Functionalization of cyclo olefin polymer substrates by plasma oxidation: Stable film containing carboxylic acid groups for capturing biorecognition elements.

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Abstract

Many current designs in biomedical diagnostics devices are based on the use of low cost, disposable, easy-to-fabricate chips made of plastic material, typically a cyclo olefin polymer (COP). Low autofluorescence properties of such material, among others, make it ideal substrate for fluorescence based applications. Functionalization of this plastic substrate for biomolecule attachment is therefore of great importance and the quality of films produced on such surface have often a significant influence on the performance of the device. In this communication we discuss the surface chemistry and some other characteristics of hydrophilic films, containing carboxylic acid functional groups, formed by plasma oxidation of COP and also films containing cross linked, polymerized acryclic acid produced by sequential deposition of tetraorthosilicate and acrylic acid by Plasma Enhanced Chemical Vapor Deposition (PECVD). Immobilization of labeled, single stranded DNA revealed high binding capacity for both coatings. To our best knowledge, this is the first example of direct immobilization of biomolecules on just plasma oxidized COP. Furthermore, more sophisticated treatment of the oxidized plastic substrate by PECVD with other organic precursors increased the binding capacity by some 40% than that of just plasma oxidized COP. The carboxy functionalized surfaces, due to the negative charge of the carboxy groups, showed very positive trends towards increasing the signal to noise ratio when charged biomolecules such as DNA, are used.

Introduction

Current trends in biomedical diagnostics are directed toward miniaturized bioassay devices that often have to measure analytes at the picomolar concentrations in blood sample volume on the microliter scale. This is exceptionally challenging considering that the same device is required to give fast, reproducible results at low cost with minimum user manipulation. Therefore, new substrate materials that can be easily fabricated with buildt-in complex microfluidics designs and functionalized with a whole library of reactive groups to specifically immobilize biorecognition elements are becoming very attractive both from scientific and technological interest. Zeonor[1, 2], a cyclo olefin polymer is one such material that has quickly found a widespread use in optics, medical and electrical devices[3-6]. Fabrication of micro-channel structures on

cyclo olefin polymer or similar substrates by MEMS technologies among others is relatively well documented[7-9]. The fabrication methods include direct bonding techniques[10, 11], for which the substrate must be photochemically activated, typically by plasma or UV/ozone oxidation[12, 13]. The surface functionalization techniques are also well known and due to the nature of the polymeric substrate, all require surface activation, usually by plasma or UV/ozone treatment[14, 15]. The major surface modification effects are seen as etching, degradation of polymer molecules and introduction of new functional groups[16]. UV/ozone oxidation, similar to plasma, is generally considered as more aggressive surface activation method since ozone is directly photolyzed with UV light, producing molecular oxygen and an oxygen atom. However, ozone oxidation in the presence of UV light induces high increase in the fluorescence background, which makes this technique inappropriate for fluorescence-based applications. Treatment of the COP in the absence of UV light represents a suitable alternative to UV/ozone, however, it may not fully match the oxidation efficiency of the more powerful methods[17, 18]. Moreover, from a high-throughput technique perspective, it only takes 1 min to oxidize plastic substrates by plasma as opposed to 1 h treatment by ozone.

Number of reports deal with the changes in chemistry and morphology of COP substrates after plasma oxidation[11-13, 19, 20]. Detailed studies by X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR) in attenuated total reflection (ATF) mode unambiguously indicate that hydroxy (-OH), carbonyls (C=O) and carboxylic acids or esters components (O-C=O) were generated by sequence of reactions of cyclopentane ring opening and dehydrogeneration, followed by oxidation. In our group, we have recently developed a one step protocol for carboxylic acid functionalization of cyclo olefin polymer (referred further in text as CA substrates) by a technique called Plasma Enhanced Chemical Vapor Deposition. This method involves an activation of the plastic substrate by plasma followed by sequential deposition of vapors of tetraorthosilicate (TEOS) and acrylic acid (AA). We reason that the resulting coating consists of thin, oxygen rich film of TEOS, onto which acrylic acid is polymerized (with plasma acting as initiator of the radical polymerization reaction) and cross-linked to form an sensing layer with high density of –COOH functionality[21].Our interest in films containing high concentration of –COOH groups is predominantly fueled by needs in biomedical diagnostics to produce coatings with low non-specific binding for the measured analyte[22]. Our recent findings showed that biomolecules that are intrinsically negatively charged, such as nucleic acids and some other proteins, expressed low non-specific binding on a carboxy modified surface due to electrostatic repulsions.

