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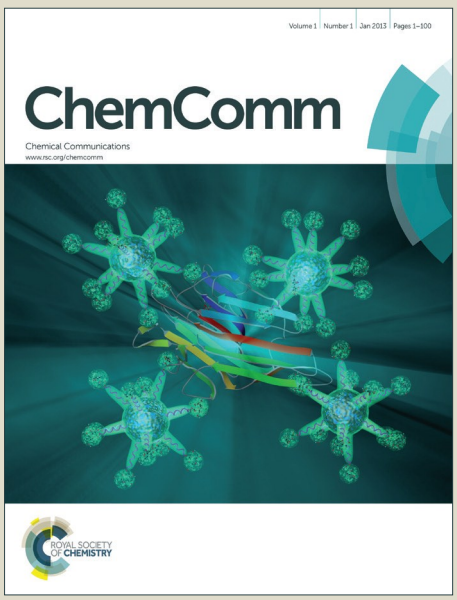

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One-post Patterning Multiple Protein Gradients by a Low-cost Flash Foam Stamp

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Printing versatile chemical and biological inks for protein and cell patterning was achieved with a simple and cost-effective flash foam stamp (FFS). The grey-scale mask fabricated stamp can generate multiple protein gradients with one-post stamping. Due to the importance of spatially controlled protein pattern in both biology and tissue engineering, this straightforward and reliable tool is an accessible solution for resource-limited laboratories conducting molecular patterning experiments.

Protein patterning is critical for the development of miniature biologically integrated devices for several biomedical applications including biosensing, tissue engineering, and drug screening.[1, 2] The spatially controlled protein pattern controls and guides cell proliferation and recruits tissue organization onto technological surfaces. The fundamental strategies of protein patterning have been well reviewed by Blawas *et al*[1] and Kaufmann et al[2]. The patterning strategies can be categorized into three types. 1) Spatially oriented chemical properties of the surface for protein seeding including UV lithography[3] and photochemistry[4, 5] methods for site-specific modified surfaces for protein anchoring. 2) Microfluidic-assisted molecular patterning including micro-transfer molding (μTM) and micro-molding in capillaries (μMIC)[6] to guide the diffusion of the bimolecular interactions and draw patterns on surfaces. 3) Contact printing including micro-contact printing (μCP) with an elastomeric stamp to transfer an 'inked' material onto a substrate. Since being introduced in the early of 1990 by Dr.Whitesides[7], micro contact printing has established its reputation for the preparation of micro- and nanostructured surfaces in a straightforward and cheap bench-top manner.[8, 9] The poly(dimethylsiloxane) PDMS stamp is the primary tool for μCP transfer of molecules of interest to the target surface. The PDMS stamp is: (a) flexible enough to make conformal contact even with a rough surface; (b) well-established micro-fabrication protocols for PDMS stamp preparation; and (c) capable of reprinting features down to the nano-meter scale. Although it is an icon printing tool, the PDMS stamp has encountered several challenges.[2, 10] First, the PDMS stamp occasionally contains a certain percentage of residual prepolymer and/or low molecular weight PDMS that is likely to leach during printing. This potentially causes artefacts on the substrates. The hydrophobic nature of PDMS makes its difficult to print polar molecules especially biological ''inks'' such as proteins or DNA. In this case, the hydrophobic nature of PDMS is associated with irreversible adsorption and denaturation of proteins. In addition, the strength of fine-featured PDMS stamp-based μCP is on the micro- and even nano-meter structure reprinting. This is not feasible for large scale patterning because of the restriction of mould fabrication and the elastomeric property of PDMS. Significant protocol optimization has to be done to ensure reliable and reproducible patterning. Last but not least, the PDMS stamp is normally replicated from a fine-featured silicon mould that is obtained via costly clean room based micro-fabrication that is not readily accessible in most biological and bioengineering laboratories. This severely limits the widespread use of micropatterning as a tool in both biology and tissue engineering.

Inspired by the personal stamp that avoids the use of an inkpad (ink is stored in the foam), we anticipated a new stamp that can get rid of expensive fabrication processes and inherit straightforward and cheap bench-top manners of contact printing. Enthusiasm will be strengthened if this approach is low-cost and feasible to generate complex protein patterns-even multiple protein gradients can be printed with a one-pot stamping.

