyethylene glycol copolymer is synthesized by reacting *N*-hydroxysuccinimide–biotin with the amine terminus of a bifunctional α -amine- ω -hydroxy-polyethylene glycol that was prepared by reducing α -amine- ω -carboxylic acid-polyethylene glycol (Nektar Therapeutics) in a 1 M tetrahydrofuran–borane mixture (Sigma). Confirmation of the amide bond between the biotin and the PEG was observed by the appearance of a triplet at 7.8 ppm in $^1\text{H-NMR}$. Lactide (Purac Biochem BV) was then graft polymerized onto the hydroxyl terminus of the α -biotin- ω -hydroxy-polyethylene glycol in the presence of a stannous 2-ethyl hexanoate initiator (Sigma). Following purification and drying, the resulting PLA–PEG–biotin was dissolved at 1 mg/ml in trifluoroethanol (TFE) and 1 ml was cast into each well of a 6-well plate and allowed to evaporate overnight to form thin films. The film is composed of a degradable block copolymer (23,400 Mw) that presents pegylated biotin groups to the aqueous phase. The PEG chain acts as a flexible linker reducing steric hindrance and enhances the ability of the biotin unit to bind the tetrameric protein avidin [24].

The avidin was patterned on the PLA–PEG–biotin using a PDMS stamp with channels ranging from 100 to 250 μm in width. The PDMS mold was placed onto the film and 1 ml of a 500 $\mu\text{g/ml}$ solution of tetramethylrhodamine conjugated avidin (av-rh, Molecular probes) in d.i. water was placed so that it wetted the PDMS molds capillary entrances. After 1 h of contact, the remaining av-rh was removed by blotting and replaced with 5 ml of distilled water. After a further 5 min, the water was removed and the washing procedure repeated another five times. The sample was then immersed in water and the mold removed by carefully peeling apart the PLA–PEG–biotin substrate and the PDMS. The sample was then washed several times with an additional 50 mL of water. The patterned immobilized avidin as illustrated in Fig. 2 provides

