



A novel tripolymer coating demonstrating the synergistic effect of chitosan, collagen type 1 and hyaluronic acid on osteogenic differentiation of human bone marrow derived mesenchymal stem cells

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ABSTRACT

The biomimetic approach mimicking *in vivo* micro environment is the key for developing functional tissue engineered constructs. In this study, we used a tripolymer combination consisting of a natural polymer, chitosan and two extracellular matrix components; collagen type 1 and hyaluronic acid to coat tissue culture plate to evaluate their effect on osteogenic differentiation of human bone marrow derived mesenchymal stem cells (hMSCs). The polymers were blended at different mixing ratios and the tissue culture plates were coated either by polyblend method or by surface modification method. hMSCs isolated from adult bone marrow were directed to osteoblast differentiation on the coated plates. Our results showed that the tripolymer coating of the tissue culture plate enhanced mineralization as evidenced by calcium quantification exhibiting significantly higher amount of calcium compared to the untreated or individual polymer coated plates. We found that the tripolymer coated plates having a 1:1 mixing ratio of chitosan and collagen type 1, surface modified with hyaluronic acid is an ideal combination to achieve the synergistic effect of these polymers on *in vitro* osteogenic differentiation of hMSCs. These results thus, establish a novel biomimetic approach of surface modification to enhance osteoblast differentiation and mineralization. Our findings hold great promise in implementing a biomimetic surface coating to improve osteoconductivity of implants and scaffolds for various orthopaedic and bone tissue engineering applications.

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1. Introduction

Extracellular matrix (ECM) is an important part of the cellular microenvironment, which along with various growth factors play a significant role in regulating differentiation and development [1]. The ECM components are considered as good candidates for fabricating scaffolds with superior bioactivity or functionalizing biomaterials to improve their performance [2]. A biomimetic surface coating to improve or enhance the osteoconductivity of biomaterials and tissue engineering scaffolds holds great promise in orthopaedic and bone tissue engineering applications.

Among the ECM, collagen type 1 accounts for 90% of organic matrix of bone and plays a significant role in mineralization [3]. Collagen promotes adhesion, osteoblast differentiation and mineralization of hMSCs [4]. Collagen based scaffolds are widely used in bone tissue engineering [5,6]. Similarly, hyaluronic acid is also a

major component of the ECM of many cells including fibroblasts [7]. They play a significant role in cell proliferation, migration [8] and wound healing [9]. Manferdini et al. reported enhanced mineralization by hMSCs on a biomimetic hyaluronic acid based scaffolds [10].

Chitosan is a natural cationic polymer with a structural similarity to the hyaluronic acid of ECM [11] and are known to accelerate wound healing and influence tissue regeneration [12]. Chitosan is widely used as a scaffold for bone tissue engineering and is shown to enhance osteogenesis [13,14].

The chitosan–collagen–hyaluronic acid based scaffolds, exploits the biomimetic approach of tissue engineering and there by promises better osteogenesis and bone regeneration. Combined chitosan–collagen matrices promoted osteoblastic differentiation of MSCs, and improved the mechanical and physical properties of the sponges [15]. Chitosan–gelatin surface modification with hyaluronic acid improved fibroblast adhesion [16].

In the present study we are trying to understand the beneficial effect of a tripolymer biomimetic coating system using a combination of chitosan, collagen type 1 and hyaluronic acid to enhance osteoblast differentiation and mineralization of human bone

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marrow derived mesenchymal stem cells [17]. Here, we optimize the concentration and ratio of mixing each component and method of coating in order to achieve an enhanced differentiation. This tri-polymer coating could be used as an osteoconductive coating for implants and scaffolds to improve their osteointegration.