Flash foam—also called photosensitive foam—is an ultra-micro bubble material typically made by polyethylene. Due to the microporous structures inside, the foam can store solution and dispense the solution by imposing pressure. Under strong

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light radiation, the flash foam absorbs light energy that can be transformed into heat energy to melt the polymer and cause fusion of the microporous structure of the foam. The diminished pore at the surface layer of the foam forms a barrier that blocks ink penetration.[11] Its low cost, ease of fabrication and ink storage advantages have been fully demonstrated for preparing microfluidic paper-based analytical devices by He et al.[11] In their work, FFS successfully printed the PDMS precursor—a viscoelastic polymer—on filter paper to form a hydroponic barrier. Motivated by this work, we investigated if this low-cost approach was feasible for printing bioink, protein solutions, and other chemical reagents. Fig. 1A lists the simple materials and fabrication process of FFS. The FFS and stamp machine were purchased from Hai Tian Electronic Information Materials Co., Ltd. (Shanghai, China). The patterns were designed on the CorelDraw (Corel Co., Ltd. Canada) and then printed on sulphuric acid tracing paper with a Xerox CP405d laser printer (Fuji Xerox, Japan) as the mask. To transfer the mask features onto the foam stamp, the tracing paper and flash foam was exposed to a flash stamp machine with xenon tubes that delivers the intense burst of light that seals the non-patterned area (Fig.1A). To test the compatibility of versatile structure reprinting, the flash foam pad (5 cm × 2.5 cm) was patterned with square array, star, straight and curling lines by a light incident (780 Joule for 30 second) (supplementary information). A model bioink, Cy3-labeled IgG (1:10000, Sigma) was filled from the backside of the foam stamp. The surface of the front side of the stamp was carefully cleaned with a wet and soft cloth to reduce the nonspecific contamination of the printing. The protein ink was printed on a clean glass slide and visualized by a microarray scanner (LuxScanTM 10K, CapitalBio Corporation, China). Fig. 1B clear shows that protein arrays can be ready patterned. Versus the fine silicon mould generated from the PDMS stamp, the resolution of the patterned structure is not so competitive the FFS fabrication and stamping indeed exempt from complicated equipment and toxic materials.

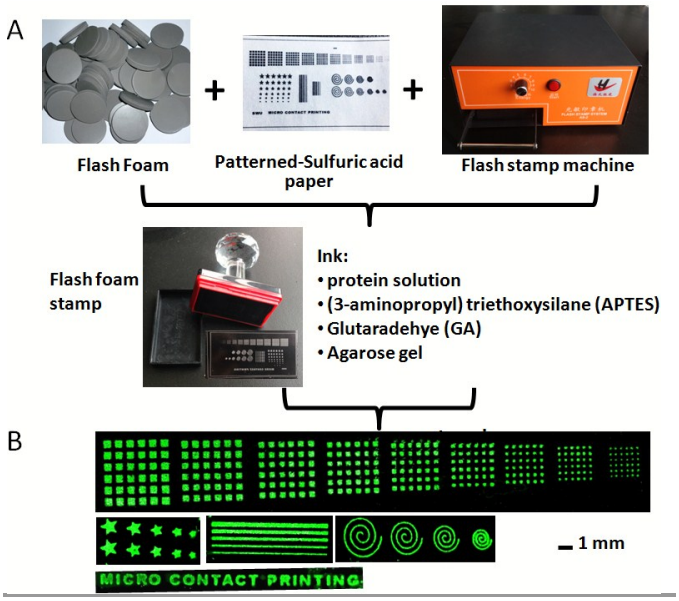

Fig. 1 Protein Patterning by Flash Foam Stamp (FFS). (A) FFS can be prepared by low-cost materials: flash foam, sulphuric acid paper and stamp machine. (B) Cy3-labelled protein was stamped on clean glass slides.

Attaching bioactive protein to surfaces can be achieved by physical adsorption and covalent immobilization. The latter is preferable because covalent bonds are mostly formed between side-chain-exposed functional groups of proteins, resulting in irreversible binding and a high surface coverage.[1] Almost all of the covalent attachments use chemical linker molecules and a number of wet-chemistry steps to facilitate the attachment. To investigate the applicability of printing versatile inks by foam stamp for protein patterning, glutaradehyde (GA)-medicated protein covalent immobilization was applied as a model system.

