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Biomineralization on enzymatically cross-linked gelatin hydrogels in the absence of dexamethasone

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Mechanical stimulus and chemical induction by dexamethasone have been an important factor in dental pulp stem cells (DPSCs) differentiation and biomineralization. We have demonstrated that the enzymatically crosslinked gelatin hydrogels are an extremely effective substrate for DPSCs differentiation towards odontoblasts. DPSCs were seeded on the crosslinked hard (~8 KPa) and soft (~0.15 KPa) gelatin hydrogels for 35 days with and without dexamethasone. Odontogenic differentiation markers such as OCN, ALP and DSPP were upregulated after 35 days of culture on crosslinked hydrogels with and without dexamethasone. SEM and Alizarin red staining of the crosslinked hydrogels showed a biomineralized sheet of hydroxyapatite deposits laid by the DPSCs on the top surface and inside the hydrogel. We found that the DPSCs differentiation and biomineralization was independent of the hydrogel stiffness and dexamethasone. We hypothesize that this biomineralization was indeed triggered by the surface chemistry of the crosslinked gelatin hydrogels since we did not observe any biomineralization on the uncrosslinked gelatin or mTG. We also showed that the DPSCs, when removed from hard hydrogel surfaces and re-seeded on a TCPS, retained their odontogenic lineage and had permanent mineralization effect. Our results show the potential of enzymatically crosslinked gelatin hydrogels as scaffolds for dentin regeneration.

1. Introduction

Teeth are susceptible to damage from mechanical trauma, chemicals, periodontal disease, caries, and pulpitis[1]. As a result, innovative new approaches have been proposed to protect enamel and dentin against corrosion. Currently, carious lesions which penetrate enamel and dentin are replaced by synthetic materials, such as titanium alloy, amalgam, composite resin, gutta percha and zirconia ceramic or new innovative approaches where materials are used to protect dentin and enamel against erosion and also aid in their remineralization[2, 3]. However, such therapies do not thoroughly restore the biological function of the tissue after significant damage or loss [4-6].

The advances in tissue engineering present a prospect for the biologic regeneration of the damaged dental tissues. Similar to the construction of other tissues, dental tissue engineering also requires an appropriate cell source, a biodegradable scaffold that can mimic the natural extracellular matrix (ECM) and bioactive molecules[7-9].

Postnatal human dental pulp stem cells (DPSCs) can be induced to undergo odontogenesis and hence can serve as replacement for damaged pulpal tissue [10-13]. Typically dexamethasone is used for induction and leads to the expression of odontogenic markers and mineralization in vitro [14, 15] although conditioned medium from tooth germ has also been used[16]. Dexamethasone is a glucocorticoid steroid that chemically induces DPSCs to differentiate into odontoblasts. However, for in vivo use steroids should be avoided as they can cause adverse side effects such as hyperglycemia and weakened immune system[17].

Scaffold materials play a critical role in determining how cells proliferate and differentiate. Those that mimic the characteristics of natural ECM can best promote appropriate cell and tissue

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maturation[18, 19]. A number of synthetic polymer scaffolds made of poly-glycolic acid (PGA), poly-L-lactic acid (PLA), copolymer of PGA and PLA (PLGA) as well as hydroxyapatite/tricalcium phosphate ceramic scaffolds (HA/TCP) and titanium scaffolds have been used as substrates for in-vitro and in-vivo DPSCs differentiation with external chemical inducers such as dexamethasone[4, 11, 20-25]. However, many of these synthetic scaffold materials have certain disadvantages because their acidic degradation products of lower pH value around the tissue after in vivo implantation can cause severe inflammation[26]. The synthetic scaffolds also lack cell recognition signals. Yang et al. tried to overcome this problem by using a blend of electrospun poly (ε-caprolactone) and gelatin with hydroxyapatite (HAP). However, they were not able to induce the DPSCs into odontoblasts phenotype without the addition of dexamethasone. Regeneration of the dental pulp/dentin complex has been reported by numerous groups, *in vivo*[27, 28]. *In vitro*, on the other hand, differentiation of DPSCs has been accomplished mostly via the addition of dexamethasone, and even in those cases, mostly along osteogenic lineage[14, 23, 29].

In this paper, we have chosen gelatin hydrogels that can be enzymatically crosslinked with microbial transglutaminase (mTG) to generate substrates of variable stiffness (hard and soft) for in vitro differentiation of DPSCs. These non-synthetic, non-cytotoxic, biocompatible hydrogels can be easily tuned to have desirable mechanical properties and have been used extensively for in-vitro and in-vivo tissue engineering applications[30-33]. Microbial transglutaminase (mTG) is a natural enzyme that catalyzes the formation of N-ε-(γ-glutamyl) lysine amide bonds between individual gelatin strands to form a permanent network of cross-linked gelatin[34, 35]. This permanent network of gelatin offers multiple focal adhesion sites for cell attachment, proliferation, and migration[36]. It was shown previously [37, 38] that in presence of external chemical inducers, the mesenchymal stem cells on polyacrylamide hydrogels with stiffness ~1KPa, ~10KPa, ~100KPa can differentiate along neuronal, muscle, and bone lineage, respectively. Here we showed that the chemistry of these crosslinked gelatin hydrogels may override the effect of the mechanical stimuli and chemical inducers. In this case, were able to induce permanent odontogenic differentiation, despite the very low moduli (<10 KPa) in the absence of external chemical inducer (dexamethasone). SEM, EDX, and Alizarin red confirmed the presence of hydroxyapatite (HAP) deposition, and RT-PCR confirmed the associated up-regulation of alkaline phosphatase (ALP), osteocalcin (OCN), and dentin sialophosphoprotein (DSPP). We speculate that this effect is due to the high density of integrin binding sites that, are cryptic in collagen and gelatin, but are exposed when gelatin is crosslinked by mTG.

2. Materials and Methods

2.1 Preparation of gelatin-mTG hydrogels

Hydrogels were formed by first dissolving gelatin (300-bloom, Porcine Type A, Sigma-Aldrich, MO, USA) in DPBS (1X pH 7.4 GIBCO, Invitrogen Co., Carlsbad, CA, USA) at 70-80^{o}C at a gelatin concentration of 0.1 g/ml (10%(w/v)) followed by sterile filtration through 0.22 μm SteriFlip (Millipore) media filters before thermally setting at room temperature for 24 h. These physically-crosslinked hydrogels are not permanent and are thermally reversible. Microbial transglutaminase (mTG) was used as a cross-linker at a stock concentration of 0.1g/ml (10 %(w/v)). Hard and soft gelatin-mTG gels were made by mixing 1.125 mL and 1.488 mL of a 10% gelatin solution in DPBS with 0.375 mL(3:1(v/v) gelatin: mTG), and 0.012 mL (125:1(v/v) gelatin: mTG) of a sterile filtered stock solution of mTG (10%, Ajinomoto LLC, NJ, USA) respectively, to obtain total of 1.5 mL hydrogel solution. The stock mTG solution was made by first dissolving mTG powder in DPBS and then sterile filtering through 0.22 μm Millex (Millipore) syringe filters. The crosslinking reaction took place in a cell culture incubator at 37^{o}C for 24h. After 24h, the hydrogels were heated at 65^{o}C for 5-7 min to deactivate mTG. The hydrogels were made in 6 well/ 12 well/ 35mm TCP/TCPS (Tissue culture polystyrene; BD Biosciences, Franklin Lakes, NJ) as per the assay to be carried out. As a control, glass coverslips were coated with gelatin and mTG separately at 37^{o}C for 2 h. mTG coated glass coverslips were heated at 65^{o}C for 5-7 min to deactivate mTG.

2.2 Dynamic Rheological characterization

Oscillatory shear rheometry tests were carried out on gelatin-mTG hydrogels with rotational rheometer (Bohlin HR Nano, Malvern Instruments Ltd., Germany) using previously described methods[39]. Briefly, all 35 mm TCPS containing the hydrogels were fixed to the bottom plate with a double-sided tape during each test method. The top plate was then lowered to a gap of 1mm from the bottom of the 35mm TCPS. Stress sweep was performed by holding the temperature (37^{o}C) and frequency (1 Hz) constant while increasing the shear stress from 1 to 5000 Pa. The stress sweep measured G' (storage/elastic modulus; index of stiffness) information on the structural behavior of the crosslinked network for all hydrogels by

shearing them until the structure broke down. All samples were tested in triplicates.

