

# ANTIBACTERIAL NITRIC OXIDE-RELEASING PLATFORMS BASED ON NATURALLY DERIVED MATERIALS FOR BIOMEDICAL APPLICATIONS

by

SAMA GHALEI

(Under the Direction of Hitesh Handa)

## ABSTRACT

Bacterial infection of biomaterials is a major clinical problem and the most important factor for failure of medical devices. Therefore, the development of multifunctional biomaterials that possess antibacterial properties and can resist infection is a continual goal for biomedical applications. Nitric oxide (NO), a free radical gas molecule, is a principal component of the innate immune system and functions as a potent antimicrobial agent. Different types of NO donors have been previously incorporated into polymers of both natural and synthetic origin for a variety of biomedical applications including drug delivery, medical device coatings, implantable biomaterials, and tissue engineering scaffolds. Due to their reproducible characteristics in terms of molecular weight, degradation kinetics, and mechanical properties, synthetic polymers are attractive for a variety of biomedical applications. However, from the biological standpoint, synthetic polymers often lack much-desired bioactivity and biocompatibility, which may translate into adverse side effects. Natural polymers on the other hand are abundant and resemble the components present in biological extracellular matrices. Thus, they are readily accepted by the body and possess high bioactivity and biocompatibility. Despite a large body of research existing on NO releasing materials, the use of natural materials for NO delivery and other biomedical

applications have not yet been extensively explored. In this dissertation antibacterial NO-releasing platforms in different formats, (i) nanoparticles, (ii) hydrogels, and (iii) nanofibers, were developed based on naturally derived materials for versatile biomedical applications.

INDEX WORDS: Biomaterials, Bacterial infection, Nitric oxide, Natural materials

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## DEDICATION

In dedication to my beloved family for their infinite love and support.

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**CHAPTER 1**  
**INTRODUCTION AND LITERATURE REVIEW - ANTIBACTERIAL SILK FIBROIN-**  
**BASED BIOMATERIALS<sup>1</sup>**

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<sup>1</sup> Ghalei S, Handa H. A review on antibacterial silk fibroin-based biomaterials: current state and prospects. *Materials Today Chemistry*. 2022 Mar 1;23:100673. Reprinted here with permission of the publisher.

## 1.1 Silk fibroin in biomedical applications

Silk fibroin is a natural macromolecular protein that has a long history of use in the textile industry and as a suture material in medicine [1]. Approved by the US Food and Drug Administration (FDA) as a biomaterial in 1993, SF has since been the subject of extensive research for different biomedical applications due to the unique combination of properties that it offers [2]. A high level of biocompatibility, tunable biodegradation, outstanding mechanical properties, processability into versatile material formats, and the presence of many accessible functional groups for chemical modifications have all rendered SF an extremely favorable material for development of various medical devices, drug delivery platforms, and tissue engineering scaffolds [3-5].

Silk is synthesized by the epithelial cells in specialized silk glands of different silkworms (mulberry and non-mulberry) and spiders [6]. The difference in silks obtained from various origins is mainly in their amino acid sequences and crystalline structure, giving rise to their distinct physical properties. While it is not feasible to obtain silk from spiders in large quantities [7], domesticated mulberry *Bombyx mori* (B. mori) silkworms provide a constant supply of silk that can be utilized in different fields [8]. Besides B. mori, silk derived from other varieties of silkworms such as non-mulberry *Antheraea mylitta* (Indian Tasar silk), *Antheraea assama* (Indian Muga silk), *Antheraea pernyi* (Chinese Oak Tussah silk), *Antheraea yamamai* (Japanese silk) and *Philosamia ricini* (Indian Eri silk) have also recently found increasing interest due to their comparatively superior mechanical properties and performance [9]. However, owing to the difficulties associated with their extraction and processing, domesticated mulberry B. mori silkworms remain the primary source of silk for textile and biomedical applications [10].

The *B. mori* silkworm cocoons are mainly composed of silk fibroin (SF) fibers covered with an adhesive protein called sericin. Sericin is a hydrophilic polymer that holds the fibroin fibers together like a glue and accounts for almost 30% of the cocoon's weight [11]. Though individually investigated for biomedical applications, the combination of SF and sericin together in raw silk fibers has been rarely used due to the concerns raised regarding their adverse immune reactions and inflammatory activities. This was reported to be caused by the possible contamination from sericin and the wax like material present on the silk fibers [12]. Therefore, for most applications, the SF fibers are purified from sericin through a degumming process by boiling the cocoons in 0.02 M sodium carbonate solution based on a previously established extraction procedure [13]. According to the extraction protocol, the degummed SF fibers can be dissolved in 9.3 M lithium bromide solution and dialyzed against deionized water to obtain aqueous SF solution that can be further processed into many different material formats such as films, hydrogels, micro/nanoparticles, and micro/nanofibers. Other SF extraction methods based on proteolytic enzymes [14, 15], acid treatments [16], and low-pressure argon plasma [17] have also been reported. However, these methods have been less commonly utilized due their high cost and harsh effects on the structure and properties of SF fibers, respectively [18].

The *B. mori* SF is composed of a heavy (~350 kDa) and a light (~25 kDa) polypeptide chain (connected with a disulfide bond) along with the P25 glycoprotein in a molar ratio of 6:6:1 [19]. The heavy chain consists of repetitive hydrophobic domains, mainly composed of glycine, alanine, and serine amino acid sequences, and random hydrophilic domains consisting of acidic or charged amino acids such as glutamic acid, aspartic acid, arginine, and lysine [20]. The hydrophobic domains in SF can adopt a  $\beta$ -sheet conformation to form water insoluble crystalline regions that are responsible for the excellent mechanical strength of SF. These crystalline structures are interspersed between hydrophilic amorphous regions with  $\alpha$ -helical conformation that provide SF with elasticity and toughness [21]. A schematic of the structure of silk fibroin is represented in **Figure 1.1**.

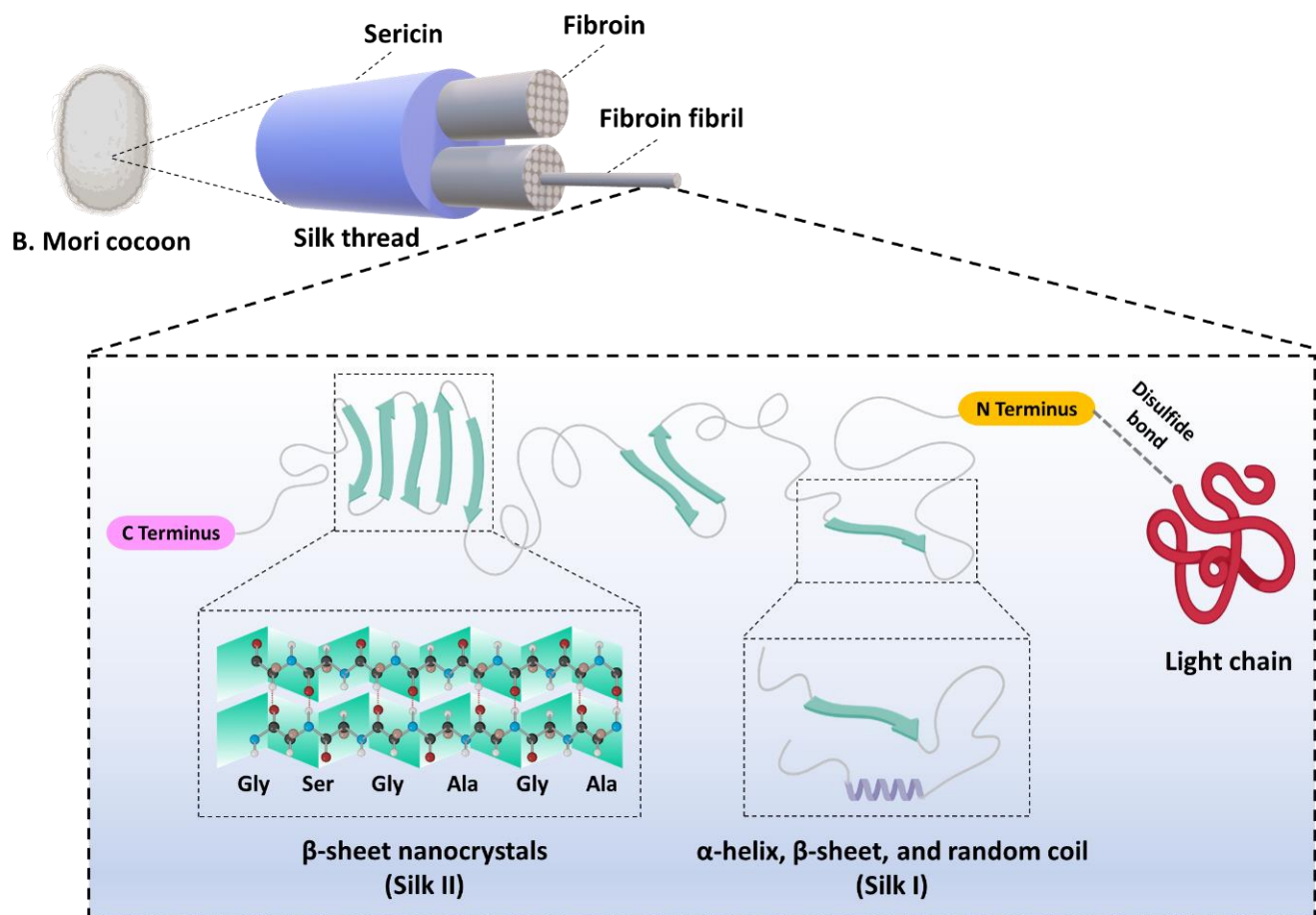


**Figure 1.1** - A schematic representation of the *B. mori* silk fibroin structure.

The superior mechanical properties of silk related to its molecular structure and packing, along with its high biocompatibility and tunable biodegradation have made SF distinctly unique among other natural and synthetic polymers [22]. The transformation of  $\alpha$ -helical conformations into  $\beta$ -sheet crystalline structures in SF can be induced by different physical and chemical treatments *in vitro* to produce highly stable hydrogels and 3D scaffolds [23]. SF in an aqueous state or when dissolved in organic solvents (e.g., formic acid, hexafluoro-2-propanol) can also be used to fabricate films, nanofibers, and other type of biomaterials for different biomedical applications (**Figure 1.2**) [24, 25]. In addition, the presence of both hydrophilic and hydrophobic domains in the SF structure endows it with a self-assembling ability in the aqueous environment, enabling the synthesis of SF micro/nanospheres using water-based approaches [26]. SF-based micro/nanospheres have been widely investigated in the past for delivery of various drugs and therapeutic agents [27-29].



**Figure 1.2** - Silk fibroin properties, different formats, and biomedical applications.

Despite the many outstanding properties of SF that qualifies it for a wide range of applications either alone or in combination with other biomaterials, a major limitation with SF biomaterials is the lack of inherent antibacterial activity. Bacterial infection is a great concern for indwelling medical devices and implantable biomaterials. Despite the standard sterilization treatments such as dry/wet heat or ionizing radiation, the surface of these materials can still be colonized by bacteria due to the possible contamination from the operating room environment, the patient's own skin, or other distal infections in the body [30, 31]. Biomaterial's contamination can give rise to difficult to treat infections that endanger the patient's life and put a burden on both the patient and healthcare budget by increasing the healing time and requiring a revision surgery to remove the infected implant [32]. In fact, biomaterial associated infections are one of the major reasons for failure of implants and medical devices. Health care-associated infections (HAIs) annually cost up to \$33 billion and pose a severe threat to society [33]. Therefore, intensive efforts have been devoted to developing biomaterials that possess antibacterial activity and can resist such infections.

Due to the wide-ranging application of SF-based biomaterials (SFBs), over the last decade, many researchers have investigated different strategies to confer these materials with antibacterial properties. Various methods such as combination, modification, and functionalization with different antibacterial agents or alteration of the surface architecture have been implemented to prevent bacterial colonization on the surface of SFBs and improve their performance. In this review, for the first time we go through each of these methods, their advantages and drawbacks, and give an overview of the recent advances and future trend in this research area.

## 1.2 Silk fibroin interaction with bacteria

While the antibacterial applications of SFBs have been extensively explored in the past decade, the study of SF as a support material for bacterial adherence, growth, and biofilm formation is a newly emerged area of research that has found application in the fields of bioelectronics and bioanalytical sciences [34]. The as-spun silk fibers in the cocoons of silkworm larvae have been shown to provide a good substrate for the growth of various bacterial species and biofilm formation. The formed biofilm will in turn act as a primary defense barrier protecting the cocoon from environmental stresses. The *Pseudomonas cepacia* bacteria present in soil have also been found to feed on the carbon and nitrogen that they obtain by hydrolyzing the degummed silk via their fibrinase enzyme [35]. In a study by Tabei et al. SF films containing 0.5% glycerol as a plasticizer were demonstrated to have great ability to collect and retain a high density of various bacterial strains on their surface after a short period of exposure [36]. The attachment of bacteria to polymeric surfaces is governed by several factors such as surface hydrophobicity, surface charge, and surface chemical composition. While the mechanism of interaction of SF with bacteria and its supporting effect on biofilm formation has not yet been completely elucidated, SF moderate wettability has been identified as one of the primary influential factors in the attachment of cells to its surface [37]. The initial bacterial adherence to a surface (docking stage) is determined by nonspecific cell-surface interactions such as van der Waals, electrostatic, and hydrophobic attractive or repulsive forces. The loosely attached bacteria to the surface then start to secrete an extracellular polymeric substance (EPS) that allows for a stronger adhesion between bacteria and the surface (locking stage). SF protein with suitable hydrophobicity and excellent bioabsorbability has been demonstrated as an effective substrate for docking and immobilization of various bacterial species. Though favorable for some applications such as biofuels and microbial sensors

[36], the supporting effect of SF in bacterial growth and proliferation is highly undesirable and must be avoided for medical devices and biomaterials related applications. In the next sections, we review on the different methods employed in the last decade to overcome the bacterial colonization of SFBs.

### 1.3 Antibacterial approaches for silk-fibroin based biomaterials

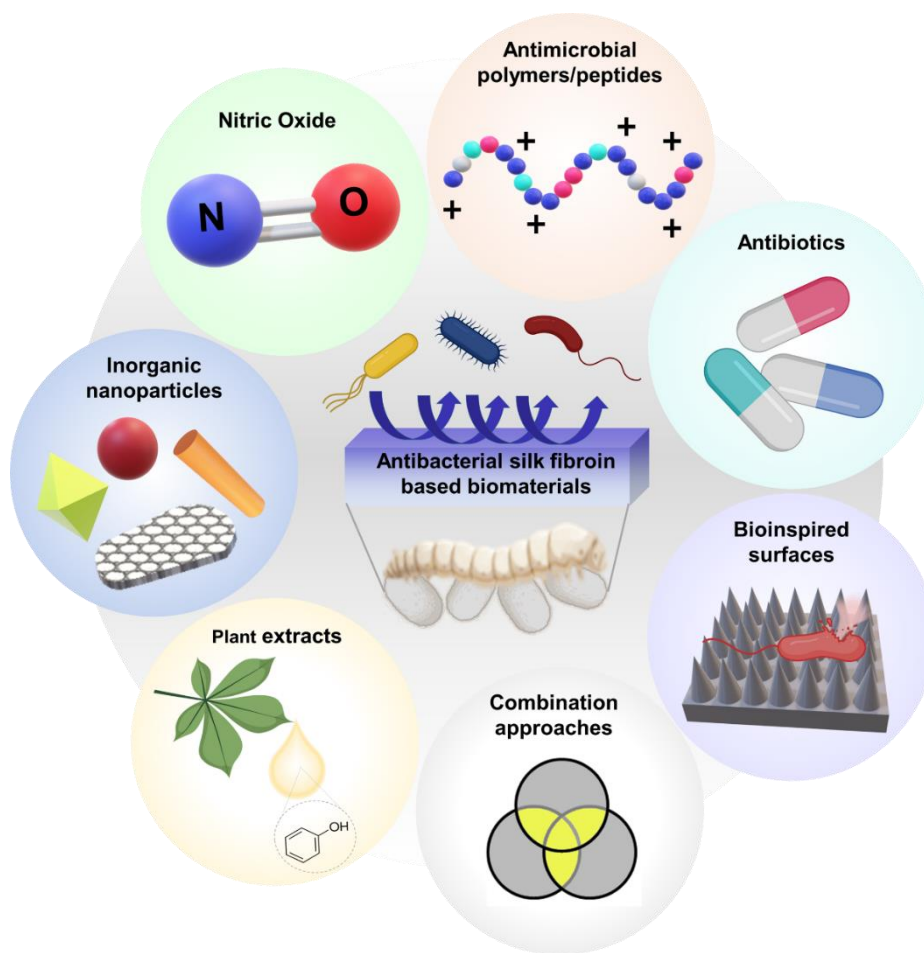


**Figure 1.3** - Different antibacterial approaches for silk fibroin-based biomaterials.

#### 1.3.1 Antibiotics

Antibiotics are one of the most commonly used agents to be combined with SF-based biomaterials (SFBs) for prevention and treatment of bacterial infections. While systemic

administration of antibiotics often results in poor tissue penetration and low bioavailability, and can cause adverse side effects, their localized delivery through implantable biomaterials and scaffolds offers several advantages. These advantages include tunable release directly at the site of infection and achieving a higher dosage with reduced side effects [38]. The release of antibiotics from SFBs can be controlled by tailoring their chemical and structural design as well as their biodegradation rate [39]. Since the bacteriostatic activity of such materials is dependent upon the release of an antibiotic from a silk fibroin-based substrate, most studies have implemented a zone of inhibition (ZOI) or disc diffusion test to evaluate the antibacterial responses (**Table 1.1**). ZOI test is a quick and rather inexpensive method that is used to determine the antibacterial activity of a material, in relation to a target microorganism, by measuring the size of the bacterial inhibition zone formed around the test material due to an antibacterial agent being released or leached out from it.

Depending on their type, antibiotics induce bacterial cell death through different approaches. For instance, aminoglycoside antibiotics (i. e. gentamicin) and tetracyclines are inhibitors of protein biosynthesis, quinolones such as levofloxacin and ciprofloxacin impede DNA replication, while glycopeptides (i. e. vancomycin) and  $\beta$ -lactam (i. e. amoxicillin) antibiotics interfere with normal bacterial cell wall synthesis [40]. In a study by Lan et al. vancomycin loaded gelatin microsphere were prepared and embedded in freeze-dried SF scaffolds for wound healing applications. According to a ZOI assay, the fabricated scaffolds were able to inhibit the gram-positive *Staphylococcus aureus* (*S. aureus*) while did not show any antibacterial activity against gram negative *Escherichia coli* (*E. coli*) bacteria after 24 h. This is because gram negative bacteria possess an outer membrane that makes them more resistant against  $\beta$ -lactam and glycopeptide antibiotics like vancomycin that act on the bacterial cell wall [41]. Therefore, for any application

the choice of antibiotic is of primary importance and should be made based upon their mechanism of action and the type of target bacterial cells that they are going to counteract [42].

So far, many different antibiotic-loaded SFBs have been developed for versatile biomedical applications such as wound healing, bone tissue engineering, treatment of osteomyelitis, and development of sutures, ear tubes, and orthopedic implants (**Table 1.1**). Various physical and chemical methods have been implemented for fabrication of these antibiotic eluting SFBs. Antibiotics can be physically blended in SF aqueous/organic solutions prior to casting, electrospinning, and freeze-drying to obtain antibiotic loaded SF films [43, 44], electrospun fibers [45], or cryogels [41, 46, 47], respectively. In addition to physical approaches, the chemical surface modification of SFBs using plasma treatment has also been reported to be a viable method for conjugation of antibiotics to the surface of SFBs [48, 49]. Choudhury et al. developed amoxicillin trihydrate (AMOX) impregnated SF sutures using oxygen plasma treatment. According to their study, the oxygen plasma treatment of (*Antheraea assama*) silk fibroin (AASF) yarn resulted in the formation of a rougher morphology and oxygen containing functional groups on the surface, leading to an improved hydrophilicity and drug-impregnation efficiency by 16.7%. The oxygen plasma treated AASF/AMOX yarns (AASF/O<sub>2</sub>/AMOX) had a sustained release of AMOX for up to 336 h and showed a clear ZOI against *S. aureus* (54.7 mm) and *E. coli* (19.3 mm) after 24 h *in vitro*. In addition, the *in vivo* analyses revealed the effectiveness of AASF/O<sub>2</sub>/AMOX in mitigating the infection and reducing the healing time in an *S. aureus*-infected mouse wound model after 14 d. Other fabrication methods such as electrophoretic deposition of antibiotic loaded SF on the surface of metallic implants [50, 51], fabrication of layer by layer/coacervate antibiotic loaded coatings on SFBs (e. g. sutures) [52], and synthesis of composite SFBs using antibiotic loaded nanofillers [53] have also been described in the literature. In a recent study by Wu et al. [53],

tetracycline hydrochloride (TCH) was first loaded into the lumen of halloysite nanotubes (HNTs) and then electrospun with SF aqueous solution to create antibacterial SF fibers. Compared to directly loading the TCH into SF fibers, the use of HNTs as an intermediary drug carrier resulted in a significantly more controlled release of TCH from SF fibers and therefore a longer lasting antibacterial effects against *S. aureus* and *E. coli* bacteria *in vitro* (**Table 1.1**).

Despite the ease of use, large availability, and low cost of antibiotics, the emergence of antibiotic-resistant bacteria over the past decades has hindered the application of antibiotic eluting SFBs. Biofilm formation on the surface of biomaterials and implants leads to development of resistant cells by providing an extracellular polymeric matrix composed of lipopolysaccharides, lipids, and proteins around the bacteria which protects them against the host immune system and antibiotics [54]. To eradicate the bacteria in biofilm and eliminate infection, remarkably higher drug doses and longer treatment periods are required compared to their free-living planktonic counterparts. This in turn prolongs and increases the patient's exposure to the drug, resulting in an enhanced risk of unwanted side effects [55].

Considering the inefficacy of the antibiotics in treatment of biofilm related infections, alternative approaches are essential to overcome the issues associated with bacterial resistance. In this regard, a fundamental understanding of antibiotics bactericidal mechanisms and drug-target interactions will be very helpful for development of new formulations and improvement of the old ones to increase their efficacy toward different types of bacteria.

**Table 1.1** - Processing and antibacterial activity of SFBs combined with antibiotics for different biomedical applications.

| Antimicrobial additive (antibiotics) | Other additive(s)    | Synthesis method                                                                                  | Content                               | Antimicrobial capability                                                                                                             | Application                                | Refs |
|--------------------------------------|----------------------|---------------------------------------------------------------------------------------------------|---------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|------|
| <b>Amoxicillin trihydrate</b>        | Chitosan             | Oxygen plasma treatment and dip-coating                                                           | 25 wt%                                | ZOI (25 d):<br><i>S. aureus</i> (5-35 mm)<br><i>E. coli</i> (15-35 mm)                                                               | Surgical suture                            | [48] |
| <b>Amoxicillin trihydrate (AMOX)</b> | ----                 | Oxygen plasma treatment and dip-coating                                                           | 50 µg/mL                              | ZOI (24 h):<br><i>S. aureus</i> (54.7 mm)<br><i>E. Coli</i> (19.3 mm)<br><br>Skin infection reduction in mouse models (after 3-14 d) | Suture/wound healing                       | [49] |
| <b>Ciprofloxacin hydrochloride</b>   | ----                 | Molded hydrogels                                                                                  | 9 mg/mL                               | Killing and growth inhibition of <i>M. catarrhalis</i> after 24 h (quantitative data N/A)                                            | Bioresorbable drug-eluting ear tubes       | [56] |
| <b>Colistin</b>                      | -----                | SF membranes                                                                                      | Log-scale dilutions (0.027-270 mg/mL) | RE (24 h):<br><i>P. aeruginosa</i> (complete elimination at 270 mg/mL)<br><br>CFU (3 d) rat burn infection: >3 log                   | Wound dressing<br>Biodegradable scaffold   | [43] |
| <b>Gentamicin</b>                    | ----                 | Electrophoretic deposition of gentamicin-loaded SF coatings on 3D-printed CoCrMo bone substitutes | 1 mg/mL                               | ZOI (7 d):<br><i>S. aureus</i> (18-22 mm)                                                                                            | Orthopedic implants                        | [50] |
| <b>Gentamicin (Gen)</b>              | Titanium (Ti) foil   | Gen loaded SF NPs deposited on chemically activated Ti foil                                       | 0.1-0.3 wt%                           | ZOI (5-120 min):<br><i>S. aureus</i> (Diameter N/A)<br><br>Reduced bacterial adhesion                                                | Metal based implants                       | [57] |
| <b>Gentamycin sulfate (GS)</b>       | Gelatin microspheres | GS-loaded gelatin microspheres embedded in freeze-dried SF scaffolds                              | 10 wt%                                | ZOI (24 h):<br><i>S. aureus</i> (8.8 mm)<br><i>E. Coli</i> (7.5 mm)<br><br>Burn infection reduction (21 d):<br><i>P. aeruginosa</i>  | Treatment of infected full-thickness burns | [47] |
| <b>Gentamicin sulphate (GS)</b>      | Dextrose             | Dextrose incorporated muga and tasar                                                              | 20-25 mg/samples                      | ZOI (24 h):<br><i>S. aureus</i> (19-21 mm)                                                                                           | Wound dressing                             | [44] |

|                                   |                                                      |                                                                                                      |             |                                                                                                                                                                                       |                              |      |
|-----------------------------------|------------------------------------------------------|------------------------------------------------------------------------------------------------------|-------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|------|
|                                   |                                                      | SF films impregnated with GS                                                                         |             | <i>E. Coli</i> (16-18 mm)                                                                                                                                                             |                              |      |
| <b>Gentamicin sulfate (GS)</b>    | -----                                                | Molded SF-GS hydrogel screws                                                                         | 1.6 wt%     | ZOI (24 h):<br><i>S. aureus</i> (22.2 mm)<br><i>E. Coli</i> (22.6 mm)<br><br>RE (4 weeks):<br><i>S. aureus</i> (97.8 ± 3.9%)<br><i>E. coli</i> (93.4 ± 7.9%)                          | Absorbable fixation screw    | [58] |
| <b>Levofloxacin (LV)</b>          | Hyaluronic acid (HA)                                 | i) Complex coacervate and<br><br>ii) LBL deposition of LV blended HA and SF coatings on CoCrMo alloy | 0.750 µg/mL | RE (24 h- Complex coacervate):<br><i>S. aureus</i> (70%)<br>RE (24 h-LBL deposition):<br><i>S. aureus</i> (30%)<br><br>SEM (24 h):<br>Reduction in <i>S. aureus</i> biofilm formation | Orthopedic implants          | [52] |
| <b>Tetracycline hydrochloride</b> | Halloysite nanotube                                  | Electrospinning                                                                                      | 2 wt%       | ZOI (24 h-7 d):<br><i>S. aureus</i> (24.2 mm-12.8 mm)<br><i>E. Coli</i> (26.8 mm-12.9 mm)                                                                                             | Wound dressing               | [53] |
| <b>Tetracycline (TC)</b>          | Titanium (Ti)                                        | Electrophoretic deposition of TC-modified SF coatings on Ti surfaces                                 | 0.15 v/v    | ZOI (24 h):<br><i>S. aureus</i> (35 mm)<br><i>E. Coli</i> (20 mm)                                                                                                                     | Drug delivery of Ti implants | [51] |
| <b>Vancomycin hydrochloride</b>   | Short-stranded poly (L-lactide) fibers<br><br>Pectin | Electrospun fibers/hydrogel composite                                                                | 3 wt%       | ZOI (24 h):<br><i>MRSA</i> (20.3 mm)                                                                                                                                                  | Drug delivery Bone TE        | [59] |
| <b>Vancomycin (VANCO)</b>         | Gelatin microspheres                                 | VANCO-loaded gelatin microspheres embedded in freeze-dried SF scaffolds                              | 10 % w/w    | ZOI (24 h):<br><i>S. aureus</i> (6.65 mm)<br>No inhibition zone against <i>E. coli</i>                                                                                                | Burn and ulcer treatment     | [41] |
| <b>Vancomycin (VANCO)</b>         | Gelatin nanospheres                                  | Colloid-electrospinning                                                                              | 0.4 wt%     | ZOI (24 h):<br><i>S. aureus</i> (Diameter N/A)                                                                                                                                        | Drug delivery system         | [45] |
| <b>Vancomycin (VANCO)</b>         | ----                                                 | VANCO loaded SFNPs embedded in freeze-dried SF scaffolds                                             | 18.8 wt%    | ZOI (26 d):<br><i>S. aureus</i> (11-14 mm)<br><br>Reducing infection in rats with severe osteomyelitis after 6-weeks implantation                                                     | Treatment of osteomyelitis   | [46] |

RE, reduction efficiency; TE, tissue engineering; LBL, layer by layer; *M. catarrhalis*, *Moraxella catarrhalis*; MRSA, *Methicillin-resistant Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; MIC, minimum inhibitory concentration; CFU, colony forming unit

### 1.3.2 Inorganic nanoparticles

Various inorganic nanomaterials with antibacterial properties such as metal (e. g. Ag, Au, Cu, Ca) and metal oxide nanoparticles (e. g. ZnO, TiO<sub>2</sub>, CaO<sub>2</sub>), transition metal dichalcogenide monolayers (e.g. MoSe<sub>2</sub>), and graphene oxide (GO) nanosheets have been combined with SFBs for different biomedical applications (**Table 1.2**). Compared to antibiotics, it is more difficult for bacterial cells to develop resistance toward these nanomaterials due to their multiple mechanisms of action. The principal mechanism that inorganic nanomaterials employ to kill the invading bacteria is the generation of reactive oxygen species (ROS) [60]. ROS produced at low levels can be neutralized by bacterial cells antioxidant defense mechanisms. However, high concentrations of ROS can overcome these defense systems, causing oxidative damage to critical intracellular components such as enzymes, proteins, DNA, and lipids [61]. In addition to ROS generation, other bactericidal mechanisms of inorganic nanomaterials include disrupting the cell wall, damaging the chromosomes and DNA, and dysregulating the metabolic activity of the microorganisms [62].

Inorganic nanoparticles are typically synthesized using physiochemical approaches which are often costly and require the use of toxic substances and harsh conditions. Therefore, recently increasing attention has been given to simple and green methods of nanoparticles synthesis using biomaterials that are eco-friendlier and more biocompatible [63, 64]. SF is one of such materials that has been used frequently as a biocompatible reducing and stabilizing agent for *in situ* fabrication of various inorganic nanomaterials such as Ag NPs [65-67], Au NPs [68, 69], and CuBTC metal–organic framework (MOF) particles [70]. Being a protein, SF is mainly composed of repeated sequences of glycine, alanine, and serine amino acids with nearly 10% tyrosine

residues evenly distributed throughout the protein sequence [71]. The ionization of tyrosine phenolic moiety at basic pH values and consequent electron transfer to the metal ions is recognized as the main mechanism of reduction by SF [72, 73]. Other reduction mechanisms such as chelation of metal ions and electron donation by unprotonated carboxylic groups at high pH [70] and reduction through the methanolic -OH groups in serine amino acids have also been reported by previous studies [74].

Moreover, the presence of hydrophilic–hydrophobic segments in the SF structure has been shown to facilitate its binding with inorganic NPs such as graphene and transition metal dichalcogenides (TMD) [75]. In a study by Huang et al. [76], carboxyl-modified silk fibroin (CMSF) was used as the exfoliating agent for preparation of MoSe<sub>2</sub> TMD nanosheets. The strong binding and interaction of CMSF carboxyl groups with the TMD atoms resulted in the formation of exfoliated MoSe<sub>2</sub> nanosheets with high efficiency and long-term dispersion stability. The MoSe<sub>2</sub>–CMSF nanosheets exhibited good biocompatibility and high peroxidase-like catalytic activity toward the decomposition of H<sub>2</sub>O<sub>2</sub> into •OH radicals therefore requiring the use of ~1000 times lower H<sub>2</sub>O<sub>2</sub> dose for *in vitro* antibacterial effectiveness against gram-negative *E. coli* and gram-positive *Bacillus subtilis* (*B. subtilis*) bacteria compared to the traditional medical therapy. At physiologically relevant levels of H<sub>2</sub>O<sub>2</sub>, MoSe<sub>2</sub>-based films were also shown to effectively promote the wound healing and prevent infections *in vivo* using Kunming mice with infected skin wounds as models.

In addition to *in situ* fabrication methods, antibacterial SFB composites containing inorganic nanomaterials can also be made *ex situ* by first synthesizing the nanoparticles and then incorporating them into the SFB substrate [77, 78]. Embedding inorganic nanoparticles in SFBs not only endow them with antibacterial activity and improve their mechanical properties but also

prevents excessive NPs leaching, resulting in prolonged antibacterial longevity, reduced cytotoxicity, and enhanced overall biocompatibility [77, 79]. Antibacterial SFB nanocomposites incorporated with *ex situ* synthesized GO [80], ZnO [81-83], TiO<sub>2</sub> [84, 85], and CaO<sub>2</sub> NPs [86, 87] have been previously developed and evaluated for diverse biomedical applications such as wound healing, bone regeneration, and urethral tissue engineering (**Table 1.2**). Dhas et al. fabricated AgNPs impregnated B. mori silk fibers using two different *in situ* and *ex situ* approaches [77]. The results of their study demonstrated that *in situ* prepared fibers had larger ZOI and a higher reduction efficiency after 24 h against both *S. aureus* and *P. aeruginosa* bacteria compared to *ex situ* fabricated fibers.

Despite their versatile biomedical applications and broad-spectrum antibacterial activity, the inorganic nanomaterials/SFBs composites have not yet found their way into clinical practice due to the unforeseen risks associated with their safety and stability in long-term use. Owing to their small size, nanoparticles can cross biological barriers, penetrate tissues, and interact with cells and intracellular organelles leading to membrane disintegration, DNA modification, mitochondrial apoptosis, and cell death. In addition, depending on their physiochemical properties such as shape, charge, and wettability nanoparticles might agglomerate and accumulate in tissues and organs inducing immune responses [88, 89]. Therefore, further research is essential to fully evaluate and identify the effect of nanoparticles size, shape, charge, and chemical composition on their toxicological properties and develop novel, green methods of synthesis to obviate the use of hazardous chemicals and enhance their biocompatibility.

**Table 1.2** - Processing and antibacterial activity of SFBs combined with inorganic nanoparticles for different biomedical applications.

| Antimicrobial additive (Inorganic nanoparticles) | Other additive(s)                                                   | Synthesis method                                                                           | Content                                                          | Antimicrobial capability                                                                                                          | Application                     | Refs |
|--------------------------------------------------|---------------------------------------------------------------------|--------------------------------------------------------------------------------------------|------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|---------------------------------|------|
| <b>AgNPs</b>                                     | -----                                                               | Reduction of Ag ions by SF solution using a microfluidic device                            | 125 $\mu\text{g/mL}$ $\text{AgNO}_3$                             | Live/dead assay:<br>Maximum bacterial death at 3 h<br><br>RE (2 h):<br><i>E. coli</i> (>75%) in an infected mouse model           | Surgical site infections        | [65] |
| <b>AgNPs</b>                                     | Poly(lactic acid (PLA) Nano hydroxyapatite (nHA) Polydopamine (PDA) | Reduction of Ag ions by SF and fabrication of SF/Ag/nHA/PLA composites                     | 50 mM $\text{AgNO}_3$ solution                                   | Turbidity test against <i>E. coli</i> and <i>S. aureus</i> (24 h):<br>Lower absorbance of the medium containing the scaffolds     | Bone TE                         | [67] |
| <b>Ag NPs</b>                                    | -----                                                               | SF as the reducing and stabilizing agent for preparation of SF-AgNPs solutions under light | 0-153.6 mg/L                                                     | MRSA:<br>MIC (19.2 mg/L)<br>MBC (76.8 mg/L)<br><br>CLSM and SEM (10 h):<br>No biofilm formation at concentrations higher than MIC | General biomedical applications | [66] |
| <b>AgNPs</b>                                     | -----                                                               | Dip-coating Tasar SF nanofibrous mats in AgNPs solution                                    | 1 mM $\text{AgNO}_3$ solution                                    | ZOI (12 h):<br><i>S. aureus</i> (10 mm)<br><i>E. coli</i> (14mm)<br><i>P. aeruginosa</i> (8 mm)<br><i>S. epidermidis</i> (10 mm)  | Skin TE                         | [78] |
| <b>AgNPs</b>                                     | -----                                                               | Dip-coating and microwave irradiation of SF fibers in $\text{AgNO}_3$ solution             | 0.5, 1 and 1.5 mmol $\text{L}^{-1}$ of $\text{AgNO}_3$ solutions | Diffusion assay (24 h):<br><i>S. aureus</i> , <i>M. tuberculosis</i> , and <i>E. coli</i> (dose-dependent antibacterial activity) | Wound healing                   | [90] |
| <b>Ag doped hydroxyapatite (HA) NPs</b>          | Chitosan (CS) Strontium (Sr)                                        | Freeze-dried AgSrHA/CS/SF cryogels                                                         | -----                                                            | Turbidity test (24 h):<br>Growth inhibition of <i>E. coli</i> and <i>S. epidermidis</i>                                           | Bone TE                         | [54] |
| <b>AgNPs</b>                                     | Strontium nanotubes (SNT) Titanium (Ti)                             | SF/Ag coating on PDA modified SNTs (created by anodizing Ti)                               |                                                                  | Turbidity test and ZOI (24 h):<br>Antibacterial activity against <i>S. aureus</i> and <i>E. coli</i><br>RE (1-5 d):               | Bone TE                         | [91] |

|              |                              |                                                                                                                                                   |                                         |                                                                                                                                                                                                                    |                     |      |
|--------------|------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|------|
|              | Polydopamine (PDA)           |                                                                                                                                                   |                                         | <i>S. aureus</i> and <i>E. coli</i> (100-70%)                                                                                                                                                                      |                     |      |
| <b>AgNPs</b> | -----                        | The SF solution containing AgNO <sub>3</sub> was exposed to light to form SF/AgNPs films                                                          | 0.1-2 % AgNO <sub>3</sub>               | ZOI (2 d): Antibacterial activity at 0.5-1% against <i>S. aureus</i> and kanamycin resistant <i>E. coli</i> (quantitative data N/A)                                                                                | Bone TE             | [92] |
| <b>AgNPs</b> | -----                        | <i>In situ</i> reduction of Ag ions and fabrication of AgNPs loaded SF gels                                                                       | 26.5±1.24 ppm                           | MIC (6±1.32 ppm) and ZOI (11±2.12mm) against <i>S. aureus</i> after 2 d                                                                                                                                            | Wound healing       | [93] |
| <b>AgNPs</b> | Carboxymethyl chitosan (CMC) | <i>In situ</i> synthesis of AgNPs in SF solution, and fabrication of freeze-dried AgNPs-SF-CMC sponges                                            | AgNO <sub>3</sub> solution (0.05 wt%)   | Turbidity test and ZOI (24 h): Significantly lower OD values and larger inhibition zones against <i>S. aureus</i> and <i>P. aeruginosa</i> compared to control without Ag NPs and compared to AQUACEL® Ag          | Wound healing       | [94] |
| <b>AgNPs</b> | -----                        | <i>In situ</i> reduction reaction between SF and silver nitrate under light                                                                       | 153.6 and 384 mg/L of SF-AgNPs solution | SEM (7 d): Significant reduction of <i>S aureus</i> biofilm at 384 mg/L of SF-NS solution compared with saline.                                                                                                    | Sinusitis treatment | [95] |
| <b>AgNPs</b> | -----                        | Impregnation of silk fibers with AgNPs using <i>in situ</i> (AgNO <sub>3</sub> solution) and <i>ex situ</i> (colloidal AgNPs solution) approaches | AgNO <sub>3</sub> solution (4 mM)       | Higher antibacterial activity of <i>in situ</i> prepared fibers after 24 h<br><i>P. aeruginosa</i> : ZOI (3.3±0.5 mm), RE (99.9%)<br><i>S. aureus</i> : ZOI (2.4±0.4 mm), RE (95%)                                 | Wound healing       | [77] |
| <b>AgNPs</b> | -----                        | Electrospinning SF/AgNO <sub>3</sub> solution and irradiation with UV to obtain SF/AgNPs nanofibers                                               | 0.1%, 0.5%, and 1% wt/v                 | No antibacterial activity at 0.1% and 0.5% against <i>S. aureus</i> and <i>S. epidermidis</i><br>ZOI (24 h) at 1%:<br><i>S. aureus</i> (6.5 mm)<br><i>S. epidermidis</i> (5.6 mm)<br><i>P. aeruginosa</i> (9.3 mm) | Wound healing       | [96] |

|                                                                      |                           |                                                                                                   |                                                                                |                                                                                                                                                                                                                                                                                                                                                                                       |                                                            |      |
|----------------------------------------------------------------------|---------------------------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------|------|
| <b>AgNPs</b>                                                         | -----                     | Dip-coating and <i>in situ</i> photoreduction of Ag ions on SF sutures                            | 0.5 wt/v % AgNO <sub>3</sub> solution                                          | Inhibition zone against <i>S. aureus</i> and <i>E. coli</i> after 24 h (Diameter N/A)<br><br>RE (24 h-bacteria in solution):<br><i>E. coli</i> (78%), <i>S. aureus</i> (81%)<br>RE (24 h-adhered bacteria):<br><i>E. coli</i> (34%), <i>S. aureus</i> (35%)                                                                                                                           | Suture                                                     | [97] |
| <b>Ag@AgCl NPs</b>                                                   | Cellulose acetate (CA)    | Photocatalytic reduction and <i>in situ</i> formation of Ag@AgCl NPs on SF/CA composite membranes | 0.1 M AgNO <sub>3</sub> aqueous solution                                       | ZOI (24 h):<br><i>E. coli</i> (13.99 mm)<br><i>S. aureus</i> (12.59 mm)                                                                                                                                                                                                                                                                                                               | Antibacterial applications                                 | [98] |
| <b>AuNPs</b>                                                         | -----                     | Reduction of AuNPs by SF solution                                                                 | 0.5 mM SF-AuNPs colloidal solution                                             | RE (24 h):<br><i>E. fecalis</i> (85.19 ± 3.24 %)<br><i>S. aureus</i> (32.34 ± 1.70 %)<br><i>E. coli</i> (77.95 ± 9.59 %)<br><i>P. aeruginosa</i> (63.38 ± 4.26 %)                                                                                                                                                                                                                     | Nanoparticles with antibacterial and anticancer properties | [69] |
| <b>AuNPs AgNPs</b>                                                   | Nano-hydroxyapatite (nHA) | <i>In situ</i> reduction of AgNPs and AuNPs by SF/nHA solution and fabrication of hydrogels       | 0, 0.1, 0.5 and 1% HAuCl <sub>4</sub> ·3H <sub>2</sub> O and AgNO <sub>3</sub> | Turbidity test and resazurin assay (24 h):<br>SF/AgNPs (0.1-1 %):<br>Antibacterial activity against MSSA, MRSA, <i>S. epidermidis</i> , <i>E. coli</i> , and <i>P. aeruginosa</i><br><br>SF/AuNPs (≥0.5%):<br>Antibacterial activity against MSSA, MRSA, <i>S. epidermidis</i> , <i>E. coli</i> , and <i>P. aeruginosa</i><br>No antibacterial activity against <i>S. epidermidis</i> | Bone regeneration                                          | [68] |
| <b>Diamino-2-pyrimidinethiol-functionalized Au NPs (DAPT-Au NPs)</b> | -----                     | Aqueous SF/Au-DAPT membranes                                                                      | 27 µg/cm <sup>2</sup>                                                          | MIC and ZOI (20 h):<br><i>E. coli</i> and MDR <i>E. coli</i> (4 µg/mL, ~14 mm)<br><br>Inhibition of growth after 20 h.                                                                                                                                                                                                                                                                | Wound healing                                              | [99] |

