Next generation nitric oxide-releasing polyurethane membranes for implantable glucose biosensors

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Abstract

Herein, fabrication of polyurethane glucose sensor membranes doped with next generation biocompatible macromolecular nitric oxide-releasing scaffolds (hyperbranched polyesters, hyperbranched polyurethanes, alginate) was reported. Nitric oxide-releasing *S*-nitrosothiol-modified hyperbranched polyesters-doped membranes achieved extended nitric oxide-release (> 48 h) and promising *in vitro* sensor performance (sensitivity 20.9 nA/mM, linear dynamic range 0 – 15 mM). Nitric oxide-releasing *N*-diazeniumdiolate-modified hyperbranched polyurethanes-doped membranes showed desirable smooth membrane morphology, but had relatively limited nitric oxide-releasing duration (up to 18 h) and half-life (~ 0.4 h). Nitric oxide-releasing *N*-diazeniumdiolate-modified alginate-doped membranes showed significantly longer nitric oxide-releasing half-life (~ 1.2 h) with hyperbranched polyurethanes, but their nitric oxide-releasing duration (< 20 h) was still limited. Challenges for future studies on this subject lie in extending the nitric oxide-releasing lifetimes of the membranes and obtaining more control over the nitric oxide-release kinetics.

Introduction

Diabetes is a worldwide problem. In 2012, there were 1.5 million diabetes-associated deaths globally, making the disease the eighth leading cause of death.[1] Since diabetes is characterized by an inability to regulate blood glucose levels, monitoring blood glucose levels is essential in the management of diabetes.[1-3] Implantable glucose sensors capable of continuous accurate glucose monitoring provide an ideal approach to this problem.[2, 3] Unfortunately, such sensors often induce a robust foreign body response resulting in local inflammation and eventual isolation (via granulation tissue) of the sensor from native tissue, significantly reducing the analytical performance of the sensor.[4] The sensors often require frequent recalibration or replacement, limiting their clinical utility.[4] Nitric oxide (NO), a diatomic free radical naturally produced in human body, is known to enhance wound healing and mitigate the foreign body response.[4-6] Recent research has focused on developing NO-releasing glucose sensor membranes to improve in vivo glucose sensor performance.[2-11] (**Figure 1**) The Schoenfisch lab has worked to dope polyurethane (PU) sensor membranes with macromolecular NO-releasing scaffolds.[5-11] The sensor membranes were shown to possess a diverse range of NO payloads and NO-release kinetics. In vivo testing revealed that PU membranes doped with NO-releasing silica nanoparticles (SNPs) enhanced overall sensor performance by decreasing the foreign body response in a 10-day study.[6] However, the SNPs leached from the PU membranes, and such particles could remain in the human body for extended periods (as long as 30 days) with potential toxicity concerns.[8,12,13] To maximize the potential of NO-releasing materials for clinical applications, a scaffold that minimizes the negative effects of leaching is desired. A new type of glucose sensor membranes utilizes novel biocompatible macromolecular NO-releasing scaffolds with potential biodegradability and tunable NO-release properties in hope of obtaining glucose sensor membranes with high biocompatibility and sustained NO-release.

