FABRICATION, CHARACTERIZATION AND WOUND HEALING EFFECT OF SILK FIBROIN-GRAPHENE NANOPLATELETS COMPOSITE FILMS

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ABSTRACT

Silk fibroin (SF) obtained from *Bombyx mori* silkworm cocoon is an insoluble protein-based polymer that widely used in biomaterial applications, including wound healing. A pristine SF film can be prepared via a solution casting method, but the film has poor strength (i.e. brittle). The main drawbacks of SF film are the brittleness of the films that can be overcame via post-treatment (ethanol immersion, methanol immersion and water annealing). Attenuated total reflectance-Fourier transformed infrared spectroscopy (ATR-FTIR) showed that SF films were presented in a more stable form after ethanol (80% v/v) post treatment. The peaks of silk II structure from amide I and II of the ethanol treated films were shifted to 1620 cm^{-1} and 1510 cm^{-1} and the crystallinity results is supporting by X-ray diffraction (XRD) analysis. The introduction of nano-sized graphene platelets (GNP) into the SF systems to form SF-GNP composite films could enhance the physical, mechanical and thermal properties of the film. Drawbacks, due to brittleness and less flexibility, the GNP fillers were poorly dispersed throughout the SF matrix. Hence, glycerol (20 wt.%) as a plasticizer was introduced into the SF-GNP system in order to increase the flexibility, thus assisting the dispersibility of the GNP fillers. In this study, the effects of SF-GNP and post-treated SF-GNP composite films at various GNP loadings (0.1 wt.%-1.0 wt.%) were investigated. Results from ATR-FTIR revealed a high absorption intensity at the peak position 1620 cm^{-1} which correspond to the β-sheet conformation of amide I (C=O stretching) for SF-GNP composite films and the post-treated composite films. The addition of GNP had increased the crystallinities of the SF-GNP composite films obtained from the plot of XRD. Comparing the results between the FTIR and the XRD findings, the relation between SF molecular chains and graphene can be connected. Thermal stabilities of the composite films were also increased with increasing GNP and the degradation rate, as measured by thermogravimetric analysis and biodegradation test. The addition of GNP and glycerol has also improved the flexibility (strain), the SF-0.7G has shown tremendously good flexibility of the composite which an increases up to 1180 % compare to pristine SF as demonstrated by tensile and the fracture surface of the films were examined using field emission scanning electron microscopy. Images from the transmission electron microscopy also shown GNP filler had been dispersed uniformly throughout the matrix. The results of in vitro cell culture displayed that the SF-GNP composite films had supported the cell survival and exhibited the optimal biocompatibility. The SF-GNP composite films showed several potentially wound healing properties where the composites have the ability to recover from wound scratching assay test. From all of the results obtained, the SF-GNP better results in mechanical and thermal properties compared to the post-treated SF-GNP. Thus, SF-0.7G revealed the best results compared to other formulation which shows good flexibility and wound healing properties compared to others. In conclusion, SF-GNP composite films offer a new option in biomaterial choice for the development of wound healing.

