Nanostructured interfaces with RGD arrays to control cell–matrix interaction

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Surface nanofeature of tissue-repairing materials is a crucial aspect which governs cellular function and healing response. Emerging studies have developed various materials coupled with cell-adhesive ligands such as the peptide of arginine-glycine-aspartic acid (RGD) to mimic extracellular matrix (ECM). Herein, we highlight the recent progress in fabrication of RGD nanoarrays on rigid and soft supports to tailor cell adhesions. These interfaces are generated via the newly-established transfer nanolithography combined with block copolymer micelle nanolithography with or without addition of order-interfering reagents. The cellular responses depend strongly on the chemo-mechanical features of such interfaces. Therefore, the novel material techniques encourage one to extensively investigate the cell-material crosstalk on the molecular level, as well as to design new nanomaterials for regenerative medicine.

1. Introduction

Living cells in vivo sense and respond to environmental signals including chemical, physical and biological cues from the surrounding tissue components. In medical applications such as tissue engineering, the surfaces of materials regulate numerous physiological actions including cell metastasis, wound healing, tissue remodeling and regeneration etc. Once loaded on a material, most of cells tend to attach and spread on the surface along with rearrangement of cytoskeleton. The specific cell adhesion is usually triggered by bioconjugation of ligands containing some peptide sequences such as arginine-glycine-aspartic acid (RGD) to their receptors such as integrins in the plasma membrane. As the first event of cellular response to a material, cell adhesion influences cell morphology, vitality, motility and their capability for proliferation, differentiation and apoptosis.

A strategy to improve biomaterials for regenerative medicine is mimicking the extracellular matrix (ECM), and RGD sequences embedded in biomimetic ECM are especially useful in biomaterial design. As previously discovered, the interface between cells and ECM is, in many cases, structured at the nanometre scale. As a consequence, prospective designs of new biomaterials rely on extensive fundamental researches of biomimetic interfaces with well-defined model nanostructures.

The nanoscale organization of ECM ligands such as RGD peptides on non-fouling background affords a valuable model to study cellular responses to ECM motifs on the molecular level. So far, it has been revealed that the interfacial arrangement and specificity of ligands exert strong effects on cell fate.
block copolymer micelle nanolithography technique affords a unique approach to make such models for mimicking ECM to detect cellular responses. Very recently, this technique has been developed to generate, in parallel, both ordered and disordered RGD nanopatterns, which enables deeper understanding of the supermolecular mechanism of specific cell adhesion; and even soft polymeric substrates have become available to provide biomimetic surfaces that are decorated by RGD nanopatterns.

The present review highlights the recent progress in the fabrication of innovative biomimetic interfaces decorated with RGD nanopatterns on both inorganic and polymeric supports. Corresponding cell studies based on these models reveal chemomechanical cues for cell adhesion. The remaining challenges will be commented as well.

2. Fabrication of ordered/disordered nanopatterns and cellular responses to the nanoscopic arrangement of RGD

While there are various ways to generate surface nanopatterns, the block copolymer micelle nanolithography technique is very efficient and precise to prepare extensive nanopatterns for tuning cell behaviors. This technique first involves the fabrication of gold (Au) nanoparticle arrays based on the self-assembly of polystyrene-block-poly(2-vinyl pyridine) (PS-b-P2VP) diblock copolymer micelles loaded with the metal precursor (HAuCl4). By dip-coating and subsequent plasma treatment, well-defined positioning of hexagonally arranged Au nanoparticles on inorganic substrates (e.g., glass, Si or mica wafers) can be achieved (Fig. 1a–c). The nanoscopic dimensions of the pattern such as the nanoparticle diameter and interparticle distance may be controlled by varying the preparation conditions such as the block lengths of PS-b-P2VP polymer, the concentration of PS-b-P2VP micelles, the pulling speed of substrates during dip-coating and the loading amount of metal precursor for micelles. So far, this approach has proven to be highly versatile to decorate gold, cobalt, platinum, palladium, iron or nickel nanoparticle patterns, with particle diameters down to 3–20 nm, onto plasma-resistant substrates. Moreover, up to now, by using the PS-b-P2VP polymers with appropriate matching of block lengths and the preparation conditions tried, a great many nanopatterns with different interparticle distances between 25 nm and 200 nm were obtained, which were usually varied in certain distance gaps for our current investigation of cellular activities.

