Fluorescent Hydrogels for Embryoid Body Formation and Osteogenic Differentiation of Embryonic Stem Cells

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ABSTRACT: Substrate mechanics (e.g., stiffness and topography of the microenvironment) are likely critical for driving normal morphogenesis and tissue development. As such, substrate mechanics imposed by hydrogels have been exploited to guide the lineage differentiation of stem cells and to drive stemness. In this work, we chemically modified gelatin hydrogels through glyceraldehyde cross-linking to render them suitable for cell culture. The modified hydrogels proved to be ideal for embryonic stem cell osteogenesis, initially providing a soft nonadhesive surface for the formation of embryoid bodies. They subsequently degraded in culture to afford a harder surface during osteoblast differentiation. The gels synthesized are highly fluorescent, relatively easy to prepare, and can potentially aid in overcoming the challenge of imaging changes to the microenvironments of cells during three-dimensional cell culture. Exploiting these materials could lead to the development of tissue-engineered products of increased complexity and rational treatment strategies.

KEYWORDS: embryonic, embryoid body, stem cells, fluorescence, gelatin, hydrogel, glyceraldehyde, cross-linking, ECM, osteogenesis, microenvironment, differentiation
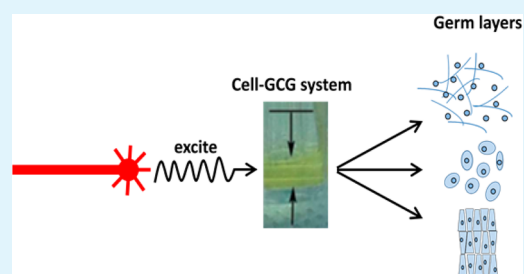
INTRODUCTION

Embryonic stem cells (ESCs) are permanent pluripotent cells with an unsurpassed promise for regenerative therapies. They have been exploited for the generation of a multitude of different cell types, among them the cartilage- and bone-forming cells of the skeleton. Such lineage induction often involves the addition of soluble differentiation triggers in conventional two-dimensional monolayer cell culture systems.[1−5] However, stem cells naturally reside in a three-dimensional (3D) extracellular matrix (ECM), a complex and dynamic network composed of fibrous matrix proteins that provides the physical structure, mechanical integrity, and biochemical activity of their environment.

To mimic this native environment, several studies have explored the culture of ESCs in defined 3D settings by using a variety of natural and synthetic scaffolds for lineage guidance.[6−11] Osteogenesis specifically has been shown to be successful when culturing was performed on stiffer matrices.[12,13] In contrast, hydrogels, 3D water-swollen polymer networks, specifically from natural materials (e.g., collagen, fibrin, chitosan, dextran, hyaluronic acid (HA), alginate, and Matrigel) are a more attractive material for the induction of early differentiation events due to their resemblance to soft tissues found in the early embryo.[14] Hydrogels have tunable properties and are stabilized through either covalent cross-linking methods such as Michael-type addition and photopolymerization or through physical interactions (e.g., ionic, self-assembly), often for cell encapsulation (for review see Nicodemus and Bryant, ref 15). For example, suspension of murine ESCs in the self-assembling hydrogel RAD-16I supports subsequent osteogenic and hepatic differentiation,[16,17] and encapsulation in HA gels is able to support growth and survival of human ESCs.[18]

Although some progress has been made in the successful culture and differentiation of stem cells using hydrogels, one challenge that still remains is the imaging of changes to the 3D microenvironments imposed by cell culture. This is grounded in the current inability to distinguish between its newly formed structural characteristics and those present initially. Chemically attaching dyes or fluorophores to various constituents of the microenvironments to obtain contrast is impractical because it is labor-intensive, requires specialized skills, and can become expensive. Simply dispersing contrasting reagents inside the microenvironments leaves inside the free-floating and potentially toxic compounds. Imaging the newly formed ECM using advanced bioimaging technologies that utilize complex endogenous contrasts such as second harmonic generation and two-photon excited fluorescence meets with little success as well. Specifically, when the newly synthesized ECM is deposited on the pre-existing collagen background that also

generates the above-mentioned endogenous contrasts, the two become indistinguishable.[19]

