Carboxymethyl kappa carrageenan-modified decellularized small-diameter vascular grafts improving thromboresistance properties

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Abstract: The development of decellularized small-diameter vascular grafts is a potential solution for patients requiring vascular reconstructive procedures. However, there is a limitation for acellular scaffolds due to incomplete recellularization and exposure of extracellular matrix components to whole blood resulting in platelet adhesion. To address this issue, a perfusion decellularization method was developed using a custom-designed set up which completely removed cell nuclei and preserved three-dimensional structure and mechanical properties of native tissue (sheep carotid arteries). Afterwards, carboxymethyl kappa carrageenan (CKC) was introduced as a novel anticoagulant in vascular tissue engineering which can inhibit thrombosis formation. The method enabled uniform immobilization of CKC on decellularized arteries as a result of interaction between amine functional groups of decellularized arteries and carboxyl groups of CKC. The CKC modified graft significantly reduced platelet adhesion from 44.53 ± 2.05% (control) to 19.57 ± 1.37% (modified) and supported endothelial cells viability, proliferation, and nitric oxide production. Overall, the novel CKC modified scaffold provides a promising solution for thrombosis formation of small-diameter vessels and could be a potent graft for future in vivo applications in vascular bypass procedures. © 2019 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 107A: 1690–1701, 2019.

Key Words: small-diameter vascular grafts, decellularization, thrombogenicity, carboxymethyl kappa carrageenan, sheep carotid arteries

INTRODUCTION

Cardiovascular disease is the main cause of death in the United States and developed countries. The number of coronary artery bypass grafting in the US is nearly a half million in every year.1 Autologous vessels grafting such as patient’s own veins or arteries remain the most common and the gold standard for vascular grafts. However, autologous grafts cannot always be harvested since they have several drawbacks such as requiring multiple surgical procedures, limited availability in the amounts of autologous vessels, and leading to morbidity at the donor site.2 As a promising alternative, tissue engineering has emerged for the fabrication of effective vascular grafts.3 Prosthetic vascular grafts based on synthetic polymers have demonstrated high long-term patency for the replacement of large diameter arteries (>6 mm).4–6 However, synthetic grafts have poor long-term patency when they are used in small-caliber (<6 mm) arterial replacements as a result of unfavorable healing reactions with surface thrombogenicity.4,7,8 Vascular grafts based on decellularized tissue are progressively attracting attention more than natural and synthetic materials.9 Extracellular matrix (ECM) proteins support cell migration and proliferation which includes cell–cell interactions and gene expression.10,11 Nevertheless, One major challenge in an acellular tissue engineered vessel is removal of endothelial cells (ECs) which is responsible for coagulation and platelet adhesion due to thrombogenic ECM proteins.12 Therefore, to address this limitation, one potential solution is to modify the acellular vascular grafts with anticoagulants inhibiting intrinsic thrombogenicity. Heparin immobilization strategies have been incorporated into vascular grafts as the most common approach for improving blood compatibility.13–17 However, several side effects such as heparin-induced thrombocytopenia, bleeding, heparin-associated osteoporosis, skin reactions, eosinophilia, and so forth do exist for the use of heparin in some clinical conditions.18 These side effects indicate that we still need
Finally, decellularization solution containing 0.1% NH₄OH, 0.02% EDTA, and 10% penicillin/streptomycin for 48 h. Subsequently, the vessels were washed in 0.05% Trypsin-hypotonic solutions (0.02% EDTA and 0.7% NaCl) for 2 h washed in a hypertonic (0.02% EDTA and 1.1% NaCl) and prior perfusion.

In this study, we developed a promising method to obtain decellularized small-diameter (<6 mm) vessel grafts isolated from sheep carotid arteries. The decellularization process was developed by using a perfusion system. Luminal surface of vascular grafts were covalently linked to CKC. The efficacy of immobilizing CKC onto ECM was evaluated by platelet adhesion analysis, histological imaging, and ninhydrin assay. Furthermore, ECs viability, proliferation, and functionality in static condition were assessed. We have also developed a custom-designed perfusion bioreactor to evaluate ECs respond in dynamic condition.

