SYNTHETIC POLYMER-COLLAGEN COMPOSITE HYDROGEL FOR DRUG DELIVERY & TISSUE ENGINEERING

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SUMMARY

Thermo or pH reversible physical in situ forming hydrogels made of either synthetic or natural/modified natural polymers have attracted much attention due to their simple formulation by solution mixing, biocompatibility and minimally invasive administration. They were thus widely explored as potential biomaterials for drug delivery or tissue engineering. In this study, however, we propose the use of pH sensitive collagen and thermo reversible PEO-PPO-PEO tri-block copolymer to achieve the resulting composite hydrogel that combines the appropriate mechanical properties of synthetic component with the biocompatibility of biological component and explore its potential use in sustained drug release as well as tissue engineering. Copolymers with EO (ethylene oxide) percentages of 30 and 80 were used with varying concentration. The data from the *in-vitro* release kinetics of FITC-dextran done in PBS pH 7.4, at 37 °C showed that both F108 based collagen and L103 based collagen composite hydrogels had similar burst release as collagen hydrogel initially and then the rate of release started to decline releasing significantly less drug than collagen gel. Both gradually returned to almost the same rate as that of collagen on day 6. However, the amount of drug being released was lower in both composite hydrogels by 3 to 4% in the former case and 8% in the latter case. Scanning electron microscopy indicated that addition of polymer at low concentration (below 5% and 10% for F108 and L103 respectively) reduced the pores but did not perturb the formation of fibers. K562 cells were cultured in this three-dimensional composite hydrogel as cellular matrix for application in tissue

engineering. Cell proliferation test revealed an increase of cell growth as presented by fluorescence development with time for C-F108 hydrogel though high polymer concentration (5%) depicted cell inhibition. Our results suggested that this biomaterial might have potential use as both drug carrier and cellular scaffold.

Another type of gel called xerogel was also developed by chemical crosslinking of collagen with hydrophobic polymer PPG diglycidyl ether in the study. This dry gel had the ability to swell in water. Series of test by the use of equipments such as NMR and DSC or reagent like TNBS were carried out to investigate the extent of successful crosslinking. Data from the TNBS test revealed a decrease of 24% of free amino groups as the polymer was increased from 61% to 86%, indicating more amino groups had reacted with the fixatives due to polymer increment. Water contact angle and cell cytotoxicity study were also conducted to examine the biocompatibility of this material. It was found that gel was more hydrophilic as compared to the control i.e. collagen with the value of 41.6° for the former to 61.1° for the latter. Moreover, fibroblast cells cultured on the matrices had comparable growth and viability to those on control, particularly when the polymer concentration used was below 10 mg. This finding could signify the suitability of collagen-PPG diglycidyl ether xerogel by proper polymer amount for use as tissue scaffold.

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND INFORMATION

Hydrogels are materials that do not dissolve but swell in water and maintain three-dimensional networks. Hydrogels with thermo-reversible property formed using novel synthetic block copolymers have been a subject of interest and widely explored as injectable drug carriers and extracellular matrices (ECM) for tissue engineering. As they are meant for prolonged human body uses, these hydrogels should have high biocompatibility as well as modulated biodegradability. Many studies have already been done to find suitable polymers or improve on the biocompatibility of these materials. Collagen as a natural biopolymer has high biocompatibility but lacks in mechanical properties. On the contrary, synthetic polymer has the required physical properties but low biocompatibility. One of the ways to improve is by solution mixing to form composite hydrogel that gives the desired properties of both materials. The method is attractive due to its simplicity of fabrication. Another method of improvement that is also studied in this project is by crosslinking and modification of collagen with the synthetic polymer of certain functional groups to form more stable hybrid gel, hereinafter referred to as ‘xerogel’. A gel whose water is removed by drying such as lyophilization/ freeze-drying and has the ability to reabsorb water by swelling is term a xerogel. It is a typical practice to cross-link collagen in order to reduce its degradation rate and minimize the immuno response of the host as well as to avoid rapid dissolution of the material when in contact with biological fluids.

1.2 OBJECTIVES

The objectives of this project were as follows:

Polymer blended hydrogel

- To design and synthesize pH sensitive composite hydrogels based on PEO-PPO-PEO tri-block copolymer and collagen
- To study the release kinetics of the hydrogels
- To conduct cell viability and proliferation tests on the hydrogels

Polymer crosslinked xerogel

- To develop collagen-PPG diglycidyl ether hybrid dry gels by crosslinking between amino groups of collagen and reactive group of PPG diglycidyl ether to form covalent attachment
- To characterize the gel properties in order to gauge the amount of crosslinking
- To study the cell cytotoxicity of the material

1.3 SCOPE

Chapter 2 gives a brief literature review on the attractiveness of hydrogels for use as biomaterials, several polymers as well as some techniques commonly used to synthesize hydrogel, certain characteristics of collagen and PEO-PPO-PEO triblock copolymer in addition to their respective drawbacks.

The fabrication procedure of collagen-PEO-PPO-PEO based hydrogels and collagen-PPG diglycidyl ether based dry gel is outlined in Chapter 3 together with the schematic diagram and tables.

The experimental procedures to characterize hydrogels can be found in Chapter 4 while the results obtained from the experiments conducted are summarized in Chapter 5, followed by analysis of results and discussion written in Chapter 6. Some of the graphs and tables are presented in the Appendix.

The subsequent chapter concludes with the findings of the potential use of hydrogen in certain applications. Finally, recommendations from the author are given which might be useful if this project is to be continued.

CHAPTER 2
LITERATURE REVIEW

Success in the application of biomaterials relies heavily on the biocompatibility of biomaterials. Hydrogel as a biomaterial especially in drug delivery has been proven by cumulative evidence that it is highly biocompatible [1]. First, it has low interfacial tension with surrounding biological fluids and tissues and that minimizes the driving force for protein adsorption and cell adhesion. Second, hydrogel simulates some hydrodynamic properties of natural biological gels, cells, and tissues in many ways. Third, the soft, rubbery nature of hydrogel minimizes mechanical and frictional irritation to the surrounding tissue. The use of biodegradable polymers that disintegrate into harmless products under physiological conditions is desirable in the development of hydrogel for advanced drug delivery systems as the need for removal of the biomaterial from the body is avoided. However, many biodegradable synthetic polymers are not water-soluble and thus do not form hydrogels even in the presence of abundant water. They can be rendered otherwise by preparing either block copolymers, polymer blends or interpenetrating polymer networks (IPN) with hydrophilic polymers that can absorb significant amount of water and swell without dissolving in water. Some of the polymer compositions that have been used to fabricate hydrogels are summarized in the table below [2].

Table 2-1
Hydrophilic polymers used to synthesize hydrogel matrices

Natural polymers and their derivatives (± crosslinkers)
Anionic polymers: Hyaluronic acid (HA), alginic acid, pectin, carrageenan, chondroitin sulfate, dextran sulfate
Cationic polymers: chitosan, polylysine
Amphipathic polymers: collagen (and gelatin), carboxymethyl chitin, fibrin
Neutral polymers: dextran, agarose, pullulan

Synthetic polymers (± crosslinkers)
Polyesters: PEG-PLA-PEG [poly(ethylene glycol)-poly(lactic acid)-poly(ethylene glycol)], PEG-PLGA-PEG [poly(ethylene glycol)-poly(lactic-co-glycolic acid)], PEG-PCL-PEG [poly(ethylene glycol)-polycaprolactone-poly(ethylene glycol)], PLA-PEG-PLA, PHB [poly(hydroxy butyrate)], poly(propylene fumarate-co-ethylene glycol)]±acrylate end groups, poly(poly(ethylene glycol)/poly(butylene- oxide) terephthalate)
Other polymers: PEG-bis-(PLA-acrylate), PEG±cyclodextrins, PEG-g-poly(acrylamide-co-Vamine), poly-acrylamide, poly(*N*-isopropyl acrylamide-co-acrylic acid), poly(*N*-isopropyl acrylamide-co-ethyl methacrylate), poly(vinyl acetate)/poly(vinyl alcohol), poly(*N*-vinyl pyrrolidone), poly(methyl methacrylate-co-hydroxyethyl methacrylate), poly(acrylonitrile-co-allyl sulfonate), poly(biscarboxy-phenoxy-phosphazene), poly(glucosylethyl methacrylate-sulfate)

Combinations of natural and synthetic polymers
Poly(PEG-co-peptides), alginate-g-(PEO-PPO[poly(propylene oxide)]-PEO), poly(PLGA-co-serine), collagen-acrylate, alginate-acrylate, poly(hydroxypropyl methacrylamide)-g-peptide), poly(hydroxyethyl methacrylate/Matrigel®), HA-g-(*N*-isopropyl acrylamide)

The use of natural polymer especially collagen as a biomaterial is currently undergoing a renaissance in tissue engineering field as well as drug delivery systems [3,4]. Collagen comprises distinct molecules with a unique triple-helix configuration of three polypeptide subunits known as α-chains. Collagen type I is the most commonly used material for biomedical applications due to its biological properties and easy availability. It has a compound of three chains, two of which are identical, termed α1(I) and one α2(I) chain with different amino acid composition [3].

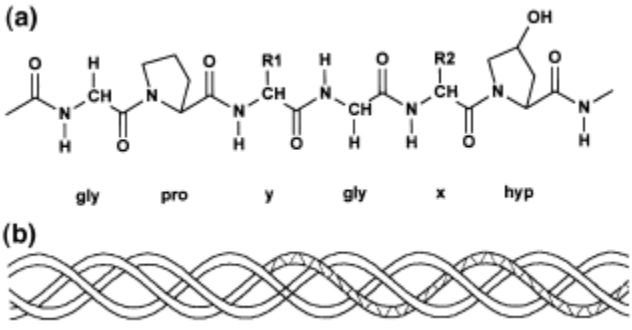


Figure 2-1. Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure

Under physiological condition, collagen molecules can self-assemble through fibrillogenesis to form microfibrils and later into fibrils, which then further organize themselves to form fibers [5]. Collagen can also be reconstituted to form hydrated gel that is similar to loose connective tissue *in vivo*. The attractiveness of collagen rests largely on the notion that it is a natural material of low immunogenicity and antigenicity and is therefore seen by the body as a native constituent rather than foreign matter.

Poly(ethylene oxide) – *block*-poly(propylene oxide) – *block*-poly(ethylene oxide) (PEO-PPO-PEO) copolymers (commercially known as Poloxamers or Pluronics®) are among the most widely used synthetic polymers and has found specialized application such as in biomedical areas owing to its low toxicity.

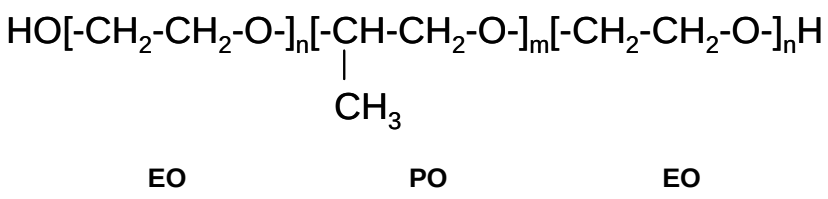


Figure 2-2. Chemical structure of PEO-PPO-PEO copolymers

Variation of this amphiphilic block copolymer composition (PPO/PEO ratio) and molecular weight (PEO and PPO block length) leads to the production of molecules with optimum properties that meet the specific requirements, for example, in pharmaceuticals (drug solubilization and controlled release [6] and burn wound covering [7]). The defining property of Pluronics® is the ability of individual block copolymer molecules or “unimers” to self-assemble into micelles in aqueous solutions as the concentration reaches that of critical micelle concentration (CMC), a process called “micellization” [8].

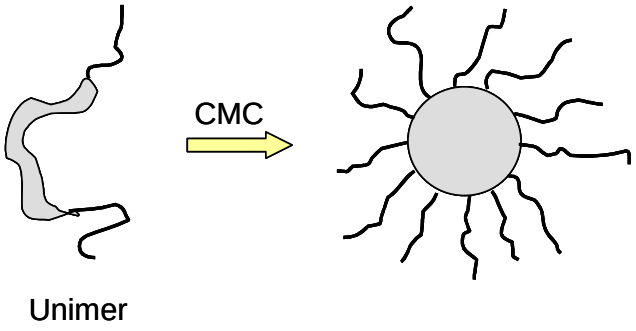


Figure 2-3. Integration of micelles above CMC

Gels are formed when PEO-PPO-PEO solutions of high copolymer concentration exhibit a dramatic change in viscosity at temperatures close to ambient, revealing a “thermoreversible gelation” property [9] with the micelles remaining apparently intact in the form of a “crystal” i.e. they lock into a crystalline structure of hard spheres [10,11].

Despite the respective benefits of natural and synthetic polymers, they are still far from being ideal hydrogel materials when used individually. For instance, collagen is often precluded by inadequate mechanical properties while the synthetic polymer has the required physical properties but is not acceptable from the point of view of biocompatibility. To circumvent the problem, they are often combined to form composites or crosslinked to improve the properties of a hydrogel [2,12].