2.3 Cell culture and Cell Seeding

Human DPSCs strain AX3 were isolated from the third molar teeth as previously described[40]. All experiments were performed in compliance with the relevant laws and were approved by Committee on Research Involving Human Subjects (CORIHS) at Stony Brook University (IRB # 20076778). The DPSCs were cultured in alpha minimal essential medium (α-MEM) (Catalog # 12571, GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen) and 1% penicillin–streptomycin (GICO, Invitrogen) in a humidified incubator at 37°C with 5% CO_2. The DPSCs monolayers were grown in tissue culture polystyrene (TCPS) dishes to nearly 80% confluence, harvested after treatment with 0.05% trypsin-EDTA (GIBCO, Invitrogen) solution, then centrifuged to obtain a pellet and finally re-suspended in α-MEM containing 10% FBS, 1% Pen-Strep, 10mM β-glycerol phosphate (Sigma-Aldrich), 200 μM L-ascorbic acid 2- phosphate (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) also known as the "base / non-induced media" for use in functional assays.

DPSCs were seeded at a density of 1500 cells/cm^2 on the hard and soft crosslinked gelatin-mTG hydrogels and TCPS in a 6 well TCPS plate and on the gelatin and mTG coated glass coverslips. Half of the samples were incubated at 37°C with 5% CO_2 and cultured with the "base/ non-induced media" and the other half with the "induced media" (base media containing 10^{-8} M dexamethasone = induced media (Sigma-Aldrich)) for 35 days. Culture medium was replaced on alternate days.

2.4 Shear Modulation Force Microscopy (SMFM)

Cell stiffness was measured on the hard and soft cross-linked hydrogels after day 1, 3 and 7 of incubation in induced and non-induced media using an atomic force microscope (AFM, Dimension 3000; Digital Instruments, Co., Ltd. Santa Barbara, CA). AFM was operated in shear modulation force microscopy (SMFM) mode [41] using a silicon nitride tip on a cantilever with a bending spring constant of 0.06 N/m. During the measurement, a force of~ 25 nN was exerted by the cantilever on the cell's perinuclear region and a sinusoidal drive signal (1400 Hz) was applied to the x-piezo controlling cantilever, inducing a small oscillatory motion of the tip parallel to the cell surface. When the drive signal amplitude was varied from 7.5 mV up to 125 mV, which corresponds to an x-piezo displacement of 1.5–25 nm, the cantilever response was recorded to estimate the stiffness of the cell surface[42]. The AFM set-up was calibrated such that a greater response amplitude indicated a more compliant surface and vice versa. The drive frequency of 1400 Hz was chosen for the measurements since it lies in the flattest region of the cantilever's response curve. A total of nine experimental points (three points per cell and three cells per sample) were obtained for each hydrogel condition.

2.5 Scanning Electron Microscopy (SEM)

On day 7, 14, 21 and 35 after cell seeding, the hard and soft hydrogels were washed in DI water twice. The samples were cut into half and scooped out of the 6 well plates and placed on a piece of Si wafer to be left for air-drying at room temperature till they got dehydrated and shrunk. The specimens were sputtered with gold and examined with an LEO/Zeiss 1550 field emission SEM (Minnesota, USA) at 20 KeV accelerating voltage using Robinson type backscattering electron detectors. The elemental contents on the surface of the scaffolds were measured by energy dispersive X-ray microanalysis (EDX) (Phoenix XEDS system). Gelatin and mTG coated coverslips were prepared and analyzed similarly on day 35.

2.6 Confocal Laser Scanning Microscopy

To determine actin cytoskeleton organization and the morphology, the cells were fixed with 3.7% (w/v) formaldehyde, permeabilized with a mild detergent (0.4% Triton, Sigma), stained for actin with Alexa Fluor-488 Phalloidin (Molecular Probes, Eugene, OR) and with Propidium Iodide (Molecular Probes, Eugene, OR) for the nucleus. The hard and soft gelatin-mTG samples were then imaged using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystem Inc., Bannockburn, IL) after 21 days of incubation.

2.7 RT-PCR

After 1, 7, 14, 21 and 35 days of cell culture, the cross-linked gelatin hydrogels were washed with DPBS and the cell layer was extracted using 3 mg/ml of collagenase type I (230 u/mg, Worthington Biochemicals Corp., Lakewood, NJ). 1ml of collagenase was added to each hydrogel sample followed by 1 h incubation at 37°C. This was followed by another 10min incubation at 37°C in 0.05% trypsin –EDTA and centrifugation in non-induced media. The total RNA was extracted from hard and soft gelatin-mTG hydrogels and TCPS using Qiagen RNeasy kit (RNeasy kit, Qiagen, Valencia, CA). 1 μg of total RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen) into cDNA using Random Primers (Invitrogen). The obtained cDNA was used as a template for PCR. The odontogenic differentiation of cells was monitored by specific markers for- osteocalcin (OCN), dentin sialophosphoprotein (DSPP) and alkaline phosphatase (ALP). The sequences of the specific

primer sets are listed below: *ALP* (sense: 5'-ACGTGGCTAAGAATGTCATC-3'; antisense:5'-CTGGTAGGCGATGTCCTTA-3'); *dentin sialophosphoprotein* (DSPP) (sense: 5'- AATGGGACTAAGGAAGCTG-3'; antisense: 5'-AAGAAG CATCTCCTCGGC-3'); *osteocalcin* (OCN) (sense 5'-CATGAGAGCCCTCACA-3'; antisense5'-AGAGCGACACCCTAGAC-3'); 18s (sense:5'-AACCCGTTGAACCCCATT-3'; anti-sense:5'-CCATCCAATCGGTAGTAGCG-3'). 18s was used as a housekeeping gene to normalize mRNA expression. Real-time PCR was performed using SYBR Green PCR kit (Qiagen, Valencia, CA) and controlled in a DNA Engine Opticon 2 Thermal Cycler with continuous fluorescence detection (MJ Research Incorporation, Union, NJ) at the Stony Brook University DNA Sequencing Facility. DNA amplifications were performed under the following conditions: 95°C (15min) followed by 94°C (30 s), 55°C (30 s), and 72 °C (30 s) for 40 cycles, with a final 10 min extension followed by data collection (Opticon Monitor 3 analysis software, MJ Geneworks, Bio-Rad Laboratories Inc.). Each sample was assessed in triplicate. All mRNA expression was absolute and is shown in units of Atta mole/20 ng RNA (amol/20 ng RNA).

2.8 Alizarin Red Staining

After 35 days of incubation, the hard and soft hydrogel constructs were rinsed twice with DPBS and then fixed with 10% (w/v) formalin for 1 day, washed with PBS and stained with 1 mg/ml (0.1%w/v) alizarin red stain (Sigma-Aldrich) for 30 sec. Stained samples were then washed with DI water 4 times; each wash is 1 min long. Pictures of the top surface and cross-section of the hydrogels were taken using an optical microscope (CK40, Olympus).

2.9 Permanent differentiation

To investigate if the DPSCs differentiation was long term and permanent, after 21 days of incubation, the cell layer was extracted from the cross-linked hydrogels with collagenase and trypsin. The extracted cell layer was suspended in non-induced media for the non-induced samples and induced media for the induced hydrogel samples. This layer was broken down by pipetting to get a homogenous cell suspension. 1500 cells/cm^2 were seeded on 6 well TCPS plates. Half of the samples were cultured in non-induced base media and another half in induced media for 21 days. TCPS with DPSCs was used as a control. SEM, EDX analysis, and RT-PCR were carried out after 21 days of incubation on all samples.

3. Results and Discussion

3.1 Influence of substrate mechanics on the DPSCs behavior

By varying the amount of mTG, we were able to generate gelatin hydrogels of variable stiffness. Logarithmic plots of G' vs shear stress shown in Figure 1A represent the storage/elastic modulus, linear viscoelastic range (LVR) profile, and the breaking stress of the hydrogels, after curing for 24 h with variable mTG concentration. From the figure, we can see that the LVR, or the region where G' is relatively constant, is between 1-900 Pa and 1-50 Pa for hard and soft hydrogels, respectively. By averaging G' in this region, we obtained the elastic moduli of 8.00 ± 0.5 KPa and 0.15 ± 0.03 KPa for the hard and soft gels, respectively. Increasing the stress amplitudes further, G' begins to deviate noticeably from the LVR plateau indicating that irreversible structural changes have occurred. The yield points for the soft and hard gels are 100 Pa and 1000Pa, respectively indicating that despite being harder, the harder gel with the higher modulus has higher elasticity.

AFM, operated in the SMFM mode, was used for in situ measurement of the DPSCs moduli after 1, 3 and 7 days of incubation. During our measurement, two simultaneous forces were exerted on the cells; first, a sinusoidal drive signal applied to the x-piezo that induced a small oscillatory motion on the cell surface, and second, a normal force of ~ 2 nN to maintain tip contact with the cell surface. The normal indenting force was applied to the perinuclear region between the cell nucleus and cytoplasmic edge to ensure reproducibility of the stiffness measurement.

