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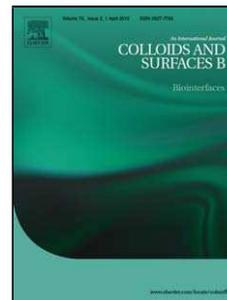
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## Accepted Manuscript

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## Deposition of chemically reactive and repellent sites on biosensor chips for reduced non-specific binding

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### Abstract

The performances of new polymeric materials with excellent optical properties and good machinability, have led the biomedical diagnostics industry to develop cheap disposable biosensor platforms, appropriate for point of care applications. Zeonor, a type of cycloolefin polymers (COP) is one such polymer that presents an excellent platform for biosensor chips. These polymer substrates have to be modified to have suitable physico-chemical properties for immobilizing proteins. In this work, we have demonstrated the amine functionalisation of COP substrates, by Plasma enhanced chemical vapour deposition (PECVD), through codeposition of ethylene diamine and 3-aminopropyltriethoxysilane precursors, for building chemistries on the plastic chip. The elemental composition, adhesion, ageing and reactivity of the plasma polymerized film were examined. The Si-O functionality present in amino silane contributed for a good interfacial adhesion of the coating to COP substrates and also acted as a network building layer for plasma polymerization. Wet chemical modification was then carried out on the

amine functionalized chips to create chemically reactive isothiocyanate sites and protein repellent fluorinated sites on the same chip. The density of the reactive and repellent sites was altered by choosing appropriate mixtures of homofunctional phenyldiisothiocyanate (PDITC), pentafluoroisothiocyanate (5FITC) and phenylisothiocyanate (PITC) compounds. By tailoring the density of reactive binding sites and protein repellent sites, the non specific binding of ssDNA has been decreased to a significant extent.

Keywords: PECVD, amine functionalisation, cyclo olefin copolymer, non specific binding

## Introduction

In point of care diagnostic (POC) device platforms, immobilization of the biorecognition reagents to the polymer surface in a rapid, repeatable and controllable fashion remains a key issue. For a biosensor to work efficiently, biomolecules have to be immobilized on surfaces in their biologically active state with low non specific binding. Zeonor, a type of cycloolefin polymer (COP) is a new generation polymer presenting excellent optical properties, good chemical resistance, ease of fabrication and cost effectiveness suitable for disposable biosensor platforms.<sup>[1,2,3]</sup> However, there is a need for surface modification of the COP substrates to create a suitably reactive and stable surface in an industrially feasible cost effective manner. The wet chemistry methods are usually time consuming and work reproducibly only in few selected solvents.<sup>[4]</sup> The choice of reaction media is further limited in case of the new polymeric material such as COP, as the chemical resistance of the plastic material is retained mostly in protic, polar solvents. All things considered, the increased demand for the quality and the amount of solvents with the resulting increase in the solvent waste makes the wet chemistry

techniques less attractive if bulk quantities of coated substrates are required. The use of gas-phase processes can overcome such drawbacks and represents a class of relatively straightforward methods to functionalize the plastic surface.<sup>[5-9]</sup>

A one step process of surface functionalization, by plasma enhanced chemical vapour deposition (PECVD), is more appealing than the multistep,<sup>[10-13]</sup> wet chemical process. In this work, we demonstrate a plasma polymerisation route to a stable, amine-functionalised surface on COP, and report detailed characterisation of the surface thus prepared. The surface functionalisation was carried out through codeposition of 3-aminopropyltriethoxysilane (APTES) and ethylene diamine (EDA) on COP. PECVD of amine functionalisation using ethylene diamine (EDA) precursor has been demonstrated by Jung et. al.,<sup>[14]</sup> however we in our earlier work have demonstrated that EDA on its own has very poor adhesion to COP substrate.<sup>[15]</sup> The need for siloxane functionality from APTES and the enhancement of amine functionality using co-deposition of APTES+EDA has been demonstrated in our earlier work. The alkoxy silane (APTES) inserted into the olefin bonding and also acted as a network builder for the coating whilst the other component provided the reactive functionality. The surface has been quantitatively characterised using X-ray photoelectron spectroscopy (XPS). An ideal surface material should have a high binding capacity but also show outstanding adhesion, stability and resistance against harsh washing and regeneration conditions. The adhesion strength of the coatings to COP surface was evaluated qualitatively by measuring the nature of chemical bondings present in the coating using Attenuated total reflection - fourier transform infrared spectroscopy (ATR-FTIR) before and after ultrasonication in water, SDS solution and PBS Tween solution. Spectroscopic ellipsometric and

fluorescence scan results confirm the reactivity of deposited APTES with both the carboxyl group of dendrimer G 4.5 and sulfonyl chloride groups of lissamine rhodamine B dyes. Chemically reactive isothiocyanate sites and protein repellent fluorinated sites were deposited on the amine functionalized COP substrates through the use of homofunctional phenyldiisothiocyanate (PDITC), pentafluoroisothiocyanate (5FITC) and phenylisothiocyanate (PITC) compounds. The density of reactive binding and repellent sites were altered by using appropriate PDITC derivatives through incorporation of fluorinated benzene rings, taking advantage of supramolecular assemblies based on  $\pi$ - $\pi$  stacking. The variation in non specific binding with surface chemical modification is reported.

