Supporting information

Nitric oxide releasing plasma polymer coating with bacteriostatic properties and no cytotoxic side effects

Thomas D. Michl[a,b], Bryan R. Coad[b], Michael Doran[c], Michael Osiecki[c], Morteza Hasanzadeh Kafshgari[b], Nicolas H. Voelcker[b], Amanda Hüsler[a], Krasimir Vasilev[b], Hans J. Griesser[b]

[a] Ian Wark Research Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
[b] Mawson Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
[c] Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, QLD 4059, Australia

Experimental details

Materials

Isopentyl nitrite 96% (IPN), sodium nitrite ≥96%, Potassium hydroxide, Griess' reagent and crystal violet solution was obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. The coverslips were made by stamping out a 0.05 mm thick PET foil supplied from Goodfellow Cambridge Ltd. (Huntingdon, England) with a 13 mm punch and were washed with Ethanol twice prior to their use. Cold filterable Oxoid ™ Tryptic Soy Broth (TSB, CM1065) and Oxoid ™ Nutrient Agar (CM0003) were obtained from Thermo fisher (Scoresby, Australia). Phosphate buffered saline (PBS, SLBB6584) tablets and crystal violet stain were obtained from Sigma Aldrich. The BacLight ™ staining kit was obtained from Invitrogen (Mulgrave, Australia) and was used according to its specifications. All chemicals listed were used without further purificatoin and Milli-Q ™ filtered water was used to prepare aqueous solutions, with the aforementioned chemicals, according to their recommended concentration. The TSB and PBS were autoclaved prior to their usage in order to ensure sterility. The bacteria strain in question was *Staphylococcus epidermidis* ATCC® 35984 ™ and the used 24 well plates were NUNC™ brand, purchased from Thermo fisher.

Plasma polymerization and post-plasma processing

The reactor design used and the means of depositing a thin plasma polymer coating was performed as previously reported.[1,2] The plasma polymer deposition was carried out as follows: PET coverslips with a diameter of 13 mmand one silicon wafer were placed into the plasma chamber; the coating on top of the silicon wafer was used for

determination of polymer thickness by Ellipsometry afterwards. First, the plasma apparatus was pumped down to its base pressure of 30 mTorr to establish vacuum. Afterwards, the inlet needle-valve was slowly opened to allow for atmospheric air flowing into the chamber and the valve was adjusted so the pressure in the plasma chamber stabilized at 200 mTorr. Afterwards, an air plasma was struck at a RF frequency of 13.56 MHz, a vapour pressure of 200 mTorr, input power of 50 W and treatment time of 1 minute. This was done to oxidize the surface of the PET coverslips in order to ensure better bonding with the subsequent plasma polymer; followed by pumping down the plasma chamber down to its base pressure of 30 mTorr. IPN vapours were introduced into the plasma chamber and by adjusting the inflow, the pressure was let stabilize at 200 mTorr for at least one minute. Plasma polymerization was initiated with a RF frequency of 13.56 MHz, at a vapour pressure of 200 mTorr, input power of 18W and deposition time of 2 minutes. Afterwards, a pulse generator was connected to generate a steady pulsed plasma. The pulse period was 20 ms with an on period of 1 ms. The pulsed plasma was continued for 90 minutes at the above described frequency, power and pressure.

Antibacterial testing

Time lapse

1 mL of a frozen aliquot (-20 °C) of bacteria were thawed, followed by plating a small portion out on an agar plate and incubated overnight at 37 °C. The frozen 1 mL aliquots were replaced new ones from the -78 °C freezer once a month in order to ensure the genetic pedigree of the bacteria. The next day a single colony was picked and seeded into 10 mL of TSB and incubated at 37 °C for at least 18 h. 10 mL of this solution were centrifugated at 1000rpm for 10 minutes, removing the supernatant and replacing it with PBS; followed by two subsequent centrifugation/wash cycles with PBS. The optical density of the bacteria in PBS solution was determined at 600 nm and adjusted with fresh PBS so its optical density corresponded to OD600=0.2 which approximately corresponds to a bacterial concentration of 10^8 CFU/mL. This solution was further diluted with PBS to 10^6 CFU/mL. The IPN plasma polymer coated and air plasma only treated PET coverslips as reference were placed in a 24-well plate and inoculated with 600 µl of the bacteria solution. The well plate was rhythmically shaken at a frequency of 2 Hz and incubated for 1h at a temperature of 37 °C to ensure the homogenous attachment of bacteria to the surfaces. Afterwards, the bacteria solution was drawn off and the samples were washed twice with 600 µl of PBS; followed by the addition of 600 µl of TSB into each well. For clarity sake, this time point was defined as “0 h” and the samples were incubated further under agitation. The samples for analysis were removed from incubation at set time intervals and placed into a separate 24 well plate and inoculated with 300 µl of BacLight ™ solution which was previously prepared according to specification. After 20 minutes of

incubation at room temperature, the samples were washed three times with an excess of deionized water and stored in 600 µl of deionized water to prevent the stain drying out, which would cause an alteration of the results. One at a time, the samples were imagined with a Nikon Eclipse Ni ™ microscope, equipped with a green/red filter and with the Nikon digital sight DS-L3 ™ at a 490 nm excitation wavelength. Imagined samples were refrigerated at 4°C to stop any growth. This procedure was repeated for all samples after the specified time interval. Upon all samples were imagined, the supernatant was removed, replaced with 600 µl of crystal violet stain and incubated for 20 minutes. This was followed by a washing with an excess of deionized water and placement into a fresh wellplate. Pictures were taken using a Samsung Galaxy S2.

Cytotoxic testing

Cell Culture

Human bone marrow aspirate were obtained with written consequent and ethical approval. Every 5 ml bone marrow aspirate was diluted to 35 ml with Phosphate buffer saline (PBS, Invitrogen). The mononuclear cells are isolated using Ficoll-Paque (GE Healthcare) density gradient at 400g for 20 minutes. The buffy layer was removed and washed in PBS and resuspended in low glucose DMEM (Invitrogen) supplemented with 10 % fetal bovine serum (Thermofisher) and 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies) referred to DMEM 10 % FBS from hereon and seeded into a single T175 flask (Nunc). The cells were then incubated in 20 % oxygen, 5 % CO_2 at 37 °C for 24 hours to allow adherent cells to attach. The media was then replaced with fresh media and the flask was placed into a hypoxic incubator (2 % oxygen and 5% CO2 at 37 °C). The media was replaced twice a week until the monolayer was 80-90 % confluent before passaging.

Kg1a myeloid leukaemia cell line was sourced from ATCC and was maintained in RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 100 units/mL penicillin, and 100 µg/mL streptomycin (referred to RPMI 10% FBS from here on) in a T25 flask and incubated at standard cell culture conditions (5% CO2 at 37 ^{0}C). The media was changed twice weekly and the cells were passage when reach 80-90% confluent.

