CHAPTER 5

Sustained Release of Growth Hormone and Sodium Nitrite from Biomimetic Collagen Coating Immobilized on Silicone Tubes Improves Endothelialization

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ABSTRACT

Biocompatibility of biomedical devices can be improved by endothelialization of blood-contacting parts mimicking the vascular endothelium’s function. Improved endothelialization might be obtained by using biomimetic coatings that allow local sustained release of biologically active molecules, e.g. anti-thrombotic and growth-inducing agents, from nanoliposomes. We aimed to test whether incorporation of growth-inducing nanoliposomal growth hormone (nGH) and anti-thrombotic nanoliposomal sodium nitrite (nNitrite) into collagen coating of silicone tubes enhances endothelialization by stimulating endothelial cell proliferation and inhibiting platelet adhesion.

Collagen coating stably immobilized on acrylic acid-grafted silicone tubes decreased the water contact angle from 102° to 56°. Incorporation of 50 or 500 nmol/ml nNitrite and 100 or 1000 ng/ml nGH into collagen coating decreased the water contact angle further to 48°. Endothelial cell number was increased after surface coating of silicone tubes with collagen by 1.6-fold, and with nNitrite-nGH-collagen conjugate by 1.8-3.9-fold after 2 days. After 6 days, endothelial cell confluency in the absence of surface coating was 22%, with collagen coating 74%, and with nNitrite-nGH-collagen conjugate coating 83-119%. In the absence of endothelial cells, platelet adhesion was stimulated after collagen coating by 1.3-fold, but inhibited after nNitrite-nGH-collagen conjugate coating by 1.6-3.7-fold. The release of anti-thrombotic prostaglandin I$_2$ from endothelial cells was stimulated after nNitrite-nGH-collagen conjugate coating by 1.7-2.2-fold compared with collagen coating.

In conclusion, our data shows improved endothelialization using nNitrite-nGH-collagen conjugate coating on silicone tubes suggesting that these coatings are highly suitable for use in blood-contacting parts of biomedical devices.

Keywords
Biomimetic coating, Endothelialization, Growth hormone, Nanoliposome, Sodium nitrite
INTRODUCTION

The biocompatibility of synthetic materials is of crucial importance in the development of biomedical devices that are in continuous contact with blood, such as artificial lungs, vascular grafts, heart valves, stents, etc. [1, 2]. Endothelial cell seeding of blood-contacting parts of biomedical devices minimizes the need for anticoagulants when using these devices [3, 4]. The surface of most commercially available synthetic materials used in biomedical devices, such as silicone, is highly hydrophobic and therefore not suitable for endothelial cell seeding. Modification of silicone surface by chemical surface treatment [5], glow discharge treatment [4], or coating with extracellular matrix proteins [6, 7], may improve endothelial cell adhesion and proliferation.

Collagen, the main protein in the extracellular matrix of connective tissue, is often used for tissue engineering purposes due to its key role in cell adhesion to the synthetic material surface, which involves integrins. The integrin family of cell adhesion receptors contains four collagen receptors that are involved in cell-matrix interactions [7, 8]. The presence of these receptors implicates that collagen has excellent properties that allow cell attachment, but also undesired platelet adhesion in areas of a collagen coating that are not fully covered by endothelial cells, which causes thrombosis [9]. A practical solution to the problem of collagen thrombogenicity is the creation of collagen conjugate coatings that release anticoagulants such as heparin [9, 10], aspirin [11], hirudin [12], thrombomodulin [13], and nitric oxide (NO) [9, 14] at the blood-polymer interface. We have previously shown that nitrite and acidified nitrite released from biomimetic sodium nitrite-collagen conjugate coatings significantly decreases platelet adhesion and increases endothelialization of silicone tubes [15]. The rate of endothelialization can be improved by immobilization of specific exogenous growth factors or growth hormones. Endothelial cells seeded at low density on a material surface can be stimulated by growth-inducing agents to rapidly form a confluent monolayer in a few days [16]. Growth hormone (GH), also known as somatropin, is a mitogen for a variety of cell types, including smooth muscle cells, fibroblasts, adipocytes, macrophages, lymphocytes, and endothelial cells [17]. It plays a role in controlling cell metabolism, balanced growth, and differentiation by modulating the synthesis of multiple mRNA species such as insulin-like growth factor 1 [17].

Biomaterials co-immobilized with different biomolecules to improve endothelialization, e.g. anti-thrombotic and cell growth-inducing biomolecules, show simultaneously anti-thrombotic and growth-inducing properties [16, 18]. Currently the use of anti-thrombotic or growth-inducing agents is limited by their short half-life, renal toxicity, physical and chemical instability, and rapid clearance. Prolonged release of anti-thrombotic or growth-inducing agents from liposomes, that function as drug delivery carriers, has gained considerable attention, since
liposomes are biocompatible, biodegradable, and capable of releasing encapsulated drugs in a sustained manner, and transporting drugs across biological membranes [19, 20].

In this study, we aimed to test whether sustained release of the anti-thrombotic agent sodium nitrite and the growth-inducing agent GH from collagen conjugate improves endothelialization by inhibiting platelet adhesion when silicone is not yet fully covered by endothelial cells, and by stimulating fast endothelial cell coverage of the silicone. First a stable collagen conjugate coating able to withstand fluid shear stress on silicone tubes was developed as described earlier [21]. Collagen solution blended with nNitrite and/or nGH, was co-immobilized on the internal surface of acrylic acid (AAc)-grafted silicone tubes. The release of nitrite and/or GH from the surface-modified silicone tubes was determined, and the effect of the released molecules on endothelial cell proliferation and prostaglandin I\(_2\) (PGL\(_2\)) release, as well as on platelet adhesion 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. Sodium nitrite and other chemicals for the Griess assay were obtained from Merck (Kenilworth, NJ, USA), and were of the highest purity available. Somatropin (GH) was obtained from Novo Nordisk (Aalborg, Denmark). 1,2 Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (amino-PEG lipid) and dipalmitoyl phosphatidylcholine (DPPC) were purchased from Lipoid Gmbh (Ludwigshafen, Germany). Cholesterol and sucrose were purchased from Sigma Aldrich (Gillingham, Dorset UK). Chloroform was obtained from Duksan (Gyeonggi, Korea).

