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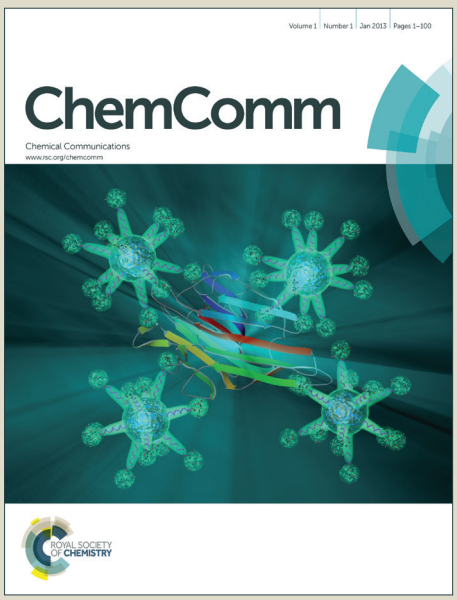

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Nitric oxide releasing plasma polymer coating with bacteriostatic properties and no cytotoxic side effects

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We report a stable plasma polymer coating, using isopentyl nitrite as a volatile precursor, which releases Nitric oxide at bacteriostatic concentrations when contacted with water, inhibiting bacterial growth without cytotoxic side effects to human mesenchymal stem/stromal cells.

Bacterial infections continue to pose serious problems in the use of biomedical implants and devices as bacteria are readily able to colonise the surfaces of synthetic materials.[1] Infection rates and severity of the outcomes vary between devices, but are still too high despite best practices in operating theatres, leading to patient trauma and enormous costs to health systems. In the US alone, approximately 1 million implant related infections (IRIs) occur per year.[2] They can lead to revision surgery and lengthy recoveries in conjunction with antibiotic treatment, in turn promoting antibiotic resistance.[3] Furthermore, antibiotics highly effective against planktonic bacteria show drastically decreased efficacy against bacteria embedded in infection biofilms, which are prevalent in IRIs.[4]

Herein we describe a novel coating that was designed to release Nitric oxide (NO), a molecule that fulfils several important signalling functions both in bacteria and in human wound healing. At low concentrations, NO "stuns" bacteria by interfering with their quorum sensing, causing suppression of bacterial growth and biofilm formation, and even induces dispersion of formed biofilms. [5] NO can also act as a signalling molecule in promoting human wound healing and stem cell differentiation.[6] Thus, NO reduces the propensity for infections to set in and assists the immune system and the efficacy of antibiotic drugs. Accordingly, there has been extensive research on NO releasing drugs, polymers and coatings.[7] However, their drawbacks are synthetic complexity, cost, decomposition upon storage, and ability to deliver NO to the affected site, due to the short life span of NO. New and facile methods for the preparation of stable NO-releasing surface coatings are required.

The coating we have developed contains NO in a latent form, stable during air storage but activated when in contact with aqueous media. Release of NO occurs upon implantation or other contact with the patient's body fluids (e.g., catheters). The coating is deposited using plasma polymerization, a process that is used widely in both research and industry for coating solid materials with thin adherent coatings. Volatile chemical precursors are excited into a plasma state and through a process of fragmentation and recombination form robust cross-linked coatings. The advantages of plasma polymerization are speed, simplicity, no need for solvents, scalability, and excellent adhesion to most substrate materials, which has prompted many studies on its applicability to biomaterials.[8]

Isopentyl nitrite (IPN) is known to produce NO upon contact with water. This compound is sufficiently volatile for use in plasma polymerization, but combining the advantages of plasma processing with the antimicrobial action of NO was contingent upon being able to retain enough nitrosoxy groups in the molecular activation in the plasma, and to obtain coatings that retain their activity even after storage. We report here that pulsed plasma polymerization of IPN leads to stable coatings that are bacteriostatic and not cytotoxic to mesenchymal stem cells.

The plasma polymerization of IPN onto polyethylene terephthalate (PET) coverslips was carried out in a previously reported, custom-built, plasma reactor.[9] In short, IPN vapours were introduced into a pre-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, reducing the fragmentation of the precursors in the gas phase and hence improving the retention of the frail nitrosoxy group (see Supporting Information).
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 a low degree of fragmentation and elimination of the labile nitrosoxy functional group.

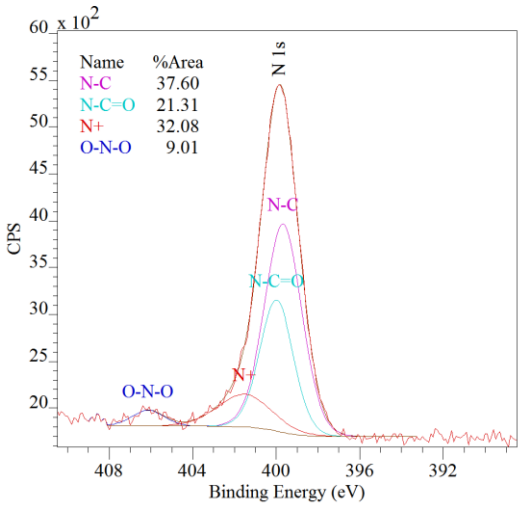

Figure 1: XPS N 1s high resolution curve fit

Curve fitting (supporting information) of high-resolution C 1s, N 1s (Figure 1) and O 1s show the majority of N species being 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 nitrosoxy 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 in a pH 13 adjusted aqueous solution at either 37 or 70 °C over the course of 72 h (Figure 2).
Evidently, the amount of NO released, which directly corresponds to the NO_2^- measured, depended upon the

