CHAPTER 4

Biomimetic Modification of Silicone Tubes Using Sodium Nitrite-Collagen Immobilization Accelerates Endothelialization

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ABSTRACT

Biomimetic coatings to increase endothelialization of blood-contacting materials in biomedical devices are promising to improve the biocompatibility of these devices. Although a stable extracellular matrix protein coating on a biomaterial's surface is a prerequisite for endothelial cell attachment, it also stimulates platelet adhesion. Therefore anti-thrombotic additives such as nitric oxide donors to a stable protein coating might lead to successful endothelialization of a material's surface. We aimed to test whether immobilized bioactive nitrite and acidified nitrite-generating sodium nitrite-collagen conjugate on silicone tubes enhances endothelialization by increasing the number of endothelial cells as well as growth hormone production, and by decreasing platelet adhesion. Stable collagen immobilization on acrylic acid-grafted silicone tubes decreased the water contact angle from 102° to 56°. Initial 25 µM sodium nitrite in conjugate resulted in maximal growth hormone production (2.5 fold increase), and endothelial cell number (1.8 fold increase) after 2 days. A 95% confluent endothelial cell monolayer on sodium nitrite-collagen conjugate coating was obtained after 6 days. Maximum (2.7 fold) inhibition of platelet adhesion was reached with initial 500 µM sodium nitrite in conjugate. Our data showing that sodium nitrite-collagen conjugate coating with 25-50 µM sodium nitrite on silicone tubes increases the number of endothelial cells attached and inhibits platelet adhesion, suggest that this coating is highly promising for use in blood-contacting parts of biomedical devices.

Keywords
Surface modification, Acrylic acid grafting, Collagen, Sodium nitrite, Endothelial cells
INTRODUCTION

Endothelial cell seeding of blood-contacting parts of biomedical devices to mimic the function of the normal vascular endothelium is promising to decrease thrombotic complications resulting from blood flows through these devices [1, 2]. The surface of most commercially available materials used in biomedical devices, such as silicone, is highly hydrophobic and does not support endothelial cell attachment [1]. To overcome this limitation, several physicochemical techniques have been employed to improve the properties of silicone through surface modification [2, 3].

A widely used surface modification method is plasma graft polymerization of acrylic acid (AAc), which introduces functional carboxyl groups on an inert silicone surface [4-7]. Nowadays biomimetic coatings incorporating bioactive molecules that can be bound successfully to the carboxyl groups of AAc, such as collagen and gelatin [4, 6], insulin [8], and anticoagulants such as thrombodulin [9], albumin [10], and heparin [9, 11, 12] are extensively used. AAc-grafted materials show improved wettability [4-7], but they negatively affect many cell types when in direct contact [4, 6]. On the other hand, covalent immobilization of collagen onto AAc-grafted materials, through chemical bonds between amino-groups of collagen and carboxyl functional groups of AAc, enhances cell adhesion, proliferation, and differentiation [3, 4, 6]. However, collagen is highly thrombogenic, and accelerates platelet aggregation in those areas of a material which are not fully covered by endothelial cells [11].

Nitric oxide (NO) is an important inhibitor of platelet adhesion [13, 14]. Recently we have shown that treatment of endothelial cells by shear stress, cold temperature, and aspirin stimulates NO production and decreases thrombus formation [15]. NO-releasing material coatings suppress thrombogenic problems in the absence of endothelial cells or in areas which are not fully covered by endothelial cells [13, 14, 16]. In addition to inhibition of platelet adhesion and activation [13, 14], NO regulates amongst others (a.o.) vascular cell proliferation and migration [17], and the production of vascular endothelial growth factor (VEGF) [18] and/or growth hormone (GH) [19]. Nitrite, the stable end-product of NO metabolism, may represent a potential source of NO in an acidic environment [20, 21]. Whether acidified nitrite indeed prevents thrombus formation and increases endothelial cell growth has not yet been unequivocally established.

In this study we aimed to investigate whether incorporation of the nitrite donor sodium nitrite in a collagen coating immobilized on AAc-grafted silicone tubes increases the number of endothelial cells as well as decreases platelet adhesion. A two-step plasma treatment was used to graft AAc on silicone [7]. Collagen and sodium nitrite-collagen conjugate with different initial concentrations of sodium nitrite were added to the lumen of AAc-grafted silicone tubes to allow
collagen immobilization. Collagen content, nitrite and acidified nitrite release, water contact angles of the surface-modified silicone tubes, as well as endothelial cell attachment were determined. The effect of nitrite and acidified nitrite release from the sodium nitrite-collagen conjugate coating on the number of endothelial cells and GH production after 2 days of culture, as well as platelet adhesion, was assessed.

MATERIALS AND METHODS

Materials
Medical grade tubular silicone rubber (inner diameter 2 mm) was kindly donated by Raumedic (Helmbrechts, Germany). AAc was supplied by Fluka (Buchs, Switzerland), and purified by distillation under vacuum to remove impurities and stabilizers. De-ionized water was used in all experiments. Chemicals for the Griess assay were obtained from Merck (Kenilworth, NJ, USA), and were of the highest purity available.