|                                                                   |                                               |                                                                                                                               |                                    |                                                                                                                                                                         |                                        |       |
|-------------------------------------------------------------------|-----------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|-------|
| <b>Ca<sup>2+</sup></b>                                            | Polyethylene glycol diglycidyl ether (PEG-DE) | Freeze-dried Ca <sup>2+</sup> loaded SF-PEG-DE porous material                                                                | 1.8, 3.6, 5.4, and 7.2%, (w/w)     | CFU and turbidity test (6 h): Inhibitory effect on the bacterial growth and propagation of <i>E. coli</i> and <i>S. aureus</i>                                          | Hemostatic material                    | [86]  |
| <b>Calcium peroxide (CPO)</b>                                     | Gelatin (GL)<br>Human keratin (HK)            | Physical blending to make composite film scaffolds                                                                            | 20% CPO                            | ZOI (24 h):<br><i>E. coli</i> (3.55 ± 0.24 mm)<br><i>S. aureus</i> (2.89 ± 0.20 mm)                                                                                     | Urethral TE                            | [87]  |
| <b>CuBTC MOF particles</b>                                        | -----                                         | Formation of CuBTC MOF on silk yarns by immersing in Cu(OAc) <sub>2</sub> ·2H <sub>2</sub> O, and H <sub>3</sub> BTC solution | 2-8 dipping cycles                 | ZOI (24 h- IV-cycle 4):<br><i>E. coli</i> (7.7 mm)<br><i>S. aureus</i> (6.5 mm)<br><br>ZOI (24 h- IV-cycle 10):<br><i>E. coli</i> (8.0 mm)<br><i>S. aureus</i> (7.5 mm) | Wound healing and medical applications | [70]  |
| <b>Graphene oxide (GO)</b>                                        | -----                                         | Electrospinning                                                                                                               | 5 wt% GO                           | Survival rate after 18 h:<br><i>E. coli</i> (35.7 ± 3.6 %)<br><i>S. aureus</i> (41.6 ± 0.3%)<br><br>SEM:<br>Shrunk morphology of <i>E. coli</i> on the SF/GO nanofibers | Wound dressing                         | [80]  |
| <b>Graphene oxide (GO)<br/>Reduced GO (RGO)<br/>Graphene (Gr)</b> | -----                                         | Electrospinning                                                                                                               | 1 wt%                              | RE (24 h):<br>Antibacterial activity of SF/GO, SF/RGO, and SF/Gr against <i>S. aureus</i> (92.8%, 98.6%, 80.6%) and <i>E. coli</i> (70.3%, 84.9%, 69.5%)                | TE                                     | [100] |
| <b>Reduced Graphene oxide (GO)<br/>TiO<sub>2</sub></b>            | -----                                         | Electrospinning                                                                                                               | 0.01 wt%                           | RE against <i>S. aureus</i> :<br>SF/RGO: 94.8%<br>SF/TiO <sub>2</sub> : 83.6%<br>SF/GO/TiO <sub>2</sub> : 84.9%                                                         | TE                                     | [101] |
| <b>MgOH<sub>2</sub> nanorods</b>                                  | Polyvinyl alcohol<br>Sodium Alginate          | Freeze-dried Hydrogels                                                                                                        | 0.3 wt%                            | Crystal violet assay (24 h):<br>Inhibition of <i>P. aeruginosa</i> biofilm formation                                                                                    | TE and general biomedical applications | [102] |
| <b>MoSe<sub>2</sub> nanosheets</b>                                | -----                                         | Exfoliation of MoSe <sub>2</sub> nanosheets by carboxyl-modified SF                                                           | MoSe <sub>2</sub> –CMSF nanosheets | Live/dead assay, SEM, turbidity test, and CFU (6 h):                                                                                                                    | Wound dressing                         | [76]  |

|                            |                               |                                                                  |                                                                             |                                                                                                                                                                                         |                            |      |
|----------------------------|-------------------------------|------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|------|
|                            |                               | (CMSF) solution                                                  | (50 $\mu\text{g mL}^{-1}$ )                                                 | Antibacterial activity against <i>E. coli</i> and <i>B. subtilis</i>                                                                                                                    |                            |      |
| <b>TiO<sub>2</sub></b>     | -----                         | TiO <sub>2</sub> /SF blend membranes                             | TiO <sub>2</sub> /SF weight ratios: 1/1000, 3/1000, 5/1000, and 10/1000     | 1/1000 samples showed the highest antibacterial activity<br><br>ZOI (24 h):<br><i>S. aureus</i> (19.0±0.2)<br><i>E. coli</i> (17.0±0.1)<br><i>P. aeruginosa</i> (13.0±0.2)              | Skin TE and wound dressing | [85] |
| <b>TiO<sub>2</sub></b>     | Chitin (Ch)<br>Glycerol (Gly) | Freeze-dried SF/Ch/TiO <sub>2</sub> wound dressings              | 0.5, 1.5, 3.0 % (w/w) TiO <sub>2</sub>                                      | ZOI (24 h):<br><i>S. aureus</i> (19 mm)<br><i>E. coli</i> (20-22 mm)<br><br>CFU (3% TiO <sub>2</sub> ):<br>5-fold decrease in number of colonies                                        | Wound dressing             | [84] |
| <b>ZnO NPs</b>             | Sodium alginate (SA)          | Dip-coating SF fibers functionalized with SA in ZnO NPs solution | 0.5% suspension of ZnO NPs                                                  | ZOI (2-6 d):<br><i>S. aureus</i> (1.9-0.5 mm)                                                                                                                                           | Suture                     | [83] |
| <b>ZnO NPs</b>             | Hyaluronic acid (HA)          | Co-axial electrospinning                                         | 1, 3, and 5% w/v ZnO NPs                                                    | ZOI (24 h) (5% ZnO fibers):<br><i>E. coli</i> (2.93±0.12 mm)<br><i>S. aureus</i> (3.37±0.12 mm)<br><br>CFU (24 h):<br><i>E. coli</i> (28±2 colonies)<br><i>S. aureus</i> (6±1 colonies) | Burn wound healing         | [82] |
| <b>Porous ZnO NPs (PZ)</b> | -----                         | Dip-coating silk thread in PZ solution                           | 50 mM Zn(NO <sub>3</sub> ) <sub>2</sub> ·6H <sub>2</sub> O initial solution | RE (6-8 h):<br><i>S. aureus</i> and <i>E. coli</i> (~80%)<br><br>Suppressing bacterial infection in a mouse model (2 w)                                                                 | Suture                     | [81] |

MDR, multi drug resistant; MSSA, methicillin-susceptible *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; MBC, minimum bactericidal concentration; SEM, scanning electron microscopy; CLSM, confocal laser scanning microscopy

### 1.3.3 Inherently antibacterial polymers and peptides

Polymers and peptides with inherent antibacterial properties have been widely used as additives for SF to develop antibacterial SFBs that can resist infection and function properly when

used *in vivo* (**Table 1.3**). Owing to its biocompatibility, biodegradability, and nontoxic origins, chitosan is the most studied antibacterial polymer in combination with SF for different biomedical applications such as wound healing, development of blood contacting devices, and tissue engineering [103]. Chitosan is a linear cationic polysaccharide produced by basic deacetylation of chitin, originally extracted from the shells of crustaceans and insects. The bactericidal activity of chitosan is believed to be mainly associated with the protonation of amino groups at low pH values ( $\text{pKa} \approx 6.5$ ) and their consequent electrostatic interaction with negatively charged bacterial cell membranes [104]. This interaction causes damages in the bacterial cell wall, impairs vital bacterial functions, and ultimately results in cell death. At higher pH values, where chitosan has a lower charge density, other bactericidal mechanisms such as hydrophobic interactions and chelation effects predominate [105, 106]. Other polymers with inherent bactericidal activity such as polyethylenimine (PEI), polypropylene (PP), and Poly (hexamethylene biguanide) hydrochloride (PHMB) have also been combined with SF for development of antibacterial wound dressings, scaffolds, and sutures (**Table 1.3**). Electrospun SF/PEI nanofibers were shown to have a strong inhibitory effect against gram positive *S. aureus* and gram negative *P. aeruginosa* bacteria after 5h and did not show any *in vitro* cytotoxicity against L929 fibroblast cells [107]. In a study by Gogoi et al. PP was grafted onto multifilament yarns ( $d \approx 100 \mu\text{m}$ ) of a non-mulberry *Antheraea assama* silk fibroin (AASF) through a plasma graft polymerization process to develop antibacterial sutures [108]. AASF yarn was first sterilized using Ar plasma and PP was grafted onto its surface using Ar/propylene plasma discharge. The resulting AASF/PP sutures showed a ZOI of  $35 \pm 0.01$  mm against gram negative *E. coli* bacteria after 24 h *in vitro* and remarkably improved the wound healing process when tested *in vivo* on rabbits, with complete healing after 16 days compared to 25 days for only Ar plasma treated AASF sutures. In another study, PHMB/SF porous sponges

were fabricated through electrostatic interactions and freeze-drying method and were shown to have antibacterial activity against *S. aureus* and *E. coli* bacteria at PHMB/SF ratios of higher than 2/100 when tested *in vitro* by a 24 h disc diffusion assay [109].

In addition to polymers, in the last decade, there has been an increasing interest into antimicrobial peptides (AMPs) as effective antimicrobial therapeutics. AMPs are a diverse class of small molecular weight proteins with broad spectrum antibacterial activity that constitute the first line of defense in the innate immune system of different organisms [110]. AMPs are mainly composed of cationic and hydrophilic regions and can acquire amphipathic  $\alpha$ -helical conformations that facilitate their penetration through the phospholipid bilayer membrane of the bacteria leading to inhibition of vital biological processes and cell death [111]. In fact, the cationic nature of AMPs is attributed to the presence of positively charged amino acids such as lysine and arginine in their structure allowing them to target the negatively charged membranes of bacteria causing perforation and cell lysis as well. Due to their disrupting effects on the bacterial cell wall and alteration of membrane permeability, AMPs are good candidates to be used in conjunction with other antibacterial agents to yield a more efficacious bactericidal activity [112]. In addition to the discussed mechanisms of action, AMPs can also exert their antibacterial effect by modulating and activating the host immune system [113]. Compared to conventional antibiotic treatments, AMPs offer many advantages including higher efficacy, environmental friendliness, and lower possibility of developing bacterial resistance due to their mainly physiomechanical membrane-targeted mechanism of action [114]. In addition, given the neutral zwitterionic composition of eukaryotic cell membranes, AMPs demonstrate minimized mammalian cell toxicity and a highly selective bactericidal activity [115].

There have been several efforts to fabricate antibacterial SFBs using both naturally occurring and synthetic AMPs (**Table 1.3**). Physical fabrication methods reported so far include dip-coating of SF sutures in AMP solution [116] and electrospinning of SF/AMP solutions [117] for development of antibacterial nanofibrous wound dressings. However, to overcome the rapid leaching of the antimicrobial agent associated with the physical methods of synthesis, chemical conjugation approaches have been proposed. Covalent attachment of AMPs to SFBs has been mainly carried out through carbodiimide chemistry. The chemical grafting of AMPs such as cecropin B [118], Cys-KR12 [119], and L-Cysteine [120] onto different SF substrates via EDC-NHS coupling have been reported for fabrication of antibacterial wound dressings and implantable biomaterials that have shown strong antibacterial activity toward both gram positive and gram negative bacteria and had no demonstrated cytotoxicity (further details can be found in **Table 1.3**). Other chemical methods such as enzymatic oxidation of SF with tyrosinase and consequent grafting of  $\epsilon$ -polylysine has also been demonstrated by Wang et al with about  $\sim 1.97$  log reduction in *S. aureus* bacteria after 18 h, however cytotoxicity assays were not performed to further evaluate the biocompatibility of these materials [121]. In addition to physical and chemical methods of synthesis, Gomes et al. employed a bioengineering approach in which they designed and cloned three fusion AMPs (human neutrophil defensin 2, human neutrophil defensins 4, and hepcidin) to modify the spider silk [122]. The genetically engineered spider silk demonstrated antibacterial activity against *E. coli* and *S. aureus* according to a 24 h radial diffusion assay. Nevertheless, some of the major limitations regarding the development of recombinant SF proteins include the poor yield and the challenging scale-up process [123].

Quaternary ammonium compounds (QACs) are another type of polymeric substances with potent antimicrobial properties that have been previously used to develop antibacterial silk sutures

[124]. QACs are cationic surfactants with a nitrogen atom, covalently bonded to four aryl/alkyl chains. Similar to the other antimicrobial polymers and peptides, QACs act on bacteria through electrostatic interactions with the membrane. The lipophilic structure of the alkyl chains enables the QAC permeation through the bacterial membrane where it will disrupt structural proteins and enzymes leading to cell death [125]. The length of the alkyl chains as well as the charge density are determining factors in the bactericidal effectiveness of the QACs [126]. Silk sutures coated with a 25% solution of K21 quaternary ammonium compound demonstrated a long lasting ZOI against *Porphyromonas gingivalis* (*P. gingivalis*) and *Enterococcus Faecalis* (*E. faecalis*) after 12 days in culture.

While the development of antibacterial SFBs using bactericidal polymers and peptides offers great promise *in vitro*, there remains several pitfalls that need to be addressed before their clinical translation. For instance, the emergence of resistant bacteria, although slower compared to antibiotic treatments, have been reported after long term use of AMPs. There are also great concerns regarding the bacteria developing resistance toward host-defense peptides after exposure to high doses of therapeutic AMPs [127]. The mechanisms underlying the evolution of bacterial resistance toward AMPs are described in a review by Sierra et al [112]. Aside from the issues related to bacterial resistance, AMPs face other limitations as well, such as high production costs, short half-life, and lack of stability *in vivo* due to degradation by proteases [128]. In addition, the adverse long-term effects of AMPs are not yet fully known, given that they can induce immunogenicity and demonstrate hemolytic activity according to previous studies [129]. The use of delivery systems such as SFBs has been proposed as a viable solution to overcome some of the mentioned limitations associated with AMPs. However, to further enable their practical clinical applications, active research into designing new AMPs with modified properties (e. g. reduced

toxicity, prolonged stability, and enhanced efficacy) and rigorous *in vivo* studies using suitable animal models are required.

**Table 1.3** - Processing and antibacterial activity of SFBs combined with inherently antimicrobial polymers and peptides for different biomedical applications.

| Antimicrobial additive (polymers and peptides) | Other additive(s)                                   | Synthesis method                                   | Content                                                | Antimicrobial capability                                                                                                                                                      | Application                 | Refs  |
|------------------------------------------------|-----------------------------------------------------|----------------------------------------------------|--------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|-------|
| <b>Carboxymethyl chitosan (CMC)</b>            | -----                                               | LBL CMC coating on electrospun SF nanofibers       | 1mg/ml (1-21 layers)                                   | RE (12 h) and SEM: <i>E. coli</i> (64%)<br><i>S. aureus</i> (67%)                                                                                                             | TE                          | [130] |
| <b>Chitosan (CS)</b>                           | Egg shell membrane (ESM)                            | CS/SF/ESM hydrogels                                | 2% w/v                                                 | ZOI (time N/A):<br><i>E. coli</i> (39 mm)<br><i>Candida albicans</i> (42 mm)                                                                                                  | Cartilage TE                | [131] |
| <b>Chitosan (CS)</b>                           | Nylon6 (N6)                                         | LBL deposition of CS and SF on N6 nanofibrous mats | 1 mg/mL (5-20 layers)                                  | RE (16 h)<br><i>E. coli</i> and <i>S. aureus</i> (> 95%)                                                                                                                      | Pelvic floor reconstruction | [132] |
| <b>Chitosan (CS)</b>                           | Heparin                                             | Heparinized SF blended with CS                     | 2% w/v                                                 | Significant inhibitory effect on <i>S. aureus</i> proliferation compared to samples without CS.                                                                               | Blood contacting devices    | [133] |
| <b>Chitosan (CS)</b>                           | Transforming growth factor- $\beta$ (TGF- $\beta$ ) | UV-crosslinked methacrylated SF NPs with CS        | 6 wt%                                                  | RE (24 h):<br><i>E. coli</i> (83%)<br><i>S. aureus</i> (67%)                                                                                                                  | Articular Cartilage TE      | [134] |
| <b>Chitosan (CS)</b>                           | Polycaprolactone (PCL) Heparin (Hep)                | LBL deposition of Hep/CS on PCL/SF nanofibers      | 1mg/ml (1-10 layers)                                   | RE (16 h):<br><i>E. coli</i> and <i>S. aureus</i> (up to 95%)                                                                                                                 | Vascular grafts             | [135] |
| <b>Chitosan (CS)</b>                           | -----                                               | Electrospinning                                    | CS/SF blend fibers: (80/20); (50/50); (20/80); (0/100) | Turbidity test (24 h):<br><i>E. coli</i> (increased antibacterial activity with increased CS content)                                                                         | Wound dressing              | [136] |
| <b>Chitosan (CS)</b>                           | -----                                               | Freeze-dried CS/SF scaffolds                       | The SF/CS blend ratios: 2:1, 1:1, 1:2, and 1:3         | Turbidity test (24 h):<br><i>S. aureus</i> (higher antibacterial activity of 1:2 and 1:3 samples)<br><br>SEM (24 h):<br><i>S. aureus</i> (fewer adherence on blended samples) | TE                          | [137] |

|                                                                                                                |                         |                                                                            |                                                                |                                                                                                                                         |                                |       |
|----------------------------------------------------------------------------------------------------------------|-------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-------|
| <b>Chitosan (CS)</b>                                                                                           | Rectorite (REC)         | LBL deposition of CS and REC on SF electrospun mats                        | 16 CS and 15 REC layers (1 mg/mL)                              | RE (24 h):<br><i>E. coli</i> (84%)<br><i>S. aureus</i> , (92%)                                                                          | Bone TE                        | [138] |
| <b>Chitosan (CS)</b>                                                                                           | Polydopamine (PDA)      | LBL CS/PDA coatings on SF nanofibers                                       | 15 layers of CS (1 mg/mL) and PDA on 2 cm <sup>2</sup> SF mats | RE (24 h):<br><i>E. coli</i> and <i>S. aureus</i> (~ 98%)                                                                               | General biomedical application | [139] |
| <b>Chitosan (CS)/ Polydopamine nanoparticles (PDA NPs)</b>                                                     | -----                   | Freeze-dried SF/CS/PDA cryogels                                            | ----                                                           | Enhanced antibacterial activity against <i>E. coli</i> and <i>S. aureus</i> (quantitative data N/A)                                     | Wound healing                  | [140] |
| <b>Chitosan NPs (NCS)</b>                                                                                      | Hyaluronic acid (HA)    | Freeze-dried NCS/HA/SF scaffolds                                           | 5% CS solution                                                 | ZOI (24 h):<br><i>E. coli</i> (16 mm)<br><i>Pseudomonas aureus</i> (14 mm)                                                              | TE                             | [141] |
| <b>Chitoooligosaccharide (COS)</b>                                                                             | -----                   | Enzymatic grafting of COS onto SF membranes                                | -----                                                          | RE (24 h):<br><i>E. coli</i> (87.62%)                                                                                                   | General biomedical application | [142] |
| <b>Quaternized chitin</b>                                                                                      | Polycaprolactone (PCL)  | LBL deposition of QC and SF on SF/PCL electrospun mats                     | 7 layers of QC (1 wt%)                                         | RE (5 h):<br>Higher antibacterial activity against <i>S. aureus</i> than <i>E. coli</i> (93.63 ± 2.09%) a                               | Skin TE                        | [143] |
| <b>Polyethyleneimine</b>                                                                                       | Polymethyl methacrylate | Electrospinning                                                            | 1% w/v                                                         | ZOI (24 h):<br><i>P. aeruginosa</i> (Diameter N/A)                                                                                      | Dental applications            | [144] |
| <b>Polyethyleneimine</b>                                                                                       | -----                   | Electrospinning                                                            | 10, 20 and 30% w/w                                             | Complete inhibition of <i>S. aureus</i> and <i>P. aeruginosa</i> growth after 5 h                                                       | Wound dressing                 | [107] |
| <b>Poly(hexamethylene biguanide)hydrochloride (PHMB)</b>                                                       | -----                   | Freeze-dried SF/PHMB sponges                                               | PHMB/SF ratios: (0/100-10/100)                                 | ZOI (24 h):<br><i>E. coli</i> and <i>S. aureus</i> inhibition zone at >2/100 ratios (Diameter N/A)                                      | Wound healing                  | [109] |
| <b>Polypropylene (PP)</b>                                                                                      | -----                   | PP grafted Muga SF sutures fabricated by argon plasma graft polymerization | -----                                                          | ZOI (24 h):<br><i>E. coli</i> (35±0.01 mm)                                                                                              | Suture                         | [108] |
| <b>Human neutrophil defensin 2 (HNP-2), human neutrophil defensins 4 (HNP-4), and hepcidin fusion peptides</b> | -----                   | Cloning and fusing spider silk                                             | 10, 50 and 100 µg/mL protein solutions                         | ZOI (24 h):<br><i>E. coli</i> and <i>S. aureus</i> (highest bactericidal activity for HNP-4 and lowest bactericidal activity for HNP-2) | TE                             | [122] |

|                                               |       |                                                                                                    |                                      |                                                                                                                                                                               |                                |       |
|-----------------------------------------------|-------|----------------------------------------------------------------------------------------------------|--------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|-------|
| <b>6mer-HNP1 antimicrobial peptide</b>        | ----- | Dip coating silk sutures (Perma-Hand®)                                                             | 2 wt%                                | CFU (24 h):<br>MRSA (~1.5 log)<br><i>E. coli</i> (~2 log)                                                                                                                     | Suture                         | [116] |
| <b>HPRP-A2 antimicrobial peptide</b>          | ----- | Electrospinning                                                                                    | 0.01% (wt/v)                         | RE (18 h):<br><i>E. coli</i> (90.39%)<br><i>P. aeruginosa</i> (72.93 %)<br><i>S. aureus</i> (98.19 %)<br><i>S. epidermidis</i> (72.05 %)                                      | Wound Dressing                 | [117] |
| <b>Antimicrobial peptide motif (Cys-KR12)</b> | ----- | Cys-KR12 immobilization onto electrospun SF mats using EDC/NHS and thiol-maleimide click chemistry | 0.15–1.43 nmol per square centimeter | RE (4h):<br>Strong antibacterial properties against <i>S. epidermidis</i> , <i>E. coli</i> , and <i>P. aeruginosa</i> (>3 log)<br><br>SEM:<br>No biofilm formation after 24 h | Wound healing                  | [119] |
| <b>Cecropin B (CB) antimicrobial peptide</b>  | ----- | CB modified SF films by carbodiimide chemistry                                                     | -----                                | RE (24 h):<br><i>E. coli</i> (93.5%)<br><i>S. aureus</i> (91.6%)                                                                                                              | Implantable biomaterials       | [118] |
| <b>L-Cysteine (L-Cys)</b>                     | ----- | Covalent immobilization of L-Cys on SF knitted fabric                                              | -----                                | RE (24 h):<br>High bactericidal efficiency against <i>S. aureus</i> at both acidic pH (78.4-89.3 %) and basic pH (77.3-98.6 %)                                                | Skin disease management        | [120] |
| <b>ε-polylysine (ε-PLL)</b>                   | ----- | enzymatic oxidization of SF with tyrosinase followed by coupling of ε-PLL                          | -----                                | CFU (18 h):<br><i>S. aureus</i> (~1.97 log)                                                                                                                                   | General biomedical application | [121] |
| <b>ε-polylysine (PLS) Chitosan (CS)</b>       | ----- | SF/PLS/CS bioadhesive composite                                                                    | 10 wt% CS/PLS                        | ZOI (120 min):<br><i>S. aureus</i> (10.2 mm)<br><i>E. coli</i> (10.4 mm)                                                                                                      | Wound healing                  | [145] |
| <b>Quaternary ammonium compound (QAC-K21)</b> | ----- | Dip-coating silk suture strips                                                                     | 5%, 10%, 20%, and 25% K-21 solutions | ZOI (12 d):<br>Inhibition zone against <i>P. gingivalis</i> and <i>E. faecalis</i> for 25% solution                                                                           | Suture                         | [124] |

#### 1.3.4 Plant extracts

Plants extracts have been used for medicinal purposes [146] since ancient times and in the last decade, they have gained attention as natural antioxidants and antibacterial agents to be used in combination with polymers such as SFBs for various biomedical applications (**Table 1.4**). Thus far, herbal extracts such as olive leaf, thymol, *Nigella sativa*, Baicalein, *Pistacia terebinthus*, *Pistacia lentiscus*, and *Hypericum empetrifolium*, as well as manuka honey have been combined with SFBs mostly through electrospinning method for wound dressing applications. The antibacterial and antioxidant properties of these plant extracts are believed to be mainly attributed to the presence of abundant phenolic compounds in their structure such as phenolic acids, tannins, flavonoids, carvacrol, and thymoquinone [147, 148]. In an attempt to determine the correlation between the phenolic content and antibacterial activity, Shan. et al studied 46 different herbal extracts by measuring their total phenolic content and evaluating their bacterial inhibition efficiency [149]. They found that the bactericidal activity of an herbal extract is directly influenced by its phenolic content and plant extracts with higher levels of phenolic compounds exhibited stronger antibacterial activity.

Phenolic compounds are important secondary metabolites and a part of defense mechanisms in plants which have potent antioxidant and antibacterial activity. Depending on their type and molecular structure, plant-derived phenolics exhibit different mechanisms of antibacterial action such as interfering with integral proteins and enzymes resulting in the alteration of cytoplasmic membrane, dysfunction in the metabolic activity, impairing of the genetic material, and affecting the electron and nutrient transfer in bacterial cells [149, 150]. Additionally, many phenolic containing herbal extracts have been reported to have anti-quorum sensing and biofilm suppressing activities as well [151]. In a study by Chan et al., baicalein (BAI), a Chinese herbal

extract, was blended with SF to electrospin antibacterial SF fibers for wound dressing applications. Polyvinylpyrrolidone (PVP) was added to the mixture to further control the release of BAI from the dressings. The SF/PVP/BAI mats were tested against *S. aureus* which is one of the most common bacteria found in the mucous membranes and soft tissue infections in humans and 88.2–98.9% of bacteria reduction was observed after 24 h contact with the mats. The SF/PVP/BAI mats were also able to reduce infection and improve the healing rate when tested *in vivo* in a *S. aureus* infected mouse wound model.

Due to their different modes of action compared to antibiotics, herbal phenolic compounds offer promising potential for treatment of the antibiotic resistant bacteria. However, a major setback regarding the use of plant extracts as antibacterial agents is the lack of established protocols and set criteria for evaluation of their antibacterial effect resulting in contradictory results. Moreover, the high diversity of phenolic compounds and their different antibacterial effects against various bacterial strains also add up to the complication [152]. Therefore, there is a need for more defined and standard methods of investigation to make direct comparison between the results of different studies possible.

**Table 1.4** - Processing and antibacterial activity of SFBs combined with plant extracts for different biomedical applications.

| Antimicrobial additive (Plant extracts)        | Other additive(s)           | Synthesis method | Content     | Antimicrobial capability                                                                               | Application    | Refs  |
|------------------------------------------------|-----------------------------|------------------|-------------|--------------------------------------------------------------------------------------------------------|----------------|-------|
| <b>Chinese herbal extract (Baicalein, BAI)</b> | Polyvinyl pyrrolidone (PVP) | Electrospinning  | 18 wt % BAI | RE (24 h): <i>S. aureus</i> (88.2–98.9%)<br><br>Reduction of infection in a wound mouse model (0-25 d) | Wound healing  | [153] |
| <b>Elaeagnus Angustifolia</b>                  | polyvinyl alcohol           | Electrospinning  | 10 v/v%     | RE (24 h): <i>S. aureus</i> (99.5±0.3 %)<br><i>E. coli</i> (99.2±0.4%)                                 | Wound dressing | [154] |

|                                                                              |                                         |                                                      |                                                          |                                                                                                                                                                                                                                                                   |                                      |       |
|------------------------------------------------------------------------------|-----------------------------------------|------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------|-------|
| <b>Manuka honey (MH)</b>                                                     | -----                                   | Electrospinning<br><br>Methanol crosslinked cryogels | 5% of MH with 5 and 20 unique manuka honey factor (UMF)  | ZOI (24 h-cryogels): <i>E. coli</i> and <i>S. aureus</i> (~ 0.16 cm)<br>ZOI (24 h-nanofibers): <i>E. coli</i> and <i>S. aureus</i> (0.5-1 cm)<br><br>CFU and SEM (4 h-adhered bacteria): Significant reduction of bacteria compared to control                    | Treatment of chronic bone infections | [155] |
| <b>Manuka honey (MH)</b>                                                     | Polyethylene oxide (PEO)                | Electrospinning                                      | 0, 10%, 30%, 50% and 70% (wt/v) MH (UMF 5 <sup>+</sup> ) | ZOI (18 h-SF/MH(70%)): <i>E. coli</i> (7.6 mm), <i>S. aureus</i> (4.5 mm), <i>P. aeruginosa</i> (8.3 mm), and MRSA (6.7 mm)<br><br>RE measured by turbidity test (24 h): <i>E. coli</i> (51%), <i>S. aureus</i> (29%), <i>P. aeruginosa</i> (57%), and MRSA (40%) | Wound dressing                       | [156] |
| <b>Nigella sativa (NS)</b>                                                   | -----                                   | Electrospinning                                      | 1 and 1.5 % NS                                           | ZOI (24 h-SF/NS1%): <i>E. coli</i> (8mm) and <i>S. aureus</i> (12mm)<br><br>ZOI (24 h-SF/NS1.5%): <i>E. coli</i> (10mm) and <i>S. aureus</i> (14mm)                                                                                                               | General biomedical application       | [157] |
| <b>Olive leaf extract (OLE)</b>                                              | Hyaluronic acid (HA)                    | Co-axial electrospinning                             | 12 and 15% (w/v) OLE                                     | RE (0-1 h): <i>S. aureus</i> and <i>E. coli</i> (99.98-100.00%)                                                                                                                                                                                                   | General biomedical application       | [158] |
| <b>Oregano essential oil (OEO)</b>                                           | Poly (L-lactide-co-caprolactone) (PLCL) | Electrospinning                                      | 2.5 and 5 v/v%                                           | Turbidity test RE (24 h): <i>S. aureus</i> (93.93±1.23 – 100±0.61%)<br><i>E. coli</i> (52.91±1.48 – 98.02±0.34%)<br><br>ZOI against <i>S. aureus</i> and <i>E. coli</i> after 24 h (quantitative data N/A)                                                        | Wound dressing                       | [159] |
| <b><i>Pistacia terebinthus</i> (PT), <i>Pistacia lentiscus</i> (PL), and</b> | Chitosan (CS)                           | Dip-coating SF/CS blend films in                     | 0.05 g of films soaked in 2 mL of                        | Turbidity test (24 h): Growth inhibition of <i>E. coli</i> and <i>S. epidermidis</i>                                                                                                                                                                              | Wound healing                        | [160] |

|                                                |                                                          |                                   |                                           |                                                                                                                           |                   |       |
|------------------------------------------------|----------------------------------------------------------|-----------------------------------|-------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|-------------------|-------|
| <b><i>Hypericum<br/>empetrifolium</i> (HE)</b> |                                                          | different plant<br>extracts       | 4% (w/v)<br>PT, PL,<br>and HE<br>extracts |                                                                                                                           |                   |       |
| <b>Thymol (THY)</b>                            | Polycaprol<br>actone<br>(PCL)<br>Hyaluronic<br>acid (HA) | Electrospinning                   | 5 mg/mL<br>THY                            | RE and SEM (24 h):<br><i>S.aureus</i> (87.42%)<br>and <i>P.aeruginosa</i><br>(58.43%)<br>No biofilm formation             | Wound<br>dressing | [161] |
| <b>Vanillin (V)</b>                            | Keratin (K)                                              | SF/K/V freeze-<br>dried scaffolds | 20 mM                                     | ZOI and RE (24 h):<br><i>E. coli</i> (93.3%)<br><i>S. aureus</i> (68.4%)<br><br>> 50% reduction<br>efficiency after 48 h. | Skin TE           | [162] |

### 1.3.5 Bioinspired approaches

Biofilm formation on the surface of indwelling medical devices and implants is a major problem leading to implant failure, significant morbidity and even mortality among the patients. The biofilm starts to form when bacteria adhere to a surface and produce an extracellular polymeric substance which secures their attachment and growth [163]. Therefore, there have been many efforts to provide the surface of biomedical materials with antibiofouling properties. In this regard, nature provides an infinite source of inspiration for researchers and scientists to design surfaces that can repel bacteria and inhibit their colonization. Several examples of natural antibiofouling surfaces can be found in animals' skin (e.g. shark, gecko), insect wings (e.g. cicada, butterfly), and plant leaves (e.g. lotus, rice) that have evolved unique topographical and chemical features to resist bacterial contamination [164]. The presence of hierarchical micro/nanoscale patterns on the surface of these living organisms endow them with low adhesion and superhydrophobic and self-cleaning properties. Micro/nano-patterned surfaces minimize the attachment of bacteria by offering a significantly smaller contact area and an intrinsically low surface energy. They can also kill the bacteria upon contact by physically rupturing or deforming their cell wall membrane [165].

Inspired by the intricate architecture of natural antifouling surfaces and with recent advances in micro/nano-fabrication techniques, many researchers have attempted to replicate these naturally occurring topographies with the hope of reproducing their behavior on the surface of biomedical devices and implants [166]. Considering that this field is still in its infancy, there are only a few number of studies regarding the fabrication of micro/nano patterned SFBs for antifouling biomedical applications (**Table 1.5**). In a study by Mehrjou et al., homogeneous nanocones were fabricated on the surface of SF films by oxygen plasma etching for orthopedic implant application [167]. The plasma etching process resulted in the formation of new hydroxyl bonds on the surface of the nanopatterned films therefore increasing the surface energy by around 176%. The hydrophilic nanopatterned SF films could reduce the adhesion of both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria by more than 90%, while increased the proliferation of osteoblast cells by 30%. In another study by Tullii et al. [168] nanostripes and microwells with different diameters were fabricated on the surface of SF films employing a soft lithography approach. The results of their study demonstrated that the patterned SF films were able to reduce the number of adhered *E. coli* by 66% compared to flat SF films and at the same time supported the adhesion and proliferation of mammalian cells (HEK-293).

Novel bioinspired development of antifouling micro/nano patterned surfaces provides an alternative drug-free route for prevention of biomaterials associated infections. In contrary to the conventional chemistry-based approaches which require the continuous low-dose release of antibacterial agents leading to the emergence of resistant bacteria, the bioinspired micro/nano scale surface topographies promise a long-term solution, killing the bacteria through a mechanical mechanism [169]. However, this also poses a limitation on the use of micro/nano-patterned surfaces for antibacterial purposes as the bactericidal effect is only achieved if the bacteria come

into direct contact with the surface. Moreover, there are concerns regarding the mechanical stability of these patterns for long-term *in vivo* applications. Physical defects in the fabricated micro/nano-structures might cause complications such as localized platelet accumulation, biofilm formation, and unwanted cytotoxic effects [170]. It is also important to note that the size, shape, spacing and type of the micro/nano-patterns as well as the type of invading bacteria dictates the bactericidal response [171]. Therefore, finding a particular surface pattern that is effective against all types of bacteria remains a major scientific challenge. In addition to the bactericidal activity, the cytocompatibility of the micro/nano-patterns should also be considered carefully. Cytotoxic responses have been reported in the past for micro/nano scale surface structures with high aspect ratio [172]. Therefore, utmost importance must be given to investigating the stability and potential short and long-term effects of these surfaces in future studies.

In addition to surface-mediated bactericidal mechanisms, many living organisms produce antibacterial molecules as part of their defense system against pathogens. One of such natural products is nitric oxide (NO), a free-radical gas molecule with manifold physiological functions [173]. NO is produced endogenously from L-arginine by nitric oxide synthase enzymes in the  $10^{-12}$ - $10^{-6}$  M range. At lower nanomolar concentration ( $10^{-12}$ - $10^{-9}$  M), NO is produced by endothelial (eNOS) and neuronal (nNOS) nitric oxide synthases to regulate vasodilation, angiogenesis, and neurotransmission and at higher micromolar concentrations ( $10^{-9}$ - $10^{-6}$  M), it is produced by inducible nitric oxide synthase (iNOS) in macrophages as a potent antimicrobial agent to eradicate foreign pathogens [174].

NO possesses a broad-spectrum antimicrobial activity, acting upon bacteria, viruses, and fungi through multiple different mechanisms [175]. Owing to its small size and lipophilic nature, NO can easily diffuse through the bacterial cell membranes where it can cause detrimental effects on

different bacterial cell components and functions. Being a free radical, NO readily reacts with oxygen and superoxides to generate reactive nitrogen and oxygen byproducts that can impose oxidative and nitrosative damage on bacterial membrane lipids, proteins, metabolic enzymes, and DNA leading to their inhibition or death [176]. The multi-mechanistic antibacterial activity of NO makes it extremely difficult for bacteria to develop resistance, therefore, offering a promising alternative to conventional antibiotics [177, 178].

In the recent years, many researchers have attempted to mimic the endogenous production of NO by developing materials that can deliver NO exogenously for different biomedical applications such as blood contacting medical devices [179-181], urinary catheters [182], endotracheal tubes [183], tissue engineering scaffolds [178, 184], and wound dressings [185, 186]. To overcome the limitations associated with direct delivery of NO including its gaseous state, high reactivity, and short half-life, various NO donor molecules (organic nitrates and nitrites, *N*-diazoniumdiolates, and *S*-nitrosothiols (RSNOs)) have been developed and incorporated into organic and inorganic substrates [187]. Incorporation of the NO donors in a substrate offers many advantages such as improving the NO release kinetics, allowing for a more sustained and tunable NO delivery, and minimizing the potential cytotoxic effects, therefore enabling the development of versatile biocompatible materials with potent antibacterial activity [188]. In addition, exogenous delivery of NO at concentrations as low as 450 pM has been shown to effectively disperse antibiotic resistant biofilms into a planktonic state by increasing the intracellular phosphodiesterase activity of the biofilm, leading to an elevated rate of cyclic di-GMP degradation, ultimately resulting in the disintegration of the biofilm [189].

Natural polymers such as SF offer an interesting platform for NO delivery due to their biocompatible degradation products, reduced immune response, large availability, and lower cost

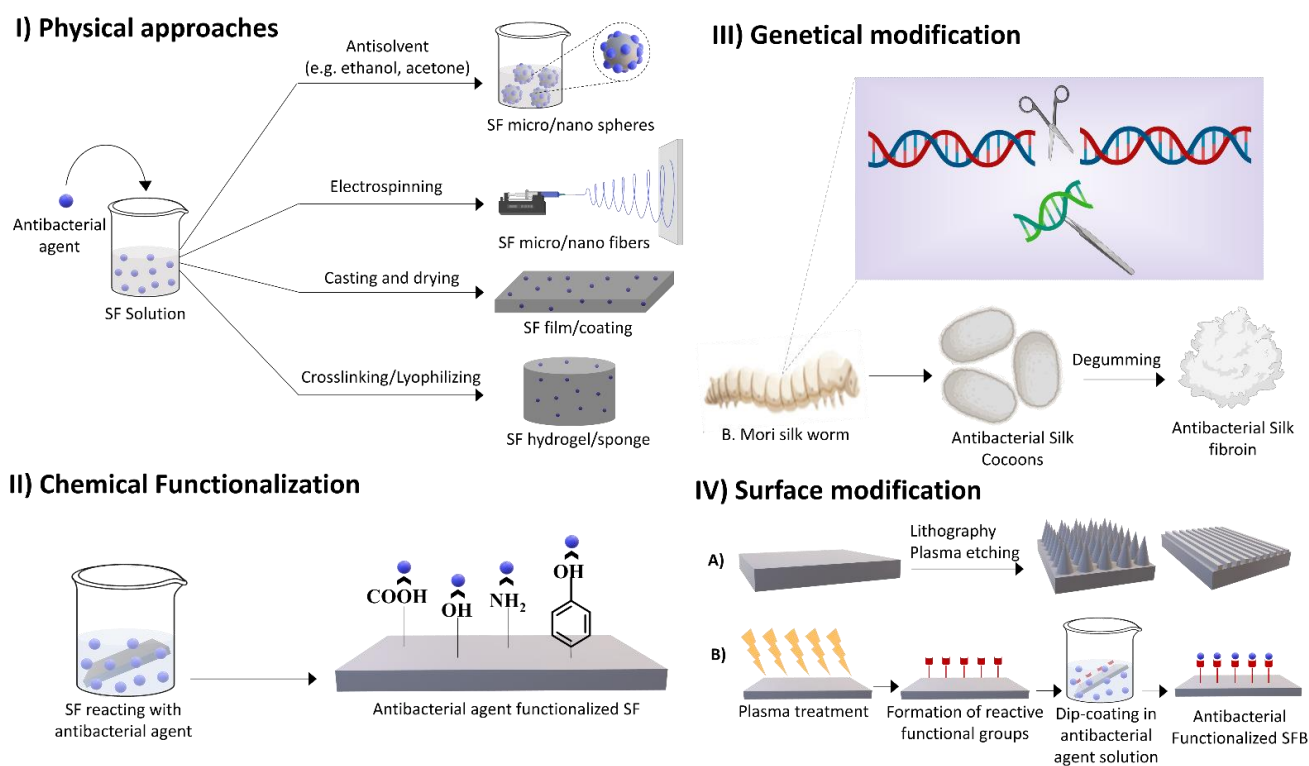


**Figure 1.4** - Different methods of developing antibacterial silk fibroin-based biomaterials.

compared to synthetic delivery platforms [190]. Our group has recently demonstrated the fabrication of NO releasing SF NPs through an antisolvent/self-assembling approach [191]. S-Nitroso-*N*-acetylpenicillamine (SNAP), a synthetic tertiary RSNO, was loaded into SF NPs by adding SNAP/ethanol solution to an aqueous SF solution and freeze-thawing the mixture. The prepared SNAP-SF NPs could release a total of  $1.31 \pm 0.02 \times 10^{-10} \text{ mol mg}^{-1} \text{ NO}$  over a 24 h period and showed a concentration-dependent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. coli*, inhibiting more than 99.9% of the bacteria at a concentration of 10 mg/mL. This study was the first to demonstrate the implementation of NO as an antibacterial additive for SF and therefore can open up new avenues for more exploration in this area and development of novel NO releasing antibacterial SFBs in the future.

