FABRICATION OF CORE-SHELL STRUCTURED NATURAL RUBBER BASED MICROFIBRES FOR ARTIFICIAL BLOOD VESSEL TISSUE ENGINEERING SCAFFOLDS

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(Master of Materials Science)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

February, 2016
I am the author of the thesis entitled

FABRICATION OF CORE-SHELL STRUCTURED NATURAL RUBBER BASED MICROFIBRES FOR ARTIFICIAL BLOOD VESSEL TISSUE ENGINEERING SCAFFOLDS

submitted for the degree of Doctor of Philosophy (Engineering)

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ACKNOWLEDGMENTS

I would like to say thanks to those who have guided and helped me during my PhD journey in Deakin University.

First of all, I would like to give my sincere thanks to my principal supervisor, Lingxue Kong, who supported me on the whole way with his abundant experience. His strict guiding leads my research toward right direction.

I would also like to express my appreciation to my associate supervisors Professor Zheng Peng, Professor Xungai Wang and Dr. Cynthia Wong, who guided me in rubber materials, fibre materials, and biological materials, respectively. Special thanks to Dr. Fenghua She for her patient support in detailed experimental design and suggestion. Special thanks to Dr. Chris Garvey from ANSTO for his help in physical characterization, especially SAXS testing in Australian Synchrotron. Also, I would like to thank Dr. Haitao Niu, Dr Ludo Dumee, Dr. Bin Tang and Dr. Xin Liu, Dr. Jinfeng Wang, Dr Pimm Vongsvivut, Dr. Jingyu Chen and Dr. Zhenyu Li for their help with my experiment.

Thirdly, I would like to acknowledge my fellow colleagues of IFM who have helped me in characterization, Chengpeng Li (DSC, FTIR, TGA), Pingan Song (Rheological testing), Zhifeng Yi (SAXS), Lijue Chen (MTT assay), Bao Lin (SEM-Quanta, AFM and SAXS), Chunfang Feng (Mechanical testing), Qi Cao (Optical microscope), and Xinchu Zhao (TEM).

In addition, I would like to show my great thanks to Deakin University for financial and technical supports. The international scholarship supported my project financially. The support from professional technicians and the availability of excellent facilities at IFM have also be invaluable to my project. Special thanks to Australian Synchrotron for the access to their facilities and the technical support from the beam scientists.

Last but not least, I would thank my family. Great thanks to my parents, my daughter and my wife and her parents for their strong support!
ABSTRACT

Vascular diseases are contributing to higher and higher morbidity and mortality to the world. Tissue engineered artificial blood vessels are a promising way to meet increasing requirement of vascular replacement. However, compliance mismatch between prosthesis material and native vessel leads to thrombogenicity issue after surgery. Natural rubber (NR), a biopolymer tapped from rubber tree, is of excellent elasticity, which can be potentially used as scaffolds materials for blood vessel tissue engineering. To mimic porous structure of extra cellular matrix (ECM) and enhance biocompatibility of NR, co-axial electrospinning of NR and biocompatible polyvinyl pyrrolidone (PVP) is introduced for fabricating core-shell structure scaffolds.

Firstly, stability control of natural rubber electrospinning process was investigated to fabricate uniform NR based scaffold mats. NR/Tetrahydrofuran (THF) properties were controlled by increasing homogeneity and rheological stability. THF was selected as solvent for electrospinning NR solution with a concentration ranging from 15g/L to 40g/L and when the concentration of 25g/L was used, fibres with a diameter ranging from 400nm to 7μm could be spun. Both filtration and centrifuge can increase the stability of electrospinning by removing gel component. The optimal volume ratio of NaCl solution to NR solution is 4:100. Processing parameters that influenced stability of electrospinning NR solution including voltage range, needle diameter and feeding rate were investigated. Characteristic length of Taylor cone increased with increase of voltage. The fibre diameter decreased and the morphology of fibres tended to be straighter with an increase of voltage. The competition between relaxation and stretching of electrospun fibres contributed mostly to the curl or straight morphologies. FTIR results showed that the solvent played a negligible role in the structure of electrospun fibres. The stretching rate of electrospun fibres were influenced by the solution concentration. XRD and DSC results demonstrated that during electrospinning process, crystallinity degree of NR materials increased. The mechanical properties of electrospun fibre mats showed high elongation at break, which can reach to 639.5% at most with tensile strength at around 132.9Mpa. Strain-induced crystal in electrospun fibres during stretching process was reflected in the SAXS pattern shape changes.
Secondly, core-shell structure NR-PVP fibres were fabricated from co-axial electrospinning. At first, the compatibility of NR and PVP materials was estimated. 20% PVP/water solution and 25g/L THF solution could achieve a steady co-axial electrospinning process. Intact core-shell structure fibres could be spun from relative shorter inner needle and non-conductive core-fluid, which was confirmed by FIB-SEM. FITC dyed PVP was used as a trace for confirming core-shell structured fibres in confocal microscope images, which showed a layer of 50 nm PVP coating outside NR fibres.

Thirdly, water stability of PVP and interfacial interaction between core and shell were enhanced by UV radiation. 254 nm UV light was used for crosslinking of PVP, which contributed to increasing water stability. Crosslinking degree in electrospun fibres increased with radiation time; however, with increase of radiation time, cylinder shape of the fibres would be damaged due to increase of temperature from radiation. 3-hour radiation would crosslink NR fibres without damaging cylinder shape of electrospun fibres. Crosslinked PVP-NR core-shell fibres showed improved degradability and oxidation stability. Compared with electrospun NR fibres coated with PVP, core-shell structure fibres possess obvious advantages in mechanical properties.

Finally, in vitro evaluation of NR based scaffolds was carried out on human arterial smooth muscle cells (HASMC). Cytotoxicity testing results from soaking solution showed that NR film was a poor scaffold for SMC culture due to its high hydrophobicity. MTT assay results showed that scaffolds from core-shell structure NR-PVP, were of highest cell viability (91.7% after 1 day culture) followed by electrospun NR fibres (71.5%), while that of PVP coated ENR fibres was only 60.1%. Cell proliferation was investigated in cell growth time points at: 1 day, 3 days and 7 days. The increase order of cells growing rate on different scaffolds was: core-shell fibres > ENR fibres > PVP coated fibres > NR film.
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Abbreviation

NR          Natural rubber
NRL       Natural Rubber Latex
PVP        Poly vinyl pyrrolidone
DPNR     De-proteinized natural rubber
DSC     Differential Scanning Calorimetry
FTIR    Fourier Transform Infrared Spectroscopy
DLS    Dynamic light scattering
XRD    X-ray Diffraction
SAXS    Small Angle X-ray Scattering
CH$_2$Cl$_2$ Dichloromethane
CCl$_3$ Chloroform
THF    Tetrahydrofuran
ABV    Artificial Blood Vessel
PCL    Poly (ε-caprolactone)
ECM    Extracellular matrix
HASMC  Human Arterial Smooth Muscle Cells


1 Introduction

1.1 Aims of the project

Blood vessels are elastic tubes in which blood undertakes the task of delivering nutrition and oxygen to the body where wastes are collected. However, aged blood vessels become stiffer as the elastic fibres because loose and the tunica intima layer thickens because of the accumulation of fat, cholesterol and calcium [1, 2]. Therefore, blood vessels tend to be narrower and stiffer with the increase of age, which in turn leads to vascular diseases. Balloon angioplasty, thrombolysis and vessel implant are common ways to cure them [3]. Long-term therapy and risk of thrombogenicity restrict application of balloon angioplasty. Thrombolysis is an efficient way to break down clot. However, damaged blood vessels and seriously blocked vessels need replacement surgery.

Artificial blood vessels (ABVs) play an important role in vascular replacement [4, 5]. Blood vessels from donators and the patients themselves, porous tubes made of synthetic polymers, and tissue engineered vascular grafts have been the main sources of vascular replacement. Autologous vessels are usually the first choice, however, twice surgery and sometimes no suitable vessels limit its application [3, 6]. Dacron and ePTFE are two synthetic polymers successfully used on larger vessels (diameters larger than 6mm). Unfortunately, they failed in being used as smaller artificial blood vessels due to compliance mismatch with native blood vessel [5, 7, 8].

To find compliant scaffold materials for artificial blood vessels, elastomers are electrospun to form scaffolds that have excellent elasticity and flexibility which are compliant to host vessels [9]. Natural elastomers such as collagen [10, 11] and elastin [12] have been electrospun for scaffolds of tissue engineering of blood vessel (TEBV). However, poor mechanical properties and premature dissolution limit their applications [13]. Electrospun polyurethane (PU) and poly isobutylene [14] are also tried in vascular graft but less proof for their clinical application prospects. Natural rubber is a biosynthesised, biodegradable and non-toxic elastomer and has excellent elasticity
under room temperature. However, its tensile strength and biocompatibility can’t meet the requirement of ABVs.

To improve tensile strength and biocompatibility of electrospun NR, core-shell structure fibres with polyvinyl pyrrolidone (PVP) outside could be a good strategy [15, 16]. The fibres coated with PVP will have enhanced tensile strength as well as biocompatibility due to PVP’s excellent biological properties. However, natural rubber is a no-polar polymer and is incompatible with PVP, which will influence mechanical properties and structure of fibres [17, 18]. Core-shell structure fibres with elastic NR in core and biocompatible PVP as the shell would be a good strategy. It has been used to increase biocompatibility of biodegradable polymers such as: poly (ε-caprolactone) (PCL) [16], Poly (L-lactide-co-ε-caprolactone) [19, 20] through a core-shell structure fibre strategy. However, the immiscibility of NR and PVP at interface will influence mechanical properties and structure of the fibres [17, 18].

To enhance interfacial action between NR and polyvinyl pyrrolidone (PVP), crosslinking would be introduced on the interface [15, 18]. PVP is water soluble and has poly vinyl structure which would be used for grafting and crosslinking with double bonds in NR. As described in the literature, PVP could be initiated to free radical by heat [21] or other methods, such as: UV radiation [22], superoxide [23], which would initiate double bonds in NR. Ideally, PVP would be initiated by free radical under radiation which would initiate free radical crosslinking with double bonds in NR. UV radiation is an ideal strategy for increasing water stability of PVP by crosslinking and interfacial enhancement of NR and PVP. Interfacial interaction is commonly investigated and improved in polymer blends. However, the interfacial interaction in core-shell structure fibres has seldom been investigated, especially the interfaces between polymer materials. It is important to investigate and improve immiscible interface of core-shell fibres because it relates to the stability of properties and structure of combined fibres. Therefore, this project will focus on investigating and enhancing interfacial interaction between NR and collagen at the interface of core-shell fibres.

1.2 Hypothesis

The project is based on several hypotheses, which were listed as follows:
1) NR fibres mimic extracellular matrix (ECM) in structure and mechanical properties;
2) Fibre morphology could be controlled by stable electrospinning;
3) Core-shell structure fibres with PVP covered outside of NR core can improve biocompatibility of ENR fibres;
4) UV radiation could crosslink PVP to improve its water stability without introducing cytotoxicity;
5) UV radiation may crosslink NR fibres instead of oxidation;
6) Mechanical properties and hydrophilicity of fibres would be improved after UV radiation;
7) Core-shell structure NR-PVP fibres showed better biocompatibility than ENR fibres.

1.3 Outlines of the chapters

This thesis consists of 8 chapters as follows:

Chapter 2 mainly reviewed project background of electrospun fibres for artificial blood vessel scaffolds. Progress and issues existed in artificial blood vessel areas were firstly presented together with the possibility of using NR materials for ABV applications. Then, the fabrication of uniform scaffolds from electrospinning and co-axial electrospinning NR and PVP was discussed. The characterization methods and strategies to enhance interfacial interaction between core and shell were looked at.

Chapter 3 listed materials and methodology used in this project.

Chapter 4 studied the stability control of electrospinning natural rubber process that decides the uniformity and mechanical properties of electrospun fibres. Optimal conditions for steady electrospinning of natural rubber were investigated from two aspects: polymer solution properties and steady process. In the first section, filtration and centrifugation were used for increasing homogeneity of the solution. In the second section, critical voltage and nozzle diameters were investigated followed by the discussion on competition between stretching and relaxation during electrospinning and
its influence on fibre morphology. In the third section, morphologies and properties of electrospun NR fibres were discussed.

Chapter 5 focused on the preparation of intact core-shell structure fibres from the steady co-axial electrospinning through the effective combination of core and shell solution properties with the processing parameters. In the first part, the compatibility of NR and PVP materials was estimated from optical microscope and DSC. Secondly, the stability of co-axial electrospinning controlled by the relative properties of core and shell fluids, such as rheological properties, concentration, feeding rate, conductivity together with the effects of processing parameters, including voltage, relative feeding rate, diameter and the relative length of inner needle were investigated. Finally, intact core-shell structure fibres were prepared from controlling the stability of co-axial electrospinning and the core-shell structure and properties were characterised.

Chapter 6 discussed UV radiation of core-shell structured NR-PVP fibres. PVP was cross-linked first and then the crosslinking was happened on the interface, which would enhance the interfacial interaction between NR and PVP. Firstly, methods for the crosslinking of PVP were investigated in terms of heat annealing and UV irradiation followed by conditions for UV radiation such as wavelength of UV light and radiation time. Secondly, the radical crosslinking and competition between crosslinking and photo-degradation were discussed in UV radiation of NR fibres. Finally, UV radiation of core-shell structured NR-PVP was investigated in terms of the changes in water stability and mechanical properties after UV radiation.

Chapter 7 reported in vitro biological evaluation of electrospun fibres with different components and morphologies, in which, cytotoxicity and cell proliferation of smooth muscle cells were tested. The cytotoxicity of ENR, NR film, PVP coated fibres and core-shell structure fibres was discussed in the first section through cell counting technique and optical microscope. Secondly, the relationship between scaffold hydrophilicity and cytotoxicity was discussed. Thirdly, cell proliferation and viability were investigated by MTT assay on ENR, NR film and core-shell structured fibres.

Chapter 8 outlined the findings of the project. The limitations and potential solutions were also discussed.
2 Literature Review

2.1 Artificial Blood Vessels

2.1.1 Function and structure of blood vessel

Blood vessels are an important part of the blood circulatory system. Blood vessels or vascular grafts can be divided into three types according to their structures and functions: veins, arteries and capillary [1]. Arteries have similar structure to veins but different functions. Both artery and vein are composed of three layers (Figure 1): the tunica intima, the tunica media, and the tunica adventitia. The tunica intima comprises a layer of endothelial cells (also known as endothelium, 1-2 μm in thickness and 10-20 μm in diameter) associated with connective tissue and an elastic laminate, which is rich in collagen type IV and elastin [19]. The endothelium participates in vascular permeability and hemostasis.

The tunica medium is the thickest layer of the three layers. It contains smooth muscle cells within a surrounding extracellular matrix (ECM) which is composed of collagen (type I and type III), elastin fibres and various proteoglycans. It provides mechanical strength and responds to external stimuli. The tunica adventitia consists of connective tissue fibres, which comprise fibroblasts and randomly arranged collagen (type I). It provides stiffness when the vessel uptakes high tensile loading[9].

Capillaries are thin vessels that are distributed in the tissues and connect arteries and veins. They are monolayer tubes with diameters ranging from 4 to 15 μm but total diameters of capillaries are much larger than those of arteries, which contributes to a lower flow rate [1]. Single endothelial cell forming wall results in a large surface to volume ratio. Low flow rate and large surface area are in favour of sufficient exchange between blood and blood vessel wall. Capillaries are the areas in which blood exchanges nutrition, oxygen, and waste with other organs.

2.1.2 Requirement of artificial blood vessels

An ideal artificial blood vessel should mimic blood vessels biologically and mechanically. Biologically, ABVs should work with native body very well without
any postoperative complications, which means that the ABVs must be biocompatible, non-thrombogenic, and infection resistant [9, 24]. There is no additional immune or tissue response caused by implanting ABVs as well as degradation products [25].

Mechanically, blood vessels have excellent elasticity and tensile strength. Blood vessels are not only the channel for blood delivery but also pump blood with the same pace of heart. They bear high pressure while expand and contract. The average pressure in arteries is 1,000 mmHg [26]. Burst pressure is used to character material’s pressure tolerance. The burst pressure of native saphenous veins is more than 2,000 mmHg [27] while for arteries it is at over 3,000 mm Hg [28].

Compliance means the tendency of a hollow organ to resist recoil toward its original dimensions [29]. Compliance is a factor which can show deformation rate of the material under certain pressure. The compliances of veins are as high as 30 times as those of arteries [30, 31]. The compliance of blood vessels ranges from 5-15%/100 mm Hg [9]. Apart from class and diameter, properties of blood vessels also depend on their components. Elastin, collagen and smooth muscle are main components in blood vessel wall. Mechanical properties of the substances in vessel wall are shown in Table 1. Collagen provides tensile strength while elastin contributes to elasticity [32].

Table 2-1 Mechanical properties of vessel wall components [9]

<table>
<thead>
<tr>
<th>Substances</th>
<th>Yong’s Modulus/MPa</th>
<th>Tensile Strength/MPa</th>
<th>Maximum Extension/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastin</td>
<td>0.3-0.6</td>
<td>0.36-4.4</td>
<td>100-220</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0×10^2-2.9×10^3</td>
<td>5-500</td>
<td>5-50</td>
</tr>
<tr>
<td>Smooth</td>
<td>Relaxed</td>
<td>6.0×10^{-3}</td>
<td>-</td>
</tr>
<tr>
<td>Muscle</td>
<td>Contracted</td>
<td>1.0×10^{-2}-1.27</td>
<td>-</td>
</tr>
</tbody>
</table>
Apart from mechanical properties, biological properties of materials and topology of the scaffold should also be considered. Like ECM, a porous scaffold provides supporting and growing environment to cells in tissue engineering [33]. Several factors should be considered when designing the ABV materials: 1) biocompatible and biodegradable; 2) no inflammation and toxic by-product; 3) cell attachable materials; 4) after the growth of functional tissues, the scaffold should degrade gradually; 5) the pores in the scaffold should be suitable for cellular infiltration and growth of uniform tissue [25, 34, 35].

The topology of scaffold refers to several physical parameters:

1) Fibres’ average diameter and diameter distribution in scaffold;
2) Average porosity and pore-size distribution in fibre mats. The diameter of fibres will determine the strategy used in cell culture.

![Figure 2-1 Different cell culture strategies on different fibres [29]](image)

From Figure 2-1, (A): cells can be attached and grow in the micro-pore; (B): they may also grow along the surface of micro-fibres; (C): they will attach on and be supported by several nano-fibres [32]. The effective pore size for cell growth ranges from 20μm to 60 μm in TEBV [36].
2.1.3 Progress of ABVs scaffold materials

To find a suitable scaffold for ABV, natural materials and biodegradable polymers were investigated. In the early period, collagens were used as scaffold [10, 37]. The first tissue engineered vessel made from collagen was reported by Weinberg and Bell [38]. However, it showed poor mechanical strength. Fibrin was considered as a scaffold material due to its role as sealant in wound healing surgery and potential of producing autologous biomaterial [26]. It alone has poor mechanical properties. However, fibrin-collagen composite showed higher tensile strength and gel compaction [39]. To improve mechanical properties, scaffolds were constructed from biodegradable polymer PGA, PCL, and PLA, and their copolymers [40-42]. However, by-products from degrading these polymers cause inflammatory response and risk of aneurismal [19].

Dacron and expanded polytetrafluoroethylene (ePTFE) are two of synthetic materials, which are most successfully used as vascular graft materials in clinical practices. Dacron fibres were developed from Polyethylene terephthalate (PET) by Dupont in 1950. PET is linear polyester with good stability and mechanical properties which contribute to its excellent durability. First artificial blood vessel made from Dacron got implanted in 1957 by Julian [43]. However, a commercial graft product from Dacron still needs several processing techniques, such as knitting, weaving, velour and crimping. Several biological reactions immediately follow the implantation of the graft. Dacron can satisfy replacement requirement of large diameter (>10mm) blood vessels [19]. Five-year patency rates are around 93% for aortic bifurcation grafts but it fails in bypass surgery [5, 44] .

Poly tetrafluoroethylene (PTFE) also known as Teflon was developed by Dupont in 1937. It was first used as heart valve in medical application. The expanded PTFE (ePTFE) is a porous structured PTFE made by a serial of processes: heating, stretching, and extruding. e-PFTE graft is a non-woven porous tube which is from cracking of stretched PTFE tubes. Some functional modifications and biological improvements are still necessary before the graft is implanted. It can be used successfully in medium diameter (6-10mm) BVs replacement and five-year patency rates range from 91% to 95% [44].
However, both Dacron and PTFE fail in small diameter blood vessel replacement. Compliance mismatch mainly causes failure of synthetic ABVs in smaller diameter blood vessels [5]. The raw materials of Dacron and ePTFE are highly crystalline and rigid. The compliances of Dacron and ePTFE are much lower than those of native blood vessels, which contributes to high risk of thrombogenicity [9]. In large-diameter blood vessels, blood flow fluent is higher, which can avoid thrombogenicity. While in small-diameter vessels, blood flows slowly, which causes calcium deposition, thrombogenicity. Long-term patency of small-diameter ABVs is lower than 40% [9], which causes disappointing results of small-diameter implant. Small diameter blood vessels (<6mm) stand for most part of total blood vessels. Therefore, it is necessary to develop elastic materials for constructing small diameter ABVs.

To sum up, Dacron and ePTFE are two of the successful ABV scaffolds for large blood vessel replacement. However, they failed in smaller artificial blood vessels due to compliance mismatch. Therefore, it is necessary to find an elastic material to construct compliant ABV.

2.2 Electrospun natural rubber for ABV materials

2.2.1 Advantages of electrospun NR as ABVs scaffold

Natural rubber (NR) can be potentially used as compliant artificial blood vessel material due to its excellent elasticity. It possesses excellent elasticity due to its chemical molecular structure and high molecular weight [45]. Chemically, NR (*Hevea*) is mainly composed of poly isoprene, in which cis 1, 4- isoprene units account for 98% [46]. The chemical structure of cis 1, 4- polyisoprene is shown in Figure3. The double bonds in molecular chain contribute to high flexibility of the molecules because the molecular chain can rotate around these double bonds. Furthermore, high molecular weight will also contribute to high elasticity. The number average molecular weight ($M_n$) ranges from $0.25 \times 10^6$ to $2.71 \times 10^6$, while the weight average molecular weight ($M_w$) is between $3.4 \times 10^6$ and $10.17 \times 10^6$ [47]. NR has a glass transition temperature ($T_g$) at -73 °C, which means that it is at high elastic state under room temperature [48, 49].
Natural rubber is cheap, non-toxic, and biodegradable when it is not vulcanized. Vulcanization changes toxicity, solubility, biodegradability of the natural rubber due to the use of toxic substances such as sulphur, zinc oxide and accelerator during the vulcanization process [50, 51]. Naturally, NR is biodegradable and can be degraded by some bacteria in soil. The price of natural rubber is much lower compared with other biological materials or natural materials such as PCL, collagen and elastin. Similar compliance to human skin and soft tissues leads to its role in biomedical applications such as surgery gloves[52], tubes and catheters [53]. Though some issues related to proteins in NR latex restricted its biomedical application in medicine and artificial organs, it still draws great interests of biomaterials scientists due to its potential applications in vascular permeability, angiogenesis and wound healing [20].

Scaffold fabricated from electrospun fibres performs better cell attachment than film scaffold. Electrospun nano-fibres or micro-fibres can be randomly aligned into mats or from an aligned nano-fibre mat. Chua et al. [41] compared cell proliferation on nano-fibre mats and film. It has been shown that scaffolds made from these fibres can reflect favourable cellular responses. Figure 2-3 shows the difference of cell attachment on film (a, b, c) and on electrospun fibres (d, e, f). It is obvious that cells connect better on electrospun fibre mats than on film.

**2.2.2 Biological issues**

Natural rubber has been widely used in medical products, such as surgical gloves [52], tubes and catheters [53], balloon and male condoms [54]. However, natural rubber will cause three distinctive types of adverse reactions: irritation, immediate hypersensitivity (type I allergy) and delayed hypersensitivity (type IV allergy). Irritation refers to non-immunologic response. Type I allergy is caused by proteins in NRL, which will result in an immediate antibody-mediated reaction minutes after exposure. Type IV allergy is an immunological reaction caused by cell mediation, which leads to contact dermatitis.
in 1 to 4 days after exposure [55]. Type IV allergy is usually due to chemical residue from the process of making NR products such as chemical additives involved in NR processing including additives for NR vulcanization, anti-ageing and reinforcement. The rate of chemical sensitivity from NR product exposure is less than 1.5%. De-proteinized natural rubber (DPNR) could be potentially used due to its low risk of allergy [56-58]. DPNR, a derivative product of natural rubber from removing most of proteins in natural rubber latex by centrifuge, is thought to be a good strategy to avoid allergy risk of natural rubber product.