The response amplitude was measured and plotted against drive amplitude, with the response amplitude being proportional to the drive amplitude, indicating that there was no slip during the cell-tip contact. Assuming a Hertzian model for this SMFM set-up, the response amplitude has previously been shown to be inversely proportional to 2/3 power of the lateral modulus of the specimen[43]. Using this calibration, we found that cell stiffness increased more than 2-folds over 7 days of incubation (Figure 1B). Despite an order of magnitude difference in the moduli of the hydrogels, the differences in the relative moduli of the cells, were modest (Figure 1B) regardless of the presence or absence of dexamethasone. This is unlike the results previously reported by Ghosh et al.[44] where a ~5 fold increase in the hydrogel moduli produced ~ 150% increase in the cell moduli. Hence, the predominant influence on the cell moduli did not appear to be mechanical in nature.

3.2 Influence of chemical and mechanical stimuli on biomineralization

It has been shown previously for mouse MC3T3-E1 cells[45] that large increases of the cell moduli within the first seven days may be

associated with biomineralized deposits. We, therefore, analyzed the hydrogels, using SEM, to determine whether the gel moduli influenced the deposition of minerals. Figure 2 shows the SEM micrographs on day 7 (first column) and 21 (third column) where we can see that biomineralized deposits are present after one week of incubation on both hard and soft gels, and with and without dexamethasone induction. The EDX spectra obtained on day 7 from the bright clusters is shown in the second column, where we can see that it corresponds to Ca and phosphorous, or hydroxyapatite (HAP). Similar early mineral deposition was also reported by Meng et al.[45], and was found to be associated with large increases of the cell moduli. The white mineralized deposits seen in the SEM micrographs, increased with increasing number of days in culture, as can be seen in Figure 2. No difference in the biomineralization was found even after 21 days between the hard and soft gels or those with and without dexamethasone. Biomineralization, with similar nodules of HAP, for cells cultured with dexamethasone, has been reported by numerous groups[46], but such extensive deposition in the absence of chemical inducers has not been observed. These results indicate that the mTG cross linked gelatin hydrogel substrates can induce DPSC to biomineralize in the absence of any additional soluble *chemical inducers (such as dexamethasone)* or substrate *mechanical stimuli (i.e. hydrogel stiffness)*.

In Figure 3, we show confocal images of the DPSCs after 21 days of incubation on hard and soft hydrogels, with and without dexamethasone. From the figure, we can see that there is no significant difference in the appearance of the individual cells or the organization of the tissue. The actin filaments were spread out and well-attached onto the hydrogel surface. There was no visible change seen in the morphology of the cells on the hard or soft / induced, or non-induced hydrogel surfaces emphasizing even more that the effect was independent of dexamethasone and mechanical stiffness.

3.3 Alizarin red staining of the biomineralized ECM

Biomineralized deposition on the hydrogels increased further with the incubation time, as shown in Figure 4 H-K. We showed that a solid sheet of biomineralized deposits covered the surface of all the samples. In Figure 4A, we show the SEM micrograph of the sheet formed on the hard hydrogel by cells cultured without dexamethasone. From the figure, we can see that the sheet is approximately 50 µm thick and the EDX spectra (Figure 4A insert) indicates that it is composed entirely of HAP. Since the SEM images are performed on dry samples, we also imaged the wet hydrogels using Alizarin red stain. Alizarin red has been used extensively to stain the calcium rich deposits by cells of osteogenic/odontogenic lineage [47]. We observed that the intensity of the red color was similar on all samples. In Figure 4B-G, we showed a cryotome's section of the DPSCs tissue on different types of hydrogel stained with Alizarin red. We observed that the biomineralized layer was formed on top of the gel, and adjacent to the cell layer (Figure 4B-C). The thickness of the biomineralized layer appeared to be constant for all samples and is not a function of hydrogel modulus or presence of dexamethosone.

3.4 Odontogenic differentiation of DPSCs

OCN, ALP, and DSPP mRNA expression from DPSCs seeded on hard (+/-) and soft (+/-) gelatin-mTG gels and TCPS (+/-) were compared on day 1, 7, 14, 21 and 35. The odontogenic differentiation of DPSCs was confirmed by RT-PCR analysis which showed almost 7-fold higher mRNA expression of DSPP on crosslinked hydrogels (highest in hard (-)) at day 35 compared to the TCPS control (Figure 5A). TCPS was used as a control since it has been used as a standard control in the literature. DSPP is used as a specific marker for odontoblast differentiation. It is highly expressed by odontoblasts and is essential for dentinogenesis [48, 49]. DSPP is the initial translational product of DSPP mRNA, which is then cleaved to dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) [48]. Both DSP and DPP play an important role in the ECM mineralization and dentinogenesis. DSPP mRNA is post-transcriptionally upregulated in a time-dependent manner when DPSCs are cultured in the induction medium containing glycerophosphate, ascorbate-2-phosphate, and dexamethasone[50, 51]. ALP is usually regarded as an early marker of hard tissue formation or odontogenic differentiation and is required for deposition of minerals in tooth and bone. OCN, a vitamin K-dependent non-collagenous ECM protein is also used as a late marker of osteogenic differentiation, but it plays an important role in dentin regeneration and, therefore, it's used as a confirmation marker for odontogenesis. Our results showed an upregulation of OCN by day 21 on all crosslinked hydrogels (highest in hard (-)) and a down-regulation by day 35 of incubation (Figure 5B). By day 35, the OCN expression level was similar on all the hydrogels indicating that odontogenesis was more natural on these crosslinked gelatin gels than osteogenesis. The upregulation was also noticed to be independent of dexamethasone as the non-induced hard gel had the highest OCN and DSPP expression. It is interesting to see that after 21 days of incubation, the cells expressed six to nine-fold more OCN on the induced crosslinked gels than the TCPS (+). On the non-induced

crosslinked hydrogels, this difference is 11 to 16 fold. Expression of ALP, (Figure 5C) increased over time on all the surfaces showing the highest expression levels on day 35 of culture (highest on hard (-)). Cells had overall higher ALP activity on the crosslinked gels compared to TCPS.

On Day 7, the DSPP, OCN, and ALP expression is low (Figure 5). However, calcified deposits were observed on day 7 as well (Figure 2). On conducting an auxiliary experiment with the hard and soft hydrogels in the absence of DPSCs for 35 days, we found that gelatin-mTG hydrogels had the capability of self-mineralization in the presence of just the media. Weekly SEM micrographs of the hydrogels from Day 7 to Day 35 showed few mineralized deposits that were laid in the absence of DPSCs and dexamethasone (Figure 6A-D). EDX spectra on Day 7 (Figure 6E) also confirmed the presence of some calcified deposits. Alizarin red staining of the hard and soft hydrogels in the absence of DPSCs by day 35, showed a weak uniform stain (Figure 6F-I), but did not show the bright red pattern of calcified deposits shown in (Figure 4D-G), indicating that it may be in part due to the physiochemical reaction of the hydrogels. However, the presence of DPSCs significantly increases the amount of biomineralization.

Dexamethasone increases the mRNA expression of ALP in all the samples which are consistent with the literature[52, 53]. However, in crosslinked hydrogels, the ALP activity seems to be independent of dexamethasone as non-induced hard gel recorded the highest ALP expression. In the absence of dexamethasone, the crosslinked hydrogels had threefold higher ALP expression than the control. Dexamethasone has been considered as an important inductive factor for stimulation of DPSCs into odontoblasts [14] which has been used in culture compositions for various scaffolds and explant DPSCs cultures [4, 10, 23, 27, 54-56]. In our study, we were able to show that DPSCs can differentiate into odontoblasts in absence of any external soluble factors (dexamethasone), producing abundant bio-mineralized CaP deposits and upregulating the OCN, ALP, and DSPP expression.

Schwab et al.[57] have shown that the presence of multiple RGD sequences or focal adhesions can induce osteogenic differentiation on different substrates. These sequences are also abundant on collagen and gelatin that is composed of collagen segments. When the chains are folded in collagen or gelatin, these sequences are not readily available. Numerous authors have previously shown that crosslinking with mTG exposes these domains and hence, provides multiple cell adhesion sites[58-60]. In particular Ito et al.[58] show that many of these sites are RGD, which in turn were shown to trigger biomineralization and differentiation on polystyrene substrates[57]. We hypothesize that this is the situation on crosslinked gelatin as well. In order to further probe this hypothesis, we also cultured the DPSCs, without dexamethasone, on glass coverslips coated with mTG, and uncrosslinked gelatin. The results (Figure 7A-B) clearly show that in contrast to non-induced crosslinked gelatin hydrogel (Figure 4I), no biomineralization was observed after 35 days of DPSCs culture on either mTG or gelatin separately. Therefore, these results confirm our observation that the chemistry of the crosslinked gelatin hydrogels drives the DPSCs differentiation towards odontogenesis. DPSCs, when seeded onto crosslinked gelatin hydrogels, see crucial binding sites for the adsorption of proteins that might be required for odontogenic differentiation and biomineralization. It also confirms that the differentiation is not entirely dependent on either the mechanical stimuli or dexamethasone.

