Fabrication and detection of tissue engineered bone aggregates based on encapsulated human ADSCs within hybrid calcium alginate/bone powder gel-beads in a spinner flask

Kedong Song^{1,*}, Yanfei Yang¹, Lili Xu¹, Jiaxin Tian¹, Jiangli Fan¹, Zeren Jiao¹, Shihao Feng¹, Hong Wang², Yiwei Wang³, Ling Wang^{4,*}, Tianqing Liu^{1,*}

¹State Key Laboratory of Fine Chemicals, Dalian R&D Center for Stem Cell and Tissue Engineering, Dalian University of Technology, Dalian 116024, China;

²Department of Orthopaedics, First Affiliated Hospital of Dalian Medical University, Dalian 116011, China;

³Burns Research Group, ANZAC Research Institute, University of Sydney, Concord, NSW, 2139, Australia,

⁴Department of Oncology, First Affiliated Hospital of Dalian Medical University, Dalian 116011, China

*Corresponding Author: Prof. K Song, Prof. T Liu and Prof. L Wang, State Key Laboratory of Fine Chemicals, Dalian R&D Center for Stem Cell and Tissue Engineering of Dalian University of Technology, 116024, China. Telephone: +86-411-84706360. Email: Kedongsong@dlut.edu.cn, liutq@dlut.edu.cn, whwl@hotmail.com

Abstract:

Traditional treatment for bone diseases limits their clinical application due to undesirable host immune rejection, limited donor sources and severe pain and suffering for patients. Bone tissue engineering therefore is expected to be a more effective way in treating bone diseases. In the present study, hybrid calcium alginate/bone powder gel-beads with a uniform size distribution, good biocompatibility and osteoinductive capability, were prepared to be used as an *in-vitro* niche-like matrix. The beads were optimized using 2.5% (w/v) sodium alginate solution, 4.5% (w/v) CaCl₂ solution and 5.0 mg/mL bone powder using an easy-to-use method. Human ADSCs were cultured and induced into chondrocytes and osteoblasts, respectively. The cells were characterized by histological staining showing the ADSCs were able to maintain their characteristic morphology with multipotent differentiation ability. ADSCs at density of 5×10⁶ cells/mL were encapsulated into the gel-beads aiming to explore cell expansion under different conditions and the osteogenic induction of ADSCs was verified by specific staining. Results demonstrated that the encapsulated ADSCs expanded 5.6 folds in 10 days under dynamic condition via spinner flask, and were able to differentiate into osteoblasts (OBs) with extensive mineralized nodules forming the bone aggregates over 3 weeks post osteogenic induction. In summary, hybrid gel-beads encapsulating ADSCs are proved to be feasible as a new method to fabricate tissue engineered bone aggregation with potential to treat skeletal injury in the near future.

Key words: bone tissue engineering; hybrid gel-beads; ADSCs; bone aggregates; dynamic condition

1. Introduction

Autogenous, allogeneic and synthetic bone grafts are widely used in treating bone loss or defects caused by trauma, congenital malformation, infection and bone tumor. However, host immune rejection of grafts, limited donor sources and great suffering to patients [1-4] limits the clinical efficacy and satisfaction of these bone grafts. Bone tissue engineering (BTE) with promising potential to regenerate bone with natural form and function, has been widely studied due to its unlimited supply and low risks of disease transmission [5, 6]. It showed the potential to pave a new way for bone healing and improve the quality of lives of patients. Therefore, it became a research hot spot in fabricating engineered bone tissue *in-vitro* with natural properties that to be used for tissue regeneration, through coupling with biomimetic scaffolds, cells and growth factors, among which the design and fabrication of scaffold is paramount [7, 8].

In fabricating clinical BTE-based scaffolds, natural polymers such as hyaluronic acid (HA) [9], fibrinogen [10], collagen [11] and chitosan [12, 13] or synthetic biodegradable polymers like PEG [14, 15], PLA [16], PCL [17] are selected and studied widely, relying on their excellent properties of good biocompatibility, controlled degradation rate, sufficient and interconnected porosity, mechanical integrity, and mechanotransduction [5, 6, 18]. Furthermore, a series of processing techniques such as solvent casting [17], phase inversion [19], fiber bonding [16], melt based technology [20], high pressure based method [21], freeze drying [22], and rapid prototyping technologies [23, 24] are introduced and modified aiming to produce scaffolds with decent properties for bone tissue engineering. However, majority of these processing technologies were inclined to introduce some toxic solvents or ingredients [17, 25-28] causing cell cytotoxicity. Available non-solvent processes are either complex or time-consuming which inhibits the incorporation of thermo-unstable biological elements such as growth factors and other bioactive molecules [18]. Alginate is a polyelectrolyte, which forms a highly versatile, biocompatible, and nontoxic matrices of gel, has been accepted in tissue engineering [29], cell immobilization [30, 31], drug delivery [32] and controlled release [33], as well as in food application [34]. Alginate is able to form the gel-beads with a microstructural feature like egg-box and semi-permeable property [35-37], providing living cells a niche-like environment [38]. Three major methods were reported and utilized for producing gel-beads or encapsulation of cells [37, 39, 40], including: 1) electro-spraying by voltage, 2) air flow using air motion under pressure, 3) droplet methods only by a syringe. In addition, the physical and biological properties of these gel-beads can be optimized for appropriated applications [41], which is superior in repairing bone injuries with a big difference in bone defect area. Bone powder sourced from natural bone contains enriched hydroxyapatite (HA) and BMP factors with promising to serve as a signal factor to promote osteoinduction and osteogenesis [31, 42, 43]. In the present study, a straight-forward, easy-to-use method (droplet method by a syringe) was adopted to form hybrid gel-beads incorporating bone powder, followed by applying to embed cells for fabricating bone tissue *in-vitro*.