## **Experimental details**

### **Compounds and materials**

DNA used in this study with following sequence 5'-ACG GCA GTG TTT AGC-3' was modified at 5'-end with Cy5 and 3'-end with C6-amine linker. The oligonucleotide was purchased from Eurofin MWG Operon (Ebersberg, Germany) and dissolved in DI water according to the manufacturer instructions before the use. Bare COP slides (Zeonor® 1060R) 75mm X 25mm were obtained from Åmic AB (Uppsala, Sweden). Gold-coated glass (Ti/Au = 2 nm/48 nm, 26 mm x 76 mm, 1 mm thick) slides were purchased from Phasis Sarl (Geneva, Switzerland). 3-aminopropyltriethoxysilane (APTES), ethylenediamine (EDA), phenyl diisothiocyanate (PDITC), acetonitrile, triethylamine, PAMAM dendrimer (generation 4.5), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), hydrochloric acid (HCl) 1.0N, N-Hydroxysuccinimide (NHS),

dimethylformamide (DMF), and xylene were purchased from Sigma Aldrich and used without further treatment. Lissamine rhodamine B sulfonyl chloride was obtained from Invitrogen (Eugene, OR, USA). ssDNA was purchased from MWG Biotech

### **Plasma deposition**

The deposition of amine functional coatings was carried out in a computer controlled PECVD reactor Europlasma, model CD300 (Oudenaarde, Ghent, Belgium). An aluminium 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.<sup>[16]</sup> The powered electrode, was placed slightly below the top of the chamber and the chamber wall was grounded. Also an electrically isolated, water cooled hollow metallic setup placed 10 cm away from the powered electrode was used as the substrate holder. During the process, electric potential from the RF generator is applied to the powered electrode, which in turn excited the gases present in the chamber to a plasma state. The RF generator was connected to the chamber through an automated matching box. The mass flow controllers (MFC) were used to control the flow of argon and oxygen gases. Before each MFC, a shut off valve was installed to avoid leakage of any gas that was not used during the process.

The bare COP slides were cleaned with dry air and then loaded in the chamber. The chamber was pumped down to a base pressure of 20 mTorr. Prior to the deposition, plasma cleaning and activation was carried out using argon (50 sccm) + oxygen (50 sccm) mix plasma (250 watt RF power). After three minutes, the oxygen flow was closed and the RF power reduced to 14 watt. A needle valve, connected to the vacuum chamber,

was used to control the flow of precursor vapours: APTES and EDA. The precursors were stored in two different containers and mixed in the plasma chamber. As the vapour pressure of APTES was less than 10 Torr at 100° C, the APTES container was heated at 80° C and to prevent condensation of APTES in pipelines, the stainless steel supply lines from source to vacuum chamber were also heated at 80° C through a temperature controlled heating tape. The vapour pressure of EDA was 10 Torr at 20° C and hence no external heating was required. The operating pressure was ~ 100 mTorr and the deposition time was 4 minutes.

### **Elemental analysis**

The x-ray photo electron spectroscopic (XPS) data were collected on a Kratos Axis UltraDLD equipped with a hemispherical electron energy analyzer. Spectra were excited using monochromatic Al K $\alpha$  X-rays (1486.69 eV) with the X-ray source operating at 100 W. This instrument illuminates a large area on the surface and then using hybrid magnetic and electrostatic lenses collects photoelectrons from a desired location on the surface. In this case the analysis area was a 220 by 220 micron spot. The measurements were carried out in a normal emission geometry. A charge neutralisation system was used to alleviate sample charge buildup, resulting in a shift of approximately 3 eV to lower binding energy. Survey scans were collected with 160 eV pass energy, whilst core level scans were collected with pass energy of 20 eV. The analysis chamber was at pressures in the 10<sup>-9</sup> Torr range throughout the data collection.

Data analysis was performed using CasaXPS ([www.casaXPS.com](http://www.casaXPS.com)). Shirley backgrounds were used in the peak fitting. Quantification of survey scans utilised relative sensitivity factors supplied with the instrument. Core level data were fitted using

Gaussian-Lorentzian peaks (30 % Lorentzian). The binding energy scale was corrected for the neutraliser shift by using the C 1s signal from saturated hydrocarbon at 285.0 eV as an internal standard.

### **Film adhesion**

The nature of chemical bonding present in the film is determined by a fourier-transform infrared spectroscopy system (Perkin Elmer – Spectrum GX FTIR) used in the attenuated total reflection (ATR) mode (Horizontal accessory equipped with a ZnSe crystal, Perkin Elmer). The detector and the sample chamber are filled with nitrogen gas during measurements. For all data presented, unmodified Zeonor slides are used as background.

### **Surface Wettability**

The film wettability is analysed by measuring the water contact angle of the film surface using the First Ten Angstroms FTA200 contact angle analyser. A high purity HPLC grade water (Sigma Aldrich) is used for the measurement.

### **Reactivity of amine groups on the functionalized COP**

To examine the reactivity of the amines of COP deposited with APTES, ellipsometric measurement and fluorescence scan were carried out. A COP slide was cut into small pieces and dissolved in xylene at 0.25 wt% w/v to make the raw COP solution. The COP solution was filtered through a PTFE filter (pore size 0.2  $\mu\text{m}$ ) (Chromafil Xtra PTFE-20/25 Macherey-Nagel, Duren, Germany) to eliminate the precipitates and dust particles. The filtered COP solution was then spin coated onto the Au-coated glass slide at 1500 rpm in 30 seconds with acceleration in 2 seconds and 2500 in 5 seconds with acceleration in 2 seconds.<sup>[17]</sup> The solvent xylene was naturally evaporated, leaving a thin COP layer of approximately 10 nm in thickness measured by a spectroscopic ellipsometer (UVISEL,

Jobin Yvon Horiba, France). After APTES deposition, a second ellipsometric measurement was carried out. A G 4.5 carboxyl-terminated dendrimer solution of 200  $\mu\text{l}$  was prepared by mixing 105.2  $\mu\text{l}$  of dendrimer, 14.6 mg of EDC, 2.21 mg of NHS, 3.2  $\mu\text{l}$  of HCl and 458  $\mu\text{l}$  of DI water. The dendrimer G 4.5 solution was deposited inside a silicone isolator (Press-to-Seal, Sigma Aldrich, MO, USA) and was left for reaction for 2 h and washed extensively with PBS Tween solution and rinsed with DI water and finally dried in a  $\text{N}_2$  stream and a third spectroscopic ellipsometric measurement was carried out.

