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Unravelling the potential of nitric acid as a surface modifier for improving the hemocompatibility of metallocene polyethylene for blood contacting devices

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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 reduced 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 notable 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 heart valves, stents and etc.
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 is 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(C₅H₅)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 HNO₃ for improving the blood compatibility of mPE. In a work done utilizing HNO₃ by Moreno-Castilla et al., dictates that the HNO₃ 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 HNO₃ 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 HNO₃ 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 HNO₃ 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 HNO₃ treatment. Then, 8- 10 ml of concentrated HNO₃ 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 shaker which moves at a constant speed. Moreover, in this work mPE sample were subjected to HNO₃ 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°C to remove the acid present on the surface of the polymer. The scheme of the experiments performed was shown in Fig. 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 untreated mPE, 30 minutes and 60 minutes HNO₃ 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 compositions or functional groups present within the polymer. There are three samples present in the study, which are untreated mPE, 30 minutes and 60 minutes HNO₃ treated mPE samples. All these samples were studied using this ATR-FTIR.

3D-Hirox Digital Microscope

The latest 3D-Hirox digital microscope model (KH-8700) was used to determine the formation of pits and pores of the samples. The 3D-Hirox digital microscopy images are very useful in determining the morphological structure of samples to determine whether the sample has pores or it has an even surface. There are two types of images which are obtained from 3D-Hirox digital microscopy either with or without profilometry line. The surface morphology of 1cm x 1cm of mPE and HNO₃ treated mPE sample was assessed at an area of approximately
5757 µ² at a magnification of 500X. The same as white light confocal profilometry, in-focus and 3D images were obtained using this 3D microscope. Slices of the image were captured at different heights acquired for the surface topography analysis (Pereira et al, 2013). Maximum of three profiling lines is chosen as the profiling value of the each sample. Each point in X, Y and Z axes of the profiling line is measured and their values can be exported in excel sheet to represent the height of the pits in the sample. Data processing was performed using the in-build 3D profilometry software. Images were recorded at standard 1,200 to 1,600 pixel resolution.

**Scanning Electron Microscope**

The surface microstructure of the samples can be critically analyzed in detail by using SEM. The SEM which is utilized to study the polymeric samples was JEOL JSM5800 SEM with OXFORD ISI 300 EDS X-ray Microanalysis System. Untreated mPE, 30 minutes and 60 minutes HNO₃ treated mPE underwent gold sputtering and then been studied using SEM at a magnification of 1500x.

**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, 30 minutes and 60 minutes HNO₃ treated mPE sample was measured by AFM in contact mode on a 10 × 10 µm² 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 the untreated mPE, 30 minutes and 60 minutes
HNO$_3$ treated mPE 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 $\mu$L at 37°C) was applied on untreated and treated polymer surface with NaCl-thromboplastin (Factor III, 100 mL, Sigma) which contains Ca$^{2+}$ 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 $\mu$L at 37°C) is incubated in prior with substrates at 37°C and followed by its activation by adding rabbit brain cephalin (100 $\mu$L 37°C). Later, the samples were incubated at 37°C for 5 minutes and followed by incubation with calcium chloride (0.025M). Inclusion of CaCl$_2$ triggers the clotting process. The time taken from the inclusion of CaCl$_2$ 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 (30 minutes and 60 minutes) and untreated samples were equilibrated with physiologic saline (0.9%w/v; 37°C, 30 minutes) 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 (60 minutes, 37°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 mPE samples were subjected to HNO\(_3\) exposure for 30 minutes and 60 minutes, later incubated along with physiological saline (0.9% w/v; 37°C, 30 minutes). This is kept on the rotary shaker for an hour to wash off the acid residues on the surface of the polymer. This is followed by immersing of untreated mPE, 30 minutes and 60 minutes HNO\(_3\) treated mPE samples in 1mL fresh PRP and the incubation was maintained at 37°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 86.06°. This was found to be far greater in comparison to the acid treated samples. The mean contact angles of 30 minutes and 60 minutes HNO\(_3\) treated samples are 72.03° and 69.73°, 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 Fig. 2. No changes were observed in the functional groups of the untreated mPE, 30 minutes and 60 minutes HNO₃ treated mPE however there is a slight decrease in the intensity of transmittance is observed. 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 3313 cm⁻¹ (O-H stretching) corresponding to hydroxyl group, 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 differ in their structures. A peak was also observed at 1020 cm⁻¹ which belongs to the C-O stretching.

