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Title

Development of a tissue engineered lymphatic graft using nanocomposite polymer for the treatment of secondary lymphedema

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Brief running title

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Lymphatic graft, lymphatic vessel, lymphedema, tissue engineering, POSS-PCU, nanocomposite

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Abbreviated Abstract

This study aims at developing a tissue engineered lymphatic graft using nanocomposite polymer and human dermal lymphatic endothelial cells for the treatment of secondary lymphedema. We demonstrated the feasibility of developing a tissue engineered lymphatic graft, offering a new treatment option for secondary lymphedema.

Abstract

Damage of the lymphatic vessels, commonly due to surgical resection for cancer treatment, leads to secondary lymphedema. Tissue engineering approach offers a possible solution to reconstruct this damage with the use of lymphatic graft to re-establish the lymphatic flow, hence preventing lymphedema. The aim of this study is to develop a tissue engineered lymphatic graft using nanocomposite polymer and human dermal lymphatic endothelial cells (HDLEC). A nanocomposite polymer, the polyhedral oligomeric silsequioxane-poly(carbonate-urea)urethane (POSS-PCU), which has enhanced mechanical, chemical, and physical characteristics was used to develop the lymphatic graft. POSS-PCU has been used clinically for the world first synthetic trachea, lacrimal duct and undergoing clinical trial for coronary artery bypass graft. Two designs and fabrication methods were used to manufacture the conduits. The fabrication method, the mechanical and physical properties, as well as the hydraulic conductivity were tested. This is followed by in-vitro cell culture analysis to test the cytocompatibility of HDLEC with the polymer surface. Using the casted extrusion method, the nanocomposite lymphatic graft demonstrates desirable mechanical property and hydraulic conductivity to re-establish the lymphatic flow. The conduit has high tensile strength (casted: 74.86±5.74MPa versus coagulated: 31.33±3.71MPa; $p<0.001$), favourable kink resistance and excellent suture retention property (casted versus coagulated, $p<0.05$). Cytocompatibility study showed that the POSS-PCU scaffold supports the attachment and growth of HDLEC. This study demonstrates the feasibility of developing a tissue engineered lymphatic graft using the nanocomposite polymer. It displays excellent mechanical property and cytocompatibility to HDLEC, offering much promise for clinical applications and as a new treatment option for secondary lymphedema.

1.0 Introduction

The lymphatic system is an extensive vascular network involved in maintaining the homeostasis of extracellular fluid. The damage or dysfunction of this system leads to accumulation of lymphatic fluid in extracellular space, leading to lymphedema. The commonest cause of lymphedema in developed countries is iatrogenic, a direct consequence of surgical resection for cancer treatment leading to the development of secondary lymphedema, giving rise to significant psychosocial distress and increased risk of cellulitis and ulcer formation [1, 2]. The current surgical management is not able to reconstruct this damaged lymphatic vessels due to unavailability of appropriate donor vessel and high risk of donor site morbidity [3]. Given that the incidence of secondary lymphedema had increased substantially in the past decade, with more than one in five breast cancer survivors developing upper limb lymphedema, there is an urgent need to look beyond native tissue to reconstruct the damaged lymphatic system [4].

Tissue engineering approach, combining biomaterial scaffold and seeded cells, offers a possible solution to reconstruct the damaged lymphatic vessels with the use of lymphatic graft to re-establish the lymphatic flow, hence preventing the development of secondary lymphedema. Although artificial lymphatic graft has yet to be successful due to several challenges, mainly involving the material selection and fabrication of the small calibre graft, the advancement in nanotechnology and better understanding of lymphatic biology offers solution to these limitations [5].

An ideal lymphatic graft should match the properties of native lymphatic vessel [5]. These features include durability with good flexibility, ability to maintain patency, non-toxic, non-allergenic and non-carcinogenic. In terms of handling, it should be kink-resistant, easy to

suture with good suture retention property, and have adequate fatigue strength. The goal of tissue engineered lymphatic graft is to reproduce the structure, function, mechanical property, and cellular organisation of a native collecting lymphatic vessel.

The aim of this study is to tissue engineer a single luminal collecting lymphatic vessel using the nano-composite polymer Polyhedral Oligomeric Silsesquioxane modified Poly(carbonate urea-urethane) (POSS-PCU) to re-establish lymphatic flow in secondary lymphoedema (Figure 1). The objectives are to fabricate the conduit and to select the most promising design and fabrication method, as well as to assess the cytocompatibility of human dermal lymphatic endothelial cells (HDLEC) with POSS-PCU in order to endothelialise the conduit. POSS-PCU is a nanomaterial that has been used for lacrimal duct [6], heart valve [7], auricular reconstruction [8], the world first synthetic trachea [9], and currently undergoing clinical trial for coronary artery bypass graft [10]. Its favourable mechanical and biochemical properties include biocompatibility, durability, thermodynamic stability, anti-thrombogenic ability, and its ability for biofunctionalisation and endothelialisation [11-13].