Plasma graft polymerization of AAc onto silicone tubes
A two-step plasma treatment was used to prepare AAc-grafted silicone (AAc Si) tubes [4-7], i.e. plasma pretreated silicone tubes were immersed in an aqueous solution of 30% AAc in water followed by plasma graft copolymerization on a reabsorbed layer of AAc on silicone. A reaction chamber (Seren R600, Anatech ltd, Union City, CA, USA) evacuated to 0.6 mbar and pretreated with 60 W of oxygen plasma was used for 0.5 min plasma pretreatment and for 3 min plasma graft copolymerization of AAc on silicone tubes. The residual monomers and homopolymers were removed by incubation in water for 24 h. The grafted amount of AAc was calculated after weighing the samples before and after graft polymerization as described by Karkhaneh et al [7].

Collagen and sodium nitrite-collagen conjugate immobilization on AAc Si tubes
Sodium nitrite stock solution (NaNO₂; Merck, Kenilworth, NJ, USA) at 0.01 M was used to prepare 5, 10, 25, 50, 100, 250, and 500 µM of sodium nitrite in water containing 1 mg/ml collagen (acid soluble collagen type I; Pasteur Institute of Iran, Tehran, Iran) and 0.02 M acetic acid. Solutions were gently shaken for 1 h at 4°C to obtain homogeneous sodium nitrite-collagen conjugates.

AAc Si tubes were immersed into 30 ml 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer solution containing 48 mg 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 15 mg N-hydroxy succinimide (NHS; Fluka, Neu-Ulm, Germany) [22]. The solution was
gently stirred for 5 h at 4°C to activate the carboxyl groups on AAc Si tubes. Then AAc Si tubes with activated carboxyl groups were filled with collagen or sodium nitrite-collagen conjugate for collagen immobilization at 4°C for 24 h. Collagen-immobilized AAc-grafted silicone (AAc Si-Col) tubes and sodium nitrite-collagen conjugate immobilized AAc-grafted silicone (AAc Si-Nitrite-Col) tubes were washed in water for 1 min to remove unbound proteins and sodium nitrates, and stored at 4°C. To assess the stability of collagen linked to the tubes, the lumen of AAc Si-Col and AAc Si-Nitrite-Col tubes was washed extensively three times with 5 ml water for 5 min by infusion from one end into the lumen of the tubes using a syringe. The amount of immobilized collagen on AAc Si-Col tubes as well as on AAc Si-Nitrite-Col tubes before and after washing was determined by using a Bradford protein assay (Bradford, MA, USA) according to the manufacturer’s instructions [4], followed by quantification in an Eppendorf biophotometer D30 (Eppendorf, Hamburg, Germany). Concentrations of immobilized collagen on surface-modified silicone tubes were assessed by comparison with a standard curve consisting of serial dilutions of collagen.

To verify indirectly that sodium nitrite-collagen conjugate was bound to the silicone, nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes was measured by Griess assay. The AAc Si-Nitrite-Col tubes were filled with Dulbecco’s minimal essential medium/F12 (1/1, v/v) (Gibco, Life Technologies, Grand Island, NY), and incubated for 4 h and 48 h. Nitrite and acidified nitrite release was measured as nitrite (NO$_2^-$) accumulation in the medium, using Griess reagent containing 1% sulfanilamide, 0.1% naphtylethelene-diaminedihydrochloride, and 2.5 M H$_3$PO$_4$ [23, 24]. Serial dilutions of NaNO$_2$ in the medium were used as standard curve. The absorbance was measured at 540 nm with a microplate reader (Stat Fax-2100, Miami, FL, USA). Nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes was also expressed as the percentage of the initial sodium nitrite in conjugate (i.e. the total amount of nitrite in the medium divided by the total amount of sodium nitrite in conjugate x100%).

Characterization of surface-modified silicone tubes
Surface-modified silicone tubes were dried for 2 h at room temperature, cut longitudinally, and glued on glass microscope slides to determine surface wettability by the sessile drop method. Five µl double-distilled water droplets were placed on each tube, and the water contact angle was recorded after 1 min using Kruss G10 goniometer contact-angle measurement equipment (Krüss GmbH, Hamburg, Germany). The results are mean values of five water contact angle measurements performed at randomly chosen areas of each silicone tube.
Endothelial cell seeding, adherence, and culture

Human umbilical vein endothelial cells (HUVECs) were obtained from the National Cell Bank, Pasteur Institute of Iran (Tehran, Iran), and used between passages 3 and 6. After surface modification, triplicates of unmodified and surface-modified tubes (inner diameter 0.2 cm, length 3 cm, surface area 1.88 cm²) were put into petri dishes, sterilized with UV light, and washed twice with PBS and once with culture medium before endothelial cell seeding. One hundred microliters of an endothelial cell suspension containing 3x10⁵ endothelial cells/ml Dulbecco's minimal essential medium/F12 medium with 10% fetal bovine serum (GIBCO, Renfrewshire, Scotland) was added to the lumen of the tubes, which were rotated every 30 min for 4 h to promote homogeneous cell adhesion. Endothelial cells were either cultured in a humidified atmosphere of 5% CO₂ in air at 37°C for 6 days, with medium replacement every 2 days, or used to determine cell attachment at 4 h.

The attached cells were fixed, dehydrated in graded ethanol series, and stained with 5% Giemsa for optical microscopic examination. The number of adhered endothelial cells was determined by using an image-processing system (Image Pro Plus, version 6, Media Cybernetics, Bethesda, MD, USA). Three objective fields were randomly chosen in central and peripheral regions of each tube and the mean number of adhered cells determined. All attachment assays were run in duplicate.