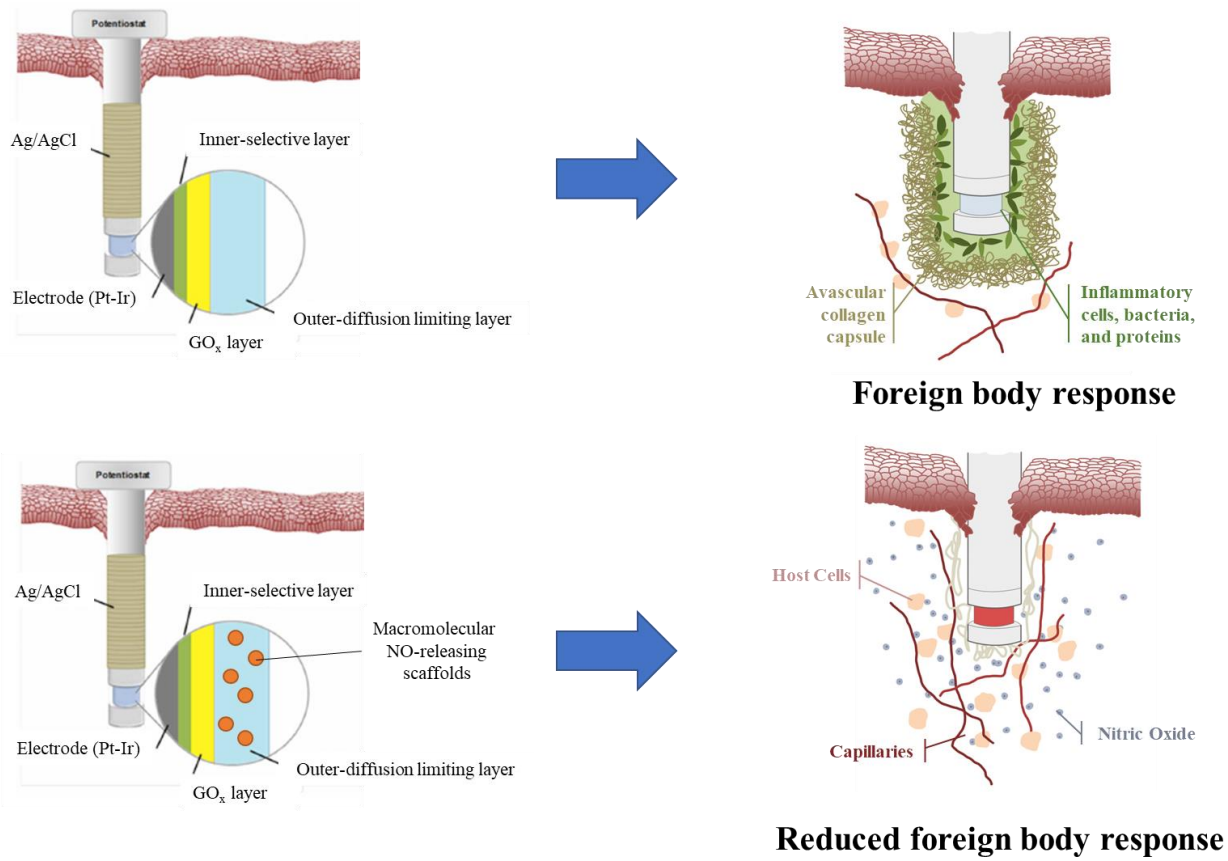


Figure 1. Implantable glucose sensors with and without NO-releasing scaffolds

Herein, we report on the fabrication of PU glucose sensor membranes doped with novel NO-releasing systems, including hyperbranched polyesters (HBPE), hyperbranched polyurethanes (HBPU), and alginate. (**Figure 1**) The NO-release characteristics, morphology of the membranes, and *in vitro* performance of glucose sensors with these membranes were assessed to determine their potential for use in implantable glucose sensor membranes.

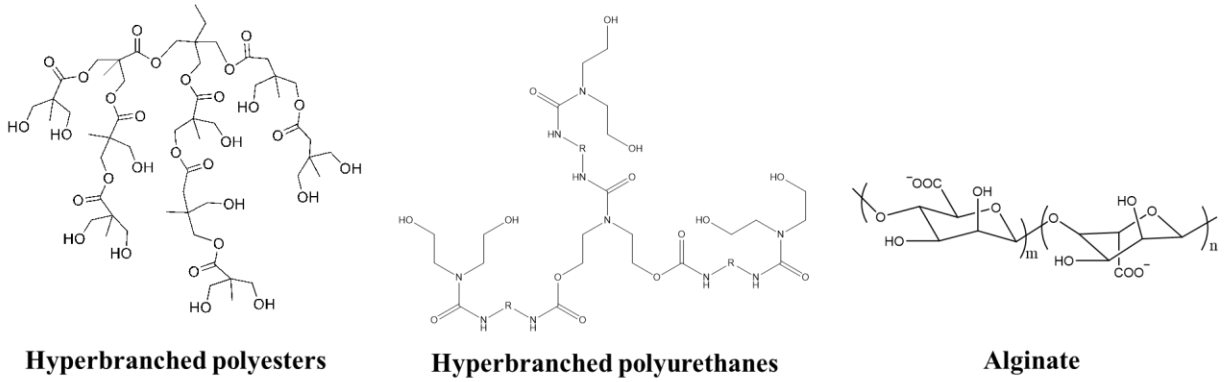
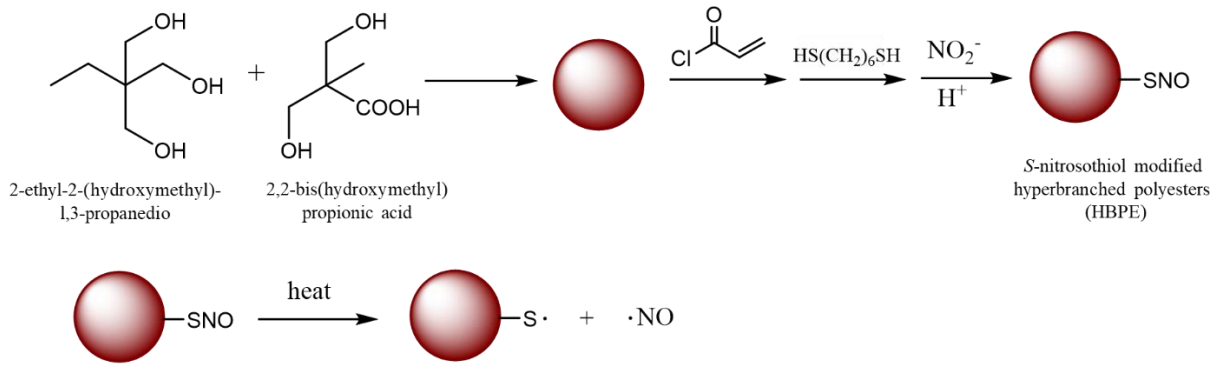


