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Printable Cellular Scaffold Using Self-Crosslinking Agents

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Abstract. *The current engineered skin substitutes for diabetic foot ulcer treatment lack effective host integration. The goal of this research is to create a wound care material that promotes integration with host tissue. We have been investigating a printable biodegradable scaffold composed of gelatin and oxidized alginate, both materials with very high biocompatibility and low toxicity. We investigated the printability of oxidized alginate and its use as an 'ink' for drop-on-demand crosslinking of gelatin. The oxidized alginate was characterized by Fourier transform infrared spectrophotometry. Crosslinking rates were investigated as a function of crosslinker concentration. Crosslinking densities were measured by trinitrobenzene sulfonic acid assay. The mechanical properties of the crosslinked gels were measured in dried samples. The biocompatibility and ability of the printed scaffolds to support fibroblast attachment and proliferation were tested. Our results show that using 15% oxidized alginate and 10% gelatin allows us to obtain skin wound dressings with better properties. © 2012 Society for Imaging Science and Technology.*
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INTRODUCTION

Tissue-engineered skin grafts represent a treatment for chronic wounds such as diabetic foot ulcers or venous leg ulcers when other treatment options have failed. These types of ulcer are the most common complication of diabetes mellitus, with a prevalence of 8.3% in the US.[1] Treatments for chronic wounds and their complications represent costs of more than 20 billion dollars in the US because these types of wound need repetitive treatments.[2] Close to 10% of patients admitted to hospitals receive skin graft treatments, bringing the market for skin grafting to 1 billion dollars per year.[3]

Current tissue-engineered skin grafts have several shortcomings. Acellular grafts need 10 to 14 days for vascularization, they may obscure the infection due to fluid accumulation under the graft, they can cause immunological rejection, or sometimes they can require a second procedure.[4,5] Cellular grafts suffer from immunological rejection due to allogeneic cells, they may be a source of disease transmission, they have a short life, are expensive, and are difficult to handle.[6,7,5]

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The main goal of this research is to create a tissue-engineered skin graft with better host tissue integration. We hypothesize that, by layering keratinocytes onto a vascularized matrix of fibroblast cells, the graft resembles the skin structure more closely. We will use inkjet printing to assemble the graft because it allows us to customize the graft, to create a complex structure, and to have homogeneous cell dispersions in the graft.

MATERIALS AND METHODS

Alginate is a polysaccharide extracted from algae which has been used extensively as a material for cell-based therapy, wound dressings, and dental impression.[8] Gelatin is derived from collagen, and it has been used in biomedical applications because it shows high biocompatibility and low toxicity.[9] Alginic acid sodium salt (sodium alginate) with medium viscosity and sodium periodate ACS reagent grade were purchased from MP Biomedical, gelatin from porcine skin (type A 300 bloom), antibiotic antimycotic solution (100×), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich, sodium tetraborate decahydrate (borax) from Fisher Scientific, phosphate buffered saline (PBS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and trypsin–EDTA from Thermo Scientific, ethyl alcohol from Pharmco AAPER, and Spectra/Por 6 membrane tubing MWCO 2000 from Spectrum Laboratories. Neonatal human dermal fibroblast (NHDF) cells were donated by Dr. Karina Arcaute at The University of Texas at El Paso. Dulbecco's modified eagle medium (DMEM) with high glucose was from Invitrogen. Live/Dead Viability/Citotoxicity Kit were from Marker Gene Technologies.

The spectrophotometric measurements were obtained by using a Perkin-Elmer Spectrum 100 series Fourier transform infrared (FTIR) spectrometer. Osmolarity analyses were done using a Fiske micro-osmometer Model 210. The absorbance was determined in a Biomate 3 Thermo Scientific spectrophotometer. During the mechanical testing, a dynamic mechanical analyzer (DOA 800, TA Instrument, New Castle, DE) was used. For imaging, a Nikon Eclipse Ti microscope and Nikon D-Eclipse C1 Confocal unit and Software were used.