There are two types of hydrogels: 1. ‘physical’ or ‘reversible’ gels when the networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces; 2. ‘chemical’ or ‘permanent’ gels when they are covalently-crosslinked networks. Several ways to synthesize hydrogels are tabulated below [2].

Table 2-2
Methods for synthesizing physical and chemical hydrogels

Physical gels
Warm a polymer solution to form a gel (e.g. PEO-PPO-PEO block copolymers in H_2O)
Cool a polymer solution to form a gel (e.g. agarose or gelatin in H_2O)
'Crosslink' a polymer in aqueous solution, using freeze-thaw cycles to form polymer microcrystals (e.g. freeze-thaw PVA [poly(vinyl alcohol)] in aqueous solution)
Lower pH to form an H-bonded gel between two different polymers in the same aqueous solution (e.g. PEO and poly(acrylic acid))
Mix solutions of a polyanion and a polycation to form a complex coacervate gel (e.g. sodium alginate plus polylysine)
Gel a polyelectrolyte solution with a multivalent ion of opposite charge (e.g. Na^+alginate$^-$ + Ca^{2+} + $2Cl^-$)
Chemical gels
Crosslink polymers in the solid state or in solution with:
Radiation (e.g. irradiate PEO in H_2O)
Chemical crosslinkers (e.g. treat collagen with glutaraldehyde or a bis-epoxide)
Multi-functional reactive compounds (e.g. PEG + diisocyanate = PU hydrogel)
Copolymerize a monomer + crosslinker in solution (e.g. HEMA (hydroxyethyl methacrylate) + EGDMA (ethylene glycol dimethacrylate))
Copolymerize a monomer + a multifunctional macromer (e.g. bis-methacrylate terminated PLA-PEO-PLA + photosensitizer + visible light radiation)
Polymerize a monomer within a different solid polymer to form an IPN gel (e.g. acrylonitrile + starch)
Chemically convert a hydrophobic polymer to a hydrogel (e.g. partially hydrolyse poly(vinyl acetate) to poly(vinyl alcohol) or polyacrylonitrile to polyacrylonitrile/polyacrylamide/poly(acrylic acid)

Polymer blending is a technique to obtain materials whose physical properties depend on blend composition and may be modulated through compositional changes. The resulting materials could combine the appropriate mechanical properties of the synthetic component with the biocompatibility of the biological components. The collagen/PVA [poly(vinyl alcohol)] blends had been investigated by Beatrice *et al* [13] to display very good film-forming properties for the development of haemodialysis membranes having taken the advantage of non-antigenicity and absorbability of collagen and water solubility and film-forming ability of PVA. On the other hand, Maria *et al* [14] blended collagen with PVA to form composite hydrogel to be used as protein-releasing matrices.

Crosslinking by condensation reactions is another frequently applied technique to modify polymer properties. Non-crosslinked collagen based materials are rapidly degraded *in vivo*. One of the ways to prolong its durability is by crosslinking which leads to desirable biochemical and structural modifications such as decreased antigenicity, increased mechanical strength, reduced solubility and a reduced rate of biodegradation [15]. The most widely applied crosslinking agent is glutaraldehyde, which results in very stable materials [16]. Nevertheless, glutaraldehyde treated materials show high cytotoxicity [17]. More recently, researchers have begun to explore the use of epoxy compounds as it is found that crosslinking of collagen with such reagent based on glycidyl ethers yielded well-stabilized tissue having comparable mechanical properties to glutaraldehyde crosslinked materials with no cytotoxic degradation products observed [18,19]. In this study, polypropylene glycol diglycidyl ether polymer for use in crosslinking with collagen will be investigated. Polypropylene glycol (PPG) has a chemical structure similar to that of PEG that has very high biocompatibility and been widely used in biomedical application. One property of PPG is that it is thermo-sensitive, being more hydrophilic at low temperature and hydrophobic at high temperature. For this reason, chemically modified collagen with PPG of bifunctional epoxy compound will be able to form stable gel at body temperature, as the hydrophobic interaction between PPG segments will provide extra force to keep the macromolecules together.

CHAPTER 3
SYNTHESIS OF HYDROGELS

3.1 POLYMER BLEND TECHNIQUE

Materials

The highly purified, pepsin-solubilized, bovine corium type I collagen marketed under the trade name of Vitrogen® 100 with the collagen concentration of 3.2 mg/ml was obtained from Cohesion Technologies, Palo Alto (USA). PEO-PPO-PEO copolymers with average molecular weight of 13,300 (F108) and 4400 (L103) were purchased from Polysciences, Inc. (USA) and Aldrich respectively. Fluorescein isothiocyanate (FITC)-dextran (M_w 21,200) was obtained from Sigma.

Procedures

1. Collagen – tri-block copolymer hydrogel preparation

The composite hydrogel was formed from collagen and varying concentration of tri-block copolymers. Briefly, Vitrogen® with pH of 2.0 was added with polymer and conjugate buffer ($Na_2CO_3/NaHCO_3$, pH $\approx$ 9.5) with a ratio of 1:1:3. The solution was then mixed homogeneously with vortex action. For preparation of hydrogel as a cell-seeding matrix, the mixture was further added with a predetermined amount of 1 M HCl to achieve neutral pH and 1% of streptomycin and penicillin to prevent contamination while maintaining the same final concentration. This was followed by incubation at room temperature for approximately one hour to form stable gels.

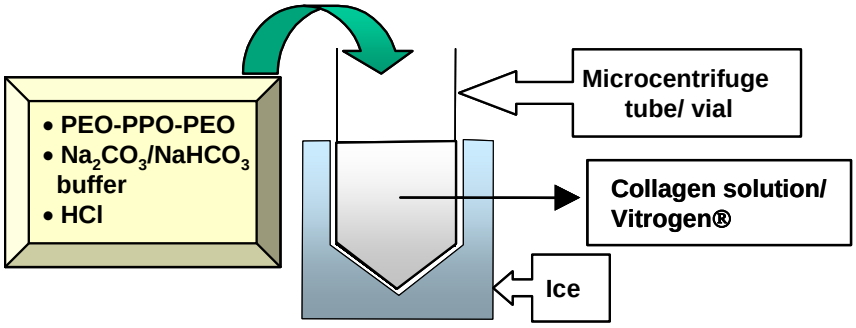


Figure 3-1. Schematic diagram of the fabrication procedure of composite hydrogel

Figure 3-2. Photo images of C-F108 (1%) hydrogel sample at different position

After left undisturbed for approximately one hour, the mixture would transform into jello-like translucent hydrogel consisting of entanglement of fibrous collagen coated with polymer (Figure 3-2)

2. Preparation of collagen – tri-block copolymer hydrogel containing FITC-dextran

Hydrogel preparations were carried out in 1 ml micro-centrifuge tubes. The model drug FITC-dextran was incorporated into the hydrogel matrix by dissolving the drug flakes (0.5%) into collagen solution prior to addition of the polymer and

buffer and allowed to gel. Control hydrogels were prepared exactly as above but with the polymer substituted by PBS.

The hydrogels were prepared as follows:

Table 3-1. Sample preparation of hydrogels

Hydrogel	Concentration		
	Collagen	PEO-PPO-PEO	
		EO 30%	EO 80%
C-L103 (1%)	3.2 mg/ml	1%	-
C-L103 (5%)	3.2 mg/ml	5%	-
C-L103 (10%)	3.2 mg/ml	10%	-
C-L103 (15%)	3.2 mg/ml	15%	-
C-F108 (1%)	3.2 mg/ml	-	1%
C-F108 (5%)	3.2 mg/ml	-	5%
C-F108 (10%)	3.2 mg/ml	-	10%
C-F108 (15%)	3.2 mg/ml	-	15%

3.2 CROSSLINKING OF SYNTHETIC POLYMER BY HYDROLYSIS

This method involved crosslinking of the synthetic polymer to collagen molecules via reaction between functional groups of the protein, that is, amino groups and reactive groups of polymer molecules to form covalent attachment in the presence of a solvent.

Materials

Table 3-2. Materials used in the fabrication of composite hydrogel by hydrolysis

1. Protein/ biopolymer	Bovine Serum Albumin (BSA)* / Vitrogen 100®
2. Polymer	PPG tolylene 2,4-diisocyanate terminated M_w 2300# / PPG diglycidyl ether M_n 380
3. Media	Conjugate buffer of Na_2CO_3 & $NaHCO_3$ pH 9.5 / 1 x PBS pH 7.4 / NaOH/ HCl

** BSA was utilized to conduct the first few trial runs in different buffers with different pH values.*
 # Due to its low solubility, PPG tolylene 2,4-diisocyanate terminated was later abandoned in favor of PPG diglycidyl ether.

Procedures

BSA / Vitrogen® 100 was dissolved in a conjugate buffer/ PBS/ NaOH on ice and mixed well. The amount of ingredients and pH values of the mixtures were shown in Table 3-3 below.

Table 3-3. A) amount of BSA & PPG in various media of different pH values B) amount of Vitrogen® 100 & PPG in NaOH of different pH values

A

Sample	BSA	PPG diglycidyl ether	Conjugate buffer (Na_2CO_3/$NaHCO_3$)	PBS	NaOH (0.1 M)	HCl (0.1 M)	pH
PG-BSA1	5 mg	5 mg	2 ml	-	-	-	9.5
PG-BSA2	5 mg	5 mg	-	5 ml	-	-	7.4
PG-BSA3	5 mg	5 mg	-	-	2 ml	1 ml	12
PG-BSA4	5 mg	5 mg	-	-	2 ml	-	14

B

Sample	Vitrogen (initial concentration = 3.2 mg/ml)	PPG diglycidyl ether	1 M NaOH	1 M HCl	pH
PG-COL1	1 ml	5 mg	0.5 ml	0.35 ml	12
PG-COL2	1 ml	5 mg	0.3 ml	-	14

PPG diglycidyl ether was then added into the solution. The mixture was stirred using magnetic bar at low temperature ($\lesssim$10 °C) for approximately 72 hours to allow reaction to take place. Afterwards, the solution was neutralized with 0.1 M HCl or NaOH to pH 7.0 and added with DI water to obtain the desired final concentration (final concentration of BSA & Vitrogen = 1 mg/ml). To remove un-reacted polymers, the solution was dialyzed against water for one week at low temperature ($\lesssim$10 °C). Xerogel was obtained by freezing the dialyzed solution at -120 °C and dried in a vacuum dessicator. The dry gel was later hydrated for testing purposes.

CHAPTER 4
EXPERIMENTATION

SCANNING ELECTRON MICROSCOPY FOR POLYMER BLENDED HYDROGEL AND CROSSLINKED XEROGEL

Collagen–tri-block copolymer hydrogel matrices were fixed, dehydrated and dried using the following methods: A few drops of sample (5 – 10 μl) – while still in liquid form – were placed on 13-mm circular glass coverslips and left undisturbed at room temperature to allow solution to gel. Gels were then fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. The specimens were washed three times with PBS prior to dehydration through a series of graded ethanol solutions. After the final dehydration wash (100% ethanol), instead of critical point drying, specialized drying agent hexamethyldisilazane (HMDS, Fluka) was put into the matrix and allowed to evaporate at ambient temperature under a fume hood overnight.

On the other hand, polymer crosslinked samples were prepared by directly freeze-drying gel solution droplets.

The resulting samples were sputter coated with gold and examined using a scanning electron microscope. Images were recorded digitally at an accelerating voltage of 5 kV using SEM-JEOL 5600.

4.1 POLYMER BLENDED HYDROGEL

4.1.1 IN VITRO RELEASE STUDY

In vitro release studies of FITC-dextran loaded composite hydrogels were carried out by placing the hydrogel-containing micro-centrifuge tubes in 1x PBS buffer, pH 7.4, at 37 °C after the opening of the tubes was first wrapped with loosely woven cloth to retain hydrogels. The buffer was changed at regular time intervals. The aliquots were then pipetted out and assayed using a Tecan Spectrafluor (λ_{ex} = 485 nm; λ_{em} = 535 nm).

4.1.2 CELL PROLIFERATION & VIABILITY ASSAYS

Cell type used was K562, a human haemopoietic cell line. These cells of myeloid origin are critically dependent on the interaction with the surrounding microenvironment i.e. cell:cell, cell: extracellular matrix (ECM) and cell:growth factor to regulate hematopoiesis. The matrix played an active role in control of cell adhesion and in proliferation processes. One of the major categories of matrix molecules was collagen. Koenigsmann *et al* had identified collagen type I as an adhesive substrate for myeloid progenitor cells [20] whereas Klein *et al* found the myeloid cell lines to adhere strongly to collagen XIV [21].