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 Fig. 3(A)- 3(F). Fig. 3(A) represents the 3D image of the control and Fig. 3(B) shows its profiling image. Likewise, the Fig. 3(C) and Fig. 3(D) depict the 3D image and its profiling image of the thirty minutes HNO₃ treated sample, respectively. Similarly, Fig. 3(E) and Fig. 3(F) elucidates the 3D image and its profiling image of one hour HNO₃ treated mPE sample. The graph plotted with the values obtained from the 3D-Hirox digital microscope is represented in Fig. 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 Fig. 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 HNO₃ treated sample has the maximum number of pores with greater depth of fissures and holes which was ascertained using the 3D profiling. Fig. 4 shows the depth of the pores formed due to the etching effect of the HNO₃ and it is evident that the control has the least pore depth, followed by 30 minutes HNO₃ treated sample and finally the one hour HNO₃ 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 42.5 µm was the highest height of the pore in the case of 60 minutes HNO₃ treated sample whereas the highest pore for the 30 minutes HNO₃ treated mPE was 30 µm and 17.5 µm for untreated mPE. This shows the numerical values, data on relative changes for clearly differentiating the etching effect of HNO₃ on mPE.

SEM imaging is another surface characterization method of the samples at the micro level (Zhao et al, 2011). Topography of the mPE was investigated as shown in Fig. 5(A-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. However, the size and the number of the pits seems to increase in case of the 60 minutes acid treated sample.

The AFM images are represented in Fig. 6 (A-C). Fig. 6(A), Fig. 6(B) and Fig. 6(C) are the AFM image of the untreated, 30 minutes and 60 minutes HNO₃ treated mPE sample, respectively. From the results obtained, it was found that the mean value of Ra of untreated mPE film, 30 minutes and 60 minutes HNO₃ treated mPE surface are 2.069 nm, 4.233 nm and 5.127 nm, respectively. The nanotopographic analysis of the samples was performed using AFM. Fig. 6(A) 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 untreated mPE sample. On the other hand, Fig. 6(B) which is the 30 minutes HNO₃ treated sample has more nano-roughness compared to the control but less roughness than the 60 minutes HNO₃ treated sample. This shows that 60 minutes HNO₃ treated sample has most 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. 7(A-C). From the tensile stress-strain curve it is palpable that the elastic
modulus of 30 minutes HNO$_3$ treated mPE is 33.01MPa (Fig. 7B) and 60 minutes HNO$_3$ treated mPE is 34.75MPa (Fig. 7C) which are slightly greater than the elastic modulus of untreated mPE 31.32MPa (Fig. 7A). 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 30 minutes and 60 minutes HNO$_3$ treated. Their results of PT and APTT were summarized in Fig. 8(A) and Fig. 8(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 minutes and 60 minutes 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 minutes 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. 8(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 minutes and 60 minutes treatment. From the results obtained, it is obvious that the 60 minutes HNO$_3$ treated mPE is the least hemolytic
compared to other samples. Moreover, it was also found that an absorbance value of 60 minutes 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 as shown in Fig. 9A. A maximum of 22 platelets was observed on the surface of the untreated samples (Fig. 9B), meanwhile the number of platelets decreased to a maximum of 18 platelets (Fig. 9C) and 15 platelets (Fig. 9D) on 30 minutes and 60 minutes HNO$_3$ treated samples, respectively. Statistical analysis of the untreated sample with the treated one (number of platelets adhered) with one-way ANOVA shown significant differences ($P < 0.05$) between them after 30 minutes and 60 minutes treatment.