**MATERIALS AND METHODS**

**Materials**

The following reagents and chemical were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Triton X-100, ethylenediaminetetraacetic acid (EDTA), trypsin, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and 2-morpholinoethanesulfonic acid mono-hydrate buffer (MES).

**Vessel harvest and decellularization**

Sheep carotid arterial segments were isolated from a local slaughter house and transported with ice to the laboratory. The blood vessels had an internal diameter of 4–5 mm and were cut into segments of approximately 3 cm in length. Vessels were freeze-thawed three times in phosphate-buffered saline (PBS) for up to 5–7 h in a freezer of –80°C. Then, the vessel was connected to a mechanical perfusion system using a peristaltic pump with two flow inlets and two flow outlets for decellularization of the vessel (Fig. 1). The pulsatile interior perfusion flow rate was adjusted to 35 mL/min and exterior perfusion flow rate to 50 mL/min and the scaffold was washed in a hypertonic (0.02% EDTA and 1.1% NaCl) and hypotonic solutions (0.02% EDTA and 0.7% NaCl) for 2 h each. Subsequently, the vessels were washed in 0.05% Trypsin, 0.02% EDTA, and 10% penicillin/streptomycin for 48 h. Finally, decellularization solution containing 0.1% NH₄OH and 1% Triton X-100 in PBS was placed and vessels were washed for two cycles of 48 h at room temperature, then washed in PBS for an additional 12 h and the vessels were stored in sterile PBS at 4°C before further use.

**Characterization of decellularized sheep carotid arteries**

**Imaging.** The decellularized sheep carotid arteries and native arteries were fixed in 10% formalin for 24 h, embedded in paraffin and sectioned into 6 μm slice using a microtome. Hematoxylin and eosin (H&E), Masson’s trichrome, and Verhoeff’s staining were performed and sections were evaluated and scanned by an Inverted Phase Contrast Microscope (Olympus, CKX41, Japan). In addition, the samples were imaged by scanning electron microscopy (SEM; AIS2100, SERONTECHNOLOGIES, South Korea). Finally, the vascular sections were stained with 4’,6-diamidino-2-phenylindole (DAPI; 1 μg/mL). Fluorescent microscopy (Olympus, BX51, Japan) was used to observe the samples.

**CKC synthesis, characterization and immobilization**

Carboxymethylation of κ-carrageenan (CKC) was performed by Fan’s group previously. Briefly, 2 g K-carrageenan was dissolved in 18 mL of isopropanol, followed by 1 h of stirring in room temperature. Then, 2.4 mL of sodium hydroxide was gradually added to the solution and stirred for 2 h at 35°C. Finally, the solution was placed in a water bath of 50°C. In this step, the solution containing 1.225 g monochloroacetic acid in 4.5 mL isopropanol was added to the previous solution followed by gentle stirring for 12 h. The resulting sediment was transferred to a –80°C freezer for 24 h and was dried by a freeze-dryer. In order to ensure the existence of carboxyl groups in carrageenan, Fourier-transform infrared spectroscopy (FTIR) analysis was carried on a PerkinElmer spectrometer. Sample spectra were obtained with 100 scans at 4.0 cm⁻¹ resolution over the range 600 and 4000 cm⁻¹. The carboxyl functional groups on CKC were activated using EDC and NHS by adding 8 mg of EDC and 16 mg of NHS to 5 mL MES Buffer (0.5 M, pH = 6.2). Then 0.01 g of CKC was added to this solution and was gently stirred on a shaker for 1 h at 37°C. To perform the surface modification, the scaffolds were put in the mentioned solution for 4 h at 37°C in a Shaker-incubator. The final step is to wash the scaffolds in solutions of NaH₂PO₄ (0.1 M) for 2 h and PBS for 12 h (PBS solution was changed every 4 h). To optimize the coating effect and anticoagulant activity of the coated CKC, the process was repeated using different amounts of EDC/NHS. Similarly, surface modification was performed on sample E1C1 using 2 mg of EDC and 4 mg of NHS, sample E2C1 using 4 mg of EDC and 8 mg of NHS, sample E3C1 using 8 mg of EDC and 16 mg of NHS, sample E4C1 using 12 mg of EDC and 24 mg of NHS, and sample E5C1 using 16 mg of EDC and...
32 mg of NHS with the same amount of CKC for each group (0.01 g).

