

Patterning Proteins and Cells Using Two-Dimensional Arrays of Colloids

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A simple method is described for controlling the organization of proteins on surfaces using two-dimensional arrays of micron-sized colloidal particles. Suspensions of colloids functionalized with proteins are deposited onto coverslips coated with gold using a combination of gravitational settling and applied electrical fields. Varying settling time and particle concentration controls the density of particles on the substrate. Surface coverage ranged from an essentially continuous coating of protein on close-packed arrays to domains of protein separated by distances as large as 16 μm . Colloidal particle arrays were also patterned into 500 μm islands on substrates using elastomeric lift-off membranes. The applicability of this approach to the promotion of fibroblast cell adhesion and spreading was demonstrated using particles coated with the cell adhesion protein fibronectin. Behavior of adherent cells varied with particle density. This method provides a general strategy for controlling the organization of functional proteins at surfaces on three length scales: the size of individual colloidal particles, the spacing between particles, and the organization of particles in patterned arrays.

Introduction

Control over the organization of proteins on surfaces on microscopic length scales is important in the development of biosensors^{1,2} and protein microarrays^{3,4} and in the organization and control of growth of cells on surfaces.^{5–7} Techniques for applying proteins to surfaces include photolithography,^{8–10} soft lithography,^{7,11} spot arraying³ and direct writing using the tip of an AFM.^{12,13} In this article, we describe a new method for the patterning of proteins on surfaces that involves the attachment of proteins to microscopic particles and the subsequent assembly of these particles on a surface. The organization of proteins is determined by both the size of particles and the spacing between particles on a surface.

For methods that depend on patterning with light (photolithography and most applications of soft lithography), the characteristic feature size of protein patterns is typically 5–500 μm . Recently, dip-pen nanolithography¹⁴ has been used to create arrays of 200 nm islands composed of cell adhesion proteins; however, the total array size was only 80 \times 80 μm .¹² Other methods of controlling the organization of proteins have focused on molecular length scales: that is, formation via self-assembly of synthetic molecules functionalized with peptide fragments. Molecules used in this approach include lipids,^{15,16} alkanethiolates,¹⁷ and polymer stars¹⁸ and brushes.^{19,20} Such techniques allow for the control over composition on the length scale of approximately 1–50 nm.

We describe a convenient strategy for producing patterned arrays of proteins that consists of the deposition of protein-coated colloidal particles onto a substrate. The use of colloids to pattern proteins builds on existing knowledge of particle functionalization and assembly. Conjugation of proteins to colloidal particles is a well-developed technology, and there are a large number of covalent and noncovalent approaches to immobilizing proteins on particles.²¹ Once on a particle, the position of proteins on a surface can be controlled by positioning the particles using physical methods of assembly.^{22–24} Arrays of particles can also be patterned on length scales ranging

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from 10 μm to 1 cm using soft lithography,^{25,26} capillary forces,^{22,26,27} and the application of electromagnetic fields.^{24,28–32} In contrast to dip-pen nanolithography, assembly of particles can simultaneously functionalize large surface areas.

Protein patterning is finding widespread use in studying the functional effects of cell adhesion to substrates, usually using patterns similar to the size of a cell (10–50 μm).^{6,7} When cells adhere to surfaces, they form discrete adhesive sites called focal adhesions: protein-rich complexes that range in size from less than 100 nm to approximately 1–2 μm .^{18,33} Therefore, to determine how the distribution of focal adhesions regulates cell behavior requires patterning on length scales much smaller than the diameter of a single cell. Protein-coated colloids are within this size range, giving us the ability to organize cell adhesion proteins on surfaces at subcellular length scales.

In this paper, we describe the preparation of arrays of particles coated with the adhesion protein fibronectin (FN) and the attachment and spreading of fibroblast cells on these arrays. A combination of gravimetric settling and electric field driven assembly is used to organize particles on surfaces. Using this method, we produce surfaces presenting 2 μm adhesive islands (particles as small as 20 nm are available commercially) with separations between islands that range from 16 μm to less than 0.5 μm . We show that fibroblast cells attach and spread on substrates functionalized in this way. We also show that this method of colloid assembly is compatible with and complementary to the techniques of soft lithography. This approach to patterning proteins therefore gives flexibility and control over both the size and spacing of adhesive contacts and over the larger-scale geometry of the patterning of cells.

