First step toward near-infrared continuous glucose monitoring: *in vivo* evaluation of antibody coupled biomaterials

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Abstract

Continuous glucose monitoring (CGM) is crucial in diabetic care. Long-term CGM systems however require an accurate sensor as well as a suitable measuring environment. Since large intravenous sensors are not feasible, measuring inside the interstitial fluid is considered the best alternative. This option, unfortunately, has the drawback of a lag time with blood glucose values. A good strategy to circumvent this is to enhance tissue integration and enrich the peri-implant vasculature. Implants of different optically transparent biomaterials (poly(methyl-methacrylate) [PMMA] and poly(dimethylsiloxane) [PDMS]) – enabling glucose monitoring in the near-infrared (NIR) spectrum – were surface-treated and subsequently implanted in goats at various implantation sites for up to 3 months. The overall *in vivo* biocompatibility, tissue integration, and vascularization at close proximity of the surfaces of these materials were assessed. Histological screening showed similar tissue reactions independent of the implantation site. No significant inflammation reaction was observed. Tissue integration and vascularization correlated, to some extent, with the biomaterial composition. A modification strategy, in which a vascular endothelial-cadherin antibody was coupled to the biomaterials surface through a dopamine layer, showed significantly enhanced vascularization 3 months after subcutaneous implantation. Our results suggest that the developed strategy enables the creation of tissue interactive NIR transparent packaging materials, opening the possibility of continuous glucose monitoring.

Keywords: *In vivo*, continuous glucose monitoring, antibody-coupled, vascular endothelial-cadherin, enhanced vascularization

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Introduction

The traditional method for diabetics to control their blood glucose levels at home is through point samples with a Finger-prick Glucometer, also referred to as self-monitoring of blood glucose (SMBG). Because pain is associated with this method, it is infrequently done, and rarely performed sufficiently to follow the blood glucose dynamics. Improved blood glucose control has been shown to reduce the risks of complications in Type 1 diabetes.

A continuous measurement and regulation of glucose through the uptake of glucose or injection of insulin improves not only the quality of life but also prolongs the life expectancy.¹ Since this is known, many technologies are being pursued to develop novel continuous glucose monitoring systems (CGMS).² CGM-technologies can be classified according to their invasiveness or the transduction mechanism of the sensor.³ The latter divides the CGMS

into three categories: electrochemical, optical, or combinatorial. The electrochemical-based sensors fail upon prolonged use due to component-based failures such as lead detachment, electrical shorts and membrane delamination, or biocompatibility-based failures such as membrane biofouling, electrode passivation, fibrous encapsulation, and membrane biodegradation.³ Another well-investigated optical glucose-sensing technology is near-infrared (NIR) spectroscopy, a technology known to be reliable to measure glucose in fluids and bio-environments.⁴

However, an implantable NIR-sensor is not yet available. The technology to develop a small, micro-scaled spectrometer, enabling implantation, is still under investigation. In the GlucoSens-project with which the authors are associated, the development of such a micro-spectrometer is ongoing. This strategic basic research project aims to develop ‘enabling technologies for CGM using implantable

Figure 1 The objective of the GlucoSens project is to develop technology platforms to enable continuous glucose monitoring based on minimally invasive optical sensors. This implies a miniature spectrometer and readout-system inside a biocompatible package. Converting spectra to glucose values needs complex tissue mathematical models. The dimensions of the doughnut were set to 16 mm of outer diameter, 3 mm of inner diameter, and a thickness of 5 mm. (A color version of this figure is available in the online journal.)

single-chip optical sensors' (Figure 1). Recently, the first data on this development have been published.^{5–7}

For implantation purposes, such a 'lab-on-chip' could be packaged in biocompatible packaging material. Besides biocompatibility, candidate biomaterials have to meet a number of other criteria: the material needs to be transparent in the NIR, not biodegradable, have enough mechanical stability to maintain sensor and receiver in the right position, and allow good interaction with the blood vessels of the host tissue. Poly(methyl-methacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS) are packaging materials fulfilling some of these criteria. They are NIR-transparent^{8,9} and have shown to possess the required biocompatibility: PMMA as a bone cement and PDMS as part of breast implants.^{10–12} However, upon implantation, fibrous encapsulation of an implant is often observed.¹³ Such an encapsulation imposes a significant diffusion barrier to small analytes such as glucose and increases the lag time of the sensor by as much as threefold.^{14,15} Equilibrium between plasma levels and interstitial fluid will eventually be reached, but in diabetes-management, a short-time lag is critical. Enabling a good vascularization at the implant site may extend sensor performance and shorten time lag.¹⁶

The purpose of this study is, therefore, to design biocompatible packaging materials for glucose sensors that can remain implanted for a prolonged time period without foreign reaction from the host. We hypothesize that both the composition of the polymer bulk and the applied surface functionalization of the packaging material favor the tissue integration and neovascularization around the implanted materials.

Materials and methods

Fabrication of the packaging material

Synthesis of PDMS-based material. A PDMS-type material was prepared using a mixture of three methacrylate-terminated siloxane polymers: 2 mol% of methacryloxypropyl terminated PDMS (MW = 10000 g/mol – ABCR, Karlsruhe, Germany), 25 mol% of methacryloxypropyl T-structured PDMS (MW = 595 g/mol – ABCR, Karlsruhe, Germany),

and 73 mol% of methacryloxypentamethyldisiloxane (MW = 232 g/mol – ABCR, Karlsruhe, Germany). These compounds were mixed with 2 mol% relative to the amount of methacrylate groups of photo-initiator (Darocur 1173 – BASF, Germany) and cured under UV-A light ($\lambda = 365$ nm) for 4 h. In what follows, these materials will be denoted as PDMS- or PDMS-type material.