K562 cells were incubated at 37 °C with 5% CO_2 and fed with RPMI media with 10% fetal bovine serum (FBS) and 1% penicillin & streptomicin to prevent contamination. Collagen–tri-block copolymer gel matrix solutions of varying polymer concentrations were prepared, distributed into 48-well plate and incubated for one hour. They were then seeded with 100 μl of cell suspension for biological testing and added with 100 μl fresh RPMI media. Cell proliferation was

assessed using alamar blue, a new non-fluorescent substrate, which after reduction in living cells yields a very strong fluorescent product. Measurements of fluorescence were then performed with Spectrafluor plate reader (Tecan Spectrafluor) at 535 nm excitation wavelength and 595 nm emission wavelength and a gain of 50. Cell viability was determined via trypan blue dye exclusion. On day 7, the hydrogel matrices were removed from the well plate while 20 μl aliquots in the plate were diluted 2x in trypan blue and counted using a haemocytometer. Percent viability was calculated using the equation listed below. This procedure was repeated at least 3 times.

$$\% \text{ Viability} = \frac{\text{Viable cells}}{\text{Total cells}} \times 100\%$$

4.2 POLYMER CROSSLINKED XEROGEL

4.2.1 GEL ELECTROPHORESIS

Gel electrophoresis is a technique used for separation of proteins on the basis of their molecular weight. The SDS – PAGE (Sodium dodecyl sulphate – polyacrylamide) gel electrophoresis was conducted on crosslinked gel solution in order to detect any property improvement by analyzing changes in its molecular weight. The procedure was carried out according to Laemmli's method [22] with some modifications. SDS – PAGE gel containing 3% stacking gel and 4% separating gel was formulated as shown in Table 4-1.

Table 4-1. Materials used in preparation of SDS – polyacrylamide gel

	3%	4%
Separating buffer (1.5 M Tris, pH 8.8)	–	2.5 ml
Stacking buffer (1.0 M Tris, pH 6.8)	1 ml	–
10% SDS	40 μl	100 μl
Deionized H_2O	2.6 ml	6.3 ml
40% Acrylamide	0.3 ml	1 ml
10% Ammonium persulphate	40 μl	100 μl
TEMED	4 μl	8 μl

Protein sample solutions were mixed with 50 mmol Tris-Cl pH 6.8, containing 2% SDS, 10% glycerol and 0.1% bromophenol blue, heated at 95 °C for 4 minutes to denature the proteins and subjected to electrophoresis with a current of 50 mA in vertical slab gel until the bromophenol blue marker reached the bottom of the gel. The gel was washed with water, covered using staining solution comprising coomasie blue R-250, ethanol and deionized water and heated in microwave on high till the solution just began to boil. It was then placed in a slow shaker for one hour at room temperature to allow diffusion staining. The gel was de-stained by repeated washing in 40% ethanol and 10% acetic acid.

4.2.2 TNBS TEST

2,4,6-Trinitrobenzenesulfonic acid (TNBS) was a rapid and sensitive assay reagent for the determination of free amino groups. Primary amines, after reaction with TNBS, formed a highly chromogenic derivative, which could be measured using UV spectrophotometer (UV-2501PC, Shimadzu) at 420 nm (see Figure 4-1).

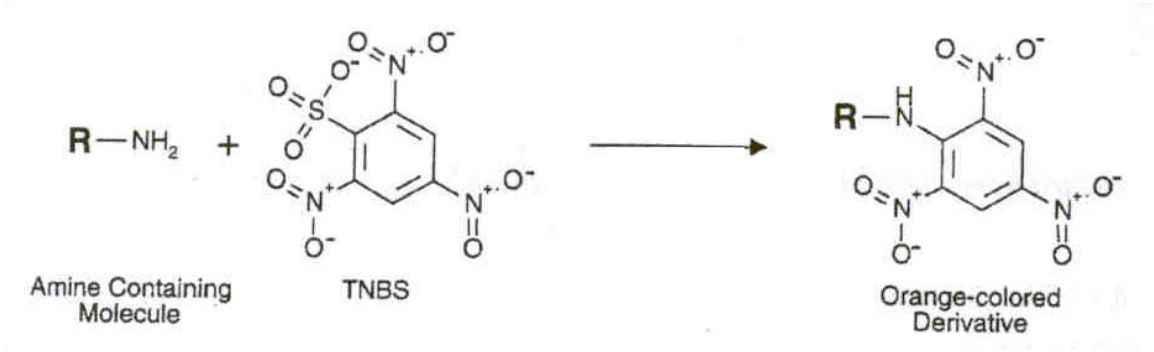


Figure 4-1. Reaction of TNBS with a primary amine-containing molecule producing a chromogenic derivative

The purpose of this experiment was to obtain the quantitative change in the number of free amino groups that accompanied the chemical modification of the amino groups in proteins (in this case collagen type I after reaction with PPG diglycidyl ether) as a measure of the degree of crosslinking. The test method was adapted from a procedure described by Habeeb [23] as briefly mentioned in the following:

Proteins to be assayed were dissolved directly in 4% sodium bicarbonate reaction buffer, pH 8.5 to the desired concentration. This was followed by the addition of 0.1 ml of 0.1% w/v TNBS to 0.5 ml of each sample solution. The mixture was incubated at 37 °C in water bath for 2 hours. 0.25 ml of 10% SDS and 0.125 ml of 1 N HCl were then added to each sample and the absorbance of the solutions was measured.

4.2.3 THERMOGRAVIMETRIC ANALYSIS & DIFFERENTIAL SCANNING CALORIMETRY

Thermogravimetric analysis (TGA-2050, TA Instruments) was conducted to determine the degradation temperature of hybrid gel before running differential scanning calorimetric (DSC) test. Approximately four milligrams of the dry gel – obtained by freezing the gel solution at -120 °C and drying in a vacuum dessicator – were placed into TGA sample pan and heated at the rate of 20 °C min^{-1} to 800 °C. This was followed by the calorimetric test performed on a TA Instruments Modulated DSC-2920 Differential Scanning Calorimetry to examine denaturation temperature of the sample, which correlated with the degree of crosslinking.

Due to its porosity and inherent hygroscopicity, the sample was infused with N_2 gas to inhibit water absorption from humid air, weighted and sealed in the hermatic pan. The adopted procedure to run DSC test was: heat the sample from -150 °C to 160 °C (below decomposition temperature) at 20 °C min^{-1} (first scan) and rapidly cool to -150 °C followed by subsequent reheating to 160 °C at 20 °C min^{-1} (second scan) and 20-minute isothermal.

4.2.4 PROTON NMR SPECTROSCOPY

Nuclear Magnetic Resonance (NMR) is the study of molecular structure through measurement of the interaction of electromagnetic radiation with a collection of nuclei matters immersed in a magnetic field. Based on hydrogen chemical shift, the chemical structure of NMR samples could be deduced from the NMR spectra. In this case, the NMR spectrum of hybrid gel was compared to that of pure

collagen as control to detect any structural changes. The test was performed on a Bruker 400 MHz NMR. Prior to spectroscopy, approximately 4-milligram sample was prepared by dissolving the analyte in an 800 μl deuterium oxide (D_2O) lock solvent. The solution was then transferred into NMR sample glass tube and run in the spectrometer.

4.2.5 CELL CYTOTOXICITY ASSESSMENT

Mouse fibroblast cell line, 3T3, was utilized due to its availability in the laboratory and could be passaged many times while retaining their principal characteristics. Moreover, it had been reported that the incorporation of fibroblasts into a collagen gel produced contraction of that gel [24].

In-vitro cytotoxicity test was conducted to evaluate the biocompatible characteristics of collagen-PPG diglycidyl ether hybrid gel as drug carrier.

Two types of test methods were adopted in accordance to ISO 10993-5: 1. Test on extracts; 2. Test by direct contact.

Test on extracts

Materials:

Sample	**Components**		**Weight (mg)**
	Collagen (3.2 mg/ml)	PPG diglycidyl ether	
Pure collagen	3.2 mg	-	4.1
PG-COL*c*5	3.2 mg	5 mg	4.4
PG-COL*c*10	3.2 mg	10 mg	4.5
PG-COL*c*15	3.2 mg	15 mg	4.7

All samples were sterilized by a 16-h treatment with UV light in a laminar flow hood.

Procedure:

Samples (≈ 5 mg) were added with 1 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% streptomycin and penicillin and then kept at 37 °C for 5 days. Extracts were then filtered with 0.22 μm PES syringe filters and measured for its cytotoxicity on 3T3 cells that had been seeded onto 96-well plates at a density of 2 x 10^4 cells/ml. Cells in culture medium served as a negative control. The extracts were loaded onto the wells and incubated for 72 h at 37 °C in a CO_2 atmosphere.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl Tetrazolium Bromide) colorimetric assay was used to verify mitochondrial functionality of the cell. After the incubation period, 20-μl MTT solution (5 mg/ ml) was added into each well and kept for 4 h. Living cells would cleave the pale yellow substrate to yield a dark blue formazan product. One hundred microliters of DMSO were added to each well in order to dissolve the crystals. The plate was placed in the incubator for 10 mins before the absorbance measurements. The optical density was then read on a multiwell microplate reader at the wavelength of 595 nm. The absorbency directly correlated with the metabolic activity and thus correlated with the number of living cells.

Test by direct contact

Material used was PG-COL*c*20. 400 μl of this sample solution was evenly spread on the circular glass coverslips and freeze dried followed by UV sterilization for 16 h. The dry gel was then placed onto the 24-well plate and seeded with 3T3-cultured cells. Blank glass coverslip as well as bottom plate plastic were used as

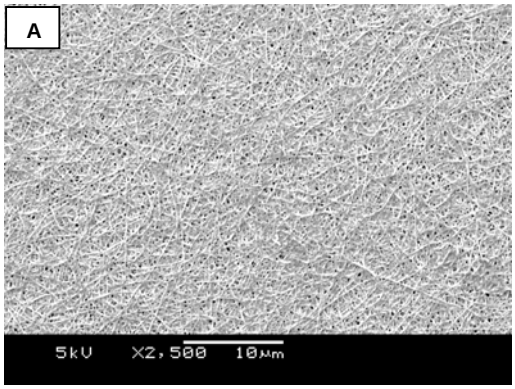
controls. Seeding density was 2 x 10^5 cells/ml. The sample was incubated at 37 °C in an air atmosphere containing 5% CO_2. Twenty-four hours after seeding the cells, culture medium was pipetted out and cells were added with 1 ml (1%) Neutral Red (NR) to assess cell lysosomal damage. The time required for staining was approximately 3 to 4 hours. Cells on sample and controls were observed under light microscope and 10 shots taken at different surface areas. Average number of cells was then quantified by using computerized image analysis system (Micro Image Lite) that had counting feature.

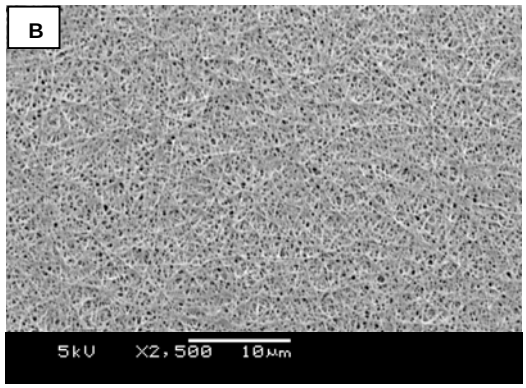
CHAPTER 5
RESULTS

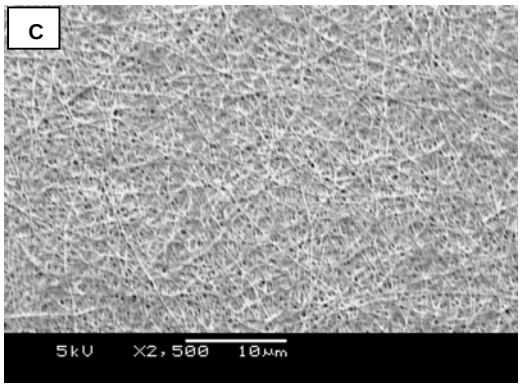
5.1 POLYMER BLENDED HYDROGEL

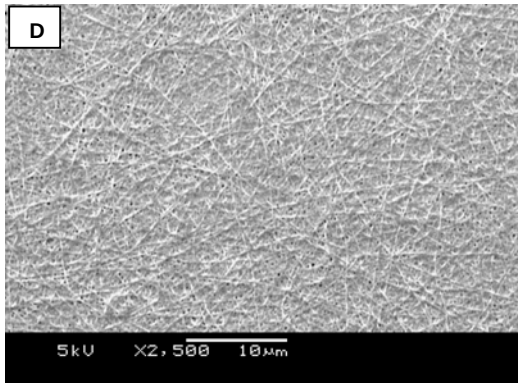
5.1.1 SCANNING ELECTRON MICROSCOPY

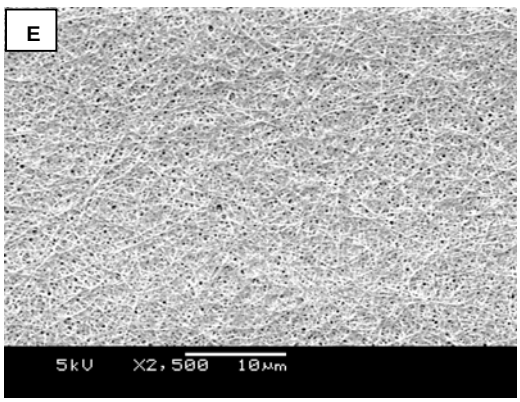
Figure 5-1 showed the surface morphology of collagen – PEO-PPO-PEO (L103 & F108) hydrogels at varying concentrations of polymer. It could be seen that collagen matrix consisted of entanglements of fibrillar structure with inter-fiber spacing. The introduction of polymer at low concentration reduced the number of spacings though not significantly in the matrix macrostructure and the formation of collagen fibers was not at all affected. However, as concentration increased, the coating of polymer onto the fibers became thicker and fewer pores were observed. When 5% concentration of F108 was used, the matrix was illustrated in the SEM picture as having fewer and shorter fibers but slightly bigger in diameter with dense coating indicated by the disappearance of pores in Figure 5-1G. The fiber formation had seemed to be perturbed by polymer at high concentration. On the other hand, the fibers were not greatly affected at 5% concentration of L103 (see Figure 5-1 D).