2.0 Materials and methods

2.1 Synthesis of POSS-PCU nanocomposite

The polymer synthesis has been previously described in detail [11, 14, 15]. In brief, polycarbonate polyol and trans-cyclohexanediolisobutyl-silsesquioxane were placed in a reaction flask with nitrogen and heated to dissolve the nanocage. Pre-polymer was then formed by adding Methylene di-isocyanate (MDI) into the mixture and reacted at 70^{o}C for 90 min. This was followed by adding N, N-dimethylacetamide (DMAC). Chain extension was

achieved by the addition of ethylenediamine and diethylamine in DMAC. Lastly, 1-butanol in DMAC was added to the mixture to form an 18% POSS–PCU solution.

2.2 Design of lymphatic graft scaffold

Two different designs were investigated in fabricating the conduit to compare the functionality in assisting the lymphatic flow.

Design 1: Non-tapered conduit - The non-tapered cylindrical conduit has internal luminal diameter of 2.0 mm along its entire length (Figure 2a). The conduit was manufactured using a stainless steel mandrel as inner mould.

Design 2: Tapered conduit - The tapered conduit is designed to explore the possibility of achieving enhanced hydraulic conductivity by capitalising on the design. The internal luminal diameter of the conduit increases in size gradually from proximal (0.8 mm) to distal (2.0 mm), whereby the lumen is wider distally (Figure 2b). A custom made stainless steel mandrel was used to achieve this design.

2.3 Extrusion of POSS-PCU to fabricate lymphatic conduits

Two methods were employed to manufacture the conduits, namely the casted extrusion (i.e. solvent evaporation) and phase inversion coagulation extrusion.

2.3.1 Development of lymphatic conduit by casted extrusion

The mandrel was dip coated in polymer solution (viscosity of 2000 centipose to 5000 centipose at 25^{o}C) and pulled out at a speed of about 4 mm/sec. The coated mandrel was then placed vertically in an air-circulating oven at 65^{o}C for 60 minutes until solid polymer was extruded by evaporation of DMAC solvent. The dip-coating process was repeated with

interval of 60 minutes between each coats and left overnight in the oven before gently removing the conduit from the mandrel by small circular and longitudinal stresses. Casted conduit with 3 coats and 4 coats were produced.

2.3.2 Development of lymphatic conduit by phase inversion coagulation extrusion

An automated bench-top polymer extrusion apparatus was used. This apparatus has a mechanical arm which holds the mandrel that passes through an extrusion die well into a cylindrical flask containing coagulation solvent (deionised water) at the speed of 10mm/s, used to develop a method for standardised and automated fabrication [16].

The polymer coated mandrel was left in the coagulation solution for an hour before gently removing the conduit from the mandrel. The conduit was kept in deionised water and put on agitation overnight to aid removal of DMAC.

2.4 Assessment and characterisation of the lymphatic conduit

2.4.1 Conduit Characterisation using Scanning Electron Microscopy (SEM)

Using SEM, the inner surface and cross section of the fabricated conduits were analysed to determine the wall thickness, wall texture and surface characteristics. The cross-sectional images of the non-tapered conduit were captured from random area along the conduit as representative of the entire structure, while for the tapered conduit, images were captured at 3 specific locations along the conduit to show the increasing luminal diameter, namely the proximal end, the middle, and the distal end of the conduit.

The conduits were washed with deionised water and cut into 2 mm thick rings, which were then cut along the longitudinal axis. The specimens were left to air dry over 24 hours. Finally, the samples were attached to aluminium stubs and coated with gold using an SC500 (EMScope) sputter coater for electrical conductance and imaged using a Carl-Zeiss Evo-HD

15 Scanning Electron Microscope. The magnifications ranged from x55, x80 and x250. Six different samples were analysed combining the different fabrication methods and designs.

2.4.2 Capillarity

The capillary action of the conduits was tested using Acid Red 37 dye (Sigma-Aldrich Company Ltd) at 37ºC. Standard glass capillary tube (Accupette Pipets 20µl from Dade Diagnostics Inc.) was used as control. The height of capillary action was measured using electronic Vernier calliper (Absolute Digimatic by Mitutoyo).

2.4.3 Hydraulic conductivity (cm/s)

The hydraulic conductivity measurement, which relates water flow velocity to hydraulic gradient under laminar flow conditions, was performed with two main objectives: to assess the ability of the fabricated conduit to allow fluid flow, and to assess the difference in flow rate between the conduits. The hydraulic conductivity was tested using a falling head permeameter developed in house. Briefly, a 50 mL burette was connected to mono-lumen silicon tubing and attached to the conduit with connectors. The fluid level in the burette was maintained at 30 mL before each test. Fluid passing through the conduit was collected in a 10 mL cylinder and the time taken to collect 10 mL of fluid was measured using a stopwatch. A total of 5 measurements were taken using each conduit.

