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Triethyl orthoformate covalently cross-linked chitosan-(poly vinyl) alcohol based biodegradable scaffolds with heparin binding ability for promoting neovascularisation

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Abstract

There is a need to develop pro-angiogenic biomaterials to promote wound healing and to assist in regenerative medicine. To this end, various growth factors have been exploited which have the potential to promote angiogenesis. However these are generally expensive and labile which limits their effectiveness. An alternative approach is to immobilize heparin onto biocompatible degradable hydrogels. The heparin in turn will then bind endogenous proangiogenic growth factors to induce formation of new blood vessels.

In this study we continue our development of hydrogels for wound healing purposes by exploring covalently cross-linking chitosan (CS) and polyvinyl alcohol (PVA) hydrogels using triethyl orthoformate (TEOF) .Two concentrations of TEOF (4 and 16 %) were compared for their effects on the structure of hydrogels -their swelling, pore size and rate of degradation and

for their ability to support the growth of cells and for their heparin binding capacity and their effects on angiogenesis in a chick chorioantoic membrane assay .

Hydrogels formed with 4 or 16% both TEOF cross-linker were equally cyto-compatible. Hydrogels formed with 4% TEOF absorbed slightly more water than those made with 16% TEOF and broke down slightly faster than non-cross-linked hydrogels. When soaked in heparin the hydrogel formed with 16% TEOF showed more blood vessel formation in the CAM assay than that formed with 4% TEOF.

Introduction

Many wounds do not heal properly and skin grafts and tissue engineered skin grafts do not "take" on wound beds because of poorly vascularized wound beds. This problem is well known as discussed in MacNeil 2007[1]. Angiogenesis is the sprouting of new capillaries from preexisting vasculature and it requires a complex multistep process involving the adhesion, proliferation, and differentiation of endothelial cells and helper cells[2].

Over the years there have been many attempts to induce angiogenesis by adding pro-angiogenic growth factors to wounds or to introduce glycosaminoglycans such as heparin which in turn will bind angiogenic growth factors (AGFs). To induce angiogenesis, AGFs must interact with pro-angiogenic receptors to induce proliferation, protease production and migration of endothelial cells (ECs)[3, 4]. Research over the last decade has shown that vascular endothelial growth factor (VEGF) has a fundamental role as a regulator of normal and abnormal angiogenesis. VEGF is essential for early development of the vasculature to the extent that inactivation of even a single allele of the VEGF gene results in embryonic lethality[5, 6]. Similarly, the Fibroblast growth factor (FGFs) family has been identified in a

variety of organisms to play a significant role in many cellular processes including angiogenesis, mitogenesis, differentiation, migration, and cell survival [7].

Heparin undoubtedly plays a pivotal role in this context since it binds to almost all the known AGFs, to several pro-angiogenic receptors and even to angiogenic inhibitors, producing an intricate network of interactions, the so called "angiogenesis glycomic interactome"[8, 9].

Many researchers have used heparin to promote angiogenesis by loading it onto different polymeric materials[10-13]. In particular they have used heparin loaded with VEGF to bind and stabilize VEGF which has a short half -life when added to wound beds on its own[14-16].

The current study continues our work in producing a hydrogel which can electrostatically bind heparin and stimulate angiogenesis while having a number of other desirable properties for clinical use. We are seeking to produce biocompatible biodegradable hydrogels which can be added to poorly vascularized wound beds which will stimulate the development of a temporary neo-dermis with new blood vessel formation. Previously, we successfully cross-linked CS and PVA using a fixed concentration of triethyl orthoformate (TEOF)[17]. In this study we have deliberately altered the concentration of TEOF to try to determine to what extent this influences material morphology, swelling behavior, the rate of degradation of the biomaterials produced, the binding of heparin and their subsequent effect on angiogenesis.