Endothelial cell proliferation and GH production

The MTT (Sigma, St. Louis, MO, USA) assay was used to evaluate endothelial cell proliferation at days 2, 4, and 6 on unmodified and surface-modified silicone tubes as described previously [15, 25]. A calibration curve with known endothelial cell numbers was used to determine the number of cells. At day 6, attached cells were stained with 5% Giemsa, and three random photographs were taken in central and peripheral regions of each tube. The surface area of 50 cells from each photograph was determined by Image Pro Plus 6 software using the pixels per micrometer provided. The area covered with cells of each silicone tubes was determined by multiplying the mean individual cell area by number of cells counted. Cell confluency was expressed as percent of tube surface covered with cells, and was calculated as follows [1]:

% Confluency = [(area covered with cells (mm²)/total surface area (mm²)) x100

GH production by endothelial cells cultured on AAc Si-Col or AAc Si Nitrite-Col tubes was determined by electrochemiluminescence. After 2 days of culture the medium was harvested, and GH concentrations quantified using an automatic analyzer (Roche Elecsys 2010, Hitachi, Tokyo, Japan).
Endothelial cell morphology
The morphology of endothelial cells attached to unmodified or surface-modified silicone tubes was assessed by using scanning electron microscopy (SEM; Essen Philips XL 30 ESEM Environmental, Philips, Amsterdam, The Netherlands). The tubes were cut longitudinally to observe cell morphology in the lumen of cell-seeded silicone tubes. After 6 days of culture, the tubes with adhered cells were rinsed with PBS, fixed with 4% (v/v) glutaraldehyde in PBS for 30 min at 4°C, washed with ultrapure water, dehydrated in graded ethanol series, and dried at room temperature. The tubes were mounted on SEM stubs, and gold-coated (10-20 nm thickness) by vapor deposition using a sputter coater with a gold (Au) target for conductance and high resolution imaging. SEM imaging was performed using 20 kV electron accelerating voltage, 15 mm working distance, and magnifications x200, and x1000.

Platelet adhesion on AAc Si-Nitrite-Col tubes
Platelet-rich plasma (PRP) was obtained from the Iranian Blood Transfusion Organization. A final concentration of 15x10⁴ platelets/mm³ was used, with a viscosity of 1 cp, which is the same as the viscosity of culture medium [26]. Polyethylene glycol-grafted silicone (PEG Si) tubes were prepared as described previously [27] and used as a negative control. In short, plasma pretreated silicone tubes were immersed in PEG/ethanol solution (20 g/l) and physically adsorbed PEG was grafted on silicone using plasma treatment for 3 min. Un-grafted PEG on the silicone surface was removed by washing tubes by methanol.

Each Si, AAc Si, AAc Si-Col, AAc Si-Nitrite-Col, and PEG Si tube added to a centrifuge tube containing 2 ml PRP, and centrifuged at 700xg for 1 h at 37°C. Each tube was run in triplicate. Then silicone tubes were removed from the centrifuge tubes. The number of platelets in the PRP solution was measured by using a blood cell counter (Medonic CA 530, E. Merck, Darmstadt, Germany). Adhesion of platelets to the silicone tubes was calculated as follows [26]:

\[
\% \text{ Platelet Adhesion} = \left( \frac{P_s - P_c}{P_s} \right) \times 100
\]

in which \( P_s \) is the number of platelets in the PRP solution before, and \( P_c \) is the number of platelets in the same PRP solution after incubation with silicone tube.

Statistical analysis
All data are expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance, and the significance of differences among means was determined by post-hoc comparisons, using Bonferroni’s method. Two way analysis of variance with pairwise comparison was used to assess differences among means between groups and over time. Differences were considered significant if \( p<0.05 \).
RESULTS

Collagen immobilization on AAc Si tubes
An AAc graft density of 420±28 µg/cm² was achieved with 0.5 min pretreatment and 3 min copolymerization. Moreover, the amount of collagen immobilized onto AAc Si-Col tubes was 19.1 µg/cm² before extensive washing with water, and 18.8 µg/cm² after washing, indicating stable collagen bonding on the AAc Si tubes (Table 1). There was no significant difference between the amount of collagen immobilized on AAc Si-Col tubes and on AAc Si-Nitrite-Col tubes with 500 µM sodium nitrite (the highest concentration used in this study) in conjugate before (p=0.36) and after (p=0.7) washing.

Table 1. Collagen adsorbed on AAc Si-Col, and AAc Si-Nitrite-Col tubes with 500 µM sodium nitrite in conjugate before and after washing. Values are mean ± standard deviation for 3 independent experiments.

<table>
<thead>
<tr>
<th>Tube, surface modification</th>
<th>Abbreviation</th>
<th>Acrylic acid graft density (µg/cm²)</th>
<th>Collagen adsorbed before washing (µg/cm²)</th>
<th>Collagen adsorbed after washing (µg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen immobilized acrylic acid-grafted silicone</td>
<td>AAc Si-Col</td>
<td>420 ± 28</td>
<td>19.1 ± 3.0</td>
<td>18.8 ± 1.7</td>
</tr>
<tr>
<td>Sodium nitrite-collagen conjugate immobilized acrylic acid-grafted silicone</td>
<td>AAc Si-Nitrite-Col</td>
<td>420 ± 28</td>
<td>21.2 ± 1.9</td>
<td>18.1 ± 2.6</td>
</tr>
</tbody>
</table>

Nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes
A scheme of the suggested mechanism for the conjugation of sodium nitrite to collagen and release of nitrite and acidified nitrite from the sodium nitrite-collagen conjugate immobilized on silicone tubes is depicted in Figure 1. The absolute amount of nitrite and acidified nitrite released from AAc Si-Nitrite-Col tubes with 0-500 µM sodium nitrite in conjugate was measured as nitrite accumulation in the medium at 4 h (Figure 2A), and 48 h (Figure 2B). At both 4 and 48 h, there was a dose-dependent relationship between the initial sodium nitrite concentration in the sodium nitrite-collagen conjugate coating of AAc Si-Nitrite-Col tubes and the amount of nitrite and acidified nitrite released (Figure 2). Nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes with 0-500 µM sodium nitrite in conjugate at 4
and 48 h was also expressed as percentage of the initial sodium nitrite concentration in conjugate (Figure 2C,D). The percentage nitrite and acidified nitrite released of the initial sodium nitrite in conjugate from AAc Si-Nitrite-Col tubes was ranging from 6% (AAc Si-Nitrite-Col tubes with 500 µM sodium nitrite in conjugate) to 12% (AAc Si-Nitrite-Col tubes with 10 µM sodium nitrite in conjugate) at 4 h (Figure 2C), and from 46% (AAc Si-Nitrite-Col tubes with 50 µM sodium nitrite in conjugate) to 57% (AAc Si-Nitrite-Col tubes with 100 µM sodium nitrite in conjugate) at 48 h (Figure 2D).

**Figure 1.** Sodium nitrite (NaNO₂) dissociates into ions (Na⁺, NO₂⁻) after dissolving in water. The acetic acid used to dissolve collagen in water causes an acidic condition (pH~3) resulting in positively charged amino acids along the collagen molecule, thereby making the entire collagen molecule positively charged. After adding sodium nitrite solution to the collagen solution, the negatively charged NO₂⁻ ions are electrostatically entrapped within positively charged collagen chain entanglements, and sodium nitrite-collagen conjugate is made. Some of the entrapped NO₂⁻ ions convert to nitrous acid (HNO₂) in the acidic environment of the collagen solution. Conjugate mixing at 4°C prevents fast decomposition of HNO₂. By adding the sodium nitrite-collagen conjugate into AAc Si tubes, the carboxyl groups of AAc generate carbodiimide bonds with collagen, and help to immobilize sodium nitrite-collagen conjugate on the silicone surface. By filling the AAc Si-Nitrite-Col tubes with aqueous cell culture medium, the polymeric collagen might swell resulting in the release of the entrapped NO₂ and HNO₂. HNO₂ can then easily decompose into NO, NO₂, and H₂O at 37°C.
Figure 2. Nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes measured as nitrite accumulation in the culture medium at 37°C after 4 and 48 h. Nitrite and acidified nitrite release (in µM) from AAc Si-Nitrite-Col tubes increased with increasing the initial sodium nitrite concentrations in conjugate at (A) 4 h and (B) 48 h. Nitrite and acidified nitrite release (in % of the amount of initial sodium nitrite in conjugate) from AAc Si-Nitrite-Col tubes ranged from 6% to 12% at (C) 4 h, and from 46% to 57% at (D) 48 h. n=3. AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone. *Significantly different from 5 µM sodium nitrite in conjugate, p<0.05, **p<0.005, and ***p<0.0005.

Wettability and endothelial cell adhesion on surface-modified silicone tubes
The wettability and cell adhesion on surface-modified tubes, e.g. AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col, were assessed and compared with Si tubes (Table 2). The average water contact angle of silicone tubes was >50% decreased after AAc grafting (Si: 102°±4°; AAc Si: 42°±2°). Collagen coating of AAc Si tubes increased the water contact angle from 42°±2° (AAc Si) to 56°±2° (AAc Si-Col). The water contact angles of AAc Si-Col tubes and AAc Si-Nitrite-Col tubes with 5-500 µM
sodium nitrite in conjugate were similar, ranging from 51° to 56°. The number of endothelial cells attached to AAc Si-Col tubes and AAc Si-Nitrite-Col tubes with 5-500 μM sodium nitrite in conjugate was also similar, ranging from 98 cells/mm² to 115 cells/mm². The number of endothelial cells attached to AAc Si-Nitrite-Col tubes with 500 μM sodium nitrite in conjugate was similar to the number of cells attached to AAc Si-Col tubes, and 1.8-fold higher (p<0.005) than the number of cells attached to Si tubes (AAc Si-Nitrite-Col tubes: 110±8 cells/mm²; AAc Si-Col tubes: 115±14 cells/mm²; Si tubes: 63±12 cells/mm², mean±SD). Only few endothelial cells were attached to AAc Si tubes; the number of cells attached to Si tubes was 3-fold higher (p<0.005) than the number of cells attached to AAc Si tubes (Si tubes: 63±12 cells/mm²; AAc Si tubes: 21±5 cells/mm², mean±SD).

Table 2. Wettability and endothelial cell attachment to Si, AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col tubes with 5-500 μM sodium nitrite in conjugate. Inner diameter silicone tube: 2 mm; length tube: 30 mm. Tubes were incubated with 3x10⁵ endothelial cells/ml, and cell attachment was determined after 4 h. Listed are also the abbreviations used for the different surface modifications used in this study. Values are mean ± standard deviation for 3 independent experiments.

