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Original Research

HEPARIN IMMOBILIZATION OF THE VASCULAR GRAFTS BY LAYER-BY-LAYER (LBL) ASSEMBLY TECHNIQUE TO IMPROVE THROMBOGENICITY

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ABSTRACT: Decellularized vascular grafts hold potential for small-diameter vascular reconstruction owing to the ECM integrity maintained and appropriate dimensions. However, the thrombogenicity of tissue-derived grafts represents a notable limitation for clinical application. This study focuses on assessing the effectiveness of heparin immobilization on cross-linked decellularized vascular grafts prepared from porcine carotid arteries. The Layer-by-Layer (LbL) assembly technique was applied to immobilize heparin on the graft surface to improve hemocompatibility. Following LbL treatment, the grafts were examined for heparin content and surface morphology using scanning electron microscopy (SEM). To assess their potential for vascular implantation, the hemocompatibility were further evaluated. SEM observations and heparin quantification confirmed successful heparin immobilization. In terms of hemocompatibility, in vitro anticoagulant activity assessment revealed a thromboresistant surface on the luminal surface of the grafts. In addition, hemolysis rates remained within the non-hemolytic threshold as defined by ISO 10993-4. In summary, the Layer-by-Layer heparin immobilization method effectively immobilized heparin onto reinforced decellularized vascular grafts, enhanced hemocompatibility, and represents a promising approach for the development of xenogeneic vascular graft materials.

Keywords: vascular graft, decellularization, heparin immobilization, thrombogenicity, Layer-by-Layer (LbL) assembly, hemocompatibility

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1. INTRODUCTION

In small-diameter vascular reconstruction (<6 mm), grafts possessing adequate mechanical strength and biocompatibility are indispensable. Decellularized xenogeneic vascular scaffolds preserve the native ECM ultrastructure and are considered promising substitutes. However, their high thrombogenic potential remains a significant obstacle because exposed collagen fibers present a risk of triggering intrinsic coagulation, accelerating thrombus formation (Cai et al., 2019; Li et al., 2025). This phenomenon leads to early graft failure and occlusion.

In the context of anti-thrombosis vascular grafts, heparin, a sulfated glycosaminoglycan, has been extensively utilized for its anticoagulant activity via antithrombin III activation (Xie et al., 2021; Yadav et al., 2024). In this study, glutaraldehyde-reinforced decellularized porcine carotid arteries were heparinized using the Layer-by-Layer (LbL) assembly technique based on dihydroxy-iron ions (so-called DHI). The grafts were evaluated for immobilized heparin content, surface morphology (SEM), and biomechanical properties. The in vitro hemocompatibility, including macroscopic anticoagulant activity and hemolysis tests, was assessed in accordance with ISO 10993-4 standards. The conducted results provide experimental evidence supporting the feasibility of this approach for improving the blood compatibility of small-diameter vascular graft materials. Further research will be conducted to determine heparin stability, endothelialization potential, and in vivo performance for clinical applications.

2. MATERIALS AND METHODS

2.1. Materials

The vascular grafts were provided by the Laboratory of Tissue Engineering and Biomedical Materials, University of Science – VNU-HCM. They were fabricated from porcine carotid arteries that underwent complete decellularization and crosslinking.

2.2. Methods

Heparin immobilization via Layer-by-Layer (LbL) assembly technique

Heparin was immobilized onto the lumen of the vascular grafts following the LbL assembly technique (Nguyen & Tran, 2022). DHI (0.05 M; Sigma-Aldrich, USA), NaCl 0.9% (Merck, Germany), and heparin (5 mg/mL; Sigma-Aldrich, USA) solutions were freshly prepared. In the initial cycle of the LbL technique, the vascular graft lumen was incubated with DHI for 10 min, followed by rinsing with NaCl 0.9% for 5 min. The graft was then incubated with heparin solution for 5 min. This alternating layer-by-layer assembly process was repeated seven cycles to prepare the vascular graft with a heparinized inner lumen (abbreviated as GDH).

