Potential of electrospun chitosan fibers as a surface layer in functionally graded GTR membrane for periodontal regeneration

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\section*{ABSTRACT}

Objective. The regeneration of periodontal tissues lost as a consequence of destructive periodontal disease remains a challenge for clinicians. Guided tissue regeneration (GTR) has emerged as the most widely practiced regenerative procedure. Aim of this study was to electrospin chitosan (CH) membranes with a low or high degree of fiber orientation and examines their suitability for use as a surface layer in GTR membranes, which can ease integration with the periodontal tissue by controlling the direction of cell growth.

Methods. A solution of CH-doped with polyethylene oxide (PEO) (ratio 95:5) was prepared for electrospinning. Characterization was performed for biophysiochemical and mechanical properties by means of scanning electron microscopy (SEM), Fourier Transform Infrared (FTIR) spectroscopy, swelling ratio, tensile testing and monitoring degradation using pH analysis, weight profile, ultraviolet-visible (UV–vis) spectroscopy and FTIR analysis. Obtained fibers were also assessed for viability and matrix deposition using human osteosarcoma (MG63) and human embryonic stem cell-derived mesenchymal progenitor (hES-MP) cells.

Results. Random and aligned CH fibers were obtained. FTIR analysis showed neat CH spectral profile before and after electrospinning. Electro spun mats were conducive to cellular attachment and viability increased with time. The fibers supported matrix deposition by hES-MPs. Histological sections showed cellular infiltration as well.

Significance. The surface layer would act as seal to prevent junctional epithelium from falling into the defect site and hence maintain space for bone regeneration.

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1. Introduction

The regeneration of periodontal tissues lost as a consequence of destructive periodontal disease remains a challenge for clinicians. Periodontists use a range of treatments to regenerate the lost periodontal ligaments (PDL), alveolar bone and cementum. However, the results are variable and unpredictable [1]. Tissue engineering (TE) using polymers and cells might provide new approaches for periodontal regeneration [2].

Use of a guided tissue regeneration (GTR) membrane has emerged as the most widely practiced regeneration procedure [3]. GTR involves placement of a barrier membrane between the gingival epithelium and connective tissues to promote regeneration of periodontal tissues [4]. For a biomaterial to function as an ideal GTR membrane, there are certain critical requirements that have to be fulfilled [5]. Firstly, the membrane has to act as an effective barrier between the periodontal tissues and epithelium. Secondly, it should promote vascularization and protect the underlying blood clot from epithelial and connective tissue ingrowth. Finally, it should degrade, without releasing any harmful byproducts, within a specific timespan to allow regeneration of periodontal tissues. GTR is aimed at selective infiltration of reparative periodontal cells into the defect site. However, currently used GTR membranes do not achieve all of the desired criteria, such as, bioactivity and unsuitable degradation rates significantly hampering the regenerative potential of currently available GTR membranes [4].

A biopolymer that has shown promising results in the field of TE for various biomedical applications is chitosan (CH). CH is a biodegradable polymer composed of distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-d-glucosamine (acetylated unit) and is present in the shells of crustaceans as well as other organisms such as the cell wall of fungi. It has been used as a scaffold material in wound healing, nerve regeneration, and cartilage and bone repair [6]. CH is soluble in dilute acids due to the presence of protonated amino acids in the chemical structure of glucosamine [7]. Overall, it produces a minimal foreign body-host tissue response and is broken down by lysozyme [8,9]. A major advantage of CH is its antibacterial effect on oral pathogens [10]. It has exhibited a potent antiplaque activity against several oral pathogens such as Porphyromonas gingivalis, Prevotella intermedia [11] and Actinobacillus actino- mycetemcomitans [12–14]. Additionally, it has also been shown to induce a high collagen turnover in vivo [15]. CH displays great diversity and has been molded into required shape and form to obtain thin films, porous membranes, hydrogels and fibers for use in a wide range of biomedical applications [16].

