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Non-Invasive Monitoring of Oxygen Tension and Oxygen Transport Inside Subcutaneous Devices After H$_2$S Treatment

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Abstract
Medical devices for cell therapy can be improved through prevascularization. In this work we study the vascularization of a porous polymer device, previously used by our group for pancreatic islet transplantation with results indicating improved glycemic control. Oxygen partial pressure within such devices was monitored non-invasively using an optical technique. Oxygen-sensitive tubes were fabricated and placed inside devices prior to subcutaneous implantation in nude mice. We tested the hypothesis that vascularization will be enhanced by administration of the pro-angiogenic factor hydrogen sulfide (H$_2$S). We found that oxygen dynamics were unique to each implant and that the administration of H$_2$S does not result in significant changes in perfusion of the devices as compared with control. These observations suggest that vascular perfusion and density are not necessarily correlated, and that the rate of vascularization was not enhanced by the pro-angiogenic agent.

Keywords
oxygen monitoring, tissue engineering, islet transplantation, diabetes, medical devices, biophotonics

Introduction
Tissue engineering approaches to create bioartificial organs have been emerging in the past few decades. In the case of the pancreas, as related to endocrine function, evidence suggests that it is unnecessary to replace the whole organ. It is shown that transplantation of the islets of Langerhans that contain the endocrine cells also results in restoration of the endocrine function. For example, transplantation of isolated pancreatic islets is used to treat type 1 diabetes (T1D). T1D is caused by autoimmune destruction of pancreatic beta cells. These cells normally synthesize and secrete insulin, which is an important hormone involved in physiological regulation of blood glucose. Self-monitoring of blood glucose (by lancet blood draw and glucose meter) in conjunction with multi-daily injections of insulin is the standard of care. However, the resulting glycemic control is imperfect and can cause harmful secondary complications such as cardiovascular diseases, retinopathy, and nephropathy. A promising alternative to daily insulin injections is transplantation of pancreatic islet cells. This transplantation can provide autonomous regulation of blood glucose concentration and therefore result in stable glycemic control, thus preventing secondary complications. Islet transplantation into the portal vein has resulted in insulin independency in the first year; however, the insulin independency was shown to decrease to less than 50% after 5 years. These findings support the potential for pancreatic islets to treat T1D; however, in consideration of the 5-year half life, inaccessibility for islet retrieval, and risk to the liver, better approaches are needed.

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Polymer devices for housing islets are being developed to improve the outcomes of islet transplantation. A variety of polymer systems as well as implantation sites have been investigated\textsuperscript{11–17}. Literature suggests that the subcutaneous space can be advantageous for cellular transplantation because of ease of access and less invasive surgical procedures, but because of low oxygen tension and vascularization under the skin, hypoxia can occur\textsuperscript{18,19}. Hypoxia can induce apoptosis and necrosis and ultimately compromise the functionality of transplanted cells\textsuperscript{20}. Multiple groups have shown that islet transplantation under the skin can be successful after modulation of the site with a device\textsuperscript{21,22}. Critical to the success of these devices is adequate supply of oxygen, which can be provided if sufficient vascular perfusion is introduced. Such perfusion can be stimulated by using microporous structures\textsuperscript{23}, inclusion of angiogenic factors\textsuperscript{24}, co-transplantation of vascular inductive cell types, or devices that can be implanted for prevascularization prior to transplantation of the cells\textsuperscript{25}. A well-vascularized device will provide reduced diffusional distances between the newly formed vasculature and the transplanted cells and result in faster transport dynamics of nutrients and oxygen, preventing the loss of cells by hypoxia. We have developed a porous poly(D, L-lactide-co-ε-caprolactone) (PDLLCL) polymer scaffold that has shown successful results in promoting islet-cell survival and achieving normoglycemia under the skin of athymic nude mice for the duration of a 70 day study\textsuperscript{26}. This porous scaffold is designed for inducing vascularization in vivo and can potentially compensate for loss of perfusion through the interpenetrating islet capillary network following isolation. Our previous results\textsuperscript{26} suggest that further increasing the vascularization of these devices may lead to a faster return to normoglycemia with lower number of islets.