3.5 Long term/permanent differentiation of DPSCs

In order to determine whether the differentiation in the absence of dexamethasone was permanent or whether it remained dependent on contact with the hydrogel surface, DPSCs were removed from the hard (+/-) and soft (+/-) hydrogel surfaces after 21 days and reseeded on TCPS (+/-) where they were incubated for another 21 days. A control set was seeded on TCPS (+/-) for 21 days. Figure 8 (top row A–E and bottom row F-K) shows the SEM micrographs and representative EDX spectra of the surfaces that were induced and non-induced with dexamethasone respectively. H (+) + /S (+) + denotes that the cells were removed from induced hard/ soft gel, re-seeded on TCPS in an induced medium. H (-) +/ S (-) + denotes that the cells were removed from non-induced hard /soft gel, re-seeded on TCPS in an induced medium. The morphology of the calcified clusters seen in Figure 8(A-D) and (F-J) appeared to be similar on all the surfaces. However, S (+) + and S (-) - had less amount of mineralized deposits than H (+) + and H (-) - indicating that the hydrogel stiffness had an influence on the long-term or permanent differentiation of the DPSCs. Corresponding EDX spectra confirms that the biomineralized deposits were calcified.

3.6 Expression of mineralized markers in permanent differentiation

Figure 9B shows the mRNA expression of OCN on induced and non-induced H (+)/H (-)/S (+)/S (-) surfaces after 21 days of culture and compares it to the mRNA OCN expression on TCPS (+) & TCPS (-). The figure shows that the extracted cells had overall

higher mRNA expression than TCPS control after 21 days of culture. Cells extracted from H (+) expressed almost eightfold higher OCN than TCPS in the induced medium whereas H (-)/S (+)/S (-) had almost fourfold higher

OCN expression than TCPS (+). In the non-induced medium, cells extracted from H (+) expressed almost thirteen-fold higher OCN expression than TCPS (-).

Cells extracted from H (-), S (+), and S (-) also had almost nine-fold, six-fold and twofold higher OCN expression than TCPS (-) respectively. All these results indicate that DPSCs that were removed from H (+) and H (-) retained their odontogenic phenotype much better than the cells extracted from S (+) and S (-), both in the induced and non-induced medium. Similar upregulation of the ALP mRNA expression (Figure 9C) and DSPP mRNA expression (Figure 9A) was also seen on the induced and non-induced surfaces that were seeded with cells extracted from the hard gels when compared to their respective TCPS control. Figure 9A shows the similar trend where a higher DSPP expression is observed on the non-induced H (-) gel. The hard gel is more robust, and the cells extracted from hard gels tend to retain the memory of prior differentiation and differentiate towards odontogenic lineage after being cultured on TCPS. As far as we know, this type of permanent differentiation has not been seen before, and it further emphasizes the effect of enzymatic gelatin crosslinking. Hard gels especially H (-) seemed to be consistent with driving the odontogenic differentiation and biomineralization permanently.

Conclusions

In this study, we have shown that for DPSCs differentiation, the crosslinking of the gelatin-mTG hydrogel and the focal adhesion binding sites are as important as the mechanical stimuli. We showed that the in-vitro differentiation can occur independently of dexamethasone and variation in mechanical stiffness. Enzymatically crosslinked gelatin hydrogels were found to be extremely effective scaffolds for odontogenic differentiation and biomineralization. Although the relative cell moduli was slightly affected the stiffness of the hydrogels by day 7, but this effect diminished towards the late stages of differentiation (day 21-35) where SEM micrographs showed biomineralized deposits on both hard and soft hydrogels. Although these hydrogels have a lower range of moduli (<10 KPa), they were successful in DPSCs differentiation towards odontogenesis. In particular, the non-induced hard gel had higher ALP and DSPP expression by day 35 indicating that the differentiation was odontogenic. When the DPSCs were removed from the hydrogel surfaces and re-seeded on the TCPS for 21 days under induced and non-induced condition, it was further noticed that the non-induced hard gel, in particular, retained its odontogenic phenotype permanently. This phenomenon of long-term differentiation has not been seen before. The cross-linked gel-mTG hydrogels facilitated the formation of dentin-like mineralized tissue on the top surface and inside the gel without dexamethasone demonstrating its use as a scaffold of transplantation of odontogenic cells for dentin regeneration.

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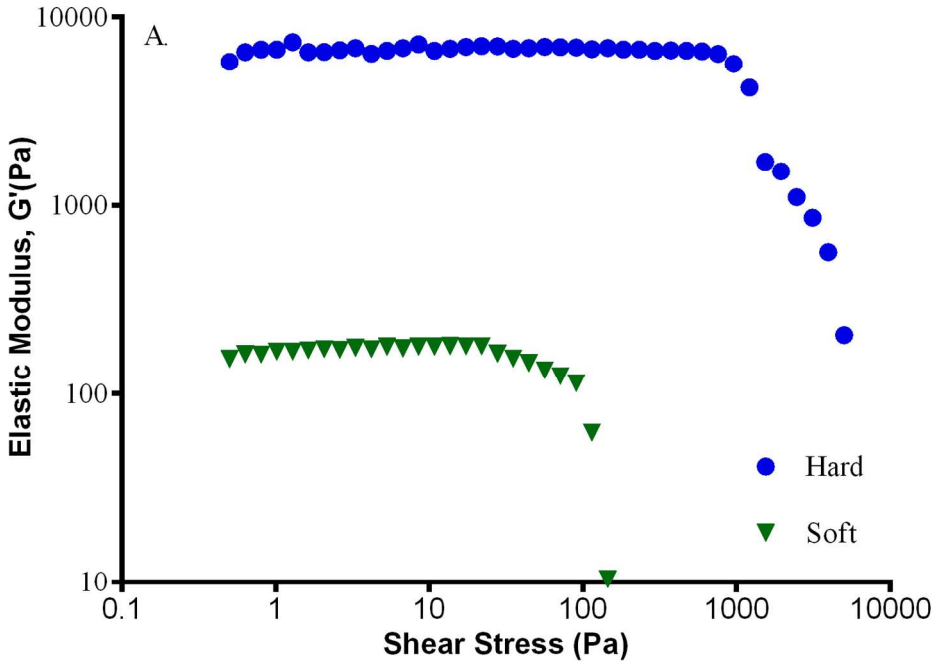
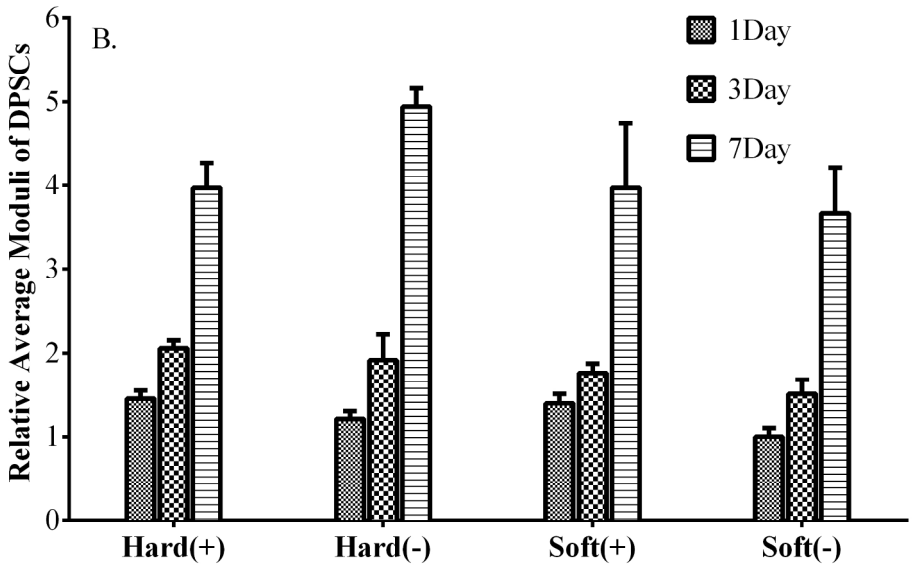
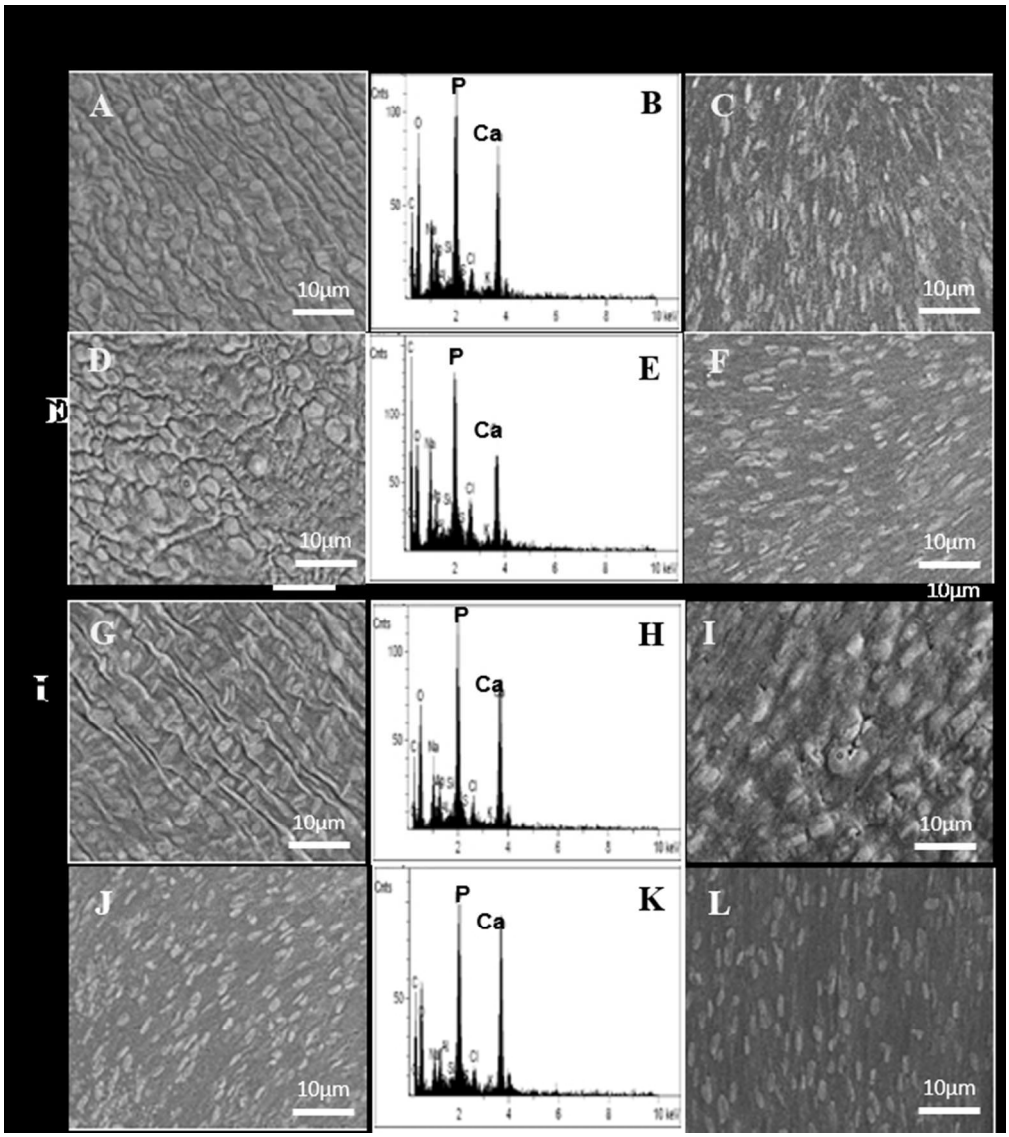