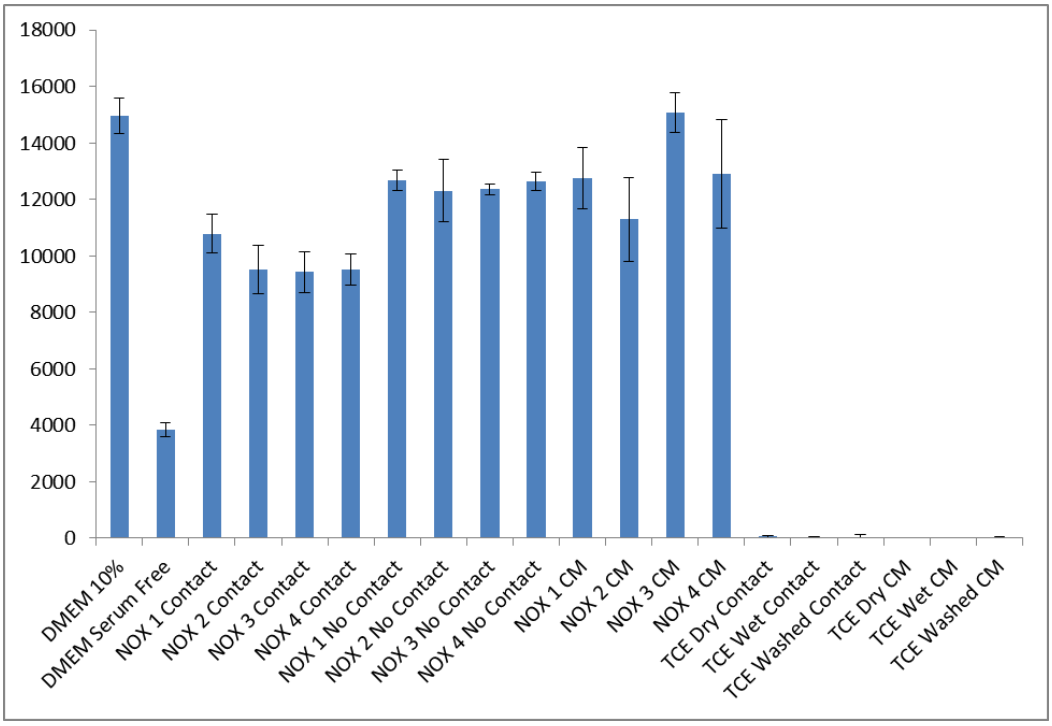
MSC cytoxicity assay

Passage 4 MSCs were seeded at 2000 cells/cm^2 suspended in DMEM 10 % FBS in a 24 well plate that had the surface modified (n=4). For the controls the 2000 cell/cm^2 are seeded into a normal tissue culture treated 24 well plate suspended in DMEM 10 % FBS for the positive growth control (n=4) and serum free DMEM for the negative control (n=4). For a the non-contact controls, 2000 cells/cm^2 were seeded in DMEM 10 % FBS into normal tissue culture

plate but with surface modified insert place vertically into the well (n=4) and 2000 cell/cm^2 were seeded in conditioned DMEM 10 % FBS that has been in contact with the modified surface for 24 hours in an incubator (5 % CO_2 at 37 °C) (n=4). The cell was allowed to grow for 4 days in standard tissue culture conditions (37 °C and 5 % CO_2). The cells were quantified by the metabolic probe AlamarBlue (Invitrogen). The media was replaced with a 1:50 dilution of AlamarBlue in DMEM 10 % FBS and incubated for 3 hours. The fluorescence signal of the AlamarBlue in media was measured in a plate reader (FLUOstar Omega, BMG Labtech, Germany) along with a cell titration to infer cell numbers using excitation and emission filters of 544 and 590.

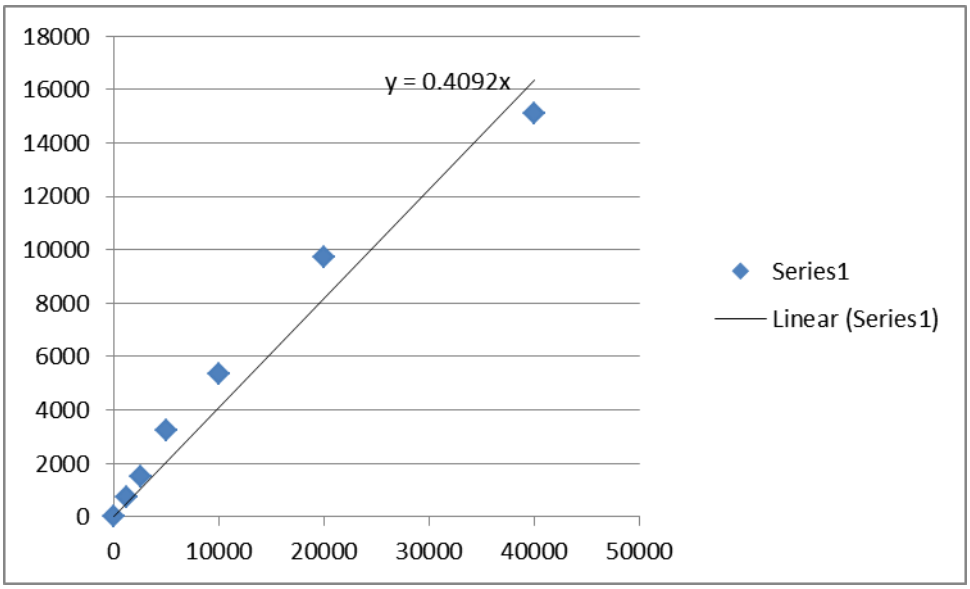
KG1a cytoxicity assay

Kg1a were seeded at 20,000 cell/ml suspended in serum free media X-VIVO 15 (Lonza) in a 24 well plate that had the surface modified (n=4). For the controls the 20,000 cell/ml are seeded into a normal tissue culture treated 24 well plate suspended in X-VIVO 15 for the positive growth control (n=4) and serum free RPMI for the negative control (n=4). For a the non-contact controls, 20,000 cells/ml were seeded in X-VIVO 15 into normal tissue culture plate but with surface modified insert place vertically into the well (n=4) and were seeded in conditioned X-VIVO 15 that has been in contact with the modified surface for 24 hours (n=4). The cells were allowed to grow for 4 days in standard tissue culture conditions (37°C and 5% CO_2). The cells were counted by flow cytometry (FC500, Beckman Coulter, USA) using Flow-CountTM Fluorospheres (Beckman Coulter) with 3 µM Propidium Iodide to gate out the dead cells.