The test fluid was made to match the viscosity of lymphatic fluid, which is 0.0018 Pa s, or 1.8 times that of water [17]. Glycerol-water mixture, with glycerol 30% by weight was used to achieve the viscosity of 0.0018 Pa s at 37ºC [18]. The pressure generated by 30 mL of fluid is about 65 cmH_20, equates to 47.8 mmHg (1 mmHg = 1.36 cmH_20), which is within the range of intrinsic pressure generated by lymphangion [19].

Using Darcy's equation of hydraulic conductivity for falling head permeameter (Equation 1), the hydraulic conductivity of the conduits was calculated [20].

Equation 1: Darcy's equation of hydraulic conductivity for falling head permeameter

$$K = \frac{aL}{At} \ell n \frac{\Delta h_0}{\Delta h(t)}$$

(K, cm/s) = Hydraulic conductivity

(*t*, s) = time taken to collect 10 mL of fluid in cylinder

(*L*, cm) = length of the conduit

(Δh_o, cm) = height of fluid in burette at the start of the experiment

($\Delta h_{(t)}$, cm) = height of fluid in burette at the end of the experiment

(A, cm^2) = cross-sectional area of the conduit

(*a*, cm^2) = cross-sectional area of the burette

2.4.4 Biomechanical evaluation

These tests were all done in accordance to the International Standard for cardiovascular implant – tubular vascular prostheses BS ISO 7198:1998 as a benchmark.

2.4.4.1 Tensile strength measurement

The mechanical testing was done using Instron 5565, which measures the longitudinal tensile stress and strain.

Polymer sheets were used for this test, instead of the fabricated conduits, to ensure accuracy of the measurement due to the small diameter of the conduit. A custom made stainless steel

plate was used as a mould for the sheets. Polymer solution was applied onto the plate and either left in the oven overnight to produce casted sheet, or left in deionised water overnight to produce coagulated sheet. Samples (n=8) were placed within the grips of the Instron 5565 and the longitudinal tensile tests were performed with crosshead speed of 50 mm/min. The maximum tensile stress at break (T max) was recorded in mega Pascal (MPa). Care was taken to ensure the samples were not stretched, twisted, or damaged by the grips, and slack was kept to a minimum.

2.4.4.2 Suture retention

Suture retention test was performed to determine the force necessary to pull a suture through and cause the conduit to fail by tear. The suture (9/0, Prolene) was passed through the graft wall, 2 mm from the edge. The conduit was placed in the lower crosshead while the free ends of the suture were placed in the upper crosshead. The specimen was pulled at the speed of 50 mm/min until either the suture ripped or the conduit failed. The force required for the suture or conduit to fail was recorded in mega Pascal (MPa).

2.4.4.3 Kink resistance

This test was intended to determine the radius of curvature required for the conduit to begin kinking, defined as 50% reduction in cross-sectional area of the conduit. Templates with increasing diameter were made for this test. The diameters ranges from 5 mm to 50 mm, each made with an increment of 5 mm. The kink resistance test method is shown in Figure 3.

2.5 Endothelialisation of POSS-PCU nanocomposite with human dermal lymphatic endothelial cells (HDLEC)

The cytocompatibility as well as the viability of HDLECs on POSS-PCU was evaluated.

2.5.1 HDLEC culture and cell seeding onto POSS-PCU

HDLEC from PromoCell GmbH, Germany were cultured in PromoCell Endothelial Cell Growth Medium MV2 consisting 5% fetal calf serum, epidermal growth factor (5 ng/ml), basic fibroblast growth factor (10 ng/ml), insulin-like growth factor (20 ng/ml), vascular endothelial growth factor 165 (0.5 ng/ml), ascorbic acid (1 µg/ml), and hydrocortisone (1 µg/ml). The cells were cultured at 37ºC with 5% carbon dioxide and used before 6 population passages. At confluence, the cell were detached by trypsinisation and subcultivated at the density of 2 x 10^4 cells/cm^2. Cell count was performed using the haemocytometer and trypan blue dye exclusion staining.

POSS-PCU sheets were manufactured and cut into 16 mm diameter discs to fit into 24-well plate (VWR International) and soaked in 70% ethanol for 30 minutes to sterilise, and then washed 3 times with phosphate-buffered saline (PBS). The polymer discs were then pre-conditioned by incubating with cell culture media for 30 minutes. Cells with density of 4 x 10^4 cells in 500µL of cell culture media were then added into each well and underwent static culture in 37ºC, humidified atmosphere containing 5% CO_2 and 95% air. Glass cover slips were used as control surface.

