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Thrombogenicity and biocompatibility studies of reduced graphene oxide modified acellular pulmonary valve tissue☆

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ABSTRACT

Current strategies in tissue engineering seek to obtain a functional tissue analogue by either seeding acellular scaffolds with cells ex vivo or repopulating them with cells in vivo, after implantation in patients. To function properly, the scaffold should be non-thrombogenic and biocompatible. Especially for the case of in vivo cell repopulation, the scaffold should be prepared in a manner that protects the tissue against platelet activation and adhesion. Anti-thrombogenicity can be achieved by chemical or physical surface modification. The aim of our study was to evaluate the platelet activation and thrombogenic properties of an acellular tissue scaffold that was surface modified with reduced graphene oxide (rGO). Graphene oxide was prepared by a modified Hummers method. For the study, an acellular pulmonary valve conduit modified with rGO was used. The rGO modified tissue samples were subjected to in vitro testing through interaction with whole blood under simulated laminar flow conditions. The following cellular receptors were then analysed: CD42a, CD42b, CD41a, CD40, CD65P and PAC-1. In parallel, the adhesion of platelets (CD62P positive), leukocytes (CD45 positive) and platelet–leukocyte aggregates (CD62P/CD45 positive) on the modified surface was evaluated. As a reference, non-coated acellular tissue, Poly-L lysine and fibronectin coated tissue were also tested. The rGO surface was also analysed for biocompatibility by performing a cytotoxicity test, TUNEL assay and Cell Cycle analysis. There was no significant difference in platelet activation and adhesion between the study groups. The only significant difference was observed for the PAC-1 receptor between Poly-L lysine group and rGO and the percentage of PAC-1 positive cells was 6% and 18% respectively. The average number of activated platelets (CD62P) in the field of view was 1, while the average number of leukocytes in the field of view was 3. No adherent platelet–leukocyte aggregates were observed. There were no significant differences in the DNA fragmentation. No significant effect of rGO on the amount of cells in different phases of the cell cycle was observed. Cytotoxicity indicates that the rGO can damage cells in direct contact but have no effect on the viability of fibroblasts in indirect contact.

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1. Introduction

There are an increasing number of patients which are rescued with prosthetic heart valves [1]. Currently, heart and cardiovascular-associated conditions are one of the main causes of death worldwide with a constantly growing number of people affected [2,3]. A large proportion of cardiovascular conditions are due to heart valve failure [2]. Heart valve replacement is a commonly accepted method for the treatment of heart valve disease [4]. Depending on the clinical indications, either biological or mechanical heart valves can be used. However, the use of commercially available valve prostheses is limited. The use of mechanical heart valves requires anticoagulation therapy, while degenerative processes and calcification reduce the durability of biological heart valve prostheses [5–7]. The limitations associated with conventional valve prostheses have led to a search for alternatives. Less invasive procedures with the use of natural-like devices would decrease the risk of complications, improve valve performance, and speed up the process of treatment, allowing more patients to receive the treatment. Tissue engineering is currently the only technology in the field to present the potential for the creation of tissues analogous to a native human heart valve, with longer sustainability and fewer side effects. In general, the more natural-like a biomaterial surface, the better it is. Because of the decreasing amount of donors with the simultaneous increasing amount of circulation problems the blood contacting material development is of the high importance. Clinically used valves for the heart assist system were optimized toward tissue analogue precursors.

☆ Contents entry: Novel aspects of tissue modification for internal blood contacting scaffolds with hemocompatible and non-toxic coatings were tested through advanced interdisciplinary investigations using a model of physiological blood flow.