Plasma graft polymerization of AAc onto silicone tubes
Silicone tubes were cleaned three times with 70% (v/v) ethanol, and one time with de-ionized water for 5 min. They were placed on the bottom of a reaction chamber (Seren R600, Anatech ltd, CA, USA), which was evacuated to 0.6 mbar, and pretreated with 60 W of oxygen plasma for 0.5 min. Plasma surface-modified tubes were immersed in a solution containing 30% AAc in de-ionized water for 30 min at room temperature. Then the tubes were removed from the solution and air-dried at 40°C for 5 min. Tubes with a pre-adsorbed surface layer of reactive AAc monomers were placed into a reaction chamber for plasma graft copolymerization for 3 min to produce AAc-grafted silicone tubes (AAc Si). The residual monomers
and homopolymers were removed by washing twice with warm de-ionized water in an ultrasonic water bath, followed by incubation in distilled water for 24 h [15, 21].

**nNitrite and nGH preparation**

Sodium nitrite solution at 0.01 M was prepared by dissolving sodium nitrite in water containing 0.02 M acetic acid at 4ºC. Some of the nitrite ions in the solution convert to acidified nitrite (nitrous acid; HNO₂) in the presence of acetic acid. Preparation of the solution at 4ºC prevents fast decomposition of HNO₂ into NO, NO₂, and H₂O [15]. From now on we refer to the total amount of nitrite and acidified nitrite as “nitrite”. GH at 90 µg/ml was prepared by dissolving somatropin in water.

nNitrite and nGH were prepared using the thin-film hydration technique as described earlier [22, 23]. A lipid phase (total weight: 10 times the sodium nitrite or GH weight) was prepared from a lipid mixture of DPPC, cholesterol, and amino-PEG (molar percentage ratio: 83:15:2), and dissolved in chloroform (1 ml chloroform/20 mg of lipid phase) in a round bottom flask. The solvent was removed in an IKA RV10 rotary evaporator (IKA, Deutschland, Germany) at 150 rpm, 50ºC, during 30 min under reduced pressure. Subsequently, nitrogen was blown over the dried lipid film for 5 min to remove residual solvent.

Dry thin lipid film was hydrated with 10 ml sodium nitrite or GH solution. Afterwards, sucrose powder (6 mg sucrose/1 mg lipid phase) was added, and the dispersion obtained was gently shaken for 30 min allowing the formation of multilamellar vesicles containing sodium nitrite or GH. The multilamellar vesicles were put in an ice bath and sonicated using a probe type ultrasonicator (Misonix sonicator s94000, QSONICA, Newtown, CA, USA) to produce nanoliposomes. Sonication was performed at a net power of 20 W and a frequency of 20 KHz, 20 sec on and 10 sec off, with a total process time of 30 min. Unwanted titanium particles that possibly disparted from the probe of the sonicator in the nanoliposome solution were removed by centrifugation in a Hettich Universal 320 R centrifuge (Buckinghamshire, UK) at 9000 rpm for 20 min.

To assess the encapsulation efficiency of nNitrite or nGH, the liposomal suspension was transferred to the upper chamber of an Amicon centrifugal filter tube (EMD Millipore, Darmstadt, Germany) and centrifuged at 5000 rpm for 30 min. The amount of non-encapsulated sodium nitrite or GH was determined by measuring the concentration of sodium nitrite or GH in the suspension in the lower chamber of the centrifugal filter tube. Encapsulation efficiency of nNitrite or nGH was calculated as the percentage of the total sodium nitrite (NaNO₂) or GH concentration using the following equation [23]:

$$\text{encapsulation efficiency} = \frac{\text{total [NaNO}_2\text{] or [GH]} - \text{non-encapsulated [NaNO}_2\text{] or [GH]}}{\text{total [NaNO}_2\text{] or [GH]}} \times 100\%$$
The mean hydrodynamic diameter of the liposomes was determined by the dynamic light scattering technique (DLS) in a Zeta sizer Nano ZS (Malvern Instruments, CO, Malvern, UK) equipped with a 633 nm laser source [23]. The nanoliposomal solution was diluted with phosphate buffered saline (PBS) to reach a viscosity of 0.933 cP. DLS analysis was performed at room temperature using a refraction index of 1.33. All liposome hydrodynamic diameter measurements were run in triplicate. Finally nNitrite and nGH were stored at 4°C until use. For cell culture experiments, nNitrite and nGH were sterilized using 0.2 µm filters (EMD Millipore, Darmstadt, Germany).

**nNitrite-collagen and/or nGH-collagen conjugate immobilization on AAc Si tubes**

nNitrite stock solution (0.01 M) and nGH stock solution (90 µg/ml) were used to prepare conjugates with 50 or 500 nmol/ml nNitrite, 100 or 1000 ng/ml nGH, a combination of 50 nmol/ml nNitrite and 100 ng/ml nGH, 50 nmol/ml nNitrite and 1000 ng/ml nGH, 500 nmol/ml nNitrite and 100 ng/ml nGH, and 500 nmol/ml nNitrite and 1000 ng/ml nGH 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 nNitrite-collagen (nNitrite-Col), nGH-collagen (nGH-Col), and nNitrite-nGH-collagen (nNitrite-nGH-Col) conjugates.

AAc Si tubes were immersed in 30 ml of 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-hydroxysuccinimide (NHS; Fluka, Neu-Ulm, Germany) [7]. The solution was stirred for 5 h at 4°C to activate the carboxyl groups on AAc Si tubes. Then collagen immobilization on AAc Si tubes with activated carboxyl groups was achieved by filling the tubes with nNitrite-Col, nGH-Col, or nNitrite-nGH-Col conjugate at 4°C for 24 h. nNitrite-Col conjugate immobilized AAc-grafted silicone (AAc Si-nNitrite-Col) tubes, nGH-Col conjugate immobilized AAc-grafted silicone (AAc Si-nGH-Col) tubes, and nNitrite-nGH-Col conjugate immobilized AAc-grafted silicone (AAc Si-nNitrite-nGH-Col) tubes were washed with water for 1 min to remove unbound collagen and/or nanoliposomes, and stored at 4°C.

**Characterization of surface-modified silicone tubes**

Unmodified and surface-modified silicone tubes, i.e. Si, AAc Si, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAC Si-nNitrite-nGH-Col, were dried with nitrogen gas, cut longitudinally, and glued on glass microscope slides to determine surface wettability by the sessile drop method [24]. 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.

The effect of nNitrite-Col, nGH-Col, or nNitrite-nGH-Col conjugate immobilization on the mechanical properties of silicone tubes was evaluated by holding both ends of each tube (length 9 mm) in a specific grip of an in-house fabricated uniaxial testing instrument, and pulling uniaxially until tension break. The ultimate tensile strength was determined based on the peak load and the initial surface area of each tube. The percent elongation-at-break was obtained from the ratio between the elongated length at the time of failure (l) and initial length (l₀) of each silicone tube [25].

