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Design of blood compatible surfaces is obligatory to minimize platelet surface interactions and improve the thromboresistance of foreign surfaces when they are utilized as biomaterials particularly for blood contacting devices. Pure metallocene polyethylene (mPE) and nitric acid (HNO3) treated mPE antithrombogenicity and hydrophilicity were investigated. The contact angle of the mPE treated with HNO3 decreased. Surface of mPE and HNO3 treated mPE investigated with FTIR revealed no major changes in its functional groups. 3D Hirox digital microscopy, SEM and AFM images show increased porosity and surface roughness. Blood coagulation assays prothrombin time (PT) and activated partial thromboplastin time (APTT) were delayed significantly ($P < 0.05$) for HNO3 treated mPE. Hemolysis assay and platelet adhesion of the treated surface resulted in the lysis of red blood cells and platelet adherence, respectively indicating improved hemocompatibility of HNO3 treated mPE. To determine that HNO3 does not deteriorate elastic modulus of mPE, the elastic modulus of mPE and HNO3 treated mPE was compared and the result shows no significant difference. Hence, the overall observation suggests that the novel HNO3 treated mPE may hold great promises to be exploited for blood contacting devices like grafts, catheters, and etc.

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Abstract

Design of blood compatible surfaces is obligatory to minimize platelet surface interactions and improve the thromboresistance of foreign surfaces when they are utilized as biomaterials particularly for blood contacting devices. Pure metallocene polyethylene (mPE) and nitric acid (HNO_3) treated mPE antithrombogenicity and hydrophilicity were investigated. The contact angle of the mPE treated with HNO_3 decreased. Surface of mPE and HNO_3 treated mPE investigated with FTIR revealed no major changes in its functional groups. 3D Hirox digital microscopy, SEM and AFM images show increased porosity and surface roughness. Blood coagulation assays prothrombin time (PT) and activated partial thromboplastin time (APTT) were delayed significantly ($P < 0.05$) for HNO_3 treated mPE. Hemolysis assay and platelet adhesion of the treated surface resulted in the lysis of red blood cells and platelet adherence, respectively indicating improved hemocompatibility of HNO_3 treated mPE. To determine that HNO_3 does not deteriorate elastic modulus of mPE, the elastic modulus of mPE and HNO_3 treated mPE was compared and the result shows no significant difference. Hence, the overall observation suggests that the novel HNO_3 treated mPE may hold great promises to be exploited for blood contacting devices like grafts, catheters, and etc.

Keywords: Nitric acid, hemocompatibility, surface modification, blood contacting device.

Introduction

The surface modification of biomaterials is a process of modifying its surface properties by changing its inherent physical, chemical or biological properties to possess desirable characteristics (John et al, 2015). Generally, the surface modification of biomaterials can be done via different techniques for the biocompatibility enhancement, which is the cornerstone property required whilst selecting a blood contacting device (Jaganathan et al, 2014a; Vellayappan et al, 2015a). There is a wide range of blood contacting devices available nowadays like grafts, catheters, hemodialysis, bypass/extracorporeal membrane oxygenation, and ventricular assist devices (VADs). Even though there is a widespread need for blood contacting devices, the formation of blood coagulation as well as commencement of thrombotic events whilst the biomaterial comes in contact with the blood, remains as a daunting challenge for researchers to decipher (Velayyappan et al., 2015). A recent statistic shows that 65-88% of aortic repair procedures performed in the US are being replaced with endovascular grafts and the thrombus formation in aortic side branches often leads to ischemia (Thompson, 2013). Another clinical study dictates that thrombus formation on the catheter surface in 50% of patients undergoing diagnostic angiography (Formanek G & Frech RS., 1970). Moreover, thrombosis are found to be the precipitating event in 30-40% of central venous catheter malfunctions (Vascular Access, 2006). Thus, prevention of thrombotic deposition and occlusion, triggered by the activation of the coagulation cascade and platelets, is a mandatory property which the implanted blood contacting devices should possess before it is recommended for clinical trials.

The advent of latest technology has paved the way for the discovery of novel polymers like metallocene which is a new class of polyolefins with superior performance characteristics like improved toughness, sealability, clarity, and elasticity. Metallocene is made up of two

cyclopentadienyl anions (Cp,) which are attached to a metal center (M) with an oxidation state II, hence resulting in a general formula $M(C_5H_5)_2$ (Kealy TJ & Pauson PL, 1951). The metallocene polyethylene (mPE) is one of the versatile polymers. The mPE has wide spectrum of applications in disposable bags, storage bottles, blood bags, and syringe tubes. Albeit mPE has an excellent permeability to oxygen and functions as an effective barricade towards ammonia and water, yet mPE poor blood compatibility hampers it from being used for blood contacting devices (Mohandas et al, 2013). Thus, different works were done for enhancing the blood compatibility of mPE recently to promote it for various biomedical applications.

In our group, we are exploring several modification techniques to improve the blood compatibility of mPE. Recently, Mohandas et al., utilized microwave radiation for surface modification of the mPE to improve its blood compatibility (Mohandas et al, 2013). Furthermore, the effect of hydrochloric (HCl) acid treatment on the metallocene polyethylene mPE depicted an enhanced blood compatibility of mPE compared to the untreated mPE sample (Jaganathan et al, 2014).

Since the HCl etching effect on mPE yielded good results, it further motivated us to find other available substitutes which are cost effective and easily available for improving the blood compatibility of mPE. Thus, being a very strong acid and oxidizing agent, we have selected HNO_3 for improving the blood compatibility of mPE. In a work done utilizing HNO_3 by Moreno-Castilla et al., dictates that the $HN0_3$ treatment affects the surface area of activated carbons and their porosity the most compared to the other treatments like hydrogen peroxide, and ammonium peroxydisulfate treatments (Moreno-Castilla et al, 1995). Moreover, Dong et al., demonstrated the HNO_3 oxidation treatment on CNT modifies the CNTs physical and chemical properties resulting in improved CNTs biocompatibility (Dong et al, 2012). For the first time the

effect of HNO_3 treatment on mPE is documented in this work. Furthermore, the present study is done to ascertain the modifications induced in mPE and its impact on the blood compatibility of the HNO_3 treated mPE samples.

Materials and Methods

Ethics Statement

The blood coagulation assays were carried out in India and the characterization tests were done in Malaysia. Prior to blood procurement, the written consent form was given to the healthy volunteers. They read the benefits and risks of participation before expressing his/her willingness by signing the form. All protocols of blood procurement and consent procedure were approved by the Pacheri Sri Nallathangal Amman (PSNA) College of Engineering and Technology Ethical Committee of Dindigul with an approved IRB number: H30114. Later, the blood was extracted via venipuncture from aspirin-free healthy adult human donor and it is prevented from coagulation with trisodium citrate at a volumetric ratio of 9:1. Newly prepared platelet rich plasma (PRP) was acquired from the Dindigul Blood Bank, Dindigul, India.

Sample Preparation and Acid Treatment

Initially, two mPE films of dimensions 10 cm × 10 cm were cut into two samples with a size of 1 cm× 1 cm. Then the samples were washed with 70% ethanol and distilled water prior subjecting it to HNO_3 treatment. Prior to HNO_3 treatment, the mPE samples were washed with 70% ethanol, then was rinsed in distilled water to remove any impurities present on the surface of the sample. Then, 8- 10 ml of concentrated HNO_3 with molarity of 15.9M was poured into petri dish which contains the square shaped sheets of mPE. The acid and sample containing dishes were later placed on the rocking shakers which moves at a constant speed. Moreover, in this work mPE sample were subjected to HNO_3 exposure for the different time durations. From

that, the optimized timings were selected by observing surface changes with an optical microscope at 40x. The samples subjected for a lesser duration didn't confer notable surface modifications when compared with control, however, during 30 minutes of exposure significant change in the surface of mPE was observed. While subjecting samples for a prolonged period, changes noted, were not significant compared to 60 minutes treated sample. Thus, for characterization studies, 30 minutes and 60 minutes treated samples alone were considered. Once the acid treatment was done, the samples were washed in distilled water and dried at room temperature overnight before performing any characterization tests. Whilst preparing the samples for blood compatibility tests, samples were kept in a beaker with physiological saline and then in a rotary shaker overnight at 37^{o}C to remove the acid present on the surface of the polymer.

