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Osteogenic behavior of alginate encapsulated bone marrow stromal cells: An in vitro study

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Abstract Sodium alginate is a useful polymer for the encapsulation and immobilization of a variety of cells in tissue engineering because it is biocompatible, biodegradable and easy to process into injectable microbeads. Despite these properties, little is known of the efficacy of calcium cross-linked alginate gel beads as a biodegradable scaffold for osteogenic cell proliferation and differentiation. In this study, we investigated the ability of rabbit derived bone marrow cells (BMCs) to proliferate and differentiate in alginate microbeads and compared them with BMCs cultured in poly-L-lysine (PLL) coated microbeads and on conventional 2D plastic surfaces. Results show that levels of proliferation and differentiation in microbeads and on tissue culture plastics were comparable. Cell proliferation in microbeads however diminished after fortification with a coating layer of PLL. Maximum cell numbers observed were, $3.32 \times 10^5 \pm 1.72 \times 103$; $3.11 \times 10^5 \pm 1.52 \times 10^3$ and $3.28 \times 10^5 \pm 1.21 \times 10^3$ for the uncoated, PLL coated and plastic surface groups respectively. Alkaline phosphatase and protein expressions reflected the stage of cell differentiation. We conclude that calcium cross-linked alginate microbeads can act as a scaffold for BMC proliferation and osteogenic differentiation and has potential for use as 3D degradable scaffold.

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1 Introduction

As an important biotechnology in tissue engineering, cell encapsulation has increasingly attracted interests in recent years. It promotes tissue regeneration by facilitating the localized retention of entrapped cells [1–3], as well as controlling the release of therapeutic agents to the host [4–6]. Microcapsules are important components for prolonging the viability and therefore sustaining the functions of confined cells [6].

The ideal characteristics of microcapsules should be suitable size for ease of injection, biocompatibility with the resident cells and surrounding tissues for ease of integration, and biodegradability to eliminate the necessity of secondary surgery for removal of materials.

Both natural materials such as collagen, fibrin, alginate, chitosan, and synthetic polymers including those based on acrylamides, 2 poly(vinyl alcohol) (PVA), 3 poly(ethylene glycol) (PEG),4–6 and (poly(ethylene glycol) fumarate) (OPF) have been developed for this application [7]. Among all the natural and synthetic materials, polyanion alginate possesses excellent biocompatibility and in vivo stability [8]. The use of sodium alginate as the polyanion and poly-L-lysine (PLL) as the polycation for microcapsule formation has been overwhelmingly explored

as an effective method of immobilizing and immunoprotecting non-autologous cells for transplantation into unmatched recipients without the need for immuno-suppression [9–16]. Therefore, in this study, alginate was selected as scaffold material and the effect of confinement within Ca-alginate microbeads of cells with osteogenic potentials and their subsequent ability to elicit osteogenic response was investigated in vitro. The ability of confined bone marrow cells (BMCs) to remain viable and secrete proteins in both PLL-coated and uncoated alginate were also compared.

2 Materials and methods

Intermediate guluronic (G) sodium alginate obtained from Sigma was purified according to the method proposed by de Vos et al. [13]. Poly-L-lysine-HCl (*M*w: 22,000) was used as received. Dulbecco's Modified Eagle Medium (DMEM) was purchased from GIBCO (NY, USA). Bradford protein assay kit was purchased from BioRad (CA, USA). Fetal calf serum (FCS) was from Biocell Laboratories (CA, USA). Dexamethasone (Dex), ascorbate-2-phosphate, sodium β-glycerophosphate (β-GP) and Alkaline phosphatase Diagnostic Kit-104LS were from Sigma (TX, USA). CellTiter 96® AQueous One assay kit was from Promega Corporation, (WI, USA). Cell-culture wares and disposables were from Corning®.

