Analyst

PAPER

Micropatterning neuronal networks†

Cite this: *Analyst*, 2014, **139**, 3256

Heike Hardelauf,‡[a] Sarah Waide,‡[a] Julia Sisnaiske,[b] Peter Jacob,[a] Vanessa Hausherr,[b] Nicole Schöbel,[b] Dirk Janasek,[a] Christoph van Thriel[b] and Jonathan West*[ac]

Received 4th April 2014
Accepted 19th May 2014

DOI: 10.1039/c4an00608a

www.rsc.org/analyst

Spatially organised neuronal networks have wide reaching applications, including fundamental research, toxicology testing, pharmaceutical screening and the realisation of neuronal implant interfaces. Despite the large number of methods catalogued in the literature there remains the need to identify a method that delivers high pattern compliance, long-term stability and is widely accessible to neuroscientists. In this comparative study, aminated (polylysine/polyornithine and aminosilanes) and cytophobic (poly(ethylene glycol) (PEG) and methylated) material contrasts were evaluated. Backfilling plasma stencilled PEGylated substrates with polylysine does not produce good material contrasts, whereas polylysine patterned on methylated substrates becomes mobilised by agents in the cell culture media which results in rapid pattern decay. Aminosilanes, polylysine substitutes, are prone to hydrolysis and the chemistries prove challenging to master. Instead, the stable coupling between polylysine and PLL-*g*-PEG can be exploited: Microcontact printing polylysine onto a PLL-*g*-PEG coated glass substrate provides a simple means to produce microstructured networks of primary neurons that have superior pattern compliance during long term (>1 month) culture.

Introduction

The ability to program the spatial arrangement of neuronal circuits has received great attention in the last 20 years, with applications spanning the elucidation of the mechanisms underlying neuron function,[1–5] to stem cell differentiation,[6] interfacing prosthetic devices[7,8] and compound screening.[9–12] *Ex vivo* neuronal circuits can be constructed by restricting neurons within microchannel architectures[13–15] or, more commonly, by micropatterning an adhesive environment against a so-called cytophobic background that resists cell adhesion. The latter approach does not physically restrict neuron development, but instead provides spatially-defined biochemical guidance cues for the directed organisation of the neuronal circuit. The literature documents a rich assortment of methods for neuron patterning,[16] with the frequent aim to provide straightforward protocols for neuroscientists to fabricate devices in their own laboratories. This trend has largely been driven by the soft lithography revolution[17,18] for the replication of high resolution patterns in poly(dimethylsiloxane) (PDMS) for microcontact printing (μCP)[19–23] or stencilling[10,24,25] approaches to biomaterial patterning.

The challenge of micropatterning primary neurons is in providing a generally accessible (*i.e.* not requiring operation in a cleanroom) and straightforward protocol that ensures maintenance of the pattern during lengthy (weeks) neuron culture. To achieve this, a polyamine anchor such as polylysine (PL) is used to tether neurons to the substrate. PL-directed neuron localisation and adhesion can be augmented by the addition of extracellular matrix (ECM) materials such as fibronectin or laminin to the patterning procedure or subsequently secreted by the neurons in the immediate locality of the polyamine pattern. These polyamine anchored ECM proteins enable the stable tethering of neurons *via* integrins that also provide life support signals. In addition, single micron resolution is essential for restricting neuron cell bodies from pathways intended for sub-cellular axon and dendritic outgrowths to interconnect the cellular nodes within the artificial circuit.[26] To illustrate this challenge, the optimal outgrowth track width for human SH-SY5Y neuroblastoma cells is 2 μm, sufficiently narrow to restrict neurons from adhering, but also sufficiently wide to support the extension of neurites and the leading growth cone.[10] Deviation from this optimum results in significant losses in the quality of the neuronal network. However, high-resolution photolithography methods for material patterning by UV disintegration[27] or by photoresist lift-off methods[28,29] are restricted to clean room environments, while direct laser writing methods[1,26] struggle to deliver the resolution and desirable write speeds with the added drawbacks of the

[a]*Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V., 44139 Dortmund, Germany*

[b]*Leibniz Research Centre for Working Environment and Human Factors – IfADo, 44139 Dortmund, Germany*

[c]*Institute for Life Sciences, University of Southampton, SO17 1BJ, UK. E-mail: J.J.West@soton.ac.uk*

† Electronic supplementary information (ESI) available as figures: Bilayer plasma stencilling [1], PLL on PEG [2 and 3], PLL mobility [4], PLL on DCDMS [5], aminosilane patterns [6], large neuronal network [7], redefining measures [8] and PEG stability [9]. See DOI: 10.1039/c4an00608a

‡ Equal contribution.

need for complex and costly instrumentation. Fortunately, μCP and stencilling methods can deliver this resolution and are suitable for device replication in biolaboratories.

Some applications are more demanding of the quality of the biomaterial patterns than others. It is trivial to provide 10's of thousands of cell adhesion sites on a substrate. For fundamental lines of inquiry, only a small percentage of these adhesion sites need to effectively accommodate neurons. However, for applications such as toxicity and compound screening it is imperative that the quality and reproducibility (across chip and chip-to-chip) of the adhesion patterns is excellent, otherwise responses to test substances can be confused with responses to artefacts introduced during surface patterning or from loss of stability during lengthy culture. For example, in neurite outgrowth inhibition assays the absence or loss of outgrowth tracks would increase the apparent toxicity of the test compound. Similarly, neuronal implants for applications in regenerative medicine require high fidelity pattern compliance to ensure effective interfacing with the host tissue.

In this contribution we have evaluated soft lithography-based stencilling and μCP methods for the preparation of high fidelity neuronal networks. The research entailed a systematic comparison of combinations of aminated cell adhesion materials and cell repellent poly(ethylene glycol) (PEG) or methylated materials. The onus of this study was the identification and refinement of reliable neuron patterning methods. Within this framework it is recognised that some groups have mastered their own methods. Ideally the micropatterning method should not require prior expertise with surface chemistry (*i.e.* they are forgiving with imperfections in the materials and procedure) and should be extremely simple to make them universally accessible. The quality and stability of the patterns was assessed using contact angle and zeta potential measurements, by imaging fluorescently-labelled material patterns and quantifying the network formation capacity of primary cortical neurons (the gold standard measure of pattern performance). From this research a variant of μCP involving polyamine patterning onto a PEGylated substrate was developed and shown to be the most straightforward, effective and reliable means for the development and long term culture (>1 month) of patterned primary neuronal networks. The method has the potential to be extended to murine and human neuronal precursor cells by the addition of an ECM material backfill step.

