Enhanced In-Vitro Blood Compatibility of 316L Stainless Steel Surfaces by Reactive Landing of Hyaluronan Ions

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Abstract: A novel dry process for immobilization of hyaluronan on stainless steel surfaces is presented. This process that we call reactive landing is based on an interaction of hyperthermal gas-phase hyaluronan ions with plasma-cleaned and activated stainless steel surfaces. Reactive landing is performed on a unique instrument that combines an in-situ plasma reactor with an electrospray ion source and ion transfer optics. Gas-phase hyaluronan anions are obtained by electrospray ionization of sodium hyaluronan solutions and immobilized by reactive landing on large-area stainless steel surfaces. The immobilized hyaluronan withstands extensive washing with polar solvents and solutions, and the washed surfaces maintain the protective properties against blood platelet activation. The mechanism of hyaluronan discharge and immobilization is discussed. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 80B: 505–510, 2007

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INTRODUCTION

Blood compatibility is often referred to as haemocompatibility and is one of the key aspects of biocompatibility. If biocompatibility is in the most general sense defined as the ability of a material to perform with an appropriate response related to a specific application,1 then blood compatibility relates to the specific interactions between biomaterials and circulating blood.

Hyaluronan (an anion of hyaluronic acid or HA) is a naturally occurring biopolymer and a major component of many cell types’ extracellular matrix (ECM).2 It forms molecular chains of varying lengths made up of repeating sugar units. Hyaluronan belongs to the polysaccharide subgroup called glycosaminoglycans that all contain an aminosugar as a part of the repeat unit, which is usually a disaccharide. Figure 1 shows the disaccharide unit of hyaluronan. It consists of alternating D-glucuronic acid and N-acetylgalactosamine, which are linked together through alternating β-1,4- and β-1,3 glycosidic bonds. Both sugars are spatially related to glucose, which in the β-configuration allows all of the substituents to be in sterically favorable equatorial positions. Thus, the structure of the disaccharide is energetically stable. Each repeating unit contains a carboxylate group, four hydroxyl groups, and an acetamido group. Unlike other important glycosaminoglycans (e.g., heparin, dermatan, chondroitin, and keratin) hyaluronan does not contain sulfate groups.

Hyaluronan is found throughout the body, from the vitreous humor of the eye to the synovial fluid of the joints.2,3 It was first isolated from the vitreous body of the eye in 1934 by Mayer and coworkers4,5 Because of its ubiquitous occurrence in the human body,6 the biocompatibility properties of hyaluronan are suggested, and as a result, there have been many attempts to apply hyaluronan in the area of biocompatible devices and implants. An extensive overview of the medical applications of hyaluronan and its derivatives is available.7 So far, the major application in the United States has been to provide a viscoelastic protective film in ophthalmic surgery.3 The interest in immobilization of hyaluronan on hard stainless steel surfaces arises from the fact that most endovascular stents are stainless steel and that coated surfaces exhibit improved compatibility with blood cells.8 The proposed mechanism suggests that hyaluronan coating inhibits the interaction of platelets with the bulk surface through interfering with the von Willebrand factor.8 Also, in general, hydrogel surfaces show low adhesive interactions with proteins and cells, and this is often associated with blood compatibility.

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For medical implants and other devices in contact with blood, it is important to minimize the tendency of their surfaces to induce blood clotting, and the resultant thrombosis. Thus, the objective of this work was to employ hyaluronan immobilization to decrease platelet adhesion on 316L stainless steel surfaces.

There are three common methods for immobilizing hyaluronan to produce biomedically useful coatings. The material can either be covalently coupled to functional groups on the surface using “wet” organic chemistry, a photoreactive group can be attached to hyaluronan which is then photocoupled to the surface, or it can be electrostatically immobilized to a positively charged surface.