Electrospinning is a relatively simple and versatile technique capable of fabricating continuous fibers from a wide range of natural and synthetic polymers with diameters ranging from a few nanometers to several microns, providing high surface area to volume ratio [17,18]. CH has been electrospun and the resultant fibers have been used for numerous applications in TE [19]. Pure CH electrospinning has rarely been reported due to the viscous cationic solutions formed by small amounts of dissolved CH, which do not achieve the critical chain entanglement. However, composite formulations of CH with other fiber forming agents, such as, polycaprolactone (PCL), polyvinyl alcohol (PVA), poly lactide-co-glycolide (PLGA) [20], cellulose and other synthetic polymers have resulted in successful generation of fibers [21]. Hybrid fibers of CH and polyethylene oxide (PEO) have also been studied in the past [22–26]. High molecular weight polymers, such as, PEO improve the spinning of the fibers by interacting with CH fibers through hydrogen bonding [22]. Addition of PEO also results in decreased CH fiber diameters, which is an advantage when electrospinning CH scaffolds for TE because the thin fibers can mimic ECM more effectively by favoring cellular infiltration and adhesion [16]. Furthermore, addition of diluents, such as, dimethyl sulfoxide (DMSO) decreases solution viscosity making it easier to electrospin as a direct result of decreased surface tension. It has been shown that cell growth is effected by scaffold-fiber orientation and cells tend to grow along the direction of the fibers [27]. Hence, changing fiber orientation could be used to control the direction of cell growth [28]. In our previously published study, we reported on the fabrication of a core layer that mimics the natural structural and functional features of tissue and bone while remaining part of a functionally graded GTR membrane [29]. It is envisaged that the fibrous mat produced in this study could act as the surface layer to the previously proposed core layer, functioning to control cell growth and regulate drug delivery at the periodontal defect [28,30].

In this study, CH fibrous membranes were prepared and fully characterize to evaluate their biophysical and mechanical properties using scanning electron microscopy (SEM), Fourier transform infrared-attenuated total reflectance (FTIR-ATR) spectroscopy, swelling studies, tensile testing and degradation profiling using pH analysis, weight profile, ultraviolet–visible (UV–vis) spectroscopy and FTIR-ATR analysis. Obtained fibers were also assessed for viability and matrix deposition using human osteosarcoma cells (MG63) and the human embryonic stem cell derived mesenchymal progenitor cell line (hES-MP). hES-MP cells are precursors for bone and ligament cells. Hence, testing a scaffold with this cell line would enable to deduce its bone regenerative potential. Cellular infiltration was assessed by histological sectioning.

2. Materials and methods

2.1. Preparation of CH solution

A solution of Low molecular weight (LMw) (50–190 kDa) (Sigma–Aldrich, UK) CH was prepared at 4.5 wt% in a combination of 3 wt% acetic acid (AA) with dimethyl sulfoxide (DMSO) (ratio 10:1). Ultrahigh molecular weight polyethylene oxide (PEO) (5,000,000 Mw) (Alfa Aesar. UK) was added to get a final CH:PEO ratio of 95:5. To ensure maximum dissolution, CH was firstly mixed in distilled water (D2O) and heated to 60 °C for 30 min followed by dropwise addition of AA and allowing a further 15 min of mixing. PEO was then added to the solution and after another 30 min of mixing, DMSO (2 ml) was finally added and left stirring for 24 h at room temperature. After 24 h, the solution was centrifuged at 45000 rpm for 5 min to remove

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undissolved impurities. Each prepared electrospinning solution was utilized within 24 h of preparation.

2.2. Electrospinning

PEO-doped CH solutions were loaded into two 1 ml syringes each with a needle gauge of 4.699 mm and placed onto an automated syringe pump (World Precision Instruments, Sarasota, Florida) and dispensed at a constant rate of 1 ml/h. Voltage at the needle was set between 17.8 kV and 22 kV. Fibers were collected on a sheet of aluminum foil wrapped around a rotating steel drum. The distance between the needle tip and the collector drum (6 cm diameter) was 15 cm. The collector was set to rotate at 160 rpm for random fibers and 2500 rpm for aligned fibers. After completion of spinning, fibers were carefully removed from the collector and placed under vacuum for 24 h at room temperature to remove any remaining solvent. They were then stored in a sealed plastic bag at room temperature (25 °C). For swelling ratio, degradation and cell culture studies, fibers were neutralized with a solution of 1 M sodium hydroxide (NaOH) and 50% Ethanol (Fisher Scientific UK) (1:1) for 45 min and followed by washing twice with distilled water for 15 min each.

2.3. Scanning electron microscopy

SEM was employed to study the surface morphology of electrospun fibers (spot size: 3.0, voltage range 5–10 kV, Philips X-L 20 microscope). Samples were mounted on aluminum stubs with double-sided carbon adhesive dots (Agar Scientific, UK) and were sputter coated under vacuum with carbon using Speedivac carbon coating unit (Model 12E6/1598). Images obtained from SEM were scaled using Image J software (NIH, USA) and fiber diameter was measured. Frequency histograms of fiber diameter were plotted using Graphpad prism software version 6.0 and directionality histograms were obtained with ImageJ2 (Fiji) (NIH, USA) software using directionality plug-in for generating orientation graphs.