Materials and methods

Surface coating and patterning

Surfaces were coated with cytophobic molecules; the cationic copolymer of poly-L-lysine grafted with poly(ethylene glycol) (PLL-*g*-PEG), PEG silanes or dichlorodimethylsilane (DCDMS). Two PEGylated silanes were used; 2-[methoxy(polyethyleneoxy)-propyl]trichlorosilane (TCS-PEG, ABCR, Germany) and methyl ether poly(ethylene glycol) triethoxysilane (mPEG, 5 kDa, Creative PEGworks). Glass substrates must first be thoroughly cleaned prior to silanisation. Preliminary experiments compared Nanostrip acid treatment (90% sulphuric acid, 5% peroxymonosulphuric acid, <1% hydrogen peroxide and 5% water) with Hellmanex II (2% v/v) alkali treatment and plasma ashing (see below). Equivalent silanisation results were obtained for the different methods. Plasma treatment was used for subsequent experiments for reasons of pragmatism and safety, especially with the view that neuroscientists may not be familiar with the practices necessary for the safe handling of aggressive acids and alkalis. Glass substrate preparation by the plasma method involved an ethanol (96% v/v) rinse, followed by a MilliQ H_2O rinse, drying with N_2 and further drying by a 100 °C bake for 10 minutes. Substrates were then plasma ashed (70 W, 40 kHz (Femto, Diener Electronic)) in a 0.2 mbar oxygen atmosphere for 60 s in readiness for coating.

PLL-*g*-PEG functionalization. Cleaned substrates were PEGylated with 100 μg mL^{-1} of PLL(20)-*g*[3.5]-PEG(5) (Surface Solutions, Switzerland) in a 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, pH 7.4) for 1 h at room temperature. Substrates were then rinsed with a sequence of 1× PBS (phosphate buffered saline), MilliQ water and a N_2 stream. Please note that the PLL-*g*-PEG batch-to-batch quality can vary. Other suppliers should be considered or the copolymer can be prepared in standard chemical synthesis laboratories.[30]

mPEG functionalization. Cleaned substrates were incubated in 2 mM mPEG in dried toluene[31] for 2–24 hours at room temperature. Critically, this silanization procedure was undertaken in a N_2 glove box to prevent O_2 and moisture interference.[32]

TCS-PEG functionalization. Cleaned substrates were incubated for 3 hours at room temperature in a 0.1 mM solution of TCS-PEG in dried toluene with 0.03% (v/v) 37% HCl catalyst. To fully dissolve the silane a 30 minute incubation is required. Following silanization the substrates were cleaned in dry toluene for 20 minutes, followed by an ethanol (96% v/v) and MilliQ H_2O wash with final drying using a N_2 stream.

DCDMS functionalization. Cleaned substrates were placed in an air-tight chamber with a 1 mL volume of DCDMS in hexane (10% v/v). The chamber was heated at 80 °C for 30 minutes, after which the substrates were rinsed in hexane and dried with a N_2 stream.

Optimal dimensions for the formation of human SH-SY5Y neuronal networks[10] were used in this study: hexagonally arranged 70 μm diameter nodes separated by 100 μm were interconnected by 2 μm-wide tracks. Array patterns contained 36 sub-arrays, each containing 202 nodes. For plasma patterning, bilayer PDMS stencils were used. The SU-8 master fabrication and PDMS replication methods have previously been described.[10] Briefly, bilayer plasma stenciling involves conformally contacting the stencil with the substrate coated with the cytophobic material and plasma etching (70 W, 40 kHz (Femto, Diener Electronic)) in a 0.2 mbar oxygen atmosphere for 60 s. The exposed substrate regions were backfilled with polylysine (100 μg mL^{-1} in 1× PBS) for 15 minutes, followed by washing with 1× PBS or culture medium for 1 hour. Alternatively, backfilling was investigated using aminosilanes purchased from ABCR, Germany; 3-aminopropyl triethoxysilane (APTES), diethylenetriaminosilane (DETA), bis(trimethoxy silylpropyl)amine (BTMSPA) and 3-aminopropyldiisopropylethoxysilane (APDIPES). Aqueous and organic solvent (*e.g.* 0.1 mM in toluene) submersion methods were

systematically evaluated. However, gas phase silanisation was the most consistent means of derivatising the substrates with aminosilanes. 100 μL volumes of the silanes were placed in a N_2 atmosphere with the substrates and heated to 100 °C for 2 hours.

In contrast to stencilling, microcontact printing requires an inverted SU-8 pattern involving a single photolithography step to produce protruding (SU-8 2, Shipley) features with a height of ~3 μm. PDMS moulding within 20 × 20 mm frames was achieved using standard methods.[24] A standard microcontact printing protocol[19–21] was optimised for this study: PDMS stamps were oxygen plasma activated (see above) to generate a silanol-rich hydrophilic surface (contact angle <5°). The stamps were then incubated for 10 min in 100 μg mL^{-1} polylysine or 50 μg mL^{-1} polyornithine in 1× PBS (Sigma-Aldrich, Germany), followed by two rinses in MilliQ distilled H_2O and thorough drying under a N_2 stream. The stamps were protected from particulates in the fume hood and left to further dry for 15 minutes, then conformally contacted (without applying pressure) to PEG or DCDMS-coated substrates for ≥30 minutes. An isopropylalcohol, MilliQ H_2O and N_2 rinse sequence was used to recycle (>20) the PDMS stamp surface for subsequent prints.

Surface analysis

Contact angle measurements were used as a rapid means to evaluate material coatings. Plasma treated glass substrates have a contact angle of <5°, PEGylated surfaces have a contact angle of ~30°, methylated (DCDMS) surfaces have a contact angle of ~105° and aminated surfaces have a contact angle of ~60°. To periodically assess the stability of coatings and interactions with polylysine a SurPASS instrument (Anton Paar, Austria) was used to measure the zeta potential. Fluorescein isothiocyanate (FITC) labeled polylysine was purchased from Sigma-Aldrich (Germany) to enable patterns to be imaged with an inverted fluorescent microscope (IX71, Olympus) equipped with a halogen lamp and FITC cut-off filters.