In this study, we investigated hydrogen sulfide (H\textsubscript{2}S) as an agent to improve vascularization of the PDLLCL device. H\textsubscript{2}S is a gaseous signaling molecule, like nitric oxide and carbon monoxide, normally produced by the body. It was first described as a neuromodulator\textsuperscript{27}, but was later recognized for its effects on vascular cells\textsuperscript{28}. Endogenous H\textsubscript{2}S is enzymatically generated in vascular cells, where it exhibits vasoactive, anti-oxidant, anti-inflammatory, and anti-apoptotic properties\textsuperscript{28}. The effects of H\textsubscript{2}S are well described, but the molecular pathways of its action are poorly understood. Cai and coworkers were the first to show that intraperitoneal injections of the exogenous H\textsubscript{2}S donor NaHS stimulate angiogenesis in subcutaneous Matrigel plugs in mice\textsuperscript{29}. Increased angiogenesis was shown by higher hemoglobin levels and the presence of more capillaries with NaHS administration at concentrations of 10 and 50 μmol/(kg*d) as compared with the untreated group. Here we investigate whether H\textsubscript{2}S will have a similar effect on vascularization of our PDLLCL devices implanted into the subcutaneous compartment as assessed by histology, CD31 expression, and an optical measure of oxygen transport between vasculature and the device.

Materials and Methods

Device Preparation

Devices were obtained from Polyganics B.V. (Groningen, The Netherlands). A 4% (w/v) PDLLCL solution was prepared in chloroform (Sigma-Aldrich, Zwijndrecht, The Netherlands). The PDLLCL solution was thoroughly mixed with sodium chloride particles (Sigma-Aldrich, Zwijndrecht, The Netherlands) of 250–425 μm (10:1 w/w) in order to create a porous structure. This solution was transferred into sterile glass Petri dishes to allow the solvent to evaporate. To remove the salt particles, the polymer sheet (5 mm thick) was extensively washed with sterile H\textsubscript{2}O. The polymer sheet was casted and resized, resulting in a 10 mm by 15 mm device. During the casting process three channels were created by introducing 400 μm diameter iron rods. These channels were created to accommodate cells, and in the current study they were used for insertion of the oxygen-sensitive tubes (OSTs) to non-invasively monitor the oxygen tension in vivo (Fig. 1A). The scaffolds were stored in 70% ethanol for several days to sterilize the scaffolds before implantation.

OST Fabrication

OSTs comprise an oxygen-permeable tube and an oxygen-sensitive dye. The selected oxygen-sensitive dye is platinum(II)- meso-tetra (4-fluorophenyl) tetrabenzoporphyrin (PtTPTBPFP), which has peak absorptions at 430 and 614 nm and emission peak at 773 nm (Frontier Scientific, Logan, UT, USA). This dye was mixed with polystyrene (MW 2500, Sigma, St. Louis, MO, USA) and dissolved into a solution using chloroform (Sigma, St. Louis, MO, USA), to form the dye solution. The dye solution contains 4 mg PtTPTBPFP, 60 mg polystyrene and 900 μL chloroform. The dye solution was flushed through an oxygen-permeable biocompatible silicone tubing (BTSIL-037, Instech Laboratories, Plymouth Meeting, PA, USA) five times to form a homogenous inner coating of PtTPTBPFP inside the silicone tubes. The tubes were stored in a well-ventilated dark environment at 24°C for a duration of 24 h to allow the remaining chloroform to evaporate. A stainless steel wire (24 Gauge A1 Wire, Kanthal, Sweden) was then inserted into each tube in order to provide mechanical stability (Fig. 1B). Afterwards the tubes containing a wire were cut to a length matching the PDLLCL device (15 mm) and the ends were sealed using medical-grade silicone adhesive (MED-1000 Nusil, Carpinteria, CA, USA). The sealed tubes were kept at room air in a dark environment for 2 days to fully cure. OSTs were sterilized by immersion in 70% ethanol for 12 h.