Chemical cross-linking is a very useful process to fabricate materials which combine the best properties of two different materials with different desirable properties. A combination of biocompatible polymers with synthetic polymers can enhance their mechanical, physical, biological and chemical properties, making them suitable for complex biological systems. Such composites reduce the disadvantages of each individual polymer whilst maximizing the

optimum properties of each in the resulting combined entity [18, 19]. One method of obtaining the required properties from natural and synthetic polymers is to blend and crosslink them [20].

Hydrogels have the capability to swell in water, trapping larger amounts within their structure without dissolving. They have physical properties similar to some aspects of the extracellular matrix and have excellent tissue compatibility. The only disadvantage of hydrogels is their poor mechanical properties after swelling. To overcome this disadvantage, hydrogels haves been modified by physical blending [21-24] or chemical crosslinking ~~methods~~ methods[25-27]. In particular chitosan in combination with other polymers has opened a new window of research for altering or tailoring the properties of materials [28-30]. Hydrogels of PVA and CS are non-toxic and biodegradable and hydrogels composed of such combinations are expected to have high biocompatibility [31].

In this study we demonstrate that the characteristics of these PVA/CS hydrogels are tunable by altering the degree of cross-linker used. We describe a TEOF cross-linked hydrogel with properties suitable for converting a poorly vascularized wound bed into a neo-dermis with new vasculature which we suggest is now suitable for evaluation in vivo.

Materials

Chitosan (CS) was purchased from Mian Scientific Company, Lahore, Pakistan and further purified in our laboratories as previously described[32, 33](degree of deacetylation (DD) 84%; Mw: 87047.26 g/mol). Poly (vinyl alcohol) (PVA) (Mw: 72,000, degree of hydrolysis 98%), hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) were purchased from Merck (Germany). Triethyl orthoformate (98%) was purchased from Alfa Aesar (Germany). Glacial acetic acid

(CH_3COOH) was purchased from AnalaR BDH Laboratory Supplies (UK). NaOH was purchased from Sigma Aldrich. Lysozyme was bought from Carbosynth Limited (UK), Toluidine Blue was purchased from Peking Chemical Works (China) and MTT was supplied by Sigma-Aldrich.

Human Breast cancer cell line MDA-MB-231 was employed to study the cytotoxicity of all the hydrogels. DMEM medium and fetal bovine serum (FBS) were purchased from GIBCO life technologies.

Preparation of CS/PVA Cross-linked Hydrogels

The CS (2.5% w/v) was dissolved in acetic acid (1%) aqueous solution and stirred magnetically for 12 hours at room temperature (RT). In a separate flask, PVA (10% w/v) was dissolved in distilled water at 80˚C under magnetic stirring for 2 hours. Then the two solutions were mixed in 80:20 (v/v) ratios of CS and PVA, and further stirred for 24 hours at RT. The resulting solution was then cast into petri dishes and the dishes were frozen at -80˚C for 24 hours. The samples were lyophilized for 24 hours in a freeze dryer at -40˚C. Then lyophilized hydrogels were rehydrated and soaked either without (Control) or with 4% or 16% TEOF w/v,-referred to as **Control, 4 CLH, 16 CLH**) in sulfuric acid (17% w/v) for 24 hours. The hydrogels were then removed from petri dishes and were neutralized with NaOH (12% w/v) for one hour then washed with distilled water three times and then lyophilized again for 24 hours.

Morphology of hydrogels assessed by Scanning Electron Microscopy

The morphology of hydrogels (before and after heparin loading) was studied~~observed~~ by SEM. The samples were mounted on a stub with conducting copper tape with a Nova NanoSEM-450. The SEM was operated at 10kV under low vacuum mode at 50Pa. The pore diameter was

calculated using image processing software (Image J). The diameter was of the pores was calculated from an average of 30 randomly selected pores.

Analysis of Chemical Structure of hydrogels

The structural analysis of the hydrogels (before and after heparin loading) was assessed by Fourier Transform Infrared Spectroscopy using Thermo Nicolet 6700P, USA. Each spectrum was recorded in the range of 4000-400 cm^{-1} and was an average of 256 scans at 8 cm^{-1} resolution.