In the next preparation step, the plain surface and the space between nanoparticles are passivated with a 2–3 nm thick protein-repellent film by covalently grafting linear poly(ethylene glycol)-silane (PEG-silane) molecules to form a self-assembled monolayer (SAM) which resists cell adhesion. Various cell-adhesive motifs for transmembrane receptor binding were anchored onto nanostructured Au. Those ligands and receptors could be found, for instance, among cyclo(arginine-glycine-aspartic acid-d-phenyl alanine-lysine-)[c(RGDfK)-j versus integrin (e.g., α5β1 and αβ3),13–15,24–26 neuron-specific DM-GRASP as one of the cell adhesion molecules of immunoglobulin superfamily (IgSF-CAM) versus another IgSF-CAM molecule in cell membrane,27,28 or the apoptotic ligand tumor necrosis factor (TNF) versus death receptors such as TNFR1 and TNFR2. The ligands flanking c(-RGDfK-) designed by Kessler et al. is especially attractive. As shown in Fig. 1d, the Au nanoparticles, usually on one side of the dipping line, is coupled with c(-RGDfK)-thiol ligands for inducing specific recognition of single integrin receptors in adherent cell membrane. Cell experiments illustrate that the PEG-passivation and RGD-functionalization are successful and a clear borderline could be visualized after cell culture (Fig. 1e). Herein, the initial arrangement of Au nanoparticles well reflects the lateral arrangement of ligands and thus receptors in focal adhesion (FA) plaques.

Cellular response to RGD nanopatterns on nonfouling background relies on many structural and chemical properties of such surfaces, e.g., ligand organization, affinity of ligands for specific receptor binding as well as the property of PEG layer. Past researches concerning randomly dispersed RGD ligands suggest that cell adhesion and spreading can be dramatically reduced when the average ligand spacing is beyond tens of nm, or even as large as a few hundreds of nm. Lately, a more conclusive criterion of the critical interligand distance for

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Presentation of the block copolymer micelle nanolithography technique. (a) Sketch of dip-coating micelles loaded with HAuCl4 precursor for making Au nanopatterns on inorganic substrates. (b and c) Scanning electron microscopic (SEM) images of a Si substrate decorated with hexagonally assembled PS-b-P2VP micelles loaded with HAuCl4 (b) and Au nanoparticles reduced after plasma treatment (c). (d) A low-magnification SEM image showing the borderline (arrow) between a nanopatterned region and a plain PEG-passivated region on glass. The space between Au nanoparticles on inorganic substrates varied in certain distance gaps for our current investigation of cellular activities. (e) A fluorescent micrograph of MC3T3-E1 osteoblasts cultured on RGD nanopattern with an average ligand spacing of 42 nm, showing the clear borderline (arrow) between the RGD nanopatterned region (left) and plain PEG region (right) as marked by adherent cells. Here, the cells were fluorescently stained for actin and nuclei after 48 h in culture.
specific cell adhesion is based upon well-defined nanopatterns obtained via block copolymer micelle nanolithography as mentioned above. By this approach, Spatz et al. reported that the interfacial patterning of c(-RGDfK-) ligands dramatically influences cell adhesion quality and dynamic cell behaviors.\(^\text{13,14,24,25}\) A very recent report indicates that the order of a nanopattern may also be adjusted by adding a specific amount of polystyrene (PS) as an order-interfering reagent.\(^\text{15}\) A series of nanopatterns with both ordered and disordered arrangements of RGD under various average densities were fabricated and associated cell adhesion behaviors were observed. The effect of order degree is closely related to the effect of RGD ligand spacing. As shown in the top row of Fig. 2a, a critical interligand distance of \(\approx 70\) nm (an average ligand density of \(\approx 231\ \mu^2\)) was found on hexagonally ordered RGD nanopatterns, above which cell attachment and spreading were significantly restricted. The negative cellular response to interligand distance beyond \(\approx 70\) nm could be ascribed to the lack of effective integrin clustering and the failure in subsequent formations of FA complexes and cytoskeleton networks (Fig. 2b).

According to this report, the impact of order degree of an RGD nanopattern on cell adhesion is not significant if the global average interligand distance is less than \(70\) nm. Interestingly, specific cell adhesion is still “turned on” on the disordered RGD nanopatterns when the global average interligand distance is larger than \(70\) nm.\(^\text{15}\) This observation indicates that

