1. INTRODUCTION

The number of polymeric materials that are used in biomedical application has increased enormously over the past decade (Kohane et al., 2008). The major applications include resorbable sutures, drug delivery systems and orthopaedic fixation devices such as pins, rods and screws (Behravesh et al., 1999; Middleton and Tipton, 2000).

Skin is the largest organ of the body with many essential functions that would help the survival. Since it is in direct contact with the external environment which renders them highly prone to damage and or injury. A quick regeneration or repair after the injury is necessary to avoid complications (Xiao et al., 2002). For the past few decades, polymeric biomaterials were developed which can act as smart skin substitutes by performing many of the functions of skin.

Skin substitutes are heterogeneous group of wound coverage materials that aid in wound closure and replace the functions of the skin, either temporarily or permanently, depending on the product characteristics. These substances serve as alternatives to the standard wound coverage in circumstances when standard therapies are not desirable (Shores et al., 2007). Skin substitutes are used to aid in wound closure, alleviate pain and replace the function of the skin. Skin substitutes have important roles in the treatment of deep dermal and full thickness wounds of various aetiologies (Halim et al., 2010). Nanocomposites of metals oxides such as zinc oxide that are proved for the antimicrobial activity are now incorporated with skin substitutes.

For the past few decades, poly(ε-caprolactone) (PCL) has drawn a lot of attention in biomedical applications. PCL has several advantages including low cost, biocompatibility, and biodegradability (Choi et al., 1999). PCL has been suggested in applications such as drug delivery system (Zhong et al., 2001; Allen et al., 2000), tissue-engineered skin (plain film) and scaffolds for supporting fibroblasts and osteoblasts growth (Woei et al., 2001; Hutmacher et al., 2001) etc.

Electrospinning is an excellent method for the fabrication of fibres with diameters from micrometre to nanometre scale (Li et al., 2013). Electrospinning technology, which can
easily mass-produce thin nanofibrous membranes with good conformability, could offer a solution for the fabrication of skin substitutes. Electrospun nanofibers resemble the native topographical features of the natural extracellular matrix and may thus promote the cell’s natural functions in a biomimetic fashion. Electrospun nanofibers have various properties that make them suitable as skin substitute materials such as high oxygen permeability, variable pore size, a high surface area to volume ratio and morphological similarity to the extracellular matrix (EM) (Smith et al., 2004; Zahedi et al., 2010). The ability to incorporate a variety of bioactive molecules (such as antimicrobials and wound healing agents) into the nanofibers can enhance the rate of wound healing. Electrospun membranes will act as a physical barrier to the invading microbes and prevent the infection. Further, the fibres will support the cell migration towards the centre of the wound from the periphery. All these together contribute to the fast wound healing while using electrospun membranes as skin substitutes.

Zinc oxide (ZnO) is an inorganic compound widely used in everyday applications. Nanoparticles of ZnO and their application in coating systems have attracted a great deal of attention in recent years because of its multifunction property, especially antibacterial activity (Li et al., 2010; Vlad et al., 2011). ZnO nanoparticles are also endowed with their excellent piezoelectric properties (Fan et al., 2006).

Thus, in this thesis, electrospun PCL/ZnO nanocomposite scaffolds were fabricated by electrospinning technique and characterized. Fabricated PCL/ZnO nanocomposite scaffolds were evaluated for the applicability as skin substitutes.

2. MOTIVATION OF WORK

Polycaprolactone (PCL) based materials as biomaterials, especially as tissue engineering scaffolds and wound coverage materials have been reported. Industrialization of electrospinning of PCL is still a challenging task due to the difficulties in the scaling up of the electrospinning process to a large scale. Clogging of the polymer at the tip of the needle is a major problem in electrospinning that hinders the scaling up. Thus, to overcome this bottleneck, strategies like use of alternative ecofriendly solvents should be tried.

Invasion and proliferation of pathogenic microorganisms into wound site and causing infections is a common but complicated phenomenon. Even though many antibiotics are effectively used in wound coverage materials to prevent the pathogenesis in wounds, antibiotic resistance and the side effects caused by them make less convenient. Since ZnO
nanoparticles are effective against many bacteria, incorporation of these nanoparticles in electrospun PCL skin substitutes eradicates the possibility of bacterial growth in wounds. Thus, skin substitute materials with tunable morphology, high mechanical stability, antibacterial property and enhanced tissue proliferation ability from electrospun mats of PCL fibers filled with ZnO nanoparticles were proposed. We hypothesized that ZnO nanoparticle can act as the generators of H$_2$O$_2$ in the tissue engineering scaffold which will act as key regulators at important pathways which enhance cell proliferation, angiogenesis and wound healing as well as attribute antibacterial property. Thus, the ZnO nanoparticles in PCL matrix can play an important role in the generation of reactive oxygen species (ROS), especially H$_2$O$_2$, which may trigger fibroblast proliferation, wound healing and the vascularization through the polymeric membrane. This will facilitate the successful integration of host tissue with the scaffold and proliferating capillaries bring oxygen and micronutrients to growing tissues and remove catabolic waste products. Since fibroblast proliferation and angiogenesis are necessary for wound healing, their induction is beneficial in skin tissue engineering for achieving fast wound closure.