**Discussion**

Blood clotting occurs when blood comes in contact with a foreign surface such as implants following platelet activation. This can be catastrophic in clinical settings, especially in case of various biomedical applications like grafts, catheters, hemodialysis, bypass/extracorporeal membrane oxygenation, and ventricular assist devices (Qi, Maitz & Huang, 2013). In order to circumvent this issue, the hemocompatibility of the blood contacting devices has to be improved and HNO$_3$ surface treated mPE holds a great potential. For ascertaining the topographical modification caused by HNO$_3$ on the mPE sample, characterization tests was performed using 3D Hirox, SEM, AFM, contact angle and FTIR. On the other hand, the blood compatibility of the sample was studied by conducting different blood coagulations assays like hemolysis assay, PT, APTT, and platelet adhesion.
The decrease in contact angle indicates the improved wettability and hydrophilicity of the mPE polymer. It is a well known fact that the surface energy is a vital parameter determining polymer’s adhesion, material wettability and even biocompatibility (Kwok, Wang & Chu PK, 2005). Thus, the assessment of contact angles is contemplated as one of the most convenient method for the determination of surface free energy of solid samples. This technique depends on the interactions between the solid sample of interest as well as liquids with well determined surface tensions. Our result is in good agreement with our previous published results of HCl exposed mPE (Jaganathan et al, 2014). Thus, the improved surface roughness is reflected in the decrease in contact angle with HNO₃ treatment time. 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 HNO₃ 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). Similarly, the improved surface roughness is also palpable from the results of AFM, SEM, Hirox 3D microscopy result. Hence, the decrease in the contact angle and increase in surface roughness are in consensus from obtained result. This is analogous to the studies that have ascertained that the increase in wettability is instigated by the increase in surface roughness and associated decrease in the contact angle (Mirabedini et al. 2004; Mirzadeh & Dadsetan 2003; Rochotzki et al. 1994).

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. The surface changes induced in the metalocene polyethylene (mPE) surface is instigated by the etching effect of nitric acid (HNO$_3$). The HNO$_3$ exposure produces pits and holes in the surface of mPE. Owing to the formation of pits and pores in mPE, the surface roughness increases. The improved surface roughness of mPE is reflected in increased intensity of absorbance in FTIR but the peaks remain almost the same, indicating no chemical changes were seen in the surface of mPE. Bergström demonstrated that absorption heavily depends upon the surface properties of the material (Bergström 2008). Since most real life surfaces are not perfectly flat and possess certain degrees of texture and roughness to them, this will influence their optical behaviour. Pits and valleys in a material may, for example, “trap” some of the light and thereby increase the intensity of absorption. However, when the absorption increases, the transmittance decreases. (Sorrell 2006) This explains the reason for the increased absorption leading to decreased intensity of transmittance in this study, as the surface roughness improves after HNO$_3$ treatment. 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 60 minutes HNO$_3$, 30 minutes HNO$_3$ and then finally the control.

It was found that the Ra value of the 60 minutes HNO$_3$ treated sample value is almost twice greater than the control and slightly higher than the 30 minutes HNO$_3$ treated mPE. 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 -5°C 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 HNO₃ 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 main advantage of the HNO₃ over HCl is the improvement of hemocompatibility without deteriorating the tensile strength of the mPE samples after treatment. The effect of HCl on the tensile strength of mPE is not yet reported, but it was found that the HCl treatment deteriorate the tensile strength of the sisal fiber (I. Oladele 2010). Similarly, in another work done by Mingchao et al., the tensile strength of basalt fiber declines after the HCl exposure (Wang Mingchao et al. 2008). However, the results of HNO₃ exposure improved the tensile strength of different polymers like polyacrylonitrile, carbon fibers, thermoplastic polyimide composite and etc (Bahl 1984; Li 2009; Nie & Li 2010). In our study HNO₃ treatment did not reduce the tensile
strength of mPE. Hence, the major advantage of HNO₃ treatment over HCl treatment is the
deterioration of tensile strength of mPE can be prevented, in addition to the enhancement the
blood compatibility properties of mPE sample.