Thermal Analysis of stability of hydrogels

The thermal stability of the samples was analyzed by Thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC) using SDT Q 600(USA). Thermograms were obtained from 25-600˚C at a heat ramp of 10 ˚C/min under inert nitrogen environment.

Swelling Properties

The swelling behavior of cross-linked hydrogels was observed by soaking them in phosphate buffered saline (PBS). The samples were cut into small pieces of approximately equal weight and weighed then submerged in PBS solution at 37 ˚C for 3 hours. The samples were withdrawn after the intervals of 15min, 1, 2 and 3 hours and blotted dry and weighed again. The percentage degree of swelling was measured by following formula:

$$\text{Degree of Swelling (\%)} = [(Ms-Mi)/Mi] \times 100$$

where, Ms is the mass of hydrogel after swelling and Mi is the initial mass of the sample taken.

In vitro degradation

Degradation tests were carried out gravimetrically for each sample composition (n = 3). The hydrogels were weighed (W1). Then the samples were kept in phosphate buffered saline (PBS) and/or lysozyme solution (1 mg/ml) at 37^{o}C for different time points (day 2 [D2], day 6 [D6], and day10 [D10]). The samples were taken out at each time point, dried at 37^{o}C for 24 hours and subsequently weighed (W2). The dried weight (without water content) remaining ratios were determined as following:

$$\text{Dry weight remaining ratio (\%)} = W2 / W1 \times 100$$

Heparin Loading

Heparin was loaded onto the cross-linked hydrogels. To load heparin, a solution was prepared containing ~~WHAT VOLUME OF~~ 10mg/mL (w/v, 10mL) of heparin. Then the cross-linked scaffolds (7 × 3 cm) ~~(WHAT WEIGHT OR SIZE?)~~were submerged in this solution~~,~~ overnight at RT. The scaffolds absorbed almost all of these solutions. The resulting heparin containing scaffolds were frozen at -20 ˚C and finally lyophilized at -40 ˚C for 24 hours.

Toluidine Blue assay

Toluidine blue (25mg/100mL) was dissolved in HCl (0.01N) with NaCl (2% w/v). Thin (0.5 mm) ~~HOW THIN?~~sections of hydrogel were cut and placed on a glass slide. The toluidine blue solution was added drop wise onto the samples and left to stand for 24 hour. After staining, the hydrogels were washed for 10min with distilled water to remove unbound toluidine blue. Then the samples were air dried and observed under a microscope for the presence and distribution of heparin in the hydrogels.

MTT Assay

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Cellular toxicity was determined using a~~by~~ 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay. Prior to cell culture, all the scaffolds (1 cm^2) included in this study, a) control hydrogels, b) 4 CLH and c) 16 CHL, were sterilized in 70% ethanol for 30 minutes. Immediately before cell seeding the scaffolds were washed 2-3 times with PBS and pre-conditioned in DMEM medium for 20 minutes. MDA-MB-231 cells were seeded in 24-well cell culture plate with 5×10^4 cells per well with or without hydrogels in 1 ml medium. Cells seeded in 24-plate wells without hydrogels were used as a positive control. On day 5 the medium was discarded and cells were washed with 1 ml PBS. 1 ml (0.5 mg/ml) MTT solution was added to each well and the plate was incubated at 37^{o}C for 3 hrs. The MTT solution was discarded and the cells/scaffolds were washed once with 1 ml PBS. To solubilize the formazan crystals 0.5 ml dimethyl sulfoxide (DMSO) was added to each well and the plate was kept under shaking conditions for 10-20 minutes. The optical density (OD) of the dissolved crystals was measured by using a microplate reader at 590 nm. The assay was set up in triplicates for each composition. % Viability is represented as the mean ± SD of 3 independent experiments. % Viability was calculated using the following formula:

$$\% \text{ Viability} = \frac{\text{Absorbance (sample)} - \text{Absorbance (Blank)}}{\text{Absorbance (control)} - \text{Absorbance (Blank)}} \times 100$$

