Alginate nanobeads interspersed fibrin network as in situ forming hydrogel for soft tissue engineering

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ABSTRACT

Hydrogels are a class of materials that has the property of injectability and in situ gel formation. This property of hydrogels is manipulated in this study to develop a biomimetic bioresorbable injectable system of alginate nanobeads interspersed in fibrin network. Alginate nanobeads developed by calcium cross-linking yielded a size of 200–500 nm. The alginate nanobeads fibrin hydrogel was formed using dual syringe apparatus. Characterization of the in situ injectable hydrogel was done by SEM, FTIR and Rheometer. The developed hydrogel showed mechanical strength of 19 kPa which provides the suitable compliance for soft tissue engineering. Cytocompatibility studies using human umbilical cord blood derived mesenchymal stem cells showed good attachment, proliferation and in situ delivery of cells to the defect area. Injectable in situ forming hydrogel could be a suitable delivery carrier for stem cells for soft tissue regeneration.

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1. Introduction

Biomaterials derived from natural sources are advantageous because of their inherent property of being recognized by the cells, which includes the presence of cell binding receptors, induction of growth factor and its binding sites, proteolytic and remodeling properties triggered by cell binding etc [1,2]. On the other hand, most synthetic polymers have the advantage of being inert, high mechanical integrity and can be easily processed unlike natural polymers. Optimizing the properties of natural polymers to provide the required mechanical strength and degradation rate to match up with the regeneration rate of tissues would be beneficial.

Injectable hydrogel provide a suitable platform for the delivery of cells, drugs, proteins etc to the site via a minimally invasive technique in addition to providing a cross-linked swollen network of biopolymer with soft compliance, mimicking soft tissues. Hydrogels also possess the advantage of having a highly permeable structure aiding in the efficient exchange of nutrients and oxygen [3]. Injectable in situ forming hydrogels acts as a suitable depot for the effective delivery of cells to the defect area. Soft tissue reconstruction is often a priority when it comes to damage or removal of soft tissue. Injectable in situ hydrogels is minimally invasive, can completely fill the defect site irrespective of its irregularity and is patient compliant that it overcomes the discomfort occurred during surgical procedures. The use of synthetic materials to augment soft tissue regeneration presents some limitations for host tissue integration [4] and thereby projects the need for blending or chemical modifications of the same. Natural polymers being biocompatible and with mechanical strength matching the tissue elasticity would be a suitable alternative. Fibrinogen, the monomeric of fibrin, is composed of two sets of three polypeptide chains named Aα, Bβ, and γ, which are joined together by six disulfide bridges [5]. Fibrin is formed after thrombin-mediated cleavage of fibrinopeptide A from the Aα chains and fibrinopeptide B from the Bβ chains. This generates the fibrin monomer that has a great tendency to self-associate and form insoluble fibrin [6]. Fibrin has been widely used in clinics as sealants. A number of allogeneic fibrin sealants such as Tisseel®, Evicel™, and Crosseal™ have been approved by the Food and Drug Administration (FDA) for clinical use as hemostatic agents [7]. Unlike a synthetic hydrogel, fibrin is not just a passive cell delivery matrix, but it binds specifically many growth factors as well as clot components, such as fibronectin, hyaluronic acid and von Willebrand factor [8]. Fibrin has two pairs of RGD sites and a pair of AGDV sites through which it can interact with integrins and has several sites that interact with...
the leucocyte integrin αmβ2 [9]. This bioactivity makes fibrin an attractive matrix for stem cell differentiation and tissue engineering. The softness and large compliance of fibrin gel make it effective for soft tissues. By modulating the mechanical and chemical properties of a fibrin-based matrix stem cell differentiation and tissue regeneration can be effectively carried out. In order to improve the low mechanical stiffness for some tissue engineering applications, fibrin hydrogel can be combined with other scaffold materials to obtain constructs with desired mechanical strength [7,10].

Alginate is a widely studied polysaccharide because of its structural resemblance to the extracellular matrix glycosaminoglycans [11]. It is a natural polymer extracted from brown algae. Water soluble alginate gelates when reacted with di- or tri-valent counter ions. Alginate gels are extensively studied for tissue engineering applications as a cell encapsulation material as well as an injectable 3D matrix for in vivo cell delivery. Several reports demonstrated that calcium alginate gel exhibits poor biodegradability, biodegradation and cell adhesion except its easy preparation. As a biomaterial alginate is used because of its biocompatibility and hydrophilic nature and also due to its easy injectability [12,13]. Alginate cannot be broken down enzymatically and thus has a regulated degradation. Concerns have also been expressed on its immunogenicity and low cell adhesiveness [3]. Hwang et al. developed alginate particle embedded fibrin injectable hydrogel which showed an improved in growth of soft tissue in vivo [4]. They had developed alginate particles of higher weight percentage yielding non homogeneous micron sized particles [4]. Although the developed hydrogel showed enhanced neo tissue formation in vivo the higher percentage of alginate would pose issues of degradation. Microgels developed using alginate and fibrin also showed enhanced cell retention and viability and showed dramatic increase in retention in vivo [14].