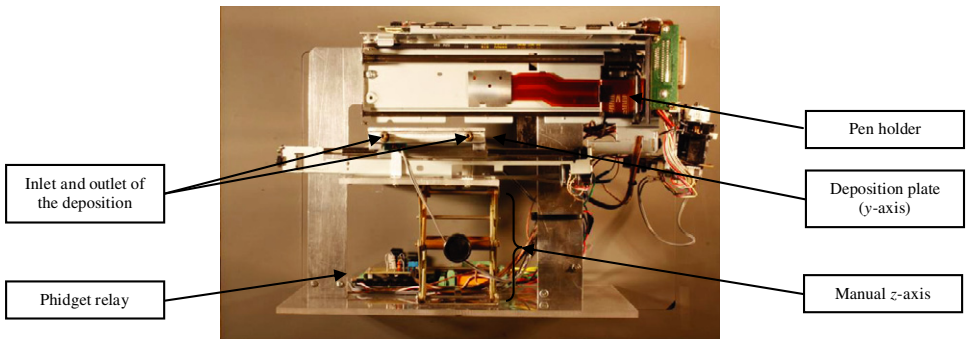

Figure 1. Photograph of the modified HP Deskjet 340 printer.

Alginate oxidation and characterization

Sodium alginate was oxidized in the presence of sodium periodate as described elsewhere.[10] Briefly, 10 g of sodium alginate was dispersed in 25 ml of ethanol in the presence of 0.4 M sodium periodate, and stirred at room temperature in the dark for 6 h. Deionized water was added to the solution to obtain 1 L, which was kept in the dark for 72 h. The precipitate solution was dialyzed until periodate was absent. The oxidized alginate (OA) was lyophilized.

The aldehyde group in OA was analyzed in the FTIR spectrometer. The spectra of the samples were measured using polytetrafluoroethylene sample holder cards in the spectral range 4000–400 cm^{-1}, and analyzing the range 2500–500 cm^{-1}.

Viscosity measurement was carried out in a Brookfield DV-E digital viscometer with a small sample adapter and reticulating ethanol–deionized-water solution through the system to vary the temperature in the testing solution. OA solutions were analyzed when dissolved in PBS and 0.1 M borax solutions varying the spindle velocity, temperature and concentration.

The osmolarity of PBS and 0.1 M borax solutions were measured to reduce swelling and gel deformation.[11]

Scaffold characterization

To measure the gelling time, 0.5 ml of 10% OA in 0.1 M borax solution was mixed with 0.5 ml of 10% gelatin in 0.1 M borax solution using a magnetic stir bar on a hot plate at 37°C and 250 rpm. The gelling time was determined by recording the time elapsed when the stir bar stopped moving.

The degree of crosslinking was determined by TNBS assay.[12] In brief, 5 mg OA was reacted with 1 ml of 0.5% TNBS and 1 ml of 4% $CHNaO_3$ at 60°C for 4 h. 1 ml of this solution was reacted with 3 ml of 6N HCl at 40°C for 1.5 h. TNBS solution reacts with the unreacted gelatin and forms a soluble solution. The absorbance (ABS) was measured at 334 nm in a spectrophotometer.[12]

Tensile testing of the OA–gelatin scaffold was measured using a dynamic mechanical analyzer (Instron 5866). Samples (10% OA 10% gelatin) were dried at room temperature. Using cardboard strips and cyanoacrylate glue, the rectangular dried samples were placed in the grips of the analyzer. Samples were stretched at room temperature with a constant deformation rate of 0.5 mm min^{-1} until fracture.