2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR-ATR spectra were obtained using a Thermo Scientific i500™ FTIR spectrophotometer, which is coupled with an ATR sampling accessory. Spectra were collected in the mid infrared region (4000-650 cm⁻¹) at 4 cm⁻¹ resolution accumulating 64 number of scans. ATR had a diamond crystal mounting. Spectral data was processed using a Thermo Nicolet OMNIC™ software (Thermo Scientific, Madison, WI, USA).

2.5. Mechanical properties

Electrospun fibrous scaffolds were mechanically tested in tension under dry and wet conditions on a BOSE ELF 3200™ (BOSE ElectroForce System groups, BOSE, Minnesota, USA) using a 22.5 N load strained at a rate of 0.1 mm/s to failure and a gauge length of 6 mm. From the obtained stress–strain curves, the point at which the samples snapped was used to calculate the ultimate tensile strength (UTS) and the strain (%), while the initial linear gradient was taken as the Young’s modulus (E). Specimens were cut into rectangles with dimensions 5 mm × 20 mm × T, where T is the thickness of the membranes under dry or wet conditions.

2.6. Swelling and degradation studies

Samples (13 mm Ø) for use in swelling studies were dried and weighed before storing in phosphate buffered saline (PBS) at 37 °C to allow any water uptake to occur. At time intervals of 0, 15, 30, 60, 120 and 140 min, specimens were removed from PBS and any excess water was removed with tissue paper before weighing samples. The swelling ratio was calculated using the formula: Swell Ratio % (Q) = (Ww − Wd)/Wd × 100. Where dry weight is given as Wd and wet weight is given as Ww.

For degradation studies, dry samples (13 mm Ø) were dried and weighed and noted as W0. These were then immersed in degradation media containing PBS and 5 mg/mL of hen egg lysozyme (Sigma–Aldrich, UK). Samples were incubated at 37 °C for 4, 7, 14, 21 and 28 days. Media was renewed after every 3 days and at each time interval samples were washed 3 times with distilled H2O and then dried out thoroughly before weighing them again as Wt. Weight loss was calculated by using the formula: Weight loss % = (W0−Wt)/W0 × 100.

Measurements of the supernatant pH were performed using a pH meter (Mettler Toledo S20 SevenEasy™). Ultraviolet–visible (UV–vis) spectrophotometry was performed (LAMBDA 950 UV Vis spectrophotometer PerkinElmer) on the sample supernatant by taking blank background and PBS was used as a reference against the sample while acquiring the spectra. Analysis was performed within the range of 200–500 nm. Samples were analyzed in quartz cuvettes (Thor Labs UK). Data of the UV–vis was processed by Vision proTM software by Thermo Scientific®. FTIR-ATR spectroscopic analysis was also carried out on each specimen at each time point.

2.7. Cell culture on fibrous mats

Cell culture was conducted using human osteosarcoma cells (MG63) and human embryonic stem cell-derived mesenchymal progenitor cells (hES-MPs). MG63’s were expanded in Dulbecco’s modified Eagle’s medium (DMEM) (Biosera, Ringmer, UK) supplemented with 10% Foetal Calf serum (FCS), 2 mM l-glutamine, 100 μg/mL of penicillin and streptomycin. hES-MPs were expanded on gelatin (0.1% w/v in distilled water) coated surfaces and cultured in Alpha Minimum essential medium (α-MEM) (Lonza, Verviers, Belgium), supplemented with 10% FCS, 2 mM l-glutamine and 100 μg/mL penicillin and streptomycin. Cells were grown in a humidified incubator at 37 °C with 5% CO2 with fresh media changes performed every 2–3 days. Cells were grown to 90% confluency and then detached via trypsin–EDTA. MG63s were used between passages 60–68 while hES-MPs were used between passages 3–7. To analyze the viability of osteoblastic cells on different fiber orientations, cells were seeded at a density of 250,000 cells per sample using a marine grade stainless steel seeding ring (internal Ø 10 mm). Prior to cell seeding, the fibers were sterilized with ethanol for 1 h, washed twice with PBS for 15 min, and then coated with either culture medium for 1 h prior to seeding MG63’s or gelatine for 1 h prior to seeding hES-MPs. Cell free fibers were included as controls. All other
reagents were purchased from Sigma–Aldrich, Life Sciences, UK.

2.8. Alamar Blue® assay

In order to quantify cell attachment and viability, fluorescent measurements of Alamar Blue were obtained after 1, 4 and 7 days of culture. For each time point, cell seeded samples were carefully washed with PBS and 0.5 ml of Alamar blue® solution (diluted 1:10 with PBS) was added followed by incubation at 37 °C for 4 h. Fluorescence was measured at 570 nm using a fluorescence plate reader (Bio-TEK, NorthStar Scientific Ltd, UK). Based on cell metabolic activity the system incorporates an oxidation–reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of the growth medium resulting from cell growth. Reduction related to growth causes the REDOX indicator to change from oxidized (blue) form to reduced (red) form. After the measurements were taken, samples were washed with PBS, fresh media was added and samples were further cultured in the incubator until the next time point.

