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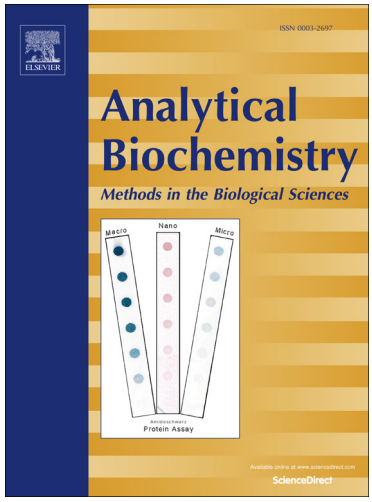

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Coordination Complexes as Molecular Glue for Immobilization of Antibodies on Cyclic Olefin Copolymer Surfaces

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List of Abbreviations

COC	cyclic olefin copolymer
COP	cyclic olefin polymer
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
PS	polystyrene
XPS	x-ray photoelectron spectroscopy
AFM	atomic force microscopy
MES	2-(*N*-morpholino)ethanesulfonic acid
PBS	phosphate buffer saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
BSA	bovine serum albumin
T20	Tween® 20
TnI	troponin I
MAb	monoclonal antibody
TNFα	MAb1 tumor necrosis factor alpha
Strep-HRP	streptavidin-horse radish peroxidase conjugate
TSH	thyroid-stimulating hormone
GAM-HRP	peroxidase-conjugated goat anti-mouse
TMB	tetramethylbenzidine
NMR	nuclear magnetic resonance

Abstract

A novel metal-based chelating method has been used to provide an order of magnitude increase in immunoassay performance on COC plastics compared to passive binding. COCs are hydrophobic and without surface modification are often unsuitable for applications where protein adhesion is desired. When interacting with the bare plastic, the majority of the bound proteins will be denatured and become non-functional. Many of the surface modification techniques reported to-date require costly equipment setup or the use of harsh reaction conditions. Here, we have successfully demonstrated the use of a simple and quick metal chelation method to increase the sensitivity, activity and efficiency of protein binding to COC surfaces. A detailed analysis of the COC surfaces after activation with the metal complexes is presented, and the immunoassay performance was studied using three different antibody pairs.

Introduction

Cyclic olefin copolymers (COC) are amorphous thermoplastics that have been used extensively recently for various applications ranging from biosensors,[1,2] biodiagnostic chips, microfluidic devices,[3,4] micro total analysis systems,[5] DNA immobilization, immunoassays and microarrays.[6] The clarity and optical resistance against commonly used sterilization regimes[7] makes COCs more attractive for many applications than polystyrene, which tends to cloud when sterilized with ethylene oxide or radiation. In addition, COCs offer high chemical resistance to acids, bases and polar organic solvents (e.g. dimethylsulfoxide, methanol, acetone), as well as thermal resistance by selecting COCs with high glass transition temperatures.[8] Furthermore COCs are photosensitive, which allows the writing of gratings for selective and label-free biosensing.[9] In contrast to poly(methyl methacrylate) (PMMA), COCs are less sensitive to humidity[10] and has orders of

magnitudes lower loss at terahertz frequencies.[11] The density of COCs is approximately half the density of glass, and COCs are less brittle, which makes them an attractive alternative to glass in optical components when weight or durability become important. By comparison, polycarbonate and PMMA, both used as alternative materials in life sciences, are brittle, prone to chemical degradation and have inferior optical properties compared with COCs.[12]

Despite the COCs offering excellent bulk properties for many applications as described above, surface modification of these materials remains a challenge. These thermoplastic materials are prepared by chain copolymerization of cyclic monomers such as norbornene with ethylene or by ring-opening methathesis polymerization of cyclic monomers followed by hydrogenation.[13] Their pure hydrocarbon composition means they lack readily accessible functional groups. The surface hydrophobicity of COC promotes fouling by proteins, a complicating factor when adhesion of proteins is unwanted. In cases where adhesion of proteins is desired, non-specific, passive immobilization of proteins often leads to loss of function. The introduction of hydrophilic polar functional groups through various surface pretreatments, to increase the surface free energy, has been suggested as a method to minimize such difficulties. Among the surface modification techniques reported on COCs are plasma treatment,[3,14,15,16,17] UV,[5] gamma or electron beam radio-sterilization[18] and chemical treatment[19,20] to oxidize the non-reactive surface.

Typically during the surface modification process, the polymer surface is modified through oxidation, degradation and crosslinking, which inevitably causes structural alterations to the first few molecular layers on the polymer surface. Plasma treatment derived from oxygen, ammonia and noble gases is one of the commonly used surface pretreatment

methods. Although the COC surfaces are successfully rendered hydrophilic by the plasma, these surfaces are unstable, as polymer chains on the surface tend to rearrange and return the surface to the native unreactive hydrophobic form. This phenomenon has been widely confirmed through increment in contact angle measurements[21] and ζ-potential decay,[22] which often occurs over a period of days. Furthermore, the source of surface charge after pretreatment with plasma, gamma or electron beam radio-sterilization is unclear. To optimize the hydrophilic functionality and energy of COC surfaces, pretreatment for long duration and high power plasma source has been conducted. Although this may increase shelf life, the surface roughness can be compromised thus rendering the materials incompatible with applications that require homogeneous surface roughness.

It is well known from several decades of experience in ELISA that plain polystyrene (PS) microtitre plates without surface modification are only suitable for a small percentage of proteins. More hydrophilic proteins such as antibodies require polar groups on the plate surface and as a consequence, there are many types of surface-modified microtitre plates. Following this learning, increasing the polarity of COC surfaces is likely to be essential to improve its ability to bind antibodies. The different types of cyclic olefin polymers (COP) and copolymers (COC) available commercially have varying abilities to bind proteins. Some workers have reported problems associated with non-specific binding to COC surfaces,[23] however there have also been reports of COP materials that are inert to biological molecules. Nile et al. reported the use of COP (Zeonor 1420R) as the base material for a high-density multiwall plate (Aurora Biotechnologies).[12] These plates were reported to be inert to biological molecules except small hydrophobic polypeptides less than 10 kDa. Based on these reports, it is difficult to achieve desirable antibody binding properties on these surfaces without applying surface treatments.

Where passive binding is undesirable or covalent linkages are difficult to achieve, a completely different approach to binding proteins via the use of metal complexes can be used.[24] Using a high throughput surface discovery approach, many thousands of metal complexes were screened for their efficiency to bind antibodies, and several particular complexes were found to be highly efficient in binding proteins. These particular metal complexes, called Mix&Go^{TM} [25,26,27] depend on two basic characteristics for binding. Using slow exchanging metal complexes such as chromium (III) in oligomeric form, there is avidity or multi-component chelation to the synthetic surface, while retaining potential to similarly bind proteins. A single metal–ligand interaction may readily break however the odds of multiple interactions breaking simultaneously are low. However, COC plastics are hydrophobic and should not have electron donating groups to chelate to metal complexes.