2. Materials and methods

2.1. Preparation of chitosan, collagen type 1 and hyaluronic acid solutions

The chitosan (CHI) powder (>87.61% degree of deacetylation, DD) which was a kind gift from Indian Sea Foods (Cochin, India) was purified before use by repeated precipitation with 10% NaOH (Merck, India). Hyaluronic acid (HA) from human umbilical cord and collagen type 1 (COL) from calf skin were purchased from Sigma Aldrich (St. Louis, MO, USA). About 1% CHI solution was prepared by autoclaving purified CHI powder in distilled water and then dissolving it by adding 1 M sterile glacial acetic acid (Merck, India). One percent of COL solution was prepared by adding 0.1 M sterile glacial acetic acid. One percent of HA solution was prepared in sterile HEPES buffered saline (Sigma Aldrich, St. Louis, MO, USA). The coating density of CHI, COL and HA, which supported hMSC adhesion and proliferation, was standardized to 25, 6 and 50 $\mu\text{g}/\text{cm}^2$, respectively (data not shown). Based on this the working solutions of CHI (0.5 mg/ml), COL (0.12 mg/ml) and HA (1 mg/ml) were prepared and used for further combination coatings under laminar flow hood in order to maintain sterility. A coating volume of 50 $\mu\text{l}/\text{cm}^2$ of the surface area was used for coating the tissue plate surface (BD Biosciences, USA).

2.2. Preparation of single polymer coated plates

The single polymer coated plates were prepared by covering the tissue culture plates with the polymer solutions of CHI (0.5 mg/ml), COL (0.12 mg/ml) and HA (1 mg/ml) to give a final coating density of 12, 25 and 50 $\mu\text{g}/\text{cm}^2$, respectively. The plates were allowed to dry at 37 °C in an incubator. The acidity of the chitosan coated plates were neutralized with 0.1 M NaOH solution. All the coated plates were first rinsed with distilled water and then with Dulbecco's phosphate buffered saline (DPBS; GIBCO, Invitrogen, NY, USA) before seeding the cells.

2.3. Tripolymer coating by polyblend method

The polyblend solution was prepared by mixing CHI (0.5 mg/ml) and COL (0.12 mg/ml) solutions at different mixing ratios given in Table 1. This solution was blended in a magnetic stirrer for 6 h at RT. To this clear solution of CHI–COL, HA (1 mg/ml) was added slowly and this tripolymer solution was blended in a magnetic stirrer for another 6 h at RT. This gave a turbid solution. The tissue culture plates were coated with the tripolymer polyblend solution and were allowed to dry at 37 °C in an incubator. The coated plates were rinsed with distilled water and then with DPBS before seeding the cells. These plates will be referred to as polyblend coated plates and represented as (CHI:COL:HA)-P henceforth.

2.4. Tripolymer coating by surface modification method

CHI–COL blend solutions were made in different mixing ratios mentioned in Table 1. This solution was blended in a magnetic stirrer for 6 h at RT to give a clear solution. Tissue culture plates were first coated with this blend solution of CHI–COL and were allowed to dry in an incubator at 37 °C. HA solution was then layered over the dry CHI–COL coated plates at a coating density of 50 $\mu\text{g}/\text{cm}^2$. The plates were allowed to dry in an incubator at 37 °C. The plates were washed with distilled water and with DPBS before seeding the cells. These plates will be referred to as surface modified plates and represented as ([CHI:COL] 1HA)-S henceforth.

Untreated normal tissue culture plates (UT), CHI coated, COL coated and HA coated plates served as the controls for the comparative study.

2.5. Culture of hMSCs on the coated plates

hMSCs were isolated from bone marrow of adult human donors after taking informed written consent by method mentioned earlier [18]. Institutional Ethics Committee (IEC) and Institutional Committee for Stem Cell Research and Therapy (ICSCRT) approvals were taken before initiating this experiment. The hMSCs were grown in Knockout-DMEM (GIBCO, Invitrogen, NY, USA) basal medium containing 10% foetal bovine serum (certified Australian, HyClone, Victoria, Australia), 2 mM L-glutamine (GIBCO, Invitrogen, NY, USA) and 1% Pen Strep (10,000 units/ml penicillin and 10,000 $\mu\text{g}/\text{ml}$ streptomycin, GIBCO, Invitrogen, NY, USA). This media will be referred to as hMSC media henceforth. hMSCs were

Table 1
Mixing ratios of the tripolymer combinations.

