Marine Environmental Research 128 (2017) 46–57

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Marine-derived collagen biomaterials from echinoderm connective tissues

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ARTICLE INFO

Article history:
Received 9 December 2015
Received in revised form
19 March 2016
Accepted 29 March 2016
Available online 31 March 2016

Keywords:
"Blue" biotechnology
Alternative biomaterial
Marine collagen
Echinoderms
Mutable collagenous tissues
Human fibroblast culture
Guided tissue regeneration
Environmental impact
By-product valorisation

ABSTRACT

The use of marine collagens is a hot topic in the field of tissue engineering. Echinoderms possess unique connective tissues (Mutable Collagenous Tissues, MCTs) which can represent an innovative source of collagen to develop collagen barrier-membranes for Guided Tissue Regeneration (GTR). In the present work we used MCTs from different echinoderm models (sea urchin, starfish and sea cucumber) to produce echinoderm-derived collagen membranes (EDCMs). Commercial membranes for GTR or soluble/reassembled (fibrillar) bovine collagen substrates were used as controls. The three EDCMs were similar among each other in terms of structure and mechanical performances and were much thinner and mechanically more resistant than the commercial membranes. Number of fibroblasts seeded on sea-urchin membranes were comparable to the bovine collagen substrates. Cell morphology on all EDCMs was similar to that of structurally comparable (reassembled) bovine collagen substrates. Overall, echinoderms, and sea urchins particularly, are alternative collagen sources to produce efficient GTR membranes. Sea urchins display a further advantage in terms of eco-sustainability by recycling tissues from food wastes.

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1. Introduction

The marine ecosystem and its inhabitants have always been sources of food, biomaterials, active compounds or simply ideas for human applications (e.g. medicine, cosmetics, biotechnology, biofuels, etc.). Many examples of sustainable exploitation of "blue resources" have been reported so far including: i) professional swimsuits inspired by shark skin, ii) algae and marine sponge bioactive substances (Gupta and Abu-Ghannam, 2011; Dembitsky et al., 2005; Rao et al., 2006; Guzmán et al., 2011) for pharmacological use (anti-cancer or anti-neurodegeneration drugs), iii) structural molecules (*i.e.* chitin and collagen) from different marine animals as alternative biomaterials for biomedical applications (Gomez d'Ayala et al., 2008; Gómez-Guillén et al., 2011). Basic research on ocean life and applied research on possible industrial applications are the key activities in terms of "blue growth" in biotechnology and bioeconomy (European Commission, 2012): the sustainable exploitation of "blue resources" and the eco-friendly management of industrial wastes are nowadays two of the most challenging aspects in this field.

To date, marine invertebrates (e.g. sponges, jellyfish and molluscs), are among the most promising groups of animals for this kind of studies because of their variety and abundance in all seas.

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http://dx.doi.org/10.1016/j.marenvres.2016.03.007
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However, a wide range of marine biodiversity is still unexplored from this point of view. Echinoderms are marine invertebrates widespread in all the oceans and employed as source of food for decades (e.g. sea cucumbers and sea urchins; Conand, 2004; Barrington et al., 2009). They are well-known also for their peculiar connective tissues, called Mutable Collagenous Tissues or MCTs, which are able to rapidly change their passive mechanical properties (stiffness and viscosity), under the nervous system control (Wilkie, 2005). MCTs are a unique feature of echinoderms and, although their presence was not described in all known species, their ubiquity throughout the Phylum is highly probable: indeed, MCTs have been described in all the five extant Classes (Wilkie, 2005) and in fossil specimens as well (Baumiller and Ausich, 1996), thus indicating they are probably an ancestral character. This type of tissue has been recently proposed as possible source of inspiration for "smart dynamic biomaterials" for tissue engineering and regenerative medicine applications (Barbaglio et al., 2012, 2013). Particularly, the sea urchin peristomial membrane (a well-known MCT) has been proposed as a sustainable and eco-friendly source of native fibrillar collagen to produce thin membranes for regenerative medicine applications (Di Benedetto et al., 2014). Indeed, the peristomial membrane is a sea urchin food industry waste that can be transformed in a highly valuable by-product.

Among the "blue biomaterials" marine collagen has the most promising perspectives as valid candidate for replacing the most commonly used mammal-derived collagen. This latter is routinely employed in a wide range of human applications (Karim and Bath, 2008; Silva et al., 2014; Silvipriya et al., 2015), from large-scale uses, such as food (Djagny et al., 2010), pharmaceutical/nutraceutical industry (Sahithi et al., 2013) and cosmetics (Buck II et al., 2009), to more targeted fields, such as cell cultures (Lee et al., 2008) and biomedical/clinical applications (Tsai et al., 2005; Glowacki and Mizuno, 2008). However, due to allergy problems (Silvipriya et al., 2015), religious and social/life style constraints (Jenkins et al., 2010), disease transmission-connected reasons (e.g. bovine spongiform encephalopathy or BSE) and high costs of recombinant technologies, collagen sources alternative to mammals are constantly investigated (Silva et al., 2014).