Figure 2-3 Comparison of cell proliferation on electrospun film and fibre mats [59]: (a-c) hepatocytes cultured on Gal-film formed rounded spheroids; (d-f) hepatocytes cultured on Gal-nano-mesh.

Vulcanization changes toxicity, solubility, biodegradability of the natural rubber [60]. Though vulcanization can enhance mechanical properties of the natural rubber, toxic substances are involved during vulcanization process such as: sulphur, zinc oxide and accelerator. Thiram, carbamate and thiazole are commonly used as vulcanization accelerator. However, they cause type IV allergy. Therefore, quantity of these additives in NR products for medical devices is limited. Electrospinning of NR involves using organic solvents, which are toxic to human body. However, most of the solvents are highly volatile and the residue is quite low due to large surface area and tiny fibre diameter. It will not cause cytotoxicity due to chemical residue. This doesn’t mean that NR fibres would be biocompatible. The proteins in NR materials are still in NR fibres, which would cause type I allergy. NR latex contains a large number of water-soluble
proteins, which are easily absorbed through skin and cause allergic response. Even DPNR with very low protein content will potentially contact with human skin or tissue due to quite large surface area of electrospun fibres.

In summary, electrospun natural rubber will not cause type IV allergy, which relates to chemical residues. However, it may cause type I allergy due to protein residues, which could be decreased by electrospinning of DPNR.

2.2.3 Blood compatibility of NR

Blood biocompatibility depends on physicochemical features of the material surface [61-66]. Natural rubber is highly hydrophobic, which would cause issue to protein adsorption and platelet adhesion. Porous structure of electrospun NR fibres would contribute to modify hydrophilicity of NR materials by the feature structure. The contact angle result of NR materials and electrospun NR fibres would support this. Surface modification of NR materials with hydrophobic materials are the mean methods to improve blood compatibility. Chemical grafting copolymerization and UV induced grafted coating with hydrophobic polymers or monomer would be the main methods to improve NR materials blood compatibility.

Surface grafting modification is the mostly used strategy for improving blood compatibility of NR materials. The first attempt was reported by Razzak et al. [64]. N, N-dimethyl acrylamide (DMAA) and N,N-dimethyl amino ethyl acrylate were polymerized onto a NR tube under radiation. The irradiation time, grafting temperature, and monomer concentration were the factors that influenced grafting yield. The blood compatibility was determined by observe the blood clotting in the modified NR tube. Higher grafting yield contributed to a better blood compatibility. When the grafting rate was beyond 30%, the clotting disappeared. The blood flow through the NR-g-DMAA tube can last for 60 min instead of 40 min for un-grafted tubes. Argon plasma treatment followed by UV radiation grafting co-polymerized with poly(ethylene glycol) methacrylate (PEGMA) was designed by Kang et al. [67]. Protein absorption and platelet adhesion of NR tubes were improved with a higher grafting rate of PEGMA. Another strategy is to graft MPC and VPy on NR latex films in the presence of initiator. This approach can be simply conducted in aqueous solution. The blood compatibility
testing results showed that surface modified NR films contributed protein adsorption and platelet adhesion without worsening mechanical properties [67].

On the other hand, large surface area provides much better chance for preparing biocompatible NR based materials with core-shell structure biocompatible coating. Efforts have been made on surface modification of NR tube, film products [61, 66, 67]. Compared with film and tube NR-product, electrospun fibres, with porous structure and microscale diameter, show more advantages over flat 2D surface area. Also, the porous structure and thinner diameter contribute to super softness. Therefore, electrospun NR fibres provide possibility to prepare biocompatible materials. In summary, blood compatibility issue of NR materials, which relates to materials physicochemical feature, would be improved by porous and fibrous structure and surface modification.

2.2.4 Progress of electrospinning cis poly isoprene

Electrospinning of natural rubber was first mentioned by Stihornkul [68] in their primary work of electrospinning NR/ABS blends in THF, in which only contents of ABS and distance between collector and needles effecting morphology of electrospun fibres were investigated [68]. Hao et al. did some primary investigation of electrospinning cis PI, which can be electrospun in chloroform and dichloromethane (CH2Cl2) and bamboo like fibres in CH2Cl2 could be got. Mixtures of natural rubber latex (NRL) and PCL solutions were electrospun for biological devices by Costa et al [69]. However, the stability of the electrospinning process was ignored in the previous work. The stability of the process refers to not only the control of morphology of electrospun fibres but also the continuous producing process, which is related to the industrial application.

In summary, attempts to electrospinning of NR or NR-like polymers have been done, but stability control of electrospinning NR process is lacking.
Table 2-2 Progress in electrospinning natural rubber / cis poly isoprene

<table>
<thead>
<tr>
<th>Material system</th>
<th>Conditions</th>
<th>Fibre diameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NR/ABS and NR/CB in THF</td>
<td>Flow rate: 30 ml/h, Voltage: 15 kV, Distance: 20 cm, Rotating circular plate at 1000 rpm.</td>
<td>20.90 ± 7.71 μm to 14.40 ± 5.13 μm</td>
<td>[70]</td>
</tr>
<tr>
<td>2 Liquid ENR and Liquid NR + PVC (70: 30) in THF</td>
<td>Voltage : 16 kv, Distance : 10 cm, 16% wt concentration</td>
<td>5-9 μm to 1-3 μm</td>
<td>[71]</td>
</tr>
<tr>
<td>3 8 % (w/v) NR/toluene +8 % (w/v) PCL/ chloroform</td>
<td>Voltage: 20 kv, Distance: 12 cm, Fixed grounded collector</td>
<td>210 nm</td>
<td>[69]</td>
</tr>
<tr>
<td>4 NR/ABS with vulcanization system dissolving in THF</td>
<td>15 kv , 30 ml/hr, 10-25 cm</td>
<td>10-30 μm</td>
<td>[68]</td>
</tr>
<tr>
<td>5 Poly cis isoprene in chloroform and dichloromethane</td>
<td>11kv and 15kv, Rotating collector 1–6 wt. %, 10-15 cm</td>
<td>20-60μm</td>
<td>[72]</td>
</tr>
</tbody>
</table>
2.3 Instability of electrospinning

Electrospinning [73] process can be divided into several stages: jet launching, jet thinning, whipping instability and solidification to fibre [7]. The distribution of electrical field among the needle tip and collector is shown in Figure 2-4(a). Normally, the instability will happen at the jet breakup point, where the straight polymer jet became bending/buckling. Also, the fibre will be branched or thinned. The polymer solution jet will be solidified with the evaporation of solvent.

![Figure 2-4 Distribution of electric line in front of the collector (a) and whipping mode of polymer jet in electrical field (b)](image)

2.3.1 Instability types

The electrospinning processing parameters and solution properties have direct influence on the stability. The whipping would occur when the electrical strength is higher than 2kv/cm. When the electrical strength ranges from 1 to 5 KV/cm, the whipping model is in the domination state [74]. Spivak and Dzenis [75] endowed a parabolic shape for the tip of cone and found a critical value, at which when the radius was less than and caused stable jet. The critical value can be calculated from the surface tension, dielectric permeability, and charge density of the jet [73, 76]. The whipping instability starts from the end of straight polymer jets during the stretching process (Figure 2-4(b)). It is nonaxisymmetric instability with the jet moving in uncontrolled and hardly quantifiable ways [77]. Bending instability of PEO solution with high molecular weight has been reported by Reneker [78] from observation to theory. The
whipping stability refers to the elastic modulus of polymer solution. The rheological properties of polymer solution can be used to investigate the creep, compliance or stress relaxation of the NR solution.

The fluid properties such as conductivity, surface tension, viscosity influenced the stability. The relationship between conductivity and whipping stability has been investigated by Hayati et al. in 1987 [79]. High surface charge density contributed to rapid whipping [76]. Changing geometry of electrical field has been used to control the non-linear whipping instability. Disc and ring electrodes instead of flat aluminium sheet have been used to improve the uniformity of electric field. However, the influence of the electrical strength difference in the different place can be neglected [80].

The whipping instability is the specific feature of electrospinning, which is not in the electro-spraying process. The formation of the whipping instability is from the force difference on the fibres, which is continuous and whose motion is not free. The moving of the point on the fibre jet is limited by the fibre shape. The ratio of fluid relaxation time to instability growth time is called Deborah number which can show the Raleigh instability [76].

\[
De = \frac{\lambda \nu_0}{R_0}
\]

where \( \lambda \) is relaxation time, \( \nu_0 \) is the initial velocity, \( R_0 \) original jet radius. For the electrospinning process, \( \lambda \) relates to the rheological properties of solution while the \( \nu_0 \) is decided by the feeding rate and diameter of nozzle.

Bending stability starts from certain distance away from droplet tip. They bend due to the Coulombic interactions discussed above in relation with the electric bending force [81]. Bending instability has been investigated by Reneker et al. [78, 82] and Shin et al [83], who proposed their own theory for instability. A linear Maxwell equation was built by Reneker et al [82] to stimulate polymer jet. Hohman et al generated an instability theory to predict a nonaxisymmetric instability [80]. The rheology of the polymer jet has been demonstrated in terms of Newtonian viscosity, a power-law viscosity, the linear Maxwell equation.
Buckling happens when the high viscosity jet flows slowly. Solutions with a high viscosity would cause the buckling if the flow speed is not high enough. The stability can be estimated from Re number and stable condition reported refers to fluid conditions with Re at higher than 1.2. When the Re number decreases, buckling increase, which means that the instability increase. Jet length L should be shorter than a certain value. If the jet length is larger than certain value, buckling will happen. They bend due to the Coulombic interactions discussed above in relation with the electric bending force. Polymer jet relaxation can be a bridge to combine the rheological properties and instability. Four different models of polymer jet instability were given in Figure 2-5 [84]:

![Polymer jet morphologies during electrospinning: a) steady model; b) rotating model; c) swing model; d) blurring](image)

**Figure 2-5** Polymer jet morphologies during electrospinning: a) steady model; b) rotating model; c) swing model; d) blurring [66].

### 2.3.2 Strategy for steady control

The stability control of electrospinning could be improved by controlling the following aspects: homogenous solutions, steady feeding flow and electrical force. The stability of electrospinning process refers to the stability of feeding, Taylor cone, stretching process and collecting pattern. Feeding pattern of electrospinning is of low speed (less than 0.001 m/s) and the flow behaviour is usually laminar flow in the narrow cylinder needle. The influence of feeding rate on instability is mainly on the Taylor cone [73]. The feeding rate should be higher than a minimum value for producing jet or drop at certain conductivity.
Stability of structured Taylor cone strongly depends on the physical properties and flow behaviour of the fluids and applied voltage [85]. When the voltage surpassed the critical value and the jet will be pulled out from the tip of the Taylor cone. The Taylor cone shape change with voltage has been reported in [86]. Reneker et al described the process of electrospinning with the increasing of electrical strength [87]. They also pointed that the static Taylor cone angle is 49.3° when the critical voltage was reached [80]. The stability of Taylor cone was discussed in theory [73] and a minimum feed rate was required for steady structure of Taylor cone. Stability of structured Taylor cone strongly depends on the physical properties and flow behaviour of the fluids and applied voltage [85].

Reneker et al developed a theory to describe the phenomenon: a linear charged jet tends to be separated to decrease the system energy [88]. This can be proved from the decrease of fibre diameter with increase of charge density. Helicoidal path is due to the bending of the jet. However, self-sticky properties and insulativity of the natural rubber will prevent the jet separation. Therefore, the diameter of NR fibres is larger, which is usually around 2-5 μm. Normally, the instability will happen at the jet breakup point, where the straight polymer jet became bending/buckling. Also, the fibre will be branched or thinned. The polymer solution jet will be solidified with the evaporation of the solvent.

A theory has been developed to discuss the entanglement in the polymer solution that influences the morphology of the electrospun fibres. Polymer chains are random coils in the solution as shown in Figure 2-6. If the concentration is too low (c<cf), entanglement in the solution is not big enough that can’t provide sufficient viscosity for electrospinning which will contribute break-up of fibre or bead. If the concentration is too high ([η]c> 10), there will be too much entanglement in molecular chain which cause difficulty in forming fibres. Therefore, a range of concentration of electrospinning can be got from the morphology of fibres.
2.3.3 Challenges of electrospinning NR

The first challenge lies in poor tensile strength of unvulcanised NR [49, 89]. Without vulcanization, tensile strength of NR cannot reach requirement of ABV. Radiation vulcanization and blending with polymers with good tensile strength would be good strategies to overcome this challenge. Y-ray and UV radiation vulcanizations have been reported and there were no toxic substances involving in these processes. Therefore, post radiation of as-electrospun fibres would be a good strategy. Another strategy is to blend NR with polymers of high tensile strength. Natural polymers like collagen, silk and synthetic polyesters such as: PLA, PGA, PCL, can be blended with NR. However, NR is an elastomer with high hydrophobic properties would have difficulty in blending with these polymers.

Secondly, high viscoelasticity influenced stability of the electrospinning process. Viscoelasticity plays three roles in the process of electrospinning: onset delay of disturbance growth, faster disturbance growth, and retardation of final jet break up [90]. The high viscoelasticity of NR solution due to high molecular weight, flexibility of NR molecules and solution concentration influence the stability of electrospinning process in the steps of feeding and stretching process. The evaporation of solvent increases the concentration and solidification of fibres from the jet. The rheological properties and surface tension change during the evaporation of solvent. The stretching process involved evaporation of solvent and the surface areas increase with the thinning of the fibres, which contributes to evaporation of solvent. The balance of force was also broken with the extension of fibre jets, which would lead to an increase of fibre stress.
If the fibres were collected on the collector before the contraction of fibres with the disappearance of Columba force and increase of molecular relaxation, straight fibres would be collected. With an increase of distance, the relaxation of the fibres due to high viscoelasticity would be larger than the faded Columba force due to the solidification of fibre jet and curl fibres would be collected. On the other hand, hollow collector will provide an additional electrical field, which would be much stronger than that existed between the needle and the collector. The fibres would be stretched under the strong electrical field and were straight and well aligned. Hong et al. [91] reported aligned fibres collected among the void window in a metal collector. However, the mechanism of formation of this type of aligned fibres was not clearly described.

Thirdly, the inhomogeneity of NR solution resulted from gels will also contribute to instability of electrospinning process and the formation of non-uniform fibres. Gels are defined as particles that formed from large polymer molecular chains with chemical connection and physical knot, which show quite different features than linear polymers [92]. Gels are mainly from chemical crosslinking, interpenetrating polymer networks (IPN), and physical crosslinking and entanglement. Gels structure and component in natural rubber have been investigated for long time, especially after demonstrating the correlation of gels and excellent mechanical properties of NR materials [46, 93-97]. It was reported that the gel phase is composed of micro-gels, which will aggregate to an apparent gel phase [93]. Impurity in NR contributes to physical crosslinking of NR molecules. Chemical crosslinking in NR can date back to the synthesis process and increases with storage. The complex component of natural rubber causes the multi-phase in natural rubber solution [98].

2.4 Core-shell fibres

2.4.1 Methods comparison

There are three main strategies to prepare core-shell micro-fibres: co-axial electrospinning, emulsion electrospinning and direct coating, which were described in Figure 2-7. Co-axial electrospinning is considered as a promising way to prepare core-shell structure fibres, which has attracted more and more attentions from researchers [41, 99, 100]. The setup of a co-axial electrospinning process is similar to a typical
electrospinning process except a special solution feeding needle which has core and shell two layers. It is composed of two syringes with different solutions (core-solution and shell-solution), which are separately pumped to two co-axial needles with different diameters. High voltage is added between needles and collector. With effect of electronic field, mixture of solutions will be electrospun together and form a co-axial cone, which leads to fibres with core-shell structure. Core-shell fibres from co-axial electrospinning of PCL and collagen have been prepared by He et al [20, 101], which can also be prepared by surface modification of electrospun fibres in collagen solutions.

![Diagram of electrospinning process](image)

Figure 2-7 Methods of preparing co-axial micro-fibres: a) co-axial electrospinning [99], b) emulsion electrospinning [102], c) coating directly [20].

Emulsion electrospinning is also considered as a simple way to prepare core-shell fibres from emulsion of polymers [102-105]. The process of emulsion electrospinning is similar to the normal solution electrospinning. The dispersed phase in the emulsion is stretched into the core of the electrospun fibres, and the continuous phase forms the shell. Xu et al. [45] reported a water-soluble polymer and an amphiphilic polymer: poly (ethylene glycol)-poly (L-lactic acid) (PEG-PLA) di-block copolymer were generally incorporated into the W/O emulsion with an emulsifying agent as a surfactant to decrease the surface tension [46].

Coating or deposition of shell material onto the electrospun nano/micro-fibres was first used to prepare core-shell fibres [103]. He et al. [20] coated plasma treated electrospun PCL with collagen solution to get core-shell structure fibres and investigated its potential application in growing endothelial cells. On the other hand, with a reverse way, carbon nanotube can be filled with suspension to get core-shell structure fibres [106].
The methods listed above all have their advantages and drawbacks. Both co-axial electrospinning and emulsion electrospinning can get core-shell fibres in one stage, while direct coating method needs two stages. Co-axial electrospinning is simple but difficult to get fibres with good concentricity. Emulsion stability will influence fibre structure of emulsion electrospinning. The difficulty of getting uniform shell layer from coating methods lies in random structure of electrospun fibres. Here, in this project, we will focus on co-axial electrospinning process.

Though core-shell structure fibre can be constructed, the interface of core-shell structure fibres will influence properties of co-electrospun fibres. Strong interfacial interaction contributes to good interfacial adhesion and excellent bulk properties of core-shell structure fibres. Therefore, investigating and improving interfacial interaction between core and shell phases would contribute to enhancement and stability of bulk properties.

2.4.2 Challenges in co-axial electrospinning

Intact core-shell structure fibres are hard to get from co-axial electrospinning due to challenges in relative properties of core and shell fluids and their cooperation with processing parameters. Relative length of inner needle diameter was considered as a factor that influenced the structure of core-shell fibres. Yu et al. [107] reported a modified co-axial electrospinning by using a shorter inner needle and demonstrated that shorter inner needle led to formation of envelop, which was in favour of core-shell structure Taylor cone. The result of Lee et al. [108] confirmed the shorter inner needle’s effect on formation of core-shell structure fibres.

To sum up, coating, co-axial electrospinning and emulsion electrospinning are three of main methods to prepare core-shell fibres. However, the interface between core and shell phases still needs to be investigated.
2.5 Enhancement of interface in polymer blend

2.5.1 Immiscible polymer interfaces

To develop a novel material that can combine excellent properties of different polymers, polymer blending is a convenient way. The polymers can be combined in several ways: mechanical blending, solution blending and melt mixture [109]. Polymer blending is a low cost and time-saving method compared with polymerizing a new polymer. As a biosynthesis polymer, natural rubber possesses some excellent properties but also has some drawbacks in other properties. However, immiscibility of polymers usually influences mechanical properties of the blended material though blending seems attractive. Blending of incompatible polymers will cause phase separation at interface. The blending properties are related to the Flory–Huggins parameter ($\chi$) via the interfacial thickness. The Gibbs mixing energy of polymer blends can be described by a lattice model. The entropic part is related to center motion of polymer chain and is also known as translational entropy. It contributes to mixing. However, it is usually very small due to high molecular weight of polymers. This part can be either positive or negative depending on compatibility of the two polymers. The interaction parameter of most polymers is usually very large, which leads to a positive Gibbs mixing energy with macro phase separation.

Table 2-3 Interface width of different type of polymer blend [71]

<table>
<thead>
<tr>
<th>Type of blend</th>
<th>Interface width(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immiscible</td>
<td>2</td>
</tr>
<tr>
<td>Block copolymer</td>
<td>4 to 6</td>
</tr>
<tr>
<td>Polymer/copolymer</td>
<td>30</td>
</tr>
<tr>
<td>Reactive compatibilization</td>
<td>30 to 60</td>
</tr>
</tbody>
</table>

The immiscibility of polymer blends can be characterized by interface width. The theory to predict the degree of interfacial mixing of polymer blends has been provided by Helfand [110]. Interface thickness is usually used to evaluate miscibility of polymer
blends [111]. It refers to the intercept of the steepest tangential with the horizontal lines. According to experimental result of Helfand and Tagami, the thickness of polymer interfaces ranged from 2-6nm [112]. Interface width of polymer blend with different compatibility was list in Table 2-3.

2.5.2 Compatibilization by addition

A variety of strategies have been developed to improve the immiscible polymer interfaces, which are also known as compatibilization. They can be divided into two types: compatibilization by addition of a third component and reactive compatibilization [113, 114]. Addition of a copolymer is an effective compatibilization of binary blends. The strategies with the aim of arranging some polymeric additive at the interfaces are commonly used. Copolymers such as di-block copolymers, triblock, comb, graft or random copolymers can all be used. The polymer chains of the additive can cross the interface more than once (as shown in Figure 2-8). For instance, the block copolymer has both repeat units of the incompatible polymers. It can appear on the interface and hold both sides by Van der Waals force [114].

![Figure 2-8 Molecular morphologies of added copolymer at interface [73]](image)

In addition, a third substance which can attract both of the components is also applied. The substance which is (partially) miscible with both of the blending polymers can also improve interfacial action between two incompatible polymers [15]. These substances can act as surfactant, which can adsorb at interface and reduce the interfacial tension. The effect of additive can be investigated by the same method as homo-polymer especially neutron reflection.
2.5.3 Reactive compatibilization

Reactive compatibilization is to integrate two different phases at interface by chemical methods. To increase compatibility of blends, reactive compatibilization is to form strong interaction at interface by chemical reaction \([18, 114, 115]\). It consists of chemical reactions between the two components by introducing either a reactive component or a catalyst.

![Figure 2-9 Reactive compatibilization during blending process [73]]

Grafting reaction taking place \textit{in situ} at the interface has been designed to improve immiscibility of polymer blending. Crosslinking has been commonly used to bond the two polymers \([109, 116]\). However, the complication of chemical reaction and selection of suitable reaction lead to the difficulties of wide application. Coupling reaction is another efficient method to enhance interfacial adhesion between immiscible polymers \([117]\). Furthermore, a compatibilizer with a low molecular weight is usually used in a typical polymer blending process.

To sum up, polymer blends can combine excellent properties of different types of polymers. However, immiscibility among blend phases causes poor interfacial adhesion which will influence bulk properties of polymer blends. Reactive compatibilization and
a third component are commonly used to modify the immiscibility of polymer interfaces.

2.5.4 Crosslinking of PVP

Polyvinyl pyrrolidone (PVP) is a water-soluble polymer, which is easily dissolved in water and other polar solvents. The chemical structure of PVP was shown in Figure 2-10. It is also a polymer surfactant, whose polarity depends on its molecular weight and distribution of molecular weight.

![Figure 2-10 Chemical structure of Poly vinyl pyrrolidone (PVP)](image)

PVP was designed to play a protective role in preventing natural rubber from contacting to the tissues. However, it will be taken away easily by the water or polar solvents in the human body and will lose its function. To protect PVP from flooding in the water, cross-linked PVP can take the place of PVP after electrospinning. Cross-linked PVP is polar but it is insoluble in water. In addition, it is biocompatible, therefore, is widely used in pharmacy. Cross-linked PVP, polyvinyl poly-pyrrolidone (PVPP), has been used as a disintegrant in tablets. Chiara et al. reported a thermal method to prepare cross-linked PVP [21] and a radical mechanism of crosslinking was given in Figure 2-11. PVP can be completely water-insoluble after the PVP film was annealed at 200 °C for less than 1 hour. K2S2O8 was used as initiator for UV crosslinking of PVP solution [23]. The chemical reaction theories of PVP aqueous solution were discussed: polymerization, oxidation, degradation and ring-opening reaction. Ring-opening reaction can only happen at high or low pH value solution. Calculation of crosslinking degree of PVP solution after UV radiation was reported by Lopérgolo [118], in which several methods and calculation methods of crosslinking were presented. UV-irradiation (λ=254 nm) could initiate crosslinking [22]. Gel content was calculated from the weight change before and after irradiation. Hydrogels prepared by this method do
not impose any toxic effect to live organisms. All samples presented inflammation indexes within a satisfactory range, i.e. as a non-irritating material. Hydrogen peroxide and photo-Fenton reactions were used to accelerate the crosslinking of PVP aqueous solution under UV radiation affection.