Cell culture condition is also known to affect cell growth, migration and infiltration [44, 45]. Under static condition, cells growth inside the scaffolds is slow due to poor mass transfer of nutrients from outside to inside of the scaffold and lack of discharge of metabolites from inside the scaffold [46]. Furthermore, non-uniform distribution of nutrients in the 3D scaffold leads to an uneven distribution of cells and a necrotic zone inside the scaffolds when cells loaded into scaffolds are cultured statically [47, 48]. To solve this problem, bioreactors such as stirred tank bioreactor, RCCS and perfused bioreactor are designed to provide a constant dynamic culture environment to enhance mass transfer of nutrients and metabolites, facilitate cell distribution and reduce dead zone at the center of scaffold [49, 50]. In addition, shear stress generated by fluid flow under dynamic condition creates a comparable *in-vivo* environment, which promotes cell proliferation and differentiation under the mechanical stimulation [51, 52]. Dynamic cultures in spinner flask facilitated multiplication of cells onto porous biopolymer microcarriers and osteoinduction, in which cell-cultured microcarrier implants were successfully fabricated for bone tissue engineering [53]. A study of engineered vessel tissue using collagen /PLGA hybrid scaffolds under a pulsatile perfusion system was reported to enhance cell expansion and facilitate a significant cell alignment in a direction of the scaffold [54].

In the present study, a calcium alginate/ bone powder gel-bead, with favorable structure and osteoinductive property, was prepared by an easy-to-use method. The hybrid gel-beads encapsulating ADSCs were allowed to culture inductively to fabricate tissue engineered bone aggregates in a spinner flask. The application of gel-beads under dynamic culture is expected to repair bone defect diseases through the feature-controlled hybrid gel-beads containing engineered bone aggregates.

2. Materials and methods

2.1 Materials

Human adipose-derived stem cells (ADSCs) were isolated from human subcutaneous adipose tissue (obtained from a women aged 35 and medically obese after the corresponding informed consent was signed) and inductive medium for ADSCs multi-differentiation was pre-prepared, using the established method as described in the past [55]. Sodium alginate and calcium chloride were used to prepare calcium alginate gel-beads, while sodium citrate was adopted for decomposing gel-beads. Hydrochloric acid, hydrogen peroxide and methanol (at analytically grade) were used for tissue processing of natural bones. Essential cell culture supplies were obtained: DMEM (Sigma, USA), FBS (Gibco, USA) and trypsin (Gibco, USA). Calcein AM, toluidine blue, saffron O, oil red O and HE staining kits were purchased from Sigma (USA). ALP quantitative kit was provided (Nanjing Jiancheng Bioengineering Institute, NJBI for short). ALP (NJBI), Alizarin red (Sigma) and Von kossa (NJBI) were obtained and used to investigate the differentiation of osteoblasts and bone aggregates formation in gel-beads.

2.2 Preparation of bone powder

All tissues attached to the bone were removed precisely and the bones were cut into small pieces. Bone pieces were then mixed and soaked repeatedly in tissue processing solution until clarification. After washing for few times, partial protein and all grease were digested from the bones and the treated bones were able to crush into smaller pieces. Finally, these bone pieces were grinded over 3 h, followed by subsequently drying via brinell filter. All bone powder was stock for later use.

2.3 Characterization of bone powder

The surface morphology of prepared bone powder was scanned under scanning electron microscopy (SEM). The size distribution of bone powder was determined by a laser particle size distribution analyzer. The main components were detected through revealing specific functional groups by infrared absorption spectrum.

2.4 Preparation of calcium alginate/bone powder gel-beads

The gel-bead precursor solution was prepared by mixing bone powder (5.0mg/ml) with alginate sodium (2.5% (w/v)). The mixed solution was dripped into calcium chloride (4.5% (w/v)) at speed of 3 mL/min, under stirring condition. After reaction for 10mins, the gel-beads were formed, followed by PBS wash to remove residual Ca^{2+} .