Method of coating	Mixing ratio, CHI:COL:HA			Plate label
	Chitosan (0.5 mg/ml)	Collagen type1 (0.12 mg/ml)	Hyaluronic acid (1 mg/ml)	
Polyblend method	1	1	1	(1CHI:1COL:1HA)-P
	3	2	4	(3CHI:2COL:4HA)-P
	3	1	5	(3CHI:1COL:5HA)-P
	3	4	2	(3CHI:4COL:2HA)-P
	3	5	1	(3CHI:5COL:1HA)-P
	2	3	4	(2CHI:3COL:4HA)-P
	1	3	5	(1CHI:3COL:5HA)-P
	5	3	1	(5CHI:3COL:1HA)-P
	4	3	2	(4CHI:3COL:2HA)-P
	4	2	3	(4CHI:2COL:3HA)-P
	5	1	3	(5CHI:1COL:3HA)-P
	1	5	3	(1CHI:5COL:3HA)-P
	2	4	3	(2CHI:4COL:3HA)-P
Surface modification method	1	0	1	([1CHI:0COL]1HA)-S
	3	1	1	([3CHI:1COL]1HA)-S
	1	1	1	([1CHI:1COL]1HA)-S
	1	3	1	([1CHI:3COL]1HA)-S
	0	1	1	([0CHI:1COL]1HA)-S

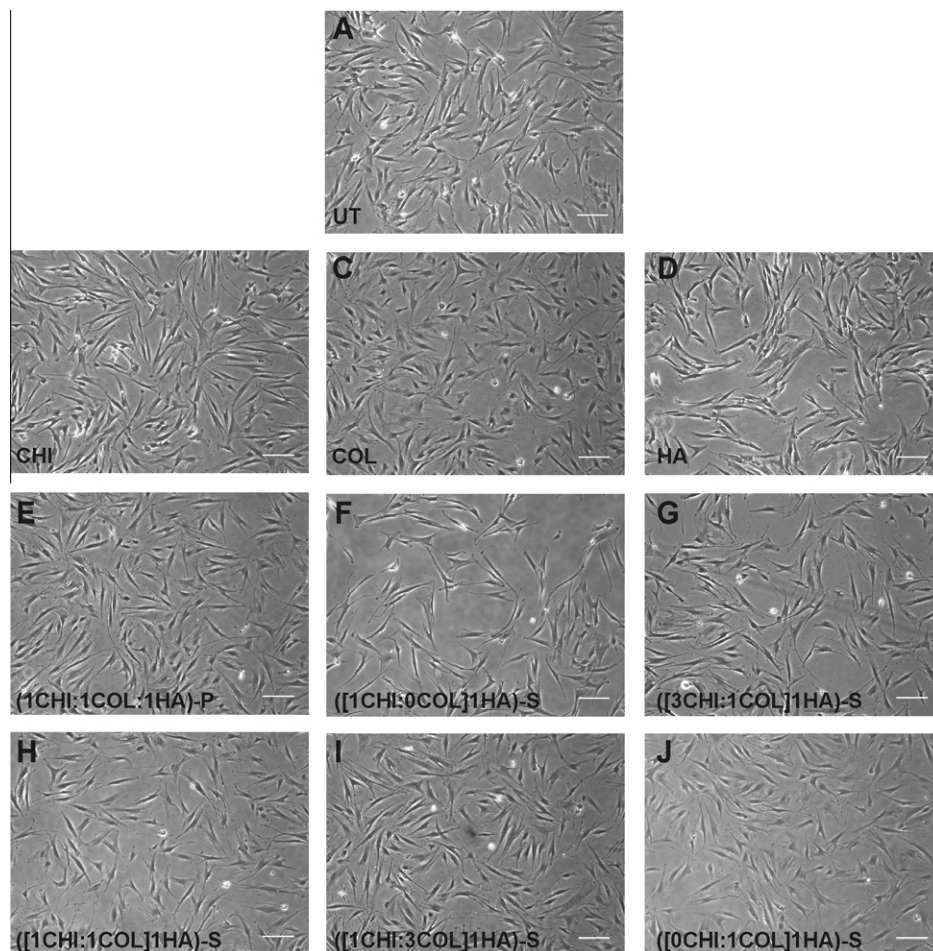


Fig. 1. hMSC culture. Representative phase contrast micrographs of the cells after 24 h of seeding. There was cell adhesion to all the coated plates and the cells showed comparable fibroblast like morphology. Scale bar – 50 μ m.

plated at a seeding density of 5000 cells/cm² on each of the treated plates. The cells were grown in hMSC media in a humidified incubator maintained at 37 °C with 5% CO₂. The plates were observed under a phase contrast microscope (Nikon Eclipse TE2000-S) for cell adherence and morphology.