![Diagram of PNVP radical formation scheme](image.png)

Figure 2-11 PNVP radical formation scheme proposed, similar to that for a chemically activated process [21]

UV-irradiation is a good sterilization method of polymeric biomedical materials. However, it is necessary to test the surface properties of the blends for their susceptibility to changes generated by the exposure to UV radiation. During UV-irradiation of polymers, the excited molecules are formed in the first step, and then the secondary processes such as chain scission, crosslinking, and oxidation take place.

In summary, PVP needs crosslinking to increase its water stability and UV (254 nm) radiation would cause crosslinking with lower by product.
3 Materials and methodology

3.1 Introduction

This chapter focuses on the materials, preparation and characterization methods used in this project. Firstly, all the chemical reagents and raw materials were specified. Then, electrospinning and co-axial electrospinning technologies were described, including preparation of electrospinnable solutions and process parameter control for steady electrospinning process. In addition, cell culture technology of smooth muscle cells was illustrated together with cytotoxicity testing methods and cell viability characterization methods. Finally, characterization methods of polymer solutions, electrospun fibres and scaffolds materials with cells were listed.

3.2 Materials

Natural rubber, STR-5L, dry solid natural rubber was supplied by Agriculture Products Processing Research Institute of Chinese Academy of Tropical Agriculture Sciences. Solid dry natural rubber is mixed in a two-roll mixer at 60 rpm for 15 minutes before use. 0.25g natural rubber was cut into small pieces and then dissolved in 10mL solvent to prepare electrospinning solutions. Tetrahydrofuran (THF) is bought from Chem-supply Pty Ltd. and stabilized with 2, 6-di-tert-butyl-4-methylphenol (BHT). Toluene, AR, is bought from Sigma Aldrich and used as solvent directly. Chloroform and dichloromethane were bought from Fisher Chemical and used as solvent of natural rubber directly. Fluorescein 5(6)-isothiocyanate (FITC) powder, ≥90% (HPLC) was bought from Sigma Aldrich, which was dissolved in alcohol solution for dyeing of PVP solution. Aluminium foil, GLAD®, 50m was cut into 20cm×50cm and attached on a metal frame for the collection of electrospun fibres. Trypan Blue Solution, 0.4%, is used as a cell stain to assess cell viability through the dye exclusion test. It is often performed while counting cells with the hemocytometer any time cell viability needs to be determined quickly and accurately. The dye exclusion test is based upon the concept that viable cells do not take up impermeable dyes (like Trypan Blue), but dead cells are permeable and take up the dye.
3.3 Methods

3.3.1 Electrospinning

1) Selection of solvent/ solvent system

Several factors should be considered for selecting suitable solvents for electrospinning natural rubber. First of all, solubility should be big enough to provide sufficient concentration and viscosity. Luo et al. [119] selected solvent for electrospinning PMSQ by mapping the solvents in Teas graphs according to the solubility parameters and found an areas of solvents/solvent system for electrospinning according to the electrospinnability. Solubility parameters of natural rubber are \((\partial_d, \partial_p, \partial_h, R)\) = (20.8, 1.8, 3.6, 14) [120, 121]. Then a circle area stands for solubility area of NR can be found in Teas graphs (Figure 3-1) with center locating at point (0.794, 0.069, 0.137).

![Figure 3-1 Teas graphs of different solvents with NR](image-url)
Several commonly reported solvents for natural rubber are tetrahydrofuran (THF), dichloromethane, toluene and chloroform [68, 72]. Some of physical properties relate to electrospinning process were listed in Table 3-1.

Table 3-1 Physical properties of potential solvents for electrospinning NR

<table>
<thead>
<tr>
<th>Solvent</th>
<th>THF</th>
<th>Dichloromethane</th>
<th>Chloroform</th>
<th>Toluene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>( \text{C}_4\text{H}_8\text{O} )</td>
<td>( \text{CCl}_2\text{H}_2 )</td>
<td>( \text{CCl}_3\text{H} )</td>
<td>( \text{C}_7\text{H}_8 )</td>
</tr>
<tr>
<td>Density/(\text{g/cm}^3)</td>
<td>0.8892</td>
<td>1.33</td>
<td>1.483</td>
<td>0.867</td>
</tr>
<tr>
<td>Flash point/(^\circ\text{C})</td>
<td>-14</td>
<td>None</td>
<td>None</td>
<td>4.44</td>
</tr>
<tr>
<td>Boiling point/(^\circ\text{C})</td>
<td>66</td>
<td>39.6</td>
<td>61.2</td>
<td>111</td>
</tr>
<tr>
<td>Toxicity</td>
<td>Relatively nontoxic</td>
<td>Inhalation hazard, carcinogenic</td>
<td>Carcinogenicity</td>
<td>Toxic</td>
</tr>
<tr>
<td>Solubility of NR</td>
<td>Gel</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Viscosity ((20^\circ\text{C}))/mPa.s</td>
<td>0.48</td>
<td>0.43</td>
<td>0.57</td>
<td>0.59</td>
</tr>
<tr>
<td>Surface tension/mN/m</td>
<td>23.97</td>
<td>28.12</td>
<td>26.67</td>
<td>27.93</td>
</tr>
<tr>
<td>Vapor pressure (20°C)/h-Pa</td>
<td>190.7</td>
<td>47</td>
<td>213.3</td>
<td>38 (25°C)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------</td>
<td>----</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>Flammability</td>
<td>Flammable</td>
<td>Non-flammable</td>
<td>Non-flammable</td>
<td>Highly flammable</td>
</tr>
<tr>
<td>Polarity</td>
<td>4.2</td>
<td>3.4</td>
<td>4.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Viscosity /cp</td>
<td>0.46</td>
<td>0.41</td>
<td>0.54</td>
<td>0.56</td>
</tr>
</tbody>
</table>

2) Preparation of NR solutions

Solid dry natural rubber is masticated in a two-roll mixer at 60 rpm for 15 minutes before dissolving. Solid natural rubber was cut into small pieces followed by dissolving in solvent to prepare electrospinning solutions. The dissolution process takes several days. 10% sodium chloride aqueous solution was added to the solution before electrospinning to increase conductivity. Gels were removed before being used for electrospinning.

3) Electrospinning process

Electrospinning of natural rubber was carried out in a closure protecting box, which was connected to the fume hood through a vent pipe. The setup of electrospinning is composed of a high voltage supplier (Gamma high voltage research ING, voltage range: 0-50kv), solution feeding system including a syringe pump (Walker), glass syringe and grounded aluminum sheet collector. A syringe pump was used to feed solution at a steady speed. The positive electrode of high voltage supply was clamped on the needle of syringe. The negative electrode was added on an aluminum sheet which was used as the collector for collecting electrospun fibres. Influence of concentration on morphology of electrospun fibres was investigated to find the range of concentration for steady electrospinning in which bead-free fibres were collected. The conductivity
density of the solutions can be adjusted by NaCl added aqueous solution.

4) Collection pattern

Electrospun fibres were collected on flat aluminium foil traditionally. In this project, fibres were collected on aluminium foil, water bath and hollow window in aluminium foil collector. Parallel aligned fibres were collected among hollow collectors with different shapes: triangle, circle, square and rectangle. The morphology of electrospun fibres can be controlled [74, 107, 122-124]. To investigate the influence of the shape of the hole on the alignment of the fibres, holes with different shapes were used. 25.0g/L NR/THF was used as the electrospinning solution. Electrospinning conditions were as follows: feed rate, 1.0 mL/h; diameter of needle, 0.7 mm; voltage, 10.0 KV; distance between tip and collector, 20cm. Aluminium foils with hollow window of different shapes: rectangle (4.6cm×1.6cm), triangle (edge length:4.6cm), round shape (diameter: 4.6cm) were used as collectors.

3.3.2 Solution homogeneity enhancement

1) Mastication to decrease molecular weight [125, 126]

The high molecular weight of natural rubber not only influences the solubility of NR but also contributes to high content of gel, high viscoelasticity, and thick fibres [127]. In industry, mastication is an efficient way to break long molecular chain of natural rubber during mixing natural rubber with other additives. There are two types of mastication methods: two-roll miller and internal mixer, in which the long molecular chain can be broken down by the mechanical force under temperature effects. After mastication, the average molecular weight decreased while molecular weight distribution became wider [126, 128]. The NR samples were masticated in this project for 10 minutes on two-roll mixer at room temperature to avoid thermo-oxidative effects [126] before being used for electrospinning.
2) Filtration of solution

![Image](image-url)

**Figure 3-2 Filtration of gel in NR solution by glass syringe with metal mesh**

Filtering gel from NR solutions will increase solution homogeneity and stability of electrospinning process. Gel in NR solution will cause instability of structured Taylor cone. Even worse, the larger gel particle will influence the fluent feeding of the solution and lead to clogging of the electrospinning nozzle. Therefore, it would be necessary to remove the gel before electrospinning natural rubber. However, selecting suitable purification method is hard for NR solution in THF due to the corruption of solvent to the plastic materials and pollution of evaporated solvent to the environment. The filter paper or membrane cannot be used for removing gel in natural rubber solution because high viscosity of solution and easy evaporation of solvent. A metal net (as shown in Figure 3-2, stainless steel woven wire, 400 meshes) can be used to filter the gel from NR solution. The gels will be filtered during extraction, which could decrease concentration change due to evaporation of solvent.

3) Centrifuge

Centrifuge was carried out in an enclosed environment for a shorter time which made that quicker than filtration and less concentration change. NR solution centrifuged at different speed can get different contents of gel. 1 mL 25g/L NR/THF solutions were centrifuged at 1krpm, 5krpm and 10 krpm for ten minutes, respectively.

3.3.3 Instability analysis

The instability of electrospinning process relates to not only polymer solution properties but also the processing parameters. Several factors were selected for
controlling the stability of electrospinning processing of natural rubber. For NR material, the molecular weight and component have been decided. In a certain solvent, the properties of solution such as viscosity, conductivity and surface tension all depend mainly on the concentration (C). The processing parameters that are used for controlling electrospinning process (depicted in Figure 3-3) include voltage (V), feeding rate (Q), distance between needle tip to collector (h), and needle diameter (R).

![Diagram of electrospinning process](image)

Figure 3-3 Processing parameters of electrospinning process

Critical voltage can be estimated from the following experimental formula [76]:

\[
V_c^2 = (2L - h)^2 \left( \ln \frac{2h}{R} - 1.5 \right) (0.117 \pi RT)
\]

L: length of needle,

R: diameter of needle,

T: temperature, and

h: distance between needle tip and collector.
These factors have been investigated separately by different researchers. Hayati et al. [79] investigated the influence of factors including surface tension, electrical conductivity of solution, and feeding rate on instability. The electrospinning process can be investigated experimentally and theoretically. The experiment is based on the observation and experimental approach while the theoretical study is based on the basic physical theories. In this project steady electrospinning conditions will be investigated firstly based on experimental observation and the results are then compared with the theoretical predictions. The processing parameters such as critical voltage for electrospinning [79], entanglement concentration [129, 130], fibre diameter [131, 132], and Q-U regime for stable electrospinning [84] can be experimental studied and the theories of electrospinning process in the view of conservation [133] and stability of Taylor cone [73] will be considered to in this work.

The change in Taylor cone shape with voltage was observed and the characteristic length was recorded (Figure 3-4). The critical voltage for electrospinning (V_c) and voltage that started causing the rotation of bending jet (V_r) were recorded at different feeding rate to find a suitable voltage range. Polymer solution properties, needle diameter and distance between needle tip and collector were kept constant during this process.

Figure 3-4 Taylor cone shape changes with voltage at feeding rate of 1mL/h, needle diameter of 0.7mm, distance between nozzle tip to collector of 25cm.

### 3.3.4 Co-axial electrospinning

A two-fluid syringe needle (Figure 3-6) with core-shell structure which can separate core fluid and shell fluid is used to feed polymer solutions during co-axial
Electrospinning process. 5 mL syringe is filled with collagen solution and is fed to the shell layer by a syringe pump at certain speed. 5 mL glass syringe with NR solution is fed to core layer of the needle, which is connected to a high voltage supply. A grounded aluminium sheet was placed at 20cm from the needle tip for the collection of fibres. NR solution is fed to outer shell of co-axial electrospinning needle while collagen solution is pumped to inner channel.

Figure 3-5 Set-up and special nozzle for co-axial electrospinning

To get core-shell structured fibres, solution properties and processing parameters require to be optimised. Ratio of core and shell fluids, concentration of core and shell solutions, and inner needle diameter can be modified to achieve steady co-axial electrospinning. The relative feeding rate of core and shell fluids can be modified by using syringes with different diameter due to the same feeding speed being used for both core and shell from the same pump.

Figure 3-6 Conditions for co-axial electrospinning of NR and PVP
Electrospun fibres collected with aluminium foil were put in a transparent glass tube, which was filled with nitrogen. Then the tube was put in UV box and radiated for 2 hours.

3.3.5 Smooth muscle cells culture

1) Preparation of supplemented medium 231

25mL smooth muscle growth supplement (SMGS) were placed in a 37° C water bath to thaw till the component melt. 500mL medium 231 were taken from cold storage and make sure that the caps of the vessels are tight. The bottle of supplement was gently swirled to avoid splashing the supplement into the cap of the bottle and causing the supplement to foam. The outside of the containers was wiped with 80% ethanol. The supplement to the medium 231 was then transferred into Class II Biosafety Cabinet (BSCII). The bottle of supplemented medium was tightly capped and the contents were well swirled to ensure a homogeneous solution.

2) Initiating culture from cryopreserved cells

A vial of HASMC was removed from liquid nitrogen storage but care should be taken to protect hands and eyes. The lower half of the vial was dipped into a 37ºC water bath to thaw. When the contents of the vial have thawed, the outside of the vial was wiped with 80% ethanol and was moved to a BSCII. The vial contents were then transferred to a 15 ml conical tube containing 9 mL pre-warmed media. They were then centrifuged for 7 min at 180 rpm to pellet cells. The cell pellet was resuspended in 10 mL of media. It was then divided into two parts of cell suspension and transferred to 75 cm² culture flasks. Following inoculation, the medium was swirled in the flasks to distribute the cells. HASMC were attached to culture surfaces quickly, and if the medium was not distributed immediately following inoculation, the cells might grow in uneven patterns. The cultures were incubated in a 37°C, 5% CO₂/95% air, humidified cell culture incubator. For best results, the culture should not be disturbed for at least 24 hours after the culture has been initiated.
3) Maintenance of stock cultures

The culture medium was changed to freshly supplemented Medium 231 for 24 to 36 hours after establishing a secondary culture from cryopreserved cells. For subsequent subcultures, the medium was changed 48 hours after establishing the subculture. The medium was changed every other day thereafter, until the culture is approximately 80% confluent. Once the culture reached 80% confluency, the medium was changed every day.

4) Passage/subculture the cells

The medium was taken from fridge to warm it up in a water bath. The TrypLE (X10, life technology) solution was diluted with PBS solution (5mL: 45mL) which was then moved to the water bath. The medium was removed from cell flask and the cells were washed with 10 mL PBS. After the PBS were removed then 3 mL TrypLE were added. And the resultant product was kept in the incubator for 3 mins. The cells were checked under microscope. 3 mL media and then 10 mL PBS were added. The tube was then transferred to centrifuge at 180 rpm for 7 minutes. The solution was removed and then 1 mL medium was used to resing the cells from tube bottom. At last, 250 μL was taken to the flask and 10mL medium was added.

5) Cell counting

10 μL well mixed cell suspension was put in 1 mL centrifuge tube followed by adding 20 μL trypan blue and 10 μL PBS. Then 10 μL was taken from the mixture and put in the interlayer between the glass cover and mirror with a square grid. It was then put under microscope and the number of cells was counted with a counter.

3.3.6 Cytotoxicity and viability

1) Cell cytotoxicity

The cytotoxicity of the scaffolds was carried out in triplicates by incubating samples with 1cm×1cm size in 24-well cell culture plate within 1mL medium 231 at 37°C, 5% CO₂ for 24 hours. Then the medium was removed and HASMC cells were cultured at
200,000 cells/mL. The cells were cultured for 3 days and the cell viability was assessed by trypan blue. Dead cells were blue while alive cells were white and bright after staining with trypan blue. Cell viability was calculated from the number of alive cells to total cells number in percentage.

2) Cell proliferation

HASMC cells were seeded on the sample at 5000 cells/well in 96-well cell plate with 100μL medium 231. The cell proliferation was carried out by using 3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide (MTT) assay at different cell growth points: day 3 and day 7. MTT, a water soluble tetrazolium salt, can yield a yellowish solution when it was prepared in media. It can become an insoluble purple formazan by dehydrogenase enzymes due to cleavage of the tetrazolium, which can be used to estimate live cell number at absorbance at 570 nm.

3) Immuno-staining

The medium was removed before the cells were washed with PBS. Then 4% paraformaldehyde (PFA) in PBS solution, was prepared by dissolving PFA powder in PBS solution at 60°C for 2 hours with stirring followed by filtration with 0.2μm filter, was used to fix cells. After the reaction at room temperature for 10 minutes, PFA solution was taken away. Before the Triton X-100 was added, the cells were washed with PBS for 3 times. After 10 minutes, the TritonX-100 was removed followed by washing with PBS. Then Dapi (1μg/mL) was added on the cells. PBS solution was used to wash the cells after around 10 -15 minutes to remove the potentially left Dapi. The ActinRed™ 555 Ready probe™ Reagent (2 drops in 1mL PBS solution) was added. The solution was incubated at room temperature for 30 minutes and washed with PBS solution for three times to remove excess fluorescent dye. The samples were stored in PBS at 4°C for confocal microscopy.

4) Cells growth evaluation by MTT assay

The MTT assay is a colorimetric assay for reflecting the number of viable cells present. MTT was dissolved in PBS to prepare 5 mg/ml solution. The solution is filtered through
a 0.22 μm filter net. The cells were cultured in a 96-well plate at a concentration of 3000 cells per well for 3 days for MTT standard curve. Cells were seeded in 96-well plate wetting in the medium and were incubated for 24 hours at 37 °C. 20 μL 5mg/mL MTT was added to each well and incubated for 4 hours. The medium was removed carefully and 200 μL DMSO were added to each well. Then the absorbance at 570 nm was read.

5) Standard curve of MTT assay

HASMC cells in medium 231 with density of 200,000 cells/mL, 100,000 cells/mL, 50,000 cells/mL, 25000 cells/mL, 12500 cells/mL, 6250 cells/mL, 3125 cells/mL, 1575 cells/mL, 788 cells/mL and medium 231 were seeded triply in a 96-well plate. Cell viability was assessed by adding 20μL MTT (5mg/mL in PBS) after 4-hour incubation in at 37 °C, 5% CO₂. After removing medium and dissolving precipitation with DMSO, the absorbance at 570 nm of the pink solution was measured on a microplate reader. The final absorbance value of each well was from the average absorbance of the measured absorbance removing background medium absorbance. Then the standard curve of MTT assay was made from changing of absorbance with cells number. At last, the linear relationship of absorbance and cells numbers was used to evaluate cells number from MTT absorbance value. Different time points were selected for getting different proliferation and viability. Cells cultured in 96-well plat with the samples were done MTT assay in the similar way as the standard curve method.

3.4 Characterization

3.4.1 Chemical structure of electrospun fibres

Fourier transform infrared (FTIR) spectra of electrospun NR fibre mats on aluminum sheet were characterized with a FTIR spectrometer Bruker Vertex-70 equipped with an attenuated total reflectance (ATR) crystal accessory operating at 64 scans in the standard wavenumber range of 600–4000 cm⁻¹ at a resolution of 4 cm⁻¹. Characteristic absorption peaks of the solvents and polymers used were shown in Table 3-2.
Table 3-2 Main absorption peaks of standard FTIR [134, 135]

<table>
<thead>
<tr>
<th>NR</th>
<th>CCl$_3$H</th>
<th>THF</th>
<th>C$_7$H$_8$</th>
<th>CCl$_2$H$_2$</th>
<th>PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>836</td>
<td>671</td>
<td>912</td>
<td>465</td>
<td>706</td>
<td>657</td>
</tr>
<tr>
<td>1129</td>
<td>760</td>
<td>1070</td>
<td>696</td>
<td>739</td>
<td>2981</td>
</tr>
<tr>
<td>1300</td>
<td>1216</td>
<td>1032</td>
<td>729</td>
<td>896</td>
<td>1279</td>
</tr>
<tr>
<td>1376</td>
<td>2400</td>
<td>1184</td>
<td>1030</td>
<td>1266</td>
<td>1296</td>
</tr>
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<td>3618</td>
<td>3423</td>
<td>3028</td>
<td></td>
<td>2960</td>
</tr>
</tbody>
</table>
Figure 3-7 FTIR-ATR result of stretched natural rubber films

FTIR can be used to investigate stretching rate of NR materials through FTIR peak central frequency shifting from 843 cm\(^{-1}\) to 833 cm\(^{-1}\) during the stretching process. According to the references [136, 137], peak at 843 cm\(^{-1}\) refers to strain induced crystalline of NR. Different stretching rate will cause different peaks at 840 cm\(^{-1}\), which can be used to estimate the electrical force during electrospinning (Figure 3-7).

### 3.4.2 Component distribution in NR-PVP mixture

Miscibility of NR and PVP in blend was investigated on a FTIR microscope (Lumos, Bruker). Film samples from NR-PVP in chloroform, NR/THF and PVP/water mixture were prepared. 100 μm×100 μm square region scan was investigated, however, there was no such difference among different points. It seems that the size of separated phase was less than 100 μm×100 μm. Then 40 μm×40 μm was scanned and still no separation. Large scale view was scanned with small point size at the 10 μm×10 μm. The integration difference from 2820 to 3050 cm\(^{-1}\) was set as the standard. In this wavelength range, natural rubber has strong sharp peaks while PVP has a relatively
weak peak. PFA-FTIR: Hyperion 3000 Focal Plane Array (FPA) FTIR microscope, Bruke, (Australian Synchrotron), was used for FTIR mapping with a 4 μm×4 μm with 256 scans. Samples were prepared on the surface of CaF₂ mirror to make it as flat as possible.

3.4.3 Crystal structure analysis

Crystal properties of electrospun fibres were investigated by X-Ray diffraction (XRD). All measurements were conducted on a Phillips PW-1729 diffractometer (35 kv, 28 mA) (Amsterdam, the Netherlands) with CuKα radiation (λ=0.154 nm). The results were recorded in the range of 2θ=5-60° (by step size of 0.05°). The tube voltage and tube current were kept at 40 kv and 40 mA, respectively. All the samples on the aluminium foil without peeling off were fixed onto a glass slide and the scanned area was kept for 5mm×5mm.

3.4.4 Solution feature testing

Conductivity meter (Mettler-Toledo) was used for measuring the conductivity of solutions at room temperature by dipping the conductivity pole in the solution. The resolution reaches to 0.01 μs/cm;

Surface tension test: follow the standard method (ISO 1409:2006): Plastics/rubber — Polymer dispersions and rubber lattices (natural and synthetic) — Determination of surface tension by the ring method;

Viscosity of solution mixture is measured by viscometer to calculate interface action as described in [15];

Rheological properties testing: NR/THF solutions were tested on rheometer (HR-3, TA). Water sealing strategy was used to prevent evaporation of THF during testing. The accessories are composed of solvent trap cover and centering ring (as seen in Figure 3-8), which can create a stable vapor barrier together with solvent trap geometry to prevent solvent evaporation during rheological properties testing. The flow model was used to test the apparent viscosity with shear rate ranging from 2.85s⁻¹ to 300 s⁻¹;
Dynamic properties testing: Viscoelasticity constitutive relationship of natural rubber solution can be tested from the oscillation model in rheological properties testing. Plate rotor and small-amplitude dynamic measurement were adopted. A sinusoidal strain $\gamma$ with an angular frequency $\omega$ is applied, in which $\gamma = \gamma_0 \sin(\omega t)$. And the complex modulus can be expressed as follow:

$$G = G'(\omega) + G''(\omega)$$
In which, $G'$ is storage modulus and $G''$ is loss modulus. When $G'(\omega) = G''(\omega)$, $\lambda = 1$ and relaxation time of the solution can be estimated. The cross over point of $G'$ curve and $G''$ curve in Figure 3-9 refers to the relaxation point and the angular frequency could be used to estimate relaxation time.