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**Fig. 2** (a) Fluorescent micrographs of MC3T3-E1 osteoblasts labelled for actin and nuclei after 24 h in culture on comparative ordered and disordered nanopatterns under various average RGD densities. All the supports for Au nanopatterns used here were PEG-passivated glass, and the Au nanoparticles were coupled with c(-RGDfK-) thiol ligands. Atomic force microscopic (AFM) images of Au nanopatterns are shown as the inset in each fluorescent micrograph. The numbers on the top left represent the average interligand distances in units of nm, where “D” indicates the disordered nanopattern. (b) Schematic illustration of the assumed cytoskeleton assembly associated with FA formation that might be regulated by integrin clustering based on RGD nanopatterns of different ligand spacings. A spacing of \(<70\) nm between neighboring ligands results in effective integrin clustering, leading to the formations of FA complex and F-actin network, whereas a spacing of \(>70\) nm results in no integrin clustering. (Reproduced with permission from ref. 15, copyright 2009, American Chemical Society).
cell adhesion on disordered nanopatterns can be activated at less global average ligand density than on ordered ones. Herein, we propose that the “turning on” of cell adhesion on large disordered RGD nanopatterns is due to the polydispersity of local interligand distances which leads to the formation of local integrin clusters in the cell membrane. Based on hierarchically nanostructured RGD patterns, Arnold et al. supposed that the minimal number of RGD enabling effective FA formation might be six per adhesion site.\textsuperscript{26} In general, these PEG-passivated RGD nanopatterns mimic ECM to a certain extent and serve as a good model system to fundamentally investigate cell-biomaterial interactions.

3. Fabrication of RGD nanopatterns on soft substrate

For mimicking the cellular environment in the most tissue analogs, a soft polymeric support is believed to be more appropriate than a rigid inorganic support, where biomechanics is concerned. As a unique type of soft matter, hydrogels have been paid much attention in the past decade.\textsuperscript{34–55} While porous scaffolds constitute the main material form in the field of tissue engineering,\textsuperscript{56–65} injectable hydrogels have recently been developed as a complementary form.\textsuperscript{42,66–70} For instance, PEG-based hydrogels have been widely used for pharmaceutical, tissue engineering and surface patterning applications, due to its biocompatibility, tunability of stiffness, and non-fouling property (resistant to nonspecific protein adsorption and thus nonspecific cell adhesion).\textsuperscript{35,71–75} Accordingly, compared with a rigid substrate passivated by PEG SAM, a PEG hydrogel is considered to be a superior platform for investigating \textit{in vitro} cellular responses. Extended effort dedicated to incorporating cell-adhesive ligands into synthetic ECM materials has led to the development of various strategies for modification of PEG hydrogels.\textsuperscript{2,35,36,72,75,76} They mostly encompass the covalent grafting of ligands to 3-D polymer networks or surface immobilization to prefabricated substrates, which, however, suffer from the precise determination of interligand distance, cluster number of ligands and the lack of ligand order. It is, so far, very difficult to straightforwardly decorate PEG hydrogels by regular RGD arrays due to the chemical inertness of PEG.

Recently, the groups of Spatz and Ding put forward a transfer lithography technique to resolve this problem.\textsuperscript{96,97} Combination of the transfer lithography and block copolymer micelle nano lithography gives an access to construct desired Au nanopatterns on polymer surfaces, and further RGD immobilization results in well-defined RGD nanopatterns. The general procedure of transfer nanolithography technique is shown in Fig. 3a. First of all, Au nanopatterns are deposited on inorganic supports (e.g., glass, Si or mica wafers) by means of diblock copolymer micelle nanolithography as described above. Then linker molecules such as 2-propene-1-thiol are chemically immobilized onto Au nanostructures through the thiol end groups. Afterwards, a layer of macromers such as poly(ethylene glycol) diacrylate (PEGDA) covers the nanostructured inorganic support, and polymerization of macromers gives a PEG hydrogel, by which the Au nanostructures are then well attached onto the polymer support. The last step is the separation of the inorganic support and the polymer layer by ways of mechanical peeling or swelling in a solvent such as water. As a result, the initial Au nanopatterns produced on plasma-resistant inorganic supports are transferred onto soft polymeric substrates.

As demonstrated in our work, this transfer nanolithography technique could be extended to various polymers, from hydrophilic PEG hydrogels to hydrophobic materials like polystyrene (PS) and poly(dimethylsiloxane) (PDMS).\textsuperscript{16} Besides, this transfer lithography technique can be applied to different patterns at both the nano- and micro-scales.\textsuperscript{77–80} Take PEG hydrogels as an example, Au nanopatterns may be well transferred onto such gels that are formed by polymerization of PEGDA macromers of different molecular weights, ranging from 700 to 10 000 (Fig. 3b–d), with elastic modulus varied among several orders of magnitude. Here as well, to promote integrin-mediated cell adhesion, c(-RGDfK-)-thiol ligands are subsequently immobilized onto interfacial Au

![Fig. 3](image-url) (a) Schematic presentation of the transfer nanolithography technique which transfers a metallic nanoarray to a polymer support. (b–d) Cryo-SEM images of Au nanopatterns transferred onto water-swollen PEG hydrogels crosslinked from (b) PEGDA 700 (c) PEGDA 4000 (d) PEGDA 10 000 macromers. All the cryo-SEM images here were scanned in EsB detector mode.
nanopatterns with PEG hydrogel as a support. Thereby, a well-controlled synthetic template which potentially mimics a physiological tissue environment has been set up, which could be used to test cellular responses to tunable ligand arrangement and substrate stiffness in a microenvironment. Hereof, the arrangement of ligands and substrate stiffness are two independent parameters that could be decoupled from each other in practice.