Characterization of surface modified scaffolds

**CKC immobilization.** The ATR-FTIR spectra of the surface modified acellular scaffolds were obtained using PerkinElmer spectrometer. In order to determine component of ECM in unmodified and surface modified vascular grafts, FTIR analysis using an attenuated total reflection system (ATR) was carried out. The presence of CKC on the luminal surface of the decellularized carotid arteries was detected by Toluidine Blue O staining which were imaged with an inverted phase contrast microscope. To determine the amount of amine group in acellular scaffolds, the ninhydrin assay was conducted. The reaction of ninhydrin with primary amine groups of the vascular scaffold produces a visible purple color which the optical absorbance was detected at 570 nm using spectrophotometer. Ninhydrin solution was prepared by dissolving 2% (w/v) of ninhydrin powder in ethanol (>96%). Decellularized grafts were immersed in ninhydrin solution and heated in hot water bath of 100°C for 16 min, then cooled down for 20 min in cold water. Finally, optical absorbance was measured via ELISA reader (Convergyx, EL-Reader 96X, Germany). The static water-in-air contact angles on the luminal surface of modified and unmodified scaffolds were measured by using a contact angle goniometer (Model CA-500, Sharif Solar, Iran) at room temperature. A droplet (3 μL) of deionized water was placed on the luminal surface of the grafts and the contact angle was determined. The procedure was repeated five times at different sites in the same surface. The contact angle of each sample was expressed as the mean value of five contact angle measurements.

**Mechanical testing.** Three groups of grafts, native, decellularized, and surface modified (n = 3) were cut into conduits with 1.5 cm in length and 4-5 inner diameter for tensile measurement. The samples were loaded on two steel rods and mounted in clamps of a uniaxial mechanical testing machine (Zwick/Roell, Z050, Germany). All samples were hydrated during testing to maintain physiologic conditions and were evaluated until failure at a rate of 5 mm/min.

**Platelet adhesion.** The grafts were evaluated for platelet adhesion using platelet-rich plasma (PRP). Fresh human blood was obtained from the Iranian Blood Transfusion Organization. To isolate PRP, whole blood was centrifuged at 225g for 10 min at room temperature. Two groups of vessel grafts, decellularized, and surface modified including E1C1, E2C1, E3C1, E4C1, and E5C1 was added to a centrifuge tube (Model CA-500, Sharif Solar, Iran) at room temperature. A droplet (3 μL) of deionized water was placed on the luminal surface of the grafts and the contact angle was determined. The procedure was repeated five times at different sites in the same surface. The contact angle of each sample was expressed as the mean value of five contact angle measurements.

**EC seeding, viability, and proliferation**

Human umbilical vein endothelial cells (HUVEC, NCBI code: C554, Pasteur Institute of Iran) were used to re-endothelialize the acellular vascular scaffolds (passage 2-5). Decellularized surface modified and unmodified vascular grafts were seeded with seeding density of 10^5 cells/cm² at 37°C and 5% CO₂ to evaluate cell attachment and proliferation. Briefly, the cell suspension was injected into the luminal surface of carotid arteries. Afterwards, grafts were rotated 90° after standing for 30 min, and seeding was completed after a rotation of 360°. Dulbecco's modified Eagle's medium (Gibco, NY) with 10% fetal bovine serum (Gibco) as a culture medium was changed every 2 days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell viability and proliferation on each group of grafts (n = 3) at days 1, 3, and 7 according to the manufacturer's instructions (ATCC 30-1010, USA). In order to quantify the number of ECs, a calibration curve with known cell numbers was plotted. A custom-designed vascular perfusion bioreactor driven by a peristaltic pump (Heidolph, 5101, Germany) was produced in our laboratory. The bioreactor consisting of an autoclavable glass chamber, silicone tubing, a periodic peristaltic pump, and a culture medium flask, were used to circulate a closed sterile flow loop (Fig. 2). For single seeding (lumen only), vascular grafts were seeded with HUVEC and incubated for 8 h for enabling cell adhesion. Afterwards, the specimens were placed in the chamber and ECs were exposed to the liquid flow of 50 mL/min (wall shear stress of 1.24 Pa for laminar flow of a Newtonian fluid) in an incubator.23