**Table 1.5** - Processing and antibacterial activity of SFBs developed through bioinspired approaches for different biomedical.

| Antimicrobial additive (Bioinspired)          | Other additive(s) | Synthesis method                                        | Content                               | Antimicrobial capability                                                                                    | Application                    | Refs  |
|-----------------------------------------------|-------------------|---------------------------------------------------------|---------------------------------------|-------------------------------------------------------------------------------------------------------------|--------------------------------|-------|
| <b>Micro-nano patterns</b>                    | -----             | Soft lithography                                        | Nanostrips and microwell morphologies | Fluorescent imaging and turbidity test (24 h): 66% decrease in the number of adhered <i>E. coli</i>         | Antifouling biomaterials       | [168] |
| <b>Nanopatterns</b>                           | -----             | oxygen plasma etching                                   | Homogeneous nanocones                 | SEM and live/dead assay (6-24 h): Reduced attachment of <i>E. coli</i> and <i>S. aureus</i> bacteria (>90%) | Orthopedic implants and TE     | [167] |
| <b>S-Nitroso-N-acetylpenicillamine (SNAP)</b> | -----             | SNAP loaded SF NPs synthesized by an antisolvent method | 9.1 ± 0.6% (w/w) SNAP                 | RE (24 h- 0.25 to 10 mg/mL): MRSA (48.17 to 99.99%)<br><i>E. coli</i> (53.77 to 99.96%)                     | General biomedical application | [192] |

### 1.3.6 Combination approaches

The multi-drug resistant bacteria, emerged by the widespread use of antibiotics and other antimicrobial agents, pose a severe threat to public health worldwide. One effective approach to combat this type of bacteria is the combinational use of different antibacterial agents [193]. Combination therapies using antibacterial agents with multiple targets of action can reduce the chances of development of resistance, decrease the use of excess drug doses and occurrence of adverse side effects, and often results in synergistically higher antibacterial activity [194].

Thus far, development of antibacterial SFBs using chitosan along with other antibacterial agents such as penicillin [195], ZnO nanoparticles [196], and water crude longan seed extract [197] have been reported in the literature. These studies are all in agreement that a higher bactericidal efficacy can be obtained by using a combination of chitosan with another antibacterial agent as additives for SFBs (**Table 1.6**). Moreover, in a study by Zhou et al. Ag NPs in combination with gentamicin

were used to fabricate antibacterial SF based coatings for Ti implants [198]. The results of the study showed a significantly higher bactericidal activity by synergistic combination of gentamicin and Ag NPs at concentrations far below their minimum inhibitory concentration. The prepared coatings were able to reduce the bacterial adhesion on the surface of Ti implants by 95%. In another study by Vieira et al. Ag NPs were reduced by SF in a basic condition to make conductive SF/Ag NPs gels [199]. By applying an electric current to the gels, 50% higher ROS generation were obtained compared to control. The SF gels made with 50% AgNO<sub>3</sub> had a conductivity of 1.5 S cm<sup>-1</sup> and were able to inhibit 80% of *E. coli* in 1 min with electric current being applied. The fabrication of electrospun SF based nanofibers containing a combination of S-Nitrosoglutathione (GSNO) NO donor molecules and type I collagen peptides (CP) has also been reported as an antibacterial wound dressing for treatment of ischemic chronic wounds [200]. According to a 24 h disk diffusion assay the fibers containing CP alone showed only moderate antibacterial activity against *E. coli* and *P. aeruginosa* and low activity against *S. aureus*, while addition of GSNO significantly increased the corresponding zones of inhibition and antibacterial activity against these bacteria.

Overall, the combination approaches offer an interesting alternative to inefficient monotherapy methods and can reduce the risks associated with the emergence of antibacterial resistance. With a multitude of antibacterial agents available and different material formats that SF can acquire, there is an opportunity to design a plethora of novel antibacterial SFBs. However, the exact molecular mechanisms of all antibacterial components and their cross interactions should be carefully considered before fabrication to obtain a highly synergistic bactericidal efficiency and prevent any undesired side effects, respectively.

**Table 1.6** - Processing and antibacterial activity of SFBs combined with multiple antibacterial agents for different biomedical applications.

| Antimicrobial additive (Combination)                                | Other additive(s)                  | Synthesis method                                                                                 | Content                                                                                           | Antimicrobial capability                                                                                                                                                                                                    | Application                          | Refs                         |
|---------------------------------------------------------------------|------------------------------------|--------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------|------------------------------|
| <b>AgNPs<br/>Gentamicin (Gen)</b>                                   | Titanium (Ti)<br>Polydopamine (PD) | Dip coating Ti implants with SF/AgNPs/Gen solutions                                              | AgNO <sub>3</sub> (2–120 mM/L)<br>Gen (200–1200 µg/L)                                             | MIC against <i>S. aureus</i> :<br>SF/AgNPs (>120 mM/L)<br>SF/Gen (1000–1200 µg/L)<br>SF/AgNPs/Gen (Removal of all bacteria at Gen (200 µg/L) and Ag <sup>+</sup> (10 mM/L))<br><br>RE of adhered bacteria after 24 h (>95%) | Osteogenetic applications            | [198]<br>[201]<br>[202, 203] |
| <b>AgNPs<br/>Electric current</b>                                   | -----                              | <i>In situ</i> reduction of Ag <sup>+</sup> by SF and fabrication of SF/AgNPs gels               | 0%, 5%, 10%, 25%, and 50% (w/w) AgNO <sub>3</sub> with electric current of 1.5 S cm <sup>-1</sup> | RE (1 min-50% AgNO <sub>3</sub> ):<br><i>E. coli</i> (80%)                                                                                                                                                                  | Wound healing                        | [199]                        |
| <b>Chitosan (CS)<br/>Penicillin (PEN)</b>                           | -----                              | Oxygen plasma treatment of <i>Antheraea assama</i> SF yarn and functionalization with CS and PEN | CS (760 ± 6 µg) on each yarn (90 cm)<br>PEN (25–250 µg)                                           | ZOI (24 h):<br><i>B. subtilis</i> (30.2±0.4 mm)<br><i>E. coli</i> (6.3±0.4 mm)                                                                                                                                              | Blood-contacting biomaterials        | [195]                        |
| <b>Chitosan (CS)<br/>Water crude longan seed extract (WLS)</b>      | -----                              | CS/SF/WLS injectable hydrogels                                                                   | ----                                                                                              | RE (2 h):<br><i>E. coli</i> (100%)<br><i>S. aureus</i> (86%)                                                                                                                                                                | Bone TE                              | [197]                        |
| <b>Chitosan (CS)<br/>ZnO</b>                                        | Polyvinyl alcohol (PVA)            | Dip-coating SF/PVA composite films in CS-ZnO NPs solution                                        | 0.2, 0.5, and 1 wt% Cs-ZnO NPs                                                                    | ZOI (24 h-1 wt%):<br><i>S. aureus</i> (4 mm), <i>E. coli</i> (5 mm), and <i>P. mirabilis</i> (4 mm)                                                                                                                         | Wound dressing                       | [196]                        |
| <b>Type I collagen peptide (CP)<br/>S-Nitrosoglutathione (GSNO)</b> | Polyvinyl alcohol (PVA)            | Electrospinning                                                                                  | 1% and 0.5% (w/v) of GSNO and CP                                                                  | ZOI (24 h-CP/SF/PVA):<br><i>E. coli</i> (18 ± 0.28 mm)<br><i>P. aeruginosa</i> (17 ± 0.31 mm)                                                                                                                               | Treatment of ischemic chronic wounds | [200]                        |

|  |  |  |  |                                                                                                                                                                                        |  |  |
|--|--|--|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|--|
|  |  |  |  | <i>S. aureus</i> ( $9 \pm 0.16$ )<br>ZOI (24 h-<br>CP/GSNO/SF/PVA)<br><i>P. aeruginosa</i> ( $22 \pm 0.24$ )<br><i>S. aureus</i> ( $20 \pm 0.27$ )<br><i>E. coli</i> ( $23 \pm 0.19$ ) |  |  |
|--|--|--|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|--|

#### 1.4 Antibacterial silk fibroin-based biomaterials in clinical trial

As an FDA approved material, SF has been the subject of considerable interest for development of cosmetics, biomedical products, and clothing. Many of these products such as silk masks, gels, sutures, wound dressings (Fibroheal™ Ag), tissue scaffolds (FibroFix™, SilkVoice®), and textiles (DermaSilk®) have already been commercialized and can be found on the market while several others are awaiting to pass clinical trials [204, 205]. The review paper by Holland et al. on biomedical uses of SF, presents a detailed overview of the previously passed and currently ongoing clinical trials of silk-based biomedical materials [206]. Owing to their low inflammatory responses, flexibility, oxygen permeability, tunable degradation, and good mechanical properties, SFBs are majorly used as scaffolds for tissue engineering and wound healing applications. Several examples of SF-based scaffolds, wound dressings, and surgical meshes (HQ® Matrix Soft Tissue Mesh) exist that their safety and efficacy have been tested through clinical trials and are soon to be commercially available [205]. In a recent study, a bi-layered wound dressing was fabricated by mixing SF, gelatin and sericin solutions and casting them on a silk fabric. The top-layer was crosslinked using glutaraldehyde and after several washing with glycine and deionized water, it was freeze-dried and gamma-sterilized. The final product was compared to the commercial Bactigras and showed satisfactory results in preclinical and the randomized clinical trials for safe and effective full-thickness skin wound healing [207]. The results of the preclinical evaluation of a three-layered SF-based nerve conduit (SilkBridge™), developed by an electrospinning method, was published recently [208]. When tested in a model of

rat median nerve injury, the SilkBridge™ conduit demonstrated great ability to repair the function and recover the morphology of the median nerve similar to the results obtained by the reference autograft nerve reconstruction method. The SilkBridge™ nerve conduits are currently undergoing clinical studies to evaluate their safety and efficacy for the healing of digital nerve defects in humans ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03673449) identifier: NCT03673449). With the increasing number of SFBs becoming clinically available, combination of antibacterial agents into these materials can be a great strategy to further improve their abilities and broaden their range of applications. Despite a large body of research regarding antibacterial SFBs in the recent years and their promising applications, these materials have scarcely found their way into clinical applications. However, due to the rapidly growing nature of this field and the myriad of potential applications of antibacterial SFBs, their emergence in clinical settings is highly anticipated in the years to come.

## **1.5 Summary**

Silk fibroin (SF) is a natural polymer that has been dominantly used for different biomedical applications owing to its unique combination of properties including super biocompatibility, excellent mechanical properties, controllable biodegradation, and versatile material formats. Many SF-based biomaterials (SFBs) have been developed so far that are used as tissue engineering scaffolds, wound dressings, sutures, and medical device coatings. Regarding the lack of inherent antibacterial activity of SF and with bacterial infection being the principal cause of biomaterials failure, there have been many attempts to endow SFBs with antibacterial properties and span their range of application. The water-based processing of SF, along with the presence of multiple functional groups (e.g. hydroxyl, carboxylic acid, and amine) in its structure have enabled the physical loading or chemical functionalization of SFBs with different antibacterial agents such as antibiotics, antibacterial polymers/peptides, and phenolic plant

extracts. In addition, the electron donating ability of SF at basic pH has made it a potent reducing and stabilizing agents for green synthesis of several bactericidal inorganic nanoparticles such as Ag NPs and Au NPs, facilitating the fabrication of antibacterial SF-based nanocomposites. However, despite a large body of studies in this area, development of bacterial resistance, low stability, high cost, and potential cytotoxic effects mark some of the major limitations associated with the use of these antibacterial agents.

Recently, increasing interest have been directed toward designing bactericidal surface patterns on SF substrates inspired by natural surfaces such as insect wings, animals' skin, and plant leaves, that can repel or kill the bacteria upon contact. These bioinspired approaches advantageously eliminate the use of toxic chemicals and have proved effective in reducing the surface fouling and mitigating bacterial infections. Although promising, this field is still in the early stage of its growth and further studies are required to assess the long-term efficacy of these patterns *in vivo* and find an optimized range of design parameters for maximal bactericidal action and minimal cytotoxic effects. Another recently emerging bioinspired technique to provide SFBs with antibacterial activity is the use of nitric oxide donor molecules. NO donors have been previously incorporated into various synthetic polymers for development of antibacterial medical devices and coatings and have recently found their way into natural polymers as well. Due to the versatile physiological roles of NO beside its antibacterial action, this method is highly promising for development of antibacterial SF-based platforms with therapeutic activity for different biomedical applications.

Considering the different bactericidal mechanisms offered by various methods, one powerful approach is to use a combination of these methods to design SFBs with strong synergistic bactericidal activity. However, most approaches lack adequate characterization information with

unknown long-term effects and are not yet ready for clinical translation. Therefore, thorough *in vivo* studies, establishment of standard verification test protocols and criteria, and full understanding of the bactericidal mechanisms of each approach are areas that need to be worked on in future.

## **1.6 Statement of dissertation defense**

Despite a large body of research existing on NO-releasing materials, the use of natural materials for NO delivery and other biomedical applications has not yet been extensively explored. The goal of this dissertation research is to develop biocompatible NO-releasing platforms such as nanoparticle delivery systems, hydrogels, and nanofibers based on natural materials for drug delivery and tissue engineering applications. This dissertation was prepared using a manuscript-style format. Each chapter is derived from previously published articles or manuscripts under revision in peer-reviewed scientific journals.

Chapter 2 and 3 describe the fabrication of nanoparticle platforms based on naturally derived materials for controlled and tunable NO delivery. In chapter 2, the NO donor molecule, SNAP, was physically encapsulated in SF NPs using an antisolvent/self-assembly approach. The hydrophobic interactions between SNAP and SF macromolecules were studied by Fourier transform infrared (FTIR) deconvolution analysis of amide I band. In addition, the fabricated SNAP-SF NPs were tested *in vitro* to assess their NO release kinetics, storage stability, antibacterial activity, and cytotoxicity. This work has been published in the journal of ACS Applied Materials & Interfaces [191].

In chapter 3, covalent immobilization of SNAP onto halloysite nanotubes (HNTs) was demonstrated thorough an aminosilane crosslinker called aminopropyltrimethoxysilane (APTMS). The chemical modification of HNTs and retention of nanotube morphology throughout the

modification were confirmed by Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Nitric oxide release under different stimuli, total NO content, and storage stability at different conditions were determined by chemiluminescence. Finally, the antibacterial properties and cytocompatibility of the NO-releasing HNTs were examined. This work has been published in the Journal of Colloid and Interface Science [191].

Chapter 4 and 5 demonstrate the development of macroscale NO-releasing materials such as hydrogels and nanofibers based on natural materials for tissue engineering applications. In chapter 4, SNAP-swelled interpenetrating network (IPN) hydrogels based on SF and gelatin methacryloyl (GelMA) were prepared. The fabricated hydrogels were observed under SEM to study their morphology and microstructure and analyzed through FTIR to assess their structural and conformational properties. The optimal swelling time, maximum SNAP loading, and NO release from the hydrogels were measured. In addition, the mechanical properties of the hydrogels along with their enzyme mediated degradation profile were examined. Finally, the *in vitro* antibacterial activity and cytocompatibility of the hydrogels were also investigated to evaluate their suitability as potential scaffolds for tissue engineering applications. This work has been published in the Journal of ACS Biomaterials Science & Engineering [209].

In chapter 5, nitric oxide (NO) donor molecule, S-nitrosoglutathione (GSNO), and the natural biodegradable polymer, zein (ZN), were combined with SF to develop antibacterial and biodegradable nanofibrous scaffolds for tissue engineering applications. The NO-releasing SF-ZN nanofibers (SF-ZN-GSNO) were observed under SEM to evaluate their morphology and size distribution. The long-term enzymatic degradability of the fibers along with their wettability and mechanical properties under uniaxial tensile loading were tested. In addition, the cumulative

leaching of GSNO from the fibers and their NO release kinetics were studied. Moreover, *in vitro* antibacterial activity of the scaffolds against gram-positive and gram-negative bacteria and their cytocompatibility with 3T3 fibroblast cells were also investigated.

## **CHAPTER 2**

### **SILK NANOPARTICLES: A NATURAL POLYMERIC PLATFORM FOR NITRIC OXIDE DELIVERY IN BIOMEDICAL APPLICATIONS<sup>2</sup>**

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<sup>2</sup> Ghalei S, Mondal A, Hopkins S, Singha P, Devine R, Handa H. Silk Nanoparticles: A natural polymeric platform for nitric oxide delivery in biomedical applications. *ACS Applied Materials & Interfaces*. 2020 Nov 18;12(48):53615-23. Reprinted here with permission of the publisher.

## 2.1 Abstract

In this study, the preparation and characterization of nitric oxide (NO) releasing silk fibroin nanoparticles (SF NPs) are described for the first time. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) loaded silk fibroin nanoparticles (SNAP-SF NPs) were prepared via an antisolvent/self-assembling method by adding a SNAP/ethanol solution to an aqueous silk fibroin (SF) solution and freeze-thawing. Prepared SNAP-SF NPs had a diameter ranging from 300-400 nm and an overall negative charge of  $-28.76 \pm 0.73$  mV. Among the different SNAP/SF ratios tested, the highest encapsulation efficiency ( $18.3 \pm 1.3$  %) and loading capacity ( $9.1 \pm 0.6$  %) values were attributed to the 1:1 ratio. The deconvolution of the amide I band in the FTIR spectra of SF NPs and SNAP-SF NPs showed an increase in the  $\beta$ -sheet content for SNAP-SF NPs, confirming the hydrophobic interactions between SNAP and silk macromolecules. SNAP-SF NPs released up to  $1.31 \pm 0.02 \times 10^{-10}$  mol min<sup>-1</sup> mg<sup>-1</sup> NO over a 24 h period. Moreover, SNAP-SF NPs showed concentration-dependent antibacterial effects against *Methicillin-resistant Staphylococcus aureus* (MRSA) and *Escherichia coli* (*E. coli*). Furthermore, they did not elicit any marked cytotoxicity against 3T3 mouse fibroblast cells at concentrations equal to or below 2 mg/mL. Overall, these results demonstrated that SNAP-SF NPs have great potential to be used as a NO delivery platform for biomedical applications such as tissue engineering and wound healing, where synergistic properties of silk fibroin and NO are desired.

## 2.2 Introduction

NO is a diatomic free radical gas molecule produced in humans and other mammals, which exhibits various functions in physiological processes including inhibition of platelet adhesion and activation, prevention of infection, regulation of angiogenesis, and accelerated wound repair [210, 211]. The multifaceted roles of NO have inspired researchers to develop NO-releasing/generating

materials for different biomedical applications such as antibacterial and hemocompatible coatings for medical devices, wound dressings, and tissue engineering scaffolds [212-215]. Despite the beneficial functions, the gaseous radical state and short half-life of NO have restricted its direct delivery [216]. To overcome this limitation, reactive precursors or NO donor molecules have been developed [217]. Among the different chemistries available, S-nitrosothiols (RSNOs) have received much attention as a potential class of stable NO donors due to their high cytocompatibility and exhibition of promising results in humans for different clinical conditions [218-220]. However, RSNOs suffer from low thermal stability and therefore have been commonly incorporated into various polymeric substrates to extend their utility and provide localized NO release at the tissue/material interface. Incorporation of RSNOs into various polymeric matrices including polyurethanes [221], polyethylene glycol (PEG) [222], Pluronic F127 hydrogel [223, 224], poly(vinyl alcohol), and poly(vinyl pyrrolidone) [225] have been reported previously. These NO-releasing polymeric materials can be potentially used for versatile biomedical applications such as wound dressings, coatings for blood-contacting devices, and urinary catheters [226].

Among the developed NO delivery systems, polymeric micro/nanoparticles have been the center of particular interest due to their low cost, controlled NO release, and their flexibility to be incorporated into different materials [227, 228]. Preparation of different NO-releasing polymeric micro/nanoparticles based on both natural and synthetic polymers have been previously reported [229-232]. Micro/nanoparticles based on poly lactic-co-glycolic acid (PLGA) are one of the most studied polymeric platforms for NO delivery due to their desirable pharmacokinetic properties and biodegradability [229] [232]. However, low colloidal stability, harsh processing conditions, and acidic degradation products of PLGA micro/nano particles may impact the stability of the loaded NO donor and is potentially undesirable at the target site [233]. Natural polymeric platforms offer

an interesting alternative to synthetic ones, due to their advantageous properties such as more biocompatible degradation products, reduced immune response, more availability, and lower cost [234, 235].

In this study, for the first time, encapsulation of a water-insoluble NO donor, SNAP, in a natural polymeric nanoparticle platform made of silk fibroin (SF) is reported. SF is a protein extracted from silkworm cocoons with an exceptional combination of properties rarely found in any other natural or synthetic polymer [236]. Due to its strong mechanical properties, biocompatibility, non-immunogenicity, diverse material formats, water-based processing, and tunable biodegradation SF has been utilized for developing various drug delivery systems [237-239]. Comprehensive *in vitro* and *in vivo* studies in the past have reported that SF displays lower immunogenic and inflammatory responses and demonstrates excellent biocompatibility compared to other commonly used biodegradable polymers such as PLGA, polylactic acid, polyglycolic acid, and collagen [240]. This is in part due to the production of nontoxic amino acids by SF upon degradation that can be metabolized *in vivo* [241, 242]. The presence of both hydrophilic and hydrophobic amino acids in the SF structure allows for preparation of SF nanoparticles (SF NPs) through a simple self-assembling process and enables the co-delivery of drugs with distinct physical properties [243, 244]. SF NPs have previously shown great promise for delivering a range of therapeutic agents in different applications such as prevention of infection and inflammation [245, 246], colitis [247], diabetes [248], and cancer treatment [249, 250]. The surface of SF NPs can also be chemically modified or functionalized with biomolecules, antibodies, or ligands to enhance cellular adhesion, active targeting, and controlled release [251, 252].

Regarding the indisputable functionality of NO and SF in various biomedical applications, this study describes the fabrication of NO-releasing SF NPs using SNAP as the NO donor

molecule. The fabricated SNAP-SF NPs were tested *in vitro* to assess their NO release kinetics, storage stability, antibacterial activity, and cytotoxicity, in addition to their other physiochemical properties.

## **2.3 Materials and methods**

### **2.4.1 Materials**

*Bombyx mori* (B. mori) silk cocoons were obtained from Paradise fibers (USA). Absolute ethanol was purchased from VWR (USA), and Spectra/Por™ 3 RC dialysis membrane tubing (3.5 kDa, MWCO) was purchased from fisher scientific (USA). *N*-Acetyl-*D*-penicillamine (NAP), sulfuric acid, lithium bromide, ethylenediaminetetraacetic acid (EDTA), collagenase type II were purchased from Sigma Aldrich (St. Louis, MO). The Cu-NPs (99 %, 40–60 nm) were purchased from SkySpring Nanomaterials, Inc. (Houston, TX). Luria–Bertani (LB) Agar was bought from Difco Laboratories Inc. (Detroit, MI) and LB broth was purchased from Fisher Bioreagents (Fair Lawn, NJ). Dulbecco’s modified Eagle’s medium (DMEM) and trypsin-EDTA were obtained from Corning (Manassas, VA 20109). Fetal bovine serum (FBS) and Penicillin-Streptomycin (Pen-Strep) were bought from Gibco-Life Technologies (Grand Island, NY). The bacterial strains Methicillin-resistant *S. aureus*, *E. coli* (ATCC 25922), and 3T3 mouse fibroblast cells (ATCC 1658) were obtained from American Type Culture Collection (ATCC).

### **2.4.2 Synthesis of Silk fibroin and SNAP**

Aqueous SF solution was extracted from B. mori cocoons following a previously established procedure [253]. Briefly, B. mori cocoons were boiled in 20 mM sodium carbonate solution for 30 min to degum and dried in room temperature. The dried silk fibroin was dissolved in 9.3 M lithium bromide at 60 °C for 4 h and dialyzed against deionized water (DIW) water in a dialysis membrane (3.5 kDa cut-off) for 48 h. The obtained aqueous SF solution was then filtered

and stored at 4 °C for further use. SNAP was synthesized using a previously reported protocol [254]. Briefly, equal molar values of sodium nitrite and *N*-acetylpenicillamine (NAP) were added to a methanol solution (50 % v/v) containing 1 M H<sub>2</sub>SO<sub>4</sub> and 1 M HCl. After being stirred for 30 min in the dark and cooling in an ice bath for 5 h the SNAP crystals were precipitated. The collected crystals were rinsed, vacuum-dried, and stored at –20 °C for use in all experiments.

#### **2.4.3 Synthesis of Silk and SNAP loaded Silk NPs**

SNAP-SF NPs were prepared using the previously described anti-solvent/self-assembling method [255]. A 30 mL solution of SNAP in a 30:70 v/v mixture of ethanol/water was prepared. This mixture was then added dropwise to 10 mL aqueous SF solution (2 % wt/v) under gentle stirring. The mixed solution was kept at –20 °C for exactly 3 h resulting in a milky emulsion after defrosting at room temperature. The NPs were collected by centrifugation at 4225 g for 10 min and were re-suspended in DIW. The NPs sediment was washed an additional 3 times to remove any unloaded SNAP then freeze-dried at –80 °C for 48 h to obtain SNAP-SF NPs powder. The particles were stored at –20 °C until use. Fabrication of pure SF NPs was carried out using the same procedure as SNAP-SF NPs. However, the freezing time was increased to 24 h to allow enough time for the formation of pristine silk NPs.

#### **2.4.4 Characterization of Silk and SNAP loaded Silk NPs**

Dynamic Light Scattering (DLS) (ZetaSizer, Malvern) was used to measure the size and surface charge of the NPs in DIW. Size analysis was also performed with ImageJ software using TEM images obtained by a JEOL JEM-2100 transmission electron microscope. Morphology of the nanoparticles was observed using a field emission scanning electron microscope (SEM, FEI Teneo, FEI Co.). The FTIR spectra of the SF NPs and SNAP-SF NPs (in the form of KBr pellets) were analyzed using a Nicolet 6700 spectrometer (Thermo Electron Corporation, Madison, WI).

128 scans with a resolution of 4 cm<sup>-1</sup> were done for each measurement, over the wavenumber range of 4000–400 cm<sup>-1</sup>. Amid I band deconvolution was done for each spectrum to measure the crystallinity (β-sheet content) using OriginPro 8 software according to previous studies [256].

#### 2.4.5 Measurement of NO loading and release

To measure the NO release from SNAP-SF NPs, a defined weight of NPs was immersed in 0.01 M PBS containing EDTA or 0.3 U/mL collagenase type II solution at 37 °C. At different time points, NO released from the NPs was recorded using a Sievers Nitric Oxide Analyzer (NOA) (model 280i, Boulder, CO). The total NO loading of SNAP-SF NPs was measured by injection of 0.25 M copper (II) chloride and ascorbic acid to trigger the catalytic release of all the NO content of the NPs within a short time and calculating the area under the obtained release rate curves. Encapsulation efficiency (EE) and loading capacity (LC) values of NO in SNAP-SF NPs were determined based on the initial amount of SNAP used to make the NPs according to Eq. 2.1 and Eq. 2.2, respectively [257].

$$EE (\%) = \frac{\text{Total amount of NO released (mg SNAP)}}{\text{Initial amount of NO (mg SNAP)}} \times 100 \quad (\text{Eq. 2.1})$$

$$LC (\%) = \frac{\text{Total amount of NO released (mg SNAP)}}{\text{Total Weight of SNAP-SF NPs (mg)}} \times 100 \quad (\text{Eq. 2.2})$$

#### 2.4.6 Cell culture and viability studies

**Cell culture:** 3T3 mouse fibroblast cells (ATCC 1658) were cultured in complete DMEM medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin and were incubated in a humidified environment at 37 °C, with 5 % CO<sub>2</sub>. The culture medium was changed every two days, and cells were checked daily for growth and contamination. At 80 % confluency, cells were detached from the T-flask using 0.18 % trypsin and 5 mM EDTA and counted under a hemocytometer after staining with Trypan blue.

**CCK-8 assay:** The effect of nanoparticles on cell viability was evaluated by the CCK-8 assay kit according to the manufacture's protocol (Sigma Aldrich). First, cells were seeded in 96-well plates (3000 cells per well) and allowed to attach in the incubator for 24 h. The media was then replaced with fresh media containing SF NPs or SNAP-SF NPs at various concentrations (0.25, 1, 2, 5, and 10 mg/mL) and incubated for another 24 h. Cells maintained in DMEM without nanoparticles were used as a control group. To avoid absorbance interference by SF and SNAP-SF NPs, the media containing nanoparticles was removed and replaced by 100  $\mu$ L of fresh media plus 10  $\mu$ L of CCK-8 solution. After incubating for 1 h at 37  $^{\circ}$ C, the absorbance was detected at 450 nm. Five replicates (n=5) were tested for each concentration and the cell viability was calculated according to the following equation:

$$Cell\ viability\ (\%) = \frac{Absorbance\ (treated\ cells)}{Absorbance\ (control\ cells)} \times 100 \quad (Eq. 2.3)$$

**Live/Dead staining:** The qualitative testing of 3T3 mouse fibroblast cell viability was performed using an EthD-III/Calcein AM staining kit (Thermo Fisher Scientific) following the manufacturer's protocol. 10000 cells per well were seeded in 48-well plates and allowed to attach in the incubator for 24 h. After cell attachment, the media was replaced with fresh media containing SF NPs or SNAP-SF NPs at various concentrations (0.25, 1, 2, 5, and 10 mg/mL) and incubated for another 24 h. Cells maintained in DMEM without nanoparticles were used as a control group. After incubation, nanoparticle/media solutions were gently aspirated and a PBS solution containing 2  $\mu$ M of Calcein-AM (green) and 4  $\mu$ M of EthD-III (red) was added to each well and incubated at room temperature for 20 min. The stained cells were observed using an EVOS FL microscope.

#### 2.4.7 *In vitro* bacterial viability

To assess the antibacterial efficacy of the SF NPs and SNAP-SF NPs, *E. coli* (ATCC 25922) and methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA 041) were used. For both, LB broth was used for inoculation and incubated for 12 h at 37 °C under shaking conditions (150 rpm). The bacterial cultures were grown to a mid-log phase. The resulting bacteria solution was centrifuged at 2500 rpm for 7 min. The supernatant was discarded, and the bacteria re-suspended in PBS and re-centrifuged to remove any dead cells and other cell debris. The resulting bacterial pellet was again re-suspended in PBS and adjusted to a bacterial concentration of  $\sim 10^8$  CFU/mL. In a 96-well plate, different concentrations of each sample were exposed to the final bacterial solution. Post-exposure, the 96-well plate was incubated for 24 h at 37 °C at 150 rpm (n=3). To measure bacterial viability reduction, the bacterial solution from each well was collected. After serial dilutions ( $10^{-1}$  –  $10^{-5}$ ), the bacterial solution was plated on LB agar and incubated for 24 h. The CFU counted provided the number of bacteria per mL of solution. The percentage of bacterial viability reduction was calculated from the following equation where  $C = \frac{CFU}{mL}$  :

$$Reduction\ of\ bacterial\ viability\ (\%) = \frac{C_{control} - C_{sample}}{C_{control}} \times 100 \quad (Eq. 2.4)$$

#### 2.4.8 Storage stability evaluation

SNAP-SF NPs were placed in vials under the following conditions: room temperature with ambient light, room temperature in the dark, 37 °C in the dark, and in the freezer (-20 °C) in the dark. At different time points, the remaining NO content in the nanoparticles was measured to determine the % SNAP remaining. This was compared to the initial amount of SNAP

loaded into the nanoparticles calculated by catalytically releasing the entirety of the encapsulated SNAP immediately after fabrication [221].

#### **2.4.9 Statistical analysis**

All data are presented as a mean  $\pm$  standard deviation collected from three data points unless otherwise noted. Statistically significant differences ( $p$ -values of  $< 0.05$ ) were compared by one-way analysis of variance.

### **2.4 Results and discussion**

#### **2.4.1 Synthesis and characterization of SNAP-SF NPs**

SF NPs were produced using a bottom-up approach called anti-solvent precipitation that builds nanoparticles by assembling silk molecular chains together (**Figure 2.1**) [258]. The addition of ethanol, which is an anti-solvent for aqueous SF, causes a change in the random coil and/or helical conformation to  $\beta$ -sheet structures in SF molecular chains to some degree. The formed  $\beta$ -sheet nanocrystals then grow into nanoparticles under shearing forces induced during the freezing step. The SF NPs produced by this method feature a hydrophobic core composed of well-ordered crystalline  $\beta$ -sheet structures. Whereas the shell of the NPs consists of mainly hydrophilic segments [255]. Therefore, the hydrophobic core of SF NPs provides an ideal environment to encapsulate water-insoluble SNAP molecules through hydrophobic interactions. This method has been previously used to load hydrophobic drugs such as Paclitaxel in the core of SF NPs for cancer treatment applications [259]. Advantageously, ethanol can both dissolve the SNAP and induce the NPs formation, making this approach simpler and more cost-effective.

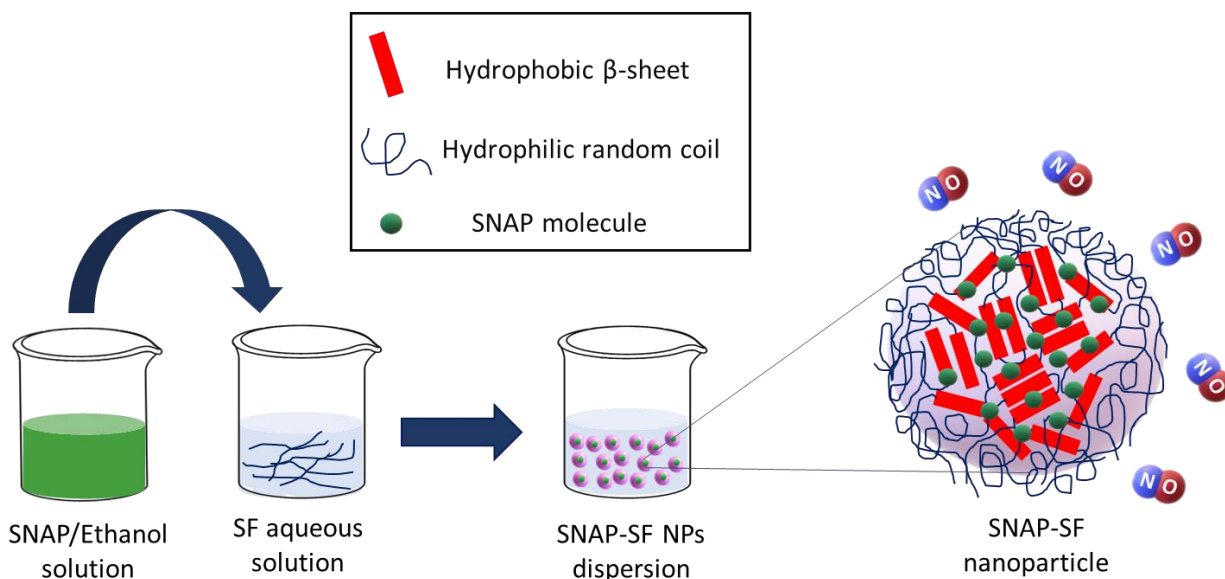


**Figure 2.1** - Schematic representing the fabrication of SNAP-SF NPs by anti-solvent precipitation method.

The production yields of SF NPs and SNAP-SF NPs produced by the described anti-solvent method were measured and found to be  $60 \pm 8 \%$  and  $50 \pm 5 \%$ , respectively. According to TEM, the SF NPs had an average diameter of  $345.1 \pm 23.3$  nm and a spherical morphology, which was observed by SEM (**Figure 2.2**). Previous studies have also reported a similar size range for SF NPs produced using this method [255]. SNAP-SF NPs showed the same morphology and were slightly larger than SF NPs with an average diameter of  $382.5 \pm 20.1$  nm, which is most likely due to the encapsulation of SNAP in the NPs. SEM imaging was also able to show no evidence of excess crystallized SNAP on the surface of SNAP-SF NPs, demonstrating efficient encapsulation. This prevents large burst releases of NO from occurring that could potentially be cytotoxic to localized cells in contact with the NPs.

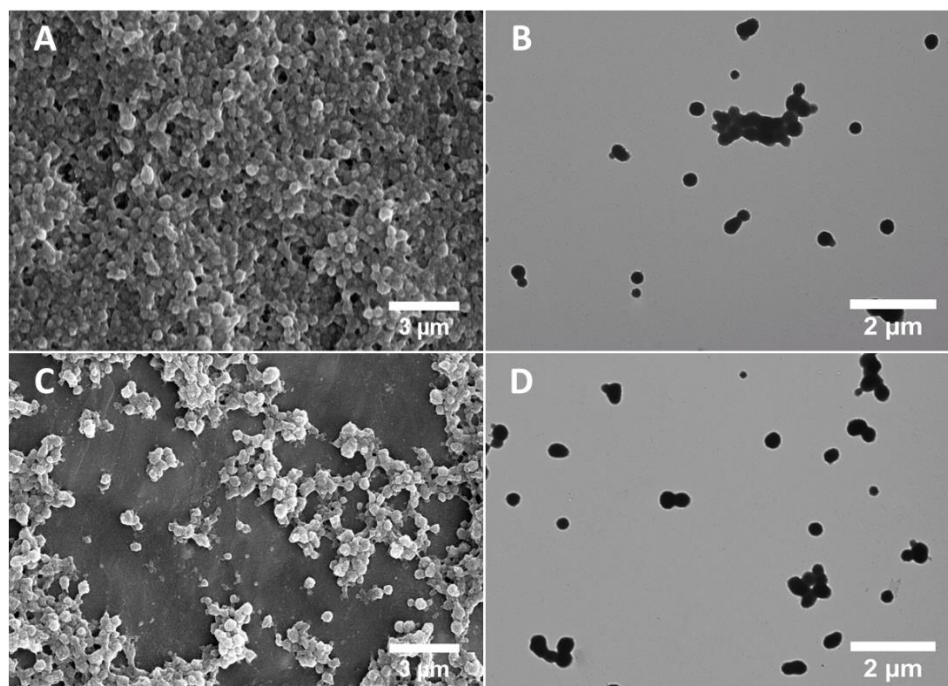


**Figure 2.2** - SEM (A,C) and TEM (B,D) images of A,B) SF NPs and C,D) SNAP-SF NPs.

The average size of NPs measured from TEM images was in the same range as those obtained from DLS studies (**Table 2.1**). The result of zeta potential analysis demonstrated that SF NPs and SNAP-SF NPs have an overall negative charge of  $\sim -26$  and  $\sim -28$  mV respectively at pH 7–8. The increase in NPs size with SNAP loading may have influenced the surface charge of the SF NPs. An increase in surface negativity has also been reported by Wang et al. for ibuprofen incorporation into SF nanoparticles, which has a similar carboxylic acid functionality as SNAP [260]. Moreover, the high negativity of the NPs surface ensured the formation of stable NPs suspension in DIW [261]. Correspondingly, it was observed that freeze-dried NPs powder could be easily re-dispersed in aqueous media.

**Table 2.1** - Zeta-potential and particle sizes of SF NPs and SNAP loaded SF NPs obtained from dynamic light scattering analysis. \* and \*\* indicate p-values  $\leq 0.05$  and  $\leq 0.01$ , respectively.

| Sample      | Zeta potential (mV)    | Size (nm)              |
|-------------|------------------------|------------------------|
| SF NPs      | $-26.41 \pm 0.87$      | $338.43 \pm 7.10$      |
| SNAP-SF NPs | $-28.76 \pm 0.73^{**}$ | $379.36 \pm 15.92^{*}$ |

Encapsulation efficiency (EE) and loading capacity (LC) values of NPs with different SNAP to SF ratios were measured using NOA, and the results are summarized in **Table 2.2**. EE and LC increased slightly by increasing the ratio of SNAP:SF from 1:4 to 1:1, however, it decreased with further increasing the ratio. That is most likely because after a certain ratio of SNAP:SF, the polymer matrix becomes saturated and cannot incorporate more drugs into it. The oversaturation of SNAP could also begin to compromise the structural integrity of the SF NPs, decreasing their ability to efficiently encapsulate the SNAP in solution. Overall, 1:1 ratio of SNAP:SF had the highest EE and LC values, and therefore, it was chosen for further studies.

**Table 2.2** - Loading capacity and encapsulation efficiency in SNAP-SF NPs with different SNAP-to-SF ratios. Increasing the ratio of SNAP:SF from 1:2 to 1:1 caused a significant increase in loading capacity ( $P \leq 0.01$ ). However further increase in the ratio of SNAP:SF from 1:1 to 2:1 caused a significant decrease in encapsulation efficiency ( $P \leq 0.05$ ).

| SNAP:SF ratio | Encapsulation efficiency (%) | Loading capacity (%) |
|---------------|------------------------------|----------------------|
| 2:1           | 10.80±1.08*                  | 3.60±0.23            |
| 1:1           | 18.31±1.32                   | 9.15±0.65**          |
| 1:2           | 16.06±1.14                   | 5.35±0.33            |
| 1:4           | 13.87±1.01                   | 2.77±0.16            |

#### 2.4.2 FTIR and deconvolution of amide I band

**Figure 2.3** shows the FTIR spectra of SNAP loaded and pristine SF NPs. The amide I, II, and III characterization peaks of silk fibroin protein are the dominant peaks in both spectra and cover the SNAP characterization peaks. The absorption at the amide I band increased with the addition of SNAP. This might be related to the increase of the  $\beta$ -sheets conformation in SNAP-SF NPs as previously reported in other studies [259, 262].

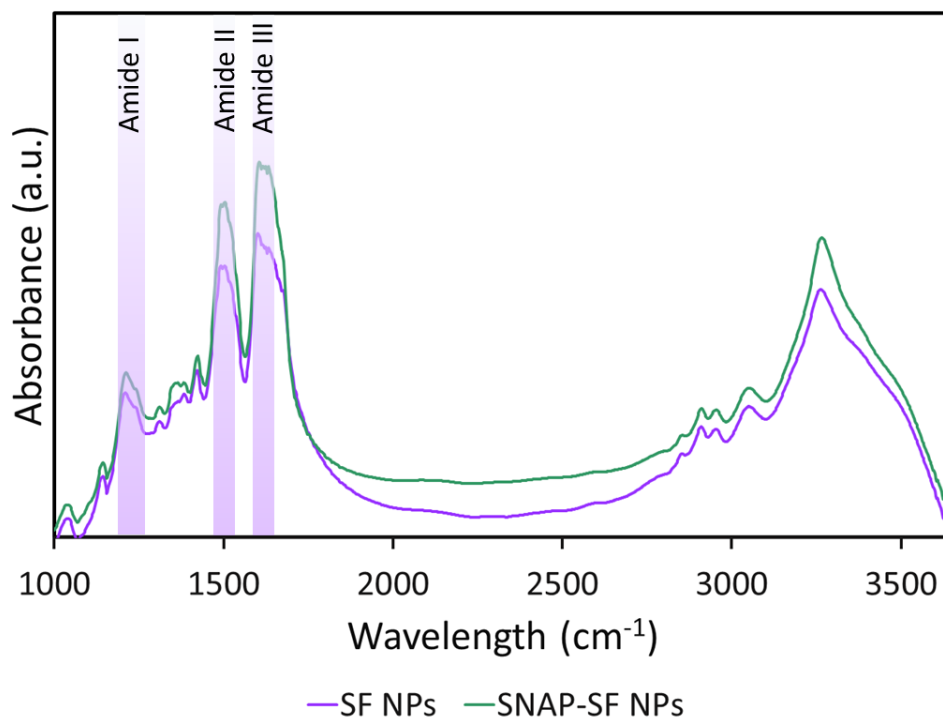


**Figure 2.3** - FTIR spectra of SF NPs and SNAP-SF NPs.

To characterize the modifications induced by SNAP addition, peak deconvolution analysis was performed on the amide I peak and shown in **Figure 2.4**. The degree of crystallinity was measured for each type of NPs accordingly. As expected, SF NPs showed an average  $\beta$ -sheets content of 16 %, while SNAP-SF NPs presented an average crystallinity degree of 23 %. This indicates that the addition of SNAP gives rise to conformational changes of SF from random coil/helix to  $\beta$ -sheet, likely due to the hydrophobic interactions between SNAP and SF molecular chains. The formation of more  $\beta$ -sheet structures after addition of SNAP might be beneficial to enhance its encapsulation in the SF NPs and tailor the release kinetics.