Synthesis of PMMA-based materials. Three different PMMA-based materials with diverse compositions and stiffness were selected for implantation. All materials were composed of methyl methacrylate (MMA) (Sigma, Sigma-Aldrich, Bornem, Belgium) and poly(ethylene glycol) dimethacrylate (PEGdiMA) (Sigma, Sigma-Aldrich, Bornem, Belgium). The materials were cured using UV-A light ($\lambda = 365$ nm) for 55 min (PMMA1 and PMMA2) and 90 min (PMMA3) in the presence of 2 mol% Irgacure 2959 (BASF, Antwerp, Belgium) as the photo-initiator. PMMA1: 50 mol% MMA and 50 mol% PEGdiMA (MW = 550 g/mol); PMMA2: 50 mol% MMA and 50 mol% PEGdiMA (MW = 750 g/mol); and PMMA3: 85 mol% MMA and 15 mol% PEGdiMA (MW = 550 g/mol).

Polymer processing for *in vitro* testing and implant production. For the required analyses, two sample shapes were required: polymer sheets and polymer doughnuts. The former was produced by molding in between two parallel glass plates separated by a silicone spacer (thickness = 1 mm). The latter material shape was realized using a specially developed mould. The dimensions of the mould were 16 mm of outer diameter, 3 mm of inner diameter, and a thickness of 5 mm.

Evaluation of the physicochemical properties

Static contact angle measurements. To evaluate the material surface wettability, a drop of water (1 μ L) is placed on a biomaterial sample (1–2 cm²), and the static contact angle is measured with the OCA-20 (Benelux Scientific, Eke, Belgium), using the sessile drop method. The contact angles were measured over a time interval of

30 s (at 1 s intervals). The contact angle was determined using the SCA20 (version 2.1.5) software using ellipse fitting, and the average over the whole time period was taken. On each sample, the contact angle was determined in three different places to determine the homogeneity of the layer.

Tensile measurements. Tensile test was performed using dog bone-shaped samples ($25 \times 4 \times 1 \text{ mm}^3$) with a load cell of 100 N on a Universal Tester 100-KM (Hounsfield Test Equipment Ltd - Tinius Olsen, Ltd, Salfords, Surrey, England). From the obtained stress-strain curves (obtained at a speed of 20 mm/min at room temperature), the E-modulus was determined using QMat software (Tinius Olsen). Before the measurements were started, a preload of 0.5 N was applied.

Swelling studies. Disc-like biomaterial samples ($h = 1 \text{ mm}$, $\varnothing = 1 \text{ cm}$) were freeze-dried and weighted. The samples were incubated in milliQ water for 24 h and weighted again. After freeze-drying and weighing, the gel fraction was calculated as $\text{gelfraction (\%)} = m_e/m_0 \times 100\%$ with m_0 = dry weight of the sample after freeze drying and m_e = dry weight of the samples after swelling in water and subsequent freeze drying.

Biomaterial optical properties: transmission toward NIR-light. Absorption of NIR-light in the 1300–2500 nm range was measured through 1-mm thick biomaterial samples. The samples were fixed in the sample compartment of an Agilent 680 Series FTIR (Agilent Technologies Belgium, Diegem, Belgium) and NIR-light was transmitted through the samples. Measurements were processed by Resolutions Pro software (Agilent Technologies Belgium, Diegem, Belgium).

Surface activation of PDMS- and PMMA-based materials to enable biofunctionalization

Activation of the surface of both material types was realized by the deposition of a polydopamine (PDA) layer: the doughnuts were incubated for 24 h at room temperature in 1.5 mL of a 2-mg/mL dopamine hydrochloride solution (Sigma, Sigma-Aldrich, Bornem, Belgium)/Tris-buffer ($\text{pH} = 8.5$). Next, samples were rinsed with milliQ water and nitrogen-dried. Biofunctionalization of the activated surfaces was achieved by coupling an anti-VE-cadherin antibody (anti-VE-cad) (Santa Cruz, Heidelberg, Germany) onto the PDA layer. The samples were incubated overnight at 37°C in 2.5 mL of a $2 \mu\text{g/mL}$ anti-VE-cad phosphate-buffered saline (PBS) solution ($\text{pH} = 7.3$). Next, samples were rinsed with PBS and nitrogen-dried. An alternative biofunctionalization strategy was developed by coupling the anti-VE-cad on a gelatin B (gelB) layer that was deposited on top of the PDA layer. Samples were incubated overnight at 40°C in a 1.5-mL solution of 1 g/v% gelB (Rousselot, Gent, Belgium) /Tris-buffer ($\text{pH} = 8$). Next, samples were rinsed with milliQ water and nitrogen dried. The coupling of the anti-VE-cad was achieved as described for the PDA-layer but is based on a

physisorption process compared to a covalent coupling for the antibody-PDA-layer. After activation, all doughnut-shaped samples were sterilized with ethylene-oxide according to standard methods¹⁷ (at AZ Maria Middelaes, Gent, Belgium). Sterilization was done after modification and prior to the antibody coupling under sterile conditions.

Quantification of VE-cadherin binding by radiolabeling using ^{125}I

To evaluate the antibody binding capacity of the biomaterials, VE-cadherin antibodies were radiolabeled using ^{125}I . Radioiodination was performed using the Iodo-Gen method as previously described.¹⁸ In brief, a mixture of sodium iodide and the antibody was added to an Iodo-Gen-coated reaction vial that was rinsed with 1 mL Dulbecco's phosphate-buffered saline (DPBS). The mixture, diluted with DPBS to a total antibody concentration of $2 \mu\text{g/mL}$, was reacted for 15 min at room temperature. The initial activity was measured as a reference. The solution ($200 \mu\text{L}$) was placed on each sample overnight at 37°C . The amount of radio-labeled antibody was obtained by counting the radioactivity with a gamma counter (Cobra II, PerkinElmer, Zaventem, Belgium). All experiments were performed in triplicate.