The purpose of our study was to develop an injectable in situ forming alginate fibrin hydrogel, which could mimic the native tissue elasticity as well as feature enhanced cell material interaction with the minimum use of components. So in this work we developed nanobeads of alginate with the aim of reducing the total alginate content in the construct without compromising the material integrity. In addition the formation of nanobeads would open up other prospect like growth factor or cell delivery. These nanobeads would be incorporated into fibrin in the form of an in situ developing hydrogel. The higher surface area to volume ratio for nanobeads would help it to interact with fibrin matrix. The developed alginate nanobeads interspersed fibrin hydrogel was characterized using SEM, FTIR, rheological analysis and its efficiency to depot cells was evaluated in vitro by live dead assay, DNA quantification and fluorescence imaging.

2. Materials and methods

2.1. Materials

Algicin acid-Sodium salt was purchased from Aldrich Chemicals, India. Calcium Chloride Dihydrate Extra Pure and Thrombin (bovine plasma) were purchased from Merck, India. Primary antibodies used were purchased from Millipore, India and stored under –20 °C. Secondary antibody and actin specific stain were purchased from Molecular Probes, India. Fibrinogen required was isolated from human blood plasma pooled from healthy volunteers after their consent by cryoprecipitation method [15]. Duplojet Applicator was purchased from BD Sciences, India.

2.2. Preparation of alginate nanobeads

Alginate nanobeads were prepared by spraying 1% w/v alginate solution into a bath containing 1 M CaCl$_2$ using a custom made atomiser. The nanobeads were then filtered out. The size range of the particles was obtained by Scanning Electron Microscopy (JEOLJSM-6490LA).

Fig. 1. Schematic diagram showing the method of gel synthesis.

Fig. 2. SEM images of alginate nanobeads (A, B); alginate nanobeads fibrin composite (C, D); fibrin (E, F).
2.3. Preparation of alginate nanobeads-fibrin composite hydrogel

The filtered alginate nanobeads suspended in distilled water/media were used for gel synthesis. 9:1 ratio of alginate nanobeads and fibrinogen (100 mg/ml) and 9:1 ratio of alginate nanobeads and thrombin (100U/ml) were respectively loaded into the two compartments of a double syringe apparatus (Duplojet Applicator) and injected to produce alginate nanobeads fibrin composite hydrogel as shown (Fig. 1). This method yields a final fibrin concentration of 10 mg/ml in the gel.

2.4. Analytical characterization

The spatial structure of the developed scaffold was studied using a Scanning Electron Microscope (JEOLJSM-6490LA). The in situ formed hydrogel was lyophilized and gold sputter coated (JEOL, JFC-1600) prior to imaging. The FTIR spectrum of the composite scaffold was analyzed using Perkin Elmer RX1 and compared with the respective controls.

2.5. Rheological studies

The mechanical behaviour of the synthesized hydrogels was determined using an oscillating rheometer equipped with peltier system for temperature control (Bohlin CVO Rheometer, Malvern Instruments, UK). Both temperature varying and isothermal mode were carried out accordingly. The contribution of elastic modulus ($G'$) being the solid like behaviour and viscous modulus ($G''$) being the liquid like behaviour were recorded with changing temperature using a parallel plate (20 mm). The frequency was set at 1 Hz. A constant strain of 0.05 was applied for the measurement in constant strain parallel plate oscillating mode. The crossover point of $G'$ and $G''$ was considered as the gelation point.

2.6. Biodegradation and swelling studies

In vitro biodegradation was studied by immersing a predetermined weight ($W_0$) of the scaffold in PBS containing lysozyme ($10^4$ units/ml) prepared at neutral pH, incubating at 37 °C for different time points and observing their respective dry weights ($W_t$). The results were compared with the analysis of controls viz alginate nanobeads and fibrin, carried out in the same way. The percentage degradation was calculated using equation (A1).

\[
\text{Percentage degradation} = \left[\frac{(W_0 - W_t)}{W_0}\right] \times 100
\] (A1)

The water uptake analysis was done at neutral pH and 37 °C. The gel formed of weight $W_0$ was injected into a tube containing PBS and equilibrated. The swollen gel was weighed ($W_1$) after wiping the excess PBS using a tissue paper. The water uptake was calculated as swelling ratio using equation (A2).