<table>
<thead>
<tr>
<th>Tube, surface modification</th>
<th>Abbreviation</th>
<th>Wettability, water contact angle (°)</th>
<th>Number of adhered cells, (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone</td>
<td>Si</td>
<td>102.1 ± 4.0</td>
<td>63 ± 12</td>
</tr>
<tr>
<td>Acrylic acid-grafted silicone</td>
<td>AAc Si</td>
<td>42.2 ± 2.0</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Collagen immobilized acrylic acid-grafted silicone</td>
<td>AAc Si-Col</td>
<td>56.3 ± 2.0</td>
<td>115 ± 14</td>
</tr>
<tr>
<td>Sodium nitrite-collagen conjugate immobilized acrylic acid-grafted silicone with different sodium nitrite concentrations in conjugate (μM)</td>
<td>AAc Si-Nitrite-Col</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM</td>
<td>53.1 ± 1.5</td>
<td>101 ± 10</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>54.4 ± 3.5</td>
<td>112 ± 5</td>
<td></td>
</tr>
<tr>
<td>25 μM</td>
<td>56.2 ± 4.0</td>
<td>104 ± 3</td>
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<td>50 μM</td>
<td>51.5 ± 3.1</td>
<td>100 ± 13</td>
<td></td>
</tr>
<tr>
<td>100 μM</td>
<td>52.2 ± 1.8</td>
<td>115 ± 9</td>
<td></td>
</tr>
<tr>
<td>250 μM</td>
<td>54.3 ± 2.7</td>
<td>98 ± 11</td>
<td></td>
</tr>
<tr>
<td>500 μM</td>
<td>55.2 ± 3.0</td>
<td>110 ± 8</td>
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</table>
**AAc Si-Nitrite-Col tubes affect GH production by endothelial cells**

AAc Si-Nitrite-Col tubes with different concentrations of sodium nitrite caused a dose-dependent increase in GH production by endothelial cells, with a maximum effect at 25 µM initial sodium nitrite in the conjugate (Figure 3). The amount of GH produced by endothelial cells on AAc Si-Nitrite-Col tubes with 25 µM initial sodium nitrite was 2.5-fold higher (p<0.005) than on AAc Si-Col tubes, 1.6-fold higher (p<0.05) than on AAc Si-Nitrite-Col tubes with 50 µM initial sodium nitrite, and 2-fold higher (p<0.05) than on AAc Si-Nitrite-Col tubes with 500 µM initial sodium nitrite in the conjugate.

**Figure 3.** Effect of the initial sodium nitrite concentration in a sodium nitrite-collagen conjugate coating of AAc Si-Nitrite-Col tubes on GH production by endothelial cells after 2 days of culture. Initial sodium nitrite concentration of 5-50 µM in sodium nitrite-collagen conjugate coating resulted in enhanced GH production, with a maximum effect at 25 µM sodium nitrite in the conjugate. AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone. *Significant effect of sodium nitrite in conjugate compared to control without sodium nitrite, p<0.05, **p<0.005, #Significantly different from 25 µM sodium nitrite in conjugate, p<0.05.

**AAc Si-Nitrite-Col tubes affect endothelial cell proliferation**

Initial sodium nitrite concentrations of 5-50 µM in the conjugate increased, but concentrations higher than 50 µM sodium nitrite did not affect endothelial cell number on AAc Si-Nitrite-Col tubes (Figure 4). Maximal stimulation of cell number...
after 2 days was obtained when cells were cultured on AAc Si-Nitrite-Col tubes with 25 µM initial sodium nitrite in the conjugate. The number of endothelial cells on AAc Si-Nitrite-Col tubes with 25 µM initial sodium nitrite was 1.8-fold higher (p<0.005) than on AAc Si-Col tubes, 1.3-fold higher (p<0.05) than on AAc Si-Nitrite-Col tubes with 50 µM initial sodium nitrite, 1.8-fold higher (p<0.05) than on AAc Si-Nitrite-Col tubes with 100 µM initial sodium nitrite, 2.1-fold higher (p<0.005) than on AAc Si-Nitrite-Col tubes with 250 µM initial sodium nitrite, and 2.3-fold higher (p<0.005) than on AAc Si-Nitrite-Col tubes with 500 µM initial sodium nitrite in the sodium nitrite-collagen conjugate.

**Figure 4.** Effect of the initial sodium nitrite concentration in a sodium nitrite-collagen conjugate coating of AAc Si-Nitrite-Col tubes on the number of endothelial cells after 2 days of culture. AAc Si-Nitrite-Col tubes with 50 µM or less initial sodium nitrite in conjugate showed an increase in the number of endothelial cells, with a maximum effect at 25 µM sodium nitrite in the conjugate. The number of cells on AAc Si-Nitrite-Col tubes with initial 25 µM sodium nitrite was 1.8-fold higher than on AAc Si-Col tubes. AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone. *Significant effect of sodium nitrite in conjugate compared to control without sodium nitrite, p<0.05, **p<0.005, #Significantly different from 25 µM sodium nitrite in conjugate, p<0.05, ##p<0.005.

Endothelial cell proliferation on Si, AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col (with 25 µM initial sodium nitrite in the conjugate) tubes was compared after 2, 4, and 6 days of culture (Figure 5A). The number of endothelial cells on AAc Si
tubes was significantly decreased compared with Si tubes by 4-fold (p<0.0005, day 4) and 3-fold (p<0.005, day 6). On the other hand, the number of endothelial cells on AAc Si-Col tubes was 3-fold (p<0.0005, day 4) and 3.4-fold (p<0.0005, day 6) increased compared with the number of cells on Si tubes. Nitrite incorporation in AAc Si-Nitrite-Col tubes further increased the number of endothelial cells by 68% at day 2 (p<0.05), 34% at day 4 (p<0.05), and 28% at day 6 (p<0.005), compared with AAc Si-Col tubes. Thus endothelial cells showed the highest proliferation rate on AAc Si-Nitrite-Col tubes at all time points measured. The number of endothelial cells increased on AAc Si-Col tubes and AAc Si-Nitrite-Col tubes with increased incubation time, while such an increase over time was not observed with Si and AAc Si tubes.