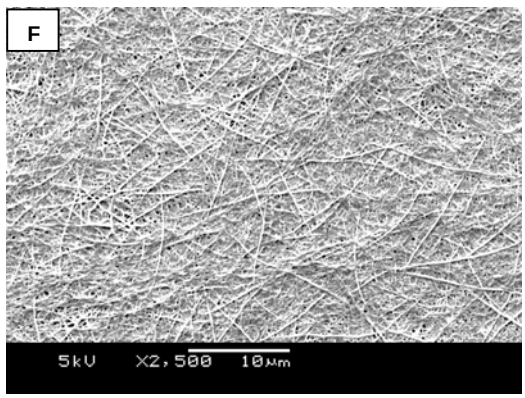












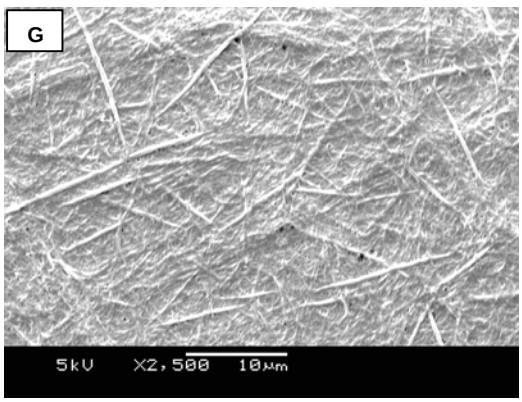


Figure 5-1. SEM images of the morphology of A) Collagen gel; B) C-L103 (1%); C) C-L103 (3%); D) C-L103 (5%) E) C-F108 (1%) F) C-F108 (3%) G) C-F108 (5%) where the collagen concentration is 3.2 mg/ml. (Original magnification: x2500)

5.1.2 IN VITRO RELEASE STUDY

The cumulative release profiles of FITC-Dextran from collagen – PEO-PPO-PEO hydrogels were shown in Figures 5-2, 5-3 and 5-4. The graph of collagen gels depicted initial burst of 15% drug for the first 12 hours followed by diffusional release of drug which slowly declined following the $t^{1/2}$ kinetics before reaching plateau. In the case of C-L103 hydrogels, all the graphs had similar initial burst release as that of collagen gel except for C-L103 (15%), which had a slightly higher burst of about 23%. Eventually the amount of drug being released from C-L103 (1%, 5%, 10%) became significantly less than that of collagen gel ($p < 0.05$ t-test) after day 6, 4 and 6 respectively by 3 to 4% while displaying almost similar collagen release pattern and subsequently reached plateau after one month. On the other hand, the diffusion of drug from C-L103 (15%) was too rapid somewhat in a zero-order fashion as compared to that of collagen gel. The graph of C-F108 (1%) in Figure 4 indicated initial burst release of 14% during 10½ hrs and then the rate of release gradually decreased, diffusing the drug significantly lower ($p < 0.05$ t-test) by 8% after day 6 than that of collagen gel, which lasted for approximately a month whereas C-F108 (5%, 10%, 15%) underwent rapid drug diffusion compared to collagen gel and released 100% drug in less than or equal to 4 days as shown on the graphs.

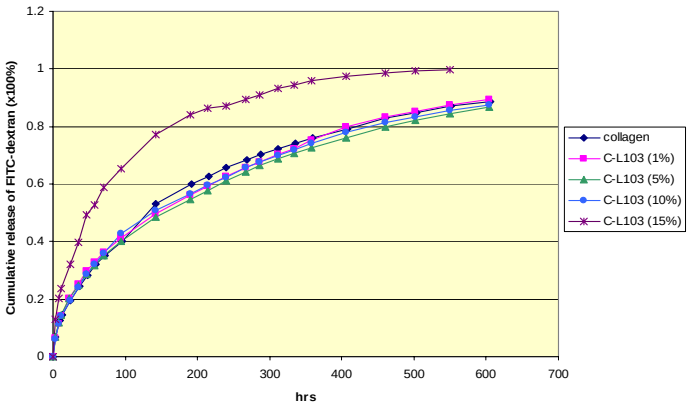


Figure 5-2. In vitro release of FITC-Dextran from collagen – PEO-PPO-PEO (EO 30%) hydrogels prepared with polymer concentration of 1, 5, 10 & 15 percents

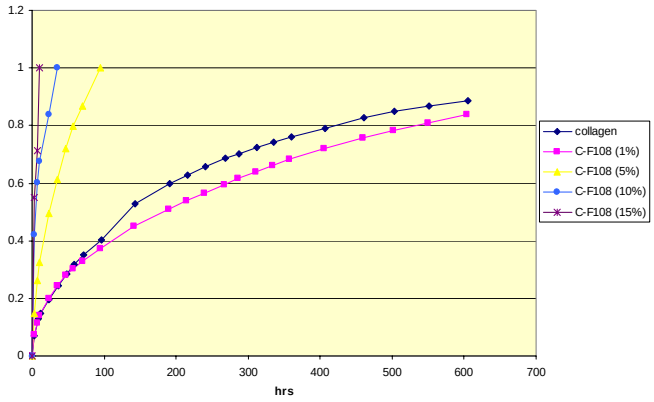


Figure 5-3. In vitro release of FITC-Dextran from collagen – PEO-PPO-PEO (EO 80%) hydrogels prepared with polymer concentration of 1, 5, 10 & 15 percents

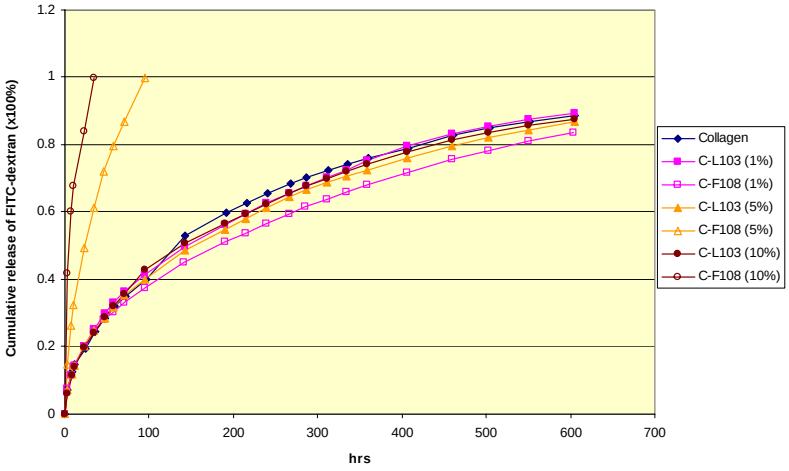


Figure 5-4. Comparison between the in vitro release of FITC-Dextran from collagen – PEO-PPO-PEO (EO 30% & 80%) hydrogels prepared with polymer concentration of 1, 5, 10 percents

5.1.3 CELL PROLIFERATION & VIABILITY ASSAYS

Cell proliferation and viability assays were evaluated in order to study the influence of the matrix on cell behaviour. As shown in Figure 5-5 below, cell growths on collagen–PEO-PPO-PEO hydrogels i.e. C-F108 (1%) and C-F108 (3%) appeared to be increasing in a similar pattern as indicated by the effect of time on fluorescence development although the growth in the latter became slightly faster after day 5. Both demonstrated a constant difference of 14% as compared to that of collagen from day 4 onwards. As the concentration of

polymer reached 5%, cell growth on hydrogel matrix was inhibited compared to that of collagen gels as observed on the graph.

In the case of C-L103 hydrogels, cells grew at rapid rate especially in C-L103 (5%) matrix before gradually declining after day 1. Although C-L103 (5%) displayed slight inclination from day 1 onwards, the variation was quite big and thus did not reflect significant increase.

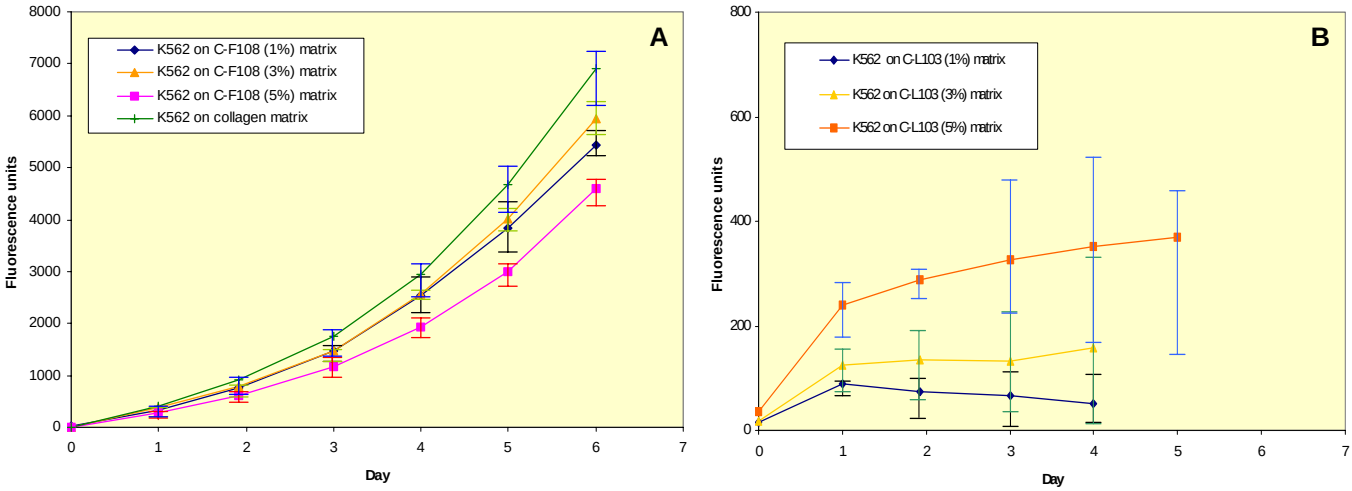


Figure 5-5. K562 cell proliferation on A) collagen – PEO-PPO-PEO (EO 80%) matrices B) collagen –PEO-PPO-PEO (EO 30%) matrices of different polymer concentration measured by the addition of Alamar blue. Mean values were used in the plot. Error bars represented the standard error of the mean (n=3)

Cell viability for C-F108 hydrogels was found to be high (see Table 5-1 below) and very close to that of collagen gels. This could indicate that tri-block copolymer PEO-PPO-PEO (EO 80%) at concentration below 5% did not significantly affect cell viability in the matrices. However, a greater concentration of polymer (5%) in the matrix resulted in lower cell viability (below 50%, results not shown here). On the other hand, PEO-PPO-PEO (EO 30%) significantly affected cell viability even at 1% concentration.