**ECs morphology, immunostaining, and nitric oxide measurement**

After 5 days of dynamic and static culture, the EC morphology was assessed by using electron scanning microscopy. The cell viability was also evaluated with regard to the quality by vital staining using Hoechst and Propidium iodide (PI). In order to characterize the re-endothelialization, and cell–cell contacts formation on the luminal surface of modified vascular grafts, immunostaining with antibodies against CD31 was carried out at day 5. Nitric oxide (NO) was estimated as nitrite (NO₃⁻), stable metabolite of NO, by using Griess reagents. Two groups of vascular grafts including unmodified and surface modified vascular scaffolds with the length of 3 cm were used to assess NO release under static and dynamic condition. The optical absorbance was measured at 540 nm using a NanoDrop spectrophotometer (NanoDrop/2000c, Thermo-Fisher Scientific, Waltham, Massachusetts, USA).24
Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. Numerical data are presented as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA to test for statistical differences. When the p value is less than 0.05, the results were considered statistically significant.

RESULTS

Verification of the decellularization process

Carotid arteries from sheep [Fig. 3(A)] were decellularized using perfusion decellularization protocol which maintains original dimension of native arteries [Fig. 3(D)]. To ensure successful decellularization, H&E [Fig. 3(B,E)] and DAPI [Fig. 3(C,F)] staining were performed to reveal the absence of cells or nuclear matter from vascular grafts. At H&E staining, cell nuclei are stained in purple and ECM in pink. At DAPI staining cell nuclei are stained in blue. The preservation of ECM after decellularization was verified by Masson’s trichrome staining for collagen [Fig. 4(A,D)], colored in blue and Verhoeff’s staining for elastin [Fig. 4(B,E)], colored in black fibers. The SEM images of the vessel grafts from luminal surface of native and decellularized scaffolds are shown in Figure 4C and 4F, respectively.

Verification of CKC synthesis and immobilization

Results for FTIR analysis are shown in Figure 5. The FTIR spectra reveal the basic characteristic of kappa carrageenan and successful synthesis of CKC. The new characteristic peak at 1746 cm\(^{-1}\) in CKC is assigned to carboxylic acid C=O stretch which indicates the introduction of carboxymethyl group to the kappa carrageenan as previously reported.\(^{21}\) Figure 6 shows the ATR-FTIR spectra of luminal surface of unmodified and the surface modified scaffold using CKC. In the spectra from ECM before surface modification two absorption peaks at 1510 and 1637 cm\(^{-1}\) existed attributing to amide II and amide I, respectively. The ATR spectra of the luminal surface of modified scaffold shows an increase in the peak intensity at 1036 cm\(^{-1}\) which attributes to sulfate group of CKC.\(^{25}\) Furthermore, an increase in peak density at 1638 cm\(^{-1}\) which assigns to amide I, demonstrates the immobilization of CKC. During the successful attachment of CKC, it is expected that the amide I formation increases because of the interaction between amine and carboxyl functional groups existing in vascular grafts and CKC.
respectively. Therefore, these two peaks demonstrate the successful immobilization of CKC on the luminal surface of the vascular scaffold. CKC covalent linkage in the luminal surface of vascular tissue was directly visualized using toluidine blue staining. Figure 7(A) reveals a blue color indicating positive staining of CKC with 10 μm thickness. In contrast, Figure 7(B) which is decellularized vessels without modification did not reveal blue color in the luminal surface of vascular tissue.