Blank COP slides deposited with APTES were used in the fluorescence scans since they are transparent. Lissamine rhodamine B fluorophore was dissolved in DMF/DI water at  $1.3 \times 10^{-7}$  M to make a dye solution. Droplets (10  $\mu\text{l}$  each) of the dye solution were pipetted onto amine functionalized COP slides. The dye droplets were allowed to react for 1 h and then the slides were washed extensively three times with PBS Tween buffer. Each time, the slides were scanned in a fluorescence microarray scanner (ScanArray Gx, PerkinElmer, USA).

### **DNA attachment**

Amino-reactive slides were prepared using APTES+EDA coated slides by incubation with 1,4-phenyldiisothiocyanate (PDITC) in DMF : pyridine mixture (9:1) for 2 hours. The slides were then washed with DMF, MeOH and dried under stream of  $\text{N}_2$ . One set of such PDITC activated slides was subsequently blocked in blocking buffer (containing 5mM of  $\text{H}_2\text{N-PEG}_4\text{-COOH}$ ) for 4 hours. Afterwards, ssDNA modified at 5'-terminus with amino modifier and 3'-terminus with FITC was diluted in printing buffer (10% glycerol, 0.1 M sodium citrate at pH=8.5) and micro spotted (50 drops, 0.6 nL each) onto both the PDITC activated slide and the blocked slide using a microarray spotter. The

coupling reaction was allowed to proceed for 4 hours at 25°C in a humid chamber. The arrayed slides were then washed with 0.1% SDS followed by extensive wash with DI water.

## Results and Discussion

X-ray photoelectron spectroscopy (XPS) was carried out for a quantitative elemental analysis of the plasma deposited coatings. The elements present in the coating C, N, O, Si were detected using the XPS survey scan, **Fig 1(left)**. To study the various bonding environments, a high resolution scan for the core level photoemission spectra of C 1s peak was carried out, **Fig 1(right)**. The C 1s spectra show a saturated hydrocarbon peak (285.0 eV) and two additional peaks to higher binding energy at 286.4 eV ( $\sim +1.4$ ) and 288.4 eV ( $\sim +3.4$ ), which are best associated to C-N bonding and  $-\text{CONH}_2$  groups, respectively.

The nature of chemical bonding present in the coatings was determined using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). The vibration bands associated to Si-O-C and Si-O-Si vibrations were observed indicating the formation of a siloxane network **Fig 2**.<sup>[18-20]</sup> The ATR-FTIR spectra also confirms the formation of amino-compounds on the Zeonor® surface. The shoulders around  $1683\text{ cm}^{-1}$  and  $1705\text{ cm}^{-1}$  could be assigned to the C-O vibration mode of amides and a broad peak centered around  $1644\text{ cm}^{-1}$  can be attributed to free amines.<sup>[21-24]</sup> Another peak around  $1554\text{ cm}^{-1}$  can be attributed to N-H bend of  $\text{NH}_2$  hydrogen-bonded to Si-OH. The ATR-FTIR measurements taken before and after ultrasonication in water, SDS and PBS Tween also demonstrated that the coating adhesion to COP substrate is good.

The surface wettability was measured using contact angle analyzer and the ageing study was carried out by measuring the contact angle variation over a period of 5 weeks. The contact angle varied from about  $58^\circ$  up to  $64^\circ$  over a period of 5 weeks, **Fig.3**.

Spectroscopic ellipsometric measurements on COP-coated Au-coated glass slide were performed to confirm the successful deposition of APTES and G 4.5 dendrimer on the COP-coated substrates. After APTES deposition, the  $\Delta$  spectrum shifted downward from “COP/Au” to “APTES/COP/Au” as shown in **Fig. 4**. The ellipsometric fitting confirmed an APTES layer of 8-9 nm was successfully deposited on the COP/Au slide. The activity of the amines functionalized COP-coated Au-coated glass slides were further demonstrated by its capability to react with the carboxyl groups of the dendrimer G 4.5 after washing extensively (**Fig.4**). The downward shift of  $\Delta$  spectrum from “APTES/COP/Au” to “G 4.5/APTES/COP/Au” corresponds to an increase in thickness of 2-3 nm which was caused by the deposition of the dendrimer G 4.5. With the successful deposition of dendrimer G 4.5, it is expected that this high density of carboxyl functional groups will be useful DNA or protein immobilization for sensor surfaces. **Fig. 5** shows the fluorescence scan images of a dye spot on the amines functionalized COP substrates. From Fig 5, the presence of fluorescence signal even after 3 washes indicates that the Lissamine Rhodamine B sulfonyl chloride dyes were covalently linked to the amine coated COP surface. The interaction between the dye and the surface is through sulfonyl chloride and amine interactions.