2.5 Morphology and biocompatibility of hybrid gel-beads

The morphologies of gel-beads with or without bone powder, were observed by inverted phase contrast microscope, respectively. According to procedures to prepare gel-beads, ADSCs were embedded into hybrid gel-beads at density of 5×10^6 cells/mL. Cell embedded gel-beads were then transferred to six-well plate for culturing for 1 week. The cell distribution and viability in the hybrid gel-beads were assessed through staining of calcein AM and analysis under fluorescence microscope. Cells embedded by gel-beads without bone powder were used as control.

2.6 Multi- differentiation potential of the ADSCs

ADSCs (5th passage) were cultured for 7 days and stained using calcein AM and HE. ADSCs (6th passage) were seeded into 24-well plate at density of 1×10^5 cells/mL and cultured for 10 hours. Cell culture medium was then replaced with osteoinduction medium. After culturing in osteoinduction medium for 2 weeks, ADSCs differentiated into osteoblasts with cell morphology changes were determined using ALP staining, HE staining and calcein AM staining. Moreover, after 2 weeks induction in chondroinductive medium, the capability of ADSCs differentiation into cartilages were also determined via toluidine blue and safranin O staining. ADSCs were also induced into adipose cells, which were verified by oil red staining.

2.7 Dynamic amplification of encapsulated ADSCs

ADSCs were encapsulated into gel-beads with bone powder at density of 5×10^6 cells/mL and then cultured in spinner flask at 30 rpm (experimental group, dynamic condition) or 6-well plate (control group 1, static condition), respectively. Gel-beads encapsulating cells with no bone powder culturing under static condition were used as control group 2. The cell growth profile was examined for all three groups. 10 days later, ADSCs were collected and harvested by breaking down gel-beads using prepared sodium citrate solution while the cell number was counted via haemocytometer .

2.8 Dynamic fabrication of bone aggregates

After culturing in general medium for a week, the encapsulated ADSCs in three different groups were transferred to osteoinductive medium for inductive culture for 3 weeks. All harvested cells were then stained by ALP (ALP content were also measured quantitatively as scheduled), Alizarin red or Von-Kossa staining kits for investigating whether the bone aggregates were formed. Detail protocols were reported in the previous studies [55, 56].

2.9 Statistical analysis

Data were presented as mean $\pm$ standard deviations. One-way ANOVA was used to evaluate the inter-group comparison using software Origin 8.0 (OriginLab Corporation, USA). $P < 0.05$ was considered as statistical difference.

3 Results and discussion

3.1 Morphology of bio-derived bone powder

From Fig.1A, Bio-derived bone retained a characteristic porous structure as same as natural bone. After being milled and dried, the bone powder presented white, smooth and loosely distributed (Fig.1B&C). Under inverted phase contrast microscope, the microstructure of bone powder was observed to be irregular and shaggy morphology (Fig.1D). This micro-morphology was further scanned through SEM (Fig.1E&F). SEM images showed that the bone powder had irregular sphere, flake, rod-like and unsmooth surface which could be beneficial to cell adhesion and growth.

3.2 Size distribution and analysis of main components of bio-derived bone powder

The particle size distribution of bone powder was measured by laser particle analyzer. Results demonstrated that the particle size of the bone powder were measured and distributed between 0.7 μ m and 95 μ m, of which approximately 70% was in the range of 20~ 50 μ m. 11.6% of bone particles were smaller than 10 μ m and only 1.9% of the particles were below 2 μ m. The average diameter of bone powder was measured at 30.8 μ m with specific surface area of 0.14m²/g. As demonstrated in Fig.2B, strong absorption peaks appeared in 1031cm⁻¹ and 561cm⁻¹, indicating the characteristic peak of stretching vibration and bending vibration of P-O bonds, respectively. Other stretching vibration bands including -OH, CO₃²⁻, N-H, C-H, and -NH₂ occurred at 3425 cm⁻¹, 1456 cm⁻¹, 2926 cm⁻¹, 2852 cm⁻¹, and 1560 cm⁻¹. This result confirmed that the main composition of bone powder is hydroxyapatite and calcium carbonate, which were proved to promote cell recognition, adhesion and osteoinduction [42, 43].

3.3 Hybrid gel-beads morphology and viability of encapsulated cells

The morphology of gel-beads was also observed to be regularly spherical and rather transparent (Fig.3A&B). The particular feature was remained when the gel-beads were incorporated with bone powder. Moreover, bone powders were well distributed through the gel-beads and no aggregation or deposition of bone powder was apparent (Fig.3C&D). In 1 week cell culture, the encapsulated ADSCs were positively stained with calcium AM, showing the live cells are uniformly distributed inside the gel-beads (Fig.3G&H) and the hybrid gel-beads (Fig.3E&F), suggesting the favorable biocompatible and safety of these gel -beads for cell growth.

