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Low-pressure plasma enhanced immobilization of chitosan on low-density polyethylene for bio-medical applications

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ABSTRACT

With the aim of improving blood compatibility of low density polyethylene (LDPE) films, an effective low-pressure plasma technology was employed to functionalize the LDPE film surfaces through *in-situ* grafting of acrylic acid (AAc). Subsequently, the molecules of poly(ethylene glycol) (PEG) and chitosan (CHI) were immobilized on the surface of grafted LDPE films. The unmodified and modified LDPE films were analyzed using various characterization techniques such as contact angle, atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FTIR) and X-ray photo electron spectroscopy (XPS) to understand the changes in surface properties such as hydrophilicity, surface topography and chemical composition, respectively. Furthermore, LDPE films have been subjected to an ageing process to determine the durability of the plasma assisted surface modification. The blood compatibility of the surface modified LDPE films was confirmed by *in vitro* tests. It was found that surface modified LDPE films show better hydrophilic behavior compared with the unmodified one. FTIR and XPS results confirm the successful immobilization of CHI on the surface of LDPE films. LDPE films showed marked morphological changes after grafting of AAc, PEG and CHI which were confirmed through AFM imaging. The *in vitro* blood compatibility tests have clearly demonstrated that CHI immobilized LDPE films exhibit remarkable anti thrombogenic nature compared with other modified films. Surface modified LDPE films through low-pressure plasma technique could be adequate for biomedical implants such as artificial skin substrates, urethral catheters or cardiac stents, among others.

1. Introduction

The low density polyethylene (LDPE) has been widely used in various industrial applications over the past decades; especially in biomedical field to produce blood contacting devices as artificial heart valves, stents, blood bags, etc., owing to its superior mechanical and favorable biocompatibility [1–5]. However, surface induced thrombus formation, due to the immediate adsorption of plasma protein and activated platelets, is the major drawback of LDPE films when in contact with blood. Hence, the material needs appropriate treatment to prevent the surface induced thrombus formation because the surface chemistry and topography of the materials play a pivotal role in determining their biocompatibility, strongly influencing the biological response and determining their long-term performance *in vivo* [6–8]. Furthermore, the biomaterials also need to possess significant physical and mechanical properties in order to function properly in the biological environment. Therefore, it is necessary to modify surface properties without affecting the bulk-mechanical properties. Recently, different common approaches such as corona treatment, conventional polymerization, radiation polymerization and plasma induced polymerization were adopted to remedy the lack of blood compatibility of the LDPE films [9].

Among the various methods, the low-pressure plasma assisted polymerization of specific monomer is a gorgeous technique to alter or provide the specific functionalities on the surface of LDPE films. The low-pressure plasma has to be generated by either direct or RF current. It contains a variety of new species such as metastable free radicals, ions, photons and neutrals which are chemically active

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and leads to induce the formation of free radicals in the polymeric chain and in this way it is possible to insert or interact with certain functional groups on the polymer surface that will enhance the surface properties of the polymeric films. In this process, the LDPE film surface is initially treated by inert gas plasma such as argon which induces chain scission and bond breaking at the surface, creating carbon radicals on the polymer surface through abstraction of C—H and C—C bonds. Thereafter argon plasma treated LDPE films were exposed to the oxygen atmosphere. Radicals which are produced by argon plasma treatment interact with oxygen atoms resulting in the formation of peroxide active species on the surface that can initiate the grafting of desired monomer. The plasma induced graft polymerization does not affect the overall product and performance of the material bulk because it is confined to only few nanometer depth. Owing to its high reproducibility, chemical stability, scalability, controlled hydrophilic/hydrophobic character, uniformity, and adaptability to various film depositions, gives rise to new surface properties improving the biocompatibility by creating interfacial layers in different deposition processes or beads for active biological compounds immobilization [10–13]. This fabulous technique can be employed to design the selective biomedical materials with tailored chemical functionalities and morphologies to interaction with blood components such as platelets, proteins, etc. Recently, acrylic grafted polymeric materials have been used in various biomedical applications because it provides active sites for immobilization of specific biomolecules for further improvement in biocompatibility.

The molecules of PEG and chitosan are the potential candidates to improve the blood compatibility of polymeric materials. Poly(ethylene glycol) is relatively non-toxic, non-flammable, non-immunogenic, non-antigenic and present protein-resistant properties. This precursor induces, on the LDPE surface, a high density of polar functional groups especially ether and carboxylic groups. Sakthi Kumar et al. immobilized PEG (MW 200) on PET film surface by radio frequency (RF) plasma induced polymerization for the improvement of hemo-compatibility [14]. Furthermore, the immobilization of chitosan molecules (which have reactive amino and hydroxyl groups) on the surface of LDPE further improves its biocompatibility and makes it a desirable candidate for biocompatible and blood compatible biomaterials [15]. Xin et al. [16] grafted acrylic acid (AAc) on the surface of polyethylene (PE) film through O_2 plasma and UV pretreatment and thereafter the molecules of O-stearoyl chitosans (OSC) were immobilized on the surface of AAc-grafted PE films. Their results clearly demonstrated that the OSC immobilized PE films exhibited anti-platelet adhesion (i.e. improved the blood compatibility) compared with that of untreated PE. The anti-platelet adhesion of PE-OSC is ascribed to the suitable balance of physico-chemical changes induced by the plasma and UV induced surface modification. Kang et al. improved the bio-compatible properties of poly(methyl methacrylate) film by immobilization of proteins on the surface of AAc grafted PMMA [17]. Aiping et al. have studied the immobilization of molecules of the chitosan (CS) and O-Carboxymethyl chitosan (OCMCHI) on poly(ethylene terephthalate) films surface-activated by argon plasma followed by graft copolymerization with acrylic acid (AAc) [18]. However, the most of the researchers reported the grafting of AAc and PEG by the direct immersion methods [19–25].

