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In-vitro Biocompatibility Analyses on Co-electrospun of Polyurethane/Chitosan and Polyvinyl Alcohol/Elastin Vascular Membrane

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Abstract:
The demand for vascular grafts and membranes is rising, particularly in Malaysia, where cardiovascular diseases are the leading cause of death. Chitosan (CS) and elastin (EL) are commonly used in cardiovascular applications for their cell adhesion and proliferation properties, promoting functional tissue formation. This study blended these natural polymers with synthetic materials, polyurethane (PU) and polyvinyl alcohol (PVA), to create co-electrospun vascular membranes. Glutaraldehyde (GA) was used as a crosslinking agent to enhance membrane stability. The study aimed to investigate the biocompatibility of co-electrospun membranes before and after GA crosslinking, focusing on cell viability and proliferation using cardiomyocytes. The results obtained from cell viability show that all co-electrospun membranes were very toxic, with the highest cell viability found in PU-CS/PVA at 26.36 ± 4.52% and PU-CS/PVA-EL at 22.27 ± 12.56%. However, in the cell proliferation analysis, the PU-CS/PVA-EL membrane was able to support cell proliferation at 85.88 ± 4.47% on day 3 and 78.34 ± 1% on day 7. After crosslinking, all the co-electrospun membranes experienced a significant reduction in cell proliferation that led to cytotoxicity. Therefore, due to their toxicity, this study did not support crosslinked PU-CS/PVA-EL membrane for cardiovascular applications.

Keywords: Vascular membrane; Co-electrospinning; Biocompatibility

1. INTRODUCTION

According to the Malaysian Ministry of Health Report, cardio vascular disease (CVD) has consistently been the primary cause of death since 1980s (1). Furthermore, the report indicates that CVD has been on the rise over the past three decades (1). Patients diagnosed with CVD undergo a range of vascular repair treatment methods, such as angioplasty, stent implantation in the clogged artery, coronary artery bypass graft surgery, and vascular regeneration (2,3). Most surgical procedures to treat vascular diseases for long term revascularisation require the use of vascular grafts (4). Electrospinning is widely utilised as an optimal technology for the production of vascular grafts and membranes due to the ability to manipulate various parameters, such as fibre fineness, thickness, three-dimensional (3D) structure, mechanical properties, and degradation rate (5).

Natural polymers are commonly used as electrospinning materials and have been recognised as being well suited for biological applications. Their composition allows them to possess qualities that closely resemble the native chemical environment, leading to a high level of biocompatibility (6). However, compared to synthetic polymers, natural polymers usually exhibit less adequate mechanical properties (7). Mixing both natural polymer and synthetic polymer will improve the properties of electrospun membrane, to be more biocompatible and have strong mechanical properties. In order to achieve an even distribution of employed materials for the fabrication of electrospun membrane with a proper blending of each material, this study implemented a co-electrospinning technique to accommodate both natural and synthetic polymers. The co-electrospinning involved the ejections of polyurethane-chitosan (PU-CS) and poly (vinyl alcohol)-elastin (PVA-EL) to form a single co-electrospun membrane.

Polyurethane is a synthetic material that has been considered as a promising candidate material for synthetic vascular grafts and membranes due to its outstanding properties, including elasticity, non-toxicity, excellent mechanical properties, and high blood biocompatibility (8). Chitosan is the natural polymer that was mixed with PU to increase the biological function of PU. Chitosan has been extensively utilised across various disciplines, owing to its notable characteristics, including biodegradability, renewability, biocompatibility, super absorbency, strong adhesion, and antibacterial qualities

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(9). Polyvinyl alcohol, another synthetic polymer, is recognized for its biocompatibility and favourable mechanical properties (10). In order to accommodate PVA with antithrombogenic behaviour, EL was blended within the PVA matrix (11). As the primary constituent of blood artery tunica intima, elastin has been shown to control platelets and have antithrombotic properties (12,13).