The Oxygen Monitor

An oxygen monitor was fabricated a previously described\textsuperscript{10}. The monitor is placed onto the surface of skin, emits light through the skin and onto the implanted OSTs, and receives luminescence to record luminescent waveforms. The
monitor comprises a printed circuit board that houses a silicon photodetector bracketed by two light-emitting diodes (LEDs) having central emission wavelength of 617 nm (Fig. 1C). An optical bandpass filter is mounted onto the detector to reject the LED wavelengths while passing the dye emission wavelengths. For each oxygen measurement both LEDs flash simultaneously 25 times at 1% duty cycle (100 μs on, 10,200 μs off) to excite the oxygen-sensitive porphyrin dye. The emission of the dye after each flash is passed through an optical filter and onto the photodiode. Photodiode electrical current is converted to voltage by an operational amplifier and sampled over a period of 200 μs at a sampling frequency of 500 kHz using a myRIO data acquisition module (National Instruments, Austin, TX, USA). LED and data acquisition timing is controlled by LABVIEW 2015 (National Instruments, Austin, TX, USA). The dye continues to emit light after the end of each LED emission period with a decay that can modeled as exponential:

$$ V = V_0 e^{\left( -\frac{t}{\tau} \right)} $$

where $V$ is the detector voltage, $V_0$ is the voltage at the start of the decay, $t$ is time in seconds, $\tau$ is the lifetime parameter reported in seconds. Lifetime values were computed from emission waveforms using the fit function in MATLAB (MathWorks, Natick, MA, USA) configured for nonlinear least squares regression. The average $\tau$ for all 25 cycles is hereafter reported as lifetime ($\tau$). The porphyrin dye emission is quenched by oxygen and consequently lifetime values ($\tau$) decrease with increasing PO$_2$.

**OST Calibration**

A custom-built glass chamber with an inlet and outlet port was placed above the oxygen monitor. OST were individually placed inside the chamber and exposed to different gases mixtures at atmospheric pressure comprising 160), 76, 38, and 0 mmHg oxygen. $\tau$ was measured at each oxygen partial pressure to generate a calibration curve (Fig. 2A). This process is repeated for each OST individually. OSTs were matched for calibration in groups of three and inserted into devices.

**Implantations and NaHS Injections in Animals**

The University of California Institutional Animal Care and Use Committee at the University of Irvine approved all described animal procedures (IACUC # 2008-2850). Animals were housed at the University of California Irvine animal facility and maintained under 12-hour light/dark cycles with ad libitum access to water and standard chow. Devices were implanted in male athymic nude mice (Foxn1$^\text{nu}$, Charles...
River, Wilmington, MA, USA) 8 weeks old. Briefly, under anesthesia a small incision was made in the skin to create a subcutaneous pocket on the back of the mouse. PDLLCL devices containing the calibrated OSTs and fibrin were placed into this pocket and the skin was closed using skin staples (Cellpoint Scientific, Gaithersburg, MD, USA). All mice received ibuprofen water (0.2 mg/mL; Banner Pharmacaps, High Point, NC, USA) as analgesic post-surgery for 2 days. Since H2S is a gas, we used a sodium hydrosulfide (NaHS) solution as a H2S donor. NaHS is unstable in solution and therefore a new solution was prepared just prior to each injection. From the day of implantation, mice received twice daily an intraperitoneal injection of NaHS until 28 days after implantation. One group of mice (n = 6) received a low dose of NaHS (25 μmol/kg), the other group (n = 6) received a high dose of NaHS (50 μmol/kg), and mice receiving saline injections served as a control group (n = 6).

**Dynamic Inhaled Gas Test (DIGT)**

To closely follow the vascularization process in vivo, DIGT measurements were performed on days 3, 7, 21, 35, 49 and 63 after implantation on each animal (n = 6 per group). For the DIGT measurements each mouse was anesthetized by 2–3% of isoflurane (Piramal Healthcare, Morpeth, United Kingdom) while breathing 760 mmHg oxygen. When the animals were under full anesthesia the gas was changed to 152 mmHg oxygen and isoflurane level was reduced to 1.5%. The optical monitor was then placed on the skin directly above the implanted device. Following 100 baseline lifetime measurements (spaced by 4.25 s for a total of 425 s), the inhaled gas was switched back to 760 mmHg oxygen. It is expected that the tissue PO2 will rise and fall as the inhaled PO2 is increased and decreased, respectively. Accordingly, once the inhaled gas is returned to 760 mmHg, lifetime values should reach a new plateau, where the time to plateau is an indication of the oxygen transport between the blood, tissue and OSTs. Once the plateau was reached, animals were taken off of anesthesia and returned to their cages. The time to plateau is calculated using custom MATLAB code (MathWorks, Natick, MA, USA) and reported here as **Risetime** (in minutes).

