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Jellyfish collagen scaffolds for cartilage tissue engineering

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Porous scaffolds were engineered from refibrillized collagen of the jellyfish *Rhopilema esculentum* for potential application in cartilage regeneration. The influence of collagen concentration, salinity and temperature on fibril formation was evaluated by turbidity measurements and quantification of fibrillized collagen. The formation of collagen fibrils with a typical banding pattern was confirmed by atomic force microscopy and transmission electron microscopy analysis. Porous scaffolds from jellyfish collagen, refibrillized under optimized conditions, were fabricated by freeze-drying and subsequent chemical cross-linking. Scaffolds possessed an open porosity of 98.2%. The samples were stable under cyclic compression and displayed an elastic behavior. Cytotoxicity tests with human mesenchymal stem cells (hMSCs) did not reveal any cytotoxic effects of the material. Chondrogenic markers SOX9, collagen II and aggrecan were upregulated in direct cultures of hMSCs upon chondrogenic stimulation. The formation of typical extracellular matrix components was further confirmed by quantification of sulfated glycosaminoglycans.

1. Introduction

Collagen is the prevailing component of extracellular matrices in connective tissues. Due to its biocompatibility, biodegradability, low immunogenicity and cell-adhesive properties, collagen is one of the most frequently utilized materials in the field of tissue engineering [1]. Collagen can be extracted from a variety of organisms. Preferential sources of collagen for tissue engineering applications are bovine skin and tendon as well as porcine skin. However, collagen of bovine origin involves the risk of infection with diseases such as bovine spongiform encephalopathy. Furthermore, mammalian collagens, especially of porcine origin, are increasingly rejected for religious reasons [2]. Marine organisms are an alternative natural source of collagen and, presumably, are safer compared to mammals. Recent publications focus mainly on the isolation and characterization of collagen from different fish species, such as salmon [3], shark [4] or deep sea redfish [5] and marine sponges [6]. Another attractive marine source for the extraction of collagen is jellyfish. The global increase in jellyfish population causes major problems in the ecological environment, and their potential use in tissue engineering, next to food industry and medicine, may help to reduce their further expansion [7]. With a collagen content of more than 60% [8], jellyfish has the potential to become a significant source of collagen in biomedical applications [9]. Isolation and molecular characterization of jellyfish collagen derived from *Stomophulus nomurai* has been reported decades ago [10,11]. More recent investigations are concerned with collagen from other jellyfish species, e.g. *Rhopilema asamushi*, *Stomolophus meleagris*, *Catostylus tagi* and *Rhizostoma pulmo* [7–9,12]. High collagen recovery rates have consistently been reported. Amino acid analyses revealed a composition similar to vertebrate collagen with, however, a lower content of hydroxyproline, which leads to relatively low denaturation temperatures between 26 and 29.9 °C. Differences in the subunit composition of collagens from different species are detectable in their respective electrophoretic pattern. Hence, it may be stated that collagens of different jellyfish species show similarities to different vertebrate collagen types. Some jellyfish collagens are comparable to vertebrate collagen IV or V [10,11], others seem to resemble vertebrate collagen I [7,12,13] and some show a unique structure with a fourth α-chain [9]. Hsieh [14] postulates that jellyfish collagen of *S. meleagris* is similar to vertebrate collagen type II according to the molecular mobility, salting-out concentration, high content of hydroxylysine, solubility properties, absence of disulfide bonds and highly hygroscopic nature. Similar findings are described by

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Bermueller *et al.* for jellyfish collagen of *Rhopilema esculentum*, which consists of only one type of α-chain and shows a degree of glycosylation similar to that of vertebrate collagen type II [15]. Since collagen II, as the main component of cartilage extracellular matrix, has been shown to support the chondrogenic differentiation and maintenance of chondrogenic phenotype to a higher extent compared to collagen I [16], jellyfish collagen derived from *R. esculentum* might be a predominant prospective material for the preparation of scaffolds in cartilage tissue engineering.

So far only a few publications have reported on the preparation of jellyfish collagen-based scaffolds for tissue engineering applications. Song *et al.* [17] generated porous scaffolds by freeze-drying and subsequent chemical cross-linking of acid-solubilized jellyfish collagen. Biocompatibility investigations comprised the attachment of human fibroblasts as well as the immune response after implantation of the scaffolds *in vivo*, being similar to other collagen sources. Another type of porous scaffold was established by the same group, combining jellyfish collagen and hyaluronic acid [18]. Tubular porous scaffolds from jellyfish collagen reinforced with poly(lactic-co-glycolic) acid fibers were developed by freeze-drying and electrospinning techniques [19,20]. Seeded with osteosarcoma cells, these constructs were cultivated to test the influence of electrospinning parameters on cell adhesion and proliferation, or, when seeded with endothelial cells and smooth muscle cells in a perfusion system, to generate vascular grafts.

There are three different types of cartilage: hyaline, fibrocartilaginous and elastic. All cartilage types consist of chondrocytes and an extracellular matrix with collagens, proteoglycans and water, differing in protein types and proportions [21]. Joint surfaces are covered by hyaline cartilage, which is predominantly collagen type II (90–95%), followed by collagen XI and IX [22,23]. On the contrary, in fibrocartilage (in knee joint menisci and intervertebral discs), which has properties of both connective tissues and hyaline cartilage, collagen type I and fewer proteoglycans are present [24]. Analogous to hyaline cartilage, elastic cartilage is mainly composed of collagen type II. The main difference to the other two types is elastin and a higher number of cells [21]. Cartilage repair is very challenging since all three types of cartilage tissue are non-vascularized and have therefore a poor intrinsic regeneration capacity.

