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Traditional bone grafting requires an open surgical approach to the graft application sites with the attendant complications of a large surgical scar, increased pain and a longer post-operative recovery. To overcome these limitations, there is a great need for the development of better bone graft substitutes. In this study, we developed a novel injectable system which was a biomimetic bone substitute consisted of Poly (L-lactide-co-ε-caprolactone) (P(LLA-CL)) nanoyarns suspended in type I collagen hydrogel (Col). A dynamic liquid support system was employed to fabricate continuous P(LLA-CL) nanoyarns. The electrospun long nanoyarns were chopped into short nanoyarns before they were incorporated into Col. The result of rheological evaluation showed that the mechanical property of Col was enhanced after the nanoyarns were incorporated into it. The mixture of Col and nanoyarn could be smoothly injected out of 16 gauge needle. *In vitro* study showed that human mesenchymal stem cells (hMSCs) proliferated well on Col with nanoyarns. Alkaline phosphatase activity and osteocalcin expression of hMSCs on hydrogel with nanoyarns were much higher than those on control groups. This study highlights the potential of using a novel injectable biomimetic scaffold for bone regeneration.

Keywords: collagen hydrogel; nanoyarns; injectable scaffold; bone regeneration

1. Introduction

The number of bone grafting procedures has been estimated to be between 0.5 million and approximately 1.5 million per year in the USA alone.[1] It has also been estimated that more than 1.4 million bone-associated procedures required bone graft materials, and 1.9 billion dollars were spent in the USA market for bone graft materials only in 2007.[2] The current 'gold standard' has been autogenous iliac crest bone grafting. However, the morbidity of iliac crest bone harvesting, including chronic donor site

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pain, infection, fracture, hematoma, increased operation time and costs, is reported to be as high as 30%.[3] In addition, traditional bone grafting requires an open surgical approach to the graft application site with the attendant complications of a large surgical scar, increased pain and a longer post-operative recovery.[4] There is a great need for the development of better bone graft substitutes to overcome these limitations.

The ideal bone graft would be the one that is not associated with donor site morbidity, yet can be applied in a minimally invasive manner to the grafting site and reliably mimics host bone tissue.[5] In certain situations of a larger bone defect such as in osteoporotic vertebral compression fractures, the grafts applied would also need to be able to provide structural support. There is still an unmet clinical need for bone graft substrates which should have proper mechanical strength to provide structural support. Such a product would find widespread applications not only in traditional indications for bone grafting, but it also would be suitable for kyphoplasty in the treatment of osteoporotic vertebral fractures and other situations.

Minimally invasive procedure (MIP) in clinical treatment reduces patient's pain and shortens the recovery. A number of injectable systems for MIP have been developed, such as Norian-SRS®, Accell 100™, Grafton®, BioSet™, AlloFuse™, etc.[2] Although each has its own advantages in bone regeneration, drawbacks of these systems should be noticed. Calcium phosphate cement (CPC) has the ability of self-hardening, but it is brittle and lacks micro pores. Its poor injectability also should be noticed.[6] A number of CPC products such as Norian-SRS® and Kryptonite® have been commercialized, but they are facing problems such as low porosity (less than 60%) and low biodegradation rate.[7,8] Healos® Fx Injectable Bone Graft Replacement which includes collagen microfibres incorporated with nano-hydroxyapatite particles has been considered as a best bone graft substrate mimicking the main components of native bone matrix.[9] This bone graft replacement has achieved success in clinical studies and been approved by Food and Drug Administration. But the diameter of needle for its injection is large, which limits its usage in clinic such as in the treatment of osteoporosis. Moreover, before it is injected to the defect sites, it should be saturated with bone marrow aspirate from patient, which increases the pain of patient and operation risks.

