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In vitro hemocompatibility testing of UV-modified hyaluronan hydrogels

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Abstract

Hydrogels (hylans) based on cross-linked hyaluronan (HA) are potentially good biomaterials for vascular tissue engineering applications because they are highly non-antigenic and -immunogenic. To facilitate surface endothelialization, vital to vascular deployment, we irradiated the gel surface with low wavelength UV light. This process micro-textures the smooth gel surface to provide sites for cell anchorage and causes limited scission of native long-chain HA yielding smaller fragments that elicit an enhanced cell response. In the current in vitro study, we assessed the effects of UV irradiation on the short-term (<45 min) interaction between hylan gels and human blood cells (RBCs, platelets) and coagulation proteins at physiologic temperature.

Although the lowered hydrophilicity of irradiated (UV) hylans elicited greater vascular cell response relative to unmodified (U) hylans, platelet deposition was unaffected and much lower compared to collagen-coated glass controls. The adhered platelets were rounded or mildly pseudopodic and did not express p-selectin, an activation marker. Both gel types induced identical, and minimal platelet release as measured using an platelet factor 4 ELISA, and identically deferred the intrinsic and extrinsic coagulation pathways. Both gel types induced elevated levels of contact activation of bound, but not plasma-phase factor XII relative to controls. Hemolysis rates were also identical and within accepted standards. We conclude that UV-treatment of hylans, useful to improve surface endothelialization, does not compromise their short-term hemocompatibility, vital to their use as vascular implant materials.

Keywords: Hylan; Ultraviolet; Blood-material interactions; Thrombosis

1. Introduction

Currently, the long-term success balloon angioplasty techniques, used to re-vascularize blood vessels closed in by atherosclerotic plaques, is limited by rapid re-occlusion (restenosis) within 6 months of the procedure. Re-stenosis is particularly severe in small (<3 mm diameter) peripheral vessels which inherently tend to re-occlude much more severely than larger vessels [1] and respond unfavorably to the struts (stents) often deployed to prevent vessel collapse and re-closure [2]. Although multiple factors contribute to restenosis, a leading cause is the disruption of the protective lining of lumenal endothelial cells (ECs) during angioplasty, which exposes the highly thrombogenic sub-endothelial matrix and collagen to blood, whereupon platelets are recruited to the vessel wall. Upon contact with the injured tissue, platelets become activated, induce fibrin to crosslink, and form a platelet-fibrin thrombus. Thrombi are potent stimuli for neointimal thickening and hyperplasia of vascular smooth muscle cells. To address this problem, several previous studies [3–5] have attempted to isolate the injured tissue from blood using inert, synthetic barrier materials. However, these materials were not expected to endothelialize to provide longer-term protection against thrombosis, nor were they intended to signal the medial SMCs at the injury site to remain quiescent; also, such synthetic scaffolds are more likely to evoke non-physiologic cell responses and induce unnatural tissue healing. To address these issues, we are exploring the use of biocompatible, non-synthetic cell-scaffolds derived using components of tissue extracellular matrix

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(ECM). Ideally, we would expect the candidate material to retain the preferred biologic properties upon chemical derivatization.

Slowly degrading hydrogels (hylans) formulated through crosslinking long-chain ($MW > 2 \times 10^6$ Da) hyaluronan (HA), a glycosminoglycan (GAG) component of connective tissues, with divinyl sulfone (DVS) are potentially excellent vascular biomaterials. Hyaluronan is highly non-antigenic and non-immunogenic, owing to its high structural homology across species [6], and poor interaction with blood components [7]. To our advantage, hylans retain many of the preferred characteristics of HA such as their poor antigenicity and non-immunogenicity [8,9], and may thus be potentially good implant materials. Although DVS-crosslinked hylans are widely used as a tissue spacer to prevent surgical adhesions, the novelty of our work with hylan lies in its intended use as a cell-interactive but biocompatible tissue engineering scaffold or cellularized vascular implant material. However, in earlier studies we showed that the highly anionic HA chains inhibit cell attachment, and may thus prevent successful endothelialization of the hylan implants to provide long-term vascular protection [10]. In previously published work, we sought to improve the endothelial cell-binding properties of hydrated hylan gels through controlled irradiation with low wavelength ($\lambda = 254$ nm) ultraviolet (UV) light [11]. Since UV is unable to penetrate hydrated gels, their bulk properties, including crosslinking, are unaffected. Rather, the effects of UV under the specified conditions of irradiation are restricted to the surface alone; the smooth, undulating surface of the native hylan gel is textured by UV into one containing a honeycomb-like network of ridges, which we previously showed using SEM to be ~1–2 μm across and ~10 μm apart from each other [11]. Likely, the anchorage provided by these ridges to contacting cells is responsible for the improved cell adhering properties of the irradiated gels. In addition, controlled irradiation with UV light also causes limited scission of long-chain HA ($MW \sim 2 \times 10^6$ Da) near the gel surface to yield shorter HA fragments and oligosaccharides that exhibit higher biologic activity and evoke enhanced cell responses such as ready attachment and proliferation [11,12]. The use of endothelialized hylan gels as vascular barrier grafts is however contingent on the poor interaction of the UV-irradiated gels with human blood. The purpose of this study was to qualitatively and quantitatively assess the relative hemocompatibility of unmodified and UV-irradiated hylan gels under static, in vitro conditions and confirm that the UV-treatment, necessary to endothelialize their surface, does not compromise their hemocompatibility.