It has been previously shown that versions of such metal complexes can bind to plain non-irradiated PS surfaces. Presumably, these metal complexes are in coordination with the π electrons of the phenyl ring and while one interaction may be weak, a multiplicity of interactions is sufficient to create a completely different surface over the previously hydrophobic non-functional surface. Considering its structure COP/COC surfaces do not have electron donating potential to bind metal complexes. However, work on enhancing adhesion of metals onto such COC/COP surfaces has been reported.[8,15] Niklova et al. studied the effects of plasma treatment on the adhesive strength of aluminium and copper on COC surfaces.[15] The adhesive strength in COC-metal composites increased with the intensity of plasma treatment however the untreated controls demonstrated ability to bind these metals as well. If a weak potential to bind metals on COC surfaces exists, a basic strategy of using slowly exchanging metal complexes and avidity of such metals using

polymeric metal constructs will potentially allow the formation of a surface that promotes protein binding. Once formed, the residual co-ordination sites remaining after forming metal complexed surfaces are chelated to small ligands such as water which undergo exchange with a specific half-life. In other words, the Mix&Go™ activated COC surfaces can be stored but remain active indefinitely. Importantly, in the presence of many classes of biomolecules (antibodies, streptavidin, Protein A or G, etc.), coordination forces bind such proteins firmly onto the surface.

In this study, the potential of the metal complexes to bind to COC surfaces to form a hydrophilic chelating surface was investigated. Three different sandwich assays were performed using one particular formulation called Mix&Go™ Biosensor, which was known to perform well on polystyrene surfaces as well as silica and other metal oxide surfaces. Two assays were known to work well by passive binding on PS microtitre plates and the third sandwich assay gave poor results under the same conditions. In order to better understand the effects of metal coordination on such hydrophobic surfaces, surface analytical techniques such as x-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and contact angle studies on these metal complex activated surfaces were performed.

Materials and Methods

Materials

2-(*N*-morpholino)ethanesulfonic acid (MES), phosphate buffer saline (PBS), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), bovine serum albumin (BSA), Tween® 20 (T20), sulfuric acid and Greiner Bio-One COC plates (Cat. No. M3812) were obtained from Sigma-Aldrich. 19C7 Troponin I (TnI) MAb, human cardiac TnI antigen and 16A11 biotinylated TnI MAb were purchased from HyTest Ltd (Finland). MAb1 tumor

necrosis factor alpha (TNFα) antibody, biotinylated MAb11 TNFα antibody and streptavidin-horse radish peroxidase conjugate (Strep-HRP), were purchased from BD Biosciences. Recombinant human TNFα was purchased from R&D Systems (USA). 057-11003 thyroid-stimulating hormone (TSH) MAb was purchased from Meridian Life Science. 5403 biotinylated TSH MAb was purchased from Medix Biochemica. Peroxidase-conjugated goat anti-mouse (GAM-HRP) was obtained from Jackson ImmunoResearch (USA). 3,3',5,5'-tetramethylbenzidine (TMB) substrate was purchased from Thermo Scientific. Mix&Go^{TM} Biosensor was obtained from Anteo Diagnostics Ltd. Buffers prepared were 50 mM MES at pH5.2 (coating buffer for TNFα and TSH assays), 10 mM PBS with 1 % BSA (assay buffer for TNFα and TSH assays), 50 mM MES with 5 % BSA (blocker for TNFα and TSH assays), 10 mM PBS with 0.05 % T20 (wash buffer for TNFα, TSH and TnI assays), HEPES at pH7.4 (coating buffer for TnI assay), 10 mM PBS with 0.1 % T20 (assay buffer for TnI) and PBS with 1 % Casein (blocker for TnI assay).

Characterization

^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AVANCE 400 MHz instrument operating at 400.13 MHz with a standard Bruker 5 mm broad band observed gradient probe. Deuterated chloroform ($CDCl_3$) was used as the solvent. Spectra were referenced to solvent signal at $\delta\ ^{13}C = 77.0$ ppm. All spectra were processed using Bruker TOPSPIN.

Contact angle measurements were carried out on an apparatus comprised of an adjustable stage and lens assembly fitted with a camera and linked to a Scion imaging software. The sample was placed on the stage and 5 µL of Milli-Q water was transferred by a 50 µL glass flat tip syringe onto the sample surface. The needle tip was removed from the water droplet

before capturing an image of the droplet. Contact angles were measured using the imaging software. All measurements were taken five times.

A Kratos Axis Ultra X-ray Photoelectron Spectrometer with monochromatic Al Kα X-rays (1486.6 eV) at 150 W (15 kV, 10mA) was used to collect XPS spectra. Photoelectron data was collected at a take off angle of theta = 90°. Survey scans were taken at an analyzer pass energy of 160 eV. Survey scans were performed over a range of 1200-0 eV binding energies with 1.0 eV steps and a dwell time of 100 ms. The base pressure in the analysis chamber was set at 1.0 x 10^{-9} torr and during sample analysis 1.0 x 10^{-8} torr. Processing was performed using CasaXPS. Binding energy corrections were made by referencing spectra to the carbon C 1s fixed at 285 eV.[28] Survey scans were taken of an area of 0.7 x 0.3 mm^2.

A MFP-3D (Asylum Research) atomic force microscope was used for all the measurements. The cantilevers used were HA_NC (Etalon) from NT-MDT, Russia having a nominal spring constant of 4.5 N/m and nominal resonant frequency of 145 kHz. All the images were obtained by employing the Tapping Mode of the AFM in air. The AFM was mounted on an anti-vibrational table (Herzan) and operated within an acoustic isolation enclosure (TMC, USA).

Immunoassays

The plates were treated by incubating each well with Mix&Go^{TM} Biosensor solution for an hour. Wells were then washed thoroughly with deionized water, followed by a wash with coating buffer. For passive binding experiments, the plates were used as received.

For the loading assays, capture antibodies were diluted in coating buffer at 1 μg/mL and incubated in plates for 30 minutes before washing with wash buffer twice on a TECAN 96 Plate Washer™. Plates were then blocked with blocking buffer for an hour. The washing step was carried out three times to remove excess blocker. This was followed by the incubation of GAM-HRP in assay buffer (0.1 μg/mL) for 30 minutes, followed by five times washing with wash buffer. The substrate, TMB, was reacted with HRP for 7 minutes before the reaction was terminated by the addition of 2 M sulfuric acid, which resulted in a yellow product.