Cytotoxicity

Cytotoxicity was evaluated according to the ISO10993 part 5 standards of extraction tests with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) as a detection test. Human Foreskin Fibroblasts (HFF) (American Type Culture Collection, ATCC) were cultured (37°C , $5\% \text{CO}_2$) in a DMEM High Glucose GlutaMAX-medium supplemented with fetal bovine serum, L-glutamine, sodium pyruvate and penicillin-streptomycin (Gibco, Life Technologies, Gent Belgium). From an ethylene oxide sterilized polymer sheet, 0.5 g was cut and placed in 1 mL of HFF culture medium at 37°C . The extraction medium was diluted after 8 days (1:1) with HFF culture medium, and then placed on top of a monolayer of HFF. After 24 h the medium was discarded and replaced by $200 \mu\text{L}$ of a 0.5 mg/mL MTT containing medium, followed by an incubation for 4 h at 37°C . The MTT-solution was discarded and replaced by $200 \mu\text{L}$ of lysisbuffer (0.1% of TritonTM-X-100 [Fluka, Sigma-Aldrich, Bornem, Belgium] in isopropanol/0.04 M HCL [Chem-Lab, Zedelgem, Belgium/UCB, Brussels, Belgium]), and incubated for 30 min at 37°C . Absorbance was measured at 570 nm with KCjunior software on a Universal Microplate Reader EL800 (BIO-TEK Instruments Inc., Bad Friedrichshall, Germany). The viability was calculated as percentage of the control.

In vivo implantation study: animal model and implantation procedure

The studies were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/051 and 2011/095). All animal care did comply

with the EC guidelines for the care and use of laboratory animals.

Animals and surgical procedure. Six adult female goats (Saanen milk goats) with ages between 3 and 6 years and mean body weight between 50 and 70 kg were used. The goats were housed in groups, and had continuous access to food and water. Twelve hours preoperatively, the goats were deprived of food and received medication (Neopen® 1 mL/20 kg subcutaneously (Intervet, Merck & Co., Inc, Brussels, Belgium) and Ketofen 10%® 2.2 mg/kg intramuscularly (Merial, Velsbroek, the Netherlands)) to assure relevant blood values during surgery. Before surgery the goats were premedicated using xylazine (Proxylaz®, 0.2 mg/kg IM [Prodivet, Eynatten, Belgium]). Anesthesia was induced with ketamine (Ketamine 1000®, 1.1 mg/kg (Ceva Santé Animale, Naaldwijk, the Netherlands)) and midazolam (Dormicum®, 0.03 mg/kg [Roche, Woerden, Belgium]) and maintained after tracheal intubation with an O₂ in isoflurane-mixture (Isoflo® [Abbott Lab, Wavre, Belgium]). Routine monitoring (ECG, pulse oximetry, capnography, direct blood pressure, and arterial blood gasses) was performed. Ringer's lactate solution (5 mL/kg/h (Baxter)) was administered intravenously during the anesthetic period. All animals received peroperative antibiotics (Neopen® 1 mL/20 kg subcutaneously) and non-steroidal anti-inflammatory drugs (NSAID, Ketofen 10%® 2.2 mg/kg intramuscular). Postoperatively, antibiotic treatment was continued for 5 days, and NSAIDs were given for 3 days.

Implantation. After induction of anesthesia, the animals were placed in lateral recumbency. The skin was sharply incised and the correct implantation depth was achieved through further blunt dissection before implant positioning. Incisions were sutured (if needed in several layers) with resorbable sutures (Maxon 2/0 skin, Vicryl 2/0 muscle [Ethicon, Johnson & Johnson Medical N.V./S.A., Diegem, Belgium]) and protected with a sterile self-adhesive bandage. When all samples were implanted, the goat was transported to the recovery box. The goats were closely monitored in the post-operative period. In the first experimental setup – intended to aid design optimization, implantation site, and packaging evaluation – three goats were used. In each goat, the above described four different materials were tested: one PDMS-based and three different PMMA-based: PMMA1, PMMA2, and PMMA3. Each material was implanted subcutaneous (para lumbar fossa), intra-peritoneal (ventral abdominal area), and intramuscular (gluteus muscle region) on both flanks of the animal. Animals were euthanized after 1 month and the doughnuts were harvested with surrounding tissue for histological analysis. In the second experimental setup – intended to evaluate the effect of the surface modifications – another three goats were used. Based on the results obtained in the first experiment, PDMS and PMMA1 were selected as materials, and only subcutaneous implants were performed. In order to obtain a better interaction with

the host tissue and host vascularization, both biomaterials were surface-modified with PDA, PDA + anti-VE-cad, and PDA + GelB + anti-VE-Cad. Non-modified materials were used as control. Each type of biomaterial was implanted in both flanks of each animal. Animals were kept alive for three months. Materials were harvested for histological processing after 1 and 3 months.

Histological analysis

Doughnuts with their surrounding tissue were placed in 4% (para)formaldehyde for 24 h at 4°C. They were dehydrated and embedded in paraffin, 7-µm sections were cut on a SLEE cut 4060 microtome (SLEE medical GmbH, Mainz, Germany). Sections in the middle of the doughnut were analyzed. These sections were stained with hematoxylin & eosin (H&E) (Hematoxylin from VWR, Leuven, Belgium and Eosin-Y w/Phloxine (Microm, Thermo Fisher, Walldorf, Germany). For histological evaluation, histological slides were viewed using a Jeneval light microscope (Carl Zeiss NV-SA, Zaventem, Belgium). From the second series, all stained slides were scanned under a 20× objective using an Olympus BX51 microscope, and captured using dotSlide software (Olympus, Aartselaar, Belgium). Eighteen square fields per image (100 µm × 100 µm) abutting the tissue-implant surface were manually selected, and the total number of capillaries was manually counted. The criteria applied for designating a capillary are explained in Figure 2.