2.9. Collagen and calcium staining

For identification of collagen deposition by cells on the CH membranes, Picro-sirius red staining was performed. Media was removed after 14, 21 and 28 days, and samples were washed with PBS and then fixed with 3.7% formaldehyde for 30 min and samples were then washed with PBS and Sirius red solution (Direct red dye 1 mg/ml in saturated picric acid, both Sigma, UK) was added to fully submerge samples and left for 18 h under mild rocking (20 rpm). Excess dye was washed with distilled H2O and samples were destained for quantitative analysis using a known volume of 0.2M NaOH and Methanol (v/v) (1:1) for 15 min. The extracted solution was read for absorbance at 490 nm in a 96 well plate reader. Total calcium deposition by hES-MPs was quantified at day 14, 21 and 28 after seeding. Samples were fixed (see collagen staining) followed by distilled H2O washes and then application of 1% Alizarin red solution (pH 4.1) (Sigma, UK) at 1 ml per sample for 20 min on a platform shaker. The unbound dye was removed with distilled water washes. For quantification, the stain was extracted using a known volume of 5% (v/v) perchloric acid to each well for 30 min. The extracted solution was read for absorbance at 405 nm. Data shown is after subtraction of the absorbance reading obtained on blank scaffolds.

2.10. Histological cryosectioning

Histological sectioning of electrospun fibers was taken as complete transverse-sections across the centre of electrospun scaffolds. Samples were fixed with 3.7% formaldehyde, washed 2 × PBS for 15 min each and then soaked in 1% sucrose solution for 30 min prior to embedding in OCT™ (Tissue-Tek®, Sakura, freezing medium) compound media. Samples were cryo-sectioned (Leica CM1860UV Ag protect) at 7 μm slices and mounted on glass slides (Thermo Scientific, Menzel-Glaser, Saarbrucken, Germany). Staining was performed with Haematoxylin (Harris) and Eosin (Sigma–Aldrich, UK). Stained sections were imaged with a light microscope (Leica, Motic) with 4×, 10× and 20× objectives. Images were scaled on Image J (NIH, USA) software.

2.11. Statistical analysis

Unless stated otherwise, all experiments were conducted at least in triplicates. All presented data refers to mean ± standard deviation (SD). In order to check for any statistically significant differences, One-way ANOVA was performed followed by Tukey’s post hoc test. Results with p-values of ≤0.05 (*) were considered statistically significant. All data was analyzed using Graphpad Prism 6.07 software. Mechanical properties were analyzed by Two-way ANOVA, which was performed using the computing environment R (R Development Core Team, 2015). R Core Team (2015).

3. Results

3.1. Scanning electron microscopy

SEM images showing CH electrospun fibrous mats are presented in Fig. 1. Fig. 1a shows fibers with an apparent high degree of orientation compared with Fig. 1b that shows fibers with no preferential direction of orientation. All fibers show an almost clean/beadless morphology. Mean fiber diameters ranged from 410 ± 163 nm to 288 ± 107 nm for highly orientated (aligned) and randomly orientated (random) fibers respectively. Orientation histograms of aligned and random fibers (Fig. 1e and f) give a clearer representation of the directionality of fibers. Random scaffolds showed an even distribution of fibers in all directions as compared to aligned scaffolds, which contained fibers with a majority orientation within 20° of one predominant direction indicating a much more unidirectional alignment of fibers compared to random fibers. The overall thickness of the random and aligned mats obtained was 150 ± 10 μm from 15 ml solution.

3.2. FTIR spectroscopy

FTIR spectra of the surface of electrospun CH mats were obtained in conjunction with an ATR accessory and are given in Fig. 2. Both random and aligned scaffolds show a typical broad band of CH around 3366–3375 cm⁻¹ and another characteristic band around 1558 cm⁻¹, these are commonly attributed to N–H and O–H stretching of primary amino groups, (due to the presence of inter molecular hydrogen bonds with O–H group). Furthermore, N–H stretching of secondary amides (Amide II) is also visible. Spectral peaks at 1150, 1029 and 1023 cm⁻¹ are attributed to stretching vibrations of the glycosidic bonds (C–O–C), peak at 1591 cm⁻¹ is ascribed to amide II (N–H in plane deformation), other significant bands were present at 1420 cm⁻¹, owing to CH₂ wagging coupled with –OH in plane deformation, 1375 cm⁻¹ due to amide III [29]. A very low quantity of PEO was added to the electrospinning solution to aid fiber formation, hence, the typical triplet peak observed in virgin PEO from 1150 to 1060 cm⁻¹ are diminished. Peak at 1092 cm⁻¹ has been attributed to typical stretching vibration of ether group (C–O–O) in PEO spectra [31]. The PEO peak at 2878 cm⁻¹ when observed in CH PEO
fibers seem to have integrated and peak occurring in neat CH around 2800 cm\(^{-1}\) due to CH\(_2\) stretching have a more defined and sharper peak, which is indicative of chemical interactions and complex formation between CH and PEO.