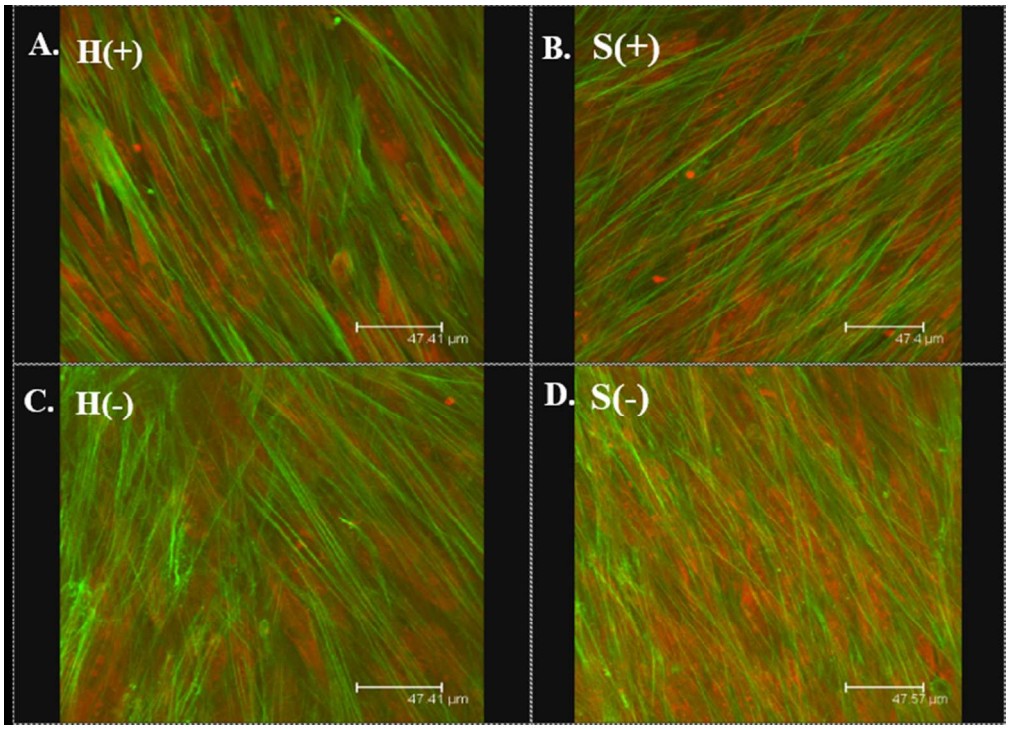
Oscillatory shear stress showing the stiffness of hard and soft hydrogel (B) Cell modulus/stiffness determined by AFM from day 1 to day 7 on induced and non-induced hard and soft hydrogels.



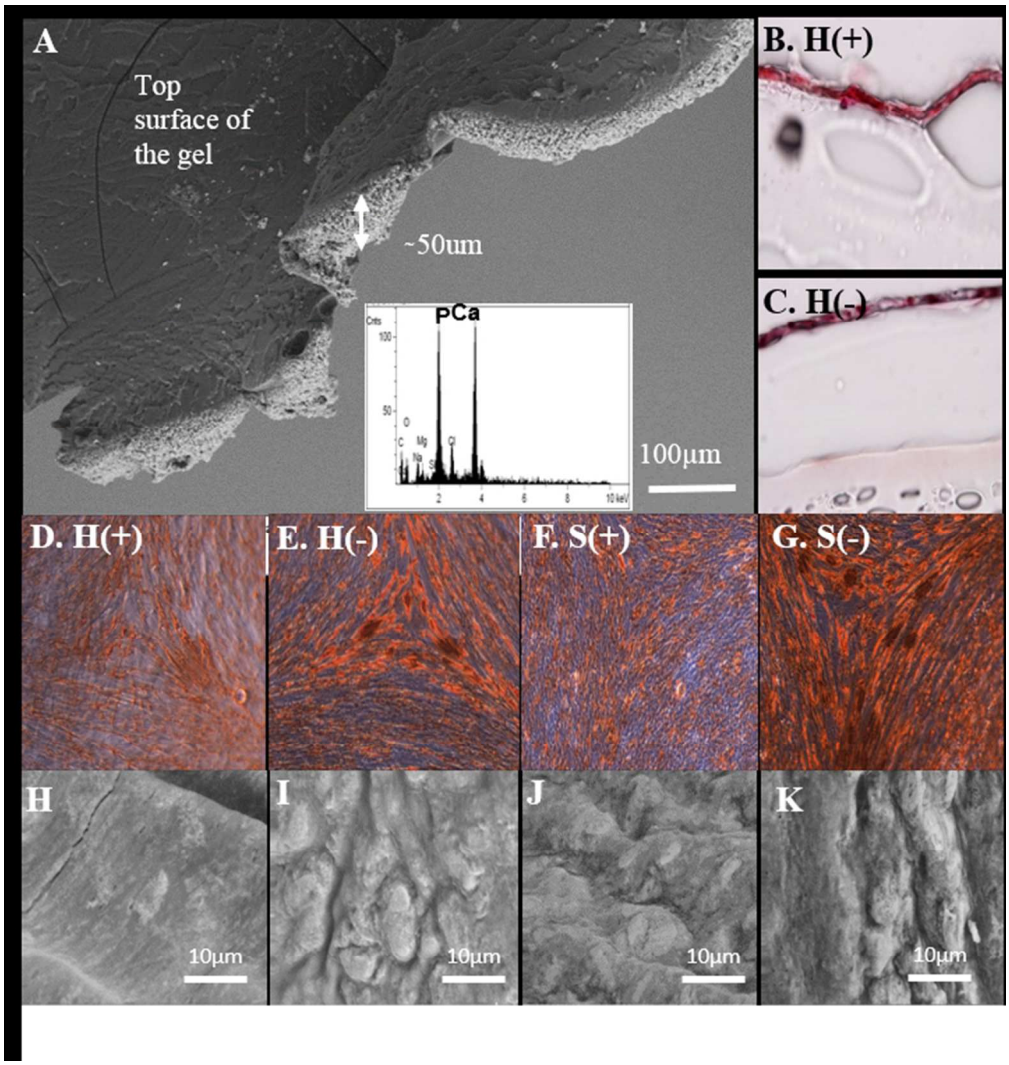
(B) Cell modulus/stiffness determined by AFM from day 1 to day 7 on induced and non-induced hard and soft hydrogels.