Statistical analysis

Statistical significance for capillary counts were assessed by one-way ANOVA with Tukey's post-test for multiple comparisons. If values were not normally distributed according to the Shapiro-Wilk normality test, a Kruskal-Wallis non-parametric test was performed to determine statistical significance. The threshold for significance was $P < 0.05$ and is shown by an asterisk above the error bars; a significance of below 0.001 was depicted as a triple asterisk above the error bars. The error bars represent the standard deviation. Statistical analyses were performed using IBM SPSS Statistics Version 20 (IBM Corporation, New York, United States) software package.

Results

Material characterization

All doughnuts under investigation were developed using UV-curing of the monomers (for PMMA type materials) or cross-linkable polymers (for PDMS type materials). The characteristics of the different biomaterials developed are outlined in Table 1.

Through static contact angles measurement, the surface wettability of the biomaterials by water was assessed. PMMA-based biomaterials showed contact angles between 50° and 70°. The PDMS-based biomaterial possessed a contact angle of 103°. The E-modulus of a biomaterial gives an insight in the elasticity, the higher the value the stronger the material. PMMA3 is the least deformable material with a value of 1053 MPa, while

Figure 2 Histological section (H&E staining) of the tissue surrounding the implant. The criteria that were used when counting the capillaries at close proximity of the doughnuts (green squares): presence of a red blood cell, presence of an endothelial cell, and presence of a lumen. Only two of the three criteria needed to be satisfied (besides diameter of the blood vessel <10 μm) for a structure to be considered a capillary (arrows ⇒). Scale bar overview = 1 mm, inserts = 50 μm. (A color version of this figure is available in the online journal.)

Table 1 ■■■■■

	PMMA1	PMMA2	PMMA3	PDMS
Contact angle	56° ± 1. 8	52° ± 1. 8	69° ± 2.9	103° ± 0. 3
E-modulus	198 MPa ± 18. 0	21 MPa ± 0. 5	1053 MPa ± 51.0	1 MPa ± 0. 1
Swelling	14%	30%	12%	0%
NIR transmission	70–80%	60–70%	50–60%	80–90%

NIR: near-infrared; PMMA: poly(methyl-methacrylate).

PDMS, with an E-modulus of 1 MPa, is the most deformable material. As a third material property, the maximum swelling ability of the biomaterials in water was evaluated. The PDMS-type material keeps the original dimensions, while PMMA-type materials swell in a composition-dependent fashion. For PMMA1 and PMMA3, a swelling of respectively 14% and 12% was observed; while a considerable swelling of 30% was observed for PMMA2. The transmittance level of NIR-light (1300–1700 nm) through all biomaterials (1 mm sample thickness) was calculated from the absorbance of the NIR-light. PDMS transmitted most of the NIR-light (80–90%) followed by PMMA1 (70–80%), PMMA2 (60–70%), and PMMA3 (50–60%).

Cytotoxicity studies

Cell viability data obtained by culturing HFF in extraction media from the different materials are given in Figure 3. None of the extraction media was cytotoxic. Compared to a control culture, cell viability was always above 80.

Histological evaluation of the implants

Regardless of the implant composition and/or surface modification, a connective tissue layer was formed around all implants (Figure 4). Based on the morphological features of the cells and the extracellular matrix, two distinct types of capsules could be described.

Figure 3 Cytotoxicity presented by the reduction of viability compared to a control culture (no contact with biomaterials). An MTT-assay was performed after the extract of the biomaterials was placed on the cells. The mean viabilities for the different biomaterials (a) and the modifications of PDMS and PMMA1 (b) were above 80% (line). Error bars = standard deviation, $N = 3$

Figure 4 Two types of capsules were observed 1 month after implantation. On the one hand, a capsule type 1 with quiescent fibroblasts (fibrocytes, inserts), a clear-cut border (insert) at the doughnut-capsule and capsule-tissue interfaces. On the other hand, a capsule type 2 with active fibroblasts (insert), a more ruffled border (insert) at the doughnut-capsule interface, and with a smooth transition at the capsule-tissue interface. Scale bar overview = 1 mm, fibroblast insert = 50 μm, and border insert = 200 μm. (A color version of this figure is available in the online journal.)

Capsule type 1. A connective tissue capsule characteristic for a final-stage of the tissue repair process. The connective tissue was characterized by an increase of fibrillar ECM (staining pink after H&E staining and correlated with the presence of parallel oriented collagen fibers), and the presence of quiescent fibrocytes: small, spindle-shaped cells with a small, dark nucleus. The capsule was sharply delineated from the implant (separating the doughnut from the capsule) and also sharply delineated from the surrounding tissue.

Capsule type 2. A connective tissue capsule corresponding to the active stage of the tissue repair process. The connective tissue was characterized by the presence of active fibroblasts, showing an abundant cytoplasm, a large oval pale-staining nucleus with fine (euchromatic) chromatin,

and a large nucleolus. The ECM was less dense and more disorganized (compared to capsule type 1) with a lower content of collagen fibers, as indicated by the decrease in eosinophilic reaction. Regarding the interface between materials and host tissue, we observed more interaction without a clear demarcation. No clear-cut demarcation was observed in the surrounding tissue. These capsules appeared to be more 'integrated'.

First implantation series: evaluating implantation site and biomaterial composition

None of the materials evoked an obvious inflammatory reaction. A minor chronic inflammation (presence of macrophages and lymphocytes) was observed around some doughnuts (17%), but never to the extent that the tissue was completely inflamed. Inflammation was observed

Figure 5 Evaluation of the capsule of all the samples in the first series (1 month of implantation with regard to the type of biomaterial regardless of implantation site (left), and implantation site regardless of biomaterial (right). As depicted in Figure 4, the capsule around the doughnuts was classified as either a type 1 (grey box) or a type 2 capsule (white box). The number of coins with the capsule type was divided by the total number of implanted coins (% of coins) to be able to evaluate differences between packaging materials or implantation sites

equally among the different implantation sites and was independent of the material used. Comparison between the different biomaterials and implantation sites with regard to the type of connective tissue layer that was formed around the implants is presented in Figure 5.