The scheme of the experiments performed was shown in Figure 1.

Characterization of the Samples

Contact Angle Measurement

The hydrophilicity tendency of the polymer was determined using Dynamic Contact Angle Analyzer (FTA200—First Ten Angstroms). Here, water droplet was placed on the surface of the sample. Water droplet of 1 μL was used and the photographs were taken in the ultra-fast mode within 30 seconds. The degree of the angle formed is determined using computer interfaced software. The contact angles were recorded and analyzed for the samples which are not treated with HNO_3 and 30min and 60min HNO_3 exposed samples (n = 3).

Attenuated Total Reflectance Fourier Transfer Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR equipment NEXUS- 870 model spectrophotometer was utilized have additional features such as extended beam splitter, two light sources, and middle band MCT detectors with various sampling options. This was used for the purpose of analyzing the chemical

120 compositions or functional groups present within the polymer. There were three samples present
121 in the study, which are untreated and 30 min and 60min HNO_3-treated samples. All these
122 samples were studied using this ATR-FTIR.

123 **3D-Hirox Digital Microscope**

124 The latest 3D-Hirox digital microscope model (KH-8700) was used to determine the
125 formation of pits and pores of the samples. The 3D-Hirox digital microscopy images are very
126 useful in determining the morphological structure of samples to determine whether the sample
127 has pores or it has an even surface. There are two types of images which are obtained from 3D-
128 Hirox digital microscopy either with or without profilometry line. The surface morphology of
129 1cm x 1cm of mPE and HNO_3 treated mPE sample was assessed at an area of approximately
130 5757μ^2 at a magnification of 500X. The same as white light confocal profilometry, in-focus and
131 3D images were obtained using this 3D microscope. Slices of the image were captured at
132 different heights acquired for the surface topography analysis (Pereira et la, 2013). Maximum of
133 three profiling lines is chosen as the profiling value of the each sample. Each point in X, Y and Z
134 axes of the profiling line is measured and their values can be exported in excel sheet to represent
135 the height of the pits in the sample. Data processing was performed using the in-build 3D
136 profilometry software. Images were recorded at standard 1,200 to 1,600 pixel resolution.

137 **Scanning Electron Microscope**

138 The surface microstructure of the samples can be critically analyzed in detail by using
139 SEM. The SEM which is utilized to study the polymeric samples was JEOL JSM5800 SEM with
140 OXFORD ISI 300 EDS X-ray Microanalysis System. All samples underwent gold sputtering and
141 then been studied using SEM at a magnification of 1500x.

142

Atomic Force Microscopy

The surface roughness of the samples can be determined with the help of AFM. The AFM model used to analyze the samples is SPA300HV with a scan rate of 1.502 Hz in tapping mode. Here, the surface morphology of mPE and HNO_3 treated mPE sample was measured by AFM in contact mode on a 10 × 10 μm^2 area, and the mean average surface roughness (Ra) and 3D pictographic view is obtained. Each AFM image was analyzed in terms of Ra (Pelagade et al, 2012). The surface roughness is calculated using the software SPIWin.

Tensile Testing

The tensile strength was tested using ZWICK Universal Tester (Z010, Germany) at a gage length of 15 mm and a speed of 10 mm^{-1} for all the specimens at a load cell capacity of 100 N with a sample thickness of 1mm. The reported tensile moduli is represented as average results of five tests.

Blood Coagulation Assays

Prothrombin Time (PT)

Prothrombin time is a valuable indicator to find the prohibition of extrinsic pathway. Platelet poor plasma (PPP) (100 μL at 37^{o}C) was applied on untreated and treated polymer surface with NaCl-thromboplastin (Factor III, 100 mL, Sigma) which contains Ca2+ ions. The time consumed for the formation fibrin clot was assessed with the help of a stopwatch and a steel hook (n = 3) (Amarnath LP, Srinivas A and Ramamurthi A et al, 2006).

Activated Partial Thromboplastin Time (APTT)

APTT is utilized for studying the propensity of the blood to coagulate via intrinsic pathway and to determine the effect of biomaterial on delaying the process. Platelet poor plasma (100 μL at 37^{o}C) is incubated in prior with substrates at 37^{o}C and followed by its activation by

adding rabbit brain cephalin (100 μL 37^{o}C). Later, the samples were incubated at 37^{o}C for 5min and followed by incubation with calcium chloride (0.025M). Inclusion of $CaCl_2$ triggers the clotting process. The time taken from the inclusion of CaCl2 up to clot formation is recorded as the activated partial thromboplastin time (APTT) (n = 3) (Amarnath LP, Srinivas A and Ramamurthi A et al, 2006).

Hemolysis Assay

The HNO_3 treated (30min and 60min) and untreated samples were equilibrated with physiologic saline (0.9%w/v; 37^{o}C, 30 min) followed by its incubation with 3mL aliquots of citrated blood diluted with saline (4 : 5 ratios by volume). This mixture of blood and distilled water was prepared at a ratio of 4: 5 by volume to result in comprehensive hemolysis which was used as the positive control. Physiological saline solution was utilized as negative control which produces no coloration. The samples were subjected to incubation in their respective mixtures (60min, 37^{o}C).These mixtures were later centrifuged and their absorbance of clear supernatant was determined at 542 nm. The absorbance of positive control was normalized to 100% and the absorbance of both the samples was ascertained as a percentage of hemolysis whilst comparing it with positive control (n = 3) (Amarnath LP, Srinivas A and Ramamurthi A et al, 2006).

Platelet Adhesion Assay

The samples were subjected to HNO_3 exposure for 60 min and later incubated along with physiological saline 0.9%w/v; 37^{o}C, 30 min) . This is kept on the rotary shaker for an hour to wash of the acid residues on the surface of the polymer. This is followed by immersing of treated and untreated samples in 1mL fresh PRP and the incubation was maintained at 37^{o}C for an hour. PRP was poured off and the membranes were rinsed in physiologic saline and dried. Ultimately, the samples were viewed using the microscope (n = 3). The polymer surface was photographed

and platelet count was determined on a region with a 40x magnification (Amarnath LP, Srinivas A and Ramamurthi A et al, 2006).

Statistical Analyses

All experiments were conducted thrice independently. One-way ANOVA was done to determine statistical significance. The results obtained from all experiments are expressed as mean ± SD. In case of qualitative experiments, a representative of three images is shown.

Results

The mean contact angle of the control was found to be 86.06º. This was found to be far greater in comparison to the acid treated samples. The mean contact angles of 30 and 60 min treated samples are 72.03º and 69.73º, respectively, were significantly lower with respect to untreated surface as shown in (Table 1).

FTIR was performed for the determination of chemical composition of untreated and treated samples as shown in Figure 2. No changes were observed in the functional groups between the treated and the untreated ones. There were alike peaks observed at wavelengths 2850 cm−1 and 2930 cm−1 belonging to the alkane group (C–H stretch). The peaks were also found at 1647 cm−1 (C=C bending), 1470 cm−1 (C–H bending) and at 725 cm−1 (C–H rocking), belonging to the alkane family but differing in their structures. A peak was also observed at 1020 cm^{-1} which belongs to the C-O stretching. Nevertheless, the intensity of the peaks associated with the treated surface was observed to slightly differ from untreated one.