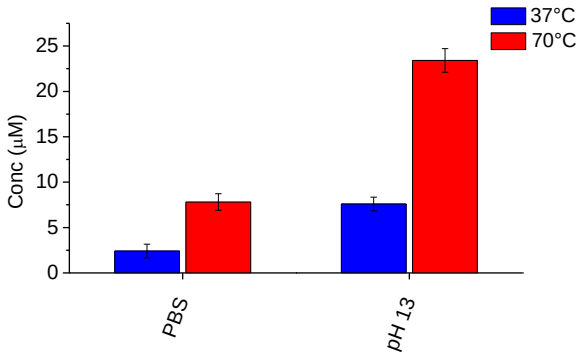

Figure 2: Total NO released (NO_2^- measured) after 72h

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 there is an increase of temperature and pH in the wound environment, possibly leading to an increase in NO release when it is needed the most. [10]

In-vitro testing was performed using a particularly vigorous biofilm forming and clinically relevant strain of *Staphylococcus epidermidis* (ATCC® 35984 ™). 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; few dead bacteria could be identified at this time point. After incubation, bacteria in the reference sample continued multiplying and formed biofilms. In contrast, the bacteria in contact with the IPNpp neither multiplied nor form any visible biofilm; this effect persisted up to 14 hours. These qualitative measurements accorded with a quantitative assessment of the total biomass with the help by 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.

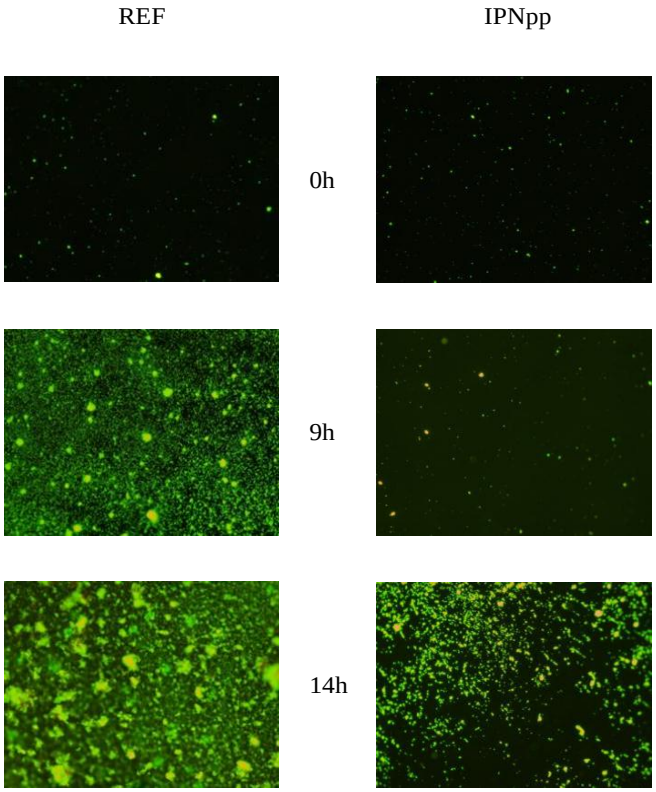

Figure 3: BacLight time lapse of Reference and IPNpp after 0, 9 and 14 h (10x magnification)

While organic nitrites can deteriorate over time, presenting a challenge for industrial manufacturing,[11] we have found that this did not pose a problem for our IPN plasma coatings, as storing samples for 2 months at room temperature and ambient atmosphere prior to use led to little to no difference in performance (Supporting Information). We conclude that the coating appears to be sufficiently stable and thus suitable for the conditions and timeframe involved in the production, distribution as well as storage of biomedical devices and implant products.

To investigate biocompatibility of the IPN coatings, cytotoxicity testing with human mesenchymal stem/stromal cells (MSC) was conducted. A known cytotoxic coating (chlorinated plasma polymer from 1,1,1-Trichloroethane (TCE)),[12] was used as the negative control. The observed cell attachment and spreading (Supporting Information) was somewhat lower on IPNpp compared with the positive control tissue culture polystyrene (TCP), whereas no viable cells were observed on the cytotoxic control. While the IPNpp coating is less supportive of cell attachment than TCP, this might not matter in practical applications; the key point is that the IPNpp surface has definitely no cytotoxic effect and enables healthy cell proliferation (Figure 4).

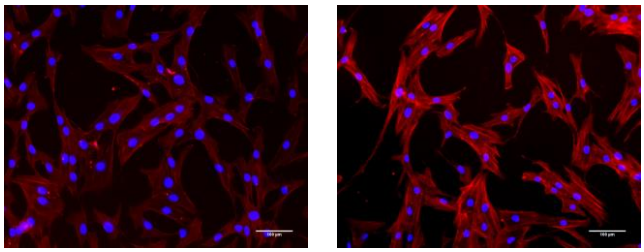

Figure 4: MSC stained with DAPI (nuclei, blue) and phalloidin (actin fillaments, red)

In conclusion, this study demonstrated for the first time that suitable plasma conditions can be derived to enable plasma deposition of IPNpp coatings without excessive cleavage of the relatively labile nitrosoxy group in the plasma, and that the resultant coatings demonstrate excellent bacteriostatic properties but no toxicity to stem cells. The production of such NO releasing coatings by a single processing step and using the readily available and affordable compound IPN should open up opportunities for the commercial-scale deposition of IPNpp coatings onto a wide variety of biomedical devices, implants, and other products that require selective prevention of bacterial colonization. This is in contrast to the many reported coatings that are bactericidal and thus cause release of bacterial endotoxins and/or being cytotoxic themselves. Our novel coating seems promising for combating infections associated with implants and biomedical devices; accordingly, ongoing research is focusing on maximising nitrosoxy retention, NO release kinetics, and animal model testing.

Notes and references

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