In total, 76% had a type 1 capsule versus 24% with a type 2 capsule. Obvious differences between the various material compositions were observed. Of all PDMS-based doughnuts, 47% had a type 1 capsule compared to 85% of all the PMMA-based doughnuts. Between the different PMMA formulations, the highest amount of type 1 capsule is associated with the PMMA3 doughnuts (94%), followed by PMMA2 (83%), and PMMA1 (78%). The amount of type 1 capsules surrounding the doughnuts was slightly lower for subcutaneous implants compared to the other implantation sites (71% subcutaneous, 82% muscular, and 75% peritoneal).

Conclusions for second implantation series. Based on the fact that similar tissue reactions were observed irrespective of the implantation site, only subcutaneous implantations were performed in the second series. On the material side, PDMS and PMMA1 were selected based on their optimal physicochemical and optical properties (low swelling and high NIR transmission) combined with the lowest type 1 capsule occurrence. Since the majority of the implants were not well integrated in the surrounding host-tissue, we performed a surface modification anticipating enhanced tissue integration and vascularization.

Second implantation series: evaluating the effect of the surface modification on the vascularization

The antibody binding capacity of the native (i.e. non-modified) and the surface-modified biomaterials was

assessed using radiolabeled anti-VE-cadherin after 24 h of incubation. For both PMMA- and PDMS-type materials, the anti-VE-cadherin binds to the highest extent on the PDA functionalized materials (38 respectively 21 ng/cm²). The antibody binding capacity on PDA is significantly higher compared to GelB (22 respectively 9 ng/cm²) and non-modified materials (9 respectively 8 ng/cm²), with *P* values smaller than 0.001. Modified PMMA (PDA and GelB) binds twice as much antibody as the modified PDMS (PDA respectively GelB), while control samples bind equal amounts (Figure 6). Preliminary studies revealed that the amount of antibody bound remained the same 50 h after incubation, and a random orientation of the antibody was obtained. From these studies (radiolabeling), we selected the bare biomaterials as a control for all conditions and the biomaterials with only PDA (without anti-VE-cad) as a control for the antibody. Other controls were omitted in this study to keep the number of coins/goat and the number of goats to a minimum. However, we are aware that gelatin alone could enhance cell interactions, but not in a way that endothelial cells would be attracted by preference.

As in the first series, histological sections were screened for the presence of inflammatory cells, tissue response type around the implants, and the encapsulation of the implants. Special attention was addressed to the presence of capillaries in close proximity to the implants. No obvious chronic inflammation was observed. Overall, 19% of the doughnuts showed minor inflammation reactions. A minor difference was observed between the two material types (17% for PMMA1 versus 21% for PDMS).

Regarding fibrous capsule formation, most of the PMMA1 doughnuts (modified and non-modified) were associated with a type 1 capsule irrespective of the implantation time (one versus three months). For PDMS

Figure 6 The antibody against VE-cadherin was labeled with ^{125}I , then non-modified and modified packaging materials of PDMS and PMMA1 were incubated with the antibody. Error bars = standard deviation, statistical significance is indicated by ***($P < 0.001$) and $N = 3$

doughnuts, more diversity was observed between modifications 1 month post-implantation. PDA and PDA + anti-VE-cad modified PDMS doughnuts had a high amount of type 2 capsules. When evaluating these modifications after being implanted for 3 months, the amount of type 1 capsules predominated. The effect of the surface modification on the mean number of counted capillaries/ mm^2 is graphically represented in Figure 7. One month post-implantation, no statistical differences were observed for the amount of capillaries between the different surface modifications, for both biomaterials (PMMA and PDMS). After 3 months of implantation, significant differences in the amount of capillaries were observed between the PDMS-based materials. Compared to the controls and the PDA modified materials, a significantly higher number of capillaries was present around the PDMS materials, modified with PDA and anti-VE-cadherin. In contrast, the amount of capillaries around the implants modified with PDA, GelB, and anti-VE-cad was comparable to that of the control (Figure 7(d)). Although no significant differences were observed between the PMMA control material and the applied modifications, the same tendencies as for PDMS were observed (Figure 7(c)).

Discussion

Why select a goat experimental *in vivo* model?

Although genetic diabetic animal models¹⁹ such as the spontaneously diabetic Goto-Kakizaki rat and genetically engineered diabetic mice are often used for studying diabetes, goats were selected as experimental model in this study. Since the goal of the GlucoSens project was to develop an NIR-sensor for CGM, the animal model had to be relevant for studying human glucose metabolism and enable the implantation of a relatively large packaging material in which the sensor elements are embedded. Although the normoglycemic blood glucose values of goats (50–75 mg/dL range; mean = 62.8 mg/dL) are lower than the human values (80–100 mg/dL range;

mean = 72 mg/dL), goats are widely accepted as an animal model for studying human diabetes.²⁰ The glucose metabolism of goats reacts in a similar fashion as the human glucose metabolism on stimuli like stress, glucose infuse, etc. Moreover, goats can be ‘made’ diabetic with the use of alloxan or streptozotocin. These compounds destroy pancreatic β -cells and induce diabetes mellitus via insulin reduction and can be used to study hyperglycemia. The size of our implants, being 16 mm in diameter, further justifies the application of a large animal model. Finally, up to 30 subcutaneous implants can be tested in one single goat, keeping the amount of animals to the strict minimum.

Why select a doughnut-shaped implant?