### Determination of the degree of crosslinking

As a result of amide type crosslinks, the concentration of free amino functional groups, existing in vascular grafts, decreased with increasing degree of crosslinking. Due to ninhydrin assay, the concentration of NH₂ in crosslinked and non-crosslinked scaffolds were measured which determines degree of crosslinking. The degree of crosslinking in each group of surface modified scaffolds including E1C1, E2C1, E3C1, E4C1, and E5C1 was determined from the amount of reacted amino groups which are shown in Figure 8. According to this figure, maximum crosslinking is occurred in E3C1 group consisting of 8 mg of EDC, and 16 mg of NHS, and 0.01 g CKC. There is no significant difference in degree of crosslinking in other four groups.

### Contact angle measurement

Surface wettability was evaluated using the water contact angles of the unmodified scaffold as a control and CKC modified samples (E1C1, E2C1, E3C1, E4C1, and E5C1). Table I shows the results of surface wettability of vascular grafts. According to the structure of ECM and its adhesion proteins, appropriate surface wettability is expected. These results show that the surface modification of vascular tissue has no significant effect on the wettability of the vascular grafts.

### Mechanical properties

In order to evaluate tensile properties of specimens (summarized in Fig. 9), stress–strain response curve for three groups of vascular scaffolds including native, decellularized, and surface modified were plotted in Figure 9(A). The results were summarized in Figure 9(B) and decellularized grafts revealed a decrease in young modulus from the native tissue 15.53 ± 0.73 MPa to 7.82 ± 0.93 MPa because of the decellularization process. In contrast, surface modified grafts having 9.85 ± 0.41 MPa were slightly stiffer than decellularized with 7.82 ± 0.93 MPa, because of the crosslinked CKC, although this difference was not significant [Fig. 9(B)].

### Adhesion of platelets

The number of platelets adhered on the different groups of CKC modified scaffolds (E1C1, E2C1, E3C1, E4C1, and E5C1), and unmodified decellularized grafts as a control are shown in Figure 10. The difference in the amount of platelet adhesion on the surface modified grafts is explained by the
degree of CKC crosslinking into the luminal surface of carotid arteries. From this figure, the platelet adhesion significantly reduces from $44.53 \pm 2.05$ (control) to $19.57 \pm 1.37$ (E3C1). In group E3C1, we have uniform layer of CKC with maximum degree of crosslinking which can inhibit platelets adhesion due to the anticoagulant properties of CKC.\textsuperscript{21} Furthermore, the morphology of platelets adhered to the control and surface modified (E3C1) was evaluated using scanning electron microscopy (Fig. 11). As expected, decellularized arteries due to its adhesion proteins such as collagen, allow platelets to adhere to the luminal surface of vascular scaffolds [Fig. 11(A,C)]. On the contrary, The CKC

![FIGURE 4. Histological and scanning electron microscopy of native (A–C) and acellular vascular grafts (D–F). At Masson’s trichrome staining (A and D) collagen is stained in blue and at Verhoeff’s elastic staining (B and E) elastic fibers are stained in black (scale bars are 100 μm). Scanning electron microscopy images of the luminal surface of the native (C) and decellularized scaffolds (F). Scale bars are 50 μm.](image)

![FIGURE 5. FTIR transmission spectra of the kappa carrageenan and carboxymethyl kappa carrageenan. The characteristic peak of carboxylic acid is shown corresponding to C=O stretch.](image)
modified vascular graft significantly decreased the number of platelets adhered to the scaffold [Fig. 11(B,D)].