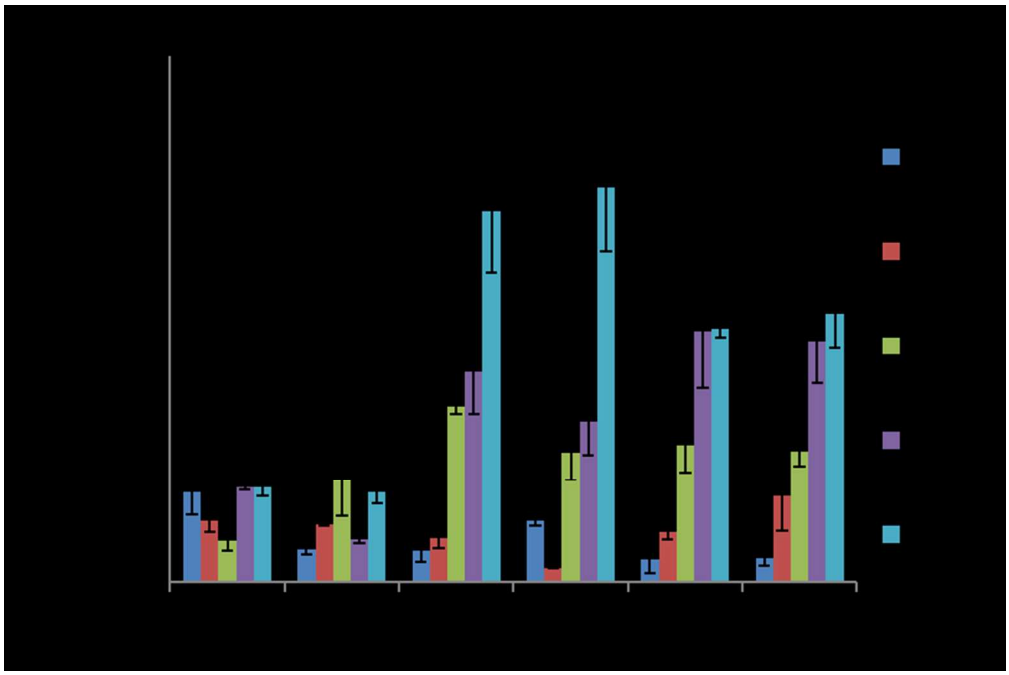
White CaP biomineralized deposits were seen in the SEM micrographs of the induced Hard gel (H (+)), non-induced Hard gel (H (-)); induced soft gel (S (+)); non-induced soft gel (S (-)) on day 7 (first column) and day 21 (last column). EDX spectra on day 7 (middle column) confirmed that the white biomineralized deposits were hydroxyapatite minerals (Ca and P peaks).
124x140mm (150 x 150 DPI)



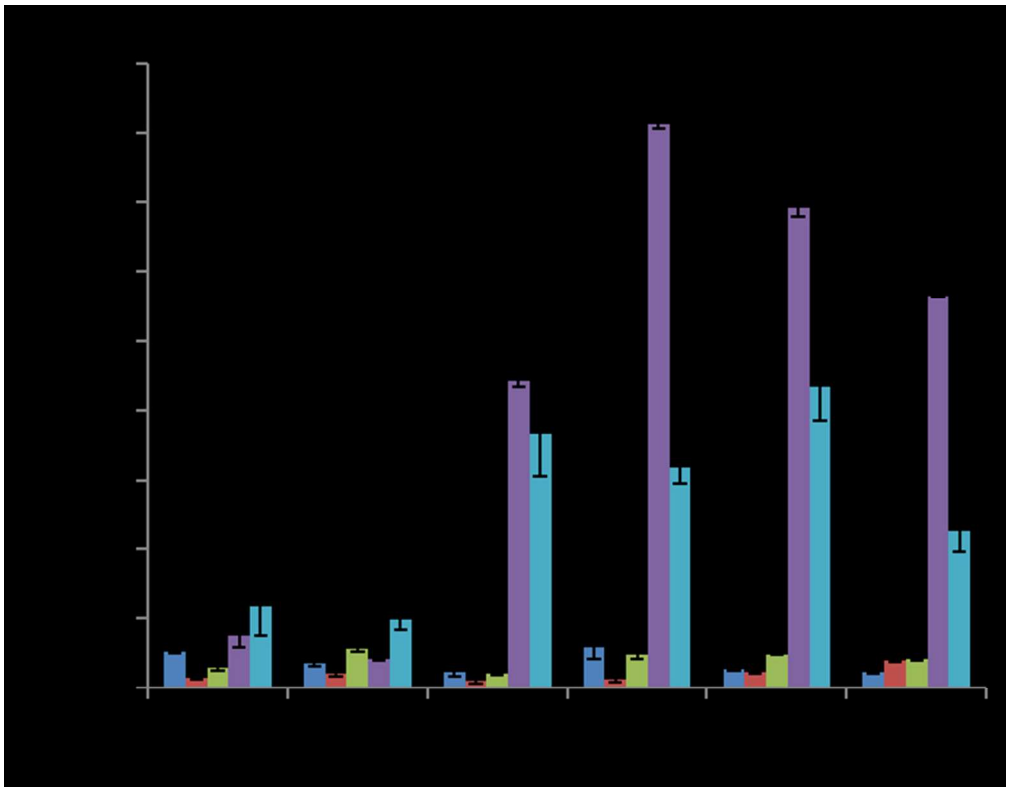
Well spread out morphology of DPSCs after 21 days of culture on induced (A) hard gel H(+) (B) soft gel S(+), non-induced (C) hard gel H(-) (d) soft gel S(-). Actin filaments were stained by Alexa Flour 488 (green), nucleus was stained by propium iodide (red); Bar= 47.41µm.
129x93mm (150 x 150 DPI)



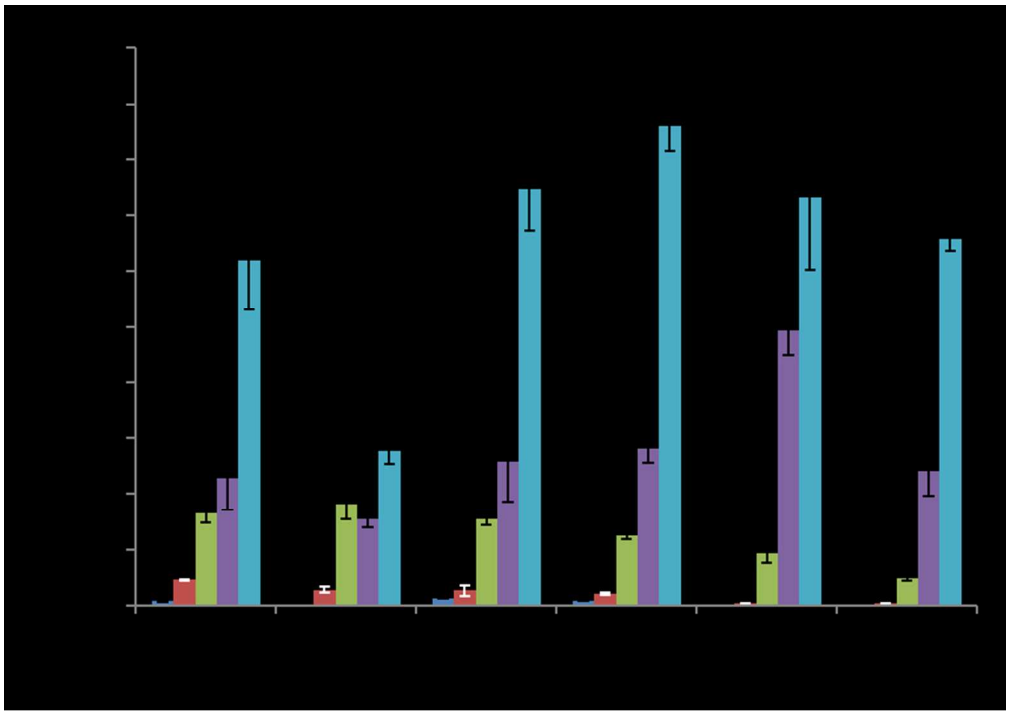
(A) Cross-section of a non-induced hard gel (H (-)) after 35 days of DPSCs culture showing a self-supporting sheet of biomineralized deposits present inside the gel. EDX spectra (insert in (A)) confirms the hydroxyapatite mineral. Cross-sectional view of the alazarin red stained calcified biomineralized deposits in the (B) Hard (+) and (C) Hard (-) gel. Top view of the alizarin stained red calcified deposits and their corresponding SEM images after 35 days of DPSCs culture on: (D, H) Hard (+); (E, I) Hard(-); (F, J) Soft (+); (G, K) Soft (-) gels. The calcified deposits laid by the cells are stained deep red and have a defined pattern.
121x128mm (150 x 150 DPI)