Hydrogel is capable of absorbing large amount of water and easily formed by cross-linking.[10] Its great injectability and easy shaping simplify the minimally invasive injection procedure. Collagen is a main component of native bone tissues. It has been manipulated to various forms and widely employed for bone regeneration.[11,12] Collagen hydrogel (Col) mimics natural extracellular matrix (ECM) and provides appropriate biological signals to cells.[13] Although the Col has many advantages, its application in tissue engineering is limited by its inherent weakness in mechanical properties. It usually works as a vehicle to deliver drugs, growth factors or cells.[14–16] For its applications in bone tissue engineering, Col has been used to repair the critical bone defects in a rabbit model and worked as a space-filling agent to repair calvarial defects in a rat model.[17,18]

Cell-induced contraction forces resulting in hydrogel deformation had great effects on cell growth, migration, proliferation and differentiation. A study showed that cell-induced contraction caused Col to contract as much as 85%.[19] One of methods to overcome the gel contraction is to improve its mechanical property. Cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, glutaraldehyde and Au nanoparticles were employed to improve its mechanical properties.[20–22] An alternative approach is to subject it to plastic compression to generate high collagen fibrillar density.[23] Another approach is to add micro/nano-fibers into Col. Gentleman et al.

incorporated short collagen fibers into collagen solution before the solution gelled. They found that the gel contraction was reduced and the permeability of the cells was enhanced after short collagen fibers were added.[24,25]

Poly(L-lactide-co-ε-caprolactone) (P(LLA-CL)) is a synthetic copolymer. It has been electrospun into nanofibers with the diameters ranging from micrometer to nanometer and applied as various tissue-engineered scaffolds for bone, skin, cartilage and blood vessel engineering applications.[26–29] Studies showed that the mechanical property of P(LLA-CL) nanofibers was much better than that of electrospun natural polymeric nanofibers.[30,31] Nanoyarn is made from electrospun nanofibers mimicking ECM and shows submicron fibrous morphology. Its higher mechanical strength makes it possible for conventional physical manipulation.[32,33] Hydrogel and electrospun nanofibers have their own advantages and disadvantages in the applications for tissue engineering. For the design of an injectable system, the nanoyarn should be further processed to maintain the injectability of the injectable system.

The aim of this study was to develop a novel injectable system which was a biomimetic substrate. The system was composed of short electrospun P(LLA-CL) nanoyarns and collagen type I hydrogel. The nanoyarns were incorporated into Col before the gelation of hydrogel. We hypothesized that short nanoyarns incorporated into Col would improve the mechanical property of Col and have positive effects on cell behaviours. Moreover, the injectability of Col would be maintained after short electrospun nanoyarns were incorporated into the hydrogel.

2. Experimental

2.1 Materials

P(LLA-CL) (75:25) was purchased from Fine Chemical Sales Carbohydrate Chemistry Team Industrial Research (New Zealand). Collagen type I (Col) (PureCol®, 3 mg/ml) was purchased from Advanced BioMatrix, Inc. (USA). Healos® Fx Injectable Bone Graft Replacement was purchased from Johnson & Johnson Pte. Ltd (Singapore). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from Daikin, Japan. The other reagents were purchased from the Sigma-Aldrich Co. (Milwaukee, Wisconsin). All of the materials were used without further purification.

2.2 Methods

2.2.1 Fabrication of P(LLA-CL) nanoyarns

P(LLA-CL) was dissolved in HFIP at 10% w/v under magnetic stirring. As shown in Figure 1(A), P(LLA-CL) nanoyarns have been fabricated by using a dynamic liquid support system.[33] Briefly, a high voltage of 14 kV was applied to the prepared solution with a 27G needle as the spinneret. A feed rate of 1 ml/h was set on the syringe pump (kD Scientific).The electrospun P(LLA-CL) nanofibers were deposited on a water vortex. The nanoyarns were fabricated as the nanofibers were flowing down with water. As shown in Figure 1(B), annular P(LLA-CL) nanoyarns floated on the surface of the below water bath collector. The nanoyarns were collected from the below water bath and freeze-dried immediately.