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\text{Swelling ratio} = \left[\frac{(W_1 - W_0)}{W_0}\right] \times 100
\] (A2)
Swelling ratio = \frac{(W_1 - W_0)}{W_0} \quad (A2)

2.7. PTAH staining

Phosphotungstic acid hematoxylin stain (PTAH) is a mix of phosphotungstic acid and hematoxylin stain used in histology for staining specific tissue proteins. To confirm the fibrin formation within the gel a fibrin specific PTAH staining were carried out. A thin section of gel in glass slide was incubated with PTAH solution overnight in room temperature. Dehydrate with 95% ethanol and absolute alcohol. This was then mounted with mounting media and viewed in bright field microscope.

2.8. Mesenchymal stem cell isolation and culture

Human umbilical cord blood was harvested during delivery after necessary consent from the institutional body of Amrita Institute of Medical Science along with the written consent from the individuals. It was immediately used for the isolation of mesenchymal stem cells by method reported earlier [16]. The cells thus obtained has a spindle morphology and they were given media change every alternate days until confluence. All cell studies were carried out with cells in the third to fifth passages.

2.9. Cell viability

Live dead assay (Molecular Probes, India) was done on the gels for determining the number of cells live and dead within the scaffold after a period of 48 h. Gels were washed with PBS and stained with Calcein-AM and Ethidium homodimer with a concentration of 2 μM and 4 μM respectively and incubated in 37 °C for 1 h. This was then washed and viewed under fluorescent microscope.

2.10. Cell proliferation

500 μl of gel along with cells at a seeding density of 15000 were injected onto coverslips placed in 24 well plates. Gel preparations were carried out with growth media to facilitate cell survival. Plates were then kept in CO₂ incubator. Samples were stained for actin filaments and nucleus after 6 h and 48 h of incubation. Firstly after discarding the media, samples were fixed with 4% PFA for 20 min. Washed with PBS and permeabilised with 0.05% Triton X. Further, phalloidin conjugated with Texas red was added with a dilution of 1: 100 and incubated for 2 h at ambient conditions. The stained samples were visualized using confocal microscope. The level of cell infiltration was also analyzed using confocal microscopy after 48 h. Further Picogreen assay (Molecular Probes, Eugene, OR) to quantify DNA of cells retained in the gel upto 14 days was also done. After predetermined incubation period the gel containing cell seeded at density of 15000 cells/well were retrieved and 3 times cycle of freeze, thaw and sonication was done under buffered condition. The sample was then incubated with picogreen reagent for 10 min and the fluorescence intensity was measured using with excitation at 490 nm and emission at 520 nm.

2.11. Statistical analysis

Results are expressed as mean ± SD of n ≥ 3. Statistical analysis was done using Student’s t-test. Values with p < 0.05 are considered significant. The error bars denote ±SD.
3. Results and discussion

3.1. Preparation and characterization of alginate nanobeads-fibrin composite hydrogel

Polydispersed distribution of alginate nanobeads with size ranging from 200 to 500 nm and spherical morphology was obtained by spraying method (Fig. 2A, B). The nanobeads were held together by fibrin to form a highly cross-linked network in the gel. The mechanical instability of fibrin gels were overcome by this, modulus of which showed a remarkable increase when compared to fibrin gel. It showed gel formation as soon as injected. Gel was found to be stable within the temperature range of 37–45 °C.

Alginate nanobeads showed a complex modulus of 17.9 kPa and were obtained by taking measurements isothermally at 37 °C since this system is not temperature responsive. When conjugated with fibrin the complex modulus of the system increased to 19.1 kPa (Fig. 3). This increase in modulus indicated that fibrin actually acted as a space filling gel system. Native soft tissue elastic modulus ranges between 10 kPa and 100 MPa (covers all types of soft tissue) [17–19]. Scaffolds available clinically for cartilage regeneration like Puramatrix have Youngs modulus of 16.7 ± 1.0 kPa [19]. Thus the developed hydrogel showed equivalent elasticity as native soft tissue and was found to be compatible with that of clinically available matrix and comparatively lower than that of whole alginate gel (36.3 ± 5.4 kPa) [19], thereby providing a suitable niche for the encapsulated cells. As fibrin formation occurs quickly no significant time was required for gelation making it more compliant [6,7].

The SEM image represents the uniform distribution of the nanobeads within the fibrin matrix forming a macro-porous structure (Fig. 2C and D). Fibrin by itself forms a fibrous structure with interconnected pores (Fig. 2E and F). The homogeneous distribution of alginate nanobeads would further enhance the mechanical compliance of the hydrogel.