![Figure 3-9 Determination of relaxation time of polymer solution from rheological properties](image)

The relationship between shear rate and viscosity can be expressed by the power law model as follow:

$$\eta = m \gamma^{n-1}$$

in which, $m$ represents consistency coefficient of the fluid, $n$ power law index[138]. The curve fitting of flow curve of NR solution can be fitted in growth exponent function and got the viscosity and shear rate relationship. It can also be fitted in the following equation:
\[ \eta = \eta_\infty + \frac{(\eta_\infty - \eta_o)}{[1+(\lambda^*\gamma)^m]} \] [139]  

Fitting of the rheological testing flow curve can get dynamic viscosity information of the solution together with relaxation time \( \lambda \).

### 3.4.5 Particle size testing

The particle size of DPNR solutions in THF with different concentrations were tested on Nano ZS DLS particle size tester (Malvern). To avoid the influence of multi-scattering, the concentration of the solution is in a low concentration. The solutions were filtrated to remove gel and larger particles. Stokes-Einstein model was selected as a testing model. The solution was filled in a glass cell and covered to avoid evaporation. It was kept equilibrium for two minutes prior to testing[140].

### 3.4.6 Mechanical properties testing

Tensile strength is tested on tensile strength tester of Anton Paar (60N) (Figure 3-10). Mechanical properties are important factors which should meet requirement of the application of electrospun fibres. According to biomedical application of the electrospun mats in tissue engineering scaffold, the fibre mats should support weight of the cells and be elastic and strong enough to follow transformation of the tissue. However, the measurement of electrospun fibre’s mechanical properties is not so easy due to the low force durability of electrospun fibres, which means the fibres are easily broken by very tiny force (several \( \mu \) N). Therefore, two strategies should be applied to measure mechanical properties of electrospun fibres. First is the protection strategy of electrospun fibres. Aligned fibres were collected among a frame made from thick paper with a regular shape window in the middle. The edges of paper frame were cut off before mechanical testing. The other strategy is that the tense sensor should have enough resolution to recognize the force change during testing process.
3.4.7 Thermal properties testing

The blend of NR and PVP were prepared from solvent casting of NR/PVP solution in chloroform. They were dissolved in chloroform and stirred for uniform mixture and then casted the solution on a glass board to remove the solvent. The casted film were put in the vacuum oven at 60 °C to remove the residual solvent overnight. The NR/THF solution were dipped on a glass slide followed by mixing PVP/water solution and filmed from solvent casting. NR/PVP blend solution was made in dissolving NR and PVP in chloroform together.

DSC experiments were performed by using a TA-DSC model Q200 instrument with protection of nitrogen gas. Electrospun fibres collected on the hollow windows were encapsulated in aluminium pans and sealed. Before the test, all the samples were dried at 50°C in an oven for overnight in nitrogen atmosphere. The specimen was first heated to 200°C at a heating rate of 20°C/min to remove heating history and then cooled down to -80°C at a cooling rate of 20°C/min, equilibrium for 5min. and then heated to 250°C at a heating rate of 10°C/min. The glass transition temperature was calculated based on the exothermic slope produced during the second heating process. Universal analysis 2000 (TA) software was used for data analysis.
3.4.8 Morphology investigation

SEM: The morphology of electrospun NR fibres were investigated by scanning electron microscopy (Carl Zeiss, Supra 55VP, Germany) at 5kv accelerating voltage. All the samples were coated with thin layers of gold at a currency of 40mv for 60s.

TEM: TEM (JEM2100, JEOL Inc.) with a LaB₆ filament at 200 KV and 108μA was used to investigate interior structure of electrospun nano-fibres. The fibres were collected on the copper sample grid during electrospinning process.

Optical microscope: Light microscope (DP 70, Olympus) was used to investigate morphology of electrospun fibres at bright field and dark background models. Lenses with magnification times: 5×, 10×, 20×, 40× and 100× were equipped with.

Confocal microscope: Confocal microscope (TCS SP5, Leica Co.) was applied to investigate FITC dyed core-shell structure fibres and cells seeded on electrospun fibres. The samples were prepared by dissolving PVP in FITC/alcohol solution for shell feeding fluid to prepare core-shell structured fibres from co-axial electrospinning. Active lasers included 488nm argon laser with 20-30% intensity for excitation of FITC, 30% level of UV for Dapi stained cell nucleus and 8% level of 605nm light for initiating TRITC dyed cytoplasm. The diameter of electrospun fibres were measured in the images with an appendix tool in Adobe Acrobat pro. PDF reader.

Inverted microscope (Eclipse Ti-S, Nikon) was used to investigate cells morphology in medium and on materials. Fluorescent light source was equipped for investigation stained cells and materials.

FIB-SEM: FEI Quanta 3D FEG FIB-SEM was used to cut the core-shell structure electrospun fibres and investigated its cross-section. The work theory was showed in Figure 3-11. An ion source together with an electron source were used to cut the core-shell structure fibre in a program. The cross section of cut fibre was investigated through SEM.
3.4.9 Microstructure investigation

Electrospun NR fibres collected among hollow window were tested under small angle X-ray scattering (SAXS) with long camera length: 730.838 mm and the samples were stretched during SAXS testing: 5s scanning time & 20s tensile. Different scanning times (1s, 2s and 5s) were tested for the damage of samples first. Raw materials of natural rubber and NR film from solvent casting were also tested by the SAXS in a static model and the samples were attached on an adhesive tape.

3.4.10 SEM samples preparation

Cells images from SEM can provide information of cell morphology and the attachment pattern of cells on the sample. The samples were soaked in 2.5% glutaraldehyde/PBS (0.1M, pH 7.2) solution for 4-5hours. Samples were washed in PBS twice and each for 30 minutes. Then they were left in PBS overnight. The samples were dehydrated in 50, 70, 80, 95, and 100% ethanol/deionized water for 30 minutes. They were dehydrated in 2 × 30mins for 95% and 3×30mins for the 100%. The samples were put in ethanol:
hexamethyldisilazane (HMDS) (1:1) two times and 30 minutes each followed by soaking in HMDS for 10 minutes and three times. Specimen were dried on filter paper in a glass Petri dish with lid ajar within a fume hood. Coat with gold prior to SEM.
4 Conditions investigation for steady electrospinning of natural rubber

4.1 Introduction

Stability control of electrospinning natural rubber process decides uniformity of electrospun fibres together with their mechanical properties. For electrospinning natural rubber, two extrusive difficulties lie in gels caused homogeneity issue and curl structure due to high viscoelasticity of the solution. Optimal conditions for steady electrospinning of natural rubber were investigated in this chapter from two aspects: polymer solution properties and steady process. In the first section, solution properties were modified by solvent, concentration, homogeneity and conductivity. Solvent was selected from solubility, polarity and electrospinnability together with hydrodynamic analysis. Then, electrospinnable concentration window was decided by the entanglement for continuous fibre formation and gel for blocking electrospinning process. Filtration and centrifugation were used for increasing homogeneity of the solution. Optimal ratio of added conductive solution to NR solution was decided by the conductivity and fibre diameter. In the second part, factors that influence instability of dynamic electrospinning process were discussed, such as processing parameters, the role of high viscoelasticity and collection pattern. Critical voltage and nozzle diameters were investigated followed by the discussion on competition between stretching and relaxation during electrospinning and its influence on fibre morphology. In the third section, morphologies and properties of electrospun NR fibres were discussed. FTIR was used for chemical structure investigation before and after electrospinning as well as the influence of solvents and concentration. Crystal structure change from rubber to fibre was characterized by DSC and XRD. Mechanical properties were tested together with SAXS testing for microstructure change during stretching process.

4.2 Homogeneous and electrospinnable solution

4.2.1 Solvent selection

Benzene is not considered for electrospinning due to its toxicity to the environment after evaporation during electrospinning. Considering the toxicity and risk of flaming,
carbon disulphide is not suitable for electrospinning either. Several solvents for natural rubber were commonly reported are tetrahydrofuran (THF), dichloromethane, toluene and petroleum spirit and chloroform [68, 72].

![Teas graph](image)

**Figure 4-1 Potential solvents of NR in Teas graph**

Each solvent can be found as a point in the Teas graph according to their solution parameters. The distance between the solvent point and natural rubber (11) in Teas graph can provide relative solubility of NR in the solvent. From the Teas graph, water (10), acetone (5) and N, N-dimethylformamide (DMF) (7) are poor solvents for natural rubber due to the long distance between them and natural rubber, which is coincident to the fact. From the Teas graph, a range of solvents for electrospinning natural rubber could be decided: chloroform (2), toluene (3), dichloromethane (12), THF (4) and ethyl acetate (6). From the Teas graph, THF and ethyl acetate showed a similar solubility to NR. However, in fact, NR is hard to be dissolved in ethyl acetate. Therefore, chloroform, toluene, dichloromethane and THF can be selected for NR solvents from the Teas graph.

Fibre morphology can be used for selecting electrospinning solvents due to its close relationship to fibre properties. The fibre morphology considered here refers to fibre
diameter and bead. The observation of electrospun fibres from different solvents was listed in Table 4-1. Beads appeared when the concentration is low and bead-free fibres formed at higher concentration. From the experimental investigation, the minimum concentration for electrospinning NR in different solvents can be set. Beads appeared from NR/THF and NR/toluene solutions when the concentration was as low as 10g/L. While in chloroform solutions, beads lasted until the concentration reached to 25g/L. Dichloromethane solution was electrospun and bead-free fibres were not got until the concentration reached 20g/L. The minimum concentration of electrospinnable solution refers to bead-free fibre morphology.

<table>
<thead>
<tr>
<th></th>
<th>10g/L</th>
<th>15g/L</th>
<th>20g/L</th>
<th>25g/L</th>
<th>30g/L</th>
<th>40g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>beads</td>
<td>fibres</td>
<td>fibres</td>
<td>fibres</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>THF</td>
<td>beads</td>
<td>fibres</td>
<td>fibres</td>
<td>fibres</td>
<td>fibres</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>beads</td>
<td>beads+</td>
<td>fibres</td>
<td>fibres</td>
<td>fibres</td>
<td>-</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>beads</td>
<td>beads</td>
<td>beads+</td>
<td>fibres</td>
<td>fibres</td>
<td>fibres</td>
</tr>
</tbody>
</table>

The average diameter distribution of electrospun fibres changing with concentration was shown in Figure 4-2. The images of fibres electrospun from different solvents were shown in appendix (Figure A-1). The concentration range selected was from 15g/L to 30g/L based on the previous observation. With increase of concentration, the average diameter of electrospun fibres from the same solvent increased. The total average fibre range was in 2.0-7.0 μm. The diameter difference among different solvents was not that obvious. Fibres from electrospinning NR/chloroform showed biggest average diameter which ranged from 3.0 to 6.5 μm while the fibres from NR/dichloromethane ranged
from 1.8 to 4.8 μm. The average diameter range of NR/THF has some overlap with those from toluene and chloroform. The electrospun fibres from synthetic poly cis isoprene in chloroform and dichloromethane have been reported by Hao et al. [72], in which thick fibres with larger diameters around 20-60 μm from dichloromethane solution with bamboo nodes along were spun. While in the chloroform solution, smooth fibres with diameter around 6-8μm were obtained. NR raw materials here were masticated before being used for electrospinning. Mastication caused molecular weight decrease, which contributed to fibres with smaller diameter. Another reason lied in the difference of NR and synthetic poly cis isoprene in non-rubber content, which can lead to entanglement of NR solution with lower molecular weight at lower concentration. It was also suggested that polar solvent was better than non-polar solvent for electrospinning non-polar polymer [72]. The strong entanglement in NR solution contributes to the difficulty of splitting NR jets and the flexible polymer chain facilitating fibre alignment. Smooth straight fibres from polar solvent are attributed from the strong Coulomb force.

![Figure 4-2 Average diameter of fibres as a function of solution in different solvents with different concentration (voltage: 8KV, feed rate: 2.0mL/h, distance between needle tip to collector: 25cm, needle diameter: 0.8mm)](image-url)
The hydrodynamic analysis can provide statistical particle size information of NR solutions, which would contribute to the electrospun fibre diameter. The hydrodynamic radius of NR in different solvents with the same concentration were investigated by dynamic light scattering (DLS). Due to the limitation of large particles during the DLS testing, solutions were diluted for testing. 1.0g/L solutions of NR in THF, chloroform, dichloromethane, toluene was tested on DLS for hydrodynamic analysis and results were listed in Table 4-2.

Table 4-2 Hydrodynamic testing of NR solutions (1.0g/L) in different solvents

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>THF</th>
<th>Chloroform</th>
<th>Dichloromethane</th>
<th>Toluene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarity</td>
<td>4.2</td>
<td>4.4</td>
<td>3.4</td>
<td>2.4</td>
</tr>
<tr>
<td>(R_h) of NR solution/nm</td>
<td>7.54</td>
<td>10.28</td>
<td>6.35</td>
<td>15.61</td>
</tr>
<tr>
<td>Flory-Huggins parameter (\chi)</td>
<td>0.45</td>
<td>0.19 to 0.01</td>
<td>0.54 to 0.41</td>
<td>0.36 to 0.32 [141]</td>
</tr>
<tr>
<td>Spinnability [142] /mg</td>
<td>0.77</td>
<td>0.55</td>
<td>0.61</td>
<td>0.86</td>
</tr>
</tbody>
</table>

The measured hydrodynamic radius of NR solutions showed obvious solvent dependence. Natural rubber molecular chains tend to be curl in the poor solvents while in the good solvents they stretched out. The hydrodynamic radius of NR solutions in different solvents has the order of: Toluene > Chloroform > THF > Dichloromethane. The solvents do influence radius of gyration of the polymer in the following relation: \(R_g \sim N^\nu\), in which, \(R_g\) is the gyration radius, \(N\) is the bond segments number and \(\nu\) is Flory exponent, which ranges from 1/3 to 5/3 and depends on the polymer-solvent interaction and concentration. The gyration radius can be estimated from the
hydrodynamic radius \( R_h \) in the following relationship: \( R_h = 0.77 R_g \) [143]. This radius can be used to express the morphology of polymer chain in a certain solvent at a concentration. The diameter of electrospun fibres that has anything to do with the hydrodynamic radius can be characterized in the particle diameter of solution with electrospinnable concentration. The spinnability can be used for estimating productivity of the solution. Toluene solution showed highest spinnability, however, considered toxicity and environmental issue, toluene was not a good choice for electrospinning NR. THF, a less toxic solvent, also showed higher spinnability together with relative good solubility.

In summary, considering the solvent properties, fibre morphology and hydrodynamic analysis results, THF was selected as solvent for electrospinning natural rubber. First, THF is of relative low toxicity and good solubility together with excellent evaporation property. In addition, electrospun NR fibres from NR/THF showed smooth surface morphology and uniform fibre diameter distribution. Finally, as a polar solvent, NR solution in THF showed lower \( R_g \), which contributed to smaller fibre diameter.

4.2.2 Concentration range selection

1) Electrospinnable concentration window

Polymer solution properties depended closely on the concentration. The limitation of concentration for electrospinning is from the lower limitation from the bead formation at lower concentration and the high limitation is from the aggregation of NR solution, at which the electrical force is not big enough to draw the fibre out. It was pointed out that beads formed under low polymer concentration [144]. As a limitation of forming uniform fibres from electrospinning, bead formation concentration is considered as the low limitation of electrospinning happening. The higher concentration limitation is decided by the polymer solubility in the solvent and the electrospinning limitation.

It was reported that the formation mechanism of beads was that [145]: solvent molecules tend to distribute over entangled polymer molecular chains at high viscosity and they would congregate to increase surface tension. The beads formed during electrospinning due to the imbalance of surface tension and electrical force. Polymer
jets tend to change shape to make up the difference between surface tension and Coulomb force. Spindle-like beads are attributed from increasing of surface tension requirement. Figure 4-3 shows optical microscope images of NR beads. Spindle-like beads were observed during electrospinning NR/THF at lower concentration (less than 15g/L). The average length of the beads was around 10μm with wide length around 7 μm. The direct distance between neighbouring beads were around 20 μm. The higher concentration limitation of NR/THF can be found from Table 4-1. When the concentration of NR/THF reached to 40.0g/L, the solution was hard to be electrospun. Therefore, electrospinnable concentration range of NR/THF solution is: 15.0g/L to 40.0g/L.

![Figure 4-3 Spindle-like beads in electrospun NR fibres: a) ×100; b) ×2000](image)

2) Effects of concentration on entanglement

Sufficient entanglement is the precondition for successful electrospinning. In polymer solutions, molecular chain entanglement increased with concentration. Uniform electrospun fibres can only be obtained in a certain concentration range, in which no beads formed and no clogging of electrospinning nozzle. Relationship between chain entanglements in the polymer solution and electrospinnability has been clearly demonstrated in the reference [146]. Number of entanglement per chain: Ne =2 ~ 3.

The concentration can be a weight that balance entanglement and network. The suitable concentration range for electrospinning should be in the range of C* and C”’. C* is the critical concentration for entanglement while C” is the concentration for network. For NR in THF the C* can be estimated from the hydrodynamic analysis. The DLS result
of 1.0g/L solution showed no entanglement while that of 2.0g/L solution showed entanglement. Therefore, we can set the range of C*: 1.0g/L < C* < 2.0g/L, though the exact value of C* was not decided.

Figure 4-4 DLS nanoparticle testing results of NR/THF solution with different concentration: a) 1.0g/L; b) 2.0g/L; c) 5.0g/L.

DLS results also showed that there were no large gel particles in NR solution at lower concentration (less than 1.0g/L). Figure 4-4 showed the particle size distribution result.
There was only one peak appeared in 1.0g/L solution. With increase of concentration, micro-gels in NR solution got aggregated and gels formed. From the result of particle size distribution, the average diameter of the particles increased with concentration of solution from: 5.615 nm to 7.531 nm, which means that increase of concentration contributed to increase of particle size due to the distance between the molecular chain decreasing with increase of concentration. Combining gel content measurement and DLS testing result, there is no entanglement in the dilute NR solution (1.0g/L) and the entanglement of the solution will happen at higher concentration (2.0 g/L and 10.0 g/L). In another way, the gels in NR solution should be composed of physical crosslinking, which can be damaged with increase of concentration.

3) Gel content and structure

Entanglement is necessary for electrospinning to prepare continuous fibres, however, with increase of entanglement, gelation of NR solution will happen. The solution of NR in THF is a combination of sol and gel [93]. It was reported that the gel phase is composed of micro-gels, which will aggregate to an apparent gel phase [93]. The component of natural rubber is complex, which causes the multi-phase in natural rubber solution. During storage, or when in contact with air and light rubber will crosslink to a certain degree. The crosslinked components will be difficult to be dissolved which will affect the dispersion in the NR solution. The particle size increases as well with increase of concentration. When the particle size reaches a certain value, light coagulation will happen and gel phase will form. Gel and sol transition will happen if the solution concentration or other external factors, such as solvent type and temperature[147, 148]. Gelation is coagulation contacts between particles and starting structure. In this sol-gel system, with the increase of bulk natural rubber, gelation will happen.

Gel content changes with concentration were depicted in Figure 4-5. The gel weight and concentration increased with concentration. There was no gel when the concentration was low (less than 1.0g/L). The gel content increased with increase of concentration when the concentration is higher than 1.0 g/L. The gel content can reach 35% when the concentration increased to 50g/L.
Figure 4-5 Gel content changes with concentration of NR/THF solution

Figure 4-6 Molecular chains statements in NR/THF solutions with different concentration
The process of gelation of NR solution with increase of concentration can be demonstrated in Figure 4-6. Micro-gels in the solution were floating in the solution without entanglement as shown in dilute solution item in Figure 4-6. The hydrodynamic diameter remains the same when the concentration is lower than the critical concentration \( C^* \). When the concentration is larger than \( C^* \), the hydrodynamic diameter increases gradually. When the micro-gels become compacted at the solution with higher concentration, polymer chain entanglement from the physical entanglement and chemical interaction will contribute to network in the solution. It was reported that proteins can provide crosslinking sites to combine NR molecules [46]. With the increase of concentration, entanglement of micro-gels contributed to the aggregation and gel formed.

In summary, NR solution concentration is a key factor that influence the electrospinning process. The electrospinning concentration window decided from no bead and being electrospinnable was from 15.0g/L to 40.0 g/L.

### 4.2.3 Solution homogeneity

1) Filtration

Floccules floating in NR/THF solution can be seen through the light (Figure 4-7). These are gels from cross-linked rubber with poor solubility in THF. In addition, the solution is not transparent due to impurity in NR. After extraction by a syringe with metal net, the gel floccules were removed and the solution became transparent (Figure 4-7). The filtration effect was shown in appendix (Figure A2).

Entanglement is necessary for electrospinning to prepare continuous fibres, however, with increase of entanglement, aggregation of NR solution will happen. There will be a competition between the stability and beads. A suitable concentration range for electrospinning natural rubber is quite narrow (15g/L to 40g/L). The concentration can influence fibres’ morphology in two aspects: 1) fibre or bead; and 2) diameter of fibres. Beads happened when the concentration was lower than entangled concentration \( C_e \) and it disappeared with increase of concentration. When the concentration reached
entangled concentration, high restrict force stopped electrospinning. Fibres with average diameter ranging from 1-3 μm were got from different solutions.

The filtration decreased concentration of concentrated NR solution and the concentration range for electrospinning NR changed as well. After gels were filtering, the larger gels were removed and NR solution became homogenous with decrease of total solid concentration. However, from the rheological property testing of NR solution before and after filtration, apparent viscosity of filtered NR solution could be higher than that of NR solution. Particles instead of fibres were collected when concentration of NR solution is 10g/L as shown in Figure 4-8. Electro-spraying instead of electrospinning happened when concentration is less than 10g/L. With increase of concentration, beaded fibres were collected. When the concentration reached 15g/L, smooth bead-free fibres with a diameter around 2μm were obtained. With increase of concentration, fibre morphology changed from typical cylinder shape to ribbon. When the concentration reached 60g/L, curl fibres with diameter around 3-5μm were gathered. Before filtration, NR solution with concentration 40g/L was even not electrospinnable. Therefore, after filtration, electrospinnable NR concentration became wider and uniform fibres were collected. On the other hand, filtered NR solution also improves the stability of the electrospinning process. After filtration, the nozzle was not clogged by the polymer solution. [149]
Figure 4-8 Electrospun fibres from filtered NR solutions (Electrospinning processing parameters: voltage: 8KV, feed rate: 1.0 mL/h, needle diameter: 0.8mm, tip to collector distance: 25cm. a) 10g/L; b) 15g/L; c) 25g/L; d) 60g/L).

The filtration of gel also reduced size limitation of electrospinning nozzle. Before filtration, nozzle with inner diameter less than 0.7 mm would be easily to be blocked. With filtering of gel, nozzle with inner diameter 0.4 mm can also be used for steadily electrospinning NR solution. With decrease of nozzle size, the diameter of electrospun fibres decreased to 0.4 μm from 15g/L NR/THF solution, in which beads can also be seen in Figure 4-9.

In summary, filtration can increase homogenity of natural rubber solution and uniformity of electrospun fibres and the electrospinnable concentration window was widen by the removal of gel.
2) **Centrifugation**

Electrospun fibre morphologies from different centrifugation speed are shown in Figure 4-10. Centrifugation speed was controlled to morphology of electrospun fibres and effect of centrifugation speed can be seen obviously. When the centrifugation speed was lower than 1.0krpm, there was no effect on NR solution and no separation. With the centrifuge rate increases, yellow precipitation appeared gradually at the bottom of the tubes. The lower limitation for gel separation from NR solution (25g/L) was 1krpm. Higher centrifugation speed would influence entanglement in the solution, which would affect fibre morphology.

To investigate the centrifugation speed effect on electrospun fibres, NR solutions purified from different centrifugation speeds were electrospun under the same processing parameters. Fibre morphologies were investigated by SEM (Figure 4-10). Centrifugation speed has a similar effect to the filtration. With centrifuge speed increased, fibre became thinner and thinner and beads started forming when the centrifugation speed was at around 5krpm. Therefore, the proper centrifugation speed for NR/THF solution (25g/L) was from 2 krpm to 5 krpm and the speed at 2.0 krpm showed best uniform electrospun fibres.
4.2.4 Salt adding

The conductivity of the solution depends on the ions in the solution and the voltage applied [150]. The electric force stretches polymer solution and forms Taylor clone at the tip of metal needle and attracts fibres flying to collector. It is the surface charge of polymer solution or jet results in static electric force in electric field. Therefore, the value of electric force depends on charge density and electric field strength. Electrical charge in a solution contributes to static electric force that plays an important role in electrospinning process. The current caused by potential difference is related to types of ions in solution and their concentrations. In very dilute solutions, Debye–Hückel–Onsager equation can be used to calculate conductivity of the solution [144]. The electrolyte has a constant molar conductivity at a higher concentration. For insulated polymer, adding a conductive salt or surfactant can provide sufficient charge for
electrospinning. Polymer jets with high conductivity have high surface charge density, which contributes to spin finer fibres. The self-repulsion of the excess charges on the surface will lead to an increase in the elongation force on the jet under a certain electric field. This will avoid the Rayleigh instability and enhance whipping, which results in uniform fibres without beads.