However, the hydrophilic nature and high swelling degree of PVA and CS may pose limitations to their biomedical application (14,15). Glutaraldehyde (GA) crosslinking, in the vapour phase can help to prevent distortion of fibre morphology while preserving the scaffold's high degree of bioactivity (16,17). Previously, it was found that using GA in the vapour phase produced non-cytotoxic scaffolds as long as the unreacted GTA was effectively eliminated (18). Furthermore, the crosslinking procedure has an ability to effectively stabilise the nanofibers and control the membrane's degradation when immersed in an aqueous environment (19, 20). In this study, the biocompatibility aspects of utilising vapour phase GA as a crosslinking agent on co-electrospun PU-CS/PVA-EL membranes were evaluated *in-vitro* with cardiomyocyte cells. The biocompatibility analyses included cell viability and cell proliferation.

2. METHODOLOGY

2.1 Sample Preparation

The initial electrospinning solutions consisted of 8% (w/v) PU solution and 3% (w/v) CS solution that are prepared at room temperature. The 8% (w/v) PU was made by stirring 1.2 g of PU beads with 15 mL of DMF for 24 hours at a stirring speed of 250 rpm (21, 22). The 3% (w/v) CS was prepared by dissolving 0.17 g of CS in 5.6 mL of N,Ndimethylformamide (DMF) solvent and stirring at 250 rpm for 24 hours (21, 22). Afterwards, the PU-CS solution was prepared by stirring 9.7 mL of 8% (w/v) PU solution with 0.3 mL of 3% (w/v) CS solution for 1 hour with the stirring speed set at 170 rpm. Furthermore, the PVA solution with a concentration of 8% was made by stirring 1.6 g of PVA with 20 mL of deionised water for 2 hours, while the temperature was consistently maintained at 80°C (21, 22). Then, 1% (w/v) EL solution was prepared by stirring 0.03 g of EL derived from bovine neck ligament with 3 mL of a 0.2M Trizma base buffer for 24 hours at room temperature (21, 22). Subsequently, PVA-EL solution was prepared by stirring 9.5 mL of 8% (w/v) PVA solution with 0.5 mL of 1%(w/v) EL solution for 1 hour at 37°C.

The production of PU/PVA, PU-CS/PVA, PU/PVA-EL, and PU-CS/PVA-EL membranes involved the utilisation of two electrospinning machines (SP20, Nanolab Instrument, Malaysia; SP102HSM, Progene Link, Malaysia) that were positioned in an opposing manner. The rotating collector was positioned at the centre of both electrospinning machines and covered by aluminium foil. Each electrospinning solution was placed in a plastic syringe with a stainless-steel needle and a volume of 6 mL prior to the electrospinning process. The nanofibers were created using a rotating collector with a rotation speed set between 50-60 rpm. The needle was set to be 15 cm away from the rotating collector. The flow rate for each electrospun nanofiber was set at 0.50 mL/h. The electrospinning solution containing PU-CS was subjected to a voltage of 12 kV, while the solution containing PVA-EL was subjected to a voltage of 20 kV (21, 22).

After 12 hours of electrospinning, the middle portion of the membranes, which was assumed to have the most equal fibres from needle 1 and needle 2, was cut into a square shape of 5 × 7 cm. Afterwards, each membrane was crosslinked with GA as the crosslinking agent through a vapour treatment. The membranes were crosslinked by exposing them to 2.5 wt% GA vapour for a duration of 15 hours in a closed container (23). Following the completion of the cross-linking process, the resulting membranes were placed in an oven at a temperature of 50°C for 24 hours to eliminate any remaining GA on the membranes.

2.2 *In-Vitro* Cell Test

The AC16 cells (CRL-3568, ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HyClone™, Cytiva, USA) containing L-glutamine, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid and supplemented with 10% (v/v) foetal bovine serum (HyClone™, Cytiva, USA) with 1% penicillin-streptomycin (100 U/100 µg/mL) (Gibco, USA). The cells were maintained in an incubator (Galaxy S Plus, Richmond Scientific, UK) at 37°C in a 95% humidified atmosphere with 5% CO_2. The media were refreshed every 72 hours. Before *in-vitro* analyses, all membranes were sterilised using ultraviolet (UV) light for 15 minutes on each side (24).