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One potential approach is tissue engineering [8–11] using an acellular tissue scaffold that can be seeded with autologous cells either ex vivo [12,13] or in vivo [14]. For the success of a tissue engineering strategy in clinical applications, the scaffold is required to provide a non-thrombogenic and biocompatible microenvironment. However, during the decellularisation process, highly thrombogenic collagen structures are exposed [15]. As a result, it may cause activation of the coagulation cascade, the complement cascade, and even adhesion of platelets and leukocytes to the collagen structure. To overcome these complications, tissue engineering strategies involving ex vivo endothelialisation of scaffolds have been proposed. Endothelial cell linings provide natural protection against platelet activation and adhesion. However, for strategies in which acellular scaffolds are implanted in vivo, where they can act as an endothelial progenitor cell capture structure, the scaffold should be protected against clot formation and thrombotic events. This may be particularly important in the cases of human valves or vascular grafts, due to lack of endothelial cell ingrowths, even after years of implantation. The main methods to improve hemocompatibility include bioactive coatings and surface modifications to inhibit platelet and leukocyte material interactions. This latter method often involves the formation of a hydrophobic layer. For this purpose, pyrolytic carbon or graphite can be applied. High expectations are also associated with graphene. Graphene is a crystalline form of carbon with a regular hexagonal pattern, which can be defined as a one-atom thick layer of graphite. Graphene is an attractive material for many applications because of its excellent electrical, optical and mechanical properties as well as its ability to form layer-by-layer structures that are similar to natural tissue. Some studies indicate good interaction between cells with the graphene. For example, the adherence of human mesenchymal stromal cells and osteoblast has been observed. The graphen nanogrids can be effectively used for the differentiation of human neural stem cells (hNSCs), in to the neural networks [16]. In other study reduced graphene oxide (rGO)/TiO_2 heterojunction film has been used as biocompatible flash photo stimulator of hNSCs into neurons [17]. The chemical role of graphen or graphene oxide is also important, toward stem cell differentiation because of different binding abilities of G and GO toward different growth agents [18]. On the other hand the studies on reduction process of GO were reported, and the biocompatible reductants have been proposed for substitution of the toxic reducing agents. The use of ginseng-rGO as more biocompatible exhibited more differentiation of hNSCs into neurons and showed better proliferation of the stem cells after 3 days, compared with other toxic reductants [19]. The differentiation and proliferation of the cells on the rGO surface can be improved by physical factors. The pulsed laser stimulation has been reported as an agent caused the differentiation of hNSCs into neurons and self-organisation of neuronal network [20]. The GO can be used for the coating of collagen scaffold to improve the cell proliferation and differentiation. But the effect depends on the GO concentration, the low concentration can stimulate the cell in-growth in vivo and increases the biodegradation of scaffold, while a high concentration causes the opposite biological effect [21]. The studies of covalently linked biocompatible graphene/polycaprolactone composites as a material for tissue engineering application are also performed. It was presented that the composite materials exhibit better homogeneity, biomechanical and electrical conductivity properties, and result in the low in vitro cytotoxicity and good biocompatibility [22]. Scaffolds used in tissue engineering should be characterized by low thrombogenicity. There are studies showing that graphene exhibit a low thrombogenicity. Graphene coatings of nitinol stents support smooth muscle and endothelial cell growth and promote serum albumin adsorption which is important for decreasing the thrombosis rate [23]. However GO or RGO was recently reported to be highly thrombogenic in vitro and induces platelet aggregation in mice, which may cause restrictions for biomedical applications. But it was found that surface chemical modification like GO amine-modified graphene (G–NH_2), significantly reduces platelet activation in vitro and prevents pulmonary thromboembolism in vivo in mice [24]. The present study focused on the effects of a reduced graphene oxide coating of acellular pulmonary valve tissue, on the platelet activation for developing a tissue engineering scaffold. Specifically, the cell surface markers CD42a, CD42b, CD41a, CD40, CD62P and PAC-1 were analysed. Platelet activation can lead to thrombosis formation and the induction of the coagulation cascade, both of which significantly affect bioprosthesis durability. Platelet activation also contributes to an inflammatory response by supporting leukocyte adhesion and monocyte recruitment. This study attempted to assess whether rGO can be a valuable material for improving the thromboresistance of biomaterials. To the best of our knowledge, there are no studies on the potential application of graphene in the surface modification of collagen tissues derived from the acellular pulmonary valve conduit, that could be potentially used in the construction of heart valve or vascular bioprostheses. Most of the research related to the evaluation of thrombogenicity and biocompatibility of graphene is performed on separate graphene layer, and is not performed on the collagen tissue–graphene composite material. It is very important for potential in vivo applications in which graphene modified surfaces will be used and not a single layer of graphene. It appears that the impact on the biological properties of graphene is strongly influenced by the substrate layer such as acellular collagen tissue. This is particularly important in the thrombogenicity study of the blood-contacting materials. It should also be noted that in the present study the estimation of platelet adhesion and activation were made in dynamic conditions, while most in vitro thrombogenicity test is performed in a static system which is far from in vivo conditions. Used in the present study dynamic model allows the simulation of flow and shear stresses similar to those that occur within a medium-sized vessels. The study design are close to the physiological conditions, which is particularly important for the study blood contacting material and make the results more valuable in the case of potential in vivo applications of the graphene modified scaffold.

2. Materials and methods

2.1. Reduced graphene oxide (rGO) synthesis

Graphene oxide was prepared by a modified Hummers method. 5 g of thermally expanded graphite (Asbury Carbons, United States, particle diameter: 300–425 μm) and 6.5 g of potassium nitrate (POCh, Polan, purum) were added to a beaker containing 200 ml of concentrated sulphuric acid (POCh, 96–98%, p.A.). The beaker was cooled to under 5 °C in an ice bath and 15 g of potassium permanganate (POCh, Poland, purum) was gradually added. After adding the last portion, the ice bath was removed and replaced by water (25 °C). The reaction was left in that state for 16 h. The beaker was once again put into an ice bath and 230 ml of deionised water (DI) was slowly poured into the suspension. The mixture was then heated to 95 °C for 15 min, further diluted with an additional 280 ml of DI water and cooled to room temperature. Finally, 5 ml of 30% hydrogen peroxide was added. The graphite oxide suspension was washed with 3% hydrochloric acid solution and, after removal of sulphate ions, continuously washed with DI water until no chloride ions were detected. The purified suspension was then ultrasonicated for 1 h to exfoliate oxidised graphene sheets inside graphite structure, producing a stable suspension of graphene oxide in water. Then the graphene oxide was reduced by sodium borohydride ($NaBH_4$) as follows. 200 ml of purified suspension of concentration 2 mg/ml was stirred and heated up to 95 °C. Next, 1.5 g of sodium borohydride was dissolved in small amounts of deionised water and added into hot graphene suspensions which rapidly darkened. The reaction mixture was maintained at the temperature of 95° C during 1 h with continuous stirring. After the reduction was completed, the product was purified by repeated centrifugation and three days of dialysis. Such purified rGO suspension was used in further in vitro experiments. A materials characteristic of the sample was made

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