When developing an NIR-sensor, it is of importance to keep the fiber optic interface constant throughout the measurement.²¹ Within the project, our aim was to create an implantable sensor without the need of frequent optical fiber realignment. This can lead to large variations in the glucose concentration measurements. Therefore, an approach was selected in which we aim to embed the light source and detector in a doughnut-shaped biomaterial, preserving a constant ‘light path’ between light source and detector. To enable this material design, a mold was developed. The external and internal diameters were chosen to be respectively 16 and 3 mm due to space requirements for incorporating the spectrometers, electronics, and radio frequency electronics for wireless communication. The thickness of the tissue within the measurement site is known to be critical regardless of the spectral range.²² The composition and thickness of the tissue probed by the transmitting radiation strongly impact analytical performance by affecting the signal-to-noise ratio of the measurement. Optical path lengths usually vary between 1 and 10 mm.²³ In our study, the optic interface will be incorporated into the packaging materials, thus path lengths of around 5 mm can be achieved.

Figure 7 Graphical representation of the amount of capillaries at close proximity of the doughnuts, measured on histological slides. The mean number of capillaries in 18 squares of $100 \times 100 \mu\text{m}$ (see Figure 2, green squares) was brought to mean capillary density/ mm^2 . A significant increase (*) to the control was counted when PDMS doughnuts that were implanted for 3 months are modified with PDA and have the anti-VE-cad – antibody coupled to this layer. Error bars = standard deviation, statistical significance is indicated by * ($P < 0.05$) and $N = 3$

Why select PDMS- and PMMA-type packaging materials?

PDMS and PMMA were selected because of their known optical transparency in the NIR range.^{8,9} Moreover, both materials are known to be biocompatible and are widely applied in medicine: PMMA as bone cement, contact and intra-ocular lenses, fixation and anchoring of artificial joints, and cranial reconstruction. PDMS is applied as breast implant, in facial reconstructive surgery, as orbital implant, in hearing aids, implantable rods for the delivery of contraceptives, and intraocular lenses.^{10–12,24} The International Organization for Standardization (ISO) 10993 provides a series of standards for evaluating the cytotoxicity of a medical device prior to clinical testing. *In vitro* testing is covered by the ISO 10993-5 norm. According to these standards, a material showing a cell viability of or above 70% is considered non-toxic. The results of our study indicate that none of the applied materials were cytotoxic (Figure 3) and that chronic inflammation was, as anticipated, mild and occurred in less than 20% of the

implants irrespective of the biomaterial composition or the implantation site. Using the two candidate implant materials, it is feasible to obtain a constant fiber optic interface. None or only limited swelling was observed for, respectively, the PDMS-type and PMMA1 materials (Table 1). This implies that all optical parts remain fixed in space after the implant production in the mold. Since the selected materials are non-biodegradable, we anticipate this to be the case throughout the life-time of the sensor.²⁴ This inflammatory response could be explained by the degradation process of the biodegradable sutures used.

Why apply biomaterial surface modification?

Although biocompatible, PDMS and PMMA are both inert and hydrophobic materials, possessing limited or no cell interactive properties. This might possibly hamper a good integration of the implant into the surrounding tissue. Recent reviews about tissue responses to biomaterials state that the end-stage healing by repair response is

generally a fibrous encapsulation of the implant. The extent and the occurrence of capsule formation depends on several factors: the physical and the chemical properties of the materials, the duration, and the site of the implantation.¹³ Also in our study, the majority (75.6%) of all native materials under study – independent of the implantation site and the biomaterial composition (Figure 5) – were separated from the surrounding tissue by a well-delineated connective tissue capsule (type 1). However, both materials seemed to evoke a different tissue response: PDMS implants – in contrast to PMMA1, PMMA2, and PMMA3 implants – seem to slow down the process of fibrosis and encapsulation. Indeed, 1 month after implantation the minority of all PDMS doughnuts evoked a type 1 capsule compared to the majority of the PMMA implants. Three months after implantation, the majority of PDMS implants also evoked a type 1 capsule. Reducing capsule formation by bio-activating the materials surface has since long been the focus of many research teams.^{3,25–28} This can be realized by applying a number of approaches including among others plasma treatment and thin film modifications.

Since the goal of our research includes the measurement of glucose levels, both integration of the implant in the surrounding tissue and improvement of the vascularization in the implant vicinity are of importance.²⁹ The latter is known to extend sensor performance and shorten the time lag.¹⁶ For this reason, we opted to modify the surface of the packaging material with the aim of increasing the vessel density at the implantation site. In disease and tissue regeneration, new blood vessels were thought to only be formed from pre-existing blood vessels by a process known as sprouting angiogenesis.³⁰ In this process, endothelial cells migrate and divide from the existing vessels, and fusion of vacuoles within the endothelial cells creates the vascular lumen. However, recent studies showed that an alternative mechanism can also lead to the formation of new blood vessels in tissue regeneration, a process called vasculogenesis or neovascularization.³¹ In this process, endothelial precursor cells (EPCs) play a key role. EPCs are formed in the bone marrow and mobilized by growth factors into the circulation, from where they home to sites of new vessel growth. Signals important to mobilize EPCs are generated, for example, by ischemic tissues. Since implantation of biomaterials is accompanied by rupture of blood vessels, the generation of ischemic tissue in the surrounding of the implant could be important to recruit EPCs (and endothelial cells) in the environment of the implant. Both late EPCs and endothelial cells express specific surface markers that can be used as targets to capture these cells. In this regard, monoclonal antibodies, aptamers, peptides, etc.³² are used to bio-functionalize materials. Although most of these materials are used in the blood stream to ensure (promote) re-endothelialization of stents, vascular grafts, we hypothesized that in ischemic tissues such materials could be beneficial in attracting endothelial-like cells, and consequently, promote neo-vascularization around an implant. The modification applied was based on coupling vascular endothelial cadherin (VE-cadherin) antibodies. VE-cadherin is a surface marker that is exclusively expressed on late EPC and endothelial cells and is also known to play an essential role

during morphogenesis of the blood vessels.^{33,34} Very recently, VE-cad antibodies have been shown to be superior for the endothelialization of stents compared to CD34, for example.^{32,35,36}

The simultaneous addition of endothelial growth factors such as VEGF or anti-inflammatory agents such as dexamethasone – who have proven their additional value to enhance neovascularization³⁷ – could be considered in future works. Since slow release of those factors is the key for prolonged effect, implementing those in the current construct could have a negative impact on its stability.