Experimental Section

Fabrication of Colloid Arrays. Our method for the patterning of arrays of colloidal particles is summarized in Figure 1. A thin film of Au (150 nm thick) was prepared by electron beam evaporation onto 12 mm diameter glass coverslips primed with a thin layer of Ti (10 nm thick). Gold surfaces were used immediately or stored in ethanol to prevent surface contamination. Particle assembly was performed in rectangular wells fabricated by inserting a poly(dimethylsiloxane) (PDMS) spacer (~1.5 mm thick) between the gold-coated coverslip and a piece of glass coated with indium tin oxide (ITO, Delta), which served as a transparent counter electrode. Particle assembly took place on the gold electrode; the apparatus was oriented so that the gold electrode was on the bottom. Assembly was visualized through the ITO-coated glass. In some preparations, elastomeric membranes (~25 μm thick, much thinner than the spacer) were fabricated in PDMS using soft lithography, as described previously,^{34,35} and placed on top of the gold electrode. Holes in the membrane defined patterns of assembled particles.

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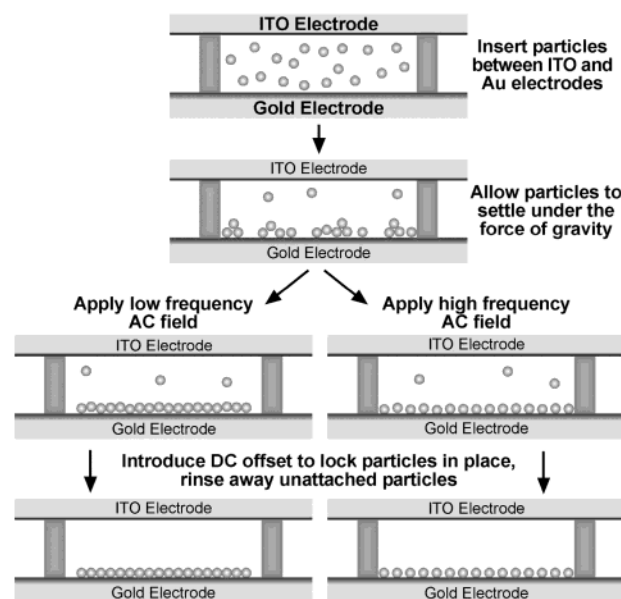


Figure 1. This schematic diagram shows the procedure for making arrays of protein-coated colloidal particles. Suspensions of colloids in water are introduced between glass coverslips functionalized with a transparent film of ITO (top electrode) or gold (bottom electrode). Particles are allowed to settle onto the gold electrode. An alternating electric field is applied. A high-frequency field results in repulsive forces between particles that helps in their distribution on the surface, while a low-frequency field results in an attractive force between particles that drives them to form a close-packed array. The application of a dc field to the assembled arrays results in irreversible immobilization of the particles to the gold.

Table 1

sample ID ^a	suspension concn (% solids)	settling time (min)	E-field (VPP, Hz)	surface coverage ^b (% area)	particle spacing (microns)
A	0.025	1	8; 40 000	0.33 ± 0.26	16.3 ± 10.3
B	0.025	3	8; 40 000	0.60 ± 0.28	11.5 ± 5.98
C	0.031	20	8; 40 000	3.9 ± 0.8	4.41 ± 2.53
D	0.025	30	8; 40 000	6.6 ± 0.7	3.44 ± 1.74
E	0.025	60	8; 40 000	12 ± 1.4	2.43 ± 1.22
F	0.05	40	8; 40 000	14 ± 1.4	2.26 ± 1.07
G	0.10	40	8; 40 000	33 ± 3.3	0.87 ± 0.81
H	0.10	60	8; 40 000	44 ± 2.6	0.27 ± 0.33
I	0.13	180	8; 500	98 ± 0.1	close packed

^a Sample ID corresponds to the identity of images in Figure 2.

^b Average coverage and standard deviation for 25 different regions, 48 × 48 μm in size.