2.5.2 Cell metabolic activity evaluation

The metabolic activity of HDLEC was evaluated using Alamar Blue assay (In Vitro Toxicology Assay Kit, Resazurin based from Sigma-Aldrich Company Ltd). Alamar Blue is metabolized from blue resazurin to pink resazurin by viable cells and the change in colour is detected spectroscopically. Alamar Blue assay was performed as per manufacturer's instruction. Briefly, 10% Alamar Blue solution by volume was added to the samples cultured in the 24-well plate (n=4) for a period of 4 hours to metabolise the dye, following which 100µl of supernatant was transferred to 96 well plate (Fisher Scientific Ltd). Fluorescence

was measured at 570 nm and 630 using Fluoroskan Ascent FL spectrofluorometer from Thermo Labsystems. Reading were taken at 6 hours, day 1, day 2, day 4 and day 6 of culture.

2.5.3 Cell adherence onto POSS-PCU

Cell adherence was analysed by using immunofluorescence staining with Rhodamine phalloidin, which preferentially stains F-actin cytoskeleton of cells, producing red fluorescence. The samples were washed with PBS to remove culture medium and residual proteins before fixing with 4% paraformaldehyde in PBS. Permeabilisation was then performed using 0.01% triton X in PBS for 5 minutes and incubated with 1% bovine serum albumin (BSA) for 30 minutes at room temperature. The samples were rinsed gently with PBS and incubated with rhodamine phalloidin (Life Technologies) diluted 50 times in PBS, for 30 minutes. Samples were washed with PBS and stained with a single drop of ProLong® Gold antifade reagent with DAPI (Life Technologies) to stain the nucleus. Imaging was performed with fluorescence microscope (EVOS® FL Imaging System, Life Technologies) at 24 hours and day 12 post seeding.

2.6 Data collection and Statistical analysis

All data are presented as means ± standard deviations. Comparison between sample groups was performed using either independent T-test or one way analyses of variance (ANOVA) test as appropriate. The mean of each group from one way analyses of variance (ANOVA) was further compared to each other using post-test Bonferroni adjustment. Statistical analysis was performed using IBM SPSS Statistics 21 software. P values < 0.05 was considered statistically significant.

3.0 Results

3.1 Conduit characterisation using SEM

SEM images of the cross-section, longitudinal section and the luminal surface of the non-tapered conduits are shown in Figure 4. The casted conduit has solid wall with etched surface, likely from the mandrel surface. The coagulated conduit displays porous wall with rough luminal surface topography.

The SEM images of cross-section and longitudinal section of tapered conduit are shown in Figure 5. Casted conduit has consistent wall thickness along its length while the coagulated conduit has inconsistent wall thickness and luminal shape. The wall thickness of the fabricated conduits are shown in Table 1.

3.2 Capillarity

Capillary action is tested to determine the ability of the conduit to assist and enhance lymphatic flow. The capillary action of the conduits was noted to be lower than the standard glass capillary tube (Table 2), with the casted conduit having the lowest capillary action (Figure 6).

3.3 Hydraulic conductivity

Significant difference was noted between the conduits ($p<0.05$, one way ANOVA), with casted non-tapered conduit having the best hydraulic conductivity (0.0419 ± 0.001cm/s) (Figure 7).

3.4 Biomechanical evaluation

3.4.1 Tensile strength measurement

The casted sheet has maximum tensile strength of 74.86 ± 5.74 MPa, which is significantly higher than the coagulated sheet which has a maximum tensile strength of 31.33 ± 3.71 MPa ($p<0.001$, independent t-test).

3.4.2 Suture retention

Suture retention is critical in the design of a lymphatic graft as it directly relates to the success of the anastomosis, hence that of the implant. The casted conduits (3 and 4 coats) has better suture retention property than the coagulated conduit ($p<0.05$, one way ANOVA). For the casted conduit with 3 and 4 coats, the suture breaks at the maximum stress of 3.82 ± 0.01 MPa and 3.83 ± 0.03 MPa respectively, while the conduit remains intact. However, for the coagulated conduit, the conduit fails at the maximum stress of 3.75 ± 0.04 MPa, while the suture remains intact.

3.4.3 Kink resistance

Overall, the coagulated conduit and tapered design demonstrates better kink resistance (Figure 8). However, since the coagulated conduits and tapered design has relatively poor tensile strength and hydraulic conductivity, the casted non-tapered conduit appears to be the ideal choice for the development of lymphatic graft. Hence, comparing only the casted non-tapered conduit, the conduit with 3 coats demonstrates a better kink resistance.