Figure 2. Novel macromolecular NO-releasing scaffolds

Experimental

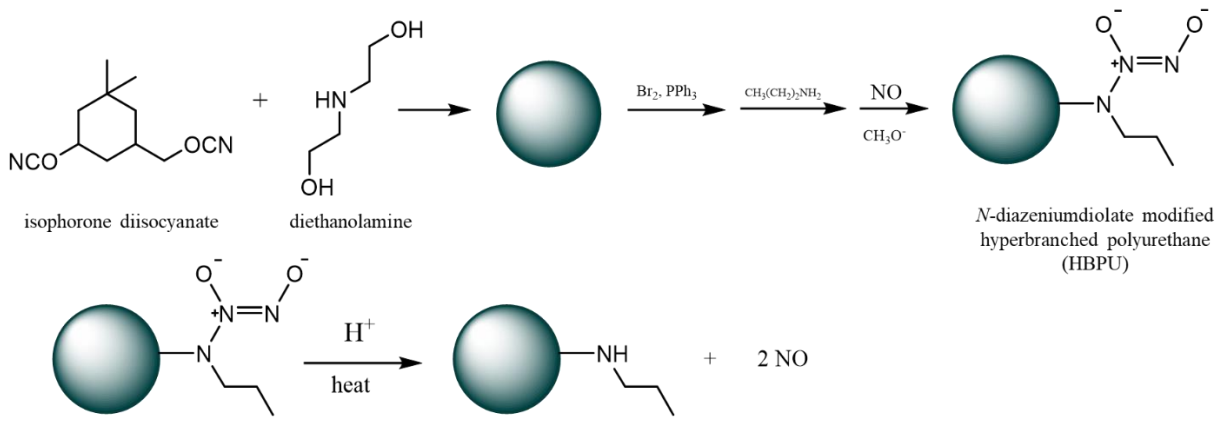
Fabrication of NO-releasing HBPE-doped PU membranes Third generation HBPE were synthesized and modified with *S*-nitrosothiol functional groups based on literature reported protocols to form NO-releasing HBPE (**Scheme 1**).[14, 15] To fabricate NO-releasing HBPE-doped PU membranes, NO-releasing HBPE (24 mg/mL) and PU (60 mg/mL) were dissolved in tetrahydrofuran (THF). The mixture was coated onto mock glucose sensors (stainless steel wires, 357 μm dia.) by the loop casting method as reported in literature.[4-8] Briefly, 6.5 μL of the mixture was coated evenly onto the sensor (or mock sensor) surface by a stainless steel wire ring (1 mm dia.). The casting process was repeated to achieve multiple coats onto the sensor surface. The sensors were left to dry briefly under ambient conditions between each coating process.



Scheme 1. Synthesis of NO-releasing HBPE

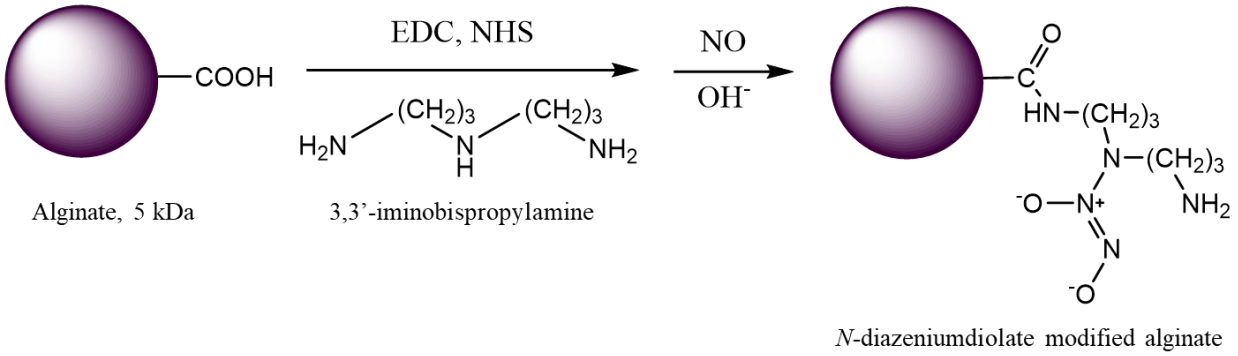
Fabrication of NO-releasing HBPU-doped PU membranes HBPU were synthesized based on literature reported protocols.[16] Then, HBPU (1.0 g) was dissolved in 10 mL N, N-dimethylformamide (DMF) with triphenylphosphine (1.538 g). Br_2 (0.30 mL) was added slowly and the mixture was allowed to react at 75 °C for 24 h. Brown product (HBPU-Br) was collected by precipitation in diethyl ether. The collected HBPU-Br was dissolved in 5 mL DMF with 3.0

mL propylamine for 72 h to form HBPU-NH. HBPU-NH was precipitated and washed in diethyl ether. HBPU-NH (10 mg/mL) was then dissolved in anhydrous methanol with sodium methoxide (94.5 mM), and the mixture was allowed to react with gaseous NO (150 psi) for 72 h. The resulting *N*-diazeniumdiolate-modified HBPU was collected by precipitation in anhydrous diethyl ether. (**Scheme 2**) NO-releasing HBPU with a range of concentrations (25, 50, 100 mg/mL) in PU (50 mg/mL) were dissolved in 3:1 DMF: THF (v/v). The solution was coated onto mock sensors by the loop casting method to form 10 coatings. To increase membrane hydrophobicity, an additional topcoat was applied to some mock sensors by loop casting a hydrophobic PU (TPU, water uptake 0.20 ± 0.18 mg water/mg PU, 50 mg/mL solution in 50 v/v% DMF, 50 v/v% THF) onto the coated mock sensors.



