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Nitric Oxide-Releasing Semi-Crystalline Thermoplastic Polymers: Preparation, Characterization and Application to Devise Anti-Inflammatory and Bactericidal Implants

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Abstract

Semi-crystalline thermoplastics are an important class of biomaterials with applications in creating extracorporeal and implantable medical devices. *In situ* release of nitric oxide (NO) from medical devices can enhance their performance via NO's potent anti-thrombotic, bactericidal, anti-inflammatory, and angiogenic activity. However, NO-releasing semi-crystalline thermoplastic systems are limited and the relationship between polymer crystallinity and NO release profile is unknown. In this paper, the functionalization of poly(ether-block-amide) (PEBA), Nylon 12, and polyurethane tubes, as examples of semi-crystalline polymers, with the NO donor *S*-nitroso-N-acetylpenicillamine (SNAP) is demonstrated via a polymer swelling method. The degree of crystallinity of the polymer plays a crucial role in both SNAP impregnation and NO release. Nylon 12, which has a relatively high degree of crystallinity, exhibits an unprecedented NO release

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Author contributions

M. Meyerhoff and Xuewei Wang conceived the project and designed the experiments. Xuewei Wang, A. Jolliffe, Q. Zhang, Y. Cui, M. Mahoney, and J. Douglas conducted *in vitro* experiments including polymer functionalization, NO test, and polymer characterization. B. Carr, M. Bilger, A. Rojas Pena, and R. Bartlett designed and conducted the animal experiment. J. Wu and C. Xi are responsible for the biofilm test. Xianglong Wang and J. Bull conducted the mechanical test. M. Hoenerhoff performed histological analysis. Xuewei Wang wrote the main manuscript. B. Carr wrote the protocol of the animal experiment. M. Meyerhoff revised the manuscript and all other authors approved or provided comments to the manuscript.

Supporting Information

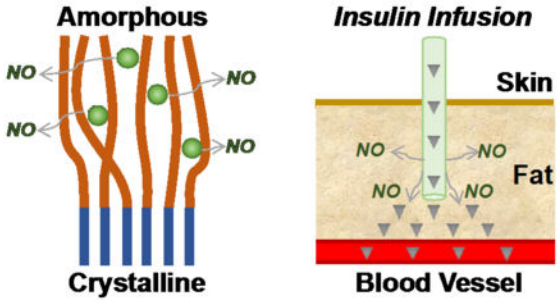
Electronic Supplementary Information (ESI) available: Experimental section and supplemental figures 1–7.

The authors declare no conflict of interest.

duration of over 5 months in a low NO level, while PEBA tubing exhibits NO release over days to weeks. As a new biomedical application of NO, the NO-releasing PEBA tubing is examined as a cannula for continuous subcutaneous insulin infusion. The released NO is shown to enhance insulin absorption into the bloodstream probably by suppressing the tissue inflammatory response, and thereby benefit insulin pump therapy for diabetes management.

Graphical Abstract

Anti-bacterial and anti-inflammatory semi-crystalline polymers are obtained by doping nitric oxide donor molecules into the amorphous polymer phase.



1. Introduction

Nitric oxide plays important bioregulatory roles in many physiological and pathological processes such as vasodilation, neurotransmission, angiogenesis, platelet activation/aggregation, immune response, and wound healing.[1] Inspired by the function of endogenous NO, release of exogenous NO has been used as a biomimetic strategy to improve performances of various biomedical implants, conduits, and dressings. Blood-contacting devices such as vascular grafts, stents, catheters, extracorporeal circuits, and intravascular sensors exhibit significantly mitigated thrombus and microbial biofilm formation in the presence of local release of NO.[2] NO-releasing subcutaneous glucose sensors elicit lower inflammatory responses and/or more new blood vessel formation compared to control sensors, which can improve the accuracy of glycemic monitoring.[3] Release of NO from skin dressings/creams has been shown to promote healing of burn/diabetic wounds, ulcers, and acne via combined functions of NO in multiple processes such as inflammation, infection, angiogenesis, and collagen formation.[2]

Because of the short half-life of NO (seconds), NO donors such as N-diazeniumdiolates and S-nitrosothiols are often used as stored pro-drugs that release NO upon activation by increased temperature, light, pH, metal ions (e.g., Cu^{+}), or nucleophilic reagents (e.g., ascorbic acid).[4] β-galactosidase was used to control NO release from β-galactose-protected diazeniumdiolates in a highly selective manner.[5] These donor molecules are often doped into polymers via physical mixing or covalent attachment. They can also be attached to various inorganic/organic/biological particles and then blended into polymers. Based on these methods, NO-bearing moieties/molecules have been successfully grafted to polyurethane, silicone rubber (PDMS), urethane-silicone copolymers, plasticized PVC,

polyester, polymethacrylate, polyethylenimine, ethylcellulose, xerogels and hydrogels, which form the basis of most biomedical NO-release devices reported to date.[2,6] A common strategy to obtain NO-releasing devices is to apply a NO-release polymer coating on the surface of the original device. This can be accomplished by dipping coating or solvent casting of the NO donor-doped polymer or pre-polymer solution. In this way, a NO-release coating can be formed on the surface of biomedical tubes (e.g., extracorporeal circuits and catheters), metal stents, wire type sensors, and dressing membranes.[2,6] However, to achieve a reasonably high level and longer duration of NO release, the coating thickness is usually hundreds of micrometers or more, which is not possible for smaller size devices such as peripheral venous catheters (*vs.* central venous catheters) and subcutaneous insulin infusion cannula. The adhesion of the NO-release coating may be another concern depending on the chemical properties of the coating and substrate material. Further, the addition of a coating on the tubing can make the device fabrication much more complicated, which may reduce commercialization interest, despite the fact that the efficacy of the NO-release coating approach has been repeatedly demonstrated over the past two decades.

Some hydrophobic polymers such as silicone-urethane copolymers blended with NO donors can also be used to fabricate single-layer tubes by dip-coating of polymer solutions on a mandrel.[7] However, the dip-coating method usually generates less uniform tubes with rougher surfaces compared to the hot melt extrusion method widely employed in industry. Notably, the low thermal stability of NO donors prohibits direct extrusion or injection molding of melted NO-releasing polymers into tubes or other shapes to create medical devices.

To enable functionalization of pre-made devices such as commercial catheters with NO donor molecules, our laboratory recently introduced a simple but effective solvent-assisted NO donor impregnation method.[8] A volatile solvent system containing the dissolved NO donor is capable of swelling the catheter tubing and the NO donor will stay within the polymer network after solvent evaporation. With appropriate solvent selection, the swelling process may not significantly change the structure and properties of the original polymer tubing. Thus far, the polymers employed to demonstrate this approach have been only silicone rubber and a silicone-polyurethane-polycarbonate block copolymer (i.e., Carbosil 20–80A).[8] Like most other polymers used in coated/cast NO-release layers/tubing, they all have very low polymer hardness (in the scale of Shore Hardness A). These soft materials typically allow a higher loading of NO donors because of the large free volume between flexible polymer chains, and crystals of the NO donor can even be observed within the polymer with increased NO donor loading.[8c] Accordingly, their applications commonly target soft implants, such as central venous catheters, urinary catheters, and catheter type intravascular oxygen sensors.