2.2.2 Preparation of injectable hydrogel system

Col was prepared on ice by adding eight parts of collagen type I solution to one part of 10 times sterilized phosphate-buffered saline (PBS) and then one part of filtered

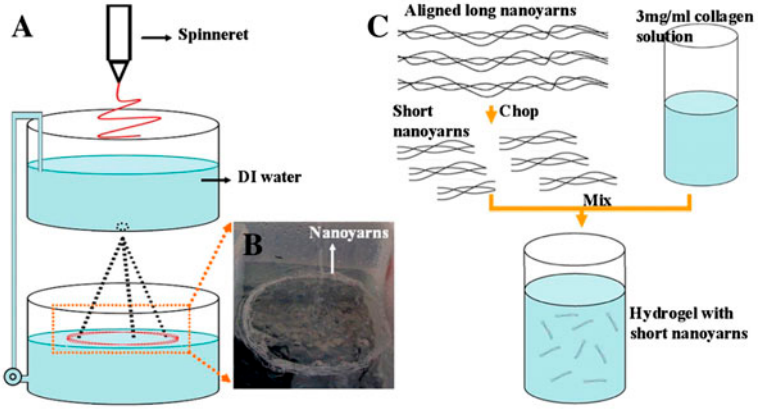

Figure 1. (A) Schematic image of the set-up for the fabrication of nanoyarn. (B) Optical image of nanoyarns with annular morphology formed on the surface of below water bath. (C) Schematic image of the fabrication of injectable hydrogel with nanoyarns.

0.1 N sodium hydroxide was added by a pipettor. The mixture solution was gently mixed to avoid bubbles, then kept at 4 °C in a refrigerator for further usage. For attaining the gelation of the mixture solution, it was kept under 37 °C for 2 h to get fully gelled.

As shown in Figure 1(C), for the preparation of Col/P(LLA-CL) nanoyarn (Col/P(LLA-CL)) sample, long nanoyarns were chopped into short nanoyarns with the length around 1 mm. Short nanoyarns were sterilized by 70% ethanol, then washed with PBS for three times. Sterilized nanoyarns were mixed with the mixture solution to make sure that the weight ratio of nanoyarns and collagen type I was 1:1. The solution containing P(LLA-CL) nanoyarn was kept under 4 °C in a refrigerator overnight to remove the remaining bubbles on the surface of the solution. Col/P(LLA-CL) hydrogel was prepared by placing the solution with P(LLA-CL) nanoyarns under 37 °C for 2 h to get fully gelled.

2.2.3 *Scanning electron microscope (SEM) analysis*

The morphorlogy of nanoyarns and the distribution of nanoyarns in Col were studied under SEM (Hitachi S-3000 N, Japan). The mixture solution and the mixture solution with P(LLA-CL) nanoyarns were injected in a 24-well plates by 16 gauge needles, respectively. They were frozen at −20 °C overnight and lyophilized to obtain porous sponges. Prior to SEM examination, the specimens were cross-sectioned and sputter-coated with gold.

2.2.4 *Rheological evalution*

The rheological properties of the injectable system were analysed by an ARES-RF rthemeter (TA Inst., USA). A mineral oil was added around the samples to avoid evaporation. Time sweep data were generated by using a frequency of 1 Hz and 5% strain. The temperature was kept at 37 °C during the process of evaluation.

2.2.5 *Cell culture*

Human mesenchymal stem cells (hMSCs) (Lonza, USA) were cultured in a 75 cm^2 cell culture flask. The basic culture medium consisted of the following constituents:

Dulbecco's modified Eagle's medium with 1% antibiotics and 10% Fetal bovine serum (Invitrogen, CA, USA). The cells were harvested by trypsin-EDTA after they were cultured until passage 4. Before cell seeding, 300 μl of mixture solution and 300 μl of mixture solution with P(LLA-CL) nanoyarn were injected into 24-well plates, respectively, then kept under 37 °C for 2 h to attain gelation. Helos® was used as a control. 3 mg Helos® material (Helos) was weighted in sterile conditions and placed in 24-well plates. 1.0×10^4 cells were seeded on each sample and supplied with basic media containing 2.5 mM L-glutamine (Invitrogen, CA, USA) and 50 mg/L L-ascorbic acid (Sigma Aldrich Chemical Company Inc., St. Louis, USA). The medium was changed every two days.