2.1 Fabrication of alginate microbeads

Alginate microbeads were produced using a semi-automatic droplet generator assembled in our laboratory based on principles described by Wolters et al. [17]. After several pilot studies, the most suitable parameters of the droplet generator were determined and summarized in Table 1.

To prepare microbeads, purified alginate was dissolved at 4°C in Krebs-Ringer-HEPES (1.5% w/v) and the solution was filtered through 0.45 μm filter. The alginate solution was converted into microdroplets with the droplet generator and these droplets were further converted into homogenous microbeads by gelation in a 100 mM $CaCl_2$ (10 mM HEPES, 2 mM KCl) solution for 5 min. Subsequently, the Ca-alginate microbeads formed were washed twice with Phosphate Buffered Solution (PBS). Half of the microbeads were coated with PLL by suspending them in 0.1% PLL solution for 5 min followed by three successive washings with PBS and a final outer layer coating with 0.2% w/v alginate solution to envelope the PLL coating.

Table 1 summarizes the air-droplet generator parameters used in generating the homogenous microbeads

Parameter	Settings
Length of alginate needle shaft	18 mm
Inner diameter of needle	0.311 mm
Outer diameter of needle	0.518 mm
Pressure on alginate in the syringe	25 kPa
Air-flow rate	2.0 L/min

2.2 Characterization of microbeads

The shape and size of microbeads were evaluated with a bright field inverted microscope (Leica DM IL) fitted with a Leica DFC 320 Digital Camera system. To examine the difference between coated and uncoated microbeads, scanning electron microscopy (SEM) was performed after fixing cell free microbeads with 2.5% glutaraldehyde in cacodylate buffer. The beads were processed through critical point drying using liquid carbon dioxide as transitional fluid and coating with carbon for viewing on Leo 1530 Field Emission Scanning Electron Microscope.

To further establish the formation of a PLL coating layer on the coated microbeads, Fourier Transforming-Infrared Spectroscopy (FT-IR) was performed. Cell free sample microbeads were thoroughly washed with double distilled water, lyophilized and grounded. FT-IR spectra were recorded using KBr pellet in a Nicolet Impact 410 Spectrophotometer.

2.3 Isolation of bone marrow cultures

Cultures of BMCs derived from four months old, New Zealand white rabbit were established in vitro as described earlier [18]. Briefly, the procedure involves the aspiration of 5 mL of unfractionated marrow in heparin. Following density gradient centrifugation on 5 mL Ficol, marrow cells were plated in a 75 cm^2 flask containing DMEM, 2 mM L-glutamine, 1 mM sodium bicarbonate, 10% heat inactivated FCS, 50 U/mL penicillin and 50 μg/mL streptomycin (hereafter referred to as the complete culture medium). The cells were then cultured at 37 °C in humidified air (95% air, 5%), CO_2 water-jacketed incubator. After 4 days, spent out medium with suspended cells were carefully removed and attached cells refreshed with fresh complete medium. Cells were trypsinized and passaged each time they reached 85% confluence. BMCs were thus prepared for in vitro study and used at passage three (3).

2.4 BMC encapsulation and culture

BMCs were suspended in the filtered alginate solution at a concentration of 1×10^6 cells/mL. These cells in alginate solution were extruded through a 25 G needle by the semi-automatic microdroplet generator before being converted into microbeads in 100 mM $CaCl_2$ bath. They were further divided into uncoated and PLL coated groups as described earlier. All three experimental groups (uncoated, PLL coated microbeads and 2D monolayer plastic surface groups) were cultured in complete culture medium supplemented with osteogenic stimulating factors consisting of 100 nM Dex, 50 mg/mL ascobate-2-phosphate and 10 mM β-GP.