In contrast to the soft implants, many biomedical devices rely on semi-crystalline polymers that have much greater hardness (in the scale of Shore Hardness D) because of highly oriented crystallites within. For example, polyethylene (PE), polyether ether ketone (PEEK), polycaprolactone (PCL), polyvinylidene fluoride (PVDF), polytetrafluoroethylene (PTFE), polyurethane (PU), poly(ether-block-amide) (PEBA), and polyamide (Nylon) in their semi-crystalline forms are employed in a wide range of biomedical applications such as heart

valves, vascular sutures, vascular stents, joint replacement implants, spinal cages, dental prostheses, implantable acoustic sensors, peripheral catheters, and subcutaneous cannulas.[9] Although NO release may protect these devices, for example, against thrombus, infection, and inflammation, functionalization of these stiffer semi-crystalline polymers with NO donors has not been clearly documented.[2] One possible reason is the difficulty in preparing solutions of most semi-crystalline polymers at room temperature which is necessary to help preserve the stability of the NO donor species. The crystalline domain of these materials function as a robust physical cross-linker and makes the dissolution of these polymers difficult. Interestingly, the solvent-assisted NO donor impregnation method, compared to previous coating/casting methods, only requires swelling rather than dissolution of polymers. This unique feature provides an excellent opportunity for the functionalization of semi-crystalline polymers since their amorphous domains allow facile swelling. In this work, we explore this direction by using PEBA, Nylon 12, and PU (Shore hardness 55D to 75D) as exemplary semi-crystalline polymers. *S*-nitroso-N-acetylpenicillamine (SNAP) can be successfully incorporated into these polymers by using a binary swelling solvent system. Depending on the chemical and crystalline properties, these polymers are shown to exhibit a NO release duration ranging from days to months. Notably, although some polymers (especially PU) that are possible to form crystalline structures have been previously investigated as NO-releasing materials,[10] information on whether they are amorphous or semi-crystalline under the employed conditions was not provided and the relationship between polymer crystallinity and NO release activity has, to date, not been explored.

As an initial application of this technology, we also provide data for using SNAP-impregnated PEBA tubing as cannula device for subcutaneous insulin infusion. Insulin infusion sets, particularly the cannula component that is implanted under the skin, is considered to be the “Achilles heel” of the insulin pump therapy for type 1 diabetic patients. Indeed, patients need to switch the infusion site every 2–3 days to obtain reliable insulin absorption into the circulating bloodstream and avoid various other adverse complications.[11] The mechanism of such site failure is not fully understood, but tissue inflammation is supposed to be a significant contributor and infection may also play a role.[11–13] Exogenous NO as an anti-inflammatory and anti-bacterial agent is a possible solution to this problem. However, most previously developed NO-release polymer platforms are not suited for this application since the tiny infusion cannula has to be rigid or semi-rigid to prevent accidental kinking during implantation. Herein, we demonstrate that the new NO donor-functionalized semi-crystalline polymer platform enables the development of NO-releasing insulin infusion cannula that exhibit improved subcutaneous insulin absorption in a sheep model in which insulin is infused following bolus injections of glucose into bloodstream of the animals.

2. Results and Discussion

2.1 Impregnation of SNAP into PEBA and Nylon 12 Tubes via Solvent Swelling

PEBA and Nylon 12 are amide-containing block copolymers characterized by excellent mechanical properties, chemical resistance, sterilization resistance, processability, durability, and biocompatibility. Herein, we examined medical grade tubing made of three grades of PEBA (Pebax 6333 SA 01 MED, 7033 SA 01 MED, 7233 SA 01 MED) and Nylon 12

(Rilsamid AESNO MED). While PEBA is composed of polyamide 12 (PA12) as the hard segment and poly(tetramethyleneoxide) (PTMO) as the soft segment, Nylon 12 only has PA12 segments.[14] Table 1 shows chemical composition and some key properties of these semi-crystalline polymers. The crystalline domain is formed solely by PA12 segments within these polymers with a low ratio of PTMO, whereas the amorphous domain consists of both PA12 and PTMO segments.[14] With an increasing ratio of PA12, the polymer has an increasing crystallinity degree and Shore hardness.

Dissolution of these semi-crystalline polymers with high percentages of PA12 segments requires harsh solvents, such as concentrated acids or phenols. In contrast, haloalkanes such as dichloromethane, dichloroethane, chloroform, and bromoform were found to swell these polymers to a significant extent (>10% tube volume increase) but not dissolve them. Dichloromethane was chosen in this work because it has a very low boiling point (39.6 °C) which facilitates solvent evaporation and minimizes residual solvent in the final NO-releasing polymer. The swelling ability of the solvent toward a polymer can be interpreted by the solubility parameter. A more pronounced swelling can be obtained when the solubility parameter of the solvent and polymer is closer to each other.[15] Dichloromethane, PA12, and PTMO have a Hildebrand solubility parameter of 20.2, 21.1, and 18.3, respectively.[16] Therefore, dichloromethane is likely able to swell both the PA12 and PTMO segments.

S-nitroso-N-acetyl-penicillamine (SNAP) is a commonly used NO donor because of its lower toxicity compared to diazeniumdiolate type donors[2a] and its higher chemical stability compared to primary *S*-nitrosothiols such as *S*-nitrosoglutathione and *S*-nitrosocysteine.[17] The solubility of SNAP in dichloromethane is very low (< 0.5 mg/mL). Therefore, methanol that has a SNAP solubility of ~280 mg/mL was mixed with dichloromethane as the swelling solvent. Interestingly, these two solvents exhibit strong synergistic interaction on both swelling of PEBA/Nylon 12 tubing and dissolution of SNAP (Figure 1). Because impregnation of SNAP into the polymer depends on the SNAP concentration in the swelling solution (solubility) as well as the volume of solution penetrated into the polymer network (swelling), the synergistic effect is highly desired to achieve a high SNAP loading. We chose dichloromethane-methanol mixture in a 7:3 volume ratio as the final swelling solvent due to its highest swelling ability and reasonable SNAP solubility. Figure 1 also shows that the overall tube swelling ratio decreases in the order of Pebax 6333, Pebax 7033, Pebax 7233, and Nylon 12, which corresponds to an increasing degree of polymer crystallinity. This is expected because solvent swelling occurs preferentially in the permeable amorphous phase rather than the crystalline domains that have strong interchain hydrogen bonding.

