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Biological surface engineering: a simple system for cell pattern formation

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Abstract

Biological surface engineering using synthetic biological materials has a great potential for advances in our understanding of complex biological phenomena. We developed a simple system to engineer biologically relevant surfaces using a combination of self-assembling oligopeptide monolayers and microcontact printing (μCP). We designed and synthesized two oligopeptides containing a cell adhesion motif $(RADS)_n$ ($n = 2$ and 3) at the N-terminus, followed by an oligo(alanine) linker and a cysteine residue at the C-terminus. The thiol group of cysteine allows the oligopeptides to attach covalently onto a gold-coated surface to form monolayers. We then microfabricated a variety of surface patterns using the cell adhesion peptides in combination with hexa-ethylene glycol thiolate which resist non-specific adsorption of proteins and cells. The resulting patterns consist of areas either supporting or inhibiting cell adhesion, thus they are capable of aligning cells in a well-defined manner, leading to specific cell array and pattern formations. © 1999 Elsevier Science Ltd. All rights reserved

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1. Introduction

The understanding of complex biological phenomena typically requires the development of new biological materials and novel technologies. The ideal biological material should be amenable to molecular design, easy synthesis and to be tailored for a broad range of applications. For example, in order to study cells in a well-controlled manner and to make precise manipulation of cell arrangement, specific materials and surface modifications are required. To achieve this goal, it is possible to select specific substrates, such as extracellular matrix proteins and specific motifs that interact with cell adhesion molecules to support cell adhesion on special designed surface patterns. We have developed a simple technology to arrange cells based on a combination of

self-assembling oligopeptides [1–3] and microcontact printing (μCP) [4, 5]. This development may lead to advances in biological surface engineering, permitting new types of experiments to probe the details of cell physiology.

Over the last few years, major advances in microcontact printing (μCP) on surfaces have been made [5–14]. Microcontact printing employs molecular self-assembly—the spontaneous association of molecules under thermodynamic equilibrium conditions—into stable and ordered structures due to the formation of noncovalent bonds [7]. This technology takes advantage of the formation of self-assembled monolayers (SAMs), which undergo molecular self-organizations on surfaces. SAMs are a structural characteristic of various organic molecules that can align on a surface into two-dimensional, quasi-crystalline domains. This technique is now widely used for surface modification and microfabrication for cell pattern formation [5–18]. There has been a wide spectrum of applications of μCP in electronics [6],

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sensors [7], surface catalysis [8], microseparation [9], adsorption of protein [10] and adhesion of cells to surfaces [11–18]. These modified surfaces can also be applied to arrange cells in specific patterns. In these applications, some regions of the surface are specifically patterned in order to present functional groups that adsorb proteins for cell attachment. The remaining regions are modified to present groups that inhibit adsorption of proteins and subsequent cell attachment [9–12].

Several investigators have developed other microfabrication methods for biological surface modification by adsorbing proteins and other substances onto solid surfaces for specific molecular recognition, biosensors [19, 20] and cell attachment [21–33]. For example, photolithography has often been used to create these micropatterns, followed by the adsorption of proteins and cells to the solid surface. Since protein adsorption is largely dependent upon non-specific interactions between the protein and the surface, these methods cannot orient the adsorbed proteins to uniformly expose the desired ligands. Many compounds have been used to generate SAMs presenting functional groups that either support or inhibit adsorption of extracellular matrix proteins for subsequent cell attachment [11–15]. The combination of SAM technology and μCP allows dictation of cell shape through the placement of cells in predetermined regions separated by defined distances [12, 13]. Recently, Chen et al. used this technique to correlate cell attachment, spreading and apoptosis [14, 15].