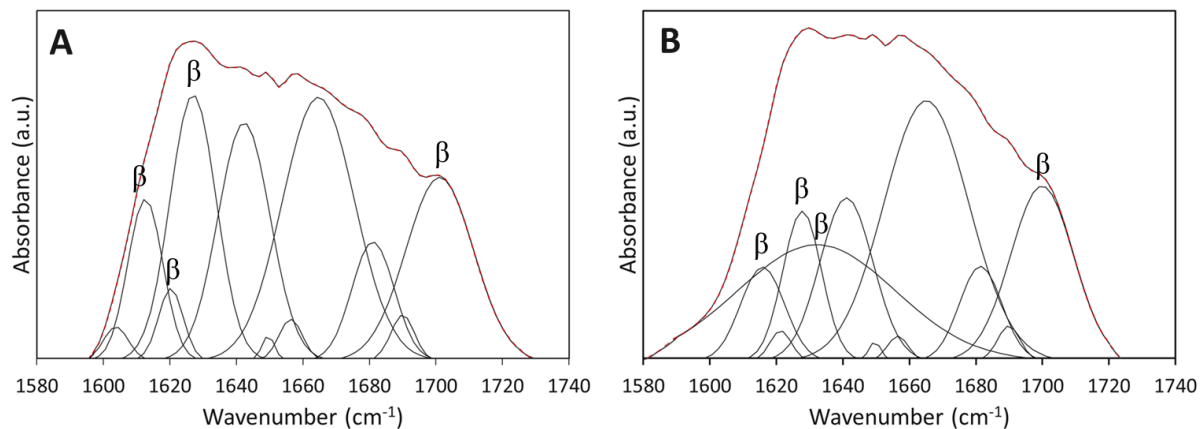


**Figure 2.4** - Deconvoluted FTIR spectra of the amide I band of A) SF NPs and B) SNAP-SF NPs. The crystallinity content for these samples are 16 % and 23 % respectively.  $\beta$  =  $\beta$ -sheets.

### 2.4.3 NO release kinetics and storage stability

NO release from RSNOs can be triggered by exposing them to heat, light, or specific metal ions with catalytic activity such as  $\text{Cu}^{2+}$  [254, 263]. In this study, to prevent the catalytic and light-induced NO release, SNAP-SF NPs were placed in an amber vial containing PBS plus EDTA at 37 °C. Therefore, the primary mechanism of NO release is the decomposition of the SNAP entrapped in the SF NPs by means of moisture/heat via homolytic cleavage of the S-NO bond resulting in disulfide species (RSSR) formation (Eq. 2.5) [264].



The NO release rate from SNAP-SF NPs at different time points is presented in **Figure 2.5** over a 24 h period. The highest release rate equal to  $0.42 \pm 0.04 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$  was observed at 2 h. Afterward, the NO release rates showed a decreasing trend over time, reaching to  $\sim 0.01 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$  at 24 h. The cumulative NO release curves shown in **Figure 2.5** were estimated by calculating the total NO release over the 24 h period. The NO release half-life was 4 h, which is a notable improvement compared to previously reported biopolymeric nanoparticle-based NO delivery systems that reportedly deliver more than half of their NO payload in less than 2 h [230, 231]. This could potentially be attributed to the hydrophobic core of SF NPs that limits

SNAP diffusion into the media by minimizing the water uptake. Therefore, SNAP molecules entrapped inside the core will release at a slower rate. SNAP-SF NPs released up to  $1.31 \pm 0.02 \times 10^{-10}$  mol  $\text{mg}^{-1}$  NO over a 24 h period before the concentration increase became undetectable.

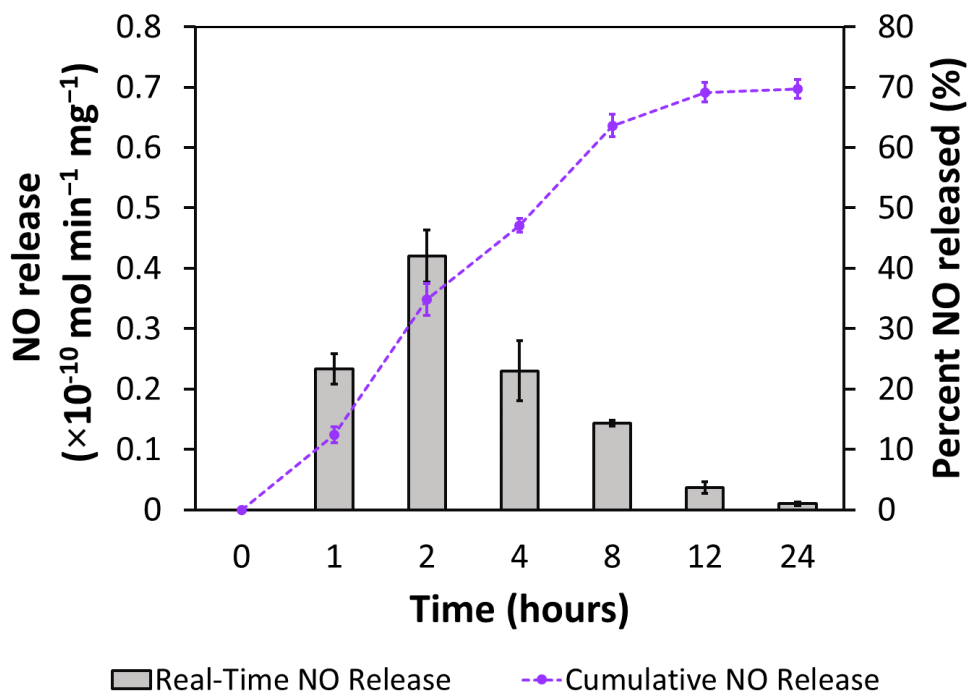


**Figure 2.5** - Cumulative and real-time NO release from SNAP-SF NPs under physiological conditions (soaking in PBS buffer with or without collagenase type II, at 37 °C in the dark). Data represent the mean  $\pm$ SD. \* and \*\* indicate p-values  $\leq 0.05$  and  $\leq 0.01$ , respectively, obtained by comparing NO release from SNAP-SF NPs in PBS with collagenase to PBS without collagenase at each time point.

The storage stability of the SNAP-SF NPs was tested under different conditions (**Figure 2.6**). SNAP-SF NPs showed excellent stability in  $-20$  °C at dark conditions maintaining their entire NO reservoir after a month. However, SNAP-SF NPs kept at room temperature either under dark or light conditions, lost more than 30 % of their NO after a week, and more than 50 % after a month. The lowest stability was observed for SNAP-SF NPs kept at 37 °C, which indicates the higher susceptibility of SNAP to release NO at elevated temperatures. Overall, the optimum

storage condition was found to be at  $-20^{\circ}\text{C}$  in the dark, where 94 % of SNAP was remaining after a month.

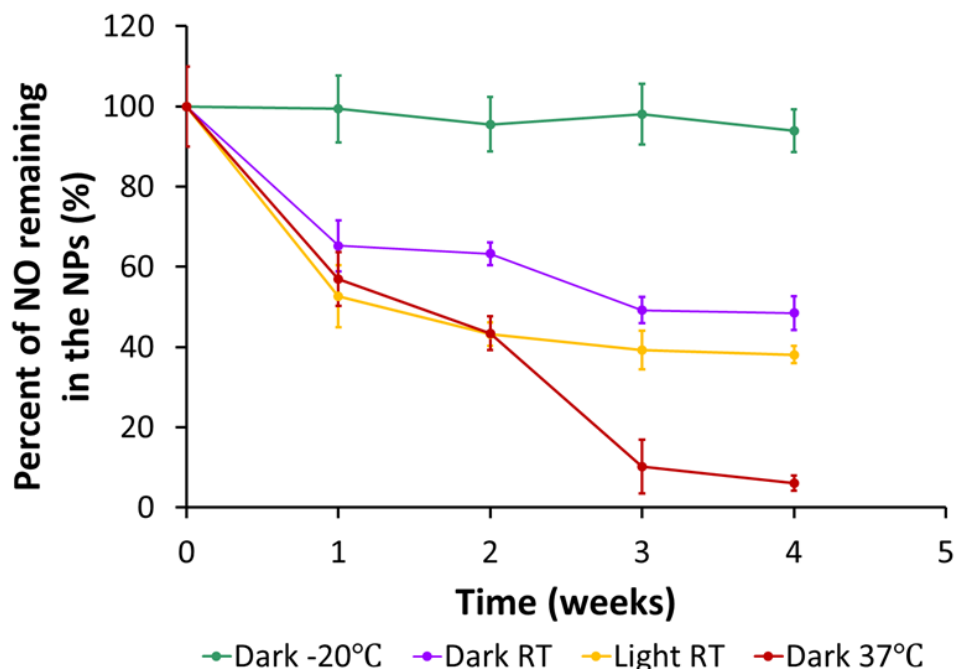


**Figure 2.6** - Storage Stability of SNAP-SF NPs at different conditions over 4 weeks.

#### 2.4.4 *In vitro* cytocompatibility assays

NO exhibits dose-dependent toxicity towards cells [265]. It has been demonstrated before that high concentration of NO in the micro- to millimolar range can create nitrosative stress leading to cell apoptosis and cytotoxicity [266]. However, lower NO concentrations (pico- to nanomolar range) result in favorable cellular responses and enhanced proliferation [267]. The cytocompatibility of SF and SNAP-SF NPs was assessed *in vitro* in direct contact with 3T3 mouse fibroblast cells (**Figure 2.7**). No apparent cytotoxicity was observed below 5 mg/mL concentrations for both SF NPs and SNAP-SF NPs. At 5 mg/mL, cells showed 65.8 % and 63.14 % viability values after treating with SF NPs and SNAP-SF NPs, respectively. The cell viability values were further reduced to 55.9 % and 53.9 % at 10 mg/mL concentration of SF NPs and

SNAP-SF NPs, respectively. There was no significant difference between the viabilities of mouse fibroblast cells treated with SNAP-SF NPs, SF NPs at all concentrations. Therefore, it can be inferred that cytotoxicity of the NPs at higher concentrations (5 and 10 mg/mL) is not correlated with NO release. Since SF has also proved to a biocompatible polymer, the cytotoxicity of SF NPs observed is most likely caused by the high number of NPs. Similar *in vitro* cytotoxic effects of polymeric nanoparticles at high concentrations have been previously demonstrated by other studies as well [232, 268].

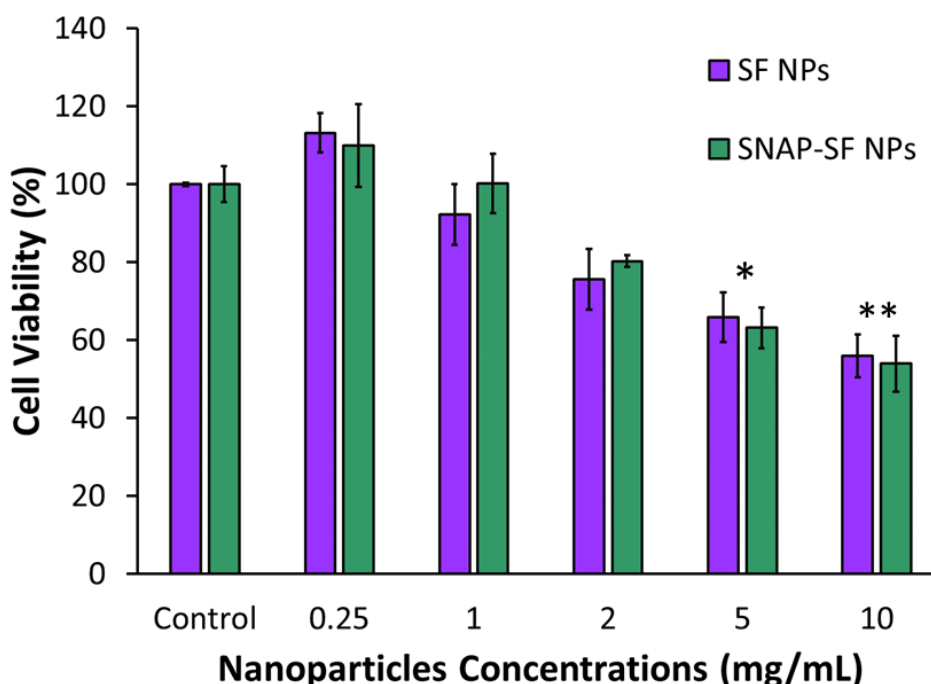


**Figure 2.7** - 24 h viability of 3T3 mouse fibroblast cells (as percentage relative to control) using WST dye-based CCK-8 assay. Error bar represents standard deviation (n = 6). \* and \*\* indicate p-values  $\leq 0.05$  and  $\leq 0.01$  compared to control, respectively.

3T3 mouse fibroblast cell viability treated with various concentrations of SF NPs and SNAP-SF NPs was also assessed qualitatively by EthD-III/calcein AM staining. As shown in **Figure 2.8**, a similar trend to the cell viability results obtained by CCK-8 assay was observed. Cells treated with lower NPs concentrations (0.25-2 mg/mL) showed minimal decrease in viability

when compared with untreated controls. However, exposure to higher concentrations of NPs (5 mg/mL and 10 mg/mL), resulted in a greater decrease in the number of live cells similar to the results obtained from the quantitative *in vitro* viability assay.

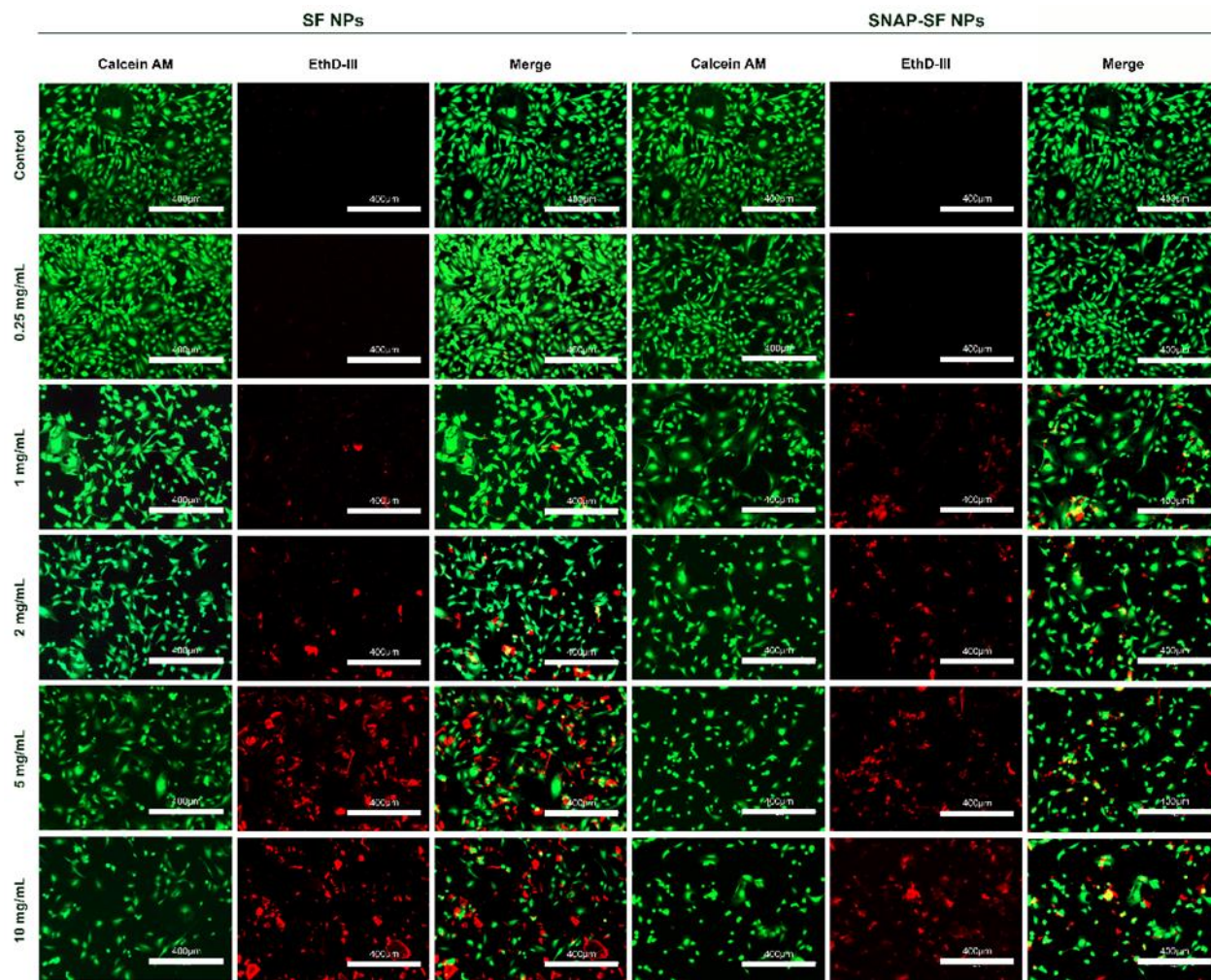


**Figure 2.8** - Live/Dead imaging of 3T3 mouse fibroblast cells at different concentrations of nanoparticles. Live cells are stained green by calcein AM and dead cells are stained red by EthD-III. Same control was used for both SF NPs and SNAP-SF NPs. Scale bar = 400  $\mu$ m.

#### 2.4.5 *In vitro* bacterial viability

NO is a small molecule with a half-life of less than a few seconds, acting through non-specific bactericidal mechanisms [269, 270]. NO can kill the bacteria by either directly modifying membrane-bound proteins, metabolic enzymes, and DNA, or by interacting with superoxides and

producing reactive nitrogen species, which further alter functions of proteins that are critical for cellular processes [270, 271]. In this study, the antibacterial activities of SF NPs and SNAP-SF NPs against Gram-positive *MRSA* and Gram-negative *E. coli* were assessed using a CFU counting method (**Figure 2.9 A and B**). SNAP-SF NPs showed antibacterial activity against both bacteria in a concentration-dependent manner. By increasing the SNAP-SF NPs concentration from 0.25 mg/mL to 10 mg/mL, the bacteria reduction efficiency was enhanced from 48.17 % to 99.99 % (> 4 log reduction) for *MRSA* and from 53.77 % to 99.96 % (> 3 log reduction) for *E. coli*. Bacterial reduction efficiency values of SNAP-SF NPs at different concentrations are presented in **Table 2.3**. However, SF NPs did not affect the viability of *MRSA* and *E. coli* at any of the tested concentrations, suggesting that antibacterial activity of the SNAP-SF NPs can be accredited merely to the NO content.

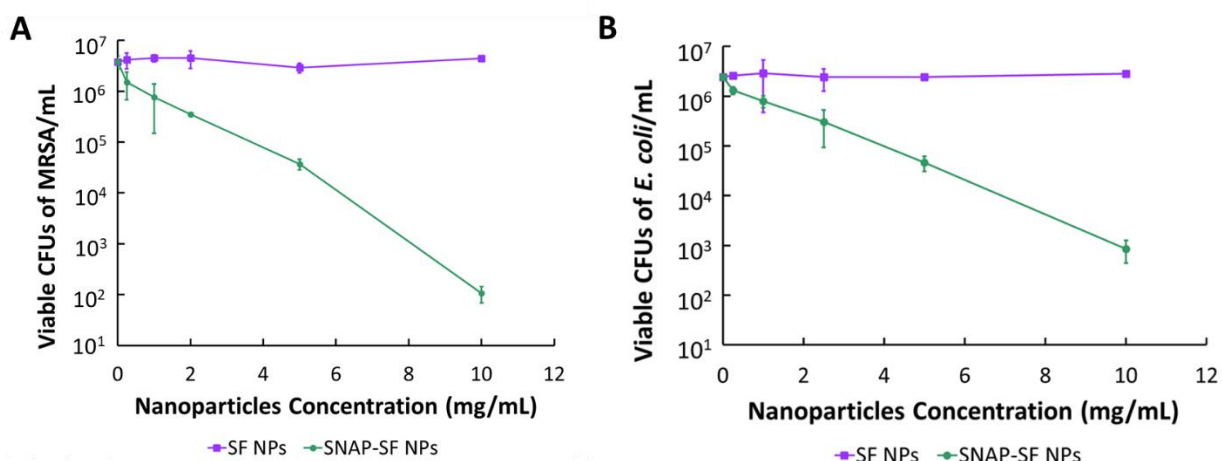


**Figure 2.9** - Antibacterial activity of SF NPs and SNAP-SF NPs against A) *MRSA* and B) *E. coli* after 24h.

**Table 2.3** - Viability reduction efficiency values of MRSA and *E. coli* bacterial strains after exposure to different concentrations of SNAP-SF NPs for 24 h. (\* and \*\* indicate p-values  $\leq 0.05$  and  $\leq 0.01$  versus SF NPs at the same concentration respectively).

| SNAP-SF NPs Concentration (mg/mL) | Reduction Efficiency (%) |                    |
|-----------------------------------|--------------------------|--------------------|
|                                   | <i>MRSA</i>              | <i>E. coli</i>     |
| 0.25                              | 48.17 $\pm$ 1.75         | 53.77 $\pm$ 8.17   |
| 1                                 | 79.63 $\pm$ 15.09**      | 71.69 $\pm$ 7.48   |
| 2                                 | 90.78 $\pm$ 0.80**       | 78.22 $\pm$ 10.88* |
| 5                                 | 99.02 $\pm$ 0.28**       | 98.07 $\pm$ 0.78** |
| 10                                | 99.99 $\pm$ 0.01**       | 99.96 $\pm$ 0.02** |

## 2.5 Conclusion

Overall, we demonstrated that silk-based NPs could serve as a biocompatible and inexpensive NO delivery platform. The amphiphilic properties of SF allow sustained release of NO by encapsulating the water-insoluble NO donor, SNAP, inside a hydrophobic core while having a more hydrophilic surface desired for cytocompatibility. The amide I band FTIR deconvolution confirmed the successful entrapment of SNAP molecules inside the core of SF NPs by hydrophobic interactions, which further resulted in increased crystallinity of the SF NPs structure. SNAP-SF NPs were able to release  $1.31 \pm 0.02 \times 10^{-10}$  mol mg<sup>-1</sup> of NO over a 24 h period and exhibited strong antibacterial properties against *MRSA* and *E. coli*, which are responsible for several difficult-to-treat infections in humans. Besides, NPs did not show cytotoxic effects toward fibroblast cells at concentrations  $\leq 2$  mg/mL. SNAP-SF NPs either alone or when incorporated into other substrates can be potentially utilized for biomedical applications such as antibacterial implantable medical devices, tissue engineering scaffolds, and wound dressings, where a combination of therapeutic and antibacterial properties of silk and NO is desired. The results of this study form the basis for further future studies in pre-clinical settings to ascertain the therapeutic potential of SNAP-SF NPs.

**CHAPTER 3**  
**NITRIC OXIDE-RELEASING HALLOYSITE NANOTUBES FOR BIOMEDICAL**  
**APPLICATIONS<sup>3</sup>**

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<sup>3</sup> Ghalei S, Hopkins S, Douglass M, Garren M, Mondal A, Handa H. Nitric oxide releasing halloysite nanotubes for biomedical applications. *Journal of Colloid and Interface Science*. 2021 May 15;590:277-89. Reprinted here with permission of the publisher.

### 3.1 Abstract

Halloysite nanotubes (HNTs) are natural aluminosilicate clay that have been extensively explored for delivery of bioactive agents in biomedical applications because of their desirable features including unique hollow tubular structure, good biocompatibility, high mechanical strength, and extensive functionality. For the first time, in this work, functionalized HNTs are developed as a delivery platform for nitric oxide (NO), a gaseous molecule, known for its important roles in the regulation of various physiological processes. HNTs were first hydroxylated and modified with an aminosilane crosslinker, (3-aminopropyl) trimethoxysilane (APTMS), to enable the covalent attachment of a NO donor precursor, *N*-acetyl-*D*-penicillamine (NAP). HNT-NAP particles were then converted to NO-releasing *S*-nitroso-*N*-acetyl-penicillamine HNT-SNAP by nitrosation. The total NO loading on the resulting nanotubes was  $0.101 \pm 0.07 \mu\text{mol/mg}$  which could be released using different stimuli such as heat and light. Qualitative (Fourier-transform infrared spectroscopy and Nuclear magnetic resonance) and quantitative (Ninhydrin and Ellman) analyses were performed to confirm successful functionalization of HNTs at each step. Field emission scanning electron microscopy (FE-SEM) showed that the hollow tubular morphology of the HNTs was preserved after modification. HNT-SNAP showed concentration-dependent antibacterial effects against Gram-positive *Staphylococcus aureus* (*S. aureus*), resulting in up to 99.6% killing efficiency at a concentration of 10 mg/mL as compared to the control. Moreover, no significant cytotoxicity toward 3T3 mouse fibroblast cells was observed at concentrations equal or below 2 mg/mL of HNT-SNAP according to a WST-8-based cytotoxicity assay. The SNAP-functionalized HNTs represent a novel and efficient NO delivery system that holds the potential to be used, either alone or in combination with polymers for different biomedical applications.

### 3.2 Introduction

Nitric oxide (NO), a gaseous diatomic free radical produced endogenously, has been shown to possess a wide variety of physiological functions. The molecule was first identified as the endothelial-derived relaxing factor in the 1980s and its many other roles in biological processes were later discovered, such as cell proliferation, inhibition of platelet activation and adhesion, prevention of bacterial proliferation and biofilm formation, immune system regulation, angiogenesis, and wound healing [272, 273]. The wide-ranging and advantageous properties of NO in physiology have prompted many researchers to develop materials that can exogenously deliver NO for different biomedical applications [219, 274].

NO is generated *in vivo* by nitric oxide synthase (NOS) enzymes at the pico- to nanomolar range depending on the physiological function or site of generation. Therefore, the effective therapeutic dose of NO is determined by the final application. Regarding this, NO dose and release kinetics should be considered carefully in developing therapeutic NO delivery systems [275]. However, storage and precise spatiotemporal delivery of NO is technically challenging due to its short biological lifetime and high reactivity [276]. Owing to the difficulties of delivering NO gas directly, several types of NO donor molecules have been employed which can store and spontaneously release NO when placed under physiological conditions [213]. S-Nitrosothiols (RSNOs) in particular are an endogenous class of NO donor molecules and have received substantial attention for biomedical applications due to their versatility when being incorporated into polymeric medical devices. S-Nitroso-N-acetyl-penicillamine (SNAP), a synthetic tertiary RSNO derived from the amino acid penicillamine, has been utilized and studied extensively in combination with biomaterials due to its relatively high stability and nontoxic origins [221, 277].

Incorporating NO donors into biomaterials not only induces the desired therapeutic effects but also allows for localized control over NO release. In recent years, various NO-releasing biomaterials have been developed by either physical blending, solvent swelling, or covalently attaching NO donors into organic and inorganic formulations such as polymeric films, hydrogels, nanofibers, and micro/nanoparticles [186, 212, 278-280]. Despite having simpler production methods, physically blended or impregnating NO-releasing materials have issues with potential leaching of the donor molecules and by-products leading to decreased NO release lifetimes and potential cytotoxic effects. These issues can be circumvented by covalently linking the NO donor molecules to their underlying substrates, therefore increasing the stability and safety of NO-releasing scaffolds [281].

NO donors can be covalently attached to polymer backbones or submicron size inorganic filler particles [282-284]. Covalent attachment of NO donor molecules to the surface of filler particles has the advantage of imparting NO release properties to a wide range of biomedical polymers by simply blending the filler into the polymer. The mechanical properties of the polymeric substrate can be enhanced by the addition of these NO-releasing filler particles while simultaneously not affecting the polymer's backbone chemistry or crosslinking process. Moreover, by adjusting the amount of filler added and the type of polymer used, the NO release kinetics can be fine-tuned for specific applications [285]. A few numbers of research groups have previously reported the development of covalently attached NO donors (N,N-diazeniumdiolates and S-nitrosothiols) to filler particles such as porous and nonporous silica [286, 287], fumed silica nanoparticles [288, 289], and diatomaceous earth silica particles [290]. However, burst NO release kinetics and low attachment efficiency of NO donors to the modified surface of these particles

mark two main factors limiting their efficacy. Therefore, seeking for novel NO carriers with improved storage and release properties remains an open challenge in the field of NO delivery.

For the first time in this study, covalent attachment of the *S*-nitrosothiol NO donor molecule, SNAP, to inorganic halloysite nanotubes (HNTs) is demonstrated. HNT [ $\text{Al}_2(\text{OH})_4\text{Si}_2\text{O}_5(2\text{-H}_2\text{O})$ ] is a naturally occurring, biocompatible aluminosilicate clay with a hollow tubular structure. The tubular structure of halloysites is composed of silica on the outer surface and alumina at the innermost surface with an inner diameter of 10–30 nm, an outer diameter of 40–70 nm, and a length of 500–1500 nm [291]. The unique hollow nanotubular structure along with other attractive features such as high length-to-diameter ratio, availability in large amounts at low cost, mechanical strength, biocompatibility, and environmental friendliness, make HNTs highly suitable for a wide range of applications [292]. Applications of HNTs include various fields such as a carrier for the loading and controlled release of guest molecules, adsorbent for pollution remediation, personal care and cosmetics, template or nanoreactor for biocatalysis, and fabrication of polymer nanocomposites [293].

Specifically, the drug delivery potential of HNTs has received much attention in the biomedical field. Guest molecules can be either loaded into the inner lumen of HNTs or grafted onto the outer surface [294-297]. Different chemistry of the inner and outer surfaces in HNTs allows for separate modification and simultaneous immobilization of two or more active agents with different biological properties which offers an additional advantage over other nanofillers [298]. The successful loading of diverse active molecules such as pharmaceuticals [299-301], biomacromolecules [302-305], corrosion inhibitors [306-310], and antibacterial agents [311-314] on HNTs has been previously demonstrated [315]. However, the delivery of NO gas using HNTs has never been explored.

In this work, the outer surface of HNTs was functionalized with the primary amine-containing silane reagent, aminopropyltrimethoxysilane (APTMS). The terminal amine groups of APTMS modified HNTs were then reacted with NAP-thiolactone, a self-protected penicillamine derivative, yielding free sulfhydryl groups on the surface. After NAP attachment, the free sulfhydryl groups were nitrosated to form the corresponding SNAP derivatized HNTs. The efficiencies of both surface silylation and NAP-thiolactone attachment were evaluated. The chemical modification of HNTs and retention of nanotube morphology throughout the modification were confirmed by Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Nitric oxide release over an 8 h period, total NO content, and storage stability at different conditions were determined by chemiluminescence. Finally, the antibacterial properties and cytocompatibility of the NO-releasing HNTs were examined.

### **3.3 Materials and methods**

#### **3.3.1 Materials**

Halloysite nanotubes (HNTs) (quality level=100), *N*-Acetyl-*D*-penicillamine (NAP) ( $\geq 99.0\%$  purity), (3-Aminopropyl) trimethoxysilane (APTMS) (97% purity), *L*-cysteine hydrochloride monohydrate ( $\geq 98.0\%$  purity), Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB) ( $\geq 98.0\%$  purity), glycine hydrochloride ( $\geq 99.0\%$  purity), 1,4,8,11-tetraazacyclotetradecane (cyclam) ( $\geq 98.0\%$  purity), and *t*-butyl nitrite ( $\geq 96.0\%$  purity) were purchased from Sigma-Aldrich (St. Louis, MO). Absolute ethanol ( $\geq 99.8\%$  purity) was purchased from VWR (USA). Toluene ( $\geq 99.5\%$  purity) and methanol ( $\geq 99.8\%$  purity) were purchased from Fischer Scientific (Waltham, MA). Sodium acetate ( $\geq 99.9\%$  purity) was obtained from EMD Chemicals, Inc. (Gibbstown, NJ). Luria-Bertani (LB) broth was obtained from Fisher Bioreagents

(Fair Lawn, NJ). LB Agar was purchased from Difco Laboratories Inc. (Detroit, MI). Phosphate-buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate, was used for all *in vitro* experiments. Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were purchased from Corning (Manassas, VA 20109). The Cell Counting Kit-8 (CCK-8) was obtained from Sigma Aldrich (St. Louis, MO 63103). The antibiotic Penicillin-Streptomycin (Pen-Strep) and fetal bovine serum (FBS) were purchased from Gibco-Life Technologies (Grand Island, NY 14072). The bacterial strain *S. aureus* (ATCC 25922) and mouse 3T3 cells (ATCC 1658) were originally purchased from American Type Culture Collection (ATCC).

### **3.3.2 Preparation of *N*-Acetyl-*D*-penicillamine (NAP) thiolactone**

Self-protected NAP-thiolactone was synthesized using the established protocol developed by Moynihan and Robert [316]. 5 g of NAP was dissolved in 10 mL of pyridine and a separate mixture of 10 mL of pyridine and 10 mL of acetic anhydride were also prepared. Both solutions were allowed to chill in an ice bath for 1 h before being combined and stirred at room temperature for 24 h. The solution was then rotary evaporated at 60 °C to remove all the pyridine. The remaining viscous, orange crude was re-dissolved in chloroform and washed and extracted three times with 1M HCl. The organic layer was then dried using anhydrous magnesium sulfate and filtered. The chloroform was removed under vacuum at room temperature to obtain a solid product. The solid material was washed and filtered with hexane and allowed to dry overnight at room temperature before being stored at 4 °C.

### **3.3.3 SNAP functionalization of HNTs**

**3.3.3.1 HNTs purification and alkali treatment:** HNTs were modified according to the previously reported literature with slight modification [317]. The as-received HNTs (3g) were

purified with 30% H<sub>2</sub>O<sub>2</sub> aqueous solution (10mL) and magnetically stirred for 2 h. The HNTs dispersion was then centrifuged and the resultant purified HNTs were first dried at 110 °C for 8 h in an oven and then dried at 60 °C in a vacuum oven for 4 h. To increase the number of hydroxyl groups on the surface, HNTs were treated with sodium hydroxide (NaOH). 2 g of HNTs were dispersed into 100 mL of 0.01 M NaOH solution and sonicated for 10 min using a water bath sonicator (Ultrasonic cleaner, Branson 2800) to obtain a homogeneous dispersion and magnetically stirred for 24 h at room temperature. The resultant hydroxylated HNTs (HNT-OH) were collected by centrifugation and rinsed several times with water until the pH reached near neutral. The prepared HNT-OH were dried at 110 °C for 8 h then at 60 °C for 4 h under vacuum.

**3.3.3.2 Surface silylation:** Surface silylation of HNTs was carried out following the previously reported literature with slight modification [318]. 0.3 g of hydroxylated HNTs were added to 10 mL of dry toluene and sonicated for 10 min to obtain a uniform dispersion. 1 mL of (3-aminopropyl) trimethoxysilane (APTMS) was added and the solution was refluxed under constant stirring for 20 h at 120 °C. The product was recovered by centrifugation and washed with toluene several times to remove the excess organosilane. APTMS modified HNTs (HNT-APTMS) were dried under vacuum at 60 °C for 24 h.

**3.3.3.3 NAP-thiolactone attachment and nitrosation:** The silanized HNTs were further reacted with NAP by slightly modifying a protocol by Frost et al [288]. Equal amounts (0.2 g) of HNT-APTMS and NAP were suspended in 15 mL toluene and allowed to stir for 24 h at room temperature to obtain NAP derivatized HNTs (HNT-NAP). Nitrosation of the formed HNT-NAP was done by adding *t*-butyl nitrite. *T*-butyl nitrite was first chelated of any copper contaminants by vortexing it with an equal volume amount of 20 mM cyclam solution and repeated three times. The organic *t*-butyl nitrite layer was then separated into an amber vial and stored at 5 °C. 500 µL

of *t*-butyl nitrite was then added to 15 mL of HNT-NAP to form a green, HNT-SNAP dispersion. The nitrosated nanotubes (HNT-SNAP) were then collected by centrifugation, washed several times with fresh toluene, vacuum-dried at room temperature, and stored in the dark at 4 °C.

### **3.3.4 Ninhydrin primary amine quantification**

The amount of amine groups bound to the surface of HNT-APTMS was estimated by using the ninhydrin assay. 2 mL of HNT-APTMS dispersions in 0.05% glacial acetic acid was combined with 1 mL of ninhydrin reagent and the mixture heated in a boiling water bath for 10 min. After cooling to room temperature, the samples were brought to a total volume of 8 mL with 95% ethanol and the absorbance was measured at 570 nm using a UV–vis spectrophotometer (Thermo Scientific Genesys 10S UV–vis). The amine content of HNT-APTMS samples was quantified using a calibration curve of known glycine hydrochloride concentrations.

### **3.3.5 Ellman's test for free sulfhydryls**

Ellman's Reagent, DTNB (5,50-dithio-bis-[2-nitrobenzoic acid]), was used to determine the amount of thiol groups on the surface of HNT-NAP nanotubes. Briefly, a stock DTNB solution containing 2 mM DTNB and 50 mM NaAc was prepared and used to create a working DTNB solution consisting of 50 µL of DTNB stock solution, 100 µL of PBS, 800 µL of H<sub>2</sub>O, and 50 µL of the sample. Absorbance values for each sample were recorded with a UV–vis spectrophotometer (Thermo Scientific Genesys 10S UV–vis) at a wavelength of 412 nm. The number of thiol groups was calculated from a standard curve of L-cysteine at pre-known concentrations.

### **3.3.6 Fourier-transform infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR)**

The successful functionalization of HNTs at each step was analyzed using Fourier transform infrared spectroscopy (FTIR). The HNT powders before and after modification were formed into a disk after mixing with potassium bromide (KBr) with a ratio of 1:100. For each measurement, the spectra were obtained from 128 scans with a resolution of  $4\text{ cm}^{-1}$  over the wavenumber range of  $400\text{--}4000\text{ cm}^{-1}$ .

Solid-state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP/MAS  $^{13}\text{C}$  NMR) was performed using a Bruker NEO 600 MHz spectrometer equipped with a 4mm rotor CMP-HRMAS probe. Due to the low carbon count with respect to the HNT element composition, final NMR spectra were obtained from the summation of 18 spectra at 256 scans at a spinning speed of 10 kHz. Carbons were detected and characterized on both the APTMS and NAP modified HNTs.

### **3.3.7 Nitric oxide release and storage stability measurements**

NO release from HNT-SNAP was measured via chemiluminescence using a Sievers nitric oxide analyzer (NOA) model 280i (Boulder, CO). Samples were weighed and incubated in 0.01 M PBS containing EDTA at  $37\text{ }^{\circ}\text{C}$  in an amber reaction vessel to protect from light. Nitric oxide released from the suspension of nanotubes was continuously swept from the vessel by an  $\text{N}_2$  purge gas at a flow rate of  $200\text{ mL min}^{-1}$  into the NOA system. The NO levels in the ppb unit measured at each time point were converted to NO release rate in  $\text{mol min}^{-1}\text{ mg}^{-1}$  using the NOA instrument constant, determined by quantitative reduction of a known amount of nitrite. Between the final and initial NOA measurements, the samples were stored in 0.01 M PBS with EDTA at  $37\text{ }^{\circ}\text{C}$ . NO release at different temperatures was measured by placing the sample vial in  $4\text{ }^{\circ}\text{C}$ , room

temperature (RT), 37 °C, and 45 °C water bath, respectively. Using the same instrument, light-triggered NO release from HNT-SNAP was also measured by placing the nanotubes in clear sample vials and irradiating them with a light-emitting diode (LED) lamp (12 W).

To measure the total NO loading, alternating injections of 0.25 M copper (II) chloride and ascorbic acid were added to the NOA reaction vessel. These injections catalytically triggered the release of all NO present in the sample within a time frame measurable by NOA. The total amount of NO released was calculated by the integration of NO release rate curves. Storage stability of the HNT-SNAP was examined by storing the samples under light and dark conditions at ambient temperature and in the freezer (-20 °C). After 4 weeks, the NO content in each sample was measured and compared to the initial total NO loading to calculate the % NO remaining.

### **3.3.8 Morphology observation**

The morphology of the nanotubes was observed by field emission scanning electron microscopy (FE-SEM). Transmission electron microscopy (TEM) images were used to measure the size of nanotubes before and after modification. Dilute HNT dispersions in DI water were dropped onto a carbon-film supported Cu grid and dried. Then the samples were observed using a JEOL JEM-2100 transmission electron microscope under an accelerating voltage of 200 kV. In order to compare the diameter of different HNT samples, ImageJ software was used to measure the widths of 50 tubes from the TEM images.

### **3.3.9 *In vitro* antibacterial assay**

To assess the antibacterial activity of the derivatized halloysite nanotubes, a 24 h antibacterial assay was performed against *S. aureus* (ATCC 6538) based on a previously established protocol [254]. Bacteria were handled in a BSL-2 facility previously approved by the University of Georgia. An isolated strain of *S. aureus* was inoculated in LB broth at 150 rpm at 37

°C. After 16 h, the bacterial solution was centrifuged at 2500 rpm for 7.5 min and washed with sterilized PBS (pH 7.4). The solution was again centrifuged at 2500 rpm for 7.5 min and resuspended in sterilized PBS through vortexing. The bacterial solution was diluted with PBS to reach a concentration of  $\sim 10^7$  colony-forming units (CFUs) per mL. Using a 96-well plate, 100  $\mu$ L of the bacterial solution was plated with 100  $\mu$ L of HNT (concentrations ranging from 0.5 mg/mL–10 mg/mL) suspended in PBS (n=5). Wells absent of nanotubes were used as control. The plate was incubated at 37 °C at 150 rpm for 24 h. After incubation, solutions in each well were serially diluted and plated on LB agar plates. Plates were incubated overnight at 37 °C. After incubation, CFUs present on the plates were counted to determine the effect of the presence and concentration of control and NO-releasing halloysite nanotubes on the viability of *S. aureus*, which represent the number of viable CFUs present per mL. The % reduction in bacterial viability was calculated according to the following equation (C = CFUs per mL):

$$\text{Reduction of bacterial viability (\%)} = \frac{C_{\text{control}} - C_{\text{sample}}}{C_{\text{control}}} \times 100 \quad (\text{Eq. 3.1})$$

### 3.3.10 Cell culture and viability studies

3T3 mouse fibroblast cells (ATCC 1658) were cultured in a 75 cm<sup>2</sup> T-flask containing complete DMEM medium with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin to prevent contamination. The T-flask with cells was incubated at 37 °C, in a 5 % CO<sub>2</sub> humidified environment to allow for monolayer formation. The culture medium was changed every two days, and cells were checked daily for growth and contamination. After the confluency reached above 80 %, cells were trypsinized (0.18 % trypsin and 5 mM EDTA) and detached from the T-flask. Finally, the cells were counted under a hemocytometer using Trypan blue (dye exclusion method). The effect of modified HNTs on cell viability was determined by the CCK-8 cell counting kit according to the manufacture's protocol (Sigma Aldrich). The CCK-8 test

is nondestructive in nature and more sensitive than other tetrazolium salts such as MTT, XTT, WST-1, and MTS. The number of living cells is directly proportional to the amount of formazan dye (orange color) generated by the interaction of the highly water-soluble tetrazolium salt, WST-8, with dehydrogenases in the cells and is detected at the absorbance maxima of 450 nm. First, cells were seeded in 96-well plates at a density of 5000 cells per well and allowed to attach in a humidified incubator with 5 % CO<sub>2</sub> for 24 h. The media were then replaced with fresh media containing unmodified HNTs or HNT-SNAP at various concentrations (0.5 mg/mL-10 mg/mL) and incubated for another 24 h. Cells maintained in DMEM without HNTs were used as a control group. To avoid absorbance interference by HNTs and HNT-SNAP, the media containing nanotubes was removed and 100 µL fresh media plus 10 µL of CCK-8 solution was added into each well and incubated for an additional 2 h at 37 °C. The absorbance was detected at 450 nm. The data were expressed as mean ± standard deviation (SD) (n=6), and the cell viability was calculated using the following equation [319]:

$$Cell\ viability\ (\%) = \frac{Absorbance\ (treated\ cells)}{Absorbance\ (control\ cells)} \times 100 \quad (Eq. 3.2)$$

### 3.3.11 Statistical Analysis

All data are reported as a mean ± standard deviation collected from three data points unless otherwise noted. A one-way analysis of variance was used to compare any significant differences. *p*-values of < 0.05 were considered statistically significant.