Cell morphology on nanostructured PEG hydrogels with hexagonally arranged RGD ligands of different lateral spacings is shown in Fig. 4. Cells adhere and spread quite well on such gel surfaces with average ligand spacing of about 40 nm (Fig. 4a). In agreement with previous cell studies on PEG-passivated rigid substrates, cell attachment and spreading become highly restricted when the average lateral spacing of RGD nanoarray is beyond 70 nm (Fig. 4b and c). After culturing fibroblasts for over 14 days on such gels that partially decorated with RGD nanoarrays, a clear borderline marked by densely adhering cells was still observed (Fig. 4d), which reveals the long-term bioactivity of the RGD ligands and the long-term nonfouling property of the PEG hydrogel.

4. Nanopatterning on a curved surface of PEG hydrogel

Most tissue engineering applications require biocompatible analogs that mimic the three dimensional (3-D) complex ECM. Over the past decades, steady progress in nanotechnology and cellular biology has enabled us to investigate the crosstalk between material nanostructure and cell activity. Various synthetic biomaterials with two-dimensional (2-D) and 3-D nanostructures have been developed intending to probe living cell functions.

Lately the construction of RGD nanopatterns onto 3-D synthetic ECM analogs is made to be available. One of the emerging developments has been realized by extending the transfer nanolithography technique from planar surfaces to curved surfaces. Fig. 5 shows a PEG hydrogel microtube with the interior channel wall decorated by Au nanopattern. During the transfer process, a glass fiber was firstly decorated with Au nanoparticles that were functionalized with linker molecules (e.g., 2-propene-1-thiol), and then embedded in PEGDA macromers. After formation of the hydrogel via polymerizing macromers, the glass fiber was removed. Then (c-RGDfK)-thiol was used to functionalize Au nanopatterns. As a result, a microchannel with its internal wall covalently patterned with RGD nanoarrays was produced. According to the preliminary cell studies, HeLa cells could adhere well on the internal wall of the microchannel.

5. Summary and perspectives

In this paper, we have highlighted the nanostructured interfaces with RGD arrays that are constructed on both rigid and soft supports. It has been demonstrated that cell activity is highly correlated with the affinity, spacing, density and even order of such RGD ligands. However, the development of nanostructured ECM-mimetic materials for probing cellular activities and in vivo applications still remain significant challenges.

Fully understanding the relationship between extracellular signals and cellular behaviors upon interaction with synthetic materials is still far away. Besides the research progress introduced in this highlight, surface topographic cues for inducing cellular responses could be seen in some recent reviews. Cellular responses to various adhesive motifs and their arrangement, substrate stiffness, external forces, chemical and topological signals is required to be further examined systematically. Besides adhesion of cells, migration, differentiation and apoptosis of different cell types (e.g., neurons, fibroblasts, osteoblasts and cancer cells) are ready to be investigated.

One important future area of research that is worthy of emphasis might be the...
examination of cellular responses to different stiffness of materials on nanostructured interfaces. It was discovered that the behavior of cells is significantly influenced by the mechanical properties of substrate. The development in fabrication techniques of nanopatterns and micropatterns on hydrogels reshape this field by affording a feasible way to adjust the stiffness of substrate while keeping the intact surface patterns. The viscoelasticity of PEG hydrogel can be adjusted by changing the molecular length of PEGDA macromers and their crosslinking density. Future work on how cells interact with patterned PEG hydrogels of varied viscoelasticity is still ongoing.

Another challenge comes from the research in cell and molecular biology. For instance, both inside-out and outside-in chemo-mechanical signals across the cell-material interface require exploration. These investigations might elucidate the details of the supermolecular network related to interactions among intracellular proteins and between those proteins and the ECM signals. The underlying mechanism of the critical interligand distance in FA formation is especially required to be revealed.

In light of material improvement and potential guidance of medical applications, various architectures of nano-, micro- or micro-nano hybrid structures would be tailored on much more complex 2-D or 3-D materials for directing specific cell functions. Besides understanding the basic cellular physiological principles, future research might be directed towards designing new integrated nanodevices serving for diagnosing, guided differentiation, anti-apoptotic, anti-cancerization and other biomedical applications.

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