We have previously created several different types of self-assembling oligopeptides [1–3]. Type I peptides are based on the intermolecular self-assembly of ionic self-complementary β-sheets in which the peptide molecules coalesce together through molecular recognition to form a matrix—a new biological material that is currently being developed as scaffolding for tissue engineering. Type II peptides employ intramolecular and intermolecular self-assemblies: this type of peptide can undergo conformational changes under various conditions and can potentially be developed as a molecular switch [34]. Type III oligopeptides spontaneously attach to surfaces to form monolayers, thereby modifying the surface properties.

Our new system using the Type III peptides as surface modifiers by means of microcontact printing has several advantages: (1) the oligopeptides can be engineered to incorporate multiple features; (2) oligopeptides can be readily synthesized by well-developed chemical methods and purified to homogeneity by HPLC; (3) the peptide sequence can control the orientation of ligands presented at the surface; and (4) provides an opportunity to study cell–material and cell–cell interactions in great detail. This technique not only eliminates the non-specific surface adsorption of extracellular matrix proteins, but it also simplifies synthesis of surface materials, therefore, represents a significant step forward in biological surface engineering.

We have additionally demonstrated the ability to generate various two-dimensional geometric configurations with specific features on the micrometer scale using a rapid prototyping technique [35]. Some patterns have linear arrays, while others have squares with connecting strips of defined shape, size, and distance. Here we report that this biological surface engineering technique leads to well-defined pattern formation in a variety of cell types.

2. Materials and methods

2.1. Gold-coated glass slides and EG_6SH

Preparation of (11-mercaptoundec-1-yl)-hexa-(ethylene glycol) $(HO(CH_2CH_2O)_6(CH_2)_{11}SH$ or EG_6SH) has been described previously [36]. The gold substrates were prepared by electronic beam evaporation of 2 nm of titanium and 12 nm of gold onto a pre-cleaned microscope glass cover slide [36].

2.2. Peptides

Reagents for materials peptide synthesis were purchased from Rainin Instrument (Woburn, MA) and Anaspec (San Jose, CA). The RADSC-14 peptide was synthesized using solid-phase t-Boc chemistry with an automated peptide synthesizer (Applied Biosystem 430A); RADSC-16 and other peptides were synthesized using solid-phase F-moc chemistry with a Rainin PS3 peptide synthesizer. The crude peptides were purified by HPLC and characterized by mass spectroscopy and complete amino acid hydrolysis. The peptides were dissolved in distilled, deionized water and filtered through a 0.22 μm filter. The solution was then adjusted to 2 mM concentration and stored at 4°C until used.

2.3. Oxygen plasma treatment

The PDMS stamp was oxidized briefly by oxygen plasma for about 10 s at approximately 0.2 Torr O_2 pressure in a Harrick plasma cleaner at the middle power setting. This procedure yielded a hydrophilic PDMS surface, which might contain silanol groups [37].

2.4. Cell cultures

Human epidermoid carcinoma A431 cells (ATCC CRL 1555) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH/3T3 mouse embryo fibroblasts (ATCC CRL 1658) were cultured in DMEM with 10% FBS. Transformed primary human embryonic kidney 293 cells (ATCC CRL 1573) were grown in MEM (modified Eagle's medium) with 10% FBS. All cells were

Table 1
Oligopeptides used for microcontact printing surfaces in this study

Name	Sequence (N–>C)	Note
RADSC-14	+ − + − RADSRADSAAAAAC	(RADS)$_{2-3}$ is the ligand,
RADSC-16	+ − + − + − RADSRADSRADSAAAC	AAAAA or AAA is the linker, C is the anchor

The oligopeptides were either synthesized by t-Boc chemistry (RADSC-14) or F-moc chemistry (RADSC-16) and purified by HPLC. They were dissolved in water at a concentration of 2 mM and filtered through a 0.22 μm filter before use.

cultured at 37°C under humidified 10% CO_2. Seeding densities for different cell types were 5×10^4 cells/6 well-cluster dish. Endothelial cells were isolated from freshly excised aortas of 3–4 week old calves (Area and Sons, Hopkinton, MA) by a previously described procedure [38]. Cell lines were cultured up to passage 7 in DMEM with 5.0% calf serum at 37°C in a humidified 5% CO_2 95% air incubator. About 2 ml of a 1×10^5 cells/ml suspension was seeded onto patterned substrates.