**Vessel Morphology**

The subcutaneous devices were removed on day 63 after implantation. OSTs were removed from the devices and devices were cut into two pieces; one half of the scaffold was processed for the histological analysis whereas the other half was used for the quantification of vessels by PCR (described below). For histology, the scaffolds were fixated in 2% paraformaldehyde and processed for paraffin embedding. Sections were stained with an overnight incubation of CD31 (1:200; R&D Systems, Abingdon, United Kingdom) after 15 min incubation at 100°C for antigen retrieval (10 mM citrate buffer, pH 6.0) and a subsequent blocking step with 5% donkey serum (Sigma-Aldrich, Zwijndrecht, The Netherlands). The secondary donkey anti-goat alkaline phosphatase conjugated antibody (1:100; Abcam, Cambridge, United Kingdom) was applied for 45 min. Alkaline phosphatase activity was demonstrated by incubating for 10 min with SIGMAFAST™ Fast Red (Sigma-Aldrich, Zwijndrecht, The Netherlands). A short incubation with hematoxylin was used as counterstain. All stained sections were scanned with...
the microscope scanner Hamamatsu Nanozoomer (Hama-
matsu, Almere, The Netherlands). Scans were analyzed
using Aperio ImageScope (Leica Microsystems B.V., Rijs-
wijk, The Netherlands). The number of CD31-positive struc-
tures was normalized by the measured scaffold area (mm²).
The fold change was calculated relative to the control.

Vascularization Quantification
To quantify the amount of vessels in the scaffolds, half of
the scaffold at day 63 was processed for real-time reverse tran-
scription polymerase chain reaction (RT-PCR) for the vas-
cularization marker CD31. RNA was isolated using Trizol
according to the manufacturer’s protocol (Invitrogen, Fisher
Scientific, Landsmeer, The Netherlands). The RNA concen-
tration was determined using a NanoDrop 1000 spectrophot-
ometer (NanoDrop products, Wilmington, DE, USA). cDNA
was reverse transcribed using a SuperScript® III Reverse
Transcriptase kit according to the instructions of the manu-
facturer (Life Technologies, USA). RT-PCR was conducted
using ViiA™ Real Time PCR system (Life technologies,
Carlsbad, CA, USA) with a primer and probe set (TaqMan
Gene Expression Assays, Thermo Fisher Scientific, USA)
for CD31 (Mm01242576_m1; Thermo Fisher Scientific,
Waltham, MA, USA) and qPCR Mastermix Plus (Eurogen-
tec, Seraing, Belgium). Reactions were performed at 50°C
for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 60 s
repeating in these last two steps for 40 cycles. Delta Ct
values were calculated and normalized against the expres-
sion of the housekeeping gene GAPDH (Mm99999915_g1;
Thermo Fisher Scientific, Waltham, MA, USA). Delta Ct
values were used to calculate the fold change compared with
gene expression of the control mice.

Statistics
Statistical analysis was carried out in GraphPad Prism (ver-

sion 7.0d; GraphPad Software, Inc., San Diego, CA, USA).
A Shapiro–Wilk normality test was performed to test the
data for normality. A Kruskal–Wallis test with Dunn’s mul-
tiple comparison was performed to study the statistically
significant differences for nonparametric distributions.
One-way ANOVA with a Tukey post-hoc was performed for
parametrically distributed data. p-values <0.05 were consid-
ered significant. The data are presented in mean ± standard
development in case of parametric distribution and median ±
terquartile range in case of nonparametric distribution.

Results

OST Calibration and DIGT
Prior to implantation, each OST (Fig. 1B) undergoes a gas
 calibration inside a glass chamber. In this calibration gases
with different oxygen levels are inserted into the chamber in
order. Fig. 2A shows a calibration process during which the
gas mixtures were cycled three times. With each gas
exchange, the corresponding τ is recorded as the mean value of
the subsequent plateau. Lifetime values at each oxygen
concentration across all three cycles show low variation
(standard deviation < 0.08 µs). This result demonstrates the
repeatability and reliability of oxygen measurements using
OST. Following the calibrations, OSTs were inserted into
devices, which were implanted as described above. Fig. 2B
shows an example of the calculated PO2 values during a
DIGT test. This curve demonstrates an increase in PO2 levels
as reported by the OSTs.

