Bulk and Surface Covalent Immobilization of ECM Molecules on Hydrogels formed by Michael-type Addition

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Statement of Purpose: Many synthetic hydrogels are resistant to protein adsorption and cell adhesion, providing the opportunity to immobilize specific adhesive ligands for directed cell-material interactions. Previously, we have shown that L1 neural cell adhesion molecule immobilized to low protein binding surfaces supports selective neuronal adhesion.[1] Our long term goal is to immobilize L1 to hydrogel coatings on polymer fibers. Toward this end, the present study investigated methods for bulk and surface protein immobilization to synthetic hydrogels and resulting bioactivity, using fibronectin as a model adhesive macromolecule.

Methods: Tetronic® T904 4-arm block copolymers (BASF) were modified with terminal acrylate groups using acryloyl chloride (T904 ACR, Figure 1) and crosslinked by Michael-type addition using dithiothreitol (DTT) or PEG-dithiol (PEGDT, Aldrich).

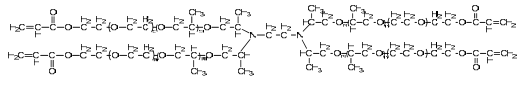

Fig 1. Tetronic T904 with terminal acrylate groups

Two approaches of protein conjugation were employed: Bulk immobilization: *1. Effect of reduction time:* 10 µl fibronectin (FN, 1 mg/ml) was reduced by mixing with 10 µl DTT (106 mg/ml) for 0-30 minutes. Reduced FN was then mixed with 80 µl 28.75% (w/v) T904 ACR in 50 mM PBS (adjusted to pH 7.2), vortexed, centrifuged, injected between glass coverslips separated by 1 mm spacers, and allowed to crosslink for 2 hrs at room temperature and 2 hrs at 37 °C. The DTT concentration was selected to provide a final 1:1 acrylate:thiol ratio. *2. Effect of pH.* Reduced FN (5 min) was mixed with T904 ACR prepared at final pH ranging from 7.0 to 8.0 and crosslinked as described above. *3. Effect of FN concentration.* Hydrogels were crosslinked as described above with T904 ACR pH 7.2 and reduced FN (5 min) at varying final concentrations ranging from 1-100 µg/ml.

Surface Immobilization: T904 ACR hydrogels were crosslinked with PEGDT at 1:1.2 acrylate:thiol ratio to leave unreacted thiol groups for subsequent protein immobilization. FN was modified with pyridyldisulfide groups using SPDP [Succinimidyl 3-(2-pyridyldithio) propionate)] (Pierce), purified by size exclusion chromatography, and applied to the surface of the gels 30 min after the initiation of crosslinking in 48 well plates and left overnight at room temperature for conjugation. Gels incubated with non-modified FN were used as negative controls.

Assay of FN bioactivity: After overnight crosslinking, gels with bulk immobilized FN were transferred to 24 well plates. All gels were rinsed with PBS, equilibrated with media at 37 °C for 1 hour, and seeded with NIH 3T3 fibroblasts. After 24 hrs culture, cells were fixed, stained with Alexa 594-phalloidin, and digitally imaged by fluorescent microscopy. Cell spread area was measured using ImagePro software.

Results: *Bulk immobilization:* Fibroblast spreading increased with increasing reduction time (Figure 2A) and decreased with increasing pH (Figure 2B). Fibroblast spreading showed a dose-dependent response to the amount of incorporated FN (Figure 2C). All gels containing FN supported cell adhesion with a minimal rounded morphology corresponding to the approximately 800 μm^2 minimum area seen in the figures. Negative controls without any FN did not support cell adhesion. *Surface Immobilization:* FN-SPDP immobilized to T904 ACR/PEGDT hydrogels supported fibroblast spreading comparable to bulk immobilized FN (data not shown). Comparable incubation of gels with native, unmodified FN did not support fibroblast adhesion, confirming the specificity of immobilization.

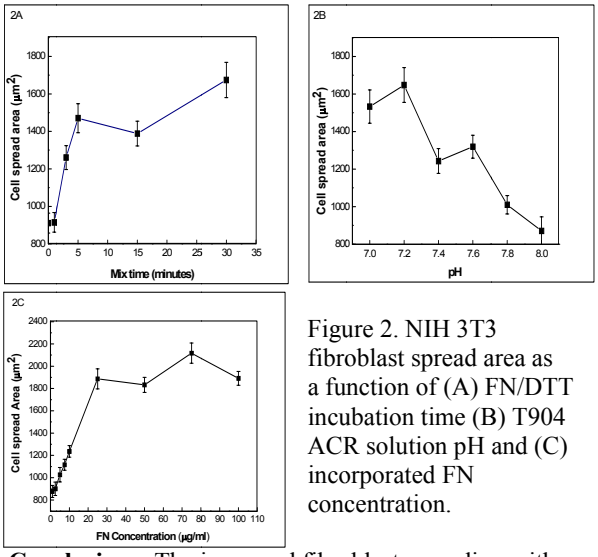

Figure 2. NIH 3T3 fibroblast spread area as a function of (A) FN/DTT incubation time (B) T904 ACR solution pH and (C) incorporated FN concentration.

Conclusions: The increased fibroblast spreading with increased reduction time and reduced crosslinking pH indicates that FN bioactivity requires covalent immobilization and not simply physical entrapment. This is based on the expectation that increasing reduction time generates an increased number of free thiols for covalent immobilization and increased pH accelerates crosslinking, limiting the reaction efficiency of a large macromolecule such as FN relative to the low MW DTT crosslinker. Preliminary studies have shown that L1 neural cell adhesion molecule does not retain bioactivity during bulk immobilization, likely due to the role of disulfide-bonded Ig domains in its bioactivity. Current studies are investigating its surface immobilization and activity.

References: 1. Webb K, et al. *Biomaterials* 2001; **22**:1017.

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