Microspheres functionalized with streptavidin (2 μm , either plain or yellow-green fluorescent, Polysciences) were rinsed and resuspended in deionized water (18 M Ω) and placed between the electrodes. The presence or absence of fluorophore did not affect the assembly process. Particles were allowed to settle before the electric field was applied. The concentration of the particles (0.025–0.21% solids) and the settling time (1 min to 3 h) were adjusted in order to produce surfaces of different coverage (see Table 1 for specific conditions). After settling, a waveform generator (Agilent 3312A) was used to briefly apply an alternating (sinusoidal) electric field (6–8 VPP) across the particle suspension. The frequency of the electric field was adjusted to either promote or prevent particle clustering.^{28,31,32,36} To form dispersed arrays, high frequencies (2000–40 000 Hz) were used to induce interparticle repulsion; to form close-packed arrays, low frequencies (500–700 Hz) were used. After particles were assembled, they were immobilized irreversibly onto the gold electrode by introducing a dc field (2.5 V) for 60 s.³² Unattached particles were washed away using phosphate-buffered saline solution

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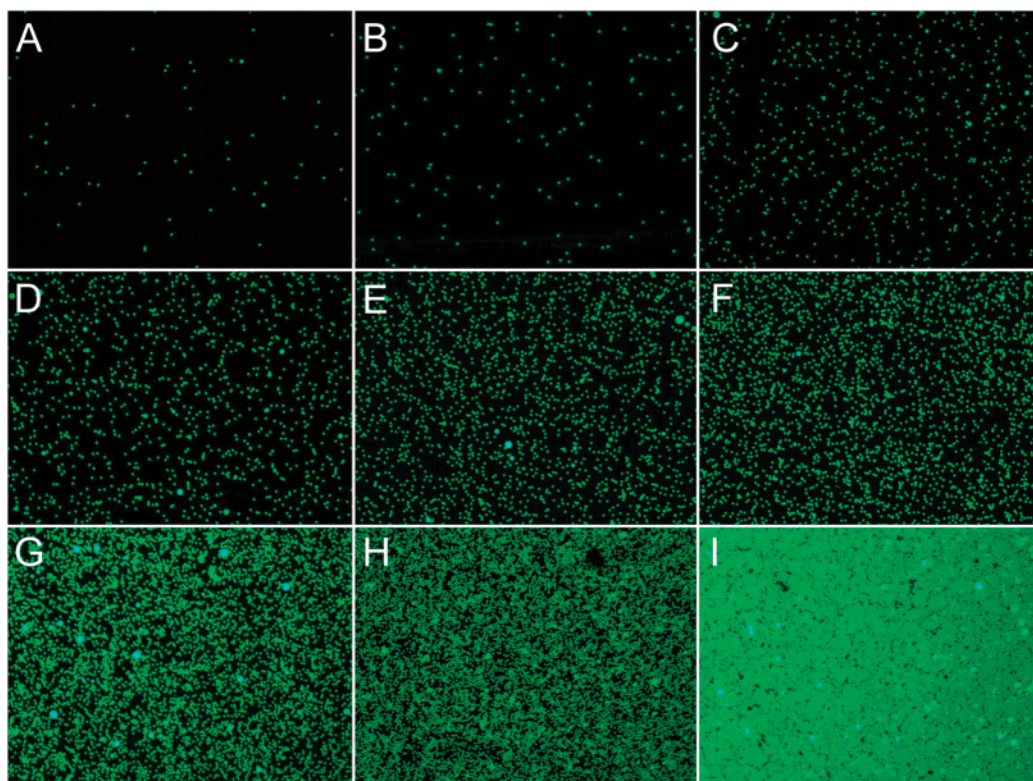


Figure 2. Varying particle concentration and settling time produced surfaces with a wide range of densities of 2 micron particles (green), functionalized with the protein streptavidin. Specific conditions for the preparation of each surface, samples A–I, and the resulting surface coverages and particle spacings are reported in Table 1.

(PBS). Areas not covered by particles were made nonadhesive to other proteins and cells by incubating at room temperature in a solution of 2% bovine serum albumin (Sigma) in PBS for 30 min.

To immobilize FN on colloidal arrays, purified rat plasma FN was first covalently modified with sulfo-NHS-biotin (Pierce). A 1 mg/mL solution of FN was dialyzed into 50 mM sodium bicarbonate, pH 8.5. Following dialysis, 40 μ L of a 1 mg/mL sulfo-NHS-biotin solution was added per 1 mg of FN. The mixture was placed on ice for 2 h and dialyzed overnight into CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11, 150 mM NaCl). Biotinylated FN was diluted to a final concentration of 50 μ g/mL in PBS. Immobilized particle arrays were incubated with biotinylated FN at room temperature for 60 min, and unbound FN was removed by washing with PBS.