Animal Study
Animals received injections of NaHS (low and high-dosage
groups of 25 µmol/kg and 50 µmol/kg) and saline (control
group) as described above. Injections occurred twice daily for
the first 4 weeks (day 28) followed by another 5 weeks of no
injections (up to day 63). The follow-up period of 5 weeks
with no injections (day 28 to day 63) aims to provide neces-
sary time for device vascularization to complete. The DIGT
was performed on all animals (n = 6 per group) on days 3, 7,
21, 35, 49, and 63. Fig. 3 (A–C) shows the Risetime values
calculated from the DIGT measurements on all animals. Sta-
tistical differences in lifetimes values were not observed either
for each treatment condition across all days, or between treat-
ment conditions on each day. Similarly, DIGT plateau oxygen
pressures do not show statistical differences or trends between
different conditions or at different timepoints (Fig. 3 (D–F)).
These observations indicate vascular perfusion of implants
was not affected by administration of the pro-angiogenic
H2S. Fig. 4 shows the histological and gene expression eva-

lutions performed on the explanted devices ( explantation on
day 63). Surprisingly, statistically different values were
observed for the expression of the endothelial cell marker
CD31 between treatment groups (Fig. 4D). This may indicate
that treatment with a low dose of H2S can possibly down-
regulate the gene expression of CD31 (fold change of 0.57
± 0.02) compared with the control group and the high-dose
group. In addition, histological analysis of CD31 expression
shows a similar trend at the protein level (Fig. 4E). Here,
CD31-positive vessels were counted in each sample. While
the control mice had a mean vessel area density of 40.5 ± 7.5
blood vessels per mm² of scaffold, both the low and high

treatment groups showed significantly decreased values of
15.0 ± 2 and 18.7 ± 8.3 blood vessels per mm² of scaffold,
respectively. These results indicate that the vascularization
inside the devices may have been negatively affected by the
H2S administrations.

Discussion
The success of subcutaneously implanted cell therapy
devices depends on the ability to transfer nutrients and oxy-
gen to implanted cells. It is shown that implantation of such
devices can trigger a series of host reactions at the implanta-
tion site, such as chronic inflammatory response, granulation
tissue development, foreign body reaction, and fibrosis and fibrous capsule development. The foreign body response often occurs in the first 3 weeks after implantation; after which, wound healing may proceed accompanied with neovascularization of the device. Invariably, this foreign body reaction can cause formation of diffusional barriers that result in lower availability of nutrients and, importantly, oxygen within the implanted devices. This low availability can result in reduced functionality of the cells and formation of hypoxic conditions, which ultimately can lead to cell death and device failure.

It is therefore necessary to ensure the availability of oxygen and nutrients as delivered by a perfused vasculature. For such vascularization purposes, porous three-dimensional biomaterials have been used as subcutaneous medical devices in the field of tissue engineering. The pore structure of these biomaterials allows blood vessel infiltration and thereby reduction of diffusional distances and barriers between the neovasculature and the cells. In this study we explore the rate of vascularization and oxygen availability within porous PDLCL devices developed by our group. These PDLCL devices have already shown success in preserving functionality of pancreatic islet cells after subcutaneous implantation, which resulted in achieving normoglycemia in type 1 diabetic animals.

In these previous studies we implanted the devices several weeks before islet transplantation to allow dampening of the foreign body response and ingrowth of blood vessels. When islets were transplanted into these devices, the vascularization supports functional islet survival in the period between transplantation and islet revascularization. However, further improving the vascularization of this device will lead to faster induction of normoglycemia with lower numbers of islets making islet transplantation available for a larger number of type 1 diabetic patients.