2.5. Design of type III oligopeptides

Type III oligopeptides used in this study consist of three distinctive features (Table 1 and Fig. 1A): (1) The ligand can be, in principle, a variety of functional groups for recognition by other molecules or cells. Since a peptide has two asymmetric N- and C-termini, the ligand can be located at either terminus of the peptide depending on how it is recognized by other biological substances. (2) A linker of variable length can be used to make the ligand free for interaction with proteins and cells. (3) The anchoring group is a chemical group on the peptide that can react with the surface to form a stable, covalent bond.

The cell adhesion motif RGD and its derivatives found in a variety of cell adhesion molecules have been used

Fig. 1. Self-assembling peptides for biological surface engineering: (A) molecular models of the oligopeptide RADSC-14 with the sequence RADSRADSAAAAAC and of ethylene glycol thiolate (EG$_6$SH). The N-terminal segment (RADS)$_2$ is the ligand for cell attachment, the five alanine segment, AAAAA, is a linker to the anchoring cysteine. The cysteine anchor is covalently bound to the gold atoms on the surface. The molecular models reveal that both molecules form self-assembled monolayers with different heights. The extended lengths of RADSC-14 and EG$_6$SH are approximately 5 and 4 nm, respectively. Color definition: carbon, light green; oxygen, red; nitrogen, blue; hydrogen, white; sulfur, yellow. (B) A schematic description of microcontact printing (μCP) using RADSC-14 oligopeptides and EG$_6$SH. A PDMS stamp (i) was oxidized using O_2 plasma, inked with a 5 mM ethanol solution of EG$_6$SH and dried under a stream of nitrogen. The inked stamp was placed on a gold substrate for approximately 1 min and peeled off carefully (ii and iii). The resulting gold substrate was immersed in 2 mM aqueous solution of RADSC-14 for 2 h, removed, rinsed with deionized and distilled water and ethanol, and dried under a stream of nitrogen (iv).
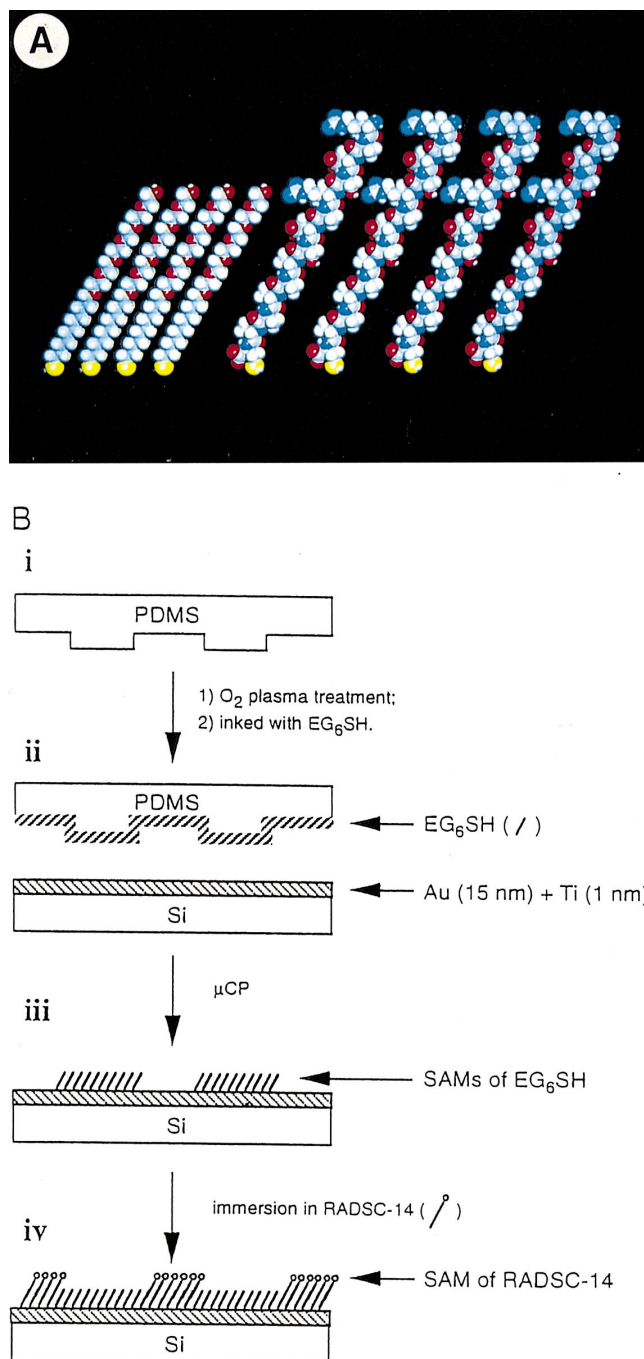
widely as substrate to support cell adhesion [39, 40]. In this study, we chose to use a ligand RADS (arginine–alanine–aspartate–serine), which appears to be a recognition motif for cell adhesion in a native extracellular matrix protein [41] found as a member of a large extracellular matrix protein family [39, 40]. In previous studies, we observed that oligopeptides containing the RAD motif are good adhesion substrates for attachment of a variety of cells [3]. We thus designed two peptides that contain two or three RADS motifs at the N-terminus (Table 1 and Fig. 1A). These peptides have a linker with either three or five alanines between the ligand and the C-terminal cysteine. The surface anchor is the thiol group on the side chain of cysteine, which can covalently attach to the gold substrate [11–13]. Other biological coating materials, e.g. poly-L-lysine, fibronectin, laminin, collagen gel, and Matrigel^{TM} are large complex molecules and their ligands for cell adhesion are not always exposed on the surfaces. The oligopeptides are short, simple and guarantee ligand exposure. These peptides can then be used as a substrate for microcontact printing along with EG_6SH as illustrated schematically in Fig. 1.