Scheme 2. Synthesis of NO-releasing HBPU

Fabrication of NO-releasing alginate-doped PU membranes Commercially available alginate (300 kDa) was degraded to 5 kDa to achieve better mixability with polyurethane. Briefly, 2.0 g alginate (300 kDa) was allowed to react with 40 mL hydrogen peroxide (4.9 M) at 85 ℃ for 1 h. Alginate (5 kDa) was collected by precipitation in ethanol. Alginate (5 kDa) was then modified by dipropylenetriamine (DPTA) through reactions with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). DPTA modified alginate (15 mg/mL) was dissolved in aqueous solution of NaOH (50 mM) and reacted with gaseous NO (150 psi) for 72 h. The NO-releasing alginate formed was collected by precipitation in anhydrous ethanol. (**Scheme 3**) Solid NO-releasing alginate was ground up and mixed with 50 mg/mL polyurethane (PU) solution in 3:1 DMF: THF to form a suspension, which was coated onto mock glucose sensors by the loop casting method to form 10 coats.



Scheme 3. Synthesis of NO-releasing alginate

Characterization of NO-release from the PU membranes The NO payload and NO-release kinetics from the PU membranes were measured under physiological conditions using Griess assay and a chemiluminescence NO analyzer (NOA). For Griess assay, mock sensors were immersed in phosphate buffered saline (PBS, pH 7.4) and incubated at 37 ℃. The NO released from the mock sensors was quantified by treating the solution with the Griess reagents.[7] For NOA, the mock sensors were incubated in pH 7.4 PBS at 37 ℃. NO released from the mock sensors was carried into the NO analyzer by a constant nitrogen flow. This NO undergoes a chemiluminescence reaction with ozone, releasing a photon that can be measured by an optical detector.[5-11] For the NO-releasing HBPE-doped membranes, 500 mM diethylenetriaminepentaacetic acid (DTPA) was added to the PBS to prevent Cu(II)-induced NO release.[15] NO payload of the membrane ($[NO]_t$) was measured as the total amount of NO released; NO-releasing duration (t_d) was measured as the time for the NO flux to drop below 1 pmol cm^{-2} s^{-1}; NO-releasing half-life ($t_{1/2}$) was measured as the time for the release of one half of $[NO]_t$.

***In vitro* sensor performance** Amperometric needle-type glucose sensors were constructed from a Ir-Pt wire working electrode and a Ag|AgCl reference electrode. Sensors coated with polyurethane membranes were soaked in PBS at 37 ℃, and glucose was added gradually to the solution to obtain a calibration curve. Linear dynamic range (LDR) of the sensor was defined as the range of glucose concentration in which the calibration curve is linear ($R^2 > 0.98$). Sensitivity of the sensor is defined as the slope of the calibration curve in the determined LDR.

Characterization of membrane morphology To evaluate the morphology of the PU membranes, mock glucose sensors coated with the membranes were characterized by scanning electron microscopy (SEM).

Results and Discussion

NO-releasing HBPE-doped PU membranes

HBPE are a type of hyperbranched organic polymers that have been found to be both biocompatible and biodegradable.[14,15,17] NO-releasing HBPE was developed by Yang et al. for therapeutic applications.[15] The high NO-payload (up to 2 μmol/mg) and relatively long NO-release half-lives (up to 3 h) made NO-releasing HBPE favorable for glucose sensor applications.[15] Additionally, NO-releasing HBPE utilizes *S*-nitrosothiol as the NO-donor group, which is the NO-donor group utilized naturally by organisms.[15, 18] Therefore, NO-releasing HBPE was chosen as the first NO-releasing scaffold to be investigated in this study.

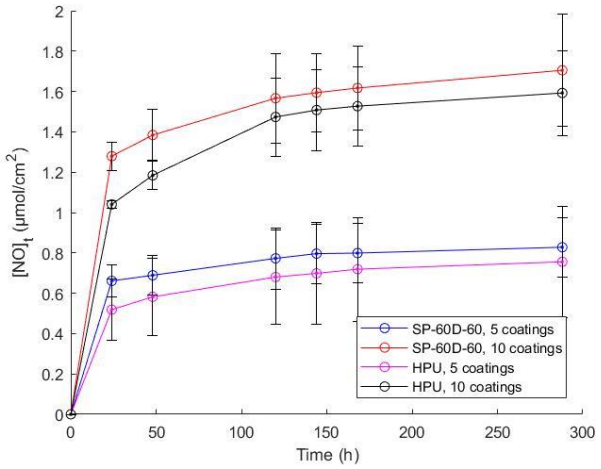


Figure 3. NO-release from NO-releasing HBPE-doped PU membranes, characterized by Griess assay

Table 1. NO payloads of NO-releasing HBPE-doped PU membranes, measured by Griess assay

PU [a]	Number of coatings	$[NO]_t$ (μmol/cm^2)
SP-60D-60	5	0.82 ± 0.15
	10	1.71 ± 0.28
HPU	5	0.76 ± 0.28
	10	1.59 ± 0.21

a. PU water uptake (mg water/ mg PU): 1.73 ± 0.26 (SP-60D-60), 0.63 ± 0.34 (HPU).