In this work, we chemically modified gelatin hydrogels through glyceraldehyde cross-linking to render them highly fluorescent and suitable for cell culture. The fluorescent materials prepared can potentially aid in overcoming the challenge of imaging changes to the microenvironments of cells during 3D cell culture by contrasting newly formed structural characteristics and those present initially. Gelatin was used as the main material as it is a hydrolyzed collagen and the main component of the ECM. It is a natural polymer with the desirable properties for the bulk hydrogel material such as transparency, limited higher ordered protein structure, easy sterilization, and low cytotoxicity. To date, different forms of gelatin scaffolds have been developed, which often require the potentially DNA-damaging photoactivation of the cross-linking process. For example, Lin and colleagues have accomplished chondrogenic differentiation of human bone marrow mesenchymal stem cells by encapsulating them in photoactivated methacrylated gelatin.[20] However, osteogenic differentiation of these cells was achieved in a 70:30 gelatin–chitooligosaccharide hydrogel cross-linked with glutaraldehyde[21] suggesting that cross-linking can be achieved chemically as well.

Glyceraldehyde seems advantageous for the chemical cross-linking as it is nontoxic and makes the gelatin structurally stable at 37 °C. As a triose sugar, it chemically cross-links proteins through a nonenzymatic cross-linking glycation process at a high rate compared to other sugars due to its open chemical frame. On the basis of its composition, such a GCG hydrogel should closely mimic a native ECM that is biodegradable. An added advantage is that glycation is visible through the formation of browning chromophores and formation of fluorescent protein cross-links. This added advantage allows for visual monitoring of the progress of the modification without employing sophisticated characterization tools.

The main objectives of this study were to quantitatively characterize the GCG hydrogel and to validate that the modification would support ESC osteogenesis. The chosen cross-linking strategy proved to be ideal for ESC osteogenesis, initially providing a soft nonadhesive surface for the formation of embryoid bodies. Because of its biodegradability the hydrogel gradually disappeared over time in culture to provide a harder surface as is preferred by osteoblasts as they differentiate. Through the monitoring of the differentiation process of the ESCs as described in this work, it might be possible to better understand the dynamic interplay between the behavior of stem cells and the microenvironment of the hydrogel. This understanding in turn can lead to successful tissue-engineered products of increased complexity and rational treatment strategies.

◼ MATERIALS AND METHODS

Gelatin Hydrogel Formation and Cross-Linking. A type A gelatin (Sigma, G1890–500G) stock solution was prepared in doubly deionized H_2O (ddH_2O) and sterilized by autoclaving. This gelatin (300 μL, 30 g/L) was pipetted into each well of 48-well tissue culture plates (Greiner bio-one Cellstar Products catalog No. 677 180) and incubated at 4 °C overnight. Glyceraldehyde (0.1 M, Sigma, 127 K1434) cross-linker was prepared in 30 mM phosphate-buffered saline (PBS) buffer that contained 0.3 M NaCl and was adjusted to pH of 7.4 with NaOH. The glyceraldehyde solution was sterilized with a 0.22 μm filter (Millipore, Millex GV Filter, catalog No. SLGV003RS) and stored at 4 °C. Cold glyceraldehyde solution was added to gelatin polymerized for ~24 h (1:1 volume ratio). The cross-linking was

performed at 4 °C in tissue culture plates covered with aluminum foil. A yellow color change (characteristic of the Millard glycation reaction) indicated cross-linking of gelatin.

In Situ Kinetic Measurements of Cross-Linked Gelatin Formation. Increase in fluorescence within the GCG hydrogels that was due to glycation was measured in situ, in a high-throughput format using a FlexStation microplate reader (Molecular Devices) in the backscattering mode. The excitation wavelength was 350 nm, and emission spectra were collected in the range from 420 to 600 nm with a 2 nm step. A spectrum was independently acquired from samples in six wells of a 96-well tissue culture plate (30 g/L prepolymerized gelatin and 0.1 M glyceraldehyde cross-linker per well at a 1:1 ratio). The background fluorescence of the non-cross-linked gelatin was subtracted from each spectrum, and the spectra were averaged. The measurements were independently repeated at least three times, and the error bars are the standard deviations from the mean.