Despite the high number of communications demonstrating that the carboxyl group is significant component of oxidized COP, there are very few reports suggesting that biomolecules could be captured directly on oxidized cyclo olefin polymers in reasonable density and good adhesion. We therefore investigated the binding capacity of plasma treated COP in a reaction with amino-modified oligonucleotide. Reaction mechanism of how DNA with amino linker binds to COOH groups to form stable amide bond differs from other carbonyls, such as aldehydes and ketones. The former group reacts with amines after activation with coupling agents, e.g. by carbodiimides. The latter can react with amines spontaneously to

form an imine. However, because the Schiff base is susceptible to rapid hydration and transimination with other amine containing molecules, it is good practice to transform imines to amines with reducing agents such as borohydrides (Scheme 1).

Scheme 1 Chemical structure of the plastic substrate (inset) and generally accepted functional groups on the surface of oxidized COP and -COOH slides. Amine-terminated DNA can be coupled to –COOH groups via stable amide bond formation catalyzed by EDC or semi-stable imines formed by Schiff base formation.

To assess the relative amount of aldehydes, ketones and carboxylic acids on oxidized COP, 15 base oligonucleotide functionalized with a Cy5 at the 5' end and an -NH_2 linker at the 3' end was immobilized on the surface. The DNA was coupled to the surface upon three different conditions: i) in a solution containing 50mM of (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride) (EDC), ii) in a solution containing no additives and iii) followed by immersion of the slide in a 20mM solution of $NaBH_3CN$. The amount of captured DNA was measured by means of fluorescence. Moreover, the data were corroborated with results obtained by Total Internal Reflection Ellipsometry (TIRE)[23, 24].

Materials and methods

Plain COP slides (Zeonor® 1060R) 75 mm x 25 mm were obtained from Åmic AB (Uppsala, Sweden) and were oxidized in oxygen plasma. The plasma oxidation of COP took place during 1 min at 200 mTorr, 200 W and with a flow of oxygen at 100 ml/min, in a an expanded plasma cleaner (PDC- 002, Harrick Science, Ossing, NY). Slides were used for spotting and for the ellipsometry measurement immediately, longer exposure to air was avoided. The deposition of -COOH functional coatings was carried out in a computer controlled PECVD reactor Europlasma, model CD300 (Oudenaarde, Ghent, Belgium). An aluminum vacuum chamber, connected to a Dressler CESAR 136 RF power source (Munsterau, Stolberg, Germany) with an operating frequency of 13.56 MHz, with an automated impedance-matching box, was used. The details of the deposition system is provided elsewhere [25]. A needle valve, connected to the vacuum chamber, was used to control the sequential flow of vapors of tetraorthosilicate (TEOS) and acrylic acid (AA).

Cy5-labeled and amino modified single stranded DNA was purchased from Eurofins MWG Operon and it was spotted on the slides from two solutions containing: i) 10 x 10^{-6} M of DNA, 50mM of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC) and 10% w/v glycerol and ii) 10 x 10^{-6} M of DNA and 10% w/v glycerol. One set of slides was subsequently immersed in an aqueous solution of 20 mM of $NaBH_3CN$. All substrates were washed 3 times with MilliQ-water and the fluorescence signal was recorded on PerkinElmer ScanArray Express (PerkinElmer, Massachusetts, USA) with laser excitation wavelength of 633 nm and emission filter wavelength of 670 nm. Then all slides were immersed in MilliQ-water for 6 hours, dried under the stream of N_2 and fluorescence was recorded again. The extra rinse in MilliQ-water was performed in order to remove any loosely bound DNA and to assess the stability of the films towards hydrolysis.