NO-release from NO-releasing HBPE-doped PU membranes was characterized by Griess assay (**Figure 3**) because it was expected to have relatively low and constant NO-flux.[8,15] The results showed that the membranes were capable of releasing NO for > 48 h. Such a relatively long NO-release duration can be promising for clinical applications. The NO payloads of the membranes were found to be tunable by varying the number of coatings. (**Table 1**) The NO payloads (**Table 1**) and NO-release kinetics (**Figure 3**) of the NO-releasing HBPE-doped membranes were not significantly influenced by the hydrophobicity of the membranes. Such an

observation was expected, because the NO-release from the *S*-nitrosothiol functional groups is a free radical process, which should not be affected by the hydrophobicity of the membranes.[15,18]

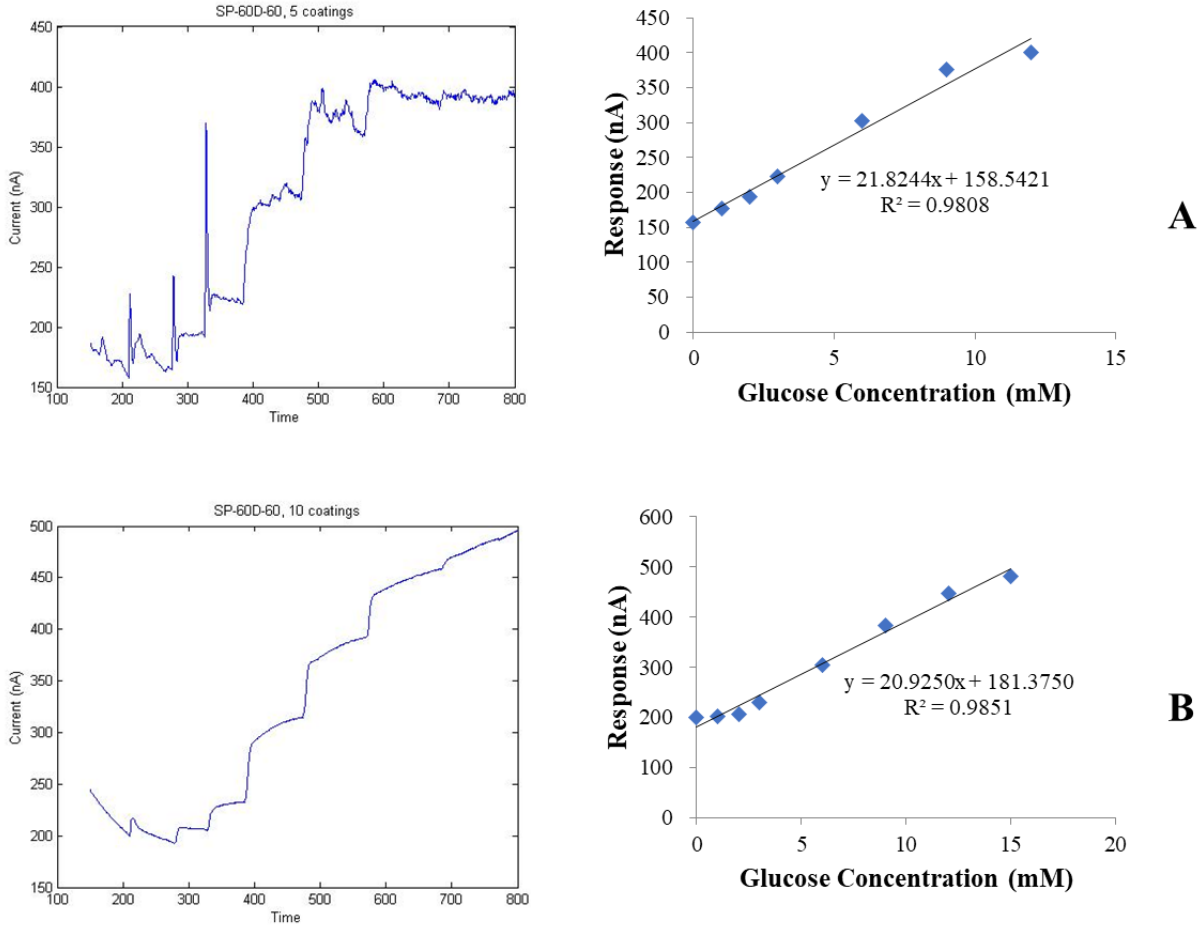


Figure 4. Amperometric sensor response of needle-type glucose sensors with A) 5 coatings, B) 10 coatings of NO-releasing HBPE-doped SP-60D-60 membranes, and the corresponding calibration curves within LDRs. Each increment of current corresponds to one increment of glucose concentration.