For the sandwich assays, capture antibodies were diluted in coating buffer at desired concentrations and incubated in plates for 30 minutes (unless stated otherwise) before washing with wash buffer twice on a TECAN 96 Plate Washer™. Plates were then blocked with blocking buffer for an hour. The washing step was carried out three times to remove excess blocker. Antigen in assay buffer was incubated in plates for an hour before washing five times with wash buffer to remove excess analytes. This was followed by the incubation of biotinylated detection antibodies in assay buffer (0.5 μg/mL) for 30 minutes, followed by five times washing with wash buffer. Strep-HRP (0.1 μg/mL in assay buffer) was then reacted with the biotinylated antibodies for 15 minutes and washed five times with wash buffer. The substrate, TMB, was reacted with HRP for 7 minutes before the reaction was terminated by the addition of 2 M sulfuric acid, which resulted in a yellow product.

Optical density (O.D.) of the colorimetric substrate for immunoassays was measured using a Tecan Infinite® 200 PRO. The measurements were conducted at 450 nm with the reference wavelength at 620 nm. The bandwidth at both wavelengths was set to 9 nm. The temperature of the measurements was between 23 – 24 ºC.

Results and Discussion

Chemistry of the Surfaces

The class of commercial materials known as COCs encompasses a large number of potential cyclic monomers prepared using various polymerization methods. The chemical structure and composition of the COC polymers are important as they determine the physical and surface properties of the materials. Changes in the copolymer content and chemical structure of the cyclic monomers can modify physical properties such as microstructure, glass-transition temperature and surface energy.[13,22,29] Shin et al. reported comprehensive ^{13}C NMR studies of the chemical structures of a series of commercial COCs and COPs and related these to the thermal and surface properties.[13] They demonstrated that with increasing amount of bulky cyclic monomer units, the glass transition temperature of the copolymers increases and the presence of ester or ether groups increases the surface energy. The chemical composition of the COC plates used in these studies was determined by ^{13}C NMR as shown in Fig. 1 and the polymer was found to be comprised of ethylene and norbornene monomers. The peaks in the NMR spectra were assigned according to the extensive NMR studies that have been reported in the literature.[13,30,31] Resonances from the ethane-1,2-diyl units (carbons 8 and 9) and the cyclic monomer (carbons 5 and 6) overlapped in the region between 29.8 to 31.8 ppm. Other peaks due to the norbornanediyl units are at 32.7 ppm (carbon 7) and between 40.7 to 41.6 ppm (carbons 1 and 4). The main peaks of interest lie in the region of 46 to 48 ppm, and are assigned to the methine carbons (2 and 3) of the norbornanediyl units. These peaks provide information on the composition and structure of the copolymer. Rische et al. showed that, in general, COCs display either of two distinct NMR spectra depending on the norbornene content.[31] Well-separated and narrow peaks were observed when the fraction of norbornene in the COC used was less than 50 %, as

opposed to broader and less separated peaks for higher norbornene contents. These observations indicate that the COC studied here has less than 50 % norbornene units and consists mainly of alternating ethanediyl/norbornanediyl and long sequences of ethylene units**.** NMR does not indicate the presence of oxygen or other groups having electron donating potential within this COC material.

The COC plates were treated with Mix&Go^{TM} simply by exposing the surfaces to the metal polymer solution for an hour under ambient conditions (M&G-COC). The presence of bound metal complexes on the COC surface was confirmed through the detection of chromium peaks by XPS. Fig. 2 shows the XPS spectra of the COC surfaces before and after treatment with Mix&Go^{TM}. Measurements were conducted on three different areas of four treated wells, which were distributed randomly in a plate (Supplementary Information).

Table **1** shows the percentages of Cr relative to other elements measured in each well. The variability in the distribution of Cr between wells was relatively high ranging from 0.43 to 2.67 % in the four wells tested. The Cr complex was stable to multiple washing steps indicating that by some mode of action, metal complexes could be bound to COC surfaces.[8,15] The untreated COC surface showed the presence of zinc, silicone and oxygen, which may be attributed to the presence of molding agents and contaminants or possibly oxidation of the surface introduced during the injection molding process. It may be possible that these oxygen species are the anchoring points for avidity binding of these metal oligomers. Once the metal complex is deposited on these sites, the complexes may aggregate or diffuse on the surface or through the bulk polymer bulk via random diffusion processes.[32] This may contribute to the variation in level of Cr detected in the wells by XPS.

The roughness of the COC surfaces before and after treatment with Mix&Go^{TM} was measured by AFM as shown in Fig. 3. On magnification (5 x 5 μm^2 area), the metal complexed COC surfaces looked distinctly different to the untreated surface confirming that a metal complex film has coated onto the COC. The roughness of the COC surfaces was measured by AFM to be 4.7 ± 0.5 nm and was 4.5 ± 1.0 nm after treatment with Mix&Go^{TM}.

These results indicate that treatment with the metal-complex solution did not alter the topography of the surfaces. In contrast, alternative surface treatments can significantly increase surface roughness. COC surfaces are commonly rendered hydrophilic through exposure to high-energy sources such as plasma and gamma-irradiation. These treatments produce unstable hydrophilic surfaces and over prolonged exposure time, surface etching occurs. Roy et al. observed increase in roughness from 11 nm to 23 nm after 120 s exposure to argon plasma treatment.[3] Further exposure up to 180 s produced COC surfaces with higher roughness and the use of oxygen instead of argon produced even rougher surfaces. Similarly, Nikolova et al. observed an increase in roughness from 4.3 nm to 8.2 nm on their COC surfaces after 60 s of oxygen plasma treatment.[15]

The hydrophilicity of the COC surfaces was also determined via contact angle measurements. The contact angle of the hydrophobic COC surface was measured to be 95.6 ± 3.9°, which was similar to previously reported values.[5] The treatment with Mix&Go^{TM} did not significantly alter the hydrophobicity of the COC surfaces as the contact angle was found to be 104.5 ± 2.6°. The detection of chromium and the underlying surface using XPS and the minimal changes in roughness in the AFM images are suggestive that the metal complex coatings are very thin films, and may not totally cover the underlying surface. The absence of significant changes in the contact angle further indicates that the metal complex coating applied was insufficient to alter the hydrophilicity of the surfaces. This shows that the application of Mix&Go^{TM} retains the original properties of the COC surfaces but is adequate to provide superior improvements to the immunological performance of the materials as discussed in the following section.