To enable the immobilization of antibodies to the materials surface, we opted to apply a thin PDA prime layer. The role of this prime layer is two-fold. First, it enhances the hydrophilicity of the material surface. Research worldwide has shown that this surface property has a profound influence on a specific protein binding.^{38,39} Second, the selected prime layer (i.e. PDA) also enables the immobilization of a large variety of (bio)molecules. In our work, we deposited a surface-adherent film through a simple incubation process. Antibodies were subsequently covalently bound either directly on the PDA⁴⁰ or by physisorption through an intermediate gelatin type B (GelB) layer.⁴¹ Radiolabeling studies revealed that the PDA-modified biomaterials bound twice as much anti-VE-cad compared to GelB (Figure 6). These data can be nicely linked to the degree of vascularization 3 months after implantation. Indeed, the highest micro vessel density was observed for PDA-anti-VE-cad. The application of the gelatin intermediate layer, with the aim to enhance the anti-VE-cad availability, thus did not improve vascularization (Figure 7). For PDMS, the capillary density is significantly higher (1.5 times) for the PDA-anti-VE-cad compared to the native materials and the materials modified with PDA only. PMMA1 displays the same trends but lacks statistical significance (1.2 times higher). Combining these findings with the fact that the anti-VE-cad density on PMMA1 was two-fold higher compared to the one on PDMS, it becomes obvious that the antibody density is not the only factor affecting the capillary density. These findings will form the basis for future research using quartz crystal microbalance coupled ellipsometry to investigate potential differences in antibody orientation between both materials under study as well as dose-response studies linking the antibody density to the capillary density in the peri-implant.

Conclusions

Although transparent in the NIR range, non-modified PMMA- and PDMS-type materials possess several drawbacks disabling application as packaging materials for implantable continuous glucose sensors. The strategies we elaborated in the present work were not successful in preventing implant encapsulation. However, PDMS seemed to delay the end-stage healing by repair response to a higher extent compared to PMMA. From the different strategies applied to enhance peri-implant vascularization, a VE-cadherin antibody coupled to polydopamine was able to stimulate capillary formation within the capsule. In our opinion, the strategy elaborated here represents an

improved environment for glucose monitoring compared to the current state-of-the-art.

Authors' contributions: VK and EVDW were responsible for creating and modifying the packaging materials. LV was responsible for the animal experiments. KK and FDV were responsible for the radio-active experiments. HD and PD were responsible for editing and writing assistance. MC is chair of the department and responsible for editing and writing assistance.

