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Decoration of silk fibroin by click chemistry for biomedical application

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ABSTRACT

Silkfibroin (SF) has an excellent biocompatibility and its remarkable structure translates into exciting mechanical properties rendering this biomaterial particularly fascinating for biomedical application. To further boost the material's biological/preclinical impact, SF is decorated with biologics, typically by carbodiimide/N-hydroxysuccinimide coupling (EDC/NHS). For biomedical application, this chemistry challenges the product risk profile due to the formation of covalent aggregates, particularly when decoration is with biologics occurring naturally in humans as these aggregates may prime for autoimmunity. Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC; click chemistry) provides the necessary specificity to avoid such intermolecular, covalent aggregates. We present a blueprint outlining the necessary chemistry rendering SF compatible with CuAAC and with a particular focus on structural consequences. For that, the number of SF carboxyl groups (carboxyl-SF; required for EDC/NHS chemistry) or azido groups (azido-SF; required for click chemistry) was tailored by means of diazonium coupling of the SF tyrosine residues. Structural impact on SF and decorated SF was characterized by Fourier transform infrared spectroscopy (FTIR). The click chemistry yielded a better controlled product as compared to the EDC/NHS chemistry with no formation of inter- and intramolecular crosslinks as demonstrated for SF decorated with fluorescent model compounds or a biologic, fibroblast growth factor 2 (FGF2), respectively. In conclusion, SF can readily be translated into a scaffold compatible with click chemistry yielding decorated products with a better risk profile for biomedical application.

1. Introduction

The structural features of silkfibroin (SF), a 370 kDa protein purified from cocoons of *Bombyx mori* (L. 1758), set this biomaterial apart from other material in nature. For example, silk threads from spiders rival material features of some of our best synthetic polymers available today such as Kevlar ® in terms of energy absorbed before break (Cunniff et al., 1994). These outstanding mechanical properties are linked to an observation already reported in 1913, namely that silk fibers diffract X-rays (Lucas et al., 1958). These diffraction patterns were typical for β-sheets and have served Pauling and Corey to build their famous model describing the secondary structure of SF (Marsh et al., 1955), one year after Pauling received the Nobel Prize for chemistry in 1954. The protein's primary sequence, within which a remarkable number of repetitive glycine alanine glycine alanine glycine serine (GAGAGS) motifs is perhaps most striking and the reason for intra- as well as inter-chain van der Waals interaction, lead to a stacked and antiparallel β-sheet structure and causal to the materials remarkable properties. For these properties along with its biocompatibility and ability to tune the geometry of manufactured implant materials, SF was increasingly profiled for biomedical application. These included tissue regeneration of vascular (Yagi et al., 2011), neural (Uebersax et al., 2007), skin (Min et al., 2006), bone (Li et al., 2006; Meinel et al., 2004; Karageorgiou et al., 2004; Meinel et al., 2005; Hofmann et al., 2007; Uebersax et al., 2006), ligament (Altman et al., 2002), myocardium (Vunjak-Novakovic et al., 2011) and other tissues (Wang et al., 2006). Beyond its scaffold function for cells, pure SF is a rather biologically inert material which by itself yields no or insufficient tissue regeneration when e.g. implanted into critical sized bone defects (Karageorgiou et al., 2004; Meinel et al., 2005). Therefore, growth factors such as fibroblast growth factor 2 (FGF2) (Wenk et al., 2010), nerve growth factor (NGF) (Uebersax et al., 2007), insulin-like growth factor I (IGFI) (Uebersax et al., 2008) and bone morphogenetic protein 2 (BMP2) (Li et al., 2006; Meinel et al., 2006) are only some examples which had been physically adsorbed or covalently bound to SF in an effort to impact the

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proliferation or differentiation of cells seeded on the decorated SF with the ultimate goal to (re-)engineer lost tissues. These growth factors were typically introduced to SF by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry by which carboxylic acid groups were covalently bound to primary amines (Sofia et al., 2001; Vepari et al., 2010; Vepari and Kaplan, 2007). For that, the SF carboxylic acid residues had been reacted with the primary amines of the target molecules, thereby forming a stable amide bond when reacted in 2-(N-morpholino)ethanesulfonic acid (MES) at pH 4.0–6.5 or phosphate buffer at pH 7.2–7.5. A striking limitation of this approach is related to SF's primary structure, as approximately only 3 percent of all SF amino acids carry free carboxylic groups, thereby limiting the functionalization rate (Zhou et al., 2001), with an estimated number of 25 aspartic acid, 30 glutamic acid, and 12 lysines (primary amines) per SF molecule. Therefore, EDC/NHS chemistry leads to additional SF/SF crosslinks, impacting the control of physical material properties, which for solid SF scaffolds may be an undesired outcome (e.g. loss of manufacturing control) or desired outcome (e.g. tailored mechanical properties through post-manufacture treatment with EDC/NHS). More alarming within the context of biopolymer decoration in liquid state is that most growth factors, cytokines, and other regulatory factors (as will be demonstrated in this manuscript for fibroblast growth factor 2 – FGF2) carry both carboxyl and amino groups. Ultimately, cross-links between these biologics are inevitable leading to covalent aggregates (Enami et al., 1998; Lopez-Alonso et al., 2009). These aggregates are a major structural concern and harbinger for antibody formation against the therapeutic molecules themselves (yielding loss of potency) and certainly more alarming, against their naturally produced equivalents (yielding to autoimmunity and reduced potency of the naturally occurring growth factors). Impaired activity of endogenous regulatory factor by scaffold induced autoimmunity would translate into a severe pharmaceutical concern substantially challenging the safe clinical use of these decorated materials (Koren et al., 2008). Further concerns are for use in women of child bearing potential who may immunologically respond to these structurally modified aggregates by formation of maternal antibodies to the regulatory molecule. If the regulatory molecule – which was used for decoration – is critical for normal fetal or neonatal development, the transmission of maternal antibodies against it may impact fetal and neonatal development during pregnancy or nursing, respectively. These and other safety concerns substantially challenge the use of EDC/NHS. Alternatives are needed in an effort to open biomaterials to novel chemistries providing less critical outcome. Consequently, and to overcome these drawbacks, we deployed Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) (commonly referred to as click chemistry) (Kolb et al., 2001; Kolb and Sharpless, 2003). CuAAC is highly specific within the context of protein coupling and covalent crosslinking among regulatory molecules or among SF molecules is unlikely.