3.4 Growth and multi-differentiation potential of ADSCs

Post 1 week cell culture, ADSCs remained fusiform and viable (strong green fluorescence in Fig.4B) with growth tendency featuring colony, eddy and radial patterns (Fig.4A&C). ALP, Calcein AM and HE stainings were used to detect osteoinduction 2 weeks after ADSCs culturing in osteoinduced medium. As ALP expression of induced ADSCs were positive detected and intensive staining area was seen in cytoplasm (Fig.4D), this finding confirmed that ADSCs have been induced into osteoblasts. The morphology of induced cells showed variation from threadiness to oval, triangle and fusiformis, and cell nucleus expanded leading to an enhanced stereo sense (Fig.4E&F) compared to non-induced cells. Chondrocyte induced ADSC was positively stained by toluidine blue and saffron O, further determining that the

induced ADSCs expressed enriched ECM of cartilage. The secretion of ECM from induced ADSCs maintained the phenotype of cartilage, as shown in positive metachromatic stained matrix (purple and red areas from Fig.4G&H) in surrounding area of the cells. After adipogenetic induction for 2 weeks, spherical red lipid droplets were observed by Oil red O staining (Fig.4I), indicating the potential of ADSCs differentiating into adipocyte.

3.5 Morphology and amplification of encapsulated ADSCs

The porous structure of hybrid gel-beads provides a three dimensional environment for cell growth. As displayed in Fig.5A&B, the encapsulated ADSCs featuring sphere or spheroidicity, distributed uniformly in the gel-beads. The cell growth of encapsulated ADSCs was significantly accelerated in spinning flask compared to those in two static control groups (Fig.5C). Expansion folds of ADSCs in experimental group were measured at 5.6 folds which was significantly higher compared to control groups which was measured at 2.2 and 2.6 folds, respectively. No difference of cell expansion folds were found between two control groups (Tab.1). These interesting findings indicated that fluid flow in spinner flask was the key factor for enhancing mass transfer of nutrients into gel-beads and generate shear stress stimuli, subsequently promoting the proliferation of ADSCs.

3.6 Morphology of encapsulated ADSCs after osteoinduction

After osteoinduction, white regions were noticed inside the gel-bead and these white regions were gradually expanded over time, suggesting that ADSCs have been induced into osteoblasts and secretion of ECM by osteoblasts appeared (Fig.6). In contrast, the osteoblast differentiation ability of encapsulated ADSCs **under dynamic condition was more obvious** compared to two control groups. This ability was also promoted due to the incorporation of bone powder.

3.7 ALP staining and quantitative determination

ALP was one of most important signals to characterize the differentiation of osteoblasts, and to identify osteoblasts. After osteoinduction for 2 weeks, the encapsulated ADSCs in all three groups are positively stained by ALP kit, showing numbers of cells in gel-beads were stained in lavender (Fig.7A-C). Furthermore, the color intensity of ALP staining was found higher in the experimental group compared to those ADSCs in two control groups. Between two control groups, the ALP expression was lower in control group 1 (hybrid gel-beads, static condition) then to control group 2 (gel-beads with no bone powder, static condition).

At first 2 days of osteoinduction, no ALP expression was tested in all three groups. 3 days later, ALP expression was first detected in experimental group with most colored region, followed by control group 1 and control group 2. However, after osteoinduction for 2 weeks, the content of ALP in three groups was decreased. This observation may be explained by that osteoblast maturation over time inhibited the expression of ALP and started mineralized nodules formation [55]. The difference of ALP expression of induced ADSCs were inferred that shear stress generated by fluid flow stimulate the secretion of osteoblast ECM due to the differentiation of ADSCs into osteoblasts that is promoted by hydroxylapatite in bone powder.

3.8 Alizarin red staining

After 3 weeks osteoinduction, the encapsulated cells were stained by Alizarin Red; positive staining showed irregular red mineralized nodules appeared in the gel-beads of three groups (Fig.8). The number of mineralized nodules was remarkably high in experimental group, but low in control group 2. It was clearly approved that the hybrid gel-beads encapsulating ADSCs osteoinduced in dynamic condition had the strongest calcification capability, attributing to double effects of the stimuli of shear stress and the acceleration of osteoinduction via bone powder.

3.9 Von-Kossa staining

The osteoinduced ADSCs secreted abundant ECM and the gel-beads were colonized by osteoblasts. The ECM and mineralized nodules, which form the bone aggregates were apparent inside the gel-beads. Comparing in three groups, the encapsulated ADSCs by osteoinduction, had been stained black particles (Fig.9A-C), illustrating that large number of ADSCs were successfully differentiated into osteoblasts. To further investigate osteoinduction inside the gel-beads, the gel-beads were cut in the radial direction. It was noticed that black nodules in the internal area of the gel-beads were abundant and the number was high in the experimental group. This result could be explained by two factors: shear stress stimuli by spinner flask and hydroxylapatite in bone powder. Therefore, bone powder containing in the hybrid gel-beads and culture under dynamic environment together formed a niche suitable for ADSCs differentiated into osteoblasts and bone aggregates fabrication.

