Electrospinning synthesis and assessment of physicochemical properties and biocompatibility of cobalt nitrate fibers for wound healing applications

SARAVANA KUMAR JAGANATHAN\textsuperscript{1,2,3} and MOHAN P. MANI\textsuperscript{4}

\textsuperscript{1}Department for Management of Science and Technology Development, Ton Duc Thang University, Ho Chi Minh City, Vietnam
\textsuperscript{2}Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam
\textsuperscript{3}IJNUTM Cardiovascular Engineering Center, School of Biomedical Engineering and Health Sciences, Faculty of Engineering, Universiti Teknologi Malaysia, Skudai 81310, Malaysia
\textsuperscript{4}School of Biomedical Engineering and Health Sciences, Faculty of Engineering, Universiti Teknologi Malaysia, Skudai 81310, Malaysia

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Abstract: The aim of this study was to develop polyurethane (PU) wound dressing incorporated with cobalt nitrate using electrospinning technique. The morphology analysis revealed that the developed composites exhibited reduced fiber and pore diameter than the pristine PU. The electrospun membranes exhibited average porosity in the range of 67% - 71%. Energy-dispersive X-ray spectra (EDS) showed the presence of cobalt in the PU matrix. The interaction of cobalt nitrate with PU matrix was evident in Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA). The contact angle results indicated the improved wettability of the prepared PU/cobalt nitrate composites (82° ± 2) than the pure PU (100° ± 1). The incorporation of cobalt nitrate into the PU matrix enhanced the surface roughness and mechanical strength as evident in the atomic force microscopy (AFM) and tensile test analysis. The blood compatibility assays revealed the anticoagulant nature of the prepared composites by displaying prolonged blood clotting time than the PU control. Further, the developed composite exhibited less toxicity nature as revealed in the hemolysis and cytotoxicity studies. It was observed that the PU wound dressing added with cobalt nitrate fibers exhibited enhanced physicochemical, better blood compatibility parameters and enhanced fibroblast proliferation rates which may serve as a potential candidate for wound dressings.

Key words: Polyurethane, cobalt nitrate, electrospun fibers, skin tissue engineering, physico-chemical characteristics, bio compatibility.

INTRODUCTION

According to the American Burns Association (ABA) statistics, the burn injury mortality was reduced from 3.2% to 2.7% in men and for women, it was from 4.6% to 3.3%. Although mortality rate was reduced, the clinical applications were still findings difficulties in the treatment of burn wounds and infection prevention (Chen et al. 2017, Peck 2011). If there is infection occurs, there might be delayed healing and in some cases, the wound were unhealed (Chen et al. 2017, Zhang et al. 2015). Hence, the main importance of the burn wound
management is the immediate caring to reduce the infection and promote quick healing. It was reported that the infection in the damaged skin tissue was mainly caused by the bacteria’s like Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli (Chen et al. 2012, 2017). In order to reduce the infection caused by the pathogen, the developed wound dressing must fight against the microbial growth. The bacterial growth could be reduced by developing a scaffold with low interconnected pore spaces which restricts the bacterial cell division (Premuzic and Woodhead 1993). Further, it could also reduce by loading any antimicrobial agents into the fabricated dressings. An ideal wound dressing must have necessary characteristics such as maintaining a moist environment, gaseous exchange property, wettability behavior and should remove excess exudates (Kamoun et al. 2017). Further, the ideal wound dressing should be easy to apply, non-allergic, non-toxic and should minimize the trauma (Manikandan et al. 2017).

Electrospinning technique has emerged as a promising method in past decades to fabricate nanofibrous structures for tissue engineering applications (Chen et al. 2017). Electrospinning technique is a simple, versatile and cost-effective system. It consists of three main components namely voltage unit, syringe pump and collector drum. The polymer solution was loaded into the syringe pump and the high voltage is supplied to the polymer solution (Pillai and Sharma 2009). When the applied voltage exceeds the certain threshold voltage, nanofibers are drawn from the polymer solution and get deposited on the collector drum (Premuzic and Woodhead 1993). Recently, the polymeric nanofiber developed through the electrospinning has gained a huge attention in biomedical applications. The nanofibers obtained from the polymeric materials were reported to have desirable characteristics like the large surface area and high porosity with a reduced pore size (Huang et al. 2003). Further, these nanofibers help to support the host cell growth on the ECM for new tissue generation (Unnithan et al. 2012a). In this research, the polyurethane was used to fabricate the wound dressing. It was reported that the PU was widely employed in wound dressing applications owing to its better barrier properties and oxygen permeability (Unnithan et al. 2012b, Lakshmi et al. 2010).