ABSTRAK

Fibroin sutera (SF) dari kepompong sutera *Bombyx mori* merupakan polimer protein tidak terlarut yang digunakan dalam pelbagai aplikasi bahan-bio termasuk penyembuhan luka. Filem asli SF boleh disediakan melalui kaedah acuan larutan tetapi filem yang dihasilkan mempunyai kekuatan yang rendah (cth. rapuh). Kekangan utama filem SF adalah kerapuhan filem yang dapat diatasi melalui proses pasca-rawatan filem (rendaman etanol, rendaman methanol dan penyepuhlindapan air). Refleksi pantulan keseluruhan – inframerah jelmaan Fourier (ATR-FTIR) menunjukkan filem SF adalah lebih stabil selepas pasca-rawatan menggunakan etanol (80% v/v). Struktur sutera II daripada amide I and II telah berinjak kepada 1620 cm^{-1} and 1510 cm^{-1} dan keputusan kekristalan disokong oleh analisis pembelauan sinar X (XRD). Penambahan platelet nano grafin (GNP) ke dalam sistem SF untuk menghasilkan SF-GNP filem komposit mampu meningkatkan sifat-sifat fizikal, mekanikal dan termal filem. Disebabkan rapuh dan ketidakbolehan mulur SF, pengisi GNP tidak disebarkan dengan baik dalam matriks SF. Maka gliserol (20 % berat) sebagai pemplastik telah diperkenalkan dan seterusnya membantu penyebaran pengisi GNP dan meningkatkan kemuluran filem komposit. Kajian ini mengkaji kesan SF-GNP filem komposit dan pasca-rawatan SF-GNP dengan pelbagai kandungan GNP (0.1% berat - 1.0% berat) Keputusan ATR-FTIR menunjukkan penyerapan yang tinggi pada puncak 1620 cm^{-1} yang merujuk kepada pengesahan lemberan-β daripada amide I (regangan C=O) bagi SF-GNP filem komposit dan pasca-rawatan SF-GNP filem komposit. Kehabluran filem komposit SF-GNP meningkat dengan penambahan grafin yang ditunjukkan dalam plot XRD. Pembezaan analisis XRD dan analisis ATR-FTIR dapat menjelaskan hubungan antara rantai molekul SF dan grafin. Kestabilan termal dan degradasi SF-GNP meningkat dengan peningkatan kandungan grafin berdasarkan analisa termagravimetrik dan ujian degradasi. Penambahan GNP dan gliserol telah meningkatkan kebolehlenturan komposit filem , dimana SF-0.7G telah menunjukkan peningkatan yang bagus, iaitu sebanyak 1180% berbanding SF asli yang didapati dari ujian regangan dan permukaan retakan telah diperiksa dengan mikroskop elektron pengimbas pancaran medan. Imej mikroskop elektron penghantar juga menunjukkan penyebaran pengisi grafin yang baik dalam matriks. Hasil kultur sel *in vitro* menunjukkan bahawa filem komposit SF-GNP dapat menyokong kemandirian sel dan memperlihatkan keserasian optimum. Filem komposit SF-GNP menunjukkan beberapa ciri keupayaan penyembuhan luka dengan komposit mempunyai keupayaan untuk pulih dari ujian penilaian gores luka. Daripada keseluruhan keputusan didapati,SF-GNP filem komposit menunjukkan keputusan mekanikal dan termal yang lebih baik berbanding keputusan pasca-rawatan SF-GNP. Maka, SF-0.7G menunjukkan keputusan yang terbaik berbanding formulasi yang lain dari segi kebolehlenturan dan penyembuhan luka. Kesimpulannya, keputusan dari kajian ini menunjukkan bahawa komposit filem SF-GNP menawarkan pilihan baharu dalam pilihan bahan-bio untuk pembangunan penyembuhan luka.

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LIST OF ABBREVIATIONS

SF	-	Silk Fibroin
RSF	-	Regenerated Silk Fibroin
GNP	-	Graphene Nanoplatelets
G	-	Graphene
GO	-	Graphene Oxide
ATR-FTIR	-	Attenuated Total Reflectance-Fourier Transform Infra-Red
XRD	-	X-ray Diffraction
TGA	-	Thermogravimetric Analysis
DSC	-	Differential Scanning Calorimetry
FESEM	-	Field Emission Scanning Calorimetry
TEM	-	Transmission Electron Microscopy
PLA	-	Polylactic acid
PTFE	-	Polytetrafluoroethylene
PU	-	Polyurethane
PVA	-	Polyvinyl Alcohol
NH_3	-	Amine
H	-	Hydrogen
HFIP	-	1,1,1,3,3,3-Hexafluoro-2-Propanol
HEMA	-	Hydroxyethyl Methacrylate
Na_2CO_3	-	Sodium Carbonate
LiBr	-	Lithium Bromide
Gly	-	Glycine
Ala	-	Alanine
Ser	-	Serine
Thr	-	Threonine
Val	-	Valine
2-D	-	Two Dimensional

LIST OF SYMBOLS

F	-	Force
A	-	Area
Δ	-	Displacement/Difference
μm	-	Micrometre
mm	-	Millimetre
nm	-	Nanometre
mL	-	Millilitre
L	-	Litre
°C	-	Degree Celsius
σ	-	Stress
ε	-	Strain
θ	-	Angel of incident
d	-	Diameter
λ	-	Wavelength

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Biomaterial is a study on materials development and modification to fit the medical application; where their physical and biological feasibility is assessed. Biomaterial can be described as any substance or a mixture of substances, that can be used as a whole or as part of a system that replaces, treats or augment any tissue, organ or function of the living body [1]. In general, biomaterials are classified to synthetic and natural. Among a range of synthetic biomaterials are ceramics, composites, metal and also synthetic polymer. Synthetic polymers are commonly selected for biomedical application, for example polyester, polylactic acid (PLA), polytetrafluoroethylene (PTFE) and polyurethane (PU). On the other hand, the natural biomaterials from plant and animal origin offer several attractive advantages such as, unique mechanical properties, excellent biocompatibility and both enzymatically and hydrolytically degradable [2].