The morphological analysis of the samples was done using the 3D Hirox Microscopy and SEM where as the nanotopographic analysis of the sample was performed with the help of AFM. The images obtained are shown in Figure 3(a)-(f). Figure 3(a) represents the 3D image of the control and Figure 3(b) shows its profiling image. Likewise, the Figure 3 (c) and Figure 3(d)

depict the 3D image and its profiling image of the thirty minutes HNO_3 treated sample, respectively. Similarly, Figure 3(e) and 3(f) elucidates the 3D image and its profiling image of one hour HNO_3 treated mPE sample. The graph plotted with the values obtained from the 3D-Hirox digital microscope is represented in Figure 4. Here, each point in the profile line is measured and computed. These points represent the height of the pits in mPE surface. Thus, in Figure 4, the height of the pits or pores is plotted against the area of the profile line. From this graph, it is palpable that there is less number of pores of pits in case of the control mPE. It is found that the number of pores, increased in the 30 minutes and found to be highest in 1 hour acid treated mPE sample. The 1 hour HNO_3 treated sample has the maximum number of pores with greater depth of fissures and holes which was ascertained using the 3D profiling. Figure 4 shows the depth of the pores formed due to the etching effect of the HNO_3 and it is evident that the control has the least pore depth, followed by 30 minutes HNO_3 treated sample and finally the one hour HNO_3 treated sample. Hence, the duration of acid treatment has an impact on the surface porosity by affecting the pore diameter or area. From the Fig. 4 , it is visible that 42.5µm was the highest height of the pore in the case of one 1 hour HNO_3 treated sample where the highest pore for the 30 minutes HNO_3 treated mPE was 30µm and 17.5µm for untreated mPE. This shows the numerical values, data on relative changes for clearly differentiating the etching effect of HNO_3 on mPE.

SEM imaging is another surface characterization method of the samples at the micro level (Zhao et al, 2011). Topography of the polymers was investigated and morphological studies of the polymer samples were made Fig. 5(a),(b) and (c). It was observed that the surface of mPE sample has very less or negligible pits under a 1500x magnification. However, on observing the SEM image of 30 minutes treated sample, it was found that the surface of the treated samples has

been etched by the acid exposure. A few number of pit formation was also observed. Moreover, the size and the number of the pits seems to increase in case of the 60min acid treated sample.

The AFM images are represented in Fig. 5 (d) and (e). Fig. 5(d) is the AFM image of the untreated mPE sample and Fig. 5(e) is the AFM image of one hour HNO_3 treated mPE sample. From the results obtained, it was found that the mean value of Ra of untreated mPE film and 1 hour HNO_3 treated mPE surface are 2.069 and 5.127 nm, respectively. The nanotopographic analysis of the samples was performed using AFM. Fig. 5(d) illustrates the 3D surface topography of the sample mPE. Here, it is observed that the surface of the sample is even with less hills and valley structures in the sample. On the other hand Fig. 5(e) which is the 1 hour HNO_3 treated sample AFM image shows more number of hill and valley structure on the surface of the sample.

The average tensile testing result of mPE before and after nitric acid treatment is represented in Fig. 6. From the tensile stress-strain curve it is palpable that the elastic modulus of HNO_3 treated mPE (34.75MPa) is slightly greater than the elastic modulus of untreated mPE (31.32MPa). The elastic modulus, maximum force, elongation at maximum force and work up to maximum force is given in table 2.

Prothrombin time and activated partial thromboplastin time tests were done on the three samples, namely, untreated and 30min and 60min HNO_3 treated. Their results of PT and APTT were summarized in Fig. 7(a) and Fig. 7(b), respectively. Both PT and APTT demonstrated an increase in their value for acid treated samples compared to the control. Mean PT of untreated sample was observed to be 19.23 s, whereas 30 and 60min HNO_3 exposed samples shown 19.86 s and 21.4 s, respectively. Likewise, mean APTT was found to be 105.66 s, 113 s and 136.33 s for untreated, 30 min and 60 min acid treated mPE, respectively. Statistical analysis of the

untreated sample with the treated ones using one-way ANOVA insinuates significant differences ($P < 0.05$) between them for both PT and APTT times after 60 min exposure.

Besides that, hemolysis is an important screening test, which provides quantification of small levels of plasma hemoglobin that may not be assessed under in vivo conditions (Schopka et al, 2010). The hemolysis test was conducted on treating samples and untreated sample for investigating the effect of polymer surface on red blood cells (RBC). Mean absorbance seemed to decrease in the case of treated samples (0.02 and 0.007 for 30min and 60min HNO_3-treated samples) compared with the untreated (0.05) mPE, indicating lesser damage incurred and interaction between the treated samples and RBC Fig. 7(c).This is because the absorbance is directly proportional to the hemolytic index (HI) of the RBC. Statistical analysis of the untreated as well as acid treated samples (absorbance at 542nm) using one-way ANOVA ascertained significant differences ($P < 0.05$) between them after 30 and 60min treatment. From the results obtained, it is obvious that the 1 hour HNO_3 treated mPE is the least hemolytic compared to other samples. Moreover, it was also found that an absorbance value of 1 hour HNO_3 treated mPE to be in similar trend compared to the one hour HCl treated mPE (Jaganathan et al, 2014).

Besides HI, the adhesion of platelets on a blood contacting device's surface could result in coagulation and thrombus formation. Hence, the platelet adhesion test has to be performed to analyze the blood compatibility of blood contacting device (Wenzhong et al, 2008). The number of platelets adhered to a surface of treated polymers was found to be reduced to a great extent compared to the number of platelets which was found in the untreated sample. A maximum of 22 platelets was observed on the surface of the untreated samples, meanwhile the number of platelets decreased to a maximum of 15 platelets on 60min treated samples as represented in Fig. 8(a). Statistical analysis of the untreated sample with the treated one (number of platelets

281 adhered) with one-way ANOVA shown significant differences ($P < 0.05$) between them after 60
282 min treatment. The image of the polymer surface further dictates the decrease in the number of
283 platelets adhered on the acid treated present in Fig. 8 (b) compared to the untreated polymer
284 surfaces as shown in Fig. 8(c).

285 **Discussion**

286 Blood clotting occurs when blood comes in contact with a foreign surface such as
287 implants following platelet activation. This can be catastrophic in clinical settings, especially in
288 case of various biomedical applications like grafts, catheters, hemodialysis,
289 bypass/extracorporeal membrane oxygenation, and ventricular assist devices (Qi, Maitz &
290 Huang, 2013). In order to circumvent this issue, the hemocompatibility of the blood contacting
291 devices has to be improved and HNO_3 surface treated mPE holds a great potential. For
292 ascertaining the topographical modification caused by HNO_3 on the mPE sample,
293 characterization tests was performed using 3D Hirox, SEM, AFM, contact angle and FTIR. On
294 the other hand, the blood compatibility of the sample was studied by conducting different blood
295 coagulations assays like hemolysis assay, PT, APTT, and platelet adhesion.