Polycaprolactone degrade chemically by hydrolytic cleavage of the back-bone ester bonds. This hydrolysis reaction can be affected by the presence of additives, polymer crystallinity and the degradation conditions. Even though the biodegradability of PCL is well established, limited studies only carried out on the effect of nanofillers on the in vitro degradability of electrospun PCL membranes. Thus, the effect of ZnO nanoparticles on the degradation of electrospun PCL membranes also should be studied.

Since any material used for biomedical applications is prone to contamination with pathogenic microbes, it is most important to sterilize such materials using the most effective method, the gamma irradiation. No one tested for the gamma sterilizability of electrospun PCL membranes containing ZnO nanoparticles. Thus, PCL membranes incorporated with various concentrations of ZnO nanoparticles were irradiated with gamma radiation of varying doses.

3. OBJECTIVES AND SCOPE OF THE WORK

The major objectives of the present study are the following:

- To find out the effect of various eco-friendly solvents on the clogging of PCL during electrospinning.
To examine the effect of ZnO nanoparticles on the antibacterial properties of the electrospun PCL membranes.

To find out the effect of ZnO nanoparticles on the morphology, crystallinity and mechanical properties electrospun PCL membranes.

To determine the biocompatibility of electrospun PCL/ZnO nanocomposite membranes.

To explore the effect of ZnO nanoparticles on the degradation of electrospun PCL/ZnO nanocomposite membranes.

To investigate the effect of gamma irradiation on the materials properties of electrospun PCL/ZnO nanocomposite membranes.

To study the cell attachment, migration and proliferation on electrospun PCL/ZnO nanocomposite membranes in vitro and in vivo.

To study the angiogenic property of electrospun PCL/ZnO nanocomposite membranes.

4. SUMMARY OF THE RESEARCH WORK

4.1. Optimization of electrospinning of polycaprolactone in acetone, acetic acid and their mixtures

Optimization of electrospinning of polycaprolactone (PCL) has been carried out. Two eco-friendly solvents, acetone and acetic acid were tried as the solvents to electrospin PCL. Various spinning parameters like polymer concentration, applied voltage, tip to collector distance and flow rate were adjusted to avoid bead formation and to get fibers with uniform fibre diameter and enough pore spacing for the cell migration. Figure 1 shows the morphology of electrospun membranes using acetone/acetic acid mixture 3:7 as the solvent which is the best optimized solvent system. Clogging of the polymer at the tip of the needle is a major problem in electrospinning that hinders the scaling up of the process at mass scale. Use of combinations of acetone and acetic acid could successfully overcome the phenomenon of clogging. Figure 2 clearly demonstrates the reduction in clogging while using acetone/acetic acid mixture as the solvent.
Figure 1: SEM images of electrospun PCL membranes using acetone/acetic acid in the ratio 3:7 as the solvent. 15 wt % PCL solution electrospun at an applied voltage 15 kV (a) and 20 kV (b), a tip to collector distance of 20 cm and a flow rate of 0.5 mL/hour. Graphs represent the frequency of diameter distribution.

Figure 2: Photographic image of the tip of the needle during the electrospinning of polycaprolactone in acetone (A), acetic acid (B) and acetone/acetic acid mixture (3:7) (C) at different time intervals 5 minutes (a), 15 minutes (b) and 30 minutes (c).
4.2. Fabrication and characterization of electrospun polycaprolactone/ZnO nanocomposite membranes