2.5 Cell viability assay

Evaluation of cell viability was conducted using the MTS colorimetric assay protocol, similar to the one used previously [19, 20]. This tetrazolium-based colorimetric test is based on the metabolic activity of the mitochondrial dehydrogenase of viable cells. In this study, tests were carried out weekly on both coated and uncoated microbead groups for 28 days starting on day 1 and results were compared with 2D plastic surface cultures of similar initial cell seeding density. Six wells of the 96 well plates (each containing 35 ± 5 microbeads or equivalent number of 2D plastic surface cell cultures) were evaluated at each time point. A predetermined linear calibration curve between optic density (OD) and different cell concentrations was used to determined viable cell numbers.

2.6 Alkaline phosphatase (ALP) assay

Samples from both microbead groups (50 microbeads ± 5) were prepared for ALP assay using the method described by Weber et al. [14] after two washings in double distilled water. To release cells from the uncoated group, microbeads were incubated in a 50 mM EDTA-solution (pH 7.0) for 4 min while clean cover slips were used to crush coated microbeads and release cells. Cells from plastic surface cultures were released by washings with cold PBS, two cycles of freezing and thawing and then scraping with a cell scraper.

Released cells were mixed with 20 μL of 0.1% triton X100. ALP activities of cell lysates were assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate using ALP assay kit according to the manufacturer's instructions. 200 μL samples from each group were analyzed by using an ELISA-reader at 405 nm wavelength. ALP activity was quantified after calibration with *p*-nitrophenol and given as ng/mg protein/cell).

2.7 Total protein assay

The ability of confined cells to secret proteins and for these proteins to permeate into culture medium was determined by monitoring total protein concentration in serum free culture medium using a Bradford protein assay kit. Approximately 70 ± 8 microbeads encapsulating 80 ± 10 BMCs per bead were washed three times in PBS to remove any traces of medium remaining on the membrane surfaces. The beads were placed in 24 well culture plates, and 1 mL of serum-free medium was added in order to ensure that any protein detected in the medium was solely from the entrapped cells. Assays were conducted 48 h later by mixing 10 μL of the culture medium from growing cells and 0.20 mL of Bradford reagent in wells of 96 well plates. The absorbance was read at 595 nm after 20 min of incubation. The concentration of total protein was normalized using a calibration between bovine serum albumin (BSA) concentration and absorbance. Results are presented as mg/mL.

2.8 Statistical analysis

Student *t*-test (assuming equal variances) was performed to determine the statistical significance of data obtained from cells in both PLL coated and uncoated alginate microbead groups and compared with 2D plastic surface cell cultures. Data were expressed as the mean ± standard deviation (SD) of at least 6 separate cultures. ($p < 0.05$ were considered significant).

3 Results

3.1 Characterization of microbeads

Figure 1 shows the surface topography and size of the alginate microbeads as revealed by SEM of uncoated (Fig. 1a) and coated (Fig. 1b) microbeads. The diameter of microbeads was 300 μm ± 50 μm. Notice the granular surface of uncoated microbeads with numerous fissures while the coated microbeads had smooth, laminated surface. Unlike uncoated beads, PLL coated beads were unable to withstand critical point drying and collapsed during the processing for SEM (Fig. 1b). This could indicate that the coated microcapsules might be hollow while the uncoated alginate might