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 HNO₃. There was a notable increase observed in the PT and
APTT of the HNO₃ 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 HNO₃
on mPE.

The improved surface roughness induces physicochemical changes which results in a
favorable impact on the hemocompatible property of mPE thereby making it more resistant to
RBCs damage. The lysis of RBCs generally occur due to the increase in osmotic pressure
triggered by the poor material surface which normally results in the rupture and release of the
cellular contents including hemoglobin (Zhang et al. 2015). Hemolysis percentage is the
representative of RBCs damage. Hence, the improved surface roughness of HNO$_3$ treated mPE decreases the RBCs damage when they come in contact with them. According to ASTM F756-00(2000) standard, both 30 and 60 min HNO$_3$ treated samples are deduced to be non-hemolytic materials since the percentage of damage falls below 2 (M. Fazley Elahi 2014). This result is in agreement to one of the research elucidating that the surface roughness plays an important role in controlling the thrombogenicity and it was demonstrated that catheters with increased roughness were found to be less thrombogenic than smooth surfaced catheters (Bailly et al. 1999). 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 is of extreme importance as the proposed mPE material will be in contact with blood for a prolonged period in the blood circulation system.

The increased surface roughness of HNO$_3$ treated mPE produces a better resistance to platelets by minimizing the platelet adhesion. The extent of platelet adhesion as well as its activation is a deciding factor of thrombogenicity of a material, as blood compatible substrates neither attracts nor activates the platelets present in the blood stream. In general the hydrophilic surface is observed to be more efficient in preventing the platelet adhesion. This is because; it has the ability to exert steric repulsion to avoid unspecific protein deposition (Gomathi et al. 2010). Moreover, the hydrophilic surface is also found to encourage the adsorption of anti-platelet adhesion proteins like albumin, high molecular-weight kininogen, etc., which further bolsters it’s shielding capacity against platelet deposition and activation. These observations were evidently replicated in the HNO$_3$ treated mPE surfaces (Gomathi et al. 2010; Lee & Lee 1998). The obtained results are in consensus with other studies conducted on hydrophilic surface dictating that improved surface roughness led to decreased platelet adhesion (Zingg et al. 1981; Zingg et
Similarly, Zhao et al., demonstrated that when the NiTi alloy surface roughness was increased, platelet activation, adhesion, as well as hemolysis were appreciably reduced (Zhao et al. 2011). The reduced platelet adhesion in the HNO$_3$ treated sample dictates the improved hemocompatibility of surface modified mPE (Gomathi et al, 2012; Habibzadeh et al, 2014).

Thus, the possible mechanism from the obtained result of this study is the surface roughness of mPE improves by the HNO$_3$ treatment. When surface roughness of mPE increases, it results in decrease in contact angle and increases the hydrophilicity of mPE. Hence, the improved surface roughness minimizes the RBCs damage by reduction of osmotic pressure triggered by the poor material surface and leads to decreased platelet adhesion by exerting steric repulsion to avoid unspecific protein deposition. This is the possible mechanism by which the blood compatibility of HNO$_3$ treated mPE improves when it is compared against the control as represented in Fig.10. Hence, this modified mPE with more surface roughness, altered wettability, and better blood compatibility may be the vital characteristics that can be utilized for construction of long-term blood contacting devices like catheters, transvenous pacing leads, stents, grafts and etc.