Immunological Performance

Binding of biomolecules such as antibodies and other proteins on untreated COC surfaces is driven by hydrophobic interactions, which often leads to conformational damage and subsequent loss of functionality. From the surface analysis studies, we have shown that treatment with Mix&Go^{TM} has created a very thin film of metal complexes that may not totally cover the underlying hydrophobic surface (by contact angle measurements). This is consistent with the lack of electron donating groups on the COC surface leading to a new surface that may still include the potential for hydrophobic binding but now augmented by metal coordination forces. This could be likened to PS microtitre plates, which have been surface treated to improve passive binding of more hydrophilic proteins such as antibodies.

In order to test antibody-binding performance on COC plates treated with Mix&Go^{TM}, three sandwich ELISA (tumor necrosis factor alpha (TNFα), troponin I (TnI) and thyroid-stimulating hormone (TSH)) were selected. The TNFα antibody pair was recommended for ELISA by the manufacturer. The TSH antibody pair has been confirmed by previous work to perform adequately on microtitre plates. The TnI antibody pair was known to work when the capture antibody was covalently coupled on particles but not by passive binding on microtitre plates. Fig. 4(A) shows the loading assay data of mouse anti-human TNFα, TnI and TSH antibodies using goat anti-mouse conjugated with horseradish peroxidase (GAM-HRP) as the secondary antibody. The binding efficiency of all three capture antibodies on untreated and Mix&Go^{TM} treated COC surfaces was different as determined by anti-species antibody binding and consistent with the differences observed by surface analysis. Using a capture antibody concentration of 1 μg/mL, TNFα and TnI antibodies can be successfully bound to untreated COC surfaces by passive binding. However, the TSH antibody either did not bind or was totally damaged such that it could not be detected by the anti-species antibody. In

contrast, there was an increase in loading for all three capture antibodies on Mix&Go™ treated COC surfaces. However, the absolute loading was different for each antibody suggesting some antibody-specific factors in this loading assay. The results of sandwich ELISAs (1000 µg/ml of TNFα and TnI antigen and 5 µIU/ml of TSH antigen) at the same capture antibody concentration are shown in Fig. 4(B). The amount of functional antibodies as evidenced by antigen binding on untreated and Mix&Go™ treated COC surfaces clearly show increased assay performance under the experimental conditions. Both TnI and TSH assays did not give any signal with passive-binding, while TNFα assays on Mix&Go™ treated surfaces gave approximately nine times greater signal.

To further understand the differences between untreated and Mix&Go™ activated COC surfaces, titrations of capture antibody as well as antigens for all three ELISAs were also conducted. Fig. 5 shows the titration curves for the TNFα system. Capture antibody titration was conducted at an antigen concentration of 1000 pg/ml and antigen titration with capture antibody at 1 µg/ml. On Mix&Go-COC, maximum signal was obtained at approximately 2 µg/mL capture antibody with no further increase in performance with increasing concentrations of capture antibody. In comparison, more than double of that concentration (approximately 5 µg/mL) is needed to reach maximum capacity of a passive well. The maximum O.D. values for the passive are approximately half of the O.D. values measured on the M&G-COC surface. The signal-to-noise ratio (S/N) in the antigen titration study also significantly improved on the M&G-COC surfaces in comparison to the untreated COC surfaces (see Fig. 5(B)). Additionally, the percent coefficient of variation (%CV) of a plate improved significantly after treatment with Mix&Go™ from 16 % (untreated) to 6 % (treated) (Supplementary Information). It is interesting to note that this 6% inter-well CV was achieved on Mix&Go™ treated COC plates having chromium ion distributions varying from

0.43 to 2.67%. This suggests greater surface uniformity to the incoming capture antibody and variations in coating thickness as opposed to percent coverage.

TNFα sensitivity on the M&G-COC surfaces was significantly better implying that the functionality of capture antibodies on M&G-COC was maintained whereas passive binding on untreated COC must have lead to functional damage. Similar improvements were also observed for the TnI and TSH sandwich assays. The TSH capture antibody titration curve reached a plateau earlier on the M&G-COC surfaces at approximately 5 μg/mL while the passive curve increased up to the concentrations studied (Fig. 6(A)). There was a clear antigen titration on the M&G-COC surface while the same assay on the untreated surface failed (Fig. 6(B)). Similarly, the TnI assay (Fig. 7) required fewer antibodies to reach a maximum with higher Ab loading capacity and obtained a measurable antigen titration on the M&G-COC surfaces compared to the untreated COC surfaces. It should be noted that the measured O.D. values for the TnI assays are relatively low but this is not surprising considering that this capture antibody was known to be sensitive to passive binding. TnI sandwich assays using this particular capture antibody on commercially-available polystyrene microtiter plates (MaxiSorp® and Greiner Bio-One) gave very poor performance (Supplementary Information). Interestingly, on both treated and untreated surfaces, there were relatively large quantities of immobilized antibodies by loading assay, indicating that the poor ELISAs were likely due to degradation of the TnI antibodies.

In addition to the improved assay performance, the binding of antibodies to M&G-COC surfaces occurred at a much faster rate compared to passive binding suggesting a completely different mode of binding. Typically, antibody coating on polystyrene plates requires an overnight incubation.[33] Fig. 8 shows the coating capture antibody time profiles

for the three sandwich assay systems. On the M&G-COC surfaces, a dramatic increase occurs in the first 10 minutes of incubation for all three systems. As expected, the passive COC surfaces required longer incubation periods for antibodies to fully coat the wells. To demonstrate the efficiency of M&G-COC over untreated COC surfaces, a 24-hour incubation time point, which is considered to be the commonly used incubation period for passive binding, was also measured for all three systems and is reported in Fig. 9. The measured signals after 24 hours show that the increased incubation period on untreated COC did not improve the activity of the assays and was incapable of reaching the efficiencies demonstrated by the M&G-COC surfaces. The lower O.D. values measured for the 24-hour time points compared to the shorter time points may be attributed to the deterioration of the biomolecules over time and the different kinetics observed at 4 °C.

Conclusions

In this study, a metal chelation system called Mix&Go^{TM} Biosensor was tested on COC plastics, a surface which was expected not to have electron donating groups for coordination of metal ions. However, surface analysis clearly shows effective binding of these metal complexes to the surface by simple addition of these aqueous solutions to the wells of the COC microtitre plate followed by incubation for 1 hour. These surface treatments were stable in their activated form and did not lead to any measurable change in surface roughness or hydrophobicity. For the three test assays, this treatment lead to significant increases in sensitivity, capture antibody savings, improved inter-well reproducibility to the point that it enabled assays that were not possible by passive coating.