In this paper, blood compatibility of the LDPE film is improved by dc excited low-pressure plasma enhanced polymerization technique. The surface properties of LDPE films were initially improved by argon plasma treatment and subsequent grafting of acrylic acid. After that the molecules of PEG were immobilized on the surface of acrylate grafted LDPE films through PEG in vapor phase. A few reports regarding the immobilization of PEG on the surface of polymeric films using low molecular weight PEG in vapor phase are available [14]. Thereafter the molecules of chitosan were immobilized covalently on the surface of PEG grafted LDPE films for further improvement in bio-compatibility. The degree of hydrophilicity of unmodified and modified LDPE films was analyzed by contact angle and surface energy analysis. The Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) were used to analyze the chemical composition and atomic force microscopy (AFM) was used to study the changes in surface topography of the LDPE films. The *in vitro* blood compatibility of surface modified LDPE films was also systematically investigated by *in vitro* test which included platelet adhesion, protein adsorption, thrombus formation and whole blood clotting time (WBCT) analysis.

2. Experimental setup and methodology

2.1. Materials

Smooth and homogeneous low density polyethylene (LDPE) films of thickness of 40 μm were procured from Reliance Petro Chemicals Ltd, Mumbai, India. Before the plasma treatment, films were cleaned ultrasonically using acetone and de-ionized water to remove organic contaminants on the surface of the LDPE films followed by drying in the oven at 50 °C for 1 h. Other chemicals such as chitosan, citric acid, poly(ethylene glycol) (MW 600) and acrylic acid were procured from Sigma-Aldrich and LOBA, India.

2.2. Plasma treatment and grafting of AAc

The plasma induced polymerization reactions were carried out using low-pressure dc plasma reactor (Fig. 1a). It consists of "D" type plasma chamber with dimension of 30 cm W × 30 cm H. The chamber is fabricated with stainless steel and the water cooling copper tube is breezed on the surface of the plasma chamber, to avoid excessive heating of the chamber during the plasma treatment. Two circular (diameter = 10 cm) water cooled electrodes were fixed parallel to each other within the chamber as shown in Fig. 1a and the upper electrode is of magnetron type, which is used to confine the plasma for homogeneous plasma surface treatment. The distance between the two electrodes was kept 5 cm and the plasma excitation occurs between two vertical electrodes using high tension dc power supply (V_{max} = 3 kV and I_{max} = 1 A). A circular sample holder and a gas shower ring are placed between the electrodes (diameter 10 cm, 2 cm vertical distance to sample holder) (Fig. 1a). The active plasma zone displays a cylindrical symmetry as shown in Fig. 1b. The gas inlet system enables gas mixing controlled by an electronic flow meter and a mass flow controller (MFC); the pressure in the chamber was measured by a Pirani gauge. Initially, ultrasonically cleaned LDPE film was kept on the sample holder and the chamber was evacuated to a low-pressure of $\sim 10^{-3}$ mbar using a rotary vacuum pump. After that, the chamber was purged with argon gas for three times and, then, the argon flow rate was adjusted to 120 SCCM. The working pressure inside the chamber was maintained at 0.2 mbar.

A dc potential was applied between the two water cooled electrodes and adjusted till stable plasma was generated. Initially, the samples were treated in the argon plasma at a fixed power, electrode separation, exposure time and pressure of 350 W, 5 cm, 5 min and 0.2 mbar, respectively. Then, oxygen was purged in the plasma chamber at the rate of 20 SCCM for 20 min in the absence of plasma which leads to incorporation of peroxides onto the oxidative LDPE films that are able to initiate the graft polymerization of acrylic acid. The formation of peroxides are mainly caused by formation of free radicals on the surface of LDPE films due to argon plasma