ECs viability, morphology, and immunostaining
Cell numbers and viability on the luminal surface of vascular grafts in static and dynamic culture were measured using MTT assay. All results are shown in Figure 12 indicating no significant change in cell numbers was detected in static condition of modified and unmodified vascular grafts. However, in dynamic condition at day 1 we can see a decreasing in number of cells because of flow shear stress which can detach ECs from the luminal surface, although at day 7 we can see a slightly increasing in number of cells because ECs were adapted to dynamic condition. Furthermore, the cell viability was evaluated qualitatively using PI and Hoechst staining (Fig. 13). After 5 days of static culture unmodified

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Contact Angle (°)</th>
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<tbody>
<tr>
<td>Unmodified</td>
<td>56.53 ± 3.1</td>
</tr>
<tr>
<td>E1C1</td>
<td>61.05 ± 4.3</td>
</tr>
<tr>
<td>E2C1</td>
<td>58.84 ± 2.2</td>
</tr>
<tr>
<td>E3C1</td>
<td>55.31 ± 1.4</td>
</tr>
<tr>
<td>E4C1</td>
<td>56.34 ± 4.7</td>
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<tr>
<td>E5C1</td>
<td>57.45 ± 2.7</td>
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[Fig. 13(A–C)] and CKC modified [Fig. 13(D–F)] vascular grafts were stained with PI (red) and Hoechst (blue). The unmodified scaffold reveals only one dead cell (PI stain) and there is no dead ECs in unmodified scaffold, which proves viability of ECs. The morphology of ECs on the luminal surface of the CKC modified vascular graft was observed using SEM imaging under static and dynamic culture. In Figure 14(A–C), the morphology of ECs is round cell shape; however, in Figure 14(D–F), ECs morphology under flow shear stress is spindle shape. Finally, EC–cell contact on two groups of scaffolds including unmodified and modified was detected by CD31 staining (Fig. 15).

**NO release**

The release of NO by HUVECs was assayed by Griess solution and the values are shown in Figure 16. From the graph, it is evident that the release of NO in static condition is more undetectable than exposure of ECs to fluid shear stress and physiological stimuli. The effect of shear stress on the NO release was reported previously by Kabirian et al. which concluded that simulating physiological shear stress can stimulate ECs to produce more NO rather than static condition.\(^{24}\) NO significant difference was observed in release of NO in unmodified and CKC modified vascular grafts.

**DISCUSSION**

The majority of research conducted on vascular tissue engineering has been focused on macromolecular synthetic materials.\(^7,8,26\) However, they have shown low patency rates in small-diameter vessels due to early thrombosis formation.\(^7\) Therefore, the current study represents our efforts to develop an off-the-shelf small-diameter bypass graft.

To achieve this, we describe a novel method for decellularizing of sheep carotid arteries using a custom-made perfusion system. We also introduce CKC as a promising material with anticoagulant activity for small-diameter vascular grafts. The number of platelets adhered to vascular grafts compared to unmodified ECM was favorably reduced by immobilizing CKC into the luminal surface of scaffolds. Therefore, this strategy can address one of the major limitations in acellular scaffolds, which is thrombosis formation due to exposure of ECM proteins to whole blood. Furthermore, we demonstrated that the CKC modified scaffold allows for ECs proliferation and function, indicating the future application of our strategy for incorporating autologous cells or stem cells.

A number of decellularization protocols have been developed including detergents, physical strategies and combination of these strategies.\(^9\) Our decellularization protocol is based on the combination of freeze–thaw steps with chemical reagent in perfusion flow system through the lumen of vessel. The process revealed positive results as no residual cells were observed in H&E staining and DAPI analysis. Additionally, the histological analysis revealed a well-maintained structure of the vascular tissue using Masson’s trichrome and Verhoeff’s staining for collagen and elastin, respectively. The duration of our protocol provides a mild condition for
preservation of important structural and biochemical components. The effectiveness of using pressure controlled perfusion system was previously reported.\textsuperscript{27–29} Mechanical properties were evaluated by plotting stress–strain curve to compare native, unmodified and modified vascular grafts. While a reduction in young modulus was observed after decellularization, due to surface modification a part of mentioned reduction was compensated by immobilization of CKC on vascular grafts, leading to suitable mechanical strength for grafting application. The effects of heparin immobilization on mechanic of decellularized aortas were previously reported, showing that modified grafts were slightly stiffer than acellular tissue.\textsuperscript{13}

Many attempts have been done to reduce thrombogenicity of grafts, including coated artificial grafts with heparin.\textsuperscript{17,30,31} Our study proves surface modification of the scaffolds reduced platelet adhesion \textit{in vitro}; however, there is no certainty how long the modified grafts would last \textit{in vivo}. Therefore, more animal studies are needed to answer this question. Previous studies have also shown that CKC, having the nearest similarity to the heparin structure, is a promising alternative and it also promotes anticoagulant activity.\textsuperscript{21,32,33} However, there are only a few studies about CKC and its anticoagulant activity. Therefore, more research should be conducted to prove the hemocompatibility of CKC.