COCs are highly hydrophobic and are known to lead to non-specific binding of proteins, which often render them non-functional. However, its superior optical properties

over other commonly used polymers has increased its popularity in various applications and where needed, different surface modification techniques have been applied to change the surface properties in order to improve binding of proteins. Methods based on covalent binding are usually preferred because they are more controllable and minimize protein denaturation. However, covalent binding often involves syntheses that require synthetic skills. The method of surface treatment with Mix&Go^{TM} implemented here is simple, economical and does not require in-depth chemistry laboratory skills or the use of any sophisticated instruments. This preliminary study did show variations in the amount of metal complexes in individual wells of a microtitre plate detected by XPS. However, these variations were not reflected in the immunoassay performance of the activated COC surfaces. The improved performance of M&G-COC compared to COC was demonstrated through assays conducted on two antibody pairs (TNFα and TSH) known to work on standard PS microtitre plates (by passive coating) and another antibody pair (cardiac biomarker, TnI) known not to work on standard PS microtire plates (by passive coating). All these capture antibodies have been shown to bind much faster and reach maximum coating at lower antibody concentrations indicating minimal functional damage.

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References

[1] G. Emiliyanov, P.E. Høiby, L.H. Pedersen, O. Bang, Selective serial multi-antibody biosensing with TOPAS microstructured polymer optical fibers, Sensors 13 (2013) 3242-3251.

[2] G. Emiliyanov, J.B. Jensen, O. Bang, P.E. Hoiby, L.H. Pedersen, E.M. Kjær, L. Lindvold, Localized biosensing with Topas microstructured polymer optical fiber, Optics Letters 32 (2007) 460-462.

[3] S. Roy, C.Y. Yue, Y.C. Lam, Z.Y. Wang, H. Hu, Surface analysis, hydrophilic enhancement, ageing behavior and flow in plasma modified cyclic olefin copolymer (COC)-based microfluidic devices, Sensors and Actuators B: Chemical 150 (2010) 537-549.

[4] W.I. Jang, K.H. Chung, H.B. Pyo, S.H. Park, Self-operated blood plasma separation using micropump in polymer-based microfluidic device, (2006) 641511-641511.

[5] C. Li, Y. Yang, H.G. Craighead, K.H. Lee, Isoelectric focusing in cyclic olefin copolymer microfluidic channels coated by polyacrylamide using a UV photografting method, ELECTROPHORESIS 26 (2005) 1800-1806.

[6] G.A. Diaz-Quijada, R. Peytavi, A. Nantel, E. Roy, M.G. Bergeron, M.M. Dumoulin, T. Veres, Surface modification of thermoplastics-towards the plastic biochip for high throughput screening devices, Lab on a Chip 7 (2007) 856-862.

[7] R.R. Lamonte, D. McNally, Cyclic olefin copolymers, Adv. Mater. Process. 159 (2001) 33-36.

[8] Z.-R. Lu, In vivo imaging of dosage forms, Drugs Pharm. Sci. 188 (2009) 181-201.

[9] I.P. Johnson, W. Yuan, A. Stefani, K. Nielsen, H.K. Rasmussen, L. Khan, D.J. Webb, K. Kalli, O. Bang, Optical fibre Bragg grating recorded in TOPAS cyclic olefin

copolymer, Electronics Letters, Institution of Engineering and Technology, 2011, pp. 271-272.

[10] W. Yuan, L. Khan, D.J. Webb, K. Kalli, H.K. Rasmussen, A. Stefani, O. Bang, Humidity insensitive TOPAS polymer fiber Bragg grating sensor, Optics Express 19 (2011) 19731-19739.

[11] K. Nielsen, H.K. Rasmussen, A.J. Adam, P.C. Planken, O. Bang, P.U. Jepsen, Bendable, low-loss Topas fibers for the terahertz frequency range, Optics Express 17 (2009) 8592-8601.

[12] W.D. Niles, P.J. Coassin, Cyclic olefin polymers: Innovative materials for high-density multiwell plates, Assay Drug Dev. Technol. 6 (2008) 577-590.

[13] J.Y. Shin, J.Y. Park, C. Liu, J. He, S.C. Kim, Chemical structure and physical properties of cyclic olefin copolymers, Pure Appl. Chem. 77 (2005) 801-814.

[14] S.-J. Hwang, M.-C. Tseng, J.-R. Shu, H. Her Yu, Surface modification of cyclic olefin copolymer substrate by oxygen plasma treatment, Surface and Coatings Technology 202 (2008) 3669-3674.

[15] D. Nikolova, E. Dayss, G. Leps, A. Wutzler, Surface modification of cycloolefinic copolymers for optimization of the adhesion to metals, Surface and Interface Analysis 36 (2004) 689-693.

[16] S. Roy, C.Y. Yue, Surface Modification of COC Microfluidic Devices: A Comparative Study of Nitrogen Plasma Treatment and its Advantages Over Argon and Oxygen Plasma Treatments, Plasma Processes and Polymers 8 (2011) 432-443.

[17] H. Steffen, K. Schröder, B. Busse, A. Ohl, K.D. Weltmann, Functionalization of COC Surfaces by Microwave Plasmas, Plasma Processes and Polymers 4 (2007) S392-S396.

[18] H. Barakat, C. Aymes-Chodur, J. Saunier, N. Yagoubi, Effect of electron beam radio sterilization on cyclic olefin copolymers used as pharmaceutical storage materials, Radiation Physics and Chemistry 84 (2013) 223-231.

[19] C.K. Dixit, S.K. Vashist, F.T. O'Neill, B. O'Reilly, B.D. MacCraith, R. O'Kennedy, Development of a High Sensitivity Rapid Sandwich ELISA Procedure and Its Comparison with the Conventional Approach, Analytical Chemistry 82 (2010) 7049-7052.

[20] C.K. Dixit, S.K. Vashist, B.D. MacCraith, R. O'Kennedy, Multisubstrate-compatible ELISA procedures for rapid and high-sensitivity immunoassays, Nat. Protocols 6 (2011) 439-445.

[21] S. Laib, B.D. MacCraith, Immobilization of Biomolecules on Cycloolefin Polymer Supports, Analytical Chemistry 79 (2007) 6264-6270.

[22] Y. Lu, J.S. Valentine, Engineering metal-binding sites in proteins, Current Opinion in Structural Biology 7 (1997) 495-500.

[23] E.K.U. Larsen, N.B. Larsen, One-step polymer surface modification for minimizing drug, protein, and DNA adsorption in microanalytical systems, Lab Chip 13 (2013) 669-675.

[24] B.W. Muir, M.C. Barden, S.P. Collett, A.-D. Gorse, R. Monteiro, L. Yang, N.A. McDougall, S. Gould, N.J. Maeji, High-throughput optimization of surfaces for antibody immobilization using metal complexes, Anal. Biochem. 363 (2007) 97-107.