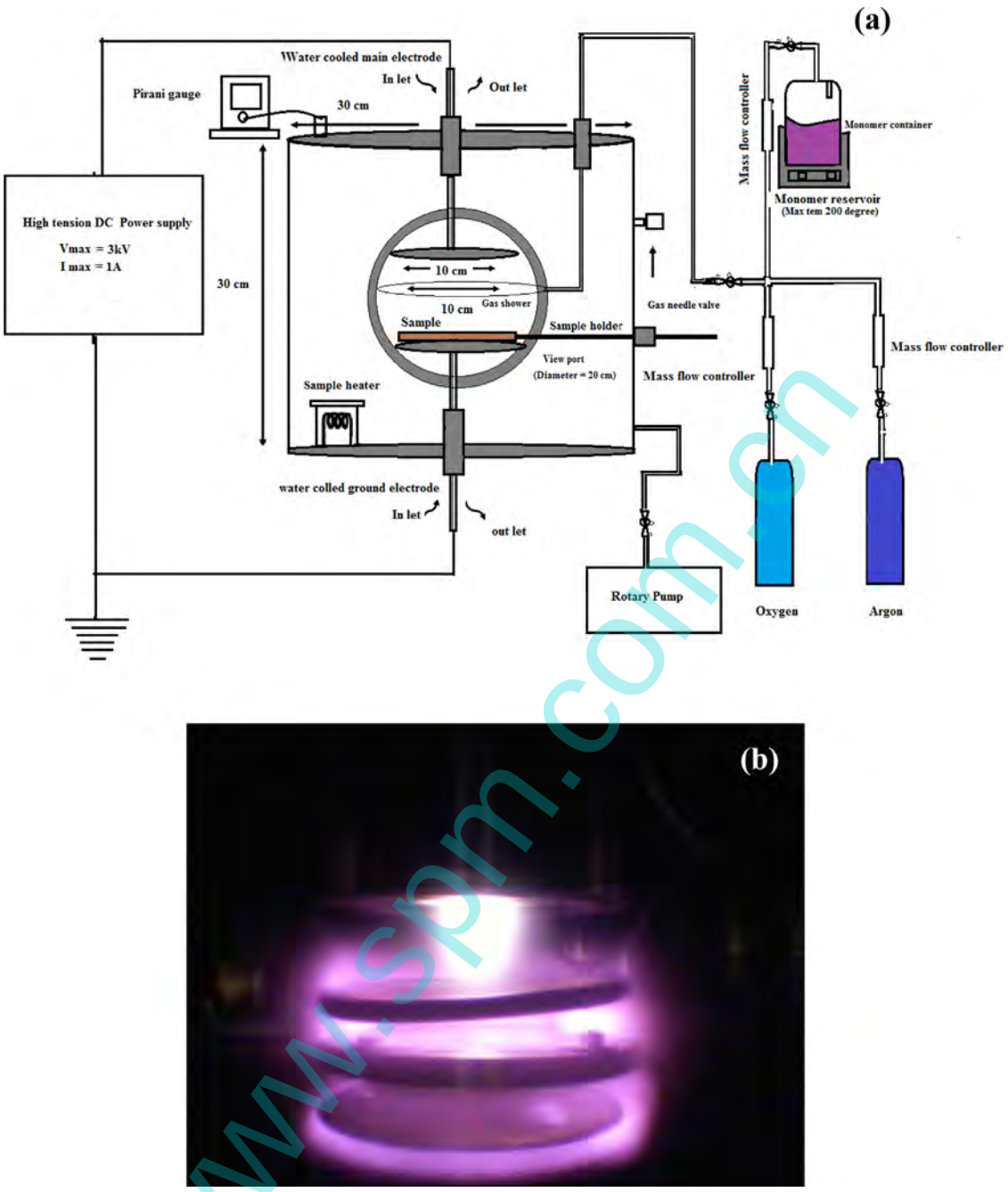


Fig. 1. (a) Schematic diagram of low-pressure plasma reactor and (b) low-pressure plasma glow observed during the surface treatment.

treatment. After that, plasma was again ignited using the above mentioned operating parameters, argon was used as a main gas with the flow rate of 120 SCCM and, at the same time, acrylic acid monomer (AAc) vapor produced by the monomer reservoir at the temperature of 80 °C. The obtained monomer vapor was fed into plasma regime as a shower by a copper ring that has perforation for uniform distribution of monomer vapor which leads to formation of acrylic coatings on the surface of LDPE films which contain high density of carboxylic functional groups [26–28]. Argon was used to carry the monomer vapor into the plasma regime and the monomer vapor flow rate was kept at 40 SCCM. The other operating parameters such as deposition time, operating pressure and discharge potentials were kept constant at 3 min, 0.2 mbar and 500 V, respectively.

2.3. Immobilization of PEG

Thereafter the molecules of PEG were grafted on the surface of AAc grafted LDPE films for further improvement of bioactivity and also PEG grated surface acts as a spacer for immobilization of biomolecules like chitosan. Initially, acrylic grafted LDPE film was washed with 0.1 wt% of Triton X-100 and with distilled water for 10 min to remove any unreacted monomers and oligomers as well as low-molecular weight polymer from the LDPE film surfaces and dried the same at reduced pressure for 12 h at RT. After that the sample, AAc grafted LDPE film was kept on the sample holder and the chamber was initially evacuated to a pressure of 10^{-3} mbar. Followed by the passage of argon (flow rate 120 SCCM) as carrier gas and PEG vapors (flow rate 40 SCCM) and the working pressure

Table 1
Typical operating parameters for plasma processing.

Plasma forming gas	Ar and O_2
Carrier gas	Ar
Precursor monomer	AAc, PEG
Ar plasma exposure time	5 min
Electrode separation	5 cm
Working pressure	0.2 mbar
Gas flow (SCCM)	Ar—120, O_2—20, AAc—40, PEG—40
Deposition time	3 min
Discharge potential	500 V
Discharge current	700 mA

was maintained at 0.2 mbar. In order to get sufficient vapor pressure of PEG, the monomer reservoir was kept at the temperature of 200 °C. Plasma polymerization results in the formation of PEG layer on the surface of AAc-LDPE films, which is caused by collisions of charged particles and plasma irradiation in the gas phase or by adsorption of PEG layer at the surface of the polymer substrate. Most probably, PEG molecules are dissociated by collision with charged particles and results in the formation of radicals and fragmentation of monomer units. These fragmentations are enforced to recombine (cross linking) on the surface of AAc grafted LDPE films by poly recombination results in immobilization of PEG molecules along with specific functional groups on the surface of LDPE films [14,29,30]. Typical operating parameters are listed in Table 1.

