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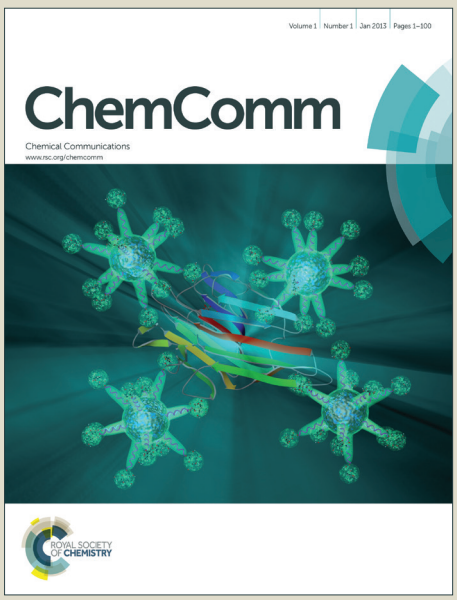

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Plasma Polymerised PolyOxazoline Thin Films for Biomedical Applications

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Poly(2-oxazoline)s are emerging revolutionary biomaterials, exhibiting comparable and even superior properties to well-established counterparts. Overcoming current tedious wet synthesis methods, we report solvent-free and substrate independent, plasma polymerised nanoscale biocompatible Polyoxazoline coatings capable of controlling protein and cell adhesion, and significantly reducing biofilm build up.

Polyoxazolines (POx) were first synthesised via ring opening solution polymerisation in 1966[1]. However, the complex and costly synthesis methods have made them unattractive for commercial production compared to competing polymers. In the last decade, the discovery of POx "stealth" properties[2] and excellent biocompatibility[3] has triggered a resurgent interest in these compounds for biomedical applications such as delivery of therapeutics, biosensors and coatings for implantable devices, detailed in several recent reviews[4]. For a number of biomaterials applications, POx needs to be placed on device surfaces in the form of coatings and thin films. Currently however, POx immobilisation onto surfaces is a tedious multistep process, in which oxazoline monomers are initially polymerised in bulk solution before the resultant polymer can be grafted to a limited selection of substrate materials using processes such as spin coating[5], grafting to[6], grafting from, photopolymerisation[7] and electrostatic interactions[8]. Overall, POx coatings generated by these methods have demonstrated good stability and low fouling properties where the nature of the monomer used and in turn the wettability of the coatings tends to control the interaction with proteins[7-8], eukaryotic cells[5, 9], and bacteria[10]. A comparative review of the antifouling properties of PEG and POx coatings by Konradi et al[10] states that despite differences in surface architectures and test conditions within the literature, both surface types are typically equally effective in respect to antifouling ability. However, POx surfaces generally show greater stability than PEG based surfaces generated in a similar manner.[10] In view of the wide range of useful properties, it is clear that from a practical perspective and use on commercial devices, the field will benefit from a facile process capable of generating strongly adherent POx coatings on a wide range of substrate materials.

Herein, we report on the first 2-methyl-2-oxazoline-based plasma polymer coatings. Plasma polymerisation is a niche technique for creating organic thin films[11]. The advantage of this facile, one-step and substrate-independent method is that it enables the formation of nanoscale coatings on any type of substrate material without the need for surface preparation[12]. Besides, the method does not require solvents or initiator, does not create liquid organic waste, and uses minimal monomer quantities which makes it cost effective and environmentally friendly.[13] We demonstrate that the unique retention of oxazoline chemical functionalities in the films obtained via this method not only retains the remarkable properties already claimed for POx coatings generated by conventional means, but also provides new routes to further functionalization with biomolecules and nanoparticles which open a vast scope of opportunities for applications in the biomedical and other fields.

Plasma polymerisation of 2-methyl-2-oxazoline (Sigma-Aldrich, Australia) was carried out in a capacitively coupled bell-chamber reactor described previously.[12a] The oxazoline precursor was deposited at a pressure of 2.3×10^{-1} mbar. Fig. 1 summarises the physico-chemical properties of POx coatings deposited using RF power of 10, 20, 40 and 50 W for 1 and 5 minutes. The thickness of the plasma deposited POx films increased with both the plasma RF power and precursor deposition time from 20 to 76 nm. The latter indicates that, during the deposition, constructive mechanisms of polymer film growth dominate over the competing etching processes (Fig 1a).