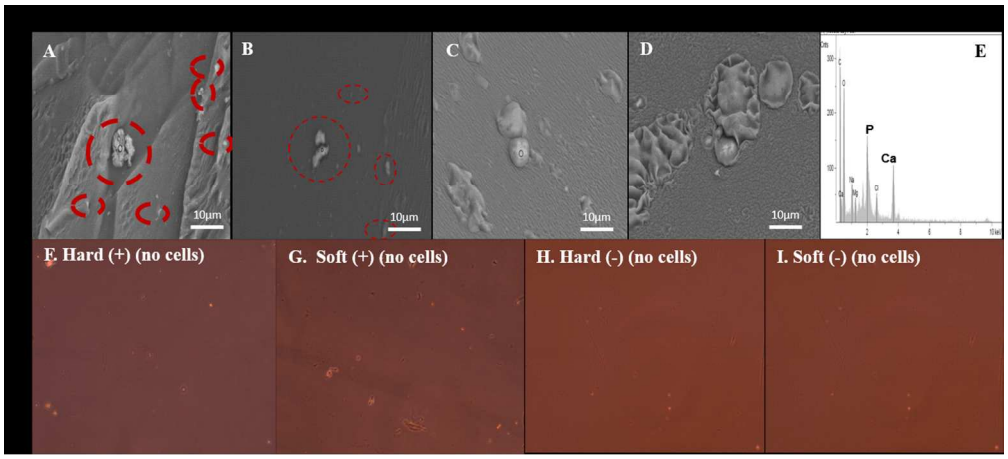
mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) and from day 1 to day 35 of DPSCs culture on TCPS (+), TCPS (-) and the hydrogels (H (+), H (-), S (+), S (-).
136x90mm (150 x 150 DPI)



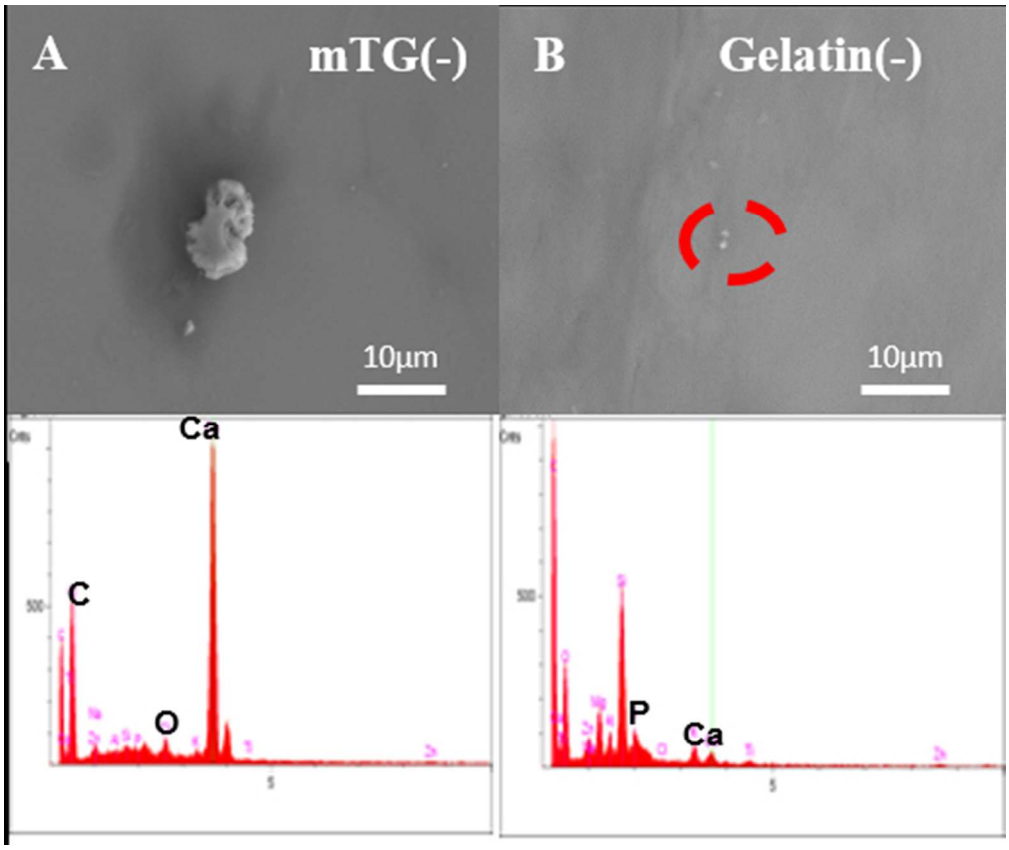
mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) and from day 1 to day 35 of DPSCs culture on TCPS (+), TCPS (-) and the hydrogels (H (+), H (-), S (+), S (-).
116x90mm (150 x 150 DPI)



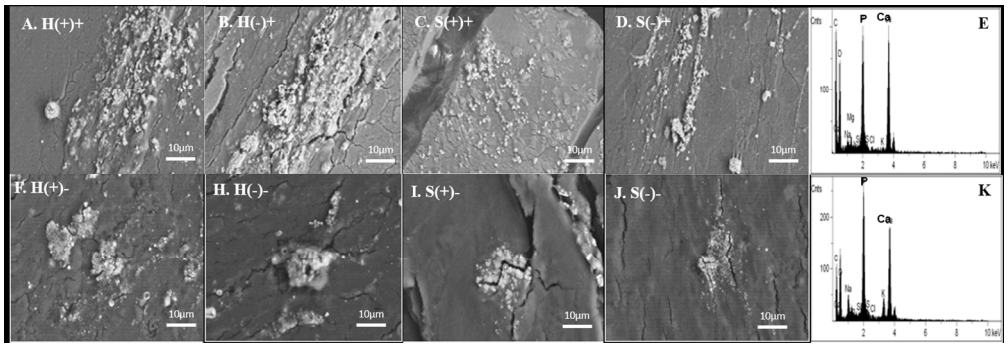
mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) and from day 1 to day 35 of DPSCs culture on TCPS (+), TCPS (-) and the hydrogels (H (+), H (-), S (+), S (-).
143x101mm (150 x 150 DPI)



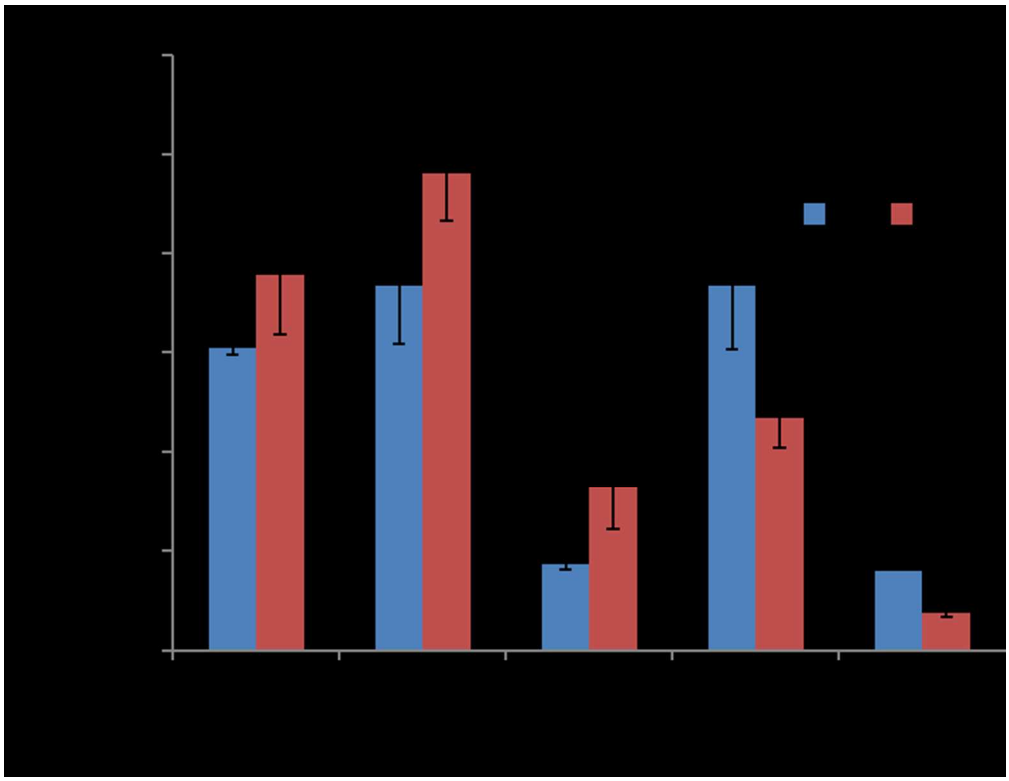
(A-D) SEM micrographs showing the small quantity of mineralized deposits formed on the hard gel in absence of dexamethasone and DPSCs. (E) EDX spectra confirms the presence of calcification. (F-I) Alizarin red stained hard and soft hydrogels without DPSCs do not show any red pattern of calcified deposits as seen with the cells.
233x104mm (150 x 150 DPI)