Assessment of Angiogenic Properties of Hydrogels using the Chick Chorionic Allantoic Membrane~~CAM~~ (CAM) Assay

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Fertilized chicken eggs were purchased from Big bird (Lahore, Pakistan) and incubated from day 2 of fertilization until day 8 at 37°C in a humidified egg incubator (R-COM Suro20). At day 8, a square window (1 cm^2) was cut into the shell and lifted, and a 1 cm^2 hydrogel piece was placed onto the chorionic allantoic membrane~~CAM~~. The hydrogel pieces implanted were squares of 1~~ 2~~

x1 cm ~~,or circular in which case 1cm diameter?)~~ Each egg was implanted with one piece of hydrogel only. The shell window was replaced with parafilm (Bemis Flexible Packaging, USA) and sealed with adhesive tape. After implantation, eggs were placed again at 37°C in a 45% humidified incubator until day 14. At day 14, the eggs were sacrificed. Angiogenesis was quantified by taking light microscope pictures of the material on the CAM and then hydrogels were removed for further analysis. Figure 1 shows a protocol of this CAM assay.

Each group contained material added to 10 fertilised eggs. Only results from surviving developing chicks were used. On average chick survival was 80%.

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[Insert Figure 1]

Figure 1: Schematic diagram of CAM Assay

Results and discussion

Chemistry

This study is an extension of our previous published work, in which we introduced triethyl orthoformate as a new cross-linking agent[34]. In this study we compared two different concentrations of cross-linker (4% and 16%) to check its effect on the morphology, thermal stability, biodegradation and biocompatibility of prepared cross-linked hydrogels. The scaffolds were then loaded with heparin to check their angiogenic behavior.

The cross-linked hydrogels were prepared by mixing the CS and PVA solutions in an 80:20 ratio and then lyophilizing at -40°C. The samples were then rehydrated and cross-linked using different concentrations of TEOF (4% and 16%) in sulfuric acid. Controls were devoid of any cross-linker. Then NaOH solution was used to neutralize the cross-linked hydrogels. The samples were again lyophilized at -40°C to gel porous hydrogels, which was confirmed by SEM images of these scaffolds. The chemical nature of cross-linking was revealed by FTIR analysis and thermal stability was investigated by TGA and DSC.

Figure 2 shows the protocol for the preparation of porous heparin-loaded hydrogels and photographs of the appearance of these.

[Insert Fig 2]

Figure 2: Schematic diagram for the preparation of control (not cross-linked), **4CLH** and **16 CLH**.

Scanning Electron Microscopy of hydrogels

The surface morphology of freeze-dried cross-linked hydrogels revealed a highly porous nature. It is known that the microstructure of hydrogels can influence cell infiltration, proliferation and function in tissue engineering [35, 36]. The morphologies of the cross-linked CS/PVA scaffolds before and after heparin loading are shown in **Figure 3**. The interconnected porous structure of the scaffolds was retained after heparin loading; however, some other ~~significant~~ changes occurred with respect to pore size and morphology. The hydrogels without TEOF were taken as

the reference control. The mean pore sizes, before drug loading, were 4.85 ~~µm~~±2.26 µm for the control, 4.73 ~~µm~~±1.92 µm for 4 CHL and 3.35 ~~µm~~±2.05 µm for 16 CLH. After heparin loading and freeze drying again, the pore sizes were increased, a little, to 5.37 ~~µm~~±2.50 µm, 5.22 ~~µm~~±2.08 µm, 4.58 ~~µm~~±1.59 µm for control, 4 CLH and 16 CLH, respectively. There was a reduction in the fibrous extensions in between pores and more sheet-like structures appeared~~ing~~ post heparin loading. The scaffolds were freeze dried again after heparin loading.