We present an alternative dry process for immobilization of hyaluronan on stainless steel surfaces. Our two-step approach is based on (1) oxygen plasma cleaning and activation of the stainless steel surface, immediately followed by (2) deposition of gas-phase hyaluronan anions. We have recently described an analogous immobilization technique for the proteins trypsin and streptavidin that were both able to retain a significant portion of their biological activity (50–60%) after dry immobilization. Immobilization is achieved by chemical interactions on the surface that are driven by the ion hyperthermal kinetic energy, and the process does not require additional solution chemistry. We have also reported immobilization of smaller molecules and suggested a mechanism for it. The dry immobilization of gas-phase ions is referred to as reactive landing.

Reactive landing uses a unique instrument that combines a plasma reactor with an electrospray ion source and ion-transfer optics. These components are combined into one instrument, which allows for fast and versatile in-situ plasma treatment of the surface prior to deposition. Preliminary results with reactive landing of hyaluronan using this instrument have been reported previously. In the present report, we are addressing more detailed questions concerning the efficiency of coating large areas, and the stability and durability of hyaluronan coated stainless steel surfaces when exposed to polar solutions and body fluids under simulated physiological conditions.

**MATERIALS AND METHODS**

**Instrument**

The instrument for reactive landing on plasma-treated metal surfaces has been described previously. Briefly, ions are produced by electrospray at atmospheric pressure and transferred to the vacuum system by a heated, glass-lined capillary of 0.8 mm i.d. Sodium hyaluronate can be effectively electrosprayed in negative ion mode after deprotonation of its carboxylic groups. To achieve sufficient desolvation when spraying large hyaluronan polymers, the temperature of the ion source was kept close to 200°C and a stream of preheated nitrogen gas was used to further promote the desolvation process. The ions are transported through an intermediate pressure region (0.5–1 Torr) by an electrodynamic ion funnel lens and pass through a 2-mm aperture to the low pressure region (1.4 × 10⁻³ Torr). Ion focusing combined with the vacuum conductance limit insures that a substantial fraction of ions pass to the low pressure region while the majority of neutral molecules including solvent are removed. Presuming that the gas flow through the apertures is proportional to their areas, we calculate that only 0.075% of neutral molecules entering the first vacuum chamber continue to the second chamber while 99.92% are pumped out. Ions passing into the second vacuum chamber are focused and transmitted by an r.f. octopole ion guide maintained at ground potential. The ion beam exiting from the octopole is intercepted by a metal target that is biased by a reverse accelerating potential through a flexible connection. Our previous results indicated that a potential bias greater than 15 V provided sufficient kinetic energy for binding hyaluronan anions to the plasma-activated surface. The metal target is mounted on a 1-m long linear motion manipulator that is inserted through a vacuum port in the plasma chamber and extends through a gate valve that separates the plasma chamber from the ion optics vacuum chamber.

Ions are deposited on the target in a round spot of a 2.7–3.0 mm radius that corresponds to an enlarged image of the ion beam in the octopole guide. This area can be extended to a 25 × 25 mm² by simultaneously moving the target along the x and y axes (z being the translation axis) during ion deposition. The motion is provided by swiveling the outside end of the probe by an electroactuated gear manipulator.

**XPS**

XPS spectra were taken on a Surface Science Instruments (SSI) S-Probe ESCA instrument. This instrument uses a monochromatized Al Kα X-ray source for photomission stimulation and a low-energy electron flood gun for charge neutralization. The Service Physics ESCAVB Graphics Viewer program was used to determine peak areas.

![Figure 1. Disaccharide structure of hyaluronan. This disaccharide unit is repeated (250–25,000 times) resulting in hyaluronan chains with molecular mass up to several millions Daltons.](image-url)
In-Situ Plasma Reactor

The custom-made plasma reactor similar to that described by Ratner was operated at 13.56 MHz. The surface treatment was carried out at 60-W rf power for 10 min in 250 mTorr of flowing oxygen gas.