4 Conclusions

In Summary, the presented study introduced an easy and promising method of the hybrid gel-beads for encapsulating ADSCs. The gel-beads with bone powder provide a better biocompatible environment to mimic the *in-vivo* niche [41] including a 3D porous structure, bone powder containing hydroxylapatite as signal factors. Moreover, a spinner flask with a simple configuration was adopted to provide a dynamic condition as biomechanical stimuli, which further enriched the niche-like environment. To fabricate the bone aggregates, the multipotential ADSCs were encapsulated in the hybrid gel-beads followed by osteoinducing for 3 weeks. The results of osteoinduction were encouraging and we found that shear stress stimuli and bone powder were paramount to promote the differentiation of ADSCs into osteoblasts and the maturation of osteoblasts during bone aggregates fabrication. This feasible approach was believed to fabricate engineered bone aggregates encapsulated by shape-adaptable gel-beads in treating bone loss and defect in the future.

Acknowledgments

This work was supported by the Fok Ying Tung Education Foundation (132027), National Science Foundation of China (31370991/31170945/81271719/81341060), the State Key Laboratory of Fine Chemicals (KF1111) and the Fundamental Research Funds for the Central Universities (DUT14YQ106/DUT15QY47) and SRF for ROCS, SEM.

Conflict of interest

The authors have no conflicts of interest.

References

- [1] R. Dimitriou, E. Jones, D. McGonagle, et al., Bone regeneration: current concepts and future directions, *BMC medicine*. 9 (1) (2011) 66.
- [2] P. Lichte, H. Pape, T. Pufe, et al., Scaffolds for bone healing: concepts, materials and evidence, *Injury*. 42 (6) (2011) 569-573.
- [3] L. Efremov, T. Kanjevac, D. Curic, et al., Perspectives on regeneration of alveolar bone defects, *Serbian Journal of Experimental and Clinical Research*. 14 (4) (2014) 14-153.
- [4] J. Wolff, M. Pyysalo, A.-K. Antalainen, et al., Removal Rates of Dental Implants Placed in Conjunction With Autologous Bone and Xenogeneic and Synthetic Alloplastic Materials in Finland Between 1994 and 2012, *Implant dentistry*. 24 (5) (2015) 552-556.
- [5] A. J. Salgado, O. P. Coutinho and R. L. Reis, Bone tissue engineering: state of the art and future trends, *Macromolecular bioscience*. 4 (8) (2004) 743-765.
- [6] A. R. Amini, C. T. Laurencin and S. P. Nukavarapu, Bone tissue engineering: recent advances and challenges, *Critical Reviews™ in Biomedical Engineering*. 40 (5) (2012)
- [7] K. Rezwani, Q. Chen, J. Blaker, et al., Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering, *Biomaterials*. 27 (18) (2006) 3413-3431.
- [8] S. Bose, M. Roy and A. Bandyopadhyay, Recent advances in bone tissue engineering scaffolds, *Trends in biotechnology*. 30 (10) (2012) 546-554.
- [9] M. N. Collins and C. Birkinshaw, Hyaluronic acid based scaffolds for tissue engineering—A review, *Carbohydrate polymers*. 92 (2) (2013) 1262-1279.
- [10] C. S. Chien, H. O. Ho, Y. C. Liang, et al., Incorporation of exudates of human platelet - rich fibrin gel in biodegradable fibrin scaffolds for tissue engineering of cartilage, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 100 (4) (2012) 948-955.
- [11] A. M. Ferreira, P. Gentile, V. Chiono, et al., Collagen for bone tissue regeneration, *Acta biomaterialia*. 8 (9) (2012) 3191-3200.
- [12] A. R. Costa-Pinto, R. L. Reis and N. M. Neves, Scaffolds based bone tissue engineering: the role of chitosan, *Tissue Engineering Part B: Reviews*. 17 (5) (2011) 331-347.
- [13] S. K. L. Levengood and M. Zhang, Chitosan-based scaffolds for bone tissue engineering, *Journal of Materials Chemistry B*. 2 (21) (2014) 3161-3184.
- [14] B. Engebretson and V. I. Sikavitsas, Long-term in vivo effect of PEG bone tissue engineering scaffolds, *Journal of long-term effects of medical implants*. 22 (3) (2012)
- [15] A. R. Shrivats, M. C. McDermott and J. O. Hollinger, Bone tissue engineering: state of the union, *Drug discovery today*. 19 (6) (2014) 781-786.
- [16] C. Zhou, Q. Shi, W. Guo, et al., Electrospun bio-nanocomposite scaffolds for bone tissue engineering by cellulose nanocrystals reinforcing maleic anhydride grafted PLA, *ACS applied materials & interfaces*. 5 (9) (2013) 3847-3854.
- [17] N. Thadavirul, P. Pavasant and P. Supaphol, Development of polycaprolactone porous scaffolds by combining solvent casting, particulate leaching, and polymer leaching