The chemical reactions can be divided into three steps: (3-aminopropyl) triethoxysilane (APTES) silanization, glutaradehye activation, and protein immobilization (Fig. 2). Briefly, NaOH (1 M) and oxygen plasma treatment hydroxylized glass slides were treated with 5% (V/V) APTES ethanol solution (Sigma-Aldrich) for 0.5 h at room temperature. The silanized slides were rinsed with ethanol and DI water, followed by drying with N_2 stream. The amino-group on the APTES-slides was activated by a homodimer crosslinker, glutaraldehyde (0.5% v/v, diluted with DI H_2O) for 1 h. After washing with DI water and drying with N_2 stream, the Cy3-labelled IgG (1:10000, Sigma) was added on the GA-activated APTES-slides and incubated at room temperature for 0.5 h. Subsequently, all slides were thoroughly washed with TBST buffer (tris-saline buffer with 0.5% tween-20) and DI flushing. Finally, the printed slides were imaged via a microarray scanner.

We studied the practicability of printing different types of ink by FFS: (1) APTES ink: APTES was directly stamped on NaOH and oxygen plasma hydroxylized glass slides by FFS. Then, GA and protein solutions were pipetted on APTES-patterned glass slides; (2) GA ink: GA was stamped on APTES silanized-slides followed by protein coating; (3) Protein ink: Cy3-labelled IgG was printed on GA-APTES-slides by FFS (supplementary information). It is encouraging that printing the three inks offers fine protein patterns (Fig. 2 a-c). The residual ink on the surface of the stamp must be removed to ensure that ink is injected from the microporous area. This minimizes cross-contamination between inks. Because APTES was diluted by organic solvent ethanol, and GA solution was prepared by DI water, the results demonstrated that FFS is not only suitable for direct printing protein, it can also be used for patterning other molecules with different chemical properties. Finally, because the hydroxylized glass is rich in Si-OH—and these are the basis of PDMS-glass bonding—the FFS-patterned glass is ready to bond with oxygen plasma-treated PDMS channels to build PDMS/glass hydride microfluidic chips.

The trend in cell biology—especially stem cell research and tissue engineering—highlights the significance of creation of physiologically relevant cellular microenvironments and three-dimensional (3-D) structures for higher protein capture capacity. Hydrogels are important soft materials that can be

fabricated in the form of micro-periodic structures to build a 3-D extracellular matrix (ECM) mimicking in vivo conditions[13]. Patterning hydrogel scaffolds can be achieved by direct-write assembling[14], electrohydrodynamic jet printing[15], interference lithography[16], contract printing[17] and microfluidic templating[18]. To investigate the possibility of patterning pre-gel bioinks by FFS, low-melt and gel temperature agarose was used as a model ink because it can remain fluid at 40–45 °C. The agarose polysaccharide is a preferred matrix for work with proteins and nucleic acids. It is also sometimes used for culturing organisms and to measure microorganism motility and mobility. In this study, Cy3-labelled IgG (1: 50000 dilution) was mixed in melted agarose (0.5% w/w, diluted by 0.9% NaCl) at 45°C. Then the pre-gel agarose solution was soaked in FFS and printed on clean slides. The gelled agarose pattern was visualized with a microarray scanner. The FFS patterning illustrates that the agarose array can be easily formed on glass slides (Fig. 2d). It is anticipated that the FFS stamp could be used for patterning thermo-sensitive and photo-sensitive hydrogels.