2.2.6 *Cell proliferation*

The proliferation of hMSCs was monitored at 7, 14 and 21 days of culture using the colorimetric 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTS) assay (CellTiter 96® Aqueous One solution, Promega, Madison, WI, USA). The cell-seeded constructs were washed with PBS for three times. 500 μl serum-free medium containing 20% MTS reagent was added into each well. The wells were incubated at 37 °C for 4 h in a humidified 5% CO_2 incubator. The absorbance was measured at 490 nm in a spectrophotometric plate reader (FLUOstar OPTIMA, BMG Lab Technologies, Germany). A calibration curve of hMSCs was established to calculate the number of living cells from the absorbance index.

2.2.7 *Alkaline phosphatase (ALP) activity*

ALP activity of hMSCs was measured using a Phosphatase Substrate Kit (Phosphatase Kit, No. 37,620, Pierce Biotechnology) at 7, 14 and 21 days of culture. Cell-seeded sample was washed with PBS for three times and incubated with 400 ml pNPP liquid at room temperature for 30 min till the colour of solution become yellow. The reaction was stopped by the addition of 200 ml of 2 M NaOH solution. The yellow colour product was aliquoted in 96-well plate and the absorbance was measured at 405 nm in a spectrophotometric plate reader.

2.2.8 *Immunocytochemistry*

Cell-seeded samples were washed with PBS for three times and fixed in 4% formaldehyde, and then exposed to BSA/PBS for 60 min at room temperature. After another wash in PBS, the wells were incubated overnight with primary antibodies against osteocalcin (Millipore Singapore Pte. Ltd). After washed with PBS, the cell-seeded samples were stained with anti-mouse-FITC secondary antibody (Sigma–Aldrich Corp., St Louis, MO) for 1 h and 4′,6-diamidino-2-phenylindole (Invitrogen Corp., Carlsbad, CA) was used to stain the cell nucleus for 30 min. The samples were viewed by LSCM (Olympus FluoView FV1000, Olympus Corp., Center Valley, PA).

2.2.9 *Stastics*

The data presented are expressed as mean ± standard deviation. Statistical analysis was done using Student's *t*-test and the significance level of the data was obtained. *P*-value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1 Characterizations of nanoyarns and nanoyarns in hydrogel matrix

Electrospinning, a convenient way to fabricate nanofibrous scaffolds mimicking ECM, has attracted many attentions. The efforts to manipulate electrospun nanofibers for the production of nanoyarn also have been made.[34–36] Nanoyarns usually were produced by special collector, such as: rotating drums or disks, parallel auxiliary electrodes, metal needles, dual metal collection rings and so on.[37–40] Circled water bath as a unique collector to fabricate continuous nanoyarns was reported by Wee-Eong Teo et al. [33]. We employed this method to produce long aligned nanoyarns by collecting the annular nanoyarns from the surface of beneath water collector (Figure 1(A) and (B)). As shown in Figure 2(A), aligned long nanoyarns were fabricated. The average diameter of nanoyarns was 16 ± 4 μm. The alignment of the long nanoyarns was greatly high. No entanglement was observed and the morphology of the nanofibers was maintained as well (Figure 2(B)). Massive continuous nanoyarns with similar diameter were produced by this method. The high alignment of long nanoyarns made them easily get separated after they were chopped into short nanoyarns (Figure 2(C) and (D)). The short nanoyarns with length around 1 mm were short enough to avoid the entanglement when they were mixed with collagen solution.

The needs for bone graft substrates have been increasing in recent years due to the ageing population. The efforts to develop ideal bone graft substrates for MIP also have been made.[41] As hydrogel and electrospun nanofiber each has its own advantages and disadvantages in the applications for tissue engineering, we incorporated electrospun nanoyarns into Col for the fabrication of a novel injectable substrate. The process for the fabrication of injectable substrate is shown in Figure 1(C). Processed short