Cell culture and pattern analysis

All experiments were undertaken in accordance with national laws for the use of animals, with the research approved by the local ethics committee. Primary cortical neurons were obtained from C57BL/6N mice, purchased from Charles Rivers Laboratories, Germany. After anaesthesia with CO_2, a pregnant mouse was sacrificed 16 days after conception by cervical dislocation. The cortices of the isolated embryos were extracted and transferred into Hanks balanced salt solution (HBSS, PANBiotech) containing 0.0125% trypsin (PANBiotech) for dissociation for 10 min, stopped by adding 0.05 mg mL^{-1} soy bean trypsin inhibitor, with free DNA destroyed using 0.01% (w/v) DNAse. The cells were further dissociated using fire-polished glass pipettes, centrifuged at 200*g* for 5 min followed by re-suspension in fresh Neurobasal media (Life Technologies) containing 2% (v/v) B27 growth supplement (Life Technologies), 10 μg mL^{-1} gentamicin and 0.5 mM stable L-glutamine (PANBiotech). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded on polylysine-coated substrates at a concentration of 2.5 × 10^5 cells per mL. The media was exchanged every 3–4 days by replacing 50% of the old media to retain sufficient autocrine and paracrine signalling.

Dorsal root ganglia (DRG) were prepared from postnatal day 1 mice (CD 1). Mice were sacrificed by decapitation. The spinal column was opened, the spinal cord removed and the dorsal root ganglia dissected. The ganglia were washed in PBS and collected in DMEM media supplemented with 1% (v/v) penicillin and streptomycin. After collection DRG were disrupted, incubated with 0.025% collagenase (Sigma Aldrich) for 45 min, dissociated using fire-polished glass pipettes, centrifuged at 200*g* for 4 minutes and resuspended in fresh F-12 medium (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin/streptomycin.

Surface patterning methods were also evaluated using LUHMES (Lund human mesencephalic) cells.[33] These cells were obtained from Marcel Leist (University of Konstanz). Cell culture flasks were coated with polyornithine (PO, 50 μg mL^{-1}) and fibronectin or laminin (1 μg mL^{-1}) in H_2O overnight at 37 °C. The solution was discarded and culture flasks were washed twice with sterile water. LUHMES cells were grown in proliferation medium consisting of advanced DMEM/F12, supplemented with 2 mM stable L-glutamine, 40 ng mL^{-1} FGF and 1% (v/v) N_2 at 37 °C in a humidified 5% CO_2 humid atmosphere. Cells were harvested using 0.025% (w/v) trypsin, centrifuged at 300*g* for 5 minutes for seeding 75 cm^2 with 2 × 10^6 cells. For differentiation 6 × 10^6 LUHMES cells were seeded into a pre-coated 175 cm^2 cell culture flask. After 24 hours the media was replaced with differentiation media, and after a further 48 hours pre-differentiation was completed and the cells were detached using 0.025% (w/v) trypsin.

CGR8 murine neuronal precursor cells were obtained from Marcel Leist (University of Konstanz). The cell line was established from the inner cell mass of a 3.5 day male pre-implantation mouse embryo (129/Ola). The differentiation media (1 L) contained 98.2 mL DMEM/F12 (Life Technologies), 98.2 mL Neurobasal media (Life Technologies), 1 mL N2 (Life Technologies), 2 mL B27 (Life Technologies), 2 mM stable glutamine (PANBiotech), 150 μL insulin (Sigma-Aldrich), 10 mg BSA fraction V (Roth) and 0.1 mM β-mercaptoethanol (Life Technologies). Media was filtered through a 0.22 μm filter. Cells were grown on surfaces coated with 10 μg mL^{-1} PO and 10 μg mL^{-1} laminin (Sigma-Aldrich). Media exchange took place every other day with pre-warmed N2/B27 media. A 20 day differentiation program is typically required.

Neurons were seeded on the arrays in a 1 mL suspension containing 2.5 × 10^5 cells and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The next day, non-adherent cells were removed by a media exchange, after which media was exchanged periodically. Neuron arrays were imaged using an inverted microscope (IX71, Olympus). A quality control threshold was used in advance of network quantification. Following the first day of culture on the patterned substrate, neuronal cultures that were estimated to have less than 10% of the nodes occupied or were randomly adhered to the surface (with a patterning efficiency[24] of ~0%) were considered unsuitable for quantitative characterisation and further experiments. Higher quality neuronal networks were quantified in

terms of the percentage of nodes occupied by neurons (occupancy (%)), the percentage of tracks occupied by neuron soma (cells in track (%)) and connections per (occupied) node (cpn). In the special case of primary neuronal networks cultured for lengthy periods we have re-defined occupancy and cpn terms (see Results, Microcontact printing).

To visualize molecular organization cells were stained by fixing with 4% paraformaldehyde with 0.5% Triton-X 100 for 10 minutes, then blocked with 5% normal donkey serum (Millipore) for at least 1 h. Primary antibodies (microtubule-associated protein 2 (MAP2; 1 : 200, Abcam) from chicken and β-3-tubulin (1 : 2000) from rabbit, Covance) were incubated for 2 h in darkness in 1% normal donkey serum. The secondary antibodies, donkey anti-chicken dylight 649 (1 : 500) or donkey anti-rabbit dylight 488 (1 : 500) were incubated for 30 min at room temperature in darkness. Lastly, nuclei were stained with DAPI (1 : 10 000) for 30 min and mounted using FluoroSave reagent (Invitrogen). A Leica DMI6000 B microscope was used to image the cells with a CCD camera (DFC 360 FX) and the LAS AF Software.

Results and discussion

Neuron patterning without polylysine

In former neurite outgrowth inhibition[9] and degeneration[10] studies, we patterned differentiated human SH-SY5Y neuroblastoma cells. Patterning was achieved by exploiting their ability to adhere to and grow on glass substrates but not methylated (thin-film PDMS) or PEGylated surfaces. Optimal patterns had 2 μm-wide outgrowth tracks that were fabricated by bilayer plasma stencilling a protein-resistant PEGylated background. The stencil, material patterns and neuronal networks are documented in the ESI Fig. 1.†

Dorsal root ganglion (DRG) neurons do not ordinarily require adhesion materials. In this study DRG neurons were reliably patterned on plasma patterned PEGylated surfaces (see Fig. 1(A)), but following 2 days culture the networked neurons were no longer registered within the material pattern. Lund human mesencephalic (LUHMES) neurons require a fibronectin or laminin-bearing surface. These cells were also readily patterned by exploiting the protein-rejecting quality of the PEGylated background to restrict laminin adsorption to the exposed areas of the glass substrate (see Fig. 1(B)). However, by the third day of culture the neuronal network began delaminating (see Fig. 1(C)), forming neurite-connected clusters (see Fig. 1(D)) that did not adhere to the pattern. By the fourth day the neuronal networks had significantly delaminated and were unsuitable for quantitative characterisation. In both cases it is probable that since the pattern limits the available adhesion area the tension forces generated by the developing network exceeded the forces coupling the neurons to the surface. In conventional LUHMES cultures a polyornithine coating precedes fibronectin deposition.[33] This highlights the need for polyamine coatings to electrostatically anchor neurons to glass surfaces. Efforts to backfill the plasma patterned PEGylated surface with polyornithine (PO) followed by passivation with laminin failed due to non-specific binding of neurons.

