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Nitric oxide releasing plasma polymer coating with bacteriostatic properties and no cytotoxic side effects

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Nitric-oxide releasing surface coatings deposited in one step retard bacterial growth whilst being non-cytotoxic

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A thin film coating was deposited by plasma polymerization (pp) of isopentyl nitrite (IPN). Contact with water caused the film to release nitric oxide (NO) which had a significant retarding effect on the growth of bacterial biofilms by up to 14 hours, while being non-cytotoxic to human mesenchymal stem cells.

Upon the insertion of an implant into the human body, a race to the surface between human cells and bacteria, with subsequent battle, begins and the winner takes all.[1, 2] Unfortunately, all too often the bacteria, such as the opportunistic *Staphylococcus epidermidis (S.epi)*, win this battle due to their fast multiplication rate and hardiness, contributing to approximately 1 million implant related infections (IRIs) per year in the United States alone.[3] These complications result in lengthy recoveries in conjunction with antibiotic treatment, in turn promoting antibiotic resistances.[4, 5] Furthermore, the conventional antibiotics used in these instances, albeit highly effective against planktonic bacteria, show drastically decreased efficacy against bacteria embedded in biofilms, which are prevalent in IRIs.[6, 7] Thus one strategy is to deliver antibacterial agents from surfaces or surface coatings.

NO has been investigated for its antibacterial properties because at low concentrations, NO "stuns" bacteria by interfering with their own quorum sensing; causing a stop in bacterial growth, biofilm formation and even induce its dispersion. [8, 9] This gives the human body additional time to launch an immune response or for circulating antibiotics to target planktonic bacteria. These properties of NO, which unite activity and selectivity towards bacteria, lead to research of NO releasing drugs, polymers and coatings.[10] However, their drawbacks are synthetic feasibility, cost, limited shelf-life and ability to deliver NO to the affected site, due to the short life span of the molecule itself. New methods are required for the straight-forward preparation of NO-releasing surface coatings.

Plasma polymerization is a manufacturing process used to deposit thin film coatings on surfaces on an industrial scale using volatile precursors. These volatile chemical precursors are excited into a plasma state and through a process of fragmentation and recombination form robust cross-linked coatings deposited on surfaces. The advantages of plasma polymerization are speed, simplicity, lack of solvents, scalability and good adhesion, regardless of the underlying substrate; sparking the interest of its application in the field of biomaterials.[11-14]

The novelty of our work is to combine the process advantages of plasma polymerization with the antimicrobial action of NO. The challenge was to retain enough nitrosoxy groups throughout the plasma process to produce NO releasing coatings with an acceptable shelf-life. Then verify that, upon contact with water, sufficient NO is released from these surfaces and test for the desired bacteriostatic and non-cytotoxic properties. All this qualities were consolidated by proper choice of process conditions and precursor.

Isopentyl nitrite (IPN) is known to produce NO and, due to its vasodilatating effects, is abused as a drug and sexual aphrodisiac. Putting IPN to more noble use, pulsed duty cycle plasma polymerized IPN coatings (IPNpp), without further treatment released NO upon contact with water. The released NO retarded the bacterial growth of the clinically relevant and vigorously biofilm forming bacterium *S.epi* by up to 14 hours. While even 2-months-old IPNpp coatings retarded *S.epi*, they did not

exhibiting cytotoxic properties towards human mesenchymal stem cells.

The plasma polymerization of IPN onto polyethylene terephthalate (PET) coverslips was carried out in a previously reported, custom-built, plasma reactor.[15, 16] In short, IPN vapours were introduced into a previously evacuated chamber and plasma deposited at a fixed monomer flow rate and power input. The plasma deposition was then changed from a continuous to a pulsed power input, lessening the fragmentation of the precursors in the gas phase and hence improving the retention of the frail organo-nitrite group.
The atomic composition and thickness of the resulting plasma polymer, as determined by XPS and ellipsometry, are summarized in Table 1.