Cross-Linking Degree Assay. To determine the extent of modification of amino groups with glyceraldehyde, we assayed for remaining free amines with the ninhydrin colorimetric method. The percentage of the remaining free amines in the cross-linked hydrogel was calculated to establish the relative degree of cross-linking. Several wells containing the GCG hydrogels prepared for cell seeding were rinsed with ddH_2O, freeze-dried, and stored at 4 °C for further use. The freeze-dried cross-linked gelatin materials were rinsed with ddH_2O three times for at least 1 h at 37 °C. After rinsing, the mix was incubated with 30.75 μg/mL of activated collagenase (Sigma) solution at 37 °C for 24 h to ensure the GCG was completely digested. The digested product was mixed with 0.7% ninhydrin solution (in ethanol) and boiled for 2 min. The absorption spectrum was collected from 300 to 900 nm using UV–vis–NIR spectrophotometer (Varian, Carry 500). Different concentrations of glycine solution mixed with collagenase and ninhydrin stock were used to prepare the calibration curve. The relative degree of cross-linking was calculated as cross-linking degree = $(1 - (\text{cross-linked absorbance}_{570}/\text{non-cross-linked absorbance}_{570}))*100\%$, where cross-linked absorbance$_{570}$ and non-cross-linked absorbance$_{570}$ values of optical absorbance at 570 nm are proportional to the free amine in cross-linked and non-cross-linked gelatin samples, respectively. The measurements were reproducible, and error is the standard deviations from the mean.

Mechanical Property Assay. GCG hydrogels cross-linked for 11 d were deactivated by rinsing with Dulbecco's phosphate-buffered saline (D-PBS, Sigma-Aldrich, catalog No. 14190) and moved to the bottom plate of a Haake RheoStress 1 temperature-controlled parallel plate rheometer interfaced with a computer. The sensor diameter was 35 mm, and the measurement was performed at 20 °C in the controlled deformation mode of frequency oscillation sweep (0.1–50 Hz). The gap between two plates was 2 mm. The strain rate was 0.05 and was confirmed to be within the linear viscoelastic range of the gelatin hydrogel materials. The measurements were reproducible, and the error bars are the standard deviations from the mean.

Murine Embryonic Stem Cell Culture. Cells of the mouse ESC line D3 (No. CRL-11632, LGC Standards GmbH, Wesel) were routinely cultured in ESC maintenance medium composed of high glucose Dulbecco's Modified Eagle Medium containing 15% fetal bovine serum (Sigma, selected batch), 50 U/ml penicillin, 50 μg/mL streptomycin, 1% nonessential amino acids, and 0.1 mM β-mercaptoethanol (all Invitrogen, Temecula). Leukemia inhibitory factor (LIF) was added freshly to each new passage at a concentration of 1000 U/ml.

Cell Seeding and Differentiation Initiation. Prior to seeding, wells containing the GCG hydrogels were rinsed with D-PBS, refilled, and incubated with D-PBS for 30 min at room temperature. D-PBS was removed, 300 μL of Tris HCl (pH 6.0) was added, and the samples were incubated in this solution for 12 h at room temperature to inactivate any adsorbed reducing sugars and/or other reactive carbonyl compounds within hydrogels. Tris-HCl quenching was repeated a total of seven times. Prior to cell culture the gels were rinsed with D-PBS seven times, ≥30 min per rinse. Cells were seeded right after equilibrating the gels with medium.
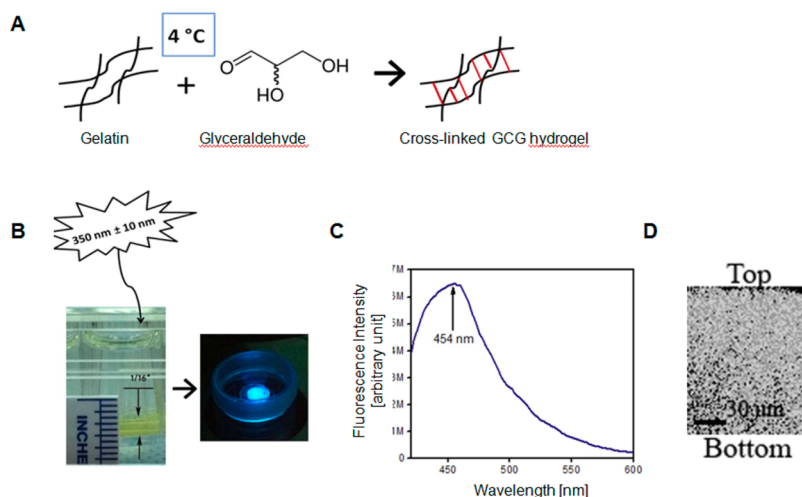
Figure 1. Overview of the cross-linking strategy. (A) Schematic illustration of material components and cross-linking reaction. (B) Schematic illustration of excitation, hydrogel fluorescence (http://www.engr.ucr.edu/~julial), and (C) emission spectrum of GCG hydrogel within the range of visible light with an emission peak centered at ~454 nm. (D) Two-photon fluorescence microscopy cross-sectional images ($\lambda_{Ex}/\lambda_{Em}$ = 720 nm/390−550 nm) of GCG hydrogel show that modification is fairly uniform throughout the thickness of the gelatin materials.