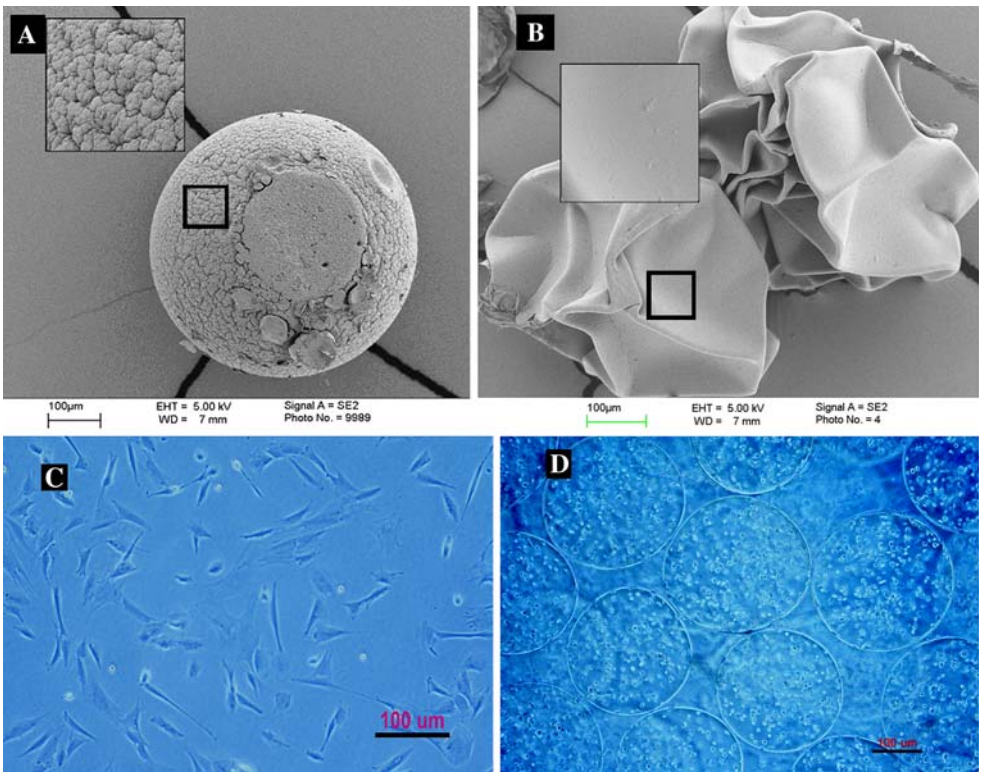

Fig. 1 Shows the morphology and surface topography of microbeads (**a** and **b**) and cells (**c** and **d**). SEM of uncoated microbead (**a**) reveals well-formed beads with rough, granular surface and numerous fissures (inset). SEM of PLL coated microbeads (**b**) reveal collapsed but intact microcapsules with smooth, laminated surface (inset). Bright field microscopy of monolayer BMC culture on 2D plastic surface reveals plastic adherent, fibroblast-like cells (**c**) and BMCs in PLL coated alginate microbeads (**d**) reveals rounded cells. Notice fairly uniformed size microbeads

be homogenously solid core, thus the two types of beads are able to provide different microenvironments for the cells.

Morphology of BMCs monitored with the bright field microscope shows fibroblast-like, plastic adherent, BMCs (Fig. 1c) established on conventional tissue culture plastic surfaces and rounded cells confined in fairly uniformed sized microcapsules in microencapsulation cultures (Fig. 1d). The thickness of the PLL coating layer was observed to be 3 µm ± 1 µm.

Figure 2 shows the FTIR spectra of uncoated and PLL-coated microcapsules. The bands near 1,610 and 1,425 cm^{-1} are assigned to the asymmetric and symmetric stretching vibrations respectively (Fig. 2a). The peak around 1,300 cm^{-1} originated from δ(OH). The characteristic band of either bonds appeared in the range of 1,176 –1,035 cm^{-1}. For PLL-coated microcapsules (Fig. 1b), in addition to characteristic adsorption peaks from alginate, there appeared amide II band near 1,542 cm^{-1}, and asymmetric stretching of CH_2 around 2,940 cm^{-1}, indicating PLL molecules did tightly bind to the alginate microcapsule despite thorough washing.