H2S has previously been shown to stimulate endothelial proliferation and migration as well as the development of a new vasculature within Matrigel constructs implanted subcutaneously. Our results from the non-invasive optical measurements to assess device perfusion in vivo showed no significant differences between the control and H2S-administered groups over the 63-day period of the study. Interestingly, histological and gene expression analysis indicate that H2S administration may have resulted in lowered formation of neovasculature, an observation not reflected in oxygen dynamics between treatments groups and across days. This suggests that any differences in the extent of vascularization may not be large enough to elicit observable changes in oxygen transport dynamics. The vascular
density as quantified with histology and CD31 expression does not necessarily have to correlate with the DIGT signal, as vascular density does not necessarily reflect the perfusion of the device. For example, consider a cross-sectional view of an oxygen-sensitive rod and its surrounding porous device and tissue. There is a no-flux condition at the tube surface (oxygen is not consumed by the dye) and \( \text{PO}_2 \) dynamics reported by the tubes are dependent upon (1) diffusional properties of the tissue and device, which do not change at the time scale of a DIGT, and (2) the net rate of transport of oxygen from the vasculature into the device or tissue. These changes are rapid, and depend on \( \text{PO}_2 \) within the vasculature and blood velocity within them. All things being equal, the primary determinants of DIGT dynamics are total available perfused vessel area, partial pressure of oxygen in the local microvasculature, and tissue or device diffusional properties. We can reliably assume that as vessels invade the device, the system will take less time to reach a new steady state. Accordingly, we anticipate \textit{Risetime} would decrease with increased vessel density and perfusion within the device. In this study, as shown in Fig. 2B for day 3, the \( \text{PO}_2 \) values rise approximately up to 20 mmHg during the DIGT. These oxygen measurements are exclusive to the surface of OSTs and do not directly measure oxygen partial pressure elsewhere in the device. The observed values are different from what our group has previously reported for DIGT experiments wherein alginate microcapsules containing oxygen-sensitive microparticles were implanted subcutaneously in Sprague-Dawley rats\textsuperscript{30}. \( \text{PO}_2 \) levels changed from 45 to 120 mmHg during a day-9 DIGT, at a rate approximately twice that of the present study. These differences indicate that spherical alginate capsules may make for a better environment for delivering oxygen as compared to the investigated device.

In addition to slow dynamics, DIGT experiments reveal low levels of oxygenation within devices measured at the surface of the inserted OSTs. Importantly, our experiment was designed to include six mice per condition, a number estimated to be sufficient when we analyzed perfusion data (total Hb) in the Cai et al. paper. However, DIGT data indicate that six mice is not nearly enough to achieve statistical power at a 95\% level of confidence. A Monte Carlo simulation based on observed mean and standard deviations in \( \text{PO}_2 \) concludes that as many as 20 times more mice would be required. Clearly any \( \text{H}_2\text{S} \) effects are small, and any detectable statistical significance will not translate to benefits to islet transplantation.

Further, our results indicate that the potentiation of vasculature growth and increased perfusion by administration of \( \text{H}_2\text{S} \) in Matrigel\textsuperscript{29} is not observed for the PDLLCL devices. A previous study by Cai et al. (2007) showed improved angiogenesis in a Matrigel plug after 7 days of intraperitoneal injections of NaHS\textsuperscript{29}. We treated our mice for 28 days with NaHS, indicating that long-term treatment with \( \text{H}_2\text{S} \)}
might not significantly affect the vascularization. Further, Dufton et al. suggest that the administration of hydrogen sulfide using NaHS can result in an increased inflammatory response and triggering of TNF-α formation. Such inflammatory response caused by the injections during the first 4 weeks of implantation, which is also accompanied by foreign body response, may then result in hampering the reported pro-angiogenic characteristics of the H₂S.

In summary, we show that the subcutaneously implanted PDLLCL devices become vascularized in such a way that is resistant to improved vascularization via H₂S treatment. However, we have used similar devices in pancreatic islet implantation studies showing maintenance of islet function with partial or total reversal of chronic hyperglycemia. This may indicate that material properties of the PDLLCL, device geometry, and pore characteristics play roles in governing vascularization, a hypothesis supported by our previous work and consistent with current thinking in the area of biomaterials. Importantly, because our technology can sustain islet function without the need for pharmaceutical factors, it may be categorized as a drug-independent device, which is preferable as a medical product for regulatory agencies.

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Ethical Approval
This study was performed with the approval of Institutional Animal Care and Use Committee (IACUC # 2008-2850) at University of California Irvine.

Statement of Human and Animal Rights
This article does not contain any studies with human. All animal subjects were treated and tested with maximum care under the supervision of Institutional Animal Care and Use Committee (IACUC # 2008-2850) at University of California Irvine.

Statement of Informed Consent
There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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