## 3.4 Results and discussion

### 3.4.1 Chemical Synthesis

**Figure 3.1** shows a schematic representation of HNT functionalization with SNAP. HNTs are first treated with NaOH to create hydroxyl groups on their external surface. It is previously reported that NaOH can effectively react with the tetrahedral silicate to create silanol (SiOH)

groups [317, 320]. After base treatment, HNTs are functionalized with aminopropyltrimethoxysilane (APTMS) by a silane coupling reaction. Silanization reactions proceed via a two-step process: (i) hydrolyzation of silanol groups in presence of water and (ii) condensation of silanol groups with SiOH groups present on the HNT-OH surface [321]. APTMS is a reactive aminosilane coupling agent that is widely used to modify the surface of HNTs [322, 323]. APTMS consists of three methoxy moieties that allow for stable surface binding and the formation of an amine rich layer on the surface. Afterward, NAP-thiolactone, a self-protected penicillamine derivative, is anchored to the amine groups by a ring-opening reaction resulting in a tertiary thiol-bearing silane. The thiol groups are then readily converted to their corresponding S-nitrosothiols by reaction with *t*-butylnitrite. The nitrosated nanotubes are referred to as HNT-SNAP.

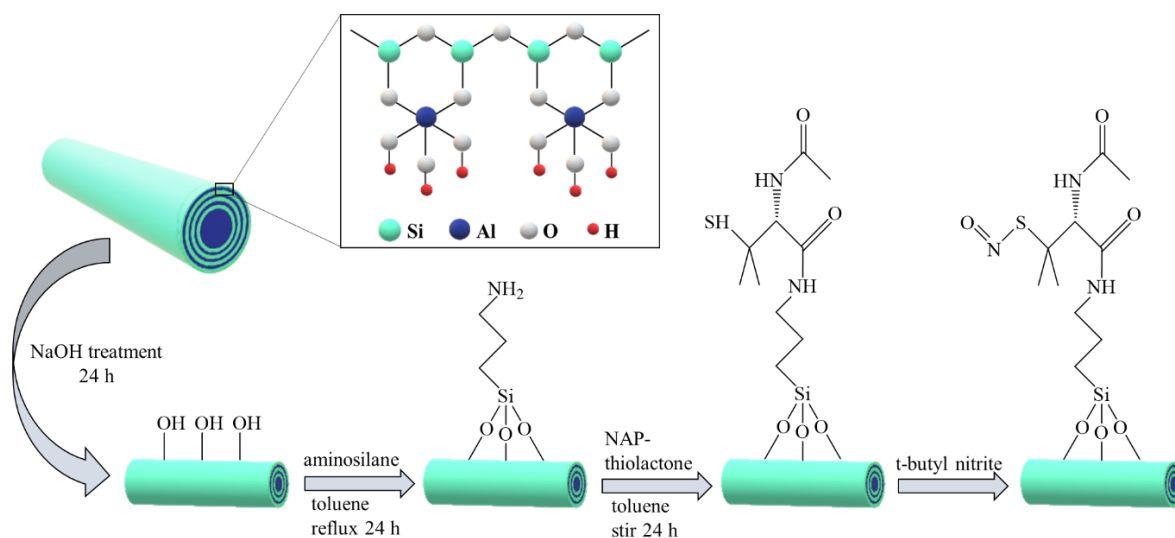


**Figure 3.1** - Schematic representing the chemical synthesis of NO-releasing halloysite nanotubes.

### 3.4.2 FTIR and NMR

The surface modification of HNT by NaOH and further chemical functionalization by APTMS and SNAP were confirmed by FTIR analysis. **Figure 3.2** shows the FTIR spectra of HNT,

HNT-OH, HNT-APTMS, and HNT-NAP. In the unmodified HNT spectrum, the peaks at 3700  $\text{cm}^{-1}$  and 3620  $\text{cm}^{-1}$  are assigned to the stretching vibrations of hydroxyl groups on the external and internal surface of HNT. The vibration at 1640  $\text{cm}^{-1}$  corresponds to O-H deformation of water and bands at 1030  $\text{cm}^{-1}$  and 910  $\text{cm}^{-1}$  exhibit the asymmetrical Si-O-Si stretching and Al-OH bending vibrations, respectively. The intensity of Si-O-Si stretching peak at 1030  $\text{cm}^{-1}$  is increased in the HNT-OH FTIR spectrum compared to the unmodified HNT. This indicates the formation of SiOH groups on the surface of HNTs according to a previous study [324]. After functionalization of HNT-OH by APTMS (HNT-APTMS), new characteristic peaks appeared at 2940  $\text{cm}^{-1}$ , 1554  $\text{cm}^{-1}$ , and 1490  $\text{cm}^{-1}$  which are respectively attributed to C-H stretching vibrations of alkyl groups, bending vibrations of  $\text{NH}_2$ , and antisymmetric stretching vibrations of C-N in APTMS. Moreover, the OH stretching band at 3620  $\text{cm}^{-1}$  is widened which might be due to the overlap of OH with the  $\text{NH}_2$  stretching vibrations signal around 3360  $\text{cm}^{-1}$ . These results indicate that APTMS has been successfully grafted onto HNT. The covalent attachment of NAP-thiolactone on HNT-APTMS was shown by characteristic peaks for amide conjugation in the respective FTIR spectrum. The new absorption band observed at 1657  $\text{cm}^{-1}$  denotes carbonyl vibrations and the signal at 1540  $\text{cm}^{-1}$  is ascribed to N-H bending vibrations. Additionally, the  $\text{sp}^3$  C-H bonds consistent with the alkyl chain of APTMS and methyl groups in NAP are also seen in subtle vibrations at 3051  $\text{cm}^{-1}$  and 2916  $\text{cm}^{-1}$ .

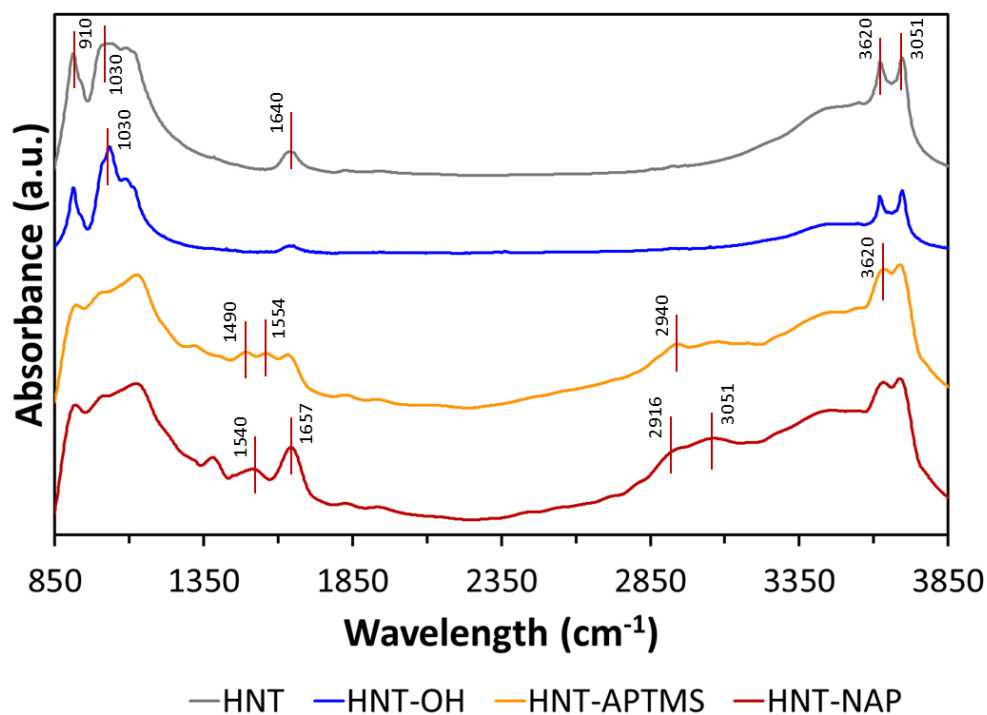


**Figure 3.2** - FTIR spectra of unmodified HNT, HNT-OH, HNT-APTMS, and HNT-NAP.

Further analysis of the surface modifications of the HNTs was done through CP/MAS  $^{13}\text{C}$  NMR. **Figure 3.3** shows the modification of APTMS to the HNTs and the subsequent attachment of NAP. The carbon peaks that form on the HNT-APTMS are identical to the ppm shifts seen from a previous study which attached a similar aminopropyl silane molecule to HNTs [325]. Signals observed at 42.58, 25.76, and 10.00 ppm match with the three carbons present on the APTMS. After the attachment of NAP, a characteristic carbonyl peak at 171.00 ppm is seen, proving there was a successful ring-opening reductive amination reaction. While some of the carbons were difficult to differentiate in the methyl region (between 15–30 ppm) due to overlapping signals, there was an overall increase in total carbons after integrating the region when compared to the HNT-APTMS spectrum. Therefore, these results strongly suggest that NAP-thiolactone is covalently attached to the HNT-APTMS.

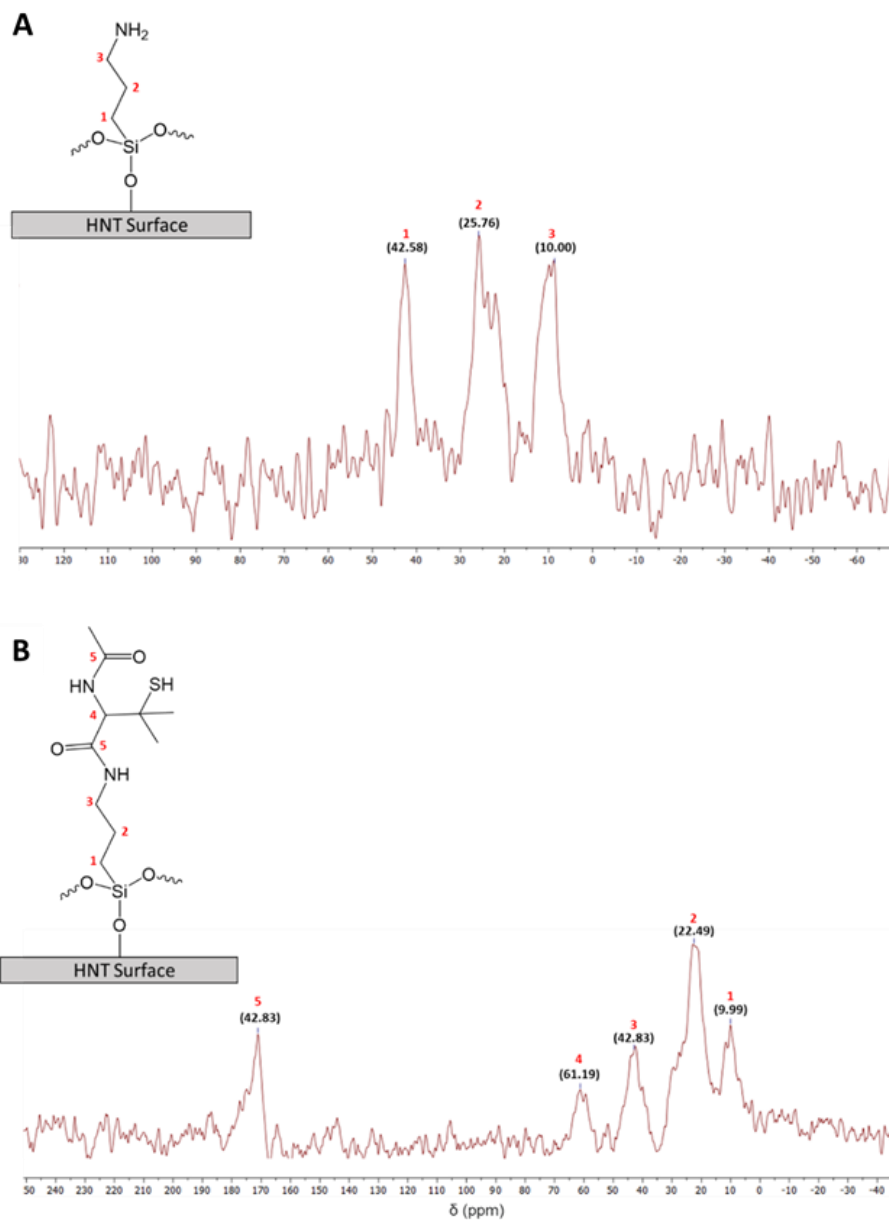


**Figure 3.3** -  $^{13}\text{C}$  CP/MAS NMR spectra of A) APTMS modified HNTs and B) NAP modified HNTs. The APTMS carbon peaks have been thoroughly investigated in previous literature [325]. While most of the carbon peaks in the NAP functionalized HNTs are overlapped in the methyl region (between 20–50 ppm), there is a distinct carbonyl peak at 171 ppm indicating successful NAP attachment.

### 3.4.3 Quantitative characterization of functionalized HNTs

To quantify HNT surface modifications and measure overall reaction efficiencies, the primary amine content, the resulting sulfhydryl content, and the total NO loading of HNTs after reaction with *t*-butyl nitrite were assessed (**Table 3.1**).

**3.4.3.1 Primary amine content:** Ninhydrin assay was performed to measure the primary amine groups of aminosilanes grafted on the surface of HNT. Ninhydrin reagent reacts with primary amines via oxidative-reductive reactions to generate a colored product known as Ruhemann's purple. The absorbance of the Ruhemann's complex at 570 nm is proportional to the surface amine density [326]. The amount of amine groups on the surface of HNT was estimated to be  $0.252 \pm 0.009$   $\mu\text{mol/mg}$ . Prior measurement, HNT-APTMS were thoroughly washed to eliminate the non-surface bound silanes. Therefore, the measured amine content is attributed to covalent aminosilanes grafting and not to physical surface adsorption.

**3.4.3.2 Sulfhydryl content:** It is previously reported that NAP-thiolactone exposes its thiol group only upon the ring-opening reaction with amine functional groups [327]. Therefore, the amount of NAP-thiolactone covalently attached to HNT-APTMS can be determined by measuring the number of primary thiols. The free thiol concentration on modified HNT's surface after NAP-thiolactone attachment was determined by means of Ellman's assay. Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with the thiol functional group to form a mixed disulfide and dianion of 2-nitro-5- thiobenzoic acid ( $\text{TNB}^{2-}$ ) in water. This solution has a distinctive yellow color that can be quantified by measuring the absorbance of visible light at 412 nm [328]. Using this method, the free sulfhydryl content of HNT-NAP was found to be  $0.202 \pm 0.015$   $\mu\text{mol/mg}$  indicating a NAP-thiolactone attachment efficiency (conversion ratio) of 80%. The conversion ratio obtained in this study is significantly higher than what was previously reported in

the literature (~ 2.5-47 %) for covalent attachment of NAP-thiolactone to aminosilane modified silica-based particles [288, 290, 329]. According to the previous studies, low values of conversion ratios are usually associated with aminosilanes self-polymerization and the formation of highly dense, interconnected silane networks causing steric congestion for NAP-thiolactone ring-opening reaction. However, in this study moderate reaction temperature, low silane concentration, and multiple washings with organic solvents advantageously minimize the formation of aminosilane-based oligomers and polymers.

**3.4.3.3 Total NO loading:** The total NO loading of HNT-SNAP was monitored via NOA using multiple injections of 0.25 M copper (II) chloride and ascorbic acid to trigger complete NO release. HNT-SNAP could store up to  $0.101 \pm 0.07$   $\mu\text{mol/mg}$  NO after nitrosation with *t*-butylnitrite demonstrating a nitrosation efficiency of ~50%. According to the previous studies, the incomplete nitrosation of HNT-SNAP is most likely caused by thiols that exist as disulfides or that are inaccessible to the nitrosating agent due to the associated steric hindrance surrounding the tertiary thiol functionality [329, 330]. Nevertheless, the total NO loading of HNTs is about three orders of magnitude larger than the previously reported SNAP functionalized diatomaceous earth silica particles and is almost equal to the total NO loading of SNAP grafted fumed silica particles [288, 290]. The obtained results after quantification of primary amines, thiol groups, and total NO content of modified HNTs along with the calculated reaction efficiencies are summarized in **Table 1**.

**Table 3.1** - Amine Content, thiol Content, conversion ratio, total NO loading, and nitrosation efficiency of modified HNTs (n=4).

| Amine content<br>( $\mu\text{mol/mg}$ ) | Thiol content<br>( $\mu\text{mol/mg}$ ) | Conv. ratio<br>(%) | Total NO<br>( $\mu\text{mol/mg}$ ) | Nitrosation<br>efficiency (%) |
|-----------------------------------------|-----------------------------------------|--------------------|------------------------------------|-------------------------------|
| $0.252 \pm 0.009$                       | $0.202 \pm 0.015$                       | 80.15              | $0.100 \pm 0.07$                   | 49.50                         |

#### 3.4.4 *In vitro* release kinetics of HNT-SNAP

NO release from S-nitrosothiols (RSNOs) such as SNAP can easily occur by cleavage of the S–NO bond in presence of either heat, light, or certain metal ions leading to the formation of free NO and disulfide species (RSSR) (Eq. 3.3) [221]. NO release from HNT-SNAP was studied under different stimuli including light and various temperatures.



The main mechanism of NO release from *in vivo* administered NO delivery systems is the thermal decomposition of SNAP via heat and moisture. Therefore, NO release measurement was first carried out at 37 °C in dark. **Figure 3.4** shows both the real-time and cumulative NO release from HNT-SNAP at different time points over the course of 8 h. The cumulative percentage was obtained by integrating the real-time NO release rates with the trapezoidal rule and dividing it by the total amount of NO initially loaded onto HNT-SNAP. The instantaneous release profile showed an initial rapid NO release over the first two hours followed by a slower release rate for 8 h. The burst effect during the first hours may correspond to the loosely bound aminosilane-SNAP complexes trapped within HNTs or less entangled SNAP functional groups that are exposed to the matrix environment. The cumulative release of NO within 8 h reached up to  $47.4 \pm 3.9\%$  meaning that the release half-life of HNT-SNAP is more than 8 h. This indicates a remarkable improvement upon the release kinetics of previously reported NO-releasing silica nanoparticles that reportedly deliver more than half of their NO payload in less than 2 hours [286, 288, 289, 331]. Covalent attachment of SNAP to HNT results in a total NO release of  $0.101 \pm 0.07 \mu\text{mol/mg}$  for over 8 h and may also decrease unwanted side effects and toxicity caused by leaching of the donor or reaction by-products commonly caused by physically blending them within a polymer.

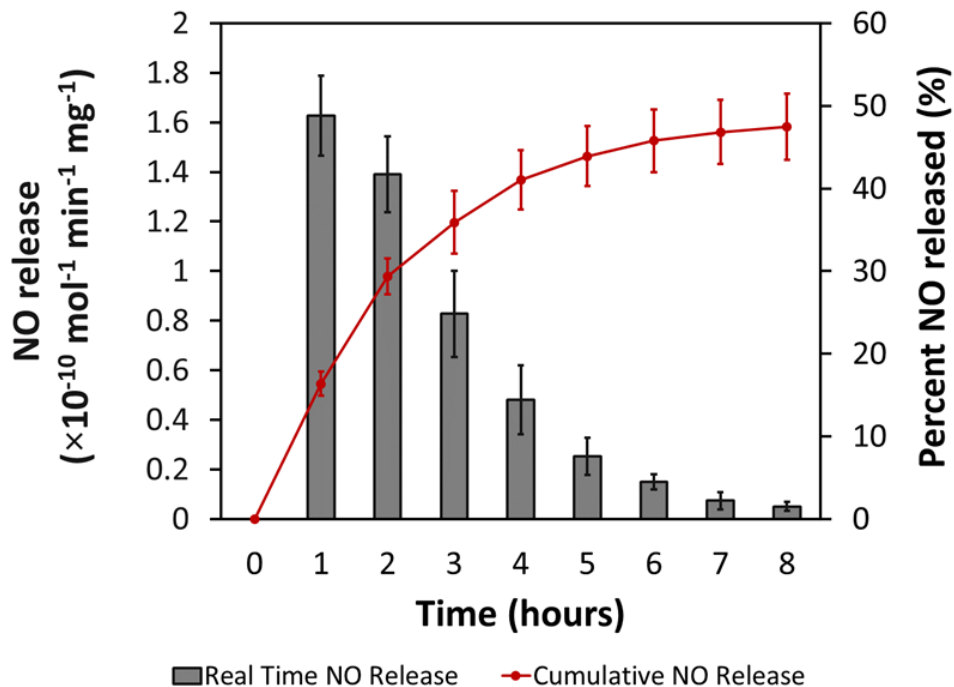


**Figure 3.4** - Cumulative and real-time NO release from HNT-SNAP soaking in PBS buffer at 37 °C in the dark. The red graph (cumulative NO release) and the black bar graph (real-time NO release) correspond to the right and left axes, respectively. Data represent the mean  $\pm$  SD (n=4).

The light-induced NO release from HNT-SNAP was also tested by irradiating the nanotubes with a white LED light (12 W) and is shown in **Figure 3.5A**. HNT-SNAP released NO at a constant rate of  $\sim 7$  ppb/mg when the light was off. However, by switching the light on, a significant increase in the NO flux was observed resulting in a sharp peak in the NO release profile (**Figure 3.5A**). This process was repeated three times and the peak intensities were decreased in time as the NO reservoir was drawn down. The light-triggered NO release properties of SNAP and S-nitrosoglutathione (GSNO) NO donors doped within polydimethylsiloxane films have been previously studied using an LED light source with different wavelengths [332].

In addition to light, the effect of temperature on NO release kinetics of HNT-SNAP was studied and the results are presented in **Figure 3.5B**. HNT-SNAP showed a very low amount of NO release at 4 °C with an average rate of 3 ppb/mg. A higher NO flux of about 7 ppb/mg was

obtained at room temperature which was further increased to 31 ppb/mg and 50 ppb/mg by elevating the temperature to 37 °C and 45 °C, respectively. By lowering the temperature back to RT, the NO flux was shortly decreased down to about 7 ppb/mg again. Overall, these results demonstrate the ability of HNT-SNAP to release tunable amount of NO by using light and adjusting the temperature which can prove useful for versatile biomedical applications.

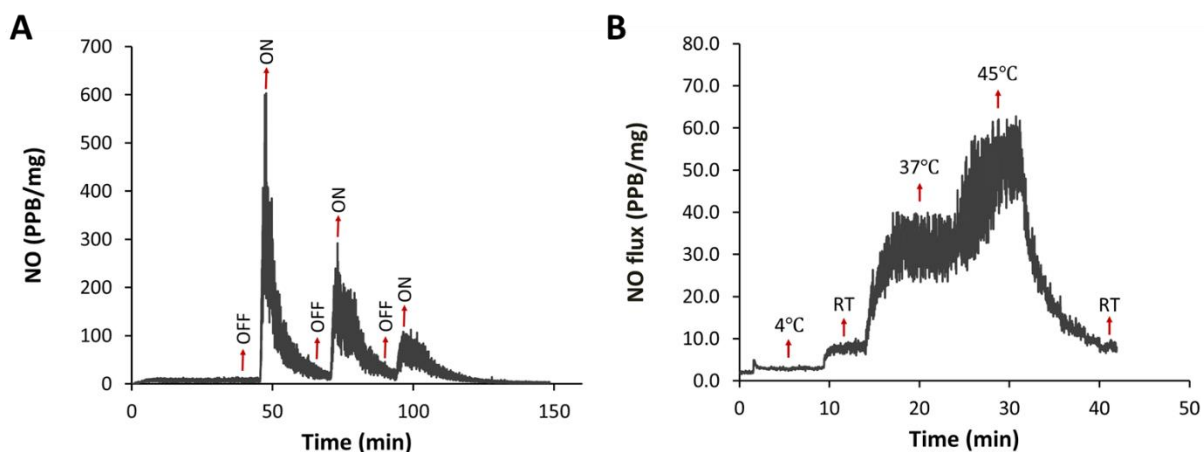


**Figure 3.5.** A) Real-time NO release from HNT-SNAP under A) light irradiation and B) different temperatures.

The kinetics of NO release from HNT can be further modulated by incorporating these inorganic fillers into polymeric substrates to limit the exposure of SNAP molecules to the media and prevent a rapid release. The fluxes of NO can be finely tuned depending upon the application by changing the polymeric matrix (and the associated water uptake properties) or altering the amount of HNT-SNAP blended into the polymer. Additionally, certain metal ions (e.g.  $\text{Cu}^+$ ) that are shown to have a catalytic effect on the release of NO from S-nitrosothiols can be loaded on the outer walls or into the inner lumen of HNTs to enhance NO release [254, 333-336]. In fact, using this approach allows for obtaining different NO release profiles for versatile biomedical applications such as platelet inhibition, bacteria-killing, and wound healing.

The stability of HNT-SNAP was evaluated by measuring the NO content after storing them at different conditions (light at room temperature, dark at room temperature, and -20 °C freezer) for a period of 4 weeks. HNT-SNAP showed the highest stability in the freezer with  $97.2 \pm 2.7$  % of NO being remained after storing at -20 °C for 4 weeks (**Figure 3.6**). While the lowest stability was attributed to HNT-SNAP stored under light at room temperature (RT) with only  $53.8 \pm 3.2$  % of NO being remained after 4 weeks. As expected, HNT-SNAP stored at dark in RT could preserve a significantly higher amount of NO ( $67.3 \pm 4.5$  %) compared to HNT-SNAP stored under light at the same temperature.

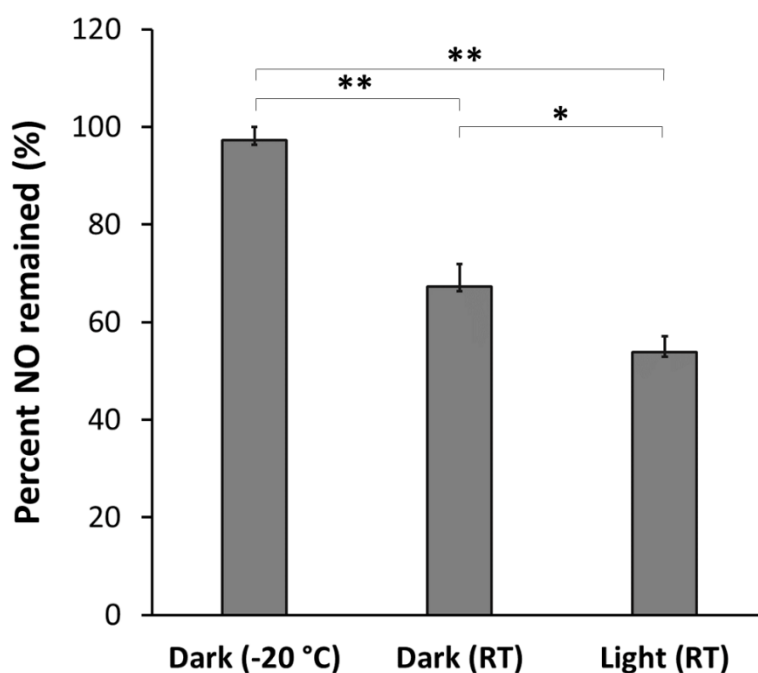


**Figure 3.6** - Storage stability of HNT-SNAP at different conditions over 4 weeks.

### 3.4.5 Morphological Analysis

The TEM and FESEM images of unmodified HNT and HNT-SNAP are depicted in **Figure 3.7**. Both FESEM and TEM images display the polydispersity and hollow tubular structure of nanotubes with an open-ended lumen along the tube. From TEM images, the size of HNTs was measured to be 100-1000 nm long, with an outer diameter of about 60 nm and a lumen diameter

of about 10 nm. After modification with SNAP, the outer walls of HNTs became slightly thicker (~70 nm), indicating that the functionalization is occurring on the outer surface of nanotubes [337].

In addition to size, the morphology of nanomaterials is also a critical factor affecting their loading capacity and release kinetics. For instance, NO-releasing fumed silica particles have been previously developed and studied for biomedical applications. However, aggregation of these particles into coarse, irregular clusters upon pyrolytic production, renders their final morphology and NO release kinetics highly unpredictable [338]. In contrast, HNTs are well known for their excellent dispersion ability originating from their stable tubular morphology, charge distribution, and unique crystal structure [339]. To obtain reliable and consistent NO release kinetics, it is imperative to maintain the original morphology of HNTs after chemical modifications. Apart from loading and controlled release of guest molecules, morphological properties of HNTs can also significantly affect their performance in other applications such as the fabrication of HNT-polymer nanocomposites, nanoreactor templates, and filtration purposes [340]. As can be seen in the FESEM and TEM images, the tubular morphology and crystal structure of HNTs are preserved after chemical modifications with APTMS and SNAP. While the original tubular morphology of the HNTs is preserved, the SEM images seen in **Figure 3.7** also demonstrate a rougher surface interface on the HNT-SNAP when compared to control HNTs. This is most likely due to a hydrolysis reaction between APTMS chains, creating a polymerized siloxane layer at the HNT interface. Since the reaction conditions mentioned previously were not perfectly anhydrous, this type of hydrolysis is commonly seen [341].

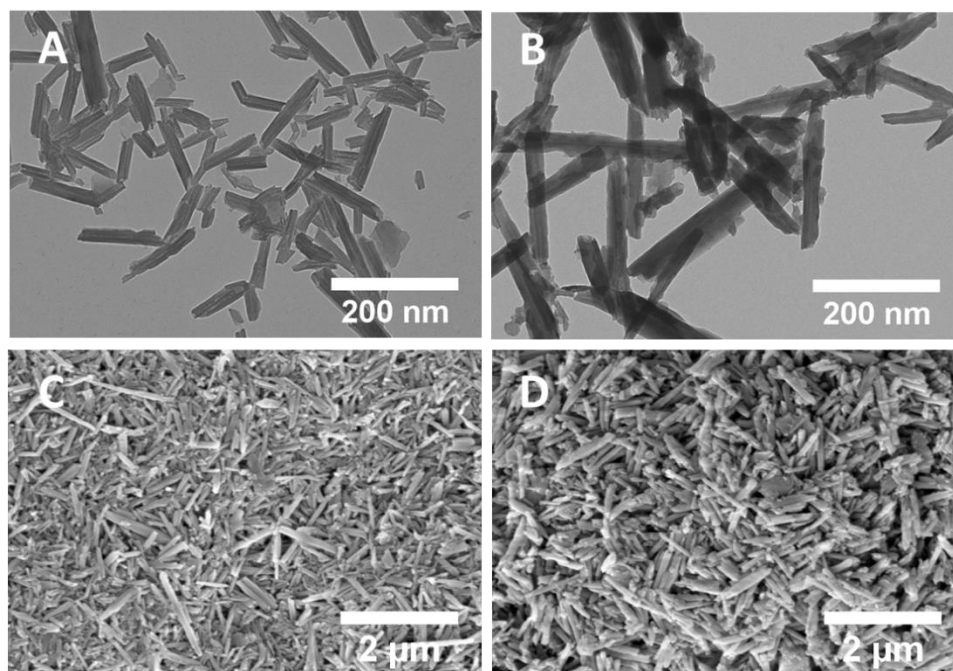


**Figure 3.7** - TEM images (A,B) and SEM images (C,D) of HNTs (A,C) and HNT-SNAP (B,D).

### 3.4.6 Bactericidal properties of HNT-SNAP

Over the past few years, increasing attention has been focused on nanoparticle-based materials in the field of antimicrobial therapies. Administration of antimicrobial agents using nanoparticles provide several benefits over free antimicrobial agents such as improved solubility, pharmacokinetics, and therapeutic index of the drug, reduced side effects, as well as controlled release and targeted delivery of the drug to the site of infection [342, 343]. One of the most common causes of hospital-acquired infections is the gram-positive *S. aureus* bacteria [344]. Currently, successful treatment of infections caused by *S. aureus* is being restricted as this microorganism has developed resistance toward silver and many antibiotics [345, 346]. Therefore, alternative therapeutic approaches are essentially needed. Nitric oxide is known to be a potent antimicrobial agent that kills bacteria through rapid and nonspecific mechanisms [269]. Therefore, the incidence of bacterial resistance is minimized as opposed to antibiotics and silver nanoparticles. According to the literature, chemical alteration of DNA, disturbing protein

synthesis, damaging cell membrane amino acids, and lipid peroxidation are the main bactericidal mechanisms of nitric oxide [174]. In this study, the antibacterial activity of HNT and HNT-SNAP toward *S. aureus* was studied as a proof-of-concept evaluation for biomedical applications. The result of bacterial viability was quantified using a CFU counting methodology and is depicted in **Figure 3.8**. The reduction efficiency values of all the tested concentrations of HNT-SNAP are also presented in **Table 3.2**. HNT-SNAP showed antibacterial activity against *S. aureus* in a concentration-dependent manner. By increasing the HNT-SNAP concentration from 0.5 mg/mL to 10 mg/mL, the bacteria reduction efficiency was enhanced from 48.9% to 99.6% (> 2 log reduction), whereas unmodified HNT showed no antibacterial activity against *S. aureus* regardless of the concentration, suggesting that the antibacterial activity of HNT-SNAP is attributed solely to the NO release. Additionally, it has been previously shown that NO donors either physically blended or covalently attached to biomaterials exhibit antibacterial effects toward different bacteria including *P. aeruginosa*, *S. aureus*, and *E. coli* [347, 348]. The previously demonstrated antibacterial properties of NO against these bacteria along with the antibacterial effects of HNT-SNAP displayed in this study provide promising perspectives for the potential application of HNT-SNAP either alone or in combination with other biomaterials. Moreover, this work marks a significant improvement upon the antibacterial properties of previously reported NO-releasing diatomaceous earth (DE) silica particles by our group [290]. HNT-SNAP at concentrations of 5 mg/mL and 10 mg/mL reduced the number of *S. aureus* bacteria by  $94.4 \pm 1.9$  % and  $99.6 \pm 0.1$  % respectively, whereas a lower reduction efficiency of  $92.9 \pm 2.6$  % was reported for SNAP modified DE silica particles at an even greater concentration of 21.5 mg/mL. The superior bactericidal efficiency of HNT-SNAP might be the result of enhanced total NO loading and higher flux of NO release. According to a study by Nichols and Schoenfisch, the bactericidal properties

of NO-releasing surfaces are dependent on the NO payload, rate of delivery, and duration of NO release [349].

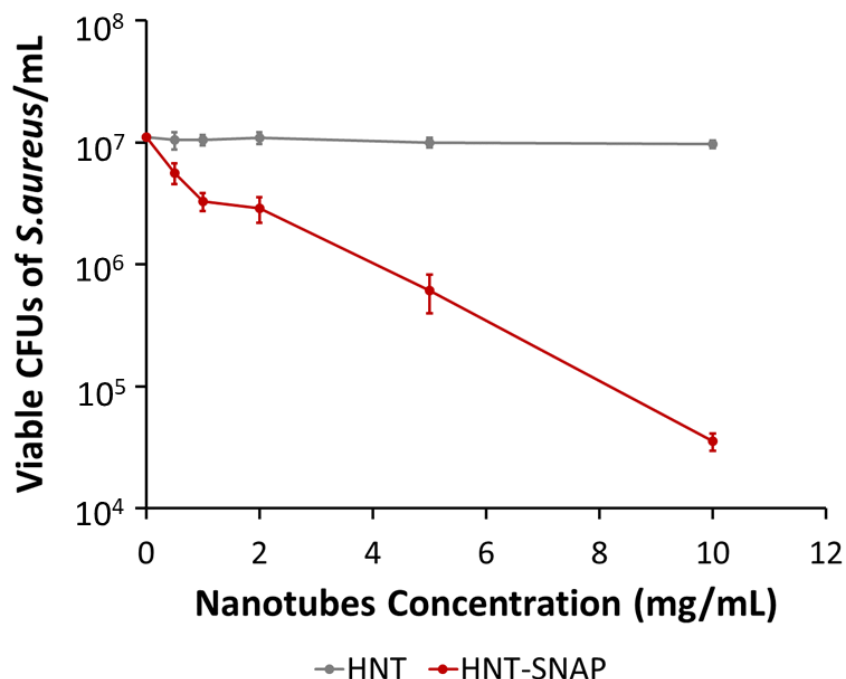


**Figure 3.8** - Antibacterial activity of HNT and HNT-SNAP against *S. aureus* after 24 h (n=5).

**Table 3.2** - Viability reduction efficiency values of *S. aureus* bacterial strain after exposure to different concentrations of HNT-SNAP for 24 h (n=5). (\*\* indicates p-values  $\leq 0.01$  versus HNTs at the same concentration).

| HNT-SNAP concentration (mg/mL) | <i>S. aureus</i> reduction efficiency (%) |
|--------------------------------|-------------------------------------------|
| 0.5                            | $48.9 \pm 3.9^{**}$                       |
| 1                              | $69.9 \pm 9.8^{**}$                       |
| 2                              | $73.8 \pm 5.0^{**}$                       |
| 5                              | $94.4 \pm 1.9^{**}$                       |
| 10                             | $99.6 \pm 0.1^{**}$                       |

### 3.4.7 Cytocompatibility of HNT-SNAP

While we were able to demonstrate a significant reduction in bacterial viability due to the NO release of the HNT-SNAP, it is imperative that they exhibit no toxic effects towards mammalian cells. To assess the cytotoxicity of HNT-SNAP, the direct contact CCK-8 (Sigma-

Aldrich) method was performed on mouse fibroblast cells (ATCC). Cells were exposed to different concentrations of nanotubes ranging from 0.5 mg/mL to 10 mg/mL. The results of the test are depicted in **Figure 3.9** and cell viability values for HNT and HNT-SNAP at all the tested concentrations are reported in **Table 3.3**. No significant cytotoxicity was observed for both unmodified HNT and HNT-SNAP at concentrations below 2 mg/mL as compared to control (wells without any nanotubes). However, by further increasing the concentration of nanotubes to higher values ( $\geq 2$  mg/mL), the number of viable cells decreased and reached less than 50% of the control at 10 mg/mL ( $p$ -value  $< 0.05$ ). It is noteworthy to mention that, at all concentrations, the viability of cells exposed to HNT-SNAP remained higher than the cells treated with unmodified HNT. The higher viability of cells in contact with HNT-SNAP can be explained by the previously described regulating effects of NO on fibroblasts proliferation. In the past, several reports have demonstrated that exogenously applied NO, at low concentrations, is able to increase the growth and proliferation of several mammalian cell lines including fibroblasts [350-352]. The *in vitro* and *in vivo* cytotoxicity of HNT have also been extensively studied and described by many research groups, all in agreement that HNT shows a high level of cytocompatibility and low cytotoxicity [315]. Therefore, the reduction in the viability of cells at higher concentrations of HNT-SNAP is most likely attributed to the high number of particles. Despite the development of many nanoparticulate NO delivery systems, only a few studies have investigated the cytocompatibility of these nanosized delivery systems in direct contact with cells. We have previously shown that regardless of the biocompatibility of the substrate and the loaded NO donor, NO-releasing nanoparticles at high concentrations (5-10 mg/mL) can induce toxicity toward fibroblast cells [353]. However, this toxic effect was found to be solely related to the high number of nanoparticles covering the cells in the well plate. Similar results have also been reported by Nurhasni et al.,

evaluating the cytotoxicity of NO-releasing poly(lactic-co-glycolic acid)-polyethyleneimine nanoparticles against mouse fibroblast cells [232]. Collectively, our results demonstrate that SNAP functionalized HNTs show tunable NO release, strong antibacterial properties, and desirable cytocompatibility which may prove useful for a range of applications. To further confirm the preclinical potential of HNT-SNAP, comprehensive *in vivo* verification of the *in vitro* results is required.

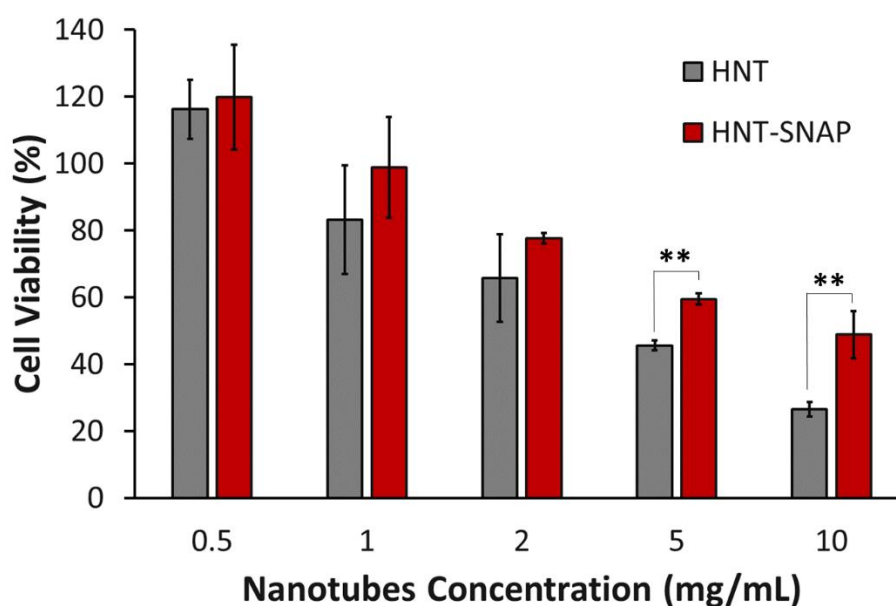


**Figure 3.9** - 24 h viability of 3T3 mouse fibroblast cells (as a percentage relative to control) using CCK-8 assay. The error bar represents the SD (n=6). *p*-values ≤ 0.01 are shown with \*\*.