Cobalt was reported to have excellent magnetic, electrical and catalytic properties. Owing to its properties, it was widely utilized in different sectors such as sensors, energy storage, heterogeneous catalysts and electrochromic devices (Ding et al. 2010). It was reported that cobalt complex could be used as an antiviral and antibacterial agent (Chang et al. 2010). To our knowledge, the use of cobalt nitrate in biomedical applications were least found. Further, to utilize the cobalt nitrate in the health and biomedicine applications, the investigation of the biocompatibility behavior plays a vital role. To our knowledge, there is no investigation of biocompatibility assessments of the cobalt nitrate fibers. In this study, a novel wound dressing based on polyurethane incorporated with cobalt nitrate was fabricated using electrospinning technique. For the electrospun PU and PU/cobalt nitrate, the various physicochemical, blood compatible and cytotoxicity properties were determined.

MATERIALS AND METHODS

MATERIALS

Tecoflex EG-80A pellets, a medical grade polyurethane was obtained from Lubrizol and the DMF obtained from Sigma Aldrich, UK. Cobalt II nitrate (Co(NO₃)₂·6H₂O) was supplied from Sigma Aldrich, UK. Phosphate buffered saline (PBS, Biotech Grade) and sodium chloride physiological saline (0.9% w/v) utilized in the coagulation studies were obtained from Sigma-Aldrich, Malaysia. All reagents used for APTT and PT assay were obtained from the Diagnostic Enterprises, India.
SOLUTION PREPARATION AND THE FABRICATION OF THE ELECTROSPUN MEMBRANES

PU pellets were dissolved in DMF at a concentration of 9 wt% and stirred overnight to obtain a homogeneous solution. Similarly, the cobalt nitrate solution was prepared at a concentration of 9 wt% and stirred for 2 hr maximum to obtain a homogeneous solution. Before electrospinning, the two prepared solutions were blended at a volume ratio of 8:1 (v/v%) respectively. Then, the prepared PU and PU/cobalt nitrate solution was loaded in a 10 ml glass syringe fitted with a stainless steel needle. The electrospun fibers were obtained at a voltage of 10 kV, with a flow rate of 0.2 ml/hr. The collector distance was placed at a distance of 15 cm. The fabricated membranes were taken out and dried under vacuum to exude any residual content.

SCAFFOLDS MORPHOLOGY

The scaffold morphology of the electrospun membranes was investigated by scanning electron microscopy. Prior to scanning, the electrospun scaffolds were coated with gold and the images were obtained. Finally, using Image J, the mean fiber diameters were calculated from the captured images.

POROSITY MEASUREMENT

Sample with a small size was cut and their thickness (t), length (l), width (w) and weight (m) were measured. The determined values were substituted in Equation 1 to determine the apparent density ($\rho_i$). Then, the determined (apparent density ($\rho_i$)) and known value (standard density ($\rho_0$)) of PU was substituted in Equation 2 to measure the average porosity percentage ($\varepsilon$).

Further, the pore size was determined through Image J software from SEM image of PU and PU/cobalt nitrate fibrous scaffold. The distance between two fibers was measured by choosing 50 locations randomly and were exported to the excel sheet to draw the graphical representation.

FTIR STUDY

The FTIR equipment was utilized to obtain the IR spectra of the electrospun fibers. Sample with a small size was placed on the measuring surface and the IR spectra was obtained at a range of 600 and 4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$ with 32 scans per minute.

CONTACT ANGLE MEASUREMENT

The water contact angles of the electrospun scaffolds were calculated through VCA Optima contact angle system mounted with a video cam. The fibrous scaffolds were cut into small piece and carefully placed on the surface. A single drop of distilled water was applied on the electrospun surface and the static image was captured using a video cam. Finally, the mean contact angle was measured through the computer integrated software.