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REFERENCES

1. The Epidemiology of Diabetes Interventions and Complications Study. Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study. *JAMA* 2003;**290**:2159–67
2. Oliver NS, Toumazou C, Cass AE, Johnston DG. Glucose sensors: a review of current and emerging technology. *Diabet Med* 2009;**26**:197–210
3. Koschwaner HE, Reichert WM. In vitro, in vivo and post explantation testing of glucose-detecting biosensors: Current methods and recommendations. *Biomaterials* 2007;**28**:3687–703
4. Olesberg JT, Liu LZ, Van Zee V, Arnold MA. In vivo near-infrared spectroscopy of rat skin tissue with varying blood glucose levels. *Anal Chem* 2006;**78**:215–23
5. Roelkens G, Green WMJ, Kuyken B, Liu X, Hattasan N, Gassenq A, Cerutti L, Rodriguez JB, Osgood RM, Tournie E, Baets RG. III-V/Silicon Photonics for Short-Wave Infrared Spectroscopy. *IEEE J Quant Electron* 2012;**48**:292–8
6. Ryckeboer E, Vierendeels J, Lee A, Werquin S, Bienstman P, Baets R. Measurement of small molecule diffusion with an optofluidic silicon chip. *Lab Chip* 2013;**13**:4392–9
7. Ryckeboer E, Gassenq A, Muneeb M, Hattasan N, Pathak S, Cerutti L, Rodriguez JB, Tournie E, Bogaerts W, Baets R, Roelkens G. Silicon-on-insulator spectrometers with integrated GaInAsSb photodiodes for wide-band spectroscopy from 1510 to 2300 nm. *Opt Express* 2013;**21**:6101–8
8. Schneider F, Draheirn J, Kamberger R, Wallrabe U. Process and material properties of polydimethylsiloxane (PDMS) for Optical MEMS. *Sensors Actuators A Phys* 2009;**151**:95–9
9. Abbasi F, Mirzadeh H, Simjoo M. Hydrophilic interpenetrating polymer networks of poly(dimethyl siloxane) (PDMS) as biomaterial for cochlear implants. *J Biomater Sci-Polym Ed* 2006;**17**:341–55
10. Lugowski SJ, Smith DC, Bonek H, Lugowski J, Peters W, Semple J. Analysis of silicon in human tissues with special reference to silicone breast implants. *J Trace Elem Med Biol* 2000;**14**:31–42
11. Race A, Mann KA, Edidin AA. Mechanics of bone/PMMA composite structures: An in vitro study of human vertebrae. *J Biomech* 2007;**40**:1002–10
12. Jongebloed WL, van der Veen G, Kalicharan D, van Andel MV, Bartman G, Worst JGF. New material for low-cost intraocular lenses. *Biomaterials* 1994;**15**:766–73
13. Anderson JM. Biological responses to materials. *Ann Rev Mater Res* 2001;**31**:81–110
14. Sharkawy AA, Klitzman B, Truskey GA, Reichert WM. Engineering the tissue which encapsulates subcutaneous implants .1. Diffusion properties. *J Biomed Mater Res* 1997;**37**:401–12
15. Wood RC, Lecluyse EL, Fix JA. Assessment of a model for measuring drug diffusion through implant-generated fibrous capsule membranes. *Biomaterials* 1995;**16**:957–9
16. Onuki Y, Bhardwaj U, Papadimitrakopoulos F, Burgess DJ. A review of the biocompatibility of implantable devices: current challenges to overcome foreign body response. *J Diabetes Sci Technol* 2008;**2**:1003–15
17. Block SS. *Disinfection, sterilization, and preservation*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2001, p.xxii, 1481
18. Fraker PJ, Speck Jr JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem Biophys Res Commun* 1978;**80**:849–57
19. Rees DA, Alcolado JC. Animal models of diabetes mellitus. *Diabetic Med* 2005;**22**:359–70
20. Haghdoust I, Ghaleshahi A, Safi S. Clinicopathological findings of diabetes mellitus induced by alloxan in goats. *Compar Clin Pathol* 2007;**16**:53–9
21. Trabelsi A, Boukadoum M, Fayomi C, Aboulhamid EM. Blood glucose sensor implant using NIR spectroscopy: preliminary design study. *Int C Microelectron* 2010;176–9
22. Burmeister JJ, Arnold MA. Evaluation of measurement sites for noninvasive blood glucose sensing with near-infrared transmission spectroscopy. *Clin Chem* 1999;**45**:1621–7
23. Hazen KH, Arnold MA, Small GW. Measurement of glucose in water with first-overtone near-infrared spectra. *Appl Spectrosc* 1998;**52**:1597–605
24. Shastri VP. Non-degradable biocompatible polymers in medicine: past, present and future. *Curr Pharm Biotechnol* 2003;**4**:331–7
25. Li AG, Quinn MJ, Siddiqui Y, Wood MD, Federiuk IF, Duman HM, Ward WK. Elevation of transforming growth factor beta (TGFB) and its downstream mediators in subcutaneous foreign body capsule tissue. *J Biomed Mater Res Part A* 2007;**82A**:498–508
26. Norton LW, Koschwaner HE, Wisniewski NA, Klitzman B, Reichert WM. Vascular endothelial growth factor and dexamethasone release from nonfouling sensor coatings affect the foreign body response. *J Biomed Mater Res Part A* 2007;**81A**:858–69
27. Zhu HG, Srivastava R, Brown JQ, McShane MJ. Combined physical and chemical immobilization of glucose oxidase in alginate microspheres improves stability of encapsulation and activity. *Bioconjug Chem* 2005;**16**:1451–8
28. Ainslie KM, Sharma G, Dyer MA, Grimes CA, Pishko MV. Attenuation of protein adsorption on static and oscillating magnetostrictive nanowires. *Nano Lett* 2005;**5**:1852–6
29. Klueh U, Dorsky DI, Kreutzer DL. Enhancement of implantable glucose sensor function in vivo using gene transfer-induced neovascularization. *Biomaterials* 2005;**26**:1155–63
30. Geudens I, Gerhardt H. Coordinating cell behaviour during blood vessel formation. *Development* 2011;**138**:4569–83
31. George AL, Bangalore-Prakash P, Rajoria S, Suriano R, Shanmugam A, Mittelman A, Tiwari RK. Endothelial progenitor cell biology in disease and tissue regeneration. *J Hematol Oncol* 2011;**4**:24
32. Avci-Adali M, Perle N, Ziemer G, Wendel HP. Current concepts and new developments for autologous in vivo endothelialisation of biomaterials for intravascular applications. *Eur Cell Mater* 2011;**21**:157–76
33. Vestweber D. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol* 2008;**28**:223–32
34. Vestweber D, Winderlich M, Cagna G, Nottebaum AF. Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player. *Trends Cell Biol* 2009;**19**:8–15
35. Lim W-H, Seo W-W, Choe W, Kang C-K, Park J, Cho H-J, Kyeong S, Hur J, Yang H-M, Cho H-J, Lee Y-S, Kim H-S. Stent coated with antibody against vascular endothelial-cadherin captures endothelial progenitor cells, accelerates re-endothelialization, and reduces neointimal formation. *Arterioscler Thromb Vasc Biol* 2011;**31**:2798–U141
36. Lee JM, Choe W, Kim BK, Seo WW, Lim WH, Kang CK, Kyeong S, Eom KD, Cho HJ, Kim YC, Hur J, Yang HM, Lee YS, Kim HS.

- Comparison of endothelialization and neointimal formation with stents coated with antibodies against CD34 and vascular endothelial-cadherin. *Biomaterials* 2012;**33**:8917–27
37. Patil SD, Papadimitrakopoulos F, Burgess DJ. Concurrent delivery of dexamethasone and VEGF for localized inflammation control and angiogenesis. *J Contr Release* 2007;**117**:68–79
38. Lee H, Dellatore SM, Miller WM, Messersmith PB. Mussel-inspired surface chemistry for multifunctional coatings. *Science* 2007;**318**:426–30
39. Elbert DL, Hubbell JA. Surface treatments of polymers for biocompatibility. *Annu Rev Mater Sci* 1996;**26**:365–94
40. Wan Y, Zhang D, Wang Y, Qi P, Hou B. Direct immobilisation of antibodies on a bioinspired architecture as a sensing platform. *Biosens Bioelectron* 2011;**26**:2595–600
41. Berneel E, Desmet T, Declercq H, Dubrueel P, Cornelissen M. Double protein-coated poly-epsilon-caprolactone scaffolds: Successful 2D to 3D transfer. *J Biomed Mater Res Part A* 2012;**100A**:1783–91

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