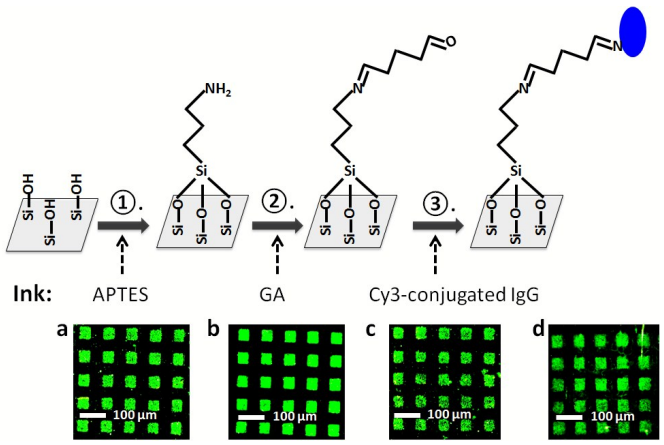

Fig. 2 Protein patterning through stamping different ink. (1) APTES silanize hydroxyl glass slides (2) one aldehyde group of GA reacts with the amino-group of APTES-glass slides; (3) the another amino-group of protein react with remaining aldehyde group of GA. Fluorescent image of protein pattern (a) stamping APTES; (b) stamping GA; (c) stamping Cy3-labelled IgG; (d) stamping agarose and Cy3-labelled protein mixture. APTES: (3-aminopropyl) triethoxysilane; GA: glutaradehye; and IgG: immunoglobulin.

The existence of protein concentration gradients and their effects have been observed in a variety of biological systems. Precise control of protein concentrations, especially extracellular proteins, is critical. These gradients are crucial for cellular responses such as migration, differentiation and ultimately tissue organization[6]. Standard µPC is capable of reprinting fine structures but is not feasible for direct printing of a protein gradient. To generate a protein gradient, laser-assisted adsorption by photobleaching (LAPAP) was established by Belisle et al.[4] Wang et al. demonstrated a gradient lithography protocol to engineer protein gradient.[19] Microfluidic assisted fluidic diffusion strategies were also utilized to build molecular gradients.[6] Those techniques either require multiple fabrication of the mould or chemical treatment. Because the incident light can seal the microporous structures of the flash foam, we argued that by adjusting the grey intensity (darkness) of the mask we could fabricate FFS features with different pore sizes. The variety of the pore sizes within the feature can deliver a pattern with different ink densities (Fig. 3A). Scanning electron microscopy characterization shows the micropores of the foam obtained from a mask featured with different grey intensity values contracted for different pore-sizes (Fig. 3B). Thus, the speed of the ink passing through the stamp would vary. During the light exposure process, light incident could pass through the mask is determined by the resolution of the structures on sulphuric acid tracing paper. Thus, we anticipated the stamp homogeneity could be improved by increasing the quality of the sulphuric acid tracing paper and resolution of printer used to generate the mask. To examine the practicability of this strategy for protein gradient patterning, Cy3-labeled IgG (1: 10000) was added to the FFS fabricated with a mask with features of different grey intensity values. The protein was printed on a GA-APTES slide (supplement information). The fluorescent micrographs obtained from the microarray scan were further quantified with a plot profile function of ImageJ (NIH free software). The fluorescent image and grey intensity plots show protein patterning with a concentration gradient was successfully established for a one-post µPC process (Fig. 3C).

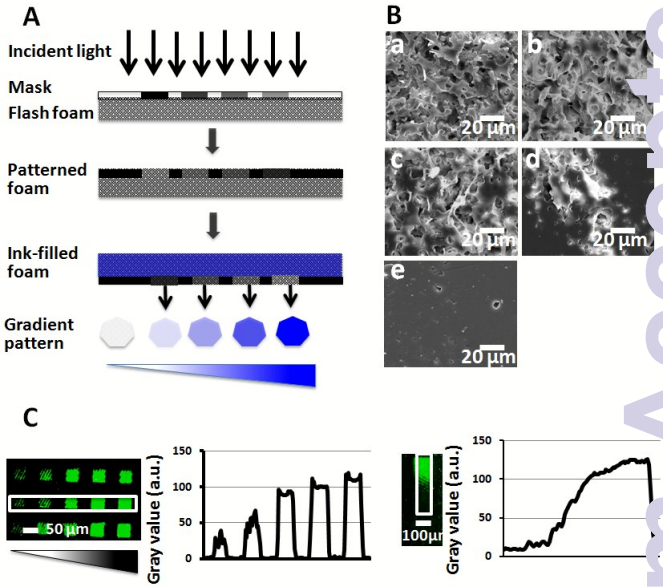

Fig. 3 Patterning a protein gradient by one-post stamping. (A) Schematic drawing of a patterning protein gradient: flash foam is covered by a computer designed gray-scale mask. (B) Scanning electron microscopy characterization of foam fabricated from a mask with: **a)**. 100% black; **b**) 80% black; **c**) 60% black; 40% black; and **e**) transparent. (C) Fluorescent image of patterned Cy3-labeld IgG and corresponding grey value analysed via plot profiling with NIH ImageJ.