Table 5-1. Cell viability of C-F108 hydrogel of varying polymer concentrations

Hydrogel	% Viability
C-F108 (1%)	77.9 ± 2
C-F108 (3%)	84.2 ± 6
Collagen	90.2 ± 1

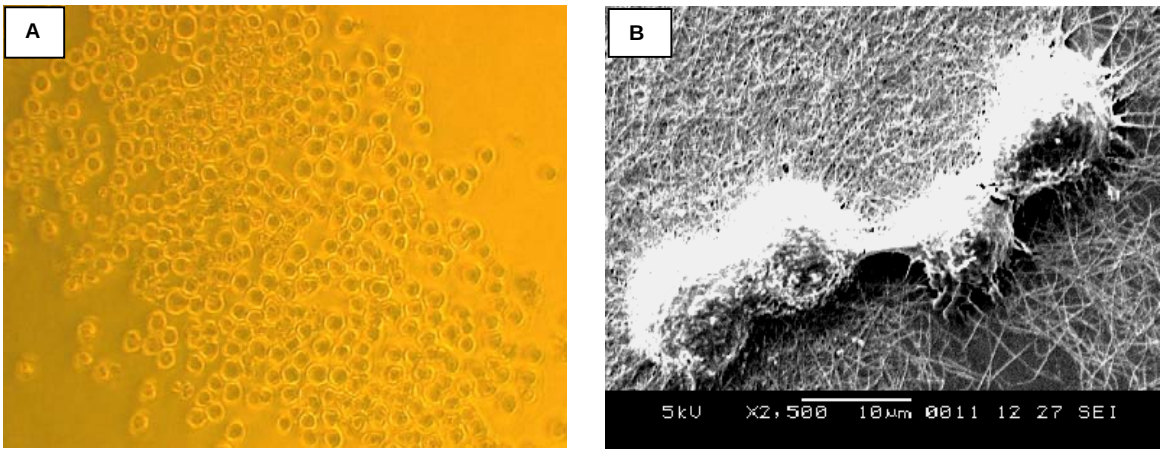
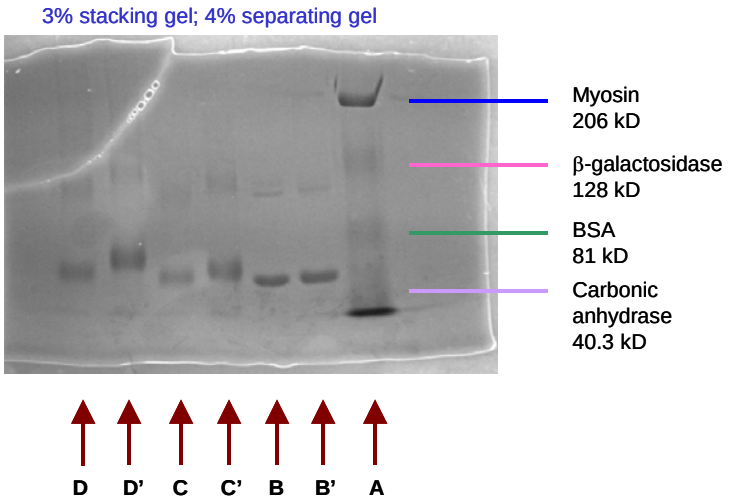


Figure 5-6. A) K562 cells on C-F108 (1%) hydrogel matrix after one week B) SEM image of K562 cells on C-F108 (1%) after fixation with glutaraldehyde (2%) (Original magnification: x2,500).

5.2 POLYMER CROSSLINKED XEROGEL

5.2.1 GEL ELECTROPHORESIS

Below was the image of protein bands of sample PG-BSA2, PG-BSA3 and PG-BSA4 that had been prepared according to the procedure mentioned earlier in section 3.2 up to step no. 3 (refer to Table 3-3A).



A: Protein marker (Kaleidoscope Prestained standard)
B: Sample PG-BSA2 in PBS, pH=7.4
B': BSA in PBS (same preparation as that of sample PG-BSA2 but without addition of PPG)
C: Sample PG-BSA3 in NaOH/HCl, pH=12.0
C': BSA in NaOH/HCl (same preparation as that of sample PG-BSA3 but without addition of PPG)
D: Sample PG-BSA4 in NaOH, pH=14.0
D': BSA in NaOH (same preparation as that of sample PG-BSA4 but without addition of PPG)

Figure 5-7. SDS-PAGE behavior of pure BSA and BSA-PPG diglycidyl ether ca 380 crosslinked hybrid in different media of different pH values

BSA was utilized as a protein model to study the feasibility of crosslinking between protein and polymer having epoxide groups at both ends. The results showed that sample PG-BSA2 had no weight change when compared with pure BSA in PBS (B') while sample PG-BSA3 and PG-BSA4 as compared to C' and D' respectively gave an indication of slight weight increase ($\Delta M_w \approx 10$ kD) suggesting an improvement in the properties of the modified BSA. It was deduced that the reaction between amino groups and end groups of polymer molecules was effective only when performed at pH value $\tilde{>}$ 12.

Under this effective alkaline condition, collagen type I – one of the main components of study – was crosslinked with PPG diglycidyl ether (see Table 3-3B) and the product was analyzed by carrying out the same test to depict protein bands as shown in the figure below.

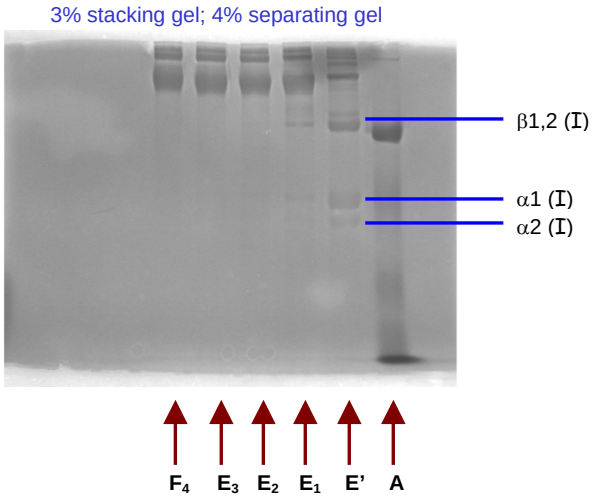


Figure 5-8. SDS-PAGE behavior of pure collagen and collagen-PPG diglycidyl ether ca 380 hybrid in media at different time interval

A: Protein marker (Kaleidoscope Prestained Standard)
E': Vitrogen dissolved in NaOH/HCl (same preparation as that of sample PG-COL1but without addition of PPG)
E_1: sample PG-COL1 in NaOH/HCl pH = 12.0 at day 1
E_2: sample PG-COL1 in NaOH/HCl pH = 12.0 at day 2
E_3: sample PG-COL1 in NaOH/HCl pH = 12.0 at day 3
F_4: sample PG-COL2 in NaOH, pH = 14.0 at day 4

There was an increase in the molecular weight of PG-COL hybrids (E_1, E_2, E_3, F_4) ($\Delta M_w \approx 90$ kD) as expected as shown by the gradual disappearance of α1, α2 and β1,2 collagen chain bands with time followed by their upward shift to form the thickening part at the second upper band as compared to those of pure collagen (E'). There was no difference between sample of pH 12 and 14.

5.2.2 SCANNING ELECTRON MICROSCOPY

Scanning electron micrographs (Figure 5-9) revealed that the pure collagen gel had extremely porous structures. This was a result of the formation of ice crystals as porogens that were removed by evaporation on freeze-drying. On the other hand, collagen-PPG diglycidyl ether of 20 mg polymer amount hybrid gel (PG-COL*c*20) showed on Figure 5-10 also consisted of porous structure that was nevertheless covered by a layer of non-porous configuration. This lamina became bulkier and thicker as the concentration of the polymer was increased.

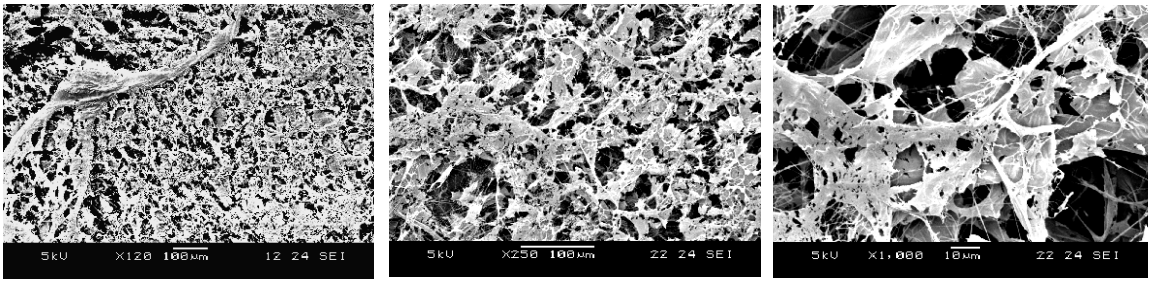


Figure 5-9. SEM images of pure collagen at different magnification

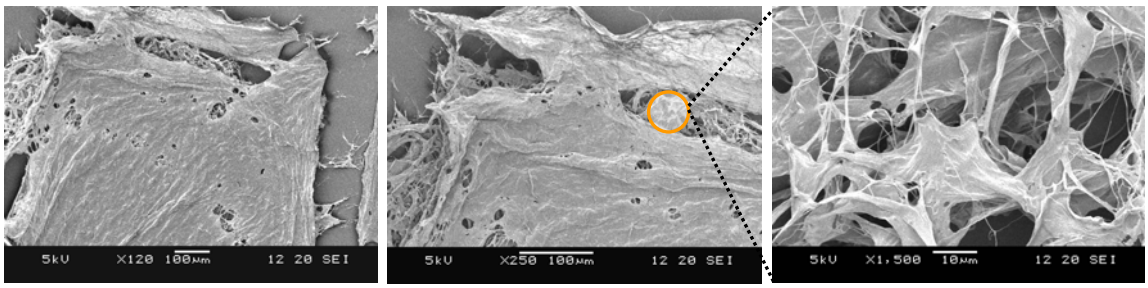


Figure 5-10. SEM images of PG-COL*c*20 at different magnification

5.2.3 THERMOGRAVIMETRIC ANALYSIS

Figure 5-11 showed the degradation temperature of PPG diglycidyl ether with a molecular weight of 380 Dalton to be 93 °C. After crosslinking with collagen, the new composite matrix had an increase in degradation temperature by ≈ 123 °C.

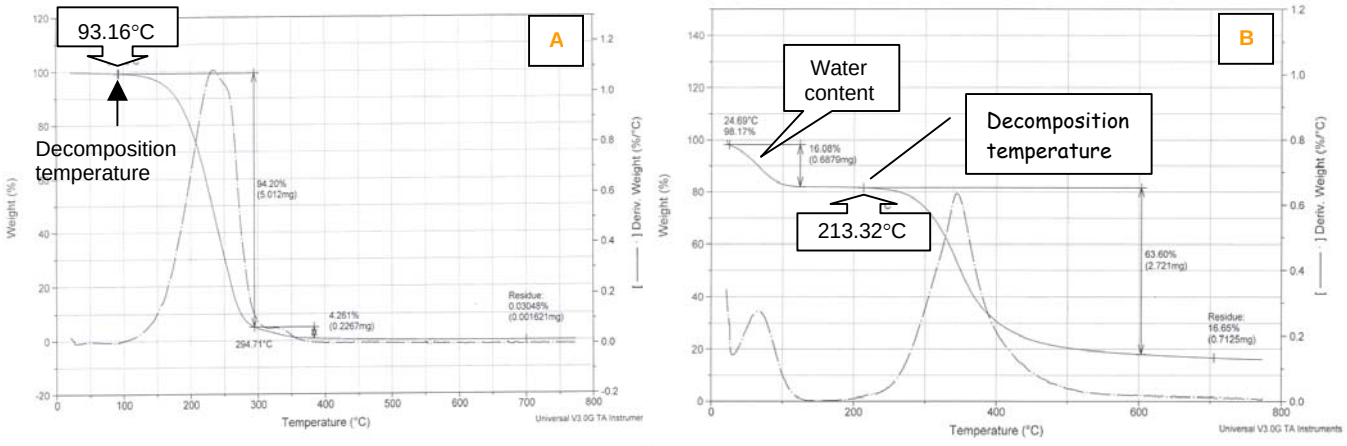


Figure 5-11. A) TGA curve of pure PPG diglycidyl ether ca 380; B) TGA curve of composite dry gel

5.2.4 DIFFERENTIAL SCANNING CALORIMETRY

The DSC calorimetric curve of pure PPG diglycidyl ether (see Figure 5-12) was used as a reference.