2.6. Osteoblast differentiation on the coated plates

hMSCs were differentiated into osteoblasts as per method described earlier [19]. Briefly, hMSCs were grown in hMSC media on coated plates and when the plates were 90% confluent, osteoblast differentiation was induced by maintaining them in hMSC media supplemented with 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate and 10⁻⁸ mM dexamethasone. This media will be referred to as osteogenic induction media henceforth. Differentiation to osteogenic lineage was evaluated at different time points by established methods like histochemical staining for secreted mineral matrix and calcium assay.

Alizarin Red S staining for calcium was used to screen the coated plates for their osteogenic potential. Only the plates which gave a positive Alizarin Red S staining after 14 days of osteogenic induction were selected for further evaluation by Von Kossa staining and calcium assay after 21 days. For staining, the cells were fixed with 10% neutral buffered formalin solution (Sigma–Aldrich, St. Louis, MO, USA). The plates were washed thrice with distilled water. For Alizarin Red S Staining, 1% of

Alizarin Red S stain was added to the plates and kept for 10–15 min at room temperature. For Von Kossa staining, the fixed cells were covered with freshly prepared 5% silver nitrate solution and exposed to ultra-violet (UV) light for 1 h. After removing the stain and washing the plates with distilled water, they were observed under a phase contrast microscope (Nikon Eclipse TE2000-S) and the images were captured using QCapture Pro 6 software. The gross view photographs were taken using Nikon D3000 digital camera.

The amount of calcium in the secreted mineral matrix of osteoblasts was quantified by the o-cresolphthalein complexone (Sigma–Aldrich, St. Louis, MO, USA) method mentioned earlier [20]. The total protein was estimated by the Bradford assay (Sigma–Aldrich, St. Louis, MO, USA) and the amount of calcium expressed was normalized to the total protein content of the cells.

2.7. Statistical analysis

The experiments were repeated with three different donor samples. Measures for each sample were performed in triplicates and the results were expressed as mean \pm standard deviation (SD). Two tailed, paired Student's *t* test was used for the statistical analysis and a *p*-value less than 0.05 was considered significant and asterisks were given accordingly to indicate the level of significance as **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