Surface Chemistry		Surface fidelity [*]			
		25 $\mu$ M	5 $\mu$ M	2.5 $\mu$ M	0.5 $\mu$ M
1st layer	2nd layer				
Amines	PDITC	8.2	8.8	8.9	12.9
Amines	PDITC:5FITC	24.6	35.5	41.5	49.3
Amines	PDITC:5FITC:PITC	36.8	53.7	84.0	165.4
Nexterion® Slide E		18.3	-	-	-

\* Calculated as a ratio between the relative fluorescence units of uncapped over capped slides

**Table 1.** Fluorescence intensity of labelled ssDNA attached to amine functionalised coatings modified by PDITC derivatives to effectively control the density of reactive binding sites.

The amino functionalized substrates were further modified with a commonly used homo-functional bilinker, PDITC, to allow immobilization of amino terminated DNA. The density of reactive binding sites was altered by using two other phenylisothiocyanate derivatives that have the potential to form well organized assemblies driven by  $\pi$ - $\pi$  stacking as depicted on **Fig. 6**. A corresponding variation in DNA attachment was observed, **Fig. 7**. The non-specific binding analysis was assessed by capping the reactive surface with a short PEG-like amino acid as blocking agent (H<sub>2</sub>N-PEG<sub>4</sub>-COOH) (**Fig.7 checked squares**). Such compound was preferred as its amine is very reactive with the isothiocyanate group on the substrate and it is possible to deprotonate the carboxylic acid forming a contact layer with negative charge. We reasoned that the charge repulsions between the surface and the negatively charged DNA are the most important interactions for the reduction of non-specific binding on DNA microarrays.

The effectiveness of the blocking reagent was demonstrated by comparing the fluorescence intensities measured from both capped and uncapped surfaces (**Table 1**).

The ratio between the covalently linked DNA on the uncapped surface and the physisorbed DNA on the capped part is shown as surface fidelity and can be also viewed as signal-to-noise ratio. As expected, the binding capacity of the surface comprised of the mixture of PDITC : 5FITC : PITC was the lowest of the measured samples, however, it also showed the highest surface fidelity throughout the whole range of DNA concentrations. We believe that the reason for further reduction in non-specific binding on this surface where the reactive site is co-diluted with fluorinated compound is the partial negative charge coming from the five electronegative fluorines attached to the conjugated  $\pi$  system. Moreover, the surfaces prepared by co-deposition of PDITC and its fluorinated derivatives compared favorably with commercially available Nexterion® Slide E (Table 1 at 25  $\mu$ M). PDITC:5FITC and PDITC:5FITC:PITC substrates showed 26% and 50% increase in surface fidelity when compared to the commercial slide. Much of this improvement is attributed to the effect of the partial negative charge of the fluorinated PDITC derivatives. Such surface repels the intrinsically, negatively charged DNA strand, thus effectively reducing the background response.

Overall, immobilization experiments using a short amino terminated oligonucleotide proved that amine functional coatings deposited using APTES+EDA have good adhesion to COP substrate and excellent binding capacity. And by tailoring the density of reactive and repellent sites, the non-specific binding could be decreased significantly.

## Conclusion

Amine functional coatings have been successfully deposited by plasma enhanced chemical vapour deposition on cyclo olefin copolymer substrates with good adhesion. X-

ray photoelectron spectroscopic measurement showed the presence of amine as well as amide functionalities. The ATR-FTIR spectroscopic measurement was in agreement with the XPS measurement and the presence of siloxane functionality, that was essential for film adhesion to COP, was also confirmed. The ATR-FTIR measurements carried out before and after ultrasonication in water, SDS solution and PBS Tween solution demonstrated good adhesion of the plasma deposited coatings to COP substrate. The ellipsometric measurement and fluorescence scans confirmed the reactivity of the amines group with both carboxyl groups of dendrimer G 4.5 and sulfonyl chloride groups of Lissamine Rhodamine B. The reactive binding sites were tailored using phenylisothiocyanate derivatives for DNA attachment. Despite the reduction in binding capacity of the mixed PDITC : 5FITC : PITC sample, the surface fidelity was the highest, representing a very promising approach to fabricate substrates for DNA hybridization assays in the next generation biodiagnostics device.

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## Figure Captions

**Figure 1.** (left) XPS survey scan collected with 160 eV pass energy, (right) C 1s corelevel X-ray photoelectron spectroscopy (XPS) of amine functionalised zeonor

**Figure 2.** ATR-FTIR spectra of amine functionalised coatings on zeonor, taken before and after sonication in water, SDS and PBS Tween.

**Figure 3.** Contact angle variation measured over a period of 5 weeks time.

**Figure 4.** Ellipsometric  $\Delta$  angle spectra of Au-coated glass substrates after COP, APTES and dendrimer G 4.5 depositions

**Figure 5.** Fluorescence scan images of APTES-functionalized COP substrate deposited with Lissamine Rhodamine 5 after three washes (a-c).

**Figure 6.** Schematic showing the surface chemistry and the supramolecular assemblies of homofunctional phenyldiisothiocyanate (PDITC), pentafluoroisothiocyanate (5FITC) and phenylisothiocyanate (PITC).

**Figure 7.** Fluorescence intensity of labelled ssDNA attached to amine functionalised coatings modified by PDITC derivatives to effectively control the density of reactive binding sites.