Various kinds of nanofillers have been introduced in polymer technology to improve the functionality of polymeric materials. The effect of zinc oxide nanoparticles on the fiber diameter, fiber morphology, antibacterial activity and the enhanced cell proliferation of the electrospun PCL woven membrane has been studied. Mechanical stability and the antibacterial activity of the fabricated material were also investigated. The effect of the ZnO nanoparticle concentration on the fiber diameter and fiber morphology was investigated using scanning electron microscope (SEM). Figure 3 shows the morphological features of electrospun PCL membranes containing ZnO nanoparticles. Presence of ZnO nanoparticles in the PCL fibers was confirmed by EDAX analysis. Fourier transform infrared spectroscopy (FTIR) analysis has been carried out to find out the nature of the interaction between the PCL and the ZnO nanoparticles. FTIR results demonstrated that there is a shift in the carbonyl peak of PCL due to the interaction of ZnO nanoparticles with PCL (Figure 4). XRD and DSC studies were carried out to determine the variation in the crystalline behaviour of PCL due to the incorporation of ZnO nanoparticles. Both X-ray Diffraction (XRD) and Differential scanning calorimetry (DSC) data showed that, low concentrations of ZnO nanoparticles increased the relative crystallinity of PCL membranes. Mechanical stability of the fabricated material was investigated by tensile testing.

Figure 3: Representative SEM micrograph of neat PCL membrane (a) and PCL membrane containing 1 wt% ZnO nanoparticles. (c) and (d) are the higher magnification micrographs of (a) and (b) respectively.
Figure 4: FTIR spectra of neat PCL, ZnO nanoparticles and the composites of both at varying concentrations of ZnO nanoparticles (a). Decrease in the intensity of peak and peak shift of carbonyl vibration as the ZnO concentration increases (b).

The typical tensile stress–strain curves for electrospun PCL/ZnO nanocomposites are presented in Figure 5. It shows that incorporation of 1 wt% of ZnO nanoparticles improved the tensile strength of the PCL membranes.

Figure 5: Stress strain curves of PCL and PCL/ZnO nanocomposites with varying concentrations of ZnO nanoparticles.

4.3 In vitro studies of PCL/ZnO nanocomposite membranes

PCL/ZnO nanocomposite membranes were also evaluated for their antibacterial property, microbial barrier property, fibroblast proliferation and biocompatibility. Since the wound coverage materials including skin substitutes are in direct contact with the blood, such materials should be blood compatible. Thus, blood compatibility of the fabricated membranes has been tested by RBC and WBC aggregation studies. Hemolysis assay and platelet activation study were also carried out. Effect of ZnO nanoparticles on the in vitro degradation
of PCL membranes was also evaluated. The results demonstrated that PCL/ZnO nanocomposites have shown good antibacterial property (Figure 6) and able to act as a barrier against invading microbes (Table 1) while being biocompatible, blood compatible and enhancing cell proliferation (Figure 7). PCL/ZnO nanocomposites membranes were able to activate platelet which is an indication of the hemostatic potential of the membranes. *In vitro* degradation study demonstrated that ZnO nanoparticle containing membranes degraded much faster than neat PCL membranes (Figure 8).

**Figure 6**: Plates showing the antibacterial activity of the fabricated PCL membranes with different concentration of ZnO nanoparticles against *E. coli* (plate (a)) and *S. aureus* (plate (b)). In both plates 2 wt% (a), 3 wt% (b), 4 wt% (c), 5 wt% (d), 6 wt% (e) ZnO nanoparticles and PCL membrane alone (f).

**Table 1**: Microbial Barrier Property with reference to positive control.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Microbial Barrier Property MBP (%) = (Control - Test) X 100</th>
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<tbody>
<tr>
<td>Neat PCL membrane</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>PCL/ZnO-1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>PCL/ZnO-2</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>PCL/ZnO-4</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>PCL/ZnO-6</td>
<td>95 ± 2</td>
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Figure 7: Proliferation of adult goat fibroblast cells through the electrospun PCL membranes. Neat PCL membranes (a and e), with 0.5 wt % ZnO nanoparticles (b and f), with 1 wt % ZnO nanoparticles (c and g) and with 2 wt % ZnO nanoparticles (d and h). The first row (a, b, c and d) is after the cell culture of 24 hours and the second row is after 72 hours of cell culture.

The variation in morphological feature of the membranes during degradation demonstrated that hydrolytic degradation starts from amorphous regions and the extent of degradation was much higher for ZnO nanoparticles containing membranes as evident from the nicks formed on individual fibers.

Figure 8: Representative SEM images of degrading neat PCL (a,d,g), PCL/ZnO-1 (b,e,h) and PCL/ZnO-4 (c,f,i) membrane before (a,b,c) and after 10 (d,e,f) and 30 (g,h,i) days of incubation in SBF. (j) and (k) represent higher magnification images of (h) and (i) respectively.

Figure 9 clearly shows the reduction in tensile strength during degradation. PCL/ZnO nanocomposite membranes containing higher concentration of ZnO nanoparticles showed higher decrease in tensile strength during degradation.
Figure 9. Stress strain curves of neat PCL membrane and PCL membrane with different wt% ZnO content after different time period of incubation (0, 10 and 30 days) in simulated body fluid.