Cytoxicity Standards

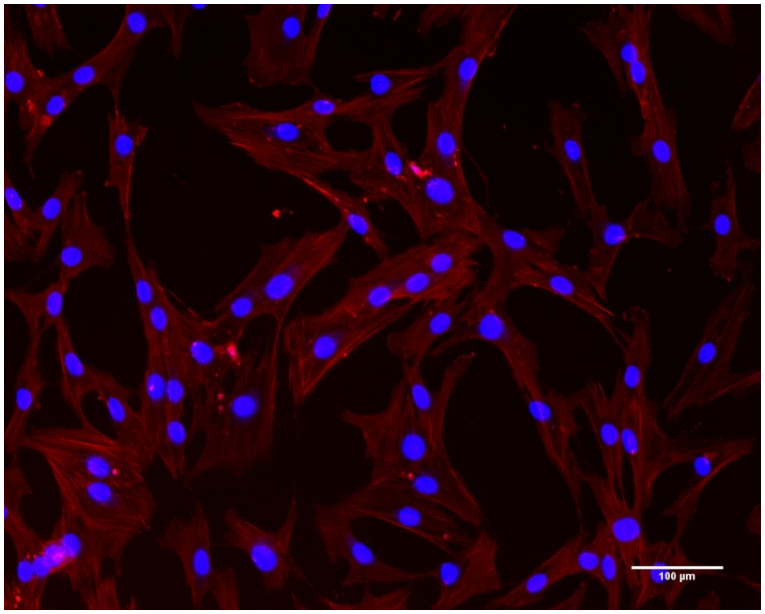
For the MSC 2000 cells/cm^2 suspended in DMEM 10% FBS in a 24 well plate was titrated with PBS and ethanol to give a dilution and toxicity curve. For the KG1a, the cells were seeded at 20,000 cells/ml suspended in X-VIVO or RPMI 10% FBS was tirated with PBS and ethanol. The cells were allowed to grow for 4 days and the cell number quantified by the AlmarBlue method for the MSC and by flow cytometry for the KG1a.



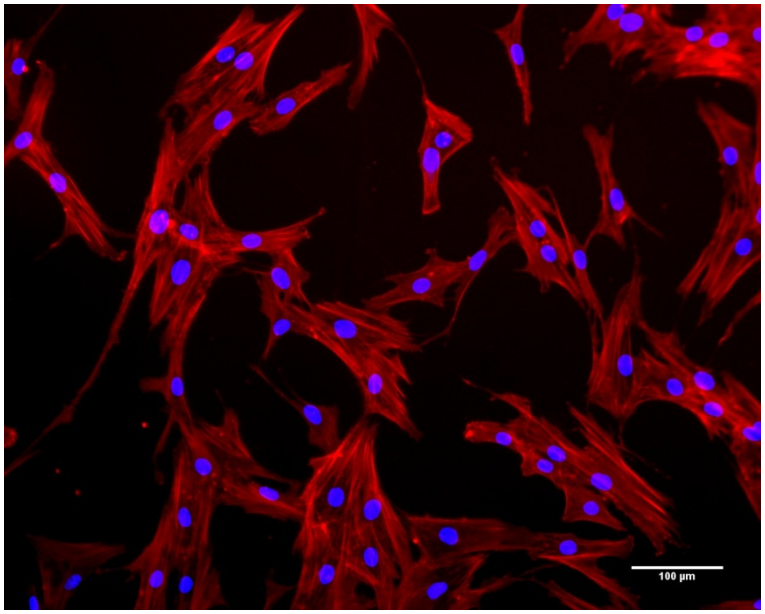
Cell imaging:

MSC monolayers were fixed by submerging in 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes at RT. Cells were washed twice with PBS, and then actin stained (red) with Alexo Fluor 594 phalloidin (Life Technologies) and nuclei stained with DAPI (Life Technologies) as per the manufacturers instructions. Stained cells were imaged using a ECLIPSE Ti epifluorescent microscope (Nikon, Japan) coupled to a Nikon DS-Qi1Mc camera.

TCP



IPN



Analytical methods

XPS

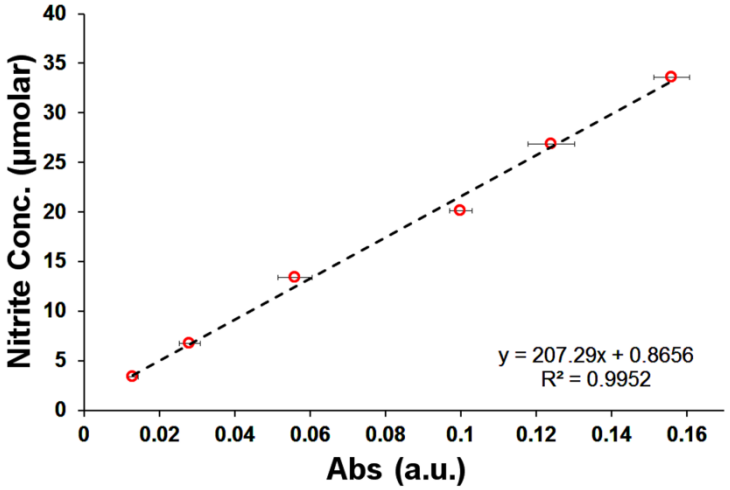
The surface analysis was done with a Kratos Axis Ultra DLD spectrometer, utilizing a monochromatic Al Kα X-ray source running at 225W, corresponding to an energy of 1486.6 eV. The area of analysis was 0.3*0.7 mm and an internal flood gun was used to surpress the charging of the samples. Survey spectra were collected at 160 eV pass energy with steps of 0.5 eV and a dwell time of 55 ms. High resolution spectra were collected at 20 eV pass energy an 0.1 eV steps for O 1s, N 1s and C 1s. The data was processed and analysed with CasaXPS (ver.2.3.16 Casa Software Ltd.) utilizing Shirley baseline correction. To compensated for charging effects, all spectra were offset the C1 peak corresponded to 284.8 eV. Atomic percentages were rounded to one decimal after the coma.

Ellipsometry

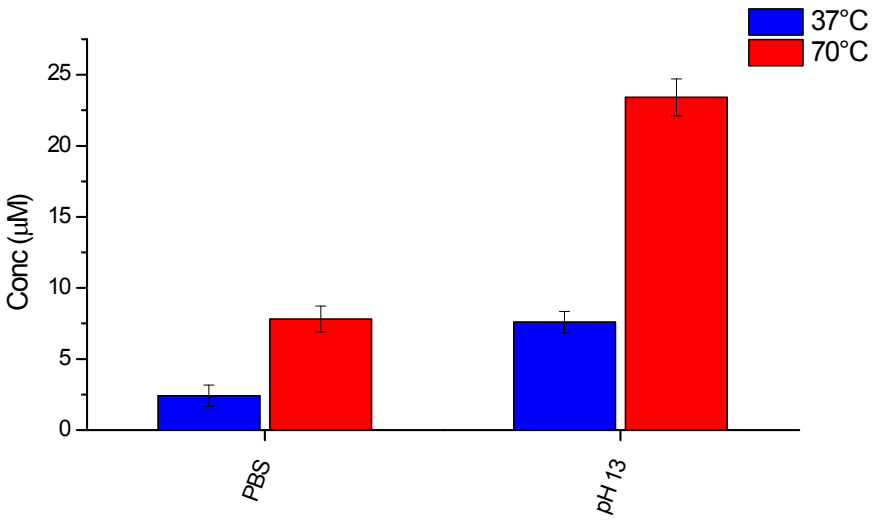
The thickness of the deposited IPN plasma polymer was determined using a J.A. Woollam (Model MC-200) V-Vase ellipsometer. For this purpose IPN was deposited onto a silicon waver under standard conditions, as described above; followed by ellipsometry measurement over a wavelength range of 400-1100 nm in 10 nm steps at alignment angles of 65°, 70° and 75°. The experimental data was fitted using the supplied modelling software WVASE32 (Ver. 3.770), utilizing a two layer Cauchy model. By refining the optical parameters, the mean squared error of the fit was minimized and the plasma polymer thickness was obtained.