3.5 Endothelialisation of POSS-PCU nanocomposite with human dermal lymphatic endothelial cells (HDLEC)

3.5.1 Cell metabolic activity evaluation

During the incubation period, HDLEC were viable on all surfaces (Figure 9). Notably, however, the HDLEC grown on casted and coagulated POSS-PCU had a lower metabolic activity as compared to glass (control) ($p<0.05$, one way ANOVA) at all-time points except at 6 hours after seeding ($p>0.05$, one way ANOVA). The difference in the metabolic activity between cells seeded onto casted and coagulated POSS-PCU was noted to be insignificant ($p>0.05$, one way ANOVA).

3.5.2 Cell adherence onto POSS-PCU

Fluorescence microscopic examination of HDLEC grown on glass slip (control), casted POSS-PCU and coagulated POSS-PCU shows the cell morphology and the differences in cell density (Figure 10). Different growth pattern was observed on the three surfaces. At 24 hours post seeding, the cells grown on glass had the highest density and widest spread, while the cells seeded onto casted POSS-PCU had the lowest cell density and were least spread. These similar findings were noted on day 12 where the cells were fully confluent on glass, and least confluent on casted POSS-PCU.

4.0 Discussion

This study has shown for the first time the feasibility of developing a tissue engineered lymphatic graft with the use of nanocomposite material and HDLEC. Nanocomposites offer superior qualities as compared to conventional materials, due to the interaction at the nanoscale level, contributing to its unique surface and bulk properties [21].

The casted lymphatic conduit was noted to be superior with uniform wall thickness and luminal shape. The tensile strength and suture retention tests show that the casted conduit is stronger and more reliable, likely due to the non-porous wall structure. These along with the hydraulic conductivity and kink resistant property leads to the conclusion that the casted non-tapered conduit with 3 coats is the best choice for a tissue engineered lymphatic graft.

There is paucity of published data on the mechanical characteristics and the tensile strength of human collecting lymphatic vessel, limiting the ability to compare and match the mechanical property of the fabricated conduit to that of the native lymphatic vessel. A study on rat model shows that the tensile strength of the submesothelial diaphragmatic lymphatic vessel is 1.7 ± 0.3 MPa [22], which is much lower than the tensile strength of the produced conduit. However, this value might not reflect the tensile strength of human lymphatic vessel which is greater in size and thickness.

The low capillary action of the conduits is contributed by two main reasons: the wide luminal diameter and the hydrophobicity of the polymer [23]. In this study, the luminal diameter of 2 mm was chosen instead of a smaller calibre to reduce the risk of blockage. On the other hand, due to the low pressure nature of the lymphatic system, a wide calibre could cause pooling of fluid in the conduit, resulting in higher pressure gradient across the conduit. This could overwhelm the system and cause flow cessation, as happens in lymphedema [24]. Hence, the luminal diameter of 2mm was chosen to match the mean diameter of lymphatic vessels [25].

We have previously described the importance of intraluminal valves in assisting the low capillary action of the lymphatic system [5]. However, in order to include intraluminal valve into the design of the conduit, a functional muscular wall is essential to ensure the patency of the valve and the conduit, which is the aspiration of an ideal lymphatic graft in the future.

The flow pressure required to transport fluid across the conduit, both horizontally and vertically, should be measured using a flow circuit equipped with pressure transducer. This measurement is crucial before pre-clinical animal testing as it will help to predict the success of the lymphatic graft. Endothelialisation is essential, not only to emulate the anatomical feature of a native lymphatic vessel, but also important to ensure the success of the lymphatic graft. Human LEC is essential in maintaining the physiological property of the lymph [26]. LEC plays a pivotal role in protein concentration of the lymphatic fluid in collecting lymphatic vessels, concentrating the lymph as it moves from the peripheral lymphatic vessels to the thoracic duct, ensuring macromolecules such as albumin remain in the lymphatic vessel and are returned to the systemic circulation [26]. Besides that, the increase in protein concentration of the lymph is also an important regulator of innate immunity as the excretion of non-selective T- and B-cell lymphocytes and natural killer (NK) cells was found to be positively correlated with protein concentration of lymph [26, 27]. To address these, we cultured HDLEC in short term culture on POSS-PCU. In vitro cell adherence test shows promising result with attachment of HDLEC on both casted and coagulated samples. Morphologically, the cells maintained their unique phenotypic morphology and continued to grow on both surfaces. The metabolic activity of the cells was also comparable on both surfaces, suggesting that the nanocomposite is conducive for HDLEC growth. However, the metabolic activity of the cells on the nanocomposite was significantly lower as compared to the control surface. Noting that the cell's metabolic activity did not reach a plateau up to day 6 suggests that the seeding density could have been higher to ensure faster confluence. In order to achieve a confluent mono-layered HDLEC on the polymer surface, the seeding density and method has to be optimised. Besides ensuring complete endothelialisation, the optimal seeding density will also ensure high cell-to-substrate strength to withstand hydrodynamic stress [28]