FTIR analysis was carried out to determine the interactions between the polymer matrix and bioactive components. Alginate beads shows characteristic peaks at 1638, 3397, 2854 and 2940 and 1036 cm⁻¹ indicating the stretching vibration of the carboxylate group, stretching vibrations of O-H bonds of alginate, stretching vibrations of aliphatic –C–H and –C–O stretching vibration of pyranosyl ring and the amide peaks of fibrin was observed at 1641, 1420, 1332 and 1255 cm⁻¹ (Fig. 4A). Alginate nanobeads fibrin (Fig. 4A(c)) shows a slight shift in the peaks at 1645 cm⁻¹ which indicates amide’s –C=O group stretching. A broadening in the 3452 cm⁻¹ peak of the composite indicates an interaction at the –OH group. There is also a slight shift in the peak at 1084 cm⁻¹ indicating the presence of C-N stretch confirming the presence of a secondary amine or an amide group. These peaks are absent in alginate indicating that fibrin has interacted with alginate to form a cross-linked network.

Alginate nanobeads showed very low rate of degradation and at the end of study 30% of sample was found to be intact (Fig. 4C). Addition of fibrin does improve the degradability of this system. Remnant sample by the end of study was reduced to 17%. Fibrin...
degrades almost completely within four weeks. The fibrin gel showed no remnants after four weeks. Moreover the addition of fibrin helped in holding water thereby showing a slight increase in swelling ratio (Fig. 4B). The swollen nature of the in situ forming gel would satisfy the requirement for a continuous supply of nutrients and gas for cells encapsulated within.

The formation of fibrin fibers was confirmed from the PTAH staining which stains proteins blue. A uniform distribution of fibrin fibers was evident from Fig. 5 which confirms that the presence of alginate nanobeads does not hinder the formation of fibrin. The interspersed alginate nanobeads provide the reinforcements for the entire gel matrix.

3.2. Cell viability and proliferation

Live dead assay was performed to analyze the amount of cells alive and dead in within the gel. Fluorescent images were taken for cells stained green with Calcein-AM indicating live cells and stained red with Ethidium homodimer for dead cells. Alginate beads alone provide very less cell retention but with the conjugation of fibrin, cells were comparatively alive (Fig. 6A). This indicates that alginate nanobeads fibrin could support cell attachment and showed low cytotoxicity. Number of live cells was greater for fibrin confirming that fibrin is not toxic to cell. Being a protein of high relevance in cell adhesion, fibrin represents an attractive substrate for cell growth due to the abundant cell attachment and growth factor binding sites [8,9].

DNA quantification of the cells seeded in the alginate nanobeads system is shown in Fig. 6B. The composite hydrogel showed an increase in DNA content compared to the controls within 1 day. DNA content kept increasing significantly which explains the proliferation of cells in the composite gel. At day 14 there was a significant enhancement in DNA content for composite gel compared to control. The composite hydrogel system showed no difference over fibrin gel in 7 days an increase was observed in 14 days which might probably be due to the low stability of the fibrin gel that would in effect fail in holding cells. This confirms that the alginate nanobeads hydrogel system could be a possible matrix for cell loading which could enhance the proliferation and spreading of the loaded cells.

3.3. Cell attachment and infiltration

Cell proliferation was analyzed from the confocal microscopic images taken for cells after predetermined time points. Initial attachment was noted by taking fluorescent images of cells stained for actin for 6 h. In the case of alginate nanobeads alone cells attached and has started spreading at 6 h (Fig. 7a). Proliferation was seen in 48 h and cells were seen to spread although not to a great extent indicating the hindrance in growth due to the presence of
beads (Fig. 7d). In alginate nanobeads fibrin composite initial attachment was high and cells showed well spread morphology. Significant proliferation was seen in 48 h and cells were showing extended morphology indicating that the cells could spread well due to the presence of fibrin which implicates the biocompatibility of the sample (Fig. 7b,e). For fibrin gels good initial attachment and cell morphology were seen. Cells showed significant proliferation in 48 h. Cells were seen to be more elongated in the fibrin gel (Fig. 7c,f). A homogeneous distribution of cells were seen in the gels within 48 h and a good three dimensional cell gel construct was formed in both alginate nanobeads fibrin and fibrin (Fig. 7g,h).

4. Conclusion

An in situ forming injectable hydrogel was developed involving simple nature inspired cross-linking chemistry. Alginate nanobeads fibrin had mechanical strength suitable for tissue engineering constructs for the regeneration of soft tissues. The in situ gel forming nature further improves the patient compliance. The biocompatible nature of the gel was demonstrated using hMSC viability and adhesion studies and the developed in situ gel showed good cell retention. The soft compliance of the gel would help in growth and proliferation of the encapsulated cells. The mechanical strength of the hydrogel is in par with soft tissue elasticity thereby forming a suitable candidate for soft tissue reconstruction. In addition the system would be even more beneficial if growth factor incorporation into the nanobeads would be done as it would provide an additional supplementation for the regeneration of soft tissue.

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