Differentiation of ESCs was induced by seeding 5000 cells per square centimeter into ESC maintenance medium without LIF either onto polystyrene plates coated with 0.1% gelatin or GCG hydrogels. Five days after seeding, specific lineage differentiation into osteoblasts was induced with the same medium that was used for cell maintenance except for a different serum lot (Atlanta) that was specifically tested for osteogenic induction efficiency. Further, medium was supplemented with 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 5 × 10^{-8} M 1,25α(OH)$_2$ vitamin D$_3$.[22] Medium was changed as needed.

Alkaline Phosphatase Activity Assay. Cells were rinsed twice in PBS and lysed in radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) with the addition of appropriate protease inhibitors (P-8340; Sigma-Aldrich, St. Louis, MO) under gentle rocking for 30 min at 4 °C. Lysates were mixed with pNPP reagent (Sigma, P7998), and the activity of the enzyme was determined by measuring the amount of p-nitrophenol formed at 37 °C after 0 and 30 min of incubation with cell lysate.[23] The increase in absorbance was monitored at 415 nm. For normalization of alkaline phosphatase activity, the total protein content in the sample was determined with the DC protein assay reagent (Biorad) as described by the manufacturer. After 15 min of incubation at room temperature, absorbance was read in an iMark microplate spectrophotometer (Biorad) at 750 nm. Protein quantities in samples were taken from a bovine serum albumin standard curve.

Calcification Assay. Using the purple substrate Arsenazo III (DCL, Toronto, Canada) total calcium content was measured from RIPA lysates as described.[23,24] Change in absorption was recorded at 655 nm in an iMark microplate spectrophotometer (Biorad). Values in cell samples were calculated from a CaCl$_2$ standard [5 mM], which was measured in conjunction with the samples and normalized to total protein content.

Statistical Analysis. Data are represented as mean ± standard deviation of three independent experiments with five technical replicates each. Statistical significance was determined with a one-way ANOVA (Vassar Stats online tool). Values of P below 0.05 were considered significant.

■ RESULTS AND DISCUSSION

Glyceraldehyde cross-links biopolymers containing amine groups according to the Millard reaction.[25] In the course of the reaction, the chain links stabilized through the covalent interactions are introduced between the protein strands as shown in Figure 1A. Arginine and lysine side chains of the
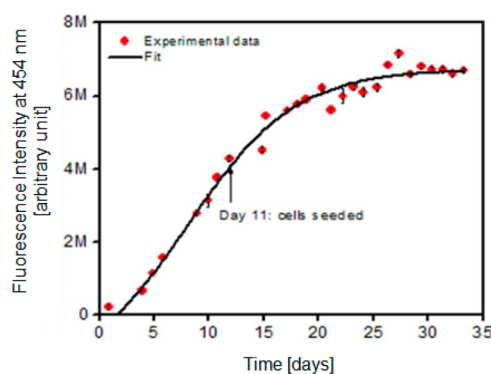

Figure 2. Fluorescence emission of GCG hydrogels as a function of cross-linking time (4 °C, 0.1 M glyceraldehyde, $\lambda_{Ex}/\lambda_{Em}$ = 350 nm/454 nm). Error bars are the standard deviations from the mean. Line is the best fit to guide the eye.