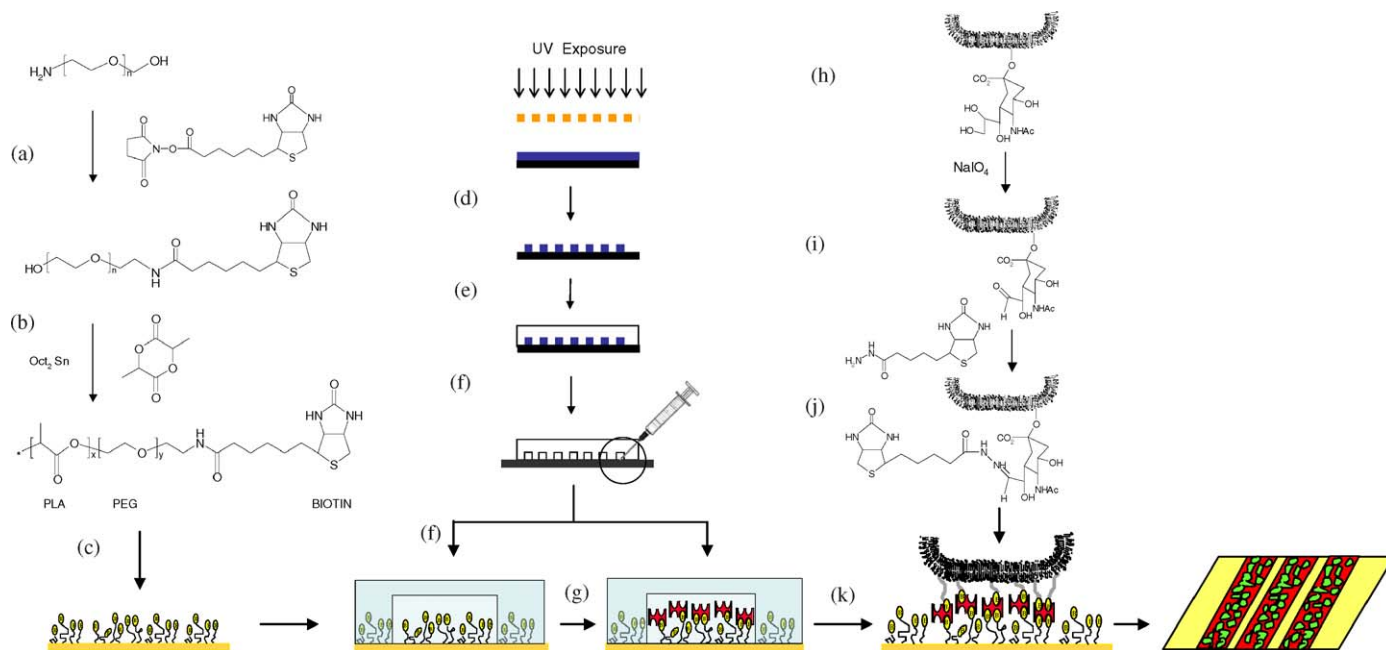


Fig. 1. Schematic of preparation of spatially controlled biotinylated cells on biodegradable templates. (a) Biotin is covalently attached to α -hydroxy- ω -amine PEG. (b) Lactide is graft polymerized onto hydroxyl terminus of biotin-PEG-OH. (c) PLA-PEG-biotin is formed into a thin film. (d) SU8 photoresist is cast over a silicon wafer and exposed to UV through a patterned master. (e) A PDMS mold is formed over the patterned template which then (f) forms a seal with the PLA-PEG-biotin film. (g) Avidin that is flowed through the microfluidic channels becomes immobilized in the predefined channel regions. (h) HDF cells are treated with sodium periodate to convert native sialic residues into non-native aldehyde groups. (i) The aldehyde groups on the surface of the cells are reacted with biotin hydrazide to produce biotinylated cells which are then (k) incubated on the avidin patterned PLA-PEG-biotin template to produce the patterned cells.