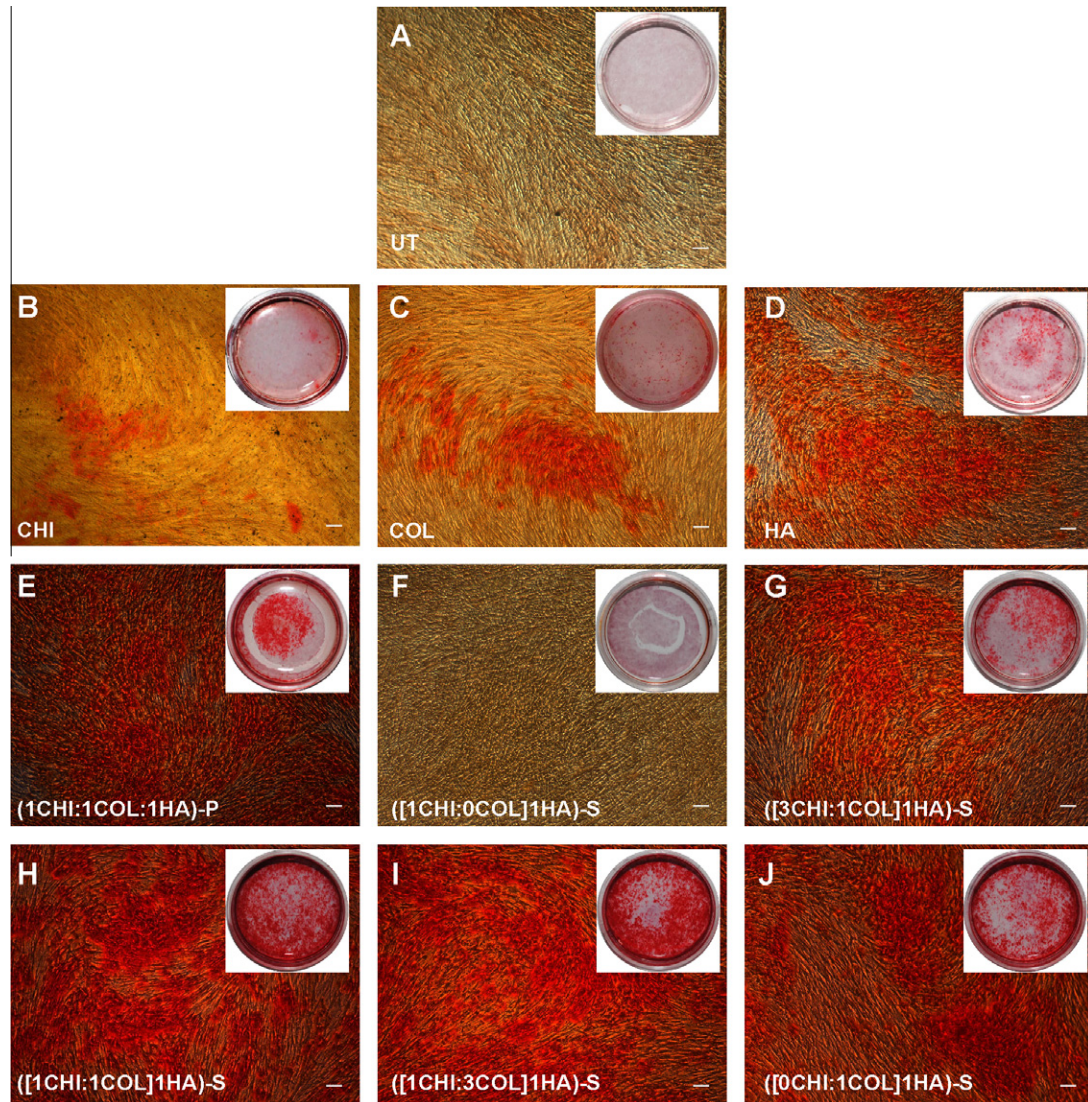


Fig. 2. Osteoblast differentiation of hMSCs (day 14). Representative phase contrast colour micrographs of calcium deposits stained by Alizarin Red S after 14 days of osteogenic induction (Inset: gross view of the corresponding stained plates). All the coated plates except ([1CHI:0COL]1HA)-S (F) showed a positive staining as well as more mineralization than the untreated plates. Note the higher intensity of staining on the tripolymer coated plates ([1CHI:1COL]1HA)-S and ([1CHI:3COL]1HA)-S (H and I). Scale bar-10 μ m.

3. Results

3.1. Osteoblast differentiation assays

A total of 13 polyblend coated plates and 5 surface modified plates with different mixing ratios of CHI, COL and HA were tested for their osteogenic potential. Only six combination plates gave a positive Alizarin Red S staining for calcium after 14 days of osteogenic induction. They included, one polyblend coated plate ([1CHI:1COL:1HA)-P), three surface modified, tripolymer plates ([3CHI:1COL]1HA)-S, ([1CHI:1COL]1HA)-S, ([1CHI:3COL]1HA)-S, and two surface modified bipolymer plates([1CHI:0COL]1HA)-S, [0CHI:1COL]1HA)-S. Only these plates were selected for further analysis in this study.

All the selected plates provided a suitable surface for hMSCs adhesion and proliferation (Fig. 1). Alizarin Red S staining done on day 14 of differentiation (Fig. 2) showed enhancement of osteoblast differentiation on all the selected plates except ([1CHI:0COL]1HA)-S (Fig. 2F) where the combination had only chitosan and hyaluronic acid. Alizarin Red S demonstrated the highest stain-

ing intensity on tripolymer coated plates, ([1CHI:1COL]1HA)-S (Fig. 2H) and, ([1CHI:3COL]1HA)-S (Fig. 2I), indicating more amount of bone matrix. The staining also revealed better mineralization on the tripolymer coated plates (Fig. 2E, G–I) when compared to the single polymer coated plates (Fig. 1B–D). The bipolymer combination of collagen type1 and hyaluronic acid (Fig. 2J) also showed more mineralization than their single polymer coated plates (Fig. 2C and D). Among the single polymer coated plates, HA showed significantly higher mineralization than CHI alone, COL alone or untreated plate (Fig. 2D).