Water contact angle measurements were carried out at room temperature using a video-based instrument (Crelab Instruments, CA). Drops with volumes of ~1 µl were deposited on the surface and left to stabilize for 15 s before the image was frozen and the instrument software was used to calculate the contact angle. Contact angle measurements were performed in triplicate, if not indicated differently.

A COP film of 22 nm in thickness was spin coated on a gold-coated glass substrate as 0.1 w % solution of a cut-up, bulk cyclo olefin polymer in xylene. It was subsequently subject of plasma treatment for 1 minute after which its thickness was reduced to 10 nm, measured by an ellipsometry[26]. After plasma treatment, the substrate was assembled in a special flow-cell containing three wells for TIRE measurements. At first, each well was filled with 30 µl of Phosphate buffered saline (PBS) and the baseline Ψ and Δ spectra were measured for oxidized COP surface. The two parameters characterize the changes in amplitude (Ψ) and phase (Δ) of the light beam and represent the quantities measured by ellipsometer. The ellipsometric angles Ψ and Δ are related to the optical properties of the whole sample[27]. Two 30 µls of aminated ssDNA 10^{-6}M in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 8.0) without EDC were then filled in well 1 and 2 and they were allowed to react with oxidized cyclo olefin polymer surface for 2h. Similarly, another 30 µl of aminated ssDNA 10^{-6}M in MES buffer (pH 8.0) with 50 mM EDC was also filled in well 3 and allowed to react for 2h. After 2h of reaction, well 1 and 3 were rinsed extensively with PBS and Ψ and Δ spectra were recorded while the well still filled with PBS buffer. In particular, for well 2, before PBS rinsing, 20 mM of $NaBH_3CN$ in DI water was passed through and kept in the well for 5 min. Ψ and Δ spectra were also recorded for well 2 while it was filled with PBS.

Results and discussion

Fluorescence scans of slides with -COOH surface groups prepared by PECVD (further referred as CA films), oxidized and plain, untreated COP show significant differences between the surface chemistry of carboxylic acids and other reactive species, which besides ketones and aldehydes might also include oxygen radicals, peroxides and epoxides (Fig. 1). To eliminate the fluorescence signal from DNA physisorbted on the surface and DNA that is coupled to the surface through unstable bonds, the slides were rinsed in DI water and immersed in PBS buffer for 6 hours prior the measurement.

Figure 1 Fluorescence images of 0.25 µL spots of 10 × 10-6 M of ssDNA (5'-Cy5, 3'-NH2) spotted on CA surface (1), Oxidized COP (2, 3) and pristine COP (4). In order to confirm the presence/absence of –COOH surface groups, the spotting solutions in top row of slides 1, 2 and 4 contained 50 mM of EDC. As a control, the bottom row of 1-4 was spotted without EDC. For the evaluation of the available aldehydes and ketones on the surface of the plastics, slide 3 was spotted without the presence of EDC. The top row of slide 3 was immersed into solution of $NaBH_3CN$ for 1 hr after DNA incubation in order to convert the formed Schiff base (imine) to stable amines.

Figure 2A illustrates the capture efficiency on the oxidized and plain COP with and without any additives. As expected, the coupling reactions of DNA with carboxylic acids through EDC and also the ones with carbonyls through Schiff base formation followed by reduction with borohydride showed significantly higher fluorescence signal than the background. It is remarkable that the amount of DNA captured on the

oxidized COP to its –COOH components was more than 65% higher than the one to the aldehydes/ketones components. The signal for DNA physisorbed on the surface remained virtually the same for both the oxidized and the plain plastic substrate. This result is suggesting that the other reactive species formed upon plasma oxidation such as radicals, epoxides and peroxides are short lived and are most likely quenched by other species in air before they can react with DNA. Also, the signal from plain COP with and without the coupling reagent stays at the same level, confirming that EDC has no effect on DNA binding in the absence of –COOH groups. The COP substrates involved in our experiment were oxidized by plasma for 1 minute. As seen on figure 2B, treatment of the plastic slides for longer time periods does not necessarily increase the concentration of the reactive groups on the surface. We reason that at longer plasma exposure time the COP slide is subject of higher degree of etching and fragmentation of the plastic material. This can lead into a formation of thin layer of highly oxidized, low molecular weight residues, loosely bound to the surface. Such film has a low adhesion to the bulk substrate and it is being washed along with the material that is deposited on it.