In this sense marine animals, and echinoderms in particular, are surely appealing (Shimomura et al., 1962; Nagai and Suzuki, 2000; Nagai et al., 2000; Swatschek et al., 2002; Song et al., 2006; Uriarte-Montoya et al., 2010; Barros et al., 2014; Di Benedetto et al., 2014). A further advantage of echinoderm MCTs is the relative easiness to obtain high amount of native collagen fibrils, which maintain their original structure (Matsumura, 1974; Trotter et al., 1994; Di Benedetto et al., 2014). Indeed, most mammalian collagen is usually employed in its hydrolized (acid-solubilized) form, a characteristic that strongly reduces the mechanical performances of the produced membrane/scaffold and that can be a limit in those biomedical applications where highly resistant materials, with fibril three-dimensional organization, are required e.g. tendon/ligament regeneration (Kew et al., 2011) or dermis re-construction (Ruszczak, 2003). Echinoderm MCTs can be useful to easily and rapidly produce fibrillar collagen membranes with a high similarity in terms of both ultrastructural and mechanical characteristics to the physiological situation of connective tissue.

A specific regenerative medicine field where fibrillar collagen membranes are commercially used is Guided Tissue Regeneration (GTR; Ferreira et al., 2012; Tal et al., 2012). One of the aims of GTR is to reduce post-surgical tissue adhesions, a common and only partially solved complication (Parker et al., 2001), which prevents proper tissue regeneration. These latter are abnormal attachments or mixture of cells forming between tissues or organs after surgery or due to local inflammation. Only recently researchers have tried to produce effective and satisfactory tools to overcome them. Indeed, barrier-membranes composed by several different biomaterials (e.g. chitosan and hyaluronic acid) have been tested for GTR but none of them displayed all the necessary functional properties, the most important of which is avoiding cell penetration into the underlying anatomical compartment (Tang et al., 2007). Collagen-based membranes seem promising from this point of view because their porosity/three-dimensional structure can be modified as desired. However, their use is still limited by the weak mechanical resistance. This, for example, reduces their efficacy in prevention of wound dehiscence or in tendon repair.

The present work was addressed to evaluate if echinoderm-derived collagen membranes could represent a valuable "blue alternative" to the commercially available (mammal-derived) membranes employed for GTR. This was done by considering different aspects, including ultrastructural properties, mechanical performances as well as the behaviour of human skin-derived fibroblasts (hSDFs) when seeded on these substrate types. Considering the high biodiversity of echinoderms, we also evaluated which animal/MCT source might be more suitable for this biotechnological application. To accomplish this, representatives of different echinoderm Classes were used: the sea urchin *Paracentrotus lividus*, the starfish *Echinaster sepositus* and the sea cucumber *Holothuria tubulosa*.

2. Materials and methods

2.1. Experimental animals

Adult specimens of the sea urchin *P. lividus*, the starfish *E. sepositus* and the sea cucumber *H. tubulosa* were collected by SCUBA divers in Paraggi (Marine Protected Area of Portofino, Ligurian Sea, Italy), transferred to the Department of Biosciences (University of Milan) and immediately dissected. Samples of sea urchin peristomial membranes (PM; Fig. 1A and B), starfish aboral arm walls (AW; Fig. 1C and D) and sea cucumber whole body walls (BW; Fig. 1E–G) were collected and stored at −20 °C for the subsequent collagen extraction protocol (see paragraph 2.2). Animal collection and experimental manipulation were performed according to the Italian law.

2.2. Echinoderm collagen extraction

Sea urchin collagen was extracted from the peristomial membranes as previously described by Di Benedetto et al. (2014). Starfish aboral arm walls followed the same protocol with only slight modifications. Briefly, the frozen tissues of both animals were dissected in small pieces, rinsed in artificial sea water and left in a hypotonic buffer (10 mM Tris, 0.1% EDTA) for 12 h at room temperature (RT) and then in a decellularizing solution (10 mM Tris, 0.1% Sodium Dodecyl Sulphate) for 12 h at RT. After several washings in phosphate-buffered saline (PBS), samples were placed in disaggregating solution (0.5 M NaCl, 0.1 M Tris–HCl pH 8.0, 0.1 M β-mercaptoethanol, 0.05 M EDTA–Na). The obtained collagen suspension was filtered and dialysed against 0.5 M EDTA–Na solution (pH 8.0) for 3 h at RT and against dH_2O overnight at RT. Starfish samples underwent an additional step in 1 mM citric acid (pH 3–4) between decellularizing and disaggregating solutions in order to remove as much as possible the calcium carbonate ossicles present in the fresh tissue. All the steps were carried out under stirring conditions.

Sea cucumber collagen was extracted from the whole body wall following a different protocol. Briefly, the starting tissue was cut into small pieces, placed in PBS and gentamicin (40 μg/mL) and left in stirring conditions at RT for at least 5 days in order to obtain a collagen suspension that was subsequently filtered. Suspensions

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