2.6. Pattern formation

The pattern master used to prepare the PDMS stamp was prepared using a rapid prototyping technique developed by Qin et al. [35]. The procedure to prepare the patterned substrates is illustrated in Fig. 1B and described briefly below. The PDMS stamp was briefly oxidized by oxygen plasma, inked with a 5 mM EG_6SH in ethanol solution, and dried with a stream of filtered nitrogen gas. The inked stamp was brought into contact with the gold substrate to transfer EG_6SH for 1 min at room temperature. The stamp was carefully peeled off from the gold cover slide. The resulting substrate was immersed in an aqueous solution of RADSC-14 for 2 h so that the SAM of oligopeptides can form on the underivatized regions. The slide was thoroughly rinsed with distilled, deionized water, followed by 70% ethanol to remove unattached peptides, then dried with a stream of nitrogen. The glass slides with patterned substrates were stored in a clean glass slide holder at room temperature until ready for use.

2.7. Photography

Photographs of patterned cells on chips were taken with an Olympus BX60F Normarski-type microscope from $50\times$–$400\times$ magnifications. Photos of patterned cells were taken with either live cells or cells that were first fixed with 4% formaldehyde in PBS. The various wavelengths were selected to enhance the contrast between cell patterns and the background.

3. Results

3.1. Formation of cell arrays

Several types of culture cells were used to study pattern formations. The human epidermoid carcinoma cells was used first. Figure 2 shows that the engineered surfaces permit the formation of cell arrays. Although the cell arrays are discontinuous, due to low-cell density at the time of image recording, the linear cell array pattern is easily recognizable in Fig. 2A. The cells on the patterned areas are confined in the peptide tracks and apparently do not cross over the tracks (Fig. 2B). The morphology of the cells appears indistinguishable from cells on non-patterned areas (not shown).