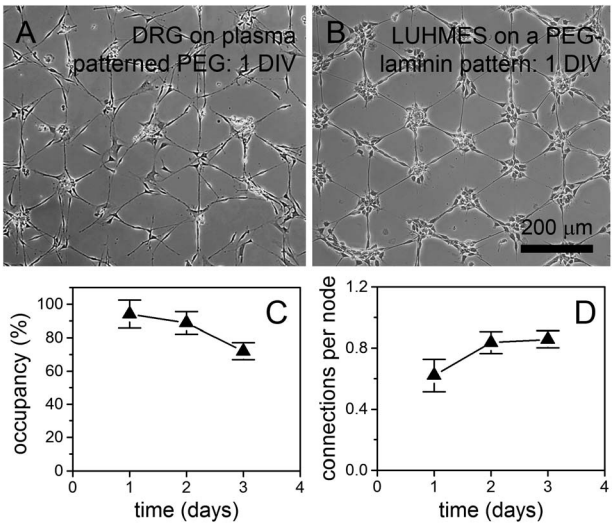

Fig. 1 Bilayer plasma stencilling PLL-*g*-PEG adlayers can be used for the formation of dorsal root ganglion (DRG) networks (A). A laminin backfill enables LUHMES cells to be patterned (B). These neuronal networks are short-lived, with occupancy declining rapidly after 3 days (C) as outgrowth connections (D) strengthen. In the absence of a polyamine anchor the networks delaminate.

Alternatively, adhesion molecules can be covalently coupled to glass surfaces, although working with reactive surface intermediates requires considerable experience to ensure reliable, stable and high quality coatings.[22,34]

Investigation of polylysine-rejecting coatings

Plasma stencilling for patterning biomaterials by backfilling relies on the surface coating preventing the adsorption of the biomaterial. PEGylated surfaces are well known for their protein rejecting qualities.[35,36] However, although the polyamines PL and PO are non-complex polypeptides they have markedly different physicochemical properties to most proteins. Zeta potential measurements were used to investigate the adsorption of PL onto plasma activated glass and substrates functionalised with a PLL-*g*-PEG adlayer. Both surfaces were coated with PL in minutes (see Fig. 2(A)). This was confirmed using plasma stencilled PEGylated substrates, with the PL having only marginally greater affinity for the exposed glass surface than the PEGylated regions (see ESI Fig. 2†). The resulting zeta potentials were similar (~20 mV), indicating that PL can become co-localised with the PLL-*g*-PEG adlayer (*i.e.* the PLL-*g*-PEG adlayer is not continuous). The structural similarity of PO with PL implies that the non-specific adhesion of LUHMES neurons on plasma patterned PEGylated surfaces may therefore have resulted from PO co-localising within the PLL-*g*-PEG adlayer.

Polylysine is a widely used neuron adhesion biomolecule and also the moiety on the PLL-*g*-PEG copolymer that enables simple electrostatic assembly on glass surfaces. To investigate whether the PL molecules were intercalating with the PLL moiety or adsorbing to or co-localising with the PEG moiety, two silane PEGs (*i.e.* without the poly-L-lysine moiety) were evaluated: methyl ether poly(ethylene glycol) triethoxysilane (mPEG)

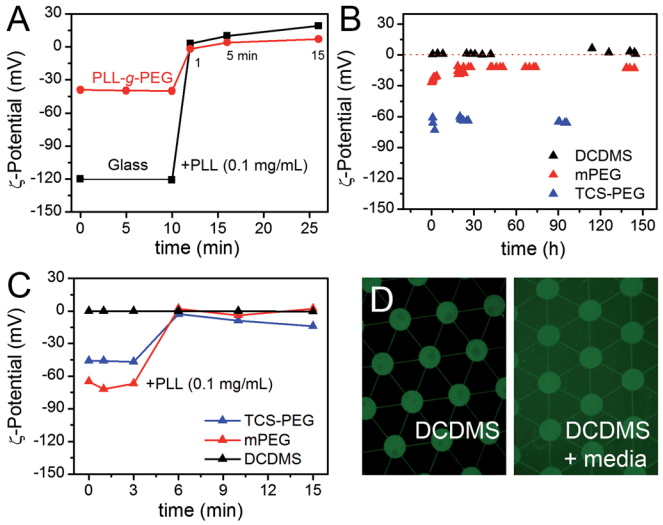

Fig. 2 Langmuir isotherms of PL (100 μg mL^{-1}) adsorption onto glass and PLL-*g*-PEG-coated glass obtained by zeta potential measurements. PL rapidly coats both surfaces (A). DCDMS, mPEG and TCS-PEG coatings are stable for at least 1 week when stored in 1× PBS at room temperature (B). The PEGylated surfaces do not resist PLL adsorption (C). DCDMS (methyl) coatings initially resist PL adsorption, producing patterns following a PLL–FITC backfill (D). However, following overnight incubation in culture media the PL is dispersed with associated loss of the PL and neuronal cell patterns.

and 2-[methoxy-(polyethyleneoxy)-propyl]-trichlorosilane (TCS-PEG), a coating reported to prevent polylysine adsorption.[37] These PEG silane coatings were stable for ≥1 week in 1× PBS (see Fig. 2(B)).

However, the PL also rapidly adsorbed to these coatings (see Fig. 2(C)), indicating an interaction with the ether repeats in the PEG moiety or incomplete surface coverage, with PL adsorbing to vacant areas. Efforts to improve the coating density using dry toluene as the carrier solvent and/or acid catalysts were unsuccessful. On occasion, in some areas the patterned TCS-PEG coating resisted the adsorption of PLL–FITC (see ESI, Fig. 3†), although incubation (hours) in 1× PBS and especially cell media resulted in extensive non-specific binding (*i.e.* pattern decay).