2.4. Immobilization of chitosan

Before the immobilization, 1 g of chitosan was dissolved in 100 mL of acetic acid aqueous solution and the mixture was stirred for 24 h at room temperature resulting 1% (w/v) of chitosan solution. After that, the surface coated LDPE films were cut into 4 cm × 4 cm and subsequently reacted with the chitosan solution keeping a constant stirring for 1 min, followed by washing with distilled water. The obtained chitosan immobilized LDPE films were washed with 0.1 wt% Triton X-100 for 10 min to remove excess chitosan from the LDPE film surfaces and dried at room conditions [31].

2.5. Surface analysis

The change in hydrophilicity induced by various plasma treatments on LDPE films was analyzed through contact angle measurements by sessile drop method [9]. Three testing liquids such as distilled water (W), formamide (F) and ethylene glycol (EG) of known surface tension components (given in Table 2) were used to measure the contact angle with respect to LDPE films and the volume of the testing liquids was fixed at 2 μL. The very representative contact angle value was an average of 10 independent measurements and an average experimental error of the measurement was ±2°. The contact angle measurements were performed under controlled room temperature and humidity conditions (55%).After that the surface modified LDPE films were stored in air up to 15 days for investigating their ageing effect.

Table 2
Surface tension components of the testing liquids.

Liquids	γ_l (mJ/m^2)	γ_l^p (mJ/m^2)	γ_l^d (mJ/m^2)
Water (W)	72.8	51.0	21.8
Formamide (F)	58.2	18.7	39.5
Ethylene glycol (EG)	48.0	19.0	29.0

The polar (γ_s^p) and dispersion (γ_s^d) components of surface free energy of LDPE films were calculated using Fowke's equation extended by Owens-Wendt as follows [32,33].

$$\left[\frac{1+\cos\theta}{2}\right] \times \left[\frac{\gamma_l}{\sqrt{\gamma_l^d}}\right] = \sqrt{\gamma_s^p} \times \sqrt{\frac{\gamma_l^p}{\gamma_l^d}} + \sqrt{\gamma_s^d} \quad (1)$$

Equation (1) is in the form:

$$Y(\text{LHS}) = m \cdot X(\text{RHS}) + C \quad (2)$$

where the value of LHS and RHS could be obtained by contact angle value (θ), surface tension of testing liquids (γ_l), and it's polar (γ_l^p) and dispersion (γ_l^d) components as given in Table 2. The plot of LHS vs RHS gave a straight line with intercept on *Y*-axis. Slope and intercept obtained from the plot were squared and added up to give a total surface free energy (γ_s) of LDPE films as given in equation (3)

$$\gamma_s = \gamma_s^p + \gamma_s^d \ (\text{mJ/m}^2) \quad (3)$$

Furthermore, the work of adhesion (W_{adh}), a quantity related to the surface wettability, was calculated using the formula [34,35]

$$W_{adh} = \gamma_l(1 + \cos\ \theta) \quad (4)$$

The surface polarity (*P*) of the plasma treated polymer films were estimated by the following formula [34,35]:

$$P = \frac{\gamma_s^p}{\gamma_s^p + \gamma_s^d} \quad (5)$$

where γ_s (mJ/m^2) is the total surface energy of the polymer film, and γ_s^p, γ_s^d (mJ/m^2) are the polar and dispersion components of surface energy of the polymer film.

Surface topography of untreated and plasma treated LDPE films was assessed using AFM. The AFM equipment was a Seiko Instruments Scanning force microscope (AFM, Ben-Yuan, CSPM 4000) and was operated in tapping mode with horizontal and vertical resolution of 0.26 and 0.10 nm, respectively. The value of Ra and RMS is an average from five independent measurements on 1 μm × 1 μm areas.

The chemical changes on the surface of modified LDPE films were assessed by Shimadzu FTIR spectroscopy at a resolution of 4 cm^{-1} and in the range of 700–4000 cm^{-1}. The changes in chemical state of LDPE films were further studied using two different XPS spectrometers. Untreated and plasma treated samples were analyzed with monochromatic Al Kα X-rays (1486.7 eV) from a Omicron Surface Science Instruments spectrometer with an EAC2000-125 Energy Analyzer. Experimental conditions were: 400-μm nominal X-ray spot size (full width at half-maximum) at 15 kV, 8.9 mA, (133.5 W) for both survey and high resolution spectra. For all the grafted samples (with AAc, PEG and chitosan) X-ray non-monochromatic Al Kα from a Kratos XSAM800 XPS spectrometer, at a TOA = 90° was used without any flood gun for charge correction. Typical operating conditions were 12 kV, 10 mA (120 W). Charge shifts were corrected taking, as reference, the binding energies (BE) for LDPE carbons centered at 285.0 eV. The C 1s and O 1s envelopes were analyzed and peak-fitted using a combination of Gaussian and Lorentzian peak shapes and Shirley background obtained from XPSPEAK41 software package. For quantitative purposes, sensitivity factors were: C 1s: 0.25; O 1s: 0.66 for the Kratos spectrometer.