2.2.1 Cell Viability Analysis

Initially, cardiomyocyte cells were seeded on 96-well plate with a concentration of 5×10^3 cells/well and incubated for 24 hours at 37°C in a 95% humidified atmosphere with 5% of CO_2. After 24 hours, the cell culture media were renewed. The 0.25 cm^2 of each co-electrospun membranes was immersed in the 96-well plate, positioned on top of the cultured cells. The cells were then incubated for 7 days at 37°C in a 95% humidified atmosphere with 5% of CO_2.

The cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. After reaching the incubation periods, the culture media were extracted and a concentration of 0.5 mg/mL of MTT solution was added to each well. Then, the cells were incubated for an additional 2 hours at 37°C. Following the 2 hours incubation period, the MTT solutions were extracted and substituted with Dimethyl Sulfoxide (DMSO) to facilitate dissolution of the crystallised purple formazan produced by the viable cells. Then, a microplate reader (The Infinite M Plex, Tecan, Switzerland) was employed to shake the 96-well plate for 10 minutes and to measure the optical absorbance (OD) values at a wavelength of 570 nm. The positive control was set to the cell medium with treated co-electrospun membranes, while the negative control was set to the cell medium with untreated co-electrospinning membranes (25). The collected data from microplate reader were processed using Equation 1.

$$\text{Cell viability (\%)} = \frac{OD_{Experimental} - OD_{Negative\ control}}{OD_{Positive\ control} - OD_{Negative\ control}} \times 1 \qquad (1)$$

2.2.2 Cell Proliferation Analysis

Initially, each co-electrospun membrane was placed inside a 96-well plate, and cardiomyocytes cells were seeded on top of each membrane at a concentration of 5×10^3 cells/well. The 96-well plate was incubated for 3 and 7 days at 37°C with 5% CO_2. Then, the cell proliferations were quantified using MTT assay by removing the culture media and replacing the media with 0.5 mg/mL of MTT solution. After 2 hours, the MTT solutions were removed and DMSO was added. The 96-well plate, with the membranes still inside, was shaken together for 10 minutes. The vascular membranes were removed prior the OD reading at 570 nm wavelength.

2.2.3 Statistical Analysis

The statistical analyses were finally conducted on cell viability and cell proliferation test. In this study, the analyses were conducted using GraphPad Prism (version 10.1.0, GraphPad Software, USA), and all values were expressed as mean ± standard deviation with 95% confidence interval. The normality of data distribution for each group was assessed using the Shapiro-Wilk test (α = 0.05), ensuring the data met the assumptions required for Analysis of Variance (ANOVA). Specifically, one-way ANOVA was used, and a *p*-value of less than 0.05 was considered statistically significant. When the ANOVA indicated significant differences, Tukey's multiple comparisons test was performed as a post-hoc analysis to identify specific pairwise group differences.

3. RESULTS AND DISCUSSION

3.1 Cell Viability Analysis

The cytotoxicity of PU/PVA, PU-CS/PVA, PU/PVA-EL, PU-CS/PVA-EL, and the corresponding GA crosslinked versions were investigated using the MTT assay. The Shapiro-Wilk test revealed that all data obtained from this analysis are normally distributed. Furthermore, the analysis demonstrates that the results are confined within a restricted range of values, all below the biocompatibility threshold of 70%. After 7 days, the data obtained from the cell viability analysis showed a significant difference ($p < 0.05$) in cell metabolic activity between the co-electrospun membranes and the control group, as shown in Figure 1.