Chemicals

The hyaluronan ions were obtained by electrospraying solution of 420 kDa sodium hyaluronate salt (Genzyme Corporation, MA Lot#9149–10A3) at a concentration of 100 nM in 85/15 methanol/water. The human blood upper physiological values for washing solutions (sodium chloride and urea) were obtained from Orten and Neuhaus and both solutions were prepared using HPLC grade water. The serum was obtained from UWEB, Seattle, WA, under the label FBC CB 7/1/03. All other solvents were purchased from Fisher, USA. Metal targets for soft landing were made of 316L stainless steel plates. The plates were mechanically polished with a diamond paste, rinsed successively with pentane, methanol, and water, sonicated in 1:1 chloroform/methanol for 15 min, rinsed with methanol, and exposed to oxygen plasma immediately after cleaning.

Cell Adhesion Test From Platelet-Rich Plasma

Whole blood was obtained from normal donors and collected into acid citrate dextrose. Citrated blood was centrifuged at 800 rpm for 20 min at room temperature. PRP was removed from the supernatant above the residual red and white blood cells. PRP (200–250 mL) of ~300,000 platelets/mL was added to the center of each of the surfaces undergoing test for platelet adhesion and incubated for 20 min at room temperature. The samples were then rinsed with phosphate-buffered saline, fixed with 3.7% formaldehyde for 15 min, and rinsed with phosphate-buffered saline. The fixed samples were stained for 90 min with 1% Coomassie Blue, destained with 5% acetic acid, and submitted for scanning electron microscopy (SEM).

RESULTS

Electrospray ionization of hyaluronan, ion deposition on a small spot, and the manipulator motion were extensively optimized prior to deposition on large areas. Following optimization, a set of large area samples (25 × 25 mm²) coated with hyaluronan was prepared. Figure 2(A) shows a single spot of landed hyaluronan and the relation between the single spot area, limit switch settings and the large coated area [Figure 2(B)]. The deposition time was 12 h for each sample chip. The highest obtained surface current was about 3 nA, which is comparable to ion currents obtained during single spot deposition without surface motion.

One sample was rinsed with the water/methanol mixture and analyzed by XPS. The results were compared with those previously obtained on single spot areas. For the large-area XPS scan obtained at low energy resolution, the comparison focused on three elements present in HA, that is, carbon, oxygen, and nitrogen. Table I shows that the relative abundance of C, O, and N in the single spot area and at the large are samples are in good agreement, if the usual error of XPS measurements (10% relative) is taken into account. The high-resolution XPS spectra of carbon 1s in hyaluronan should contain four characteristic peaks, one for each kind of carbon that is present. Again, Table I reports very good agreement between results obtained on large areas and on single spot areas.

To test for stability of the reactively landed material, the other large area samples were exposed to the following solvents/solutions for the given time periods: deionized water (5 min), methanol (5 min), 1% sodium chloride solution (30 min), 7 mM urea solution (30 min) and bovine serum (30 min). In addition to large area samples, one single spot sample was prepared as well and washed with water for 30 min. After exposure to solvents/solutions, all samples were rinsed with deionized water and submitted to XPS analyses. The XPS results are summarized in Table II.

<table>
<thead>
<tr>
<th>Atomic %</th>
<th>Single Spot</th>
<th>Large Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>31.8</td>
<td>32.6</td>
</tr>
<tr>
<td>N</td>
<td>3.7</td>
<td>2.8</td>
</tr>
<tr>
<td>C</td>
<td>55.2</td>
<td>57.9</td>
</tr>
<tr>
<td>Carbon 1s 285.0 eV</td>
<td>37.0</td>
<td>36.8</td>
</tr>
<tr>
<td>Carbon 1s 286.7 eV</td>
<td>39.5</td>
<td>42.4</td>
</tr>
<tr>
<td>Carbon 1s 288.3 eV</td>
<td>19.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Carbon 1s 289.8 eV</td>
<td>4.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Selected samples were submitted for platelet adhesion studies that were run at Genzyme research facilities using standard blood–materials interaction tests based on cell adhesion from platelet-rich plasma (PRP). The test results are presented as scanning electron microscopy (SEM) images that allow for comparison of platelet adhesion on different sample surfaces. Figure 3 shows activated platelets on the plasma-treated blank surface (no hyaluronan deposited) at different SEM magnifications. The blank sample clearly shows dense coverage of the surface with the platelets. In contrast, Figure 4 shows the results of the platelet adhesion test on a hyaluronan-coated surface that had been washed with urea solution prior to the test. The results of the test show no activated platelets on the surface of this sample. Figure 5 compares platelet adhesion inside the spot coated with reactively landed hyaluronan (Figure 5, Panel A) and outside this spot on the surrounding blank surface (Figure 5, Panel B). The uncoated outside area shows abundant platelet adhesion, while there are almost no platelets inside the hyaluronan deposited area.