2.6. Invitro hemocompatibility analysis

2.6.1. Thrombus formation

Initially the surface modified LDPE films were cut into 4 cm × 4 cm which were attached to a watch glass and subjected

to thrombus formation analysis. After that 200 μL ACD-blood was placed on the samples and incubated at 37 °C. Moreover the clotting mechanism was investigated by adding 0.1 M aqueous solution of $CaCl_2$ to the blood and the samples were agitated gently to react the blood and chemical homogeneously. After 30 min of incubation time, the formed thrombus was extracted and transferred into the 5 ml distilled water on a watch glass. The obtained thrombus was fixed by 37% of formaldehyde solution and dried under lower pressure until the sample weight did not change. The percentage of formation of thrombus was calculated using the following relation [36,37].

$$\text{Formation of thrombus (TF)}(\%) = \frac{W_{TFUT} - W_{TFSM}}{W_{TFUT}} \times 100$$

where W_{TFUT} and W_{TFSM} are the weight of formation of thrombus on the surface of untreated and surface modified LDPE films.

2.6.2. Protein adsorption analysis

Human albumin and fibrinogen were used to study the adsorption behavior of proteins on the film surfaces. All blood proteins were purchased from Sigma. Small disks (15 mm in diameter) of the polymer films were prepared with a punch and immersed in 1 mg/mL protein solutions in phosphate-buffered saline (PBS, pH 7.3–7.4) at 37 °C for 1 h. The disks were then recovered, and changes in the protein concentrations of the solution were determined using a UV-spectrophotometer.

2.6.3. Platelet adhesion studies

Platelet-rich plasma (PRP) was prepared by collecting human blood in plastic syringes containing anticoagulation agents. The blood was centrifuged at 1300 rpm for 10 min at 4 °C and the supernatant was collected. Polymer disks were washed with PBS for 24 h and placed in the bottom of the wells of a multiwell tissue culture plate; the PBS solution was removed from the multiwell tissue culture plate by pipetting. PRP (1 mL) was then seeded and incubated at 37 °C for 30 min. After incubation, the disks were recovered and rinsed three times with PBS to remove any weakly adsorbed platelets. After fixation in 2.5% glutaraldehyde PBS solution, the morphology of the adsorbed platelets was observed using a JEOL JSM-7000F scanning electron microscope (SEM).

2.6.4. Whole blood clotting time (WBCT)

0.1 mL of blood was deposited onto each sample (untreated and treated polymer surfaces) and incubated at 37 °C in a constant temperature bath. The clotting time was recorded manually by deepening a stainless-steel needle into the drop to detect any fibrin formation. The time of detection of the first fibrin filament adhering to the needle was considered the coagulation time [37].

3. Results and discussion

3.1. Surface hydrophilic analysis: contact angle, surface energy results

The extent of hydrophilicity of the surface modified LDPE films was examined by contact angle analysis which further provides the information about the homogeneity of the surface modifications. Table 3 shows the contact angle of water, formamide and ethylene glycol on the surface of LDPE films. It clearly revealed that the contact angle of the unmodified LDPE film was 95.0°, 82.4° and 76.1° for water, formamide and ethylene glycol, respectively. After plasma treatment the contact angle value decreased and further decreased significantly by *in-situ* AAc grafting on the LDPE film surfaces. The *in-situ* grafting of PEG on thus obtained AAc grafted LDPE film showed more hydrophilic nature than AAc deposited films.

Table 3
Contact angle and surface energy of the unmodified and surface modified LDPE film w.r.t water (W), formamide (F) and ethylene glycol (EG).

Treatment conditions	Contact angle (Degree)			Surface energy (mJ/m^2)		
	W	F	EG	γ_s^p	γ_s^d	γ_s
UT	95.0	82.4	76.1	4.7	14.1	18.8
Ar plasma	56.9	49.2	40.1	29.2	13.3	42.5
AAc	29.7	25.1	20.7	52.2	10.4	62.6
PEG	22.0	17.4	12.2	56.8	10.2	67.0
Chitosan	16.5	13.4	9.87	60.2	9.6	69.8

Subsequently, the lowest value of contact angle was observed for chitosan immobilized on such plasma processed LDPE film surfaces.

The polar and dispersion components of the total surface energy of unmodified and surface modified LDPE films were calculated from contact angle data using modified Fowkes equation (Table 3). It was found that the polar component of the untreated LDPE was just 4.7 mJ/m^2 and increased with the argon plasma treatment. After that, the polar component value of the LDPE increased significantly again by the grafting of AAc and PEG. Finally, by the immobilization of chitosan on the modified surface of LDPE films, it reached 56.8 mJ/m^2. Similar trend was observed in the increase of total surface energy of the surface modified LDPE films as shown in Table 3. However remarkable changes were not observed in dispersion components of the surface modified LDPE films. Thus, the decrease in contact angle and the increase in surface energy of plasma processed LDPE films are mainly due to the formation of hydrophilic groups such as ether, carboxyl, hydroxyl and amino groups on the surface of LDPE film by successful grafting of AAc and PEG followed by immobilization of chitosan. The above analysis clearly shows that the surface-modified LDPE film exhibits hydrophilic behavior.

