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Creation of a blood-compatible surface: A novel strategy for suppressing blood activation and coagulation using a nitroxide radical-containing polymer with reactive oxygen species scavenging activity

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ABSTRACT

Various polymeric materials have been used in medical devices, including blood-contacting artificial organs. Contact between blood and foreign materials causes blood cell activation and adhesion, followed by blood coagulation. Concurrently, the activated blood cells release inflammatory cytokines together with reactive oxygen species (ROS). We have hypothesized that the suppression of ROS generation plays a crucial role in blood activation and coagulation. To confirm this hypothesis, surface-coated polymers containing nitroxide radical compounds (nitroxide radical-containing polymers (NRP)) were designed and developed. The NRP was composed of a hydrophobic poly(chloromethylstyrene) (PCMS) chain to which 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) moieties were conjugated via condensation reaction of the chloromethyl groups in PCMS with the sodium alcoholate group of 4-hydroxy-TEMPO. Blood compatibility was investigated by placing NRP-coated beads in contact with rat whole blood. The amount of ROS generated on PCMS-coated beads used as a control increased significantly with time, while NRP-coated beads suppressed ROS generation. It is interesting to note that the suppression of inflammatory cytokine generation by NRP-coated beads was shown to be significantly higher than that by PCMS-coated beads. Both platelet and leukocyte adhesion to the beads were suppressed with increasing TEMPO incorporation in the polymer. These results confirm that the suppression of ROS by NRP prevents inflammatory cytokine generation, which in turn results in the suppression of blood activation and coagulation on the beads.

1. Introduction

Various materials are employed in blood-contacting implantable and extracorporeal medical devices, such as artificial hearts, artificial blood vessels, hemodialyzers and apheresis columns. Since most of those medical devices have poorly biocompatible surfaces, anticoagulants such as ethylenediamine tetraacetic acid (EDTA), hirudin, heparin, and warfarin are utilized to prevent thrombosis and embolism induced by contact between blood and these medical devices [1]. Given the side-effects of these anticoagulants, such as heparin-induced thrombocytopenia [2,3], however, numerous efforts have been made to reduce the activation of blood in response to contact with material surfaces. Suppression of such blood activation can effectively reduce the amount of anticoagulant required. In order to improve the blood compatibility of material surfaces, a number of versatile methods have been applied. One of the most simple and important techniques is polymer coating using biocompatible polymers such as poly(ethylene glycol) [4], zwitterionic polymers [5–7], microphase-separated polymers [8,9], and poly(2-methoxyethyl acrylate) [10]. These approaches can drastically reduce the adsorption of serum proteins. Protein adsorption triggers the activation of blood cells and blood coagulation on material surfaces through a complex series of events, including the activation of platelets, leukocytes, complement, and the fibrinolytic system [11]. Thus it has long been thought that the suppression of protein adsorption is highly important in the design of blood-compatible surfaces. Nevertheless, even today, all blood-contacting devices cause thrombosis with long-term usage. In fact, synthetic vascular grafts with inside diameters of less than 6 mm cannot be used because they are prone to early thrombotic occlusion [12]. In the

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case of cardiac stents, drug-eluting stents have been developed to inhibit endothelial cell proliferation and restenosis, but even these stents were found to be thrombogenic [13,14].

Recently, gaseous molecules such as oxygen, nitric oxide, carbon monoxide and hydrogen sulfide have been reported to play important roles in numerous biological events. New strategies utilizing these gaseous molecules have been proposed. For example, a polymer which releases nitric oxide has shown good suppression of blood activation [15–18]. Interestingly, it has been revealed that blood–material interactions cause an increase in the levels of reactive oxygen species (ROS) and inflammatory cytokines, which is brought about by the activation of blood cells; this leads to blood coagulation and whole body inflammation (see Fig. 1a) [19–21]. The excess ROS continuously amplify inflammation, thereby increasing the risk of potentially life-threatening disorders [22]. Indeed, long-term hemodialysis therapy induces cardiovascular disease and atherosclerotic complications, which result in high morbidity and mortality in chronic renal failure patients [23]. However, there have been few reports with experimental evidence that ROS generation is related to inflammation in blood when in contact with the material surface, and the extent of ROS involvement in inflammation is not yet clear.