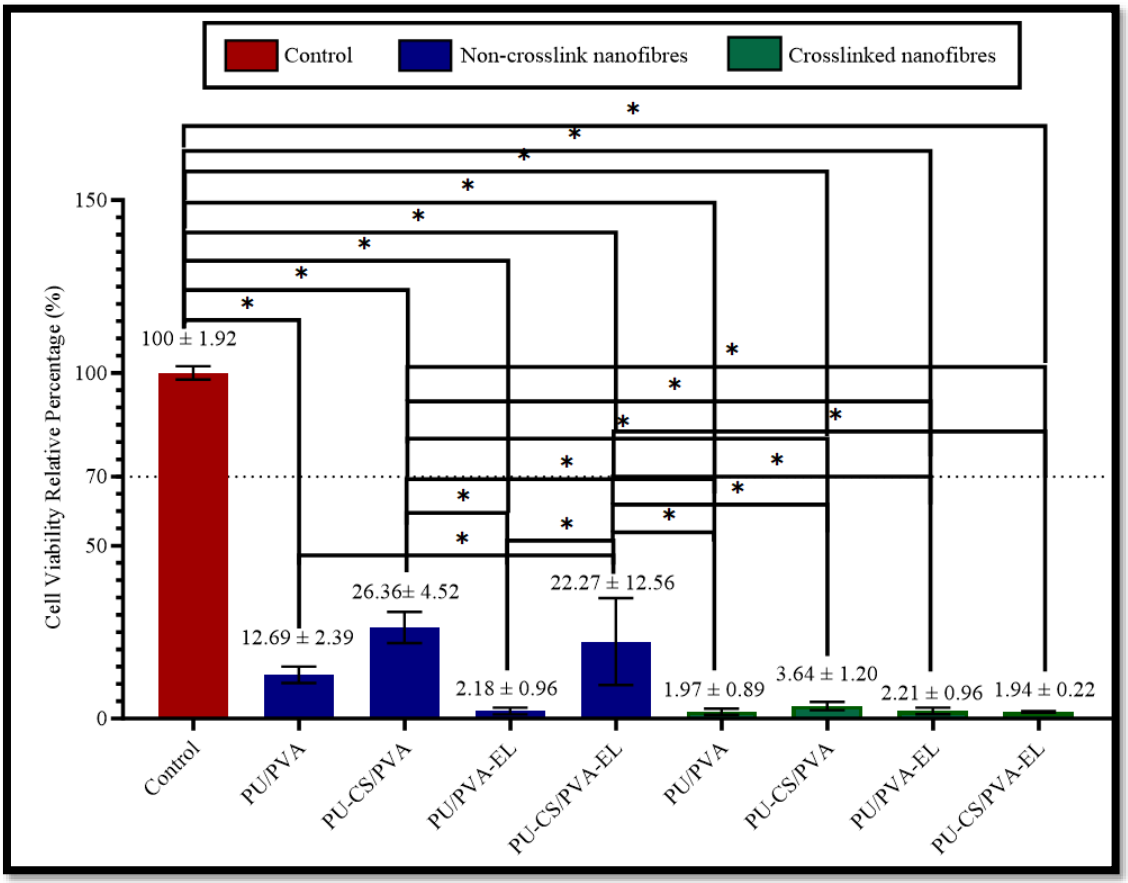

Figure 1. Cardiomyocyte cells viability at day 7 (*indicates $p < 0.0001$).

The post-hoc statistical analysis using Tukey's multiple comparison tests indicated that the cell viabilities induced by all co-electrospun membranes were significantly lower than the control group. Initially, the percentage of cell viability of cardiomyocytes in the control group was 100 ± 1.92%. However, all co-electrospun membranes resulted in a notable reduction in cell viability. Prior to crosslinking, the membranes containing CS exhibited the highest cell viability, PU-CS/PVA at 26.36 ± 4.52% and PU-CS/PVA-EL at 22.27 ± 12.56%.

After the crosslinking process, the cell viability started to decrease, and the highest observed value was found on the PU-CS/PVA at 3.64 ± 1.20%. Therefore, the cell viability percentages in all the crosslinked membranes were significantly differed with the PU-CS/PVA and PU-CS/PVA-EL. The decrease in cell viability on day 7 could be attributed to the relatively low initial cell concentration seeded on the 96-well plate. Additionally, in this *in vitro* setup, the positioning of the co-electrospun membranes over the cardiomyocytes might limit essential gas exchange to the cells underneath, potentially impairing cell proliferation and triggering stress responses, thus ultimately leading to increase cell death.