Four types of PEBA/Nylon tubes of the same wall thickness (0.01”) were soaked in dichloromethane-methanol solvent mixture containing 250 mg/mL SNAP at 37 °C for 3 h, and then dried under vacuum. We further used methanol to rinse any residual SNAP powders/crystals on the inner and outer tube surfaces. The tubes become greenish from the original clear or translucent appearance because of the green color of SNAP. To further characterize the SNAP-impregnated tubing, solid-state NMR, wide angle X-ray diffraction (WAXD), and atomic force microscopy (AFM) were employed for the Nylon 12 and Pebax 7033 tubes as the representative polymer tubing. As shown in Figure 2, for the SNAP-

impregnated Nylon 12 tubing, the characteristic resonance peak of SNAP was observed at *ca* 60.0 ppm which is an overlapped peak from the quaternary carbon next to the -SNO group (59.2 ppm, carbon “d”) and the adjacent tertiary carbon (60.2 ppm, carbon “c”). For the original Nylon 12 tube, the comparable peak intensity of all-trans methylene (33.4 ppm, carbon “j trans”) to gauche methylene (30.8 ppm, carbon “j gauche”) indicates a ϒ’ crystal form, which can be obtained by cryogenic quenching of Nylon 12 melt.[17] This all-trans to gauche methylene ratio significantly increases after SNAP impregnation, and the resonance peak of all-trans methylene and gauche methylene is downfield shifted (from 33.4 to 33.6 ppm) and upfield shifted (from 30.8 to 30.4 ppm), respectively. These changes have been attributed to the ϒ’ to ϒ crystallite modification.[18] The ϒ form Nylon 12 was obtained by annealing of quenched ϒ’ form Nylon 12 or Nylon 12 melt and may have more perfect crystallites than ϒ’ form.[18,19] One dimension WAXD pattern (Figure 3) further confirms that both tubes have hexagonal ϒ’ or ϒ crystal lattices but does not involve any monoclinic α crystallites.[19] Similar NMR and WAXD results were obtained for the Pebax 7033 tubing, suggesting crystallite transformation similar to that of Nylon 12 upon SNAP impregnation. This type of swelling-induced polymorphic transition has been observed in other polyamide species like Nylon 6,6,[20] but knowledge on the mechanism in a molecular level is limited and we didn’t try to pursue the mechanism in this work. PeakForce Quantitative Nanomechanical Mapping (PeakForce QNM) AFM was used to map “adhesion” of the outer surface of the polymer tubing. The amorphous domains should have higher adhesion property than the crystalline domain as well as the rigid interphase. As can be seen from Figure S1 in the Supporting Information, the phase separation is of similar nanometer scale before and after SNAP impregnation.

To calculate the amount of SNAP impregnated in the tube, SNAP within the tube was extracted back into the dichloromethane-methanol solvent mixture and the generation of NO catalyzed by Cu(I) was quantitated by a chemiluminescence-based nitric oxide analyzer (NOA). As shown in Table 1 (column 5), the SNAP loading decreases with increasing crystallinity degree of polymer due to the decreasing swelling ratio. Based on the area of the peak at 59.2/60.2 ppm (carbon “c/d” from SNAP) and 40.6 ppm (carbon “h” in PA12 segment) in a 1D $^{13}C\{1H\}$ NMR spectrum that provides quantitative information by using direct polarization rather than cross polarization, the molar ratio of SNAP to PA 12 is calculated to be *ca* 0.04 in Nylon 12 (spectrum not shown). This result agrees with the 1:23.2 ratio obtained by the extraction method, confirming its accuracy. Moreover, WAXD spectra shows a larger decrease in diffraction intensity after the swelling-based impregnation process on Pebax 7033 compared to Nylon 12 (Figure 3), suggesting that a less crystalline polymer may even lose more crystallinity because of the more aggressive swelling. This cascade effect is likely to be the reason that the difference in SNAP loading is more pronounced than that of the original crystallinity degree between different types of tubes.

Based on X-ray diffraction spectroscopy, our group has previously found that SNAP is able to form crystals in amorphous Carbosil and the crystal formation is the main reason for the excellent SNAP stability in that polymer.[7a] In contrast, no characteristic diffraction signals of SNAP crystals were observed for the SNAP-doped PEBA and Nylon 12 tubes (see Figure S2 for WAXD pattern of SNAP powder). Since both SNAP and PA12 have hydrogen bond-donating and hydrogen bond-accepting moieties, the formation of hydrogen bonding

between the SNAP and the polymer is expected. Moreover, as shown in Table 1 (column 6), PA12 in the amorphous phase (i.e., the available amide groups for intermolecular interaction with SNAP) are in large excess relative to the amount of impregnated SNAP molecules for all four types of polymers, which may disfavor the association between different SNAP molecules and the formation of SNAP crystals. We attempted to use solid-state NMR or ATR-FTIR to confirm and characterize the hydrogen bonding between SNAP and PA12 but this was not successful probably due to the relative small percentage of SNAP present.

By using Nylon 12 and Pebax 7033 tubes as examples, we further compared mechanical properties of the original tubes and the SNAP-doped tubes. As shown in Figure 4, SNAP impregnation doesn't induce any significant change in the ultimate tensile strength, ultimate tensile strain (maximum elongation), and Young's modulus for Nylon 12. Changes of the tensile strength and tensile strain of Pebax 7033 are not significant as well, whereas the Young's modulus has an average decrease of 13% after SNAP impregnation. The lowered elastic modulus of Pebax 7033 is consistent with its more pronounced crystallinity loss which makes the tube less stiff. However, this level of change may not be problematic for applications such as biomedical implants.

2.2 Nitric oxide release from the SNAP-doped PEBA and Nylon 12 tubes

Phosphate buffered saline with 0.1 mM EDTA (PBSE) at pH 7.4 is commonly used to characterize NO release of biomedical implants intended for use in an aqueous environment.[21] Figure 5 shows the NO release over time for the various SNAP-doped tubes studied here in PBSE at 37 °C. As can be seen, small Pebax 6333, Pebax 7033, Pebax 7233, and Nylon 12 tubes of the same wall thickness (0.01") have NO release above 0.01×10^{-10} mol/min/cm^2 over 4 days, 30 day, 58 days, and 155 days, respectively. While these fluxes are lower than desired to prevent clotting on the surface of intravascular catheter and other blood contacting devices (since NO is consumed quickly by oxyhemoglobin), such lower levels of NO with long half-life time may be desired for long-term implants and prostheses that are not continuously in contact with oxyhemoglobin. In addition, since these rigid semi-crystalline polymers in biomedical grade already exhibit high intrinsic biocompatibility (USP Class VI, from Arkema website), further enhancement of biocompatibility is possible even with a low level of NO. Notably, each data point in Figure 6 is an averaged flux of 10 tubes of 2 cm length, but we did not run triplicate tests. Therefore, there may be errors in the absolute NO flux values reported in Figure 6 originating from factors such as sensitivity fluctuations of the NOA instrument between daily calibrations.

To understand the dramatic difference in the NO release profile of the different polymer matrices, three processes are considered: 1) decomposition of SNAP in the tubing to generate NO; 2) leaching of SNAP from the tubing; and 3) diffusion of NO from the polymer into the aqueous solution. In the absence of light and catalyst, SNAP primarily undergoes thermal decomposition. Since all samples were at 37 °C, decomposition rates should be identical for the different tubes. Leaching of NO donors is a critical limiting factor to sustainability of the NO release. Figure 6A shows leaching of chemicals (SNAP and its decomposition products) from the SNAP-doped tubes into PBSE as determined by HPLC-MS. There is a clear correlation between the chemical leaching and the NO release duration.

Taking advantage of the green color of SNAP, we also examined the color change of the tube soaked in PBSE at 37 °C to directly evaluate the amount of SNAP within the polymer. As shown in Figure 6B, the rate of the green color loss is in an order of Pebax 6333 > Pebax 7033 > Pebax 7233 > Nylon 12, which is consistent with the order of chemical leaching.