- techniques for bone tissue engineering, *Journal of Biomedical Materials Research Part A*. 102 (10) (2014) 3379-3392.
- [18] Y. Liu, J. Lim and S.-H. Teoh, Review: development of clinically relevant scaffolds for vascularised bone tissue engineering, *Biotechnology advances*. 31 (5) (2013) 688-705.
 - [19] D. Huang, Y. Zuo, Q. Zou, et al., Reinforced nanohydroxyapatite/polyamide66 scaffolds by chitosan coating for bone tissue engineering, *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 100 (1) (2012) 51-57.
 - [20] N. R. Washburn, C. G. Simon, A. Tona, et al., Co - extrusion of biocompatible polymers for scaffolds with co - continuous morphology, *Journal of biomedical materials research*. 60 (1) (2002) 20-29.
 - [21] L. D. Shea, D. Wang, R. T. Franceschi, et al., Engineered bone development from a pre-osteoblast cell line on three-dimensional scaffolds, *Tissue engineering*. 6 (6) (2000) 605-617.
 - [22] L. Nie, D. Chen, J. Suo, et al., Physicochemical characterization and biocompatibility in vitro of biphasic calcium phosphate/polyvinyl alcohol scaffolds prepared by freeze-drying method for bone tissue engineering applications, *Colloids and Surfaces B: Biointerfaces*. 100 (2012) 169-176.
 - [23] M. T. Arafat, C. X. Lam, A. K. Ekaputra, et al., Biomimetic composite coating on rapid prototyped scaffolds for bone tissue engineering, *Acta Biomaterialia*. 7 (2) (2011) 809-820.
 - [24] T. Billiet, M. Vandenhaute, J. Schelfhout, et al., A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering, *Biomaterials*. 33 (26) (2012) 6020-6041.
 - [25] M. I. Sabir, X. Xu and L. Li, A review on biodegradable polymeric materials for bone tissue engineering applications, *Journal of Materials Science*. 44 (21) (2009) 5713-5724.
 - [26] H. Seyednejad, D. Gawlitta, W. J. Dhert, et al., Preparation and characterization of a three-dimensional printed scaffold based on a functionalized polyester for bone tissue engineering applications, *Acta Biomaterialia*. 7 (5) (2011) 1999-2006.
 - [27] J. Minton, C. Janney, R. Akbarzadeh, et al., Solvent-free polymer/bioceramic scaffolds for bone tissue engineering: fabrication, analysis, and cell growth, *Journal of Biomaterials Science, Polymer Edition*. 25 (16) (2014)
 - [28] J. R. Venugopal, S. Sridhar and S. Ramakrishna, Electrospun plant-derived natural biomaterials for Tissue engineering, *Plant Science Today*. 1 (3) (2014) 151-154.
 - [29] J. L. Drury and D. J. Mooney, Hydrogels for tissue engineering: scaffold design variables and applications, *Biomaterials*. 24 (24) (2003) 4337-4351.
 - [30] O. Smidsrød and G. Skja, Alginate as immobilization matrix for cells, *Trends in biotechnology*. 8 (1990) 71-78.
 - [31] K. Song, X. Yan, S. Li, et al., Preparation and detection of calcium alginate/bone powder hybrid gel-beads for in vitro culture of ADSCs, *Journal of microencapsulation*. 32 (8) (2015) 811-819.