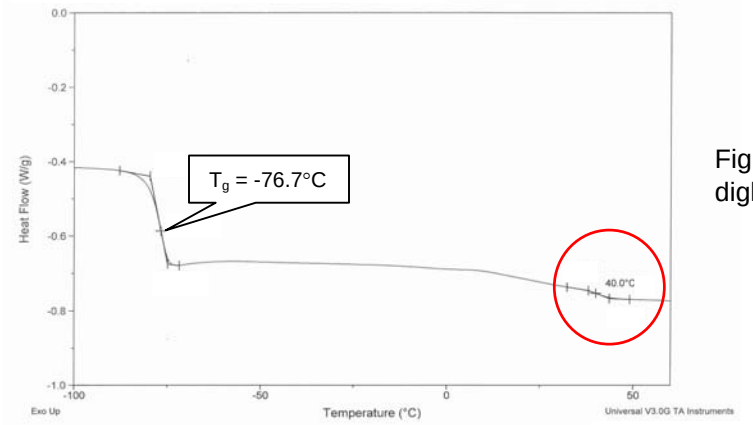


Figure 5-12. DSC graph of pure PPG diglycidyl ether ca 380; T_g = -76.7 °C

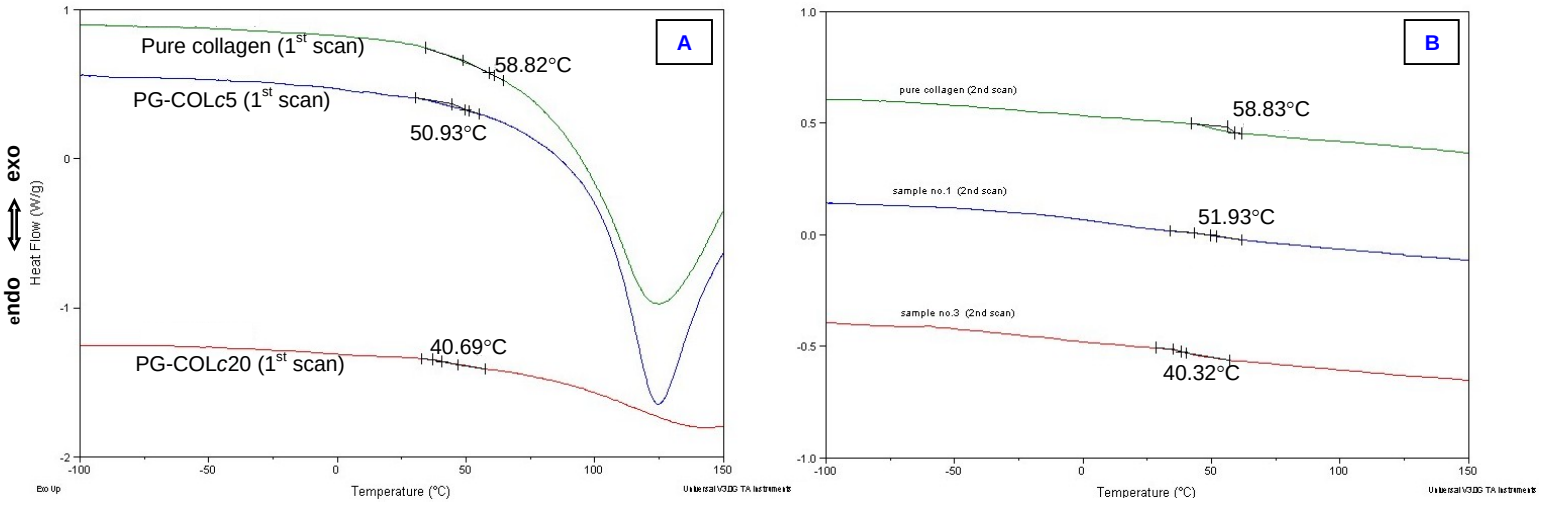


Figure 5-13. Calorimetric curve of pure collagen, PG-COL*c*5 and PG-COL*c*20. A) first scan B) second scan

The endotherm of pure collagen and PG-COL*c*5 (hybrid gel of 5 mg polymer amount added to 3.2 mg collagen) both occurred at the T_d (denaturation temperature) approximately equal to 124 °C as indicated by the curves in Figure 5-12A. The enthalpy associated with denaturation endoterm of the two was also similar i.e. about 2.6 Wg^{-1}. On the other hand, a slight shift of T_d was observed in PG-COL*c*20, which could be attributed to a certain degree of crosslinking. Thus, the concentration of polymer was likely to have an influence in the reaction. In DSC calorimetric curves of samples after second scan (Figure 5-13B), the denaturation endotherm had disappeared from the curves of collagen and composite dry gel, indicating that ordered structure had been lost. The only phenomenon observed was the T_gs.

5.2.5 PROTON NMR SPECTROSCOPY

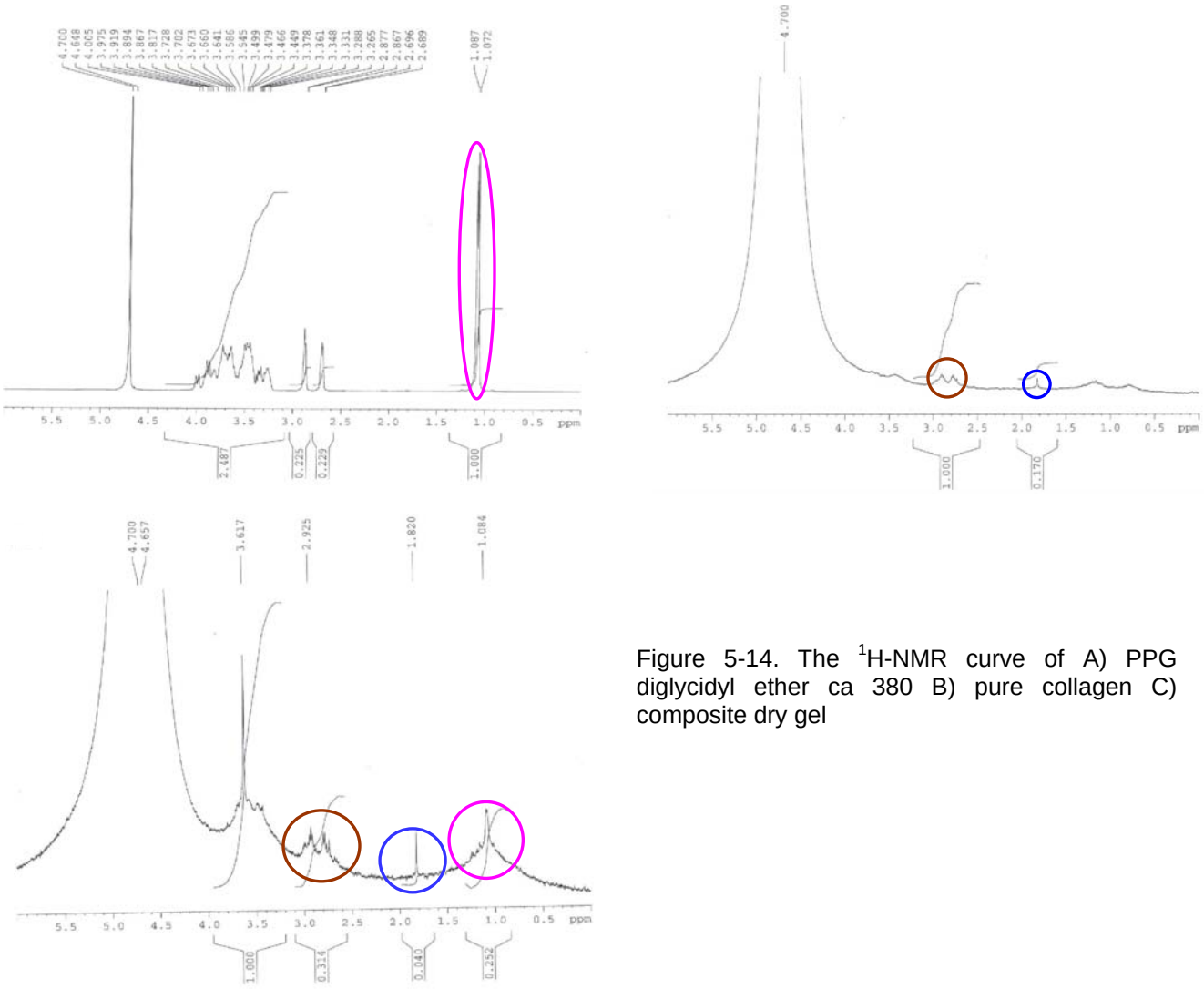


Figure 5-14. The ^{1}H-NMR curve of A) PPG diglycidyl ether ca 380 B) pure collagen C) composite dry gel

The chain irregularities of PPG diglycidyl ether were apparent in the signal range of 4 – 3.2 ppm (see Figure 5-14A) with a sharp resonance at 1.09 ppm indicating the methyl protons of PPG. In Figure 5-13C, mixture of signals from polymer and collagen were spotted.

5.2.6 TNBS TEST

The free primary amine group content (lysine residues) of samples was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS). The lower the value of amino group, the more was the protein reacted/crosslinked with polymer. Quantitative determination of the number of amines contained within a sample was accomplished through comparison to a standard curve generated by the use of any amine-containing compound – i.e BSA (Bovine Serum Albumin) in this case – dissolved in a series of concentrations as tabulated below.

Concentration (μg/ml)	BSA-mol/l	NH_2-mol/l	Absorbance
400	6.06E-06	3.64E-04	0.809
300	4.55E-06	2.73E-04	0.611
200	3.03E-06	1.82E-04	0.41
150	2.27E-06	1.36E-04	0.305
100	1.52E-06	9.09E-05	0.209

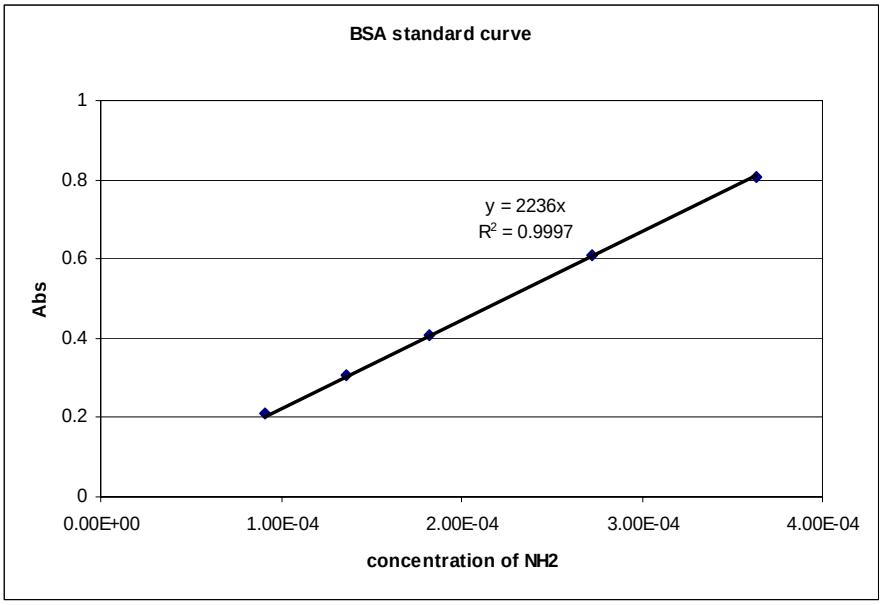


Figure 5-15. Plotted graph of concentration of amine vs. UV-absorbance

The samples were measured at wavelength of 420 nm and the data were listed below

Table 5-2. Number of free amino groups in hybrid solution of varying polymer amounts. n=2

Sample			Concentration of sample (μg/ml)	Collagen* (mol/l)	Abs	Concentration of NH_2 (mol/l) from standard curve	No. of amino& groups
Code	collagen	PPG					
[1]PG-COL*c*5	3.2 mg	5 mg	200	6.67E-07	0.399	1.78E-04	267.67
[1]PG-COL*c*15	3.2 mg	15mg	200	6.67E-07	0.327	1.46E-04	219.36
[1]PG-COL*c*20	3.2 mg	20 mg	200	6.67E-07	0.287	1.28E-04	192.53
[2]PG-COL*c*5	3.2 mg	5 mg	200	6.67E-07	0.391	1.75E-04	262.30
[2]PG-COL*c*15	3.2 mg	15 mg	200	6.67E-07	0.311	1.39E-04	208.63
[2]PG-COL*c*20	3.2 mg	20 mg	200	6.67E-07	0.308	1.38E-04	206.62

[1]batch 1; [2]batch 2

**Molecular weight of collagen ≈ 300 kD*

[&]No of amino groups in collagen ≈ 284 (from experiment)

5.2.7 CELL CYTOTOXICITY ASSESSMENT

Test on extracts

Table 5-3: Cell growth percentage of hybrid gel compared to control

	Pure collagen	PG-COL*c*5	PG-COL*c*10	PG-COL*c*15	Control
Average Absorbance (per 3 wells)	0.429	0.428	0.463	0.164	0.597
Relative cell growth*	71.8%	71.6%	77.6%	27.5%	-

*Relative cell growth = [A]test / [A}control ; [A] = absorbance

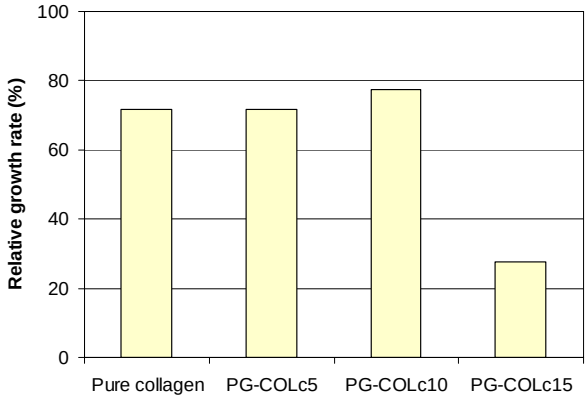


Figure 5-16. Cytotoxicity of 3T3 mouse fibroblasts on collagen-PPG composite gel, measured as percentage of the living cells, 72 h after seeding

Statistical analysis using paired T test was also conducted with 95% confidence that the difference in cell numbers on PG-COL*c*15 (hybrid gel of 15 mg polymer amount added to 3.2 mg collagen) as compared to that of control was significant while the rest were not.

Test by direct contact

Substrate	Average no. of living cells after 24 h
PG-COLc20	11
Blank glass coverslip (control)	14
Bottom well plate (control)	27

The number of fibroblasts that adhered on the PG-COL*c*20 matrix was less than that on blank glass coverslip and bottom well plate but the difference was small and not significant.