The PEG silanes were substituted with dichloro-dimethylsilane (DCDMS), an inert, highly hydrophobic coating that has a 0 mV zeta potential. Titration from pH 3 to pH 11 does not affect the zeta potential. The addition of PLL did not alter the zeta potential and PLL–FITC was observed to selectively adsorb only on the plasma activated regions and not the DCDMS regions of the substrate (see Fig. 2(D)). Storage of the DCDMS–PLL patterns in the DMEM cell culture medium for 18 hours resulted in only minor losses in the fluorescent intensity and contrast of the pattern. However, these material patterns decay during rigorous washing and are not stable when stored in cell culture media supplemented with essential growth factors (10% (v/v) FCS or Lipumin). This is surprising given the typical stability of cationic polyelectrolyte layers on negatively charged surfaces (*i.e.* plasma activated glass (ζ-potential = −120 mV)). The growth supplements resulted in pattern decay within 30 minutes and extensive non-specific PLL distributions following incubation for 18 hours (see ESI, Fig. 4†). Most probably it is the presence of ionic surfactants that are able to sequester and mobilise the PLL across the substrate surface. Isolated areas of partially patterned cortical neurons were observed following 5 days *in vitro* (DIV) culture (see ESI, Fig. 5†). This was an infrequent observation with the pattern quality being unsuitable for quantitative characterisation.

Aminosilanes as a polylysine substitute

Aminated surfaces are required for long term (>2 DIV) adhesion and survival. The mobility of polyamines when submerged in media supplemented with growth factors and the affinity of polyamines for PEGylated surfaces prompted an investigation to identify a substitute for the aminated polypeptide coatings. Silane chemistries offer the promise of covalent attachment. Aminosilanes have previously been used to pattern neurons.[3,4,38] We investigated four different silanes; APTES, DETA, APDIPES and BTMSPA. Submersion silanisation methods were not reproducible, whereas gas phase silanisation proved more reliable. A 2 hour 100 °C post-silanisation bake was used to dehydrate the coating and reduce hydrolysis.

Analysis of cured APTES, DETA and APDIPES coatings showed that a further 3 days was required to stabilise the zeta potential to approximately −20 mV (see Fig. 3). This indicates continued hydrolysis and loss of the aminosilanes from the surface. The trifunctional surface grafting silane APTES is especially prone to hydrolysis with additional leaching a consequence of the formation of non-covalently bound multilayers. Consistent with the findings of Kleinfeld[3] neuron death was evident following 2 DIV culture on these surfaces, most likely in response to the loss of an adhesive cue since the ethoxy groups have low toxicity. The other aminosilanes are more suitable for stable, monolayer deposition. Nevertheless, we were unable to replicate Kleinfeld's 12 DIV neuron culture results and Ravenscroft's[4] one month hippocampal cultures on patterned DETA surfaces. Despite the three-fold increase in

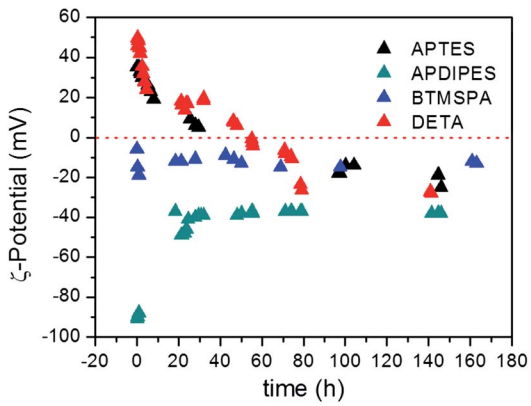

Fig. 3 The stability of several aminosilanes was evaluated using zeta potential measurements. APTES, DETA and APDIPES require ~3 days to form stable layers on glass substrates. A coating of BTMSPA, a dipodal silane presenting two amino groups, is immediately stable following preparation.

available amino groups and associated increase in surface density, in our experiments neurons only remained viable on DETA-coated surfaces for 3–5 DIV. This experience highlights the difficulty of silane-based chemistries. The alternative amino-silane APDIPES includes hydrophobic isopropyl groups that sterically shield the ethoxy tether to reduce hydrolysis and increase layer stability.[39] However, APDIPES was also unsuitable for the lengthy maintenance of neuron adhesion. The final amino silane, BTMSPA, a bipod trimethoxysilane presenting two amino groups, was immediately stable (−10 mV) following silanisation and curing (see Fig. 3) and was suitable as a glass coating for neuron adhesion.

Backfilling against a cytophobic background provides a straightforward means to pattern cells (without the complications of photoresists and without the need for operating in a clean room). Methyl-coated surfaces (*i.e.* DCDMS), but not PEGylated surfaces, resisted the adsorption of aminosilanes. Methylated glass surfaces were plasma patterned using the bilayer stencil and backfilled with BTMSPA and DETA. Shown in ESI Fig. 6,† after 5 DIV culture pattern compliance by the cortical neurons was greatly reduced, indicating loss and mobilisation of the aminosilanes (plasma patterned DCDMS coatings resist cell adhesion[24]). The patterning efficiency of these surfaces was too low to warrant quantitative analysis. An alternative to the aminosilane and polyamine backfilling approaches was therefore required.

Microcontact printing polylysine on PEG adlayers

Microcontacting printing (μCP) is superior to selective molecular assembly patterning (SMAP) and molecular assembly patterning by lift-off (MAPL) methods[40] and is by far the most popular technique for biologists to pattern proteins and cells. μCP can also be extended to the patterned transfer of PL or PO. To ensure long-term pattern compliance backfilling with cytophobic molecules such as BSA is commonly used. Alternatively reactive surfaces can be used to tether PEG species.[34,41] However, in both cases coatings were not of uniform quality resulting in limited pattern compliance. Reactive surfaces are difficult to maintain in a pristine condition prior to backfilling. To refine the microcontacting printing (μCP) protocol, to make it more straightforward and reliable, we chose to exploit the strong interaction of polyamines (PL and PO) with PLL-*g*-PEG coatings.