In the same linear cell arrays, mouse fibroblast cells with elongated processes exhibited general alignment along the tracks coated with the oligopeptide (Fig. 3A). They have a tendency to avoid attachment to the alternate EG_6SH tracks, but some cells cross over these tracks (Fig. 3). More details of these linear cell arrays are shown in Fig. 3B. The fibroblast cells cross the EG_6SH
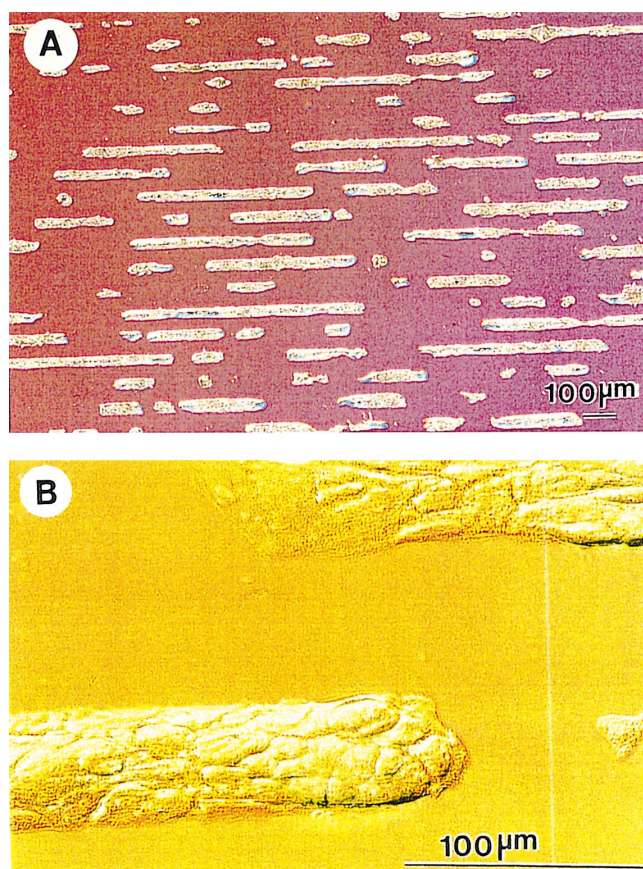

Fig. 2. Human epidermal carcinoma A431 cells on a linear array pattern. The images of cells were taken with a Normarski microscope at (A) $50\times$ and (B) at $400\times$. Individual cells can be readily distinguished in (B). The scale bar is 100 μm.
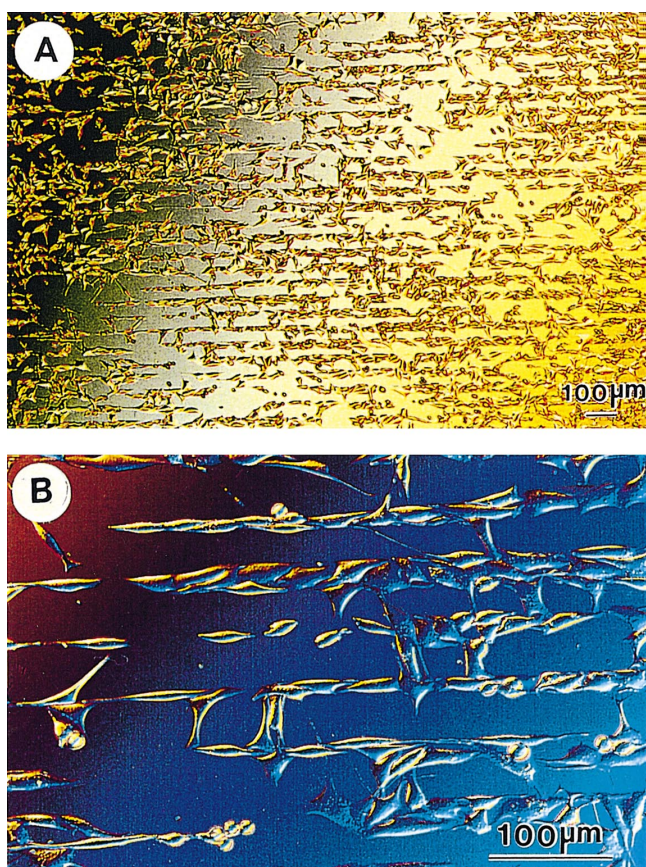
Fig. 3. Mouse fibroblast NIH 3T3 cells in linear arrays: (A) the images of cells were taken with a Normarski microscope at 50×, (B) at 200×.