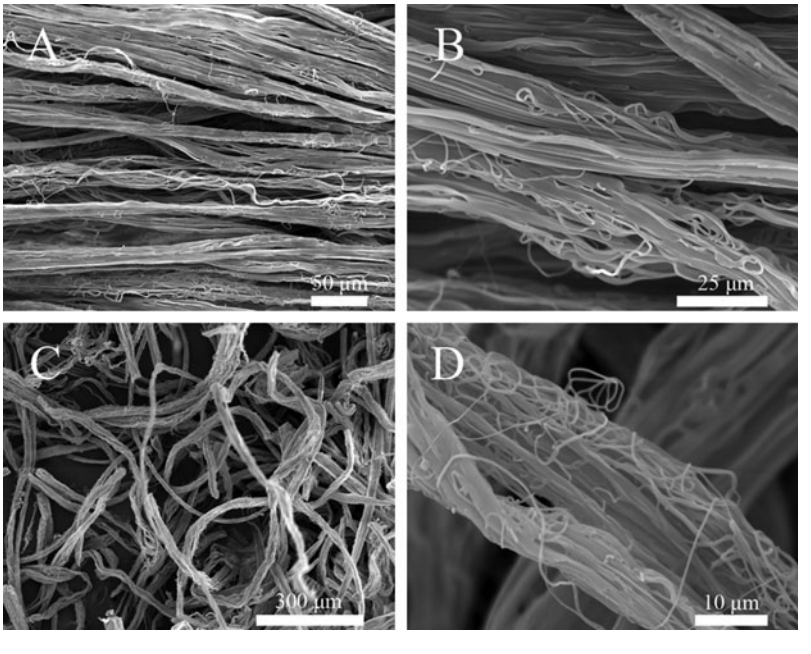

Figure 2. (A) and (C) are the SEM images of long and short nanoyarns, respectively. (B) and (D) are the SEM images of the higher magnification of (A) and (C), respectively.

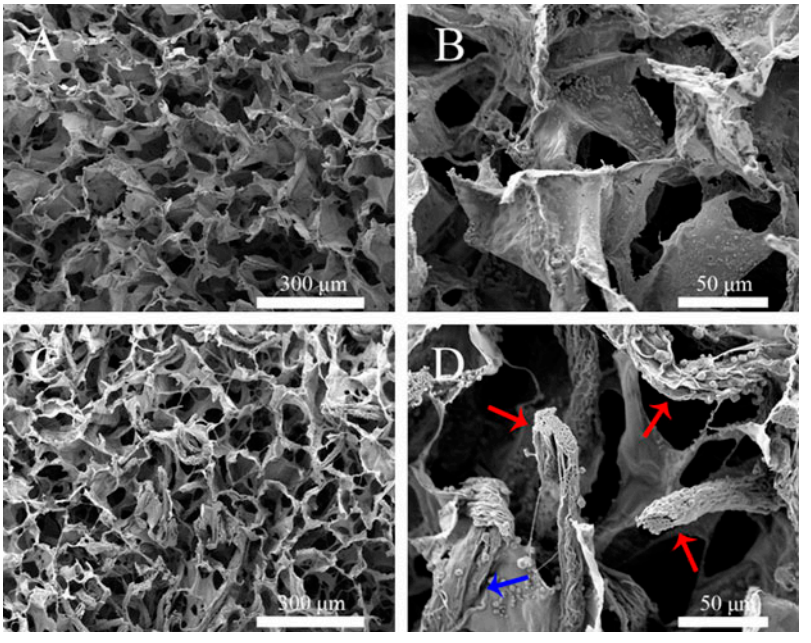

Figure 3. (A) SEM image of freeze-dried Col; (C) SEM image of freeze-dried Col/P(LLA-CL); (B) and (D) represent the higher magnification of (A) and (C), respectively. Red arrows indicate the nanoyarns penetrating through the pore walls. Blue arrow indicates nanoyarn being a part of the pore wall. (Please see the online article for the colour version of this figure: http://dx.doi.org/10.1080/09205063.2013.848326)

P(LLA-CL) nanoyarns were added in collagen solution and mixed with it properly. For the characterization of the distribution of nanoyarns in Col, P(LLA-CL) nanoyarns incorporated with collagen solution were freeze-dried and observed under SEM. As shown in Figure 3(C), the distribution of P(LLA-CL) nanoyarns in Col was homogenous. Figure 3(D) showed that some P(LLA-CL) nanoyarns were penetrating through the pore wall as indicated by red arrows and the others were a part of the pore wall as indicated by blue arrow.