**Table 3.3** - Viability values of 3T3 mouse fibroblast cells after direct exposure to different concentrations of HNT and HNT-SNAP for 24 h. (\*\* indicates *p*-values ≤ 0.01 obtained by comparison of HNT-SNAP and HNT at the same concentration).

| Nanotube concentration (mg/mL) | Cell viability (%) |              |
|--------------------------------|--------------------|--------------|
|                                | HNT                | HNT-SNAP     |
| 0.5                            | 116.2 ± 8.7        | 119.8 ± 15.6 |
| 1                              | 83.1 ± 16.2        | 98.8 ± 15.0  |
| 2                              | 65.8 ± 13.0        | 77.6 ± 1.6   |
| 5                              | 45.5 ± 1.5         | 59.4 ± 1.6** |
| 10                             | 26.4 ± 2.1         | 48.8 ± 7.0** |

### 3.5 Conclusion

Covalent attachment of the NO donor molecules to inorganic filler particles is an effective approach to impart NO release properties to a wide range of biomedical polymers and enhance their safety and stability by reducing the potential leaching of the donor molecules and by-products. However, burst NO release kinetics and low attachment efficiency of NO donors to the modified surface of these particles mark two main factors limiting their efficacy. With the goal of improving these properties, this paper aimed to develop and characterize a novel NO delivery system based on HNTs. APTMS functionalization of HNT facilitated the covalent attachment of NAP which was further converted to SNAP, the NO donor molecule. The amount of primary amine groups after APTMS modification and thiol groups after NAP attachment were estimated to be  $0.252 \pm 0.009 \text{ } \mu\text{mol/mg}$  and  $0.202 \pm 0.015 \text{ } \mu\text{mol/mg}$  respectively, indicating a favorably high conversion ratio of 80%. In total, each mg of HNTs was loaded with  $0.101 \pm 0.07 \text{ } \mu\text{mol}$  of NO which were released sustainably over an 8 h period. The high conversion ratio and NO release half-life of more than 8 h obtained for HNT-SNAP demonstrated a significant improvement upon the previously reported NO-releasing silica particles [286-290]. Light-triggered NO release from HNT-SNAP was also studied using a light-emitting diode source and it was found that NO fluxes were notably increased immediately upon irradiation. The release of NO from HNT-SNAP could also be fine-tuned by adjusting the temperature. The successful modification of HNT was further confirmed and studied using Fourier transform infrared spectroscopy and  $^{13}\text{C}$  Nuclear magnetic resonance. The SEM images showed that the unique morphology of HNTs remains intact throughout the synthesis. However, according to TEM images, the outer walls of HNTs became slightly thicker after surface modification. Additionally, the bactericidal efficiency of HNT-SNAP towards gram-positive *S. aureus* and biocompatibility with mammalian cells were demonstrated

at different concentrations ranging from 0.5 mg/mL to 10 mg/mL. Based on the promising results obtained in this study and the myriad of potential applications of NO-releasing materials, it can be concluded that modification of HNTs with NO donor molecules open up the use of these nanomaterials for several applications in the biomedicine field such as implants, catheters, tissue engineering scaffolds, wound dressings, biosensors, and medical device coatings. The results of this study form the basis for further future studies in the pre-clinical settings to ascertain the therapeutic potential of NO-releasing HNTs.

**CHAPTER 4**

**NITRIC OXIDE-RELEASING GELATIN METHACRYLOYL/SILK FIBROIN  
INTERPENETRATING POLYMER NETWORK HYDROGELS FOR TISSUE  
ENGINEERING APPLICATIONS<sup>4</sup>**

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<sup>4</sup> Ghalei S, Douglass M, Handa H. Nitric Oxide-Releasing Gelatin Methacryloyl/Silk Fibroin Interpenetrating Polymer Network Hydrogels for Tissue Engineering Applications. *ACS Biomaterials Science & Engineering*. 2021 Dec 10;8(1):273-83. Reprinted here with permission of the publisher.

## 4.1 Abstract

Bacterial infection is one of the principal reasons for the failure of tissue engineering scaffolds. Therefore, the development of multifunctional scaffolds that not only are able to guide tissue regeneration but also can inhibit bacterial colonization is of great importance for tissue engineering applications. In this study, a highly antibacterial, biocompatible, and biodegradable scaffold based on silk fibroin (SF) and gelatin methacryloyl (GelMA) was prepared. Sequential crosslinking of GelMA and SF under UV irradiation and methanol treatment, respectively, resulted in the formation of interpenetrating network (IPN) hydrogels with a porous structure. In addition, impregnation of the hydrogels with nitric oxide (NO) donor molecule, S-nitroso-N-acetylpenicillamine (SNAP), led to the development of NO-releasing scaffolds with strong antibacterial properties. According to the obtained results, the addition of SF to GelMA hydrogels caused an enhancement in the mechanical properties and NO release kinetics and prevented their rapid enzymatic degradation in aqueous media. Furthermore, swelling the GelMA-SF scaffolds with SNAP resulted in a bacteria reduction efficiency of >99.9% against gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*) bacteria. The scaffolds also showed great cytocompatibility *in vitro* by increasing the proliferation and supporting the adhesion of 3T3 mouse fibroblast cells. Overall, GelMA-SF-SNAP showed great promise to be used as a scaffold for tissue engineering and wound healing applications.

## 4.2 Introduction

Tissue engineering (TE) scaffolds aim to regenerate or restore the function of damaged tissues and organs, offering a promising alternative to organ transplantation. Ideally a TE scaffold should be biocompatible with a porous structure to provide a suitable microenvironment for cells to adhere, grow, migrate, and build their own extracellular matrix. In addition, a scaffold must be

of sufficient mechanical integrity to be able to support cells through the whole healing process, while also degrade at an optimal rate to allow cells to replace it over time [354]. A great challenge, however, is to develop scaffolds that are not only able to promote cell-material interaction but also inhibit bacterial colonization. Despite the use of different antimicrobial approaches, currently one of the main reasons of scaffolds failure in TE is their ineffectiveness in controlling infection [355].

Infection starts with bacteria adhering to the surface of the scaffold, proliferating, and producing an extracellular polymeric biofilm matrix that will further protect them from the surrounding environment by hindering the permeation of antibiotics and phenotypic alterations in the bacterial cells [356]. Bacteria in biofilms are extremely resistant to antibiotics compared to their free-floating planktonic counterparts. Therefore, biofilm formation on the surface of implantable medical devices and scaffolds can give rise to persistent infections that endangers the patient's life and requires a revision surgery to remove the infected material [357]. Implant-associated infections with a prevalence of approximately 2 million cases per year in the United States account for nearly half of all the nosocomial infections, putting a huge burden on both the patient and health care budget [358, 359]. With antibiotics falling short of treating biofilm-related infections, there is an urgent need to develop novel antibacterial approaches with enhanced efficacy that can be used to prevent bacterial infection in implantable medical devices and scaffolds.

In recent years, there has been an increasing interest in the incorporation of nitric oxide (NO)-releasing molecules in different polymeric biomaterials for application in wound healing and TE [174, 360]. Nitric oxide (NO) is a gaseous free radical, endogenously released by macrophages as a potent antimicrobial agent to fight with pathogens. NO kills bacteria through multiple nonspecific mechanisms such as reacting with oxygen and superoxides and generating reactive

intermediate byproducts that can cause lipid peroxidation, DNA alteration, and disruption of critical proteins and enzymes in the bacterial cells [361]. It has also been shown previously that NO, even when released at low concentrations (pico-nano molar), is able to disperse biofilms into a planktonic state, making them more vulnerable to the conventional antibiotic therapies [362]. Therefore, the fast-acting and multi-mechanistic bactericidal activity of NO along with its biofilm dispersal ability make it a promising alternative for antibiotics to combat biofilm-related infections. In addition to its antibacterial activity, NO is also known to play important roles in the wound healing process by enhancing angiogenesis and collagen deposition [363].

NO donors such as diazeniumdiolates (NONOates) and S-nitrosothiols (RSNOs), either physically blended or covalently conjugated to polymeric fibers [184, 364, 365], hydrogels [366-369], and nanoparticles [370-372] have been used to fabricate NO-releasing scaffolds and wound healing materials. In particular, hydrogels with hydrophilic 3D porous structures resembling the native tissue, are of great interest for scaffold related applications due to their ability to provide a highly hydrated substrate that facilitates the transport of gases and nutrients and allow cells to infiltrate, grow, and reconstruct or repair the damaged tissue [373]. Different NO-releasing hydrogels have been developed so far for application in wound healing and TE [367, 374]. Rapid leaching and a high initial burst release profile mark the main limitations associated with these hydrogels. Nevertheless, as TE scaffolds, NO-releasing hydrogels can provide a highly antibacterial platform that initially inhibits bacterial colonization in the first few hours/days after implantation while yet provide a suitable longer-term substrate for attachment and proliferation of tissue cells after the NO supply is depleted.

In this study, we developed NO-releasing interpenetrating network (IPN) hydrogels based on gelatin methacryloyl (GelMA) and silk fibroin (SF), through a two-step crosslinking/swelling

method. GelMA is a semi-synthetic polymer obtained by methacrylic anhydride (MA) modification of amine groups on gelatin. This modification does not affect the advantageous properties of gelatin such as biocompatibility, arginine–glycine–aspartic acid (RGD) sequences, gel forming ability, and biodegradability while also confers it with a photo-crosslinking property. In presence of a photo-initiator, MA monomers photopolymerize under UV light at ambient temperature to form hydrogels that are stable at higher temperatures (37 °C). This ability allows for fabrication of spatially tailored TE constructs, eliminates the need of using toxic chemical agents, and further improves the biocompatibility of these materials as TE scaffolds [375]. Despite many advantages, low mechanical properties and rapid degradation kinetics associated with gelatin and GelMA hydrogels often limit their application as TE scaffolds. This has directed researchers into developing GelMA based composite hydrogels with improved properties [376].

In this study we used SF, a natural protein widely used for TE and biomedical applications [237, 377] due to its unique properties such as excellent mechanical strength, resistance to enzymatic degradation, water based processing, and high biocompatibility to fabricate GelMA-SF IPN hydrogels. The fabricated hydrogels were swelled with *S*-nitroso-*N*-acetylpenicillamine (SNAP), an *S*-nitrosothiol NO donor molecule that has previously demonstrated high stability, good biocompatibility, and strong antibacterial and antithrombotic properties when incorporated in different biomaterials [277, 378, 379]. Considering the complementing properties of GelMA and SF, their combination in an IPN hydrogel can result in synergistically enhanced properties such as higher mechanical strength, slower degradation rate, and more controlled NO release kinetics.

The SNAP-swelled GelMA-SF IPN hydrogels prepared in this study were observed under scanning electron microscopy (SEM) to study their morphology and microstructure and analyzed

through Fourier-transform infrared spectroscopy to assess their structural and conformational properties. The optimal swelling time, maximum SNAP loading, and NO release from the hydrogels were measured. In addition, mechanical properties of the hydrogels along with their enzyme mediated degradation profile were examined. Finally, the *in vitro* antibacterial activity and cytocompatibility of the hydrogels were also investigated to evaluate their suitability as potential scaffolds for TE applications.

### **4.3 Materials and methods**

#### **4.3.1 Materials**

*N*-acetyl-*D*-penicillamine (NAP), sodium nitrite, sulfuric acid, phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), lithium bromide, Gelatin methacryloyl (80% degree of substitution), Irgacure 2959, and collagenase type II were purchased from Sigma-Aldrich (St. Louis, MO). Bombyx mori (*B. mori*) silk cocoons were obtained from Paradise Fibers (USA). Spectra/Por 3 regenerated cellulose dialysis membrane tubing (3.5 kDa, molecular weight cut-off), hydrochloric acid, and methanol were obtained from FisherScientific (Hampton, NH). Cu-NPs (99%, 40–60 nm) were purchased from SkySpring Nanomaterials, Inc. (Houston, TX). LB Agar was acquired through Sigma-Aldrich and LB broth was purchased from BD Difco. Fetal bovine serum (FBS) was acquired through VWR. Staphylococcus aureus (ATCC® 6538™, *S. aureus*), Escherichia coli (ATCC® 25922™, *E. coli*), and 3T3 mouse fibroblast cells (ATCC 1658) were obtained from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium and Pen-strep were purchased from Sigma-Aldrich. Cell Counting Kit-8 (CCK-8) was acquired from Enzo Life Sciences.

### **4.3.2 Material synthesis**

#### **4.3.2.1 Silk fibroin**

Aqueous SF solution was obtained according to a standard extraction procedure [253]. *B. mori* cocoons were first degummed by boiling in 20 mM sodium carbonate solution for 30 min. The degummed SF fibers were dried at room temperature overnight and then dissolved in 9.3 M lithium bromide solution for 4 h at 60 °C. The dissolved SF solution was dialyzed against deionized water (DIW) using a 3.5 kDa cut-off dialysis membrane for 48 h, filtered, and stored at 4 °C for further use.

#### **4.3.2.2 SNAP**

SNAP was synthesized following a modified version of a previously reported protocol [221, 281]. Briefly, 5 g of NAP plus equimolar amount of sodium nitrite were dissolved in pre-chilled 55% methanol. After complete dissolution, 20 mL and 5 mL of concentrated hydrochloric acid and sulfuric acid were added to the solution, respectively, and allowed to stir for 10 min. The reaction mixture was then placed in an ice bath and air was gently blown into the mixture to speed up the solvent evaporation and SNAP crystal formation. After 6 h, the formed SNAP crystals were vacuum filtered, washed vigorously with chilled DIW, and allowed to dry in a vacuum desiccator overnight. The reaction mixture was protected from light at all times to prevent light induced NO release. Dried SNAP was stored in dark at -20 °C. The purity of the synthesized SNAP (>95%), was verified using a Chemiluminescence NO Analyzer.

#### **4.3.2.3 Preparation of SNAP-swelled GelMA-SF IPN hydrogels**

Gelma-SF IPN hydrogels were fabricated by initially mixing aqueous solutions of GelMA (10 wt%) and SF (5 wt%) in a 3:2 v/v ratio. The Irgacure 2959 photoinitiator (1 wt%) was added to the mixed polymer solutions and stirred until fully dissolved. After transferring the solutions to

cylinder-shaped molds, they were crosslinked sequentially by first irradiating with UV light (350-500 nm, 36 W) for 5 min to initiate the photo assisted radical polymerization of the GelMA and then immersing the formed semi-IPN hydrogel in 80% methanol solution for 2 h to induce  $\beta$ -sheet formation in the SF. To fabricate the GelMA-SF-SNAP hydrogels, UV-cured GelMA-SF was immersed in 80% methanol containing 25 mg/mL SNAP for the same 2 h period. After SNAP impregnation, the hydrogels were rinsed thoroughly with DIW to remove excess SNAP on the surface and residual methanol, and then freeze-dried. GelMA and GelMA-SNAP hydrogels were prepared following the same method without adding SF to the initial polymer solution. A similar 25 mg/mL SNAP solution in 80% methanol was used for SNAP impregnation into GelMA-SNAP hydrogels. In addition, to keep consistency among all samples GelMA hydrogels without SNAP underwent the same methanol-treatment as GelMA-SF hydrogels for 2 h. The final prepared samples were stored at -20 °C until use.

#### **4.3.3 SNAP loading and NO release measurement**

NO release from the SNAP-swelled hydrogels was measured using a Sievers nitric oxide analyzer (NOA) model 280i (Boulder, CO). Pre-weighed samples were soaked in 3 mL PBS (with 100  $\mu$ M EDTA) and placed in an amber sample holder to protect from light. Using a water bath, the temperature was set at 37 °C. NO released from the samples (n=3) was continuously swept and transferred to the chemiluminescence chamber by a constant purge of high purity N<sub>2</sub> gas at a rate of 200 mL min<sup>-1</sup>. Real time NO release levels displayed on the analyzer screen in the ppb unit were converted to NO release rate in mol min<sup>-1</sup> mg<sup>-1</sup> using the NOA instrument constant. Between each measurement, the samples were incubated in 0.01 M PBS with EDTA at 37 °C in the dark. To measure the SNAP loading, 0.25 M copper (II) chloride and ascorbic acid were alternatively injected into the release media to trigger the catalytic release of all the NO content of the samples

within a short timeframe. The total moles of NO (equal to moles of SNAP) present in the samples were calculated by integrating the area under the NO release curves. To cross-verify the obtained results, a UV–vis spectroscopy method was also employed to measure the total SNAP content of the samples. Known weights of GelMA-SNAP and GelMA-SF-SNAP hydrogels were submerged in 2 mL of chilled methanol and placed on a shaker for 4 h to fully extract the encapsulated SNAP. The absorbance of the resulting extract was recorded at 340 nm to determine the amount of SNAP loaded into the hydrogels.

#### **4.3.4 Morphological analysis**

The cross-sectional morphology of the freeze-dried hydrogels was observed using a scanning electron microscope (FEI Teneo, FEI Company, Hillsboro, Oregon) with a 5.00 kV accelerating voltage. All samples were coated with gold (10 nm thickness) via a Leica Sputter Coater (Leica Microsystems, Wetzlar, Germany) prior to imaging. The pore sizes were analyzed using ImageJ software based on the SEM images obtained from at least 3 different samples of a type.

#### **4.3.5 Fourier-transform infrared spectroscopy (FTIR)**

The FTIR spectra of the scaffolds were obtained using a Nicolet 6700 spectrometer (Thermo Electron Corporation, Madison, WI). Known weights of the samples were mixed with potassium bromide (KBr) at a ratio of 1:100 and formed into a disk. All the spectra were acquired in a range of 900–4000  $\text{cm}^{-1}$  from a total of 128 scans with a resolution of 4  $\text{cm}^{-1}$ . The deconvolution of amide I peak was carried out using OriginPro 8 software as previously described [370, 380]. The number and location of the peaks were determined from the second derivative of the spectra. A Gaussian model was selected for the band shape with equal fixed width for all the considered

peaks. The crystallinity degree was calculated by measuring the percentage ratio of the  $\beta$ -sheet peaks area to the total area of amide I peak.

#### 4.3.6 Swelling capacity

The swelling property of the scaffolds was evaluated by measuring their water retention ability. The freeze-dried scaffolds with known weights ( $W_{dry}$ ) were submerged in PBS and incubated for 24 h at 37 °C to reach equilibrium water absorption. After removing the scaffolds from PBS, the excess water on the surface was gently absorbed using Kimwipes, and their weight ( $W_{wet}$ ) was measured immediately. The wet and dry weights of the scaffolds were used to calculate their water swelling ability according to the following equation [257]:

$$Swelling\ capacity\ (\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100 \quad (Eq. 4.1)$$

#### 4.3.7 Mechanical testing

Mechanical compression testing was performed on the hydrogel scaffolds based on ASTM standard F2900-11 using a universal testing machine (Mark-10 ESM303) [65, 381]. Uniaxial compression force was applied on cylindrical shaped scaffolds (7 mm diameter  $\times$  5 mm height) at room temperature, through a 1.5 kN load cell at a cross head speed of 2 mm min<sup>-1</sup>. Prior to measurement, the samples were submerged in PBS at 37 °C to reach equilibrium water uptake. The ultimate compressive strength, modulus, and the corresponding strain at yield point (fracture strain) were measured in triplicates for each sample.

#### 4.3.8 *In vitro* enzymatic degradation

The accelerated *in vitro* enzymatic degradation of the hydrogel scaffolds was tested using collagenase type II (125-250 U/mg, in PBS). A known weight of freeze-dried hydrogels (n=3) was incubated in collagenase (0.05 mg/mL) solution at 37 °C. To prevent the effect of the high amount of SNAP leached in the first few hours on reducing the pH, and consequently the enzyme activity,

the media was refreshed every two hours in the first 12 hours and every 8 hours until the end of the experiment. At predetermined time intervals the hydrogels were taken out, rinsed thoroughly with DI water, and freeze-dried. The percentage of difference in weight of the dried hydrogels before and after enzymatic degradation was calculated and plotted against time to obtain the biodegradation profile.

#### **4.3.9 Bacteria studies**

##### **4.3.9.1 Antibacterial assay**

To evaluate the antimicrobial effect of the synthesized hydrogels, the viability of planktonic bacteria exposed to the hydrogels was quantified using a standard plate count method. First, isolated colonies of *S. aureus* and *E. coli* were inoculated in 10 mL of sterile LB broth for 14 h at 37 °C and 150 rpm. The resulting bacterial culture solution centrifuged at 2500 rpm for 7 min and resuspended with sterile PBS to rinse. The solution was again centrifuged at 2500 rpm and resuspended in PBS to achieve a final concentration of  $\sim 1 \times 10^8$  CFU/mL. Using a 24-well plate, each hydrogel was exposed to 1 mL of the final bacteria solution for 24 h in separate wells at 37 °C and 150 rpm. After incubation, the solution was removed, serially diluted in fresh PBS, and plated on LB agar plates. The plates were incubated overnight at 37 °C prior to quantifying the number of colony-forming units (CFUs). Percent reduction in viable bacteria compared to control hydrogels were calculated according to the following equation, where C = CFUs per mg of hydrogel exposed:

$$\% \text{ Reduction in Viable Bacteria} = \frac{C_{\text{control}} - C_{\text{test}}}{C_{\text{control}}} \times 100 \quad (\text{Eq. 4.2})$$

##### **4.3.9.2 Zone of inhibition**

An adapted zone of inhibition test was performed according to the ASM Kirby-Bauer Disk Diffusion Susceptibility Test Protocol [382]. First, isolated colonies of *S. aureus* and *E. coli* were

inoculated in 10 mL of sterile LB broth for 14-16 h at 37 °C and 150 rpm. The bacterial solution was then centrifuged at 2500 rpm for 7 min, rinsed with sterile PBS, centrifuged again, and resuspended in sterile PBS to obtain a final bacterial concentration of  $\sim 1 \times 10^8$  CFU/mL. 100  $\mu$ L of the resulting bacterial solution was plated on a large LB agar plate (100 mm diameter) and spread evenly across the plate using an L-shaped spreader. Each of the hydrogels were carefully placed into separate quadrants of the plate, and the plate was inverted and incubated at 37 °C for 24 h. The resulting zone of inhibitions were measured using ImageJ.

#### **4.3.10 *In vitro* mammalian cell studies**

##### **4.3.10.1 Cytotoxicity**

3T3 mouse fibroblast cells were seeded in a 96 well plate (5000 cells per well) using complete DMEM supplemented by 10% FBS and 1% penicillin/streptomycin as media. The plate was incubated in a humidified environment at 37 °C, with 5% CO<sub>2</sub> to allow cells to form a monolayer overnight. To evaluate the cytotoxicity of the scaffolds, a calorimetric CCK-8 assay was conducted according to the manufacturer's protocol. Sample leachates were obtained by first UV-sterilizing the scaffolds on both sides for 30 min, and then soaking them in complete DMEM (10 mg/mL) for 24 h, at 37 °C, in dark. Complete DMEM incubated without any sample was used as control. The sample leachates were exposed to the seeded cells by replacing the media in each well with 100  $\mu$ L of the leachates. After 24 h exposure in an incubator, 10  $\mu$ L of CCK-8 dye was added to each well and allowed to react for 3 h. The dehydrogenases in live cells reduce the water-soluble tetrazolium salt, WST-8, to form an orange-colored formazan product that is detectable at 450 nm wavelength [148]. Using a microplate reader (Biotek Cytation 5), the absorbance was measured at 450 nm (n=6) and viability was calculated as relative to control according to the following equation:

$$\text{Cell viability (\%)} = \frac{\text{absorbance (treated cells)}}{\text{absorbance (control cells)}} \times 100 \quad (\text{Eq. 4.3})$$

#### 4.3.10.2 Cell adhesion and proliferation

Hydrogel scaffolds with equal surface area were sterilized under UV for 30 min on each side. 3T3 fibroblast cells (15000 cells/cm<sup>2</sup>) were seeded on each sterilized scaffold and allowed to adhere for 48 h in an incubator (T = 37 °C, 5% CO<sub>2</sub>). To observe the morphology of the attached cells to the scaffolds, DAPI and phalloidin fluorescent dyes were used to stain the cell nucleus and cytoplasm, respectively. The cell-seeded scaffolds were washed with PBS, fixed in formaldehyde solution (4%) for 10 min, and permeated using 0.2% Triton X-100 for another 10 min. The permeated cells were then stained using phalloidin and DAPI reagents and imaged under a fluorescent cell imaging system (AMG EVOS FL digital inverted microscope). The number of cells attached to each scaffold (n=3) was also quantified by treating the adhered cells with 0.18% trypsin for 5 min to detach them from the scaffolds and counting them using an EVE automated cell counter [383].

#### 4.3.11 Statistical analysis

All values are presented as mean  $\pm$  standard deviation with n=3 unless otherwise noted. The statistical significance was determined using Student's t-test with a threshold of  $p < 0.05$ .

### 4.4 Results and discussion

#### 4.4.1 SNAP impregnation

To impregnate the scaffolds with the NO donor molecule, SNAP, the hydrogels were soaked in a SNAP swelling solution for two hours. **Figure 4.1** shows the schematic illustration of the SNAP-swelled IPN hydrogels synthesis. Methanol (80% v/v) was used as the swelling solvent in this study due to its great ability to impregnate the hydrogels' structure and replace with water, crosslink the SF component in the IPN hydrogels, easy removal after fabrication, and SNAP's high

solubility in this solvent. After rinsing the samples to remove excess SNAP on the surface and completely drying the SNAP loaded hydrogels, the SNAP content of each sample was measured using NOA. At two hours of swelling, GelMA and GelMA-SF hydrogels had a maximum SNAP content of  $15.22 \pm 0.56$  wt% and  $8.05 \pm 0.09$  wt%, respectively. After two hours no increase in the SNAP content of both samples was observed. Similar results were obtained after measuring the total SNAP content of the hydrogels via UV-vis spectroscopy. Using this method, GelMA-SNAP and GelMA-SF-SNAP hydrogels showed a SNAP content of  $15.30 \pm 0.66$  wt% and  $8.23 \pm 0.99$  wt%, respectively, which was no longer increased by further soaking of the hydrogels in methanol. **Figure 4.2** shows the optical photographs of the SNAP swelled GelMA and GelMA-SF hydrogels.

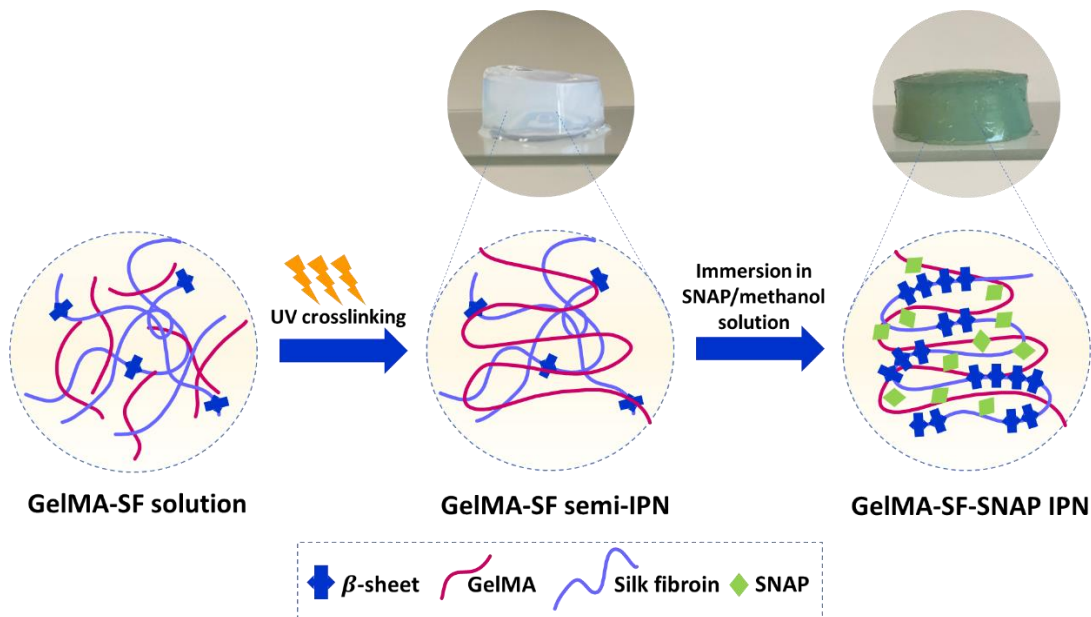


**Figure 4.1** - Schematic representing the synthesis of SNAP-swelled GelMA-SF IPN hydrogels.

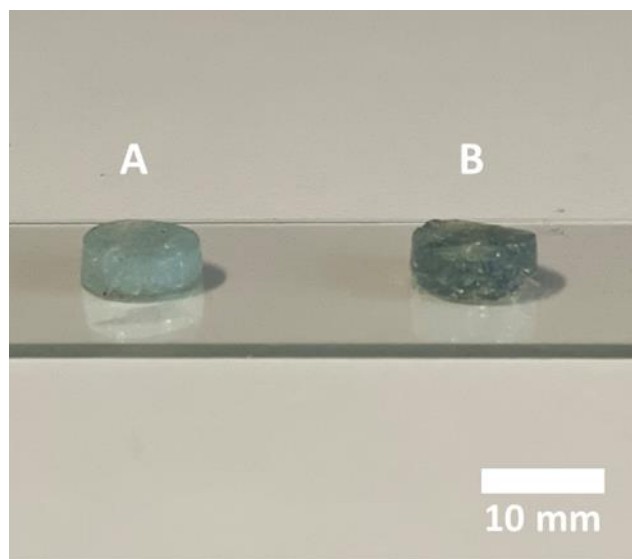


**Figure 4.2** - Optical photographs of the A) GelMA-SF-SNAP and B) GelMA-SNAP hydrogels.

#### 4.4.2 SEM analysis

Porosity and pore size are among the key parameters determining the functionality of tissue engineering scaffolds. An optimal pore size is required to facilitate the efficient transport of gases and nutrients while providing a large specific surface area for enhanced cell attachment and migration [384]. Depending upon the cell type and the tissue being regenerated, the optimal mean pore size of the scaffolds may vary. For instance, pore sizes in the range of 100-350  $\mu\text{m}$  are reported to be suitable for bone regeneration, while for neovascularization, fibroblasts ingrowth, and regeneration of adult skin 5  $\mu\text{m}$ , 5–15  $\mu\text{m}$  and 20–125  $\mu\text{m}$  sized pores are required, respectively [385]. **Figure 4.3** shows the cross-sectional microstructure of the prepared GelMA-SF scaffolds. The pure GelMA scaffolds had a porous structure with a mean pore size of  $37 \pm 6 \mu\text{m}$ . After addition of SF, the porous structure was retained with a shift in morphology from honeycomb to sheet-like structures as previously seen in other studies [386]. No visible phase separation was observed for GelMA–SF IPN hydrogels. However, the mean pore size of the scaffolds was decreased to  $24 \pm 3 \mu\text{m}$  ( $p < 0.05$ ).

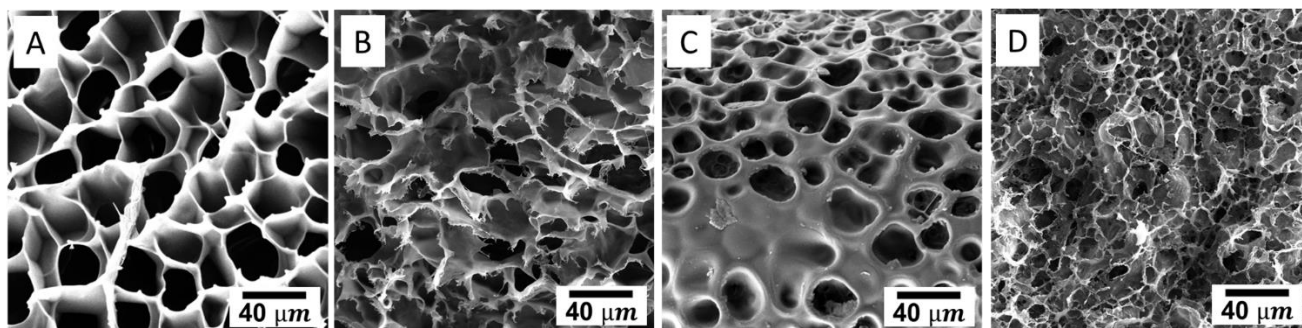


**Figure 4.3** - Cross-sectional SEM images of freeze-dried A) GelMA, B) GelMA-SF, C) GelMA-SNAP, and D) GelMA-SF-SNAP.

After SNAP impregnation, there were no significant differences in the pore sizes of the scaffolds (**Table 4.1**). No excess crystallization or aggregation of SNAP was observed on the surface of freeze-dried scaffolds indicating the homogenous incorporation of SNAP in the hydrogel's matrices. In addition, the SNAP swelling process did not cause any irregularities in the morphology of the hydrogels that otherwise could lead to adverse effects on their mechanical properties and cellular interactions. While SNAP-swelled GelMA hydrogels had honeycomb like circular pores with smooth morphology, GelMA-SF-SNAP scaffolds demonstrated pores with sharper edges due to the presence of SF in the structure. It has been reported before that circular honeycomb like pores with wide angled corners can more easily be covered by cells as they provide a more favorable surface for cells to elongate and span. Therefore, these pores undergo a faster reduction in size compared to pores with sharper edges that can delay the pore size reduction by cells and maintain a more efficient transport of nutrients and gasses over time [387]. The obtained SEM images can further help us gain a better understanding of the structure–function relationship of the GelMA–SF scaffolds particularly in terms of their physical properties and biological function.

**Table 4.1** - Mean pore size, swelling capacity, and SNAP loading of hydrogel scaffolds. Data are reported as mean  $\pm$  standard deviation.

| Samples       | Mean Pore Size ( $\mu\text{m}$ ) | Swelling Capacity (%) | SNAP Loading (wt%) |
|---------------|----------------------------------|-----------------------|--------------------|
| GelMA         | $37 \pm 6$                       | $929.1 \pm 25.5$      | ----               |
| GelMA-SNAP    | $31 \pm 3$                       | $895.0 \pm 16.2$      | $15.22 \pm 0.56$   |
| GelMA-SF      | $24 \pm 3$                       | $406.1 \pm 15.8$      | ----               |
| GelMA-SF-SNAP | $21 \pm 5$                       | $359.6 \pm 10.8$      | $8.05 \pm 0.09$    |

#### 4.4.3 FTIR and deconvolution of the amide I band

FTIR was carried out to study the structure and conformational transitions induced in GelMA-SF scaffolds, before and after methanol treatment, and after swelling with SNAP. As shown in **Figure 4.4**, the characteristic peaks of protein backbone regarding GelMA and SF polymers were observed in all spectra [388]. A broad peak attributed to the O–H and N–H stretching vibrations was identified in the  $3200\text{--}3600\text{ cm}^{-1}$  region. The absorption bands appearing at  $2800\text{--}3100\text{ cm}^{-1}$  were ascribed to the stretching vibrations of C–H groups. In the spectrum of non-treated GelMA-SF semi-IPN, the amide I, amide II, and amide III absorption bands were observed at  $1640\text{ cm}^{-1}$ ,  $1543\text{ cm}^{-1}$ , and  $1235\text{ cm}^{-1}$ , respectively. The intensity center of amide I band was slightly shifted to lower wavenumbers ( $1630\text{ cm}^{-1}$ ) after treatment with methanol and SNAP/methanol solutions, suggesting an increase in the  $\beta$ -sheet crystalline structures as previously reported by other studies [389, 390].

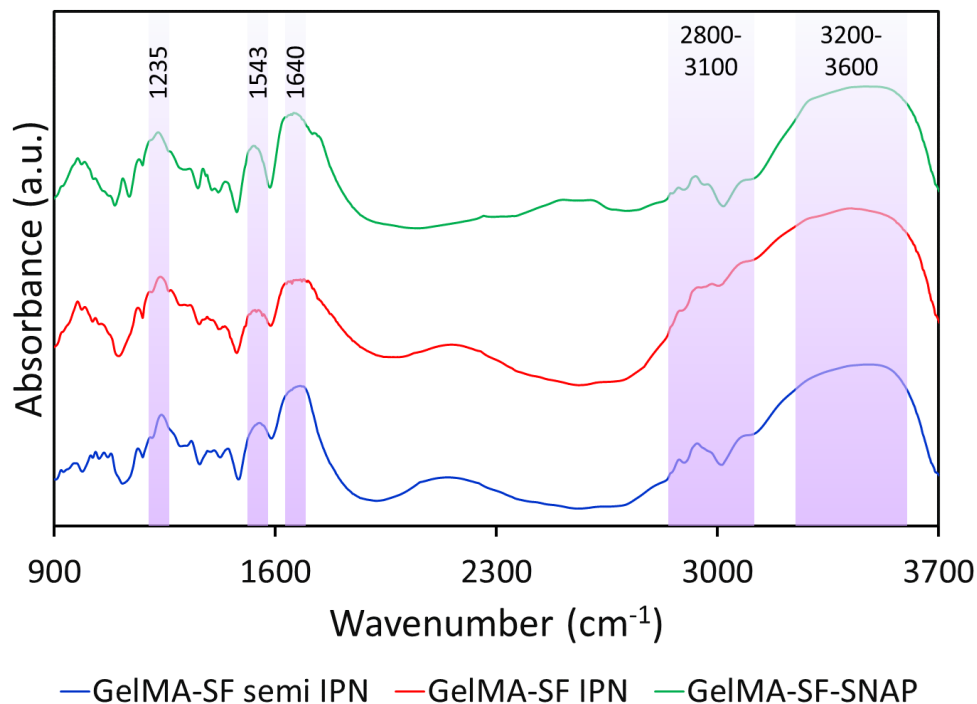


**Figure 4.4** - FTIR spectra of GelMA-SF semi-IPN before methanol treatment, GelMA-SF IPN after methanol treatment, and GelMA-SF-SNAP IPN scaffolds.

To further investigate the degree of crystallinity in each sample, deconvolution analysis of the amide I peak was carried out and the results are presented in **Figure 4.5**. As expected, the non-treated GelMA-SF semi-IPN had a  $\beta$ -sheet content of 19.9% that was notably increased to 31.2% for methanol treated GelMA-SF IPN. In addition, GelMA-SF-SNAP IPN showed a slightly higher  $\beta$ -sheet content of 34.1% that is most likely due to the hydrophobic interactions of SNAP and SF molecular chains as previously reported [370].

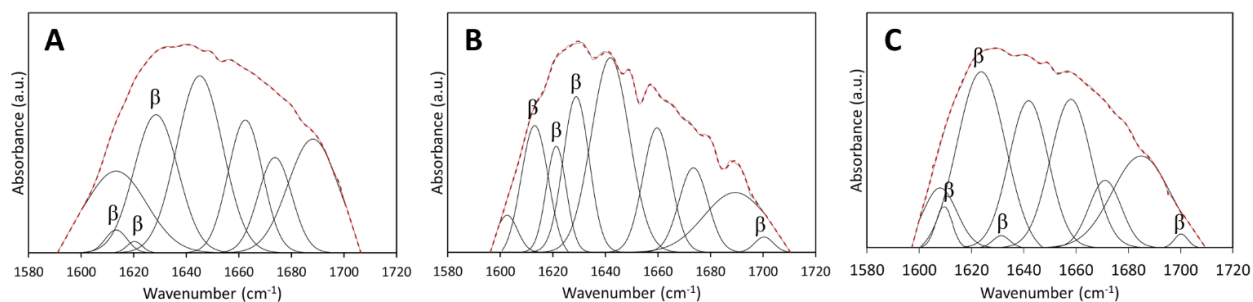


**Figure 4.5** - Deconvoluted amide I band of A) non-methanol treated GelMA-SF semi-IPN, B) methanol treated GelMA-SF IPN, and C) GelMA-SF-SNAP IPN.  $\beta$  =  $\beta$ -sheets.

#### 4.4.4 Compression strength

Although having a porous microstructure is of great importance for successful scaffold biointegration, it should not compromise the mechanical integrity of the scaffold. From a practical point of view, scaffolds should be of adequate strength and maintain their mechanical integrity over a desired period of time in order to protect cells from applied mechanical stresses and provide them a stable substrate to adhere and grow [391]. The mechanical properties of the scaffolds in this study were evaluated by a uniaxial compression test and the results are summarized in **Table 4.2**. The GelMA-SF IPN hydrogels had over 10 times higher compressive modulus and a remarkably enhanced compressive strength compared to GelMA hydrogels either with or without SNAP ( $p < 0.01$ ) (**Figure 4.6A**). Incorporation of SNAP in fact did not cause any significant changes in the mechanical properties of the scaffolds ( $p > 0.05$ ). Mechanical properties are mainly determined by the scaffold material and microstructure [391, 392]. SF is known to undergo a conformational transition from random coils to  $\beta$ -sheets when treated with organic solvents such as methanol [393]. Herein, the crystalline  $\beta$ -sheet structure of SF induced by methanol treatment and the presence of smaller pores leading to a denser structure are the main reasons behind the reinforced mechanical properties of the IPN hydrogels.

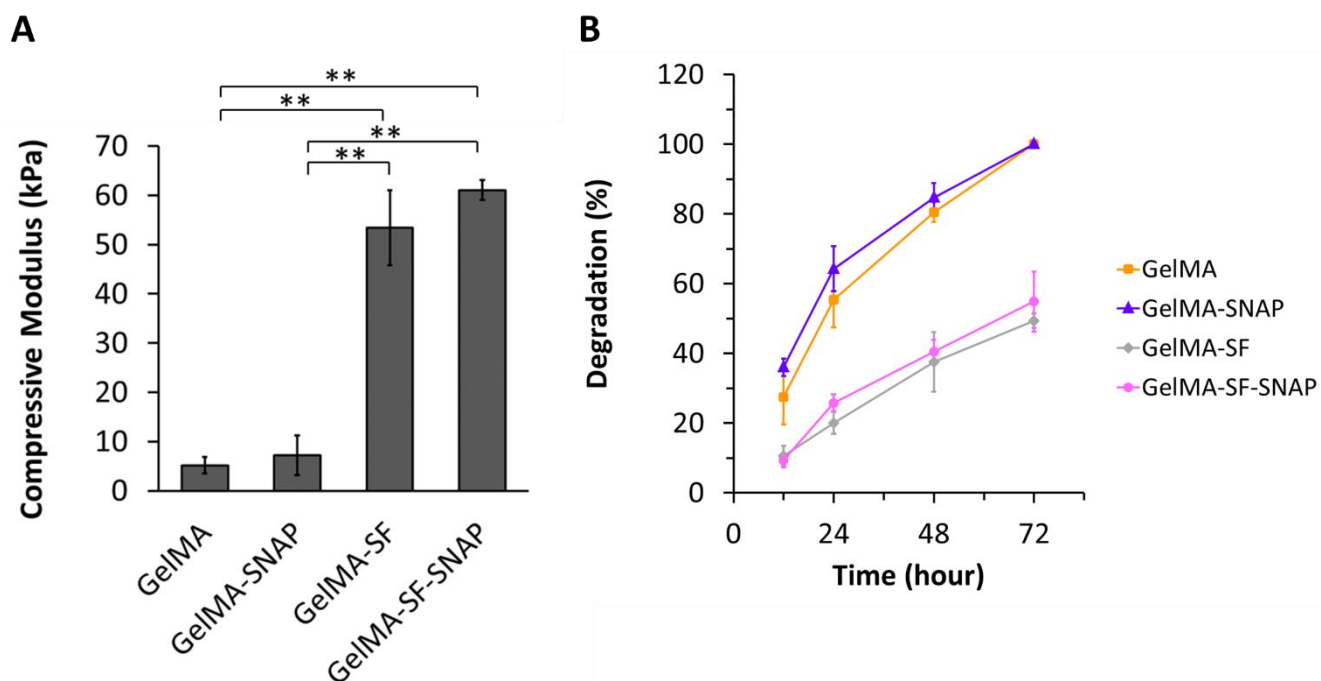


**Figure 4.6** - A) Wet-state mechanical properties and B) enzyme-mediated degradation profile of the hydrogel scaffolds in collagenase II solution at 37 °C in dark. \*\* indicate  $p < 0.01$ .