Characterization of Colloidal Arrays. Image analysis, using the IPLab software package (Scanalytics), was used to determine the average spacing, surface coverage, and uniformity of the colloidal particle arrays. The average particle spacing was found by locating the centroid of the individual colloids and then calculating the distance from each particle to its nearest neighbor. The average over the entire image of the distance between a particle and its nearest neighbor was taken to be the average particle spacing. Surface density was determined by thresholding a fluorescence image of the particle array and then calculating the percentage of pixels above the threshold value, which represents the percent coverage of the surface. The uniformity of the surface was determined by placing a square mesh over each image and calculating the surface coverage of particles for each partition. A mesh spacing of 48 microns was used in this analysis as this spacing produced partitions that just encompassed the largest spread cells. There were a total of 25 equal-sized regions for each image. Average values and standard deviations over the 25 different regions were determined.

Cell Adhesion Studies. NIH-3T3 fibroblasts were cultured at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL) supplemented with 10% (v/v) bovine calf serum (Sigma). Cells were prepared for adhesion as described³⁷ and seeded on particle arrays immobilized on glass coverslips in a 24 well plate at a density of 1×10^5 cells/well. Following a 2

h incubation, unattached cells were removed and the coverslips were rinsed with PBS. Cells were fixed in 3.7% solution of formaldehyde in PBS and stained with rhodamine-conjugated phalloidin (Molecular Probes) as described.³⁷ Images of cells were obtained using an Olympus BX60 microscope with a Photometrics CoolSnap camera.

Results and Discussion

Particles (2 μ m in diameter) conjugated with the protein streptavidin were deposited on glass coverslips coated with gold using a combination of gravity and applied electric fields (Figure 1). The presence of streptavidin allows the subsequent attachment of additional proteins that have been conjugated with biotin, a ligand that binds streptavidin with high strength and selectivity. Surfaces with different densities of colloidal particles were produced by altering both the concentration of particles in suspension and the amount of time particles were allowed to settle (Table 1). For a fixed value of settling time, the surface coverage of colloids increased approximately linearly with the density of particles in the suspension. For samples A–H, the application of a high-frequency ac field produced a net repulsion between particles that assisted in the dispersion of particles over the surface. For sample I, the application of a low-frequency ac field produced a net attraction between particles that resulted in their assembly into a close-packed array.^{31,32,36} In this way, colloidal arrays were produced that had values of surface coverage that ranged from 0.3% to 98% coverage (Figure 2). The uniformity in surface coverage was determined by measuring the area covered by colloids at 25 adjacent regions of a coverslip. Surface coverage was quite uniform with variations of 10% or less for most densities of colloids. Not surprisingly, however, for the lowest density of coverage, variation increased to 80% (Table 1).