3.3. Mechanical properties

Tensile tests conducted on dry and wet CH fibrous mats are summarized in Table 1 and representative stress–strain plots are shown in Fig. 3a and b. Dry aligned fibers showed a statistically significant higher modulus (E) compared with dry random fibers. The ultimate tensile strength (UTS) of dry aligned fibers was 13.58 ± 3.68 which was also statistically higher than the UTS of random fibers (7.5 ± 3.84). Random fibers failed at half the strain of aligned fibrous mats. Under

|                | E (MPa)       | UTS (MPa)    | Strain (%)   |
|----------------|---------------|--------------|--------------|
| Dry fibers     |               |              |              |
| Aligned        | 357.31 ± 136.22 | 13.58 ± 3.68 | 0.11 ± 0.04  |
| Random         | 259.39 ± 192.75 | 7.50 ± 3.84  | 0.05 ± 0.04  |
| Wet fibers     |               |              |              |
| Aligned        | 22.39 ± 4.06  | 4.95 ± 1.41  | 0.23 ± 0.05  |
| Random         | 6.25 ± 1.01   | 1.95 ± 1.15  | 0.13 ± 0.11  |
Fig. 2 – FTIR-ATR spectra of neat Chitosan and neat UHMWPEO are shown in the top image, aligned and random electrospun fibers are shown in bottom image. Composite spectra of aligned and random fibers show alterations in the molecular finger print region.

Fig. 3 – Example of stress strain relationship of random (A) and aligned (B) chitosan fibers during dry and wet conditions.
Swelling and degradation studies

Swelling ratio performed on CH electrospun fibers for both orientations up to 48 h is shown in the Fig. 4aii. Within the first 15 min an equilibrium state is reached at a swelling percentage of 70 to 80%. Weight loss analysis was used to assess the degradation profile of CH (Fig. 4ai) fibers. This revealed an initial weight loss of around 20% at day 4 for both random and aligned fibers. At day 14, random fibers show 10% weight loss, which is significantly higher than weight loss of aligned fibers on the same day. Changes in pH value were monitored over the experimental period showing increase overtime from pH 6.7-6.8 to pH 7.5 for random fibers and 8.5 for aligned fibers (Fig. 4aiii). In vitro degradation by-products were assessed by analyzing the supernatant of CH fibers using UV-vis spectroscopy and the obtained spectra are shown in Fig. 4bi and bii. UV-vis spectral data of degradation media gave rise to two new absorption bands at 230 and 290 nm respectively. Neat CH was known to exhibit a strong absorption band at 200 nm, which is visible in all spectra. Data collected from Day 4 to 28 shows that the bands intensity has changed with time. Degradation can also be observed in the SEM of random and aligned fibers immersed in the lysozyme solution for 14 days. Although the fiber network is somewhat still intact, the fibers have become less well-defined and swollen compared to the non-degraded fibers in Fig. 1a and b.

Cell viability and matrix deposition

Alamar blue assay conducted on CH fibers seeded with MG63 or hES-MPs is shown in Fig. 5a and b. Viability increased for both cells over time, with MG63’s showing doubling values at each time point. Statistically significant results were obtained when comparing day 1 to day 7 for each type of fiber orientation. Although no significant difference was noted on day 7 between fiber groups. Mineral deposition on electrospun CH fibers was assessed by quantifying total Collagen and calcium deposited at day 14, 21 and 28 of culturing with hES-MPs, shown in Fig. 5c and d. When calcium deposition by hESMPs was assessed, random fibers showed a statistically significant higher amount when compared at day 14 with aligned fibers. At day 21, calcium deposition was similar between fiber groups. By day 28, Cells cultured on random fibers exhibited twice the amount of deposited calcium compared to day 21 while those on aligned fibers showed no further increase. Collagen deposition assayed at day 14 showed significantly higher values than day 28 in aligned fibers. From day 14 to 28 there is an increasing trend in the absorbance values for collagen deposition. Aligned CH fibers show a significantly higher value at day 28.