A low leaching rate of a specific NO donor from amorphous polymers was previously attributed to a low polymer water uptake because NO donors have to be dissolved in water to diffuse from the polymer bulk into the aqueous phase.[21a] However, as shown in Table 1, water uptake of these PEBA and Nylon 12 polymers are all low (≤ 2.0%) and Pebax 6333 even has the lowest water uptake. Rezac et al. also observed that the water sorption of Pebax films slightly decreases when the PA12 content is increased (Pebax 2533 < 3533 < 5533 < 6333).[22] To understand the relationship between the water uptake and chemical leaching from the PEBA and Nylon 12 tubes, microphase separation of the semi-crystalline polymer needs to be taken into account. It is generally accepted that water molecules reside in amorphous rather than crystalline phases.[23] However, the amorphous phase consists of soft domains that are distant from the crystalline phase and rigid domains such as the interlamellar regions and crystallite surfaces. Water molecules in the rigid amorphous region are primarily bound to PA12 chains and have lower diffusivity.[24] In the soft amorphous phase, a portion of water is also bound to polymer chains.[24] The rest of the water molecules exist as water clusters in the free volume between the soft chains and exhibit the highest diffusivity. [24,25] In the order of Pebax 6333, Pebax 7033, Pebax 7233, and Nylon 12, the increasing crystallinity brings an increasing amount of rigid amorphous domain, and the increasing ratio of PA12 segments yields more bound water molecules in the soft amorphous domain because of the higher affinity of amide groups toward water. Consequently, the highly diffusive water population (unbound water clusters in the soft amorphous phase) of these four polymers may exhibit an order opposite to that of the total polymer water uptake. Transport of solutes like NO donors in the polymer matrix primarily rely on the unbound water population and therefore, is more favored in Pebax 6333 than Nylon 12 as observed in the HPLC-MS leaching experiments. We also confirmed this unbound water-based chemical diffusion mechanism by a dye sorption experiment. When the polymer is soaked in an aqueous dye solution, the dye penetration measured by the color change of the polymer is indeed in the order of Pebax 6333 > Pebax 7033 > Pebax 7233 > Nylon 12 (see Figure S5 for exemplary images).

Another feature of semi-crystalline polymers compared to amorphous polymers is their low gas permeability because of the barrier effect of crystallites. It has been found that the permeability of PEBA and Nylon 12 toward gases like oxygen and carbon dioxide decreases significantly with an increasing degree of polymer crystallinity.[14c] Although there is no data for NO permeability, it is reasonable to assume a similar relationship with the polymer crystallinity. In a polymer with a low gas permeability, NO gas released via thermal decomposition of SNAP within the polymer is difficult to diffuse into the aqueous solution, which causes a low observed NO flux. However, the confined NO molecules are possible to react with NAP radical to regenerate SNAP via a radical recombination mechanism like other *S*-nitrosothiols.[26] This is likely to be another reason for the extremely long NO release duration of highly crystalline polymers like Nylon 12. Such “cage effect” has been utilized to increase the stability of other *S*-nitrosothiols[26] or *S*-nitrosothiol-modified

dendrimers[27] in aqueous solutions but was not explored to tune NO release from polymer matrices.

To understand the versatility of this crystallinity-controlled NO release mechanism, we compared one more series of semi-crystalline polymer tubes made of Pellethane 2363–55D and 2363–75D. A solvent system consisting of dichloromethane and methanol in a 1:1 volume ratio was used to impregnate SNAP into these polymers. Indeed, Pellethane 2363–55D does not have any detectable NO release after 48 h of soaking in PBSE, whereas Pellethane 2363–75D with a higher crystallinity has a NO duration of 1 week (see Figure S6 in Supporting Information). Examination of other semi-crystalline polymers is currently underway in our laboratory.

2.3 Nitric Oxide-Releasing Pebax Tubes as Insulin Infusion Cannulas

Although the limitation of insulin infusion cannula in CSII therapy is well recognized and the mechanism underlying the cannula failure is starting to be examined, strategies to improve the cannula performance are rare. Becton, Dickinson and Company (BD) developed a plastic cannula with an extra side port (BD FlowSmart™ technology) and found that occurrence of flow interruption events was decreased in a short-term study (2.5 h to 4.5 h) on healthy humans.[28] However, there is no evidence suggesting any advantages in a longer wear time when most adverse events happen. Indeed, this product is designed for 3-day use just like traditional Teflon cannula. The Raad group coated cannula with an antimicrobial agent, gendine, and demonstrated that biofilm formation is considerably inhibited in a 2-week *in-vitro* study.[13b] However, this strategy does not help cannula problems other than infection (e.g., inflammation at the implant site), and such tubes have not been tested *in vivo* as insulin infusion cannulas.

The potent bactericidal function of NO has been demonstrated in many NO-releasing biomedical devices, such as intravenous catheter.[2] Again, the NO flux of the semi-crystalline thermoplastic tubing is lower than those of the larger intravascular implants. Herein, we examined the potency of a low level of NO release on inhibition of biofilm formation on the Pebax 7233 cannula by using a CDC biofilm reactor. *Staphylococcus epidermidis (S. epidermidis)* and *Staphylococcus aureus (S. aureus)* were tested since they are the most prevalent strains observed on subcutaneous insulin infusion cannula.[29] As shown in Figure 7, the total viable bacteria adhered on the NO-releasing Pebax tube is >3 logarithmic units lower than that on the control tube for both strains over both the 3 day and 7 day test periods. Representative fluorescence images (using live/dead stains) also show a much higher coverage of bacterial film on control tubes compared to the NO-releasing tubes. Notably, the outer diameter of this tube is only 0.58 mm (24-gauge), which is even smaller than those of commercial Teflon cannulas (e.g., 0.68 mm for the Comfort™ Infusion Set from Animas and the MiniMed Silhouette™ Infusion Set from Medtronic). The NO flux is only ~0.2×10^{-10} mol/min/cm^2 at the end of the 1-week experiment (see Figure S7 in Supporting Information for the NO release profile), suggesting that such a low level of NO is still able to mitigate microbial biofilm formation.

The role of NO in the immune system can be either pro-inflammatory or anti-inflammatory.[30] However, it has been repeatedly demonstrated that the presence of exogenous NO

around subcutaneous implants is able to suppress the implant-associated inflammation layer and improve the implant performance (e.g., accuracy of continuous glucose monitoring).[3,31] Herein, we implanted sterilized NO-releasing and control Pebax 7233 tubes (2 cm length under the skin, ~30° insertion angle) into subcutaneous tissue of the sheep and used these tubes as cannula to deliver insulin. Like many other projects on diabetes management technologies,[12, 31ab] this work employed healthy rather than diabetic animals as the first step because diabetic animals are difficult to handle and also much more expensive to use as test animals. However, the anti-inflammatory property of NO in type 1 diabetic animals has been confirmed very recently by the Schoenfisch group,[31c] suggesting that benefits originating from suppressed inflammation may not be limited to only non-diabetic animals.