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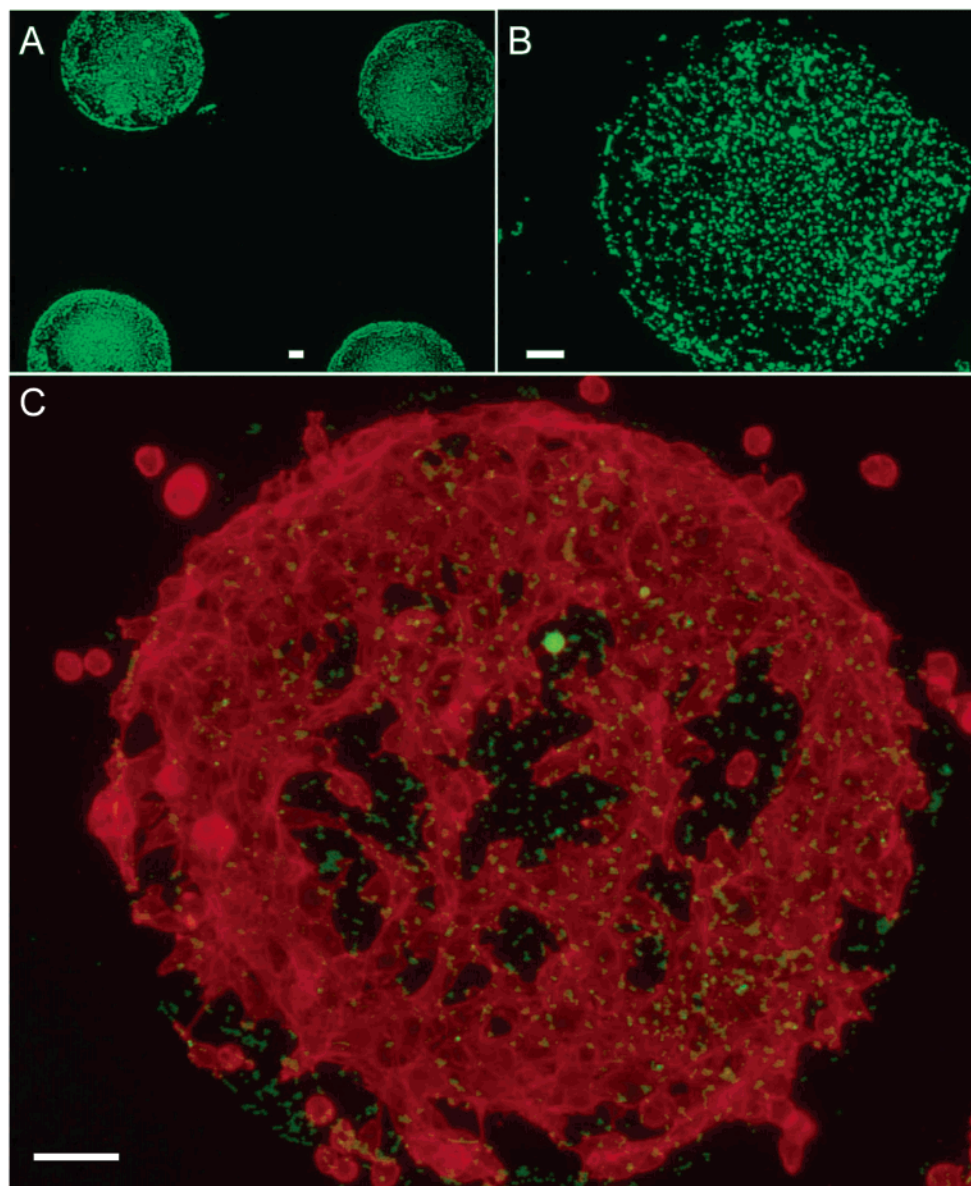


Figure 3. Particle organization and patterning. (A) Microsphere arrays were patterned into grids of 500 micron diameter circles. (B) Detail of a single circle showing the organization of particles. (C) Fibroblasts stained with rhodamine-conjugated phalloidin attached and spread on patterned circles but not on surrounding particle-free surfaces. All scale bars are 50 microns.

The average spacing between particles varied with the concentration of colloids in suspension and is reported in Table 1. The separation between particles ranged from 16.3 μm for the most sparsely covered surface to 0.27 μm for a surface coverage of 44%. Because the arrangement of particles is disordered, these values represent average spacings. The close-packed array (sample I) represents an ordered surface where the protein coating is essentially continuous.

An advantage of our approach is that the assembly of colloidal particles can be combined with other patterning techniques at larger lengths scales to produce hierarchical protein organizations. We combined colloidal assembly with soft lithography to construct 500 μm circular islands of particles by using a PDMS membrane to mask attachment of particles to the gold electrode during the assembly process (Figure 3A,B). Removal of the membrane left islands of assembled particles on the substrate; the shape and size of the islands were defined by the holes in the membrane. Thus, this approach could produce arrays of protein-coated islands where each element in the array

differs in the composition of attached proteins, as well as in the size and spacing of the protein sites within each element.

Patterned arrays of proteins have been used as substrates to direct cell adhesion and define cell shape.^{7,11,35,38} As cells attach and spread on surfaces, they apply tensile forces to sites of adhesion.³⁹ To support cell adhesion, particles must be attached to the surface with a strength sufficient to withstand cell-generated forces during adhesion and spreading and to prevent endocytosis of the particles by the cells. Direct current fields can be used to force particles into close contact with an electrode surface. It is proposed that doing so pushes the particle into a "primary minimum" and thereby results in attachment of particles that is irreversible after removal of the applied field.³² Therefore, a dc field was applied after the particle arrays were formed to "lock" the particles onto the surface.