### DISCUSSION

Hyaluronan anions that have been electrosprayed into vacuum and reactively landed with hyperthermal energy on plasma-treated metallic surfaces retain their biological activity. This opens several questions of charge neutralization, bond formation, and structure retention upon the ion impact on the plasma-treated surface. We have previously shown that ions must be efficiently discharged upon landing to allow continuous landing of further incoming ions. Even a monolayer of surface charges at a density of one elementary charge per 50 Å would produce an electrostatic field on the order of $10^8$ V/m that would be impenetrable to gas-phase ions landing at a 15 V potential applied to the landing plate. In the same work, we also reported SEM images of landed multiple layers of hyaluronan on the stainless steel surface. As expected, the images show typical three-dimensional entangled structures of polysaccharide chains and indicate that HA is deposited in several compact layers on the surface. This is possible only if the charge brought in by HA anions is efficiently neutralized by a nondestructive mechanism. The plasma oxidized surface of the landing plate consists mainly of Fe$_2$O$_3$ (and possibly Fe$_3$O$_4$ as well) and Cr$_2$O$_3$, which are

### TABLE II. XPS of Hyaluronan-Coated Surfaces After Washing

<table>
<thead>
<tr>
<th></th>
<th>Water (5 min Washing)</th>
<th>NaCl Solution (30 min Washing)</th>
<th>Water [30 min Washing (Single Spot Deposition)]</th>
<th>Blank (30 min Washing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>63.9</td>
<td>54.1</td>
<td>66.9</td>
<td>33</td>
</tr>
<tr>
<td>O</td>
<td>27.7</td>
<td>38.8</td>
<td>26.8</td>
<td>52.4</td>
</tr>
<tr>
<td>N</td>
<td>2.7</td>
<td>3.1</td>
<td>1.3</td>
<td>4</td>
</tr>
<tr>
<td>Fe</td>
<td>3.8</td>
<td>2.1</td>
<td>2.3</td>
<td>12</td>
</tr>
<tr>
<td>Cr</td>
<td>0.2</td>
<td>0</td>
<td>0.7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Figure 3. SEM images of activated platelets on a blank plasma-treated surface that was exposed to the platelets adhesion study. Magnification: 200× (Panel A), 500× (Panel B), 2000× (Panel C), and 5000× (Panel D).

Figure 4. SEM of a hyaluronan covered plasma-treated surface that was washed by urea solution and exposed to the platelet adhesion study. No activated platelets are visible.
amphoteric oxides. Thus, charge transport can be established on the surface by protonation or deprotonation of the surface hydroxyl groups. The source of protons and hydroxyl groups on the surface is water, which is a major vacuum contaminant at the pressures used in reactive landing (\(10^{-3}\) Torr). An equilibrium on the surface corresponds to an acid–base exchange that can be represented by the following dissociation reactions (\(\equiv\) denotes surface groups)

\[
\begin{align*}
\text{FeOH}_2^+ + B &\leftrightarrow \text{FeOH} + \text{HB}^+ \\
\text{FeOH} + B &\leftrightarrow \text{FeO}^- + \text{BH}^+
\end{align*}
\]