Von Kossa staining which demonstrates the calcium in the secreted mineral matrix towards the final phase of osteoblast differentiation was used to confirm and further compare the mineralization on the selected plates. All the selected plates showed positive Von Kossa staining after 21 days of osteogenic induction (Fig. 3). The staining revealed enhancement of mineralization on all the coated plates when compared to the untreated plate. However, the surface modified ([1CHI:1COL]1HA)-S plate showed remarkably higher mineral deposition. Among the individual polymer coated plates, HA coated plate showed relatively higher

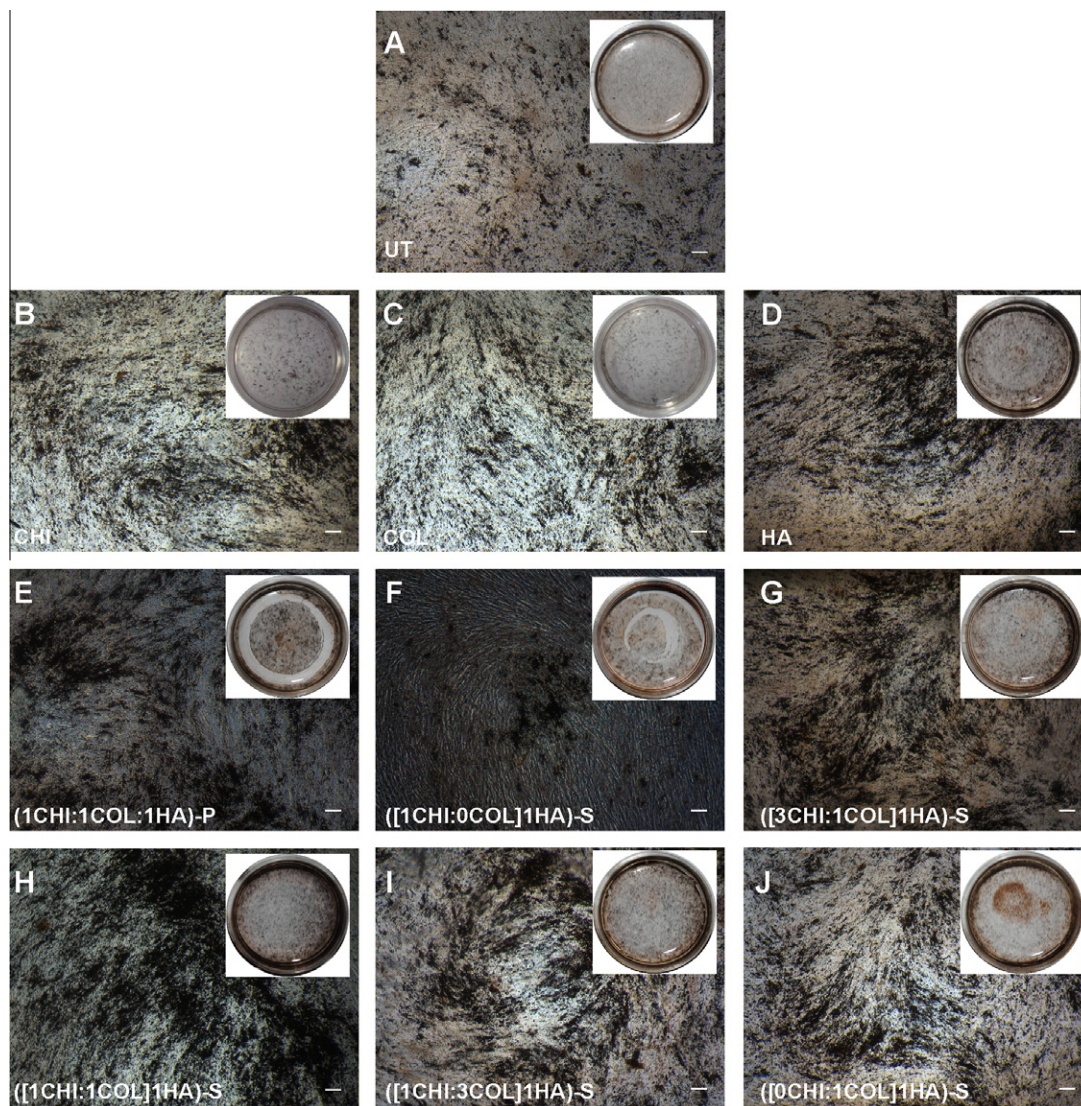


Fig. 3. Osteoblast differentiation of hMSCs (day 21). Representative phase contrast colour micrographs of calcium deposits stained by Von Kossa staining method after 21 days of osteogenic induction (Inset: gross view of the corresponding stained plates). Enhancement of mineralization is appreciated in all the coated plates. Scale bar-10 μ m.

mineral deposition than CHI, COL and untreated coated plate (Fig. 3A–D).