Quantification of immobilized heparin (Toluidine Blue O assay)

Heparin content was quantified using the TBO method (Liu et al., 2014, Nguyen & Tran, 2022). Standard heparin solution (0–200 µg/mL) was added with 0.04% TBO and incubated at 37 °C for 4 h under continuous agitation at 60 rpm. The resulting reaction was measured at 530 nm using a microplate reader (Biochrome, UK). The GDH samples were also subjected to the TBO assay, in which the heparin content (µg/cm^2) was calculated according to the established heparin standard curve.

Surface morphology by Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM), a widely used technique for material characterization in biomedical engineering, was employed to assess the surface morphology of the vascular grafts. (Dijkman et al., 2012; Negishi et al., 2017; Lee & Pham, 2023). Samples were fixed with 5% glutaraldehyde for 2 h, washed with PBS, and dehydrated through graded ethanol (35–100%). After 10 min in HMDS (Merck, Germany), specimens were air-dried, vacuum-dried at 50–70 °C for 1–2 h, sputter-coated with platinum before surface image detection using a scanning electron microscope (JEOL, Japan).

Anti-thrombotic activity test

Each 1 cm GDH sample was incubated in 600 µL fresh whole blood with 60 µL $CaCl_2$ 0.1 M (Merck, Germany) at 37 °C for 2 h (Nguyen & Tran, 2022; Kim et al., 2025). Surfaces were rinsed with 0.9% NaCl, blotted, and visually

assessed for thrombus formation. Glass and polyethylene served as positive and negative controls, respectively.

Hemolysis test

The GDH samples of 0.5 × 0.5 cm were immersed in 5 mL of 0.9% NaCl solution, followed by the addition of 0.1 mL of red blood cells obtained from whole blood (centrifuged at 3000 rpm for 5 minutes). The mixtures were incubated at 37°C for 60 minutes, centrifuged, and the supernatants were collected to measure optical density at 540 nm. The hemolysis ratio (%) was calculated according to the standard formula. A hemolysis rate below 5% was considered non-hemolytic and compliant with the hematological safety requirements of the international standard ISO 10993-4, which is widely used to evaluate the blood compatibility of biomaterials (Zhu et al., 2021). In this assay, distilled water served as the positive control (complete hemolysis), and 0.9% NaCl solution served as the negative control (no hemolysis).

2.3. Statistical analysis

All experiments were performed in at least three independent replicates. The results were presented as mean ± standard deviation (mean ± SD). Statistical analysis was conducted using GraphPad Prism 10.6 software, and differences were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Heparin quantification

The efficiency of heparin immobilization on the vascular graft surface was preliminarily evaluated through macroscopic observation after Toluidine Blue O (TBO) staining, as shown in Figure 1. In the non-heparinized sample (Figure 1A), the surface exhibited a light blue coloration. In contrast, the heparinized sample (as GDH) (Figure 1B) displayed multiple irregular dark violet regions after TBO staining, indicating the formation of Heparin/TBO complexes. These areas correspond to the presence of heparin, which specifically binds to TBO through electrostatic interactions between the cationic groups of TBO and the anionic sulfate groups of heparin.

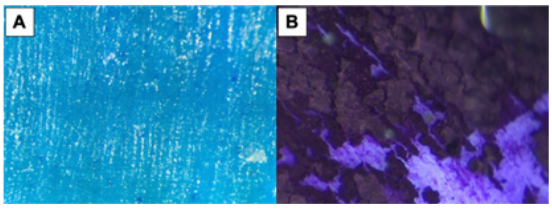

Figure 1. Macroscopic observation of TBO-stained vascular grafts

(A): Non-heparinized sample (G–); (B): Heparinized sample (GDH)

The calibration curve was established using heparin concentrations ranging from 0 to 200 μg/mL, showing a strong linear correlation between optical density (OD) at 530 nm and heparin concentration (R^2 = 0.9997). The obtained regression equation was subsequently used to calculate the amount of immobilized heparin on the sample surface.