Printer modification

An HP Deskjet 340 printer and HP 33 pen were modified. The HP Deskjet 340 printer was modified to control the deposition plate, and the HP 33 pen was modified to print small sample volume. An aluminum plate was adapted to the printer using the paper feeding sensor and two switches along the *y*-axis. Temperature control of the deposition plate is achieved by using a heat exchange hydraulic system connected in a closed loop to a chiller/heater device with a resolution of 0.1°C (Thermomix/Frigomix 1460/1495, Braun). The geometry of the internal serpentine of the deposition plate allows the temperature to remain homogeneous with only a 0.4°C maximum difference across the plate in a working range from 4°C up to 90°C. The deposition plate can be adjusted along the *y*-axis, using the paper feeding sensor and two switches. This movement is controlled using a Phidget relay (interfacekit 0/0/8), connected to the four phases of a PC357XLG half stepper motor. The *z*-axis is regulated manually with an adjustable jack allowing one to vary the distance between the pen and the plate (see Figure 1).

Cell maintaining

NHDF cells were maintained with DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Cells were incubated at 37°C in a 5% CO_2 environment. Cells at passage 10 were used in the experiment after 80–90% confluence was reached.

Cell printing

200 μL of 10% OA in 0.1 M borax solution mixed with NHDF cells (1×10^6 cells/ml) and placed into the modified HP 33 pen. 800 μL of 10% gelatin in 0.1 M borax solution was used as bio-paper on a microscope slide. The biological ink was printed onto the gelatin. Once the cell–scaffold was fabricated, it was removed from the glass slide and placed in a sterile Petri dish with DMEM. The scaffold was incubated at 37°C in a 5% CO_2 environment. The medium was changed 24 h later and then every other day. Figure 2 shows the scaffold obtained by inkjet printing.

Imaging

Cell viability of the encapsulated cell was tested with Live/Dead assay (Live/Dead Viability/Cytotoxicity Kit) at 72 h. Briefly, old medium was aspirated and the samples were

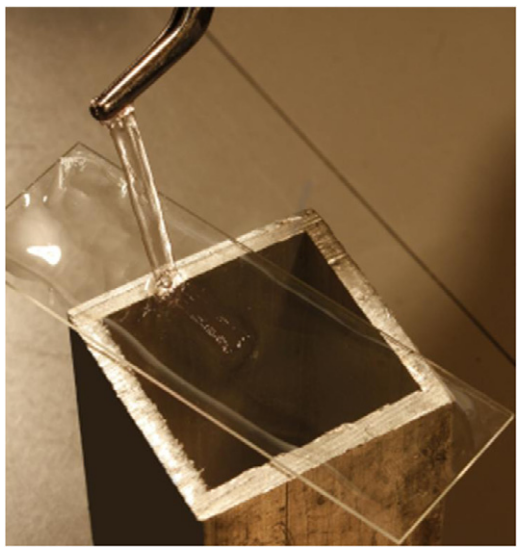

Figure 2. Photograph of a printed scaffold using a modified HP Deskjet 340 printer.

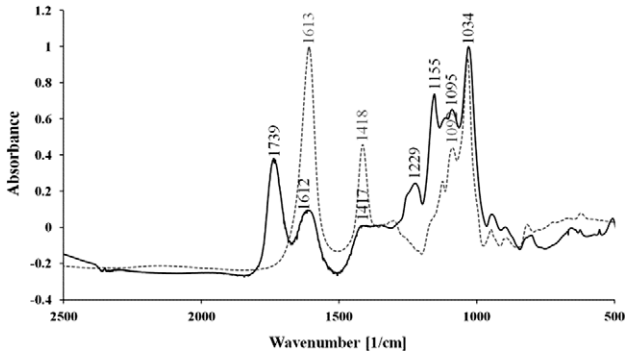

Figure 3. FTIR spectrum of sodium alginate (dashed line) and OA (solid line). The characteristic peaks are labeled.

washed with PBS to remove remaining DMEM. 20 μL of Ethidium homodimer-1 (EthD-1) and 1 μL of calcein were diluted in 10 ml of PBS. Samples were place in a 6 multi-well plate and EthD-1–calcein–PBS solution was added until the solution was 1 mm level above the scaffold. The samples were incubated at 37°C in a 5% CO_2 environment for 30 min. The stained gels were observed using a confocal microscope with fluorescence capabilities.