It was observed that the pore size decreased slightly, in both the unloaded and heparin-loaded scaffolds, as the amount of cross-linker was increased in the samples. The pore sizes decreased as follows: Control > 4 CLH >16 CLH

[Insert Fig 3]

Figure 3: Scanning Electron Microscope micrographs of cross-linked CS/PVA hydrogels, before and after heparin loading and control (without TEOF) (M~~All images m~~agnification~~s~~ bars are given with each image~~s~~). Unpaired t-test was performed to check whether the difference between pre-size values was significant or not. It was found that the pore-size difference~~s~~ between control and 4 CLH were~~as~~ not significant (p=0.7699), again between 4 CLH and 16 CLH the difference~~s~~ were~~as~~ not significant (p=0.0741) b~~. B~~ut the difference between the pore-size of the control and 16 CLH was significant (p=0.0018).

Chemical Structure Analysis by FTIR

The FTIR spectra of all PVA and CS samples are shown in **Figure 4 (a-f)**. FTIR spectra of all the samples confirmed their chemical structure. For CS and PVA, a broad peak from 3500-3200

cm^{-1} is assigned to O-H and N-H stretching vibrations.[37, 38] The sharp peaks around 2900 cm^{-1} in spectra are assigned to C-H stretching vibrations.[39] A peak observed at 1530 cm^{-1} in PVA spectrum (Fig. 3f) was assigned to O-H bending vibrations.[40] The bands at 1113 cm^{-1} and 1047 cm^{-1} are assigned to C-O and C-O-C stretching vibrations respectively.[41]

The FTIR spectra of all cross-linked composites of PVA and CS with different ratios of TEOF (**Figure 4, b-e**) were found to possess all the major spectral peaks of CS and PVA. All the samples showed a broad peak from 3500-3200 cm^{-1} for O-H and N-H stretching vibrations. The peaks near 2900 cm^{-1} were assigned to C-H stretching vibrations. The bands appeared near 1400 cm^{-1} was due to C-H bending vibrations. The peaks at 1099 cm^{-1} are due to C-O-C stretching vibrations.

In the cross-linked hydrogel spectra (**Figure 4, b-d**), a broad peak for O-H and N-H stretch also appeared at 3400-3200 cm^{-1}, but the intensity of this peak was decreased significantly due to a decrease in number of $-NH_2$ groups on the CS skeleton. This can be further explained on the basis of chemical crosslinking of the CS. This was due to a reaction between $-NH_2$ groups of CS and TEOF resulting in a Schiff base, which was verified by the presence of a new band at 1630 cm^{-1}, associated with the –C=N imine group in cross-linked hydrogels.[42, 43]

[Insert Figure 4]

Figure 4: FTIR results of PVA, chitosan and cross-linked hydrogels. FTIR spectra in the spectral region (4000-600 cm-1) for (a) Chitosan (b) Control (c) 4 CLH (d) 16 CLH (e) PVA

Thermal Analysis of hydrogels

It is known that PVA is a semi-crystalline polymer in which high physical interactions between the polymers chains, due to hydrogen bonding between the hydroxyl groups exist. Similar

bonding is also present in chitosan polymer chains. The introduction of a crosslinking agent affects both, crystallinity and physical network, originating variations in the Tg values of the system. It can be seen from **Figure 5 (B)**, T_g value of control shifted from 58 to 63 °C in case of cross-linked hydrogels. An endothermic peak at 190°C, in the control hydrogel thermogram, that represented the melting point (T_m) of PVA, was also shifted to higher temperature in the case of cross-linked hydrogels. Similarly, the thermal degradation of chitosan was at 270°C, while in cross-linked hydrogels it shifted to 300 °C. The endothermic peak after 450 °C represented the thermal degradation of all polymeric content. The shifting of peaks of cross-linked hydrogels toward higher temperatures indicated that the cross-linked hydrogels are thermally more stable than the control one.