4. Discussion

Chitosan and its composite forms have been utilized in the development of various micro-architectural features, such as, films or fibers to obtain different characteristics aimed at being used for periodontal GTR membranes [32,33]. While combing through the literature, it became apparent that the use of this relatively low ratio of UHMWPEO to achieve aligned CH fibers for periodontal GTR has not been reported to date and has great potential for fabricating functionally graded GTR membranes.

Electrospinning is an attractive technique to fabricate scaffolds that could mimic the extracellular matrix (ECM) in which cells exist under physiological conditions [34]. We have demonstrated that CH fibers with both low and high degrees of orientation can be obtained using very low concentrations of non-fouling (UHMWPEO). Although the use of fiber forming agents, such as, PEO has been discussed in the literature before [35–37], this agent was used at a very low concentration in our study. It is well known that PEO is biologically inert and hence, it is commonly used as a copolymer in electrospinning [24,25,38]. Moreover, it is frequently considered when using natural polymers to obtain fibers [39] and it is known to act as a plasticizer in the electrospinning procedure [22]. A study conducted by Jeong et al. reported that PEO inclusion of up to 5% was required for uniform nanofiber production [39]. This is in contrast to what we were able to achieve as beadless fibers were generated using a relatively low concentration (0.045 g) of PEO to CH (5:95).

CH is highly crystalline in nature and its ability to form hydrogen bonds leads to poor solubility in organic solvents making the electrospinning of neat CH even more complex. It is soluble in solvents, such as, acetic acid, dichloromethane (DCM) and trifluoro acetic acid (TFA), which help with the electrospinning process by breaking the inter-chain interactions [16]. However, DCM and TFA are both considered toxic to humans and traces may remain in the resulting fibers. Although solvents such as TFA (C₂H₅O₂)₂ and hexafluoro isopropanol (HFIP) for CH electrospinning have been extensively discussed in the literature, their toxicity remains a concern [40,41]. Frohbergh et al., have reported that they were able to optimize conditions for production on beadless CH fibers. Conditions such as humidity, temperature and DD of CH were looked into and a CH solution with 7 wt% was used for
electrospinning random fibers [41]. However, they used TFA and genipin as cross-linking agents in their study. Although genipin could be a more biocompatible alternative when compared to solvents, such as TFA for cross-linking CH and PEO fibers, it has been suggested that such cross-linked electrospun fibers are generally more hydrophobic than conventional fibers [42], which could impede cell attachment and tissue regeneration adjacent to the scaffold. The primary solvent used in our study was acetic acid, which does not have any reported toxicity or harmful effects on human cells to date.

Studies [43] conducted previously reported an average fiber diameter of 40–200 nm. In addition, in an earlier study by Sarkar and co-workers [36] achieved a fiber diameter of 80–180 nm using PEO/CH composites. Our fiber dimensions varied from 288 to 400 nm (Fig. 1c and d) which could be due to the restricted ratio of CH:PEO. Klossner et al. have demonstrated that if polymer concentration was increased, the number of beads and fiber diameter decreased [26,44]. The variations in the thickness of aligned and random fibers could be due to the effect of relative humidity and temperature as reported earlier by Pelipenko et al., and Huan et al., Humidity causes alterations in the fiber diameter by controlling the solidification process of the charged gel [45,46]. Moreover, it can dry the fibers faster or precipitate them in flight. However this is also correlated with the chemical nature of the polymer itself as natural polymers are very sensitive. Pelipenko et al., [45] studied the effect of changes in fiber diameters with respect to humidity of various formulation including CH/PEO and reported that a change in humidity from 4% to 50% decreased the diameters from 231 nm to 46 nm, which could also be the case in the current study.

![Degradation of Fibers](image)

