Rediscovering Chemical Gardens: Self-assembling Cytocompatible Protein-Intercalated Silicate-Phosphate Sponge-Mimetic Tubules

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General Information: Sodium Silicate was obtained from Ward’s science, Gelatin was procured from Kraft foods Inc.; Dipotassium hydrogen phosphate, and Calcium Chloride pellets (anhydrous, 4-20 mesh) from Fisher Scientific. D(+) - Biotin was obtained from Acros Organics,
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) from Advanced Chemtech Inc., N-Hydroxysuccinimide from Chem Impex Int’l Inc., Avidin from EMD Millipore Corp, Amino dextran, Alexa Fluor 555 Phalloidin and TO-PRO-3 from Molecular Probes, Fluorescein diacetate and Chitosan from Alfà Aesar and Dextran coated streptavidin beads from GE healthcare. Nuclear magnetic resonance (NMR) spectra were recorded on an Oxford NMR 600 (600 MHz) spectrometer. An Agilent Technologies 845x UV-Vis spectrophotometer equipped with ChemStation Rev. A.10.01 software was used to record the UV/Vis spectra. Transmission electron microscopy (TEM) images were recorded using a FEI Tecnai G2 Twin microscope. An Amray 1910 Field Emission Scanning Electron Microscope was employed; digital images were obtained using SEM Image Display software (SEMTeck Solutions, North Billerica, MA). X-ray microanalysis and mapping of elements in the samples was done using the SEM microscope above equipped with an Energy-dispersive X-ray spectroscopy (EDS) detector (ThermoNoran, Madison, WI). Infrared spectra were recorded using a Thermo Scientific, Nicolet 6700 Fourier Transform spectrometer. Powder X-ray diffraction (XRD) analyses were performed using a PANalytical X’Pert PRO diffractometer at a wavelength of 1.5418 Å. A Zeiss Live-Cell Imager and a Leica SP2 scanning confocal laser scanning microscope were used for the imaging experiments.

1. Sponge Mimic Scaffolds

Video VS1. Growth of Crystal Garden based sponge mimic protein intercalated tubular scaffolds constructed by seeding CaCl$_2$ in a solution composed of 3M Sodium silicate, 0.5M K$_2$HPO$_4$ and 15% solubilized gelatin.

2. Transmission electron Microscopy (TEM) Analysis

Transmission electron microscopy (TEM) images were recorded using a FEI Tecnai G2 Twin microscope operated at an accelerating voltage of 120 kV. The samples were first
sonicated in ethanol, and then drop-casted on carbon-coated copper grids and dried under vacuum before the analysis.

3. Scanning electron Microscopy (SEM) Analysis

The samples were also characterized by scanning electron microscopy (SEM). Samples were placed on the surface of two-sided carbon adhesive tabs on aluminum SEM specimen holders and then coated with palladium for 10 minutes using a Med20 Sputter coater (Baltec-Leica, Buffalo Grove, IL). Samples were then analyzed using an Amray 1910 Field Emission Scanning Electron Microscope at the operating voltage of 10kV. Digital images were obtained using SEM Image Display software (SEM Tech Solutions, North Billerica, MA).

X-ray microanalysis and mapping of elemental components in samples was performed using an Amray 1910 Field Emission Electron Microscope equipped with an Energy-dispersive X-ray spectroscopy (EDS) detector (ThermoNoran, Madison, WI). Samples were tilted at 30 degrees towards the detector, the final focal length was set to 15.4 mm, and a 1-um aperture was used. Accelerating voltage used to obtain the analysis was 15 kV. Data was acquired using Noran System Six software. The 0% gelatin sample has only trace amounts of carbon (Supporting Information Figure S1).
**Figure S1.** EDS spectrum of control crystal garden with 0% gelatin (red), 3% gelatin (black) and 5% gelatin sample (green). Note that controls (lacking gelatin) have low Carbon.

**4. Infrared Spectroscopy**

The incorporation of gelatin in the constructs is proved by clear-cut evidence from infrared spectroscopy: the 15% gelatin construct has Amide I peak (C = O stretch) at 1650 cm$^{-1}$, amide II peak (N-H bend and C-N stretch) at ~1600 cm$^{-1}$, and amide A peak (N-H stretch) at 3300 cm$^{-1}$ which overlaps with the OH peaks (Supporting Information Figure S3). The protein peaks are absent in the control 0% gelatin sample (Supporting Information Figure S2). The peak at 1050 cm$^{-1}$ is from Si-O bond stretching originating from the silicate ions.$^{1}$ The peak at 3650 cm$^{-1}$ corresponds to a O-H bond stretch. Unlike the normal broad peaks for most O-H stretches this peak is shifted to a higher wave number and is significantly sharper.$^{1}$ This is specific to a “free” or non-hydrogen bonded hydroxyl group. Normally these peaks are not seen in most samples due to prevalence of hydrogen bonding in hydroxyl containing compounds. Given the composite nature of the sample it is possible that a sub-population of the hydroxyl groups within the solid do not participate in hydrogen bonding.

