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Krisitan Stipe
University of Montana, Missoula

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CHARACTERIZATION OF TETRAETHYL ORTHOSILICATE-BASED DYNAMIC HYDROGELS FOR USE AS REVERSIBLE 3-D CELL CULTURE MATRICES

By

KRISTIAN TYLER STIPE

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Thesis

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Approved by:

Scott Whittenburg, Dean of The Graduate School
Graduate School

Christopher Palmer, Chair
Chemistry and Biochemistry

David Macaluso
Physics & Astronomy

J.B. Alexander ‘Sandy’ Ross
Chemistry and Biochemistry

Monica Serban
Biomedical & Pharmaceutical Sciences
CHARACTERIZATION OF TETRAETHYL ORTHOSILICATE-BASED DYNAMIC HYDROGELS FOR USE AS REVERSIBLE 3-D CELL CULTURE MATRICES

Chairperson: Christopher Palmer

Cell culture trends have shifted in recent years towards techniques that allow cells to be grown in physiologically representative, three dimensional (3D) environments. In response numerous cell culture matrices were developed and commercialized, and while they do provide extracellular matrix-like environments for the cells to grow in, they lack reversibility, hindering the recovery of cells for downstream analyses and passaging. To address this unmet need of the 3D culture market, we sought to develop a cell culture matrix with intrinsic reversibility, based on tetraethyl orthosilicate (TEOS), hyaluronic acid, and gelatin. Our results indicate that the generated hydrogels are stable under physiological conditions, present unique thixotropic properties, and have the ability to transition between liquid and gel states in response to gentle shear, thus imparting reversibility to the matrix. However, our tests indicate that the cytocompatibility of the hydrogels present challenges as the metabolic activity of encapsulates cell was impacted. Further experimental assessments we conducted suggest that these negative cellular effects are associated with the nanoparticles formed by the polymerization of TEOS and that these effects can be modulated through the addition of high molecular weight polymers such as polyethylene glycol (PEG).
Introduction

Historically, three dimensional (3D) cell cultures have been implemented to study mammalian cells in physiologically representative environments.\textsuperscript{1-4} The literature includes several studies where cells grown in two dimensional (2D) cultures yield irrelevant results for in vivo studies, especially in the context of drug screening.\textsuperscript{15-9} Cells found in nature produce extracellular matrices (ECM) comprised of proteoglycans, hyaluronic acid, collagen, elastin, other glycosaminoglycans (GAGs), and proteins.\textsuperscript{10,11} Many commercially available 3D cell culture matrices are produced by mimicking the physiological ECM.\textsuperscript{10} One such product, Purecol, consists of 99.9% pure bovine type I collagen.\textsuperscript{8} Another product, Matrigel, is composed of a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells.\textsuperscript{12} HyStem-C is a third example of a commercially available 3D cell culture matrix made of in situ crosslinkable thiol-modified hyaluronic acid, thiol-modified gelatin, and polyethylene glycol diacrylate (PEGDA).\textsuperscript{13,14} All three of the aforementioned materials are used for 3D culturing for mammalian cells, even though they have different formulations. However, they all have the same shortcoming: the need for potentially harmful conditions, such as enzymatic digestion or addition of reducing agents, in order to break apart the matrix for recovery or analysis of the cells.\textsuperscript{9,15}

This project was aimed at solving the issue of not being able to recover 3D encapsulated cells without using potentially harmful conditions. In order to accomplish less harsh recovery, a hydrogel made from tetraethyl orthosilicate (TEOS) was used as a base scaffold, and hyaluronic acid (HA) and gelatin were included to provide a physiological condition similar to the secreted ECM of the fibroblast cells that were studied in the literature.\textsuperscript{11,13,16,17} TEOS was chosen as a hydrogel scaffold for its rheological characteristics, specifically for its thixotropic behavior.\textsuperscript{18,19} Thixotropy is the property of a material becoming less viscous when subjected to an applied stress and then returning to the original state once the stress is removed.\textsuperscript{20} This provides the ability to separate the hydrogel matrix from the cells simply using a gentle vortexing followed by centrifugation. Once the cells have been pelleted the hydrogel can be removed by simply pouring or pipetting away the soft hydrogel. Gelatin was chosen to be included in the hydrogel for its ability to provide cellular attachment sites, which can promote cell proliferation throughout the hydrogel itself.\textsuperscript{21,22} Hyaluronic acid was chosen because it is a natural component of cellular ECM and can contribute to cell proliferation and migration.\textsuperscript{10,23}

Project Goals

The goal of this project is to characterize the individual components of the hyaluronic acid and gelatin adducts (HAG) thixotropic hydrogels composed from TEOS and HAG, and then characterize and optimize the hydrogel as a cell culture medium.