Blood glucose changes during a 2-h intravenous glucose tolerance test were used to assess the absorption of a rapid-acting insulin analogue, insulin lispro, through the Pebax cannula. Except when conducting the glucose tolerance test, saline rather than insulin was infused through the cannula at the typical basal rate of insulin infusion employed during clinical insulin pump therapy (10 μL/h). Each cannula was tested every other day during the 2-week experiment. Figure 8A shows the change of blood glucose concentration after administration of a dextrose bolus via an intravenous catheter. A 20-min insulin infusion was initiated, through the cannula placed subcutaneously, at the same time as the intravenous dextrose injection. The NO-releasing cannula (green lines and symbols) tend to yield lower glucose concentrations compared to the control cannula (red lines and symbols). The integrated area under the glucose concentration-time curve was further calculated (Figure 8B) to enable a quantitative comparison. We did not observe a clear cannula failure which is characterized by a high blood glucose level resistant to insulin infusion and is often observed after 2–3 days of cannula use in type 1 diabetic patients/animals. This is because non-diabetic animals are able to produce endogenous insulin in response to the glucose challenge. Indeed, as is shown in Figures 8A and 8B, blood glucose returns back toward baseline after glucose dosing in a blank test without any insulin infusion, although with a glucose concentration area greater than most tests with infused insulin. However, the endogenous insulin production may vary at different times, which results in fluctuation of the rate of glucose decrease during different glucose tolerance tests, and this makes it difficult to correct for the contribution from the endogenously produced insulin. Rather, the areas of all tests for each cannula were averaged to gain a statistical measure of the cannula potency (Figure 8C). By using this method, averaged areas for 6 pairs of NO-releasing and control cannulas in 5 animals were obtained. As shown in Figure 8D, the NO-releasing cannula yielded a lower glucose concentration area than the control cannula for 5 of the 6 pairs. Paired student's t-test (Figure 8E) indicates that such a difference is significant, suggesting that the SNAP-impregnated Pebax tube with NO release improves the subcutaneous insulin absorption.

At the end of each experiment, skin and subcutaneous tissue in the implant region was harvested, fixed in 10% neutral buffered formalin, and assessed histologically for inflammation associated with the periphery of the cannula by routine hematoxylin and eosin (H&E) staining method. Figure 9A and 9B shows representative images of H&E stained tissues. To quantitatively compare the inflammatory response between animals, an inflammation score was calculated by multiplying a score generated for inflammation density (severity) by a score generated for inflammation extent (see Experimental Section

for scoring criteria). Figure 9C shows the final tissue inflammation score for 6 pairs of cannulas. Because of the large variation between different animals, and low sample numbers, we did not obtain a statistically significant difference. However, as can be seen from the much lower averaged score for the NO-releasing cannula (Figure 9D), there is a trend that the presence of NO yields reduced inflammation. Therefore, the enhance insulin adsorption may be related to the suppressed inflammation although more comprehensive histological examination (e.g., collagen deposition and angiogenesis) is needed to fully elucidate the mechanism.

The Pebax 7233 material has a Shore hardness of 72D, which is greater than that of Teflon (50D-60D) which is used to make commercial insulin infusion cannula. This is beneficial for the insulin infusion application since a higher hardness can reduce the risk of kinking and crimping, which is actually a limitation of the current “soft” Teflon cannula compared to stainless steel needles.[11f] Moreover, biocompatibility of the medical grade Pebax material is of the highest level (USP Class VI, from Arkema website), which is the same as Teflon. Therefore, the benefit of using the combination of NO release and the Pebax cannula makes the proposed technique promising for application in subcutaneous continuous insulin infusion. Based on *in vitro* leaching experiment, the amount of leached chemicals is only ~ 0.4 mg for the 2-cm long Pebax 7233 cannula over a duration of 2 weeks. However, systematic assessment of the safety of SNAP and its decomposition products may be required for translation of this technology to clinical practice.

3. Conclusion

SNAP-impregnated semi-crystalline amide-containing polymers exhibit unique crystallinity-related NO release properties. The difference in loading of NO donors, leaching of NO donors, and diffusivity of NO molecules between different polymers is responsible for the observed highly diverse NO release magnitude and sustainability. Solvent-assisted impregnation of other types of semi-crystalline polymers with appropriate NO donors may further expand the current spectrum of NO-releasing materials and research in this direction is underway in our lab. The distinct chemical leaching property of the Pebax/Nylon polymers may also be utilized for tunable release of other drugs such as antiproliferative drug paclitaxel in drug-eluting Pebax balloons.[32]

Enhancement of subcutaneous insulin infusion/delivery via mitigated tissue inflammation is a new application of NO release materials. With these encouraging preliminary results in non-diabetic sheep models, we are planning to further test the Pebax cannula in type 1 diabetic animals that better mimic human patients. We are also pursuing alternate strategies to provide NO to the infusion site with precisely tunable fluxes and durations and thereby hope to establish the relationship between NO dose and insulin absorption efficacy. More histological analysis such as characterization of collagen deposition via Masson’s Trichrome staining and examination of new blood vessel formation via immunohistochemical staining will hopefully provide additional insight into the mechanism of the enhanced insulin absorption in the presence of NO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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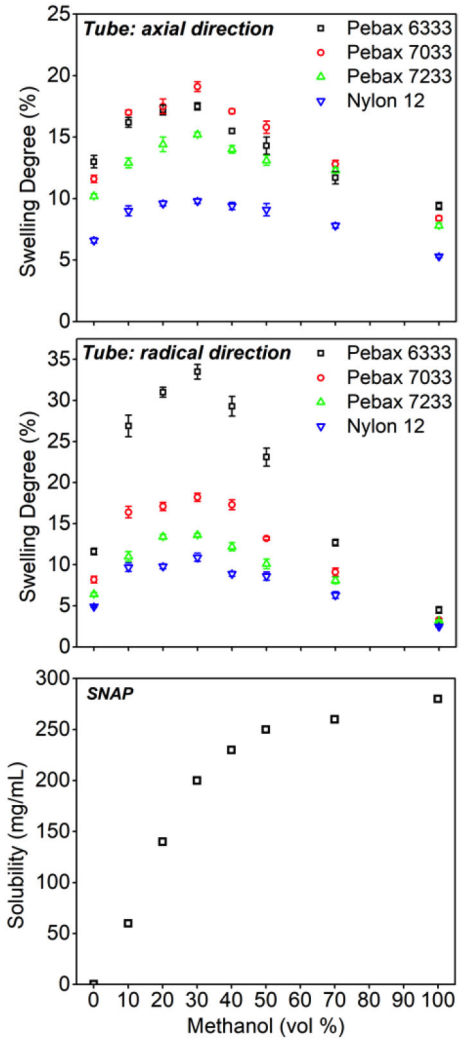

Figure 1.

Swelling ratio of four types of tubes at 37 °C and solubility of SNAP at 25 °C in a series of dichloromethane-methanol solvent mixtures. The length and outer diameter of the tubing was measured by an electronic micrometer. The swelling ratio was calculated by dividing the increase in tube length (axial direction) or outer diameter (radical direction) after 3h soaking by the original length or outer diameter. Standard deviations were obtained from n=3 tests. The solubility only has an accuracy level of 10 mg/mL.