3.2 Mechanical property and injectability of injectable system

To investigate the mechanical properties of Col and Col/P(LLA-CL), rheological evaluation was performed. As shown in Figure 4(A) and (B), the storage modulus (G') of Col and Col/P(LLA-CL) were much larger than their loss modulus (G''), respectively, which indicated that they showed an elastic behaviour.[42] After the incorporation of nanoyarns into the Col, G' and G'' of Col/P(LLA-CL) both were increased drastically even at the initial stage. The results indicated that the mechanical property of Col was enhanced by incorporated nanoyarns. For the injectability test of Col/P(LLA-CL), 16 gauge needle was used. As shown in Figure 4(C), Col/P(LLA-CL) was smoothly injected out of the needle. The result indicated that the incorporation of 3 mg/ml nanoyarns in collagen solution had no great effect on its injectability, and it would be suitable for MIP procedures in clinic.

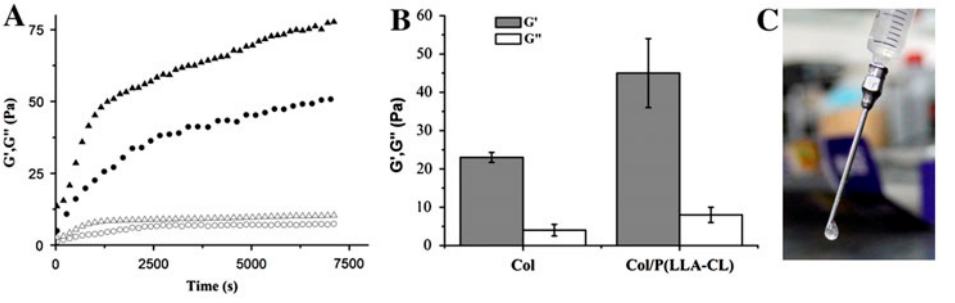

Figure 4. (A) Rheological evaluation of Col (circles) and Col/P(LLA-CL) (triangles) at 1 Hz at 37 °C. Solid symbols indicate storage modulus G', and open symbols indicate loss modulus G''. (B) The values of G' and G'' at 1000s. (C) Optical image of injectability test of Col/P(LLA-CL) by a 16 gauge needle.

3.3 *Morphology, proliferation and differentiation of hMSCs*

To observe the morphology of hMSCs on Col and Col/P(LLA-CL), optical images were taken at day 14 and day 21. No great difference was observed at day 14 (Figure 5(A) and (B)). At day 21, clustered morphology of hMSCs was observed on Col as seen in Figures 5(C) and 8(A) as indicated by white arrows, but no such obvious phenomenon was observed on Col/P(LLA-CL) (Figure 5(D)). Cell-scaffold mechanical interplay might result in this phenomenon. The contraction forces induced by cell had great effects on hydrogel matrix, which has been reviewed by Dekel Dado et al. [43]. Studies