RESULTS AND DISCUSSION

Sodium alginate is a heteropolysaccharide composed of 4-linked β-mannuronic acid and its C-5 epimer, α-guluronic acid.[15] The hydroxyl groups of the guluronic acid in the alginate are oxidized with sodium periodate, by the rupture of the carbon–carbon bond, forming aldehyde groups in each oxidized monomeric unit.

The oxidation of sodium alginate has been previously studied by several groups,[12–15] and nearly identical reaction conditions were used in this work.

FTIR spectra of sodium alginate and OA are shown in Figure 3. The stretching C–O peak appeared at 1034 cm^{-1} in both sodium alginate and OA. The FTIR spectra for OA shows characteristic peak at 1739 cm^{-1} for aldehyde group C=O, which is absent in unmodified sodium alginate spectra.[14] The aldehyde group of OA makes it possible to crosslink with the amino acid group of gelatin, creating a non-thermoreversible gel.

Table I. Viscosity [cP] of 10% OA in PBS at different temperatures varying the spindle velocity.

	Temperature [°C]			
Spindle velocity [rpm]	25	30	35	40
10	20.1	17.7	15.3	13.2
12	20	17.3	14.7	13.2
20	22.9	19.9	17.2	14.9
30	24	20.7	18.2	15.8
50	24.5	21.12	18.54	16.14
60	24.7	21.15	18.65	16.2
100	25.02	21.42	18.93	16.5

Table II. Viscosity [cP] varying the concentration of OA in 0.1 M borax solution and the spindle velocity (the temperature remained constant at ~25°C).

	OA concentration [%]		
Spindle velocity [rpm]	5	10	15
30	1.2	3.6	5.6
50	1.62	4.14	6.36
60	1.75	4.25	6.35
100	2.13	4.53	6.63

We also determined the viscosity of 10% OA in PBS at different temperatures (the results are shown in Table I). The viscosity of 10% OA in PBS is in the range 25.02–13.2 cP, where the highest is at 25°C and the lowest at 40°C. Previous studies have shown that the viscosity range needs to be between 2 and 30 cP to obtain a printable ink.[16] Thus, one is unlikely to obtain good ink formulations with OA dissolved in PBS because solutions close to the highest range are more difficult to print. Lower concentrations of OA in PBS could be used; however, the resulting gels will be too weak. Table II shows the viscosity for different concentrations of OA in 0.1 M borax. The viscosity for OA solution is in the range 1.2–6.63 cP. For this reason, 0.1 M borax solution was employed to dissolve OA and obtain the biological ink.

The osmolarities of the ink and the substrates were measured to minimize scaffold deformation.[11] Osmolarity is very important in cell culture because, when the osmolarity is too high or too low, the cell growth rate decreases and the cell death rate increases.[17] The typical osmolarity range for cells is 260–320 mOsm/kg.[18] The osmolarity for 0.1 M borax solution is 366 mOsm/kg, for PBS solution is 291 mOsm/kg, and for DMEM is 324 mOsm/kg. To prevent scaffold deformation and cell death, it will be necessary decrease the borax concentration. The osmolarity was measured for different borax concentrations; the optimal concentration was 0.09 M (330 mOsm/kg).