intermolecular strands in protein-based biopolymers can engage in the cross-linking reaction with glyceraldehyde. The cross-linking takes place as a result of Schiff base formation and Amadori rearrangements and leads to the formation of nonenzymatic glycation products collectively known as advanced glycation endproducts (AGEs). AGEs absorb in the near-UV (320−370 nm) and fluoresce in the 380−460 nm range. Our prior studies characterized the excitation wavelength dependence of two-photon fluorescence of glyceraldehyde-treated collagen hydrogels and outlined a strategy to image materials modified with this cross-linker.[26−28]

The volumes used created a GCG hydrogel ~1/16 in. in height. As seen in Figure 1B, GCG hydrogels display a yellow tint under visible light and generate strong fluorescence emission (λ_{em} = 454 nm) when excited with a mercury lamp at 350 ± 10 nm (Figure 1C). The two-photon fluorescence microscopy cross-sectional images collected at 720 nm near-infrared excitation wavelength within ~390 to 550 nm spectral emission window showing that modification is fairly uniform throughout the thickness of the gelatin materials and confirming absence of fibrous microstructure (Figure 1D).

To quantitatively monitor the progress of cross-linking, we followed a buildup of fluorescence intensity at 454 nm from
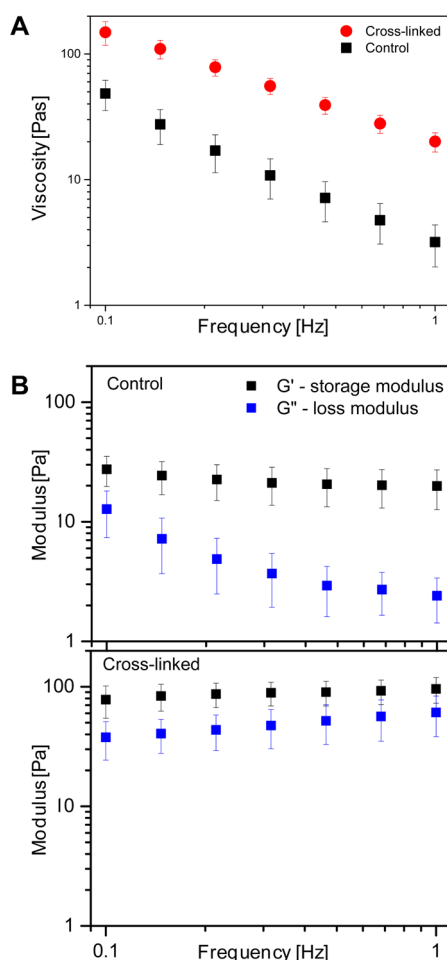
Figure 3. Dynamic mechanical properties of gelatin hydrogels (A) viscosity, (B) storage modulus G' and loss modulus G'' as a function of frequency sweep. Error bars are the standard deviations from the mean.

GCG hydrogels as they underwent modification. As seen in Figure 2, it took nearly 30 d to completely cross-link all gelatin within the hydrogels. The new equilibrium state representing

the cross-linked gelatin gels is exemplified by the flat part of the graph at longer incubation times. The midpoint transition occurs at ∼8 d. Assuming a two-state model for cross-linked transition this indicates that after ∼8 d, the degree of the stabilizing covalent cross-linking within the hydrogels is at ∼50%. The cross-linking degree assay confirmed that the 10 d cross-linked gels had a degree of gelatin cross-linking at 45% ± 8%. Consequently, we observed that after ∼10 d our gelatin cross-linking conditions would stabilize the gelatin gels and make them suitable for cellular work at 37 °C.