The difference in cell adhesion density on these surfaces is likely due to the hydrophobic nature of POSS-PCU [23]. The endothelial cell growth involves a multistep process which includes protein adsorption, cell contact with substratum, formation of bonds of attachment and cell spreading on the surface [29]. Cell adhesion is dependent on the wettability of the surface as it influences the protein adsorption [30]. Plasma modification can improve the surface hydrophilicity for enhanced cell adhesion by rendering the surface positively charged, increasing the affinity of the cells as they carry a net negative charge at physiological pH [29, 31]. This can be further enhanced with the introduction of functional groups such as hydroxyl- and carbonyl-end groups by plasma modification [32]. Moreover, besides modifying the surface chemistry, plasma modification also alters the nanotopography by increasing the surface roughness [32]. The positive effect of surface roughness in enhancing cellular adhesion has been previously described [33, 34]. This correlates with the better adhesion and spreading of HDLEC seen on the coagulated POSS-PCU which has rougher surface as compared to the casted POSS-PCU in this study.

5.0 Conclusion

We have outlined, for the first time, the feasibility of developing a tissue engineered lymphatic graft with clinically desirable biomechanical and functional properties.

The assessment and characterisation tests have highlighted that the best fabrication method and design for a lymphatic graft is the casted, non-tapered conduit with 3 coats. The ability of HDLEC to adhere and grow on POSS-PCU has brought us one step forward in realising the vision of producing a tissue engineered lymphatic graft using the nanocomposite material. This offers a new treatment option for the management of secondary lymphedema.

In a whole, this study has highlighted the ability to produce a tissue engineered lymphatic graft, the elementary step for a better healthcare in the management of lymphedema.

6.0 References

1. Fu MR, Ridner SH, Hu SH, Stewart BR, Cormier JN, Armer JM. Psychosocial impact of lymphedema: a systematic review of literature from 2004 to 2011. Psycho-Oncology. 2013;22:1466-84.

2. Robless P, Lim J, Geroulakos G. Lymphoedema. Surgery (Oxford). 2010;28:268-72.

3. Campisi C, Boccardo F, Zilli A, Maccio A, Napoli F. The use of vein grafts in the treatment of peripheral lymphedemas: long-term results. Microsurgery. 2001;21:143-7.

4. DiSipio T, Rye S, Newman B, Hayes S. Incidence of unilateral arm lymphoedema after breast cancer: a systematic review and meta-analysis. Lancet Oncol. 2013;14:500-15.

5. Kanapathy M, Patel NM, Kalaskar DM, Mosahebi A, Mehrara BJ, Seifalian AM. Tissue-engineered lymphatic graft for the treatment of lymphedema. J Surg Res. 2014 Dec;192(2):544-54.

6. Chaloupka K, Motwani M, Seifalian AM. Development of a new lacrimal drainage conduit using POSS nanocomposite. Biotechnology and applied biochemistry. 2011;58:363-70.

7. Rahmani B, Tzamtzis S, Ghanbari H, Burriesci G, Seifalian AM. Manufacturing and hydrodynamic assessment of a novel aortic valve made of a new nanocomposite polymer. Journal of biomechanics. 2012;45:1205-11.

8. Nayyer L, Birchall M, Seifalian AM, Jell G. Design and development of nanocomposite scaffolds for auricular reconstruction. Nanomedicine : nanotechnology, biology, and medicine. 2014;10:235-46.

9. Elliott MJ, De Coppi P, Speggiorin S, Roebuck D, Butler CR, Samuel E, et al. Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study. Lancet. 2012;380:994-1000.

10. University College London. Clinical Study of POSS-PCU Vascular Grafts for Vascular Access. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US) 2014. Cited 2015 July 22. Available from: https://clinicaltrials.gov/ct2/show/NCT02301312 NLM Identifier: NCT02301312.

11. Kannan RY, Salacinski HJ, Odlyha M, Butler PE, Seifalian AM. The degradative resistance of polyhedral oligomeric silsesquioxane nanocore integrated polyurethanes: an in vitro study. Biomaterials. 2006;27:1971-9.

12. Kannan RY, Salacinski HJ, Sales KM, Butler PE, Seifalian AM. The endothelialization of polyhedral oligomeric silsesquioxane nanocomposites: an in vitro study. Cell biochemistry and biophysics. 2006;45:129-36.

13. de Mel A, Punshon G, Ramesh B, Sarkar S, Darbyshire A, Hamilton G, et al. In situ endothelialization potential of a biofunctionalised nanocomposite biomaterial-based small diameter bypass graft. Bio-medical materials and engineering. 2009;19:317-31.