TGA ANALYSIS

Samples with a weight of 3 mg were placed on the aluminum pan of the TGA unit and the heating was performed under nitrogen atmosphere. The thermal stability of the samples was recorded at a heating rate of 10°C/min with temperature range from 30°C to 1000°C.

AFM ANALYSIS

The surface topography of the electrospun membranes was analyzed through atomic force microscopy (AFM) equipment. Samples to measured were placed on the surface and were scanned in 20 $\mu$m $\times$ 20 $\mu$m size. The high quality 3D image was
captured through JPKSPM data processing software with pixels of around 256 * 256 pixels, respectively. The average surface roughness is calculated from the three individual locations.

**MECHANICAL TESTING**

The tensile strength of the electrospun scaffolds was determined through a uniaxial testing machine. The samples to be tested were cut into the size of 40 mm × 15 mm and mounted vertically on the tensile machine. The load deformation data was recorded at a rate of 5 mm/min with a load of 500 N. Finally, the tensile strength of the electrospun scaffolds was determined from the constructed stress strain curves.

**COAGULATION STUDIES**

*APTT and PT assay*

The anticoagulant behavior of the developed composites was determined through prothrombin time (PT) and activated partial thromboplastin time (APTT) approved by the Chairman, Ethical and Medical Researcher Committee, Universiti Teknologi Malaysia, with the ref no UTM.J.45.01/25.10/3Jld.2(3). To begin the assay, the tested scaffolds with a size of 0.5 cm × 0.5 cm were incubated with PPP for 1 min at 37°C followed by adding the APTT and PT reagents. For APTT assay, the initiation of the blood clot was done by adding rabbit brain activated cephaloplastin and CaCl₂. Similarly, for the PT assay, the blood clot formation was done through thromboplastin (Factor III). The clotting times were measured using a stop watch and the experiments were performed in triplicate (Balaji et al. 2016).

*Hemolysis assay*

Hemolysis assay was carried out to determine the toxicity of electrospun membranes with red blood cells. To begin the assay, the electrospun membranes were cut into a size of 1 cm × 1 cm and soaked in 0.9% w/v of physiological saline for 30 min at 37°C. After, the soaked samples were added with blends of citrated blood and diluted saline set at a ratio of 4:5 v/v% for 1 h at 37°C. Then, the samples were extracted and centrifuged at 3000 rpm for 15 min. Finally, the absorbance was recorded at 542 nm for the aspirated supernatant which denotes the release of hemoglobin. The percentage of hemolysis or hemolytic index was determined as discussed previously (Balaji et al. 2016).

**CYTOCOMPATIBILITY STUDIES**

HDF cells obtained from American Type Culture Collection (ATCC) were cultured in DMEM and supplemented with 10% Bovine Serum and maintained at 37°C in 5% CO₂. For every 3 days, the culture medium was refreshed. Before cell seeding, the electrospun scaffolds were cut into round pieces and placed in the 24 well plates. Then, the samples were sterilized with 75% alcohol for 3 h and then washed with PBS. After, HDF cells with 10 × 10³ cells/cm² density were seeded on the scaffolds in each well placed and cultured for 3 days. The MTS assay was used to determine the cell viability rates of the electrospun membranes and monitored for 72 h. After 3 days, the culture medium was retrieved and added with 20% MTS reagent and incubated for 4 h. Finally, the medium was removed and the absorbance at 490 nm was recorded to determine the toxicity rate of the fabricated membranes with fibroblast cells.

**STATISTICAL ANALYSIS**

All experiment results were analyzed using GraphPad prism. Unpaired t-test was utilized to assess the statistical significance and the value of significance was set at p<0.05.