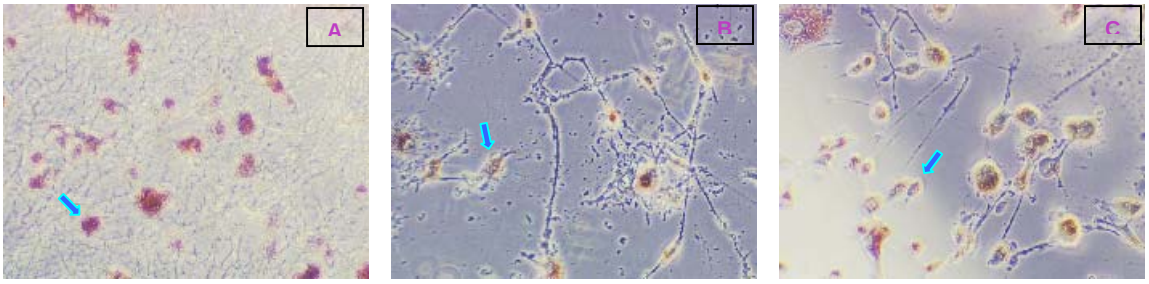


Figure 5-17. At day 1 under light microscope, fibroblasts (arrows) in: A. PG-COLc20 B. blank glass coverslip C. bottom well plastic. Original magnification x10

To study the cellular morphology, dry gel seeded with fibroblasts was subsequently assigned for scanning electron microscopic (SEM) examination. Specimen was fixed for one hour at room temperature in a 2% glutaraldehyde and dehydrated through a series of graded ethanol solutions.

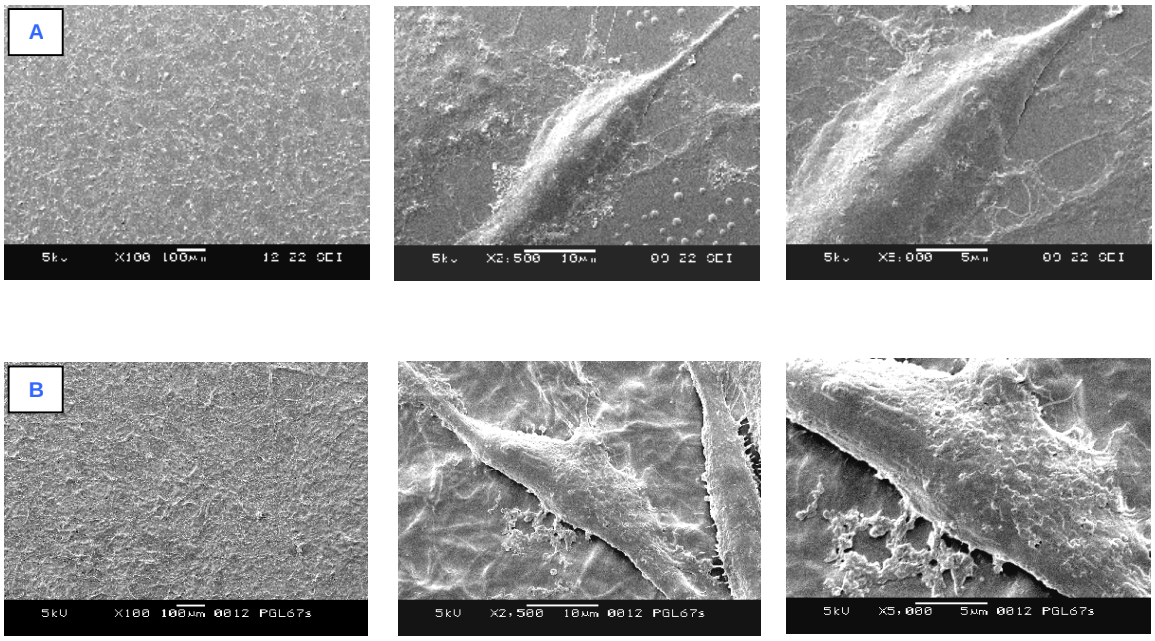


Figure 5-18. SEM images of 3T3 fibroblasts on A) glass coverslips and B) PG-COL*c*20 at different magnification

It was observed that the cells stretched out to form polygons showing normal cellular morphology on both glass coverslips and hybrid sample.

CHAPTER 6
DISCUSSION

6.1 POLYMER BLENDED HYDROGEL

The sustained release of drug from collagen gel was attributed to the inter-fiber spacing formed by collagen fiber entanglement as well as dissolution of the drug particles near the surface thus exposing more pores. However, the addition of tri-block copolymers served as coating to the collagen fibers by adsorption of hydrophobic blocks (PPO in the order of M_w 3000 for both L103 and F108) that sufficiently anchored the copolymer chain to hydrophobic side groups of amino acids in collagen molecules thus reducing the pores between fibers and allowing long term controlled release (refer to Figure 5-1). The higher the concentration of polymers, the thicker were the coatings. Nevertheless, it could reach to a point where the influence of tri-block copolymers became stronger with increasing concentration as collagen and tri-block copolymers interacted resulting in increase and ultimate hindrance to the formation of self-assembled collagen fibers, which was contrary to the study that tri-block copolymer did not interfere with fiber formation [25]. This was reflected in the graphs of C-L108 (5%, 10%, 15%) in which case the release of drug was fast as less entanglement of collagen fibers was present to sustain the drug whereas C-L108 (20%) released the entire drug in less than 6 hours (figure not shown here).

The hydrophobic PPO chains are the important factors to provide necessary anchor for the polymer molecules to remain adsorbed at the substrate for coatings and the PEO chains extend into the aqueous phase. As polymer

concentration increases, the PEO chains are getting more crowded on the surface and this somehow leads to the formation of collagen fibers being affected in evidence. Fewer fibers in turn will mean reduced surface area for PPO chain anchorage and thus decreased effectiveness of polymer coating with increment of concentration. How PEO chains interact with collagen molecules in fiber formation is not yet fully understood but it is speculated that the chains might interfere with the alignment of collagen molecules thus obstructing the formation of intermolecular crosslinks that yield microfibrils, then fibrils to become fibers. More detailed study still needs to be done. The length of the PEO chain may also be a contributing factor as the tendency to interfere during fibrillogenesis is higher with longer PEO chains resulting in less collagen fibers. This explains for the case of F108 (80% PEO) where the drug started to release at approaching zero order (see Figure 5-4) when the concentration was increased to 5%, 10% as compared to that of L103 (30% PEO) with equal concentrations.

Results from the cell proliferation test suggested that cells seemed able to live very well within the C-F108 hydrogel matrix surroundings when polymer concentration used was low. This could also be partially attributed to the functionality of PEG end groups that resulted in high blood compatibility [26] especially for this type of blood cells. However, as the concentration of polymer reached 5% or greater, the overall hydrogel became rather toxic and thus the interaction between cells and matrix decreased which was illustrated by slower growth rate (refer to Figure 6). On the other hand, cells were greatly inhibited in C-L103 hydrogel matrices. This could be due to lower affinity of cells to stronger

hydrophobic character as percentage of EG decreased thereby affecting cell viability as well as proliferation.

6.2 POLYMER CROSSLINKED XEROGEL

Crosslinking of collagen using a diglycidyl ether involved reaction of the epoxy groups with amine groups of (hydroxy)lysine residues when alkaline conditions were applied or with two carboxylic acid residues if crosslinking was performed under acidic conditions [27] (see Figure 6-1). In this event, one molecule of PPG diglycidyl ether having two functional groups reacts with two amino groups in a collagen forming a bridge as shown.

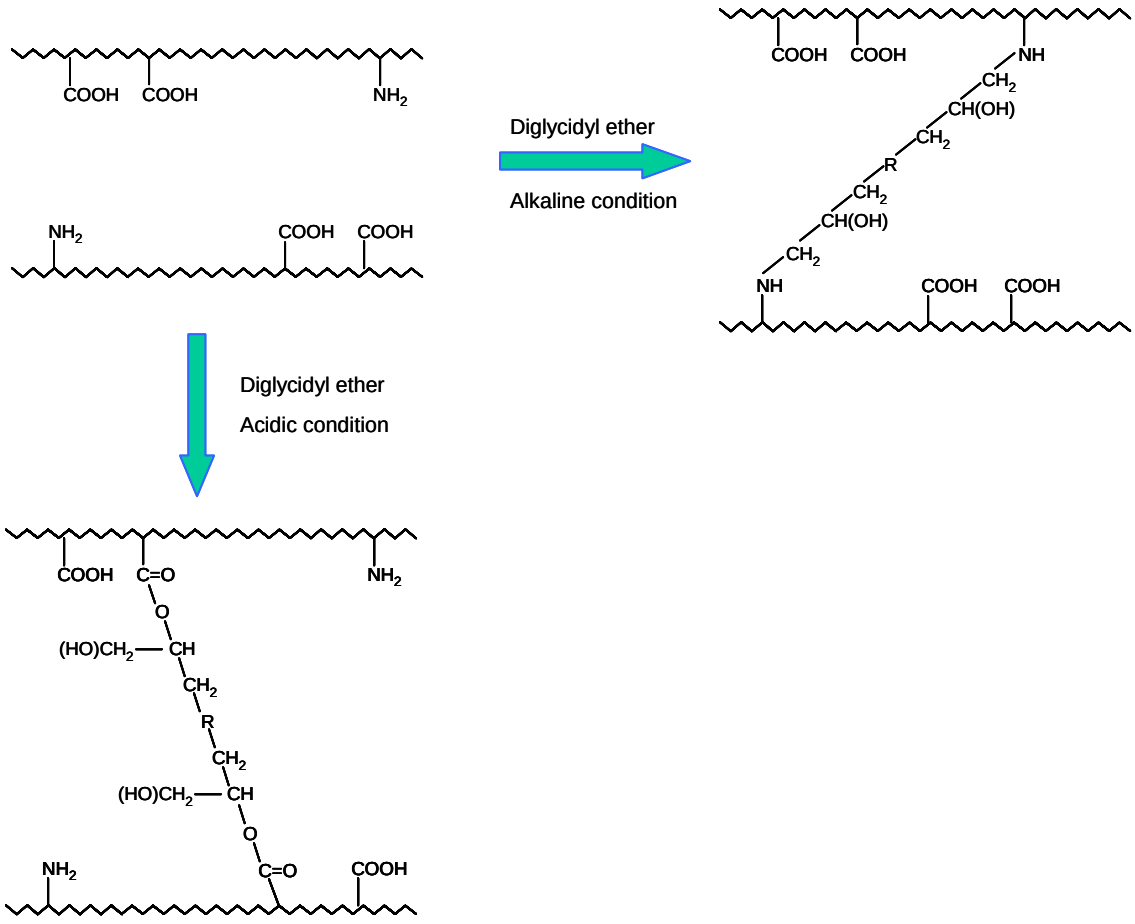


Figure 6-1. Schematic diagram of crosslinking of collagen with diglycidyl ether

The epoxy groups in diglycidyl ether, with their high reactivity due to the ease of opening of the strained three membered ring, can be cleaved under both acidic and alkaline conditions [28]. In the acid-catalyzed cleavage, an epoxide is converted by acid into a protonated epoxide, which can then undergo attack by any of a number of nucleophilic reagents. In the base-catalyzed cleavage of epoxide, it is the epoxide itself, not the protonated epoxide, that undergoes nucleophilic attack. The reaction of the epoxy and the amino groups under alkaline conditions most likely involved a nucleophilic substitution reaction at the $\delta+$ center of the epoxy group.

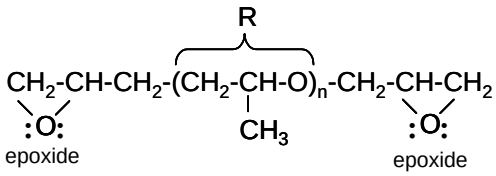
Some studies have shown that while crosslinking of collagen in acidic environment resulted in a material with higher tensile strength and elongation, the *in vitro* stability was rather poor [27,29]. When implanted, this collagen-based material showed a faster rate of biodegradation. Owing to the project application, material with delayed degradation was essential. Therefore crosslinking of collagen was carried out at high pH to obtain a material with better degradation resistance and higher stiffness for better matrix function.