![Graph](image)

Figure 4-11 Conductivity effects on electrospinning of natural rubber solution and electrospun fibres (25g/L NR/THF, 10KV, needle diameter: 0.8mm, distance between nozzle tip to collector: 25cm, feeding rate: 1.0mL/h)

10% NaCl/H$_2$O solution was added to 100mL NR/THF solution to investigate the conductivity change with adding volume. The change in solution conductivity with the amount of added NaCl solution was shown in Figure 4-11. The conductivity of the solution was not showed before the volume is less than 2mL. The smallest conductivity can be tested is 0.01μs/cm. The average diameter of electrospun fibres decreased gradually with an increase in NaCl/H$_2$O solution. As NR is insoluble in water, with adding of aqueous solution, the mixture would be separated into two phases with NR solution on the top. The best ratio of volume of NR solution to conductive solution is 4: 100.
4.3 Stability of the electrospinning process

4.3.1 Processing parameters

When the voltage surpasses the critical value, the jet will be pulled out from the tip of the Taylor cone. It has been reported by Sill and Recum [86] that the Taylor cone shape changes with voltage. Reneker et al. described the process of electrospinning with the increasing of electrical strength [87]. They also pointed that the static Taylor cone angle is 49.3° when the critical voltage was reached [80]. The stability of Taylor cone was discussed in theory [73] and a minimum feed rate was required for steady structure of Taylor cone. Stability of structured Taylor cone strongly depends on the physical properties and flow behaviour of the fluids and the voltage applied [85].

1) Critical voltage

Taylor cone will form at the nozzle tip when voltage increases and reaches certain value, which is called initial voltage for electrospinning. For electrospinning of natural rubber solution, when the distance between needle tip and collector is 25 cm, the initial voltage is 3.6 KV. Uniform fibres were collected but the distribution of fibres was very interesting: the fibres were distributed between needle clamp and collector. With increase of voltage, the characteristic length (from Taylor cone to jet) changed and the cone were elongated.

Actually, Taylor cone is not necessary for the occurring of electrospinning, especially after the initial pulling-out process, but the flow behaviour of cone jet did influence bending behaviour of polymer jet. With increase of voltage, Taylor cone will be extended as seen in Figure 4-12. The change in shape of cone jet would contribute to instability of bending behaviour. From the observation, the characteristic length change of Taylor cone was recorded and its change with increase of voltage is shown in Figure 4-12. The increase of cone characteristic length was from high viscoelasticity of polymer jet and in turn it would cause unsteady bending behaviours, such as rotating, swing, blurring and even branching [84].
Figure 4-12 Characteristic length change with increase of voltage

Figure 4-13 Range of voltage for steady electrospinning at different feeding rate: 25g/L NR/THF solution, needle diameter: 0.8mm, distance between needle tip to collector: 25cm
The voltage range for steady electrospinning was shown in Figure 4-13. $V_c$ is the minimum voltage that caused happening of electrospinning and $V_r$ is the minimum voltage that leads to unsteady bending of polymer jet. From the result we can see that, with increase of feeding rate, $V_c$ and $V_r$ decreased and the voltage range for steady electrospinning becomes smaller.

2) Effects of voltage on fibre morphologies

![Fibre diameter changes with voltage: 25.0g/L NR/THF solution, distance between needle and collector: 25cm, feed rate: 1mL/h, needle diameter: 0.8mm. a) 8.0 kV; b) 9.0 kV; c) 10.0 kV; d) 12.0 kV.]

The effects of voltage on the morphology of electrospun fibres can be seen in Figure 4-14. At lower voltage, the fibres tend to be curl structure while with increase of voltage, the fibres become straight. The possible reason lies in that with increase of voltage, electric strength increased, which would provide larger Coulomb force. The accelerating speed of polymer jet increased and so did the inner repulsive force, which would offset more polymer relaxation effect. During electrospinning, Taylor cone may disappear and the critical voltage is the minimum voltage for electrospinning happening.
3) Needle diameter

To investigate the influence of nozzle diameter on the fibre morphology and alignment, metal needles with different inner diameters were used for electrospinning. The other conditions were kept the same: 12 kV voltage, 25 cm tip to collector distance, feed rate of 1.0mL/h.
The nozzle diameter not only influences the average diameter of electrospun fibres but also the alignment. As seen in Figure 4-15, with increase of nozzle diameter, the average diameter of electrospun NR fibres increased from 1.08μm of 0.5mm nozzle to 2.80μm of 0.9mm nozzle. On the other hand, the smaller nozzle diameter contributes to curl fibres. The alignment of fibres can be expressed by the FFT results from ImageJ.
software. As described by Ayres et al [151], the alignment of fibres in different angles can be calculated from the frequency plots.

The reason that smaller needle syringe contributes to curl fibres may lie in: 1) smaller needle diameter contributes to high compression and larger shape change during feeding through the needle, which will cause larger stress due to high elasticity of NR solution; 2) smaller needle diameter corresponds to higher initial speed of polymer jet, which will cause the instability of Taylor cone; 3) smaller diameter contributes to smaller jet diameter, which can accelerate the evaporation speed of the solvent. The faster evaporation of solvent leads to earlier loss of electrical repulsion force along the fibre length and increase of elasticity, which are attributed to bending and contraction of polymer jets.

4.3.2 Dynamic process analysis

The factors in dynamic process of stretching step of electrospinning will influence the final morphology of electrospun fibres. The force, rheological properties and time will decide the final diameter and morphology of collected fibres. The force reacted on the stretched jet is from the electrical strength (E) and the surface conductivity of polymer jet. The rheological properties of jet relate to the instantaneous concentration (C) and shear rate (\( \gamma \)). The most key role is the time at which the jet was stretched. The concentration and solidification of the jet will also be decided by the time and the space factor. The instability of polymer jet concludes axisymmetric disturbance and break up. The viscoelasticity can prevent the break-up of polymer jet and contribute larger amplitude of the disturbance wave.

Polymer entanglement is considered as the key role for preventing breaking up of polymer jets and making sure continuous fibres. Viscoelasticity plays three roles in the process of electrospinning: onset delay of disturbance growth, faster disturbance growth and retardation of final jet break up [90]. Polymers with high molecular weight especially partially cross-linked show high viscoelasticity in solution. Natural rubber solutions are of high viscoelasticity. The role of high viscoelasticity on stability of electrospinning process has been investigated [90, 152]. It was demonstrated that viscoelasticity contributes three effects to electrospinning process: Onset delay of
disturbance growth due to unrelaxed stress at needle, faster disturbance growth and retardation of final break up. In which, disturbance growth delay and retardation of final break up contribute to jet stability while faster disturbance growth will cause jet instability.

The high viscoelasticity of NR solution due to high molecular weight, flexibility of NR molecules and solution concentration influence the stability of electrospinning process in the steps of feeding and stretching process. The evaporation of solvent caused increase of concentration and solidification of fibres from the jet. The rheological properties and surface tension change during evaporation of solvent. The stretching process involved evaporation of solvent and surface areas increase with the thinning of the fibres, which contributes to evaporation of solvent. The balance of force also was broken with the extension of fibre jets, which will lead to increase of fibre stress. If the fibres were collected on the collector before the contraction of fibres with the disappearance of Columba force and increase of molecular relaxation, straight fibres would be collected. With increase of distance, the relaxation of the fibres due to high viscoelasticity would be larger than the faded Columba force due to solidifying of fibre jet and curl fibres would be collected. On the other hand, the hollow collector will provide additional electrical field, which would be much stronger than that existed between needle and collector. The fibres would be stretched under the strong electric field and are straight and well aligned. Hong et al. reported aligned fibres collected among the void window in the metal collector [91].

Travelling of polymer jet between nozzle tip and collector involved two reverse effects on the polymer jet: stretching under reaction of Coulomb force and relaxation due to viscoelasticity of polymer. Polymer relaxation, the response of polymer to the external effect from electrical force, runs through the whole process of polymer jet travelling if the travelling time is not long enough for the complete relaxation of polymer. The stretching of polymer jet happens when the forepart of electrospun fibres was under bigger force than the coming part. Stretching process and shrinking process involve competition between relaxation and repulsion of polymer jet. In the stretching process, repulsion is the leading force, which cause elongation and the polymer jet, in which polymer jet became thinner and surface area increased. The evaporation of solvent contributed to solidification of fibres. In this process, the relaxation of the fibres
increases while the electrical repulsion decreased. When the fibres attached on the collector, the relaxation was stopped by the interaction between fibres and the collector. Therefore, the fibre morphology can keep most of the pattern of the polymer jet. The morphology control of curl or straight fibres from electrospinning of elastic polymers have been studied before [107, 122].

1) Relaxation time

Under the same external conditions (voltage, distance between needle tip to collector, feeding rate), the final morphology of electrospun fibres would depend on the properties of polymer jets and solid materials’ response to the external force. Relaxation of polymer solution and polymer jet will contribute to viscoelastic properties of NR solutions, which cause the curl structure in electrospun fibres. The relaxation behaviour of polymer solution can be tested from the rheometer testing. As seen from Table 4-3, relaxation time of NR solution reduced with increase of concentration.

<table>
<thead>
<tr>
<th>Concentration /g/L</th>
<th>25</th>
<th>20</th>
<th>10</th>
<th>5</th>
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<tbody>
<tr>
<td>Relaxation time/s</td>
<td>0.005</td>
<td>0.008</td>
<td>0.010</td>
<td>0.019</td>
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</table>

Fibre relaxation behaviour related to the fibre flying time during electrospinning, Coulomb force and modulus of solid polymer material. The modulus of solid materials has been decided. Therefore, the factors influencing fibre morphology are the flying time and Coulomb force, which will be influenced by the solvent evaporation, distance and voltage. Dynamic modulus of NR solution was shown in Figure A-5. Both the storage modulus and loss modulus increased with increase of angular frequency (ω) in the range of 1 to 100 rad/s and the two curves crossed at ω =200 rad/s. The relaxation time of the solution can be estimated from: \( \lambda * \omega = 1 \) at crossing point. The relaxation time of 25g/L NR/THF solution is 0.005s. The fitting and calculation process of relaxation time was shown in appendix (Figure A-4).
Relaxation time can be a bridge to connect dynamic properties of polymer solution and polymer jet with morphology of electrospun fibres. Relaxations that influence the morphology of electrospun fibres refer to solution relaxation time and relaxation of elastic fibres. The relaxation of electrospun fibres after solidification on the collector influence mainly on the morphology of electrospun fibres. The relative length of relaxation time will influence the final straight or curl fibres on the collector. The travel time depended on the feeding rate together with the distance between nozzle tip and collector.

2) Relaxation and stretching competition

Competition of stretching and relaxation of electrospun fibres during the polymer jets flying between needle tip and the collector depended on the flying time and relaxation time. Distance was decided and the electrical force and feeding rate were selected for controlling alignment of the electrospun fibres. The stretching rate of the fibres can be estimated from the FTIR and fibre diameter [153]. The flying time between the collector and needle tip depended on the distance and the initial speed of the polymer jet. Big feeding rate and short distance contribute to smaller travelling time. The morphology of electrospun fibres would be decided by the comparison of the relaxation time and flying time. If the flying time is much bigger than the relaxation time, the fibres would be relaxed fibres with curl structure. While when the flying time is shorter than the relaxation time, the fibres would be stretched and showed straight structure. The relaxation time of polymer would be the main decided factor for the relaxation time.

The competition between relaxation and stretching of the polymer jets along time and space contributed to the curl structure and straight morphologies of electrospun fibres, which could be used to control fibre morphology. The stretching rate difference of NR fibres could be revealed from FTIR spectra peak intensity at 837 cm\(^{-1}\). Aligned fibres were usually stretched more than curl fibres. The stretching rate can be estimated from the diameter and the peak intensity at 837 cm\(^{-1}\) in FTIR spectra [153]. Comparison of fibre diameter difference along one fibre in collected on the edge of hollow window and stretched straight fibres among the hollow window would provide the stretching rate of the fibres together with mechanical properties of the fibre.
It was reported that additional force from outside or two different polymers can cause the spring-like structure, however, in this case, no additional outside force and only one polymer caused spring-like structure. Possible reason is that polymer chain relaxation time is shorter than the evaporation of solvents or solidification of electrospun fibres before it attaches the collector. Spring-like fibres usually need an external force [124, 154-157]. Relaxation after stretching and mechanical instability while landing on the collector will contribute to curly structure in electrospun fibres (Figure 4-16). Helical or spring-like structure fibres depends on additional efforts from outside, such as: relative forces or relative moving. Therefore, two factors will contribute to the curly fibres, internal cause: viscoelasticity from polymer materials and relative force or moving from outside. The basic reason lies in viscoelasticity of polymer jet. Viscoelasticity contributes to stretching and relaxation during electrospinning. The curl structure NR fibres were from initial force, which was different from additional force added in the references [121, 153-156].

Figure 4-16 Curl electrospun NR fibres

4.3.3 Collection patterns

1) Hollow window

Figure 4-17 showed enlarged images of aligned fibres collected among hollow window. The fibres were not completely parallel to each other under microscopes though they tended to be parallel to each other in the photo. However, they were all straight fibres, and were all stretched.
In the SEM image of aligned NR fibres, fibres tended to be sticky to the nearby fibres and the fibres were stretched. The average diameter of single fibres was around 1.23 micrometer. The fibres on the edge of hollow window kept the parallel alignment pattern and the average diameter of the single fibres are similar (1.29 μm) to the parallel aligned fibres (1.23 μm) among the hollow window. Compared with the random fibres collected on the other part of the collector, the average diameter of parallel aligned fibres was smaller as seen in Figure 4-17. The straight and thinner morphology of the parallel aligned fibres should be resulted from the stretching of fibres by a stronger force or electric field that contributes to much stronger force to stretch the fibres.

Figure 4-17  Electrospun fibres collected: a) over hollow window and b) on the edge of hollow window
High viscoelasticity and sticky properties of elastomers will contribute to non-uniformity of electrospun fibres during collection process. Coiled nano-/micro-fibres can be obtained from electrospinning [74, 88, 122-124, 154, 156-162] based on relaxation after stretching and mechanical instability while landing on the collector. Helical or spring-like structure fibres will depend on additional efforts from outside, such as: relative forces or relative moving [122-124, 159, 161]. The non-uniformity can be divided into three types: twisting or buckling, diameter variety and cylinder shape damage. The non-uniformity is attributed to three main unstable transformation processes. First process refers to the stretching of polymer jet in the electric field. Electrical force difference in different position along the polymer jet causes stretching and turning of polymer jet. Second process refers to fibre solidification process. The floating fibres may not be completely dry when they reach collector, which damage fibre’s cylinder shape. Thirdly, when the fibres attached on the collector, the electric force disappeared and the fibre may contract if the elastic stress is bigger than the attachment between fibre and collector. The different contact points cause different degree of contraction, which contribute to diameter variety along the fibre.

The collecting pattern can be modified to improve uniformity of electrospun fibres. Dynamical instability during the collection of elastic fibres on the aluminum sheet collector mainly contributed to the non-uniformity of electrospun NR fibres. The high elasticity of natural rubber is dependent on its molecular structure, which is hard to change. To decrease reactions between collector and electrospun fibres, water bath collector was used. Uniform fibres can be got from an aluminum collector with a hollow window in the middle. Between the parallel edges of hollow window, fibres were parallel aligned (Figure 4-17). This can be a good strategy to avoid twisting, bending and crossing. The fibres collected on the water surface showed uniform diameter and less twisting due to reduced friction during fibre contraction.

The electrical field line tends to be perpendicular to the metal surface. For the flat Al foil, the electrical field line will be parallel to each other and perpendicular to the collector surface. Therefore, the fibres would be aligned randomly on collector surface. When the collector was void, the electrical field line also follows the rule that it will be perpendicular to the metal surface. Tiny surface around the void area will be bent over the hollow window. The electrical field line distribution among hollow window area
was stimulated by the ANSYS software. The effect of hollow window on the electrical field line could be seen in Figure A-14. The thickness of collector contributed to tiny surface of void window, which would lead to electrical field line bending. The bending of electrical field will cause stretching force and attaching force to the collector on fibres. Fibres collected on the hollow window with different shapes have been shown in appendix (Figure A-13).

2) Water bath

![Image](image_url)

Figure 4-18 Uniform film collected on water surface and buckle structure showed in microscope images

Fibres collected on the water surface showed some knot from the fast relaxation of polymer jet after being attached on the water surface (Figure 4-18). The stress from the stretching will contribute to the relaxation of the polymer jet. On the aluminium foil, the relaxation is usually stopped by the stickiness between NR fibres and Al foil, which contribute to the big buckle in the fibres. The fibres collected on the water suffered much smaller friction to stop the relaxation. With increase of the thickness, fibres will stick to the previously collected fibres and their relaxation would be stopped by the stickiness.

4.4 Characterization of electrospun NR fibres

4.4.1 Chemical structure of electrospun fibres

1) Solvent effects
Figure 4-19  FTIR of electrospun NR fibres from different solvents with the same concentration at 20g/L

The electrospun NR fibres kept most FTIR absorption features of solid natural rubber materials. Fibres from electrospun NR with different concentrations in four solvents were characterized by ATR-FTIR and the results were shown in Figure 4-19. The characteristic absorption bands of natural rubber: 2850 cm\(^{-1}\), 2917 cm\(^{-1}\) and 2964 cm\(^{-1}\) can be found in all the results.

NR fibres electrospun from different solvents with the same concentration at 20g/L were used for investigating solvent’s influence on electrospun fibre structure. As shown in Figure 4-19, there was no much difference from 3400 cm\(^{-1}\) to 2500 cm\(^{-1}\) except a tiny difference at around 2916 cm\(^{-1}\). Several peaks from impurities have been seen in that of NR in toluene at 1074 cm\(^{-1}\) and 695 cm\(^{-1}\), which were attributed from residue of toluene in the fibres. Therefore, solvent does not play an important role in the structure of electrospun fibres.
2) Concentration effects

NR solution with higher concentration has a higher crystallinity degree in electrospun fibres. Obvious difference in electrospun NR fibres from different concentrations lied in the peak position and height at 837 cm\(^{-1}\) as seen in Figure 4-20. Electrospinnability of NR solution with different concentrations was different. Therefore, the stretching rate during electrospinning process would be different, which would lead to different molecular regularity in NR fibres. Peak at around 837 cm\(^{-1}\) corresponding to C-H wag in -CH\(_3\) is related to crystal behaviour of NR. The strained induced crystal behaviour of NR could bridge FTIR peak at 837 cm\(^{-1}\) and stretching rate during electrospinning process.
FTIR spectra of electrospun NR fibres with different concentrations from toluene were shown in Figure 4-21. The FTIR absorption pattern is similar for the electrospun NR fibres from chloroform with different concentrations except NR/chloroform at 15g/L and 10g/L. The peaks at 1718 cm\(^{-1}\) and 1737 cm\(^{-1}\) were just found in the lower concentration 10g/L and 20g/L, respectively, which were assigned to free and hydrogen-bonded ester C=O group, respectively. The possible reasons for these two peaks were due to oxidation of electrospun NR fibres in the air and double bonds in NR molecules were oxidized to C=O with attending of oxygen in the air.

Natural rubber is easy oxidated by O\(_3\), UV and thermal conditions. The surface area of natural rubber increased dramatically after electrospinning, which also increased contact area of natural rubber with the environment and in turn caused more oxidation. To protect electrospun NR fibres from oxidation, several strategies would be feasible.
First, store the fibres under water. Natural rubber is insoluble in water and the water can separate natural rubber fibres with air. Second, store the fibres in vacuum conditions. The fibres on Al foils can be stored in vacuum dryer to prevent them contacting with air. Thirdly, the fibres can also be stored under low temperature such as in fridge or freezer. The fibres can be put in a selfhealed bag and stored in the fridge would be an effective and convenient method.

![FTIR result of electrospun fibres from NR/toluene solutions with different concentration](image)

Figure 4-21 FTIR result of electrospun fibres from NR/toluene solutions with different concentration

Different stretching rates will cause different peaks at 844 cm\(^{-1}\) [153], which can be used to estimate final stretching rate of the fibres after electrospinning. NR, especially for the crosslinked NR, has strain induced crystal (SIC) behaviour, which has been widely reported [163-165]. During electrospinning process, NR molecular chains were stretched and oriented and crystallinity will change during stretching and relaxation process. The change of crystallinity can be reflected in FTIR spectra at around 835 cm\(^{-1}\) to 845 cm\(^{-1}\).
4.4.2 Crystal structure analysis of electrospun fibres

XRD was used for characterising crystal behaviour of electrospun fibres. The comparison of XRD pattern of NR and ENR is shown in Figure 4-22. The blue line stands for NR without electrospinning, in which a large peak appears between 5° to 30°. The broad peak between 12° and 22° is considered from amorphous structure of NR molecules [166]. However, this broad peak disappeared in electrospun NR fibres. During electrospinning process, regularity of NR molecules in polymer jet increases due to applied strong stretch force [167]. The increase in regularity contributes to decrease of amorphous region in electrospun fibres. In addition, peaks appearing at 31.5° and 38.5° are considered as crystal structure of NaCl. Peak at 45° is from aluminium sheet.

![Figure 4-22 Comparison of XRD results of NR and electrospun NR: NR refers to solid NR, NR-chloroform, NR-dichloromethane and NR-THF refer to electrospun NR fibres from different solvents.](image)

The crystal rate of NR has been calculated from XRD data [163, 168].
$A_c$ is the peaks area of crystal NR; $A_a$ refers to the peak area of amorphous NR which is the diffraction peak areas below planes (200) and (120) and corresponded to 2θ value at 12° and 18° respectively. $A_a$ decreased dramatically after electrospinning, which contributed to increase of $X_c$. Therefore, the calculation of crystal rate from XRD results showed increase of crystallinity degree after electrospinning.

4.4.3 Microstructure investigation

1) Static testing

The SAXS scattering pattern of NR and ENR was shown in Figure 4-23. The scattering pattern of natural rubber was a round shape and showed that NR is isotropy, which means the molecular arrangement is random. In the scattering pattern of electrospun natural rubber, the scattering pattern is ellipse, which means that the electrospun natural rubber is orientated to certain direction. As the diameter of fibre is around 2-3μm, the SAXS testing window is on the surface of the fibre. Therefore, the orientation should be from the molecular orientation of natural rubber along fibre length direction.

Figure 4-23 Comparison of SAXS scattering pattern (a): NR and (b): electrospun NR.
2) In situ SAXS testing

X-ray dose was estimated from burning sample with X-ray at different exposure time, which was digitized by a flatbed scanner at 12,800 dpi (EPSON Perfection 1660). The image was recorded in Figure 4-24. 1s, 2s and 5s were used as scanning time on sample to investigate X-ray damage on the sample. The effects of scan time at 1s and 2s were below zero and when the scan time reached to 5s, the damage to the sample is nearly zero, which can be neglected. The longer scanning time can get stronger information about the scattering. Therefore, 5s was selected as the scanning time during stretching.

![Figure 4-24 Sample damage testing under different scanning time](image)

Stretched NR fibres showed different SAXS pattern (Figure 4-25). The number after ENR was the scanning order. Oblate shape of the SAXS pattern became flat with stretching. During the stretching process, ENR10 to ENR 11 was the fibre alignment direction change, which caused a big change of SAXS pattern tilt angle. This change is from the movement of fibre. From ENR11 to ENR16, the fibres were stretched along a certain direction and the tilt angle remained the same. The elongation of the fibres caused the molecular alignment in the fibre changed, which resulted in sharper SAXS pattern. The Oblate shape transferred from roughly round shape (ENR 11) to sharp flat pattern (ENR 16). From ENR 17, the elliptical SAXS pattern was pierced by a “sharp thorn”, which was resulted from highly aligned structure in the sample. With stretching, fibre was elongated first followed by polymer chains’ movement. NR materials have a strain-induced crystal behaviour. The sharp “thorn” was from scattering pattern of
strain-caused crystal structure. The scattering pattern of crystal structure can also be seen in the angular average SAXS pattern results (Figure 4-26). ENR 17-19 showed two scattering peaks at $90^\circ \sim 100^\circ$ and $-90^\circ \sim -100^\circ$ range respectively. These scattering peaks were from regular X-ray scattering from regular structure. With elongation of fibre (from ENR17 to ENR19), regularity of crystal structure increased with stretching and the scattering intensity at these directions.