296 The decrease in contact angle indicates the improved wettability and hydrophilicity of the
297 mPE polymer. It is a well known fact that the surface energy is a vital parameter determining
298 polymer's adhesion, material wettability and even biocompatibility (Kwok, Wang & Chu PK,
299 2005). Thus, the assessment of contact angles is contemplated as one of the most convenient
300 method for the determination of surface free energy of solid samples. This technique depends on
301 the interactions between the solid sample of interest as well as liquids with well determined
302 surface tensions. Our result is in good agreement with our previous published results of HCl
303 exposed mPE (Jaganathan et al, 2014). Furthermore, in a recent work, Gomathi et al., had

performed surface modification of polypropylene by nitrogen containing plasma improved the polymer's wettability by decreasing the water contact angle and resulted in enhanced biocompatibility and blood compatibility further corroborates our results (Gomathi et al, 2012). According to Wenzel model, the improvement in the surface roughness of mPE contributes to the reduction in the water contact angle of mPE (Chau et al, 2009). Thus, it indirectly shows that the surface roughness of the mPE sample are improved by the HNO_3 treatment, thereby decreasing the contact angle. Ultimately, the hydrophilicity and mPE hemocompatibility is improved where it can serve as a putative blood contacting device (Zhao et al, 2011).

The intensity of absorption was found to be improved with respect to all peaks in the acid treated mPE. These observations insinuate that there are modifications in the surface morphology. There is no alteration in the functional groups of the treated and untreated samples which is done by Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) studies. However, the variation in the intensity of the peaks depicts that there is some morphological changes occurred. Hence, this elucidates that there is no change in the functional groups in mPE surface, even after HNO_3 treatment of mPE which is similar to our HCl exposed mPE (Jaganathan et al, 2014). Thus, HNO_3 treated mPE sample would have enhanced blood compatibility without affecting the chemical structure of mPE since the surface roughness of the mPE is increased by the HNO_3 treatment rather than modifying the chemical structure of mPE. The percentage of weight loss study was also performed, but the change in the weight of the sample after HNO_3 treatment was not significant which ascertains there is no strong oxidation have occurred to increase the weight of the HNO_3 treated mPE samples (result not shown). This is in accordance with the FTIR result which didn't show appreciable changes in surface functional groups. Thus, it can be elucidated as the improved surface roughness resulted in better

hydrophilicity and hemocompatibility of HNO_3 treated mPE rather than the change induced in the sample surface functional group by HNO_3 treatment.

The 3D Hirox Microscopy images can be interpreted as HNO_3 etches the surface of mPE, and one hour acid treatment must have etched the mPE surface more than the 30 minutes treated sample, thereby resulting in mPE with more pits and pores with higher depth compared to the control. These observations can be compared to a later work of Vital et al., where the amplitude of depressions formed in the surface of polymer increased after tetrahydrofuran (THF) and acetic acid surface treatment whereas thickness of the polymer film remained unchanged (Vital et al, 2015). Hence, the etching effect of the acid has a favorable impact on the final surface wettability of the polymer, thereby making it more hydrophilic similar to the other surface treatments like plasma treatment to make it blood compatible for various blood contacting device applications (Yue et al, 2014).

Similarly, a larger surface disorientation and improved surface roughness were noticed in 60 min treated sample using SEM images. The SEM images of a recent study show the morphology of isotactic polypropylene (IPP) surfaces of Argon plasma treatment, showing amorphous region is etched on the surface of IPP and the etching depth was found to be increasing with an increase in the time of plasma treated thereby improving its biocompatibility (Ma et al, 2011). Likewise, SEM images of the treated and untreated samples to clearly show that there are pits formed in the surface of the mPE polymer when it is treated with HNO_3 and the pit size is also increased with the increase in the time of acid treatment. Since pits were formed, the morphological characteristic such as roughness was also observed to be increased in case of the HNO_3 treated samples. It is obvious from the Fig. 5 that the number of pits formed in the

sample is in the descending order of 60minutes HNO_3, 30minutes HNO_3 and then finally the control.

It was found that the Ra value of the 1 hour HNO_3 treated sample value is almost twice greater than the control. This is because of more number of hills and valley nano-topographic structure in the mPE sample resulted due to the etching effect of the HNO_3 on the mPE sample. This result is found to be similar to a latest study done by Cesca et al., where the AFM result obtained after poly-3-caprolactone (PCL) etched using mixed gas SF6/Argon at -5ºC has an improved surface roughness resulting in improved biocompatibility (Cesca et al, 2014). The roughness values obtained using AFM also evidenced the surface structuring after subjecting the sample to surface modification techniques to produce a rougher surface (Tverdokhlebov et al, 2014; Wanke et al, 2014). Similarly, there were other studies carried out show increase in surface roughness of sample results in improved biocompatibility (Slepicka et al, 2013). Hence, AFM nano imaging further bolsters the concept of nanotopographic surface modification caused by the acid etching effect on mPE analyzed using Hirox microscopy and SEM. This formed nanotopographic surface result in improved wettability and hydrophilicity ascertained by contact angle analysis, thereby improving the blood compatibility of mPE which is the cornerstone for blood contacting devices.

Since nitric acid improves porosity and blood compatibility of mPE, the elastic modulus of mPE was studied to make sure that the nitric acid treatment does not deteriorate the elastic modulus of mPE. There was no significant change was observed and minor improvement in the elastic modulus of HNO_3 treated mPE which may have resulted due to increase in the roughness of the surface in mPE. This result is in accordance with a recent study where the impact strength of nitric acid treated polyoxymethylene improved compared to those untreated samples (Zhang et

al, 2014). The surface of the carbon fibers synthesized from polyacrylonitrile was subjected to HNO_3 treatment and it was found that the tensile strength of the fibers increased compared to untreated samples (Bahl OP, Mathur RB & Dhami TL, 1984).

Coagulation system activation is triggered by implanting blood contacting device-protein interaction. The activation of factor XII is the first step in this activation process. Reciprocal as well as auto activation will in turn cause the amplification of activated factor XII, where this will initiate the intrinsic coagulation pathway through activation of factor XI, and finally lead to the production of fibrin. Similarly, the activation of platelets by artificial surfaces occurs due to the contact of platelets with artificial surfaces, in terms of ligand expression (GP IIb/IIIa). Ultimately, these activated platelets either adhere to the surface of blood contacting devices through proteins like fibrinogen or aggregate (Schopka et al, 2010). In order to function as a viable blood contacting devices, the implanted blood contacting device should not elicit any unwanted reactions leading to blood clot. In order to investigate that, the blood coagulation assays were carried out in the mPE treated with HNO_3.There was a notable increase observed in the PT and APTT of the HNO_3 treated mPE sample compared to the control. Changes in surface morphology of mPE by acid treatment helped in improving the blood compatibility of the polymers (Pandiyaraj et al, 2008). Thus, as discussed earlier, the increased PT and APTT is may be attributed by improved surface roughness by the formation of nanotopographic surface by HNO_3 on mPE.

This means that the surface modification of mPE with HNO_3 does not induce any damage in erythrocytes' membranes that could lead to their lysis. Albeit some literatures indicate that it is not possible to define a universal level of acceptable or unacceptable hemolysis values, a blood-compatible material must inhibit hemolysis (Wenzhong et al, 2008). In this study, this parameter

395 is of extreme importance as the proposed mPE material will be in contact with blood for a
396 prolonged period in the blood circulation system.

397 The reduced platelet adhesion in the HNO_3 treated sample dictates the improved
398 hemocompatibility of surface modified mPE (Gomathi et al, 2012; Habibzadeh et al, 2014).
399 Hence, this modified mPE with more surface roughness, altered wettability, and better blood
400 compatibility may be the vital characteristics that can be utilized for construction of long-term
401 blood contacting devices like catheters, transvenous pacing leads, stents, grafts and etc.