3.2 Cell Proliferation Analysis

The objective of the cell proliferation analysis was to evaluate the proliferation of cardiomyocyte cells on the co-electrospun membranes at 3 and 7 days of incubation. The proliferation percentages of the cells were quantified and observed by seeding them atop each membrane. Other than that, all data obtained from this analysis has a normal distribution. Initially, after 3 days, the cell control group had the highest cell proliferation at 100 ± 14.21%, followed by the PU-CS/PVA-EL membrane at 85.88 ± 4.47%, as shown in Figure 2.

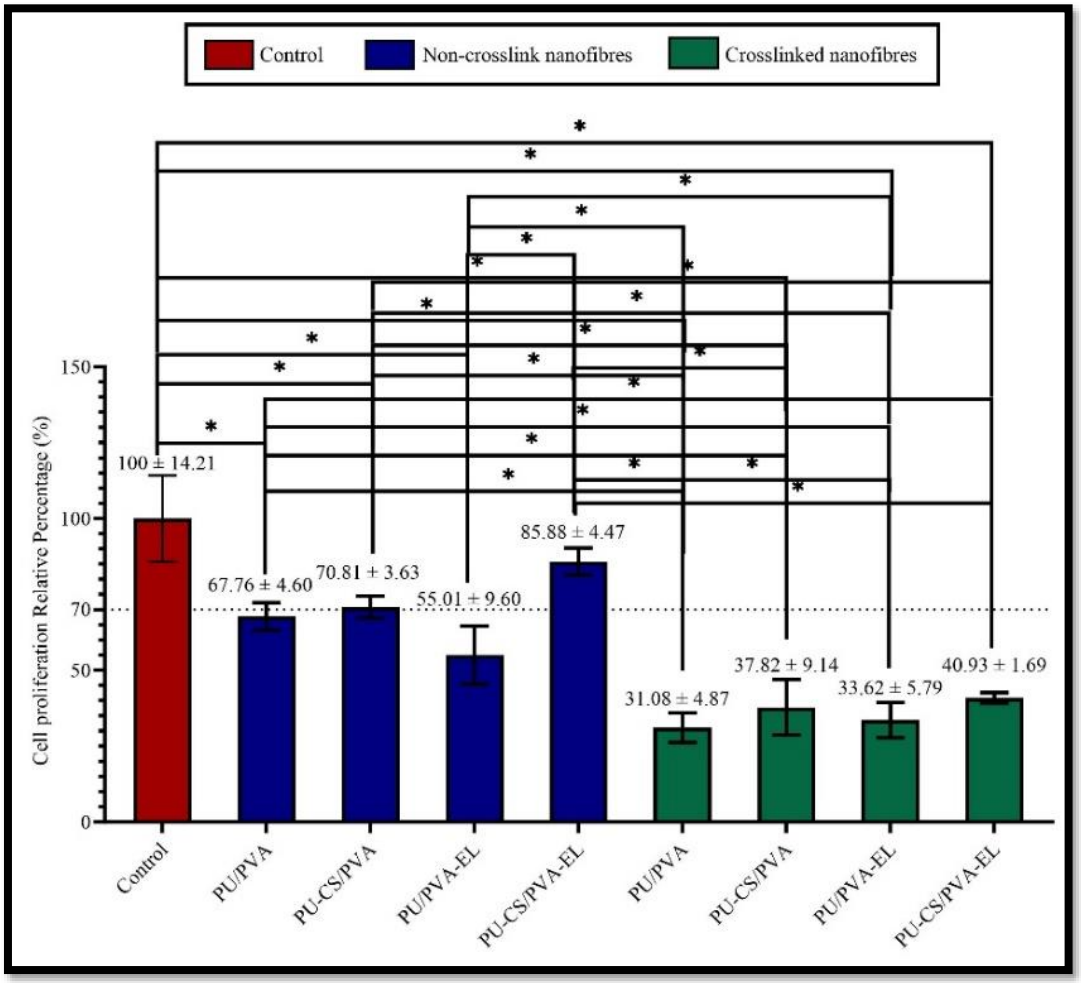

Figure 2. Cell proliferation percentages of non-crosslink and crosslinked co-electrospun membranes at day 3 (*indicates $p < 0.0001$).