Our revision to the standard (μCP) protocol involves first derivatizing a plasma treated glass substrate with PLL-*g*-PEG, followed by μCP deposition of a PL or PO pattern. The resulting material pattern is highly stable, with patterns maintained while incubated in supplemented culture media (see Fig. 4(A)). Importantly, and unlike silane methods to derivatize surfaces with PEG, PLL-*g*-PEG can be straightforwardly applied to negatively charged substrates without prior expertise with surface chemistry (*i.e.* suitable for neuroscientists). In contrast, attempts to backfill PL patterns with PLL-*g*-PEG are unsuccessful: PLL-*g*-PEG co-localises with PL, while also binding to exposed glass regions to prevent neuron adhesion.

The most telling evidence of the quality and stability of the PLL prints on the PLL-*g*-PEG adlayer was the large area patterned

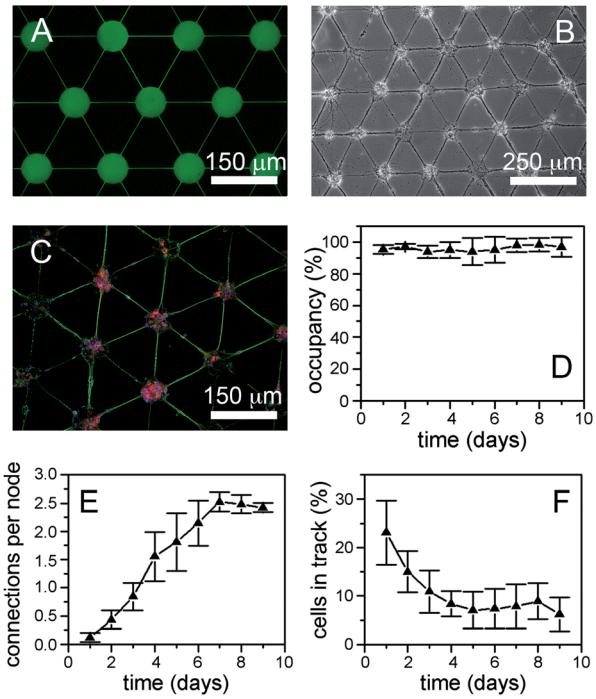

Fig. 4 PL patterns prepared by microcontact printing on PLL-*g*-PEG. High signal to noise and precise PLL–FITC patterns can be prepared (A). Primary neurons localised to the adhesion nodes and extended neurites for the formation of internodal connections, producing high occupancy neuronal networks over large areas (B). Fluorescent immunostaining shows nuclei stained with DAPI (blue), β-III-tubulin (green) and MAP-2 (red) (C). The high fidelity neuron networks had mean node occupancy values of >95% during 9 DIV (D), attained maximal network levels (~2.5 cpn) at 7 DIV (E). Neuron clustering in the first 4 days results in the number of tracks occupied by neurons reducing from 23% to >8% (F).

culture of primary cortical neurons for >1 month (see ESI, Fig. 7†), beyond the 2 weeks required to develop spontaneous electrical activity.[3,4] Documented in Fig. 4(B–F), neuronal networks developed with high fidelity to the underlying pattern. Node occupancy was >90% for the 9 day experiment, with connection per node (cpn) values developing to (near-maximal) ~2.5 levels following culture for 7 DIV. These occupancy and connection levels were maintained throughout a 33 DIV patterned culture experiment (*i.e.* until discarded). In contrast, silane–PEG coatings fail following 25 DIV[41] and only 12% of covalently coupled PEGylated areas are free of neurites following 29 DIV patterned culture.[34] These experiments validate the performance and stability of the material patterns and provide the first definitive analysis of neurite outgrowth and spatially standardized network formation rates by primary cortical neurons. The rate of development was less than with the differentiated SH-SY5Y cell line that requires 3 days for cpn values to plateaux, but the extent of interconnection was ~70% greater[10] (extensive interconnection is a feature of healthy primary neurons).

The exact nature of the highly stable coupling between PL prints and the PLL-*g*-PEG adlayer is unknown, but is unlikely to be simply a consequence of the strong electrostatic coupling of the positively charged PL (>20 mV) with the negatively charged

PEG moiety (approximately −70 mV, see mPEG analysis in Fig. 2(C)). In comparison, the zeta potential of glass is approximately −120 mV, yet polyamine coatings are still mobilised by supplements in the media. Therefore additional coupling modes may exist. Other possibilities are that PL can displace PLL-*g*-PEG or that vacancies in the PLL-*g*-PEG adlayer enable the intercalation of PL molecules (see Fig. 2(A)) that extend sufficiently to support neuron adhesion but are sufficiently protected, perhaps by steric hinderance, to prevent media supplements from dissociating the PL molecules from the surface. Further experiments are required to fully elucidate the mechanisms underpinning the stability of PL patterns printed on PLL-*g*-PEG adlayers. Nevertheless, these results demonstrate the importance of surface derivatization with PLL-*g*-PEG in advance of microcontact printing PL or PO.

Also in contrast to the SH-SY5Y cell line, primary neuronal networks initially had a large number of tracks occupied by neurons ($cit_{mouse} \approx 23\%$; $cit_{SH\text{-}SY5Y} \approx 15\%$). By 4 DIV patterned culture the pattern compliance was greatly improved (cit ≈ 7%). Migration and clustering is a characteristic of embryonic neurons. After 5 days there was a drop in occupancy levels as some neurons vacated their original nodes to co-localise with neurons on neighbouring nodes, in many cases developing neurites that extended over nodes to connect with more distant neurons. As a consequence a small number of nodes are vacated of cell soma, producing a significant impact on the occupancy levels and especially the cpn value (previously determined using a network probability simulation and a cell seeding dilution experiment[9]). These depleted values suggest network deterioration that is contrary to the true quality of the neuronal networks. For instance, the immunostaining image (see Fig. 4(C)) was taken at 9 DIV and documents the healthy molecular organisation (MAP-2 and β-III-tubulin) of the neuronal network. To ensure network measures appropriately account for inherent cellular clustering, the node occupancy and connections per node definitions were revised: a vacant node with overlapping outgrowths connecting other nodes was also classified as an occupied node. This subsequently elevates the connection per node values. The effects of this revision are shown in ESI Fig. 8.†

For the adhesion and differentiation of murine (CGR8) and human (LUHMES) neuronal precursor cells, poly-lysine (PL) is substituted with poly-ornithine (PO), a structurally and chemically similar molecule (see Fig. 5(A)). Zeta potential measurements show that PL and PO have near identical Langmuir isotherms resulting in a steady-state surface charge density of ∼20 mV (see Fig. 5(B)), a value significantly higher than with aminosilane surface derivatisation. Microstamped PL has been shown to have the same layer thickness and capacity for neuron adhesion as adsorbed PL (using a photoresist lift-off strategy[22]). Analysis of polyamine adsorption using zeta potential measurements therefore provides a useful indication of the surface quality following μCP. A 7 DIV pattern compliance experiment was used to compare PL and PO prints. Occupancy levels (∼95%) and cpn development rates were also near identical, demonstrating that the two polyamines are functionally equivalent in terms of neuron adhesion, viability and pattern compliance (see Fig. 5(C and D)).