![Figure 4-25 Fibre morphologies corresponds with SAXS pattern: stretching rate 0.5mm/min](image)

4.5 Conclusions

In this chapter, we investigated the conditions for steadily electrospinning natural rubber and the properties of electrospun NR fibres. THF was selected as solvent for electrospinning NR solution with a concentration ranging from 10 g/L to 40g/L and 25g/L solution was mostly used in the project. The formation of gels in NR/THF and the effects on stability of electrospinning have been investigated. Both filtration and centrifuge can increase stability of electrospinning by removing gel component. The optimal volume ratio of NaCl solution to NR solution is 4:100.
Processing parameters that influenced stability of electrospinning NR solution were investigated in terms of voltage range, needle diameter and feeding rate. Steady electrospinning process would happen when the critical voltage was 3.6kV. Characteristic length of Taylor cone increased with increase of voltage. The fibre diameter decreased with increase of voltage and the morphology of fibres tended to be straighter. Smaller needle diameter will generally lead to the electrospinning of curl fibres.

The competition between relaxation and stretching of electrospun fibres contributed mostly to the curl or straight morphologies. When the relaxation is stronger than the stretching, the electrospun fibres showed as curl structure and while stretching is stronger than relaxation, electrospun fibres would be stretched and were straight. Elastic resilience and viscoelasticity of NR lead to transform between curl and straight morphologies based on competition result between relaxation and stretching. The fibres collected among hollow window collector were straight due to high stretching effect from additional electrical field. The fibres collected on the water surface were curl with small knots due to the relaxation effect being much stronger than that of stretching. FTIR results showed that the solvent played a negligible role in electrospun
fibres structure and the stretching rate of electrospun fibres were influenced by the solution concentration. XRD and DSC results demonstrated that during electrospinning process, crystallinity degree of NR materials increased. In situ SAXS testing of electrospun fibres during mechanical testing results demonstrated molecular structure changes in NR fibres. Strain-induced crystal in electrospun fibres during stretching process was reflected in the SAXS pattern shape changes.
Core-shell structure NR-PVP micro-fibres from co-axial electrospinning

5.1 Introduction

In this chapter, we will focus on the preparation of intact core-shell structure fibres from the steady co-axial electrospinning through the effective combination of core and shell solution properties with the processing parameters. In the first part, the compatibility of NR and PVP materials was estimated from optical microscope and DSC. Secondly, the stability of co-axial electrospinning controlled by the relative properties of core and shell fluids, such as rheological properties, concentration, feeding rate, conductivity together with the effects of processing parameters, such as voltage, relative feeding rate, diameter of inner needle and the relative length of inner needle were investigated. At last, intact core-shell structure fibres were prepared from controlling the stability of co-axial electrospinning involved with the characterization of the core-shell structure and properties.

5.2 Compatibility of NR and PVP

Compatibility of NR and PVP can be studied by direct observation and indirect estimation by glass transition temperature of the amorphous phases in the sample. Direct observation includes visual or microscopic observation, which is based on different refractive indices. Indirect study of polymer-polymer miscibility involves observation of glass transition temperature ($T_g$) in the mixture [169].

5.2.1 Morphology analysis

Morphology of NR-PVP film from solvent evaporation of NR-PVP blend in chloroform solution provides miscibility of NR and PVP. NR/PVP (1:3 in weight) blend film showed phase separation (Figure 5-1). PVP and NR could dissolve in chloroform and got uniform solution. The micro-phase separation could be investigated under optical microscope (400× and 1000×). As seen in Figure 5-1, NR and PVP distributed uniformly in the blend. From dissolving film in water, PVP phase could be removed and NR micro-
particles left. The micro-phase separation in NR-PVP composite film showed that NR and PVP were miscible. The phase size is around 1~2μm.

5.2.2 DSC analysis

DSC testing is an effective method for estimating compatibility of solid mixture of NR and PVP. The phase separation process would be a de-mixing process. The heat change (ΔH) would be corresponding to that of the mixing process. Therefore, miscibility can be assessed by the heat during mixing. The miscibility of polymer blends, strictly means a single phase system in thermal dynamics. While in the practice, the miscibility of polymer blends means that they are homogenous.

Figure 5-1 Optical microscope images of NR-PVP film surface morphology
The glass transition temperature ($T_g$) of the blend is usually measured to assess the miscibility. The resolution of the methods is listed in Table 5-1. The $T_g$ distance should be big enough to use the $T_g$ method. $\Delta T_g$ should be larger than 20 K in binary system, especially for the polymer with a wide $T_g$ range. The narrow glass transition means a miscible system. In reverse, wide glass transition is usually due to the immiscibility of the blend. The sample tested by DSC was composed of 3.59mg PVP and 7.91mg NR.

As can be seen in the DSC curve of NR-PVP (Figure 5-2), there were 3 glass transition processes in NR-PVP blend, in which two of the $T_g$s (-63.75 °C and 182.09 °C were corresponding to that of NR and PVP, respectively. The middle one (42.55 °C) should be from the miscible blend part of NR and PVP, which means that NR and PVP could be miscible.

<table>
<thead>
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The $T_g$s of NR-PVP blend with different ratio could indicate the compatibility of NR and PVP in the solid mixture state. As can be seen in Figure 5-3, the third $T_g$ increased with weight ratio of PVP in the component. Zheng et al. [170] pointed that composition influence on $T_g$s is related to miscibility. Phase separation happened during the mixing of NR and PVP and the third $T_g$ showed that a miscible uniform phase made of NR and PVP formed in the mixture. It demonstrated that NR and PVP were partially miscible.
5.3 Stability control of co-axial electrospinning

5.3.1 Feeding rate selection

Phase separation happened during the mixing of core and shell fluids under proper volume ratio. It was observed that the mixture phase changed with the ratio. Phase separation happened when the volume ratio of NR solution to PVP was more than 1:3 and PVP: NR was less than 1:4 (Table 5-2). The homogenous mixture of NR solution and PVP solution, the volume ration of NR to PVP was either less than 1:4 or more than 5:1. Two phases were observed when the volume ratio of NR to PVP was more than 1:3 and less than 4:1. Miscibility of core and shell is crucial to make core-shell structure fibres. Immiscible core and shell fluids contributed to intact separation of core and shell layer [99]. Otherwise, mixing of core and shell layer would happen. The introduction of core-shell structure of NR-PVP fibres is to protect NR materials from being in directly contact with the environment when used as blood vessel. The core and shell should be separated perfectly and no exposure of NR materials, which means that phase separation should happen between core and shell fluids. Therefore, the volume ratio of core fluid and shell fluid should be more than 1:3 and less than 4:1.

Table 5-2 Phase number of NR/PVP solution mixture at different volume ratio

<table>
<thead>
<tr>
<th></th>
<th>1:5</th>
<th>1:4</th>
<th>1:3</th>
<th>1:2</th>
<th>1:1</th>
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<tr>
<td>NR:PVP</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PVP:NR</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Microstructure of emulsion was investigated under microscope as shown in Figure 5-4. From the microscope images of the emulsion, the particles with diameter around 3.17μm were from PVP/water solution. Also some smaller particles were observed. Larger particles with an average diameter around 21.12μm are observed from NR/THF in water, which were estimated from the light transmission of particles.
In summary, the influence of volume ratio of NR/THF solution and PVP/water solution on the miscibility of the mixture solution has been investigated. It showed that the ratio range of phase separation was more than 1:3 and less than 4:1.

5.3.2 Relative rheological properties

1) Rheological properties of core and shell fluids

The factors that influence stability of co-axial electrospinning have been discussed by Moghe and Gupta [99] and Elahi et al [171]. The miscible solvents of core and shell will contribute to core and shell mixing. In our system, THF and water are miscible in any ratio. Generally, the electrospinnability of shell fluid will influence the co-axial electrospinning process most. However, elastic natural rubber will cause instability of core electrospinning, which will influence stability of co-axial electrospinning.

The rheological behavior of NR and PVP solutions demonstrated flexible NR solution and rigid PVP solution. The flow curve of PVP solution with different concentrations and NR solutions are shown in Figure 5-5. All the solutions were of good shear thinning behavior, in which dynamic viscosity decreased with increase of shear rate. The PVP solution showed better stability with increase of shear rate than the NR solutions, whose viscosity decreased dramatically from 100 pas to around 0.01pas. Modulus testing showed higher elasticity of NR solution than PVP solution, which was selected for electrospinning. Also, zero shear viscosity of NR solution (25g/L, around 2.46%, wt %) was similar to that of PVP solution (30%, wt %, Figure 5-5a) due to more entanglement in NR solution from high molecular weight. In the solution with higher concentration,
viscosity decreased continuously with increase of shear rate. It demonstrated that thicker solutions were of better dynamic stability. The relation between concentration and fibre diameter was shown in appendix (Figure A-3). It showed that with increase of concentration, fibre diameter increased.

The relationship between shear rate and viscosity follows the power law in flow curve. A widely accepted experimental description of the shear thinning of polymer solutions, Carreau model was used for fitting shear thinning viscosity. Apparent viscosity can be expressed from the viscosity ~ shear rate curve got from flow model testing [172, 173].

\[ \eta = \eta_{\infty} + (\eta_0 - \eta_{\infty})/(1 + (\dot{\gamma}/\dot{\gamma}_0)^m) \]

<table>
<thead>
<tr>
<th>Table 5-3 Parameters for fitting function of flow curve</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td>DPNR-1</td>
</tr>
<tr>
<td>NR-1</td>
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<tr>
<td>DPNR-2</td>
</tr>
<tr>
<td>DPNR-25g/L</td>
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<tr>
<td>DPNR-40g/L</td>
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</tbody>
</table>
Figure 5-5  Rheological behaviour of PVP (a) and NR (b) solutions
The fitting of flow curve was described in appendix in detail and the fitting results of the NR solutions were listed in Table 5-3. The fitting process was shown in appendix (Figure A-5).

2) Mixture solution properties of core and shell

Rheological properties of NR solution and PVP solution can demonstrate the interfacial interaction and the miscibility of the core and shell fluids. PVP solutions with different concentrations were mixed with 25g/L NR/THF solution. The flow curves of these solution mixture were depicted in Figure 5-6. Viscosity changed faster with shear rate, especially when the shear rate is around 5 s\(^{-1}\) to 10s\(^{-1}\). The viscosity decrease slope with shear rate happens at bigger than 100s\(^{-1}\). The mixture showed viscosity fluctuation from 1 s\(^{-1}\) to 10 s\(^{-1}\) when PVP solution concentration was less than 20%. While PVP concentration reached 20%, viscosity of the mixture decreased gradually as a single solution, which demonstrated that 20% PVP/water solution and 25g/L NR/THF solution reached rheological stability.

![Figure 5-6 Flow curves of NR solution mixture with PVP solution](image-url)
Stability of solution mixture of NR/THF and PVP/water was investigated by the modulus stability with increase of angular frequency. PVP solutions with different concentration were mixed with NR/THF solution (25g/L) for rheological testing. The rheological testing results were shown in Figure 5-7. Under frequency sweep testing model, modulus $G'$ and $G''$ both increased with frequency under stable state. While when the strain reach to 10%, elastic modulus ($G'$) of 10% PVP and 15% PVP with NR solution showed instability when the frequency is more than 10rad/s and 20rad/s respectively. While in the 20%PVP mixing with NR solution, elastic modulus ($G'$) increased gradually at 10% strain rate due to increase of entanglement in the solution.
Figure 5-7 Stability of emulsion of NR/THF and PVP/water
5.3.3 Relative length of inner and outer needle length

When the inner needle is longer than the shell needle, the Taylor cone could cover the longer part of the inner needle. Therefore, the longer part of the inner needle should be less than the jet length of shell fluid. From electrospinning of PVP, the jet length of PVP is usually not so long due to low viscoelasticity of PVP solution. Considering the high viscoelasticity of core fluid-NR solution, the longer inner needle even the equal length of the inner and outer needle is not suitable for the co-axial electrospinning NR and PVP. Yu et al. [107] reported a method that improved co-axial electrospinning, in which the inner needle used was 1.0mm shorter than the outer shell. The differences of this modification with traditional co-axial electrospinning lie in: 1) Core solution is a principal role, while shell fluid plays a supporting role in smooth and stable process. 2) Thinning of jet and part of instable process would happen in 1.0mm indented region and were surrounded by solvent instead of air, which will contribute to the difference in fibre morphology. 3) Nano-fibre products produced in solvent environment were much more stable and robust than the ones produced in air, which were sensitive to environment [174]. It also emphasized the influences of solution properties and feed rate. Lee et al. [108] pointed that the shorter inner needle contributed to formation of an envelope in shell solution and reduced instability during co-axial electrospinning PAN and SAN solutions. Encapsulation should happen while the thinning of core and shell jets.

5.3.4 Relative diameter of inner needle

Needles with different diameter were used for inner needle of co-axial electrospinning. The diameter of inner needle influenced the flow behaviour of core and shell fluid and structure of co-axial electrospun fibres in terms of average thickness of shell layer.
Core-shell structured fibres from co-axial electrospinning with different inner nozzle were showed in Figure 5-8. With increase of the inner nozzle diameter, the diameter of core fibres increased while those of the shell fibres decreased. NR fibres showed bigger diameter than the PVP fibres at the same electrospinning conditions. When the inner nozzle diameter is around 0.6mm, 0.7 mm and 0.8 mm the fibres collected showed similar diameter due to smaller needle diameter of inner needle contributed to smaller core fibre diameter. With increase of the needle diameter, the diameter of core fluid increased, which would lead to thicker fibres as shown in the image of co-axial electrospinning process. In the 0.9mm nozzle, the core-shell structured fibres were much thicker than PVP fibres. The phase contrast module of optical microscope result as seen above can see core-shell structure in the thicker fibres - thicker NR fibres inside the core, while the thinner PVP fibres were in the even thinner fibres due to increase of core nozzle caused the similar effects as diameter decreasing to the shell fluid, which would contribute to diameter decrease of shell fibres (PVP). This is why there were two types of fibres in 0.9mm inner diameter image.
5.3.5 Conductivity of core and shell fluids

Electrohydrodynamic (EHD) force just acted on one fluid. If the natural rubber solution is uncharged, the EHD will mainly added on the PVP fluid. NR solution will be driven by the PVP jet to encapsulate non-charged NR solution. NR solution which got less electric force than the PVP will be slower than the PVP solution to make sure the encapsulation. Then the friction between the two solutions will drive NR into thinner fibres. Liquids satisfying the condition $\sigma_i > \sigma_o$, where $\sigma$ is the liquid-dielectric atmosphere surface tension. Surface tension inside larger than that of outer fluid is a condition for steady structured Taylor cone. The solvent of shell fluid has a lower evaporation ability which could keep the flow behaviour of shell to level on the surface of core jet. It’s better to form an encapsulation during the co-thinning of shell and core fluid [85].

5.4 Characterization of core-shell fibres

5.4.1 Cross section investigation

FIB-SEM was used to analyse the cross section of the core-shell fibres. FIB is a very powerful technology to prepare the samples for more precise analysis of the fine areas such as the cross section in NR-PVP fibres with SEM. The surface of sample was damaged by atom sputtering. Then the inside information of the sample can be investigated by electron.

Figure 5-9 SEM images of core-shell fibres and cross-section investigation by FIB-SEM
After cutting with FIB, a nice cross-section of the fibre can be seen clearly in Figure 5-9. The average diameter of the fibre is around 2μm. Core-shell structure with white bright shell and dark core was found. The colour difference was from the conductivity difference of NR and PVP. SEM image of NR-PVP-collagen fibres was shown in appendix (Figure A-7). However, the collagen was lack of electrospinnability and it was shown as particles.

5.4.2 TEM images of core-shell structure fibres

![Figure 5-10 TEM images of core-shell structure fibres](image.png)

The morphology of co-axial electrospun NR/PVP fibres observed under TEM is shown in Figure 5-10. The contrast created by the electron beam showed distinct core-shell structure. The dark shell layer was around 50nm in thickness the diameter of core layer was around 70 nm. To make sure the eccentricity of the core-shell structure fibres, cross-section of piece sample from cryostat section of resin embodied core-shell structure fibre were investigated under TEM. Core and shell layer could be separated clearly from the electron beam contrast. The average diameter of the fibre is around 640nm, while that of the core was around 420 nm, the thickness of shell is around 110nm, which was measured by Adrobe PDF associated measurement tool.
5.4.3 Confocal microscope

![Confocal microscope images](image)

Figure 5-11 Confocal microscope images of co-electrospun PVP/FITC with NR fibres: scale bar: 5μm.

FTIC dyed shell layer core-shell structured fibres were imaged with confocal microscope (Figure 5-11). Core-shell structure was clearly seen in the confocal image of NR-FITC dyed PVP fibres. PVP dyed by FITC was green while NR fibre was black. The average diameter of core-shell structure fibre was 1.49μm. The average thickness of the shell was around 0.6 μm. According to the working theory of confocal microscope, the result was an instantaneous scanning result of core-shell fibres along fibre thickness direction, from which we can just decide that the thickness of shell was no less than 0.6 μm and the core diameter was no less than 0.4 μm. The combination of 488 nm FITC provocative image and visible light image result also showed core-shell structure.

5.4.4 Phase contrast images

Phase contrast images of core-shell structured fibres with different morphologies were shown in Figure 5-12. The phase contrast images of co-axial electrospinning fibres with FITC can describe the core-shell structure in co-axial electrospun fibres clearly. The straight fibres, curl fibres, beads are all of core-shell structure. FITC dyed PVP shells showed green color with the initiation of the light.
Figure 5-12 Phase contrast images of microscope from dark field microscope

5.4.5 FTIR mapping

Figure 5-13 FTIR mapping result of core-shell structure fibres: at 1660 cm\(^{-1}\)

FTIR mapping results of core-shell structured fibres at 1660 cm\(^{-1}\) were depicted in Figure 5-13. The peak at around 1660 cm\(^{-1}\) corresponded to stretching of carbon-carbon double bonds [175], which were characteristic functional groups in NR instead of PVP. Several points (3\(\mu\)m\(\times\)3\(\mu\)m) were selected along fibre surface. Carbon-carbon double bonds stretching signal could be seen in every points with different intensity. Light blue, green and yellow points were centred on the shell of the fibre while dark blue and cyan points were centred on the core of the fibre. Peak intensity at around 1660 cm\(^{-1}\) showed difference in different position of the fibres. Stronger peak intensity was shown in light
blue, green and yellow points while much weaker peaks appeared in dark blue and cyan points. The peak intensity difference referred to signal collection difference. Due to coverage of PVP outside, the signal reflected from core part could be weakened during signal transmitting from the shell layer. When the detection centre located on the top of the fibre, less signal inside could be transmitted. Therefore, the peak intensity at 1660 cm⁻¹, from core material, was weak on fibre centre part.

5.4.6 Water washed core-shell fibres

The co-axial electrospun NR-PVP fibres were washed with water or ethonal for investigating core and shell. From Figure 5-14, after treatment with water, the PVP shell was damaged and NR materials can be seen clear in the core. Rough surface of electrospun NR fibres can be seen after removing water-soluble PVP. Rigid PVP contributed fibre formation of NR and the curl structure of the fibre demonstrated elasticity of core-shell structure fibres.

![Microscope images of co-axial fibres after washing](image)

Figure 5-14 Microscope images of co-axial fibres after washing

5.4.7 Thermal analysis

As seen in Figure 5-15, electrospun NR fibres started thermal degradation earlier than NR solid material. NR fibres have much more surface area and micro-structure and are more sensitive to the thermal effect. However, after the initiation of the thermal degradation, weight loss rate of electrospun fibres was smaller than that of NR. This may be because inner thermal conduction of NR is better than porous NR fibres. Another
reason would lie in that the thermal degradation mechanism of solid NR materials was different from that of electrospun NR fibres due to different structure.

![Figure 5-15 Thermal analysis properties of NR and electrospun NR](image)

The DSC results of NR and its electrospun fibres were showed in Figure 5-16. The $T_g$ temperature of NR (-61.7°C) is a little lower than that of electrospun NR fibres (-61.3°C). It can be explained by the molecular chain change during electrospinning. NR molecules are coiled random thread in the solid bulk at room temperature because they are in the high elastic state and their $T_g$ is much lower than the room temperature. During the dissolving process, the coiled NR molecules were dissolved in the solution and also kept the coiled feature. While the solution was stretched, NR molecules were aligned under the reaction of electric force. With the disappearing of stretching force, the molecular chain will contract to certain degree due to molecular relaxation. However, due to crystal behaviour and strong intermolecular interaction, NR molecules were difficult to move, which caused the $T_g$ increase. On the other hand, before the NR molecules can move freely in bulk solid while in electrospun fibres, the molecular movement are limited by the fibre shape.
Crystal rate of NR increased after electrospinning. The equilibrium heat of fusion of crystal NR is around 64.8 J/g [176] (endothermic peak is at around -35°C), which could be used as a standard for calculation of crystal ration of NR materials. The average equilibrium heat of fusion (∆H) of NR raw material is around 1.78J/g, which corresponded to crystal rate at 2.75%. ∆H could reach 3.31J/g after electrospinning, which corresponded to crystal rate at around 5.11%. Stretching process during electrospinning contributed to molecular orientation.
To investigate interfacial interaction between core-shell structured fibres, $T_g$ of the polymers can be investigated by DSC and the result was showed in Figure 5-17. Co-axial electrospun NR-PVP on aluminium sheet were used for DSC testing directly without pilling them. Heat flow change with increase of temperature during heating process were recorded in Figure 5-17. Glass transition process of natural rubber happened at round -75ºC, which was shown as a sudden drop of heat flow at around -70ºC. A second glass transition process happened at around -35ºC should be corresponding to the $T_g$ of NR/PVP miscible part. The peak at around 87ºC referred to water bonded molecules [177]. Glass transition of PVP was around 180ºC. Natural rubber has no obvious melt point. It melts completely at around 130ºC. Therefore, there was no melt heat absorption peak in NR’s DSC curve. When the temperature reached 300ºC, natural rubber began to degrade.

5.5 Conclusions

It demonstrated that NR and PVP were partially compatible through $T_g$ analysis and morphology observation of NR/PVP blend. Emulsion made from immiscible NR/THF and PVP/water showed phase separation when volume ratio was more than 1:3 and less than 4:1, based on which relative feeding rate was selected as 1:1. Dynamic stability of NR/THF and PVP/water solution were investigated by rheological analysis of core and shell fluids. Flexible NR solution and rigid PVP solution could have steady rheological properties when concentration of PVP reached 20%. Relative shorter inner needle could form encapsulation, which could contribute to stable co-axial electrospinning. Inner needle diameter influenced morphology of the co-axial electrospun fibres and uniform core-shell structure fibres appeared when 0.8mm inner needle was used. Charged rigid PVP solution drove non-charged NR solution to avoid fibre separation. Core-shell structure was directly confirmed by FIB-SEM and TEM. Confocal microscope images and phase contrast images of FITC dyed PVP/NR fibres showed core-shell structure with color contrast. FTIR mapping indicated chemical component distribution in the fibre and PVP covered outside of core-shell structured fibres. Thermal properties testing showed that co-axial electrospun fibres has a third $T_g$ at around -35 ºC, which meant that NR and PVP formed a compatible layer on the interface.
UV radiation crosslinking to enhance interfacial interaction between core and shell

6.1 Introduction

Crosslinking of NR and PVP on the interface would enhance interfacial interaction of NR-PVP core-shell structure fibres. Interfacial interaction of polymers are of interest in many applications such as adhesives, packaging and biomedical plant [178]. The interfacial interaction will influence mechanical properties directly. PVP was first cross-linked and then the crosslinking happened on the interface, which would enhance the interfacial interaction between NR and PVP. Disinfection application of UV light with wavelength is at around 250 nm. The crosslinking in natural rubber contributes to high elasticity. Natural rubber could be crosslinked due to double bonds in the molecular chain. Traditional methods of crosslinking NR includes chemical vulcanization under catalysis of sulphur, superoxide, persulfide and physical crosslinking methods, such as thermal treatment / irradiation using UV and Y-ray [179]. Residue of toxic materials added from traditional vulcanization methods restricted application of cross-linked materials in biomedical applications. To decrease the toxicity, radiation methods were selected for crosslinking NR and PVP. Ideally, the radiation can cause crosslinking in PVP layer together with NR-PVP interface without adding toxic additives. Firstly, methods for crosslinking PVP were investigated in terms of heat annealing and UV irradiation followed by conditions for UV radiation such as wavelength of UV light and radiation time. Secondly, radical crosslinking and competition between crosslinking and photo-degradation were discussed in UV radiation of NR fibres. Finally, UV radiation of core-shell structure NR-PVP was investigated in terms of water stability and mechanical properties change after UV radiation.