**Table 4.2** - Mechanical properties of the hydrogel scaffolds. Data are reported as mean  $\pm$  standard deviation.

| Samples       | Compressive Strength (kPa) | Compressive Modulus (kPa) | Fracture Strain (%) |
|---------------|----------------------------|---------------------------|---------------------|
| GelMA         | 66.0 $\pm$ 22.5            | 5.1 $\pm$ 1.6             | 9.9 $\pm$ 1.4       |
| GelMA-SNAP    | 84.6 $\pm$ 49.2            | 7.2 $\pm$ 4.0             | 9.6 $\pm$ 1.0       |
| GelMA-SF      | 893.3 $\pm$ 45.9           | 53.4 $\pm$ 7.6            | 15.1 $\pm$ 4.2      |
| GelMA-SF-SNAP | 794.4 $\pm$ 137.1          | 61.0 $\pm$ 2.0            | 7.7 $\pm$ 3.6       |

#### 4.4.5 Swelling capacity

The swelling capacity (SC) of scaffolds is an important factor affecting their biodegradation [394]. An optimum SC is crucial for the transport of nutrients and metabolites to cells, inhibiting tissue dehydration and improving cell growth and tissue regeneration while not adversely affecting the degradation kinetics of the scaffold [395]. After reaching equilibrium for 24 h in PBS at 37 °C, the SC of the hydrogels were measured by comparing their wet and dry

weights. Pure GelMA scaffolds had a high SC of  $929.1 \pm 25.5$  % (**Table 4.1**). However, GelMA-SF IPN hydrogels which were shown to have a compacter structure with smaller pores had a significantly decreased SC of  $406.1 \pm 15.8$  % ( $p < 0.05$ ). In addition, the water insoluble  $\beta$ -sheets in silk, formed after methanol treatment, act as crosslinking sites limiting the diffusion of water into the structure of IPN hydrogels. While methanol treatment resulted in a significantly lower SC value, addition of SNAP did not significantly influence the water retention ability of the scaffolds (**Table 4.1**).

#### **4.4.6 *In vitro* enzymatic degradation**

For successful tissue regeneration, the degradation rate of the implanted scaffold should match that of the new tissue formation. Too fast or slow degradation rates may cause the structure to collapse, leading to necrosis, or hinder tissue biointegration by fibrous capsule formation [396]. Therefore, the degradation kinetics of the scaffold needs to be carefully optimized for clinical applications. Owing to their 3D hydrophilic structure, hydrogels can uptake high amounts of aqueous media and degrade over time, going through different degradation mechanisms including erosion, enzymatic cleavage, hydrolysis, and solubilization. **Figure 4.6B** shows the enzymatic degradation behavior of the hydrogel scaffolds in collagenases solution at a concentration of 0.05 mg/mL or 10 U/mL. Collagenase is an important degradation enzyme *in vivo* with great ability to degrade GelMA. A gradual increase in the mass loss was observed over time for all the hydrogel scaffolds. After 72 h in collagenase solution, the GelMA hydrogels with or without SNAP were completely degraded while GelMA-SF and GelMA-SF-SNAP IPN hydrogels only lost around 50% of their initial mass. No further noticeable weight loss was seen after 72 h for any of the scaffolds. GelMA hydrogels with a high degradation rate in aqueous enzymatic media are at increased risk of undergoing premature collapse and deformation interfering with neo-tissue

development. According to the obtained results, addition of SF significantly slowed down the degradation rate of GelMA scaffolds, rendering them more suitable for applications where longer times are required for proper healing and reconstruction of the damaged tissue.

The significant decrease in the degradation rate ( $p < 0.01$ ) demonstrated by the GelMA-SF IPN hydrogels compared to GelMA hydrogels can be associated with the differences in their microarchitecture and structure. The formation of hydrophobic  $\beta$ -sheet crystallites with strong inter-chain interactions in SF after methanol treatment, endows it with high mechanical strength, non-solubility, and low swelling ratio in aqueous media as observed in previous sections [397]. The high crystallinity and lower SC of SF containing GelMA hydrogels limit the retention and permeation of collagenase solution into these hydrogels leading to decreased degradation rates compared to GelMA hydrogels. In addition, the smaller pore size of the IPN hydrogels provides less surface area and active sites to be exposed to the enzymatic media contributing to lower degradation rates of these hydrogels in collagenase solution [398].

#### **4.4.7 NO release kinetics**

A controlled NO release kinetic is of great importance for practical clinical application of NO-releasing biomaterials and scaffolds [281]. Due to usually having a high SC and loose structure, hydrogels commonly show rapid release profiles in aqueous media. A similar trend was observed for GelMA-SNAP hydrogels in this study with a burst NO release of  $5.3 \pm 1.2 \times 10^{-11}$  mol min<sup>-1</sup> mg<sup>-1</sup> in the first hour, followed by lower release rates at 4 h ( $0.12 \pm 0.04 \times 10^{-11}$  mol min<sup>-1</sup> mg<sup>-1</sup>) and 8 h ( $0.013 \pm 0.001 \times 10^{-11}$  mol min<sup>-1</sup> mg<sup>-1</sup>) (**Figure 4.7**). This was most likely caused by the extensive diffusion of water into the hydrogel structure and the rapid leaching out of SNAP molecules. Despite having a lower SNAP content, the IPN hydrogels with a reduced SC and compacter structure demonstrated a more prolonged NO release profile for 24 h. GelMA-SF-

SNAP IPN hydrogels exhibited an initial burst release of  $2.06 \pm 1.46 \times 10^{-11} \text{ mol min}^{-1} \text{ mg}^{-1}$  in the first hour that gradually decreased to  $0.026 \pm 0.009 \times 10^{-11} \text{ mol min}^{-1} \text{ mg}^{-1}$  at 12 h and  $0.011 \pm 0.003 \times 10^{-11} \text{ mol min}^{-1} \text{ mg}^{-1}$  at 24 h. The NO release levels for GelMA-SNAP and GelMA-SF-SNAP hydrogels were no longer detectable after 8 h and 24 h in media, respectively. A sustained release of NO in the first 24 h is essential for immediate and long-term antibacterial effects of the scaffolds as most post-operative infections occur within 12-24 h [364, 399]. Therefore, GelMA-SF-SNAP IPN hydrogels with a controlled NO release over 24 h provide a promising platform to be used as antibacterial scaffolds in tissue engineering and biomedical applications.

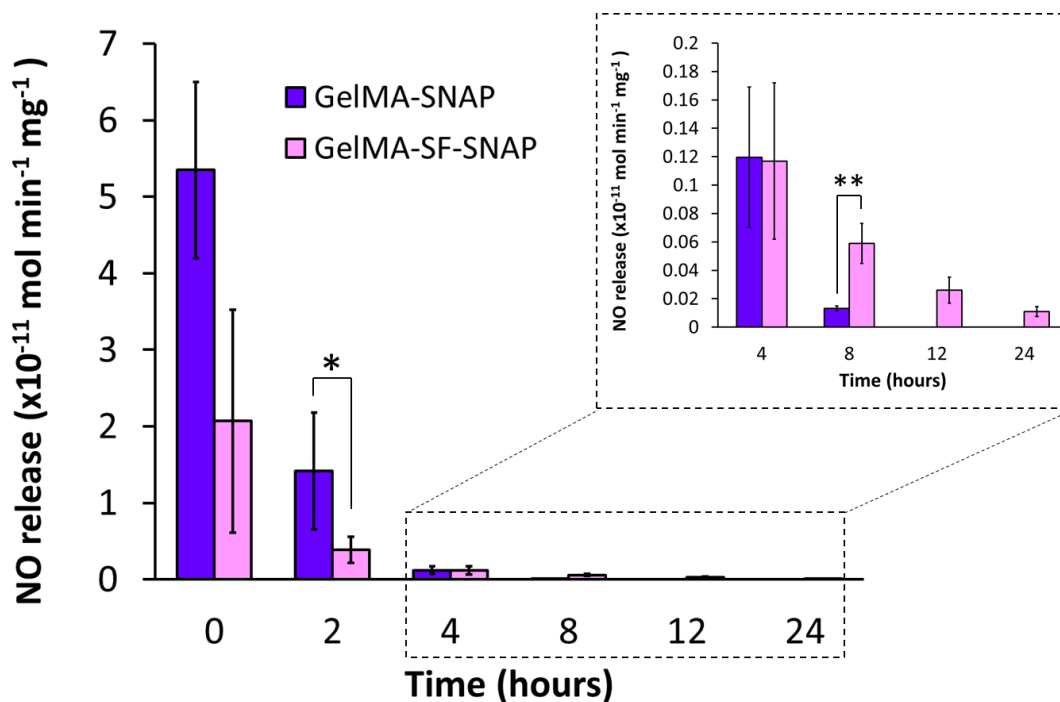


**Figure 4.7** - Real-time NO release profile of the GelMA-SNAP and GelMA-SF-SNAP hydrogels in PBS with EDTA at 37 °C in dark.

#### 4.4.8 Antibacterial properties

Infection associated with surgically implanted scaffolds is a significant clinical problem compromising the patients' safety or the treatment outcome [400]. Therefore, there is an increasing interest into developing antibacterial scaffolds that can resist bacterial colonization and further infection. NO-releasing materials have been previously shown to exhibit strong antibacterial activity against different bacterial strains [401, 402]. NO as a free radical can readily react with oxygen and superoxides to create reactive intermediate byproducts such as peroxynitrite ( $\text{OONO}^-$ ) and nitrogen dioxide ( $\text{NO}_2$ ) through which it can impose an oxidative damage on the bacterial cell membranes, proteins, and DNA [403]. The multi-mechanistic mechanism of action and broad-spectrum antimicrobial activity of NO impedes the development of resistance by bacteria therefore offering a promising alternative for conventional antibiotics. To investigate the antibacterial activity of the hydrogel scaffolds a zone of inhibition (ZOI) assay was performed against gram-positive *S. aureus*, and gram-negative *E. coli*, two commonly found pathogens in hospital acquired infections. After 24 h of incubation, the ZOI against both bacteria for GelMA-SNAP and GelMA-SF-SNAP hydrogels were measured to be 2 mm and 4 mm, respectively (**Figure 4.8**).

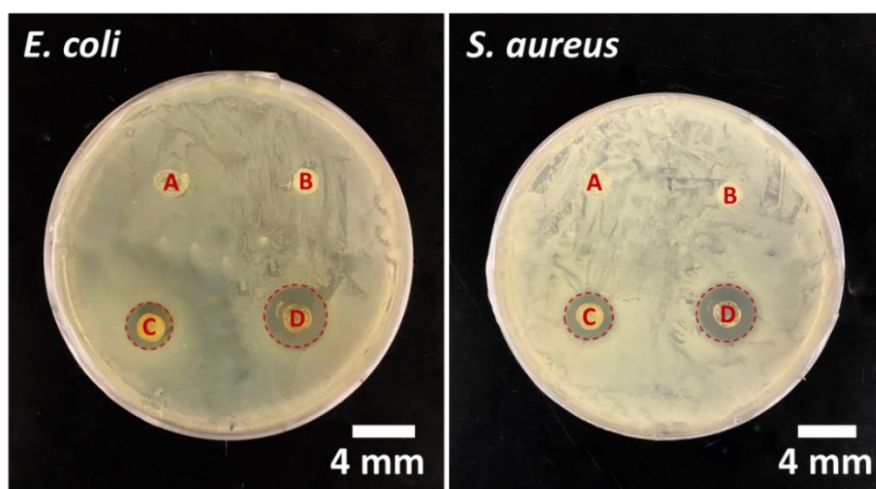


**Figure 4.8** - Antibacterial activity of A) GelMA, B) GelMA-SF, C) GelMA-SNAP, and D) GelMA-SF-SNAP against *E. coli* and *S. aureus* evaluated by measuring the diameter of inhibitory zones.

The antibacterial activity of the scaffolds was further quantified using a CFU counting method (**Figure 4.9**). After 24 h of exposure, SNAP-swelled GelMA hydrogels reduced the viability of *S. aureus* by  $99.5 \pm 0.2 \%$  and *E. coli* by  $99.3 \pm 0.3 \%$ , ( $p < 0.01$ ). GelMA-SF-SNAP hydrogels also showed a remarkable bacteria reduction efficiency of more than 99.9 % against both *S. aureus* and *E. coli* ( $p < 0.01$ ). Despite having a lower SNAP content, GelMA-SF-SNAP IPN hydrogels showed a significantly higher bactericidal activity compared to GelMA-SNAP hydrogels ( $p < 0.05$ ). This can be attributed to the more controlled release of NO from the IPN hydrogels that lasted for 24 h or the entire duration of the study. While SNAP-swelled GelMA hydrogels still showed a strong antibacterial activity, the rapid liberation of NO from these hydrogels over 8 hours led to a decreased bactericidal efficiency compared to SNAP-swelled GelMA-SF IPN hydrogels. Therefore, the NO-releasing GelMA-SF hydrogels show great promise as antibacterial scaffolds to reduce infection frequently associated with implants and TE scaffolds.

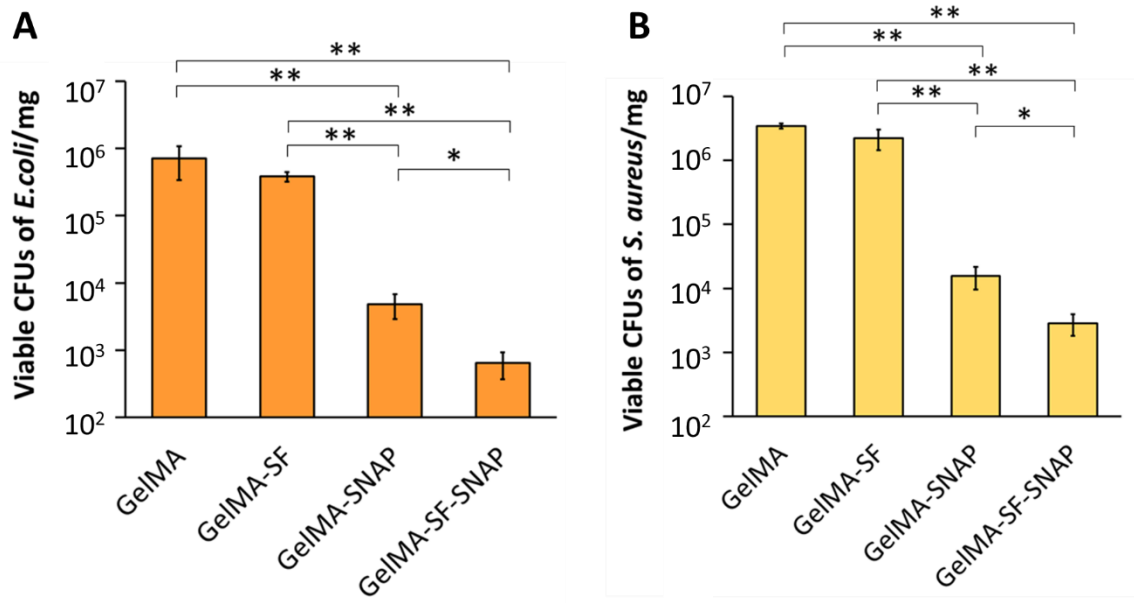


**Figure 4.9** – Antibacterial activity of hydrogel scaffolds against A) *E. coli* and B) *S. aureus* evaluated by a CFU counting method.

#### 4.4.9 *In vitro* cytocompatibility

To evaluate the cytocompatibility of the scaffolds that is a critical attribute for proper tissue regeneration, the cytotoxicity, cell adhesion and proliferation studies were conducted. According to the cytotoxicity results (**Figure 4.10A**), the leachates obtained from the samples after 24 h of incubation did not show any cytotoxic effects toward fibroblasts. The cytocompatibility of GelMA and SF has already been shown by numerous studies [404, 405]. Moreover, the leaching of SNAP, NAP, or dimers of NAP from the hydrogels is not supposed to cause any cytotoxicity as the ultimate hydrolyzation product of these molecules is penicillamine, which has been long used as a medication for clinical treatment of heavy metal poisoning [254, 406].

To investigate cellular responses in direct contact with scaffolds, cells were seeded on each hydrogel and were fluorescently stained with phalloidin and DAPI. A good cell spreading and adhesion on all samples were observed after 24 h (**Figure 4.10C-F**). The presence of RGD motifs in GelMA hydrogels are known to provide a favorable biological environment and facilitate cellular adhesion [407]. In addition, the number of cells adhered on each hydrogel was measured after 48 hours of being in culture and the results are depicted in **Figure 4.10B**. The SNAP-swelled GelMA-SF hydrogels showed the highest number of adhered cells (~106000) compared to other samples, most likely due to the regulating effects of NO on fibroblast proliferation, as previously seen in other studies [212]. However, similar enhancing effect on fibroblast proliferation was not seen in GelMA-SNAP when compared to pure GelMA scaffolds. This can be associated with the fast release of NO in the first few hours. While the cellular adhesion to the scaffolds was investigated over 48 h, the SNAP-swelled GelMA hydrogels were not able to maintain a sustained release of NO beyond 8 h. In samples that did not contain SNAP, a significantly higher number of cells was counted on GelMA (~78000) compared to GelMA-SF (~57000) scaffolds ( $p < 0.05$ ).

Consistent with the results of other studies [408], this finding is reported to be potentially caused by an increase in the stiffness of the scaffolds (**Table 4.2**) with addition of SF which is less desirable for cell proliferation. Nevertheless, GelMA–SF-SNAP IPN hydrogels demonstrated improved cell adhesion and proliferation with no apparent cytotoxicity of the leachates, indicating their cytocompatibility as potential TE scaffolds.

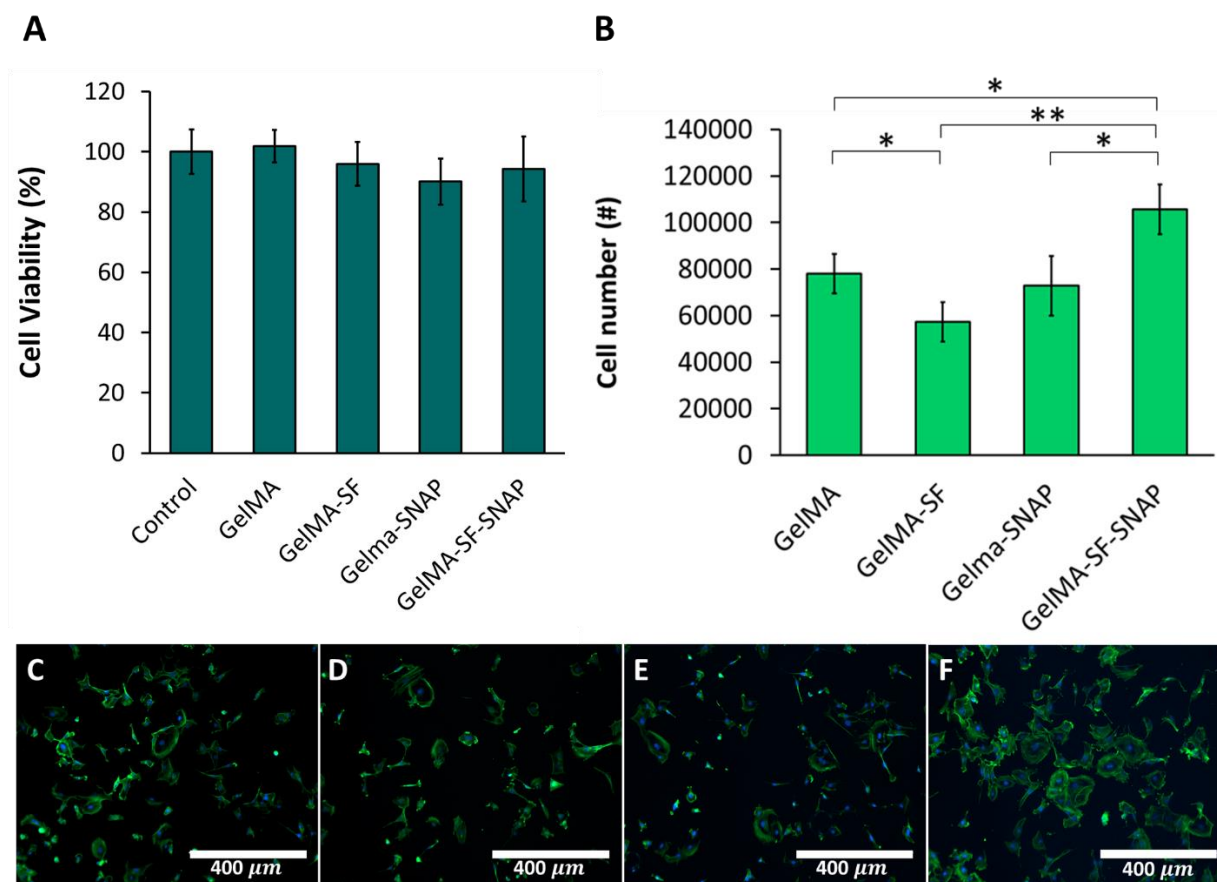


**Figure 4.10** - A) Graph representing the cell viability of 3T3 mouse fibroblasts treated with 24 h leachates from hydrogel scaffolds. B) Number of 3T3 mouse fibroblast cells detached from the scaffolds after 48 h of culture (\* and \*\* indicate  $p < 0.05$  and  $p < 0.01$ , respectively). Fluorescence microscopy of 3T3 fibroblasts grown on C) GelMA, D) GelMA-SF, E) GelMA-SNAP, and F) GelMA-SF-SNAP. Blue: DAPI; green: F-actin.

## 4.5 Conclusion

In this study, the fabrication and characterization of a NO-releasing IPN scaffold based on GelMA and SF was reported for potential tissue engineering applications. SNAP-impregnated GelMA-SF IPN hydrogels possessed a porous microarchitecture desired for cellular infiltration and growth. FTIR deconvolution analysis indicated an increase in the  $\beta$ -sheet content of the GelMA-SF hydrogels from 19.9% to 34.1% that was induced by methanol treatment and hydrophobic interactions of SNAP and SF macromolecules. The IPN scaffolds showed improved compression strength, slower biodegradation rate, and a more controlled NO release compared to pure GelMA and GelMA-SNAP hydrogels. This indicated that addition of SF with a more hydrophobic and crystalline structure can reinforce the GelMA scaffolds, making them practically more stable for potential clinical applications. A high -initial NO release from the SNAP-swelled GelMA-SF hydrogels followed by a more sustained release over 24 h led to strong antibacterial responses (>99.9% reduction efficiency) against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria, that were assessed both quantitatively by a CFU counting method and qualitatively through ZOI assay. In addition, a good adhesion, spreading, and high proliferation rate observed for 3T3 mouse fibroblast cells in contact with the NO-releasing IPN hydrogels, indicated their excellent cytocompatibility. This is while the SNAP-swelled GelMA hydrogels with larger pore sizes, higher degradation rate, and a loose network structure exhibited a mainly burst release profile over 8 h, leading to lower antibacterial efficiency and cellular proliferation rate *in vitro*. These results indicate the promising potential of GelMA-SF-SNAP hydrogels as scaffolds for tissue engineering or wound healing applications.

**CHAPTER 5**

**NITRIC OXIDE-RELEASING NANOFIBROUS SCAFFOLDS BASED ON SILK  
FIBROIN AND ZEIN WITH ENHANCED BIODEGRADABILITY AND  
ANTIBACTERIAL PROPERTIES<sup>5</sup>**

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<sup>5</sup> Ghalei S, Douglass M, Handa H. Nitric Oxide-Releasing Nanofibrous Scaffolds based on Silk Fibroin and Zein with Enhanced Biodegradability and Antibacterial Properties. Submitted to *ACS Biomaterials Science & Engineering*.

## 5.1 Abstract

Clinical application of scaffolds and implants has been associated with bacterial infection resulting in impaired tissue regeneration. Nanofibers provide a versatile structure for both antimicrobial molecules delivery and tissue engineering. In this study, the nitric oxide (NO) donor molecule, S-nitrosoglutathione (GSNO), and the natural biodegradable polymer, zein (ZN), were combined with silk fibroin (SF) to develop antibacterial and biodegradable nanofibrous scaffolds for tissue engineering applications. The incorporation of ZN increased the hydrophobicity of the fibers and resulted in a more controlled and prolonged NO release profile lasting for 48 h. Moreover, the degradation kinetics of the fibers were significantly improved after blending with ZN. The results of tensile testing indicated that the addition of ZN and GSNO had a positive effect on the strength and stretchability of SF fibers and did not adversely affect their mechanical properties. Finally, due to the antibacterial properties of both NO and ZN, the SF-ZN-GSNO fibers showed a synergistically high antibacterial efficacy with  $91.6 \pm 2.5 \%$  and  $77.5 \pm 3.1 \%$  reduction in viability of adhered *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) after 24 h exposure. The developed NO-releasing fibers were not only antibacterial but also non-cytotoxic and successfully enhanced the proliferation and growth of fibroblast cells which was quantitatively studied by a CCK-8 assay and visually observed through fluorescent staining. Overall SF-ZN-GSNO fibers developed in this study were biodegradable, highly antibacterial, and showed great cytocompatibility with fibroblasts, indicating their promising potential for a range of tissue engineering and medical device applications.

## 5.2 Introduction

Biodegradable polymeric scaffolds play a critical role in regenerative medicine by providing temporary structural support for cells to reside in and reconstruct the damaged tissue.

For efficient cell binding, proliferation, and neo tissue formation, scaffolds must be biocompatible and of close similarity to the native extracellular matrix (ECM) [409]. Electrospinning is a popular, low cost, and versatile technique for fabricating micro/nanofibrous scaffolds that can mimic the fibrillar structure of the ECM. Electrospun fibers offer many advantages for tissue engineering including having a porous network, high specific surface area favorable for cell attachment, and allowing for efficient diffusion of nutrients and oxygen [410]. These fibers can also be incorporated with different drugs, therapeutics, and antibacterial agents for specific applications.

Nitric oxide (NO) donor molecules have recently found increasing interest to be incorporated into polymeric scaffolds for wound healing and tissue engineering applications [174, 363]. NO is a gaseous molecule endogenously produced by nitric oxide synthase enzymes in the pico-nano molar range and plays important roles in the wound healing process by regulating the inflammatory response, enhancing cell proliferation, collagen formation, and angiogenesis [411]. In addition, NO is part of the innate immune system and can effectively neutralize infectious bacteria and viruses when released from macrophages and other immune cells [412].

Scaffolds can get infected by opportunistic bacteria at the time of implantation or later by the spread of infection from other parts of the body leading to serious consequences such as tissue damage, prolonged healing time, and patient morbidity and mortality [355]. The adherence of bacteria to the scaffolds may lead to the formation of biofilms on the surface, rendering treatment with antibiotics barely effective. Biofilms are multicellular communities of bacteria that have encased themselves in a matrix composed of extracellular polymeric substances (EPS) and are highly impermeable to antibacterial agents. NO has been previously shown to have the ability to disperse biofilms into more vulnerable planktonic state bacteria by decreasing the levels of c-di-GMP messenger molecule that is responsible for regulating the biofilm integrity [362]. Therefore,

due to its strong antibacterial activity, biofilm dispersal ability, and multifaceted roles in regulating the wound healing process, NO is an ideal candidate to be incorporated into scaffolds to prevent infection and promote tissue regeneration.

In the past, NO donor molecules such as diazeniumdiolates (NONOates) and S-nitrosothiols (RSNOs) have been incorporated into different polymeric fibers for tissue engineering, wound healing, and other biomedical applications [364, 365, 413-415]. The previously developed NO-releasing fibers were mostly made of synthetic polymers to avoid limitations frequently associated with the use of hydrophilic natural polymers such as high leaching of the donor molecule, burst NO release kinetics, and poor mechanical properties. However, despite their disadvantages, biopolymers are of greater popularity for tissue engineering applications owing to their lower immunogenicity, higher biocompatibility, biodegradability, and biological recognition compared to synthetic polymers [416, 417]. Therefore, further research is required into designing biodegradable and biocompatible scaffolds based on naturally derived polymers with improved NO release kinetics and good mechanical properties.

Silk fibroin (SF) is a protein that can be obtained from the cocoons of the silkworm. SF is widely used in biomedical applications due to its outstanding properties such as high mechanical properties, excellent biocompatibility, water-based processing, and ability to be shaped into different material formats [237, 418]. When in a liquid format, SF macromolecules are randomly dispersed bearing helical or random coil conformations [370]. However, for most applications, SF is treated either physically (i.e. sonication, water vapor annealing) or chemically (i.e. methanol, acetone, or ethanol treatment) to induce a conformational transition in its structure into crystalline  $\beta$ -sheets that make SF non-soluble and mechanically strong but also highly resistant to degradation [419]. Crystallized SF has been shown to have a very slow degradation rate in aqueous media

[420]. As tissue engineering constructs are required to be degraded gradually to allow the regenerating tissue to be replaced with them over time, it is important to enhance the degradability of SF-based scaffolds. One simple approach to improve the degradation kinetics of SF for tissue engineering applications is to blend it with other more biodegradable polymers [421, 422].

Zein (ZN) is a naturally derived protein obtained from corn that has been widely used for wound healing and scaffold applications due to its biodegradability and biocompatibility [319]. However, ZN suffers from poor mechanical properties and therefore, for most biomedical applications, it needs to be combined with other more mechanically robust polymers or filler particles to enhance the mechanical properties [257]. Using a single jet electrospinning method, in this study, we fabricated composite nanofibers based on SF and ZN to develop mechanically strong yet biodegradable scaffolds.

To endow the scaffolds with antibacterial activity and bioactivity, a NO donor molecule, S-nitrosoglutathione (GSNO), was embedded into these fibers. GSNO is an endogenous RSNO, naturally available *in vivo*, that is known for its great biocompatibility and excellent stability among other RSNOs [369, 423]. The NO-releasing SF-ZN scaffolds (SF-ZN-GSNO) were observed under SEM to evaluate their morphology and size distribution. The long-term enzymatic degradability of the fibers along with their wettability and mechanical properties under uniaxial tensile loading were tested. In addition, the cumulative leaching of GSNO from the fibers and their NO release kinetics were studied. Finally, antibacterial activity of the scaffolds against gram-positive and gram-negative bacteria and their cytocompatibility with 3T3 fibroblast cells were also investigated.

## 5.3 Materials and Methods

### 5.3.1 Materials

*Bombyx mori* (*B. mori*) silk cocoons were obtained from Paradise Fibers (USA). Zein from maize (Z3625), DAPI, hydrochloric acid, sodium nitrite, methanol ( $\geq 99.8\%$ ), lithium bromide, Cell Counting Kit-8 (CCK-8), and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin (1:250) for the degradation test was purchased from GoldBio. Acetone was purchased from VWR (Radnor, PA). l-Glutathione (reduced 98+%) was obtained from Alfa Aesar (Ward Hill, MA). Spectra/Por 3 regenerated cellulose dialysis membrane tubing (3.5 kDa, molecular weight cut-off) and Alexa Fluor 488 Phalloidin were purchased from Thermo Fisher Scientific (Waltham, MA). Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were purchased from Corning (Manassas, VA). The penicillin-streptomycin (Pen-Strep) antibiotic and fetal bovine serum (FBS) were bought from Gibco-Life Technologies (Grand Island NY 14072). LB broth was obtained from Fisher Bioreagents. LB Agar was obtained from Difco Laboratories. The bacterial strains *Staphylococcus aureus* (*S. aureus*) (ATCC 6538) and *Escherichia coli* (*E. coli*) (ATCC 25922) and 3T3 mouse fibroblast cells (ATCC 1658) were purchased from American Type Culture Collection (ATCC).

### 5.3.2 GSNO synthesis

GSNO was synthesized following a previously established protocol with slight modification [424]. First, glutathione (2.93 mmol, 900 mg) was dissolved in 4 mL deionized (DI) water combined with 1.25 mL of 2 M HCl. The solution was allowed to chill in an ice bath before the addition of NaNO<sub>2</sub> (3 mmol, 207 mg) for nitrosation. Afterward, the solution was allowed to chill in the ice bath for an additional 40 min, and then 5 mL chilled acetone was added to it and

stirred for 10 min. The formed dark pink GNSO precipitates were collected by vacuum filtration, rinsed with cold DI water and acetone, and allowed to dry in a vacuum desiccator overnight.

### **5.3.3 SF extraction**

Aqueous SF solution was extracted from *Bombyx Mori* silkworm cocoons according to a previously described procedure [253]. Briefly, the cocoons were first degummed by boiling in 20 mM sodium carbonate solution for 30 min. The degummed cocoons were rinsed thoroughly with DI water and allowed to dry at room temperature. The dried SF was then dissolved in 9.3 M lithium bromide at 60 °C for 4 h and dialyzed against DI water in a dialysis membrane (3.5 kDa cut-off) for 48 h. The obtained aqueous SF solution was then filtered to remove any contaminants and lyophilized to obtain SF sponges.

### **5.3.4 Fabrication of fibers**

The fibers were prepared by a single jet electrospinning method. The prepared SF sponge was first dissolved in HFIP to obtain a 10 % w/v SF solution. The solution was then transferred into a plastic 3 mL syringe with an 18-gauge stainless steel blunt-tip needle and mounted on a custom-made electrospinning setup. The needle tip was set at a 10 cm distance from a grounded collector and the fibers were spun at a flow rate of 1 mL/h with an applied voltage of 14 kV. The composite fibers were made from 10 % w/v solutions containing SF and ZN at a ratio of 2:1, respectively. To fabricate the NO-releasing fibers, 15 % w/w GSNO was added to the electrospinning solutions with respect to polymer weight and sonicated to obtain a homogenous mixture. The as-spun scaffolds were finally immersed in methanol for 5 min to induce  $\beta$ -sheet formation in SF and were placed in a vacuum desiccator to dry at room temperature for 24 h.

### **5.3.5 Scanning electron microscopy (SEM) imaging**

The morphology of the fibers was observed using SEM (FEI Teneo, FEI Co.). The fibers were sputter-coated with a 10 nm thick gold layer prior to imaging. The images were taken at an excitation voltage of 5.00 kV. The average fibers diameter was measured using Image J software by analyzing at least 50 random fibers in different SEM images.

### **5.3.6 *In vitro* biodegradation**

To evaluate the enzyme-mediated degradation of the scaffolds, known weights of the fibers were submerged in 1 mg/mL trypsin (1:250) solution in PBS and incubated at 37 °C. At different time points, the fibers were taken out, rinsed with DI water, and freeze-dried. The percentage of difference in weight of the dried scaffolds before and after enzymatic degradation was calculated and plotted against time to obtain the degradation profiles.

### **5.3.7 Tensile testing**

The mechanical properties of the nanofibrous scaffolds were investigated using a universal testing machine (Mark-10 ESM303). The fibers were cut into 50 mm long T-bar strips, fixed between the grips, and stretched at a rate of 1 mm min<sup>-1</sup>. The young's modulus, tensile strength, and elongation at break for each specimen were measured according to the obtained stress/strain profiles.

### **5.3.8 Contact angle measurement**

Static contact angle measurements were carried out using an Ossila contact angle goniometer (Sheffield, UK). 5 µl of DI water was dropped onto flat films of each sample type secured on a glass slide, and then contact angles for each sample was recorded from the frame captures using the sessile drop method.

### 5.3.9 NO release measurement

NO release from GSNO loaded fibers was measured via chemiluminescence using a Sievers nitric oxide analyzer (NOA) model 280i (Boulder, CO). The fibrous scaffolds were weighed and submerged in 0.01 M PBS containing EDTA at 37 °C in an amber reaction vessel to protect from light. A continuous flow of nitrogen gas at a rate of 200 mL min<sup>-1</sup> carried the NO released from the sample to the NOA system. The NO levels at each time point were recorded in ppb unit and converted to a standard NO release rate ( $\times 10^{-10}$  mol mg<sup>-1</sup> min<sup>-1</sup>) using the NOA instrument constant.

### 5.3.10 GSNO Leaching

The leaching of NO donor, GSNO, from the SF-GSNO and SF-ZN-GSNO fibers was measured by UV–vis spectroscopy. Fibers were weighed and incubated in a known volume of PBS (pH 7.4) at 37 °C. At different time points, the absorbance was recorded at 340 nm which is the S-NO bond characteristic peak present in S-nitrosothiols. A GSNO calibration curve in PBS was used to determine the concentration of the leached GSNO from the fibers. The percent of GSNO leached at each time point was calculated by dividing the cumulative amount of GSNO leached out to the initial amount of GSNO loaded into the fibers. Both SF-GSNO and SF-ZN-GSNO had similar GSNO content of 15% w/w with respect to the polymer weight.

### 5.3.11 Evaluation of the antibacterial activity

To determine the antimicrobial efficacy, synthesized fibers and their controls were exposed to *S. aureus* and *E. coli* for 24 h, and the viability of adhered bacteria was quantified. First, isolated *S. aureus* and *E. coli* were separately inoculated in LB media at 150 rpm at 37° C for 12-14 h. The resulting liquid culture was centrifuged at 2500 rpm for 7.5 min, rinsed with sterile PBS, and centrifuged again at 2500 rpm for 7.5 min. The pellets were subsequently resuspended in sterile

PBS to reach a final bacterial concentration of  $\sim 10^8$  CFU mL<sup>-1</sup>. In a 24 well plate, individual samples (SF, SF-GSNO, SF-ZN, and SF-ZN-GSNO) were placed in separate wells. Samples were massed prior to exposure. The well plates were then incubated at 37° C at 150 rpm for 24 h. After the 24 h exposure period, samples were removed, rinsed with sterile PBS, and homogenized for 60 s at 25,000 rpm in a 15 mL falcon tube containing 1 mL PBS to detach adhered bacteria. The resulting bacterial solution was serially diluted and plated on LB agar plates, and the agar plates were incubated at 37 °C overnight. Colony-forming units were quantified to determine the number of viable bacteria per mg of each sample, and the percentage reduction with respect to control samples according to the following equation (where C = CFU mg<sup>-1</sup>):

$$\% \text{ Reduction in adhered bacterial viability} = \frac{C_{ctrl} - C_{test}}{C_{ctrl}} \times 100 \quad (\text{Eq. 5.1})$$

### 5.3.12 Cell viability and morphology assessment

An *in vitro* CCK-8 assay was performed to evaluate the potential cytotoxicity of the scaffolds toward mammalian cells. Briefly, cell culture was carried out by seeding 3T3 mouse fibroblast cells (ATCC 1658) in a 75 cm<sup>2</sup> T-flask containing complete DMEM medium with 10 % fetal bovine serum (FBS) and 1 % Pen-Strep. The flask with cells was incubated in a humidified environment at 37 °C with 5 % CO<sub>2</sub>. After reaching > 80 % confluency, cells were detached from the flask using 0.18 % trypsin and 5 mM EDTA, stained with Trypan blue, and counted using a hemocytometer. 100 µL of the diluted cell suspension was then transferred to each well of a 96 well plate, with a density of 5000 cells per well and allowed to form a monolayer overnight. Sample leachates were prepared by soaking the fibers in DMEM medium at a concentration of 10 mg/mL and incubating at 37 °C for 24 h. The obtained leachates were then replaced with the media in each well and exposed to cells for another 24 h. The effect of the leachates on cell viability was determined by the CCK-8 cell counting kit based on the manufacturer's protocol (Sigma Aldrich).

According to the protocol, 10  $\mu$ L of a highly water-soluble tetrazolium salt, WST-8, was added to each of the wells and incubated for 2 h. WST-8 is reduced by the dehydrogenases in the living cells to produce an orange-colored formazan dye that can be detected at 450 nm. After measuring the absorbance at 450 nm using a spectrophotometer microplate reader, the relative cell viability was calculated using the below equation. Cells maintained in DMEM with no leachates were used as control (n=6).

$$\text{Cell viability (\%)} = \frac{\text{absorbance (treated cells)}}{\text{absorbance (control cells)}} \times 100 \quad (\text{Eq. 5.2})$$

The cytocompatibility of the scaffolds was further assessed by investigating cell morphology and adhesion on the samples using fluorescence microscopy. The fibers were first electrospun onto glass slides and sterilized by UV radiation for 30 min. The fibroblast cells were then seeded on the fibers at a density of 15,000 cells/cm<sup>2</sup> and placed in a humidified incubator with 5 % CO<sub>2</sub>, at 37 °C, for 48 h. To observe cell morphology on the scaffolds, DAPI and Alexa fluor-488 conjugated phalloidin dyes were used to stain the nuclei and cytoplasm, respectively. First, the cell-seeded scaffolds were rinsed with PBS, fixed with 4 % formaldehyde for 10 min, and permeabilized with 0.2 % Triton-X-100 for another 10 min. Then, a 1:500 dilution of phalloidin in PBS was applied to cells for 20 min followed by DAPI (300 nM in PBS) for 3 min. The stained cells were finally imaged using a fluorescent cell imaging system (AMG EVOS FL digital inverted microscope) and studied for overall attachment and growth.

### 5.3.13 Statistical analysis

A one-way analysis of variance was used to compare any significant difference. All data are reported as a mean  $\pm$  standard deviation (SD) collected from three data points unless otherwise

noted. A probability value ( $p$ ) of less than 0.05 ( $p < 0.05$ ) was considered to be statistically significant.

## 5.4 Results and discussion

### 5.4.1 Fibers morphology and size analysis

The fibers' size and morphology were studied using SEM. **Figure 5.1** shows the SEM images of different fibers and their corresponding size distribution graphs. The electrospun fibers had all a ribbon-shaped morphology that was attributed to the highly volatile nature of the HFIP solvent used. It has been demonstrated previously that fast and non-homogenous evaporation of the electrospinning solvent results in the formation of a shell on the cylindrical polymer jet causing it to further collapse into a flat ribbon-shaped fiber due to atmospheric pressure [425-428]. SF fibers fabricated in this study had an average diameter of  $1744.1 \pm 553.6$  nm. After the addition of GSNO, narrower size distribution and slightly decreased fiber diameter ( $1135.2 \pm 362.4$  nm) were observed, though not significant ( $p > 0.05$ ). SF-ZN and SF-ZN-GSNO fibers displayed significantly reduced fiber diameters of  $491.5 \pm 129.5$  nm and  $527.8 \pm 119.5$  nm ( $p < 0.001$ ), respectively. Consistent with the results of other studies, the same effect has been seen previously, where the addition of ZN to polymeric electrospinning solutions such as polyvinyl alcohol [429], dextran [430], and gelatin [431], caused a drop in the viscosity, favoring the formation of fibers with smaller diameters. In fact, thinner fibers with diameters in the nano-scale are desired for scaffold-related applications due to their enhanced cellular responses and closer similarity to the nanofibrous structure of the native ECM [432, 433]. Therefore, SF-ZN-GSNO fibers can provide an ideal substrate for tissue growth and regeneration.