Methods

Carboxymethyl Hyaluronic Acid Modification

Carboxymethyl hyaluronic acid (CMHA) synthesis was achieved through a heterogeneous phase reaction.\textsuperscript{24} Briefly, 250 mg of hyaluronic acid (HA) was dissolved into 2.5 mL of 45% w/v sodium hydroxide (NaOH) and stirred for two hours at room temperature. The viscous solution was transferred to a beaker containing 180.75 mL of isopropanol. A chloroacetic acid/isopropanol solution was prepared by dissolving 250 mg chloroacetic acid into 6.25 mL isopropanol. The solution containing HA and isopropanol was added in the isopropanol/chloroacetic acid mixture for 1 hour at room temperature. The sample was filtered with a #2 Whatman paper and a Büchner funnel. The HA was insoluble during this whole process. The collected CMHA was then dissolved in
25 mL of deionized water and the pH of the solution adjusted to ~pH 7.0 using 6M HCL. The solution was dialyzed against deionized water for 24 hours with 4 changes of water using dialysis cassettes with a molecular weight cutoff of 3500 Da.25

**Carboxymethyl Hyaluronic Acid/Gelatin Coupling**
The CMHA and gelatin coupling was performed by dissolving 10 mg of CMHA in 10 mL of 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer at pH 4.75 (0.5 mmol CMHA). Then 10 mg gelatin (Bloom 75) was added and allowed to dissolve. Next, 2.4 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCL (EDC) (0.25 mmol EDC) was added to the solution. The reaction was stirred for 4 hours while a pH between 4.75 and pH 6 was maintained (HCl was used for pH adjustment). After 4 hours the pH was adjusted to pH 8.5 with NaOH, then dialysis of the reaction mixture was performed against water in 50,000 Da molecular weight cutoff dialysis tubing. Seven water changes over 48 hours were performed. After dialysis was complete the solution was frozen at -80°C for 4 hours then lyophilized.10,26 This reaction scheme is shown in Figure 1.

![Chemical Scheme of the EDC Crosslinking Process](image)

**Figure 1**: Chemical scheme of the EDC crosslinking process.

**Biuret Colorimetric Assay**
The Biuret reagent was used to measure the amount of gelatin present in hyaluronic acid/gelatin (HAG) and carboxymethyl hyaluronic acid/gelatin (CMHAG) via a colorimetric assay. The Biuret assay is a copper (II) complex that will bind between two peptide bonds to give a colorimetric change.

The Biuret reagent was made by dissolving 6.0 g sodium potassium tartrate tetrahydrate in 500 mL dH2O. CuSO4 pentahydrate (1.5g) was then added, followed by slow addition of 300 mL of 10% (w/v) NaOH. The resulting solution was diluted to 1 liter with dH2O.

**Hydrogel Formation**
Hydrogels were formed by first hydrolyzing 2 mL of tetraethyl orthosilicate (TEOS) in 18 mL of 0.15 M acetic acid, which gives a 1:9 v/v ratio. The solution was vigorously mixed for 1 hour until a homogeneous solution was obtained. The resulting acid-hydrolyzed tetraethyl orthosilicate (HTEOS) was used to make hydrogels. Formulations of 1:1 and 1:2 v/v water or phosphate-buffered saline (PBS) to HTEOS ratios were tested. For hydrogel formation to occur the water or PBS and HTEOS mixture was adjusted to ~ pH 2 by using 3 M acetic acid and left to stand for 3
hours on the bench. After 3 hours the pH was adjusted to ~pH 8.5 with 1.5 N ammonium hydroxide. Gelation of hydrogels was observed within 30 seconds to 30 minutes, depending on the ratio of water or PBS to HTEOS used. Hydrogel samples were then left overnight to allow the gel to completely form. The hydrolysis reaction scheme is shown in Figure 2.

For CMHA acid crosslinked with gelatin (CMHAG) hydrogels, a solution of CMHAG was dissolved in PBS and used in place of regular PBS as described above.

![Diagram of hydrolysis reaction scheme]

**Figure 2**: Chemical scheme for hydrolysis of tetraethyl orthosilicate (TEOS).

**Cell Assays - Cell Viability and Proliferation/Metabolic Activity**
Primary adult human dermal fibroblasts (HDF) (Lonza) were used to assay the cytocompatibility of the TEOS hydrogels. Multiwell (96 or 24 well) plates were coated with 100 mL or 250 mL of hydrogel respectively, then seeded with 10,000 cells/well or 20,000 cells/well respectively. The cells were cultured in serum-free fibroblast conditioned media (Lonza) and incubated for 24 hours at 37°C and 5% CO₂ prior to assaying. Cell viability was determined by using a Live/Dead cytotoxicity assay kit (ThermoFisher) for mammalian cells. All images for the Live/Dead assay were taken on a Leica DMI3000 B brightfield/fluorescent microscope at 10X magnification, unless otherwise stated. Cellular proliferation/metabolic activity was quantified with a Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) (MTS assay) and detected via absorption measurements at 450 nm on a Molecular Devices FilterMax F5 microplate reader (Molecular Devices).

**Rheological Characterization**
The rheological properties of all hydrogels were characterized with a Discovery HR2 hybrid rheometer (TA Instruments). The tests were conducted within the materials’ pseudo-linear viscoelastic range with a 1.00 mm gap, at 20°C, unless otherwise specified. Oscillatory strain sweeps for thixotropy investigation were conducted with an 8-mm parallel plate geometry within a strain range of 1–250% and an angular frequency of 10 rad/s. The wait time between the cycles was 30 seconds. For temperature-dependent hydrogel behavior evaluation, samples were loaded onto the Peltier plate and a 20-mm parallel plate geometry (to provide a larger temperature exchange surface) was used for characterization. The hydrogel samples were equilibrated to 4°C then subjected to a temperature ramp of 2°C/min up to 37°C, at a strain rate of 0.4 % and an angular frequency of 10 rad/s. Swelling tests were performed with the 20 mm flat plate geometry and an active axial force control of 0.1 N. Hydrogels were loaded onto the Peltier plate and
equilibrated at 37°C for 6 min. Phosphate-buffered saline (PBS), pre-warmed to 37°C, was then added and the gap distance between the Peltier plate and the 20 mm parallel plate geometry was monitored for 30 min post addition. Initial gap heights varied from 1000 to 2000 μm depending on sample loading variations.19