**RESULTS AND DISCUSSION**

The SEM morphologies of the electrospun pure PU and PU/cobalt nitrate fibrous membrane were
shown in Fig. 1a and 1b. It was observed from the SEM images that the electrospun membranes possess bead free fibers. The PU/cobalt nitrate composite fibrous membrane showed the fiber diameter of 604 ± 155 nm, while the pristine PU showed diameter of 1159 ± 147 nm respectively. The fiber diameter distribution curve for the electrospun PU and PU/cobalt nitrate composites were depicted in Fig. 2a and 2b. The electrospun PU/cobalt nitrate composite fibrous mats showed reduced fibers compared to the PU fibers. The reduction in the fiber diameter of electrospun PU/cobalt nitrate composite was due to the decrease in polymer concentration when adding cobalt nitrate into the polyurethane matrix. Further, EDS spectra of the electrospun composite were indicated in Fig. 3. EDS spectra of the prepared PU/cobalt nitrate composites indicated the presence of carbon, oxygen, cobalt and gold. The average weight percentage of carbon, oxygen, cobalt and gold in the electrospun composites were found to be 63.850%, 28.777%, 4.113% and 3.260% as shown in Table I.

Unnithan et al. 2012a prepared electrospun scaffold based on polyurethane blended with the emu oil for wound dressing applications. It was observed that the addition of emu oil into the PU matrix reduced the fiber diameter and exhibited enhanced fibroblast cell adhesion and proliferation. Our developed composites showed reduced fiber diameter than the pristine PU which might favor the enhanced fibroblast adhesion and proliferation for new skin tissue growth.

The porosity of the electrospun membranes was determined through density bottle method. It was observed that the electrospun PU membranes showed average porosity of 71%, while the PU/cobalt nitrate showed average porosity of 67% respectively. Chakrapani et al. 2012 fabricated electrospun scaffold based on polycaprolactone added with collagen fibers. They reported that the fabricated nanofibrous scaffold showed porosity in the range of 60% and suggested a potential candidate for tissue engineering applications. Our fabricated fibrous scaffold showed porosity with those reported values and indicating its suitability for the tissue engineering applications. Further, the pore size measurements for the electrospun pure PU and PU/cobalt nitrate fibrous scaffolds were discussed. The electrospun PU/cobalt nitrate composite fibrous membrane showed the pore size of 753 ± 74 nm, while the pristine PU showed size of 1087 ± 63 nm respectively and their pore size distribution curve for the electrospun PU and PU/cobalt nitrate composites were depicted in Fig. 4a and 4b. It was reported that the low pore size might restrict the bacterial growth (Premuzic and Woodhead 1993) and our pore size of the fabricated membranes was observed to be reduced which might be suitable for the skin tissue engineering.

To investigate the possible interactions between PU with cobalt nitrate, the FTIR spectra of the fibrous mats were measured as shown in Fig. 5. In the spectra of PU, a wide band observed at 3328 cm⁻¹ represents the stretching mode of NH group and their vibrations were indicated at 1596 cm⁻¹ and 1530 cm⁻¹. The other peaks observed at 2938 cm⁻¹ and 2853 cm⁻¹ were attributed to stretching mode of CH group and their vibrations were seen at 1413 cm⁻¹ (Kim et al. 2009, Li et al. 2014). In the spectra of PU/cobalt composite fibrous mats, the
peaks were similar to the pure PU mat, but the peak intensity was broadened and increased with the formation of hydrogen bond (Zhou et al. 2011, Pant et al. 2010). However, the band seen at 3328 cm$^{-1}$ in the neat PU fibrous mat assigned to the stretching of the N–H group was observed to slightly shift to 3326 cm$^{-1}$ in the PU/cobalt nitrate composite mats indicating the presence of cobalt nitrate in the PU matrix the form of hydrogen bonding (Tijing et al. 2012).

The contact angle measurements of the pure PU and PU/cobalt nitrate composite mats were measured. Contact angle measurements determine the wettability of surface materials. From the results obtained, the neat PU fibrous mat exhibited hydrophobic behavior with a contact angle of 100° ± 1, while the addition cobalt nitrate, the composite fibrous mats exhibited hydrophilic behavior with a contact angle of 82°± 2. Hence, the addition of cobalt nitrate improved the wettability of the PU membranes. This was due to the presence of OH groups in the fabricated composites which were evident in FTIR analysis as identified by the broadening of a peak at 3328 cm$^{-1}$. Kim et al. 2014 prepared polyurethane scaffold blended with propolis for the biomedical applications. It was observed that the addition of propolis into the polyurethane improved the hydrophilic nature of the PU membrane and also exhibited improved fibroblast adhesion and proliferation. In our developed composites, the addition of the cobalt nitrate improved the hydrophilic nature of the PU membrane which might be suitable for the wound healing application.