[25] E. Chung, S.J. Cooper, B.T. Ohse, Y. Gao, D. Jennins, M.M. Koudijs, L. Yang, T. Ling, P. Vukovic, A. Wong, N.J. Maeji, Simple Methods for Forming Protein Monolayers, Lab-on-a-chip European Congress, Barcelona, Spain, 2013.

[26] N. Abernethy, E. Chung, B.T. Fontanelle, Y. Gao, D. Jennins, M.M. Koudijs, D. Lim, L. Yang, T. Ling, P. Vukovic, A. Wong, N.J. Maeji, Metal polymers, a glue to

immobilise proteins onto synthetic surfaces, 44th Oak Ridge Conference - Emerging Technologies for 21st Century Diagnostics, San José, USA, 2012.

[27] B.W. Muir, M.C. Barden, D.B. Rylatt, N.J. Maeji, C.J. Hillyard, A.-D.J.-P. Gorse, R.L. Monteiro, Use of metal complexes, in: U.S.P.a.T. Office (Ed.), Bio-Layer Pty Limited, United States, 2012.

[28] G. Beamson, D. Briggs, High resolution XPS of organic polymers. The Scienta ESCA300 database, Wiley, Chichester, 1992.

[29] C. Boiziau, G. Lecayon, Adhesion of polymers to metals: A review of the results obtained studying a model system, Surface and Interface Analysis 12 (1988) 475-485.

[30] J.E. Hale, Irreversible, Oriented Immobilization of Antibodies to Cobalt-Iminodiacetate Resin for Use as Immunoaffinity Media, Analytical Biochemistry 231 (1995) 46-49.

[31] K.L. Mittal, The role of the interface in adhesion phenomena, Polymer Engineering & Science 17 (1977) 467-473.

[32] F. Faupel, V. Zaporojtchenko, T. Strunskus, J. Erichsen, K. Dolgner, A. Thran, M. Kiene, Fundamental Aspects of Polymer Metallization, in: E. Sacher (Ed.), Metallization of Polymers 2, Kluwer Academic/Plenum Publishers, New York, 2002.

[33] V. Mailaender, K. Landfester, Interaction of Nanoparticles with Cells, Biomacromolecules 10 (2009) 2379-2400.

Tables and Figures

Table 1. Distribution of chromium on different COC wells

Sample	Average % of Cr	Standard Deviation
1	2.67	0.15
2	0.69	0.12
3	0.43	0.01
4	1.32	0.24

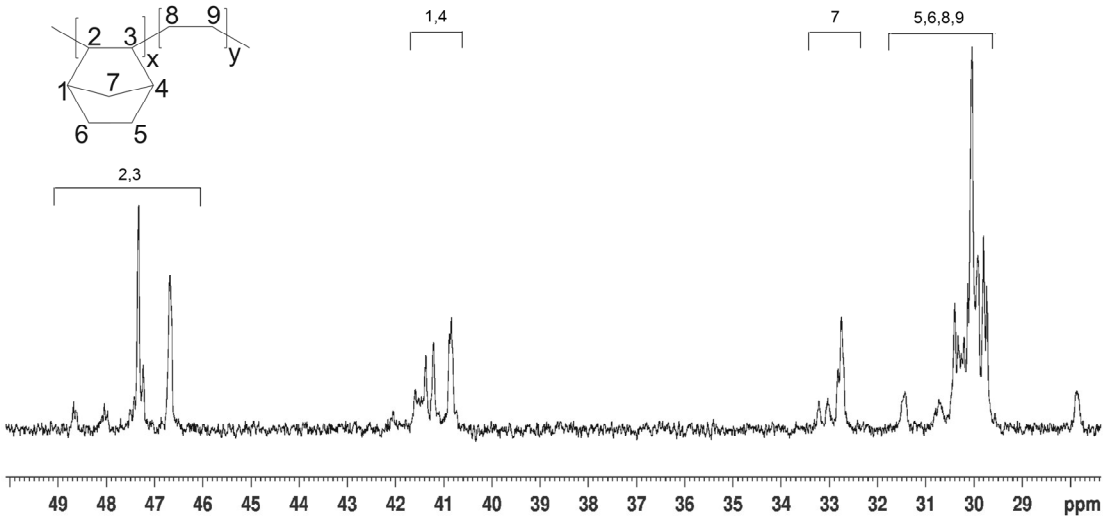

Fig. 1. ^{13}C NMR spectrum of the COC plate in $CDCl_3$.

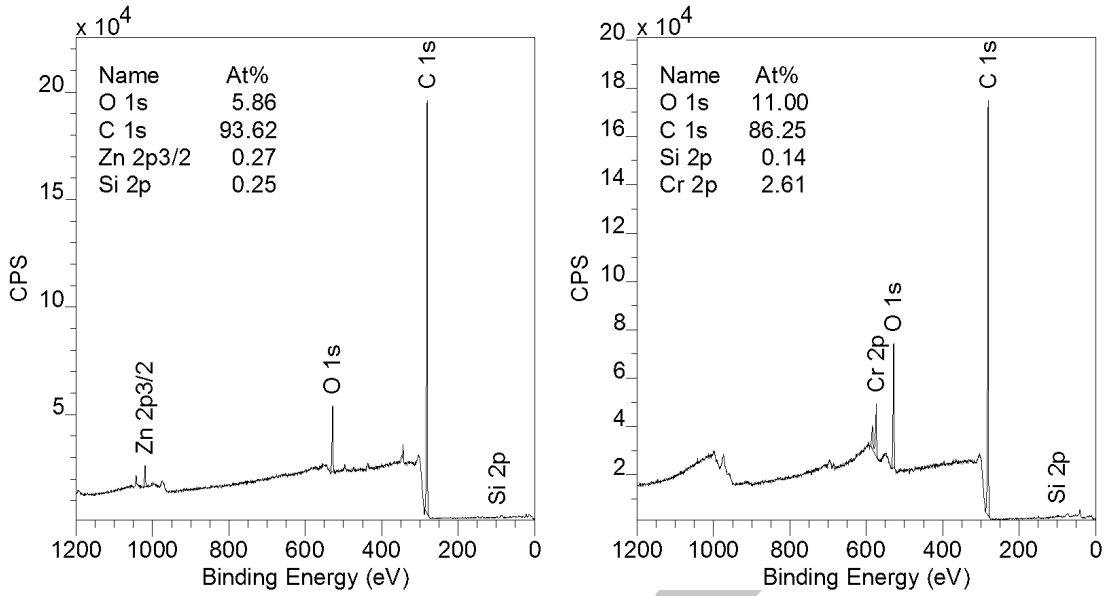

Fig. 2. XPS spectra of untreated COC (left) and M&G-COC (right).