Optical micrographs of endothelial cells attached to Si, AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col tubes were used to assess endothelial cell confluency at day 6 (Figure 5Ba-d). Cell confluency differed dependent on the surface modification used. The number of cells on Si tubes was very low, as well as cell confluency (22%) within 6 days (Figure 5Ba). Most cells did not adhere while attached cells showed poor proliferation on AAc Si tubes in the absence of collagen coating, resulting in increased cell death (data not shown). As a result, cells were only scarcely covering the silicone surface (7%; Figure 5Bb). Cell layers were more confluent (74%) on AAc Si-Col tubes than on Si and AAc Si tubes (Figure 5Bc). Although endothelial cells on AAc Si-Col tubes proliferated well, they did not form a confluent monolayer on the silicone surface. The high rate of endothelial cell proliferation on AAc Si-Nitrite-Col tubes resulted in 95% cell confluency within 6 days (Figure 5Bd).

Morphology of endothelial cells on surface-modified silicone tubes
The morphology of attached endothelial cells on unmodified and surface-modified silicone tubes was different after 6 days of culture, dependent on the type of surface modification (Figure 6). SEM images revealed that cells on Si tubes did not spread well (Figure 6a). Only few cells were attached on AAc Si tubes, exhibiting "round" morphology (Figure 6b). In contrast, cells attached onto AAc Si-Col tubes kept their natural spindle-shaped morphology (Figure 6c). This suggests that a collagen coating provides a highly compatible substratum for endothelial cells. Endothelial cells on AAc Si-Nitrite-Col tubes displayed a flat, cobble stone-shaped morphology, while no "round" cells were seen (Figure 6d).
Figure 5. Endothelial cell proliferation and confluency on Si, AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col tubes with 25 µM sodium nitrite in conjugate after 2, 4, and 6 days of culture. (A) Endothelial cell proliferation at days 2, 4, and 6. Cell number on AAc Si tubes decreased compared with that on Si tubes at all time points. Cell number on AAc Si-Col tubes increased compared with Si tubes, and even further increased on AAc Si-Nitrite-Col tubes. (B) Optical micrographs showing endothelial cell confluency after 6 days. (a) Si, (b) AAc Si, (c) AAc Si-Col, (d) AAc Si-Nitrite-Col tubes. Magnification x200. Cell confluency was low (22%) on Si tubes, and even lower on AAc Si tubes (7%) in the absence of a collagen coating. Cell coverage was rather high on collagen-coated tubes (74% confluency), and even higher when sodium nitrite was incorporated in the collagen coating (95% confluency). Si, silicone; AAc Si, AAc-grafted silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone. **Significantly different from Si tubes, p<0.005, ***p<0.0005, #Significant effect of sodium nitrite in conjugate, p<0.05, ##p<0.005.
Figure 6. Scanning electron microscopy (SEM) showing morphology of endothelial cells seeded on Si, AAc Si, AAc Si-Col, and AAc Si-Nitrite-Col tubes with 25 µM sodium nitrite in conjugate after 6 days of culture. (a) Si, (b) AAc Si, (c) AAc Si-Col, (d) AAc Si-Nitrite-Col. Magnification x200. Insert: SEM image of endothelial cells, magnification x1000. Endothelial cells on Si tubes and on AAc Si tubes exhibited “round” cell bodies (a, b). Endothelial cells on AAc Si-Col tubes displayed a flat, spindle-shaped morphology while no “round” cells were present (c). Cell morphology was changed to cobblestone-like morphology on AAc Si-Nitrite-Col tubes (d). Si, silicone; AAc Si, AAc-grafted silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone. Single arrow: round cell; double arrows: spindle-shaped cell; triple arrows: cobblestone-shaped cell.

Platelet adhesion on surface-modified silicone tubes
Platelet adhesion was decreased by 20% on AAc Si tubes compared with Si tubes (p<0.05). In contrast, platelet adhesion on AAc Si-Col tubes was increased by 27% compared with Si tubes (p<0.05; Figure 7). AAc Si-Nitrite-Col tubes with 25 to 500 µM initial sodium nitrite suppressed platelet adhesion compared with AAc Si-Col tubes. The higher the initial concentration of sodium nitrite in sodium nitrite-collagen conjugate, the more platelet adhesion on AAc Si-Nitrite-Col tubes was reduced. Platelet adhesion to AAc Si-Nitrite-Col tubes with an initial sodium nitrite concentration of 500 µM provided maximal inhibition by 63% (p<0.005) compared with adhesion to AAc Si-Col tubes. Platelet deposition onto PEG Si tubes, that
were used as a reference matrix, was very low compared to other unmodified and surface-modified silicone tubes (p<0.0005).

Figure 7. Effect of surface modification on platelet adhesion onto silicone tubes. Adhesion of platelets on the silicone tubes was expressed as percentage of the total number of platelets present in the PRP solution. AAc Si-Nitrite-Col tubes with 25-500 µM initial sodium nitrite in conjugate decreased platelet adhesion compared with AAc Si-Col tubes. Si, silicone; AAc Si, AAc-grafted silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-Nitrite-Col, sodium nitrite-collagen conjugate immobilized AAc-grafted silicone; PEG Si, polyethylene glycol-grafted silicone. *Significant effect of sodium nitrite in conjugate, p<0.05, **p<0.005, #Significant effect of AAc grafting or collagen immobilization on silicone tube, p<0.05.