% C	% N	% O	Thickness (nm)
66.2	11.3	22.4	42.7±0.5

Table 1: XPS and Ellipsometry data

The obtained atomic percentages correspond well with the theoretical values of a sufficiently thick IPN coating which would amount to 67.5 % C, 12.5 % N and 25 % O. The slightly lower percentage of nitrogen and oxygen is an indicator of the fragmentation and elimination of the labile organo-nitrite functional group.
Curve fitting (supporting information) of high-resolution C 1s, N 1s (Figure 1) and O 1s show the majority of N species being

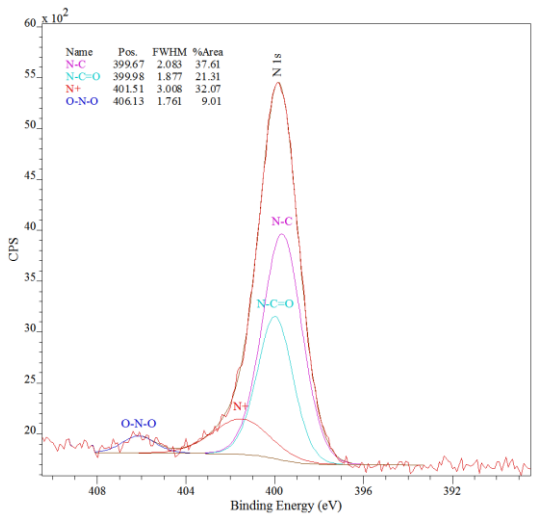

Figure 1: XPS N 1s high resolution curve fit

found in C-N bonds, which is possible after the fragmentation of the nitrosoxy group (O-N-O) and recombination with C species . However, all spectra support the presence of the nitrosoxy group at a non-negligible level. Hand in hand, the N 1s as well as O 1s spectra suggest that 9 % of Nitrogen and 24 % of Oxygen were bound in form of the desired nitrite group; pointing towards an overall retention of roughly one tenth of the functional group stemming from the IPN precursor.
This signifies that even low plasma powers, as they were used in this instance with pulsing, some nitrosoxy functional groups were lost through fragmentation; however, a portion remained evident in the surface coating.

To quantify the release of the short lived NO, it is necessary to capture the molecule in the more stable oxidized form of NO_2^- and quantify through the colorimetric Griess assay. NO in coatings was hydrolysed in either phosphate buffered saline (PBS) or a to pH 13 adjusted aqueous solution at either 37 or 70 °C over the course of 72 h (Figure 2).

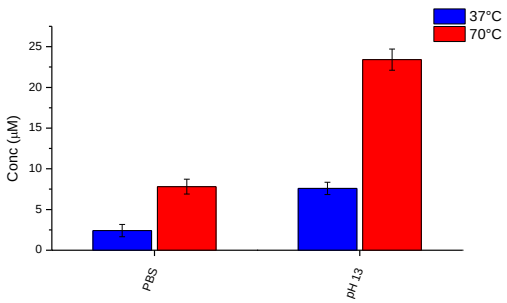

Figure 2: Total NO released (NO_2^- measured) after 72h

Evidently, the amount of NO released, which directly corresponds to the NO_2^- measured, depended upon the immersion media and temperature. A temperature increase in either case led to an almost three times higher amount of released NO; the same roughly applied when the pH of the solution was increased. Albeit the elevated pH and temperature are extreme examples, it points towards potentially using them to advantage in a real post-operational infection, as it has been shown that these go herein with an increase of temperature and pH in the wound environment, possibly leading to an increase in NO release when it is needed the most. [17]

In-vitro testing, using a particularly vigorous biofilm forming and clinically relevant strain of *S.epi* was performed. BacLight staining was employed for qualitative evaluation of the proliferation shown in time lapse results (Figure 3). Upon seeding, bacteria densities did not markedly differ between the reference and IPNpp; only few dead bacteria could be identified. After incubation, bacteria in the reference sample continued multiplying and formed biofilms. In contrast, the bacteria in contact with the IPNpp did neither multiply nor form any visible biofilm; this effect persisted up to 14 hours. These qualitative measurements matched with a quantitative assessment of the total biomass with the help of crystal violet staining of the samples (supporting information). Even by the naked eye a striking difference could be seen at the 14 hour mark between the reference and the IPNpp coated sample. The test was repeated at different time-points (supporting info) and yielded similar results

in all cases; namely the markedly delayed onset of bacterial multiplication and biofilm formation by at least 8 hours or more in all cases.