The mechanical properties of the electrospun PU and PU/cobalt nitrate fibrous scaffolds were determined through a uniaxial testing machine and the obtained stress–strain curves were shown in Fig. 6a and 6b. It is observed that the tensile strength of pristine PU was found to 7.12 MPa, while for the PU/cobalt nitrate composites the tensile strength was increased to 19.50 MPa. The obtained results clearly indicated that the addition of cobalt nitrate improved the mechanical
strength of the PU membrane significantly. It was interesting to compare our results with the metallic salt impregnated polymer matrix composites. In one of the works performed by Li et al. 2017 investigated PU/PVA membrane added with silver nitrate. It was observed that the addition of silver nitrate into the PU/PVA membrane resulted in the enhancement of the tensile strength which correlates with our findings.

The thermal behavior of the electrospun PU and PU/cobalt nitrate fibers were determined through thermogravimetric analysis and the obtained results were shown in Fig. 7. It was observed that the electrospun PU membrane exhibited initial onset degradation occurs at 276°C, while in the case of PU/cobalt nitrate fibrous mat, the initial onset thermal degradation was decreased to 168°C which indicating lower thermal stability compared the pure PU membrane. The initial degradation temperature at 168°C occurred in the PU/cobalt nitrate fibers membranes was owing to moisture evaporation (Abdelrazek Khalil et al. 2013). Moreover, at 1000°C, remaining weight percentage for the PU membrane was observed to 0.47%, while the electrospun PU/cobalt nitrate fibers exhibited weight percentage of 2.75% which was higher compared to the pure PU indicating the existence of cobalt nitrate in PU matrix. However, the obtained experiment weight residue of the PU/cobalt nitrate does not correlate with the theoretical weight residue of 11%. One of the reasons for the low weight residue exhibited in the experiments results may be due to the evaporation of water molecules present in the PU/cobalt nitrate as stated previously. Also, the early decomposition of cobalt nitrate may be contributing to this anomaly.
Figure 5 - IR spectrum of PU membrane and PU/cobalt nitrate composites.

Figure 6 - Mechanical testing of a) PU membrane and b) PU/cobalt nitrate composites.

Figure 7 - TGA analysis of PU membrane and PU/cobalt nitrate composites.

(Cerkez et al. 2018). Pant et al. 2017 prepared PU scaffold incorporated with the silver modified graphene oxide for biomedical applications. It was observed that the PU scaffold incorporated with graphene oxide showed enhanced weight residue percentage than the pristine PU. In our study, the developed composites showed enhanced residue weight percent compared to the pristine PU which can be attributed to the presence of the cobalt nitrate in the PU matrix. The results of derivative weight loss curve for the electrospun PU and PU/cobalt nitrate membranes were shown in Fig. 8. From the results obtained it was observed that pure PU showed three weight loss in which two major weight loss and one minor loss. The first major weight loss seen at 223°C to 348°C and second weight loss occurs at 348°C to 446°C respectively. The third minor weight loss was seen at small peak seen at 557°C to 684°C. In the case of electrospun PU/cobalt nitrate fibers, it was observed four weight loss in which first occurs from 112°C to 218°C, the second from 218°C to 307°C, the third loss from 307°C to 468°C and the final loss occurs at 468°C to 684°C. The occurrence of the first and second loss was due to moisture vaporization. Further, it was noted that the first weight loss peak
of PU was observed to disappear in the PU/cobalt nitrate indicated low weight loss compared to the PU membrane.

The measured surface roughness of the electrospun PU and PU/cobalt nitrate composite were shown in Fig. 9a and 9b. From results obtained, it was revealed that the surface roughness of the pristine PU was found to be increased with the addition of the cobalt nitrate. The average surface roughness of pristine PU was found to 216 ± 14 nm and for the electrospun composite membrane, the average surface roughness was observed to be 270 ± 10 nm (Ra) respectively. Sharifi et al. 2016 investigated the adhesion of fibroblast cells in the surface modified polycaprolactone nanofibrous membrane. It was observed that the plasma modified PCL (10, 40 and 70 min) showed enhanced surface roughness of 205, 225, and 243 nm. Further, the membranes with increased surface roughness favored the enhanced fibroblast adhesion and proliferation. In our study, the surface roughness of our developed composites falls within these reported values which might be conducive for the enhanced fibroblast adhesion.