DISCUSSION

Endothelial cell seeding on a silicone surface is generally known to improve blood compatibility of silicone-based medical devices. Silicone is a hydrophobic and inert material that does not facilitate endothelial cell adhesion and/or proliferation. In general, surface coating of biomaterials with extracellular matrix proteins such as collagen enhances cell growth [4, 6]. Collagen has excellent properties that allow cell attachment, but also undesired platelet adhesion causing thrombosis [11]. To suppress the thrombogenic properties of collagen, several anti-thrombotic factors such as heparin, aspirin, or NO donors have been immobilized on collagen [8-12].
Nitrite, the stable end-product of NO, has also been shown to have anti-thrombogenic properties in acidic environments [13-15], and therefore we investigated whether the combination of sodium nitrite, as a nitrite donor, with collagen in the presence of acetic acid improves endothelialization of silicone tubes by increasing the number of endothelial cells and GH production, as well as by decreasing platelet adhesion. The sodium nitrite concentrations were chosen based on the following published data. NO-generating sodium nitroprusside (SNP) at low concentration (1 µM) [28] but not at high concentration (>100 µM) [29] increases endothelial cell proliferation. Also, endothelial cells exposed to 0.1-100 µM SNP show increased proliferation with a maximal effect at 10 µM [30]. Finally NO release by NO-generating S-nitroso-N-acetylpenicillamine (SNAP; 50 µM) also stimulates endothelial cell proliferation [31]. Therefore, we used sodium nitrite-collagen conjugates with 0.5-500 µM initial sodium nitrite for coating of silicone tubes. Since the amount of nitrite and acidified nitrite released from coated silicone tubes containing less than 5 µM initial sodium nitrite in the conjugate was negligible, we used >5 µM initial sodium nitrite in the conjugate in the current study.

To study the interaction between a sodium nitrite-collagen conjugate coating with endothelial cells, a well-defined and stable collagen coating is required. A stable collagen coating can be obtained by carbodiimide bonds between the functional groups of collagen and the functional groups introduced to the surface of silicone tubes by plasma graft polymerization of a specific monomer [4-7]. Different functional groups, i.e. amine, carboxyl, and hydroxyl groups, have been introduced to the surface of silicone to facilitate collagen immobilization [4, 6]. In this study, collagen and sodium nitrite-collagen conjugate were immobilized onto silicone tubes by ionic interaction with a pre-determined AAc graft density. The amount of collagen immobilized on AAc Si tubes before washing and after washing was not significantly different, indicating that the carboxyl groups of AAc Si were tightly bound to the amino groups of collagen. In addition, incorporation of sodium nitrite (at all concentrations tested) in the collagen conjugate did not change the amount of immobilized collagen.

Nitrite and acidified nitrite release from AAc Si-Nitrite-Col tubes increased with increasing initial sodium nitrite concentrations in the conjugate. However, the amount of nitrite and acidified nitrite released from the AAc Si-Nitrite-Col tubes was low compared with the amount of initial sodium nitrite in conjugate at 4 h. At 500 µM initial sodium nitrite (the highest concentration used in this study), only 7% of the initial nitrite was released. This release was low in comparison to the 50% released from initially loaded diazeniumdiolate NO donor in the backbone of polyurethane after 4 h in another study [16]. The difference in release kinetics between the studies might be explained by the differences in the NO donor used (NO gas and nitrite-donor), the hydrophilicity/structure of the substratum used (AAc-grafted silicone and backbone of polyurethane), and the chemistry utilized to
bond NO/nitrite-donor to the substratum. The AAc grafting on silicone tubes before sodium nitrite-collagen conjugate immobilization, and the hydrogel-like nature of polyAAc might help the immobilized sodium nitrite molecules to retain their biological activity for a prolonged period of time [32]. Our study shows that almost half of the initial sodium nitrite in conjugate was released at 48 h (46-57%) at all initial sodium nitrite concentrations tested. This is comparable to the 70% released from initially loaded diazeniumdiolate NO donor in the backbone of polyurethane after 48 h as has been reported by others [16].

AAc grafting has been shown to create a hydrophilic surface with a low water contact angle, while linking collagen increases the water contact angle [4-7]. Our results are in accordance with these observations; we found that the water contact angle was considerably decreased on AAc Si tubes. The wettability of AAc Si-Col and AAc Si-Nitrite-Col tubes was even less than that of AAc Si tubes. This is inherent to the fact that collagen is more hydrophobic than AAc [4, 22]. Our data showing that collagen immobilization on AAc-grafted silicone tubes led to moderate wettability is a favorable condition for endothelial cell attachment, since a material surface with either a very high or a very low contact angle is not suitable for cell attachment [4-6].

Several reports claimed that, carboxyl functional groups create a higher negative surface charge than other commonly used surface functional groups, which causes inhibition of cell attachment [4, 6]. We found that endothelial cells in direct contact with carboxyl groups of AAc did not attach and showed poor proliferation. On the other hand, the immobilized collagen on the tubes created an adequate environment for endothelial cell attachment. Collagen is a main protein in the extracellular matrix responsible for cell binding to the material surface. The integrin family of cell adhesion receptors contains four collagen receptors that are involved in cell-matrix interactions [22, 23]. The presence of these receptors implicates that collagen is suitable as a matrix for endothelial cell growth in vitro, and might be the reason for increased endothelial cell attachment on AAc Si-Col tubes compared with AAc Si tubes. Although AAc Si tubes had toxic effects on endothelial cells in the absence of collagen immobilization, our results also indicate that these tubes can be used as substratum for collagen immobilization and then provide a favorable surface for endothelialization (AAc Si-Col tubes).