Figure 1

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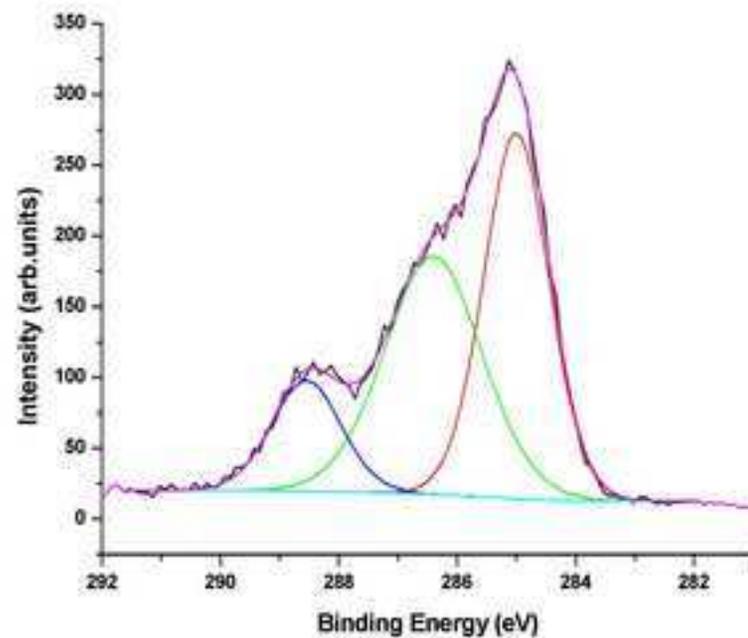
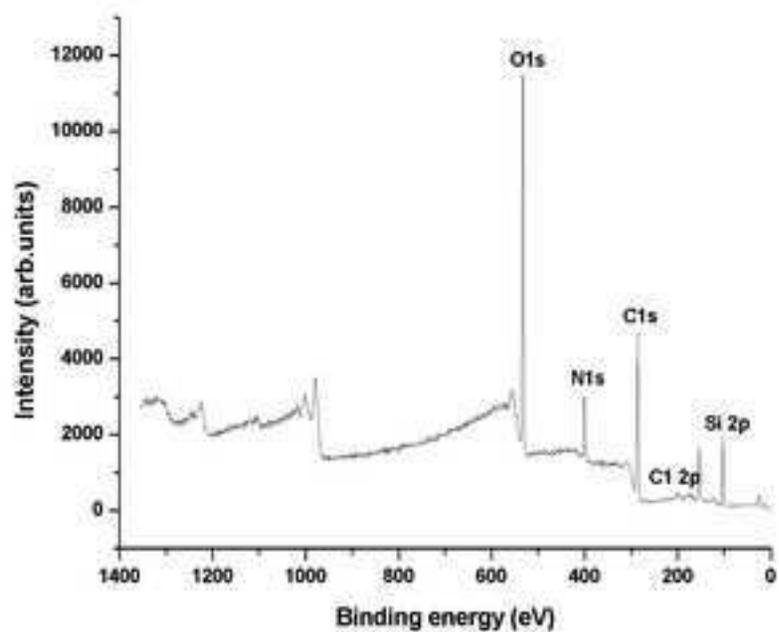