Natural biomaterial is often presumed to exhibit enhanced compatibility with human hosts, which signifies the ability to demonstrate bioactivity, and to undergo biodegradation. As such, natural materials and particulate materials can exhibit these characteristics in situations where synthetic materials have not met clinical expectations [3]. The natural materials' inherent biocompatibility and healing properties have therefore attracted the interest of medical practitioners and scientists, who endeavoured to repair, restore or enhance the human body. Their availability, ease of processing and relatively low cost ensured their prevalence in many biomedical fields, from burn treatments to the design of drug capsules, to tissue replacement [4].

Main criteria needed for wound healing from the natural biomaterials are that the materials able to support tissue formation retain their shape and volume, appearance and most importantly shorten the time of wound healing. An ideal regenerative biomaterial stimulates wound healing and initiates cells to create a new feasible tissue during *in vivo* [5]. During the healing, the chemistry and biology have indicative impact on the biomaterial properties. However, biological repairing does not focused on the physical improvement of the biomaterials that experience chemical and mechanical disruption due to degradation effects *in vivo* [6]. In wound healing, it is particularly important that the developed biomaterials have the capacity to improve mechanical, chemical damage and are capable of self-repair.

Protein based materials are favourable in biomedical engineering. Protein biomaterials consist of the sequence of monomers in the polymer chain as specified by the polymerization reamers which are unique. Protein polymer can be described as polypeptides comprising up to 20 different building blocks of amino acid [7]. One class of biomaterials, the silk proteins, have been shown to offer non-immunogenic responses upon implantation *in vivo*, controllable degradation rates with native tissue replacement, tuneable mechanical properties, and ambient processing conditions (i.e. water solvation, physiological pH and temperature) [8-11]. These properties demonstrate silk's potential to be used as biomaterial especially in wound healing.

Silk fibre is a common fibrous protein produced by a variety of insects consisting silkworm, spiders, mites, scorpions and flies. Among the native silk proteins, the silkworm silk, in specific that of the domesticated *Bombyx mori* has been utilized as high-quality textile fibre and suture for a long time. Silk fibre contains sericin and fibroin, where the silk fibroin (SF) is the main structural protein of silk fibre. Regenerated silk fibroin (SF) solution can be applied in numerous forms, such as fibres, gels, powders or membranes, depending on function and application [12-14]. Many scientists have recently examined SF as one of the potential materials for biomedical studies, since it has several useful properties, consisting of excellent biocompatibility, great permeability of water vapour and oxygen, biodegradability and minimal inflammatory reaction [15-20].

The molecular fibroin chains are developed by a structure of two components connected by a disulfide bond, which is a complete protein fibroin chain, that mostly hydrophobic and create anisotropic nanocrystals, which high in β-sheet, as well as a second small protein fibroin chain, that is relatively elastic and hydrophobic [21]. Various secondary structures have been found in silk fibroin, including β-pleated sheets, twisted – helices or coiled coils, and that gives the biopolymer material with distinctive physical properties such as elasticity, extensibility, and high strength [21,22] . As a result of the outstanding versatility in processing with excellent physical properties, silk proteins have been utilized in biomedical engineering. It was also reported that silk fibroin matrices are useful for culturing fibroblasts, osteoblasts, and stem cells [23-25].

Plasticizers are additives that increase the flexibility and processability of polymers. In general, plasticizers low the intermolecular forces along the polymer chains, and therefore increasing chain movements and free volume. Plasticization can modify the mechanical and thermal properties of polymeric materials when they are added to the polymer matrix. In biopolymer-based films and coatings, plasticizers are the common additives used since it can enhance the flexibility and ease the handling of the films, retain integrity and avoid cracks and pores of the film [26]. General plasticizers for biopolymers are monosaccharides, oligosaccharides, polyols, lipids, and derivatives. Glycerol has been identified to be exceptionally effective for use in plasticized hydrophilic polymers such as SF [27-29]. Appreciable properties for plasticizers are miscible and compatible in all proportions with plastic components, and they may be added to polymers in solution or after solvents have been removed [30]. The properties of composites are closely related to their structure and therefore, the improvement in the properties is mainly attributed to the high interfacial area between particle and biopolymer matrices and the dispersion of particle.

Incorporation of second phase as reinforcement by introducing the filler could potentially improve the mechanical properties of the natural polymer [31]. Among popular and known fillers is graphene oxide (GO) and graphene (G), which exhibit huge potential due to their outstanding mechanical properties, high binding potential, high aspect ratio, high flexibility, and superior processability [22,32,33].