**Fig. 4** – (ai) Degradation profile by % weight loss analysis of CH random and aligned fibers assessed over a period of 28 days using lysozymes. Values shown are mean ± SEM (α*) statistically significant difference was noted between time points D4 and D28. (β*) statistically significant difference observed in between aligned and random for D28 (aii) Swelling ratio of CH random and aligned fibers over a period of 48 h (n = 3). (aiii) Change in pH value of degraded fibers over a period of 28 days. (bi and bii) UV–Vis spectroscopy of degraded solution conducted for degradation media of aligned and random CH fibers for day 4, 11, 21 and 28 between a range of 200 to 400 nm. (f and g) SEM micrographs of random (biii) and aligned (biv) fibers after immersion in Lysozyme solution for 14 days.
FTIR spectra of fibers showed that PEO had successfully integrated with CH. A peak at 892 cm$^{-1}$ is pertinent to saccharide structure [23,36]. Other peaks observed at 1063, 1026 and 1150 cm$^{-1}$ in spectral data of random and aligned fibers are suggestive that PEO formed a complex with CH. PEO spectra shows a strong triplet at 1144, 1092 and 1059 cm$^{-1}$, associated with asymmetric C=O–C stretching vibration (Fig. 2 bottom image) [35,36]. These triplet peaks are strongly dependant on the crystallinity of PEO and intermolecular interactions in between C=O–C groups of CH as reported by Mehrali and co-workers [47]. Changes in the molecular fingerprint region are also indicative that CH chains were encouraged to form hydrogen bonds with PEO, by reducing the polyelectrolyte effect. The shifts of the peaks in the –NH and –OH stretching vibration regions are suggestive of partial miscibility, which is in agreement with the literature [48]. Another study performed on CH and PEO have demonstrated that using PEO as a copolymer tends to disrupt the self-association of CH chains by creation of H-bonding between its hydroxyl groups and H$_2$O molecules [23]. Consequently, this results in reduced repulsive forces between polycationic groups of CH and promotes chain entanglements which encourage fiber formation [25,26].

The results from the mechanical testing show that aligned fibers had a higher E and UTS values when compared to other commercially available resorbable GTR membranes [4]. Moreover, wet random fibers also displayed mechanical properties that are comparable to those of other commercially available GTR membranes [28,30]. Dry aligned fibers exhibited a tensile strength of 28.76 MPa which is higher than that of collagen GTR membranes [4,49,50]. The results obtained in the current study were similar to the work performed by Cooper et al. on aligned and random fibers [51]. They also observed a significantly higher E and UTS for aligned orientation. These high values could be due to the fiber density in the direction of the loading force is a primary factor to determine the mechanical response. Moreover, fiber alignment causes more force resistance along the loading direction [52]. Other relevant factors are fiber size, porosity, individual fiber orientation in the mesh, fiber-fiber interactions and entanglements of the fiber. Moreover, aligned fibers offer a higher degree of shear induced molecular chain alignment and larger fiber with uniform diameters are more compliant and strong but more ductile [53,54]. This could be favorable for periodontal GTR applications and will be strong enough to resist masticatory forces while maintaining regenerative space as well.

Swelling ratio of CH fiber is usually dependent on the concentration and type (M$_{w}$ and D$_{a}$) of CH present. [55–58]. It has been reported by Tasseli et al. that CH fibers swell most in
acidic conditions when compared to saline solution or distilled water. They mentioned that CH, being a weak base with a pK\textsubscript{a} of 6.4, the amine groups present in CH are ionized to ammonium ions (\text{NH}_{4}^{+}) at lower pH [59]. Hence, the cationic charges present in the fiber structure act as repulsive forces between the polymer chains inducing more swelling. Jankovic et al. recorded the swelling profile of CH-PEO fibers over 60 min and observed that within the first 15 min the swelling profile reached 100% and after 60 min a reduced profile was observed at an overall swelling ratio of 80% being attained [37]. It has been reported in another study that crosslinking CH fibers can enhance the mechanical strength and inhibit the swelling ratio to some extent [48]. In this study, no significant differences between the swelling profiles of the aligned and random fibers were observed. The reasons for the similar swelling ratios are not clear. However, a previous study conducted on PLGA/gelatin fibers suggests that random fibers have a higher swelling ratio than aligned fibers [60]. Hence, further studies are required to ascertain the similarity of swelling characteristics among aligned and random fibers in our study. Nevertheless, the 80% swelling ratio observed in this study is consistent with previous study [37]. Swelling of CH is likely due to the absorbance of water because of its hydrophilic nature. Indeed, increased swelling of electrospun fibers has been previously observed to impede drug release in a previous study [61]. Further studies are warranted to optimize the swelling ratios if our fibers are to be used for drug delivery to periodontal tissues.