2. Materials and methods

Hylan gels evaluated in this study were formulated and hydrated as per previously described protocols [13]. One sub-set of gels was surface-irradiated with UV light (UV gels) and the other set of gels was left untreated (U gels; control). Contact angle experiments were used to assess the surface wettability of both the gel types. Platelet attachment on the U and UV gels was evaluated by estimating platelet loss from a stock solution. Platelet activation and morphology were assessed using scanning electron microscopy (SEM) and confirmed through immunofluorescence detection of p-selectin expression. Hylan-induced damage to contacting erythrocytes was assessed by quantifying the degree of hemolysis. Thrombus formation was quantified by a global thrombus formation test (TFT). Coagulation assays for estimation of prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) were used to detect material-induced abnormalities to the intrinsic and extrinsic coagulation pathways. An enzyme immunoassay (EIA) was performed to compare materials-induced activity of free- and bound factor Xlla, and kallikrein-like activity in plasma.

2.1. Formulation of hylan gels

Hylan gels were formulated using methods loosely based on previously described protocols [13]. Briefly, 330 mg of long chain HA (Molecular weight $\sim 1.5 \times 10^6$), obtained as a sodium salt (Genzyme Biosurgicals, Cambridge, MA), was thoroughly mixed with 2 ml of 1 M sodium hydroxide (NaOH), pH 13.0 (4 °C; 30 min) and 7.6 g of 1 M sodium chloride (NaCl). Mixing was effected by repeated transfer of the mixture between two sterile syringes through a 3-way valve. The mixture was then stored over ice for 2 h and centrifuged for 10 min at 1000 g to remove air-bubbles. The mixture was then homogenously crosslinked with an emulsion of divinyl sulfone crosslinker (DVS; 64 μl; Sigma Chemicals, St. Louis, MO) in 1 ml of 1 M NaCl with continuous mixing. The crosslinked mixture was cast in 12-well plates ($A = 2\,cm^2$) and gelled over 2 h. The gels were equilibrated with an excess of 1 M NaCl to remove NaOH and then rinsed with three changes of a 70:30 volume ratio of isopropanol and 1 M NaCl to leach out unreacted DVS and also limit swelling. Finally, the gels were equilibrated in 1 M NaCl. The hydrated gels contained roughly 4% by weight of the HA and a HA: DVS weight ratio of 3:1. Half of each set of gels was irradiated with UV light ($\lambda = 254$ nm) for 24 h in a biological hood. As described in an earlier study [11], this treatment minimally alters the physical and chemical properties of the hylan gel surface to evoke enhanced cell responses necessary for use as tissue engineering scaffolds.

2.2. Procurement and processing of blood perfusates

All protocols pertaining to the use of blood were approved by the *Institutional Review Board* at the Medical University of South Carolina. Blood (30 cm^3) was drawn by venipuncture from aspirin-free healthy adult human donors and anti-coagulated with tri-sodium citrate in a 9:1 volumetric ratio. Whole blood was used for the hemolysis and thrombus formation tests. Coagulation- and factor XII-assays were performed with platelet-poor plasma (PPP) isolated from whole blood by centrifugation at 3000 g (10 min, 25 °C). For platelet adherence studies, plasma was volume adjusted in BSGC buffer, pH 7.3 [218 mg of K_3PO_4, 1.2 g of NaH_2PO_4, 7.0 g of NaCl, 4.0 g of sodium citrate, and 2.0 g of glucose in one litre of deionized water] to obtain final physiologic stock platelet counts of 1.5×10^5 platelets/μl.

2.3. Platelet deposition studies

A stock suspension containing 1.5×10^5 platelets/μl was overlaid atop U and UV gels, glass cover slips coated with rat tail collagen (control 1; 100 μg/100 μl; BD Biosciences, Bedford, MA) or inertized with siliconizing solution (Control 2; Sigmacote, Sigma). Test and control substrates were 2 cm^2 in area. Aliquots of the platelet suspension were incubated with the substrates for 15, 30, or 45 min at 37 °C ($n = 3$/substrate/contact time). The incubation volume was kept low (300 μl) to (i) minimize the floating (non-surface contacting) sub-population of platelets, and thus potentially increase and more reliably quantify the fraction of platelets in suspension lost through adhesion, and (ii) maintain the total platelet count at levels