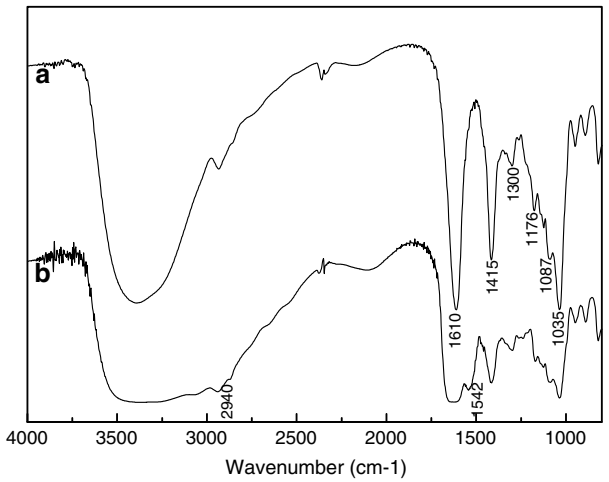

Fig. 2 Showed the FTIR spectra of Ca-alginate and PLL-coated beads. As shown in (**a**), the bands near 1,610 and 1,415 cm^{-1} were assigned to the asymmetric and symmetric stretching vibrations, respectively. The peak around 1,300 cm^{-1} was originated from δ(OH). The characteristic bands of ether bonds appeared in the range of 1,176–1,035 cm^{-1}. For PLL-coated beads (**b**), in addition of characteristic adsorption peaks from alginate, there appeared amide II band near 1,542 cm^{-1}, and asymmetric stretching of CH_2 around 2,940 cm^{-1}, indicating PLL molecules have tightly bound to the alginate though with thorough water washing

3.2 Cell viability and proliferation

To examine the influence of alginate entrapment on cell viability and metabolic activity, confined cells were monitored for a period of 28 days and compared with 2D plastic surface culture. Throughout the culture period, cells remained viable in all groups (Fig. 3). Cells in

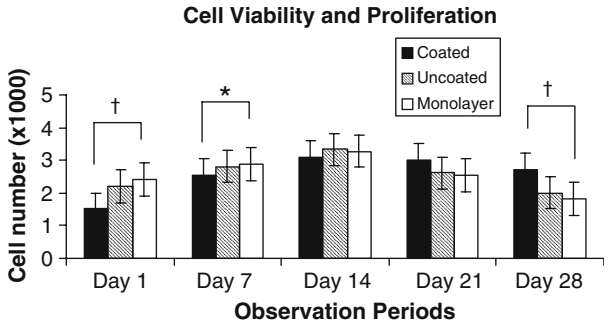

Fig. 3 Viability of BMCs inside microbeads was compared with 2D plastic surface culture for 28 days. There was a significant increase in cell number in all three groups and this therefore revealed cell proliferation and thereby, increased metabolic activity in all groups within the first 14 days of culture. Following this, a significant decrease in cell number is observed for both the 2D plastic surface and uncoated beads group while there was no significant difference in cell number between day 14 and 21 in the coated group. However, on day 28, there was a significantly lower cell number in the coated group when compared with day 21. All groups had higher cell population than day one (*$p = 0.05$; †$p = 0.001$ $n = 8$)

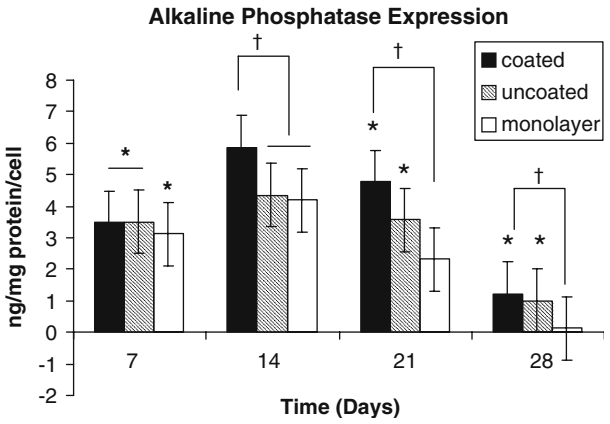

Fig. 4 The mean values of specific alkaline phosphatase (ALP) activity were highest in each of the encapsulated cell groups, than the 2D plastic surface cultures through out the study period. There was no significant difference between both groups of microbeads on day seven but PLL coated microsphere group had significantly higher activity on subsequent measurements. Specific ALP activity was significantly lower in all groups on day 21 and day 28 compared to day 14. ALP was almost unnoticeable on day 28 in the 2D plastic surface group and barely active in both microsphere groups. (*$p = 0.05$ †$p = 0.001$)