The thermal stability of cross-linked hydrogels was also confirmed by thermogravimetric analysis. The first degradation step, at 60–85 °C, could be ascribed to the removal of traces of water or solvent vapor which showed a 10% weight loss in control but only a 2-5% loss in the cross-linked hydrogels. The second degradation step was between 190-300 °C in the case of control and 200-350 °C in cross-linked hydrogels. This resulted in the highest residual weight loss and this was due to the decomposition and volatilization of organic components of polymer. In this step, control showed 50% weight loss but cross-linked hydrogels showed only a 30-40% weight loss. The thermal decomposition temperature (T_d) range of cross-linked hydrogels shifted significantly towards temperature ranges higher than those of control. The third decomposition step was after approximately 450 °C. This was mainly the organic residues that were completely volatilized. In this step too, the control showed more weight loss than the cross-linked hydrogels.

It was interesting to see that as the percentage of cross-linker is increased in the hydrogels, the weight loss decreased. This indicated that the hydrogels are becoming thermally more stable as

the amount of cross-linker was increased in the samples. In TG thermograms, controls showed a total weight loss of around 85% while hydrogels with 16% cross-linker showed less than 60% total weight loss.

The overall order of thermal stability of hydrogels was:

[Insert Figure 5]

Figure 5: (A) Thermogravimetric and (B) Differential Scanning Calorimetry thermograms of hydrogels (control and cross-linked) in the temperature range of 0-600°C.

Swelling Properties of hydrogels

The ability of a scaffold to attract water is an important property for tissue engineering particularly skin tissue engineering. The prepared hydrogels were soaked, in triplicates, in phosphate buffer saline (PBS) solution for one hour to 24 hours, to check their swelling behavior. The swelling ratios of the scaffolds are shown in **Figure 6.** The swelling of the control (without cross-linker) scaffold was clearly more thanalmost double that of the TEOF treated scaffolds. There was no significant difference in swelling between those with 4 or 16 % crosslinker.

The water-binding ability of the chitosan/PVA scaffold could be attributed to both their hydrophilicity and the maintenance of their three-dimensional structure. TIn general, the ability of the hydrogels to swell ing ratio decreased dramatically when hydrogels wereas the cross-linked. degree increased because of the decrease of the hydrophilic groups. The results in **Figure 4** suggest that the primary factor affecting the swelling property is the degree of cross-linking. The swelling studies were well supported by SEM results which showedtated that as the degree

of crosslinking was increased the average pore-size ~~was~~ decreased slightly ~~.which in result affected the swelling (solution absorption) of hydrogels.~~

~~Thus the cross linker has affected the pore size (**Figure 3**) and hydrogel swelling.~~ It is well-known that cross-linkers improve the mechanical strength of hydrogels [44-48] and help maintain their physical structure in media. ~~As expected therefore in this case the hydrogel with 16% crosslinker showed slightly less swelling compared to the one with only 4% crosslinker.CANNOT BE SIGNIFICANT AND MISLEADING TO SUGGEST IT IS>>>~~

[Insert Figure 6]

Figure 6: Solution absorption (%) of control and cross-linked CS/PVA hydrogels. Values shown are means with 1 standard deviation either side of n=3~~22~~ r~~R~~eplicates.

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Degradation Studies

Lysozyme is an enzyme which is known for its degradation of natural polymers (chitosan, chitin and collagen etc.) which contain a 1,4-β-linkage. Our in vitro degradation results are demonstrated in **Figure 7**. To mimic the in vivo degradation process, lysozyme (1 mg/ml) was added to PBS. However, our data indicated that hydrogels showed comparable degradation rates irrespective of the presence or absence of lysozyme. In control samples (**Figure 7A**), the dry weight ratio was decreased progressively from 100 % to 72 % (± 4 %) in PBS and to 71 % (± 3 %) in PBS / lysozyme solution during the 10 days of degradation. The 4 CLH showed a decrease from 100 % to 56 % (± 21 %) and 49 % (± 12 %) in PBS and PBS / lysozyme solution respectively during 10 days of degradation (**Figure 7B**). H~~Similarly h~~ydrogels with a~~n increased~~ cross-linker concentration of 16 % showed a decrease in dry weight ratio from 100 % to 56 % (±

12 %) in PBS to 63 % (± 6 %) in PBS / lysozyme solution during 10 days of degradation (**Figure 7C**). Overall, the decrease in degradation was slightly not-significantly) greater for the cross-linked hydrogels than for the non-cross linked hydrogels by 10 days .~~which confirms that cross-linking is affecting the rate of degradation of these materials but~~ This breakdown of hydrogels was not accelerated by the addition of lysozyme.