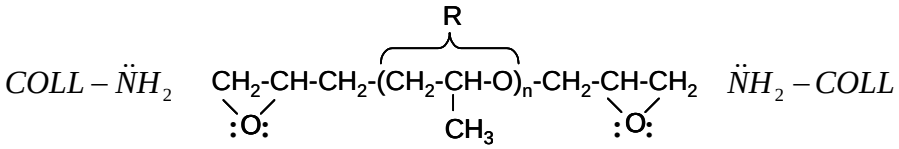
Possible chemical structure of collagen/polymer conjugate

Collagen: $COLL-\ddot{N}H_2$

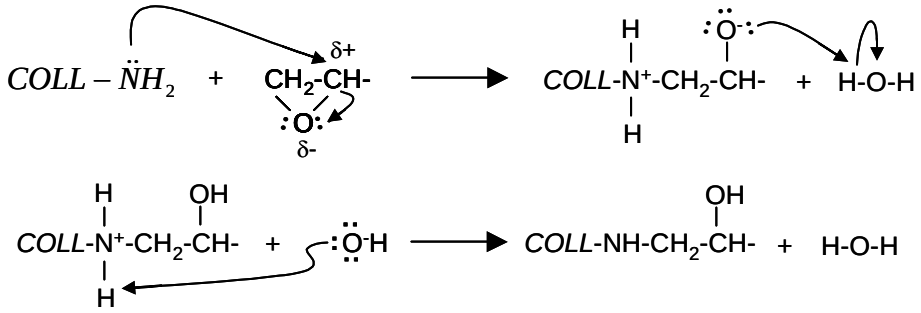
lysyl
ε-amino group

PPG diglycidyl ether:





Before crosslinking



After crosslinking

Figure 6-2. Reaction mechanisms of epoxide with lysyl ε-amino group under basic conditions (pH ≈ 12)

The fixation of collagen with diglycidyl ether was conducted at high pH, in which case the value of 12 was found to be the minimum requirement to initiate nucleophilic substitution reaction to epoxy group resulting in property change as shown by the increase of molecular weight in gel electrophoresis test (see Figure 5-8). Reaction under basic condition with pH less than 12 had also been

conducted and proven to indicate no changes though their protein band images were not illustrated here due to poor gel documentation. Evidence of occurrence of the reaction was further supported by other tests such as NMR and TNBS, the respective graphs and data of which were illustrated in earlier chapter (refer to Figure 5-14 and Table 5-2). From the NMR spectra, functional group of PPG diglycidyl ether as well as collagen was observed while the data acquired from TNBS test showed a decrease of amine groups with the increase of polymer concentration as more fixative reacted with amino groups. The content of amino groups was about 200 for 86% of synthetic polymer (0.86 mg in 1 mg of sample) as compared to 264 for 61% of polymer and 284 for 0%.

However, the yield of the product was rather low i.e. only maximum 50% could be retrieved after dialysis and freeze-drying. Beside the loss in dialyzing the sample, it might be attributed to less than the expected crosslinking with some polymer. As the degree of crosslinking was related to the increase of the shrinkage (denaturation) temperature, thermal analysis using differential scanning calorimeter was carried out and the thermograms were depicted in chapter 5 (see Figure 5-13). The denaturation temperature is that required to break the hydrogen bonding in the collagen triple helix and change it to a random coil configuration. It is therefore an indicator of those crosslinks which stabilize the triple helix against the uncoiling necessary to produce a random coil.

The denaturation temperature of sample PG-COL*c*5 and pure collagen was similar. This might indicate very little occurrence of crosslinking or possible collagen branching. Branching means that a fixative reacts with a single amino

group, resulting in a branch coming from the reacted amino acid. Usually branching was evidenced in lower denaturation temperature as compared to native collagen. Since the decrease of T_d was not significant, this could be a minor factor. However, little or no crosslinking did not indicate the absence of interaction between polymer and collagen. There could be a possibility of masking in which the fixed single amino group was masked from reacting further with another active fixative. In fact, study by Tu [30] had shown that masking was much more prevalent especially when fixation was conducted at higher pH. Crosslinking of collagen with bifunctional polyepoxy compound involved a two-step reaction sequentially, with the reaction of the first epoxy group with an amino group, followed by the reaction of the second epoxy group with another amino group in the vicinity to form a cross-link. In the intermediate between these two steps, if the unreacted second epoxy group of the partially linked polyepoxy compound was deactivated by a side reaction, no subsequent cross-linking could occur. The deactivation of the intermediate was more likely, by the side reaction with the hydroxyl ions at a higher pH as it was affected by the relative pKa values of the amino acid species and the solution environment. Therefore, relatively more masking could be expected for fixation under more alkaline conditions. The evidence of masking was supported by the TNBS test data which showed a significant decrease in the amino groups for PG-COL*c*5 for the same denaturation temperature as that of pure collagen in DSC calorimetric curve. Another contributing difficulty in achieving significant crosslinking could be due to the structure of the PPG that was highly unstable in water. In addition, the

conformation of the collagen was likely changed significantly as the polar/non-polar interaction was altered in the organic salt solution used (NaCl) with certain polarity. It was possible that the crosslinks which were able to form in this new conformation were different from those which would have formed in the aqueous environment. Conformational changes might both have brought additional pairs of amino groups into sufficient proximity for crosslinking to occur while other pairs might have been separated and crosslinking inhibited.

To study the feasibility of this PPG diglycidyl ether-collagen crosslinked dry gel as tissue scaffold specifically for skin burn, fibroblasts – cells responsible for producing extracellular matrix in wound healing and tissue remodeling – was chosen for cell seeding purposes on the matrix. Referring to Figures 5-17 and 5-18, it was observed that fibroblasts were alive and adhered well onto the collagen-polymer hybrid matrix after 24 hours and did not show any deviant cell morphology. In fact, the property of the surface of hybrid gel was tested by measurement of the water contact angle. It has been widely accepted that cells prefer to attach to hydrophilic surface than hydrophobic surface [31]. The lowered water contact angle of gel formed between the tangent plane to the surface of deionized water and tangent plane to polymer/collagen sample increased hydrophilicity of this material. Briefly, samples were prepared by spreading the gel solution over glass coverslips to form a very thin film and allowed water from the solution to evaporate slowly under a laminar flow in a hood. The morphology of the coating was observed by SEM whose images were shown below.

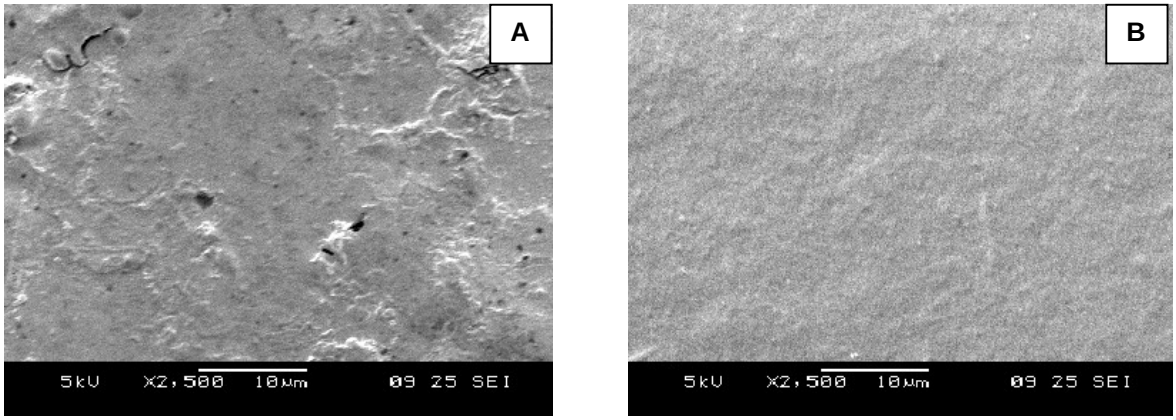


Figure 6-3. A) Surface film of collagen-PPG diglycidyl ether composite B) Surface film of pure collagen

It turned out that the contact angle of crosslinked hybrid was lower than that of pure collagen (see Table 6-1 below), thus likely to indicate high hydrophilicity, which explained strong attachment of the cell.

Table 6-1. Water contact angle of the control and conjugate film. The test repetition, n=4

Sample	Sessile drop contact angle
Control collagen	61.1 ± 3.1
PPG-collagen	41.6 ± 3.0

However, the rate of cell proliferation was very low and the number of dead cells increased with time. This could be attributed to the remnants of epoxy that induced cytotoxicity or release of cytotoxic products from cell-biomaterial interactions. The level of cytotoxicity was also dependent on the amount of polymer concentration as illustrated in Figure 5-16. As the amount of polymer added into the collagen was greater than 15 mg, the cell growth dropped drastically.

Porosity

The pure collagen matrix was porous whereas the collagen-polymer composite matrix was bulky with porous layer being covered by another less porous layer as seen in Figure 5-10. However from the SEM pictures in Figure 5-18 the matrix after cell seeding showed a marked reduction in its pore size. This could be due to the contraction of collagen substrates by fibroblasts *in vitro*, which was a well-recognized phenomenon [32]. It might also represent contraction of the collagen matrix in response to cytokines and/or growth factors present in the serum in DMEM – a medium used for maintaining the cells. Contraction had been known to be responsive to the presence of growth factors and cytokines in media [32]. Another possible factor of pore size decrease was that ethanol organic solvent used for the dehydration process in SEM sample preparation could dissolve the polymer thus reorganizing the ultrastructure of the matrix.

CONCLUSIONS

From the results it could be concluded that by the addition of a proper amount of PEO-PPO-PEO tri-block copolymer into collagen to form hydrogel, it was possible to improve or even programme the release of FITC-Dextran to slower rate than hydrogel comprising only one component i.e. collagen. This hydrogel matrix was also found cell compatible particularly when using polymer with EG of 80% at concentration below 5%.

In the case of synthetic copolymer PPG diglycidyl ether-collagen composite, the results of the study showed that crosslinking was achieved with the extent of crosslinking became higher as the polymer concentration was increased. This material was also found to be more hydrophilic than the gel made of collagen alone and served as a substrate for the fibroblast cells to adhere and proliferate. This dry gel could make a promising candidate for application as cellular scaffold.

RECOMMENDATIONS/ FUTURE WORK

Since polypropylene diglycidyl ether is highly unstable in water, certain methods or means are needed to enhance stability and promote adequate penetration into water-rich environment to stimulate crosslinking. In fact, the use of surfactant has been chosen in an effort to suspend the polymer, which will result in the formation of micelles of PPG diglycidyl ether within the aqueous surroundings. The detergent utilized was PEG-PPG-PEG triblock copolymer (Pluronics®). Nevertheless, the end product of this method after four weeks of dialysis showed extra weight that was likely to be contributed by the pluronic indicating the possibility of binding between protein and surfactant besides PPG diglycidyl ether. The unknown effects of the detergent may arise such as potential for molecular extraction or alteration of protein charge and conformation due to residual binding of a surfactant. Further study needs to be done on this aspect.

The cytotoxic effect of the biomaterial can also be studied using methylcellulose culture gel mixed with fibroblasts which was developed by Luyn *et al* [33]. The cells then migrate towards the bottom of the well and adhere and thereafter, test samples are placed on top of the gel. The advantage of this test system is the possibility of evaluating cytotoxicity for a period of up to 7 days without renewing the culture gel, thus allowing the accumulation of (possibly delayed) released cytotoxic (such as secondary cytotoxicity) product within the test system leading to more accurate cytotoxicity scoring. Furthermore, it is possible to both quantitatively evaluate by counting cell numbers and to qualitatively evaluate by

studying cell morphology with light microscope as this method eliminated the sample problem thickness.

Another tests that can be conducted in the future are the swelling studies and *in vitro* drug release to examine the potential use if this hybrid biomaterial as drug carrier as well. The swelling study was carried out in order to determine the amount of gel water uptake by immersion in deionized H_2O and left to swell to their equilibrium state overnight. Swelling measurements are relatively simple means to characterize crosslinked polymer networks and also helpful in the interpretation of drug release and diffusional transport processes through the macromolecular material. This test will be done at various temperatures in order to investigate the sensitivity of PPG diglycidyl ether to the temperature changes and their influence to the swelling ability. The *in vitro* drug release test using BSA-FITC as protein model is performed to obtain the kinetic release profiles of the hybrid to recognize the nature of its sustained release.

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APPENDIX

Conjugation of Modified Pluronic® to Collagen with EDC coupling

Materials:

1. Vitrogen (Collagen type I in acid solution) 3.2 mg/ml, Mw~300,000 daltons
2. OH-PEG-PPG-PEG-COOH (Modified from BASF Pluronic® PE6400 Mn~2900), Mn~ 2750
3. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), Mw 191.7
4. 2 M Na2CO3 aqueous solution.
5. 2 M HAc aqueous solution.

Procedures:

1. Dissolve polymer (51.2 mg, 1.86 x 10^{-5} mol) in 4 - 6 ml of PBS buffer (pH 7.4) at ~4 °C.
2. Add EDC crystals (3.56 mg, 1.86 x 10^{-5} mol) directly into the above solution, followed by stirring for 4 hours at ~4 °C.
3. Take 2 ml of Collagen (6.4 mg, 2.13 x 10^{-8} mol) and adjust the pH to 4.0 using Na_2CO_3. Then add collagen solution into the solution with the activated polymer.
4. Stir the mixture overnight at ~4 °C, and occasionally test the pH of the mixture. If the pH is not within the range of 4.5 – 5.5, adjust it to ~5.0 using 2 M Na_2CO_3 or 2 M HAc.
5. Remove un-reacted polymers in the solution through dialysis in pure water for at least one week. Change the waste at least two times a day.