microbeads showed a proliferation pattern comparable to 2D plastic surface cultures in the first 14 days. All three groups demonstrated their highest viable cell population on day 14 as $3.11 \times 10^5 \pm 1.52 \times 10^3$; $3.32 \times 10^5 \pm 1.72 \times 10^3$ and $3.28 \times 10^5 \pm 1.21 \times 10^3$ for the coated, uncoated and 2D plastic surface groups respectively. Following this, a significant decrease in cell number is observed in both 2D plastic surface and uncoated alginate groups. However, there was no significant difference in cell population in the PLL coated beads between day 14 and 21 ($p = 0.001$). Compared to day 1, coated beads had significantly higher cell numbers on day 28. On the contrary, both uncoated alginate beads and 2D plastic surface cultures showed significant decrease in cell numbers on day 21 when compared to day 14. Both groups also showed significant decrease in cell numbers on day 28 when compared to day 1.

3.3 ALP differentiation

Specific ALP levels were detectable on day 7 in all groups with 2D plastic surface and uncoated alginate microcapsule groups expressing significantly higher levels than the coated group (Fig. 4). Thereafter, expression in the coated group was consistently higher than both the uncoated and 2D plastic surface groups. Maximum expression was observed in all groups on day 14. Specific ALP expression levels decreased significantly on day 21 in all three groups with 2D plastic surface groups expressing lower levels than the encapsulated cells. There was a further decrease on day 28 when ALP levels declined to less than 25% of their maximum values in the encapsulated groups and were barely detectable in 2D plastic surface cultures.

3.4 Total protein release

The quantity of protein secreted by differentiating cells into surrounding serum free medium was evaluated at various time points. Results are presented in Fig. 5, which represents the protein secreted by cells in 70 ± 7 microbeads or the equivalent number of cells on 2D plastic surface cultures at various time intervals. Expression levels from 2D plastic surface and uncoated microbeads were significantly higher than coated beads on day 7 and 14. Between day 21 and 28, maximum protein levels were observed in the confined cells as 10.5 ± 0.17 mg/mL and 11.3 ± 0.08 mg/mL for coated and uncoated microbeads respectively. There appeared to be a surging release from accumulated intra-capsular protein with time as this upsurge was absent in the 2D plastic surface cultures. Between the two types of microbeads, the PLL coated beads persistently expressed significantly lower levels of protein. Minimal proteins were detected in the culture medium of monolayer cultures at this stage. This could be explained by the level of cell viability and the fact that no protein accumulation occurred as they were possibly washed away by the repeated PBS wash.

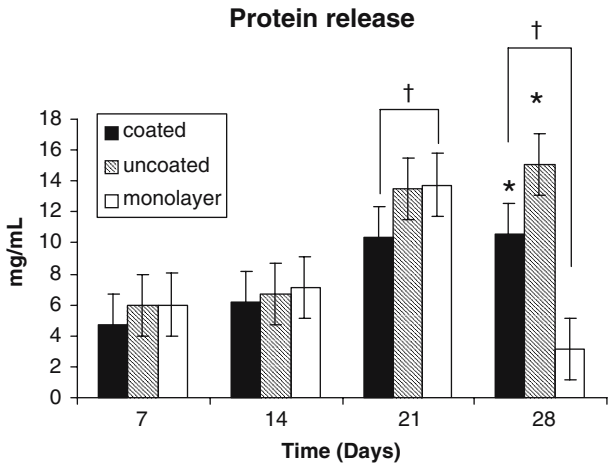

Fig. 5 Total protein in serum free medium was significantly higher in the 2D plastic surface and uncoated beads culture groups than in PLL coated alginate microbeads on day 7. There were no significant differences between the uncoated beads and 2D plastic surface groups on days 7 and 21. However, there was a significantly higher secretion between the alginate microbeads and 2D plastic surface groups on day 28. ($^{*}p = 0.05$ $^{\dagger}p = 0.001$)