To verify (indirectly) the binding of nNitrite and nGH to silicone, and to assess the continuous release of nitrite and/or GH, the amount of nitrite released from AAc Si-nNitrite-Col tubes, and the amount of GH released from AAc Si-nGH-Col tubes were determined after 4, 12, 24, 48, 72, 96, and 120 h of incubation with Dulbecco’s minimal essential medium (DMEM/F12 (1/1, v/v) (Gibco, Life Technologies, Grand Island, NY, US). To investigate a possible effect of nanoliposomes on sodium nitrite or GH release, the amount of nitrite or GH released from nNitrite or nGH was compared with that from the free form of sodium nitrite or GH in the conjugates with collagen. The free form of sodium nitrite at 50 or 500 nmol/ml, or GH at 100 or 1000 ng/ml were mixed with collagen, gently shaken for 1 h at 4°C to obtain homogeneous sodium nitrite-collagen (Nitrite-Col) or GH-collagen (GH-Col) conjugates, and immobilized on AAc-grafted silicone tubes to prepare AAc Si-Nitrite-Col or AAc Si-GH-Col tubes. Nitrite release from AAc Si-nNitrite-Col and AAc Si-Nitrite-Col tubes was measured as nitrite (NO₂⁻) accumulation in the culture medium, using Griess reagent containing 1% sulfanilamide, 0.1% naphtylethenediamine-dihydrochloride, and 2.5 M H₃PO₄ [15, 26]. Serial dilutions of NaNO₂ in medium were used as standard curve. The absorbance was measured at 545 nm with a microplate reader (Stat Fax-2100, Miami, FL, USA). Nitrite release was 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%). GH release from AAc Si-nGH-Col and AAc Si-GH-Col tubes was determined by electrochemiluminescence. The GH concentration in the medium was quantified using an automatic analyzer (Roche Elecsys 2010, Hitachi, Tokyo, Japan).

The stability of the nNitrite or nGH on surface-modified silicone tubes was assessed after storage for 1, 2, and 3 months at 4°C, to assure that storage after preparation until clinical use does not affect nanoliposome stability. For this purpose, the AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate were stored. After 1, 2, and 3 months of storage at 4°C, the tubes were put in culture medium at 37°C, and the release of nitrite and GH
was determined after 4, 12, 24, 48, 72, 96, and 120 h of incubation with culture medium and calculated as a function of the storage time [23].

**Endothelial cell seeding, viability, and proliferation**

Human umbilical vein endothelial cells (HUVECs) from the National Cell Bank, Pasteur Institute of Iran (Tehran, Iran), were used between passages 3 and 6 to evaluate cell attachment and proliferation on modified silicone tubes. After surface modification, triplicates of unmodified and surface-modified silicone tubes were put into petri dishes, sterilized with UV, washed twice with PBS solution, and washed once with culture medium before cell seeding. Hundred µl of endothelial cell suspension with $3 \times 10^5$ cells/ml DMEM/F12 medium containing 10% fetal bovine serum (Gibco, Renfrewshire, Scotland) was infused from one end into the lumen of each sterile silicone tube using a syringe. Silicone tubes with endothelial cells were then rotated every 30 min for 4 h to promote homogeneous cell adhesion to the inner surface of the tubes. Then cells were cultured for 6 days in a humidified atmosphere of 5% CO$_2$ in air at 37°C, with medium replacement every 2 days.

The cytotoxicity of nanoliposomes was assessed by a live/dead assay. Unmodified silicone tubes were seeded with endothelial cells, and either filled with culture medium or with 10% (v/v) empty nanoliposomes in culture medium. After 48 h of culture, cell viability was observed using acridine orange-propidium iodide staining. Silicone tubes containing endothelial cells were incubated with an acridine orange-propidium iodide mixture for 10 min, and monitored under a fluorescence microscope. Live cells stained green while dead cells stained red [27].

Endothelial cell proliferation on surface-modified silicone tubes was estimated at days 2, 4, and 6 by using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) as described elsewhere [11, 27]. The absorbance was measured at 545 nm using an ELISA reader (Stat Fax-2100, Miami, FL, USA). The number of endothelial cells was quantified using a calibration curve with known cell numbers. At day 6, the attached cells were fixed, dehydrated in graded ethanol series, and stained with 5% Giemsa for optical microscopic examination. Three random photographs were taken in central and peripheral regions of each tube, and the surface area of 50 cells from each photograph was determined by an image-processing system (Image Pro Plus, version 6, Media Cybernetics, Bethesda, MD, USA) using the pixels per µm provided. The area covered with cells on each silicone tube was determined by multiplying the mean individual cell area by the number of cells counted. Cell confluency was expressed as percent of the tube surface covered with cells, and calculated as follows [28]:

$$\text{Confluency} = \left[ \frac{\text{area covered with cells (mm}^2\text{)}}{\text{total surface area (mm}^2\text{)}} \right] \times 100\%$$
NO and PGI₂ release by endothelial cells cultured on surface-modified silicone tubes

After 2 days of endothelial cell culture on unmodified or surface-modified silicone tubes, i.e. Si, AAc Si-Col, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAc Si-nNitrite-nGH-Col, 0.5 ml medium was harvested for NO and PGI₂ determination. NO release by endothelial cells was measured as nitrite (NO₂⁻) accumulation in the medium, using the Griess method. PGI₂ production was determined by measuring the concentration of its stable metabolite 6-keto-prostaglandin F₁α (6-keto-PGF₁α) using a 6-keto-PGF₁α enzyme immunoassay kit (Enzo, Lorrach, Germany) according to the manufacturer’s protocol [29].

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 the probability value (p)<0.05.

RESULTS

Biomimetic modification of silicone tubes using nNitrite-Col, nGH-Col, and nNitrite-nGH-Col conjugates was successfully established. Figure 1 shows the possible mechanism for the immobilization of nNitrite-Col, nGH-Col, and nNitrite-nGH-Col conjugates on silicone tubes, and the release of nitrite and/or GH from surface-modified silicone tubes. The abbreviations used for surface-modified silicone tubes are provided in the legend of Figure 1.