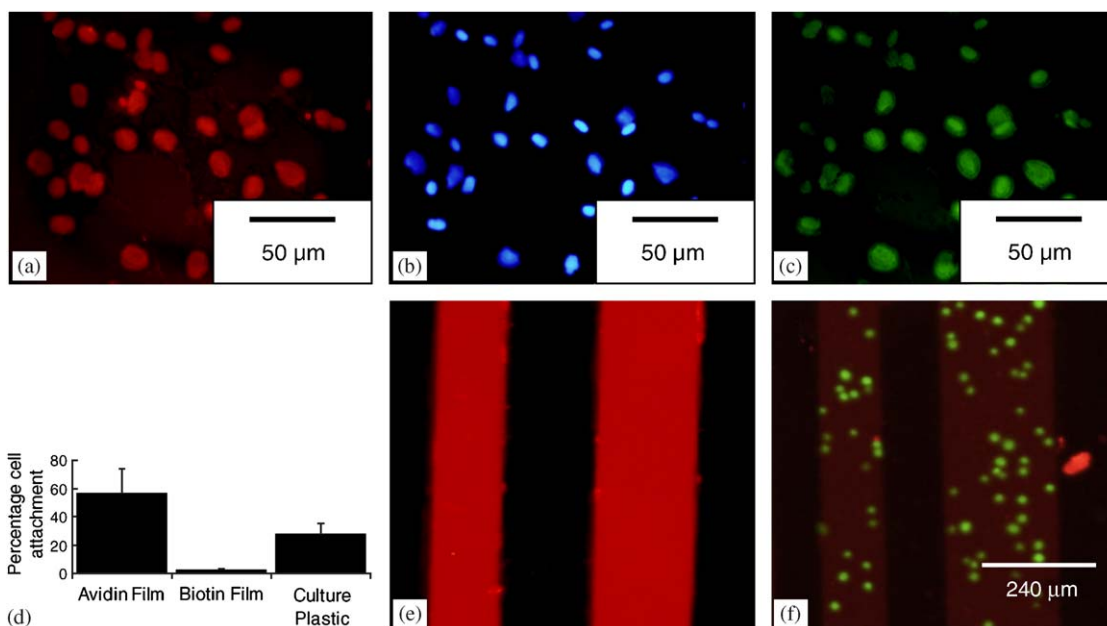


Fig. 2. (a) Fluorescent microscopy image (Olympus BX40, 555/580 nm) showing celltracker red-stained avidin-treated biotinylated HDF cells with corresponding images, (b) showing a fluorescent microscopy image of hoescht (350/450 nm) staining and (c) fluorescence emanating from the FITC-avidin (494/518 nm) bound to the biotinylated HDF cells. Fig. 2d is a bar chart comparing cell attachment of biotinylated cells on avidinylated PLA-PEG-biotin substrates, PLA-PEG-biotin substrates and TCP with $n = 9$. Fig. 2e shows a fluorescent microscopy image of the patterned tetramethylrhodamine-avidin (av-rh, 555/580 nm) immobilized on the PLA-PEG-biotin substrate. Fig. 2f shows biotinylated cells stained with celltracker green (494/518 nm) bound specifically within the av-rh (555/580 nm) patterned channels.

spatially defined binding sites for biotinylated cells to bind to.

HDF were selected as a model cell line for biotinylation based on previous reports that have shown the importance of spatial control over fibroblast attachment in maintaining viability of cocultures [6]. To prove that we could biotinylate the HDF cells, they were grown to 65–70% confluence in 12-well plates. Cell culture media in the wells was removed. Celltracker RedTM (Molecular Probes) that had been reconstituted in 11 μ l of dimethylsiloxane (DMSO) and mixed into media warmed to 37 °C was added to the wells and incubated for 45 min at 37 °C, 5% CO₂. The media was then removed and replaced with fresh warmed media and incubated for a further 1 h. The cells were washed twice with phosphate buffered saline (PBS) and incubated with a 1 mM solution of sodium periodate in cold PBS for 15 min in the dark at 4 °C. The HDF cells were then washed with buffer 1 (PBS, 0.1% Bovine Calf Serum, pH 6.5) at room temperature and then incubated with a 5 mM solution of biotin hydrazide (Sigma) in buffer 1 for 90 min at room temperature. Cells were then washed twice in buffer 2 (PBS, 0.1% BCS, pH 7.4) and incubated with a 5 μ g/ml of FITC-avidin (molecular probes) in buffer 2 solution for 15 min at 4 °C. The samples were incubated with a 0.01 mg/ml solution of Hoechst 33258 (Aldrich) in PBS. All the cells were then washed twice in buffer 2 prior to imaging by fluorescent microscopy (Olympus BX40). Fig. 2a shows an image of celltracker red-stained cells with the corresponding images showing the same cells stained with hoescht (Fig. 2b) and fluorescence (Fig. 2c) emanating from the FITC-labeled avidin that has bound specifically to the biotinylated cells. The degree of biotinylation was calculated to be $1.9 \pm 0.19 \times 10^9$ biotin moieties/cell. Control experiments in which the cells were treated with every step except the periodate treatment and control experiments in which the cells were treated with every step except for the biotin functionalization did not show any fluorescence through the FITC channel confirming that the fluorescence observed was due to a specific avidin–biotin receptor mediated interaction. The biotin–avidin interaction has been reported to primarily increase cell attachment in the first hour [18,19]. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of the long-term culture of another cell type (chondrocytes) has shown that this exceptionally strong biological binding interaction ($K_a = 10^{-15} \text{ M}^{-1}$) does not interfere with the cells long-term ability to proliferate or produce extracellular matrix proteins in comparison to untreated cells [19].