Based on the results of statistical analysis, all co-electrospun membranes, except for the PU-CS/PVA-EL membranes, showed significant differences compared to the control group ($p < 0.05$). This may result from the combination of synthetic and natural polymers in PU-CS/PVA-EL, which supports membrane stability and provides biological cues for cell proliferation (26,27). Prior to crosslinking, the PU/PVA-EL membranes have the lowest cell proliferation compared to other membranes, at 55.01 ± 9.60%. This is likely due to the fact that the reaction of elastin peptides varies depending on the scaffold's concentration and the type of cells (28,29). For instance, in the previous study, the incorporations of CS with EL with the concentrations of 2% and 4% led to greater fibroblast cell proliferation compared to the concentration of 1% EL (30). However, the addition of 5% EL to PU did not result in a significant increase in smooth muscle cell proliferation, as observed in PU-containing membranes (31).

Furthermore, the cell proliferation of PU/PVA-EL membranes showed significant differences compared to the PU-CS/PVA-EL, crosslinked PU/PVA, and crosslinked PU/PVA-EL. Following the crosslinking, all co-electrospun membranes experienced a significant reduction in cell proliferation. Therefore, all co-electrospun membranes, except for the PU/PVA-EL, were significantly different from the crosslinked membranes. Based on the results, it appeared that the vapour phase method has left residual GA on the membranes, which in turn decreased cell proliferation (32). After 7 days, the control group exhibited the highest cell proliferation at 100 ± 8.27% and significantly different ($p < 0.05$) with the cells that were proliferated on top of the co-electrospun membranes, as shown in Figure 3.