**Conclusions**

The surface modification induced by HNO$_3$ on mPE and its effect on mPE blood compatibility was assessed. Contact angle analysis depicts a decrease in the contact angle elucidating increase in the wettability of the HNO$_3$ treated samples. There were no prominent qualitative changes in the functional groups were observed by FTIR studies. The 3D Hirox microscopy imaging also confirms the improved surface roughness by formation of more pits and bumps in the acid treated sample than the control. SEM images of treated samples further substantiate that acid treated sample surface possess more pits and pores compared to the control. AFM topographical analysis shows an improved surface roughness in the 30 minutes and 60 minutes acid treated sample compared to the control due to the etching effect of the acid. Blood
coagulation assays like PT and APTT ascertains a notable delay in the clotting mechanism on the surface of acid treated samples. The result of hemolysis assay shows a minimum damage to red blood cells (RBC). Platelet adhesion assay elucidates that the number of platelets adhered to the surface of acid treated polymer was appreciably less in comparison to the untreated surface. The HNO$_3$ 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 HNO$_3$ treated mPE.
**Figure 3** Different three-dimensional representations using 3D Hirox digital microscopy. (A) Untreated mPE (B) Untreated mPE with profiling (C) 30 minutes HNO₃ treated mPE (D) 30 minutes HNO₃ treated mPE with profiling (E) 60 minutes HNO₃ treated mPE (F) 60 minutes HNO₃ treated mPE with profiling.
Figure 4 The representative height of the pores of different samples measured using 3D-profiling of 3D Hirox digital microscopy.
Figure 5 Representative SEM micrographs of untreated and HNO$_3$ treated mPE. (A) Untreated mPE (B) 30 minutes HNO$_3$ treated mPE (C) 60 minutes HNO$_3$ treated mPE.
Figure 6 Representative AFM images of untreated and HNO$_3$ treated mPE. (A) Untreated mPE (B) 30 minutes HNO$_3$ treated mPE (C) 60 minutes HNO$_3$ treated mPE
Figure 7 Representative tensile testing result of untreated and HNO$_3$ treated mPE. (A) Untreated mPE (B) 30 minutes HNO$_3$ treated mPE (C) 60 minutes HNO$_3$ treated mPE.
Figure 8 Comparison of prothrombin time (PT), activated partial thromboplastin time (APPT) and absorbance of untreated and HNO$_3$ treated mPE. (A) The PT of control, 30 minutes and 60 minutes HNO$_3$ treated mPE ($n = 3$) (B) The APPT of control, 30 minutes and 60 minutes HNO$_3$ treated mPE ($n = 3$) (C) The absorbance of control, 30 minutes and 60 minutes HNO$_3$ treated mPE ($n = 3$). For all three panels, values shown are mean ± SD and * indicating differences in the mean are significant ($P < 0.05$).
**Figure 9** Platelet adhesion assay of untreated and HNO$_3$ treated mPE. (A) Comparison of the number of platelets adhered untreated, 30 minutes and 60 minutes HNO$_3$ treated mPE. Values are expressed as mean ± SD and * indicates difference in the means are significant with $P < 0.05$ (B) Number of platelets adhered on untreated mPE (C) Number of platelets adhered on 30 minutes HNO$_3$ treated mPE (D) Number of platelets adhered on 60 minutes HNO$_3$ treated mPE.

**Figure 10** Mechanism of improved hemocompatibility by HNO$_3$ treatment
Table 1 Contact angle measurement of the mPE before and after HNO$_3$ treatment.

| S. No | Sample  | Average contact angle |
|-------|---------|-----------------------|
|      |         |                       |
| 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 HNO₃ (30 minutes)         | 33.01         | 120.63   | 2510.40   | 31114.00         |
| 3     | mPE treated with HNO₃ (60 minutes)         | 34.75         | 121.85   | 2510.39   | 32513.68         |

**Table 2** Tensile testing result of untreated, 30 minutes and 60 minutes HNO₃ treated mPE.
Schematic representation of series of characterization and blood compatibility experiments done

