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Fabrication of injectable high strength hydrogel based on 4-arm star PEG for cartilage tissue engineering

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ABSTRACT

Hydrogels prepared from poly(ethylene glycol) (PEG) are widely applied in tissue engineering, especially those derived from a combination of functional multi-arm star PEG and linear crosslinker, with an expectation to form a structurally ideal network. However, the poor mechanical strength still renders their further applications. Here we examined the relationship between the dynamics of the pre-gel solution and the mechanical property of the resultant hydrogel in a system consisting of 4-arm star PEG functionalized with vinyl sulfone and short dithiol crosslinker. A method to prepare mechanically strong hydrogel for cartilage tissue engineering is proposed. It is found that when gelation takes place at the overlap concentration, at which a slow relaxation mode just appears in dynamic light scattering (DLS), the resultant hydrogel has a local maximum compressive strength ~20 MPa, while still keeps ultralow mass concentration and Young's modulus. Chondrocyte-laden hydrogel constructed under this condition was transplanted into the subcutaneous pocket and an osteochondral defect model in SCID mice. The *in vivo* results show that chondrocytes can proliferate and maintain their phenotypes in the hydrogel, with the production of abundant extracellular matrix (ECM) components, formation of typical chondrocyte lacunae structure and increase in Young's modulus over 12 weeks, as indicated by histological, immunohistochemistry, gene expression analyses and mechanical test. Moreover, newly formed hyaline cartilage was observed to be integrated with the host articular cartilage tissue in the defects injected with chondrocytes/hydrogel constructs. The results suggest that this hydrogel is a promising candidate scaffold for cartilage tissue engineering.

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1. Introduction

Articular cartilage has very limited self-repair ability due to the absence of innervation and vascularization, as well as the intense extracellular matrix (ECM) which impedes cell migration [1,2]. Trauma caused articular cartilage defect is one of the major difficult clinical situations in sports medicine. Incomplete repair of the defect is associated with formation of fibrocartilage and subsequent degeneration of the adjacent hyaline cartilage. It is important to repair cartilage defect in its initial stage before it further progresses, which would lead to osteoarthritis. Current therapies for articular cartilage repair include micro-fracture of subchondral bone [3], autologous chondrocyte transplantation [4], osteoarticular transfer system [5] and local delivery of hyaluronic acid or glucocorticoid [6]. However, the clinical efficacy of the above therapies remains unsatisfied. Tissue engineering approach emerges as a promising therapy for functional cartilage repair or regeneration [7]. Particularly, the matrix-assisted autologous chondrocyte transplantation (MACT) has achieved great development during the last decade [8,9]. This technique involves the encapsulation of autologous

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chondrocytes into a carefully designed scaffold, in which chondrocytes can proliferate and secret ECM. The whole cell/scaffold construct is then transplanted into the cartilage focal lesion. Various types of materials, both derived from modified natural products and synthetic materials have been examined as the potential scaffold matrix [10–12].

Among the numerous materials, synthetic hydrogel has been one of the most promising candidates due to its high water content, good mass transportation property, soft tissue like elasticity and biocompatibility [13]. It can be well tuned via various chemistries to satisfy the clinical needs. For instance, by using bio-orthogonal chemistry, the injectable hydrogel allows the minimally invasive surgery possible [14–16]. To synchronize the degradation rate of the scaffold with the matrix deposition rate by the encapsulated cells, matrix metalloproteinase (MMP) peptides have been adopted as the crosslinker [2,17–19]. Among the synthetic materials, poly(ethylene glycol) (PEG) has been the most widely used building block for hydrogel. Basically two gelation mechanisms have been employed. The first one involves the chain polymerization of PEG dimethylacrylate [20–23]. Due to the fast kinetics and random crosslinking process, the resulting hydrogel has an irregular structure and a weak mechanical strength. The alternative method uses the step growth polymerization, where functional multiple-arm star PEG reacts with crosslinkers bearing at least two reactive sites, typically using functional 4-arm star PEG and difunctional linear crosslinkers, i.e., 4 + 2 system [17–19,24–30]. This method is expected to result in a much more ideal hydrogel structure comparing to the chain polymerization counterpart [31]. Moreover, it allows the modularization design of functional hydrogel. The incorporation of important biological signals or cues [31,32] and introduction of controlled degradation profile [2,33,34] have been realized by simply varying the functionality of the linear crosslinkers.

However, one major problem has rarely been clarified despite the wide usage of this step growth strategy, i.e., how to achieve the ideal network structure? The hydrogel with ideal network structure is expected to have a high mechanical strength due to the elimination of the structural defects, which would overcome the longstanding major drawback of hydrogel, *viz*., the poor mechanical properties [7,35]. Hydrogel with high strength is more suitable for earlier implantation after the cell/hydrogel hybrid construction, which will help shorten the *in vitro* culture period, and provides sufficient load-bearing capacity for mechanical loading *in vivo* before the secretion of ECM [36]. It will survive from the harsh environment in the diarthrodial joints and be a promising candidate for the MACT. Moreover, the optimization of the hydrogel formulation would also decrease the material dosage and minimize the introduction of exogenous materials.