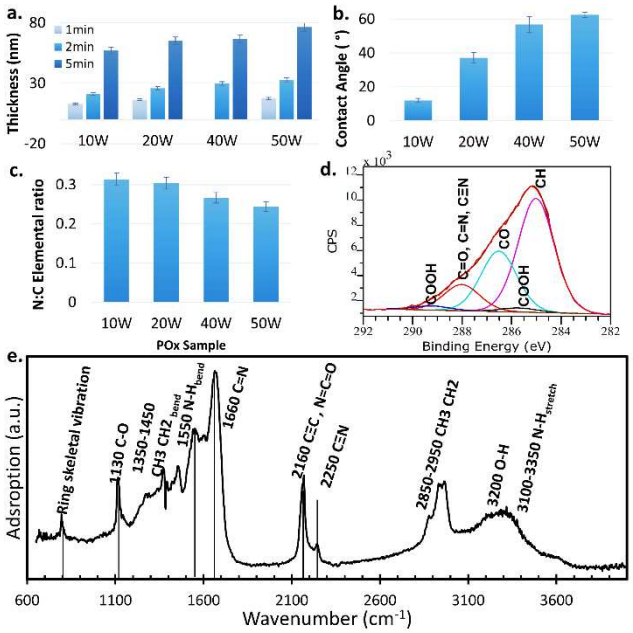

Fig. 1 (a) Plasma deposited POx films thicknesses. (b) Water advancing contact angles of POx films. (c) N/C atomic ratio measured via XPS. (d) High resolution C1s spectrum (50W5min) (e) FTIR spectrum (50W5min).

All POx films formed were hydrophilic with water contact angle increasing with RF powers, from 12° to 62° as shown in Fig. 1b. Fig. 1c shows the nitrogen to carbon ratio of POx coatings. The higher fragmentation of the oxazoline precursor occurring at higher RF power is likely to be responsible for the loss of nitrogen observed. Analysis of the high resolution C1s XPS spectra revealed a complex carbon chemistry with the presence of about 30 and 10 % of C-O and C=O environments, respectively. A typical IR spectra revealing the complex chemistry of a typical plasma deposited POx films (50W5min) is shown in Fig. 1e. All POx film present a broad –OH stretching band (3200-3400 cm^{-1}) originating from substrate hydration, typical C-H vibrations (2950 and 1370cm^{-1}) and also a very intense band at 2170 cm^{-1} with a shoulder at 2250 cm^{-1} which are attributed to alkyne C≡C and/or isocyanate O=C=N and nitrile CΞN liaison respectively. The formation of these reactive chemical functions within POx film can be attributed to the structural fragmentation and recombination of the oxazoline precursor occurring during plasma polymerisation. More interestingly, several signals specific to the oxazoline ring are detected. Namely, the strong bands observed at 1657 and 1130 cm^{-1} are associated with the stretching of the C=N and C-O bond constituting the oxazoline ring [9]. It is worth noting that in Polymethyloxazoline formed using conventional ring opening polymerisation technique the C-O band is absent from the FTIR spectrum (see figure 2 in [9]) which means lack of functionality retention. Finally a sharp band is present at 800 cm^{-1}, where para substituted aromatic ring skeletal vibration are typically found, could be indicative for the skeletal vibration of substituted oxazoline rings. To date, the common feature in studies on polyoxazolines has been the opening of the oxazoline ring which their polymerisation relies on. Yet, the retention of the oxazoline ring, could be highly beneficial for selected biomedical applications such as antibody, protein, ligand and nanoparticle. Indeed, the reactivity of the oxazoline ring is known to lead to the formation of a covalent amide bond by reaction with the carboxylic acid group[14] and thus could serve selective biomolecular binding purposes.

Assessment of the biocompatibility of the plasma polymerised POx films is essential should these coatings have a future in the biomedical field[15]. Primary human derived dermal fibroblast cells (HDF) were chosen to evaluate the possible cytotoxic effects of the plasma deposited POx coatings *in vitro*. This cell type was selected as it contribute to wound healing[16]. Plasma Polymerised Allylamine (ppAA) films were chosen as a positive control as they are known to encourage cell adhesion due to their high nitrogen content[17]. Cells were cultured on uncoated, POx and ppAA coated glass coverslips for 3 days prior to analysing fibroblast viability using an alamar blue assay. Results of HDF metabolic activity on POx coating relative to ppAA substrate are shown in Fig. 2a.

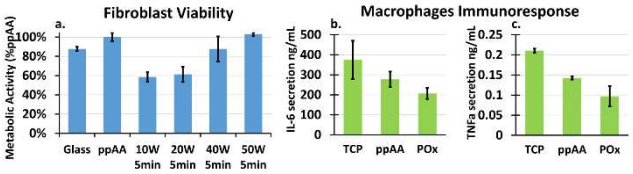

Fig 2 (a) Normalised metabolic activity of HDF grown on ppAA and 10W - 50W plasma deposited POx films. (b) IL-6 cytokine and (c) TNF-α cytokine expression of BMDM grown on POx, tissue culture plates and ppAA surfaces. All error bars show ±SEM.

An increase in the HDF viability is seen as the plasma power increases. Coatings deposited using plasma power of 40W showed similar viabilities as the untreated glass surfaces while those derived using 50W showed comparable to and even slightly better viabilities than the biocompatible ppAA surfaces[18]. Acceptably These results suggest that the plasma polymerised POx coatings derived at plasma power of 50W are non-cytotoxic and could constitute excellent platforms for cell culture, cell therapies and bioreactors.The lower viability of the cells on coating generated using lower input power i.e. 10W and 20W can be explained by instability of films prepared at these conditions when placed in solvents. When the power of deposition was increased to 40 W the film stability greatly increased which also resulted in increased cell viability. Films deposited at power of 50 W were very stable when immersed in solvent and had highest cell viability. For this reason all studies presented below are carried out with coatings prepared using input power of 50W.

Any potential inflammatory response to the coating was assessed by measuring, *in vitro*, cytokine secretion from bone marrow-derived primary macrophages (BMDM) which have the capacity to mediate early innate immune inflammatory responses. BMDM were incubated on the respected surfaces and stimulated for the secretion of pro-inflammatory cytokines as described in detailed elsewhere[19]. The level of TFN-α, IL-6 secretion are shown in Fig 2b. and 2c. A marked reduction in the expression of TFN-α and IL-6 was observed on the plasma deposited POx coating compared to a TCP control and ppAA. Overall, the biocompatibility studies support the fact that the plasma deposited POx coatings developed in this study do not impede the functionality nor the viability of primary mammalian cells. Furthermore, the reduced expression of pro-inflammatory cytokines from BMDM suggests a reduced inflammatory response which can benefit numerous medical devices such as implants, catheters and wound dressings.

The capacity of the plasma deposited POx films unique chemistry to covalently bind biomolecules was examined in real time using QCM-D measurements. The experiments were conducted under flow conditions in physiological buffers and amine group-rich ppAA coatings were used as controls. Exposure to a 0.1 mg/mL albumin (BSA) solution resulted, in a sharp decrease in frequency, corresponding to a mass increase due to protein attachment for both control ppAA and POx coated sensors (Fig. 3a and 3b, respectively). After equilibration, the sensors were subjected to thorough *in situ* rinsing and washing steps, first with PBS, then with concentrated SDS (10%) and finally, with PBS again. The purpose of the SDS was to remove from the surface proteins that were physically adsorbed only. Analysis of the frequency trace throughout these washing steps revealed differences in the protein binding behaviour dependent on the nature of the plasma polymer film. The frequencies of ppAA coated sensors increased after SDS wash and PBS rinse recovering completely their initial values, which reveals that all adsorbed protein had been washed off the sensor surface. On the other hand, no frequency change was observed for POx coated samples following PBS rinse relative to the change in frequency after initial adsorption. The permanent negative deflection remained after washing with SDS and PBS wash. This suggests that irreversible attachment indicative for covalent protein binding occurs on the POx plasma films, as opposed to the reversible physisorption of protein observed on ppAA surfaces. The average amount of BSA covalently bound to POx plasma film was estimated using the Sauerbrey equation and found to be 70 ± 7 ng.cm^{-2}. This corresponds to approximately 25% surface coverage which is appropriate for biosensor platforms as it allows sufficient spacing between protein molecules for conducting binding reactions. The protein binding ability of POx plasma deposited films does not appear to depend on the nature of the protein. Indeed, adsorption of streptavidin, fibronectin anti-human podocalyxin antibody were also achieved suggesting that the mechanism responsible for POx binding with biomolecule can apply to a broad range of compounds. It is important to note that the POx plasma polymer coatings reported here provide a different mechanism for covalent protein attachment. Common surface chemistries used for covalent attachment such as epoxy and aldehyde target the amine groups of the proteins. The POx coatings bind the carboxylic acid groups. This is an alternative approach which may be beneficial for preserving the bioactivity of some proteins. Complementary experiments featuring the covalent binding of carboxyl-functionalised gold nanoparticles and polyacrylic acid to the same coatings (Fig. S1 and S2, ESI) support the hypothesis that the binding mechanism consist of a reaction between the coated substrate and the carboxylic acid groups of the ligand. As a result, there is a broad range of potential ligands such as molecular probes, growth factors, nanoparticles and cell adhesion peptides that could be irreversibly bound to plasma deposited POx coating for targeted applications in cell culture, biosensing, implant coatings or tissue engineering