- [32] H. L. Lai, A. Abu'Khalil and D. Q. Craig, The preparation and characterisation of drug-loaded alginate and chitosan sponges, *International journal of pharmaceutics*. 251 (1) (2003) 175-181.
- [33] V. R. Babu, M. Sairam, K. M. Hosamani, et al., Preparation of sodium alginate–methylcellulose blend microspheres for controlled release of nifedipine, *Carbohydrate Polymers*. 69 (2) (2007) 241-250.
- [34] C. Valenzuela, V. Hernández, M. S. Morales, et al., Preparation and characterization of heme iron-alginate beads, *LWT-Food Science and Technology*. 59 (2) (2014) 1283-1289.
- [35] C. Bienaimé, J. N. Barbotin and J. E. Nava - Saucedo, How to build an adapted and bioactive cell microenvironment? A chemical interaction study of the structure of Ca - alginate matrices and their repercussion on confined cells, *Journal of Biomedical Materials Research Part A*. 67 (2) (2003) 376-388.
- [36] A. J. Ribeiro, C. Silva, D. Ferreira, et al., Chitosan-reinforced alginate microspheres obtained through the emulsification/internal gelation technique, *European Journal of Pharmaceutical Sciences*. 25 (1) (2005) 31-40.
- [37] K. Song, Y. Yang, S. Li, et al., In vitro culture and oxygen consumption of NSCs in size-controlled neurospheres of Ca-alginate/gelatin microbead, *Materials Science and Engineering: C*. 40 (2014) 197-203.
- [38] T. M. Chang, Semipermeable microcapsules, *Science*. 146 (3643) (1964) 524-525.
- [39] S. B. Huang, M.-H. Wu and G.-B. Lee, Microfluidic device utilizing pneumatic micro-vibrators to generate alginate gel-beads for microencapsulation of cells, *Sensors and Actuators B: Chemical*. 147 (2) (2010) 755-764.
- [40] E.-S. Chan, T.-K. Lim, P. Ravindra, et al., The effect of low air-to-liquid mass flow rate ratios on the size, size distribution and shape of calcium alginate particles produced using the atomization method, *Journal of Food Engineering*. 108 (2) (2012) 297-303.
- [41] O. Gryshkov, D. Pogozykh, N. Hofmann, et al., Encapsulating Non-Human Primate Multipotent Stromal Cells in Alginate via High Voltage for Cell-Based Therapies and Cryopreservation, (2014)
- [42] R. Gepstein, R. Weiss and T. Hallel, Bridging large defects in bone by demineralized bone matrix in the form of a powder. A radiographic, histological, and radioisotope-uptake study in rats, *The Journal of Bone & Joint Surgery*. 69 (7) (1987) 984-992.
- [43] Masinaei L, Wolfinbarger Jr L. Tissue repair of bone defects; injectable as gel, paste or freeze drying; mixing demineralized bone fragments and a carrier: U.S. Patent 7,498,040. 2009-3-3.
- [44] Y. Liu, S. Bharadwaj, S. J. Lee, et al., Optimization of a natural collagen scaffold to aid cell–matrix penetration for urologic tissue engineering, *Biomaterials*. 30 (23) (2009) 3865-3873.
- [45] N. L. Nerurkar, S. Sen, B. M. Baker, et al., Dynamic culture enhances stem cell infiltration and modulates extracellular matrix production on aligned electrospun nanofibrous scaffolds, *Acta biomaterialia*. 7 (2) (2011) 485-491.

- [46] P. Pisanti, A. B. Yeatts, S. Cardea, et al., Tubular perfusion system culture of human mesenchymal stem cells on poly - L - lactic acid scaffolds produced using a supercritical carbon dioxide - assisted process, *Journal of Biomedical Materials Research Part A*. 100 (10) (2012) 2563-2572.
- [47] A. Teo, A. Mantalaris, K. Song, et al., A novel perfused rotary bioreactor for cardiomyogenesis of embryonic stem cells, *Biotechnology letters*. 36 (5) (2014) 947-960.
- [48] I. Martin, D. Wendt and M. Heberer, The role of bioreactors in tissue engineering, *TRENDS in Biotechnology*. 22 (2) (2004) 80-86.
- [49] J. E. Hambor, Bioreactor design and bioprocess controls for industrialized cell processing, *BioProcess Int*. 10 (6) (2012) 22-33.
- [50] C. H. Mun, Y. Jung, S. H. Kim, et al., Effects of Pulsatile Bioreactor Culture on Vascular Smooth Muscle Cells Seeded on Electrospun Poly (lactide - co - ϵ - caprolactone) Scaffold, *Artificial organs*. 37 (12) (2013) E168-E178.
- [51] C. M. Valmikinathan, J. Hoffman and X. Yu, Impact of scaffold micro and macro architecture on Schwann cell proliferation under dynamic conditions in a rotating wall vessel bioreactor, *Materials Science and Engineering: C*. 31 (1) (2011) 22-29.
- [52] A. B. Yeatts, D. T. Choquette and J. P. Fisher, Bioreactors to influence stem cell fate: augmentation of mesenchymal stem cell signaling pathways via dynamic culture systems, *Biochimica et Biophysica Acta (BBA)-General Subjects*. 1830 (2) (2013) 2470-2480.
- [53] G.-Z. Jin, J.-H. Park, S.-J. Seo, et al., Dynamic cell culture on porous biopolymer microcarriers in a spinner flask for bone tissue engineering: a feasibility study, *Biotechnology letters*. 36 (7) (2014) 1539-1548.
- [54] S. I. Jeong, S. Y. Kim, S. K. Cho, et al., Tissue-engineered vascular grafts composed of marine collagen and PLGA fibers using pulsatile perfusion bioreactors, *Biomaterials*. 28 (6) (2007) 1115-1122.
- [55] Y. Zhu, T. Liu, K. Song, et al., Adipose-derived stem cell: a better stem cell than BMSC, *Cell biochemistry and function*. 26 (6) (2008) 664-675.
- [56] K. Song, L. Li, W. Li, et al., Three-dimensional Dynamic Fabrication of Engineered Cartilage Based on Chitosan/gelatin Hybrid Hydrogel Scaffold in a Spinner Flask with a Special Designed Steel Frame, *Materials Science and Engineering: C*. (2015).