To assess the potentials of the sensor membranes in clinical sensor applications, *in vitro* glucose calibrations were performed using needle-type glucose sensors. (**Figure 4**) While both the sensor with 5 coatings (sensitivity 21.8 nA/mM, LDR 0 – 12 mM) and the sensor with 10 coatings (sensitivity 20.9 nA/mM, LDR 0 – 15 mM) showed similar sensitivity and LDRs, the sensor with 5 coatings had a much smaller S/N ratio. This was likely due to the fact that 5 coatings of PU membranes were not enough to prevent the sensor from Reacting to fluctuations in local glucose concentrations. Therefore, it was concluded that 10 coatings of PU membranes were more preferable for sensor applications. The sensitivity of the sensors was comparable to that of the sensors coated with NO-releasing materials-doped PU membranes developed in previous studies.[8] The LDR of the sensor with 10 coatings (0 – 15 mM) was able to cover the

physiological blood glucose range (1 – 15 mM)[19], proving that glucose sensors coated with NO-releasing HBPE-doped PU membranes meet the minimum requirements for clinical application and can be pursued further for this application.

NO-releasing HBPU-doped PU membranes

Though NO-releasing HBPE-doped PU membranes can achieve necessary properties for clinical applications, the free radical nature of NO-releasing HBPE posed the challenge of controlling the NO-release kinetics of the membranes. Therefore, NO-releasing HBPU, a new type of macromolecular NO-releasing scaffold utilizing *N*-diazeniumdiolate NO-donor groups (**Scheme 2**) was investigated for sensor membrane applications. NO-release from *N*-diazeniumdiolates is triggered by protons.[18] So the kinetics of N-diazeniumdiolate-based NO-releasing scaffolds can theoretically be tuned by varying PU membrane hydrophobicity. HBPU is a type of highly biocompatible hyperbranched polymer.[16] It is also relatively hydrophobic, making it unlikely to leach from the PU membranes.[16] As such, NO-releasing HBPU was chosen to be the first *N*-diazeniumdiolate based NO-releasing scaffold investigated in this study.

Table 2. NO-releasing characteristics of NO-releasing HBPU-doped PU membranes, measured by NOA

PU (10 coatings) [a]	[HBPU] (mg/mg PU)	$[NO]_t$ (μmol cm^{-2})	t_d (h)	$t_{1/2}$ (h)
HP-93A-100	0.5	0.52 ± 0.34	16.0 ± 5.1	0.35 ± 0.18
HPU/topcoat [b]	0.5	0.85 ± 0.30	17.5 ± 5.2	0.40 ± 0.29
HP-93A-100/topcoat [b]	0.5	0.69 ± 0.27	13.9 ± 2.6	0.41 ± 0.21
	1	1.23 ± 0.11	15.6 ± 2.3	0.42 ± 0.17

a. PU water uptake (mg water/ mg PU): 2.56 ± 0.31 (HP-93A-100), 0.63 ± 0.34 (HPU); b. With one TPU topcoat containing no HBPU

Because *N*-diazeniumdiolates tend to show high and variable NO-flux at the early stage of their NO-release,[8,18] the NO-release from NO-releasing HBPU-doped PU membranes was measured by NOA, which allows real-time monitoring of NO-release. (**Table 2**) The NO-release characteristics ($[NO]_t$, t_d, $t_{1/2}$) of NO-releasing HBPU-doped PU membranes were evaluated as a function of the concentrations of NO-releasing HBPU in the membranes, as well as membrane hydrophobicity. It was found that the $[NO]_t$ is tunable by varying the concentration of NO-releasing HBPU in the membrane. Thus, the number of coats on the sensors can be held constant at the number that yields the most preferable sensor performance (10 coatings). The t_d of the NO-releasing HBPU-doped membranes was relatively short (< 20 h), which may limit their clinical applications. Of note, the expected control over the NO-release kinetics of the membranes by varying membrane hydrophobicity was not achieved. After varying the hydrophobicity of PU, changing the concentration of NO-releasing HBPU in the membranes, or applying hydrophobic

TPU topcoats, the $t_{1/2}$ of the membranes remained relatively constant (~ 0.40 h). A possible explanation of such discrepancy between expectation and observation is that 10 coatings of PU might not be sufficient to show a significant impact on the rate of proton diffusion to the scaffold. Future study need to be conducted to test the validity of this hypothesis.