Table 4 shows the changes in work of adhesion (W_{adh}) and polarity (P) of the modified surface of LDPE films which were calculated from contact angle measurements with respect to water. It is seen that the values of W_{adh} and P increases following the order UT < Ar plasma < AAc < PEG < CHI. The remarkable increase in these parameters again reveals that there is a formation of high density of polar functional groups on the LDPE film surface. The formation of the functional groups by the plasma induced graft polymerization will be clearly explained in the section 3.4. The strong interaction

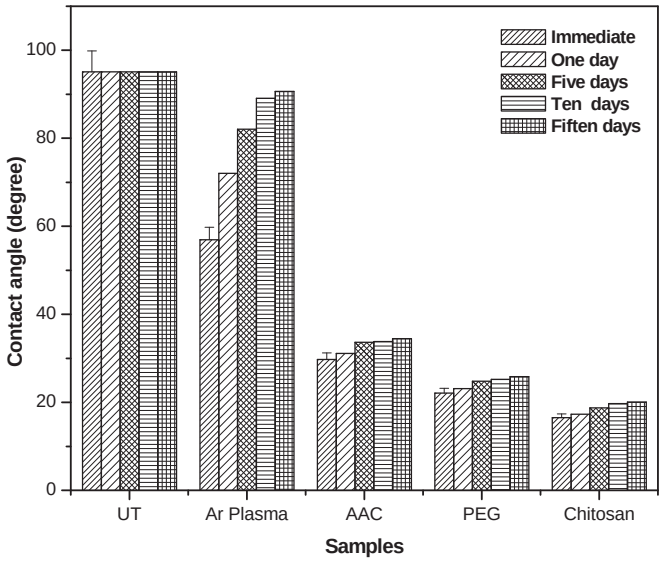


Fig. 2. Evaluation of the effect of aging on unmodified and surface modified LDPE films using water contact angle.

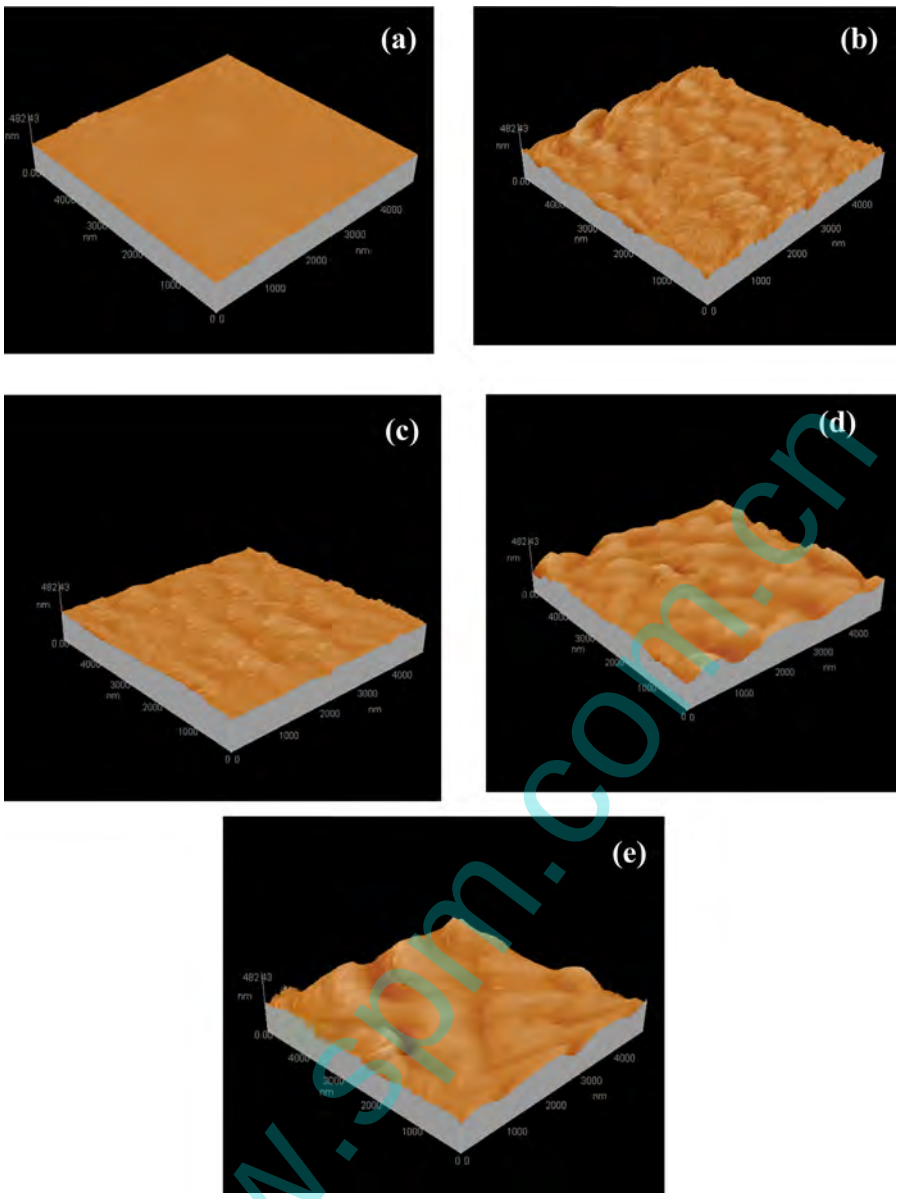


Fig. 3. AFM micrographs of (a) untreated, (b) Ar plasma treated, (c) AAc grafted, (d) PEG immobilized and (e) chitosan immobilized LDPE films.