Cell viability measurements were carried out using trypan-blue measurements. Biotin functionalized cells demonstrated an 88.3% viability in comparison to a 95.33% for untreated cells.

To evaluate the binding affinity of biotinylated cells with an avidin-immobilized PLA–PEG–biotin substrate, the biotinylated cells were washed with 5 mM EDTA and then

trypsinized and washed. A total of 5×10^5 cells were added in serum-free medium to PLA–PEG–biotin films in each well of a 6-well plate that had been saturated with avidin (Sigma) and washed three times with PBS. Cultures were allowed to attach for 15 min before washing with PBS. Attached cells were observed by light microscopy. No difference in viability was observed after exposure to the avidin-coated polymer substrate. As illustrated in Fig. 2d, biotinylated cell attachment observed on the avidinylated substrates was 25-fold higher than biotinylated cell attachment on non-avidin-coated PLA–PEG–biotin substrates and 2-fold higher than TCP. When adherent cells were washed with avidin made up to $5 \times 10^{-7} \text{ M}$ in dibasic phosphate buffer (10 mM, pH 7.4), the binding process was not reversed.

Next, HDF cells grown to 65–70% confluence in T75 flasks were incubated for 45 min at 37 °C, 5% CO₂ with Celltracker GreenTM (Molecular Probes) that had been reconstituted in 11 μ l of DMSO and mixed into media warmed to 37 °C. The HDF cells were biotinylated as described earlier and then 5×10^5 cells in serum-free media were added onto the PLA–PEG–biotin substrates that had been patterned with av-rh. After 15 min of incubation with the cells, the patterned templates were washed with PBS. Attached cells were observed by fluorescent microscopy. As illustrated in Fig. 2f, cells labeled with celltracker green were found to be bound specifically within the patterned avidin lanes. Cell binding was minimized outside the avidin-patterned channels by PEG presented from the copolymer.

4. Conclusion

In summary, we have demonstrated rapid and facile spatial control over cell attachment using avidin–biotin-specific interactions, microfluidic patterning, cell surface derivatization and functional polymer synthesis. This approach has potential for multiple applications ranging from cell sensor-based assays, immobilization of non-adherent cells in defined regions for lab on chip applications and engineering of tissues that require a hierarchical structure of cells.

Acknowledgments

We thank the Biological Sciences Funding Program from the Office of the Vice President for Research, University Of Iowa, the Pharmaceutical Research and Manufacturers of America Foundation, the National Science Foundation AGEP program and partial support by the Public Health Service Grant no. P50 CA097274-04 from the University of Iowa/Mayo Clinic Lymphoma Specialized Program of Research Excellence (UI/MC Lymphoma SPORE) and the National Cancer Institute for funding.