The dynamic mechanical measurements (Figure 3A) show that the viscosity of gelatin materials increases from ∼50 Pas in unmodified samples (controls) to nearly 200 Pas in the samples stabilized through covalent cross-linking. Both the storage modulus G' and the loss modulus G'' also increase (Figure 3B). At low frequency (low stress), for both controls and covalently cross-linked samples, the storage modulus G' is higher than the loss modulus G'' thus indicating solidified gels. For example at 0.1 Hz frequency, in controls, G' is ∼30 Pa and G'' is ∼12 Pa. In the cross-linked materials G' is ∼80 Pa and G'' is ∼40 Pa. The storage/loss modulus curves run nearly parallel over the low-frequency range. G' and G'' remain relatively linear at low frequencies (0.1−1 Hz) for both control and modified samples but become highly nonlinear at higher frequencies possibly due to sample slipping. The control gelatin modulus values reasonably agree with previously established numbers for gelatin gels.[29]

On the basis of the results of the kinetic cross-linking study, single-cell suspensions of ESCs were seeded onto GCG hydrogels on the 11th day of the cross-linking reaction. Controls on gelatin-coated polystyrene (PS) plates were run alongside. Five days postseeding, ESCs seeded onto gelatin-coated polystyrene (PS) plates were attached to the bottom of the plate and evenly dispersed throughout the well (Figure 4A). In contrast, ESCs aggregated into ∼40 to ∼125 μm large spheroids on the GCG hydrogels loosely held atop the material, but did not invade it or adhere to it (Figure 4A) by 5 d after seeding, suggesting that the material decreased the adhesion of the cells.

Indeed, pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, have the ability to
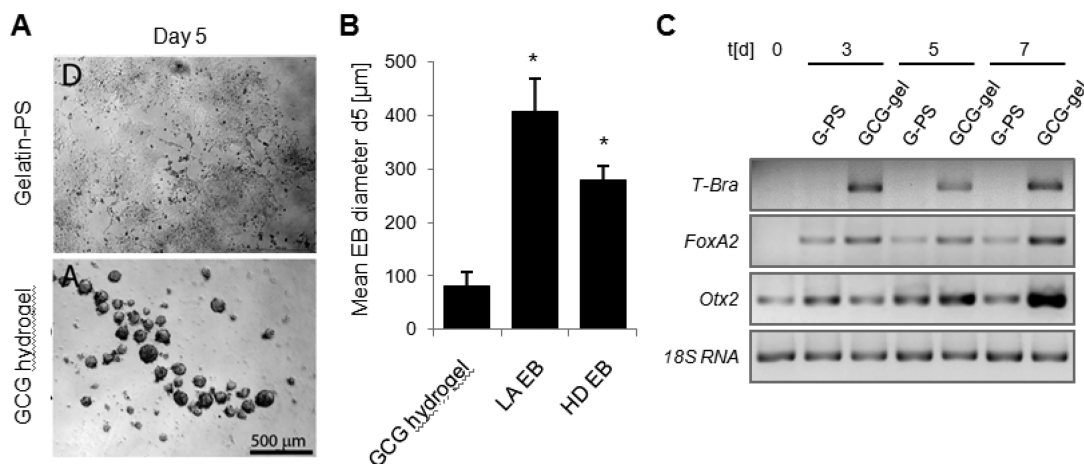