Scanning Electron Microscopy (SEM)
The hydrogel constituent nanoparticles were investigated with a Hitachi S-4700 Field Emission Scanning Electron Microscope (Hitachi Instruments). The hydrogel samples were made in 15 mL conical tubes, frozen at -80°C for 4 hours, and then lyophilized for 24 hours. The dry particles were vortexed to break up nanoparticle aggregates. The dried samples were placed on a conductive carbon tab and sputter coated with gold in a Denton Desk V sputter coater (Denton Vacuum). Images were taken at 45,000X magnification.

Dynamic Light Scattering (DLS)
To confirm the nanoparticle size non-gelling hydrolyzed TEOS formulations (1:0.1 v/v Water/HTEOS ratio) were investigated at 25°C using a Zetasizer Nano ZS DLS spectrometer (Malvern Instruments).

Proton Nuclear Magnetic Resonance (1H-NMR)
Proton nuclear magnetic resonance (1H-NMR) spectral data were obtained using a Varian INOVA 400 (400 MHz, Agilent Technologies). All water peaks were set to a reference value of 4.5 ppm. Solvents for all samples were either deuterated water (D2O) or deuterated dimethyl sulfoxide (DMSO).

Fourier Transform Infrared (FTIR)
Fourier transform infrared analyses were conducted on a Nicolet™ iS5 Spectrometer with iD5 ATR accessory from ThermoFisher Scientific.

Results & Discussion:
The obtained data are discussed in three individual subsections: the analytical characterization of the individual hydrogel constituents, the effects of hydrogel composition on cellular viability, and the rheological characterization of hydrogels. The first step was to characterize and quantify the amount of hyaluronic acid and gelatin present in the HAG adduct, then to quantify the byproducts of TEOS hydrolysis reaction; ethanol and nanoparticles. The second step was to investigate the cellular effects of the hydrogels and the cellular interaction mechanisms of HTEOS hydrogel components. The final step was the rheological characterization of the TEOS hydrogel containing the HAG component and polyethylene glycol (PEG).

Analytical Characterization of Hydrogels and Constituents

Carboxymethyl HA
Proton nuclear magnetic resonance (1H NMR) in deuterated water (D2O) was performed to confirm and quantify carboxymethyl modification of HA. The carboxymethyl modification can be observed via NMR by monitoring the chemical shift at 3.87 ppm. This chemical shift corresponds to the CH2 group that was added as part of the chemical modification. Figure 3A shows the NMR spectrogram of unmodified HA and Figure 3B shows the NMR spectrogram of CMHA. Evidence of modification appears at 3.87 ppm in the modified HA and not in the unmodified HA spectrogram. In addition to identification of modification, the degree of modification can be determined by using peak
integration of the NMR spectrograms. The peak at 1.85 ppm corresponds to the CH$_3$ group of the N-acetyl functional group. The integration of the CH$_2$ group from the carboxymethyl modification can be compared to the CH$_3$ group of the N-acetyl functional group in HA to determine modification efficiency. When the integration is performed it appears that the modification efficiency is ~40%. This corresponds to ~40% of the HA being modified with carboxymethyl functionalities.

**Figure 3**: $^1$H NMR of hyaluronic acid (A) and carboxymethyl modified hyaluronic acid (B). The red square (■) correlates the added CH$_2$ group from the modification to the peak on the NMR. The structures of hyaluronic acid and carboxymethyl hyaluronic acid are shown in (C).

**CMHA-Gelatin Crosslinking**
The successful crosslinking of CMHA and gelatin was confirmed by $^1$H NMR. Figure 4 (A) shows the spectrogram of the reaction control, for which CMHA and gelatin were combined under the same condition as the crosslinked compound but without EDC, the crosslinking reagent. After the purification process no gelatin specific signals were visible in the spectrogram. Figure 4 (B) shows the NMR spectrogram of carboxymethyl hyaluronic acid crosslinked to gelatin (CMHAG) in the presence of EDC. The end product, after purification, shows NMR resonances at 4.5 ppm and 7.5 ppm associated with gelatin. Samples were analyzed in deuterated DMSO.
Gelatin Quantification in HAG

The quantification of gelatin in the HAG adduct was determined using a colorimetric biuret assay, which binds copper (II) to peptide bonds to produce a colorimetric change.

To construct a gelatin standard curve a 1:1 volumetric ratio of biuret to gelatin solution was used to prepare the standard curve. A serial dilution of gelatin was made with concentrations between 4 mg/mL and 18 mg/mL and the absorbance of each solution at 540 nm was measured with a SpectraMax Plus 384 plate reader.

\[
y = 0.0062x + 0.0102 \quad R^2 = 0.9813
\]

**Figure 5**: Gelatin standard curve obtained by using the Biuret assay.

Once the standard curve was constructed, known concentrations were used to validate the accuracy of the curve. In Table 1 the known concentrations are shown on the left and the concentrations calculated using the standard curve are on the right.
<table>
<thead>
<tr>
<th>Known Gelatin conc. (mg/mL)</th>
<th>Calculated Gelatin conc. (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>7.142 ± 0.004</td>
</tr>
<tr>
<td>13</td>
<td>13.780 ± 0.003</td>
</tr>
<tr>
<td>15</td>
<td>16.144 ± 0.001</td>
</tr>
</tbody>
</table>

**Table 1**: Known concentrations of gelatin compared to concentrations calculated by using the absorption values and the linear regression line from the standard curve.