Colorimetric assay of nitrite

The amount of nitrite compounds (oxidization product of the nitric oxide) was measured by the Griess reaction. To detect released nitrites, sulfanilic acid of the Griess reagent reacts with nitrites to form a diazonium salt, which subsequently reacts with N-alpha-naphthyl-ethylenediamine to express a final pink azo dye [1]. A calibration curve was prepared using dilutions of sodium nitrite between 0.43-65 μM in PBS (pH 7.4, ambient temperature) mixed with equal volumes of the prepared Griess reagent according to the manufacturer's instructions.

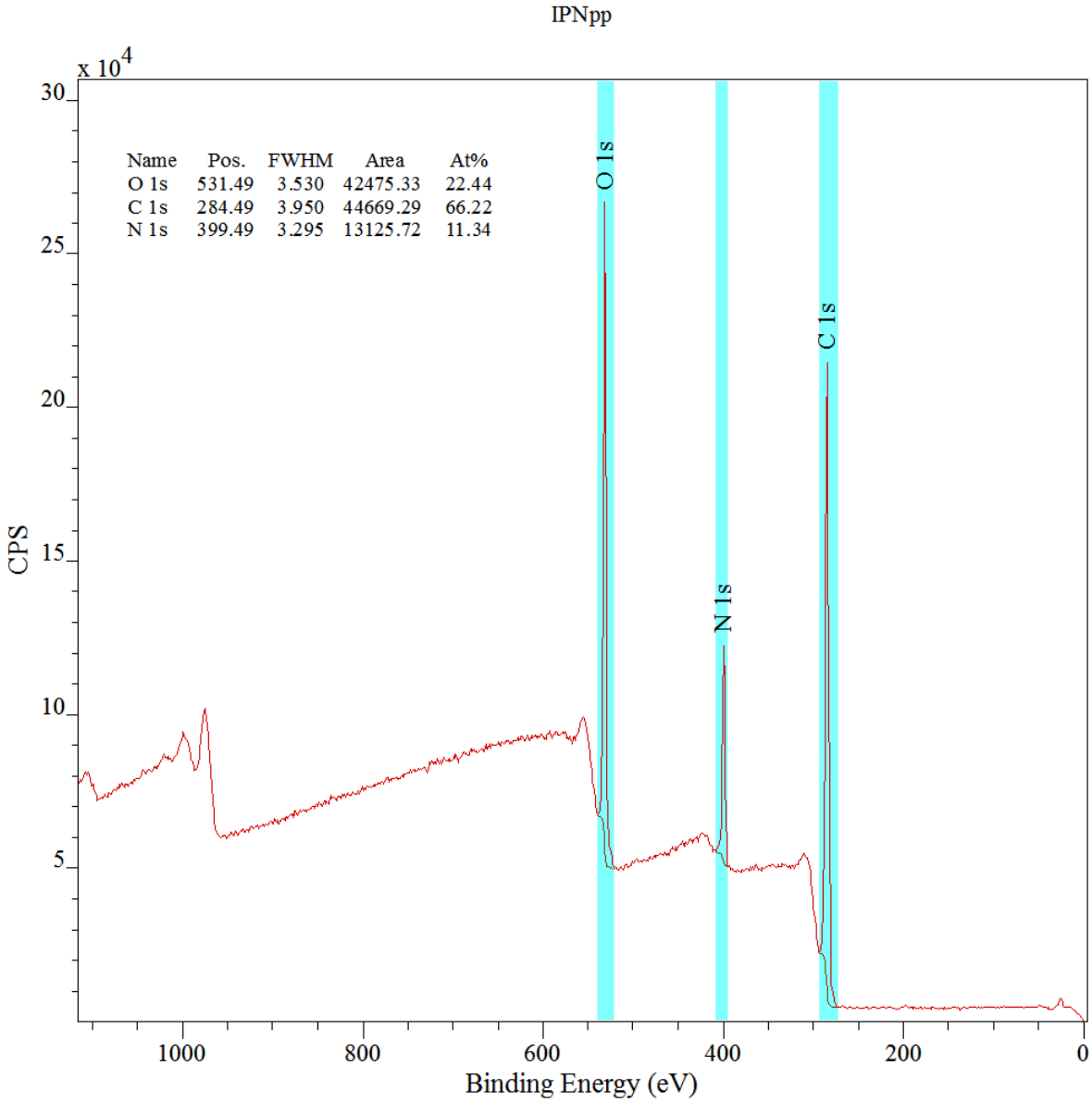


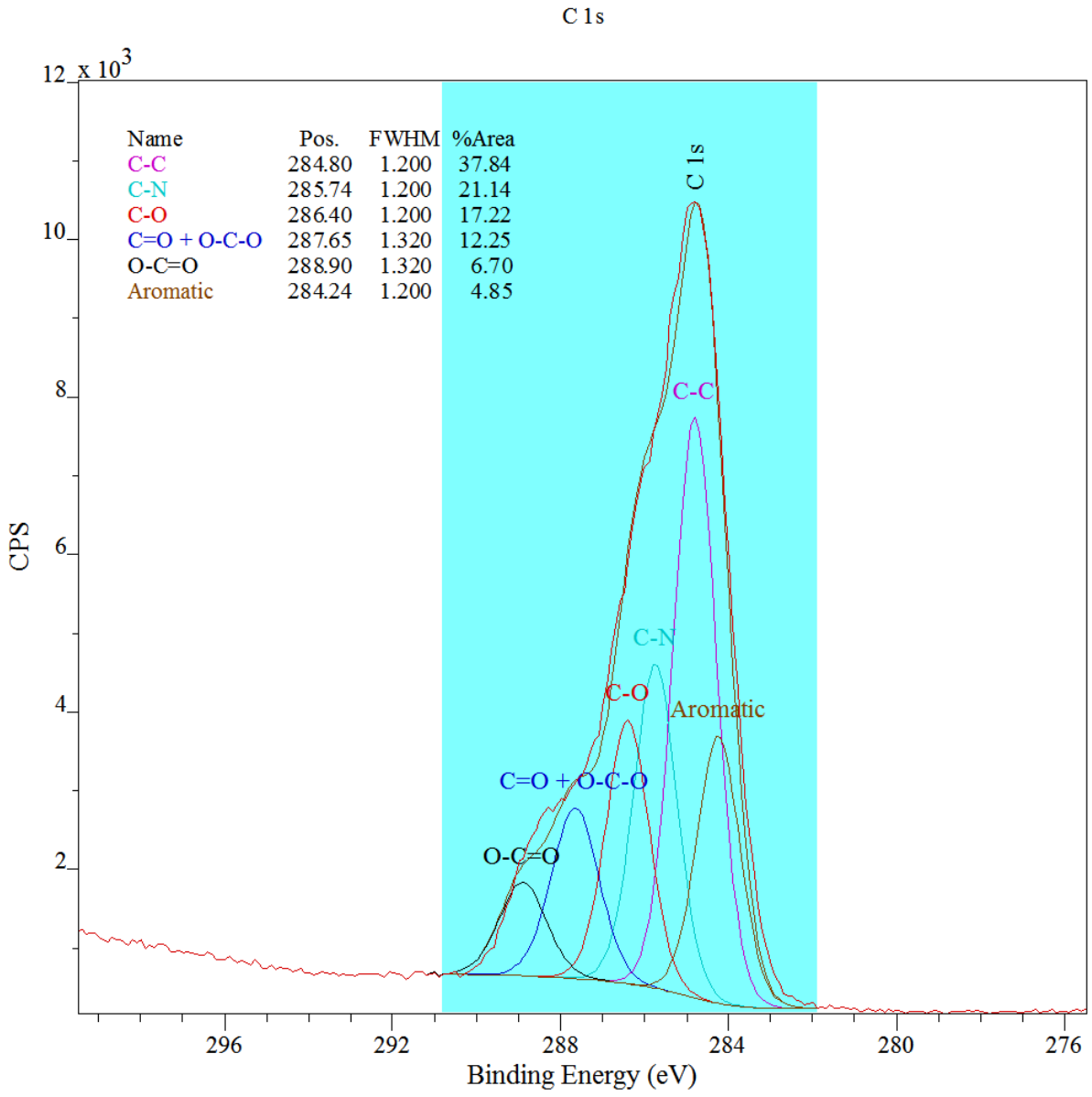
Coverslips coated with IPNpp were immersed either into 1 mL PBS or an aqueous solution with a pH 13 (made by addition of potassium hydroxide into deionized water), enclosed and let stand at either at 37 °C or 70 °C for a duration of 72 h. Upon mixing with the Griess' reagent, the absorbance of the solutions was measured at 540 nm by means of NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., DE, USA). The test was conducted in quadruplicates and the concentrations were calculated herein.