6.2 Crosslinking of PVP

Methods for crosslinking PVP can be divided into three types: chemical crosslinking, thermal crosslinking and irradiation [21, 23, 118]. Chemical crosslinking refers to adding initiator of radical polymerization, such as peroxide, persulfide, which can indicate crosslinking of double bonds in PVP. Thermal crosslinking involves thermal
annealing of PVP at certain temperature. Irradiation is another method to provide energy for crosslinking of PVP. As mentioned in the reference, UV and γ ray are two of the commonly used irradiation methods. Lilian et al. prepared PVP hydrogels by direct UV radiation under low pressure Hg lamp (maximum emission wavelength 254 nm) for wound dressings and drug delivery devices [118]. However, this process is inefficient due to the competition of crosslinking and photo-degradation. Fechine et al. crosslinked PVP efficiently by radiation under a UV-C or UV-A source with acceleration of hydrogen peroxide and photo-Fenton reactions [22]. Andrew et al. prepared crosslinked PVP by thermal annealing at 200°C [21]. After crosslinking, PVP is insoluble in water and ethanol when the crosslinking density reached certain degree.

6.2.1 UV crosslinking

After UV cross-linking, fibres’ cylinder shape disappeared/faded. The average size of holes on the surface is around 0.89 μm. The fibres seemed to be melt and lost their height compared to the electrospun fibres without UV radiation. Electrospun fibres fused after UV radiation, which contributed to the decrease of pore size formed between fibres. The clear fibre boundaries become indistinctive. The holes on the surface may be caused by the giving out of hydrogen gas. Therefore, the crosslinking has been achieved in electrospun PVP fibres together with emotion of hydrogen gas.

The process of heating and annealing of PVP was following free radial mechanism, which also matched with UV radiation process [21]. The crosslinking happened intra-molecularly and inter-molecularly as shown in Figure 6-1. Due to different radical initiated points in PVP molecules as described in Figure 2-11, intermolecular crosslinking of PVP would be of different types (red line in Figure 6-1). The morphology of fibre mats after UV radiation was shown in Figure 6-2. The holes on the surface of electrospun PVP fibre mats are from giving out of hydrogen (Figure 6-1).
Electrospun PVP fibres were easily water-soluble after contacting with water. After 1 hour UV radiation, the fibres could be partially dissolving in the water after immersing in water and the fibres become water insoluble gradually with increase in radiation time, which could be seen from microscope images of UV radiated fibres immersing in water. After 3 hours radiation, the electrospun PVP fibres were completely water insoluble as seen in the third image in Figure 6-5.
Figure 6-3 compared FTIR-ATR spectra for electrospun PVP fibres radiated with UV light under different radiation time. The differences lied in the peak of amide carbonyl (1600cm⁻¹ to 1700cm⁻¹) and the peak width increased with increase of radiation time. Single peak was shown in this area in the spectra of electrospun PVP fibres without UV radiation. However, two or three peaks were in that of electrospun PVP fibres after UV radiation. It indicated that amide bonds in PVP were partially oxidated to carboxylic group. It also showed change of C-H bonds, which corresponded to the peak around 1650 -1655 cm⁻¹ C=O.
1424 cm\(^{-1}\) and 1440 cm\(^{-1}\). The difference in FTIR spectra between PVP and UV crosslinked PVP was shown in Figure 6-3. The peak areas at around 1424 cm\(^{-1}\) and 1440 cm\(^{-1}\) decreased after UV radiation, which meant that C-H bonds decreased after crosslinking. The new peak appeared at around 1657 cm\(^{-1}\) refers to C=O stretching due to influence of hydrogen bond. After UV radiation, hydrogen bonds increased.

6.2.2 Comparison with heat annealing

The electrospun PVP fibres were annealed at 160°C, 170°C and 180°C in the oven for 0.5 hour, respectively. The changes in chemical structures of electrospun PVP at different annealing temperature could be seen in FTIR spectra (Figure 6-4). FTIR adsorption peak at 2923 cm\(^{-1}\) refers to C-H stretching. With increase of temperature (from 160°C to 180 °C), this peak becomes sharper, which means that the ratio of C-H decreased. Strong peak at about 1651 cm\(^{-1}\) corresponded to C=O stretching. The increasing peak area at 1651 cm\(^{-1}\) with temperature reflected possible oxidation during heat annealing. It seems that the fibres dissolved in water after annealing. The fibres were not completely broken after annealing at 180 °C. Then the electrospun fibres were annealed at 200 °C for half an hour, the fibres were not dissolved in water. However, the melt temperature of natural rubber is around 130 °C, at which the electrospun PVP were not crosslinked at all. According to the reference, C-H bonds broke after annealing and the hydrogen content decreased [21]. The breaking of C-H and formed radical could contribute to hydrogen formation.
Figure 6-4 Normalized FTIR results of heat annealing effects on electrospun PVP at different temperature: PVP-160, PVP-170 and PVP-180 refer to annealing at 160 °C, 170 °C, and 180 °C, respectively.

Due to the melt temperature of natural rubber (around 130 °C) is much lower than the annealing temperature of electrospun PVP, which is also close to the degradation temperature of natural rubber (230 °C), thermal crosslinking may be not a good strategy to decrease water solubility of electrospun PVP. As the chemical crosslinking methods usually involve toxic initiator, irradiation would be the suitable method for crosslinking electrospun PVP to increase its water stability. In addition, the double bounds in natural rubber can also be crosslinked by the irradiation, such as UV and $\gamma$ ray. On the other hand, UV irradiation may also cause crosslinking of natural rubber. It is possible that crosslinking will happen between NR and PVP due to the similarity of double bonds, which would increase interfacial interaction between NR and PVP.

6.2.3 UV crosslinking time

UV crosslinked electrospun PVP fibres were found to be resistant to water due to molecular network formed intra and inter PVP molecules and the water solubility
depended on radiation time. Water solubility change of crosslinked electrospun PVP fibres could be estimated by optical microscope observation. Electrospun fibres were partially dissolved in water when they were radiated under UV for 1 hour. When radiation time increased, soluble degree decreased as can be seen in Figure 6-5. Electrospun PVP fibres with high crosslinking degree was not water soluble after being immersed in water. The fibre morphology remained well for up to 2 weeks.

![Figure 6-5 Water stability testing of fibres with different UV radiation time: a) 1 hour; b) 2 hours; c) 3 hours](image)

6.2.4 Chemical reaction

Change in chemical structure of electrospun NR fibres was characterized with FTIR spectra. The results of FTIR of ENR with different radiation time were shown in Figure 6-6. After UV radiation, structure of NR would change from double bonds (C=C) to single bonds (C-C). The FTIR change of electrospun NR during UV radiation can be used to estimate chemical structure change. With increase of radiation time, more and more C=C bonds were broken with formation of C-C bonds. The FTIR absorption peak intensity at around 840 cm\(^{-1}\), which was related to C-H wagging, decreased due to the chemical structure change.
Table 6-1 Peaks corresponding in chemical groups of electrospun NR

<table>
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<tr>
<th>No.</th>
<th>Bond</th>
<th>Stretching</th>
<th>Rocking</th>
<th>Twisting</th>
<th>Waggling</th>
<th>Deformation</th>
<th>In plane bending</th>
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<td>1,2</td>
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<td>1089-1100</td>
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<td></td>
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<tr>
<td>3, 4, 5</td>
<td>CH$_2$-C/CH$_3$/</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>C=CH</td>
<td>1665</td>
<td>-</td>
<td>-</td>
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<td>6</td>
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<td>2855</td>
<td>740, 764</td>
<td>1242</td>
<td>1130/1311</td>
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<td>-</td>
<td></td>
<td>837</td>
<td>1286</td>
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</tr>
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</table>
Figure 6-6 FTIR of UV radiated electrospun NR fibres with different radiation time
Two steps of NR oxidation during UV radiation were described by dos Santos et al [180]. For the radiation at 254 nm, crosslinking reaction dominated while chain scission prevailed when the wavelength ranged from 300 nm to 350 nm. Crosslinking prevailed to photo-degradation at 254 nm, while wavelength is larger than 300 nm, chain scission dominated. The effects of irradiation time on crosslinking can be seen from intensity change at 836cm⁻¹ (=C-H wag). 254 nm UV light was used for pre-irradiation for 10 minutes and 300 nm UV light was used for radiation. 254 nm was just used for irradiation of the reaction, while the reaction can last at 300 nm wavelength UV light.

![Figure 6-7 Functional bonds in Natural rubber](image)

6.2.5 Competition between crosslinking and photo-degradation

Competition between crosslinking and photo-degradation exist in UV radiation of NR materials. Santos et al. reported pre-irradiation with short wavelength UV ray (254 nm) can stop further oxidation of NR film[180]. Dramatic increase in surface areas after electrospinning raises oxidation risk on NR fibres. After UV radiation, structure of NR would change from double bonds (C=C) to single bonds (C-C). The wag modes were observed at 837 for NR, which decreased after UV exposure when the wavelength is longer than 300nm. It kept the same under UV exposure with wavelength at around 254 nm. For the radiation at 254 nm, crosslinking reaction dominate while chain scission prevailed when the wavelength ranged from 300 nm to 350 nm. The obvious change
happened at between 1500 to 2000 cm\(^{-1}\) (around 1700 cm\(^{-1}\)). Different radiation time influences the peak intensity at around 1750 (C=O stretching in carboxylic acid) or 1715 cm\(^{-1}\), which is corresponding to stronger C=O stretching in ketone[180]. Different radiation time influence the peak intensity at around 1750 or 1715 cm\(^{-1}\), which correspond to carboxylic acid or ketone.

### 6.3 UV radiation of core-shell structure fibres

#### 6.3.1 Stability in water

An optical microscope image of half washed UV radiated core-shell structured fibres was shown in Figure 6-8. UV radiated core-shell fibres were found to be resistant to water. The change of solubility could be easily estimated from the morphology when the fibres were immersed in water. Shell of core-shell structure fibres was partially dissolved in water after 2-hour UV radiation while the shell of one that was UV radiated was completely insoluble in water.

![Figure 6-8 UV radiated core-shell fibres after water washing](image)

UV radiated electrospun PVP fibres were found to be resistant to water. The change of solubility could be easily estimated. After UV radiation, shell layer (PVP) was easily
crosslinked under UV radiation due to its larger contact area and thinner thickness. Therefore, the shell layer was insoluble in water. The core layer (NR) was insoluble in water, no matter it was crosslinked or not. Therefore, after UV radiation, the core-shell structure fibres were water insoluble.

6.3.2 Peeling issues

Core-shell structured NR-PVP fibres are much easier to be peeled from Al foil than NR fibres (Figure 6-9). Peeling electrospun fibres from Al foil is hard for many electrospun fibres due to strong attachment and weakness of the fibres. Electrospun fibres are useless unless they are peeled from the supporting aluminium foil, thought they have many advantaged features for advanced applications [76, 181]. Here two methods would be used for collecting electrospun fibres: core-shell structure fibres and non-solvent surface collection. This could be used to peel a fibre mat which was attached very tightly to the Al foil. For core-shell structure NR-PVP fibres, NR is insoluble in water, while PVP is water soluble. Then after peeling, washing away shell material with
water can get ENR fibres. Interaction between PVP and aluminium foil is much weaker than that between NR and aluminium.

Polymers with bad interaction between Al foil would be potentially used as the shell materials for peeling. Al sheet is commonly used as collecting medium for electrospinning. However, some of the polymers are difficult to be removed from Al sheet due to their good attachment to the aluminium material and weak mechanical properties of fine fibres. Therefore, peeling issue of electrospun fibres from aluminium restricts application of electrospinning. To investigate influence of topology of electrospun fibres on adhesion to Al foil, Ballarin et al. used T-peel test to bulk spin PCL and Al [182]. ToF-SIMS and XPS were used to investigate interfacial interaction between aluminium and PMMA by Kyoko et al. [183]. Equilibrium swelling was used to investigate the interaction between natural rubber and aluminium in their composite [184].

6.3.3 FTIR analysis of UV radiated samples with different time

FTIR spectra at 3100-2700 cm⁻¹ of NR-PVP core-shell structure fibres with different UV radiation time: after UV radiation for 4 hours, the FTIR absorption peak appeared at around 2720 cm⁻¹ and peak at around 3050 cm⁻¹ became bigger and bigger with increase of radiation time. Figure 6-10 gave out FTIR features in electrospun fibres with different radiation time at 1100 to 600 cm⁻¹. Peak appeared at around 1047 cm⁻¹ in the longer radiation samples (0, 2hours, 3hours and 4 hours), but not in the shorter radiated samples (less than 2 hours). C=C become weak and C=O, C-O became stronger, which means that peaks at 837 cm⁻¹, 1650 cm⁻¹ and 3036 cm⁻¹ will decrease while the peaks at 1765, 1166 and 3450 cm⁻¹ would become stronger.
Figure 6-10 FTIR-ATR spectra of UV radiated fibres with different radiation time
6.3.4 Degradability

Electrospun NR fibres were easily degraded in air because they were fully exposed to oxygen. The degradation of electrospun NR fibres started from the fibre surface and the fibres degraded in the small pores on the surface and gradually, the degradation caused breaking of fibre and the fibres were divided into fragment. As shown in Figure 6-11, when the fibres were degraded for 10 days, the surface of the fibres became porous (as shown in a). Fibre continuity was damaged when the degradation time reached 20 days (as shown in b).

![Electrospun NR fibre morphology change during degradability testing process: a) 10 days; b) 20 days.](image)

Core-shell structure fibres were degradable as well. As shown in Figure 6-12, with increase of time, fibres became degraded gradually and the fibres were completely degraded after 60 days. Comparing 10-day and 20-day degradation with ENR fibres, core-shell structure fibres were much more stable. Possible reason may lie in the PVP shell stopped air exposure from NR core, which delayed degradation.
6.4 Comparison of core-shell fibres with PVP coated fibres

6.4.1 Structure difference

Direct exposure of NR fibres to UV will cause ageing of NR. Coating of NR with PVP will protect NR fibres from UV crosslinking. However, direct coating of PVP on electrospun NR fibres will cause porosity loss and crack during extension as shown in Figure 6-13.
Comparing PVP coated NR film, core-shell structure fibres possess structure advantage with mechanical properties mismatching NR and PVP: curl structure of core-shell fibres will contribute elasticity at low stretching rate without relative movement of core and shell materials.

6.4.2 Comparison of mechanical properties

1) Electrospun fibre mats

Tensile strength properties of electrospun NR fibre mats were tested at different stretching rate: 0.5mm/min, 1.0 mm/min and 5.0mm/min. The strain-stress relationship
curve of tensile strength testing with different stretching rates were showed in Figure 6-14. The biggest elongation 639.5% was got at 5.0mm/min stretching rate with a tensile strength 132.9Mpa. The smallest elongation was about 438.0% at 0.5mm/min with a highest tensile strength 198.5Mpa. The testing at 1.0mm/min got a 470.1% elongation and 132.8Mpa tensile strength. Higher extension rate contributed to larger elongation rate because that the fast extension caused longer elongation without bearing damage from sample stress. The slow extension may cause the regular alignment of polymer chain and possible crystal, which would result in higher tensile strength.

![Graph showing tensile strength testing result of electrospun NR fibres mats](image)

Figure 6-14  Tensile strength testing result of electrospun NR fibres mats

2) Single electrospun fibres

Mechanical property of a single electrospun NR was measured by tensile strength testing (Agilent T150). The fibre diameter was obtained from SEM image. The fibres were easily to be broken. Only one fibre was set on the clamp. The tensile testing result was listed in Figure 6-14. Mechanical properties of single fibre were tested on a nano tensile strength tester. The elongation of the fibre can reach 47.4%. There is a big difference between extension properties of single fibre and that of electrospun fibre mats. The fibre collected on the hollow window was already stretched during collection.
process which was completely straight while fibres in fibre mats are curl and random aligned.

Table 6-2 Mechanical properties of single electrospun fibres

<table>
<thead>
<tr>
<th>Modulus/Gpa</th>
<th>Offset Yield Stress/Mpa</th>
<th>Offset Yield Strain/mm/mm</th>
<th>Stress At Break/ MPa</th>
<th>Strain At Break/ mm/mm</th>
<th>Toughness/ MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.269</td>
<td>6.111</td>
<td>0.023</td>
<td>8.275</td>
<td>0.144</td>
<td>1.076</td>
</tr>
</tbody>
</table>

Possible reason lies in that: electrospun fibres were extended during electrospinning process. Especially, when they were collected among the window on the collector, the fibres were extended more, because the fibres collected on the collector were curl while the fibres collected between the two edges of gap area were straight, which was resulted from fibre stretching. The stretched fibres showed less extension due to the longer original length. On the other hand, single fibre was easily broken under the same outside interruption, such as moving distance of clamp and air flow. Therefore, the final extension rate was only 47.4%.

Mechanical testing results of fibre from ENR, PVP coated fibres and core-shell structured fibres were shown in Figure 6-15. Comparing with PVP coated ENR fibres, core-shell structure fibres showed much better tensile strength and extension. PVP coated ENR fibres showed worst mechanical properties, which means that coating made the mechanical properties of ENR poorer. Core-shell structure fibres enhanced mechanical properties of ENR.
Figure 6-15 Mechanical properties of ENR fibres, PVP coated fibres and core-shell fibres

6.5 Conclusions

UV radiation was selected as the method for crosslinking electrospun PVP. The crosslinking mechanism of PVP fibres was considered as free radical process. UV wavelength was selected as 254nm, under which photo-degradation was resisted and crosslinking happened. 3 hours radiation would cause crosslinking of NR fibres without damaging cylinder shape of electrospun fibres. Crosslinking degree in electrospun fibres increased with radiation time, however, with increase of radiation time cylinder shape of the fibres would be damaged due to increase of temperature from radiation.

Crosslinking happened in both shell and core layers in core-shell structure fibres under 3 hours radiation at 254nm UV light. Water stability of shell layer (PVP) was improved and mechanical properties of the fibres enhanced a lot compared with UV radiated ENR fibres. Degradability of UV radiated fibres was improved by the protection of crosslinked PVP shell. Compared with PVP directly coated electrospun NR fibres, core-shell structure fibres possess obvious advantages in structure and properties. UV
radiation crosslinking contributed to improving water stability of PVP layer and easy peeling of core-shell structure fibres.
7 In vitro biological evaluation of scaffolds from NR based materials

7.1 Introduction

In vitro biological testing is critical for estimating biological compatibility before the scaffold is used in vivo. In vitro biological testing preliminarily evaluates the biocompatibility of materials. Basically, cytotoxicity and cell proliferation are necessary for material biological testing. Electrospun fibres with different components and morphologies were used for the test of biological compatibility, in which, cytotoxicity and cell proliferation of smooth muscle cells were tested. Smooth muscle cells, a kind of cells growing attached on the bottom of the culture flask, were selected due to structural feature of scaffolds made from electrospun fibres and the application of scaffold. Cytotoxicity of ENR, NR film, PVP coated ENR fibres and core-shell structured NR-PVP fibres was investigated by cell counting technique, optical microscope and MTT assay. Cell proliferation and viability were investigated by MTT method. The absorbance at 570nm was used for cell number due to their linear relationship at low cell number range.

Cell-scaffold interaction involves a series of proper biological responses of cells to the passive substratum, including adhesion, cell morphology, growth, differentiation, and migration [16]. Cells distribution and interfacial interaction between cell and substratum materials were investigated by optical microscope, confocal microscope and SEM. As one of the factors that influence cell-scaffold interaction, hydrophobility was investigated in terms of water contact angle testing of the materials. Crosslinking effects on cell-scaffolds interaction was also investigated. The influence of fibre morphology on cell distribution was investigated in terms of pore size, which relates to fibre diameter and fibre thickness.

Cell viability and proliferation were investigated at culture time points: day-1, day-3 and day-7. MTT assay was used for estimating cell growth curve quantitively. Cell imaging techniques such as UV microscope, confocal microscope and SEM were used for monitoring cell growing behaviour.
7.2 Fabrication and treatment

7.2.1 Fabrication of scaffolds

The electrospun scaffolds with uniform fibre diameters and thickness at around 10 μm were collected on the hollow window of Al foil. The samples were cut from Al foil with a little bit of Al foil on the edge to support and keep the scaffold unfolded. Electrospun fibres samples (0.5cm×0.5cm) were purified under high vacuum condition at room temperature overnight to make sure there was no solvent residue in the scaffold. Then the scaffolds were attached to the bottom of the cell culture flask and the Al foil edge was removed gently.

7.2.2 Treatment of scaffolds

Autoclave, ultraviolet ray and alcohol disinfection methods were investigated and UV and alcohol combination disinfection method was selected. The use of 121°C steam damaged electrospun fibres shape, especially the core-shell structure NR-PVP fibres which could be in contact with water due to water solubility of PVP. Ethanol sterilisation (for the NR-PVP core-shell/coated fibres UV crosslinking first and then ethanol) was also used followed by washing with PBS solution. It is not sufficient to disinfect scaffolds for culturing cells. UV radiation was selected for alcohol sterilized scaffolds sterilization. It showed that after 3 hours of UV light radiation, there was no contamination on the scaffolds. The UV radiation affects the solubility and cytotoxicity of the fibres [185]. Cytotoxicity was carried out after the materials were radiated with UV radiation. Therefore, UV radiation following alcohol washing was selected as sterilization methods for scaffold materials.

7.3 Cytotoxicity

It is essential that materials used for tissue engineering should be biocompatible, at least, it should be non-toxic. The cytotoxicity of scaffold materials was tested from two aspects: soak solution cytotoxicity and contact cytotoxicity. 10,000 smooth muscle cells were seeded in each well on different materials. To investigate cytotoxicity influence factors of electrospun fibres, NR film, electrospun NR (ENR) fibres, PVP coated
ENR(P-ENR) fibres and core-shell structure NR-PVP fibres (NR-PVP) were selected for cytotoxicity testing.

7.3.1 Soak solution estimation

Soak solution result could be estimated from trypan blue counting method and the dead cell number can be estimated as well. Dead cells would be blue while the alive cells were bright brown. The soak solutions were seeded SMC with a cell density at 10,000 cells/mL in incubator (37°C, 5% CO₂) for 24 hours. 0.01 mL from 1mL cell suspension was used for determining cell density by trypan blue counting after cell harvesting. The results were listed in the following table.

Table 7-1 Cells density from soak solution of different scaffold samples

<table>
<thead>
<tr>
<th></th>
<th>NR film</th>
<th>ENR fibres mats</th>
<th>PVP coated ENR fibre mats</th>
<th>Co-axial electrospun NR-PVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive cell density/(cells/mL)</td>
<td>13500</td>
<td>13800</td>
<td>13000</td>
<td>13600</td>
</tr>
<tr>
<td>Dead cell density/(cells/mL)</td>
<td>200</td>
<td>150</td>
<td>150</td>
<td>100</td>
</tr>
</tbody>
</table>

Soak solution result could be estimated from trypan blue counting method and the dead cell number can be estimated as well. Dead cells would be blue while the alive cells were bright brown.
Cell viability of NR latex is 9.14% after 2-day culture [13, 16] and 8.15% after 7 days culture. It was reported that cytotoxicity of NR film can be enhanced by coating with PMMA nanoparticles or PMMA-chitosan nano-particles [186]. It showed that all the cell viability was higher than 98% (Table 7-1), which meant that all the scaffolds used for culturing SMCs were non-cytotoxicity. All the scaffolds were non-toxic to the cells after treatment (Figure 7-1).

### 7.3.2 Optical microscope investigation

Cell viability would be estimated from optical microscope observation. The observation of the cells focused on the fibres, which may just take up quite low percentage of the total cell number. The cells growing directly on the surface of the fibres were only done on the surface of ENR fibres. The result showed that the cells can grow easily on the surface of the fibre mats. At the beginning, cells tended to be planted on the flat area while there were seldom cells on the fibre covered area. With the growing of the cells, some cells were found in the fibre mat area, which means that the fibres were not toxic. The possible reason lies in that electrospun fibres were not as flat as the plastic box bottom. The feature of HASMC cells decides that they tend to spread on the flat area after passage. As seen in Figure 7-2, round dots on the surface of NR film (a) would
be the nuclei of dyed dapi cells. It showed that cells would not be able to grow on the NR film. Stretched cells could be seen in other three images (b, c, d). Scaffolds fabricated from core-shell structure fibres showed highest cell density.

Figure 7-2 Optical microscope images of cells on different scaffolds samples after 24 hours culture: a) NR film; b) electrospun NR fibres; c) PVP coated fibres; d) core-shell structure fibres.