For the current 4 + 2 system, it is necessary to prepare the hydrogel at a suitable concentration. When the concentration of star polymer is too low, the difunctional crosslinker cannot link them efficiently; when the concentration is too high, there will be penetration and entanglement between the arm chains. The most suitable concentration should be around overlap concentration (C^*), where polymer chains start to touch each other [37]. In this study, we started from an investigation on the dynamics of star PEG in phosphate-buffered saline (PBS) solution at different concentrations with dynamic light scattering (DLS) to find the optimal concentration, given that there would be dynamics change around C^*. After that, we prepared a mechanically strong hydrogel for the cartilage tissue engineering using the 4 + 2 system based on the DLS result. Murine chondrocytes were encapsulated in the hydrogel prepared at this concentration and the resultant chondrocyte/hydrogel constructs were transplanted into severe combined immunodeficiency (SCID) mice up to 12 weeks to allow the engineered cartilage tissue to develop. The cell morphology, ECM synthesis, expression of chondrogenic marker proteins and mechanical properties of the newly formed cartilage tissue derived from the chondrocytes/hydrogel constructs were examined. At last, an osteochondral defect model in SCID mice was performed and the capacity of cartilage defect repair was tested.

2. Materials and methods

2.1. Materials

Ethylene oxide was purchased from Hong Kong Specialty Gases, HK. Potassium, naphthalene, diphenyl methane, pentaerythritol, anhydrous DMSO, divinyl sulfone, sodium hydride, acetic acid and 2, 2'-(ethylenedioxy) diethanethiol were purchased from Sigma-Aldrich, US. 4-arm star PEG (M_n = 1.6 × 10^4 g/mol, PDI = 1.03) was synthesized according to Wang et al. by high vacuum living anionic polymerization of ethylene oxide in a home-designed glassware using pentaerythritol as the initiator and diphenyl methyl potassium as the deprotonating agent [38].

2.2. Synthesis of star PEG vinyl sulfone (sPEG-VS)

*s*PEG-VS was synthesized by coupling *s*PEG-OH with an excess of divinyl sulfone following the procedure by Lutolf et al. [39]. *s*PEG-OH was dried by azeotropic distillation in toluene using a Dean Stark trap before starting the reaction. To the *s*PEG-OH dissolved in anhydrous dichloromethane, NaH was added under nitrogen at 5-fold molar excess over OH group. After hydrogen evolution, the suspension was added dropwisely to a solution of divinyl sulfone in anhydrous dichloromethane via syringe. The reaction was carried out at room temperature for 3 days in dark under nitrogen atmosphere. After the reaction solution being neutralized with acetic acid, filtered through celite and concentrated, the product was recovered by repeated precipitation in cold diethyl ether and dried in vacuum. The degree of end modification was checked by ^{1}HNMR and found to be quantitative. ^{1}HNMR ($CDCl_3$): δ = 3.2–3.3 (−O−*CH*$_2$−CH_2−SO_2−), δ = 3.41 (−C−*CH*$_2$O−), δ = 3.6–3.7 (−O−*CH*$_2$−*CH*$_2$−O), δ = 3.8–3.9 (−O−CH_2−*CH*$_2$−SO_2−), δ = 6.07–6.10 (*CH*$_2$=CH−), δ = 6.37–6.41 (*CH*$_2$=CH−), δ = 6.79–6.85 (CH_2=*CH*−).

2.3. Experimental determination of overlap concentration

Dynamics of the 4-arm star PEG in PBS solution was studied by DLS on a commercial LLS spectrometer (ALV/DLS/SLS-5022F) equipped with a multi-τ digital time correlator (ALV5000) and a vertically polarized 22 mW He−Ne cylindrical laser (λ_0 = 632.8 nm, Uniphase). The details on the light scattering instrumentation, theory and data treatment can be found elsewhere [40–44]. The star PEG PBS solutions were pre-treated by repeated filtration through a 0.45 μm hydrophilic PTFE filter to remove the nanobubbles stabilized by the PEG chains, which would result in an apparent slow mode in the dilute solution regime [45–47]. The corresponding correlation function was resolved by CONTIN program provided by ALV. The diffusion coefficient (D) of the star PEG in PBS solution at every concentration was determined from $\Gamma = Dq^2$, where Γ is the line-width obtained from the CONTIN analysis and q is the scattering vector.

2.4. Isolation and culture of murine chondrocytes

Murine chondrocytes isolation and culture were performed following established method [48,49]. Briefly, primary chondrocytes were isolated from the ribs of C57BL/6 new-born mice

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