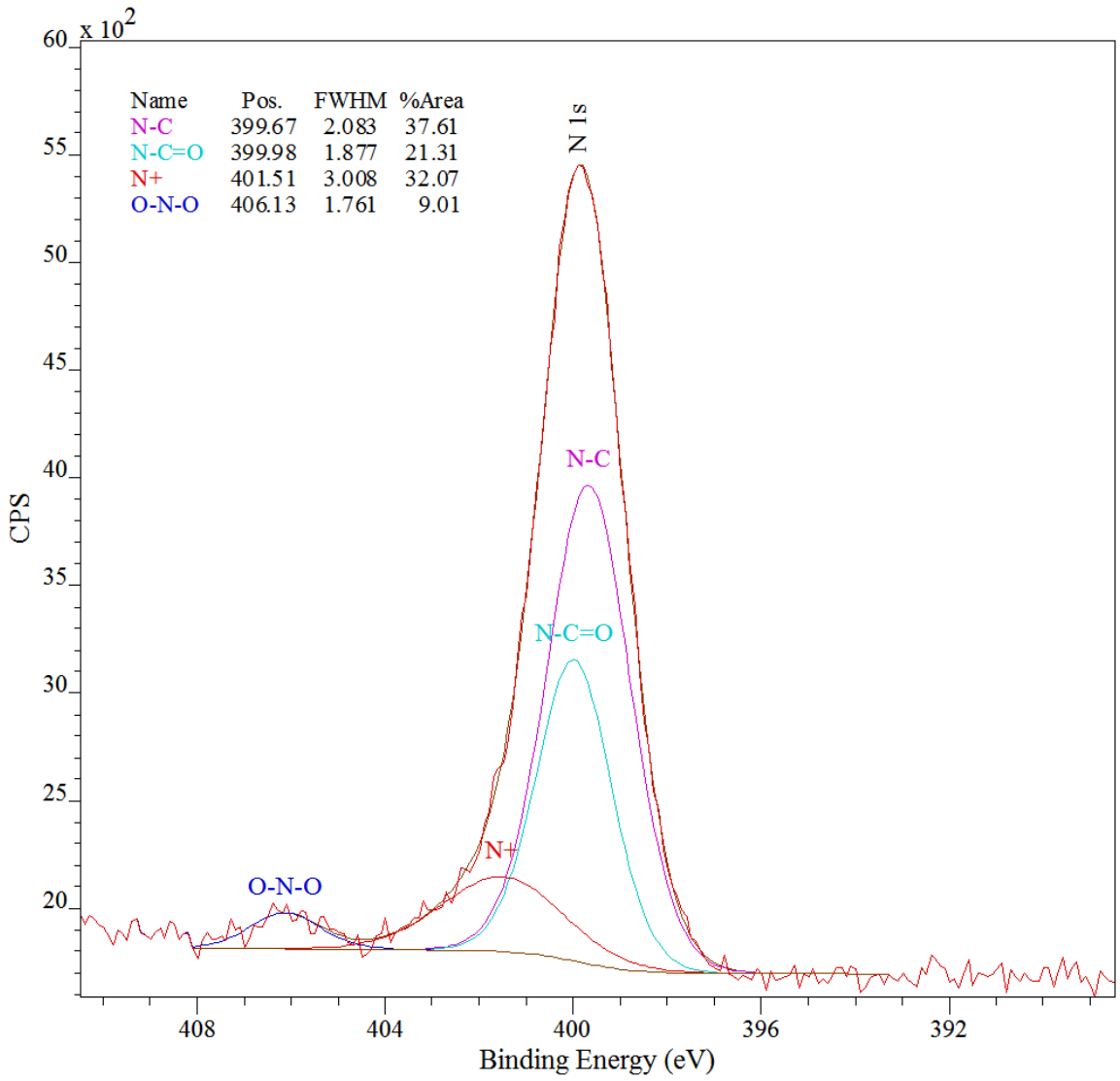


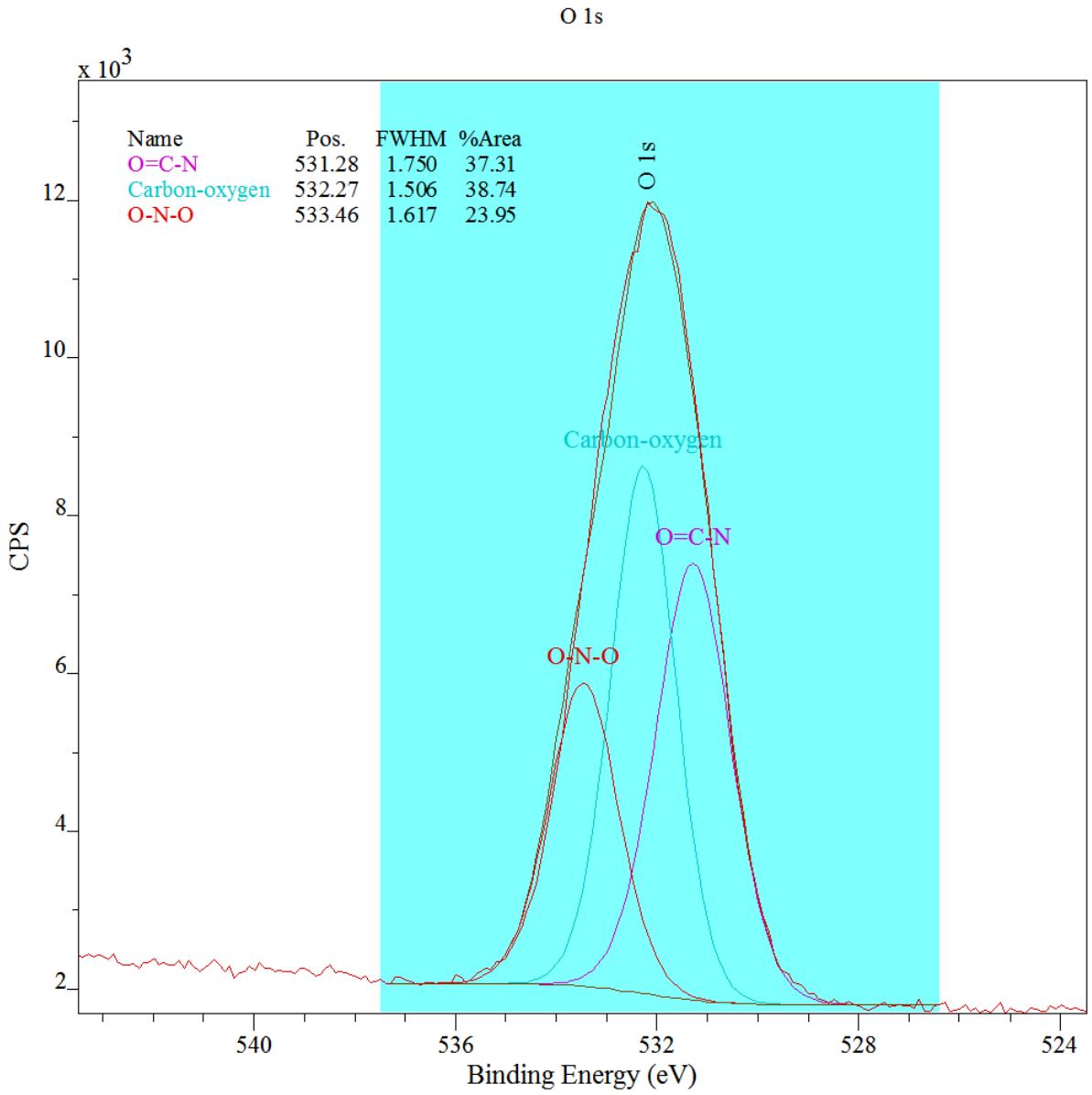
Spectra

XPS

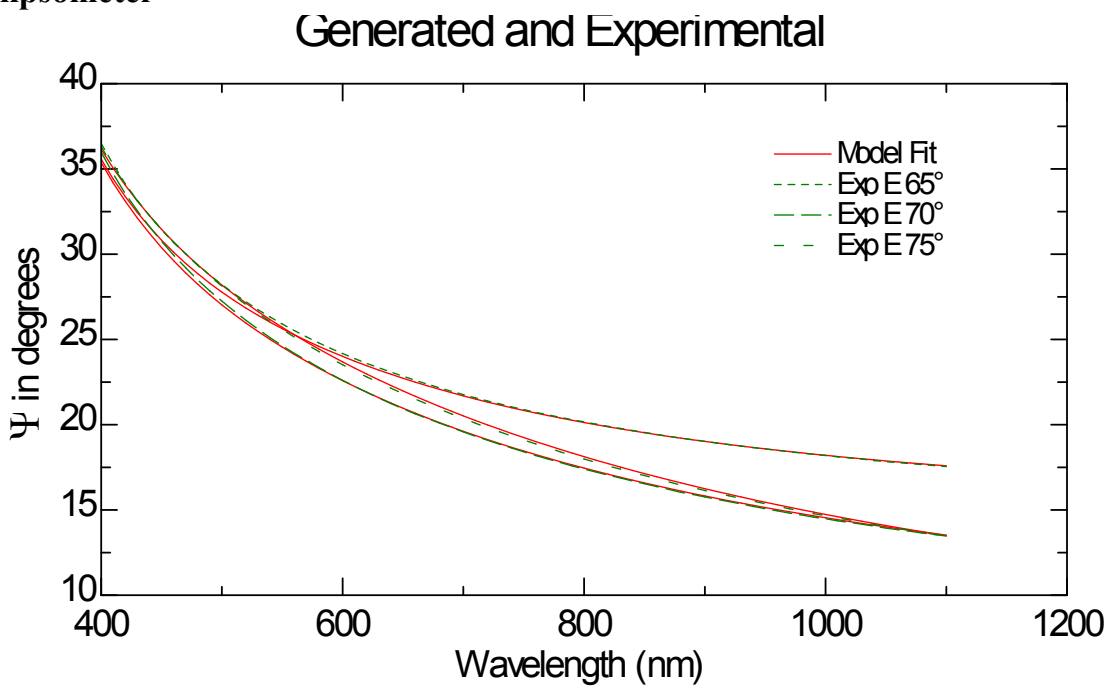

IPNpp
x 10^4
30
25
20
15
10
5
CPS
Name Pos. FWHM Area At%
O 1s 531.49 3.530 42475.33 22.44
C 1s 284.49 3.950 44669.29 66.22
N 1s 399.49 3.295 13125.72 11.34
O 1s
N 1s
C 1s
1000
800
600
400
200
0