tracks after extended incubation following an initial seeding at 5×10^4 cells/ml. This appearance is likely due to cells laying down their own extracellular matrix proteins to deviate from the linear patterned surfaces and bridge between the tracks. This observation is consistent with a previous report that mouse fibroblasts cross over inhibitory tracks presenting oligo-(ethylene glycol) groups [24]. Our findings are in accord with the concept that different cell types may respond disparately to a given surface in terms of migration behavior even if they each exhibit positive adhesive interactions with the same particular surface-coated molecules [42].

3.2. Formation of complex cell patterns

We also constructed specific, complex patterns of cells to address some important biological questions, e.g., how one cell group communicates with another. In a step toward this long-term goal, we designed patterns with square stations connected with narrow tracks of variable width and length. To test our designs with cells, we added cell suspensions to culture dishes containing the patterned substrates. After one day, pattern formation was incomplete. After 2–3 days, cell density increased and the

patterns were readily recognized. We tested several types of cells including mouse fibroblast 3T3 cells, human epidermoid carcinoma cells and bovine aortic endothelial cells. All of the three cell types readily formed defined patterns. The specific patterns with endothelial cells are shown in Fig. 4A. Figure 4B shows an isolated well-defined cell pattern where the individual cells can be distinguished in two squares connected by two cells. It should be pointed out that the endothelial cells do not have elongated processes; thus are completely confined in the printed areas. This again demonstrates that different cell types behave differently on the same surface. Human epidermoid carcinoma cells generated similar well-defined patterns (Fig. 4C and D).

4. Discussion

4.1. Type III surface self-assembling peptide materials

We have employed a new type of synthetic biological material based on oligopeptides to include three distinctive features: a ligand, a linker, and an anchor. All three groups can be tailored to specific purpose. In this study, we made two peptides with similar characteristics: one has two copies of the RADS cell adhesion motif and the other has three copies. The length of the linker was varied from 5 to 3 alanine residues. Because of the sequence similarity between these two peptides, their cell adhesion properties were indistinguishable. A variety of cell types formed patterns using a general cell adhesion motif as shown in this study. In future studies, we could change the ligand to include a specific motif that would have high affinity for a particular receptor on the cell surface so that specific cell types can be identified from a pool of cells. Likewise, the linker region can also be varied to accommodate any interaction. Properties such as length, hydrophobicity and flexibility can be altered through changes in the linker sequence. For example, we can change alanine to glycine creating more flexibility, or change alanine to valine to increase stiffness. The anchors can also be modified for different surfaces. For example, we can change cysteine to other residues, such as aspartate or glutamate. If these carboxylic groups are activated to become esters, they would readily react with amine-coated surfaces. In summary, each one of the three features can be engineered for a particular purpose.