1.2 Problem Statement of Study

SF films produced at room temperature by solution casting are brittle and water soluble, which can compromise many applications. This is due to the dominant structures of random-coil. Thus, the molecular conformation of SF, the elasticity and malleability of the films are important parameters of SF films that should be controlled for biomaterial application, since they are stiff when dried [34]. The brittleness of the films is primarily determined by the secondary structure. Therefore, by introducing post-treatment using solvents or water annealing, the secondary structure (high beta sheet) of SF can be modified [35,36]. Post-treatment by methanol immersion, ethanol immersion and water vapour annealing was intended to regenerate construction of the β-sheet structure in order to improve the insolubilities and the stabilities properties of the silk fibroin film [37,38].The study of structural changes of SF films by these methods, immersion in organic solvents and water annealing, are very interesting subject either for fundamental or applied researches from the standpoint of exploiting this biopolymer for new applications.

Glycerol has high hygroscopicity, and therefore polymers show softness when mixed with glycerol and also act as plasticizer to the materials to form more flexible systems [27,39]. Many researched evaluated the improvement in mechanical properties and flexibility when glycerol is added as a plasticizer, without affecting the other properties of the original material such as microstructure or biological response [27,34].For these reasons; glycerol was introduced into the system to improve the physical and mechanical properties of the synthesized film. Polymers show softness when mixed with glycerol and act as plasticizer to the materials to form more flexible systems [27,39].

New found materials such as graphene have triggered interest in researcher to construct of diversity of new composites due to its considerable application in biomedical. Recently, it was discovered that silk has potential to be bound with graphene, thanks to the π-π stacking and H-bonding [40,41]. This indicates that silk fibroin solution could be promising natural materials for dispersing graphene and definitely for developing silk fibroin-graphene composites. Silk fibroin solution and graphene dispersion produce miscible solution but produce non-homogenous composite films [21]. Addition of glycerol improved the homogeneity of the composites and thus, enhanced the mechanical properties of the composites due to strong interfacial adhesion with the matrix [28,30,42].

It is very interesting to investigate silk fibroin and graphene. A number of researchers have prepared SF / GO - based materials before this research. Hu et al. fabricated a new layer - by - layer ultrathin SF / GO nanocomposite membrane with high tensile strength, modulus, and toughness [32] . Huang et al. were inspired by the natural nacre and prepared strong composite films with layered structures by simple solution casting of SF / GO hydrogels [43]. Recently, Wang and friends [44] produced a flexible and biocompatible SF / GO composite film using a simple and environmentally friendly method without perceptible reagents. The mechanical properties and thermal stability of the composite films increased at fairly low concentrations of GO, and the incorporation of GO also improved the resistance of these films to degradation from an enzyme solution.

Although the development of the SF/GO blend composites has been explored, only a few researches have been based on SF/G composites. Herein, Wang et al. reported the first fabrication of green graphene-silk fibroin (G-S) composite films prepared from facile photo thermal reduction technique using varied amount of graphene fillers (0.1 wt.% to 10 wt.%). These green graphene - silk composites have been shown to be completely miscible in the nanoscale. The synergistic improvement of graphene and silk, facilitated by the interaction of hydrophobic-hydrophobic between graphitic surfaces and hydrophobic silk blocks is achieved through a very low concentration of graphene in silk matrix. The 0.5 wt. % GS composite elastic module is astounding, which is six times higher than the pure silk film due to its fast and simple manufacture and low graphene nanofiller used [21]. Unfortunately, the understanding of SF/G composites with glycerol as plasticizer remains unclear and limited.

The usual technique practiced for the incorporation of graphene filler into biopolymers are solution mixing, in-situ polymerization and melt blending [45]. Solution mixing is among the most favourite and simplest ways for the fabrication of graphene/polymer composites. The easy and fast preparation of the plasticized silk fibroin-graphene composite films prepared by solution casting technique with adding very low loading of graphene could be an advantage for the composite films. Therefore, in this study the understanding of the structural changes, mechanical properties, thermal properties, morphology and biological properties of silk fibroin film with the addition of graphene nanoplatelet (GNP) as reinforce and glycerol as plasticizer was explored. Although several studies on SF-GNP composites have been done, there have been no reports on the study of the incorporation of plasticizers on those composites.