Encapsulation efficiency and size of nanoliposomes

The encapsulation efficiency of sodium nitrite or GH into nanoliposomes was 91.3±1.4% for sodium nitrite, and 98.1±2.1% for GH. The average size of nNitrite (diameter 112 nm) and nGH (diameter 117 nm) was similar.
Figure 1. Schematic illustration of biomimetic modification of silicone tubes. Plasma surface-modified silicone tubes were immersed in an aqueous solution of AAc followed by plasma graft polymerization on a reabsorbed layer of AAc on silicone. AAc Si tubes with activated carboxyl groups were filled with nNitrite-Col conjugate to produce AAc Si-nNitrite-Col tubes, or with nGH-Col conjugate to produce AAc Si-nGH-Col tubes or with nNitrite-nGH-Col conjugate to produce AAc Si-nNitrite-nGH-Col tubes, and stored for 24 h at 4ºC for conjugate immobilization. AAc provides reactive carboxyl groups for generating carbodiimide bonds with amine groups on both nanoliposomes and collagen molecules. By filling the AAc Si-nNitrite-nGH-Col tubes with aqueous culture medium, the polymeric collagen might swell resulting in the release of the entrapped nGH and nNitrite particles. Si, silicone; AAc Si, acrylic acid-grafted silicone; AAc Si-Col, collagen immobilized acrylic acid-grafted silicone; AAc Si-nNitrite-Col, nanoliposomal sodium nitrite-collagen conjugate immobilized acrylic acid-grafted silicone; AAc Si-nGH-Col, nanoliposomal growth hormone-collagen conjugate immobilized acrylic acid-grafted silicone; AAc Si-nNitrite-nGH-Col, nanoliposomal sodium nitrite-nanoliposomal growth hormone-collagen conjugate immobilized acrylic acid-grafted silicone.

Wettability and mechanical properties of surface-modified silicone tubes
The hydrophilicity of unmodified and surface-modified silicone tubes, e.g. Si, AAc Si-Col, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAc Si-nNitrite-nGH-Col, was assessed by measuring the water contact angles (Table 1). We found that the
water contact angles decreased after collagen immobilization via AAc grafting (Si: 102±4º; AAc Si-Col: 56±2º). nNitrite-Col conjugate coating with 500 nmol/ml nNitrite in conjugate, and nGH-Col conjugate coating with 1000 ng/ml nGH in conjugate significantly decreased the water contact angle compared with AAc Si-Col tubes (AAc Si-nNitrite-Col: 51±2º; AAc Si-nGH-Col: 50±2º, p<0.05). The water contact angles of AAc Si-nNitrite-nGH-Col tubes with different nNitrite and nGH concentrations were similar, ranging from 47º to 49º. These water contact angles were slightly lower than those of tubes with nNitrite-Col or nGH-Col coatings.

The ultimate tensile strength of AAc Si-nNitrite-nGH-Col tubes with different nNitrite and nGH concentrations ranged from 3.9 to 4.1 MPa, and was not significantly different from the ultimate tensile strength of AAc Si-Col tubes (3.6±0.5 MPa, p<0.05; Table 1). The percent elongation at break was more than 100% for Si, AAc Si-Col, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAc Si-nNitrite-nGH-Col tubes, with no statistically significant differences observed between coatings (Table 1).

Table 1. Wettability and mechanical characteristics of unmodified and surface-modified silicone tubes, i.e. Si, AAc Si, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAc Si-nNitrite-nGH-Col tubes. Values are mean ± standard deviation for 3 independent experiments. *Significantly different from AAc Si-Col tubes.

<table>
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<th>Tube, surface modification</th>
<th>nNitrite (nmol/ml)</th>
<th>nGH (ng/ml)</th>
<th>Wettability, water contact angle (º)</th>
<th>Ultimate tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
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<td>4.1 ± 0.3</td>
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Nitrite and/or GH release from nanoliposomes immobilized on silicone tubes

To assess the sustained release ability of nNitrite, the percentage nitrite released from AAc Si-nNitrite-Col tubes with 500 nmol/ml nNitrite in conjugate was measured, and compared with the percentage nitrite released from AAc Si-Nitrite-Col tubes with 500 nmol/ml of the free form of sodium nitrite in conjugate (Figure 2a). For AAc Si-Nitrite-Col tubes, most nitrite was released during the first 24 h, but not anymore thereafter. The nNitrite on AAc Si-nNitrite-Col tubes gradually released nitrite during 72 h to a lower amount than from AAc Si-Nitrite-Col tubes. However, after 72 h the release of nitrite from AAc Si-nNitrite-Col tubes increased and was even higher than the release from AAc Si-Nitrite-Col tubes. After 120 h incubation, 58% nitrite was released from AAc Si-nNitrite-Col tubes and 48% from AAc Si-Nitrite-Col tubes. By adding 100 or 1000 ng/ml nGH to the conjugate in AAc Si-nNitrite-nGH-Col tubes, the release of nitrite slightly, but not significantly, decreased compared with AAc Si-nNitrite-Col tubes (data not shown).

The percentage of GH released from AAc Si-nGH-Col tubes with 1000 ng/ml nGH in conjugate was compared to that released from the AAc Si-GH-Col tubes (Figure 2b). nGH on AAc Si-nGH-Col tubes gradually released GH during 120 h of incubation. This GH release was higher than from AAc Si-GH-Col tubes. The release of GH from AAc Si-GH-Col tubes stopped at 72 h. After 120 h incubation, 22% GH was released from AAc Si-nGH-Col tubes, while only 10% GH was released from AAc Si-GH-Col tubes. By adding 50 or 500 nmol/ml nNitrite to the conjugate in AAc Si-nNitrite-nGH-Col tubes, the release of GH was not significantly changed compared with AAc Si-nGH-Col tubes (data not shown).

Stability of nanoliposomes on AAc Si-nNitrite-nGH-Col tubes during a 3 months storage period

A possible effect of storage of AAc Si-nNitrite-nGH-Col tubes at 4°C for 1, 2, or 3 months on the release potential of nNitrite and nGH was assessed (Figure 2c,d). There were no changes in the percentage of nitrite released from nNitrite as a result of storage at 4°C for 1 month (Figure 2c, p>0.05). After 2 months of storage, the percentage of nitrite released from nanoliposomes significantly decreased compared with the release from fresh nanoliposomes (p<0.05, at all-time points measured). After 3 months of storage, the percentage of nitrite released did decrease even more compared with the release from fresh nanoliposomes, with no release anymore after 48 h (Figure 2c). The percentage of GH released from AAc Si-nNitrite-nGH-Col tubes after storage showed that nanoliposomes loaded with GH were stable until 2 months (Figure 2d). After 2 months of storage, GH was continuously released until 96 h, but not anymore thereafter. After 3 months of storage, the release of GH significantly decreased compared with that from fresh nanoliposomes. This indicates that the nanoliposomes in nNitrite-nGH-Col conjugate coating of AAc Si-nNitrite-nGH-Col tubes was not stable after storage for
2 and 3 months at 4°C. Therefore, these surface-modified tubes should only be stored for a period up to 1 month at 4°C.