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Arrays of particles presenting FN supported the attachment and spreading of NIH-3T3 fibroblasts (Figure 3C). The adhesive force between the particles and the gold surface was strong enough to prevent the removal of particles by the cells as they attached and spread on the particle arrays. Cells attached and spread on particle islands only when fibronectin was present on the particles; no cell adhesion was observed in the absence of FN. On the areas between the islands that had been blocked with albumin, no cell attachment was observed. These results show that cell adhesion is the result of specific interactions between cells and the adhesion protein FN on the particles.

Our patterning method allows deposition of FN at length scales approximating the size of focal adhesions. Varying the densities of particles on the surface should allow us to examine whether separation between adhesive sites affects cell morphology. We examined cell spreading on three different surfaces: sparse (10% coverage), intermediate (43% coverage), and close-packed (96% coverage) (Figure 4). Cells did not attach on surfaces with less than $\sim 5\%$ coverage by particles.

Cell spreading and morphology varied depending on the density of FN-coated particles. Cells on a sparse array of particles had an average spread area of $997 \pm 314 \mu\text{m}^2$ (99 cells measured). These cells extended membrane processes that appear to follow pathways directed by the underlying particles (Figure 4A). One reason for this branching could be to allow cells to maximize the surface contact when adhesive sites are limited.

To quantify and compare cell morphologies, we measured cell perimeter and calculated the cell morphology index,⁴⁰ equal to the area of a circle with the same perimeter as the cell divided by the actual area of the cell. A morphology index of 1 indicates a round morphology; values greater than 1 indicate elongated or branched morphologies. NIH-3T3 cells spread on a surface coated with a $10 \mu\text{g/mL}$ solution of FN have a morphology index of 1.7 ± 0.3 (50 cells measured). In our experiments, cells on sparse arrays had an average value of 8.1 ± 4.3 (99 cells measured), reflecting the branched morphology of these cells.

On arrays of intermediate particle density, cells had an average spread area of $791 \pm 353 \mu\text{m}^2$ (32 cells measured) and a morphology index of 7.6 ± 5.6 , similar to cells on sparse arrays. It is clear from Figure 4, however, that cell morphology on intermediate arrays differs from that on sparse arrays. Membrane protrusions that resembled filopodia were observed for cells on the intermediate array. These protrusions appeared much thinner than those on the sparse arrays, although they also were formed along linear arrays of particles (Figure 4B).

Cells on a close-packed array of particles were compact and had an average spread area of only $266 \mu\text{m}^2$ (270 cells measured). Cells on this surface had few or no membrane protrusions (Figure 4C); this cell shape is reflected in an average morphology index of 2.3 ± 0.6 . Together, these results suggest that the distance between adhesive sites can regulate cell area and morphology.

Our results show that functionalized colloids can be used for protein patterning and for controlling the size and spacing of adhesive contacts. Colloid-based processes offer a number of advantages over traditional methods in protein and cell patterning. A major advantage is the ability to control protein placement at multiple length scales. We have demonstrated that colloidal assembly can control the composition of biomolecules on three different