Once the standard curve was confirmed to be accurate, samples of HAG adducts were tested to determine their gelatin content (Table 2). Specifically, three HAG concentrations were tested (10, 20, and 40 mg/mL) to determine their gelatin content. A HAG concentration of 10 mg/mL yielded 4.062 ± 0.002 mg/mL gelatin, a HAG concentration of 20 mg/mL yielded 9.383 ± 0.002 mg/mL gelatin, and a HAG concentration of 40 mg/mL yielded 17.347 ± 0.003 mg/mL gelatin. Based on these results, the percent concentration of gelatin in the HAG samples were determined to be in the 40-47% range (Table 2). Pure hyaluronic acid was also tested (data not shown) to ensure that there was no background absorbance from the unmodified HA.

<table>
<thead>
<tr>
<th>HAG conc. (mg/mL)</th>
<th>Calculated conc. (mg/mL)</th>
<th>% Gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4.062 ± 0.002</td>
<td>40.62 ± 0.16</td>
</tr>
<tr>
<td>20</td>
<td>9.383 ± 0.002</td>
<td>46.92 ± 0.23</td>
</tr>
<tr>
<td>40</td>
<td>17.347 ± 0.003</td>
<td>43.37 ± 0.31</td>
</tr>
</tbody>
</table>

**Table 2**: Measured concentrations and percentages of gelatin in HAG samples at different initial concentrations.

When carboxymethyl modified hyaluronic acid/gelatin adduct (CMHAG) was analyzed under the same conditions it was shown that more gelatin was bound to CMHAG compared to HAG. This is displayed in Table 3.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Conc. (mg/mL)</th>
<th>Gelatin Conc. (mg/mL)</th>
<th>% Gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMHAG</td>
<td>20</td>
<td>13.53 ±0.0012</td>
<td>67.64 ± 0.12</td>
</tr>
<tr>
<td>HAG</td>
<td>20</td>
<td>9.38 ± 0.0023</td>
<td>46.92 ± 0.23</td>
</tr>
</tbody>
</table>

**Table 3**: Comparison of gelatin content in CMHAG and HAG.

**Ethanol Quantification in TEOS Hydrogels**

**Ethanol Standard Curve:**

A Fourier transform infrared (FTIR) assay was developed to determine the concentration of ethanol in HTEOS gels. The C-O bond of the ethanol was monitored at 1044 cm⁻¹ to construct the standard curve and perform quantitative analyses. The standard curve is presented in Figure 6.
Figure 6: Ethanol standard curve based on C-O stretch vibration at 1044 cm\(^{-1}\) using FTIR.

**Amount of Ethanol in Hydrogel:**
Once the standard curve was constructed, 4.5 ml of a 1:1 v/v water/HTEOS control hydrogel was washed several times until ethanol was no longer detectable via FTIR. The volume used corresponds to those used for cell cultures. The amounts from each wash were added up and compared to theoretical values in 4.5 mL of hydrogel and 100 μL of hydrogel in Table 4.

<table>
<thead>
<tr>
<th>Parameter determined</th>
<th>Measured</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume of EtOH in 4.5 mL of hydrogel (μL)</td>
<td>129.7</td>
<td>156.9</td>
</tr>
<tr>
<td>Amount of EtOH in 100μL of hydrogel (μL)</td>
<td>2.88</td>
<td>3.49</td>
</tr>
</tbody>
</table>

Table 4: Calculated and theoretically determined ethanol volumes in a hydrogel.

**Nanoparticle Characterization**
Quantification of nanoparticles in a 1:1 control hydrogel was done by lyophilizing five replicates of 100μL of hydrogel and determining the resulting mass of silica nanoparticles. The end result was 1.648 ± 0.081 mg in 100 μL of hydrogel. The dried samples were analyzed using scanning electron microscopy (SEM). Dynamic light scattering (DLS) was performed on a 1:0.1 v/v water/HTEOS control hydrogel to determine shape and size of the produced nanoparticles.

**Scanning Electron Microscopy (SEM):**
The dried mass of nanoparticles obtained through the lyophilization of hydrogels was analyzed by SEM. Figure 7A shows the appearance of hydrolyzed TEOS, with a smoother surface with no major signs of nanoparticles. In Figure 7B, a 1:1 control hydrogel is shown, and appears that there are nanoparticles smaller than 100 nm. In Figure 7C, a 1:0.1 control hydrogel is shown, and it has a much looser network than the 1:1 hydrogel, which is expected, but still has nanoparticles smaller than 100 nm.
Figure 7: Scanning electron microscope (SEM) images at 45,000X magnification. (A) HTEOS. (B) 1:1 v/v water/HTEOS hydrogel. (C) 1:0.1 v/v water/HTEOS hydrogel. The scale bar is 1 μm.