Figure 4. Early differentiation events in murine ESCs seeded on GCG hydrogels. (A) Phenotypic appearance of mESC cultures 5 d after seeding. Scale bar = 500 μm. (B) Mean diameter of resulting spheres made with various techniques; $n = 100−241 ± SD$ *$P < 0.05$ compared to GCG hydrogels, one-way ANOVA. (C) RT-PCR for lineage genes in differentiating cells. G-PS: gelatin-polystyrene; GCG-gel: glyceraldehyde-cross-linked gelatin; LA EB: low attachment EB; HD EB: hanging drop EB.
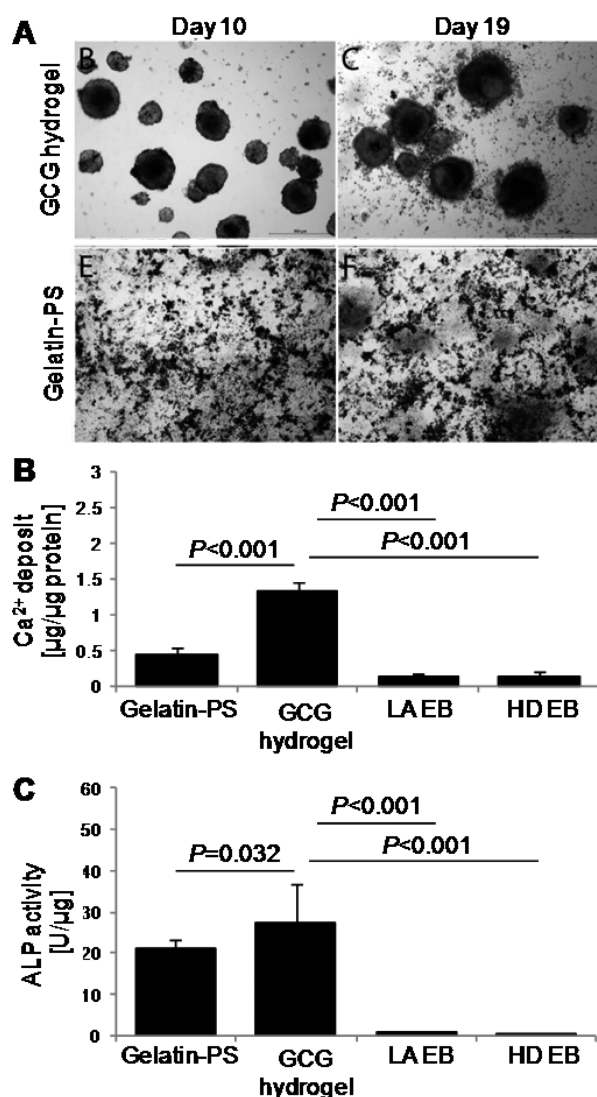
Figure 5. Effect of culture surface on osteogenic differentiation yield. (A) Phenotypic changes in cells cultured in monolayers on gelatin−PS and GCG hydrogels. (B) Deposition of calcium ions into ECM was measured with a calcium assay based on changes in absorption when reacted with Arsenazo III; values represent means of three independent experiments including five technical replicates each ± SD; one-way ANOVA. (C) Alkaline phosphatase activity assay, $n = 3$, five technical replicates each ± SD; one-way ANOVA. Gelatin−PS: gelatin-polystyrene; GCG: glyceraldehyde-cross-linked gelatin; LA EB: low-attachment EB; HD EB: hanging drop EB.

self-assemble into unique 3D structures when cultured in suspension.[30−32] In shear-activated cultures, such as in bioreactors, and with the addition of LIF, those types of spheres are made up of undifferentiated cells.[33,34] In contrast, upon removal of LIF, so-called embryoid bodies (EBs) form that, by definition, manifest all the three germ cell layers. In static culture, the intended aggregation of PSCs into EBs permits the study of early development and differentiation events of pluripotent cells and is often used to induce differentiation. Widely used is the labor-intensive hanging drop method, whereby cells suspended in media are separated in droplets and suspended from the lid of a Petri dish.[35] Natural gravity pulls the cells down, and they collect at the bottom of the droplet causing them to form aggregates. At the same time, EBs grow larger in size due to the proliferative capacity of the

aggregated cells. Static suspension is another way of culturing EBs. This is a significantly more effortless process as it is performed by adding a desired amount of ESCs per milliliter of medium to dishes that have been rendered nonadhering[31,36] by means of low-attachment solutions, such as Ultra Low Attachment. Formed through these processes, EB size varies substantially, as seen in Figure 4B, which in turn leads to asynchronous differentiation across a population of EBs in a dish.[37] Instead, and therefore advantageous over classic nonadherent surfaces, the GCG hydrogel seems to provide a surface on which aggregates form within a certain size range with less variability (i.e., smaller error bars, Figure 4B). The GCG hydrogel seemed to be partially cell-repellant, and the encapsulation/entrapment of small clusters of ESCs forming on the hydrogels possibly limited the size of EBs generated compared to other EB preparation methods.

The EBs formed on the GCG hydrogels were indeed capable of producing all three germ layers. Messages for T-Brachyury and FoxA2, representative of the mesodermal and endodermal germ layers, were elevated in the GCG hydrogels over the gelatin−PS samples (Figure 4C). In comparison to the gelatin−PC cultures, cells from the GCG hydrogels expressed Otx-2 slightly later, however, at increased levels at day 5 and day 7 after seeding (Figure 4C). In fact, Otx-2 seemed to be more abundant than the other two messages. Otx-2 expression is associated with cranial neural crest cells that have the ability to give rise to osteoblasts,[38] potentially suggesting that GCG hydrogel cultured cells had a higher potential for neural crest-derived osteogenesis. Together, these results indicated that the EBs formed on the hydrogels contained differentiating cell types of all three germ layers and should be capable of undergoing osteogenic differentiation.