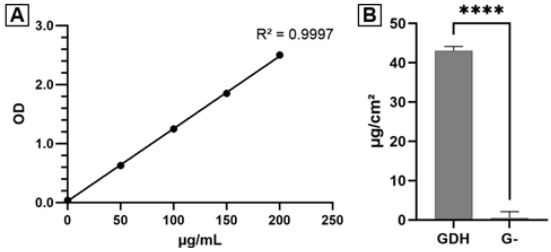

Figure 2. Quantification of immobilized heparin using the TBO assay

(A): Calibration curve between OD and heparin concentration (530 nm); (B): Heparin concentration (μg/mL) from the TBO assay was converted to surface heparin content (μg/cm^2) based on the extraction volume and the luminal surface area of each sample : GDH and G– samples.

The signal from the negative control group remained at a negligible baseline level. Based on the heparin calibration curve, OD values were interpolated to determine surface heparin content. The GDH group exhibited values ranging from 75 - 78 μg/cm^2, significantly higher than the negative control ($p < 0.0001$), confirming effective and stable heparin immobilization.

3.2. Surface morphology (SEM observation)

SEM images revealed notable differences between the non-heparinized and heparinized reinforced decellularized

vascular grafts. In the non-heparinized sample (G–, Figure 3A), the fibrous ECM structure was clearly visible. In contrast, the GDH samples (Figure 3B) exhibited a completely heparinized surface as a dense coating over the sample lumen. This result indicated that heparin was successfully immobilized on the inner lumen of the vascular graft.

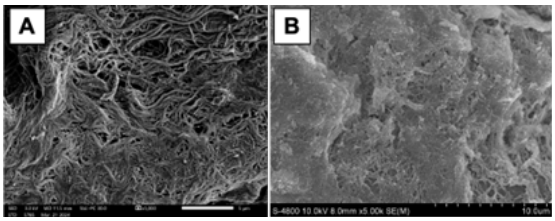

Figure 3. SEM images of the vascular graft surface

(A): Non-heparinized sample (G–); (B): Heparinized sample (GDH)

Based on the quantification results and SEM observations, the Layer-by-Layer (LbL) assembly technique effectively immobilized heparin onto the surface of decellularized vascular grafts. These findings are consistent with previous studies on heparinized biomaterials. In non-heparinized samples, the ECM fibers remained distinctly visible with an interwoven structure. In contrast, in the heparinized samples, the observed regions displayed heparin/DHI coating layers covering the ECM, as evidenced under SEM (Tao et al., 2012).

3.3. Anti-thrombotic activity

For the two control samples, visual observation revealed distinct differences. The glass sample, characterized by a smooth and rigid surface, served as the positive control (Figure 4A) and exhibited extensive formation of red thrombus. In contrast, the polyethylene (PE) sample, used as the negative control (Figure 4B), had an inert and hydrophobic surface, showing no evidence of clot formation.

For the experimental samples, the non-heparinized graft (G–, Figure 4C) displayed a dense coverage of dark-red thrombus across its surface. Conversely, the heparinized graft (GDH, Figure 4D) maintained a bright, uniform, and clot-free surface, exhibiting a stable and homogeneous morphology.

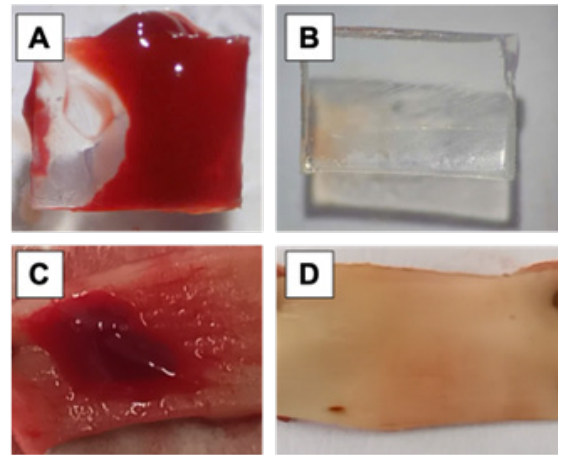

Figure 4. Macroscopic anticoagulant activity test

(A): Positive control (glass); (B): Negative control (polyethylene, PE); (C): Non-heparinized sample (G–); (D): Heparinized sample (GDH)