Binding Energy (eV)

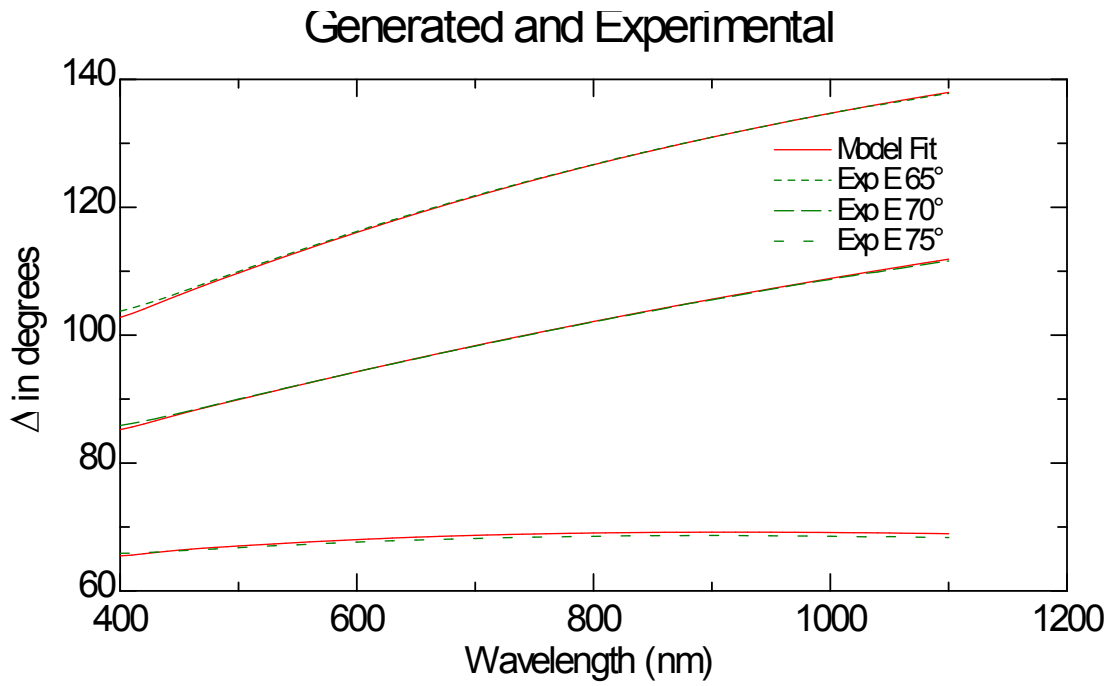

C 1s
x 10^3
12
10
8
6
4
2
CPS
Name Pos. FWHM %Area
C-C 284.80 1.200 37.84
C-N 285.74 1.200 21.14
C-O 286.40 1.200 17.22
C=O + O-C-O 287.65 1.320 12.25
O-C=O 288.90 1.320 6.70
Aromatic 284.24 1.200 4.85
C 1s
C-C
C-N
C-O
Aromatic
C=O + O-C-O
O-C=O
296
292
288
284
280
276
Binding Energy (eV)