1

Contact Angle

Tensile Testing

ATR-FTIR

Atomic Force Microscopy

3D-Hiiox

Scanning Electron Microscopy

Prothrombin Time (PT)

Platelet Adhesion Assay

Activated Partial Thromboplastin Time (APPT)

Hemolysis Assay

Characterization tests

Blood coagulation assays

Untreated mPE

HNO₃ treatment on mPE

A. 30 minutes HNO₃ treated mPE
B. 60 minutes HNO₃ treated mPE
A representative FTIR spectra of untreated and HNO3 treated mPE.
Different three-dimensional representations using 3D Hirox digital microscopy.

(A) Untreated mPE (B) Untreated mPE with profiling (C) 30 minutes HNO3 treated mPE (D) 30 minutes HNO3 treated mPE with profiling (E) 60 minutes HNO3 treated mPE (F) 60 minutes HNO3 treated mPE with profiling.
Manuscript to be reviewed

A

B

C

D

E

F
The representative height of the pores of different samples measured using 3D-profiling of 3D Hirox digital microscopy.
Representative SEM micrographs of untreated and HNO3 treated mPE.

(A) Untreated mPE (B) 30 minutes HNO3 treated mPE (C) 60 minutes HNO3 treated mPE.
Representative AFM images of untreated and HNO3 treated mPE.

(A) Untreated mPE (B) 30 minutes HNO3 treated mPE (C) 60 minutes HNO3 treated mPE
Representative tensile testing result of untreated and HNO3 treated mPE.

(A) Untreated mPE  (B) 30 minutes HNO3 treated mPE  (C) 60 minutes HNO3 treated mPE.
Comparison of prothrombin time (PT), activated partial thromboplastin time (APPT) and absorbance of untreated and HNO3 treated mPE.

(A) The PT of control, 30 minutes and 60 minutes HNO3 treated mPE (}
Platelet adhesion assay of untreated and HNO3 treated mPE.

(A) Comparison of the number of platelets adhered untreated, 30 minutes and 60 minutes HNO3 treated mPE. Values are expressed as mean ± SD and * indicates difference in the means are significant with
Mechanism of improved hemocompatibility by HNO3 treatment.
Improved Surface Roughness and Hydrophilicity

- Water Droplet (Higher Contact Angle)
- Water Droplet (Decreased Contact Angle)
- Decreased Platelet Adhesion
- Undamaged RBCs
- Steric Repulsion
Table 1 (on next page)

Contact angle measurement of the mPE before and after HNO3 treatment.
Table 1 Contact angle measurement of the mPE before and after HNO\textsubscript{3} treatment.

| S. No | Sample                        | Average contact angle in degrees* |
|-------|-------------------------------|-----------------------------------|
| 1     | Untreated mPE                 | 86.06±1.15                        |
| 2     | mPE treated with HNO\textsubscript{3} (30 minutes) | 72.03±2.05                        |
| 3     | mPE treated with HNO\textsubscript{3} (60 minutes) | 69.73±1.41                        |
Table 2 (on next page)

Tensile testing result of untreated, 30 minutes and 60 minutes HNO3 treated mPE.
Table 2 Tensile testing result of untreated, 30 minutes and 60 minutes HNO$_3$ treated mPE.

| S. No | Sample                          | E-Modulus (MPa) | Fmax. N (N) | E-FMax.% | W up to Fmax. Nmm (Nmm) |
|-------|---------------------------------|-----------------|-------------|----------|-------------------------|
| 1     | Untreated mPE                   | 31.32           | 119.09      | 2510.82  | 30680.25                |
| 2     | mPE treated with HNO$_3$ (30 minutes) | 33.01           | 120.63      | 2510.40  | 31114.00                |
| 3     | mPE treated with HNO$_3$ (60 minutes) | 34.75           | 121.85      | 2510.39  | 32513.68                |