402 **Conclusions**

403 The surface modification induced by HNO_3 on mPE and its effect on mPE blood
404 compatibility was assessed. Contact angle analysis depicts a decrease in the contact angle
405 elucidating increase in the wettability of the HNO_3 treated samples. There were no prominent
406 qualitative changes in the functional groups were observed by FTIR studies. The 3D Hirox
407 microscopy imaging also confirms the improved surface roughness by formation of more pits
408 and bumps in the acid treated sample than the control. SEM images of treated samples further
409 substantiate that acid treated sample surface possess more pits and pores compared to the control.
410 AFM topographical analysis shows an improved surface roughness in the 1 hour acid treated
411 sample compared to the control due to the etching effect of the acid. Blood coagulation assays
412 like PT and APTT ascertains a notable delay in the clotting mechanism on the surface of acid
413 treated samples. The result of hemolysis assay shows a minimum damage to red blood cells
414 (RBC). Platelet adhesion assay elucidates that the number of platelets adhered to the surface of
415 acid treated polymer was appreciably less in comparison to the untreated surface. The HNO_3
416 treatment of the mPE induces a surface modification in mPE and improves its porosity without

much effect on its tensile strength. Hence, HNO_3 treated mPE sample can be exploited for various blood contacting biomaterial applications due to its improved blood compatibility.

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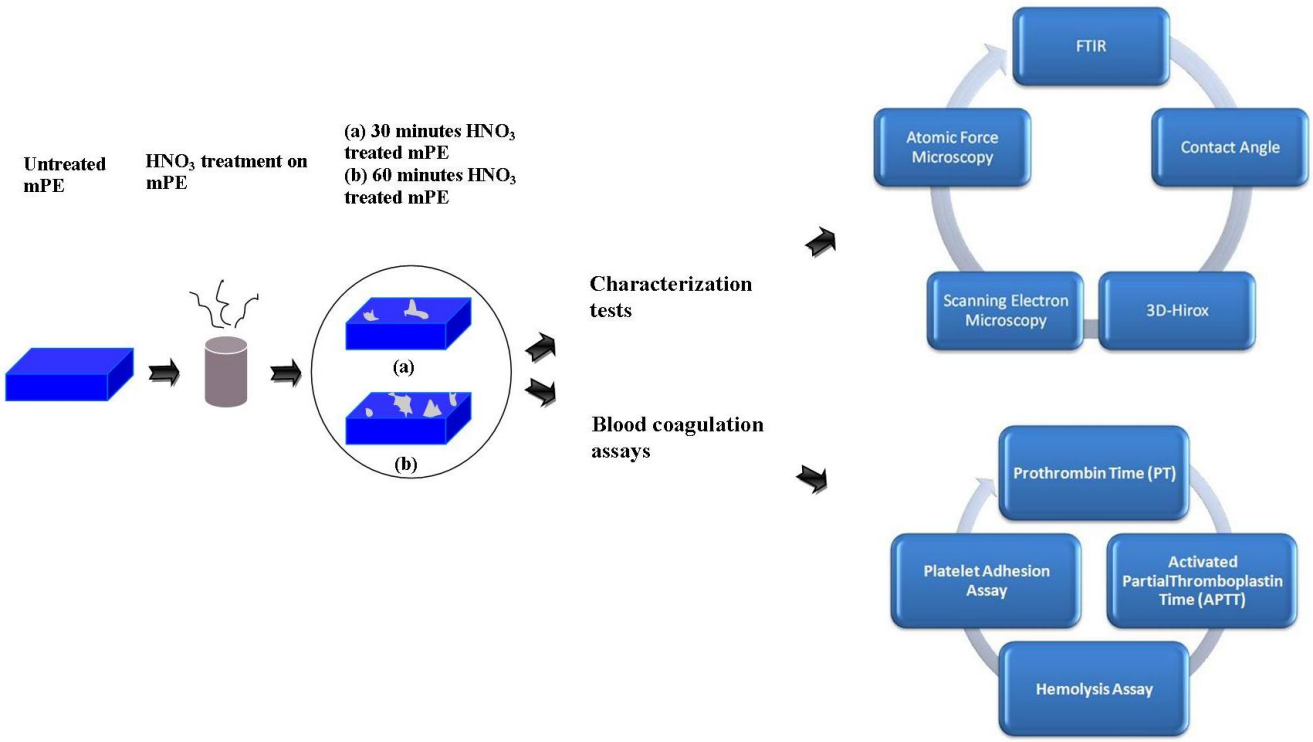


Figure 1 Schematic representation of series of characterization and blood compatibility experiments done

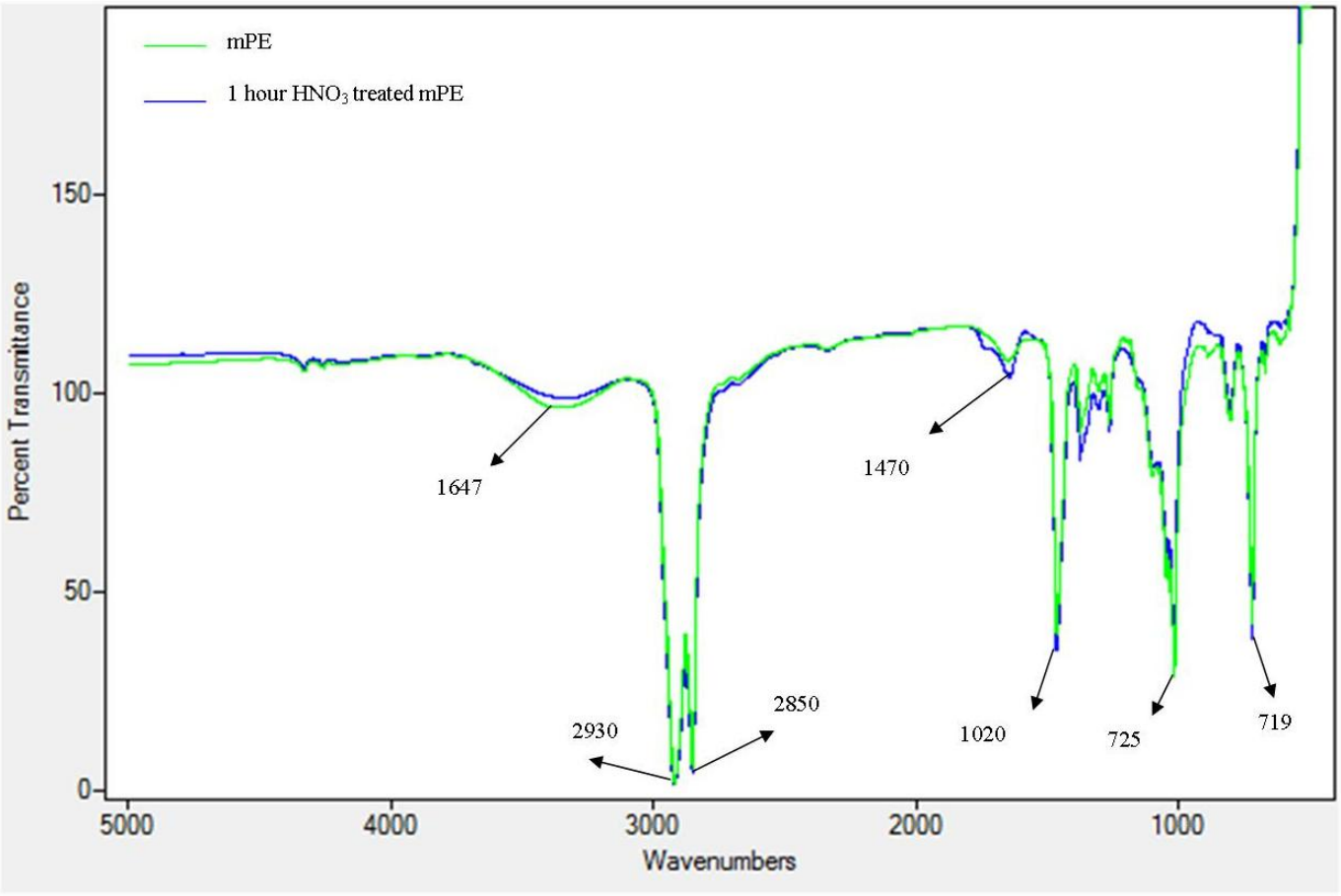


Figure 2 A representative FTIR spectra of untreated and 60 min HNO_3-treated metallocene polyethylene