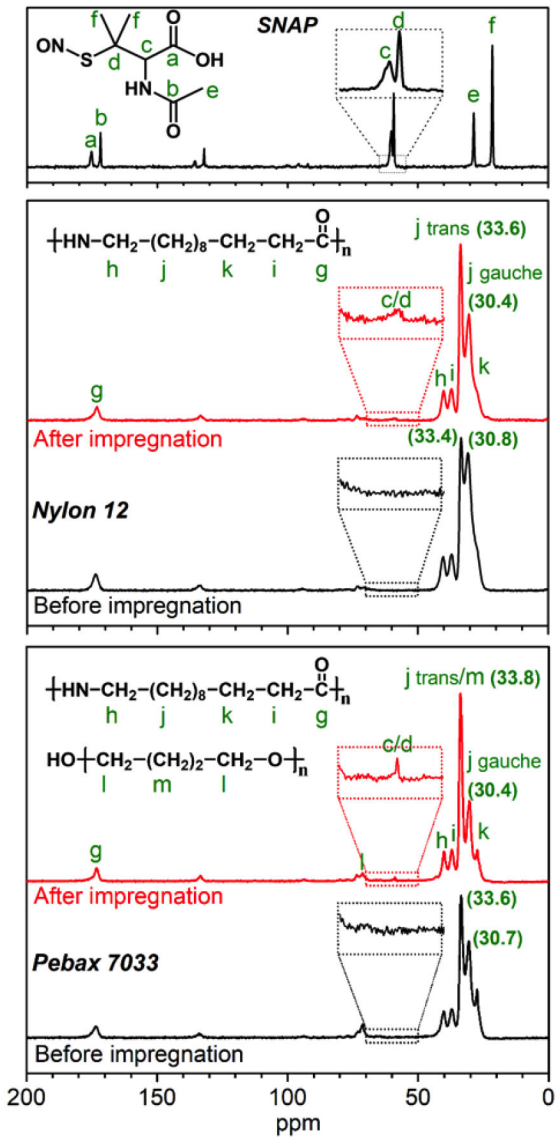

Figure 2.

^{13}C solid state NMR spectra of SNAP powder co-packed with Al_2O_3, and Nylon 12 and Pebax 7033 tubing before and after SNAP impregnation. Peaks at 130–140 ppm are spinning side bands.

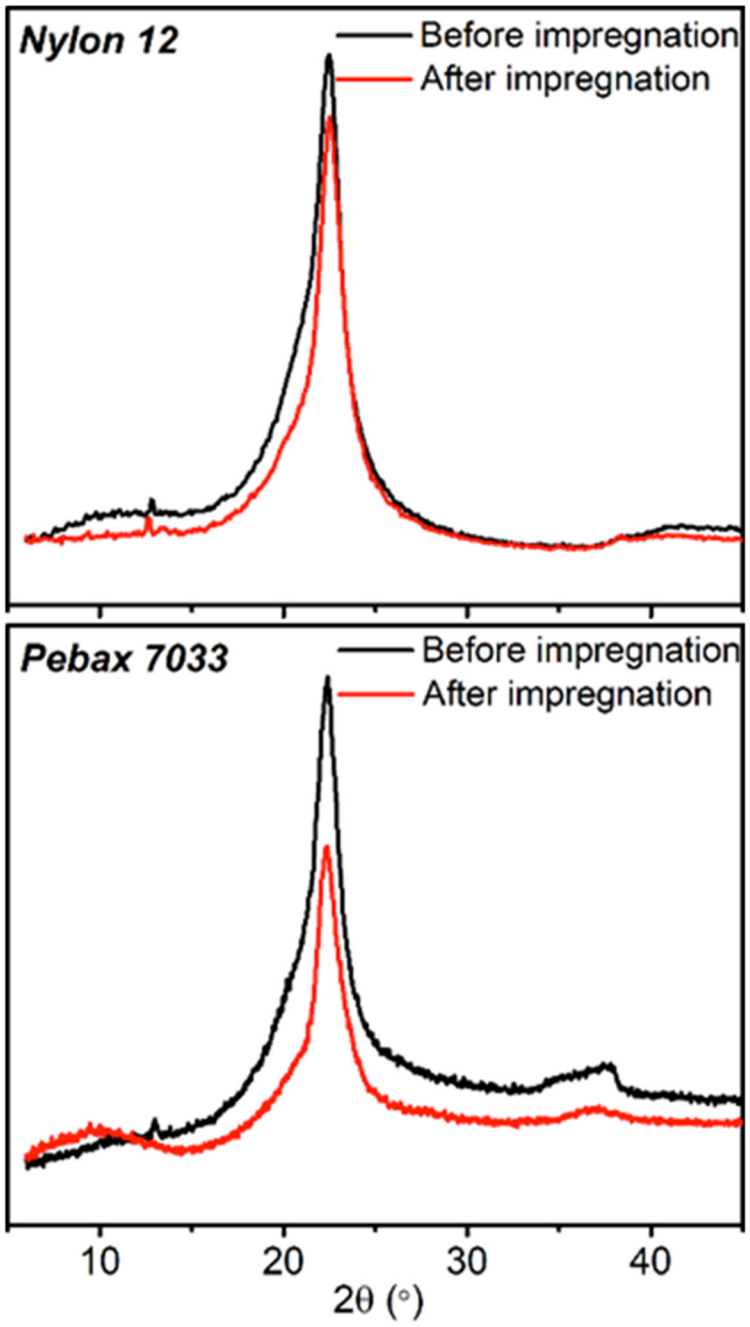

Figure 3.
1D WAXD spectra of Nylon 12 and Pebax 7033 tubing before and after solvent-assisted SNAP impregnation.

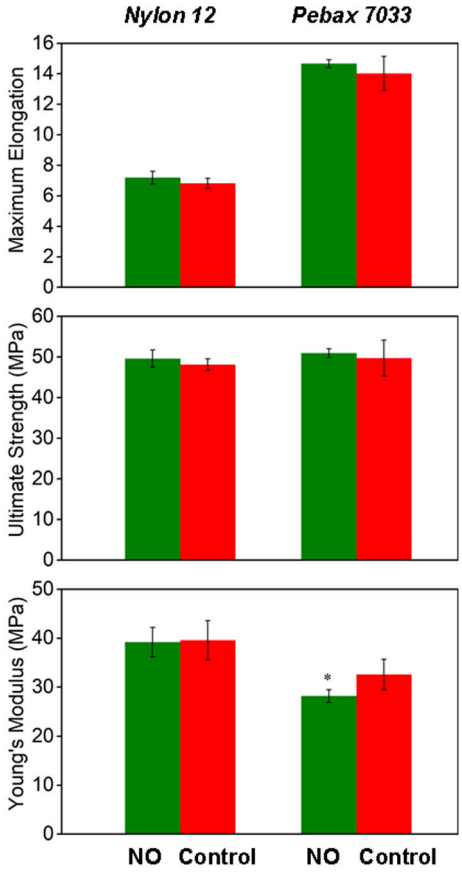

Figure 4.

Comparison of mechanical properties of commercial tubes and SNAP-doped NO-releasing tubes. Five tests were performed for each type of tubing. * $p<0.05$. See Figure S3 in Supporting Information for a representative strain-stress curve.