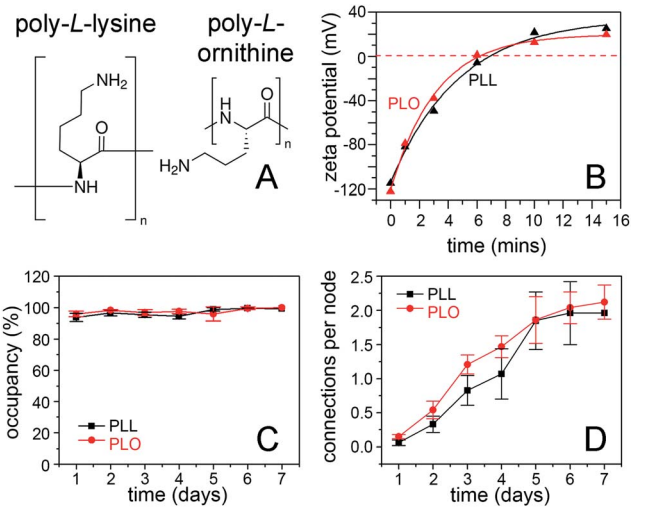

Fig. 5 Impact of substituting poly-L-lysine (PLL) with poly-L-ornithine (PLO). PLL and PLO differ by a single atom in the length of their primary amino group carbon chain (A). Zeta potential measurements demonstrate that this does not affect the rate of adsorption onto glass substrates, and result in equivalent surface charge densities (∼20 mV (B)). The different adhesion polyamines produce the same occupancy levels (C) and the same rates and quality of network formation during 7 DIV culture (D).

This line of work was extended to evaluate the addition of laminin, an adhesion factor used for neuronal precursor cells. A printed mixture of PO and laminin did not support the patterned culture of neurons, perhaps as a result of the formation of hetero-aggregates that are less tightly tethered to the glass surface. The protein rejecting quality of the PLL-*g*-PEG adlayer can be used to solve this problem: as before PO can be printed on the PEGylated surface to provide a stable anchor, followed by a backfill with laminin (overnight incubation with 10 μg mL^{-1}) to co-localise with the PO. In preliminary experiments, this approach was used for patterning neuronal precursor cells (CGR8) with high pattern compliance. Further studies are required to fully evaluate the robustness of this method and the longevity of the neuronal network patterns.

Surface stability during long term storage is desirable for distributed testing. The PLL-*g*-PEG coated surfaces are sensitive to atmospheric O_2, decaying by −50 mV in 2 months. Storage in a 100% N_2 atmosphere greatly prolongs the shelf-life with only a ∼15 mV reduction in the zeta potential during storage for over a year (*i.e.* ∼20-fold increased stability; see ESI, Fig. 9†). However, the straightforward PLL-*g*-PEG surface derivatization method and the simple polyamine printing method (with or without a protein backfill step) enable the end-user to prepare biomaterial microarrays in their own laboratories. With this strategy only the highly stable mould master or a polymer replica need to be distributed to neuroscience laboratories.

Conclusions

Microcontact printing polyamines onto PEGylated glass substrates is a straightforward and highly effective method for

the micropatterned culture of primary neurons. The coupling of the polyamines with the PEGylated glass surface provides a stable material contrast for high pattern compliance during long-term (>1 month) culture. By the addition of an ECM backfill, the method has the potential to be extended to the micropatterned culture of murine neuronal precursor cells to eliminate the need to sacrifice animals and human neuronal precursor cells to provide authentic models of the human nervous system. The method has been used for the quantitative assessment of the development of primary neuronal networks, demonstrating the potential for screening the neurotoxic and pharmaceutical effects of test substances, and also the realisation of advanced implantable neuronal interfaces.

Acknowledgements

The authors are grateful to Ulrich Marggraf (ISAS) for SU-8 fabrication. The LUHMES and CRG8 neuronal precursor cells used in this research were a kind gift from Marcel Leist (Universität Konstanz). The research was financially supported by the Deutsche Forschungsgemeinschaft (DFG WE3737/3-1), a Bundesministerium für Bildung und Forschung grant (BMBF 0101-31P6541) and by the Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen. Heike Hardelauf thanks the International Leibniz Graduate School "Systems Biology Lab-on-a-Chip" for financial support.