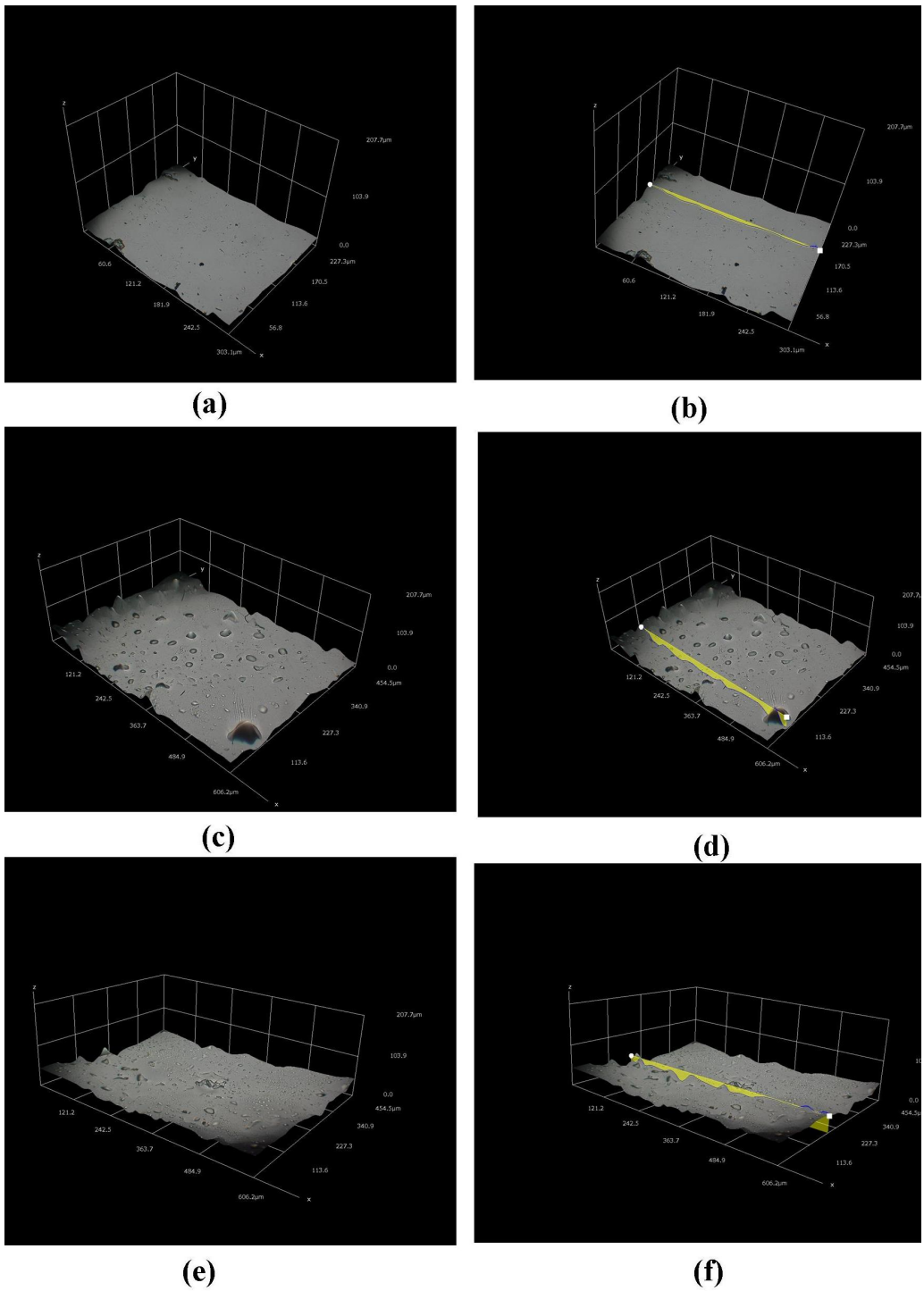


Figure 3 Different three-dimensional representations using 3D Hirox digital microscopy (a) Untreated mPE (b) Untreated mPE with profiling (c) 30 minutes HNO_3 treated mPE (d) 30 minutes HNO_3 treated mPE profiling (e) 60 minutes HNO_3 treated mPE (e) 60 minutes HNO_3 treated mPE profiling

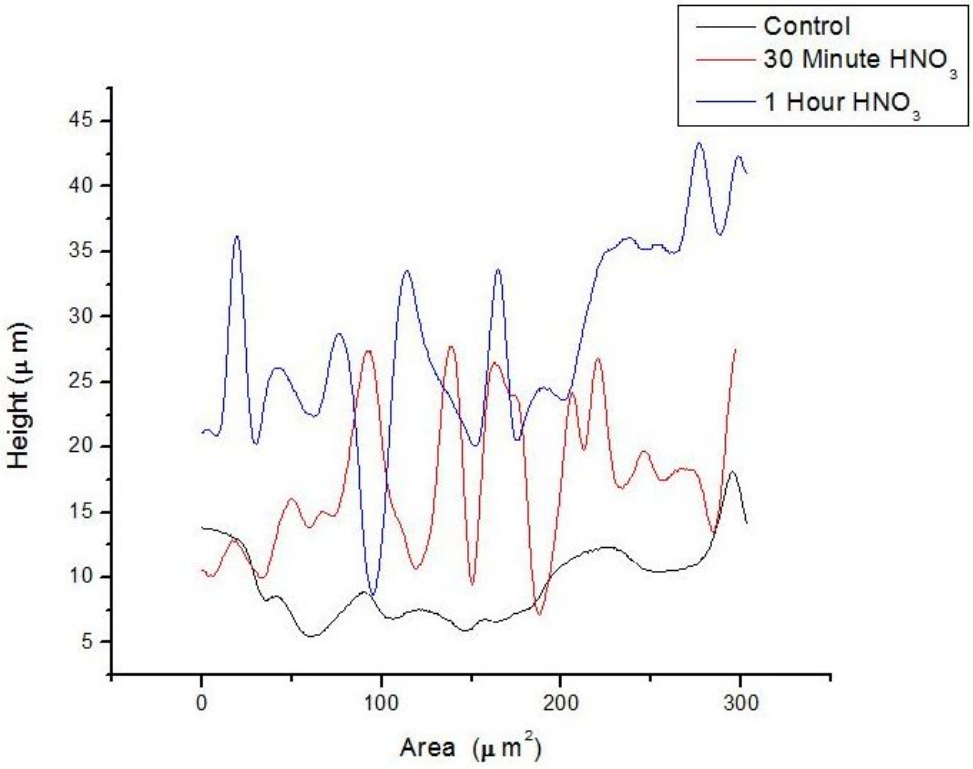


Figure 4 The representative height of the pores of different samples measured using 3D-profiling of 3D Hirox digital microscopy