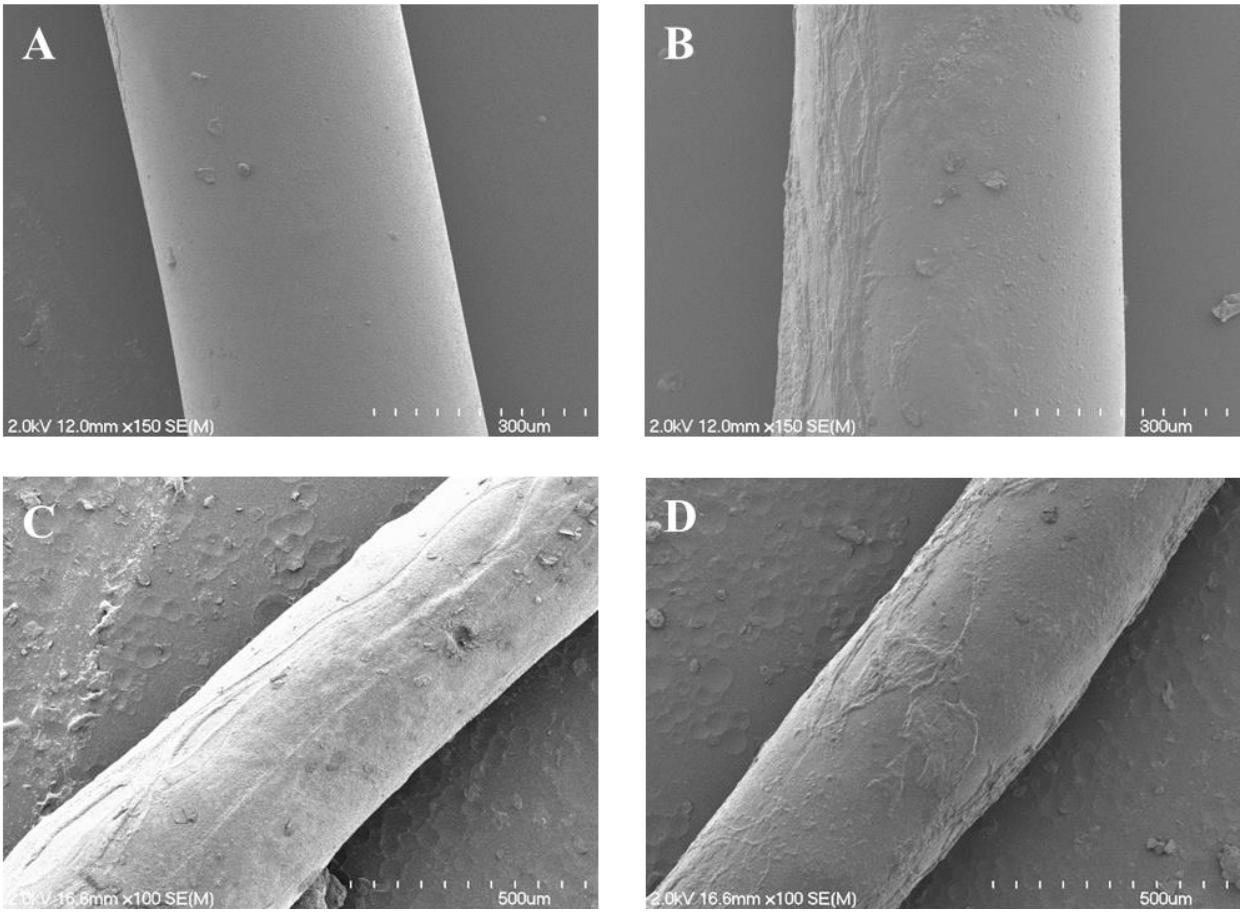


Figure 5. Morphology of A) HP-93A-100, B) HP-93A-100 with one TPU topcoat, C) HPU, D) HPU with one TPU topcoat, doped with NO-releasing HBPU (0.5 mg HBPU/mg PU), 10 coatings on mock glucose sensors