4.4 Sterilizability of electrospun PCL/ZnO nanocomposite membranes by Gamma irradiation

It is important to sterilize biomaterials that are intended to be used in clinical applications, using the most accepted method, the gamma irradiation. The gamma sterilizability of electrospun PCL/ZnO nanocomposite membranes has been performed. The PCL/ZnO nanocomposite membranes were irradiated with gamma radiation of varying doses. The irradiated materials were assessed for the sterility, changes in crystallinity, surface degradation, hydrophilicity and variation in mechanical strength. Obtained results demonstrated that electrospun PCL/ZnO nanocomposite membranes can be effectively sterilized by gamma irradiation, however a higher dose of radiation affect the materials properties. The irradiated membranes showed improved hydrophilicity and fibroblast cell proliferation.

Irradiation did not alter the morphology of the membranes (Figure 10).
Figure 10: SEM images of neat (a, b, c, d) and PCL/ZnO-1 (e, f, g, h), before gamma irradiation (a, b) and after gamma irradiation with 25 kGy (b, f), 35 kGy (c, g) and 65 kGy (d, h) doses.

Figure 11 shows the sterilization efficiency of the PCL/ZnO nanocomposite membranes sterilized by gamma radiation of various doses. From the figure it is evident that irradiation dosage of 35 kGy is required to completely sterilize both PCL and PCL/ZnO nanocomposite membranes.

Figure 11: Plate showing the growth of microbes after 14 (with sample) + 4 (without sample) days of incubation in thioglycollate medium. Streaks (a) is neat unirradiated membrane, (b) is 1% ZnO containing unirradiated membrane, (c) is neat membrane irradiated with 15 kGy Gamma, (d) is 1% ZnO containing membrane irradiated with 15 kGy Gamma, (e) is neat membrane irradiated with 25 kGy Gamma, (f) is 1% ZnO containing membrane irradiated with 25 kGy Gamma, (g) is neat membrane irradiated with 35 kGy Gamma, (h) is 1% ZnO containing membrane irradiated with 35 kGy Gamma, (i) is neat membrane irradiated with 65 kGy Gamma, (j) is 1% ZnO containing membrane irradiated with 65 kGy Gamma.
4.5. In vivo evaluation of Electrospun polycaprolactone membranes incorporated with ZnO nanoparticles as skin substitutes

Extensive *in vivo* study on the ability of PCL/ZnO nanocomposite membranes for its cell proliferation, wound healing and angiogenic properties has been carried out. ZnO nanoparticles are well known for its ability to generate Reactive Oxygen Species (ROS) which has a potential role in biological system. ROS can enhance wound healing by improved cell adhesion, migration and vascularization through growth factor mediated pathways. The plain or ZnO nanoparticle incorporated PCL membranes were implanted subcutaneously in guinea pigs. Immunological, macroscopical and histological evaluations have shown that the use of membranes containing ZnO nanoparticles enhances the cell adhesion and migration (*Figure 12*). The chicken chorioallantoic membrane (CAM) assay as well as the in vivo implantation studies confirmed the ability of the fabricated material to promote vascularization (*Figure 13*). The ZnO nanoparticles embedded membranes does not show any significant sign of inflammation. Use of PCL membranes incorporated with ZnO nanoparticles enhanced the wound healing compared to both positive and negative controls (*Figure 14*).

![Figure 12: Cross sections (H & E stained) of the neat PCL membranes (a, b, c) and membranes containing 1% ZnO nanoparticles (d, e, f) showing the migration and proliferation of fibroblasts after 5 days (a, d), after 10 days (b, e) after 20 days (c, f).]
Figure 13: CAM assay of neat PCL membrane (a), PCL membrane containing 0.5 wt% (b), 1 wt% (c), 2 wt% (d) and 4 wt% (e) ZnO nanoparticles. Arrows indicate newly formed capillaries.

Figure 14: Wound healing activity of the membrane on the first day (a, f, k and p), on 5th day (b, g, l and q), on 10th day (c, h, m and r), on 20th day (d, i, n and s) and on 30th day (e, j, o and t) of implantation. The first column (a, b, c, d and e) indicates neat PCL membranes, the second column (f, g, h, i and j) indicates PCL membrane incorporated with 1 wt% ZnO nanoparticles, the third column (k, l, m, n and o) indicates povidone –iodine treated wounds (positive controls) and the fourth column (p, q, r, s and t) indicates negative controls.