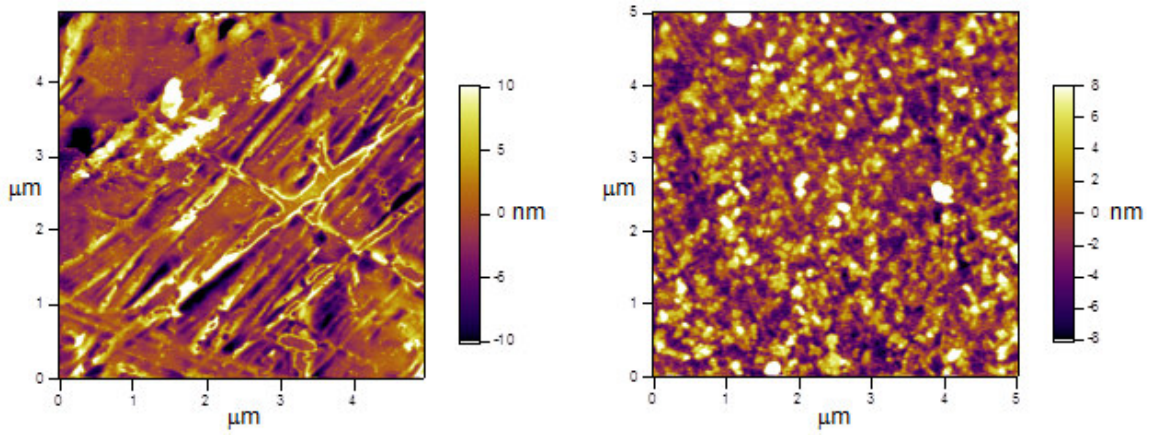

Fig. 3. AFM images of (A) untreated COC and (B) M&G-COC surfaces.

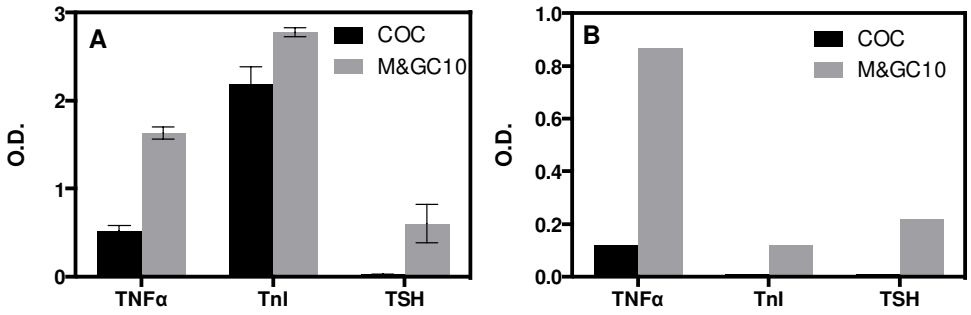

Fig. 4. (A) Loading assay of mouse anti-human TNFα, TnI and TSH Ab (1 μg/mL) with GAM-HRP as detection Ab (0.1 μg/mL) on untreated COC and M&G-COC plates. (B) Sandwich assay of mouse anti-human TNFα, TnI and TSH Ab (1 μg/mL), antigens (TNFα and TnI at 1000 ug/ml and TSH at 5 μIU/ml) and biotin mouse anti-human as detection Ab.

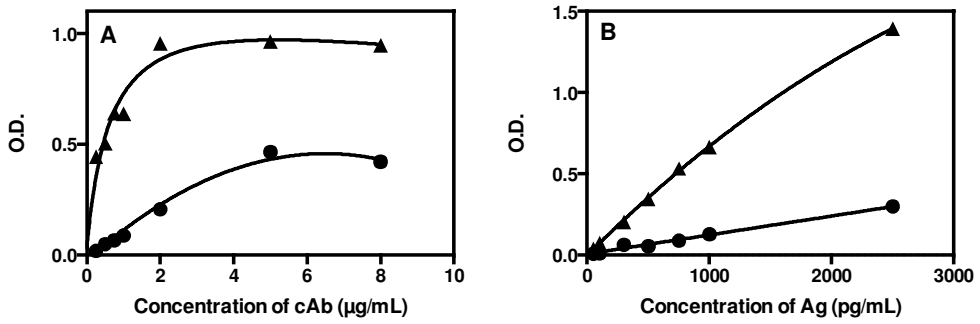

Fig. 5. TNFα sandwich assay: (A) Titration of cAb (Ag = 1000 pg/mL) and (B) Antigen (cAb = 1 µg/mL) on COC (●) and M&G-COC surfaces (▲).

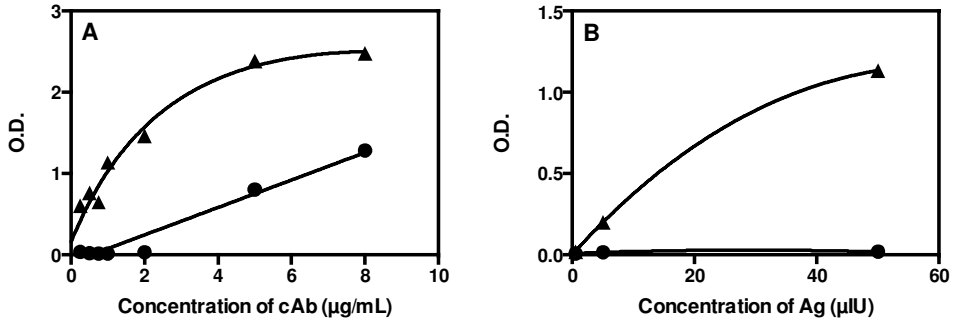

Fig. 6. TSH sandwich assay: Titration of cAb (Ag = 50 µIU/mL) and antigen (cAb = 1 µg/mL) on COC (●) and M&G-COC surfaces (▲).