**Figure 2.** Nitrite and GH release from surface-modified silicone tubes in the culture medium at 37°C, immediately after preparation and storage for 1, 2, or 3 months at 4°C. (a) Nitrite release from AAc Si-nNitrite-Col and AAc Si-Nitrite-Col tubes with 500 nmol/ml nNitrite or Nitrite in conjugate immediately after preparation. (b) GH release from AAc Si-nGH-Col and AAc Si-GH-Col tubes with 1000 ng/ml nGH or GH in conjugate immediately after preparation. (c) Nitrite, and (d) GH release from AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate after storage for 1, 2, or 3 months at 4°C. The nanoliposomes in nNitrite-nGH-Col conjugate coating of AAc Si-nNitrite-nGH-Col tubes were stable after 1 month storage at 4°C, but not after longer storage periods. Nitrite and GH release were expressed as % of the initial amount of sodium nitrite and GH in nanoliposomes or in conjugate. n=3. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; AAc Si-nNitrite-Col, nNitrite-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nGH-Col, nGH-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nNitrite-nGH-Col, nNitrite-nGH-collagen conjugate immobilized AAc-grafted silicone.
No cytotoxic effects of nanoliposomes on endothelial cells
The cytotoxicity of prepared nanoliposomes was assessed by live/dead assay after 48 h incubation with seeded endothelial cells (Figure 3a). Cell viability on silicone tubes filled with culture medium containing 10% (v/v) empty nanoliposomes was 96%, which was comparable with cell viability on silicone tubes filled with pure culture medium (98%; Figure 3a).

Nitrite and/or GH release from surface-modified silicone tubes affected endothelial cell proliferation and confluency
Collagen immobilization on silicone tubes (AAc Si-Col) increased endothelial cell numbers by 1.6-fold compared with unmodified silicone tubes after 2 days of culture (p<0.05, Figure 3b). Fifty nmol/ml nNitrite in conjugate stimulated by 1.7-fold (p<0.05), but 500 nmol/ml nNitrite did not change endothelial cell number on AAc Si-nNitrite-Col tubes compared with AAc Si-Col tubes. More endothelial cells were present on AAc Si-nGH-Col tubes compared with AAc Si-nNitrite-Col tubes (Figure 3b). By increasing the concentration of nGH from 100 to 1000 ng/ml on AAc Si-nGH-Col tubes, the endothelial cell number increased by 1.4-fold (p<0.005) after two days of culture. The number of endothelial cells on AAc Si-nGH-Col tubes with 1000 ng/ml nGH in conjugate was 2.6-fold higher (p<0.0005) than on AAc Si-Col tubes, 1.5-fold higher (p<0.005) than on AAc Si-nNitrite-Col tubes with 50 nmol/ml nNitrite, and 2.8-fold higher (p<0.0005) than on AAc Si-nNitrite-Col tubes with 500 nmol/ml nNitrite in the conjugate. The presence of 100 ng/ml nGH in nNitrite-nGH-Col conjugate coating with 50 or 500 nNitrite did not affect endothelial cell number on AAc Si-nNitrite-nGH-Col tubes compared with AAc Si-nNitrite-Col tubes. However, 1000 ng/ml nGH in nNitrite-nGH-Col conjugate increased cell number compared with AAc Si-nNitrite-Col tubes with 50 nmol/ml nNitrite (1.4-fold, p<0.05) or 500 nmol/ml nNitrite in conjugate (2.3-fold, p<0.005; Figure 3b).

Optical micrographs of endothelial cells attached on Si, AAc Si-Col, and AAc Si-nNitrite-nGH-Col tubes with 50 or 500 nmol/ml nNitrite, and 100 or 1000 ng/ml nGH in conjugate, were used to assess endothelial cell confluency after 6 days of culture (Figure 4a-f). The level of cell confluency was dependent on the surface modification used. The number of endothelial cells on Si tubes was very low, as well as cell confluency (22%) after 6 days of culture (Figure 4a). Cell confluency was 74% on AAc Si-Col tubes (Figure 4b). Although endothelial cells on AAc Si-Col tubes did show excellent proliferation, they did not form a confluent monolayer on the silicone surface. The level of cell confluency on silicone tubes with a collagen coating containing 50 nmol/ml nNitrite and 100 ng/ml nGH increased to 98% (Figure 4c). By increasing the amount of nNitrite to 500 nmol/ml, cell confluency decreased to 83% (Figure 4d). Increasing the initial amount of nGH to 1000 ng/ml in conjugate coating of AAc Si-nNitrite-nGH-Col tubes resulted in >100% cell confluency within 6 days (Figure 4e,f).
Figure 3. Cytotoxicity of nanoliposomes and effect of nNitrite and/or nGH conjugation with collagen coating on endothelial cell numbers after 2 days of culture. (a) Acridine orange-propidium iodide staining of endothelial cells cultured for 2 days on silicone tubes filled with culture medium with or without nanoliposomes. (A) Endothelial cells incubated with culture medium, and (B) Endothelial cells incubated with 10% (v/v) empty nanoliposomes in culture medium. (b) Effect of surface-modified silicone tubes with different nNitrite and/or nGH in the conjugate coating, i.e. AAc Si-Col, AAc Si-nNitrite-Col, AAc Si-nGH-Col, and AAc Si-nNitrite-nGH-Col, on the number of endothelial cells after 2 days of culture. Maximum cell number was observed on AAc Si-nGH-Col and AAc Si-nNitrite-nGH-Col tubes with 1000 ng/ml nGH in conjugate coating. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; Si, silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-nNitrite-Col, nNitrite-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nGH-Col, nGH-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nNitrite-nGH-Col, nNitrite-nGH-collagen conjugate immobilized AAc-grafted silicone. *Significantly different from AAc Si-Col tubes, p<0.05, **p<0.005, ***p<0.0005; #Significantly different from AAc Si-nNitrite-Col tubes, p<0.05, ##p<0.005.
Figure 4. Optical micrographs showing endothelial cell confluency after 6 days culture. (a) Si, (b) AAc Si-Col, (c) AAc Si-nNitrite-nGH-Col with 50 nmol/ml nNitrite and 100 ng/ml nGH in conjugate, (d) AAc Si-nNitrite-nGH-Col with 500 nmol/ml nNitrite and 100 ng/ml nGH in conjugate, (e) AAc Si-nNitrite-nGH-Col with 50 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate, and (f) AAc Si-nNitrite-nGH-Col with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate. AAc Si-nNitrite-nGH-Col tubes with 50 or 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate resulted in >100% cell confluency within 6 days. Magnification x200. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; Si, silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-nNitrite-nGH-Col, nNitrite-nGH-collagen conjugate immobilized AAc-grafted silicone.
Platelet adhesion on unmodified and surface-modified silicone tubes