Quantification of calcium also showed interesting results (Fig. 4). Among the individual polymer coated plates, there was no significant difference in calcium deposition in CHI and COL as compared to the untreated. Whereas, HA coated plates significantly improved calcium deposition. Surface modified plates showed significantly high amount of calcium deposition when collagen was present (3.21 ± 0.00 mg in ([1CHI:1COL]1HA)-S and 3.00 ± 0.33 mg in ([1CHI:3COL]1HA)-S as compared to no collagen (1.25 ± 0.32 mg in ([1CHI:0COL]1HA)-S, $p < 0.05$). ([1CHI:1COL]1HA)-S plate showed the highest amount of calcium which was significantly higher than the calcium on CHI, COL, HA and untreated plates. Absence of CHI ([0CHI:1COL]1HA)-S or addition of high concentration of CHI ([3CHI:1COL]1HA)-S also affected calcium deposition.

4. Discussion

Composite polymers have always attracted the researchers due to their ability to achieve better mechanical and biological proper-

ties than their individual polymers [21,22]. Chitosan, collagen and hyaluronic acid are widely investigated for their potential use in bone tissue engineering [23]. To our knowledge, this is a first study using the combination of these three osteogenic biomaterials for a biomimetic coating of normal tissue culture plates to evaluate their combined effect on the osteogenic differentiation and mineralization of hMSCs. Here we have evaluated the synergistic effect of these polymers at different mixing ratios by two different coating methods.

We have developed a simple but novel approach of modifying the tissue culture plate which resulted in the synergistic enhancement of mineralization by a tripolymer combination. First, we optimized the coating density of the individual polymer chitosan, collagen type1 and hyaluronic acid which favoured hMSC adhesion, proliferation and osteoblast differentiation (data not shown). This approach helped us in stringent evaluation and comparison of the osteogenic potential of different tripolymer combinations with respect to the contribution of individual components. We also created an apt platform for obtaining a positive complementary effect on osteogenic differentiation.

Out of the 13 plates with different combinations and concentrations, coated by the polyblend method, only one plate which had

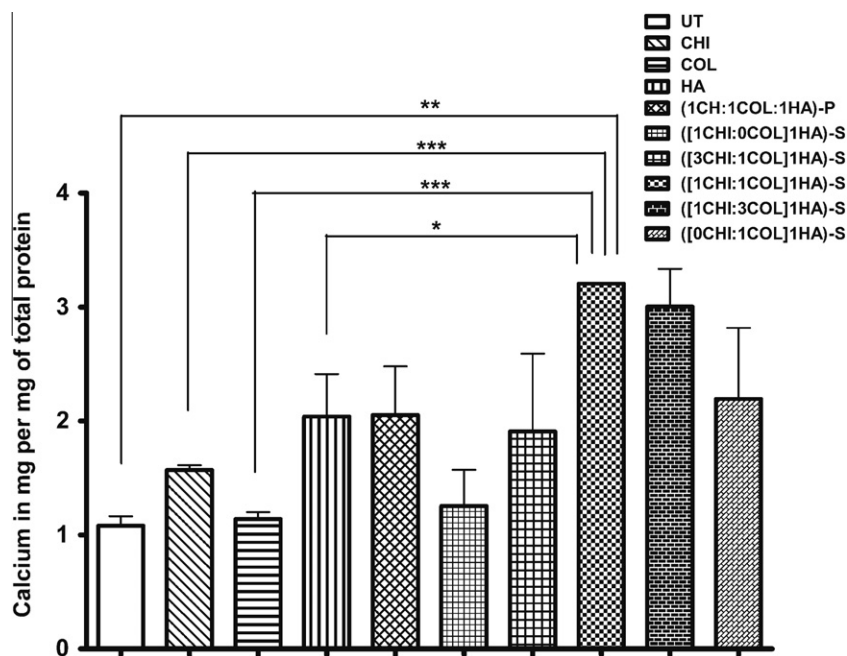


Fig. 4. Calcium assay. Graph showing the mean with SD of a representative experiment done in triplicates. The amount of calcium in the secreted matrix was quantified after 21 days of osteogenic induction. There was more amount of calcium in the mineral matrix of all the coated plates when compared to the untreated plate. The tripolymer plate, ([1CHI:1COL]1HA)-S showed the highest amount of calcium which was significantly higher than the calcium on untreated or individual polymer coated plates.

equal mixing ratio of CHI, COL and HA (1CHI:1COL:1HA)-S showed enhanced mineralization. These plates had a final coating density of 8.5, 2, 16.5 μg of CHI, COL and HA/ cm^2 , respectively. This finding shows that the chemical nature of the contacting surface has a strong impact on cell behaviour. Schneider et al. [24] also reported that the cell adhesive property of a cross-linked multilayer film of chitosan–hyaluronan was significantly regulated by the chemical properties of the outermost layer of the film. These finding emphasizes the importance of optimizing the concentration and fabrication method in developing multi-polymer composites.