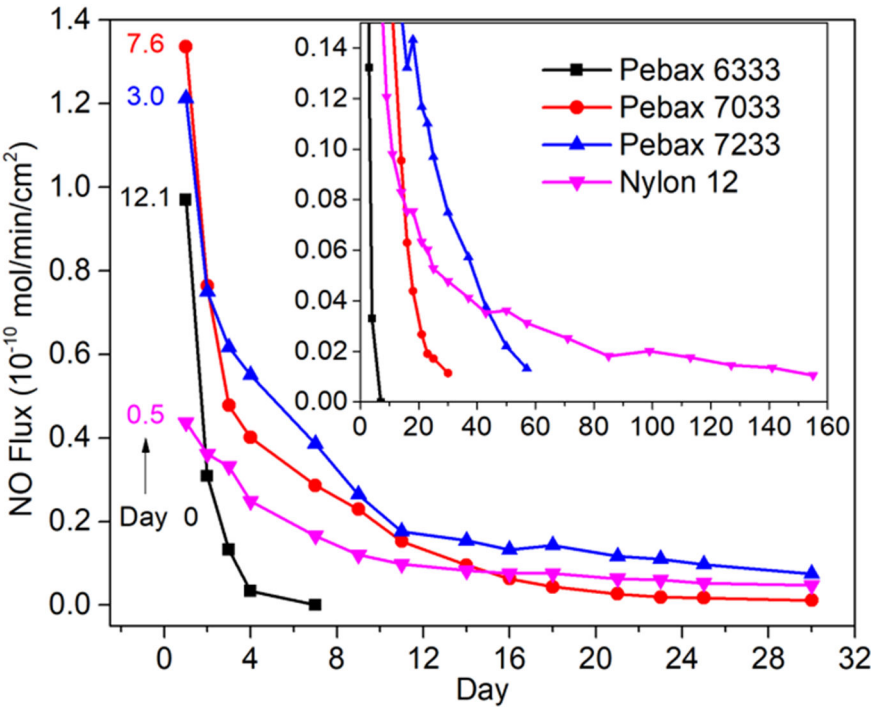

Figure 5.

Nitric oxide release from four types of polymer tubes soaked in PBSE at 37 °C. Data points are shown from Day 1 (after 24 h of soaking). Nitric oxide flux on Day 0 (before soaking) is labeled as numbers. Ten tubes of 2 cm length were tested in the same cell and the reported data point is their averaged NO flux.

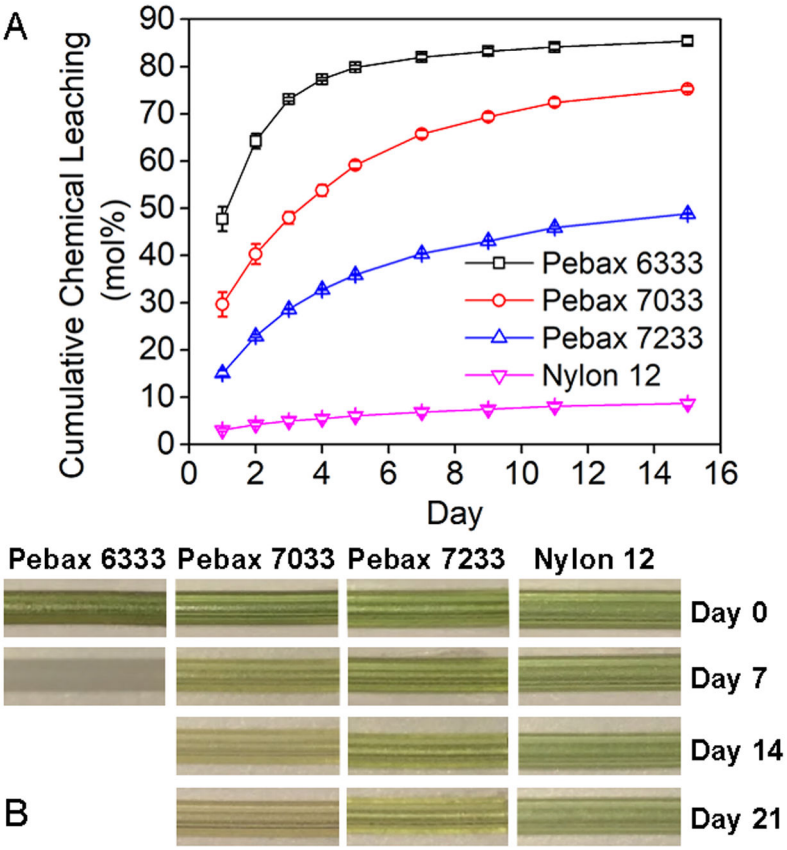

Figure 6.

A: Total cumulative leaching of SNAP and its decomposition products, N-acetylpenicillamine (NAP) and N-acetyl-penicillamine disulfide (NAP dimer), from four types of tubes soaked in PBSE at 37 °C. Molar percentages are relative to the original impregnated SNAP in the tubing before soaking. See Figure S4 in Supporting Information for the leaching curve of each species. Each leaching experiment used 8 tubes of 1 cm length. Standard deviations were obtained from n=3 tests. B: Color change of the four types of SNAP-impregnated tubes before soaking (Day 0) and after different days of soaking in PBSE at 37 °C.

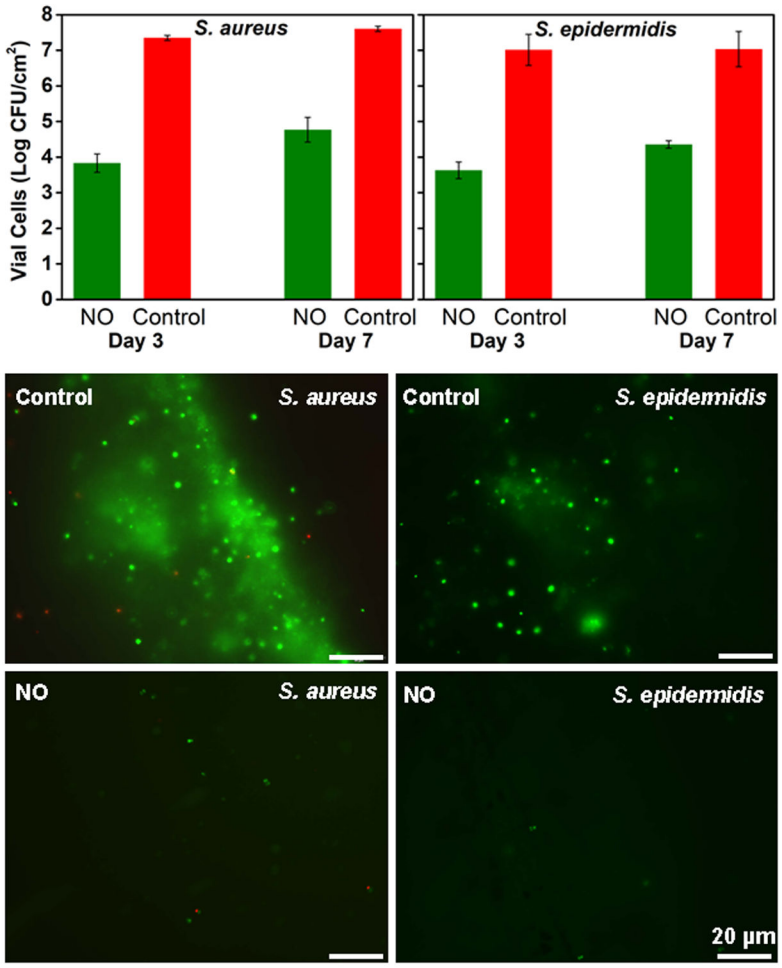

Figure 7.

Viable bacteria counts on the surface of the 24-gauge Pebax 7233 tubing in the presence and absence of NO release in a 3 day and 7 day period, and representative fluoresence images of the biofilm on the tube surface after 7 day incubation.