Table 4
Work of adhesion and polarity of the surface modified LDPE films.

Treatment conditions	W_{adh} (mJ/m^2)	Polarity
UT	66.34	0.25
Ar Plasma	112.52	0.68
AAc	136.00	0.83
PEG	140.20	0.84
CHI	143.61	0.86

between plasma particles and the surface of polymeric films leads to different concurrent processes such as surface activation, etching, grafting, etc. The change in surface morphology induced by the plasma processing might affect the surface properties by simple physical adsorption or/and by diffusion in various mechanical interlocking or linkage to biological species of interest in medical usages.

3.2. Effect of aging

The low-pressure plasma induced polymerization introduced specific functional groups with the significant morphological changes resulting in enhanced hydrophilicity and surface energy thereby improving bio-compatibility of LDPE films. There are reports indicating that the increase in hydrophilicity and surface energy achieved by gaseous plasma treatment of polymers is not permanent [38–44]. Depending upon the type of plasma forming gas, substrate polymer, time of treatment and storage conditions, hydrophobic recovery has been observed. Therefore, the stability of the plasma processing is the main concern. The hydrophobic recovery is mainly due to surface contamination, saturation of active sites and conversion of functional groups by reactions with the ambient atmosphere, re-orientation of polar groups and mobility of small polymer chain segment in to the bulk [38–44]. Thus, the stability of the plasma effect needs to be investigated properly for further biomedical applications. In the present study, stability of the plasma processed LDPE films was investigated by measuring of contact angle with storage time of first fifteen days. As it can be seen that the contact angle of the Ar plasma treated (fresh sample) LDPE film reduces from 95.09° to 56.93°. When the sample was kept for ageing, we observed hydrophobic recovery with time as shown in Fig. 2. Therefore, it is advisable to use gaseous plasma treated (in our case argon plasma treated) LDPE films immediately for the next

step of plasma polymerization / grafting of AAc. Here, it is important to note that the effect of ageing is negligible in the case of plasma polymerized/grafted samples of AAc and PEG. Similarly negligible ageing was observed for CHI immobilized LDPE films. Plasma grafted AAc and PEG and CHI immobilized LDPE films do not show significant change as that of argon plasma treated LDPE films stored in air for 15 days, which may be due to functional groups that are created by the plasma polymerization are effectively cross linked on the surface of LDPE films which lead to hindered reorientation of functional groups into the material bulk results in hydrophilicity of the materials and is not altered by the storage time. Thus, such type of surface engineering techniques and materials can be suitable for further application in biomedical industry.

3.3. Morphological analysis: AFM results

The AFM is used to obtain detailed information about the topographical changes of LDPE film induced by plasma treatment and the subsequent grafting process. It clearly shows that the surface of the unmodified LDPE film is relatively smooth with moderate surface roughness (Fig. 3a). The surface of the argon plasma treated LDPE film exhibited the rougher morphology, which is clearly related to the etching phenomenon induced by the plasma treatment (Fig. 3b). The change in surface morphology induced by the plasma processing might affect the surface properties by simple physical adsorption or/and by diffusion in various mechanical interlocking or linkage to biological species of interest in medical usages. After that, the surface of the LDPE films got flatter by grafting of AAc, leading to a smoother morphology (Fig. 3c). The results confirm the incorporation of AAc homogeneously over the surface of plasma treated LDPE films. However, the surface roughness of the film was increased substantially by the grafting of PEG which was caused by the formation of its own domains and morphology at the surface (Fig. 3d) and, consequently, the CHI immobilized LDPE film surface shows lower roughness due to higher extent of homogenization of CHI content on the surface (Fig. 3e). The R_a and RMS values of unmodified and surface modified films are shown in Fig. 4. It is seen that the R_a and RMS values of unmodified LDPE are 1.85 nm and 2.35 nm, respectively, which increased significantly after the argon plasma treatment. These values decreased by the AAc grafting and increased for PEG grafting. Finally, the R_a and RMS values of the CHI immobilized LDPE films decreased as compared to the PEG grafted surfaces. However, the final surface of such modified LDPE films revealed increased roughness compared to the untreated LDPE film. The above topographical changes induced by the low-pressure plasma clearly revealed that the surface of LDPE

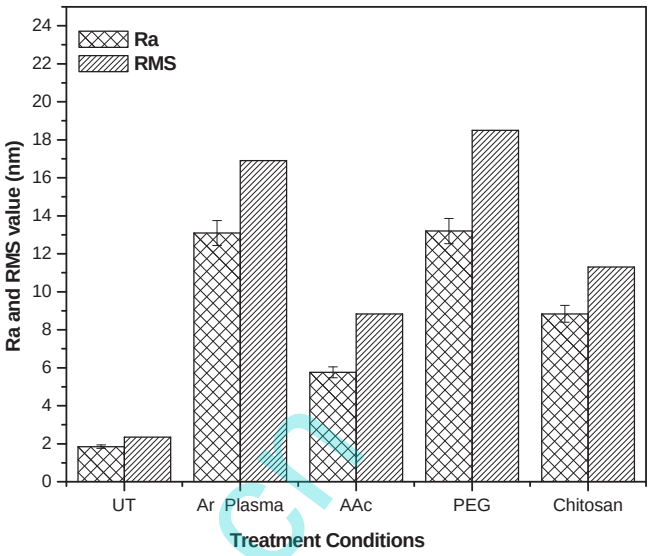


Fig. 4. Ra and RMS values of the surface modified LDPE films.