Dynamic Light Scattering (DLS):
The DLS was performed on a 1:0.1 v/v water/HTEOS control hydrogel to allow for slower gelation times (Figure 8). At the initial starting point (t=0 minutes), the average diameter of the nanoparticles is 22.4 nm, which agrees with the estimation found using SEM. Sample was placed in the Zetasizer for analysis immediately after increasing the pH to ~8.5, representing time zero in the gelation process. The secondary peak in Figure 8A at 141.6 nm can be correlated to the polymerization of the TEOS hydrogel and formation of nanoparticle aggregates. The instrument cannot provide accurate particle size after time zero, since the sample becomes a solid hydrogel within 30 seconds.

Figure 8: Dynamic light scattering analysis of a pre-gelling 1:0.1 v/v water/HTEOS mix. (A) Size distribution by intensity of a 1:0.1 control hydrogel. (B) Diameter size distribution of each peak.
Hydrogel Composition and Cell Viability
Published data on TEOS hydrogels indicate that they have cytotoxic effects on primary cells by inducing cytoplasmic vacuolation.\textsuperscript{19} The same study showed that the addition of polyethylene glycol (PEG) with molecular weights ranging between 200 and 600 g/mol attenuates the cytotoxic effects of TEOS hydrogels. In order to develop a cytocompatible thixotropic 3D culture matrix, it is important to identify the source of vacuolation and metabolic activity reduction, and the mechanism of PEG-induced cytoprotection.\textsuperscript{19,27} To this end, the effects of the hydrogel components on cell viability were investigated. Based on the well documented cytocompatibility of both HA and gelatin, the HAG was excluded from further evaluation.\textsuperscript{8,11,17} For the TEOS component, it has been shown above and in published work that ethanol and silica nanoparticles are released during the hydrolysis and polymerization processes.\textsuperscript{28} Both ethanol and nanoparticles have been reported in the literature to have cytotoxic effects.\textsuperscript{29,27,30} Therefore, for the hydrogels, the individual effect of nanoparticles and ethanol on cell viability and metabolic activity needed to be assessed.\textsuperscript{27,31–33} Human dermal fibroblasts were exposed to nanoparticles or ethanol at relevant concentrations, plated, and incubated for 24 hours before being assayed. These assays determine cell viability, proliferation, and activity of the cells under different conditions. Additionally, the mechanism of PEG-provided cytoprotection had to be determined\textsuperscript{19,27,30}

Nanoparticles
The nanoparticles for this assay were suspended in a phosphate-buffered saline (PBS) solution at the concentration specific to the measured amount of nanoparticles in a 1:1 v:v hydrogel (1.648 mg silica nanoparticles in 100 μL of hydrogel). Figure 9 shows a Live/Dead assay to determine the effects of nanoparticles on the cells. In Figure 9A, the control, there appears to be no cell death. In the presence of $\frac{1}{2}$ the concentration of nanoparticles found in 100 μL of hydrogel signs of vacuolation begin to appear, which can be seen in Figure 9B. Figure 9C shows the normal concentration for 100 μL of hydrogel and Figure 9D shows twice the measured amount of nanoparticles. The large bright red regions on the images in Figure 9 are large conglomerates of nanoparticles, and the smaller red dots are the dead cells.
Figure 9: Live/Dead assay for nanoparticles. (A) Control (B) 0.825 mg/mL nanoparticles (½x nanoparticles found in a normal hydrogel) (C) 1.65 mg/mL nanoparticles (1x nanoparticles found in a normal hydrogel) (D) 3.30 mg/mL nanoparticles (2x nanoparticles found in a normal hydrogel). Green represents an intact cell membrane, where red represents a burst cell membrane. The scale bar is 200μm.

The results of the MTS assay, a colorimetric assay of metabolic activity of the cells, are presented in Figure 10. These results show that the cells have virtually no metabolic activity in the presence of the nanoparticles, compared to the control.

Figure 10: MTS assay on nanoparticle effects. From left to right, control with no nanoparticles, ½ the concentration of nanoparticles (0.825 mg/mL), standard concentration of nanoparticles found in a 1:1 hydrogel (1.65 mg/mL), and twice the concentration of nanoparticles (3.30 mg/mL).
Ethanol
The effect of ethanol on cell behavior was studied using the same method as the nanoparticles, and the volumes used were based on results from Table 4. The results appear to indicate that ethanol, even at twice the theoretical volume measured, did not affect cell viability when observed with the Live/Dead assay (Figure 11) or the MTS assay (Figure 12).

**Figure 11**: Live/Dead assay for the effects of ethanol. (A) Control (B) 1.75 uL ethanol/mL (½x ethanol found in a normal hydrogel) (C) 3.49 uL ethanol/mL (1x ethanol found in a normal hydrogel) (D) 6.98 uL ethanol/mL (2x ethanol found in a normal hydrogel). The scale bar is 200 μm.

The MTS for ethanol treatment also supports the results from the Live/Dead. The metabolic activity of the cells in the presence of ethanol did not deviate from the control sample.
Figure 12: MTS assay on ethanol effects. From left to right: Control, 1.75 μL ethanol/mL (½x ethanol found in a normal hydrogel), 3.49 μL ethanol/mL (1x ethanol found in a normal hydrogel), and 6.98 μL ethanol/mL (2x ethanol found in a normal hydrogel).