[Insert Figure 7]

Figure 7: Measurement of rate of breakdown of hydrogels in PBS and lysozyme. Dry weight remaining ratio (%) chitosan/PVA hydrogels in PBS and 1mg / ml lysozyme / PBS at 37°C at different time points: A) Chitosan/PVA (control), B) 4 CLH, C) 16 CLH. Graphical data is presented as mean (n=3)~~2~~ ± s~~t~~andard deviation of three independent experiments.

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Toluidine Blue assay

A toluidine blue assay was performed to confirm the presence of heparin in the samples (**Figure 8**). For this purpose, very thin (0.5 mm) ~~HOW THIN???~~sections of hydrogels (control, 4 CLH and 16 CLH) were cut and stained with toluidine blue for 24 hours. Then the samples were washed with distilled water for 10min to get rid of excessive toluidine blue~~heparin~~. Then the images were captured using an inverted microscope (Euronext, IN-2053). Heparin forms a purple complex with toluidine blue while toluidine itself is blue. Thus~~So~~ the formation of a purple complex confirms the presence of heparin. From the images it can be seen that heparin was uniformly distributed in the samples. The control samples were blue in color while the heparin-loaded scaffolds changed towards shades of purple (4CLH and 16 CLH). From **Figure 8** it can be seen that 16 CLH had a darker shade of purple indicative of retaining more heparin than 4 CLH.

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These results were supported by the effects of the hydrogels in the CAM assay (**Figure 10, 11**) where 16 CLH was seen to be ~~showed~~ more angiogenic activity than 4 CLH.

[Insert Figure 8]

Figure 8: Inverted m~~M~~icroscope images of control (without heparin), 4 CLH and 16 CLH.

Use of MTT Assay to demonstrate cell compatibility of hydrogels

The MTT assay was used to examine the viability and growth of the breast cancer cell line MDA-MB-231 grown for 5 days with control (hydrogel without cross-linker, TEOF) 4 CLH and 16 CLH. Results demonstrate no significant difference in % viability of cells grown with the different hydrogels compared with control (tissue culture plate-TCP) (**Figure 9**) showing that none of these hydrogels alter the proliferation capacity and viability of the MDA-MB-231 cell line.

[Insert Figure 9]

Figure 9: Effect of hydrogels on cell viability. Viability of breast cancer cell line (MDA-MB-231) seeded in tissue culture plate controls (TCP) (no hydrogel), with control hydrogel (no cross-linker), and with 4 CLH & 16 CLH for 5 days as determined by MTT assay. Bars represent mean cell viability normalized to control cells and error bars depict the standard deviation of duplicate experiments (n = 2). Statistical analysis (one-way ANOVA, Sidak's multiple comparisons test) showed no-significant difference (p= 0.0901) between the TCP and tested materials (control, 4 CLH and 16 CLH).

Assessment of Angiogenic Properties of Hydrogels using CAM Assay

The chick chorionic allantoic membrane (CAM) was performed to explore the angiogenic response to the hydrogels. A small window was made in the shell on day 7 of chick embryos and a 1 cm^2 hydrogel piece was placed onto the CAM. Each egg was implanted with one piece of

hydrogel only (n=5or 10 ??). The chick embryos were incubated with the hydrogels grafts until day 14 of development.