4 Discussion

We observed that although, cell numbers in the alginate trapped groups were lower on day 1, possibly due to cell death during encapsulation [17], cells in the uncoated alginate group subsequently attained viable populations comparable to those on 2D plastic surface cultures signifying that the rate of cell proliferation in the uncoated bead were higher than those on 2D plastic surface cultures or in other words, a catch up growth could have taken place. This might be due to a more suitable microenvironment on 3D cultures compared to 2D. Similar phenomenon has been noted in long-term chondrocyte culture [21]. This is an interesting finding because BMC cultures normally require regular passaging to maintain prolong growth on 2D surfaces and this may explain the fall in cell numbers on plastic culture surface after 14 days without trypsinization. However cells in the 3D cultures, even though had falling in number after day 14, exhibited higher numbers than the 2D culture surface.

Comparing coated with uncoated alginate microencapsulation cell cultures, we observed that cells within uncoated beads maintain rapid cell proliferation, similar to 2D plastic surface cultures while coating with PLL inhibited the proliferation of these cells (Fig. 3). This suggests that even though PLL coating has been reported to stabilize the beads mechanically [22], a 3 μm thick PLL coating may have inhibitory effect on the rate of cell proliferation and the corresponding population of differentiated cells. Proliferation of mesenchymal stem cells has been earlier reported to exhibit varying rates on different 3D porous scaffolds [23]. Barralet et al. [24] had earlier reported that both the thickness and geometery of alginate gel influenced the rate of proliferation of entrapped BMCs but that the geometry had no influence on the differentiation. In this study, we report that with microbeads of same geometry and fairly uniform size, a decrease in rate of rabbit BMC proliferation was seen after coating the microbeads with PLL and dilute alginate. This could be as a result of limited nutrient diffusion and waste removal at the PLL-alginate membrane boundary.

Expression of ALP, an early osteoblastic marker, reflected both the population of viable cells and the stage of cell differentiation for the different time points. It was interesting to note that even though the population of PLL coated microencapsulation cultures where lower than uncoated and 2D plastic surface cultures, ALP expressions were higher from day 14. This could be explained by the population of cells at different stages of differentiation in the three different groups indicating that coated microbeads may have a higher number of newly differentiated osteoblasts when compared to the other two groups.

Based on the quantity of protein permeating into surrounding culture medium, uncoated (rather than PLL coated) alginate beads may provide a more suitable vehicle for the delivery of osteogenic factors in situations where immuno-isolation is not very necessary. This is due to many factors: uncoated beads offer lower resistance to permeating proteins through fissures on the surface of the beads. Cells in this group could also exhibit better metabolic activity because diffusion might proceed without hindrance. However, it must be stated that coated beads, even though did not release proteins to the level demonstrable with uncoated beads at any time point in this study, they showed a more stable number of viable cells with longer observation period. This may have advantage when the required quantity of protein is low and protein release period required is long.

5 Conclusions

This study shows, for the first time that purified alginate gel beads with intermediate guluronic acid content were able to support the proliferation of rabbit marrow cells and their differentiation along the osteoblastic lineage. We have demonstrated that calcium cross-linked alginate microbead is superior for rabbit marrow cell proliferation and osteogenic differentiation

in vitro when compared to 2D monolayer cultures. PLL coated beads ensured a more stable proliferation on a longer term and thus, release of proteins from confined cells could be prolonged. Based on the use of sodium alginates as cell encapsulation materials, this work demonstrates that it has potential to act as a biodegradable, cell immobilizing and biocompatible scaffold in bone tissue engineering.

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