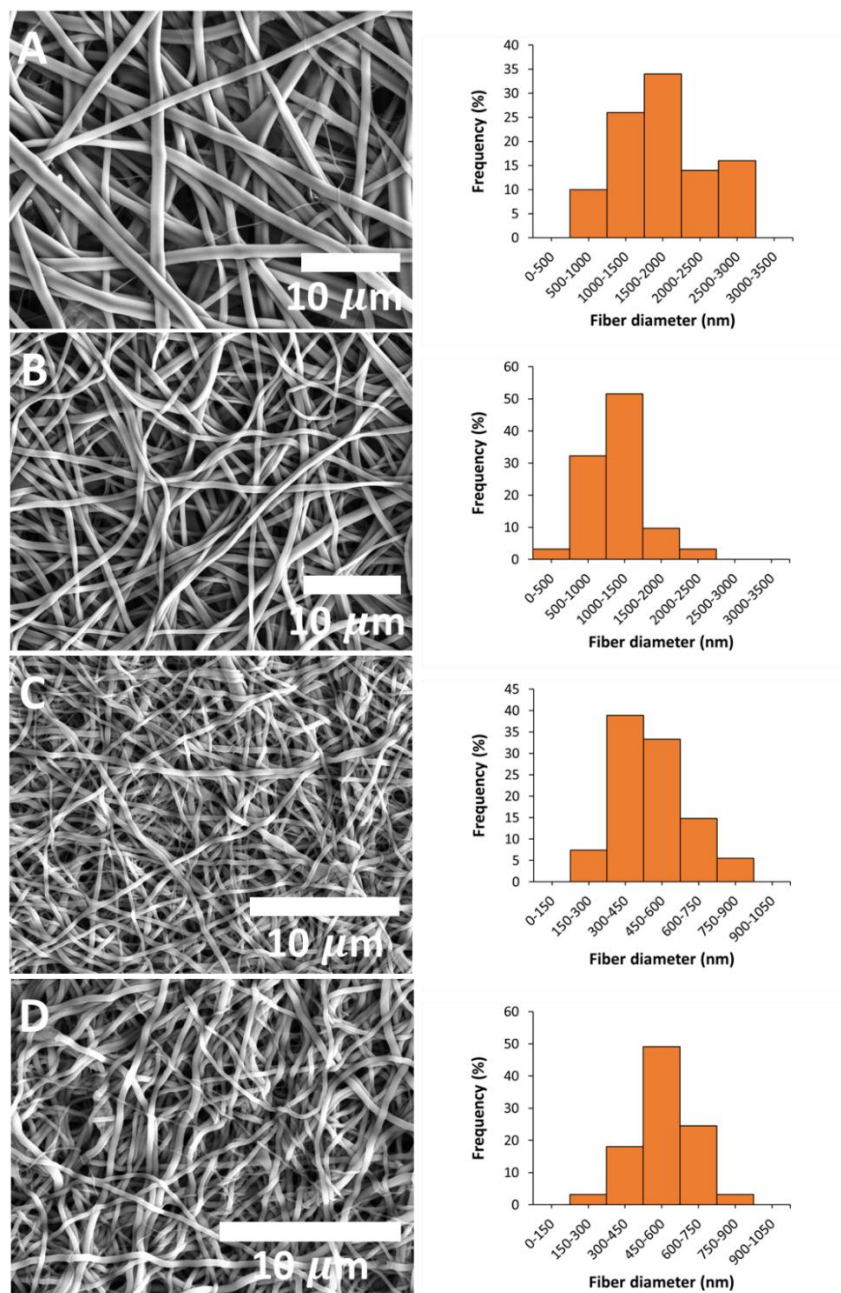


**Figure 5.1** - SEM images of A) SF, B) SF-GSNO, C) SF-ZN, and D) SF-ZN-GSNO fibers and their corresponding diameter distribution graphs.

#### 5.4.2 *In vitro* biodegradation

Degradability is an essential feature for tissue engineering scaffolds. Scaffolds provide a temporary substrate for cells to adhere, grow, and repair or reconstruct the damaged tissue. For cells to be able to replace the scaffold over time, degradation should take place at an optimal rate,

matching that of the new tissue being regenerated [434]. The degradation kinetics of the materials are mainly dictated by their structure. SF is composed of amorphous hydrophilic random coils and helical structures known as Silk I, as well as hydrophobic crystalline  $\beta$ -sheets known as Silk II structure. Certain physical and chemical treatments such as sonication, water vapor annealing, methanol, and heat treatment can induce a conformational transition from Silk I to Silk II structure. The dominance of packed crystalline  $\beta$ -sheets in the SF structure makes it non-soluble and highly resistant to degradation in aqueous media [435]. In the current study, the accelerated enzyme-mediated degradation profiles of the fibers were obtained using trypsin (1:250) solution and the results are depicted in **Figure 5.2**. Methanol treated SF fibers had a very slow rate of degradation, losing only  $19.6 \pm 5.7$  % of their initial mass after 30 days in trypsin media. The addition of ZN to the SF electrospinning solutions caused a significant shift in the biodegradation kinetics of the fibers. ZN is a hydrophobic yet biodegradable polymer that has been increasingly explored for the development of scaffolds and implantable biomaterials in the last decades due to its biodegradability and biocompatibility [436, 437]. Despite undergoing the same methanol treatment, SF-ZN fibers displayed a notably higher rate of degradation ( $p < 0.01$ ), with  $66.1 \pm 1.5$  % of their initial weight being lost during the 30-day period. The presence of GSNO did not cause any significant differences in the degradation rates of the fibers. Overall, the composite fibers showed enhanced biodegradation kinetics compared to pure SF and SF-GSNO fibers. SF and ZN are naturally derived proteins, and their degradation products are biocompatible amino acids that can be metabolized by the body [435]. Therefore, the developed SF-ZN-GSNO fibers have great potential to be used as biocompatible and biodegradable scaffolds for tissue engineering applications.

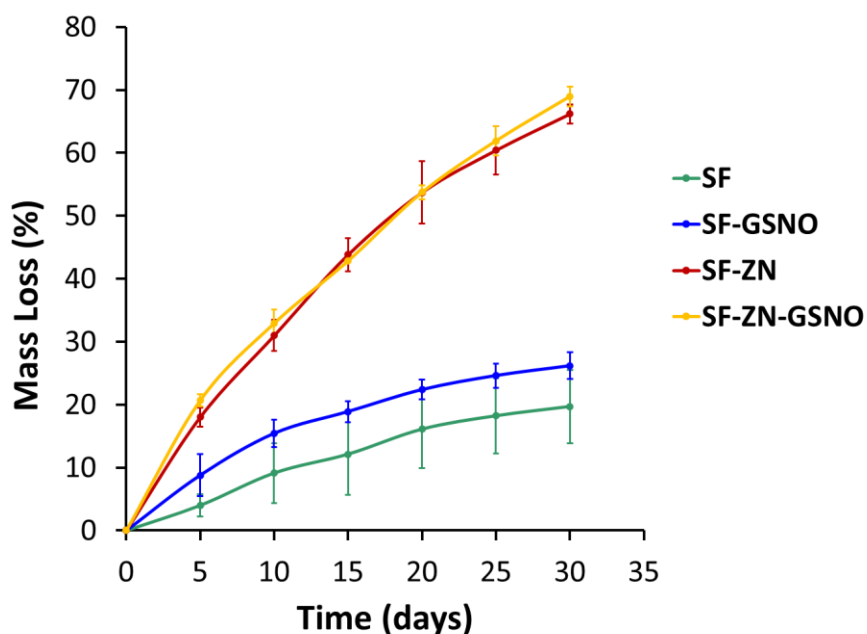


**Figure 5.2** - Enzyme-mediated degradation profile of the fibrous scaffolds in trypsin (1:250) solution at 37 °C in dark.

### 5.4.3 Mechanical properties

The mechanical behavior of biomaterials is of great importance determining their stability and biological performance for different applications. SF has uniquely high mechanical properties among other natural polymers, owing to its ability to undergo conformational transitions to form well-ordered crystalline  $\beta$ -sheets in its structure. In this study, uniaxial tensile testing was performed to investigate the effects of adding ZN and GSNO on the mechanical properties of the SF fibers. The results of the test are displayed in **Figure 5.3** and summarized in **Table 5.1**. The SF fibers had an ultimate tensile strength (UTS) of  $13.65 \pm 0.99$  MPa and an elongation at break (EB) of  $10.23 \pm 0.87$  %. After the addition of GSNO, a significant increase in the UTS of SF fibers was observed ( $p < 0.05$ ) that is consistent with the results of other studies blending NO donors into polymeric fibers [364]. However, no statistically significant difference was found between the EB of SF fibers with and without GSNO. Moreover, the composite SF-ZN fibers demonstrated a significantly enhanced EB ( $p < 0.05$ ) that can be attributed to an increase in molecular

entanglements between SF and ZN macromolecules, facilitating the slippage of polymer chains under stress as observed in previous studies [431]. While the fibers showed different UTS and EB values, no significant changes in the young's modulus were found between any of the samples. Overall, the results indicated that the addition of ZN and GSNO improved the strength and stretchability of SF fibers and did not cause any adverse effects on their mechanical properties.

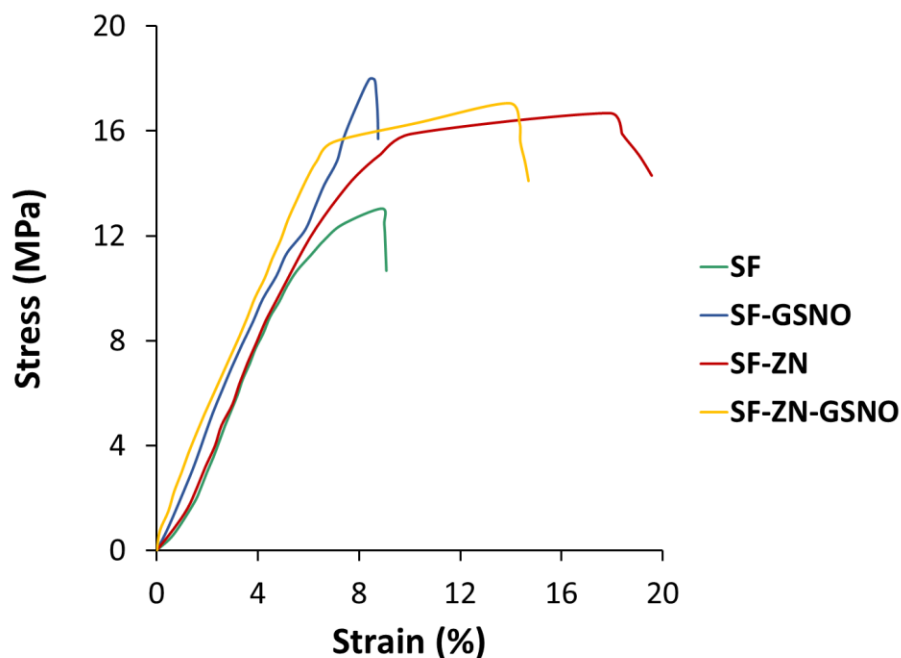


**Figure 5.3** - Stress/strain diagram of SF, SF-GSNO, SF-ZN, and SF-ZN-GSNO fibers.

**Table 5.1** - Mechanical Properties of SF, SF-GSNO, SF-ZN, and SF-ZN-GSNO fibers. \* shows ( $p < 0.05$ ) compared to SF fibers, respectively.

| Samples    | Ultimate Tensile Strength (MPa) | Elongation at Break (%) | Young's Modulus (MPa) |
|------------|---------------------------------|-------------------------|-----------------------|
| SF         | $13.65 \pm 0.99$                | $10.23 \pm 0.87$        | $2.40 \pm 0.03$       |
| SF-GSNO    | $20.65 \pm 4.05^*$              | $9.85 \pm 1.41$         | $2.43 \pm 0.08$       |
| SF-ZN      | $18.61 \pm 0.83$                | $17.13 \pm 3.96^*$      | $2.24 \pm 0.08$       |
| SF-ZN-GSNO | $17.26 \pm 0.58$                | $13.15 \pm 1.83$        | $2.36 \pm 0.09$       |

#### 5.4.4 Surface wettability

The surface wettability of biomaterials is an important factor influencing their interactions with cells. A scaffold surface with moderate wettability is favorable for initial attachment and migration of cells leading to enhanced healing rates [438]. The surface wettability of the samples was studied by measuring their water contact angle (WCA) and the results can be seen in **Figure 5.4**. To avoid the porous network of fibers affecting contact angle measurements, polymeric films of each sample were prepared and used for this study. SF films showed an average WCA of  $56.3 \pm 0.3^\circ$  consistent with the values reported by previous studies. ZN is known to be a hydrophobic protein composed of mainly non-polar amino acids. Therefore, after the addition of ZN, the WCA of SF films was significantly increased to  $83.2 \pm 2.3^\circ$  ( $p < 0.001$ ). However, the blending of GSNO caused a slight reduction in the WCA of both SF and SF-ZN films. SF-ZN-GSNO films showed moderate wettability with a WCA of  $78.6 \pm 0.9^\circ$  suggesting their suitability as scaffolds for cellular adhesion and growth.

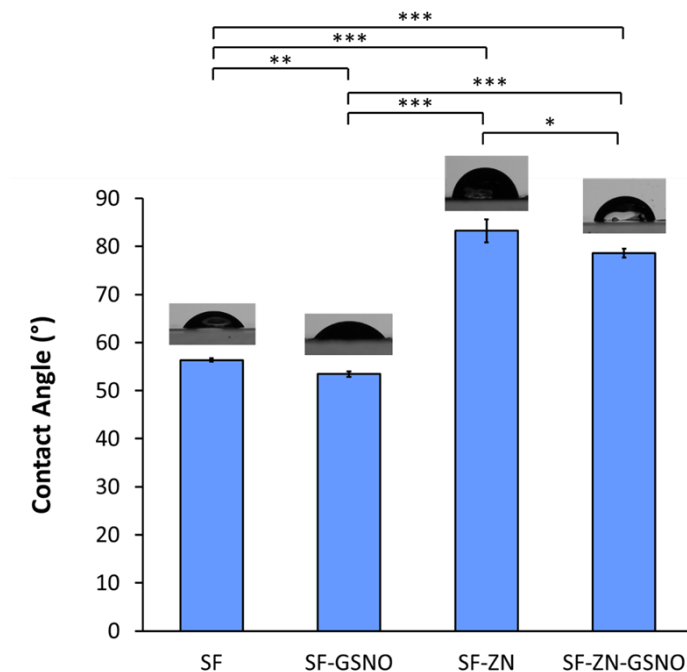


**Figure 5.4** - Static water contact angle of SF, SF-GSNO, SF-ZN, and SF-ZN-GSNO.

#### 5.4.5 *In vitro* NO release kinetics and GSNO Leaching

In order to exert effective antibacterial and therapeutic activity, NO-releasing biomaterials with sustained release kinetics are demanded. Despite the increasing number of NO-releasing scaffolds and wound dressings being developed, only a few are reported to provide steady and prolonged NO release profiles lasting for more than 24 h. In this study, the release kinetics of NO from GSNO blended fibers were tested using NOA and the results are presented in **Figure 5.5**. SF fibers had an initial burst release of NO with an average rate of  $1.39 \pm 0.26 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$  in the first hour that was gradually decreased to  $0.09 \pm 0.01 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$  at 24 h. After 24 h, the NO release rates from SF fibers were not detectable by the NOA anymore. As can be seen in **Figure 5.5**, the burst release of NO from SF fibers during the first hour was notably mitigated in the SF-ZN-GSNO fibers ( $p < 0.01$ ). The highest release rate ( $0.93 \pm 0.26 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$ ) for SF-ZN-GSNO fibers was observed at 4 h whereafter the NO release rate was declined over time and finally reached  $0.09 \pm 0.01 \times 10^{-10} \text{ mol min}^{-1} \text{ mg}^{-1}$  at 48 h. The SF-ZN-GSNO fibers released their NO content in a more controlled manner and demonstrated significantly higher release rates compared to SF-GSNO fibers after 12 h ( $p < 0.05$ ) and 24 h in media ( $p < 0.01$ ). This is most likely caused by the enhanced hydrophobic properties of SF-ZN-GSNO fibers leading to reduced water uptake and consequently slower release of NO in the media.

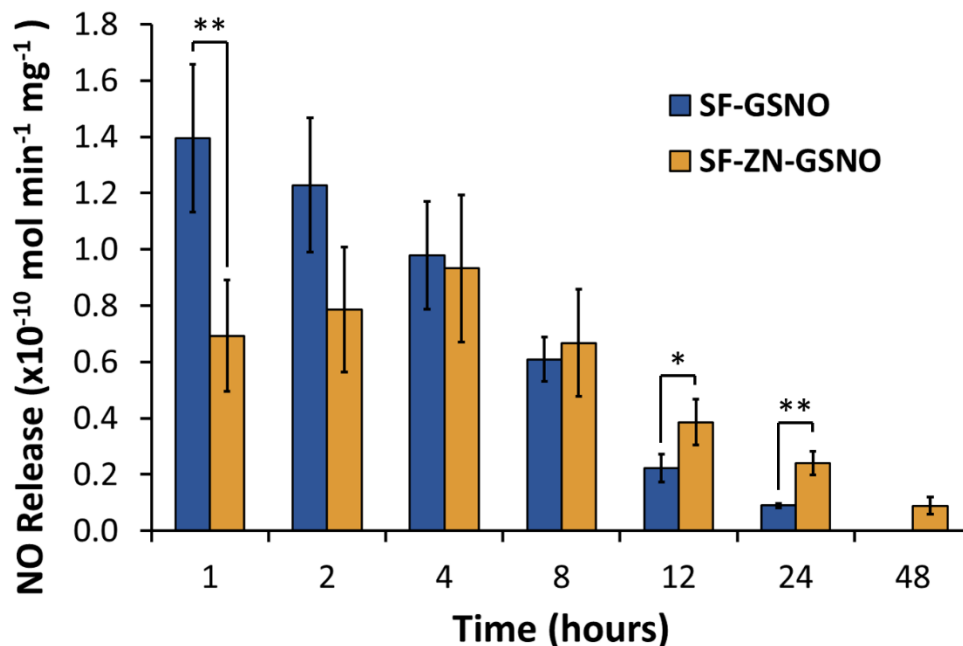


**Figure 5.5** - Real-time NO release profile of SF-GSNO and SF-ZN-GSNO fibers in PBS with EDTA at 37 °C in dark.

Excessive leaching of the NO donor molecule can adversely affect the longevity and cytocompatibility of the NO-releasing biomaterials. The cumulative amount of GSNO leached from the GSNO loaded fibers into the media was measured at different time points through UV-vis spectroscopy and the results are depicted in **Figure 6**. SF-GSNO fibers leached out  $41.0 \pm 1.5$  % of their GSNO content after 24 h while SF-ZN-GSNO fibers had a significantly reduced GSNO leaching of  $26.5 \pm 2.2$  % ( $p < 0.001$ ). This is associated with the presence of hydrophobic ZN in the structure that limits the diffusion of GSNO molecules into the aqueous media. According to these results, the SF-ZN-GSNO fibers offer a promising NO-releasing platform for biomedical applications as they minimize the leaching of GSNO from SF fibers and provide a sustained release of NO for over 48 h.

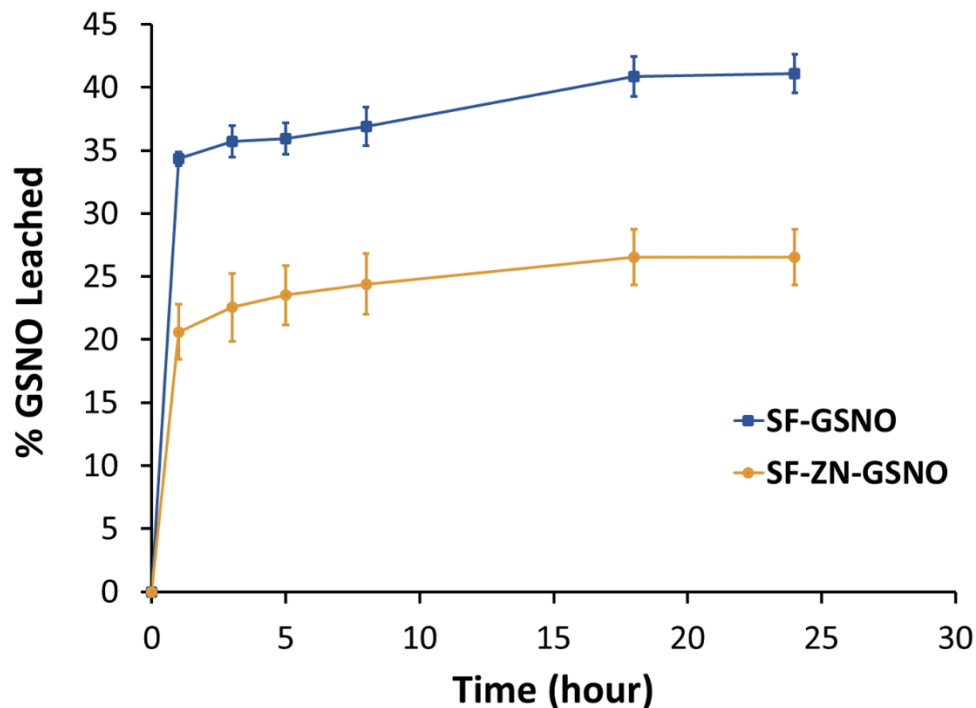


**Figure 5.6** - Cumulative leaching of GSNO from SF-GSNO and SF-ZN-GSNO fibers over the course of 24 h when submerged in PBS at 37 °C.

#### 5.4.6 Antibacterial activity of the fibers

Bacterial infection is one of the main reasons for the failure of implantable biomaterials and may lead to serious consequences such as tissue damage, systemic infections, and patient morbidity and mortality [385]. Bacteria can adhere to the surface of medical devices upon implantation and encase themselves in extracellular polymeric substances to form 3-dimensional multi-cellular communities called biofilm. Bacteria in the biofilm are extremely difficult to treat as they are more resistant to host immune defenses and antibiotic treatments compared to their free-living counterparts [439]. According to previous reports, biofilms can form as quickly as 10 h, underscoring the importance of having an efficient antibacterial treatment in the first few hours of implantation [277, 364]. NO-releasing biomaterials have shown effective antibacterial activity against different bacterial strains in the past [209, 372]. NO targets the bacteria through various mechanisms including inhibition of protein synthesis, alteration of DNA, and damaging the

membrane. The small size, short half-life, and non-specific multi-mechanistic bactericidal activity of NO make it an ideal antibacterial agent to combat antibiotic-resistant bacteria [176]. NO is also known to be able to disperse the bacteria in biofilms into a more vulnerable planktonic state by elevating the bacterial phosphodiesterase activity and therefore accelerating the degradation of c-di-GMP messenger molecules in charge of regulating the biofilm integrity [440]. In this study, an *in vitro* bacterial adhesion assay was performed to evaluate the antibacterial activity of the fibers against *S. aureus* and *E. coli*, two bacterial strains commonly found in nosocomial infections. The results are displayed in **Figure 5.7**. While pure SF fibers showed no antibacterial activity, SF-ZN fibers demonstrated a slight reduction in the viability of adhered *S. aureus* ( $32.5 \pm 9.1$  %) and *E. coli* ( $24.2 \pm 3.6$  %). The presence of abundant carotenoids such as lutein and zeaxanthin in ZN has been reported to endow it with antimicrobial activity to some extent [441]. Furthermore, the NO-releasing SF-GSNO fibers reduced the viability of *S. aureus* and *E. coli* by  $69.9 \pm 9.9$  % ( $p < 0.01$ ) and  $68.6 \pm 4.7$  % ( $p < 0.001$ ), respectively. Addition of both ZN and GSNO to SF fibers led to synergistically higher bacteria reduction efficiencies of  $91.6 \pm 2.5$  % against *S. aureus* ( $p < 0.001$ ) and  $77.5 \pm 3.1$  % against *E. coli* ( $p < 0.05$ ). These results indicate that SF-ZN-GSNO employ both active and passive strategies to inhibit bacteria, therefore offering promising potential to be used as antibacterial scaffolds for prevention of infections.

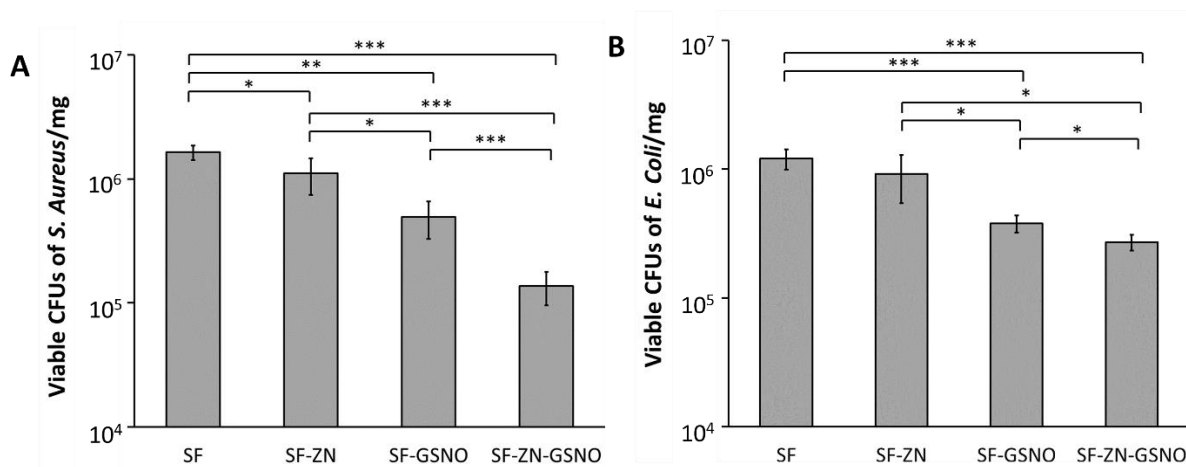


**Figure 5.7** - Antibacterial activity of fibrous scaffolds against viable adhered A) *S. aureus* and B) *E. coli*, measured by a CFU counting method after 24 h exposure.

#### 5.4.7 Cytocompatibility of the fibers and morphology of adhered cells

Despite its strong antibacterial activity, NO at high levels has been shown to be cytotoxic toward mammalian cells due to excessive nitrosative and oxidative stress that it can impose on cells. GSNO is an endogenously produced NO donor molecule in the human body that has previously shown excellent cytocompatibility with mammalian cells when incorporated in polymeric fibers [364, 415]. To evaluate any possible cytotoxicity of the GSNO blended fibers, 3T3 fibroblast cells were exposed to the 24 h leachates obtained by soaking the fibers in media. As seen in **Figure 8**, no cytotoxic response was observed for any of the samples with or without GSNO. All samples maintained cell viability at a similar level or higher than control (cells exposed to no fiber) ( $p > 0.05$ ). SF and ZN are natural polymers with high biocompatibility that have been extensively studied in the past for tissue engineering and biomedical applications. As shown in this study, the leaching of GSNO did not affect the cytocompatibility of these fibers, demonstrating the safe application of SF-ZN-GSNO fibers as a biomaterial.

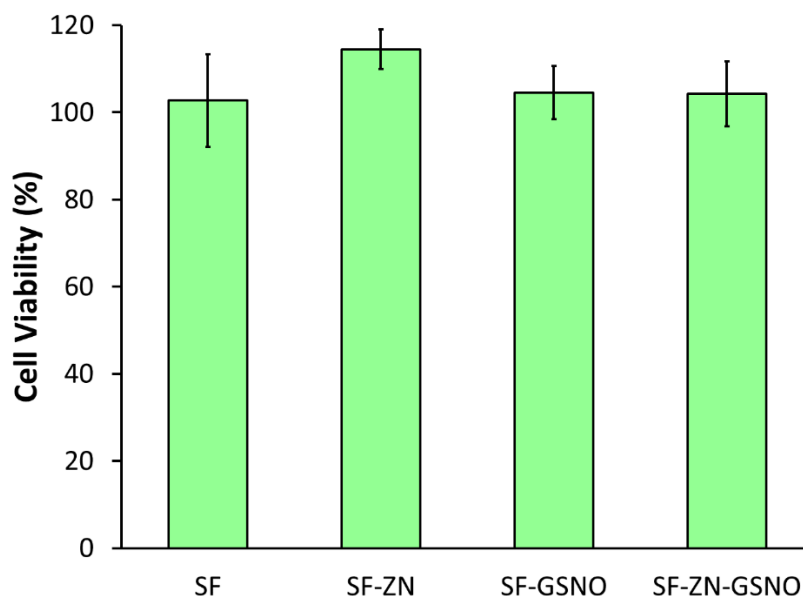


**Figure 5.8** - Viability of 3T3 fibroblast cells after 24 h exposure to the leachates of fibrous scaffolds ( $p > 0.05$ ).

A qualitative fluorescence staining assay was performed to further assess the cytocompatibility of the fibers in direct contact with cells. Fibroblast cells play a critical role in wound healing by producing collagen, creating a new matrix, and regulating wound contraction [442]. Therefore, their proliferation and successful attachment to tissue engineering scaffolds are of great importance for proper healing and tissue regeneration. The delivery of NO through polymeric platforms has been reported to have an enhancing effect on fibroblast proliferation, growth, and collagen deposition. In this study, the morphology of adhered cells to the NO-releasing fibers was studied after 48 h exposure (**Figure 5.9**). Fibroblast cells successfully attached to all fibrous mats and displayed intact nuclei and nanofibrous actin cytoskeletons as stained by blue DAPI and green phalloidin reagents, respectively. A higher density of cells with more stretched and expanded morphologies and pronounced actin filaments were observed on GSNO blended samples, implying that the NO release from the fibers provides a favorable environment for cells to attach and grow. These results are consistent with previous studies emphasizing the important

roles of NO in modulating and improving the wound healing process [443, 444]. Therefore, the NO-releasing SF-ZN-GSNO fibers can be used as a non-toxic and cytocompatible substrate for promoting wound healing and tissue repair.

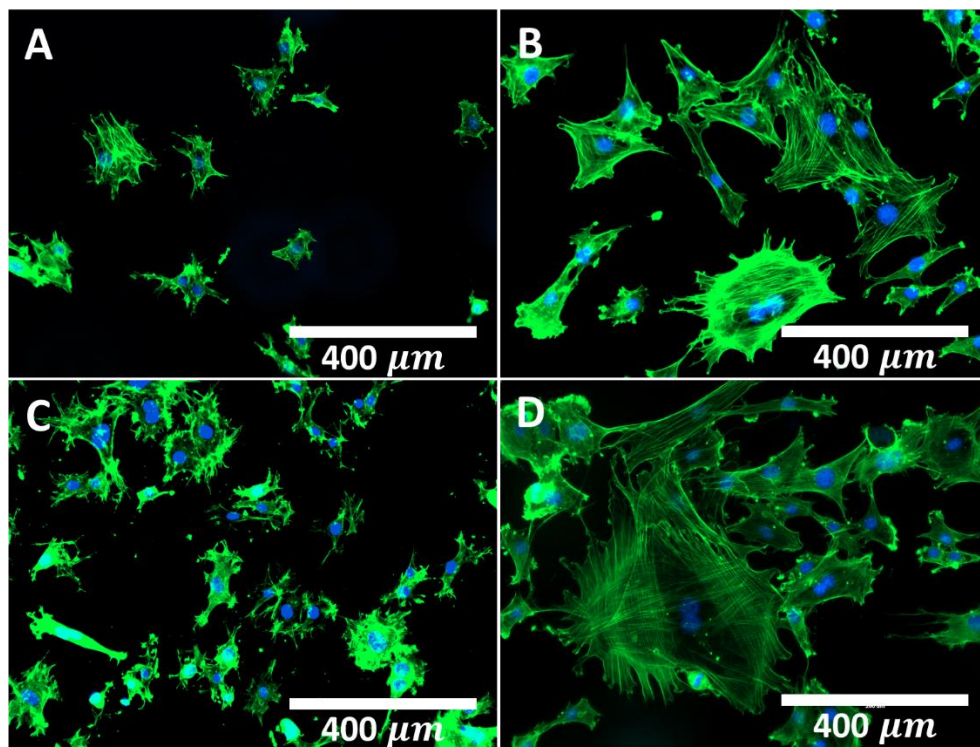


**Figure 5.9** - Fluorescence microscopy images of 3T3 fibroblast cells seeded on A) SF, B) SF-GSNO, C) SF-ZN, and D) SF-ZN-GSNO fibrous scaffolds after 48 h.

## 5.5 Conclusion

In this work, two naturally-derived proteins, silk fibroin (SF) and zein (ZN) were combined and electrospun with the NO donor molecule, *S*-Nitrosoglutathione (GSNO), to fabricate novel antibacterial, biodegradable, and NO-releasing fibers for biomedical applications. The incorporation of ZN into SF fibers reduced the diameter of the fibers and enhanced their enzymatic degradation making them more suitable for scaffolding applications. The tensile testing showed an increase in the elongation at break and ultimate tensile strength of SF fibers with the addition of ZN and GSNO, respectively. Moreover, blending of ZN into SF increased the hydrophobicity

of the fibers by introducing more non-polar amino acids into the structure, thereby decreasing the GSNO leaching amount. The increased hydrophobicity of the SF-ZN-GSNO compared to SF-GSNO fibers also led to a more controlled NO release profile with mitigated burst release and a more prolonged duration of 48 h. The continuous release of NO along with the inherent antibacterial properties of ZN endowed SF-ZN-GSNO fibers with synergistic active and passive antibacterial activity resulting in  $91.6 \pm 2.5 \%$  and  $77.5 \pm 3.1 \%$  reduction efficiency values against *S. aureus* and *E. coli*, respectively. The 3T3 fibroblast cell viability and adhesion assays also demonstrated that SF-ZN-GSNO provided a non-cytotoxic ECM-like substrate for cells to adhere to and grow on. Overall SF-ZN-GSNO fibers demonstrated good cytocompatibility, enhanced biodegradation rate, controlled release kinetics, great mechanical properties, and high antibacterial activity that make them ideal to be used for tissue engineering and wound healing applications.

## **CHAPTER 6**

### **CONCLUSIONS AND FUTURE DIRECTIONS**

#### **6.1 Conclusions**

In the past decade, NO-releasing polymeric materials have found increasing interest for antibacterial biomedical applications. The majority of these research efforts has been focused on the use of synthetic polymers due to their reproducibility, high mechanical properties, and low water uptake leading to higher stability and prolonged NO release profiles. While suitable for use in certain biomedical applications such as medical tubing and their coatings, synthetic polymers lack the desired bioactivity, biocompatibility, and biodegradability for wound healing and tissue engineering applications. In addition, the synthesis and processing of these materials are often costly and require the use of toxic substances and harsh conditions. In contrast, natural polymers possess high bioactivity and biocompatibility, are abundantly available at low cost, have similarity to the extracellular matrix of native tissues, and can be degraded into harmless byproducts that are accepted by the body. The focus of this dissertation is to develop antibacterial NO-releasing platforms such as nanoparticles, hydrogels, and nanofibers based on naturally derived materials for controlled NO delivery, tissue engineering, and wound healing applications.

In the second chapter, a natural polymeric platform based on silk fibroin was developed for NO delivery. The water-insoluble NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), was loaded into silk fibroin nanoparticles using an antisolvent precipitation method. The amide I band FTIR deconvolution confirmed the successful entrapment of SNAP molecules inside the core of SF NPs by hydrophobic interactions resulting in a continuous and controlled release of NO for 24

hours. SNAP loaded SF NPs demonstrated strong antibacterial properties against MRSA and *E. coli* through different range of concentrations while remaining cytocompatible at concentrations  $\leq 2$  mg/mL. This study was the first to investigate the use of NO-releasing SF based materials forming the basis for further future studies to develop antibacterial SF based implantable medical devices, tissue engineering scaffolds, and wound dressings [191].

In the third chapter, covalent immobilization of SNAP onto a natural clay mineral, halloysite nanotube (HNT), was investigated to develop a novel delivery platform with tunable NO release. HNT-SNAP demonstrated significantly improved conversion ratio (NAP conjugation efficiency) and NO release kinetics compared to previously reported NO-releasing silica-based particles. NO release from HNT-SNAP could be triggered by light irradiation or fine-tuned by adjusting the temperature, confirming a tunable NO release from these particles. In addition, the antibacterial activity of NO releasing HNTs toward gram-positive *S. aureus* and their cytocompatibility with fibroblast cells were demonstrated at different concentrations [178].

In chapter 4, SNAP-swelled interpenetrating network (IPN) hydrogels based on gelatin methacryloyl (GelMA) and SF were developed as biodegradable antibacterial scaffolds for tissue engineering application. The prepared scaffolds had a porous morphology and addition of SF in the IPN scaffolds resulted in significantly enhanced properties such as extremely higher mechanical strength, slower biodegradation rate, and a more controlled NO release compared to pure GelMA and GelMA-SNAP hydrogels. The SNAP swelled hydrogels demonstrated a very strong antibacterial activity (>99%) against *E. coli* and *S. aureus*. In addition, a good adhesion, spreading, and high proliferation rate was observed for 3T3 mouse fibroblast cells in contact with the NO-releasing IPN hydrogels, indicating their excellent cytocompatibility [209].

In chapter 5, two naturally-derived proteins, silk fibroin (SF) and zein (ZN) were combined and electrospun with the NO donor molecule, S-Nitrosoglutathione (GSNO), to fabricate novel antibacterial and biodegradable NO-releasing fibers for tissue engineering applications. The incorporation of ZN into SF fibers resulted in enhanced mechanical properties, degradation kinetics, and hydrophobicity which led to a prolonged and more controlled NO release profile. The continuous release of NO along with the inherent antibacterial properties of ZN endowed SF-ZN-GSNO fibers with synergistic antibacterial activity resulting in  $91.6 \pm 2.5 \%$  and  $77.5 \pm 3.1 \%$  reduction efficiency values against *S. aureus* and *E. coli*, respectively. In addition, SF-ZN-GSNO nanofibers strongly supported the attachment and proliferation of fibroblast cells, demonstrating their great potential for tissue engineering applications.

## **6.2 Future Directions**

This dissertation demonstrated the development of biocompatible nitric oxide-releasing platforms based on naturally derived materials such as silk fibroin, zein, and gelatin biopolymers and inorganic halloysite nanotubes. These materials can be used for a variety of biomedical applications specially for wound healing, tissue engineering, and implantable medical device coatings due to their high biocompatibility and biodegradability. The main challenge limiting the practicality of NO-releasing medical devices and biomaterials is their finite NO reservoir and short NO release longevity leading to loss of antibacterial activity in the long run. Natural polymers in particular, usually suffer from burst release kinetics and rapid leaching of the NO donor molecules loaded onto/into them. These issues are associated with their often high hydrophilicity and water uptake, loose molecular structure, and rapid degradation kinetics.

While maintaining the antibacterial effects in the first few hours after implantation is essential to prevent bacteria from adhering, proliferating, and forming biofilm on the surface of

biomaterials, a long-term antibacterial activity is desired to ensure the prevention of post-operative infections due to possible occurrence of hematogenous bacteremia, or direct spreading from a nearby infection site [355]. This is not only important for implantable biomaterials and indwelling medical devices but also for non-implantable medical devices such as wound dressings and sutures that are exposed to the open environment and are in higher risk of getting contaminated.

Moreover, despite its bactericidal activities, NO cannot prevent surface fouling leading to non-specific attachment of physiological proteins to the biomaterials surface that in turn facilitates bacterial adhesion [445]. One promising approach to address this limitation is the utilization of an antifouling surface coating along with NO, to synergistically avert both bacterial adhesion and proliferation on the surface of biomaterials [446].

One of the most interesting and recent methods of creating antifouling surfaces are bioinspired approaches. Several examples of natural antifouling surfaces can be found in animals' skin (e.g. shark, gecko), insect wings (e.g. cicada, butterfly), and plant leaves (e.g. lotus, rice, rose petal) that have evolved unique topographical and chemical features to resist bacterial contamination. The presence of hierarchical micro/nanoscale patterns on the surface of these living organisms endow them with usually low adhesion, superhydrophobic and self-cleaning properties. Micro/nano-patterned surfaces minimize the attachment of bacteria by offering an unfavorable textured interface as opposed to a smooth surface and by alteration of the surface energy leading to super hydrophobic/hydrophilic surfaces [447, 448]. Surfaces with high aspect ratio textures have also shown to be able to kill the bacteria upon contact by physically rupturing or deforming their cell wall membrane [449]. Therefore, in the recent years, many researchers have attempted to replicate these naturally occurring topographies with the hope of reproducing their behavior on the surface of biomedical devices and implants [450]. Antifouling micro/nano

patterned surfaces present a longer-term solution by providing an alternative drug-free route for prevention of biomaterials associated infections and obviating the need for continuous low-dose release of antibacterial agents.

The fabrication of micro/nano patterns on the surface of natural polymeric platforms such as silk fibroin (SF) has previously been shown to prevent bacterial adhesion while maintaining the bioactivity and biocompatibility of the SF substrate [167, 168]. The combination of nitric oxide and surface texturing is a recently emerged field of study that has not yet been explored with natural polymers and can be a very promising strategy to synergistically combat bacterial infection by providing different means of antibacterial action.

**Figure 6.1A** shows textured NO-releasing silk fibroin films mimicking the surface topography of rose petals. These films were fabricated using a soft lithography approach by first preparing polydimethylsiloxane (PDMS) master templates (**Figure 6.1B**) as negative replicas and then casting SF solution containing the NO donor molecule, GSNO, on them to produce positive rose petal replicas. The rose petal surface is covered with micropapillae structures with a diameter of about 20  $\mu\text{m}$  and a height of about 7  $\mu\text{m}$ . These microstructures are covered with tiny cuticular folds of approximately 730 nm in width on their top, rendering the surface ultra-hydrophobic and antibacterial [451]. While the antibacterial mechanisms of rose petal has not yet been completely understood, rose petal mimicked surfaces have been shown to possess antibiofilm activity against both rod shaped gram-negative bacteria and spherical shaped gram-positive bacteria. This activity was reported to be likely caused by restricting the initial attachment and direct contact of bacterial cells with the surface and through isolation of bacterial clusters and preventing the formation of a fibrous biofilm network [452]. The combination of NO with these surface patterns using natural

polymeric platforms like silk fibroin holds great promise for development of novel materials with long-term antibacterial activity and high biocompatibility for future biomedical applications.

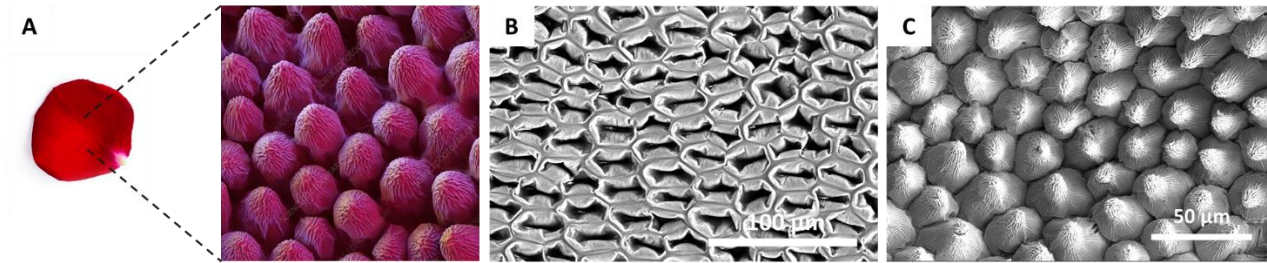


**Figure 6.1** – A) The surface topography of rose petal [453], B) the PDMS negative replica of rose petal, and C) the positive replica of rose petal on silk fibroin films.

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