Platelet adhesion on unmodified and surface-modified silicone tubes was investigated before and after endothelialization (Figure 5). Without endothelial cell seeding, platelet adhesion on AAc Si-Col tubes was increased by 27% compared with Si tubes (p<0.05, Figure 5a). AAc Si-nNitrite-Col tubes suppressed platelet adhesion compared with AAc Si-Col tubes by 2.1-fold when 50 nmol/ml nNitrite was present in nNitrite-Col conjugate (p<0.005), and by 3.5-fold with 500 nmol/ml nNitrite in conjugate (p<0.005). There was a linear relationship between the initial concentration of nNitrite in nNitrite-Col conjugate with the degree of platelet adhesion on AAc Si-nNitrite-Col tubes. Platelet adhesion to AAc Si-nGH-Col tubes was slightly but not significantly lower than AAc Si-Col tubes. The combination of nNitrite and nGH at different concentrations in the collagen coating decreased platelet adhesion compared with collagen coating alone. AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH inhibited platelet adhesion by 78% (p<0.005) compared with AAc Si-Col tubes. Platelet adhesion onto PEG Si tubes, used as controls, was low compared to other unmodified and surface-modified silicone tubes (p<0.05, Figure 5a).

Endothelialization of silicone tubes decreased the percentage of platelet adhesion on unmodified and surface-modified tubes by 50-75% compared with silicone tubes without endothelial cells (Figure 5b). Platelet adhesion to AAc Si-Col tubes and Si tubes after endothelialization was similar. AAc Si-nNitrite-Col tubes suppressed platelet adhesion compared with AAc Si-Col tubes by 1.8-fold when 50 nmol/ml nNitrite was present in the nNitrite-Col conjugate (p<0.05), and by 2.3-fold with 500 nmol/ml nNitrite in conjugate (p<0.005). Platelet adhesion to AAc Si-nGH-Col tubes with 100 ng/ml nGH decreased slightly, but not significantly, by 13% (p>0.05), and with 1000 ng/ml by 31% (p<0.05) compared with AAc Si-Col tubes. Platelet adhesion to AAc Si-nGH-Col tubes with both 100 and 1000 ng/ml nGH in conjugate before endothelialization was similar (Figure 5a). AAc Si-nNitrite-nGH-Col tubes with different nNitrite and nGH concentrations in conjugate suppressed platelet adhesion compared with collagen coating alone in the presence of endothelial cells. Platelet adhesion to AAc Si-nNitrite-nGH-Col tubes with 1000 ng/ml nGH decreased by 76% (p<0.005) when 50 nmol/ml nNitrite was used, and by 73% (p<0.005) when 500 nmol/ml nNitrite was used compared with AAc Si-Col tubes.
Figure 5. Effect of surface modification on platelet adhesion onto silicone tubes in the absence and presence of endothelial cells. (a) Platelet adhesion (expressed as % of the initial amount of platelet numbers in PRP solution) on surface-modified silicone tubes in the absence of endothelial cells. (b) Platelet adhesion (expressed as % of the initial amount of platelet numbers in PRP solution) on surface-modified silicone tubes in the presence of endothelial cells. AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate inhibited platelet adhesion by 79% in the absence of endothelial cells, and by 73% in the presence of endothelial cells compared with AAc Si-Col tubes. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; Si, silicone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-nNitrite-Col, nNitrite-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nGH-Col, nGH-collagen conjugate immobilized AAc-grafted silicone; AAc Si-nNitrite-nGH-Col, nNitrite-nGH-collagen conjugate immobilized AAc-grafted silicone. *Significantly different from AAc Si-Col tubes, p<0.05, **p<0.005, ***p<0.0005, #Significantly different from Si tubes, p<0.05.
GH release from AAc Si-nGH-Col or AAc Si-nNitrite-nGH-Col tubes stimulated endothelial cell-derived NO

Silicone tubes with coatings containing nNitrite (an anti-thrombotic agent) reduced platelet adhesion in the absence or presence of endothelial cells (Figure 5). However, unmodified or surface-modified silicone tubes without nNitrite in the coating, i.e. Si, AAc Si-Col, and AAc Si-nGH-Col tubes, also decreased platelet adhesion in the presence of endothelial cells. Since NO inhibits platelet aggregation and adhesion, the ability of endothelial cells seeded on these tubes to secrete NO indicates the anti-thrombotic property of these cell-seeded tubes. Endothelial cells cultured on Si tubes released low amounts of NO (0.51 nmol/ml; Figure 6). Collagen immobilization on silicone tubes increased NO release by endothelial cells by 3.5-fold compared with NO release by endothelial cells on Si tubes (p<0.05). GH released from AAc Si-nGH-Col tubes stimulated NO release by endothelial cells. NO release by endothelial cells cultured on AAc Si-nGH-Col tubes with 1000 ng/ml nGH, but not 100 ng/ml nGH in the conjugate significantly increased by 4.5-fold (p<0.005) compared with AAc Si-Col tubes.

Figure 6. Effect of GH release from AAc Si-nGH-Col tubes on endothelial cell-derived NO production after 2 days cell culture. AAc Si-nGH-Col tubes with 1000 ng/ml nGH increased NO release by endothelial cells by 4.5-fold compared with AAc Si-Col tubes. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-nGH-Col, nGH-collagen conjugate immobilized AAc-grafted silicone. **Significantly different from AAc Si-Col tubes, p<0.005, #Significantly different from Si tubes, p<0.05, ##p<0.005.
Nitrite released from AAc Si-nNitrite-nGH-Col tubes stimulated PGF$_{1\alpha}$ production by endothelial cells

AAc Si-nNitrite-Col tubes with 500 nmol/ml, but not 50 nmol/ml nNitrite in conjugate increased PGF$_{1\alpha}$ production by 1.8-fold (p<0.05) in endothelial cells, compared with AAc Si-Col tubes (Figure 7). GH released from AAc Si-nGH-Col tubes with both 100 and 1000 ng/ml nGH in conjugate had no significant effect on PGF$_{1\alpha}$ production. AAc Si-nNitrite-nGH-Col tubes stimulated PGF$_{1\alpha}$ production by 1.7-fold (AAc Si-nNitrite-nGH-Col tubes with 50 nmol/ml nNitrite and 100 ng/ml nGH in conjugate; p<0.05) to 2.2-fold (AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate; p<0.005) compared with AAc Si-Col tubes.