Here we report that the surface modification method of coating was more effective in combining the cell adhesive and osteogenic properties of CHI, COL and HA leading to a significant enhancement of mineralization. Mao et al. also found the surface modification method more effective than polyblend method for immobilizing hyaluronic acid on the surface of chitosan–gelatin membrane [16]. The mixing ratio and the method of coating changed the surface property of the biomaterial which altered the osteogenic property of the tripolymer coating. Enhancement of mineralization was very evident on plates coated by the surface modification method. These plates had varying concentrations of CHI and COL but a fixed concentration of HA. So the difference in the osteogenic potential among the different plates was mainly due to the difference in the concentration of CHI and COL as well as their interaction with the over layered HA. Interestingly, the maximum enhancement of osteoblast differentiation and highest amount of calcium in the secreted matrix were seen on surface modified tripolymer plate, ([1CHI:1COL]1HA)-S. The calcium assay also revealed better mineralization on the bipolymer combination of HA and COL than HA and CHI.

Collagen synthesis is the primary function of differentiated osteoblast. The $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are the major collagen binding integrins. $\alpha 2\beta 1$ has a higher affinity for the fibrillar collagen type 1, the major protein constituent of bone. The $\alpha 2\beta 1$ integrin interaction with collagen type 1 is a crucial signal for the induction of osteoblastic differentiation and matrix mineralization [25,26]. Therefore in our study, the plate which had collagen

showed marked increase in the differentiation potential and calcium mineralization. The plate without collagen showed less osteoblast differentiation and lower calcium mineralization ([1CHI:0COL]1HA)-S (Figs. 3F and 4). Addition of HA and CHI further enhanced both osteoblast differentiation and mineralization. This hypothesis is further clarified in Fig. 4 when surface modified plates having collagen showed three fold higher calcium deposition ([1CHI:1COL]1HA)-S or ([1CHI:3COL]1HA)-S as compared to that of plate with no collagen ([1CHI:0COL]1HA)-S. This shows that COL is a crucial component for osteoblast differentiation that can be further enhanced by addition of HA and CHI. MSCs and osteoblasts express CD44 and HA act as the major ligand for CD44 [27]. CD44–HA binding mediates cell adhesion, migration [28] and increases the cellular response to BMP7 [29] which, together could contribute to the enhancement of mineralization by HA. CHI which is considered osteocompatible and has got good film forming capacity [30] also contributed in creating a biomimetic coating for enhancing osteoblast differentiation and mineralization. Thus CHI, COL and HA when combined in the right proportion by an appropriate method showed synergistic effect in enhancing osteoblast differentiation and mineral deposition.

In bone healing process, adequate mineralization of the osteoid is essential for the formation of hard bone and healing of the fracture [31]. So our tripolymer combination ([1CHI:1COL]1HA)-S with adequate osteoconductivity and enhanced mineralization hold great promise for treating bone fracture. There are many reports showing enhanced osteointegration by surface modification of titanium implants with collagen type 1 [32,33]. As our tripolymer coating showed three fold higher mineralization than collagen type 1 coating, it could be a better option for the surface modification of orthopaedic implants.

In summary, we have successfully demonstrated the synergistic effect of chitosan, collagen type 1 and hyaluronic acid on human osteogenesis as the combination enhanced the osteoblast differentiation and calcium deposition more effectively than the individual polymers. Our result affirms that this synergistic effect is stringently regulated or controlled by the mixing ratio and method of

coating. The excellent osteogenic potential of this tripolymer coating can be exploited in therapeutic avenues like bone regeneration. We propose that this biomimetic coating could be employed effectively for the surface modification of implants and scaffolds used in orthopaedic applications to improve the osteoconductivity and osteointegration. They can also be adopted for fabricating three dimensional scaffolds for cell based bone tissue engineering.

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