Another important factor for proper functioning of vascular grafts is an EC lining for preventing coagulation and preserving formation of blood vessel. A vascular graft should support ECs lining and inhibit over proliferation of smooth muscle cells, which can cause intimal hyperplasia.\textsuperscript{24,35} Furthermore, it was observed that the CKC modified scaffold helps re-endothelialization and supports the EC viability and proliferation using MTT assay and immunostaining. As reported by others, the effect of similar coating like heparin immobilization on the proliferation of ECs is uncertain.\textsuperscript{36,37} Our results of unmodified vascular grafts after 7 days of static and dynamic culture are consistent with the report described by Dahan et al.\textsuperscript{38}

NO is produced by vascular endothelium which serves many important functions in the cardiovascular system such as vasodilation, inhibition of platelet adhesion, anti-proliferative, and anti-inflammatory effects.\textsuperscript{39} Applying pulse-like shear

\textbf{FIGURE 11.} SEM imaging of platelets adhesion on unmodified (A and C) and CKC modified (B and D) carotid arteries. Platelets are shown by arrows.

\textbf{FIGURE 12.} Static versus dynamic endothelial cell growth and proliferation on unmodified (control) and CKC modified vascular grafts. All data are given as mean ± SD.
stress on ECs closely correlates with their NO produc-
tion.\textsuperscript{24,40,41} Our study shows under flow shear stress, HUVECs release NO. However, the amount of NO release in static condition was not significant. Recently, the acellular organs such as heart, liver, and bladder have been reported to provide proper scaffolds which can be seeded with appropriate cell types in order to support cell functions.\textsuperscript{42–45} The CKC modified vascular grafts exhibit excellent biocompatibility toward ECs (HUVECs) seeded onto the luminal surface of acellular vascular tissue, performing endothelialization and maintaining phenotypical characteristics. The current study represents the first use of CKC in vascular tissue engineering. This strategy using

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure13.png}
\caption{Endothelial cell viability at day 5 on the luminal surface of unmodified and CKC modified vascular grafts visualized with Propidium iodide (red) and Hoechst (blue). Scale bars: 200 \textmu m.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure14.png}
\caption{Morphology of endothelial cells on the luminal surface of surface modified vascular grafts after 5 days. Endothelial cells in static condition exhibiting round shape (A–C) and in dynamic culture (B–D) exhibiting spindle shape. Endothelial cells are shown by arrows.}
\end{figure}
CKC to coat ECM scaffolds opens up a broader application of this methodology to further modify ECM-based scaffolds with anticoagulants, however, further long-term studies are still required to better evaluate the anti-thrombotic property of CKC coated on small-diameter vessels.

**CONCLUSION**

The ultimate goal of this report was to develop a small-diameter vascular graft using acellular matrix from sheep carotid arteries for preclinical research. We describe a new approach using CKC with thromboresistance property and vascular cell compatibility to vascular grafts. CKC immobilization with EDC/NHS onto the luminal surface and throughout thickness of blood vessels was characterized by FTIR, ninhydrin assay, and histological observation. The CKC modified scaffold showed a substantial reduction in platelet adhesion and supported HUVECs proliferation, viability, and production of NO in both dynamic (custom-made perfusion bioreactor) and static culture. Our bioreactor allows for decellularization and recellularization of vascular grafts. Hence, this surface modified scaffold has great potential for using as small-diameter vascular grafts or may be applied more broadly to reduce thrombogenicity in the vasculature of engineered organ. However, future *in vivo* studies will reveal more detailed information of CKC modified vascular grafts for vascular grafting and transplantation.

**REFERENCES**