x 10^2
60
55
50
45
40
35
30
25
20
CPS
Name Pos. FWHM %Area
N-C 399.67 2.083 37.61
N-C=O 399.98 1.877 21.31
N+ 401.51 3.008 32.07
O-N-O 406.13 1.761 9.01
N 1s
N-C
N-C=O
N+
O-N-O
408
404
400
396
392
Binding Energy (eV)


O 1s
x 10^3
Name Pos. FWHM %Area
O=C-N 531.28 1.750 37.31
Carbon-oxygen 532.27 1.506 38.74
O-N-O 533.46 1.617 23.95
O 1s
Carbon-oxygen
O=C-N
O-N-O
CPS
12
10
8
6
4
2
540
536
532
528
524
Binding Energy (eV)

Ellipsometer





The Cauchy equation:

$$n(\lambda) = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4}$$

Fitting Parameter	Constant	Software
Wavelength independent index of refraction	A	An
Wavelength dependent index of refraction	B	Bn
Wavelength dependent index of refraction	C	Cn

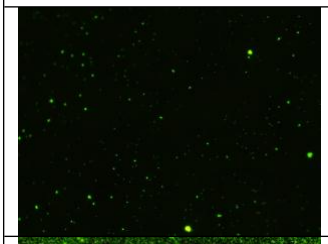
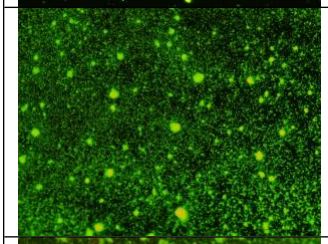
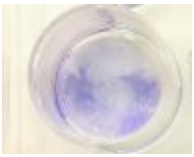
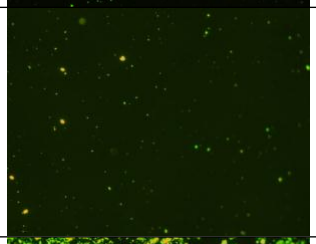
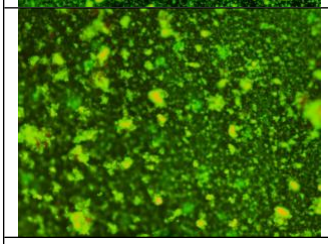
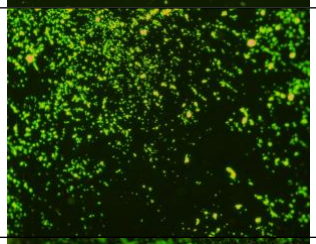
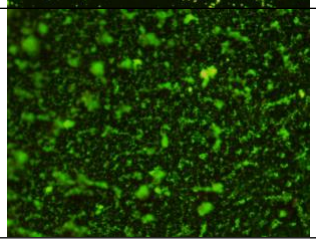
MSE=10.77

Thick.1	42.692±0.53 nm
An.1	1.6229±0.0232
Bn.1	-0.027903±0.00561
Cn.1	0.0038859±0.000644

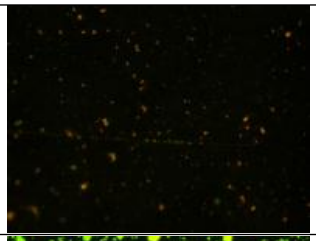
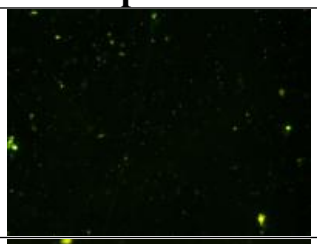
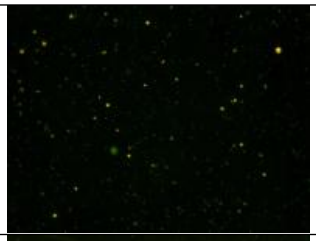
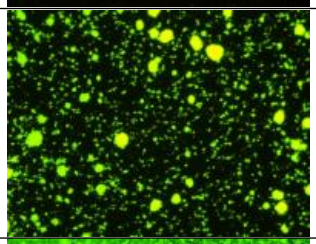
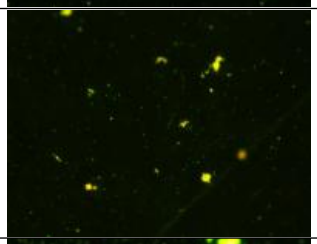
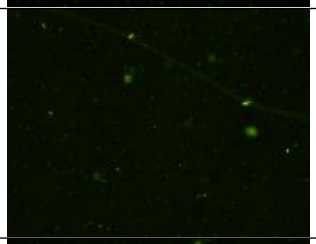
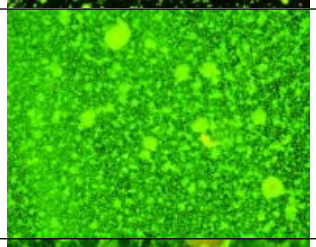
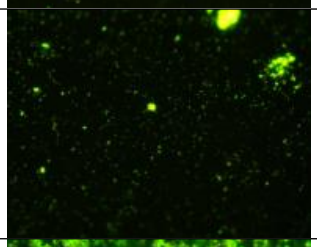
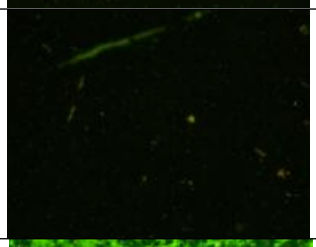
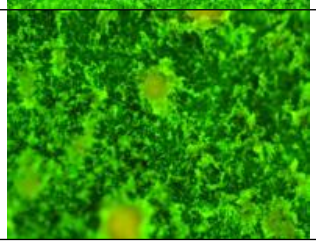
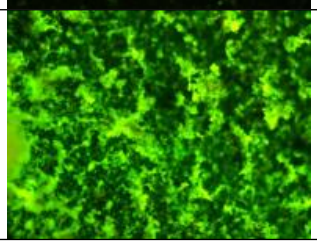
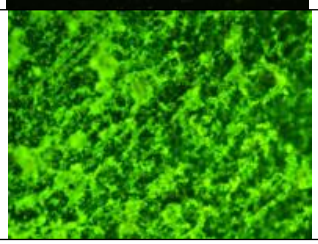
Images