We have focused on the effect of ROS-scavenging materials in vivo and have used nitroxide radicals, such as 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), which catalytically react with ROS [24–26] and can be used as electron spin resonance (ESR) probes in vivo [27,28]. Core–shell type polymeric micelles containing nitroxide radicals have already been developed for the treatment of oxidative stress injuries and in vivo ESR imaging [29,30]. It has been confirmed that they not only strongly scavenge ROS in vivo and in vitro but also significantly prevent oxidative stress damage in a cerebral ischemia–reperfusion injury model in rats [31], renal ischemia–reperfusion injury model in mice [32] and a neuron cell line used as a model for Alzheimer's disease [33,34], in which excess ROS is generated. In the process, we have hypothesized that ROS-scavenging characteristics play a crucial role in blood activation and coagulation when blood comes into contact with material surfaces. To validate our hypothesis, we have designed and developed a hydrophobic nitroxide radical-containing polymer (NRP) composed of hydrophobic poly(chloromethylstyrene) (PCMS) chains to which TEMPO moieties were conjugated via condensation reaction of the chloromethyl groups in PCMS with the sodium alcoholate group of 4-hydroxy-TEMPO (TEMPOL) (see Fig. 1b). In this paper we describe synthesis of the NRP homopolymer and its characterization in terms of blood compatibility.

2. Experimental section

2.1. Materials

2,2′-Azobisisobutyronitrile (AIBN) (Kanto Chemical Co. Inc., Tokyo, Japan) was purified by recrystallization from methanol. Chloromethylstyrene (CMS) (>95%), which was kindly provided by Seimi Chemical Co. Ltd (Kanagawa, Japan), was washed three times with 20% NaOH aqueous solution to remove inhibitors, washed three times with water, and dried using sodium sulfate, followed by vacuum distillation under a nitrogen atmosphere (2.0 mm Hg, 56 °C). N,N-Dimethylformamide (DMF) (Kanto Chemical Co. Inc., Tokyo, Japan) was purified by vacuum distillation under a nitrogen atmosphere using a molecular sieve. Tetrahydrofuran (THF), methanol, benzene, sodium hydride, 1,4-dioxane, n-decane (Kanto Chemical Co. Inc., Tokyo, Japan), TEMPOL, hypoxanthine (HX), xanthine oxidase (XOD) (Aldrich Chemical Co. Inc., USA), and 2-methyl-6-p-methoxyphenylethynylimidazopyrazinone (MPEC) (ATTO Co. Inc., Tokyo, Japan), and heparin (Mochida Pharmaceutical, Tokyo, Japan) were used without further purification.

2.2. Synthesis of NRP

After 1 mmol of AIBN was weighed into a flask, the inside of the reactor was degassed and filled with nitrogen. The degassing–N_2 purge cycle was repeated three times. 14.2 ml of CMS (100 mmol), 50 ml of 1,4-dioxane, and 3 ml of n-decane as an internal standard for gas chromatography were then added to the flask under a nitrogen atmosphere. Polymerization was conducted for 18 h at 65 °C in an oil bath. After the reaction the polymer obtained was recovered three times by precipitation into 1 l of methanol, followed by freeze-drying with benzene. The yield of the polymer obtained was 63.2% (9.6 g). Conversion of CMS was 61.5%, as determined by gas chromatography. After 20 mg of the PCMS obtained

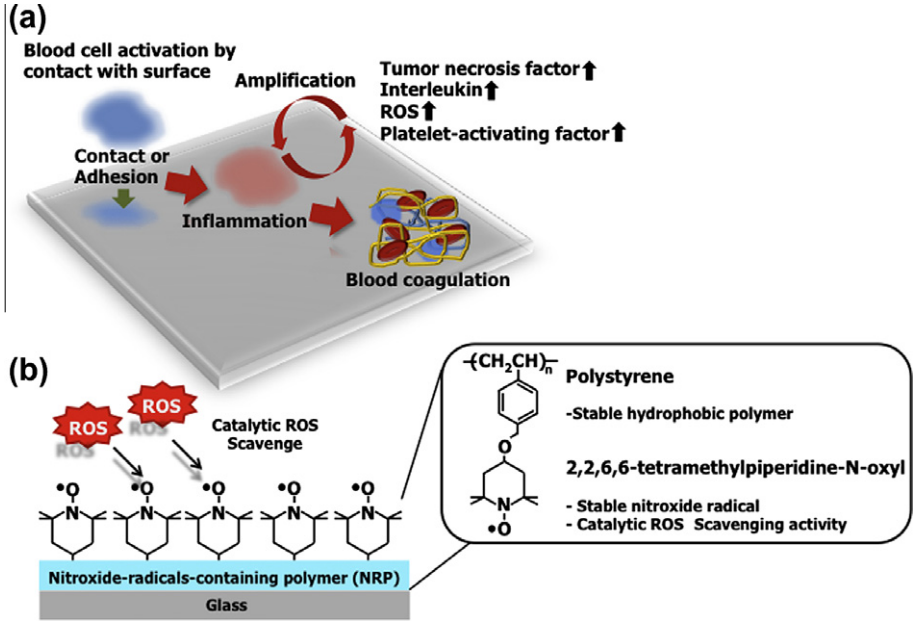

Fig. 1. Schematic illustration of (a) the mechanism of blood activation and coagulation and (b) the NRP-coated surface, which can scavenge ROS and inhibit blood coagulation.

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