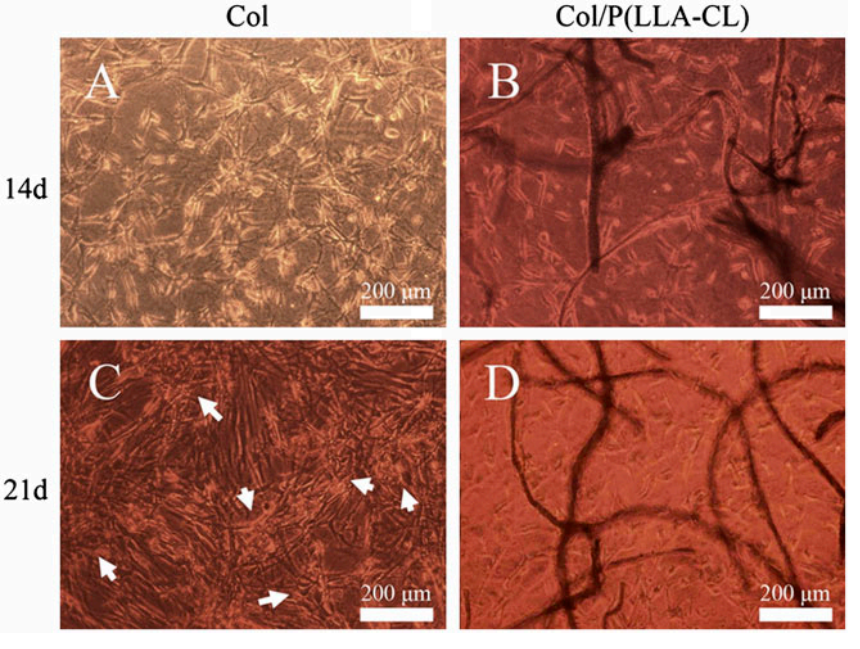

Figure 5. Optical images of morphology of hMSCs on Col and Col/P(LLA-CL) at day 14 and 21. White arrows indicated the clustered overlapping hMSCs on Col.

showed that hydrogel matrix deformed due to the cell-induced contractile forces after days of cell culture.[19,44] In our case, at day 21, with the proliferation of hMSCs on Col, cell-induced contraction forces might be one of the causes which resulted in this clustered phenomenon. Moreover, the other cause might be the mechanical weakness of Col matrix which was deformed by the cell-induced contract forces. The morphology of hMSCs on Col/P(LLA-CL) at day 21 was homogenous fibroblastic-like morphology (Figures 5(D) and 8(B)). P(LLA-CL) is a synthetic polymer showing better mechanical properties when compared with natural polymers such as collagen.[45] In this study, P(LLA-CL) nanoyarns incorporated into Col matrix might serve as back bones to prevent the deformation of Col and resist the contraction forces induced by hMSCs.

As shown in Figure 6, the number of hMSCs on Col and Col/P(LLA-CL) increased steadily and it was much more than that on Helos at all time points. The data showed that hMSCs grew well on Col/P(LLA-CL). At day 21, the number of hMSCs on Col exhibited significant increase when compared to that on Helos and Col/P(LLA-CL). The surface of Col might be deformed due to the cell-induced contraction forces. It might be one of the reasons why the cells on Col showed overlapped and clustered morphology at day 21. As shown in Figure 7, along with the cell proliferation, ALP activity of cells on Col and Col/P(LLA-CL) increased steadily. It almost stayed the same on Helos at all time points. Interestingly, at day 21, ALP activity level of cells on Col/P(LLA-CL) increased abruptly and was significantly higher than that on other groups. ALP is a cell membrane bound enzyme and its activity is utilized as a marker to estimate the osteoblastic differentiation.[46,47] The result indicated that hMSCs had differentiated and undergone osteogenic process. Scaffolds provide the support for cell attachment, proliferation and differentiation. Studies showed that mechanical property of scaffolds had significant effect on cell differentiation.[48,49] The adding of P(LLA-CL) nanoyarns in Col maintained the architecture of hydrogel matrix and provided a much better environment for cell spreading, growth and differentiation. It might be the reason why ALP activity level of hMSC significantly increased on Col/P(LLA-CL) at

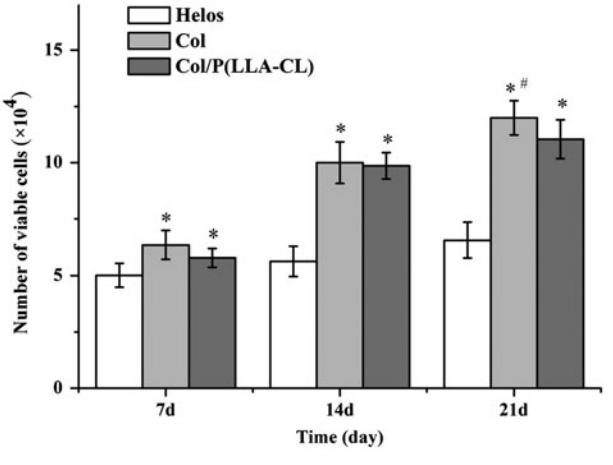

Figure 6. Cell proliferation of hMSC on Helos, Col and Col/P(LLA-CL) after 7, 14 and 21 days of culture; *$p < 0.05$, significant against Helos; #$p < 0.05$, significant against Helos and Col/P (LLA-CL) at day 21.