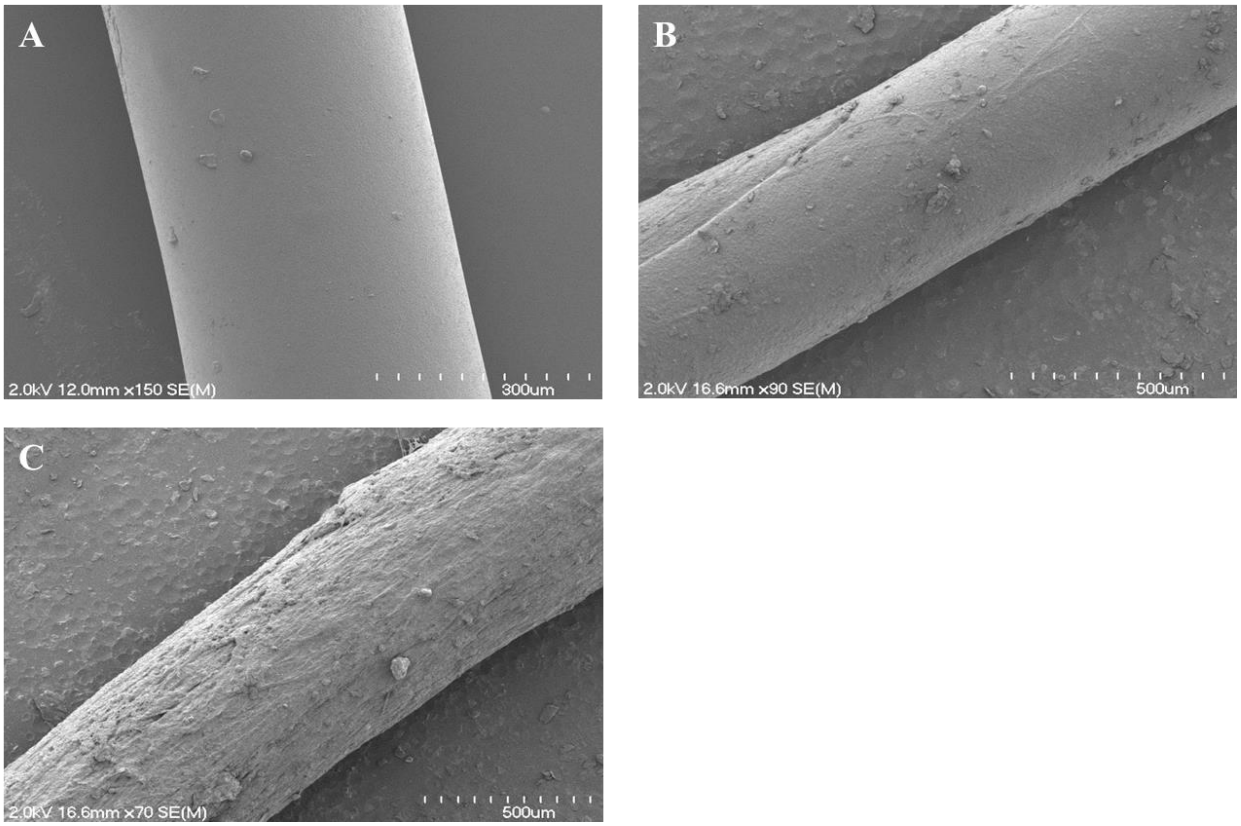


Figure 6. Morphology of HP-93A-100 membranes doped with A) 0.5 mg HBPU/mg PU, B) 1 mg HBPU/mg PU, C) 2 mg HBPU/mg PU, 10 coatings on mock glucose sensors

Previous studies have shown that smooth sensor membranes tend to reduce foreign body response in human body.[6,8] Therefore, morphology of NO-releasing HBPU-doped PU membranes was evaluated as a function of PU type (**Figure 5**) and HBPU concentration (**Figure 6**). Smooth membranes preferable for clinical applications were achieved with HP-93A-100 at low HBPU concentration (**Figure 5A**). The morphology of the HPU membrane was slightly less smooth than that of the HP-93A-100 membrane, but the difference was not significant (**Figure 5C**). Introducing TPU topcoats interrupted the smoothness of the membranes (**Figure 5B&D**), which was likely due to the fact that the viscosity of TPU solutions prevented it from being uniformly coated onto the mock sensors by the loop casting method. Such a problem should be resolvable by optimizing the solvent composition for TPU during the loop casting process, and is unlikely to negatively impact the potentials of the membranes for clinical applications.

When increasing the concentration of NO-releasing HBPU in the membranes, the membrane morphology deteriorated significantly. (**Figure 6**) It is likely that the high concentration of HBPU in the PU matrix disrupts the polymeric structure of the membrane. Such an observation suggests that, for potential applications of these membranes, the concentration of NO-releasing scaffolds in the membranes need to be limited at $\leq$ 1 mg scaffold/mg PU.

NO-releasing alginate-doped PU membranes

Although NO-releasing HBPU-doped PU membranes were capable of achieving desirable membrane morphology, their future applications were limited by the relatively short NO-release durations of these membranes. Therefore, NO-releasing alginate, a novel macromolecular NO-releasing scaffold utilizing *N*-diazeniumdiolate NO-donor groups, was investigated. Alginate is a naturally produced linear polysaccharide extracted from brown algae (*Phaeophyceae*).[21-23] Alginate has been found to be highly biocompatible and biodegradable, and has been utilized in a range of biomedical applications, including drug delivery, wound dressing, therapeutic cell entrapment.[21-23] The Schoenfisch group has been working on developing NO-releasing alginate for the treatment of cystic fibrosis, and it was found that NO-releasing alginate can achieve relatively long NO-releasing durations and half-lives. It was hypothesized that NO-releasing alginate-doped PU membranes could achieve extended and tunable NO-releasing lifetimes for application in glucose sensor membranes.

Table 3. NO-releasing characteristics of NO-releasing alginate-doped PU membranes, measured by NOA

PU (10 coats) [a]	$[NO]_t$ (μmol cm^{-2})	t_d (h)	$t_{1/2}$ (h)
HP-93A-100	1.30 ± 0.08	18.8 ± 1.0	1.23 ± 0.14
HPU	1.13 ± 0.32	19.9 ± 1.3	1.15 ± 0.18

a. PU water uptake (mg water/ mg PU): 2.56 ± 0.31 (HP-93A-100), 0.63 ± 0.34 (HPU)

The NO-release kinetics from NO-releasing alginate-doped PU membranes were characterized by NOA. (**Table 3**) The NO-releasing half-lives of NO-releasing alginate-doped membranes (~ 1.2 h) were significantly longer than those of NO-releasing HBPU-doped membranes (~ 0.4 h, **Table 2**). However, the NO-releasing durations of these membranes were still relative short (< 20 h). Of note, the control over NO-release kinetics by changing membrane morphology was not achieved, which was likely due to the same reason as the similar trends observed in NO-releasing HBPU-doped membranes.

Conclusions

This study demonstrated the possibility of fabricating PU glucose sensor membranes doped with novel biocompatible macromolecular NO-release scaffolds. These membranes were found to be able to achieve tunable NO payloads and smooth membrane morphology. Sensors with such membranes showed good sensor performance *in vitro*. However, the relatively short NO-releasing lifetimes will likely limit the clinical applications. Future studies on fabrication of biocompatible macromolecular NO-releasing scaffolds therefore need to be focused on extending the NO-releasing lifetimes of the membranes and achieving more control over the NO-release kinetics from these membranes.

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