NO is known to inhibit platelet adhesion, and to inhibit or stimulate endothelial cell proliferation dependent on the concentration [28-31]. We found that sodium nitrite-collagen conjugate coating of AAc Si-Nitrite-Col tubes increased the number of endothelial cells. Interestingly, GH production by endothelial cells was stimulated on AAc Si-Nitrite-Col tubes containing 5-50 µM initial sodium nitrite in conjugate. Twenty-five µM initial sodium nitrite in the conjugate maximally stimulated GH production. GH is known to stimulate endothelial cell growth [34, 35]. This might explain the maximal stimulation by sodium nitrite-collagen
conjugate coating of AAc Si-Nitrite-Col tubes on the number of endothelial cells with 25 µM initial sodium nitrite in the conjugate. Endothelial cell proliferation on different surface-modified silicone tubes at 2, 4, and 6 days also showed that AAc decreases the number of endothelial cells at all time points, probably by causing an acid environment [4, 6]. This was in contrast to the high increase in cell number observed on tubes with immobilized collagen. The number of endothelial cells on AAc Si-Nitrite-Col tubes with 50 µM or less initial sodium nitrite in the conjugate was increased compared with the number of cells on AAc Si-Col tubes after 2 days of culture, with a maximum effect at 25 µM initial sodium nitrite. Therefore sodium nitrite-collagen conjugate coating with 5 to 50 µM initial sodium nitrite accelerates endothelialization of silicone tubes even more than collagen coating alone.

Endothelial cells cultured on AAc Si-Nitrite-Col tubes formed a perfect confluent (95%) monolayer, but confluency was not achieved on AAc Si-Col tubes after 6 days of culture. The morphology of endothelial cells cultured on AAc Si-Col tubes was spindle-shaped, while a round morphology was observed on AAc Si tubes. Endothelial cells on AAc Si-Nitrite-Col tubes had changed to cobblestone-like morphology. Thus sodium nitrite-collagen conjugate coating was compatible with endothelial cells providing the possibility of improved endothelialization. AAc grafting increases material surface hydrophilicity thereby preventing platelet adhesion [36]. Collagen is highly thrombogenic and induces platelet adhesion on a material surface [11]. Our data agree with these observations, since we found reduced platelet adhesion on AAc Si tubes, and increased platelet adhesion on AAc Si-Col tubes. We also observed that after incorporation of sodium nitrite into collagen in the presence of acetic acid, the use of this nitrite and acidified nitrite-generating coating with 25 to 500 µM initial sodium nitrite significantly suppressed the thrombogenic properties of collagen by decreasing platelet adhesion on the silicone surface, especially when a high initial concentration of sodium nitrite in conjugate was used. The presence of acetic acid in the sodium nitrite-collagen conjugate might change some nitrite molecules to nitrous acid, that easily decomposes into NO; that is known to inhibit platelet adhesion [20, 21]. Although AAc Si-Nitrite-Col tube with 500 µM sodium nitrite in conjugate prevented more platelet adhesion, it did not affect endothelial cell growth. These results agree with data reported by others showing that NO inhibits as well as stimulates endothelial cell proliferation dependent on the concentration [28-31]. Low NO concentrations (µmolar or sub-mmolar range) stimulate cell proliferation of a.o. fibroblasts, pancreatic tumours, myoblasts, keratinocytes, and endothelial cells [28, 30, 31, 37], while NO in the mmolar range inhibits cell proliferation [29, 38, 39]. The reason for this biphasic effect is not well understood, but it has been suggested that the NO-mediated increase in endothelial cell proliferation requires the production of reactive oxygen species (ROS), since superoxide dismutase and catalase suppress in part the stimulatory effect of NO on cell proliferation [28, 39]. NO at a
low concentration (1 µM) increases endothelial cell proliferation [28], whereas NO at a high concentration (>100 µM) has no effect [29]. SNP at 0.1-100 µM increases endothelial cell proliferation, with a maximal effect at 10 µM [30]. The release of NO by SNAP at 50 µM, but not 10 µM, stimulates endothelial cell proliferation [31]. Overall, these studies support the concept that the effect of NO on endothelial cell proliferation is concentration-dependent [39].

CONCLUSIONS

Our data shows that a nitrite and acidified nitrite-generating sodium nitrite-collagen conjugate coating of AAc Si-Nitrite-Col tubes with 5 to 50 µM initial sodium nitrite increased the number of endothelial cells, more than collagen coating alone, probably via GH production, with a maximum effect at 25 µM initial sodium nitrite, resulting in a confluent endothelial cell monolayer on a silicone surface. In addition, AAc Si-Nitrite-Col tubes with 25 to 500 µM initial sodium nitrite suppressed platelet adhesion in areas that were not fully covered with endothelial cells. Since AAc Si-Nitrite-Col tubes with 25 to 50 µM initial sodium nitrite increases endothelial cell proliferation and inhibits platelet aggregation, this suggests that sodium nitrite-collagen conjugate coatings are highly promising to promote endothelialization of silicone materials in blood-contacting devices.

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