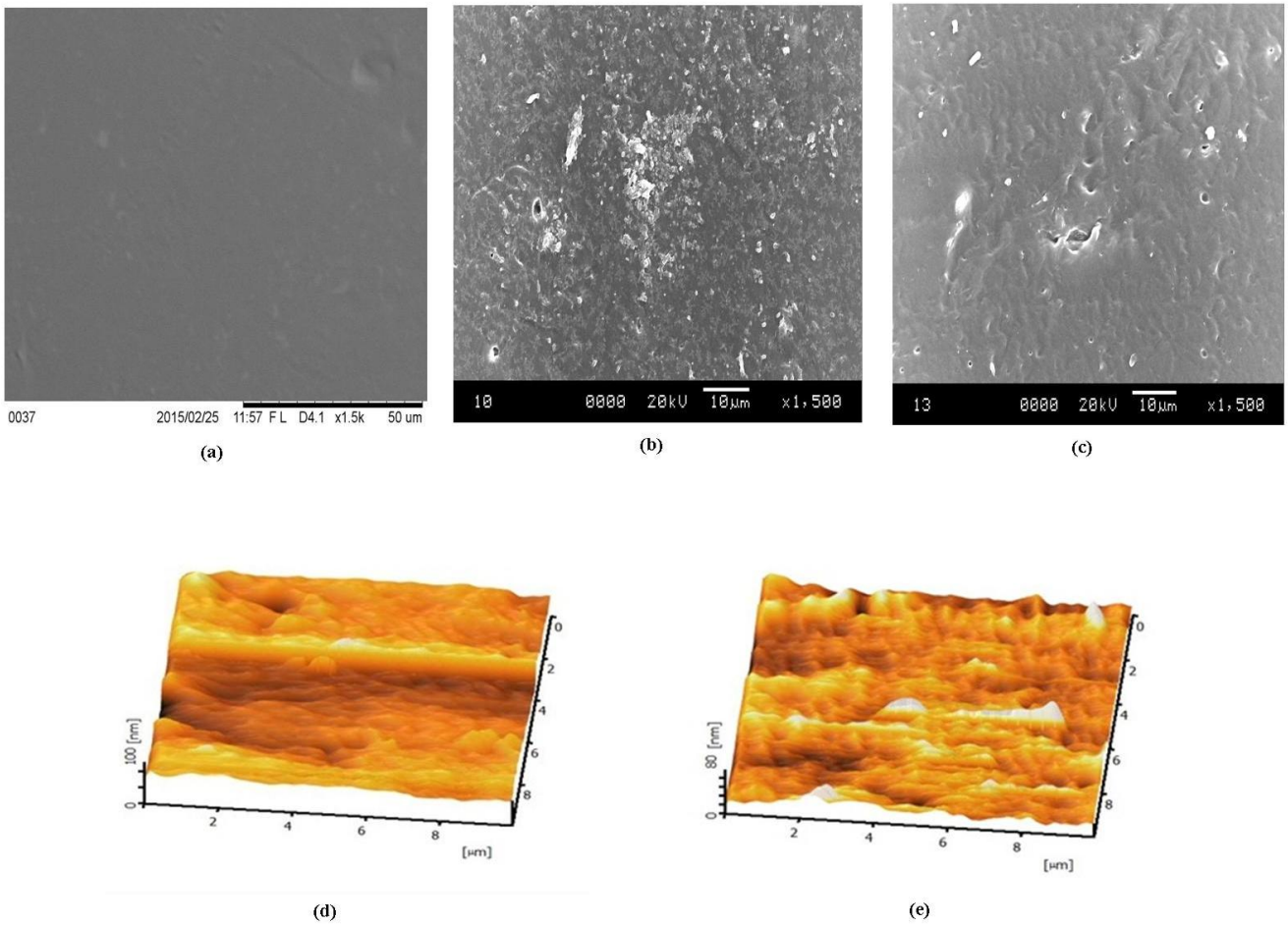


Figure 5 (a), (b) and (c) are representative SEM micrographs of untreated and HNO_3 treated metallocene polyethylene. Here, (d) and (e) are representative AFM images of untreated metallocene polyethylene and one hour HNO_3 treated metallocene polyethylene respectively