In this study, we directly compared the decoration of SF by means of CuAAC and EDC/NHS chemistry, respectively. For that, we modified the tyrosine groups of the SF primary sequence with azido (azido-SF) or carboxyl groups (carboxyl-SF) by means of a diazonium coupling reaction. Secondly, we decorated this modified SF (either azido-SF or carboxyl-SF) with a fluorescent dye by means of click (SF-click-dye) or EDC/NHS chemistry (SF-EDC-dye), respectively. These results were translated to the bioconjugation of SF with fibroblast growth factor 2 (FGF2), a potent stimulator of various cell types primarily of mesodermal origin but also to cells of the ecto- and/or endoderm and impacting cell migration, proliferation or differentiation (Bikfalvi et al., 1997; Burgess and Maciag, 1989). Furthermore, FGF2 is an endogenous growth factor in humans in fetal, neonatal and in adult life. Therefore, a functionalization of SF with this regulator molecule by means of CuAAC may provide the necessary level of control regarding site specific coupling and avoiding FGF2/FGF2 covalent aggregate formation and it is this novel chemistry which is presented within the context of SF here within.

2. Experimental details

2.1. Materials

Bombyx mori (L., 1758) cocoons were from Trudel Silk (Zürich, Switzerland). 5/6-carboxyrhodamine 110-PEG$_4$-alkyne (MW: 587.62 g/mol, $C_{32}H_{33}N_3O_8$) and azido-PEG$_3$-amine (MW: 218.25 g/mol, $C_8H_{18}N_4O_3$) were purchased from Jena Bioscience (Jena, Germany). Acetonitrile, 4-azidoaniline hydrochloride, 4-aminobenzoic acid, tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine, tert-butanol (tBuOH), dimethyl sulfoxide (DMSO), 1,4-dithiothreitol (DTT), copper(II) sulfate, phenylmethanesulfonylfluoride (PMSF), sodium L-ascorbate, tris [(1-benzyl-1H-1,2,3-triazol-4-yl)-methyl]amine (TBTA) were purchased from Sigma–Aldrich (St. Louis, MO). α-Iodoacetamido-PEG-ω-alkyne (MW: 3317 g/mol) was from Rapp Polymere (Tuebingen, Germany) and iodoacetyl-PEG-acid (MW: 3400 g/mol) was from Nanocs (New York, NY). Restriction enzymes were from New England Biolabs (Ipswitch, USA). Anti-FGF2/basic FGF monoclonal antibody, clone bFM-2, was from Merck Millipore (Darmstadt, Germany) and anti-mouse IgG peroxidase conjugate was purchased from Sigma Aldrich, (St. Louis, MO). All other reagents were from Sigma–Aldrich and of analytical or pharmaceutical grade.

2.2. Preparation of the SF solution

SF solutions were prepared as described before (Meinel et al., 2005). In brief, cocoons were boiled for 1 h in 0.02 M Na_2CO_3 and rinsed with distilled water. The purified SF was dissolved in 9 M LiBr at 60 °C for 45 min. This solution was filtered through a 5 μm syringe filter (Versapor, Pall Life Sciences, Washington, NY), dialyzed (SpectraPor, MWCO 6000–8000 g/mol, Spectrum Laboratories, Rancho Dominguez, CA) against ultrapure water and finally against borate buffer (300 mM borate, 450 mM NaCl, pH 9.0). The final SF solutions had a concentration of 50 mg/mL (300 mM borate, 450 mM NaCl, pH 9.0).

2.3. Diazonium coupling reaction

Two commercially available anilines were used to decorate the SF tyrosine groups as described before with modifications (Murphy et al., 2008). In brief, a SF decorated with azido functional groups (azido-SF) was synthesized as follows. 4-Azidoaniline hydrochloride (0.360 mmol) was dissolved in 1 mL of a 1:1 acetonitrile/water solution, which was then mixed with 0.5 mL of a p-toluenesulfonic acid monohydrate (1.430 mmol) solution dissolved in water. Finally, sodium nitrite (0.715 mmol) dissolved in 0.5 mL of water was added to the mixture. All the solutions were prepared in an ice bath before mixing. The diazonium coupling reaction was started by adding 2 mL of a 50 mg/mL borate buffered SF solution and the reaction was allowed to proceed for 30 min at room temperature, and dialyzed against ultrapure water. Carboxylated SF (carboxyl-SF) was synthesized identically, with 4-aminobenzoic acid (0.360 mmol) being used instead of 4-azidoaniline hydrochloride (Scheme 1). In order to modify the number of azido or carboxyl groups per SF molecule, different molar equivalents of diazonium salt were added to the SF solution relative to the total number of approximately 280 tyrosine residues per molecule (Table 1).