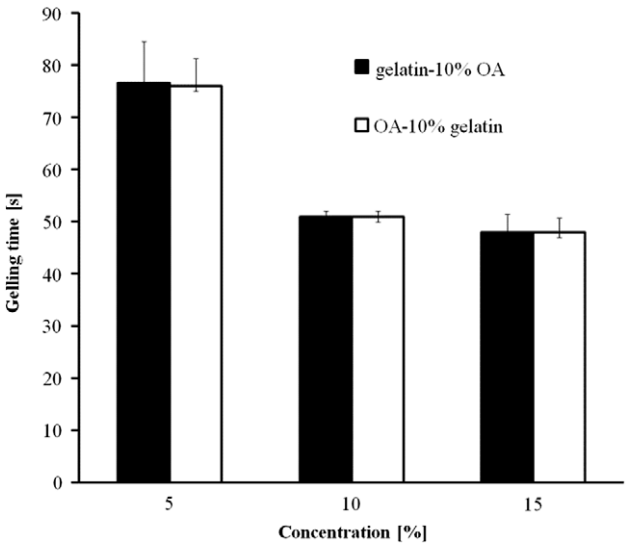

Figure 4. The black bar indicates the gelling time for OA (10% in 0.1 M borax) when the concentration of gelatin is varied (5%, 10%, and 15%). The white bar indicates the gelling time for gelatin (10% in 0.1 M borax) when the concentration of OA is varied (5%, 10%, and 15%).

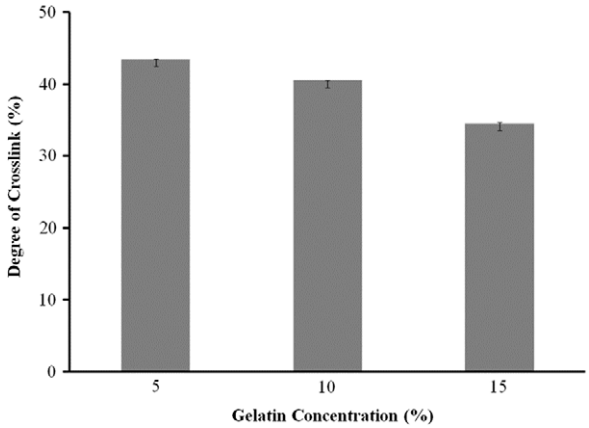

Figure 5. Degree of crosslinking for 10% OA in 0.1 M borax at different gelatin concentrations.

The gelling time was determined for gelatin and OA solutions at different concentrations. It is observed in Figure 4 that, as the concentration of OA increases (white bar), the gelling time decreases; however, the difference in gelling time between 10% and 15% OA in 0.1 M borax is not significant. The gelling time for OA (constant at 10%) dropped from 80 s to 60 s when the gelatin concentrations increased from 5% to 10%, but remained constant for higher concentrations (black bar).

Figure 5 shows the degree of crosslinking determined by TNBS assay when the OA remained constant (10% in 0.1 m borax solution) and the concentration of gelatin was varied (5%, 10%, and 15%). Crosslinking between the amino groups of gelatin and the aldehyde groups of OA is due to Schiff's base formation.[12] As expected, the degree of crosslinking decreases when the gelatin concentration increases because there are many amino groups and not enough aldehyde groups to react.

The tensile stress average for dried gels (10% OA with 10% gelatin in 0.1 M borax) is 7.12 MPa while the ultimate tensile stress average is 5.09 MPa. Previous studies have reported that the OA improves the mechanical properties of gelatin in rehydrate samples (10% gelatin in distilled water and 1–3% OA).[19] The reported tensile stress for 10% gelatin is 2 MPa, and for 10% gelatin with 1% and 3% OA it is 2.6 and 2.9 MPa, respectively.[19] The difference between those stresses and the ones reported here is attributed to the fact that dried samples were used and in previous studies rehydrated samples were used.

NHDF cells were encapsulated inside the OA–gelatin scaffold by mixing cells with the bio-ink (10% OA in 0.1 M borax) and printing the mixture onto gelatin 36°C ± 1°C. Approximately 2 min after printing, the scaffolds were soaked in DMEM medium and placed in the incubator. Cells in the scaffold were stained with the LIVE/DEAD assay at 72 h, and observed using a confocal microscope. Figure 6(a) shows the NHDF encapsulated in the scaffold