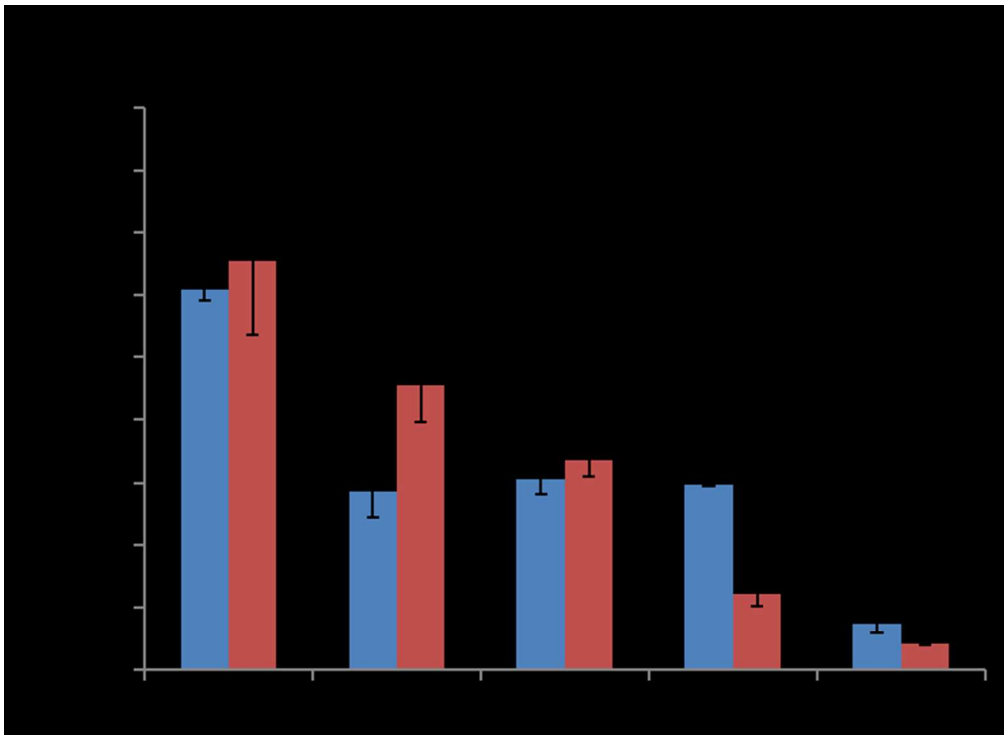
Day 35 SEM micrographs and corresponding EDX spectra of the (A) mTG coated non-induced coverslips and (B) uncrosslinked gelatin coated non-induced coverslips. No biomineralization was seen on uncrosslinked gelatin and mTG.
86x72mm (150 x 150 DPI)



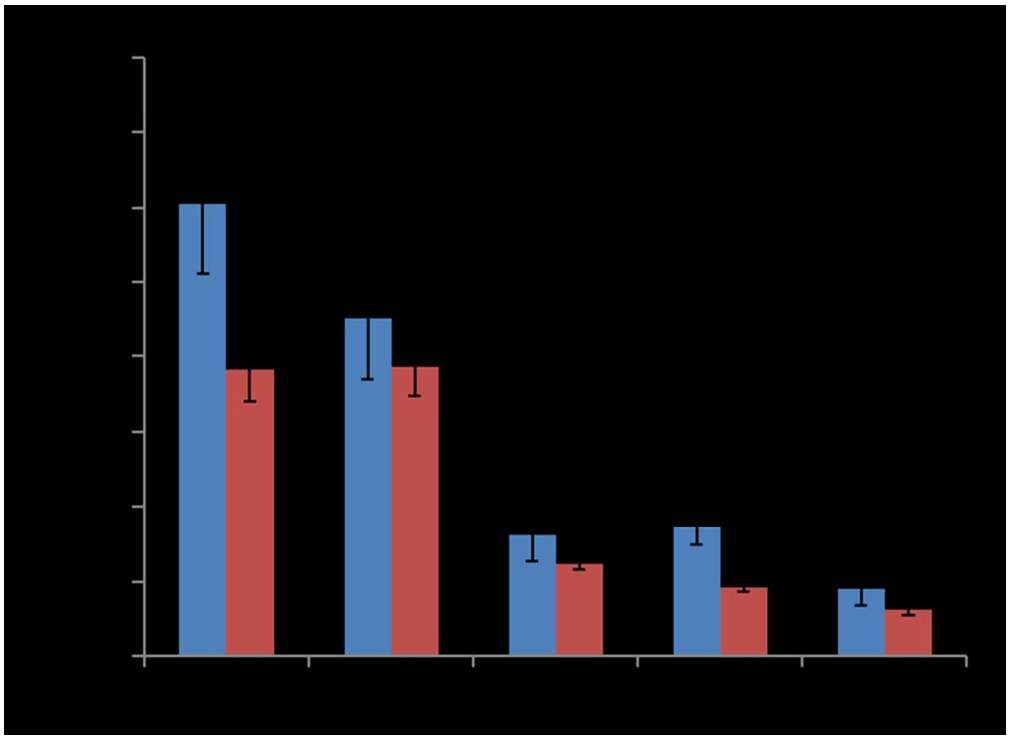
SEM micrographs and representative EDX spectra of the calcified deposits on the TCPS surfaces that were (A-E) induced with dexamethasone and (F-K) non-induced for 21 days. H (+) + denotes the surface that was seeded with DPSCs that were removed from an induced hard gel and cultured in induced medium for 21 days. H(+)+ and H(-)+ surface appear to have more bio-mineralized HAP deposits indicating a more permanent differentiation of DPSCs.
259x87mm (150 x 150 DPI)



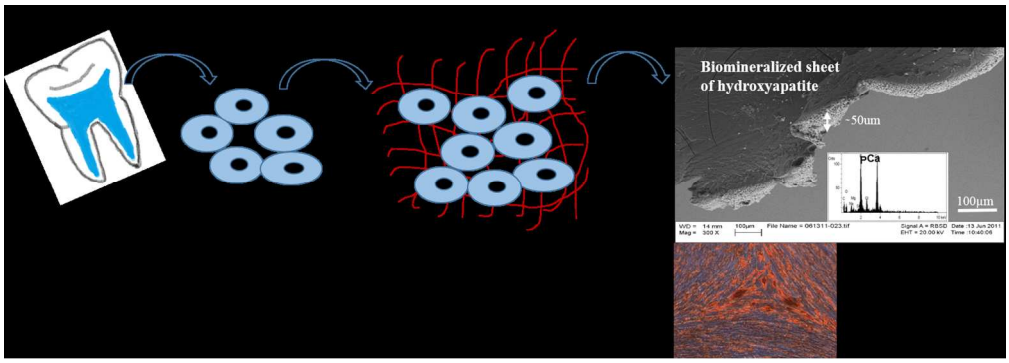
mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) after 21 days of culture. The DPSCs were removed from the H (+), H (-), S (+), S (-) after 21 days of culture and re-seeded on TCPS (+/-) for another 21 days. Blue colored histogram denotes the samples that were induced and red colored histogram denotes the samples that were non-induced.
126x97mm (150 x 150 DPI)



mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) after 21 days of culture. The DPSCs were removed from the H (+), H (-), S (+), S (-) after 21 days of culture and re-seeded on TCPS (+/-) for another 21 days. Blue colored histogram denotes the samples that were induced and red colored histogram denotes the samples that were non-induced.
124x91mm (150 x 150 DPI)



mRNA expression of (A) Dentin Sialophosphoprotein (DSPP) (B) Osteocalcin (OCN) (C) Alkaline Phosphatase (ALP) after 21 days of culture. The DPSCs were removed from the H (+), H (-), S (+), S (-) after 21 days of culture and re-seeded on TCPS (+/-) for another 21 days. Blue colored histogram denotes the samples that were induced and red colored histogram denotes the samples that were non-induced.
122x89mm (150 x 150 DPI)



Biomineralization on enzymatically crosslinked gelatin hydrogels without dexamethasone
272x96mm (150 x 150 DPI)