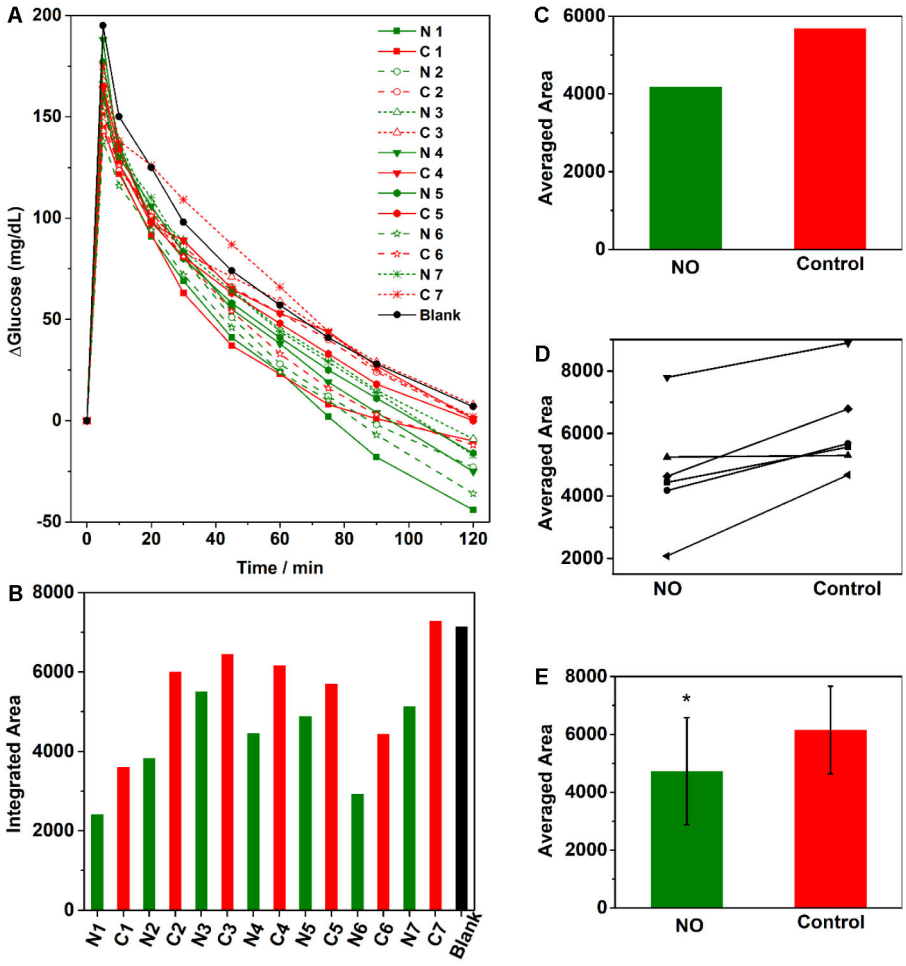

Figure 8.

A: Blood glucose values at various time points subtracted by those right before glucose injection (0 min). Green curves indicate tests on the NO-releasing cannula (N1–N7 on 7 different days). Red curves indicate tests on the control cannula (C1–C7 on 7 different days), and the blank curve indicates a test without any insulin infusion. B: The area under the glucose concentration curve for each intravenous glucose tolerance test. C: The averaged area obtained on seven different days for the NO-releasing and control cannula. D: The averaged area for 6 pairs of cannula. E: Statistical comparison of the averaged area from the 6 pairs of cannula indicates a significant difference between the control and NO-releasing cannula (*$p<0.05$ in paired t-test).

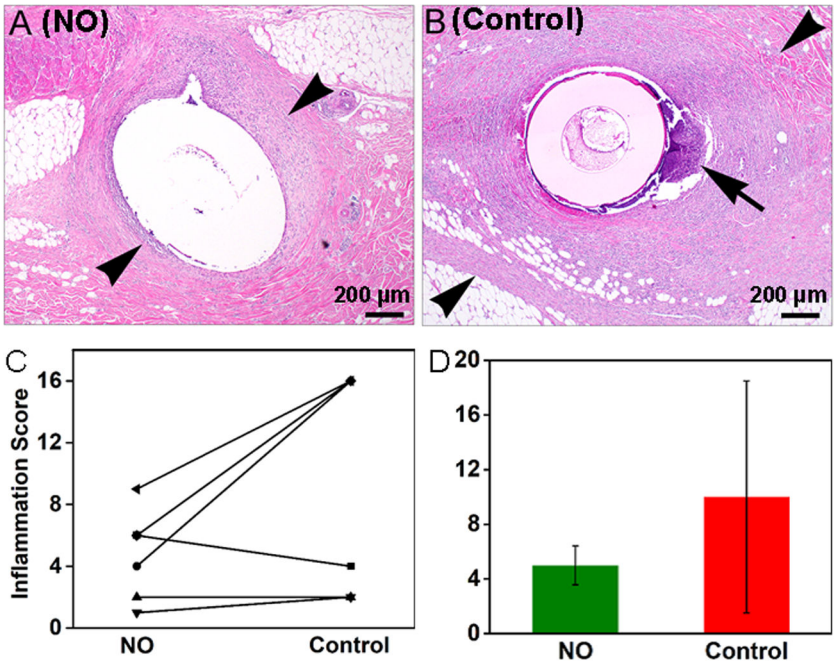

Figure 9.

Representative H&E-stained histology images for the NO-releasing cannula (A) and the control cannula (B). The NO-releasing cannula exhibits minimal to mild amounts of granulation tissue (denoted by arrowheads) and minimal inflammation, compared to increased amounts of granulation tissue (arrowheads) and accumulation of neutrophilic inflammation and cellular debris (arrow) indicative of bacterial contamination, in control cannula. C: The inflammation score of tissue samples for 6 pairs of cannula. D: Statistical comparison of the inflammation score for the NO-releasing and control cannula.

Table 1.

Composition and property of the four types of PEBA/Nylon polymers.

	Shore D hardness[a)]	PA12 Percentage (wt%; mol %)[b)]	Crystalline degree (wt%)[c)]	SNAP loading (wt%; mmol/g)	SNAP/ amorphous PA12/PA12 (molar ratio)	Water uptake (wt%)[d)]
Pebax 6333	63	82; 63	16.4 (± 0.2)	11.4 (± 0.5); 0.52	1: 6.4: 8.1	1.51 (± 0.06)
Pebax 7033	70	89; 75	18.0 (± 0.1)	8.1 (± 0.2); 0.37	1:9.7:12.2	1.69 (± 0.09)
Pebax 7233	72	92; 80	18.3 (± 0.1)	7.3 (± 0.3); 0.33	1:11.2:14.2	1.82 (± 0.06)
Nylon 12	74	100; 100	20.3 (± 0.2)	4.8 (± 0.3); 0.22	1:18.2:23.2	2.07 (± 0.04)

a): from website of Arkema;

b): molar ratio is from reference 14c; weight ratio is calculated by using a molecular weight of 197.3 and 72.1 for PA12 and PTMO, respectively;

c): obtained from differential scanning calorimetry (DSC) using 246 J/g as the melting enthalpy of the perfectly crystalline Nylon 12[14c];

d): water uptake after soaking tubes in DI water at 37 °C for 24h measured by gravimetry plus water content of the unsoaked tubing (stored at ambient humidity) determined by thermogravimetric analysis (TGA). Standard deviations were all obtained from n=3 tests.