**Figure S2.** IR spectrum of silicate-phosphate control scaffold without gelatin
Figure S3. FTIR spectrum of 15% gelatin-silicate-phosphate tubular scaffold

5. Biotinylation of gelatin incorporated calcium silicate-phosphate constructs

Programmable stigmergy scaffolds were synthesized in accordance with Scheme 1 in the manuscript. Biotinylation of gelatin incorporated calcium silicate phosphate constructs was done by dissolving biotin (17.5mg, 0.0716mmol) in 500µL DMSO and adding it to a solution of N-Hydroxysuccinimide (12.4mg, 0.1074mmol), and EDC.HCl (22.2mg, 0.1432mmol) dissolved in 2.5mL water. The constructs (grown with 15% gelatin, 3M sodium silicate, 0.5M potassium phosphate and seeded with CaCl₂) were added to the above reaction mixture and agitated gently using a shaker at room temperature for 3h; this was followed by washing the final biotinylated constructs with PBS (pH=7.4).
6. Avidin attachment on the biotinylated calcium silicate phosphate constructs

A solution of avidin (2mg, 30µmol) in 1mL PBS (pH=7.4) was added to the biotinylated gelatin constructs and agitated gently at room temperature for 12h using a shaker; then, the avidin modified constructs were washed with PBS (pH=7.4).

7. Synthesis of biotinylated amino dextran

Amino dextran 70KD MWT with ~15 amino groups per Dextran molecule from Molecular Probes Inc. (100mg, 1.428µmol), N-Hydroxysuccinimide (3.7mg, 0.0321mmol), and EDC.HCl (6.6mg, 0.428mmol) were dissolved in 3mL water and degassed with N₂. A solution of biotin (5.2mg, 0.0214mmol) in 3mL DMSO was added to the above reaction mixture and allowed to stir for one day at room temperature. The reaction mixture was then dialyzed using Spectra Por 6 dialysis membrane (MWCO 3.5 KD) in water for 48h. The product was further purified using a Sephadex LH20 column with water as eluent, lyophilized and further characterized via proton NMR.

\[ ^1H \text{ NMR} (600 \text{ MHz}, \text{ D}_2\text{O}): [a \text{ from the dextran component } \delta(\text{ppm})4.87-4.98 (\text{d, 1H})], [b \text{ from attached biotin component } \delta(\text{ppm})4.52-4.57 (\text{dd, 1H})], [c \text{ from attached biotin component } \delta(\text{ppm})4.35-4.40 (\text{dd, 1H})], \delta(\text{ppm})3.89-4.00 (\text{brd, 2H}), \delta(\text{ppm})3.73-3.82 (\text{brd, 1H}), \delta(\text{ppm})3.51-3.70 (\text{dd, 1H}), \delta(\text{ppm})3.42-3.52 (\text{dd, 1H}), \delta(\text{ppm})3.36-3.42 (\text{d, 1H}), \delta(\text{ppm})3.19-3.22 (\text{dt, 1H}), \delta(\text{ppm})2.84-2.91 (\text{dd, 1H}), \delta(\text{ppm})2.51-2.55 (\text{t, 1H}), \delta(\text{ppm})2.0-2.2 (\text{t, 2H}), \delta(\text{ppm})1.43-1.64 (\text{m, 4H}), \delta(\text{ppm})1.24-1.32 (\text{q, 2H}).\]

The extent of biotinylation of the amino dextran was determined to be 9 biotins per dextran molecule by comparing the integration of the proton peak (a) arising from dextran component and the proton peaks b and c from attached biotin. (Supporting Information Figure S4)
Figure S4 $^1$H NMR spectrum of biotinylated amino dextran.

8. Coupling of biotinylated amino dextran with the avidin modified constructs

The avidin attached constructs were incubated in a solution of biotinylated amino dextran in PBS (10mg/mL) for 24h; the final constructs were washed with PBS. Dextran analysis was performed by treating a few pieces of the constructs with 1mL of 5% phenol and 6mL of concentrated sulfuric acid for 1h to ensure complete color development. The UV absorbance was measured at 480nm. Color development was observed in the dextran coated sample but was absent in the control sample (Supporting Information Figure S5).

Figure S5. The UV-visible absorption spectra of the Dextran coated constructs and the 15% gelatin constructs (control).
9. Chitosan cue modified 15% gelatin incorporated calcium chloride scaffolds for H9C2 cell attachment

The 15% gelatin calcium chloride scaffold constructs were submerged in 1% ascorbic acid solution for 10 minutes and then thoroughly rinsed with PBS (pH 7.4) to adjust the pH. To facilitate cell binding, the constructs were then coated with chitosan by submerging them in 1% chitosan solution in 2.5% acetic acid for 30 seconds. The chitosan solution was then removed and a thin layer of the chitosan is precipitated onto the surface of the constructs by adding isopropanol. The scaffolds were rinsed with deionized H₂O. The successful coating of chitosan was confirmed via the phenol-sulfuric acid assay for carbohydrates; UV absorbance at 480nm confirmed the presence of chitosan on the constructs.

10. H9C2 Cell Culture and Live Cell Imaging

H9C2 cells were used for 3D cell culture on the surface of chitosan coated 15% gelatin incorporated calcium chloride scaffolds. The cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium), Nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 0.1% penicillin, and 25mM HEPES at 37°C, 5% CO₂. The chitosan coated constructs were sterilized by submerging them in 70% ethyl alcohol under UV light for 1h and then washed with fresh cell culture media before commencing the 3D cell culture experiments. Cells were plated in the medium in sterile 24 well plates at a density of one million cells/cm² along with the constructs; the cells were left to adhere to the scaffold and proliferate for 24 h at 37°C, 5% CO₂. The cells were labeled with cytoplasmic live cell indicator Fluorescein diacetate and viewed using a Zeiss live cell imager (Supporting Information Figure S8); from the figures it is evident that the construct is completely covered with viable cells which fluoresce green.
**Figure S6.** H9C2 cells on the chitosan coated scaffold labeled with cytoplasmic live cell indicator Fluorescein diacetate and imaged using a Zeiss live cell imager, the live H9C2 cells fluoresce green.

References: