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Properties and Reactivity of Polyoxazoline Plasma Polymer Films

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Polyoxazolines arise as a promising new class of polymers for biomedical applications, but creating oxzoline-based coatings via conventional methods is challenging. Herein, nanoscale polyoxazoline coatings were generated via a single step plasma deposition process. The effects of plasma deposition conditions on the film stability, structure and chemical group density were investigated. Detailed examination of the physical and chemical properties of plasma deposited polyoxazoline via XPS, FTIR, contact angle and ellipsometry unravels the complex functionality of the films. Partial retention of the oxazoline ring facilitates covalent reaction with the carboxylic acid groups present on nanoparticulates and biomolecules. Surface bound proteins effectively retain their bioactivity, therefore a vast range of potential applications unlocks for plasma deposited polyoxazoline coatings in the field of biosensing, medical arrays and diagnosis.

Introduction

Polyoxazolines (POx) are an important class of polymers that have attracted substantial attention recently and are emerging as potential contender to the gold standard polyethylene glycol (PEG) in the field of biomaterials. Polymer synthesis from oxazoline monomers is typically carried out via a complex cationic ring opening polymerization method. Although early research on POx compounds demonstrated their potential for use as stabilizers, adhesives, surfactants and dispersants, the cost associated with their production hindered the development of industrial applications.[1] However, recent work revealing POx low biofouling properties [2, 3] and good biocompatibility [4] has triggered a regained interest in this class of polymer, especially in the fields of biomaterials, polymer therapeutics and implant technologies. The potential of POx for biomedical applications has been reviewed extensively elsewhere. [1, 5, 6]

Most studies focus on POx in suspension [7-9] while reports on their grafting to surfaces and organization in thin films are sparse. This is most likely due to the fact that the immobilization of POx onto surfaces is a tedious process, in which the polymer has to be initially synthesized in solution before being somehow grafted onto a compatible substrate in subsequent steps. Moreover, this multistep organic chemistry process is to date still conducted in organic solvents (typically acetonitrile) and consequently generates side waste products and impurities.[1] So far, the different techniques which have been employed to generate POx coated surfaces include spin coating [10], grafting from and to,[11] photopolymerization[12] and electrostatic interactions.[13] Since the initial work of Konradi and co-workers in 2008 on POx bottle brush brushes,[13, 14] these surfaces have been generated by a few other groups who investigated their passive non-fouling and antimicrobial properties.[11, 12, 15, 16] The general consensus to date seems to be that the nature of the monomer, and in turn the wettability of the coatings, tends to control their anti-fouling efficiency. Indeed, hydrophilic poly-2-methyl-2-oxazoline (PMeOx) suppresses protein,[12] cell [15] and bacteria [16] adsorption while more hydrophobic poly-2-ethyl-2-oxazoline (PEtOx) [10] and poly-n-propyl-2-oxazoline (PnPrPOx) [15] promotes cell adhesion and growth. Besides their anti-fouling functions, POx coated surfaces are also considered for new areas of application such as biosensing.[17] In view of all these useful properties, it does not come as a surprise that POx homo and co-polymers are currently experiencing an exponential growth in patent registration numbers, in particular for biomedical applications.[6] As of today, POx are still not approved by the FDA for medical purposes. Nevertheless, industries and research bodies all around the world are taking strategic positions in this niche research area, seemingly, in the aim to secure a determining role in the near future when POx approval by the FDA will trigger a sky rocket development of POx based biomaterials and their introduction on the market.

Interestingly, the common feature to all research on POx so far has been the opening of the oxazoline ring on which conventional polymerization methods rely. Yet, for selected biomedical applications, such as antibody immobilization for biosensing purposes, it would be beneficial to retain the oxazoline ring to act as a reactive agent in selective biomolecule binding. The reactivity of the oxazoline ring present on the w-terminus of POx have formerly been used for conjugation with protein and drugs in solution.[18] The oxazoline ring does indeed form a covalent amide-ester bond by reaction with carboxylic acid functions as per the chemical reaction sketched in Fig. 1.[19, 20]

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Electronic Supplementary Information (ESI) available: Contact angle data and XPS survey , C1s and N1s spectra for the range of PPOx plasma deposition conditions. See DOI: 10.1039/x0xx00000x

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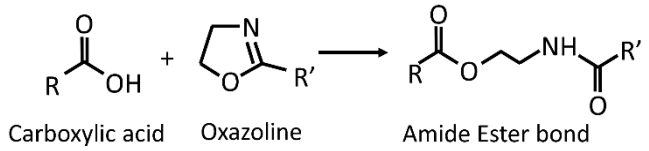

Fig. 1: Oxazoline ring reaction with carboxilic acid function leading to the formation of a covalent amide-ester bound.

In the present work we demonstrate that a facile and straightforward method developed by the authors [21] enables the creation of thin POx coatings with partial retention of the oxazoline precursor chemical functionality, in turn allowing for the irreversible binding of bioactive biomolecules. We recently reported on the first plasma polymerization of 2-methyl-oxazoline.[22] Plasma polymerization is a simple, versatile and environmentally friendly technique.[23] Indeed, unlike multi-step conventional polymerization and grafting methods, plasma deposition enabled the production of nano-thin POx coating in a hassle-free and rapid single step. Furthermore, this deposition technique is substrate independent.[24] As a result, POx coatings can be successfully deposited on any type of material without prior substrate modification. This unique advantage of plasma deposition will effectively facilitate the utilisation of POx coatings in many field of applications. Finally, plasma deposition is by essence, a solvent free technique, and as such, has the benefit to be considered a green, essentially zero-waste method for the generation of advanced organic coatings. The POx coatings produced via plasma deposition (PPOx) in our pioneer work featured useful properties comparable to POx coatings generated using conventional grafting methods. More specifically, we demonstrated that the PPOx coating were biocompatible, and significantly reduced the formation of biofilms. The present study expands these original findings and corroborate the partial retention of the oxazoline functionality, which sets PPOx aside from its counterparts generated via conventional cationic ring opening polymerization.

However, as most plasma deposited organic thin films, the chemistry and reactivity of the films is not trivial. Indeed, a complex and unorthodox film chemistry is generated by the fragmentation and recombination events occurring during plasma deposition. As pioneers in plasma polymerization of polyoxazolines, we deliver here a thorough examination of PPOx film chemistry and physical properties necessary to enable further advanced applications of these coatings. This work is not merely a summary of an extensive set of data on this new coating, but aims at providing insights on the mechanism underlying the complex chemical reactivity of PPOx, in particular, with carboxylic acids groups. We further demonstrate, by covalent immobilization of nanoparticles and proteins, the potential of these novel coatings for applications in the biomedical industry.

Experimental

Materials

2-methyl-2-oxazoline, NaOH pellets, acetic acid, NaCl, SDS 10v%, Phosphate Buffer Saline (PBS) tablets, Streptavidin, Bovine Serum Albumin, and common solvents were purchased from Sigma-Aldrich Australia and used without further purification. Goat anti-human podocalyxin antibodies were purchased from Alpha Diagnostic International. Hydrogen tetrachloroaurate (99.9985%, ProSciTech), trisodium citrate (99%, BHD Chemicals, Australia Pty. Ltd.), and 2-mercaptosuccinic acid (97%, Aldrich), were used as received. Q-Sense Gold QCM sensors were purchased from ATA scientific. Ultra high purity water obtained from a MilliQ system with resistivity greater than 18 MΩ.cm, was used for all experiments and rinsing steps.

Methods

Plasma polymerization was performed in a custom built capacitively coupled bell-chamber reactor based on previous designs.[25] Solid substrates (glass coverslips, silicon wafers and QCM crystals) were cleaned with acetone and ethanol and dried with nitrogen flow. Clean substrates were added to the plasma reactor and the chamber brought to a vacuum. A 3 minute air plasma was used to further clean and prime the samples. When the chamber reached a base pressure of $3.5x10^{-2}$ mbar the 2-methyl-2-oxazoline precursor was introduced via a needle valve until the desired precursor flow was achieved and the working pressure in the chamber steady. $2.3x10^{-1}$ mbar and $1.1x10^{-1}$ mbar working pressures were used for the deposition of “high” and “low” precursor flow rates samples, respectively. Plasma was ignited using RF powers varying from 10 to 50 W in continuous mode. The monomer deposition time was varied from 1 to 7 minutes.

Static advancing and receding contact angles of water (MilliQ, $\rho = 10^3$ kg m^{-3}, $\eta = 8.9 \cdot 10^{-4}$ Pa s) were measured on plasma polymer coated silicon wafers using a sessile drop apparatus and OCA, SCA20, dataphysics software. All measurements were conducted on a specially isolated, vibration free bench. Reproducible static contact angle data were obtained from a minimum of 5 measurements on each sample. Plasma polymer film thickness measurements were conducted with an imaging ellipsometer A J.A Woolam Co. Variable Angle Spectroscopic Ellipsometer (VASE). Data were analyzed using WVASE32 software. XPS analysis was conducted on a SAGE X-ray photoelectron spectrometer with a monochromatic Mg radiation source operating at 15 kV and 10 mA. Survey spectra acquired at a pass energy of 120 eV over the range energy range 0-1100 eV, and with a resolution of 1eV were used to determine the atomic composition of the films. The chemical state of nitrogen and carbon atoms were investigated from high resolution spectra recorded at 20 eV pass energy. The charge compensation effects were corrected by calibrating all spectra to the neutral carbon peak at 285 eV. CasaXPS was used for spectra analysis. Time of Flight Secondary Ion Mass spectrometry (ToF SIMS) measurement were conducted on a PHI TRIFT V nano ToF instrument (Physical Electronics, Chanhassen, MN, USA). +SIMS spectra were acquired at 3 different locations on each sample, with a pulsed liquid metal $^{79+}$Au primary ion gun operating at 30 kV under a vacuum of

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5.10^{-6} Pa. Fourier Transform Infra-Red spectroscopy (FTIR) was conducted with a Nicolet spectrometer. For FTIR analysis, 50 mg of potassium bromine (KBr) powder was coated in the same plasma reactor and using the same experimental conditions as the other solid substrates. The coated KBr powder was analyzed using a smart diffuse reflection accessory. All spectra were collected at room temperature over the spectral range 650-4000 cm^{-1} with a resolution of 4 cm^{-1} and 512 scans. Omnic software was used for spectra analysis.

AuNPs were synthesized following established methods previously described.[26] Briefly, nanoparticles 16 nm in diameter were produced by citrate reduction of $HAuCl_4$. The carboxylic acid functionality was provided via surface modification with 2-mercaprosuccinic acid. After rinsing with MilliQ water, plasma coated glass coverslips were incubated with AuNP suspension for 6 hours in 24 well plates. The AuNP suspension was then aspirated and the coverslips rinsed 3 times with MilliQ water. In order to challenge the bonds between the plasma polymer coating and the functionalized AuNP, SDS 1 v% or 5 M NaCl solution were then added and left for 1 hour. Finally, the substrates were thoroughly rinsed with large amounts of MilliQ water and dried with nitrogen flow prior to chemical analysis.

Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) is a surface sensitive technique used to evaluate changes in thin films mass and viscoelastic properties. It was used to assess, in real time, the plasma polymer thin films interaction with proteins in a stimulated physiological fluid environment under flow conditions. QCM-D measurements were performed with a Q-Sense E4 instrument (Q-Sense, Sweden) offering sensitivity in the $ng.cm^{-2}$ range. POx plasma deposited films were deposited onto gold QCM sensors and tested in parallel with 2 control sensors coated with allylamine plasma deposited (pAA) films. The fluid flow over the sensors was set to 0.1 $mL.min^{-1}$ using a variable-speed peristaltic pump (ISM 935, IDEX Health & Science, Germany) and kept constant for the whole experiment. All 4 sensors were initially equilibrated in MilliQ water, and subsequently in PBS, until both dissipation and frequency trace stabilized. The sensors were then exposed to 0.1 $mg.mL^{-1}$ protein solution (BSA, fibronectin, SAV or podocalyxin) for 90 minutes, before rinsing with PBS for 15 minutes, washing with SDS 1% for 15 minutes, and further rinsing and re-equilibrating in PBS for a minimum of 30 minutes or until the trace stabilised.

Results and discussion

Films thickness and stability.

The thickness and stability of the PPOx films were dependent on the plasma RF power, monomer deposition time and precursor deposition pressure. Ellipsometry measurements of film thicknesses measured on silicon wafers, are summarized in Fig. 2. Using different deposition conditions the PPOx films can be varied in a controlled fashion. When the monomer pressure was $2.3x10^{-1}$ mbar, film thicknesses ranging from 20 to 76 nm were formed. Film thickness increased with deposition time and RF power (Fig. 2a.). One must stress, however, that the films prepared using lower RF power, bear non-negligible thickness losses after extended exposure to water. For example, a 90% thickness loss was recorded for the 10 W 5min films. Using lower monomer pressure, with longer deposition times enables the formation of films ranging from 30 to 60 nm thick, with increased stability in not only water but also salt (5 M NaCl, artificial urine), acid (CH_3COOH, pH 4) and base (NaOH, pH 10) solutions as shown in Fig. 2b. In particular, films generated using higher RF powers (30 W and above) exhibit very good stability to water, with negligible film thickness loss, even after 24h of solvent exposure (dark blue bars). PPOx films formed at 20 W RF power did bare a 20% thickness loss within the first hour of exposure to water but no significant further loss was observed after 24h soaking.

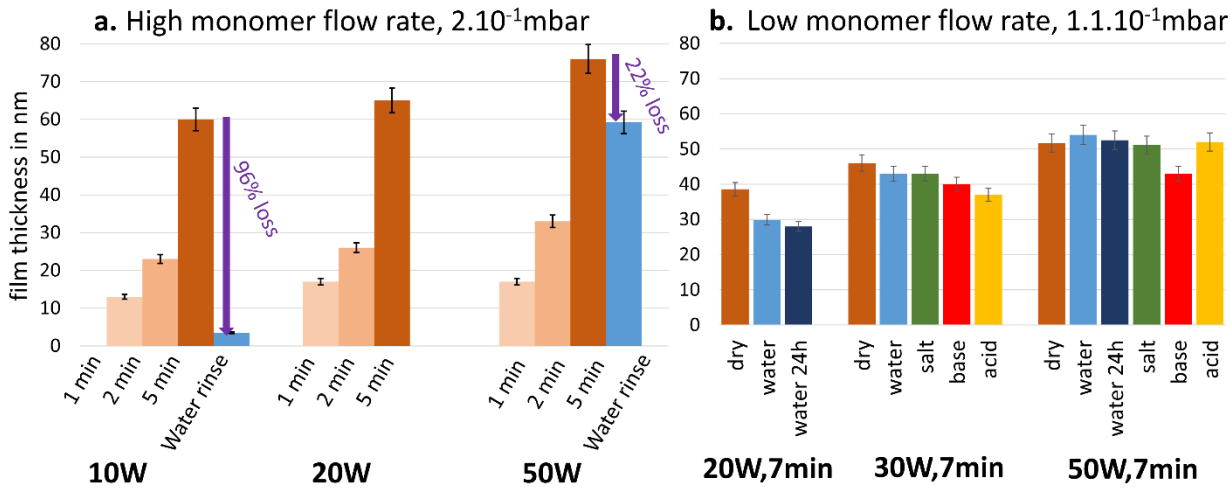

Fig. 2: PPOx films film thicknesses as measured by ellipsometry for a range of substrate, exposed or not to water, acid, base and salt solution. a. high precursor deposition pressure, variable deposition times b. low precursor deposition pressures, 7 min deposition.

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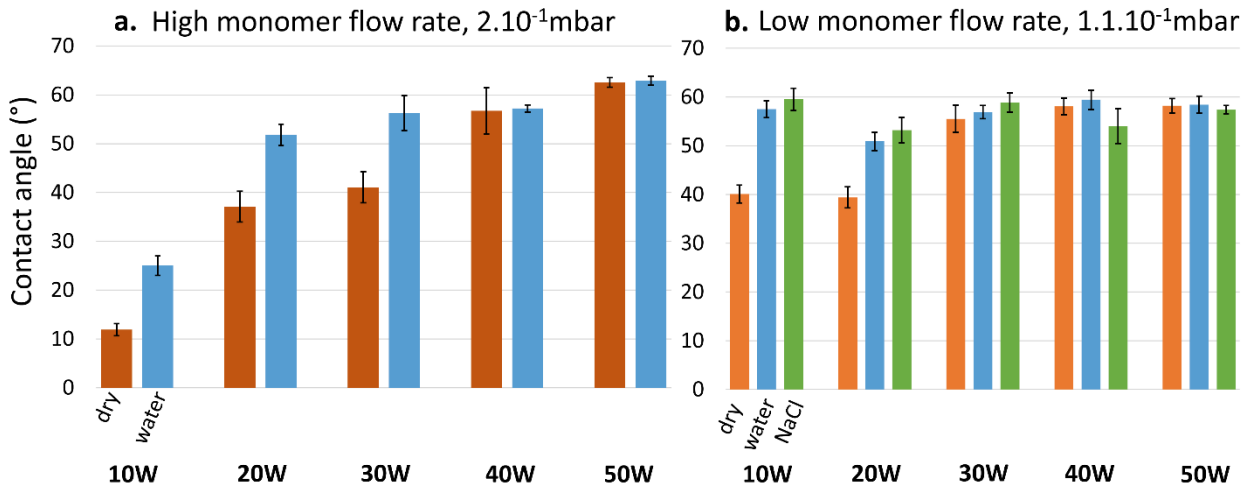

Fig. 3: Water contact angles on PPOx thin films at 10, 20, 30, 40 and 50W. a. Water contact angle on film deposited at high precursor deposition pressure, dry and after 1h exposure to water. b. Water contact angle on film deposited at low precursor deposition pressure, dry and after 1h exposure to water or 1M NaCl solution.

In this precise case (20W, low monomer flow), since the loss occurs very rapidly after exposure to water, it may be due to the desorption of loose monomer fragments which were only physically deposited on the substrate at the end of the plasma polymerization process from species remaining in the chamber after the RF power had been turned off. The typical difference in stability observed between films deposited at low and high monomer flow rate for identical plasma discharge power (e.g. films deposited at 50W in Fig. 2a. and b.) is directly correlated to changes in the monomer flow/plasma power (MF/P) ratio. Indeed, the lower the MF/P ratio, the more crosslinked is the plasma polymer film. This phenomena is common to most plasma deposited coatings and has been described in details elsewhere. [27, 28] Briefly, for a given plasma discharge power, there is more energy available per molecule of precursor for its fragmentation and recombination, when the latter is in default rather than in excess. Effective fragmentation results in the formation of a variety of reactive species which can then trigger crosslinking reactions. This allows the formation of strong stable films.

The stability of PPOx films was further confirmed via static water contact angle measurements. Contact angle measurements were conducted on dry PPOx films, films rinsed with water, and films incubated in MilliQ water, acid and basic solutions for 1h. Both glass coverslips and silicon wafers substrates were tested but no differences in the measured contact angle were observed. As expected, the PPOx film wettability was independent of the nature of the substrate. All films studied are hydrophilic with advancing contact angle not exceeding 62°. The water contact angle measured on plasma deposited POx films appeared to be an increasing function of the RF powers as shown in Fig. 3. For low power films, typically below 30 W, a significant increase in water contact angle occurs immediately after substrate exposure to water, as shown for both high and low precursor deposition pressure in Fig. 3a. and b. respectively. This initial change is not accentuated further by prolonged exposure to water (data not shown) or incubation in salt solution (Fig. 3b.). The water contact angle of films deposited at higher power was not affected by incubation in salt solution, acid or base (supporting information Fig. S1) thus supporting the stability observations. The difference in intrinsic wettability for films formed using different plasma RF powers (see Fig. 3a. for example) hint that the chemistry of the films may differ depending on the coating deposition conditions. This is not unexpected for plasma polymer thin films. Indeed the structural fragmentation and recombination of the precursor molecule occurring during plasma polymerization inevitably leads to a complex and rather unorthodox film chemistry which is known to be strongly dependent on the plasma power and precursor flow rate used.[29, 30] Typically, reactive functional groups tend to be lost by high power plasma polymerization while gentler conditions are more likely to enable the retention of the precursor chemical functionality. In the case of 2-methyl-2-oxazoline, it is of particular interest to assess whether or not the oxazoline ring is retained throughout the plasma polymerization process. Although only very few experimental data on the wettability of polyoxazoline films have been released, the work of Zhang et al on poly(2-oxazoline) bottle brush brushes (BBB) provides interesting insight.[15] Poly-2-oxazoline BBB, featuring an open ring configuration (PMeOx) have rather low water contact angle of 38°, while PiPOx (Poly-2-isopropenyl-2-oxazoline) brushes were reported to have a contact angle of 51°, which is comparable to the contact angle value obtained for the stable plasma deposited POx films, at 40 and 50 W.

Overall the most stable films are formed using the highest plasma ignition powers and the lowest precursor deposition pressure.

Film chemistry.

We now turn our attention to the effect of the deposition conditions on the film chemistry. The atomic compositions of PPOx films deposited onto glass coverslips and silicon wafers

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were determined by XPS. Once again, the nature of the underlying substrate did not influence the results: no difference in atomic composition were observed for PPOx deposited on different substrates. This is in good agreement with observation made in the wettability study, and confirms that, in the experimental conditions investigated, the plasma deposition of oxazoline, is a substrate independent method. This finding is not unexpected. Indeed, it is known that for film thicknesses greater than 5 nm, the chemistry of plasma polymer coatings is substrate independent.[24, 25, 31]

Survey spectra analysis A typical survey spectra is shown in Fig. 4a. and reveals the presence of carbon, nitrogen and oxygen species, as expected from the oxazoline precursor structure. The detailed atomic composition for the full range of PPOx film is provided in supplementary information (Fig. S2).

Although the proportions in C, N and O are comparable over the range of experimental conditions investigated, several consistent trends are noticeable. 1) The nitrogen to oxygen ratio is always slightly higher than that of the monomer (1/1). This indicates that the plasma treatment does not cause significant oxidation of films but rather yields volatile oxygenated species which are not retained in the final film. 2) A systematic decrease in nitrogen content is observed as the plasma power increases, as shown in Fig. 4b. for both precursor deposition pressures investigated. Namely, the nitrogen content decreases from 18% at 10 W to 15% at 50 W at low precursor deposition pressures, and from 21% to 18% at high deposition pressures. This type of trend has been reported before for other nitrogen rich precursor such as allylamine. Allegedly, higher amounts of nitrogen-rich functionality are typically retained when low plasma deposition powers are used because less fragmentation of the monomer occurs in these conditions.[29] 3) Finally, at comparable power, the nitrogen content is globally higher in the film deposited at higher precursor deposition pressures (Fig. 4b.). Indeed, as mentioned earlier, increasing the discharge MF/P ratio leads to a decrease in fragmentation, and in turn to increased functionality retention. As a result however, the films generated at high precursor deposition pressure suffer from a lesser crosslinking density. The rather low density of crosslinking within these films was, for example, responsible for the instability observed in both ellipsometry and wettability analysis. Further XPS investigation on PPOx film stability revealed that a systematic decrease in nitrogen content occurs after film exposure to water, as illustrated in Fig. 4c. for the samples deposited at high precursor deposition pressure. The dissolution of the films deposited at low deposition power can be attributed to phenomenon observed with other plasma deposited coatings. At conditions where the MF/P ratio is high the crosslinking between the binding block of the coatings is low. Upon immersion in solvents, low molecular weight soluble fractions of the film are easily eluted contributing to film mass loss. [32] Unlike conventional polymerisation approaches, the exact nature of the crosslinking reactions at play in the plasma deposition of oxazolines is not *a priori* known. Indeed, the plasma deposition process inherently leads to random cross linking reactions occurring between the original oxazoline precursor and its fragments generated within the plasma. The latter consist of a variety of ionised species and radicals.The following detailed analysis of the PPOx film chemistry provides insights on the nature of the radical and ionised species that may have formed and recombined during the plasma deposition process.

C1S spectra analysis Overall analysis of the XPS survey spectra indicate a rather complex film chemistry on which further insight was obtained via analysis of the corresponding high resolution C1s spectra. The presence of both oxygen and nitrogen in the oxazoline precursor requires the consideration of a range of possible carbon atomic environment such as C-OR, C=O, C-N and C=N. More precisely, the PPOx formed via plasma deposition are likely to contain a mixture of chemical structure such as those found in polymethyloxazoline produced by conventional methods, as well as intact oxazoline rings, and also more complex plasma generated nitrogen containing functions. A conventional polymethyloxazoline consist of repeated units of amides. In this configuration one would expect the C1s carbon environment to consist of C-C, C-N and N-C=O. On the other hand, oxazoline rings contain C-C, C-O, C-N, and N=C-O. Additionally, oxidation processes occurring during the plasma deposition could result in the formation of COOR functionality. Yet the signals corresponding to these components overlap because the exact binding energy of an electron is an intricate function of both the atom oxidation state and its chemical environment. As a consequence attempts at resolving these components individually would introduce great uncertainty in the curve fitting results.

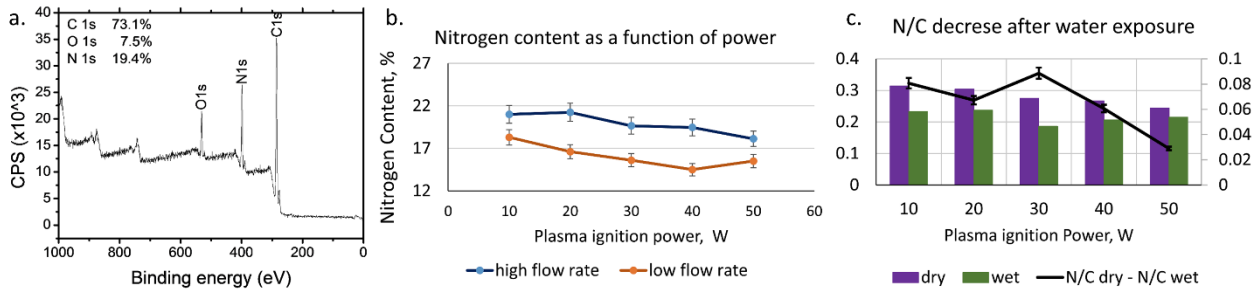

Fig 4: a. Typical PPOx XPS spectra confirming the presence of oxygen, nitrogen and carbon element, deposition condition: 40W, 2.3x10-1 mbar. b. Comparison of the evolution of the PPOx nitrogen content as a function of plasma ignition power, for two different precursor deposition pressures. c. Changes in the nitrogen to carbon ratio in PPOx films before and after exposure to water.

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Thus, in the following analysis the C1s spectra were fitted with four distinct components consisting of C-C (285 eV), COOR (289.2 eV with beta-shift peak at 285.7 eV), (C-N, C-O) and (N-C=O, N=C-O). The binding energies of these last two components were not arbitrarily set as small differences are expected between oxazoline ring and amide structure. For the single bond heterojunction, the binding energies for C-N is slightly less than for C-O (0.3eV difference). For the double bond heterojunctions, amide structures are typically found around 288.2 eV, while "oxazoline structures" have historically been reported at 287.8 eV. The authors would like to stress here, however, that although several groups have cited the work of Beamson and Briggs [33, 34] to attribute the binding energy of 287.78 eV to oxazoline structure, it is rather unclear in this original work whether the reported binding energy corresponds to a closed ring configuration (as in the illustration) or open polymerized amide bond (as in the text).

The C1s spectra of the range of samples investigated and corresponding binding energies are provided in the supporting information (Fig. S3) and confirm a complex carbon environment, consisting of C-H, C-O/C-N, C=O/C=N and COOR.

The C1s spectra analysis confirmed that low precursor deposition pressure, high plasma ignition power and exposure to water are contributing factors to functionality (C-N/C-O and C=N/C=O) loss. More interestingly, the C-O/C-N peaks are slightly, but systematically shifted toward higher binding energy for the higher deposition pressure, compared to the samples obtained at low deposition pressure at identical power. Since the binding energy of C-O is slightly higher than that of C-N bond, this shift indicates that a greater proportion of C-O bound; present in the oxazoline ring; appear to be retained in high precursor deposition pressure condition. (Fig. 5a.). Similarly, a systematic shift towards higher binding energies occurs for the NCO component at high precursor deposition pressure. These results provide a good indication of the film chemical configuration, but due to the binding energy overlap mentioned earlier, it cannot be relied on to impartially quantify the contribution of the different chemical function.

N1s spectra analysis. In order to further interrogate the complex atomic environment of PPOx films, N1s high resolution spectra were also analyzed. After careful charge correction of the spectra, N1s peaks were fitted with three plausible components, namely amine at 399.1±0.1 eV, amide at 399.9±0.2 eV and imine which is expected to be found slightly below the amine because of an increase electron density due to conjugation. [29] Since the exact binding energy of imine bonds is still debated, even among experts in the fields, its value was left as a free parameter in the fitting routine. In Fig. 5b. we compare the N1s spectra of pAA films with the one of two typical PPOx films. There is no imine component to the N1s spectrum of allylamine, while imine is clearly present in both PPOx spectra. From the detailed analysis of the high resolution N1s spectra of all the PPOx substrates analyzed (provided in supporting information with corresponding binding energy and atomic percentage, Fig. S4) the following conclusions can be drawn: the N1s atomic environment consist of a majority of amide bonds (52-98%), with also imine (2-17%) and a fraction of amine species. The imine bond is found at 398.3±0.3 eV in good agreement with the literature.[35, 36] The differences observed between the N1s peak fitting for the various deposition conditions investigated, do not appear to follow any particular trend with power or precursor pressure increase. The large overlap between the different components is most likely masking the changes occurring in the nitrogen atom environment.

ToF SIMS The molecular structure of the outer 1-2 nm of the PPOx film was further probed via ToF SIMS. In all the positive ion spectra, signals ascribed to CxHyNO fragments were detected (Fig. 6a.). In particular, the C3H4NO fragment corresponding to the oxazoline ring is present in all coatings.

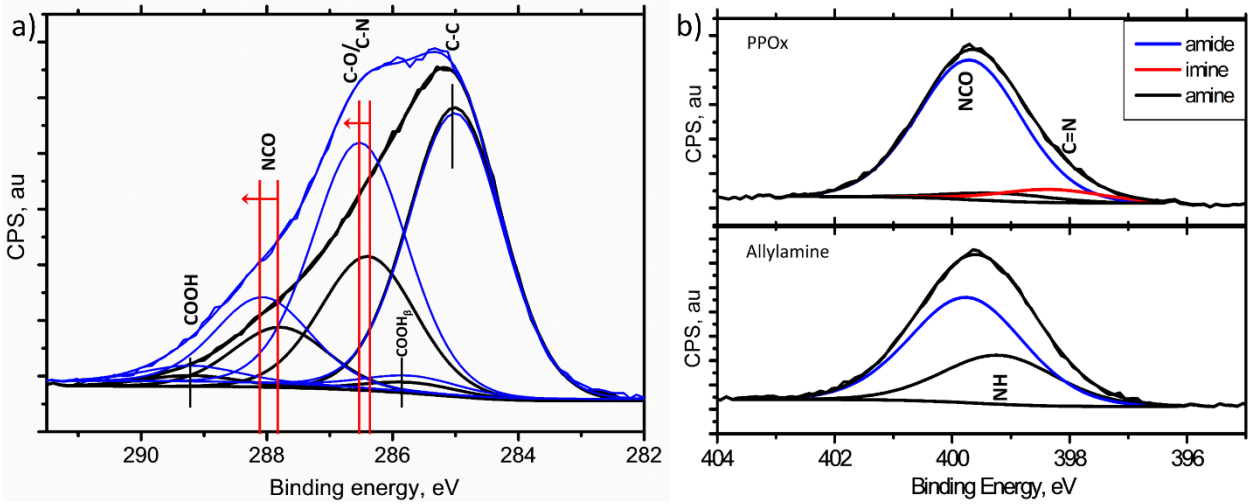

Fig. 5: a. C1s spectra of PPOx film deposited at 50Wlow precursor deposition pressure (black), and 20W high precursor deposition pressure (blue).b. the Top panel shows a typical N1s spectra of PPOx (50W high precursor deposition pressure) where imine functions are present, the bottom panel correspond to a plasma deposited allylamine film, where there is no imine.

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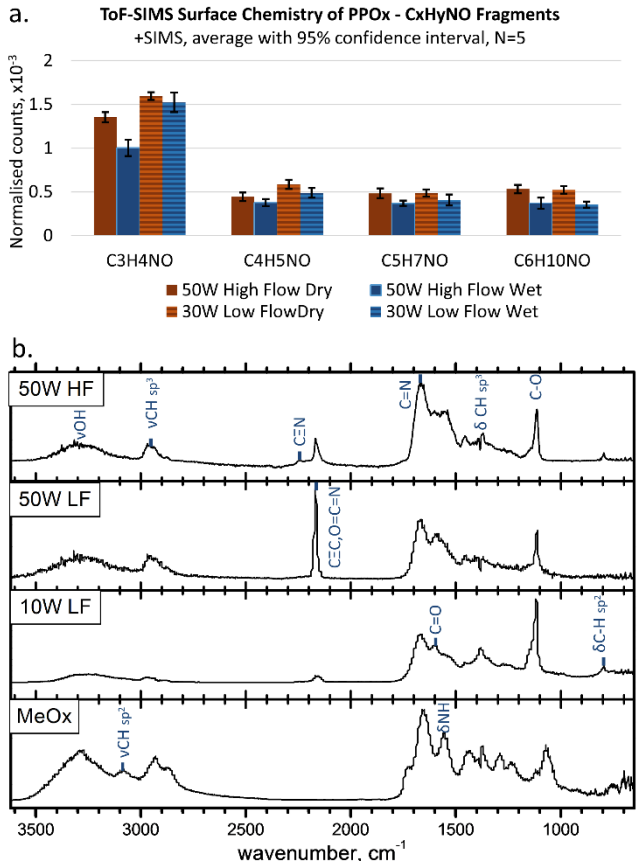

Fig. 6: a. +SIMS average response for CxHyNO fragments normalised to the total spectral count for PPOx 50W high flow and PPOx 30W low flow, before and after exposure to water. b.FTIR spectra of MeOx, bottom, and representative PPOx thin films, deposited at from top to bottom at 50W high precursor pressure, 50W low precursor pressure, and 10W low precursor pressure.

FTIR analysis The IR spectra of PPOx films deposited in different experimental conditions on KBr powder are shown in Fig. 6., together with the spectra of air dried 2-methyl-2-oxazoline monomer Several FTIR adsorption bands were common to both PPOx films and MeOx. The broad –OH stretching bands between 3200 and 3400 cm^{-1} are due to substrate hydration. The medium peaks present at 2950 and 1370 cm^{-1}, are attributed to C-H sp^3 stretching and bending modes respectively. Finally, the bands observed around 1100 and 1650 cm^{-1} are associated with the stretching of C-O and C=N bonds, which are both present in the oxazoline ring structure. The intensity of the C-O band in the PPOx films varies significantly with the plasma power and is stronger at lower power and higher precursor deposition pressure. Several FTIR adsorption bands were common to both PPOx films and MeOx. The broad –OH stretching bands between 3200 and 3400 cm^{-1} are due to substrate hydration. The medium peaks present at 2950 and 1370 cm^{-1}, are attributed to C-H sp^3 stretching and bending modes respectively. Finally, the bands observed around 1100 and 1650 cm^{-1} are associated with the stretching of C-O and C=N bonds, which are both present in the oxazoline ring structure. The intensity of the C-O band in the PPOx films varies significantly with the plasma power and is stronger at lower power and higher precursor deposition pressure. This is in good agreement with the XPS results presented in the previous section which indicated that both low power and high precursor pressure favored functionality retention. The presence of these signals in the spectra of the PPOx films appears to verify the retention of the oxazoline ring functionality. Indeed, according to the literature, the C-O band is absent from the FTIR spectra of polymethyloxazoline prepared by conventional ring opening polymerization, while a resolved C=O peak is observed at 1627 cm^{-1}.[15] However, both C=N and C-O peaks, are found at slightly higher wavenumber for the PPOx films compared to that for MeOx, indicating a difference in the molecular and/or electronic environment of these bonds. For example, a C-O band is generally present at higher wavenumber in conjugated systems such as acyl and phenyl function (1100-1350 cm^{-1}) as compared to the C-O present in pure alkoxy functions (1050-1150 cm^{-1}). In the PPOx films, the C-O signal is at 1130 cm^{-1}, as opposed to 1090 cm^{-1} in the air dried MeOx. This could be due to the presence of unsaturation in the vicinity of the C-O retained via plasma polymerization. Furthermore, rather complex signals are visible in the range 1500-1700 cm^{-1}. For MeOx, two resolved peaks are found at 1550 and 1650 cm^{-1} corresponding to NH bend and C=N stretching respectively. The PPOx films, on the other hand, present a strong and rather wide band with a maximum at 1657 cm^{-1} which is attributed to C=N present in the oxazoline ring.[37] However, the asymmetric shape of this peak hint that an overlap occurs with the C=O stretching band expected to be at 1627 cm^{-1}, for the amide function of PMeOx.[15] In some cases, the C=O band is more resolved but the intensity ratio between these two signals quickly decrease as the power increases, confirming that the chemistry of the PPOx films is dependent on the RF powers. The N-H bend signal is only visible as a broad shoulder of the C=O peak.

It is also noteworthy that a small but sharp band is present at 800 cm^{-1} for PPOx films deposited in conditions that favor functionality retention (low power/high precursor pressure). Alkene and aromatic sp2 bending modes are typically found in this wavelength range and can be attributed to the ring skeletal vibrations. The footprint signals found in this wavelength range for the air dried MeOx samples are intricate, indicating difference in the C-H sp2 substitution state. However, C-H sp2 stretching modes are present at 3080 cm^{-1}.

Finally, an intense band is observed for all PPOx samples at 2170 cm^{-1} with a shoulder at 2250 cm^{-1}. These signals are characteristic of alkyne (C≡C) and/or isocyanate (O=C=N) and nitrile C≡N functions respectively. These reactive functions are the direct results of the plasma deposition process, and their formation during the plasma deposition of precursor containing nitrogen rich ring structures have been documented in the past.[38] These chemical group do not form via conventional oxazoline grafting methods: they are exclusive to PPOx films.

Overall, the FTIR and XPS chemical analysis of the PPOx films reveal a very complex film chemistry consisting of a variety of reactive chemical functions whose presence/absence and

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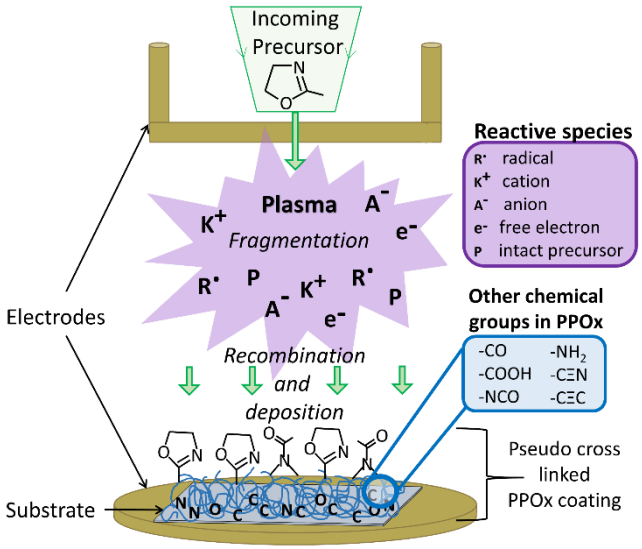
concentration varies with the plasma deposition conditions used. In particular, in view of the present results, the retention of the oxazoline ring cannot be ruled out. Scheme 1 summarises the insights gained from the physico-chemical analysis of the PPox films on the mechanism driving the plasma deposition of MeOx.

Scheme 1: Summary of the formation mechanism and chemistry of plasma deposited 2-methyloxazoline coatings



The fragmentation of the precursor and recombination processes occurring within the plasma result in the deposition of a partially cross-linked PPox films in which both closed and open oxazoline functionality are present, together with other chemical functions such as amines, nitrile, alkyne, isocyanate and carboxyl.

The presence of oxazoline rings and nitrile reactive groups is a distinctive feature which sets PPOx coating appart from conventionaly grafted PMeOx. One aspect of the potential of this unique feature is explored in the following section.

Film Reactivity

Reaction with COOH. In a preliminary study,[22] insights on the reactivity of PPOx plasma polymer films with carboxylic acid chemical groups were obtained by incubating PPOx coated glass coverslips with dilute polyacrylic acid solution. Results proved that PPOx surfaces did adsorbed polyacrylic acid (representing up to 13% of the carbon content from C1s high resolution spectra analysis) and did not bear any significant loss after washing with SDS solution, thus indicating an irreversible binding mechanism.

Here, the reactivity of PPOx films with carboxylic acid groups, as per the reaction shown in Fig. 1, is further investigated by incubating PPOx coated glass coverslips with COOH-functionalized gold nanoparticles in 24 well plates. The 16 nm AuNP used in this experiment absorb light at about 520 nm due to the characteristic plasmon resonance phenomenon. As a result, these gold nanoparticles appear pinkish in colour. Thus in Fig. 7a., a pinkish color of the samples substrate is indicative of gold nanoparticles adsorption on the otherwise transparent glass coverslips. The nature of the bond between the COOH functionalized gold nanoparticles and the PPOx coating was challenged by several rinsing and washing steps with not only water and surfactant (SDS 1v%), but also with concentrated salt (1 M, 3 M, and 5 M NaCl) and base solutions. The visual proof of AuNP binding on PPOx thin films deposited at 10, 30, and 50 W, for 5 minutes at high monomer pressure, is shown in Fig. 7a.

From the intensity of the pink coloration it appears that the AuNP did not adsorb on the 10 W substrates. This is not surprising in view of the poor stability of the films already evidenced via contact angle, ellipsometry and XPS analysis. On the other hand, both 30 W and 50 W adsorbed gold nanoparticles as well as the control pAA coated substrate. Equivalent experiments were conducted with PPOx films deposited for 7 minutes at high precursor pressure and returned similar results: in all cases, samples deposited at high power (typically above 30 W and above) successfully bind gold nanoparticles. Also, for all PPOx substrates investigated, it is visually not possible to observe any clear changes in coloration between the various samples before and after the different washing steps. In contrast, AuNP release in solution was observed when washing the pAA coated samples with SDS and concentrated NaCl salt solutions.

In order to quantitatively determine gold nanoparticle binding and desorption, the surface were analyzed via XPS. The poor stability of the 10 W films was once again confirmed, with nitrogen atomic percentage decreasing from 19 to 1% after 6 hours incubation in the AuNP solution. For this reason, in Fig. 7b., we report only the atomic percentage of gold for control pAA coated substrate, and PPOx 30 W and 50 W deposited this time at low monomer pressure for 7 minutes. The practical observation of gold nanoparticles being removed from pAA surfaces by NaCl and SDS washes is clearly confirmed by the XPS measurements, where, for example, a 50% loss of adsorbed gold is recorded after SDS wash and 25% after salt wash. This result indicates that some of the gold nanoparticles binding on the pAA samples are doing so via reversible electrostatic interactions which are readily disrupted by exposure to surfactants and concentrated salt solutions. Due to the size of the COOH-functionalized nanoparticles, it is possible that multiple concurrent electrostatic binding sites prevent the remaining gold particles to desorb from the substrate, at once.

On the contrary, the gold nanoparticles binding to PPOx surfaces do not seem to be affected by the ionic strength or pH of the washing solution, thus confirming the irreversible binding of PPOx with carboxylic acid functions. Interestingly, a slight decrease in the number of gold nanoparticles is observed after SDS rinse for 50 W samples, while in this condition the nanoparticle binding is unaltered on the 30W films which suggest a stronger, possibly fully covalent binding. In the case of the 50 W films, it could be that two different adsorption mechanisms are at play, namely electrostatic adsorption and covalent irreversible binding. On the other hand, we have shown that 30 W sample did retain more of the original oxazoline structure, and it could be that most or all binding to carboxylic acid function is in this case covalent.

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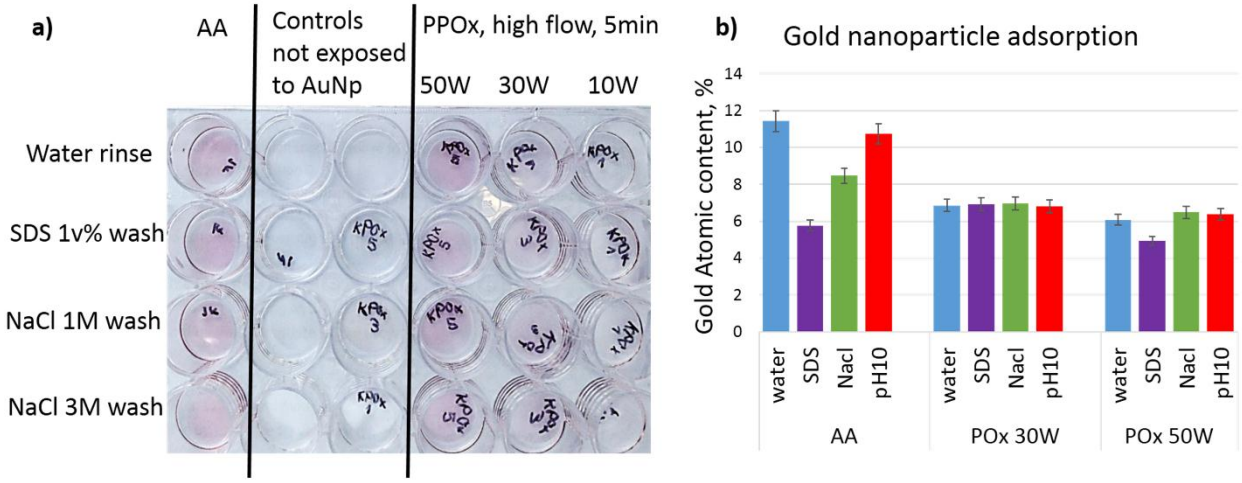

Fig. 7: a. Qualitative visual evidence of gold nanoparticles binding on PPOx substrate and control pAA substrate, subjected to different washing steps. b. Gold atomic concentration in% for pAA, PPOx 30W and 50W deposited with low precursor pressure samples after rinsing with water, SDS 1v%, NaCl 5M, or NaOH pH10 solutions.

Although further investigations are required to fully confirm the nature of the bond formed, these initial set of results do indicate that the reaction of PPOx films with COOH-groups result in irreversible nanoparticle binding.

Capacity of plasma deposited Pox coatings to covalently bind proteins Typically, biomaterials used for protein and antibody binding rely on the formation of covalent bonds between the amine groups present on the biomolecules and carboxylic acid or aldehyde groups grafted to the biosensing platform.[39] Biomaterial conjugation with biomolecules involves intermediates and several surface reaction steps, including carboxylic acid group activation. The unique chemistry of the PPOx films, namely reactive oxazoline ring retention, offers significant advantages for simple, convenient and single step biomolecular binding, without catalysts being needed.

In order to evaluate the protein binding efficiency of plasma deposited PPOx films, real time QCM-D experiments were conducted under flow conditions in physiological buffer. In a previous study, we have shown that exposure to 0.1 mg.mL^{-1} albumin solution resulted in the irreversible immobilization of 70 $\pm$ 7 ng.cm^{-2} of protein onto PPOx substrate. [22] The fact that the adsorbed protein did not wash away following several rinsing and washing steps with PBS and SDS suggested that the proteins bound to PPOx in a covalent manner. Here we tested the ability of PPOx film to bind other biomolecules relevant to biomedical application, including common a blocking media (skim milk), proteins (streptavidin and fibronectin) and antibodies (anti-podocalyxin). All experiments were conducted with PPOx film deposited directly onto gold QCM crystals. The thickness of the PPOx films deposited on this substrates was measured via ellipsometry and comparable to films deposited on silicon wafers in the same experimental conditions. In all cases, an initial sharp drop in frequency occurs shortly after exposure to the biomolecule solution, and is followed within minutes by a much slower negative change. Subsequent rinsing and washing steps with PBS and SDS solution did not result in significant change in the frequency trace, thus indicating that the bound species were not washed off the sensor surface (Fig. 8a. and b.)

The relationship between the frequency change and the quartz electrode mass is given by the Sauerbrey equation. For the Q sense e4 QCM-D apparatus, the relationship is: [40]

$$\Delta m = -C\Delta f/n$$

where Δf is the measured frequency shift (Hz), C=17.7 ng.Hz^{-1}.cm^{-2} for a 5 MHz quartz crystal and n, is the overtone number. In order to gain some insights on the rate of reaction between PPOx films and biomolecules, a 0.1 w% skim milk solution was left to bind on PPOx overnight at room temperature. The net frequency deflection remaining after washing correspond to an irreversible adsorption of 90$\pm$10 ng.cm^{-2} of skim milk protein (Fig. 8a.). However, this net mass gain correlates to what had been adsorbed within the first hour of exposure to the protein solution. This result indicates that the covalent reaction occurs rather quickly at room temperature. In the field of biosensing and bioassays, the cost of the antibodies limits considerably the amount of biomolecule that can be used to functionalize biomaterial substrates. With these types of applications in mind, irreversible biomolecule binding to PPOx substrate from very dilute solutions was tested and achieved (Fig. 8b.). Within 2 hours, 19±4 ng.cm^{-2} of anti-podocalyxin antibody were irreversibly immobilized on PPOx substrate from a 10 µg.mL^{-1} solution. Assuming a molecular weight of 100 KDa, this corresponds to an antibody surface density of 10^{11} cm^{-2}, which is comparable to the typical antibody density used in ELISA (5x10^{11} cm^{-2}). It is also worth noting that, in this experiment, antibody immobilization was achieved on the most stable, but "less functional" PPOx substrate investigated (50 W, low precursor pressure). This shows that enough functionality was retained throughout the plasma polymerization to allow the irreversible binding of satisfactory amount of antibody to the substrate. Recent work conducted by the authors, which will be presented elsewhere, has shown that the antibody coverage was sufficient to achieve selective cell capture from samples of mixed populations.

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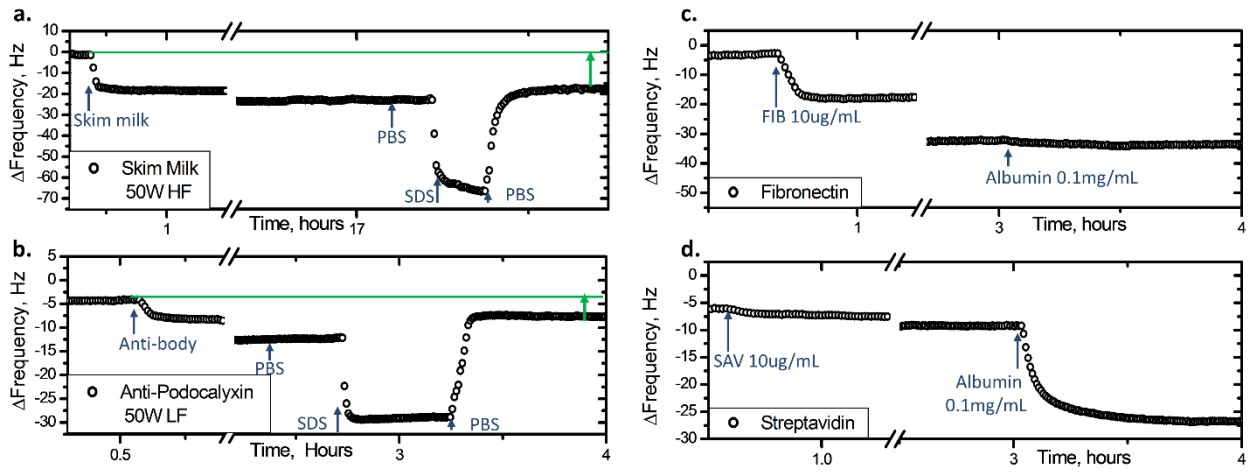

Fig. 8: Real-time QCM-D measurements of the ΔF response of a PPOx thin film to exposure to biomolecule solutions, followed by rinsing and washing step. a. skim milk solution 0.1mg.mL-1. b. Anti-human podocalyxin antibody, 10ug.mL-1 c. Fibronectin 10ug.mL-1 d. Streptavidin 10ug.mL-1.

Finally, fibronectin was used as a model protein in order to compare the protein binding potential of PPOx with POx films made via conventional methods The average amount of Fibronectin binding to PPOx is 80± 7 ng.cm^{-2}.(Fig. 8c.) This value is significantly larger than what has been reported in the literature for poly-2-methyl-2-oxazoline coated surface (<6 ng.cm^{-2}),[13, 15] thus indicating that PPOx protein binding capacity exceed that of polymethyloxazoline films produced via conventional cationic ring opening polymerization. Fibronectin molecules have a molecular weight of 440kDa and an average hydrodynamic radius of 11 nm [41-43] corresponding to an occupancy of 380 nm^2. In these conditions, the maximum theoretical packing density is 192 ng.cm^{-2}.[39] The amount of protein bound to the PPOx film roughly corresponds to a 42% coverage. Further exposure to a more concentrated solution of albumin did not induce any further mass gain, thus indicating that the substrate had reached saturation.

Bioactivity retention

In order for PPOx substrates to be used as a biosensing platform, it is essential for the biomolecules to maintain their biological activity after the immobilization process. Streptavidin was successfully immobilized on PPOx modified substrate (30± 7 ng.cm^{-2}) from a low concentration solution (Fig. 8d.) and biotinylated gold nanoparticles were used to probe its bioactivity. PPOx blocked with albumin were used as control and the presence of gold on the SAV functionalized surface was investigated via XPS. The XPS survey spectra shown in Fig. 9 demonstrate that gold is present only on the SAV functionalized surfaces. Although the amount of gold detected via XPS is small, it is in good agreement with previous reports.[44] This suggests that after immobilization to PPOx coatings, the streptavidin molecules are in their functional, bioactive conformation.

Conclusions

Stable plasma deposited oxazoline films were created with controllable thicknesses adjustable by the deposition time, precursor pressure and RF power used for plasma deposition. The films are overall hydrophilic. The chemistry of the coatings is rich and complex, as evidenced by XPS and FTIR analysis. From the present results it appears that various different functional and reactive chemical functions are formed during plasma polymerization including amide, isocyanate, and carbonyl, and that the reactive oxazoline rings are retained to some extent.

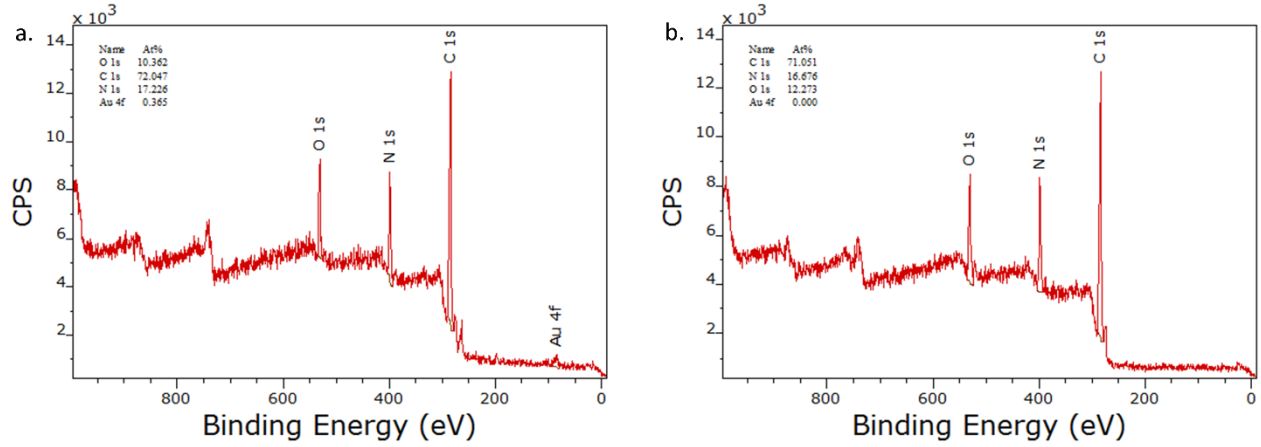

Fig. 9: XPS survey spectra of PPOx functionalized with streptavidin (a.) and albumin (b.) after incubation with biotinylated gold nanoparticles

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The reactivity of the PPOx films with carboxylic acid function was demonstrated using COOH terminated gold nanoparticles and biomolecules. Real time QCM-D analysis revealed that irreversible protein binding occurs on the PPOx films, and that the surface coverage obtained are suitable for application in biosensing. Most importantly the unique film chemistry and reactivity of PPOx has been achieved using plasma deposition: a technique with numerous inherent advantages.

Oxazoline based coatings deposited by plasma polymerization may become an attractive alternative to conventional methods. The reason for this is the ease of coating preparation and the possibility to deposit on any type of substrate material. Furthermore, plasma deposited coatings seem to allow the retention of intact oxazoline rings at the surface, which is typically not the case when other techniques for surface preparation are used. Retention of such reactive chemical functionalities is of interest for a number of applications in the biomedical space since it allows convenient and rapid covalent coupling of proteins, antibodies and other ligands or nanoparticles that carry carboxylic acid groups.

Acknowledgements

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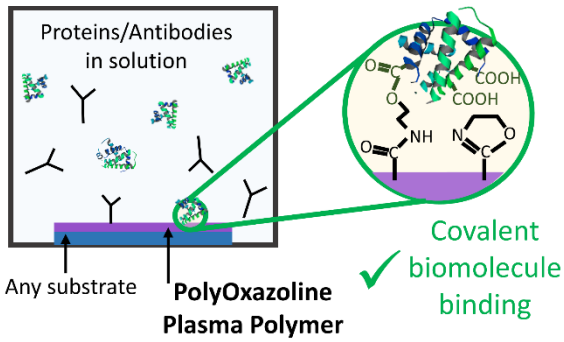
Properties and Reactivity of Polyoxazoline Plasma Polymer Films

Melanie N. Macgregor-Ramiasa[a], Alex A. Cavallaro[a], Krasimir Vasilev[a]*

Graphical abstract



Nanoscale polyoxazoline coatings generated via a single step plasma deposition process are investigated. The complex functionality of the film can be controlled by varying the deposition conditions. Partial retention of the oxazoline ring facilitates covalent binding of nanoparticules and biomolecules.

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