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Cross-linking of a biopolymer-peptide co-assembling system

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ABSTRACT

The ability to guide molecular self-assembly at the nanoscale into complex macroscopic structures could enable the development of functional synthetic materials that exhibit properties of natural tissues such as hierarchy, adaptability, and self-healing. However, the stability and structural integrity of these kinds of materials remains a challenge for many practical applications. We have recently developed a dynamic biopolymer-peptide co-assembly system with the capacity to grow and undergo morphogenesis into complex shapes. Here we explored the potential of different synthetic (succinimidyl carboxymethyl ester, poly (ethylene glycol) ether tetrasuccinimidyl glutarate and glutaraldehyde) and natural (genipin) cross-linking agents to stabilize membranes made from these biopolymer-peptide co-assemblies. We investigated the cross-linking efficiency, resistance to enzymatic degradation, and mechanical properties of the different cross-linked membranes. We also compared their biocompatibility by assessing the metabolic activity and morphology of adipose-derived stem cells (ADSC) cultured on the different membranes. While all cross-linkers successfully stabilized the system under physiological conditions, membranes cross-linked with genipin exhibited better resistance in physiological environments, improved stability under enzymatic degradation, and a higher degree of *in vitro* cytocompatibility compared to the other cross-linking agents. The results demonstrated that genipin is an attractive candidate to provide functional structural stability to complex self-assembling structures for potential tissue engineering or *in vitro* model applications.

Statement of Significance

Molecular self-assembly is widely used for the fabrication of complex functional biomaterials to replace and/or repair any tissue or organ in the body. However, maintaining the stability and corresponding functionality of these kinds of materials in physiological conditions remains a challenge. Chemical cross-linking strategies (natural or synthetic) have been used in an effort to improve their structural integrity. Here we investigate key performance parameters of different cross-linking strategies for stabilising self-assembled materials with potential biomedical applications using a novel protein-peptide co-assembling membrane as proof-of-concept. From the different cross-linkers tested, the natural cross-linker genipin exhibited the best performance. This cross-linker successfully enhanced the mechanical properties of the system enabling the maintenance of the structure in physiological conditions without compromising its bioactivity and biocompatibility. Altogether, we provide a systematic characterization of cross-linking alternatives for self-assembling materials focused on biocompatibility and stability and demonstrate that genipin is a promising alternative for the cross-linking of such materials with a wide variety of potential applications such as in tissue engineering and drug delivery.

1. Introduction

In biological systems, molecules can tidily bind one to another by non-covalent interactions (*e.g.* hydrogen bonds, van der Waals,

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electrostatic and hydrophobic interactions), forming complex hierarchical structures with outstanding functions. The last two decades have seen a remarkable increase in studies aiming to exploit supramolecular chemistry for the fabrication of materials that attempt to recreate the molecular organization found in Nature [1]. A wide variety of natural and synthetic molecules are being used as self-assembling building-blocks of hydrogel materials with promising bioactive and mechanical properties [2–4] for applications in bioactive surfaces [5], devices [6], and implants [7].

While the potential of self-assembling biomaterials has been well demonstrated [1,8], guiding molecular organization through non-covalent interactions across scales in order to recreate the structural complexity and functionality of natural tissues has been challenging [9]. An increasing number of strategies aim to fabricate, through molecular self-assembly, hierarchical structures with precision and tuneability [1,3,9–12]. Our group has recently reported a co-assembling system based on the conformational modification of elastin-like recombinamers (ELRs) by peptide amphiphiles (PAs) that enables the fabrication of membranes with the capacity to undergo morphogenesis into complex tubular micro/macro structures [10]. When a drop of PA is injected into a larger volume of ELP, an interfacial assembly spontaneously develops, forming a membrane that opens and adheres upon contact to any surface. The process leads to the formation of tubular membranes that display controlled assembly and disassembly capabilities, adhesion and sealing to surfaces, self-healing and the capability to undergo morphogenesis into complex structures with high spatiotemporal control (Fig. 1B). However, as most self-assembled biomaterials [13], these membranes lack the mechanical properties required for many biomedical applications due to the nature of non-covalent interactions involved in the self-assembly of molecules, which limits their applicability. Indeed, hydrogen bonds have very low association strength in hydrogels due to competition of water for binding sites, hydrophobic interactions are highly limited by solubility of the hydrophobes in solution [14], and hydrophilic polymers tend to dissolve into the aqueous phase [15]. All these phenomena tend to weaken the hydrogel network. Therefore, there is a need for cross-linking strategies that can enhance the mechanical properties of these materials in a practical and biocompatible manner in order to maximize the benefits of the self-assembling process such as bioactivity, complexity, and structural hierarchy.