The rate at which a GTR membrane degrades plays an essential role for in periodontal regeneration, as the rate of fiber degradation should be able to match the rate of new tissue formation. Therefore, it is important to understand the mechanism underlying electrospun fiber degradation in order to tailor scaffolds for periodontal applications [62]. Lysozyme is the most commonly used enzyme for studying degradation of CH based scaffolds for in vitro analysis [63]. Degradation rate is inversely proportional to the degree of deacetylation. A previous study assessed the effect of scaffold fiber orientation on degradation of other polymers with similar findings, whereby aligned fibers showed a more stable profile as compared to random fibers [64]. Subramanian and co-workers have reported the degradation of aligned fibers to be slower than randomly oriented fibers [64]. However, they used poly(lactide-co-glycolide) fibers for their study and carried out the
degradation study using PBS as the degradation media. In this study, lysozyme solution in PBS was used as the degradation media, which was rejuvenated after every 3 days to mimic normal physiological conditions. Another study conducted by the same author monitored the degradation rates of random and aligned fibers showing similar outcomes [65]. Subramaniam et al., have postulated that the slower degradation rate of aligned fibers is due to decreased pore sizes found in aligned mats, hence; decreasing the diffusion rate of lysozyme solution [65]. Furthermore, they stated that axial alignment improves the compactness of fibers and results in decreased pore size. This is also indicative that random CH fibers in our study might have higher porosity as compared to aligned orientation. As seen in the SEM’s of random (4e) and aligned (4f) fibers after 14 days of degradation, many fibers have significant morphology changes after 14 days of degradation in lysozyme. pH value has been reported to be as a key element for metabolism during wound healing. It is also regarded as a pivotal parameter for therapeutic interventions in wound care procedures [66]. pH analysis showed a gradual increment with respect to time for random and aligned CH fibers, reaching a peak of 8.5 at 28th day of degradation. A study conducted by Zhang and co-workers, focused on pH change and mass loss of plain CH. The results obtained were similar to those that were observed in this study although at different time points. At the end of 16 weeks of degradation, Zhang et al., showed that using a PBS solution the pH value remained around 7.4–7.6 over the experimental period [67]. UV–vis spectroscopy has been used whilst performing CH degradation studies to identify certain functional groups in molecules. The absorption of this UV or visible light by organic molecules is restricted to specific functional groups or chromophores that exhibit valence electrons of low excitation energy [68]. Studies performed on characterization of degraded supernatant in the past have reported two absorption bands. A band at 230 nm might occur due to \( \pi^* \) transition of amino group and \( \pi-\pi^* \) transition of carbonyl and carboxyl groups. Another band at 280 nm is assigned to \( \pi-\pi^* \) transition of carbonyl or carboxyl group. The increase in intensity of these bands with respect to time as observed for random and aligned fibers has been assigned to scission of 1,4-glycosidic bonds caused by rearrangement of radicals. Moreover, it is due to the formation of carbonyl and carboxyl groups as reported by Ulsaniker et al., [69]. The upward curvature of the bands with time could be due to increasing efficiency of \(-\text{OH}\) reaction with CH upon reduction of molecular weight (MW) [69]. As shown in the FTIR spectra, all the wavenumbers characteristic of CH and PEO were present indicating presence of both polymers in the fibers. Furthermore, the spectra also showed progressive decrease in intensity of amide, CH and glycosidic bands indicative of CH main chain scission.

Electrospun fibers are reported to mimic extracellular matrix and are conducive to support cell attachment, proliferation and matrix deposition [34]. Aligned fibrous scaffold offer a more suitable interface for cells to deposit collagen such that the cellular response mimics that of a cell in its native environment as reported earlier by Delaine-Smith et al., [70] and Shang et al., [52]. Furthermore, Shang and co-workers also mentioned that cellular migration on aligned fibers was significantly faster than random fibers. Significantly higher production of collagen and glycosaminoglycan was also observed by Liu and coworkers on aligned fibers compared to randomly oriented scaffolds. They also reported that that when progenitor cells were cultured on aligned and random orientations, no apparent difference was noted on cellular proliferation [71]. Hence, there are reports of mixed results in the differentiation of stem cells when cultured on aligned and random fibers. Yin et al., reported that randomly oriented fibers induced higher calcium deposition, which was correlated to integrin and myosin mediated mechanotransduction, while the aligned fibers hindered the process [72]. Mesenchymal stem cells (MSCs) are known to be able to differentiate into bone tissue, neural tissue, cartilage, muscle and fat, they are also representative of the cells found at a healing wound site [73]. Results of H & E sectioning indicated that the CH fibers were conducive to allow cellular attachment and penetration within the fibrous mats, this was also observed by extracellular matrix (collagen) coverage.

5. Conclusion

In the current study, a solution of chitosan and UHMwPEO in a ratio of 95:5 was successfully electrospun to obtain fibrous membranes with either highly aligned or randomly orientated fiber configurations. Aligned and random fiber morphologies could be combined to develop structural gradients when designing a trilayered construct to enhance regeneration of specific periodontal tissues, e.g. aligned for ligament, random for bone. The results from the mechanical data propose that fibers, dry or wet, can be handled with ease at chair side in a clinical setup. Cellular viability and mineral deposition response indicate that randomly orientated fibers promoted proliferation of osteoblastic cells and matrix turnover. Both orientations could serve the purpose of surface layers that could mimic local tissue structure and function while regenerating periodontal defect site.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dental.2016.10.003.

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