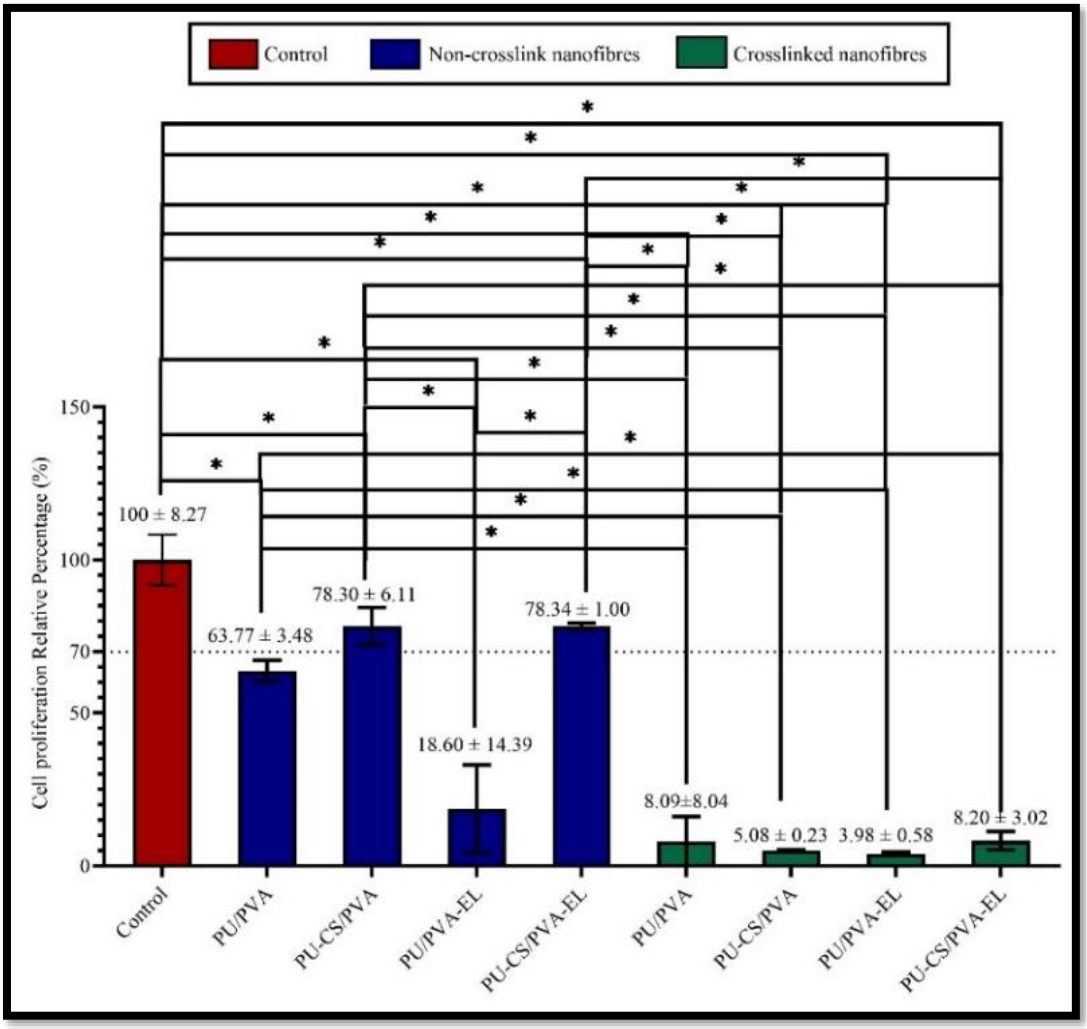

Figure 3. Cell proliferation percentages of non-crosslink and crosslinked co-electrospun membranes at day 7 (*indicates $p < 0.0001$).

On the non-crosslink membranes, the highest cell proliferation was induced by the PU-CS/PVA-EL and PU-CS/PVA membranes at 78.34 ± 1.00% and 78.30 ± 6.11%, respectively. These results suggested that chitosan in each membrane could enhance cell proliferation and promote cell interconnectivity due to the opposite charge on its surface (33-35).

Other than that, the PU/PVA-EL membranes have the lowest capability to induce cell proliferation, where the OD value was detected at 18.60 ± 14.39%. According to the Tukey's analysis, the PU/PVA-EL was significantly different from all membranes in the non-crosslinked group ($p < 0.05$) as the membranes only contained 1% elastin, which was lower than the 3% concentration of chitosan. Therefore, the absence of chitosan may have hindered the membrane's ability to promote cell proliferation.

All crosslinked membranes became significantly different from all non-crosslinked membranes except for the PU/PVA-EL membranes. The highest cell proliferation after the crosslinking process was observed on the PU-CS/PVA-EL at 8.20 ± 3.02%, influenced by the greater sensitivity of cardiomyocytes to GA (36). Overall, based on the data obtained from the cell proliferation analysis, the crosslinked membranes were unable to support cell proliferation even in the first three days of incubation, with values continuously decreasing in the following days up to the seventh day.

4. CONCLUSION

In conclusion, this research thoroughly analysed the biocompatibility of PU-CS/PVA-EL membranes before and after GA crosslinking. The cell viability analysis clarified that after 7 days, all co-electrospun membranes reduced cell viability below the threshold standard of 70%. The toxicity of these membranes was even worse after the crosslinking process, with the

highest value found in the PU-CS/PVA at 3.64 ± 1.20%. The decrease in cell viability at day 7 could be attributed to the relatively low initial cell concentration seeded on the 96-well plate. Furthermore, cell proliferation analysis showed that the PU-CS/PVA-EL membranes were able to promote cell proliferation up to seven days. However, due to the sensitivity of cardiomyocytes towards GA treatment, the crosslinked PU-CS/PVA-EL membranes failed to promote initial cell proliferation on the co-electrospun membranes. The use of GA crosslinking presented challenges that needed to be addressed for medical applications, particularly as vascular grafts that come in contact with cardiomyocytes. Further research is needed to explore alternative crosslinking methods or modifications to enhance the cell viability and functionality of these membranes.

AUTHORSHIP CONTRIBUTION STATEMENT

Fakhira Alanna Shabira: formal analysis, investigation, writing – original draft; Kugambikai Vangetaraman: methodology, validation; Sarawut Kumphune: supervision, methodology, resources, writing – review & editing; Sivakumar Sivalingam: conceptualization, funding acquisition, writing – review & editing.

DATA AVAILABILITY

Data are available upon request.

DECLARATION OF COMPETING INTEREST

No conflict of interest.

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