![Figure 7. Effect of surface modification of silicone tubes on endothelial cell-derived PGF$_{1\alpha}$ production.](image)

PGF$_{1\alpha}$ production was quantified by measuring the concentration of its stable metabolite 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$). PGF$_{1\alpha}$ production by endothelial cells on AAc Si-nNitrite-nGH-Col tubes with 500 nmol/ml nNitrite and 1000 ng/ml nGH in conjugate was 2.2-fold higher than on AAc Si-Col tubes. nNitrite, nanoliposomal sodium nitrite; nGH, nanoliposomal growth hormone; AAc Si-Col, collagen immobilized AAc-grafted silicone; AAc Si-nNitrite-nGH-Col, nNitrite-nGH-collagen conjugate immobilized AAc-grafted silicone. *Significantly different from AAc Si-Col tubes, p<0.05, **p<0.005.
DISCUSSION

Endothelium is the perfect natural blood compatible surface which secretes various substances affecting platelet adhesion and aggregation [3, 4]. Therefore, creating a functional lining of endothelial cells on blood-contacting parts of medical devices might increase the biocompatibility of these devices. Currently available synthetic materials used in medical devices, such as silicone, are unsuitable as a substrate for endothelial cell seeding and need to be surface modified [3]. The immobilization of different biomolecules on material surfaces improves blood compatibility [12-14], and enhances cell attachment and proliferation [16, 18, 21]. Anti-coagulant molecules act as anti-thrombotic agents, but are not suitable for endothelial cell attachment and growth. Promotion of endothelial cell attachment and growth by extracellular matrix molecules causes deterioration of blood compatibility [18]. Thus a biomaterial surface immobilized with different biomolecules possessing anti-thrombotic and growth-inducing properties might improve both anti-coagulation and endothelialization, thus providing a potential application for long-term use of blood-contacting medical devices [16, 18].

It is generally accepted that collagen, the main protein in the extracellular matrix, is suitable as a coating for endothelial cell attachment and growth [8]. However, collagen is highly thrombogenic, and accelerates platelet aggregation in those areas of a material which are not fully covered by endothelial cells [16]. Therefore, suppression of the thrombogenic properties of collagen by using anti-thrombotic agents on the one hand, and promotion of endothelial cell growth using growth-inducing agents to achieve a confluent cell layer on the material surface on the other hand, may result in improved blood-contacting medical devices performance. We aimed to develop a collagen coating on silicone tubes that prevents platelet adhesion by sustained release of nitrite, and that induces endothelial cell growth by sustained release of GH.

Nanoliposomes loaded with sodium nitrite or GH were used to control the sustained release of these anti-thrombotic and growth-inducing agents. The encapsulation efficiency achieved was high, i.e. >90% for both sodium nitrite and GH, showing the effectiveness of the thin-film hydration technique used for the preparation of nanoliposomes. The presence of PEG in the nanoliposome structure increased the hydrophilicity of the nanoliposomes, which efficiently hinders hydrophilic drug escape to outer water, and results in a high encapsulation efficiency [30]. The slightly higher GH encapsulation efficiency than sodium nitrite encapsulation efficiency is probably due to the higher molecular weight of GH (sodium nitrite: 69 g/mol; GH: 22124 g/mol), that likely hindered its escape to the outer solution during solvent evaporation [30].

To study the interaction between endothelial cells and nNitrite-nGH-Col conjugate coating, a well-defined and stable coating is required. Therefore, AAc
was graft polymerized on silicone tubes, providing a surface with reactive carboxyl groups to form carbodiimide bonds with amine groups on both collagen and nanoliposomes. A covalent immobilization of biological agents through carbodiimide bonds is more stable and shows improved resistance to fluid shear stress, and its biofunction lasts longer than with other bonding methods, such as physical adsorption, entrapment, etc. [18, 31, 32]. Therefore, in this study nNitrite-nGH-Col conjugate was co-immobilized on AAc-grafted silicone tubes to provide a stable coating that offers both anti-thrombotic and growth-inducing properties.

Drug liposomal formulations provide improved drug solubility, longer drug circulation times, and focused drug delivery, compared to free drugs [19, 22, 23, 33]. Our observations on nitrite released from nNitrite-Col conjugate agree with observations by others on the interaction between a lipid bilayer of nanoliposomes and collagen fibrils, that offers improved stability and decreased permeability of nitrite compared with nitrite released from Nitrite-Col conjugate [33]. AAc Si-nGH-Col tubes released GH at a higher rate than AAc Si-GH-Col tubes. This can be explained by the fact that GH is a high molecular weight peptide, and when in free form combined with collagen, the chains of GH get completely stuck with the chains of collagen, which makes it hard for GH molecules to be released. Encapsulation of GH in nanoliposomes hindered the direct mixing of GH with collagen, which led to more rapid release of GH from the AAc Si-nGH-Col tubes than from AAc Si-GH-Col tubes. The faster release of sodium nitrite than GH from nanoliposomes can be explained by the lower molecular weight of sodium nitrite than GH.

The surface-modified silicone tubes containing nNitrite and nGH were stable for 1 month storage at 4°C. The presence of cholesterol and amino-PEG in the lipid phase of nanoliposomes increases nanoliposome stability, and controls the release rate [34]. Rapid leakage of the low-molecular weight sodium nitrite from the nanoliposomes during storage for 2 months at 4°C significantly decreased nitrite released from nanoliposomes. After 3 months of storage at 4°C, the release of nitrite and GH from the nanoliposomes decreased significantly, which indicates that the surface-modified silicone tubes can be stored for 1 month at 4°C, but not for longer time periods.