4.2. Role of hexa-(ethylene glycol)

In the development of cell patterns, it is essential to have a surface that resists non-specific adsorption of proteins and cells. This is similar to the production of integrated circuit boards, where both conductors and insulators are needed. For cell patterns, the peptide is like the conductor and EG_6SH the insulator. We have
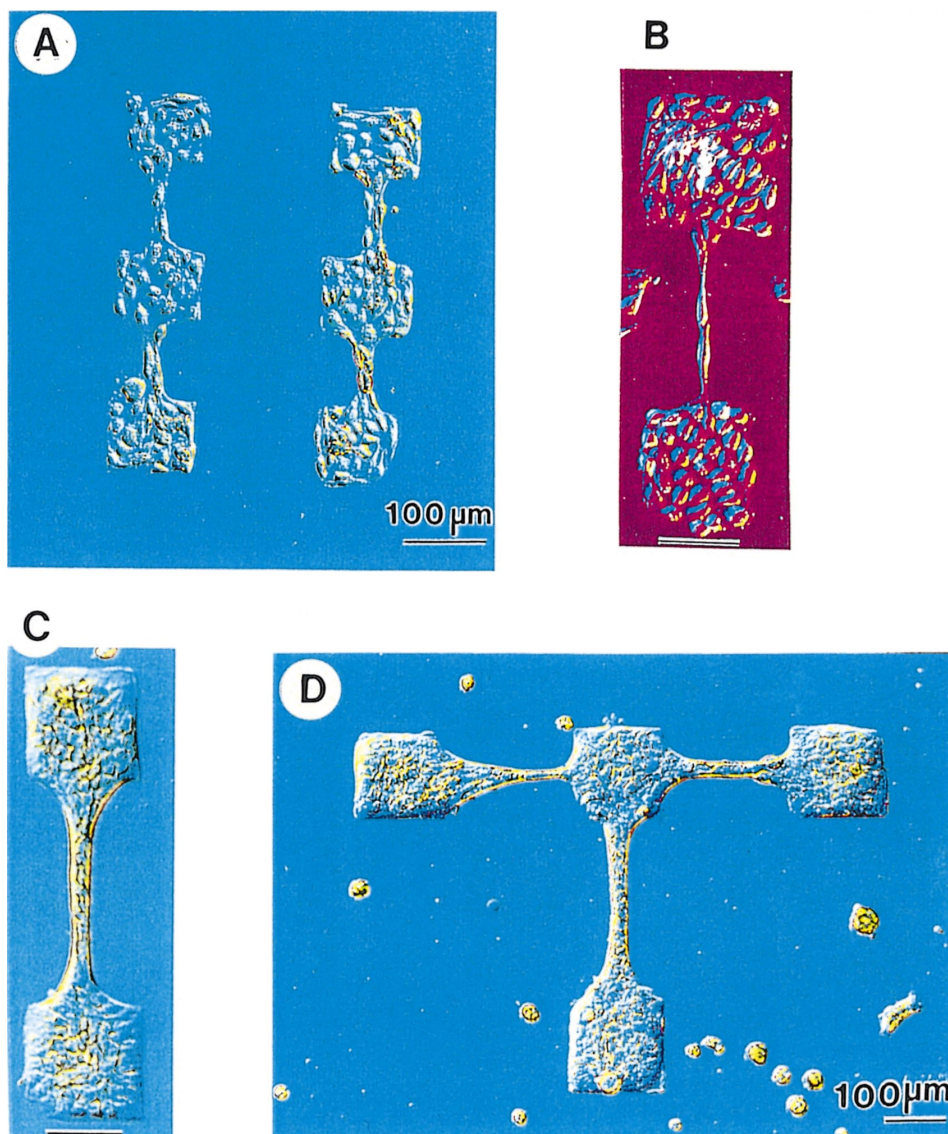
Fig. 4. Bovine aortic endothelial cells and human epidermal carcinoma A431 cells growing on designed patterns: (A) bovine aortic endothelial cells were confined to the patterns of squares connected with linear tracks. The patterns were made with an oxygen gas treated PDMS stamp to increase the surface hydrophilicity to facilitate EG_6SH wetting. (B) When the track is narrow, only single cells could fit. There are only two cells that connect the two cell communities. (C) and (D) Human epidermal carcinoma cells forming an I and T shape, making a two or three-way connection. The square areas in the letters have a high density of large number of cells. In (D), the extra-unpatterned cells are not attached to the surface, but are free-floating. The scale bar is 100 μm.