Protonation of iron oxides is known to be exothermic with reported \(\Delta H\) ranging between \(-25\) and \(-48.8\) kJ/mol for different oxide structures.\(^{27-29}\) For a review of the kinetics, thermodynamics and mechanism of the acid–basic reactions on the surface of iron oxides are reported earlier.\(^{30}\) The acidity of carboxylic groups in hyaluronan has been estimated as \(p_{K_a} = 3-4\),\(^{31}\) so that the conjugate base has a \(p_{K_b} = 10-11\). This implies that carboxylate groups in hyaluronan anions are by far the strongest Brønsted bases present on the oxide surface and they can get protonated with the surface iron hydroxide groups:

\[
\begin{align*}
\text{FeOH} + (\text{OOC---Hyal})^- &\rightarrow \text{FeO}^- + (\text{HOOC---Hyal}) \\
\text{FeOOH} + (\text{OOC---Hyal})^- &\rightarrow \text{FeOO}^- + (\text{HOOC---Hyal})
\end{align*}
\]

Because the ion current measured on the surface is practically independent of the landing potential,\(^{19}\) the discharge process must also be independent of the landing energy. However, the ion kinetic energy is important for further reactions on the surface,\(^{19,20,22}\) because immobilization of the reactively landed material shows a threshold with respect to the ion acceleration potential. The nature of the kinetic-energy induced surface reaction is currently under study. The process of hyaluronan immobilization on the surface is coupled with a redox reaction that is compensated by electron transfer between the oxide surface and the conduction band of the bulk metal. This electron current, as measured with a nanoamperemeter connected to the metal collector plate, is typically on the order of \(10^{-9}\) A, which is consistent with the amount of landed material according to Faraday’s law.\(^{19,20}\) Thus, continuous electron transfer from the surface to the bulk metal drains the negative charge that is brought to the oxide surface by landing hyaluronan anions from the gas phase.

As mentioned earlier, while multiple layers of landed material are deposited in a typical landing process, only the first one or two layers can interact directly with the surface and form bonds that allow them to survive extended washing by polar solvents and solutions. The additional layers land onto the previously deposited material and are only loosely deposited (soft landed) on the surface. As a result, the loosely bound layers can be washed from the surface and recovered intact into solution, as shown previously.\(^{19,20}\) Thus, the demonstrated extended blood compatibility of HA-treated stainless steel clearly results from surface protection that is provided by a thin layer, possibly even a monolayer, of hyaluronan.

In solution, the hyaluronan chains form random coils that interact with each other and cause gelation at \(\sim 1\%\) concentrations.\(^{6}\) The helical configuration of the hyaluronan polymer is due to hydrogen bonding between hydroxyl groups in the main chain and is also responsible for the overall hydrophilicity of the polymer. The random coil formed by a hyaluronan polymer is able to trap 1000 times its weight in water.\(^{6,32}\) Although surface immobilization reduces the degrees of freedom in comparison with free molecules in solution, the parts of a hyaluronan chain that are facing away from the surface can be expected to interact with solvent. We envision that hyaluronan ions, which were originally desolvated by electrospray ionization at high temperature, quickly resorb water once they are discharged and cooled on the surface. Water resorption starts inside the instrument (at \(10^{-3}\) Torr) as the landing process continues.
torr, if water is the primary species in the gas phase, a water monolayer could form in $10^{-3}$ s and it is completed once the surface is brought to ambient air conditions and exposed to wash solutions. As a result of water sorption, the immobilized hyaluronan molecules restore whole or at least significant parts of their natural solvated structure and form an entangled protective layer analogous to the protective layers previously described by Burns et al. and Rowland et al. This layer swells due to water sorption and forms large domains with the actual mass of hyaluronan within domains being very low. The domains of individual hyaluronan molecules overlap each other. Small molecules such as water or electrolytes can freely diffuse through the layer but large molecules such as proteins are excluded because of their hydrodynamic size in solution.

**CONCLUSIONS**

The new results reported here show that it is possible to immobilize gas-phase hyaluronan anions on large-area plasma-treated stainless steel surfaces through a dry process referred to as reactive landing. The automatic mechanical motion of the target surface is independent of the ion discharge or the immobilization process. The elemental composition of the hyaluronan-modified large-area surfaces is identical to that reported previously for single-spot modified surfaces. The immobilized hyaluronan survives extensive washing in polar solvents and solutions, and the washed surfaces maintain protective properties against blood platelet activation. The new dry *in-situ* process has the potential to improve blood compatibility of stainless steel surfaces in medical implants.

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