The samples retrieved from the CAM assay, after sacrificing the eggs, were then fixed with paraformaldehyde and then dehydrated using gradient solutions of ethanol (50-100%). A small portion of the hydrogels was cut to see the cross sectional area of the scaffold and to see the blood vessel ingrowth. The results are shown in Figure 10.

[Insert figure 10]

Figure 10: Colorimeatric quantification of blood vessels from images of scaffolds retrieved from CAM assay. The upper images show light microscopic images of control (without heparin) and heparin-loaded hydrogels CLH 4 and CLH 16. These were than photographed and the histograms show the amounts of red and blue color in these samples where the red color intensity representings the number of blood vessels.; 1 way ANOVA Dunnett's Multiple comparison test (**** p< 0.0001). Statistical analysis (1 way ANOVA Dunnett's Multiple comparison test) showed significant difference (**** p< 0.0001) between the tested materials (control, 4 CLH and 16 CLH).

NEED MORE EXPLANATIONStatistical analysis T Results Graphical results are presented as means ±1 standard deviation of n=8 ??? rReplicates (10 fertilized eggs were used per sample and on average 8 were survived per batch)..

The ability of heparin to support blood vessel growth was also evaluated by SEM. The samples were retrieved at day 14 and assessed for vessel ingrowth. Cross sections of hydrogels showedverified the ingrowth of blood vessels and the heparin-loaded cross-linked hydrogels

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displayed greater angiogenic activity than the control hydrogels. The blood vessels penetrating the scaffolds appeared to be round as shown in Figure 11.

It was also observed from SEM images that erythrocytes adhered to the hydrogel surface of both control and heparin-loaded hydrogels-this also confirmed the non-toxic behavior of these biomaterials.

[Insert Figure 11]

Figure 11: SEM images of paraformaldehyde fixed explanted scaffolds from CAM at day 14. Yellow arrows ~~are~~ indicate the ~~pointing~~ blood vessels.

Conclusions

The hydrophilic nature of chitosan and PVA was used to develop cross-linked hydrogels using two~~variable~~ concentrations of triethyl orthoformate (TEOF) as a cross-linker -4 and 16%~~(4 CLH and 16 CLH.)~~. The resulting cross-linked hydrogels were then loaded with heparin, by physical adsorption, to evaluate their pro-angiogenic behavior. The hydrogels were characterized by SEM, FTIR and DSC/TGA to assess the effect of cross-linker on the morphology, porous and chemical structure and thermal stability of the cross-linked hydrogels. ~~The hydrogels showed swelling properties and bio-compatibility. Degradation studies were also performed in PBS and using lysozyme which showed that the cross linking somehow facilitated the degradation of these hydrogels.~~Increasing the concentration of cross-linker reduced the extent of swelling of the hydrogels, had a very small effect on pore size (reducing these slightly at 16%) but had no appreciable effect on the rate of breakdown of the hydrogels which lost approximately 37% in weight by 10 days in PBS and this was not affected much by the addition of lysozyme where there was a 44 % loss in weight over 10 days. ~~NOT REALLY~~ The presence and uniform

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distribution of heparin in hydrogels was confirmed by the toluidine blue assay. The CAM assay showed the pro-angiogenic behavior of these cross-linked hydrogels and~~where~~ SEM of CAM retrieved hydrogels showed clear growth of blood vessels into **4 CLH** and **16 CLH** scaffolds. ~~Although crosslinker did not affect much the physio-chemical properties however the~~ The greatest effect of the crosslinking was seen in the ability of the cross-linked hydrogels to bind heparin and induce angiogenesis compared to the non-crosslinked materials. Here there was a significant effect of using 16% rather than 4% cross-linker. In conclusion, we have prepared new cross-linked, biodegradable and non-toxic hydrogels which bind heparin, and which without the need to add any further costly growth factors, show pro-angiogenic activity. We suggest these highly cross-linked hydrogels will be of value for skin tissue engineering applications as they will allow tissue ingrowth to form a neo-dermis while promoting new blood vessel formation.

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