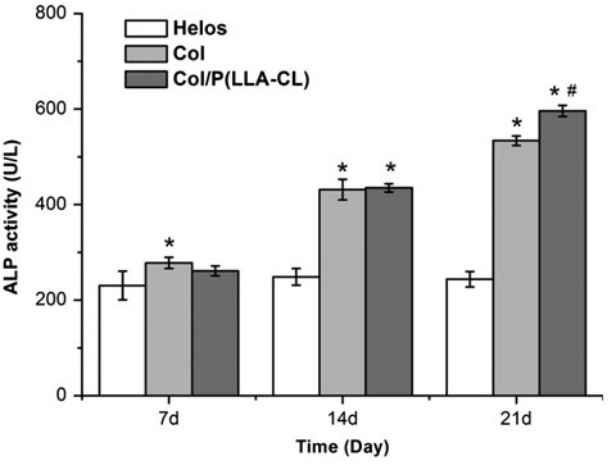

Figure 7. ALP activity of hMSC on Helos, Col and Col/P(LLA-CL) after 7, 14 and 21 days of culture; $^{*}p < 0.05$, significant against Helos; $\#p < 0.05$, significant against Helos and Col at day 21.

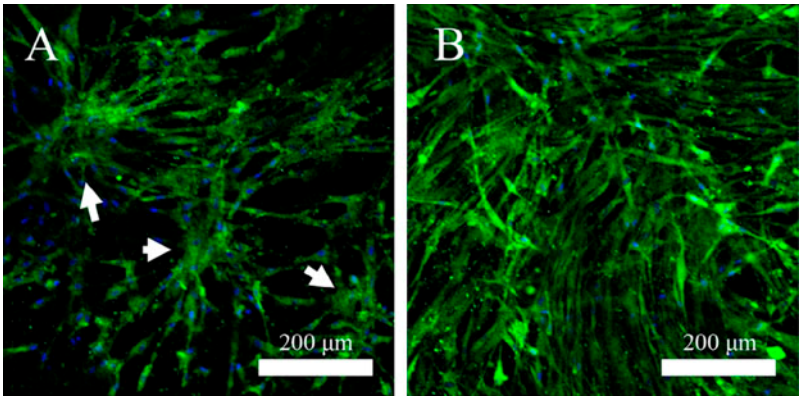

Figure 8. Immunofluorescence staining of osteocalcin with FITC-labelled secondary antibody at day 21 in (A) Col; (B) Col/P(LLA-CL).

day 21. Osteocalcin is a specific ECM protein related to bone formation.[50] The higher intensity of the fluorescent at day 21 indicated the expression of osteocalcin of hMSCs on Col/P(LLA-CL) was much higher than that on Col as shown in Figure 8(A) and (B). The P(LLA-CL) nanoyarns not only maintained the structure of Col matrix, but it also played an important role in stem cell differentiation.

All the results above proved our hypotheses. The mechanical property of Col was improved, and hydrogel incorporating with nanoyarns still could be injected out by using 16 gauge needles. Moreover, the morphology, proliferation and differentiation of cells on hydrogel with short nanoyarns were greatly influenced. Nanoyarns incorporated in hydrogel might be an effective way to decrease the hydrogel contraction and maintain the hydrogel structure. Our novel injectable system could be useful in the treatment of osteoporosis or bone fracture.

4. Conclusions

We fabricated the long and short nanoyarns using a dynamic liquid support system.The hydrogel incoporating with P(LLA-CL) nanoyarns could fully attain gelation in 2 h at human body temperature and could be injected out with 16G needle before it was gelled. The *in vitro* results showed that hMSCs proliferated well on the novel injectable system, and the system also promoted the differentiation of hMSCs. It suggests that Col incorporating with nanoyarns has a great potential in bone regeneration.

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