Overall, these results indicate that nanoparticles, but not the ethanol, are responsible for vacuolation. The amount of ethanol found in each hydrogel is not sufficient to cause negative effects on the cells. Nanoparticles, even at half the concentration found in gels, eliminate the metabolic activity of the cells.

Improving Cell Viability
Having determined that the nanoparticles are causing vacuolation and reducing metabolic activity of cells, the next step is to try to reduce or eliminate these negative effects. Based on previous data from the Serban Laboratory and others, it appears that the inclusion of a large molecular weight polymer, such as polyethylene glycol, in the thixotropic hydrogels may protect cells and lead to improved cell viability.

In the current study, inclusion of two PEG polymers of different molecular weights was assessed. Incubation studies were performed to determine the mechanism of protection of the cells. It was determined that PEG 600 performed better than PEG 200 as a protecting agent, and that it was most effective if the PEG was used to encapsulate the nanoparticles.

Hyaluronic acid gelatin (HAG) adduct was introduced to the PEG 600 hydrogels, and it was found that the HAG material containing the highest amount gelatin performed somewhat better than samples without gelatin. This effect can be related to the fact that gelatin provides cellular attachment sites that encourage cell proliferation through the matrix.

The cell protective effects of PEG were tested as a solution and as a hydrogel. The concentration of the PEG solution was mirrored to be the same end concentration of PEG in the hydrogel. In the hydrogel a 5%, 12.5%, 25%, and 50% PEG solution was used, then diluted by TEOS and the required acid and base to form the hydrogel. Taking this dilution into account, the final PEG concentrations in solutions and gels were 1.3%, 3.2%, 6.4%, and 12.8% respectively.

PEG 200:
In Figure 13 the Live/Dead assay can be seen for the different concentrations of PEG 200. The 1.3% and 3.2% PEG solution treated cells do not appear visually different from the control tissue culture plate (TCP) cultured cells, whereas 6.4% and 12.8% PEG elicit a rounded, non-specific cellular...
phenotype. One explanation for the loss of the normal cellular phenotype would be due to the viscosity of PEG solution restricting the spindle shaped characteristic.

![Figure 13](image)

**Figure 13**: Live/Dead assay for the effects of PEG 200 in solution. (A) 1.3% PEG 200 (B) 3.2% PEG 200 (C) 6.4% PEG 200 (D) 12.8% PEG 200 and (E) tissue culture plate (TCP). The scale bar is 200 μm.

The Live/Dead assay results for PEG 200 TEOS hydrogels are shown in Figure 14. The concentration of PEG 200 is the same as the PEG solutions shown in Figure 13. Since the samples are now 3D gels the resolution of the images is slightly influenced by the presence of the hydrogels. Furthermore, the fact that none of the typical fibroblast morphology is seen is not unexpected as it is widely reported in the literature for 3D substrates lacking cell attachment sites, because in a 3D environment the cell attachment sites will be different than on a 2D surface.5,8,9,36 This is true for all of the hydrogels examined in this study. The cells plated on the hydrogels appear to maintain an intact membrane, as shown in in Figure 14D. However, at 20X magnification of Figure 14D, shown in Figure 14E, evidence of vacuolation can be seen in the presence of PEG 200.
Figure 14: Live/Dead assay for the effects of PEG 200 in TEOS hydrogel. (A) 1.3% PEG 200 (B) 3.2% PEG 200 (C) 6.4% PEG 200 (D) 12.8% PEG 200 and (E) 12.8% PEG 200 at 20X magnification, instead of 10X. The scale bar is 200μm.

The results are further complemented and corroborated with a cellular metabolic activity assay as illustrated in Figure 15. In the presence of PEG 200 solutions, MTS assay data indicate that there is a reduction of metabolic activity as the concentration of PEG increases, compared to the tissue culture plate. The PEG 200 TEOS hydrogels results in Figure 15 show virtually no difference compared to the control without any PEG 200, regardless of concentration.
Figure 15: MTS assay for the effect of PEG 200 solution (top) and PEG 200 hydrogels (bottom) for 5%, 12.5%, 25%, and 50%, which correspond to the end concentration of 1.3%, 3.2%, 6.4%, and 12.8% PEG 200. The control sample was TEOS hydrogel with no PEG included, and the last sample shown is tissue culture plate (TCP). All samples are normalized to TCP.

PEG 600:
When treated with PEG 600 and PEG 200, in Live/Dead assays (Figure 16) cells appear to have similar phenotypes, except for 6.4% PEG 600 (Figure 16C), where the traditional cellular phenotype of fibroblast cells for two dimensions is observed.
Figure 16: Live/Dead assay for the effects of PEG 600 in solution. (A) 1.3% PEG 600 (B) 3.2% PEG 600 (C) 6.4% PEG 600 (D) 12.8% PEG 600 and (E) tissue culture plate (TCP). The scale bar is 200 μm.

Similar to the PEG 200 hydrogels, the PEG 600 hydrogels (Figure 17) have cells growing on the Z-axis, which causes some image distortion, as there are cells growing above and below the plane of focus and are no longer coplanar. In the PEG 600 hydrogel, there doesn’t appear to be any vacuolation readily present.
Figure 17: Live/Dead assay for the effects of PEG 600 in TEOS hydrogel. (A) 1.3% PEG 600 (B) 3.2% PEG 600 (C) 6.4% PEG 600 (D) 12.8% PEG 600. The scale bar is 200 μm.