Table 1 Expansion of encapsulated ADSCs in different groups

Group	Density(cells/mL)		Expanding fold
	Initial state	After 10 days	
Experimental	5×10^6	2.8×10^7	5.6
Control I	5×10^6	1.1×10^7	2.2
Control II	5×10^6	1.3×10^7	2.6

Fig.1 In vitro preparation and morphology assay of bio-derived bone powder. Bulk of bio-derived bone scaffold wash of distilled water (A); bone powder of bio-derived bone after ball-milling treatment (B) and drying treatment (C); morphology of bone powder (D) under an inverted microscope in bright field; E-F: morphology examination of bone powder under a SEM; E: $\times 200$, F: $\times 10000$.

Fig.2 Size distribution and infrared spectrum assay of bio-derived bone powder. Size distribution of treated bio-derived bone powder (A); Infrared spectrum assay of treated bio-derived bone powder (B).

Fig.3 External morphology of calcium alginate gel-beads and calcium alginate/bone-powder hybrid gel-beads and cell viability of encapsulated ADSCs within calcium alginate/bone-powder hybrid gel-beads after 7 days of fabrication. External morphology of calcium alginate gel-beads (A&B) and calcium alginate/bone-powder hybrid gel-beads (C&D); Cell morphology and distribution of encapsulated ADSCs in calcium alginate gel-beads in a light field under an inverted microscope after 7 days of fabrication (E&F, $\times 40$); cell viability of encapsulated ADSCs within calcium alginate/bone-powder hybrid gel-beads after 7 days of fabrication (G&H, $\times 40$).

Fig.4 Assays for ADSCs and multi-differentiation potential of ADSCs after two weeks of induction. Cell morphology (A), cell viability assay (B) and H&E staining (C) of adipose-derived stem cells at passage 5 after 7 days of culture; ALP staining (D) and H&E staining (E) for osteogenic differentiation and cell morphology detections after 2 weeks of induction, respectively; cell viability assay (F), Toluidine blue staining (G) and Safranin O staining (H) for chondrogenic differentiation detection of ADSCs after two weeks of induction; Oil red O staining (I) for adipogenic differentiation detection after 2 weeks of induction; A, D and I: $\times 200$; B, C, E-H: $\times 100$.

Fig.5 Cell morphology and distribution of ADSCs within calcium alginate/bone-powder hybrid gel-beads in experimental group after 7 days of microencapsulation and proliferation status of encapsulated ADSCs within gel-beads in different culture groups. Cell morphology and distribution of P5 ADSCs on the 7th d (A, $\times 40$) and (B, $\times 100$), respectively; (C) The proliferation status of encapsulated ADSCs within gel-beads in different culture groups, $**P < 0.01$, N.S.: not significant.

Fig.6 Cell morphology of encapsulated ADSCs after osteoinduction for 3 days, 7 days and 11 days, respectively in different culture groups. Encapsulated cell distribution, morphology and differentiation in control group I (A-C), control group II (D-F) and experimental group (G-I), on 3th d (A, D and G: $\times 100$), 7th d (B, E and H: $\times 100$), 11th d (C, F and I: $\times 40$), respectively.

Fig.7 ALP qualitative staining and quantitative determination of encapsulated ADSCs within calcium alginate/bone-powder hybrid gel-beads after two weeks and 16 days of fabrication, respectively. ALP qualitative staining of encapsulated ADSCs in control group I (A), control group II (B) and experimental group (C), respectively; A-C: $\times 40$; (D) Comparison of quantitative determination of ALP of encapsulated ADSCs in three different groups.

Fig.8 Alizarin Red staining of encapsulated ADSCs within calcium alginate/bone-powder hybrid gel-beads after three weeks of fabrication. The Alizarin Red staining of encapsulated ADSCs in control group I (A), control group II (B) and experimental group (C), respectively; A-C: $\times 40$.

Fig.9 Von-Kossa Staining of encapsulated ADSCs in calcium alginate/bone-powder hybrid gel-beads after three weeks of fabrication. Von-Kossa Staining pictures of ADSCs within hybrid gel-beads in control group I (A), control group II (B) and experimental group (C), respectively. The profiles of von-Kossa Staining of ADSCs within hybrid gel-beads in control group I (D), control group II (E) and experimental group (F), respectively; A-F: $\times 40$.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Fig.7

Fig.8

Fig.9