In the monolayer culture, cells evenly populated the plate by day 10 (Figure 5A). Dark areas in the gelatin−PS samples, indicative of beginning calcification,[24] became visible and were interdispersed throughout the culture. On day 19, the uniformity and spread of calcification was very similar to day 10 with additional presence of calcified areas. On the GCG hydrogels, the spheroids had grown substantially in size (~250 μm) by day 10, got darker, and seemed lightly attached to the gel. As noted, all the spheroids become darkened in appearance, and a reduction in gel height was noticeable. By day 19, the spheroid size had increased to ~400 μm, and the spheroids had attached. The hydrogel was completely gone at this time. This allowed us to conclude that the GCG hydrogels retained structure when initially kept at 37 °C in the CO_2-based cell culture incubator and then degraded over time. In any case, the choice of the material and its properties seemed beneficial for the initial trilineage differentiation of the cells and also supported the subsequent osteogenic differentiation of the ESCs.

To monitor this later lineage differentiation, calcium deposition and alkaline phosphatase (ALP) activity were measured for the gelatin−PS samples and the cells grown on GCG hydrogels (Figure 5B,C). ALP is a membrane-bound enzyme required for matrix mineralization. It is expressed in preosteoblasts in vivo and is an early indicator of osteoblast activity.[39,40] As cells mineralize, they deposit calcium ions into their ECM, a hallmark feature of osteoblast differentiation. In correlation with the increase in dark appearance in both culture systems (Figure 3), calcium was detected in both samples; however, it was ~threefold higher ($P < 0.0001$) in GCG hydrogels than in gelatin−PS cultures (Figure 5B). Compared

to EBs formed in low-attachment plates (LA EBs) and EBs formed from hanging drops (HD EBs), GCG hydrogel EBs yielded significantly more calcium deposit. Correspondingly, ALP activity was also higher in GCG hydrogels than in gelatin–PS cultures ($P = 0.032$) as well as LA EBs and HD EBs ($P < 0.001$).

Material chemistry,[41] biofunctionalization,[42] mechanical properties,[13] and structure[43,44] are all described as important factors that determine cellular responses to biomaterials. From the structural perspective, differences in surface topography of materials influence proliferation and differentiation of mesenchymal stem cells.[45] Moreover, the effects of materials on differentiation of stem cells are often combined. For example, it was found that osteogenesis was regulated by both stiffness and ligand type during human mesenchymal stem cell differentiation on polyacrylamide gels coated with different ECM proteins. Osteogenic differentiation was observed only on collagen I-coated gels of the highest modulus of 80 kPa.[46] For ESCs, the effect of biomaterials in directing their differentiation is less well-established compared to mesenchymal stem cell.[47] The work in the field includes a study by Evans et al.[48] reporting that genes T-Brachyury, Mixl1, and Eomes expressed during gastrulation are upregulated in cells cultured on stiffer polydimethylsiloxane substrates with covalently immobilized type I collagen compared to softer analogues. A more recent study by Candiello et al.[47] describes a spontaneous differentiation of murine ESCs toward lineage specific cell types on soft alginate substrates coated with fibronectin. In summary, our studies constitute an initial step in understanding stem cell responses to mechanically strengthened hydrogels prepared from natural materials. Additional studies are needed to fully understand the influence of physicochemical aspects of cross-linked GCG hydrogels on the biology of ESCs.

CONCLUSIONS

Glyceraldehyde-modified gelatin hydrogels induced ESCs to form clusters as compared to the lawns observed in the controls. Furthermore, the GCG hydrogels were able to support ESC osteogenic differentiation in a manner superior to current protocols with the additional advantage of being less labor-intensive (in the production of EBs) as well as being fluorescent. This manuscript reports the structural and mechanical parameters leading to the observed biological effects along with the preparation process of the GCG hydrogels.

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Author Contributions
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Notes
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ABBREVIATIONS

ESC, embryonic stem cell
GCG, glyceraldehyde-cross-linked gelatin
G-PS, gelatin-polystyrene
LA EB, low attachment EB
HD EB, hanging drop EB

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