Figure 2

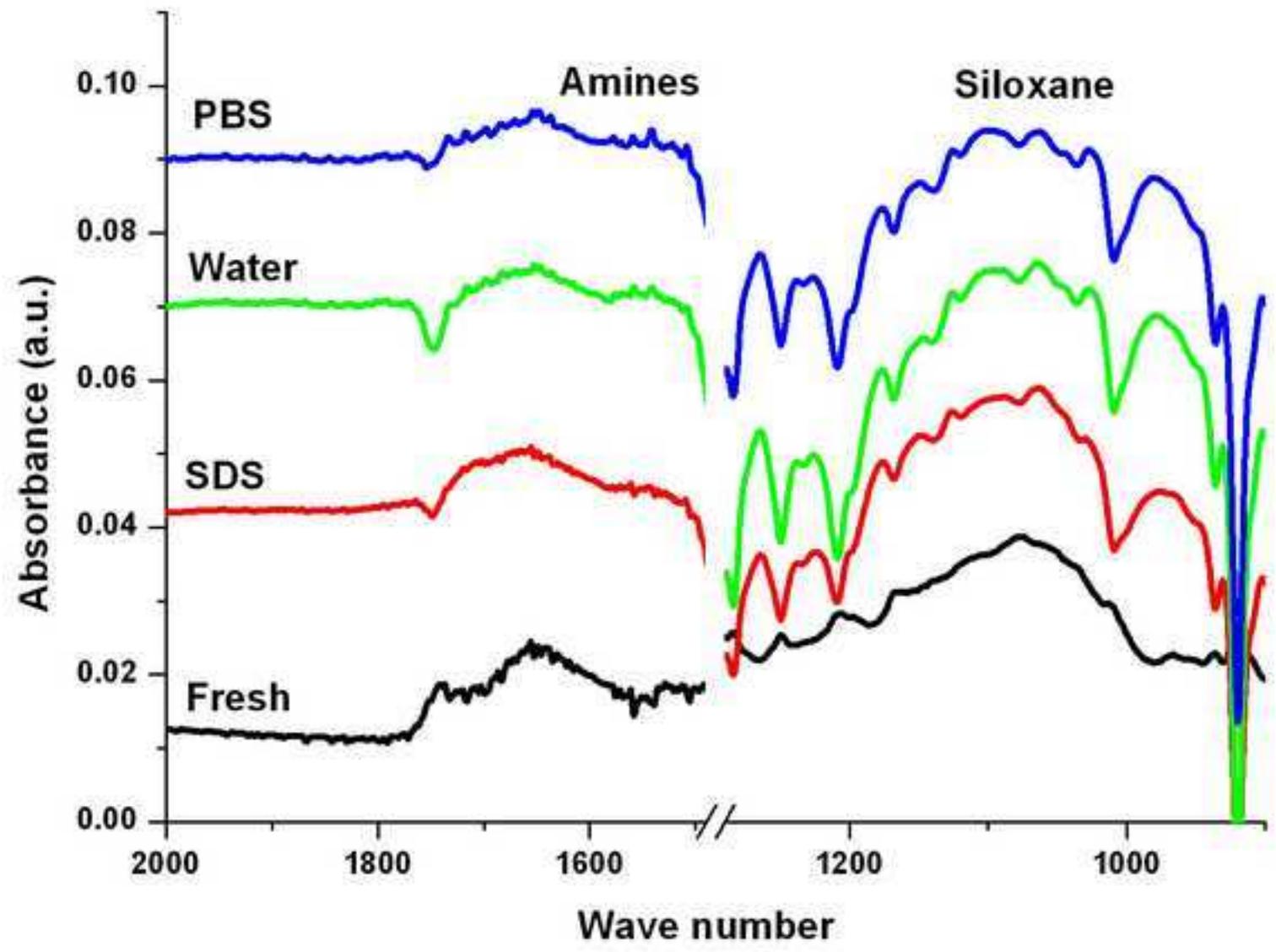


Figure 3

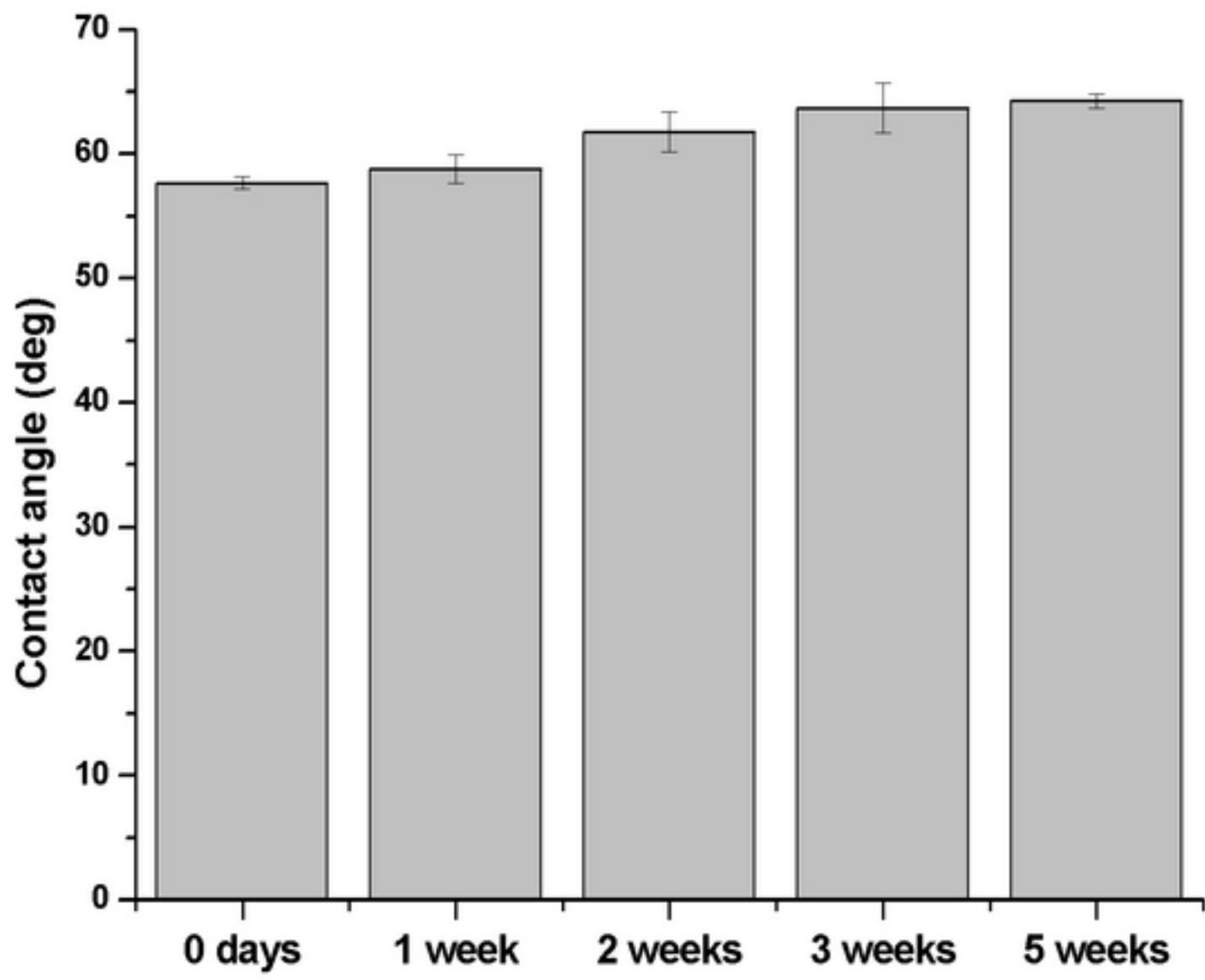