Figure 2 (A) Average mean fluorescence of ssDNA (5'-Cy5, 3'-NH_2) measured on plasma oxidized and pristine (as control) COP slides with and without EDC. The fluorescence of plasma activated COP was also measured after incubation of ssDNA followed by reduction with solution of $NaBH_3CN$ for 1 hr (white column, center). The inset illustrates the chemical reactions on oxidized COP substrate. No reaction resulting in covalent bond between the ssDNA and the substrate was expected for the pristine COP. (B) Relative fluorescence of Cy5-labeled ssDNA measured on COP slides plasma treated for 1, 2, 3, 4 and 5 minutes

Water contact angles on plasma oxidized COP show that the film is relatively hydrophilic, indicating a formation of highly polar goups, such as ethers, aldehydes, ketones and carboxylic acids. Similarly, the CA film prepared by sequential deposition of TEOS and AA by PECVD is also hydrophilic, suggesting a high concentration of carboxylic acids. Moreover, X-ray reflectometry (XRR) measurements (data not shown) revealed high degree of swelling of such coating, which can be attributed to the presence of high concentration of silanols and silyl-ethers, formed upon reaction of tetraorthosilicate with the polymerized acrylic acid. Exposure of both the plasma oxidized COP slides and the CA slides to air for up to 4 hours resulted in increase in the contact angles by more than 10 degrees. Such change can be partially attributed to air contamination and also to repeated contact with drops of water, after which some highly oxidized polymeric material or some loosely bound residues were removed[6, 17]. Immersing the slides in aqueous solution for 6 hours caused further increase in their contact angles to 47.1° for the oxidized COP and 32.2°.for the CA slide, what is in concert with the reported values for similar types of films[17]. Apart from the aforementioned removal of low molecular weight residues from the surface by water, XRR experiments on CA film upon exposure to water vapours followed by subsequent drying propose a possible surface groups rearrangement triggered by the water swelling-drying cycle.

Table 1 Water contact angles on Plasma oxidized COP and PECVD treated COP slides immediately after treatment, upon exposure to air over the course of 4 hours and immersion in water for 6 hours. The water contact angle of pristine cyclo olefin polymer is 92.35° ± 1.55°.

Total Internal Reflection Ellipsometry (TIRE) was used to qualitatively corroborate the fluorescence data. As shown in Figure 3, the trends observed by fluorescence measurement are also seen by TIRE. The binding of ssDNA catalyzed by EDC and also through Schiff base formation followed by reduction by borohydride showed a large shift in both Ψ and Δ spectra from the initial signals of the oxidized COP surface when the wells were filled with PBS buffer. In agreement with fluorescence data, it can also be seen that the binding of ssDNA to –COOH components of oxidized COP is relatively higher than to aldehydes/ketones components. On the other hand, without EDC activation, only a small amount of ssDNA seemed to physisorb to the oxidized COP surface. Fitting of Ψ and Δ spectra gave corresponding thickness of ssDNA bound to the surface of three wells[24]. The fitting results were summarized in table 2 confirming that the amounts of ssDNA bound to the plasma treated surface through carboxyl and aldehydes/ketones species were substantially larger than the amount of the physically adsorbed DNA. It can also be confirmed from the fitting results that the binding through carboxyls is higher than that of aldehydes/ketones in excellent agreement with fluorescence data.

Figure 3 Ψ and Δ spectra measured on plasma treated COP surface first filled with PBS (representative from well 1) and then on the same surfaces bound with ssDNA (measured when filled with PBS) through carboxyl, physical absorption and aldehyde/ketone interactions, respectively.

Table 2 ssDNA thicknesses obtained from ellipsometric data fitting

Conclusions

We have shown that simple plasma treatment can facilitate the formation of readily reactive species on the surface of thermoplatics, which can be used directly for the specific immobilization of biomolecules through carboxyl or aldehyde/ketone interactions. Obviously, the density of such reactive groups can be easily varied by altering the plasma oxidation parameters[19]. We believe that this result could be very useful for fast attachement of biorecognition elements in experiments that do not require sophisticated surface treatment. Nonetheless, we have also shown that films with carboxyl groups prepared by PECVD deposition have significantly higher binding capacity when compared to the oxidized substrate. It should also be noted that the reactive species on plasma treated COP surface are prone to degradation in air so it is essential to bind molecules instantaneously after plasma treatment to achieve satisfactory results. Overall, oxidation of COP by plasma represents an alternative and very straightforward method for surface functionalization for specific attachment of biomolecules.