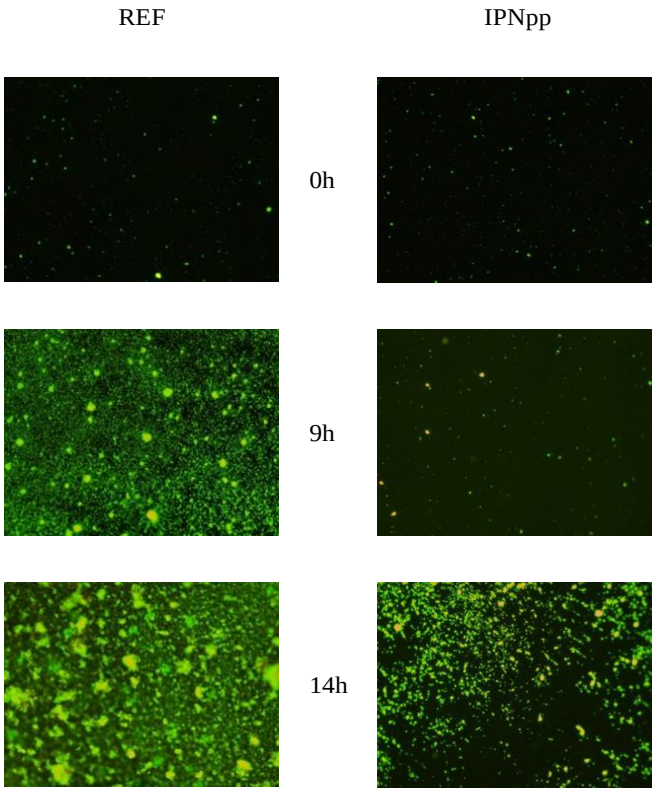

Figure 3: BacLight time lapse of Reference and IPNpp after 0, 9 and 14 h (10x magnification)

Organic nitrites deteriorate over time, presenting a challenge for industrial manufacturing.[18] We found that storing the samples in the fridge overnight or even 2 months at room temperature prior to their use, caused little to no difference in the observed results; hinting towards the robustness of the coatings stored under dry conditions (supporting Information).

To investigate the biocompatibility, cytotoxicity testing with human mesenchymal stem cells was conducted. A known cytotoxic surface (chlorinated plasma polymer coating TCE), was used as the negative reference.[19] The observed cell spread (supporting information) was lower on IPNpp compared to tissue culture polystyrene (TCP, positive reference), however, no viable cells were observed on the cytotoxic coatings. This hints that, albeit IPNpp being less ideal for cell attachment than TCP and hence the decreased cell spread, it does not exhibit any imminent cytotoxic effect and enables healthy cell-proliferation as can be seen in Figure 4.

In conclusion, this study showed that "one step" plasma polymerization of IPN leads to coatings which release NO. Coatings have bacteriostatic qualities whilst not exhibiting cytotoxicity towards human mesenchymal stem cells. These biological properties, in conjunction with the industrial manufacturing method, present a combination of desirable qualities and could be used on a commercial scale for novel implant coatings; tilting the favour of the battle between us and the bacteria to our side and ultimately lowering the incidence of IRIs.

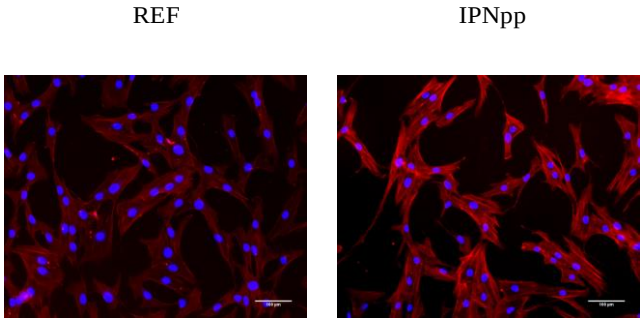

Figure 4: MSC stained with DAPI (nuclei, blue) and phalloidin (actin fillaments, red)

Additional vital questions remain, regarding the NO release kinetics, other NO releasing precursors, the optimization of the retention of the functional nitrosyl group as well as in-vivo studies. Answers to these questions will be published in a follow up study to improve further the coatings unique qualities with the ultimate aim of better patient outcomes.

Notes and references

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