The novelty of the study is the combined effect of both graphene nanoplatelet filler and glycerol plasticizer on SF composite films in several fields especially in biomaterial, but its performance as good flexible material with good dispersion has not been addressed yet. Not only that, there are limited research on the post treated of composite films. This study could be relevant, as silk fibroin composite films have a wide scope of applications in biomedical application especially in tissue engineering in the development of wound healing application Furthermore, wounds treated with these materials have shown to promote the healing by enhanced cellular proliferation, growth and differentiation and, reduced inflammation when applied to *in vivo* models. In this work, silk fibroin/graphene composite films were developed. The resulting materials were characterized by their physical-mechanical properties and the effect of wound healing properties was evaluated.

1.3 Objectives of Study

In this study, the silk fibroin/graphene composite films were discovered in order to evaluate its potential use in biomedical engineering. This study focused on characterization, physical properties, mechanical properties, thermal properties, degradation rate, biocompatibility and wound healing effect with the influence of different series of graphene content. The main aim of this research is to fabricate strong and robust silk fibroin composite films. The other objectives to achieve the main of this research include:

1) To determine the effect of the post treatment (water vapour annealing, ethanol and methanol immersion) on physical characterization , morphology, crystallinity, thermal properties and mechanical properties of the silk fibroin films.

2) To analyse the effect of the preparation of processing glycerol plasticized silk fibroin/graphene composite films using easy solution mixing with casting method and also the effect of adding different series of graphene loading on the physical characterization, morphology, crystallinity, mechanical properties, thermal properties, biodegradation rate, biocompatibility and wound healing effect of the SF-GNP composite films.

3) To evaluate the effect of post treatment of the plasticized SF-GNP composite films on the physical characterization, morphology, crystallinity, mechanical properties and thermal properties.

1.4 Question of Study

The following research questions are used to achieve the objectives of the study:

1) What is the relationship between physical, structural and morphology of the silk fibroin films and what are the effects on films robustness through the post treatment by water vapour annealing, ethanol and methanol immersion?

2) How the solution mixing and solution casting method impacted the characterization, secondary structure, morphology, crystallinity, mechanical properties, thermal properties, biocompatibility and biodegrading rate of the SF/GNP composite films and how effective are the wound healing effect of the SF/GNP composite films?

3) What are the effect post treatment on the characterization, secondary structure, morphology, crystallinity, mechanical properties and thermal properties of the SF/GNP composite?

1.5 Significance of Study

In this study, we try to understand the structural changes, mechanical and characterization of the silk fibroin film with the addition of graphene. This fact opens a set of applications of graphene / silk fibroin composites films via solution casting in several fields especially in biomedical application, due to its performance as a strong and robust material. This study could be relevant, as composite films have a wide scope and promising applications in biomaterials, membranes and tissue engineering with the use of facile preparation techniques that are easy, low cost, quick and environmentally friendly.

1.6 Scope of Study

The scopes of this research are identified and divided into few parts.

(a) Preparation of silk fibroin solution by degumming the silk cocoon in sodium carbonate (Na_2CO_3) and dried at room temperature. The silk fibre was dissolved in salt solution and was dialyzed against distilled water. The silk fibroin films were prepared through solution casting. The effects of post treatment of the silk fibroin films by water vapor annealing, ethanol and methanol immersion on the structural, thermal properties, mechanical properties, were examined via attenuated total reflectance - Fourier Transform infra-red (ATRFTIR) spectroscopy, X-ray diffraction (XRD),UV/vis spectroscopy, differential scanning calorimeter (DSC), thermogravimetric analysis (TGA) and tensile tests.

(b) Incorporation of graphene with different loading homogenously dispersed in silk solution was prepared through easy solution mixing with the help of vortex mixer and solution casting on Teflon mold. The effects of plasticized SF/graphene on the structural, thermal properties, mechanical properties, biodegradation rate and biocompatibility of the SF/graphene composite films were investigated via ATR-FTIR, XRD, DSC, TGA, tensile tests, field emission scanning electron microscope (FESEM) and transmission electron microscopy (TEM).

(c) The biological properties of the SF/G as potential used in wound healing were determined by in vitro biodegradation test, in vitro cytotoxicity and wound healing assay.

1.7 Organization of Thesis

Chapter 1 provides the overview, problem statement of the study, the objectives, research question and scope of this study.

Chapter 2 is a review the literature related to the research such as the silk fibroin, graphene, and wound healing properties.

Chapter 3 provides the research methods including the materials used, sample preparation, characterization, mechanical characterization, thermal characterization and bio-properties.

Chapter 4 presents the results and discussion of the analysis.

Research conclusions are provided in Chapter 5.

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