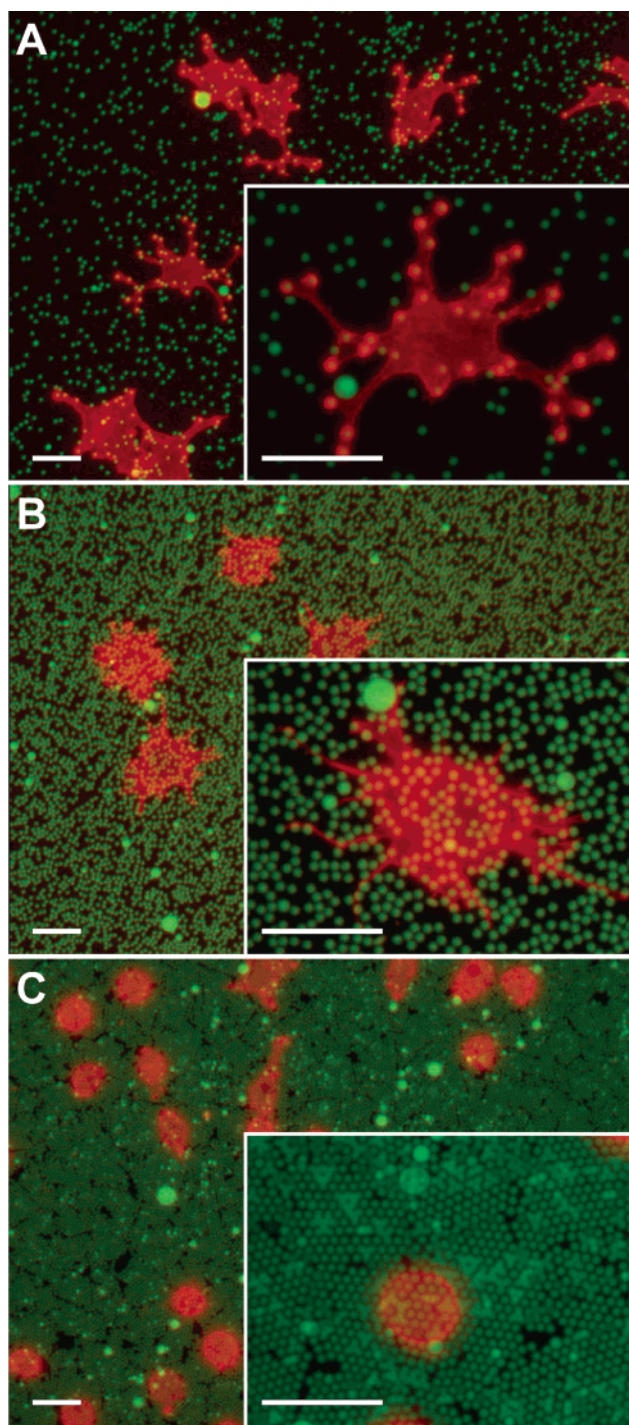


Figure 4. Spreading and morphology of fibroblast cells on different densities of colloidal particles. Fibroblasts attached and spread on particles presenting immobilized FN (green). Actin filaments in the cells were stained with rhodamine-phalloidin (red). Cell morphologies differ with particle organization: (A) array with a sparse distribution of particles, 10.2% surface coverage, 2.19 micron particle spacing; (B) array of intermediate density, 42.5% coverage, 0.59 micron particle spacing; and (C) close-packed array of particles, 96.5% coverage. All scale bars are 25 microns.

length scales: the size of individual particles, the spacing between particles, and the length scale of the patterning process. For example, the surface shown in Figure 4B has an average spacing between the particles, and therefore distance between adhesive sites, of 0.59 microns. Such spacing is difficult to achieve with techniques for patterning proteins that are based on photolithography. It

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has been shown by others that control on a fourth length scale, the size of particle clusters, may be possible by varying the solution properties of the particle suspension during deposition, for example, by varying pH.⁴¹ A second advantage is that the deposition of colloidal particles on a surface is scalable. Processes for coating large surface areas (square meters) with colloidal particles, such as spray-drying or electrodeposition, are rapid and inexpensive. A final advantage is that mixtures of different colloids can be used to form a variety of different surface compositions. While we report our work with streptavidin-coated particles in this study, this technique has been applied in our lab to colloids functionalized with several other proteins and small molecules.

This approach presents a convenient way to create complex protein-coated surfaces which will be particularly useful in determining the effects of extracellular matrix (ECM) protein organization on cell behavior. Changes in FN matrix architecture have been shown to regulate cell growth rate.⁴² Clustering of the cell adhesion peptide YGRGD on a surface increased fibroblast adhesion strength and actin stress fiber formation, relative to

randomly distributed peptides.¹⁸ Modulation of spread cell areas using different patterns of FN showed increased cell proliferation and reduced cell death in more highly spread cells.⁴³ Taken together, these results indicate that organization of ECM proteins on subcellular length scales provides regulatory information to cells. Colloidal particle patterning gives us the ability to manipulate protein organization on subcellular length scales so as to determine pattern-specific cell responses.

We believe the strategy described here will open new opportunities for controlling the behavior of cells by controlling the size and spacing of adhesive sites. It will also be useful in mediating interactions with and improving biocompatibility of synthetic materials.

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