Different chemical, biological, and physical cross-linking strategies have been proposed to increase the mechanical properties of self-assembled materials [13,16]. The ideal cross-linker should be capable to covalently link molecular groups of these building-blocks without compromising their hierarchical order, or eliciting cytotoxic, or immune reactions [17]. Natural and chemical cross-linkers can therefore enhance hydrogel stability and mechanical performance by introducing covalent bonds within the hydrogel, preventing the dissolution of the molecules into the aqueous phase.

Glutaraldehyde (GTA) has been widely used to cross-link biomaterials due to its efficacy in enhancing their mechanical properties [17]. However, GTA has been associated with dystrophic calcifications, pro-inflammatory response by activation of macrophage-like cells, and cytotoxic effects when used to cross-link implanted materials [16,17]. Therefore, cross-linking strategies based on multifunctional poly (ethylene glycol) (PEG) systems and plant extracts have been proposed as alternative cross-linker candidates. Multifunctional PEG cross-linking systems are either linear or exhibit branched chains with terminal active ester groups that react with the primary amine groups of the building blocks [13,18]. Poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG), a multi-armed functional PEG molecule, has been reported as an effective cross-linker of hydrogels [18,19] and implantable fibres [13]. The resulting scaffolds demonstrated improved stability and mechanical properties in physiological environments while exhibiting good biocompatibility [18,19]. Similarly, succinimidyl carboxymethyl ester (SCM-PEG-SCM), a linear functionalised PEG cross-linker, has been shown to improve stability and mechanical properties of collagen hydrogels with good biocompatibility [17,20]. Natural molecules from plant extracts have also been used as cross-linking agents for decades to enhance stability of materials for biomedical applications. Genipin (GNP), a natural cross-linker agent derived from the fruit of *Gardenia jasminoides Ellis*, reacts with primary amine groups of the building blocks *via* its hydroxyl and carboxyl reactive groups [21,22]. GNP has been shown to exhibit lower cytotoxicity and inflammatory responses compared to GTA, and to possess antiphlogistic, anti-inflammatory, diuretic, choleretic, and haemostatic properties [23].

In this study, we investigated possible exogenous cross-linking strategies (4S-StarPEG, SCM-PEG-SCM, GNP, and GTA) for the enhancement of stability and structural integrity of self-assembling systems with potential biomedical application. Specifically, we explored the performance of these cross-linkers in the biopolymer-peptide self-assembling system (ELR/PA membrane) previously reported by our group as ELP/PA system [10]. We compared cross-linking efficiency, resistance to enzymatic degradation, and potential toxicity of these cross-linkers, in order to identify an effective cross-linker agent that does not compromise hierarchical nano/microstructure and bioactive properties.

2. Experimental section

2.1. Materials

Peptide amphiphile (PA) molecules ($C_{15}H_{31}$CONH-VVVAAAKKK-$CONH_2$) were obtained from Cambridge peptides (Birmingham, UK). Elastin-like recombinamers (ELR) molecules (MESLLP-$[(VPGVG\ VPGVG\ VPGEG\ VPGVG\ VPGVG)_{10}$-$(VGIPG)_{60}]_2$-$[(VPGIG)_{10}$-AVTGRGDSPASS$(VPGIG)_{10}]_2$-V) were provided by Technical Proteins NBT S.L. (Valladolid, Spain). Glutaraldehyde (GTA), collagenase (Cg), pronase (Pn), proteinase K (PK) and trypsin (Tp) were purchased from Sigma Aldrich (Dorset, UK). Genipin (GNP) was purchased from Cambridge Bioscience (Cambridge, UK). Succinimidyl carboxymethyl ester (SCM-PEG-SCM, molecular weight 2000 Da) and poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG, molecular weight 10,000 Da) were purchased from JenKem Technology (Texas, USA). Elastase (Et) was purchased from Elastin Products Company (Missouri, USA). 2,4,6 Trinitrobenzenesulfonic acid (TNBS) assay was purchased from Thermo Scientific™ Pierce™ (Paisley, UK).

2.2. ELR/PA membrane fabrication

ELR and PA molecules were dissolved in ultrapure water to a final concentration of 0.1 mM and 8.7 mM, respectively. 10 μl drop of PA solution was added in 190 μl of ELR solution (Fig. 1A, B). The membranes were allowed to form at 23 °C for 48 h. Membranes were then cross-linked overnight at 37 °C with 3.15 mM GNP, 1.66 mM SCM-PEG-SCM, 0.87 mM 4S-StarPEG (concentration chosen to obtain a ratio 1:1 amine groups to cross-linker reactive groups) and 62.50 mM GTA (standard concentration) (Fig. 2). After the cross-linking process, membranes were washed three to five times in ultrapure water to remove excess of cross-linkers from the membranes.