The capacity of the plasma polymerised POx films to resist bacterial colonisation was tested using *S. epidermidis* as a model pathogen. This organism forms very robust biofilms and was chosen for its medical relevance.[20] For this experiment, half of a 12-well tissue culture plate was coated with plasma deposited POx using power of 50W for 5 minutes (Fig. 4). 1mL of 1x10^6 CFU\ml of bacteria was allowed to interact with the plate and to form biofilms overnight. Note that this is very high initial concentration of bacteria. Such contamination would rarely occur in a clinical setting but it is a good demonstration of the capacity of the coatings to resist biofilm formation. Samples were then washed with PBS and stained with safranin-O. Excess safranin stain was washed off the samples with Milli-Q water to ensure that the only remaining stain was that absorbed by bacteria. The difference between the highly fouled uncoated half of the well surface (left) and a low-fouled plasma deposited POx surface (right) can be easily observed in the microscopy image shown in Fig. 4f. On the magnified image of the coated (right)\uncoated (left) interface (Fig. 4g) it is striking that on the uncoated surface bacteria readily adhere, proliferate and express extracellular matrix to form a strong biofilm as expected [21]. In comparison, the bacteria attached to the POx coating do not seem to form a well adhered biofilm. Although individual bacteria and small colonies are observed, the bacteria do not seem to be capable of forming an intact biofilm. Although the complete picture as to why plasma deposited POx coatings resist biofilm development is yet to be understood, one may hypothesise that the plasma deposited POx disrupts signalling pathways between bacteria, and in turn impede the bacteria intercommunication necessary for biofilm formation. Another plausible hypothesis is that the coatings are able to hydrate in aqueous solvents. This would lead to softening of the film which may result in weaker bacterial adhesion.

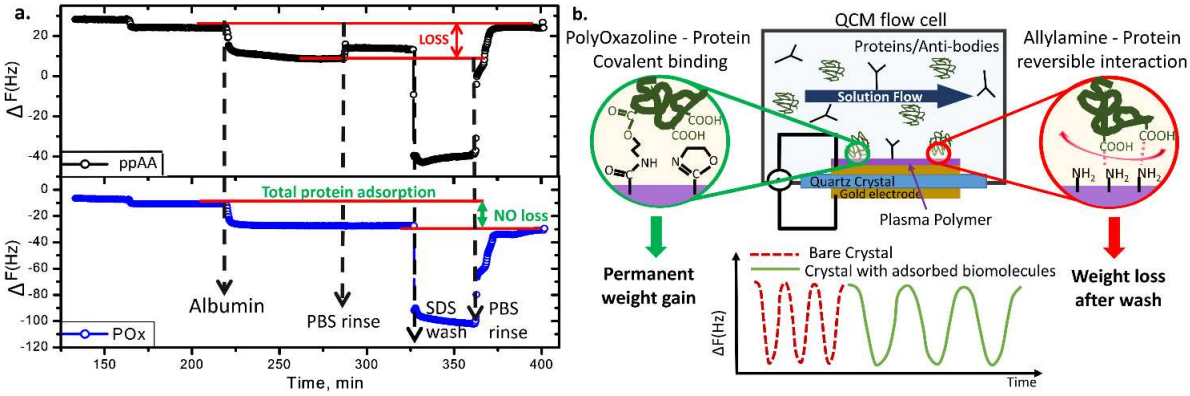

Fig. 3 (a) Real time QCM frequency trace demonstrating that the mass gained almost instantly following exposure to albumin solution (negative deflexion) is retained after PBS rinse and SDS wash (10 v% in PBS) on the POx coated QMC crystal (bottom trace), while it is lost for the allylamine equivalent (top). (b) Schematic of the different protein adsorption mechanism occurring on the POx and Allylamine coated QCM crystals.