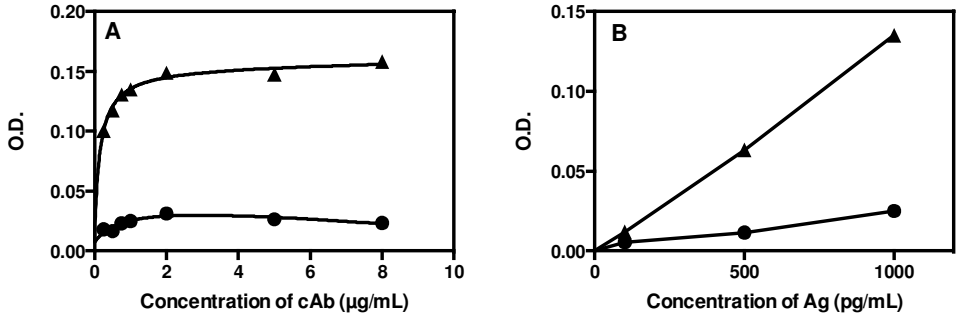

Fig. 7. TnI sandwich assay: Titration of cAb (Ag = 1000 pg/mL) and antigen (cAb = 1 µg/mL) on COC (●) and M&G-COC surfaces (▲).

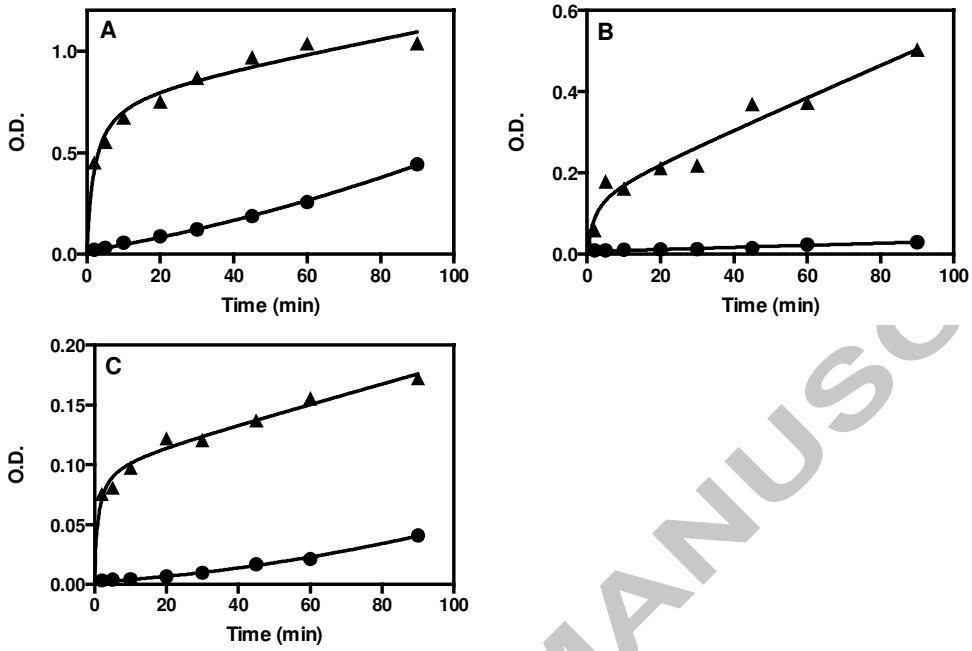

Fig. 8. Coating capture antibody (1 μg/mL) time profiles for (A) TNFα, (B) TSH and (C) TnI measured from sandwich assay on COC (●) and M&G-COC surfaces (▲).

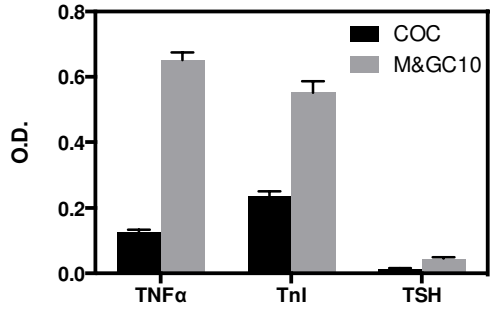

Fig. 9. Performance of TNFα, TnI and TSH sandwich assays on COC and M&GC-COC surfaces over cAb incubation time of 24 hours.

Supplementary Information – Ooi et al.

Table 1. Distribution of carbon, oxygen and chromium components in four wells (Samples 1-4) at three different areas (a = middle, b and c = sides) measured by XPS. The oxygen species is also contributed by the metal complex and increases with chromium content.

Sample	**At. %**		
	C 1s	**O 1s**	**Cr 2p**
1a	86.89	10.54	2.57
1b	85.38	11.78	2.84
1c	86.37	11.02	2.61
2a	94.81	4.36	0.83
2b	96.16	3.21	0.63
2c	95.6	3.78	0.62
3a	97.27	2.29	0.44
3b	95.79	3.79	0.42
3c	97.12	2.43	0.44
4a	92.49	6.42	1.09
4b	91.55	7.15	1.3
4c	90.97	7.46	1.57

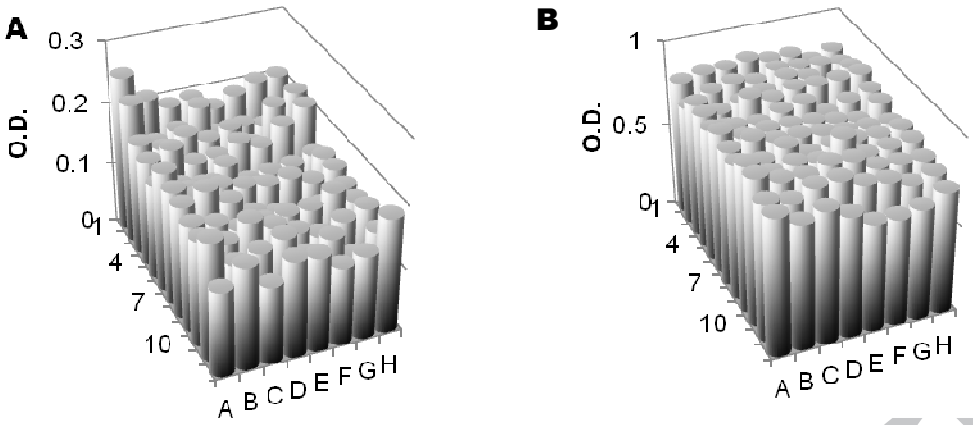

Fig. 1. O.D. measurements across wells depicting the CV of (A) passive COC and (B) M&G-COC plates.

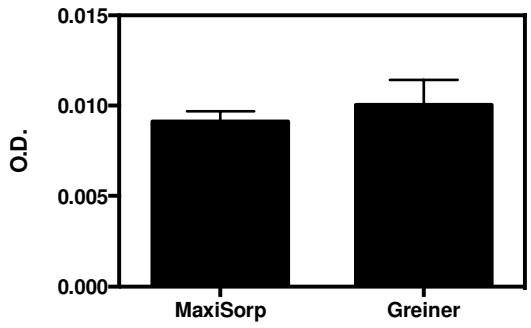

Fig. 2. TnI sandwich assay results on polystyrene microtiter plates, MaxiSorp® and Greiner Bio-One.