14. Salacinski HJ HS, Seifalian AM. Polymer for use in conduits and medical devices 2005. World Pat WO2005070998. 2005

15. Alobaid N, Salacinski HJ, Sales KM, Ramesh B, Kannan RY, Hamilton G, et al. Nanocomposite containing bioactive peptides promote endothelialisation by circulating progenitor cells: an in vitro evaluation. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery. 2006;32:76-83.

16. Sarkar S, Burriesci G, Wojcik A, Aresti N, Hamilton G, Seifalian AM. Manufacture of small calibre quadruple lamina vascular bypass grafts using a novel automated extrusion-phase-inversion method and nanocomposite polymer. Journal of biomechanics. 2009;42:722-30.

17. Bouta EM, Wood RW, Brown EB, Rahimi H, Ritchlin CT, Schwarz EM. In vivo quantification of lymph viscosity and pressure in lymphatic vessels and draining lymph nodes of arthritic joints in mice. The Journal of physiology. 2014;592:1213-23.

18. J. B. Segur, Oberstar HE. Viscosity of Glycerol and Its Aqueous Solutions. Industrial & Engineering Chemistry.1951;43, 2117-2120.

19. Olszewski WL, Engeset A. Intrinsic contractility of prenodal lymph vessels and lymph flow in human leg. The American journal of physiology. 1980;239:H775-83.

20. McWhorter DB, Sunda DK. Ground-Water Hydrology and Hydraulics: Water Resources Publications. 1977;3, 65-91.

21. Kannan RY, Salacinski HJ, Butler PE, Seifalian AM. Polyhedral oligomeric silsesquioxane nanocomposites: the next generation material for biomedical applications. Accounts of chemical research. 2005;38:879-84.

22. Moriondo A, Boschetti F, Bianchin F, Lattanzio S, Marcozzi C, Negrini D. Tissue contribution to the mechanical features of diaphragmatic initial lymphatics. The Journal of physiology. 2010;588:3957-69.

23. Tan A, Farhatnia Y, Goh D, G N, de Mel A, Lim J, et al. Surface modification of a polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane (POSS-PCU) nanocomposite polymer as a stent coating for enhanced capture of endothelial progenitor cells. Biointerphases. 2013;8:23.

24. Rahbar E, Weimer J, Gibbs H, Yeh AT, Bertram CD, Davis MJ, et al. Passive pressure-diameter relationship and structural composition of rat mesenteric lymphangions. Lymphatic research and biology. 2012;10:152-63.

25. Lu Q, Delproposto Z, Hu A, Tran C, Liu N, Li Y, et al. MR lymphography of lymphatic vessels in lower extremity with gynecologic oncology-related lymphedema. *PLoS One*. United States 2012:e50319.

26. Kawai Y, Kaidoh M, Yokoyama Y, Ohhashi T. Pivotal roles of lymphatic endothelial cell layers in the permeability to hydrophilic substances through collecting lymph vessel walls: effects of inflammatory cytokines. Lymphat Res Biol. 2014;12:124-35.

27. Quin JW, Shannon AD. The influence of the lymph node on the protein concentration of efferent lymph leaving the node. J Physiol. 1977;264:307-21.

28. Salacinski HJ, Tai NR, Punshon G, Giudiceandrea A, Hamilton G, Seifalian AM. Optimal endothelialisation of a new compliant poly(carbonate-urea)urethane vascular graft with effect of physiological shear stress. European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery. 2000;20:342-52.

29. De S, Sharma R, Trigwell S, Laska B, Ali N, Mazumder MK, et al. Plasma treatment of polyurethane coating for improving endothelial cell growth and adhesion. Journal of biomaterials science Polymer edition. 2005;16:973-89.

30. Baier RE, Shafrin EG, Zisman WA. Adhesion: mechanisms that assist or impede it. Science. 1968;162:1360-8.

31. Srinivasan S, Sawyer PN. Role of surface charge of the blood vessel wall, blood cells, and prosthetic materials in intravascular thrombosis. Journal of colloid and interface science. 1970;32:456-63.

32. Desmet T, Morent R, De Geyter N, Leys C, Schacht E, Dubruel P. Nonthermal plasma technology as a versatile strategy for polymeric biomaterials surface modification: a review. Biomacromolecules. 2009;10:2351-78.

33. Wan Y, Wang Y, Liu Z, Qu X, Han B, Bei J, et al. Adhesion and proliferation of OCT-1 osteoblast-like cells on micro- and nano-scale topography structured poly(L-lactide). Biomaterials. 2005;26:4453-9.

34. Kim DH, Han K, Gupta K, Kwon KW, Suh KY, Levchenko A. Mechanosensitivity of fibroblast cell shape and movement to anisotropic substratum topography gradients. Biomaterials. 2009;30:5433-44.

Table 1: Wall thickness of the fabricated lymphatic conduits.