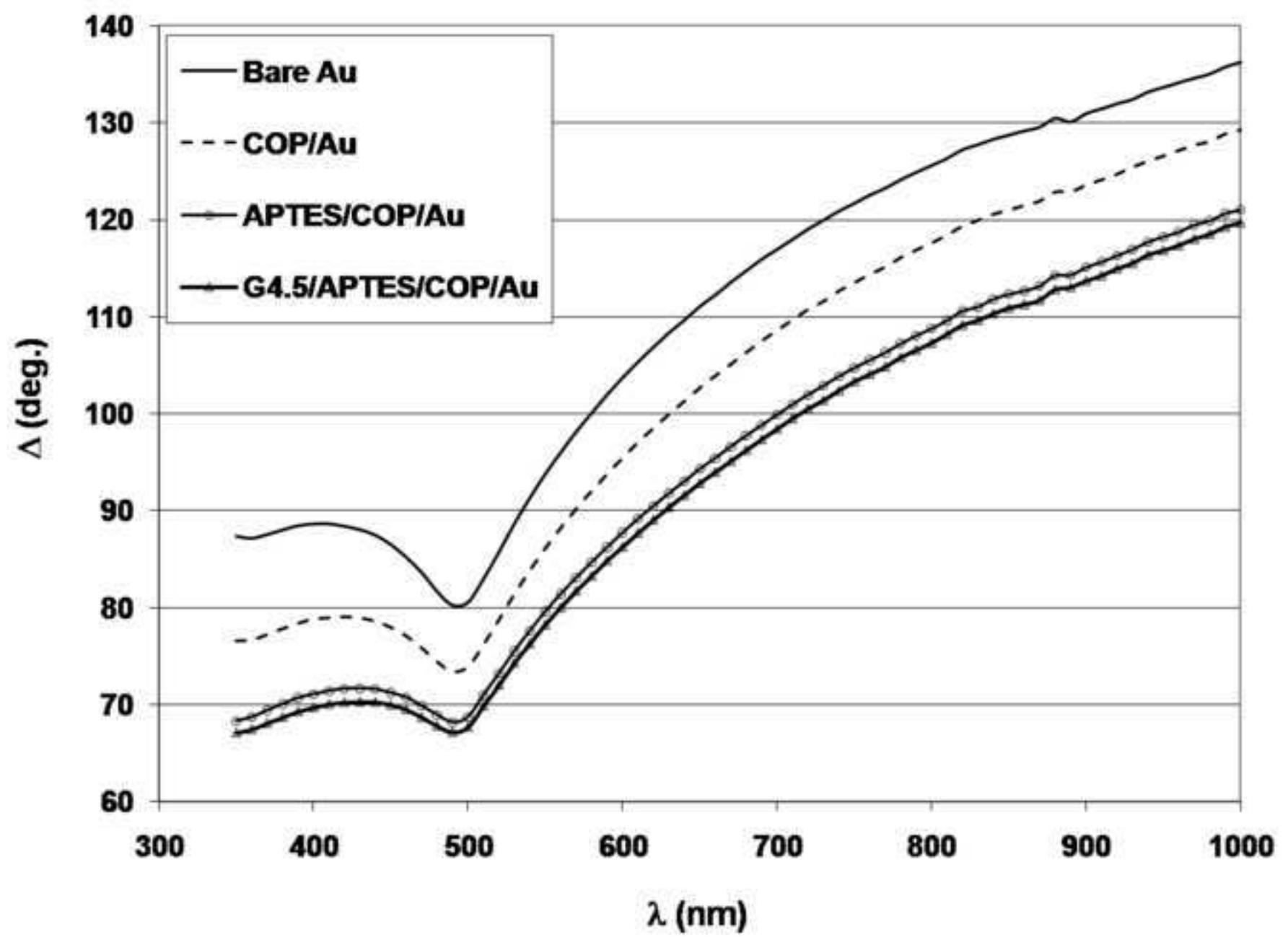
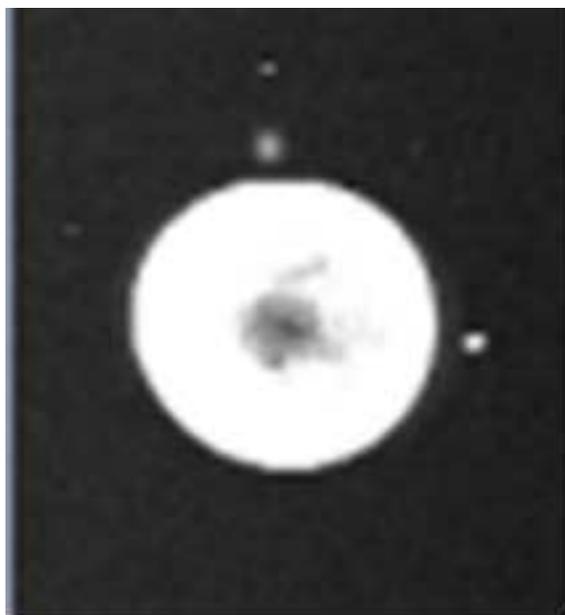