In the non-heparinized sample (G–), the presence of numerous red thrombus indicated that the material surface still exhibited a tendency to activate the coagulation cascade. In contrast, the heparinized sample (GDH) maintained a bright and uniform surface with no visible clots, highlighting the role of heparin in reducing coagulation activity. Heparin prevents plasma protein adsorption and limits platelet adhesion on the material surface, thereby creating a thromboresistant interface. Furthermore, the gradual release of heparin from the graft may inhibit thrombin activity, contributing to the maintenance of a localized anticoagulant microenvironment around the sample (Nguyen & Tran, 2022).

In a previous study, both the non-heparinized material and the positive control (glass) exhibited evident thrombus formation on their surfaces. In contrast, samples heparinized using the Layer-by-Layer technique showed no clot formation. This result confirms the anticoagulant effectiveness of the heparin immobilization method compared to untreated materials (Nguyen et al., 2022).

The macroscopic anticoagulant test allows direct observation of thrombus formation on the material surface. This method reflects the tendency for coagulation activation when there is direct contact with whole blood. The obtained results demonstrate the usefulness of this test in comparing the anticoagulant

properties among different material groups.

3.4. Hemolysis test of heparinized vascular grafts

The visual results are shown in Figure 6. The heparinized GDH sample (A) and the negative control (C) exhibited clear supernatants, indicating a low level of hemolysis visible to the naked eye, whereas the positive control (B) showed a distinctly red-colored supernatant.

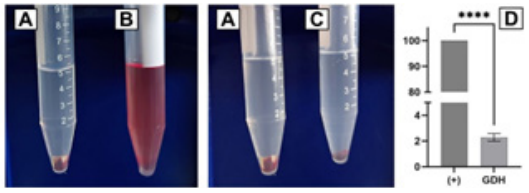

Figure 5. Hemolysis evaluation

(A): Heparinized sample; (B): Positive control (distilled water); (C): Negative control (0.9% NaCl); (D): Hemolysis ratio (%) of the heparinized GDH vascular graft was calculated using the standard ISO 10993-4 formula.

The hemolysis values of the GDH samples ranged from 1.868% to 2.998%, with an average of 2.287 ± 0.296% (Figure 5D). These results meet the safety threshold defined by ISO 10993-4 (<5%), and the slight standard deviation indicates high consistency and repeatability. This demonstrates that the heparinized material caused no significant disruption to erythrocyte membranes and exhibited good hemocompatibility.

Similarly, a study on heparinized decellularized vascular grafts reported a hemolysis ratio of 0.4%, confirming excellent hematological safety (Tardalkar et al., 2022,). These findings further support that surface heparinization effectively maintains a remarkably low level of hemolysis and ensures hematological biocompatibility.

The hemolysis test reflects the hematological safety of the material, whereas the macroscopic anticoagulant test demonstrates its ability to limit coagulation activation and thrombus formation. Together, these two assays provide a comprehensive evaluation of the material's hemocompatibility. The results indicate that heparin immobilization not only maintains a low hemolysis level but also enhances anticoagulant properties, thereby improving the application potential of the vascular graft.

In order to clearly address the use of the fabricated graft for vascular surgery, further research is required, including a detailed investigation of the material properties, in vivo biocompatibility, and vascular grafting trials in animal models. The heparin-releasing period and anti-thrombosis during the releasing phase are the two key features of this material for predicting in vivo blood-contacting behaviour . In vivo investigation is also necessary to assess biocompatibility and evaluate its efficiency in vascular grafting.

4. CONCLUSION

The study demonstrated that the Layer-by-Layer assembly technique provided a successful approach in immobilizing heparin onto the fabricated vascular grafts. The heparinized grafts displayed uniform coatings observable by SEM, demonstrated antithrombogenic luminal surfaces, and maintained low hemolysis ratios within ISO-defined safety limits. These findings support LbL-based heparinization as an effective and scalable strategy for enhancing hemocompatibility of xenogeneic small-diameter vascular grafts.

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APPENDIX

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