References

1 Z. M. Wissner-Gross, M. A. Scott, D. Ku, P. Ramaswamy and M. F. Yanik, *Integr. Biol.*, 2011, **3**, 65–74.
2 A. K. Vogt, G. Wrobel, W. Meyer, W. Knoll and A. Offenhäusser, *Biomaterials*, 2005, **26**(15), 2549–2557.
3 D. Kleinfeld, K. H. Kahler and P. E. Hockberger, *J. Neurosci.*, 1988, **8**(11), 4098–4120.
4 M. S. Ravenscroft, K. E. Bateman, K. M. Shaffer, H. M. Schessler, D. R. Jung, T. W. Schneider, C. B. Montgomery, T. L. Custer, A. E. Schaffner, Q. Y. Liu, Y. X. Li, J. L. Barker and J. J. Hickman, *J. Am. Chem. Soc.*, 1998, **120**(47), 12169–12177.
5 K. Czöndör, M. Garcia, A. Argento, A. Constals, C. Breillat, B. Tessier and O. Thoumine, *Nat. Commun.*, 2013, **4**, 2252.
6 A. Ruiz, L. Buzanska, D. Gilliland, H. Rauscher, L. Sirghi, T. Sobanski, M. Zychowicz, L. Ceriotti, F. Bretagnol, S. Coecke, P. Colpo and F. Rossi, *Biomaterials*, 2008, **29**, 4766–4774.
7 J. C. Clarke, B. W. Tuft, J. D. Clinger, R. Levine, L. Sievens Figueroa, C. A. Guymon and M. R. Hansen, *Hear. Res.*, 2011, **278**, 96–105.
8 B. W. Tuft, S. Li, L. Xu, J. C. Clarke, S. P. White, B. A. Guymon, K. X. Perez, M. R. Hansen and C. A. Guymon, *Biomaterials*, 2013, **34**, 42–54.
9 J. P. Frimat, J. Sisnaiske, S. Subbiah, H. Menne, P. Godoy, P. Lampen, M. Leist, J. Franzke, J. G. Hengstler, C. van Thriel and J. West, *Lab Chip*, 2010, **10**, 701–709.
10 H. Hardelauf, J. Sisnaiske, A. Taghipour-Anvari, P. Jacob, E. Drabiniok, U. Marggraf, J. P. Frimat, J. G. Hengstler, C. Neyer, C. van Thriel and J. West, *Lab Chip*, 2011, **11**, 2763–2771.
11 P. Shi, M. A. Scott, B. Ghosh, D. P. Wan, Z. Wissner-Gross, R. Mazitschek, S. J. Haggarty and M. F. Yanik, *Nat. Commun.*, 2011, **2**, 510.
12 R. Buckmaster, F. Asphahani, M. Thein, J. Xu and M. Q. Zhang, *Analyst*, 2009, **134**(7), 1440–1446.
13 A. M. Taylor, M. Blurton-Jones, S. W. Rhee, D. H. Cribbs, C. W. Cotman and N. L. Jeon, *Nat. Methods*, 2005, **2**(8), 599–605.
14 H. Francisco, B. B. Yellen, D. S. Halverson, G. Friedman and G. Gallo, *Biomaterials*, 2007, **28**(23), 3398–3407.
15 N. D. Dinh, Y. Y. Chiang, H. Hardelauf, J. Baumann, E. Jackson, S. Waide, J. Sisnaiske, J. P. Frimat, C. van Thriel, D. Janasek, J. M. Peyrin and J. West, *Lab Chip*, 2013, **13**, 1402–1412.
16 M. Textor, D. Falconnet, G. Csucs and H. M. Grandin, *Biomaterials*, 2006, **27**(16), 3044–3063.
17 G. M. Whitesides, E. Ostuni, S. Takayama, X. Y. Jiang and D. E. Ingber, *Annu. Rev. Biomed. Eng.*, 2001, **3**, 335–373.
18 A. Folch and M. Toner, *Annu. Rev. Biomed. Eng.*, 2000, **2**, 227–256.
19 C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides and D. E. Ingber, *Science*, 1997, **276**(5317), 1425–1428.
20 A. Bernard, J. P. Renault, B. Michel, H. R. Bosshard and E. Delamarche, *Adv. Mater.*, 2000, **12**(4), 1067–1070.
21 R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber and G. M. Whitesides, *Biomaterials*, 1999, **20**, 2363–2376.
22 D. W. Branch, J. M. Corey, J. A. Wehenmeyer, G. J. Brewer and B. C. Wheeler, *Med Biol Eng Comput*, 1998, **36**(1), 135–141.
23 R. Fricke, P. D. Zentis, L. T. Rajappa, B. Hofmann, M. Banzet, A. Offenhäusser and S. H. Meffert, *Biomaterials*, 2011, **32**, 2070–2076.
24 J. P. Frimat, H. Menne, A. Michels, S. Kittel, R. Kettler, S. Borgmann, J. Franzke and J. West, *Anal. Bioanal. Chem.*, 2009, **395**(3), 601–609.
25 A. Tourovskaia, T. Barber, B. T. Wickes, D. Hirdes, B. Grin, D. G. Castner, K. E. Healy and A. Folch, *Langmuir*, 2003, **19**(11), 4754–4764.
26 J. Corey and E. L. Feldman, *Exp. Neurol.*, 2003, **184**, 89–96.
27 A. Azioune, M. Storch, M. Bornens, M. Théry and M. Piel, *Lab Chip*, 2009, **9**(11), 1640–1642.
28 H. Sorribas, C. Padeste and L. Tiefenauer, *Biomaterials*, 2002, **23**, 893–900.
29 J. Corey, B. C. Wheeler and J. G. Brewer, *J. Neurosci. Res.*, 1991, **30**, 300–307.
30 G. L. Kenausis, J. Vörös, D. L. Elbert, N. P. Huang, R. Hofer, L. Ruiz-Taylor, M. Textor, J. A. Hubbell and N. D. Spencer, *J. Phys. Chem. B*, 2000, **104**, 3298–3309.
31 J. R. Li and J. C. Garno, *Nano Lett.*, 2008, **8**(7), 1916–1922.
32 F. Cecchet, B. De Meersman, S. Demoustier-Champagne, B. Nysten and A. M. Jonas, *Langmuir*, 2006, **22**, 1173–1181.
33 D. Scholz, D. Poltl, A. Genewsky, M. Wenig, T. Waldmann, S. Schildknecht and M. Leist, *J. Neurochem.*, 2011, **119**(5), 957.

34 D. W. Branch, B. C. Wheeler, G. J. Brewer and D. E. Leckland, *IEEE Trans Biomed Eng*, 2000, **47**(3), 290–3000.
35 N. P. Huang, R. Michel, J. Vörös, M. Textor, R. Hofer, A. Rossi, D. L. Elbert, J. A. Hubbell and N. D. Spencer, *Langmuir*, 2001, **17**, 489–498.
36 S. Pasche, S. M. De Paul, J. Vörös, N. D. Spencer and M. Textor, *Langmuir*, 2003, **19**, 9216–9225.
37 M. A. Scott, Z. D. Wissner-Gross and M. F. Yanik, *Lab Chip*, 2012, **12**, 2265–2276.
38 J. M. Corey, B. C. Wheeler and G. J. Brewer, *IEEE Trans Biomed Eng*, 1996, **43**(9), 944–955.
39 F. Zhang, K. Sautter, A. M. Larsen, D. A. Findley, R. C. Davis, H. Samha and M. R. Linford, *Langmuir*, 2010, **26**(18), 14648–14654.
40 J. W. Lussi, D. Falconnet, J. A. Hubbell, M. Textor and G. Csucs, *Biomaterials*, 2006, **27**, 2534–2541.
41 D. W. Branch, B. C. Wheeler, G. J. Brewer and D. E. Leckland, *Biomaterials*, 2001, **22**, 1035–1047.