7.3.3 Evaluation of Cytotoxicity by MTT Assay

Scaffold cytotoxicity was evaluated by cell viability of SMC cells after being incubated for 24 hours from MTT assay. It could be used for estimating cell number due to the linear relationship between absorption of light at around 570nm and cell number. Live cell number can be investigated by a maximum absorption at 570 nm after MTT treatment. The absorbance at 570 nm is directly proportional to live cell number. The viability of treated cells could be expressed by absorbance at 570 nm ratio to that of control group without scaffolds.
Standard curve of MTT assay for SMC was carried out by the known number cell culture. To determine the appropriate cell number for cell cytotoxicity, SMC cells just harvested were diluted into a series of concentrations (200,000 cells/mL, 100,000 cells/mL, 50,000 cells/mL, 25,000 cells/mL, 12,500 cells/mL, 6,250 cells/mL, 3,125 cells/mL, 1,562 cells/mL, 781 cells/mL), which were seeded into a 96-well microplate at 70 μL/well. MTT was added after 48-hour culture and the absorbance at 570 nm was measured. As shown in Figure 7-3, the absorbance at 570 nm increased when the initial cell number increased from 781 cells/well to 6,250 cells/mL. The absorbance decreased when cell concentration was over 6,250 cells/mL due to limitation of space and nutrition (Table 7-2). The standard curve can be fitted as:

\[ y = -0.0176 + 6.887 \times 10^{-5}x \ (x < 1000). \]

<table>
<thead>
<tr>
<th>Table 7-2</th>
<th>Correlation of cell density and cell number: Cells used in standard curve: HASMC (passage 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td></td>
<td>Cells density/cells/mL</td>
</tr>
<tr>
<td></td>
<td>Cells number</td>
</tr>
</tbody>
</table>
Cytotoxicity of different scaffolds was shown in Figure 7-4. 5000 cells were seeded on different scaffolds and cultured for 24 hours before MTT was added. MTT result showed that much less cells were alive on NR film, on which there was around 500 cells (Figure 7-4). Scaffolds from core-shell structure fibres had highest cell number followed by ENR fibres and PVP coated fibres. The reason could be that the cells tend to attach on core-shell structure fibres. This could indicate that core-shell structure fibres showed better cell attachment. The result showed that electrospun fibres were non-toxic to the cells and the fibres with coating and core-shell fibres showed some certain toxicity to the cells. PVP coating could influence cell attachment, which contributed less cells growing on the surface.

To get the influence of crosslinking on cytotoxicity, scaffolds from core-shell structured NR-PVP fibres radiated with different crosslinking time were recorded in Figure 7-5. It showed that cell numbers reached max value when the radiation time is 3 hours. Therefore, 3 hours was selected for UV radiation of the core-shell fibres before cell culture experiment.
Figure 7-4  MTT assay results of cells on different scaffolds

Figure 7-5  Radiation time influenced on cytotoxicity: cytotoxicity was carried out on UV radiated NR-PVP core-shell fibres and cytotoxicity was estimated by MTT assay.
7.4 Cell-scaffolds interaction

7.4.1 Immunostaining

Cell-scaffold interaction was investigated by SEM. The distribution and attachment of the cells can be seen clearly under SEM investigation. Cell-scaffold interaction included a series of biological response: adhesion, cell morphology, growth, differentiation and migration [1]. Adhesion to the surface was one of the important factors which were used for determining biocompatibility of the materials. The contact of cells with scaffold surface included attaching, adhering and spreading, which depended on cell-scaffold interaction [2]. Canbolat et al [187] studied the influence of cell attachment on the cell viability. It showed that core-shell structure fibres are of the best cell biocompatibility because of improvement in cell-scaffold contact.

Figure 7-6 Cells imaging for cell morphology and cell-scaffold relative position: a), c) cells morphology without scaffolds background; b), d) demonstrated relative position between cell and scaffolds (blue)
Two groups of confocal images in Figure 7-6 show the similar area in cells on PVP coated ENR fibre sample with different Z position. Left images can demonstrate clear structure of cells while right images could give useful information about the cells and the background materials. A fibril structure can be seen in the material and also some black dots or particles which may be from the dissolving of coated PVP. The relative position between cells and scaffolds were shown in appendix (Figure A-16).

### 7.4.2 Microstructure observation

Cells attached on fibres could be investigated by SEM imaging technology. The uneven surface of cells on electrospun fibres caused difficulty of focusing. Cells with tens micro meters in size could be easily seen under SEM at around magnification of 500 times. The cell morphology on electrospun fibres can provide cell attachment information. As seen in Figure 7-7, cells have better attachment on PVP coated fibres and core-shell fibres. While on electrospun NR fibres, cells did not show good attachment when compared to coated fibres. In addition, the relative position of cells and electrospun fibres was determined: the cells were growing on the surface of the electrospun fibres.

![SEM images](image)

Figure 7-7 Cell-scaffolds interaction observation from SEM images: a) ENR fibres; b) PVP coated fibres; c) core-shell fibres.
7.4.3 Hydrophilicity

Table 7-3 Water contact angle of electrospun mats with different thickness

<table>
<thead>
<tr>
<th>Thickness</th>
<th>E-NR 1</th>
<th>E-NR 2</th>
<th>E-NR 3</th>
<th>E-NR 4</th>
<th>E-NR 5</th>
<th>E-NR 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle/°</td>
<td>75</td>
<td>90</td>
<td>95</td>
<td>102</td>
<td>115</td>
<td>118</td>
</tr>
</tbody>
</table>

Water contact angle of electrospun fibres related closely to the thickness of the fibre mats. NR is a highly hydrophobic material, which has a higher water contact angle around 120°. A group of electrospun NR fibres were tested with different thickness and the contact angle data are as shown in Table 7-3. The electrospun NR fibres are of porous structure, which would influence water contact angle in the surface smooth rate. With increase of thickness, water contact angle increases as well.

Hydrophilicity can be used to estimate cell attachment of the materials. The cell surface is hard for artificial materials to be attached through the weak chemical interaction such as hydrogen bond, electrostatic force, polar or ionic force [188]. Hydrophilicity of the scaffold materials would influence cell-scaffolds interaction. Smooth muscle cells can stretch on the flat bottom of culture flask surface. Water wettability of scaffolds has close relationship with cellular behaviour, such as cell proliferation, cell adhesion to the scaffolds, cell morphology and cell orientation [189]. The hydrophilicity of the electrospun fibres with different morphologies was measured on water contact angle and the results were shown in Figure 7-8. Blood biocompatibility depends on physicochemical features of the material surface. Natural rubber is highly hydrophobic, which would cause issue to protein adsorption and platelet adhesion. The water contact angle is around 115° for NR film. After electrospinning, it decreased to around 54° due to porous structure of electrospun fibres. PVP coated ENR fibres showed lowest water contact angle around 30°.
Water contact angle required for cell culture is between $30^\circ$ and $100^\circ$ [190]. When the contact angle was around $30^\circ$, smooth muscle cells showed best attachment. Cells showed worst cell proliferation on highly hydrophobic surface of NR film, on which cells were unable to attach and rolled up as balls. On the other hand, cells also showed worse cell proliferation on scaffolds with lower water contact angle (around $10^\circ$). Cells could grow well on the electrospun fibres with contact angle around $30$ to $50^\circ$ and they could unfold on the scaffold as shown in Figure 7-9. The relationship between cell viability and scaffolds water contact angle can be used for explaining low cell viability on non-toxic NR film. From the cytotoxicity result of soaking solution, NR film was non-toxic to SMC cells. High hydrophobicity of NR film would be the problem that cause cell death. Cells were not able to attach on surface with such a high hydrophobicity. The water contact angle results of materials used were depicted in Figure 7-8. After electrospinning, NR mats possess porous structure on the surface and water contact angle decreased from $115^\circ$ to $57^\circ$ and the cell viability increased from around $10\%$ to more than $90\%$. Therefore, scaffolds morphology influenced the hydrophobicity, in turn, caused different cell attachment.
Figure 7-9 Cells distribution on scaffolds with different morphologies: a), e) and f) were optical microscope images of scaffolds materials; b), f) and j) were confocal microscope images; c), g) and k) were microscope images excited from UV light; d), h) and l) were SEM images.

7.4.4 Crosslinking effects

The effects of crosslinking influenced cytotoxicity in two ways: contact angle and possible toxic subjects produced during UV radiation. Compared with the non-cross-linked fibres, toxic effect of soaking solution could be used for estimating whether toxic subjects formed during UV radiation process. UV crosslinking influenced chemical component of the scaffolds together with their water wettability. After UV radiation, contact angle of the electrospun fibres became smaller, which means that the crosslinked fibre mats became more hydrophilic. Crosslinked core-shell structure scaffolds were assessed for toxicity to make sure that UV radiation crosslinking process was non-toxic to cells. As the uncrosslinked PVP is water soluble and the comparison was carried out between crosslinked core-shell fibres and ENR fibres. The crosslinking process would form some toxic chemicals, which would influence cytotoxicity of the materials. The cytotoxicity was measured before and after the materials were crosslinked with UV radiation for 2-5 hours. The core-shell structure fibre mats were radiated under UV ray before they were used for cell culture. Radiation time was
selected as a control factor for investigating cytotoxicity of radiation effect. As seen in Figure 7-9, higher cell density was imaged on core-shell structured fibres, which were crosslinked before used as scaffolds. This trend was identical to the result showed in Figure 7-8. Therefore, crosslinking of PVP caused no extra cytotoxicity.

7.4.5 Pore size

Figure 7-10 Cells cultured on electrospun fibres with different pore size: a) average size, 120μm; b) average size, 5 μm

The pore size could have potential effects on cell growth [16, 91]. Normally, the pore size of electrospun fibres were much smaller than the cellar size, which caused difficulty in cell ingrowth. The physical structure of scaffolds would change during the process of cell growing. As seen in Figure 7-10, if the cells were much bigger than the pore size of nanoscale electrospun fibres, the cells could be growing on the surface of the scaffold. While the pore size was bigger than the cellar size, cell could grow inside the pore and the scaffold will lose its supporting function. Therefore, the pore size would be smaller than the cellar size (several tens μm). When the average pore size was much smaller than that of cellar size, cells would grow on the surface of the scaffolds as shown in Figure 7-10 (b).

7.5 Cell viability and proliferation
7.5.1 MTT assay result

Live cell number at 24-hours, 72-hours and 168-hour culture estimated from MTT assay was used for estimation cell viability. Cells number of negative control (without scaffolds) was selected as a standard for the cell viability. The cell viability was calculated from the ratio of cell number to that of negative control. The calculated cell viability of SMC on different scaffolds were listed in Table 7-4.

Table 7-4 Cell viability of scaffolds from different materials

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell number</td>
<td>Cell viability/%</td>
<td>Cell number</td>
</tr>
<tr>
<td>NR film</td>
<td>368</td>
<td>7.0</td>
<td>936</td>
</tr>
<tr>
<td>ENR</td>
<td>3808</td>
<td>72.5</td>
<td>6477</td>
</tr>
<tr>
<td>PVP coated NR fibre</td>
<td>3160</td>
<td>60.1</td>
<td>4370</td>
</tr>
<tr>
<td>Co-axial electrospun fibres</td>
<td>4821</td>
<td>91.7</td>
<td>7101</td>
</tr>
<tr>
<td>Negative control / without scaffold</td>
<td>5252</td>
<td>-</td>
<td>7804</td>
</tr>
</tbody>
</table>

It showed that NR film is of the worst cell biocompatibility and the core-shell structure fibres were the best cell-compatible materials. The initial cell number was 5,000 cells.
on each sample. After 3 days incubation, only around 10% of cells were alive on NR film. Fewer cells on coated fibres died than the uncoated ENR fibres. The possible reason would be that porous structure on the surface was damaged after coating with PVP. ENR fibres and core-shell fibres had higher cell viability, which were more than 90% and 80% respectively, while PVP coated ENR fibres possessed lower cell viability rate which was less than 50%.

Figure 7-11 shows cell proliferation of SMC on different scaffolds at day1 and day 3 points. Compared with the control, cell cultured with scaffolds showed a decrease in cell proliferation. Scaffolds fabricated from NR film showed significant decrease in cell proliferation up to 88% (day1), followed by PVP coated ENR fibres with decrease rate at 48% (day 1). ENR fibres based scaffolds showed insignificant decrease in cell proliferation compared with the control at decrease rate of 27.5% (day 3). Core-shell structure scaffolds possessed most insignificant decrease in cell proliferation at rate of 12% (day 3).
7.5.2 Immunostaining

Cell proliferation can also be recorded by immuno-staining. Cells growth showed cell density increase in the confocal microscope images. As shown in Figure 7-12, left images (a, b, e, f) were just the UV laser (blue) and dyed Tapi cell nucleus could be seen as bright blue oval while right images (c, d, g, h) were combination of UV laser channel (blue colour) and TRITC fluorescent channel (red colour). The bright nucleus images also illuminated fibril background and the porous fibre structure could be seen and the cells attached on the top of porous fibre mats. The cells dyed with Dapi and TRITC provided clearly relative position of cells on scaffolds. The fibril structure in cells would be red, while fibres from NR would be blue under excitation of UV (405nm). Then the structure of fibres and cells would separate obviously.

Figure 7-12  Cells number and density on the core-shell structure scaffold increased with growth time: a), b), e) and f) were UV excited microscope images of dyed Dapi cells and the bright light was open for imaging fibre background; c), d), g) and h) were confocal microscope images of dyed dapi nucleus.

7.6 Conclusions

1) Scaffolds fabricated from different morphologies of NR based materials were purified and sterilized with combination of alcohol washing and 3-hour UV light radiation before they were used for cell culture.
2) Scaffolds cytotoxicity was evaluated in three methods: soaking solution, microscope observation and MTT assay. Soaking solution methods showed that all the scaffolds were of no cytotoxicity effect.

3) MTT assay demonstrated cell viability of different scaffolds after 1 day incubation. Quite low cell viability on NR film scaffold was observed from both microscope observation and MTT assay results, which were around 10%. Core-shell structure fibres showed highest cell viability (91.7% after 1 day culture) from MTT assay result. Electrospun NR fibres showed lower cell viability (71.5%) than core-shell structure fibres and the cell viability decreased after coating with PVP (60.1%).

4) Hydrophobicity of scaffolds surface contributed to cell viability. Hydrophobic NR film surface was difficult for cell attaching, which contributed to quite low cell viability. Hydrophilic PVP coated ENR fibres (contact angle at around 10°) and ENR fibres (contact angle at around 57°) were also against cell growth, which corresponded cell viability at 60.1% and 72.5%, respectively. Core-shell structure fibres with water contact angle at around 30° possessed higher cell viability at 91.7%.

5) Cell proliferation was investigated in cell growth time points at: 1 day, 3 days and 7 days. The increase order of cells growing rate on different scaffolds was: core-shell fibres > ENR fibres > PVP coated fibres > NR film.
8 Conclusions

The conditions for steady electrospinning natural rubber and properties of electrospun NR fibres were investigated. THF was selected as solvent for electrospinning NR solution with a concentration ranging from 15 g/L to 40g/L and 25g/L solution was mostly used in the project. The formation of gels in NR/THF and the effects on stability of electrospinning have been investigated. Both filtration and centrifuge can increase the stability of electrospinning by removing gel component. The optimal volume ratio of NaCl solution to NR solution is 4:100.

Processing parameters that influenced the stability of electrospinning NR solution were investigated in terms of voltage range, needle diameter and feeding rate. With increase of feeding rate, Vc and Vr decreased and the voltage range for steady electrospinning becomes smaller. Characteristic length of Taylor cone increased with increase of voltage. The fibre diameter decreased with increase of voltage and the morphology of fibres tended to be straighter. Needle diameter caused fibre alignment change in the forms of curl fibres from smaller needle diameter.

The competition between relaxation and stretching of electrospun fibres contributed mostly to the curl or straight morphologies. When the relaxation is stronger than the stretching, the electrospun fibres showed as curl structure while stretching is stronger than relaxation, electrospun fibres would be stretched and were straight. Elastic resilience and viscoelasticity of NR lead to transform between curl and straight morphologies based on competition result between relaxation and stretching. The fibres collected among hollow window collector were straight due to high stretching effect from additional electrical field. The fibres collected on the water surface were curl with small knots due to the relaxation effect was much stronger than that of stretching. FTIR results showed that the solvent played a negligible role in electrospun fibres structure and the stretching rate of electrospun fibres were influenced by the solution concentration. XRD and DSC results demonstrated that during electrospinning process, crystallinity degree of NR materials increased. In situ SAXS testing of electrospun fibres during mechanical testing results demonstrated molecular structure changes in NR fibres. Strain-induced crystal in electrospun fibres during stretching process was reflected in the SAXS pattern shape changes.
It demonstrated that NR and PVP were partially compatible through $T_g$ analysis and morphology observation of NR/PVP blend. Emulsion made from immiscible NR/THF and PVP/water showed phase separation when volume ratio was more than 1:3 and less than 4:1, based on which relative feeding rate was selected as 1:1. Dynamic stability of NR/THF and PVP/water solution was investigated by rheological analysis of core and shell fluids. Flexible NR solution and rigid PVP solution could have steady rheological properties when concentration of PVP reached 20%. Relative shorter inner needle could form encapsulation, which could contribute to stable co-axial electrospinning. Inner needle diameter influenced morphology of the co-axial electrospun fibres and uniform core-shell structure fibres appeared by 0.8mm inner needle. Charged rigid PVP solution drove non-charged NR solution to avoid fibre separation. Core-shell structure was directly confirmed by FIB-SEM and TEM. Confocal microscope images and phase contrast images of FITC dyed PVP/NR fibres showed core-shell structure with color contrast. FTIR mapping indicated chemical component distribution in the fibre. The measurement of thermal properties showed that co-axial electrospun fibres had a third $T_g$ at around -35 ºC, which meant that NR and PVP formed a compatible layer on the interface.

UV radiation was selected as the method for crosslinking of electrospun PVP. The crosslinking mechanism of PVP fibres was considered as free radical process. UV wavelength selected was 254nm, under which photo-degradation was resisted and crosslinking happened. 3-hour UV radiation would cause crosslinking of NR fibres without damaging cylinder shape of electrospun fibres. Crosslinking degree in electrospun fibres increased with radiation time, however, with increase of radiation time cylinder shape of the fibres would be damage due to increase of temperature from radiation.

Crosslinking happened in both shell and core layers in core-shell structure fibres under 3-hour radiation at 254nm UV light. Water stability of shell layer (PVP) was improved and mechanical properties of the fibres were enhanced a lot compared with UV radiated ENR fibres. Degradability of UV radiated fibres was improved by the protection of crosslinked PVP shell. Compared with direct PVP coated electrospun NR fibres, core-shell structure fibres possess obvious advantages in structure and properties. The
mechanical properties of electrospun fibre mats showed high elongation at break, which can reach 639.5% at most with tensile strength at 132.9 Mpa.

Scaffolds fabricated from different morphologies of NR based materials were purified and sterilized with combination of alcohol washing and 3-hour UV light radiation before they were used for cell culture. Scaffolds cytotoxicity was evaluated in three methods: soaking solution, microscope observation and MTT assay. Soaking solution methods showed that all the scaffolds were of no cytotoxicity effect.

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Limitation and future work

1) The scaffold that we fabricated from electrospun fibres were 2D scaffolds, in the future 3D scaffold could be fabricated from rotating collector;

2) The biological testing was just in vitro, in vivo testing was necessary before using in clinic;

3) We investigated the cytotoxicity of the materials after electrospinning instead of the fibre structure effects on the scaffold feature;

4) We just did the cell experiment on smooth muscle cells instead of all types of cells;

5) The allergy issues of electrospun fibres was not studied in this project and should be appropriately investigated as rubber materials could represent potential issues for some people who are allergic to rubber.

6) Structure of the scaffolds such as porosity, fibre thickness and fibre alignment influences cell culture conditions. In this study, porosity and alignment influence on cell growth was ignored.
Future work and feasible suggestions

1) Due to limitation of the PVP being investigated as the only biocompatible polymer, natural elastic materials such as collagen, elastin and synthetic elastomers including PCL, PU could be also used as shell layer of core-shell structured fibres made from co-axial electrospinning with natural rubber solutions. In addition, ageing issues of NR fibres could be avoided by coating NR fibres with stable biopolymer, which would protect NR from contacting with oxygen. The degradation period of the scaffolds could be designed by material type and scaffold structure.

2) Interfacial interaction between cells and scaffolds should be investigated. Cells used in this project refer to human arterial smooth muscle cells (HASMC). Interfacial interaction between cells and scaffolds is related to cell types and scaffolds structure, which needs to be further studied before in vivo application.
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Appendix

A1 NR fibre morphologies from different solvents

Figure A-1 Solvents and concentration effects on fibre morphologies
Figure A-2 Rheological behaviour of natural rubber solution in THF
A3 Rheological properties of NR and PVP solutions with different concentrations

Figure A-3 Rheological behavior: viscosity changes with shear rate
A4 Relaxation time of DPNR solutions with different concentrations

The modulus ~ angular frequency relation can be fitted as exp grow1 function.
Calculation of cross X:

\[ Y_1 = -0.69797 + 0.49248 \exp\left[\frac{x+16.8679}{48.29889}\right] \]

\[ Y_2 = -11.73963 + 4.20806 \exp\left[\frac{x+1321.188}{1284.2424}\right] \]

\[ Y_1 = Y_2 \quad X = 180 \text{ rad/s} \]
Figure A-5 $G'$ and $G''$ of DPNR solutions with different concentration: a) 25g/L; b) 12.5g/L

Table A-1 Relaxation time and cross $X$ from fitting of $G'$ and $G''$ of DPNR solutions with different concentration

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Relaxation time/s</th>
<th>Cross X/ rad/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>50g/L</td>
<td>0.005s</td>
<td>180</td>
</tr>
<tr>
<td>25g/L</td>
<td>0.0101s</td>
<td>98.3</td>
</tr>
<tr>
<td>12.5g/L</td>
<td>0.019s</td>
<td>52</td>
</tr>
</tbody>
</table>
A5 Fitting steps of flow curve

1) Select fitting item

Figure A-8 Fitting setting in origin

2) Set function

Tool --- fitting function organizer
\[ \eta = \eta_\infty + \frac{\eta_c - \eta_\infty}{1 + (\lambda \gamma)^m} \]

Figure A-9 Edition of new function for flow curve fitting

3) Fit till converged
Figure A-10 Modification of parameters for fitting: a) modification  b) change parameters  c) change number of points

6) Get fitted
Figure A-11 Getting fitted curve
A6 Estimation of Mc in 25g/L NR solution in THF

Volume of NR: $\frac{2.5\text{g}}{0.92\text{ g/mL}} = 2.72\text{ mL}$

$\phi_r = \frac{2.72}{102.72} = 0.026 = 2.6\% \quad \chi_{NR/THF} = 0.45$

$V_0 = 81.1\text{ mL/mol}$

$n = -\ln 0.974 - 0.026 - 0.45 \times 0.026^2 / (81.1 \times (0.0261/3 - 0.013))$

$= (0.00034 - 0.0003 \sqrt{22.97})$

$= 1.74 \times 10^{-6}$

$Mc = \frac{0.92\text{g/mL}}{1.74 \times 10^{-6}}$

$Mc = 5.29 \times 10^5\text{ g/mL}$

(Mc is the average mass of network chains).

A7 Co-axial electrospinning of NR/PVP/collagen

At first, we designed to do co-axial electrospinning of NR/collagen for improving biocompatibility of NR with PVP as surfactant. However, poor electrospinnability of collagen caused failure of co-axial electrospinning and collagen showed round balls in the electrospun fibres.
Figure A-12 SEM images of co-axial electrospun NR-collagen/PVP fibres: Beads are all spindle-like, while the collagen particles are perfect round ball.

A8 Fibres collected on the hollow window with different shapes

Rectangle shape

Square shape window

Figure A-13 Fibres collected on the hollow window with different shapes
A-9 Electrical field line stimulation of collectors with hollow window

The electrical field line tends to be perpendicular to the metal surface. For the flat Al foil, the electrical field line will be parallel to each other and perpendicular to the collector surface, therefore, the fibres would be aligned randomly on collector surface. When the collector was void. The electrical field line will be bent over the hollow window. The electrical field line distribution among hollow window area was stimulated by the ANSYS software. The effect of hollow window on the electrical field line could be seen in Figure A-14.

A-10 Confocal microscope images of cells attached on scaffolds

Figure A-14 The electrical field distribution change around the hollow window on collector
Figure A-15 Fibres attached on electrospun NR fibres

Figure A-16 Cells on crosslinked PVP-NR core-shell structure fibre mats