The PEG 600 solution MTS (Figure 18, left) shows a similar trend as the PEG 200 solution MTS, where the metabolic activity of the cells decreased with increasing concentration of PEG 600, predominantly at 12.8% PEG 600. The PEG 600 hydrogel MTS (Figure 18, right) shows that, in the presence of PEG 600, the fibroblasts had an increased metabolic activity compared to the control hydrogel.
Figure 18: MTS assay for the effect of PEG 600 solution (top) and PEG 600 hydrogels (bottom) for 5%, 12.5%, 25%, and 50%, which correspond to the end concentration of 1.3%, 3.2%, 6.4%, and 12.8% PEG 200. The control sample was TEOS hydrogel with no PEG included, and the last sample shown is tissue culture plate (TCP). All samples are normalized to TCP.

**PEG Protection Mechanism**

Having shown that inclusion of PEG 600 eliminates cell vacuolation and increases cell metabolic activity in the hydrogels, we investigated the mechanism of cell protection. There were two possible mechanisms of action; the PEG could encapsulate the cells themselves, or the PEG could encapsulate the nanoparticles responsible for negative cellular effects and vacuolation. To test this hypothesis cells were plated with different pre-treatments of PEG 600 50%. All incubations were accompanied by a control in PBS instead of PEG. Cells were incubated or washed for 10 minutes on the bench, plated on the multiwell plate, and incubated for 24 hours before assays were performed.

Figure 19 shows MTS assay results for different pre-treatment and hydrogel conditions. In the case of hydrogel PEG wash, 12.8% PEG 600 solution was introduced into the preformed hydrogel, left to incubate for 10 minutes, and then removed. This pretreatment resulted in some cell metabolic activity. In the case of Cell PEG wash, the cells were suspended in a 12.8% PEG solution and left to incubate for 10 minutes before being re-suspended in cell growth media. This sample showed virtually no metabolic activity. The control hydrogel, which is just a TEOS hydrogel with no PEG,
shows no metabolic activity. The PEG hydrogel is made under the same conditions as the rest of the gels, with a 50% PEG 600 solution in place of water/PBS to form a PEG 600 50% (12.8%) hydrogel. The PEG hydrogel sample shows moderate metabolic activity just as in Figure 18 (right). The last two samples are controls on tissue culture plate. The first control is plated with the cells that were incubated in the 12.8% PEG solution, which is why there is an observed decrease in metabolic activity compared to the sample seen in Figure 18 (right). The second control is non-PEG incubated cells. Cells that were not incubated in PEG 600 were instead incubated in PBS for the same amount of time.

Figure 19: Protection mechanism of PEG on cells and hydrogels based on metabolic activity. From left to right samples are as followed: Hydrogel that was washed and incubated in 12.8% PEG 600, cells incubated in 12.8% PEG 600 before being seeded on a TEOS hydrogel, TEOS hydrogel with no PEG, TEOS hydrogel with 12.8% PEG 600, tissue culture plate (TCP) with cells incubated in 12.8% PEG 600, and tissue culture plate (TCP) that had un-incubated cells.

The TEOS hydrogel treatments that showed metabolic activity were those where the hydrogels were incubated or formulated with PEG. This supports the conclusion that PEG is actually interacting with the nanoparticles in the hydrogel to prevent negative cell effects instead of the cells being surrounded by the PEG.27

CMHAG/PEG Hydrogels
With the optimal PEG molecular weight, concentration, and mechanism of protection determined, hyaluronic acid/gelatin (HAG) adduct was added to the PEG 600 hydrogels to test the material as a thixotropic 3-D hydrogel. The Live/Dead assay of 50% (12.8%) PEG 600 TEOS hydrogel with three different hyaluronic acid to gelatin ratios, 1:1, 1:0.5, and 1:0, each at three concentrations of 0.5%, 0.25%, and 0.1%, were tested. Assay results are shown in Figure 20. The distorted images shown in Figure 20 can be attributed to the 3D nature of the hydrogel that is allowing for cells to be above and below the plane of focus. The 1:0 HAG 0.1% appears to have decreased cell viability. This can be attributed to a lower concentration of HA and the absence of gelatin. Gelatin provides cell attachment sites, and the lack of binding sites will inhibit cell growth.
Figure 20: Live/Dead assay to address the effects of hyaluronic acid/gelatin (HAG) adduct on 50% (12.8%) PEG 600 TEOS hydrogels at different ratios of HA to gelatin (left axis) and difference concentrations of adduct (top axis). The scale bar is 200 μm.

The MTS assay result of the HAG/PEG/TEOS hydrogels, shown in Figure 21, supports the results from the Live/Dead assay. Most of the samples were within error of the control hydrogel with no HAG, except for 0.1% 1:0 HAG hydrogel.
Figure 21: MTS of different ratios of hyaluronic acid to gelatin (1:1, 1:0.5, and 1:0) and different concentrations of the HAG adduct (0.5%, 0.25%, and 0.1%).

CMHAG/PEG Hydrogel Rheology

With studies of the hydrogel-induced cellular behavior complete, rheological characterization of the CMHAG/TEOS/PEG hydrogel was needed. The effects of PEG addition on hydrogels' thixotropy, temperature stability, and swelling effects were studied to test if the addition of PEG changed the rheological characteristics of the hydrogel.  