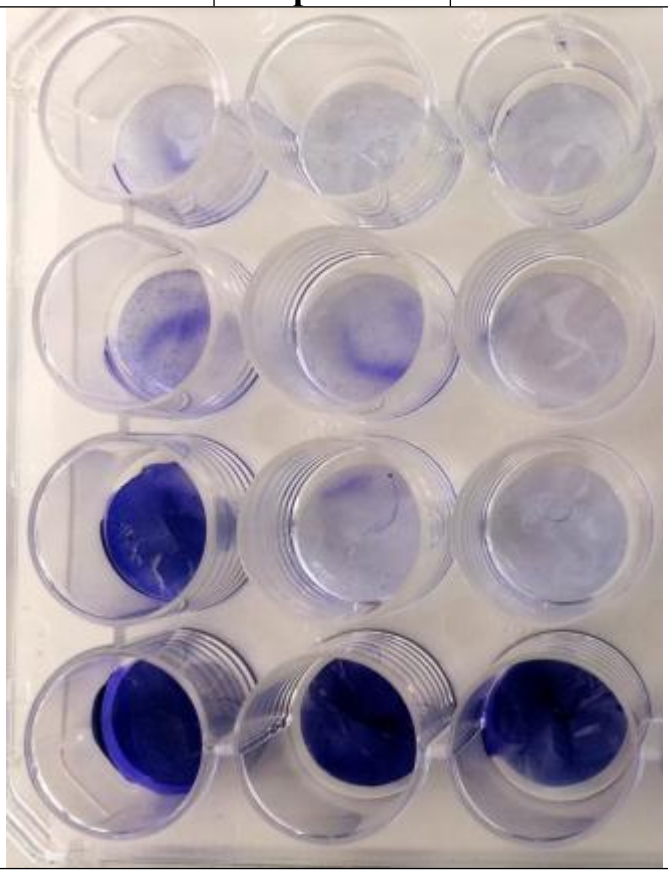
Bacterial testing

1)

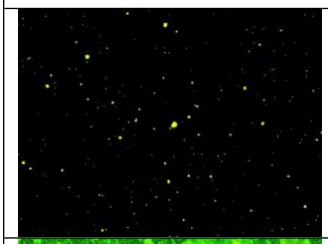
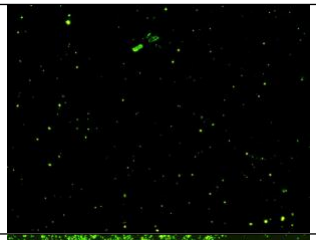
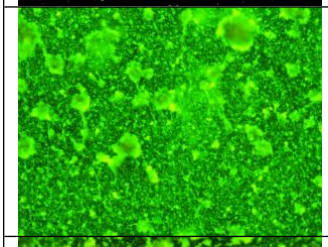
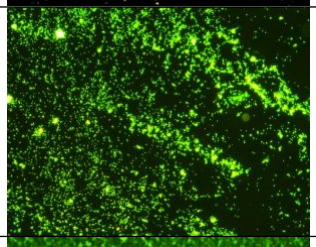
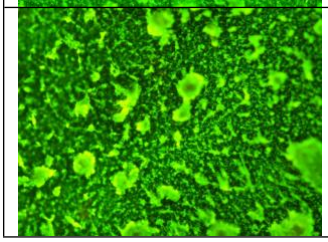
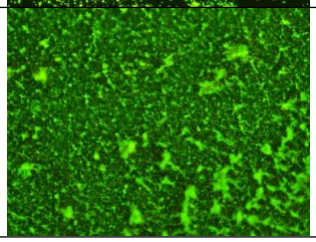
Reference		IPNpp		Time (h)
				**0**
				**9**
				**14**
				**18**

2)

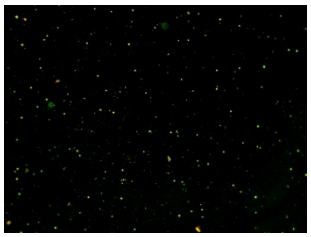
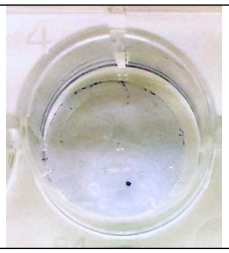
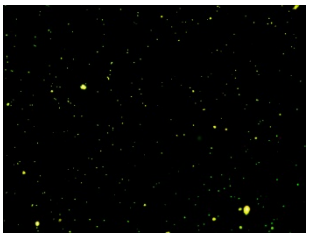
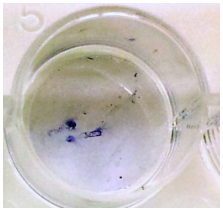
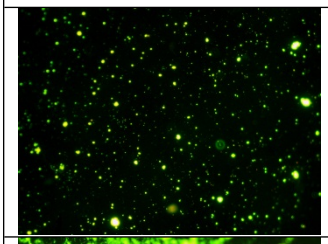
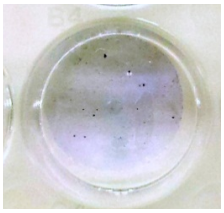
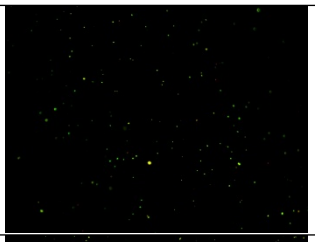
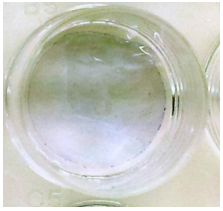
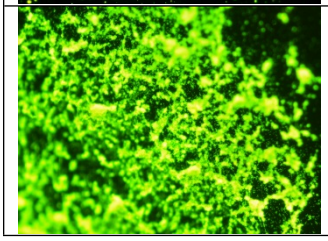
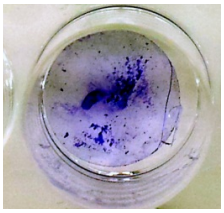
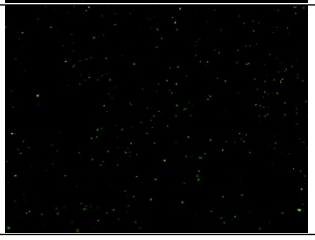
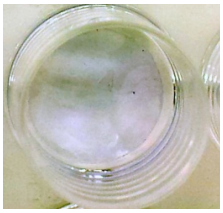
Reference	IPNpp stored at room temperature	IPNpp stored at 5°C	Time (h)
			0
			4
			8
			26

Reference	IPNpp stored at room temperature	IPNpp stored at 5°C	Time (h)
			0
			4
			8
			26

3)

Reference		IPNpp		Time (h)
				0
				11
				15

4)

Reference		IPNpp (2 months old)		Time (h)
				0
				3
				8

(1) Griesser, H. J. *Vacuum* 1989, *39*, 485.

(2) Coad, B. R.; Scholz, T.; Vasilev, K.; Hayball, J. D.; Short, R. D.; Griesser, H. J. *ACS Applied Materials & Interfaces* 2012, *4*, 2455.