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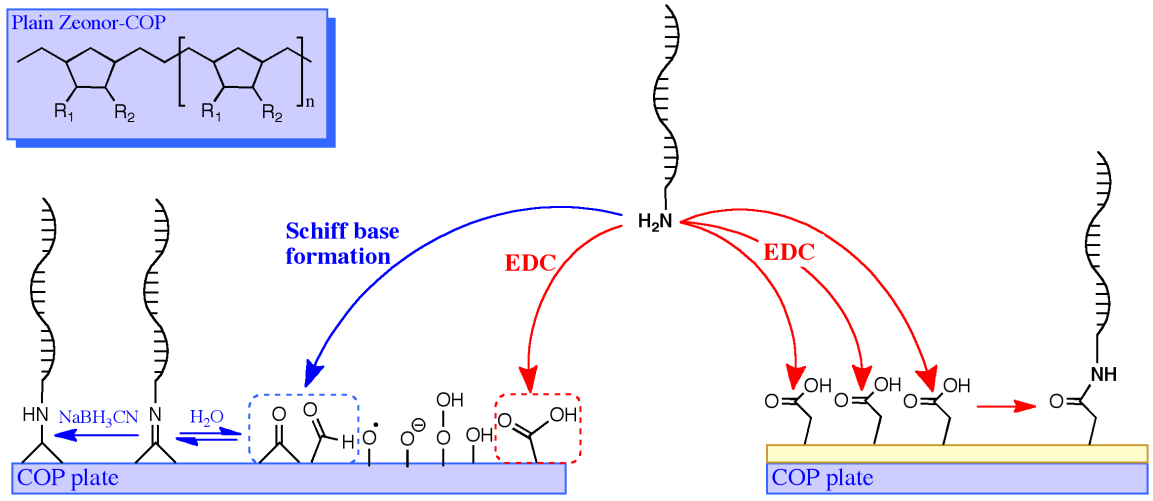
Figure(s)