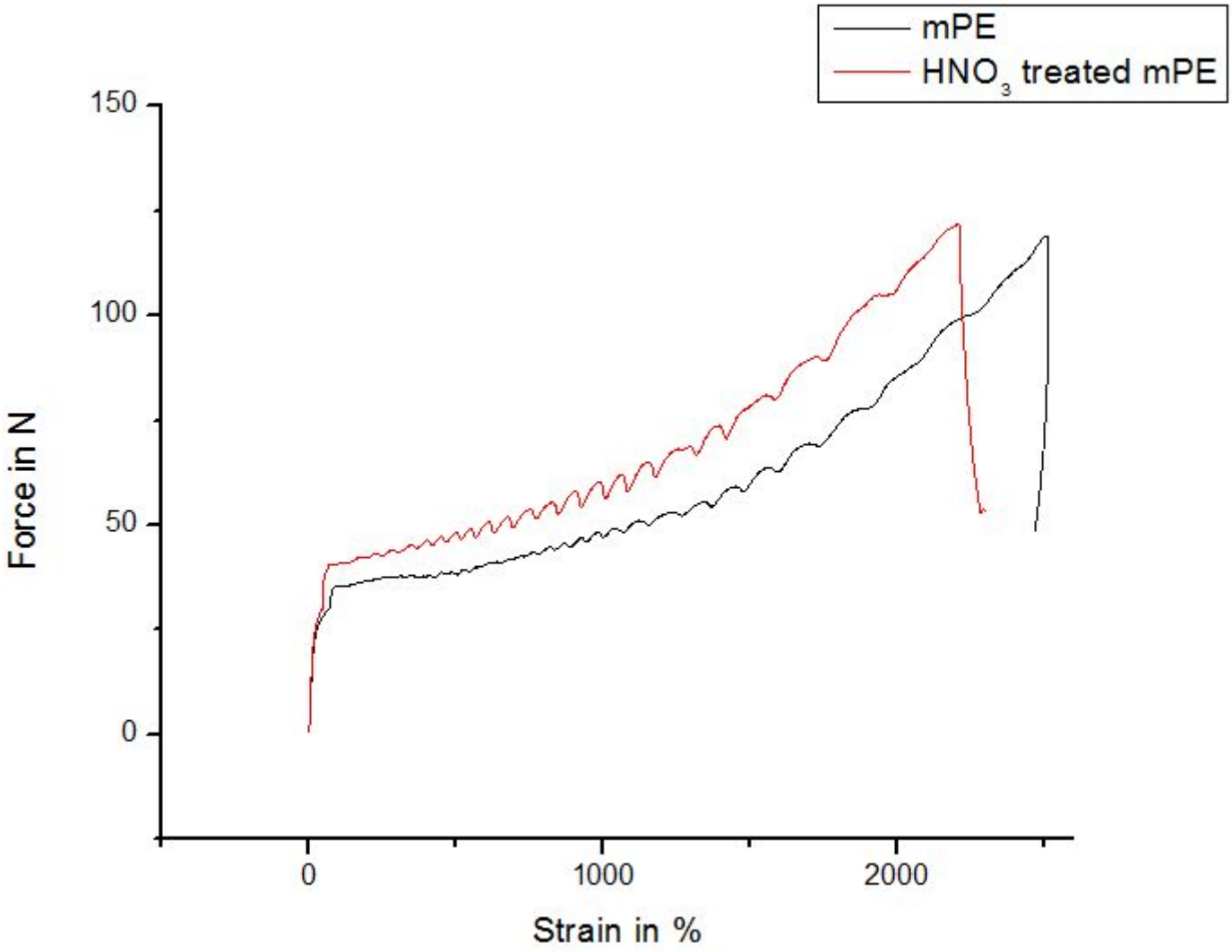


Figure 6 Tensile testing result of mPE before and after nitric acid treatment

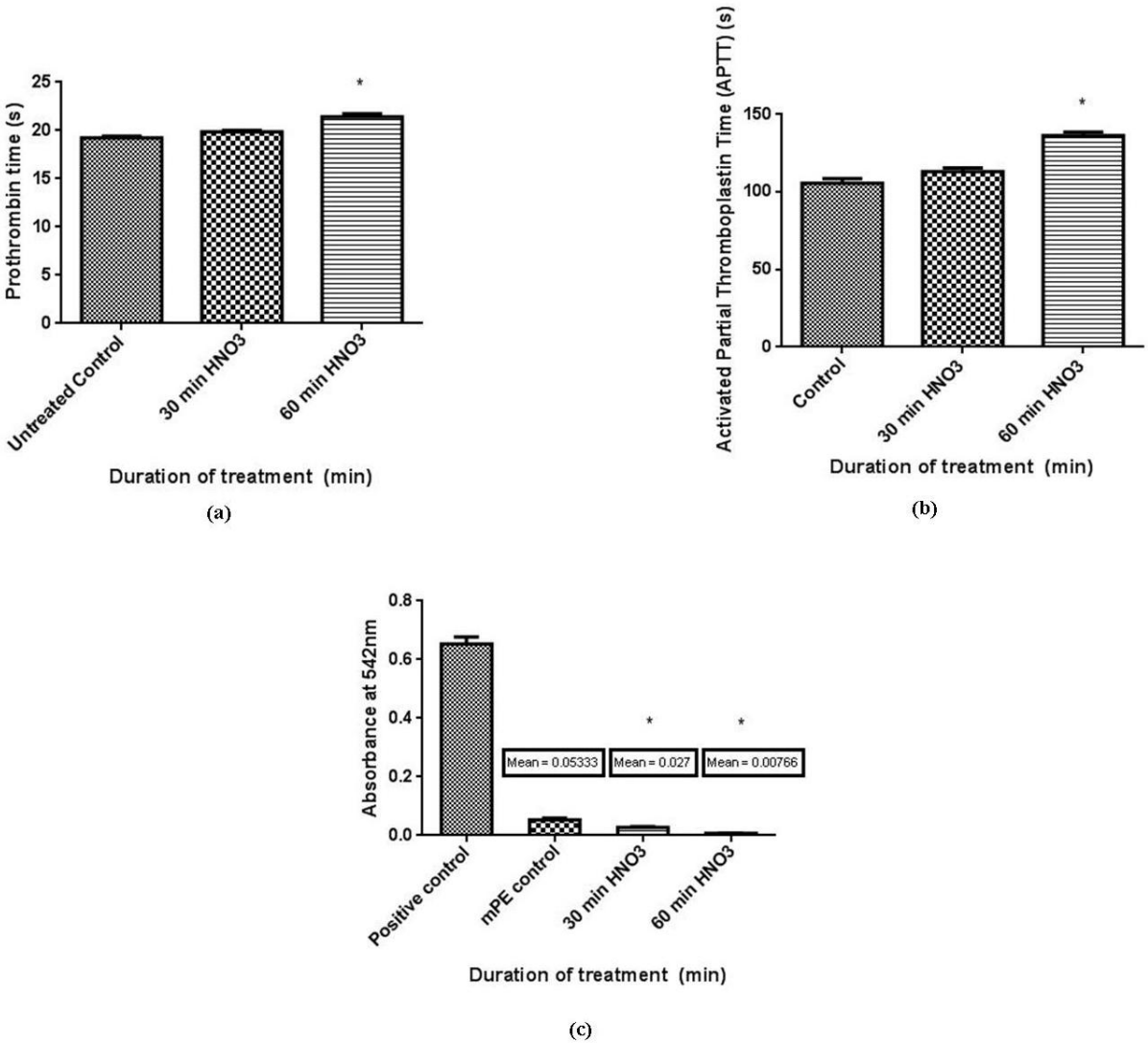
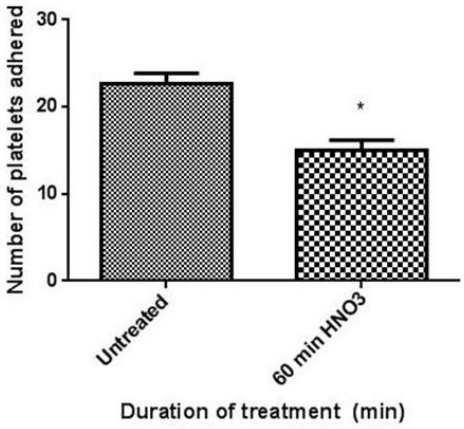
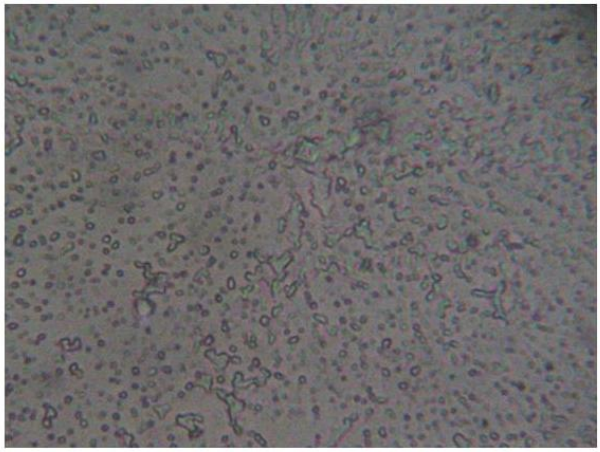


Figure 7 (a) Comparison of prothrombin time (PT) of control and HNO_3-treated metallocene polyethylene (n = 3) (b) The activated partial thromboplastin time (APPT) of control and HNO_3-treated metallocene polyethylene (n = 3 (c) The absorbance of control and HNO_3-treated metallocene polyethylene (n = 3) for all three panels, values shown are mean ± SD and * indicating differences in the mean are significant ($P < 0.05$)



(a)



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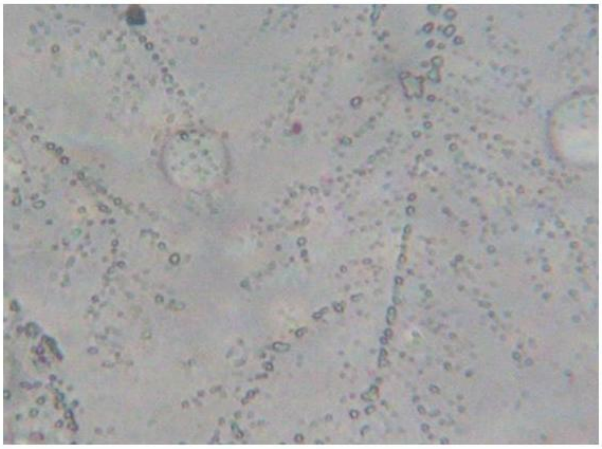

(c)

Figure 8 Platelet adhesion assay of untreated and HNO_3-treated metallocene polyethylene (n = 3), a shows the number of platelets adhered on the untreated and 60 min HNO_3 exposed surface and values are expressed as mean ± SD. * Differences in the means are significant with $P < 0.05$. Figure b and c delineates photomicrograph of platelets adhered on untreated and 60 min HNO_3-treated mPE at 40x magnification

733
734 **Table 1** Contact angle measurement of the mPE before and after HNO_3 treatment

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S. No	Sample	Average contact angle in degrees*
1	Untreated mPE	86.06±1.15
2	mPE treated with HNO_3 (30 min)	72.03±2.05
3	mPE treated with HNO_3 (60 min)	69.73±1.41

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740 **Table 2** Tensile testing result of mPE before and after 1 hour nitric acid treatment

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S. No	Sample	E-Modulus MPa	Fmax. N	E-FMax.%	W up to Fmax.Nmm
1	Untreated mPE	31.32	119.09	2510.82	30680.25
2	mPE treated with HNO_3 (60 min)	34.75	121.85	2510.39	32513.68

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