It is generally accepted that hydrophilic materials support cell adhesion and consequently improve their biocompatibility [21, 24]. The hydrophilicity of silicone tubes improved after collagen immobilization, especially with collagen conjugates with nNitrite, and/or nGH. A material surface with either a very high or a very low contact angle is not suitable for cell attachment [24]. Therefore the moderate wettability of the surface-modified silicone tubes in this study makes them suitable substrates for endothelial cell attachment.

Not only cell attachment onto a material, but also cell growth is important to reach cell confluency on material surface. NO has been shown to stimulate
endothelial cell proliferation, while reducing platelet adhesion [15, 35]. Nitrite, the
stable end-product of NO metabolism, may represent a potential source of NO and
can be used as a NO donor under hypoxia conditions or in an acidic environment
[15, 36]. We have shown previously that nitrite-generating sodium nitrite-collagen
conjugate coating of silicone tubes with 5 to 50 µM sodium nitrite increases the
number of endothelial cells more than collagen coating alone, probably via GH
production [15]. In this study, we show that conjugation of collagen with 50 nmol/ml
nNitrite increased the number of endothelial cells after 48 h of culture. Although the
amount of nitrite released from nNitrite-Col conjugate was lower than from Nitrite-
Col conjugate during 72 h, the slow but continuous release of nano-sized nitrite
was more effective to stimulate endothelial cell proliferation.

GH treatment of endothelial cells reduces intracellular reactive oxygen
species (ROS) production and regulates the synthesis of multiple mRNA species,
including that of insulin-like growth factor-1 (IGF-1) and endothelial nitric oxide
synthase (eNOS) [17, 37]. GH (200 pg/l) stimulates proliferation of cultured human
retinal microvascular endothelial cells but not HUVECs [37]. Our study also shows
that endothelial cells respond to GH with enhanced proliferation. The stimulatory
effect of GH released from AAc Si-nGH-Col tubes with 1000 ng/ml nGH in
conjugate on endothelial cell proliferation was higher than that nitrite released from
AAc Si-nNitrite-Col tubes with 50 nmol/ml nNitrite in conjugate. Combination of
nNitrite with nGH-Col conjugate in AAc Si-nNitrite-nGH-Col tubes did not hamper
GH release from nGH as indicated by the increased number of endothelial cells on
AAc Si-nNitrite-nGH-Col tubes compared with AAc Si-Col tubes.

The adhesion and activation of platelets on a biomaterial surface often
leads to coagulation and thrombus formation [13]. NO is widely recognized as a
potent inhibitor of platelet adhesion and activation [13, 14]. The extraordinarily
thromboresistant nature of blood vessel inner walls is, in part, due to the
continuous production of NO by the endothelial cells lining the blood vessels [13].
Improved blood compatibility of NO-releasing polymeric materials has been shown
in animal studies [13, 14]. Previously we showed that sodium nitrite-collagen
conjugate coating of silicone tubes with 500 nmol/ml sodium nitrite exerts strong
anti-platelet activity [15]. Interestingly, sustained release of nitrite from nNitrite-Col
conjugate more strongly inhibited platelet adhesion than nitrite release from Nitrite-
Col conjugate. Sodium nitrite stimulates NO production by endothelial cells lining
the vessel wall in a NOS-independent manner [38]. Therefore by endothelial cell
seeding of AAc Si-nNitrite-Col tubes, not only nitrite released from nNitrite-Col
conjugate, but also NO release by endothelial cells helps in increased anti-platelet
activity of these tubes.

GH released from nGH-Col conjugate in AAc Si-nGH-Col tubes did not
significantly inhibit platelet adhesion in the absence of endothelial cells. The
presence of the anti-thrombotic polymer PEG in the nanoliposome's structure
prevents platelet adhesion [39]. Therefore, all surface-modified silicone tubes containing nanoliposomes in their coatings, even without anti-thrombotic agent (e.g. AAc Si-nGH-Col tubes), showed reduced platelet adhesion. Although platelet inhibition caused by nanoliposomes themselves on AAc Si-nGH-Col tubes was not significant. After endothelialization of surface-modified silicone tubes, platelet adhesion was decreased on AAc Si-nGH-Col tubes compared with platelet adhesion on the same tubes in the absence of endothelial cells. This indicates that GH might increase the anti-thrombotic function of endothelial cells, which includes anti-coagulation, prevention of inflammatory cytokines production, and adhesion of the inflammatory cells, which is mostly controlled by the release of functional factors, such as NO, PGI₂, and thrombomodulin, by endothelial cells [13, 16].

The GH released resulted in increased NO release by endothelial cells on AAc Si-nGH-Col tubes. This indicates that GH not only affects endothelial cell proliferation, but also the anti-thrombotic function. Our results agree with data by others showing that somatropin treatment of endothelial cells increases NO production through eNOS activation [40]. High somatropin concentrations (100-1000 ng/ml) enhance eNOS mRNA expression, whereas NO production was only increased with GH at the highest dose of 1000 ng/ml [40]. We have shown earlier that nitrite release, which can continuously convert to NO, stimulates GH production by endothelial cells [15]. Since nitrite stimulates GH production by endothelial cells, and because GH released enhances NO production by endothelial cells, preparation of a surface with the ability to release nitrite and GH may play a significant role in endothelialization of blood-contacting parts of biomedical devices.

GH released from nGH-Col conjugate on AAc Si-nGH-Col tubes did not significantly affect PGF₁₀₀ production, independent of the concentration in the conjugate. nNitrite at 500 nmol/ml in AAc Si-nNitrite-Col tubes significantly increased the production of PGF₁₀₀ by endothelial cells, which agrees with data published by others [41]. Conjugation of nNitrite with nGH and collagen in AAc Si-nNitrite-nGH-Col tubes stimulated PGF₁₀₀ production by endothelial cells. This shows excellent functionality of endothelial cells on AAc Si-nNitrite-nGH-Col tubes, which is important when endothelial cell seeding is used to improve the biocompatibility of blood-contacting medical devices.

CONCLUSIONS

Encapsulation of sodium nitrite or GH in nanoliposomes followed by addition of nNitrite and/or nGH to the surface coating of silicone tubes increased the sustained release as well as the effectiveness, and bioavailability of these bioactive compounds. Nanoliposomes also allowed stability of surface-modified silicone
tubes after storage. AAc Si-nNitrite-nGH-Col tubes with nNitrite (500 nmol/ml) and nGH (1000 ng/ml) in conjugate coating provided full endothelial coverage, and low platelet adhesion even in the absence of endothelial cells, suggesting that nNitrite-nGH-Col conjugate coatings are highly promising to promote endothelialization of silicone materials in blood-contacting devices.

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