One approach to the regeneration of cartilage defects is the implantation of a tissue-engineered scaffold colonized with cells. In clinical practice autologous chondrocytes are used for this purpose. There are, however, limitations to this procedure due to the induction of morbidity at the donor site and instability in monolayer cultivation [25]. Mesenchymal stem cells from various tissues, such as bone marrow or adipose tissue [25], represent an alternative source of superior availability and are for that reason the object of research in cartilage tissue engineering [26,27].

Materials for scaffolds in cartilage tissue engineering consist mainly of natural or synthetic polymers [26], both in the shape of either hydrogels [26,28] or porous matrices [27,29,30]. Natural polymers include agarose, collagen, silk, alginate or chitosan [28,30–32]. In the clinical environment, mammalian collagen is already used in the form of membranes, e.g. CaReS (Arthro Kinetics AG, Germany), Chondro-Gide® (Geistlich Pharma AG, Switzerland), Cartimaix® (Matricel GmbH, Germany), Novocart® 3D (TETEC® Tissue Engineering Technologies AG, Germany) or hydrogels, e.g. ChondroFiller® (Amedrix, Germany).

The aim of the present study was to characterize fibril formation parameters of jellyfish collagen to find optimal parameters for the fabrication of stable porous scaffolds from refibrillized jellyfish collagen. Furthermore we wanted to evaluate the suitability of the scaffolds for potential usage in cartilage tissue engineering.

2. Materials and methods

2.1. Collagen

Collagen was extracted by pepsin digestion from cured jellyfish *R. esculentum* (LiroyBV, Rotterdam, The Netherlands) as described before [33]. Briefly, salted jellyfish was cut into pieces and extensively rinsed with cold water until salinity was $\leqslant$0.01. After equilibration in 0.5 M acetic acid for at least 30 min, pieces of jellyfish were homogenized. Following 60 h of pepsin digestion at 4 °C, the solution was centrifuged for cleaning. Collagen was then precipitated from the supernatant by adding NaH_2PO_4 and KCl at pH 7 for 12 h. After another centrifugation step the collected collagen was redissolved in 0.05% acetic acid and dialyzed against 0.05% acetic acid. It was stored at −20 °C and lyophilized only when required to avoid unwanted cross-linking. Prior to use, a stock solution was generated under constant stirring at 4 °C with a concentration of 5 mg ml^{-1} lyophilized collagen dissolved in 0.01 M HCl.

2.2. Fibril formation

For reassembly analysis, 500 μl collagen stock solution in graded concentrations of 5, 4, 3, 2 and 1 mg ml^{-1} were thoroughly mixed with 500 μl of 50 mM tris-(hydroxymethyl)-aminomethane (Tris) buffer containing graded sodium chloride concentrations of 20, 40, 60, 80 and 100 mM; final pH was adjusted to 7.4. Turbidity was measured at 313 nm over 60 min at 4 °C or 25 °C using a UV–Vis spectrophotometer Cary 50 Bio (Varian, Germany). Fibril formation was quantified indirectly by measuring the collagen concentration in the supernantant using the modified Bradford assay, as described previously [34,35]. In brief, upon completion of the fibril formation process over 4 h at 4 or 25 °C, the suspension was centrifuged for 15 min at 10,000*g*, at 4 °C. 5 μl of the supernantant were mixed with 250 μl Bradford reagent (Sigma–Aldrich, USA) containing 0.035 mg ml^{-1} SDS (Sigma–Aldrich). Absorbance was measured after 15 min at 590 nm using a microplate reader Infinite M200Pro (Tecan, Switzerland). For calibration, a graded series of jellyfish collagen stock solution was used. Finally, the ratio of fibrillized to total initial collagen was calculated, revealing the degree of fibril formation. The graphs show the mean ± standard deviation ($n = 6$).

2.3. Scaffold preparation

Based on the results of the fibril formation studies we developed the following protocol for scaffold preparation. A 5 mg ml^{-1} stock solution of jellyfish collagen was merged 1:1 with 50 mM Tris buffer, pH 8. This preparation with a resulting pH 7.4 was stirred for 12 h at 4 °C. Upon centrifugation at 5000*g* and 4 °C, the pellet was resuspended in a small amount of supernantant and transferred to 96-well cell culture dishes. Three-dimensional (3-D) sponge-like, porous scaffolds were obtained after freezing at a speed of 1 K min^{-1} and subsequent freeze-drying (Alpha 1–2, Christ, Germany) for 24 h. The scaffolds were chemically cross-linked in a 1 wt.% solution of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, Fluka, Germany) in 80 vol.% ethanol for 2 h. After careful rinsing in deionized water, in 1 wt.% glycine solution, and once more in deionized water, a final freeze-drying step concluded the procedure.

2.4. Atomic force microscopy (AFM)

Droplets of refibrillized jellyfish collagen suspended in 50 mM Tris buffer, pH 8, were transferred onto the surface of mica discs.