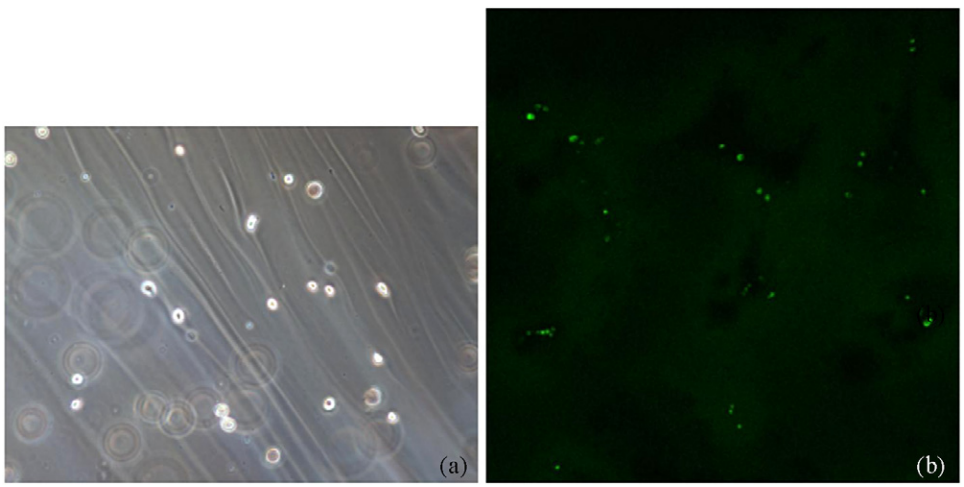

Figure 6. (a) 10× bright field image of NHDF on OA and gelatin scaffold at 72 h, and (b) 10× confocal image of NHDF.

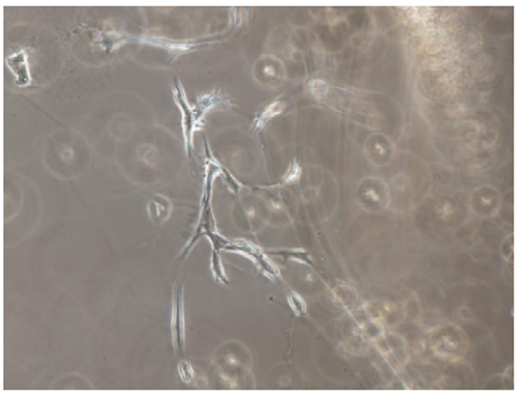

Figure 7. 10× bright field image of the encapsulated cell in 10% OA and 10% gelatin scaffold after 7 days of culture.

at 72 h, revealing that the cell morphology remains round (adhesion stage). The cell morphology might be due to the presence of the aldehyde group of OA or to the low cell density. Other investigators have found that fibroblasts are alive with regular fibroblast morphology when are seeded onto OA–gelatin gels.[15] However, this study did not report fibroblast morphology when they were encapsulated inside the gel. Fig. 6(b) shows confocal images depicting live cells in strongly green fluorescent calcein.[20] The amount of live cells found in the gels indicates the non-toxic nature of OA–gelatin gels.[12] Figure 7 shows a 10× bright field image of the NHDF cells in the 10% OA in borax and 10% gelatin scaffold after 7 days post printing. The characteristic NHDF phenotype is seen clearly demonstrating cell attachment between 2 and 7 days of culture. These data indicate that the gels are non-toxic and allow attachment and proliferation of fibroblast cells.

CONCLUSIONS

In summary, oxidized alginate was characterized with respect to printability and its ability to crosslink gelatin. When 0.1 M borax solution is used to dissolve gelatin and OA, biodegradable hydrogels can be created by inkjet printing. Adding NHDF cells to the biological ink (OA in borax solution) allows one to construct gels with embedded cells. Cytotoxicity analysis shows the survival of the cells after printing, and the characteristic fibroblast morphology after 7 days of cell encapsulation. Control over the concentration of OA as well as the spatial distribution of the cells and crosslinkers throughout the scaffold should allow us to create customizable constructs with improved properties.

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