Thixotropy is characterized by the crossing of the storage modulus (G'), which represents the "solid" state, and the loss modulus (G''), which represents the "liquid" state. Once the loss modulus is greater than the storage modulus the sample has liquid characteristics. After the sample is in a liquid state, to be thixotropic, it must be able to return to the "solid" or hydrogel state, and this is achieved by giving the sample a 30 second rest and then running the same experiment. As demonstrated in Figure 22, the storage modulus drops below the loss modulus, then returns to its original state after the applied force is removed, which is indicative of a thixotropic material. The material tested is a 50% (12.8%) PEG 600 TEOS hydrogel with a 0.5% concentration of 1:1 ratio of hyaluronic acid to gelatin (1:1 HAG). This sample is the highest concentration of HAG adduct, as well as the highest hyaluronic acid to gelatin ratio, making it the most extreme condition, and it still maintained the thixotropic characteristic. The fact that this material is thixotropic means that the hydrogel could be used to recover cells without the need to enzymatically digest or add reducing agents.
Figure 22: Thixotropic test, oscillatory strain % vs time of a 50% (12.8%) PEG 600 TEOS hydrogel with a 0.5% concentration of 1:1 ratio of hyaluronic acid to gelatin (1:1 HAG)

The next property tested is temperature stability of the hydrogel, because if the sample degrades within the working temperature range it would not be useful. The characterization showed that the sample was relatively stable, with a less than 1% change in storage modulus over a temperature range of 4 to 37°C.

Figure 23: Temperature dependence, oscillatory strain vs temperature of a 50% (12.8%) PEG 600 TEOS hydrogel with a 0.5% concentration of 1:1 ratio of hyaluronic acid to gelatin (1:1 HAG)

The final physical characteristic test is if the sample swells in the presence of liquid, specifically PBS. The PBS was equilibrated to 37°C prior to the experiment. The hydrogel sample was loaded onto the Peltier plate; the upper geometry was lowered and set to maintain an applied force of 0.1N. Any change in force produced by swelling of the sample would be adjusted for by increasing or decreasing the gap the gap between the two plates to maintain a constant applied force. The warmed PBS was added at two minutes and the hydrogel sample was submerged. After 7 minutes the sample had swollen to its maximum volume, which resulted in a 1.4% change in the gap.
Figure 24: Swelling test, gap vs time of a 50% (12.8%) PEG 600 TEOS hydrogel with a 0.5% concentration of 1:1 ratio of hyaluronic acid to gelatin (1:1 HAG)

Overall, the physical and viscoelastic characteristics of the CMHAG/PEG/TEOS hydrogels are comparable to the hydrogels without PEG and without CMHAG.\textsuperscript{19,27}

Conclusion

This work summarizes the characterization tests performed to address the feasibility of using tetraethyl orthosilicate (TEOS) based thixotropic hydrogels as reversible 3D cellular matrices. The goal of the project was to supplement existing data from the Serban Laboratory and to gain an understanding of the mechanism of TEOS-based hydrogel induced cytotoxicity. It was shown that, of the two reaction products of TEOS hydrolysis (ethanol and nanoparticles), the nanoparticles were causing cytotoxic effects. PEG was shown to have some protecting effects against the vacuolation of cells from nanoparticles created by the hydrogel.\textsuperscript{19,30,34} The mechanism for protection was determined to be facilitated by incubation of the hydrogel itself, not the incubation of cells before being added to the hydrogel, indicating that the PEG is most likely coating the nanoparticles to prevent vacuolation.

Overall, the acquired results indicate that although the TEOS hydrogels could support cell viability and metabolic activity their performance does not compare to the tissue culture plate or to commercially available products like HyStem.\textsuperscript{16,17,37} Cytotoxicity is likely still occurring because the PEG does not fully prevent interactions between the nanoparticles and cells. Since it appears that nanoparticles may still be a concern, a new method or a new hydrogel that will not produce nanoparticles needs to be considered.
**Future Experiments**

The data demonstrates that thixotropic hydrogels as a 3D cell culture system have merit based on their viscoelastic properties; however, the issue of cytotoxicity needs to be eliminated. The next step would be to eliminate nanoparticle formation completely and replace it with an alternative material that can form a thixotropic hydrogel but does not form nanoparticles.\textsuperscript{33,38} For such an approach, hyaluronan could be chemically modified with functionalities capable of thixotropy. Two possible materials would be N-(6-aminohexyl)aminomethyltriethoxysilane (AAS) and 4-aminobutyltriethoxysilane (ABS). These materials have a similar structure to TEOS (see Figure 25) but have only three hydrolysable ethoxy groups and a fourth, stable pendant group incorporating primary amine functionality. This amine group could be used to modify hyaluronic acid with the AAS or ABS using the same EDC chemistry that was used to crosslink hyaluronic acid to gelatin.\textsuperscript{26,39} Hypothetically, a thixotropic hydrogel could be formed using triethoxy silane-modified hyaluronic acid which would be too large to produce nanoparticles that could be internalized by cells.\textsuperscript{27,40,41}

![Chemical structure of 4-aminobutyltriethoxysilane (ABS) and N-(6-aminohexyl)aminomethyltriethoxysilane (AAS)](image)

**Figure 25:** Chemical structure of 4-aminobutyltriethoxysilane (ABS) and N-(6-aminohexyl)aminomethyltriethoxysilane (AAS)
References