Finally, the compatibility of this protein patterning strategy for guiding cell adhesion and growth was investigated. First, the strip line array (line: 200 µm X 2.5 cm, gap: 200 µm X 2.5 cm) was patterned on flash foam. To pattern the ECM protein on the glass slide, protein ink (collagen, 10 µg/mL) were added to the sterilized stamp and printed on a clean glass slides. Human umbilical vein endothelial cells (HUVEC, 1 X 10^5 cell/mL) in serum-free RPMI 1640 medium (Gibco) were placed on collagen-patterned slides and incubated at 37°C for 1 h. The non-adherent cells were removed by gentle washing. Then Serum-free medium was then replaced with RPMI 1640 plus 10% foetal bovine serum (Gibco). Cells only can be observed

on patterned area (Fig. 4a). The cell density significantly increases after 24 h growth, indicting FFS printing does not interrupt cell proliferation (Fig.4c). Since the serum protein in culture medium and cell secreted ECM protein would adherent on no collagen-patterned area, cell start to growing into the gap area. The cells were stained with FITC-labelled phalloidin and DAPI (Beyotime Biotechnology, China) to visualize the cell cytoskeleton and nuclei (supplement information). The fluorescent images show cells forming a line array on the collagen-patterned glass slide (Fig. 4 e and f). The experiment results indicate FFS is capable for printing functional protein to control cell adhesion and guide cell growth.

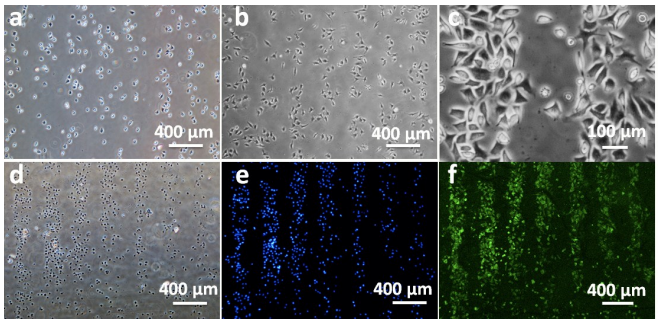

Fig. 4 Patterning of extracellular protein for guiding cell adhesion and growth. HUVEC cells (1 X 10 5 cell/mL) in serum-free RPMI 1640 medium was placed on collagen-patterned slides and incubated at 37°C for 1 h. Then non-adherent cells were removed with serum-free RPMI 1640 washing. **a).** residual cell after washing**; b)**. residual cell grow for 10 h; **c**) residual cell after 24 h; **d**) cells were fixed by 4% paraformaldehyde; **e**) cell nuclei staining by DAPI; **f**) F-actin of cell staining by FITC- FITC-phalloidin.

In this study, we demonstrated versatile protein patterning via a low-cost flash foam stamp. Protein patterning was achieved by patterning the organic reagent APTES, crosslinker GA, protein solution and agarose gel, respectively. Features down to 50 μm can be safely, reproducibly and easily pattered by FFS. Most importantly, the porous structures of the foam stamp can be controlled during the light exposure process by tuning the grey intensity of the mask structure. The pore size/density of the stamp restrained the speed of ink leakage. This made one-post printing of protein gradient possible. The FFS-patterned ECM protein can control the cell adhesion and growth, demonstrating its potential for preparing a cell pattern. Because it is crucial to establish bimolecular gradients *in vitro* to mimic the cellular microenvironment for research into cell motility, differentiation and organization, we anticipate that FFS stamping will be a candidate tool for delivery or might generate more complex protein distributions and combinations.

In conclusion, we report a novel assay for the flexible generation of protein distributions on glass substrates. Flash foam stamping provides a relatively straightforward, flexible, reliable and accessible tool that allows the generation of graded distributions of substrate-bound protein. The low-cost stamp enriches its accessibility to resource-limited laboratories conducting molecular patterning experiment in both biology and tissue engineering.

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