References

- [1] Tien J, Chen CS. Patterning the cellular microenvironment. *IEEE Eng Med Biol Mag* 2002;21(1):95–8.
- [2] Hyun JH, Ma HW, Zhang ZP, Beebe TP, Chilkoti A. Universal route to cell micropatterning using an amphiphilic comb polymer. *Adv Mater* 2003;15(7-8):576–9.
- [3] Raghavan S, Chen CS. Micropatterned environments in cell biology. *Adv Mater* 2004;16(15):1303–13.
- [4] Patel N, Padera R, Sanders GHW, Cannizzaro SM, Davies MC, Langer R, et al. Spatially controlled cell engineering on biodegradable polymer surfaces. *Faseb J* 1998;12(14):1447–54.
- [5] TessierLavigne M, Goodman CS. The molecular biology of axon guidance. *Science* 1996;274(5290):1123–33.
- [6] Bhatia SN, Balis UJ, Yarmush ML, Toner M. Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions. *Biotechnol Progr* 1998;14(3):378–87.
- [7] Chen CS, Jiang XY, Whitesides GM. Microengineering the environment of mammalian cells in culture. *MRS Bull* 2005;30(3):194–201.
- [8] Kane RS, Takayama S, Ostuni E, Ingber DE, Whitesides GM. Patterning proteins and cells using soft lithography. *Biomaterials* 1999;20(23-24):2363–76.
- [9] Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science* 1997;276(5317):1425–8.
- [10] Tan W, Desai TA. Microfluidic patterning of cells in extracellular matrix biopolymers: Effects of channel size, cell type, and matrix composition on pattern integrity. *Tissue Eng* 2003;9(2):255–67.
- [11] Tourovskaia A, Figueroa-Masot X, Folch A. Differentiation-on-a-chip: a microfluidic platform for long-term cell culture studies. *Lab on a Chip* 2005;5(1):14–9.
- [12] Patel N, Bhandari R, Shakesheff KM, Cannizzaro SM, Davies MC, Langer R, et al. Printing patterns of biospecifically adsorbed protein. *J Biomater Sci—Polym Ed* 2000;11(3):319–31.
- [13] Mrksich M, Dike LE, Tien J, Ingber DE, Whitesides GM. Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold and silver. *Exp Cell Res* 1997;235(2):305–13.
- [14] Lin CC, Co CC, Ho CC. Micropatterning proteins and cells on polylactic acid and poly(lactide-co-glycolide). *Biomaterials* 2005;26(17):3655–62.
- [15] Liu VA, Jastromb WE, Bhatia SN. Engineering protein and cell adhesivity using PEO-terminated triblock polymers. *J Biomed Mater Res* 2002;60(1):126–34.
- [16] Bhat VD, Klitzman B, Koger K, Truskey GA, Reichert WM. Improving endothelial cell adhesion to vascular graft surfaces: clinical need and strategies. *J Biomater Sci—Polym Ed* 1998;9(11):1117–35.
- [17] Bhat VD, Truskey GA, Reichert WM. Fibronectin and avidin–biotin as a heterogeneous ligand system for enhanced endothelial cell adhesion. *J Biomed Mater Res* 1998;41(3):377–85.
- [18] Bhat VD, Truskey GA, Reichert WM. Using avidin-mediated binding to enhance initial endothelial cell attachment and spreading. *J Biomed Mater Res* 1998;40(1):57–65.
- [19] Tsai WB, Wang MC. Effect of an avidin–biotin binding system on chondrocyte adhesion, growth and gene expression. *Biomaterials* 2005;26(16):3141–51.
- [20] Miyata T, Conte MS, Trudell LA, Mason D, Whittemore AD, Birinyi LK. Delayed exposure to pulsatile shear-stress improves retention of human saphenous-vein endothelial-cells on seeded Eptfe grafts. *J Surg Res* 1991;50(5):485–93.
- [21] Prescher JA, Dube DH, Bertozzi CR. Chemical remodelling of cell surfaces in living animals. *Nature* 2004;430(7002):873–7.
- [22] Luchansky SJ, Goon S, Bertozzi CR. Expanding the diversity of unnatural cell-surface sialic acids. *ChemBiochem* 2004;5(3):371–4.
- [23] De Bank PA, Kellam B, Kendall DA, Shakesheff KM. Surface engineering of living myoblasts via selective periodate oxidation. *Biotechnol Bioeng* 2003;81(7):800–8.
- [24] Salem AK, Cannizzaro SM, Davies MC, Tendler SJB, Roberts CJ, Williams PM, et al. Synthesis and characterisation of a degradable poly(lactic acid)-poly(ethylene glycol) copolymer with biotinylated end groups. *Biomacromolecules* 2001;2(2):575–80.
- [25] Salem AK, Chao J, Leong KW, Searson PC. Receptor-mediated self-assembly of multi-component magnetic nanowires. *Adv Mater* 2004;16(3):268–71.
- [26] Salem AK, Rose F, Oreffo ROC, Yang XB, Davies MC, Mitchell JR, et al. Porous polymer and cell composites that self-assemble in situ. *Adv Mater* 2003;15(3):210–3.