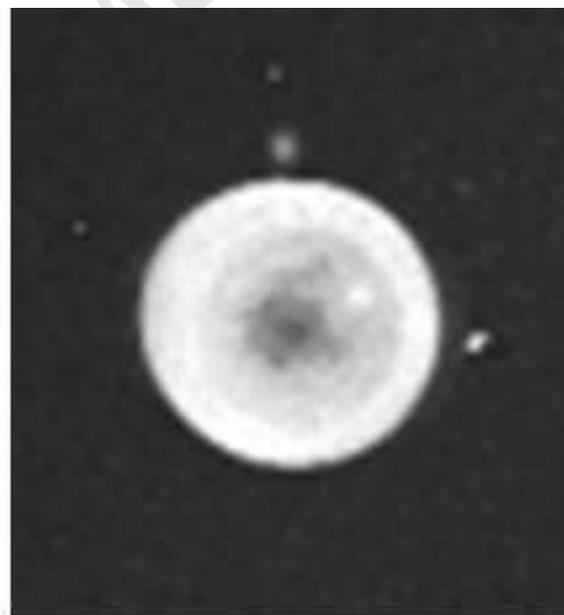
Figure 4



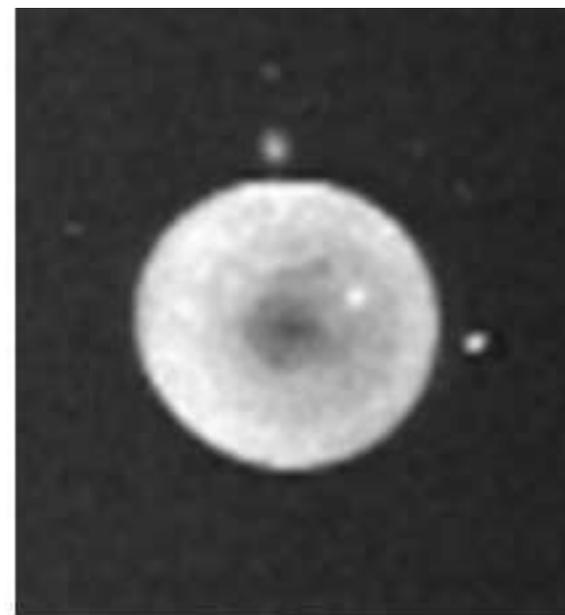
Manuscript



**(a)**



**(b)**



**(c)**

Figure 6

IScript

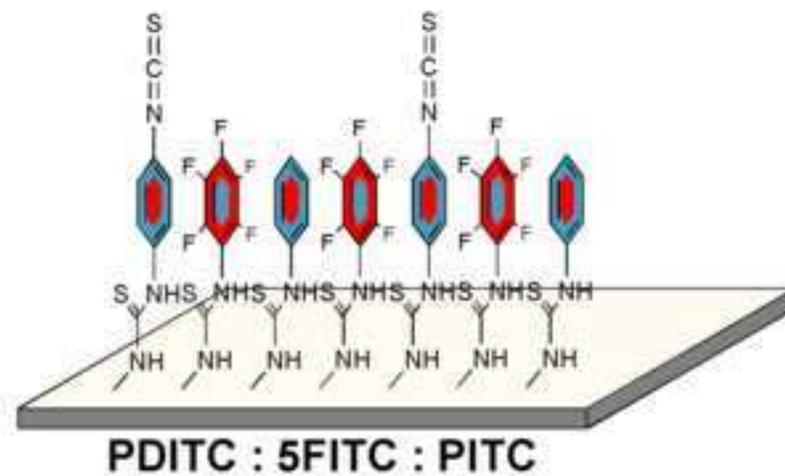
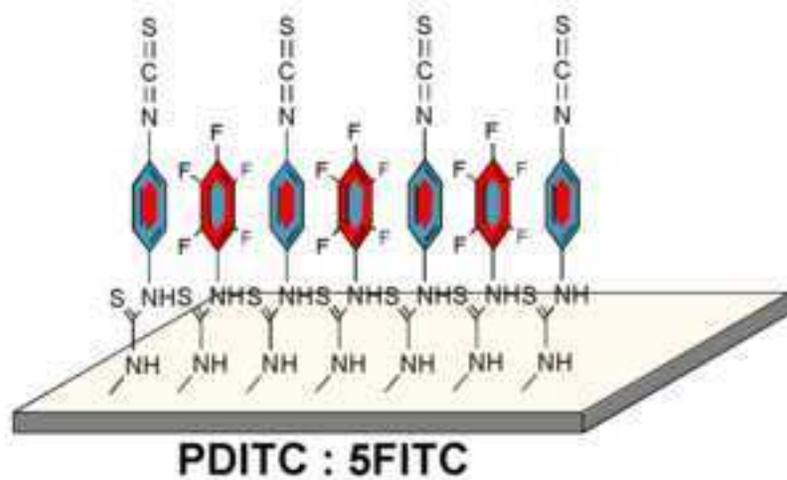
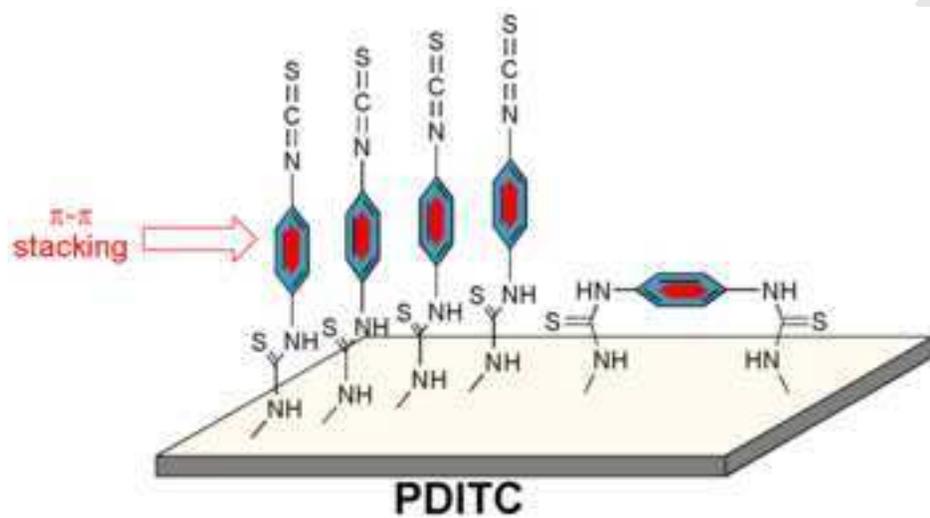


Figure 7