The blood compatibility assessments for the pure PU and PU/cobalt nitrate fibers were carried out using APTT and PT to determine the intrinsic and extrinsic pathways of the blood clotting. From the APTT and PT results, it was observed that the blood clotting time of PU/cobalt nitrate composites was observed to be higher than the pure PU. The electrospun PU/cobalt nitrate composites exhibited blood clotting time of 174 ± 4 s, while for pure PU membrane, the blood clotting was found to be 148 ± 4 s as measured via APTT assay as shown in Fig. S10 - Supplementary Material. Similarly, for PT assay, the electrospun PU/cobalt nitrate composites exhibited blood clotting time of 108 ± 3 s and for pure PU membrane, it was observed to be 85 ± 3 s as indicated in Fig. S11. Further, the hemolytic percentage was measured for electrospun PU and PU/cobalt nitrate composite to investigate their safety with red blood cells. From hemolytic assay, the index of electrospun PU/cobalt nitrate composites was observed to be lower than the pure PU. The prepared PU/cobalt nitrate composite exhibited a hemolytic percentage of only 1.73% while for pure PU membrane the index was observed to be 2.56% as shown in Fig. S12. According to ASTM F756-00(2000) standard, if the hemolysis index was above 2%, the developed material is hemolytic and the percentage below 2%, the material is non-hemolytic material. Our developed PU/cobalt nitrate composites showed a hemolytic percentage of 1.73% which was observed to be below 2% and hence it behaves like non-hemolytic material (Balaji et al. 2016). It was reported that the rougher surfaces exhibiting less thrombogenic nature than the smooth surfaces (Baily et al. 1999). Further, Vincent et al. 2012 suggested that the blood compatibility was greatly influenced by the small fiber diameters which favor the prolonged blood clotting time. In our study, the developed PU/cobalt nitrate composites showed improved surface roughness and smaller fiber diameter than the PU membrane which might have influenced the improved blood compatibility.
The proliferation of HDF cells on pure PU and PU/cobalt nitrate fibers were evaluated through MTS assay after 3 days cell culture. PU composite membranes showed enhanced cell proliferation rates than the PU membranes. The cell viability of the pure PU was found to be 132 ± 4% and the electrospun PU/cobalt nitrate fibers showed cell viability of 146 ± 2% respectively as presented in Fig. S13. The presence of cobalt nitrate enhanced the HDF cell adhesion and proliferation. It was reported that the more cell will adhere and spread on the membrane with hydrophilic nature (Wei et al. 2007). Hence, our developed composites with hydrophilic nature enhanced HDF cell adhesion and proliferation.

CONCLUSION

In this work, the PU wound dressing incorporated with cobalt nitrate was successfully fabricated using electrospinning technique. SEM morphology revealed that the developed PU and blended composites exhibited bead free fibers. The developed composites exhibited reduced fiber and pore diameter than the pristine PU. The electrospun membranes showed sufficient porosity needed for the new tissue formation. The interaction of cobalt nitrate with PU matrix was evident by the hydrogen bond formation and enhanced residue percentage as observed in FTIR and TGA analysis respectively. The contact angle results indicated the improved wettability of the prepared composites than the pure PU. The incorporation of cobalt nitrate into the PU matrix enhanced the surface roughness and mechanical strength evident in the AFM and tensile analysis. The blood compatibility assays revealed the anticoagulant nature of the prepared composites by displaying prolonged blood clotting time than the PU control. Further, the developed composite exhibited less toxicity nature revealed in the hemolysis and cytotoxicity studies. It was observed that the PU wound dressing added with cobalt nitrate fibers exhibited enhanced physicochemical and better blood compatibility parameters which may serve as a potential candidate for wound dressings.

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AUTHOR CONTRIBUTIONS

Mohan Prasath Mani performed the experiments, analyzed the data, wrote the paper, and prepared figures and/or tables. Saravana Kumar Jaganathan conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables and reviewed drafts of the paper.
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SUPPLEMENTARY MATERIAL

**Figures:** S10, S11, S12 and S13.