demonstrated previously that SAMs presenting hexa-(ethylene glycol) groups effectively prevent non-specific adsorption of proteins and cells to the surface [36]. We found that direct inking of a hydrophobic polydimethyl-siloxane (PDMS) stamp with EG_6SH in an ethanol solution did not yield well-defined patterned surfaces (data not shown): cells attached not only to the areas in which peptides are immobilized, but also on the contacted areas in which only hexa-(ethylene glycol) groups should be presented. Brief treatment of the PDMS stamp with an oxygen plasma generated a more hydrophilic surface that can be readily wetted by EG_6SH. The oxygen plasma-

treated PDMS stamp gave clean patterns (Fig. 4A). It is possible that the hydrophobic PDMS stamp could not be completely wetted by EG_6SH to form a thin layer and therefore was unable to transfer a continuous, complete pattern of EG_6SH to the surface. Oxygen plasma treatment oxidized the hydrophobic PDMS surface so that it became hydrophilic (presumably by formation of silanol groups on the surface) [37] and readily wetted by an ethanol solution of EG_6SH to support a continuous thin film. This thin film allows the complete EG_6SH coverage on the areas that contacted the PDMS stamp, and resulted in well-defined cell patterns.

4.3. Cell responses to other surfaces

We also tested various surfaces, such as plastic Petri dishes with and without coating of poly L-lysine, glass cover slides coated with gold, and gold substrates coated with EG$_6$SH, hexadecanethiol (CH$_3$(CH$_2$)$_{15}$SH or C$_{16}$SH). We also tested self-assembling peptides EFK and ELK that do not have RAD motifs. Low-cell density was observed on the peptide surface (data not shown) suggesting that these peptides are less ideal substrates for cell attachment. The qualitative results showed that the cells prefer to attach on surfaces coated with the oligopeptides RADSC-14 and RADSC-16, for the entire surface was covered with cells at high density. Few cells attached to the C$_{16}$SH surface and on the gold surface alone.

4.4. Biological surface engineering

This simple system using self-assembling peptides and other materials to modify surfaces could have a variety of applications in biomaterials, biomedical engineering, and biology. For example, we can design various materials and patterns to address specific questions in cell biology on how a community of cells may communicate through inter-cell connections (Fig. 4B). The letter I and T patterns in Fig. 4C and D demonstrate different connections making them useful in the future study of cell–cell communication. Applying external stimuli, e.g. calcium, potassium, hormones, growth factors, cytotoxic substances or electrical impulses, to one cell community, should permit the study of responses from the other linked community. This technology may also be useful in biomedical research and clinical applications to enable specific molecular detection. For example, we can design a specific ligand as a 'molecular hook' that can interact with specific cell surface molecules with high affinity thereby anchoring them onto engineered surface. This type of diagnostic device might be mass manufacturable as a chip for rapid and highly sensitive detection. Furthermore, μCP using self-assembling peptides is very repetitious allowing for a robotic system to be developed for printing specifically designed patterns. It should be emphasized that our approach is only one of the growing family of techniques to engineer surfaces for biological applications. As one example, Cannizzaro et al. [43] recently reported the use of biotinylated degradable polymer PLA–PEG–biotin–avidin (G)$_{11}$ with cell adhesion motif GRGDS peptide as the ligand to facilitate cell–surface interactions. They demonstrated that the new materials promote interaction between cells and the linked biopolymer containing cellular ligand materials.

The development of new biological materials and techniques often broadens the questions we can address, and thus deepens our understanding of seemingly intractable biological phenomena. We believe that this simple and versatile biological surface engineering system, using self-assembling oligopeptide and microcontact printing, will open new research opportunities including the further study of cell–material interactions, cell migration, cell mechanical compliance, cell–cell communication, and cell behavior.

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