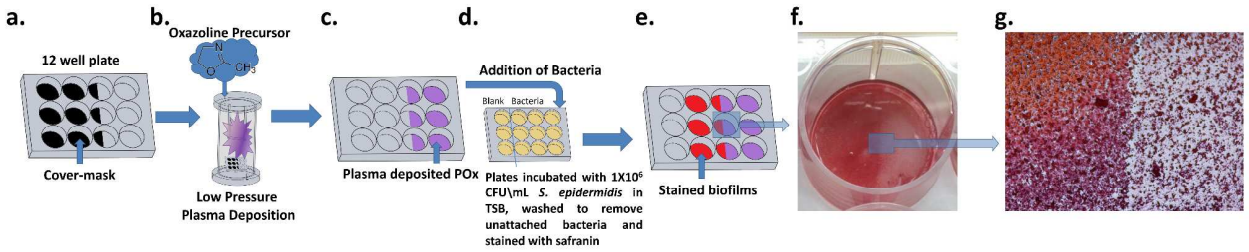

Fig. 4 (a). Schematic of cover-masks added to wells of a 12-well tissue culture plate covering full wells and half wells. (b) POx was deposited onto the exposed areas. (c) S. epidermidis was added to each well. (d) biofilms were grown and (e) stained with safranin. (f) Photo of half coated well showing high fouling on the uncoated region (left) and low fouling on the coated region (right). (g) microscopy image of uncoated\coated regions of the half coated plate post treatment with bacteria.

However, since not all hydrating and swelling polymers are low-fouling, it is more likely that the low fouling behaviour of plasma polymerised POx coatings is the result of a complex interplay between chemistry, wettability and mechanical properties at the nanoscale. In particular, the unique chemistry of the plasma deposited POx film is likely to be essential to this type of coating low fouling properties, as other amine rich plasma deposited films such as ppAA are very easily fouled. [19]. The same coatings were also placed on glass surface and demonstrated comparative performance to those on cell culture plates and petri dishes. These results demonstrated that coatings of the same quality can be deposited in the same way on glass and polymeric substrates.

The capacity of the POx coatings to concomitantly reduce both biofilm formation and inflammatory response but in the same time support the growth of HDFs can enormously benefit many implantable device technologies. Indeed, devices such as artificial heart valves, catheters or join implants all require coatings which simultaneously prevent infection, limit biofouling, and yet do not cause adverse inflammatory response. Combining these important properties is an essential advantage of plasma deposited POx films.

In summary, we report on the generation and properties of the first plasma deposited 2-methyl-2-oxazoline based coatings. The method of preparation is attractive for its simplicity and versatility. The coatings can be applied on any type of substrate material via the same one step protocol. The deposition process is solvent free, initiator free, does not create liquid organic waste and involves minimal precursors quantities, which makes it cost effective and environmentally friendly. Although, the chemistry of the resulting coatings is complex and not trivial to define (common for all plasma deposited films) compared to similar coatings obtained via conventional polymerisation, a range of beneficial properties such as hydrophiliicity, biocompatibility and low biofouling are well retained. Importantly, the coating process allows for retention of the oxazoline functionality which facilitates covalent coupling of molecules and nanoparticulates possessing carboxyl acid groups. This is a significant advantage which provides a vast range of opportunities for further surface functionalization to suit a particular application. Collectively, the combination of useful properties that can be achieved in the same coating as well as the solvent free and substrate independent nature of the preparation method will open new horizons in many fields and in particular those concerned with biomedical technologies.

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Notes and references

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Electronic Supplementary Information (ESI) available: Reactivity with carboxylic acids and topographic profile. See DOI: 10.1039/c000000x/

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