Sample	Mean (µm)	Standard Deviation (µm)
Casted with 3 coats - non-tapered	119.3	7.6
Casted with 3 coats - tapered	117.6	4.0
Casted with 4 coats - non-tapered	146.1	2.3
Casted with 4 coats - tapered	171.8	3.4
Coagulated - non-tapered	534.8	91.3
Coagulated - tapered	708.6	329.3

Table shows the mean and standard deviation of wall thickness (µm) of the fabricated conduits.

Table 2: Height of capillary action (mm) of the conduits and standard glass capillary tube.

Sample	Height of capillary action (mm)
Coagulated, tapered	2.0 ± 0.1
Coagulated, non-tapered	1.5 ± 0.1
Casted, tapered	1.5 ± 0.1
Casted, non-tapered	1.0 ± 0.1
Standard glass capillary tube, (20µl) (control)	21.1 ± 0.1

Table shows the height of capillary action (mm) of the fabricated conduits and standard glass capillary tube (20µl).

Figure 1: Illustration of lymphatic network and application of lymphatic graft. Figure (a) shows the extensive network of human lymphatic system. Surgical resection for cancer treatment such as breast cancer resection damages the lymphatic vessels (b), disrupting the lymphatic flow from the distal part of the limb towards the heart, results in lymphoedema. Figure (b) highlights the surgical scar and the damaged lymphatic network after surgical resection. A possible solution for this is to reconstruct the damaged lymphatic vessels with the use of lymphatic graft to re-establish the lymphatic flow (c).

Figure 2: Illustration and dimension of the fabricated conduit. (a) Illustration of the non-tapered conduit with luminal diameter of 2.0 mm along its entire length. (b) Illustration of a tapered conduit with proximal luminal diameter of 0.8 mm and distal luminal diameter of 2.0 mm.

Figure 3: Kink resistance test method. The conduits were curved around the templates having a predetermined diameter, D. The diameters ranges from 5 mm to 50 mm, each made with an increment of 5 mm. The outer surface of the graft is configured to contact the template at two tangential locations so that the graft defines a curve, with the apex of the curve at a distance, L from the closest surface of the template, where L is approximately the same as D.

Figure 4: Scanning electron microscopy images of non-tapered conduit. Images (a), (b) and (c) shows the cross-section of the conduit taken at magnification x55. Images (d), (e) and (f) shows the longitudinal-section of the conduit taken at magnification x55. Images (g), (h) and (i) shows the inner surface of the luminal wall taken at magnification x250.

Figure 5: Scanning electron microscopy images of the tapered conduit. Images (a), (b) and (c) shows the cross-section of the conduits at the proximal end (luminal diameter of 0.8mm) taken at magnification x55. Images (d), (e) and (f) shows the cross-section of the conduits at the middle taken at magnification x55 while images (g), (h) and (i) are the cross-section of the conduits at the distal end of the conduit (luminal diameter of 2.0mm) taken at magnification x55. Images (j), (k) and (l) are the longitudinal section of the conduits showing the inner wall surface taken at magnification x80.

Figure 6: Height of capillary action shown by the conduits. (a) Coagulated, tapered; (b) Coagulated, non-tapered; (c) Casted, tapered; (d) Casted, non-tapered; (e) standard glass capillary tube.

Figure 7: Hydraulic conductivity (cm/s) of the conduits. n=5 was used for each conduit. Significant difference in hydraulic conductivity was noted between the conduits ($p<0.05$, one way ANOVA). Error bars represent the mean ± SD.

Figure 8: Bar chart showing the kink diameter of the conduits. Highlighted in red are the hydraulic conductivity of the casted non-tapered conduits that have a better mechanical property for the development of the lymphatic graft.

Figure 9: Line graph shows the metabolic activity of the HDLEC seeded on the different polymer surfaces up to day 6. The metabolic activity of HDLEC was evaluated using Alamar Blue assay (In Vitro Toxicology Assay Kit, Resazurin based from Sigma-Aldrich Company Ltd). Error bars represent the mean ± SD, n=4 for each data. The metabolic activity of HDLEC grown on casted and coagulated POSS-PCU was lesser as compared to glass (control) ($p<0.05$, one way ANOVA) at all-time points except at 6 hours after seeding ($p>0.05$, one way ANOVA). The difference in the metabolic activity between cells seeded onto casted and coagulated POSS-PCU was insignificant ($p>0.05$, one way ANOVA).

Figure 10: Immunofluorescence staining of HDLEC grown on different surfaces at 24 hours and day 12 post seeding. Red fluorescence highlights the actin within the cells (Rhodamine phalloidin) while blue fluorescence highlights the nucleus of the cells (DAPI). The increased blue and red fluorescence on all samples at day 12 shows the increase in cell density on all surfaces. (Scale=200μm)