Scheme 1

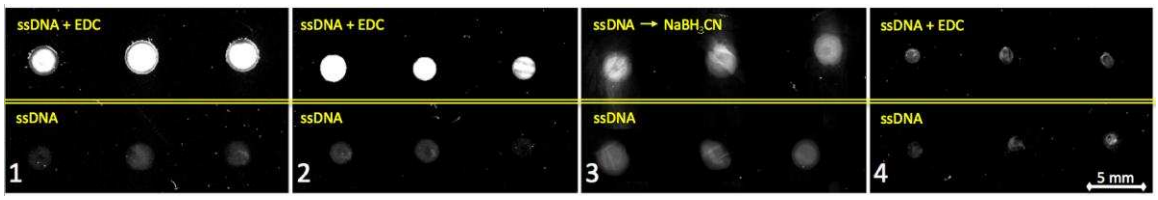


Figure 1

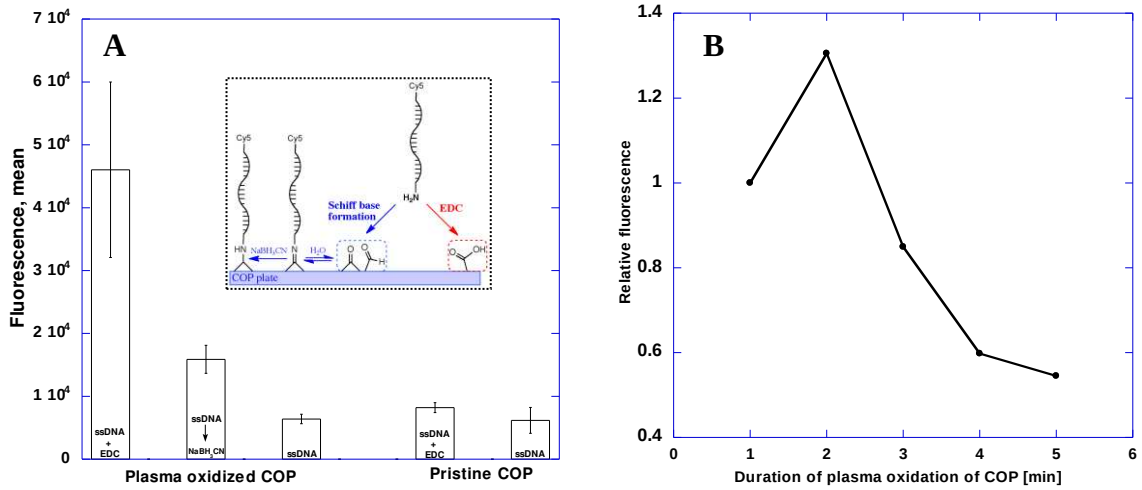


Figure 2

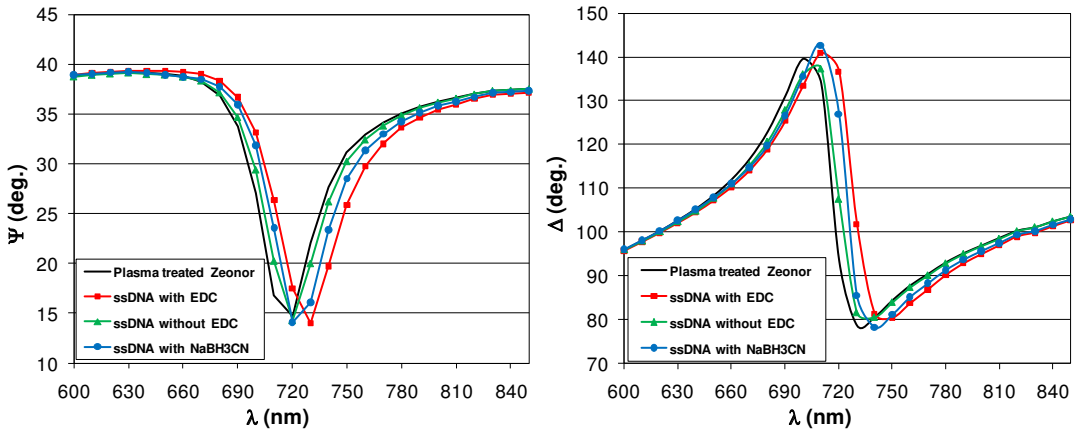


Figure 3

Scheme 1 Chemical structure of the plastic substrate (inset) and generally accepted functional groups on the surface of oxidized COP and -COOH slides. Amine-terminated DNA can be coupled to –COOH groups via stable amide bond formation catalyzed by EDC or semi-stable imines formed by Schiff base formation.

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Table(s)

Water Contact angle		Plasma Oxidized COP	PECVD treated COP
Immediately after treatment		17.0º ± 1.2º	8.4º ± 1.4º
Exposed to air for:	0.5 h	20.8º ± 2.0º	20.6º ± 0.6º
	1 h	28.8º ± 1.2º	20.0º ± 1.1º
	2 h	29.9º ± 3.5º	21.4º ± 2.1º
	3 h	33.2º ± 2.1º	22.6º ± 3.0º
	4 h	31.9º ± 2.0º	23.2º ± 1.7º
Exposed to water for:	6 h	47.1º ± 2.8º	32.2º ± 1.0º

Table 1

Binding layers on plasma oxidized COP	ssDNA thicknesses (Å)
Phosphate buffered saline	0
ssDNA with EDC	16.8 ± 2.6
ssDNA without EDC	2.9 ± 1.3
ssDNA with $NaBH_3CN$	11.6 ± 5.2

Table 2

Table 1 Water contact angles on plasma oxidized COP and PECVD treated COP slides immediately after treatment, upon exposure to air over the course of 4 hours and immersion in water for 6 hours. The water contact angle of pristine cyclo olefin polymer is 92.35º ± 1.55º.

Table 2 ssDNA thicknesses obtained from ellipsometric data fitting