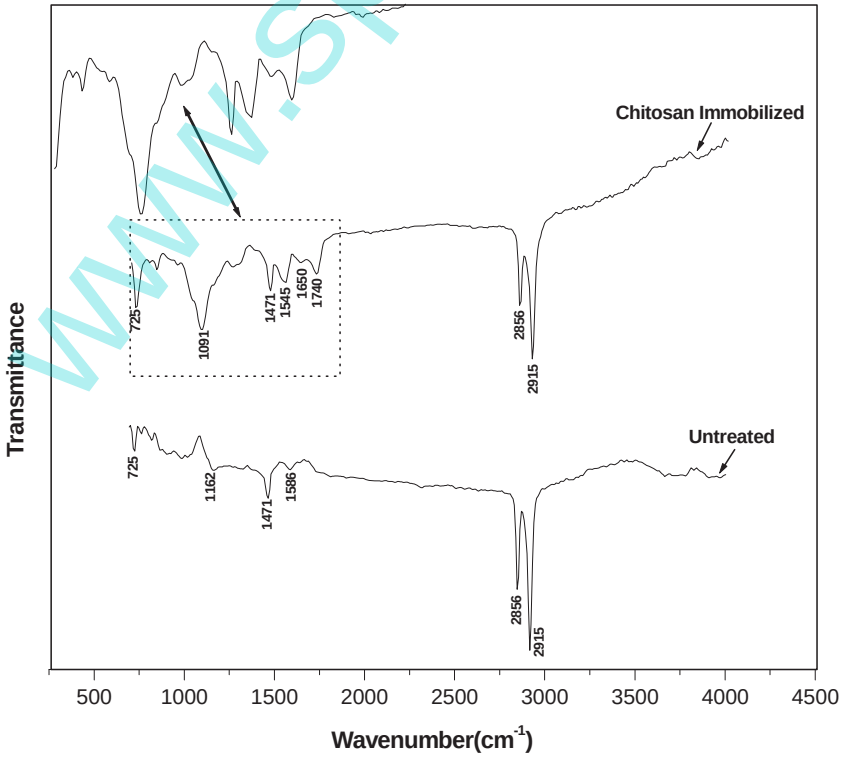


Fig. 5. FTIR spectrum of untreated and chitosan immobilized LDPE films.

films is noticeably affected by the successive grafting of AAc and PEG and immobilization of CHI.

3.4. Chemical composition analysis

3.4.1. FTIR results

An FTIR spectrum was employed to investigate the changes in chemical structure of the chitosan immobilized LDPE films. Fig. 5 shows the FTIR spectra of the untreated and chitosan immobilized LDPE films. It was found that FTIR characteristic spectrum of untreated LDPE films exhibits peaks at 2915 cm^{-1} attributed to C–H stretching, 2856 cm^{-1}, 1586 cm^{-1}, 1471 cm^{-1}, 1162 cm^{-1} and 725.89 cm^{-1} attributed to CH_2 bending (Fig. 5) [45]. However, chitosan immobilized LDPE exhibits three new peaks at 1740 cm^{-1} attributed to C=O stretching of the ester or in a carboxylate group, 1545 cm^{-1} attributed to N–H bending in O=C–NH, 1650 cm^{-1} attributed to C=O in O=C–NH and 1091 cm^{-1} attributed to C–N stretching vibration (Fig. 5) [46,47,1]. Hence the presence of new peaks clearly confirms the immobilization of chitosan on the surface of LDPE films through covalently bonding.

3.4.2. XPS results

Newly formed functional groups on LDPE films surface, as a consequence of plasma surface modification, were studied by XPS which provided further insight into the surface of LDPE films. The C 1s core level spectra of the untreated LDPE film has one main peak centered at 285.0 eV attributed to sp^3 carbon bound to carbon and hydrogen (Fig. 6), as expected. Moreover, the small component centered around 288.0 eV, corresponding to a carbon doubly bound to one oxygen or singly bound to two oxygen atoms, is most probably due to incorporation of oxygen atoms during the cleaning process of LDPE films with acetone and de-ionized water. After the argon plasma treatment, the groups formed by the local oxidation disappeared (Fig. 6) due to desorption of water molecules from the surface of LDPE films by ions, electrons, and neutral species and UV radiations in the plasma. In the O 1s region (not shown here) the components attributed to acetone or water are also detected in the untreated LDPE and decrease or even disappear with the argon plasma treatment. The C 1s spectra of the acrylic acid grafted LDPE film is fitted with three components centered at 285.0 (83%), 286.4 ± 0.1 (9%) and 288.7 ± 0.2 eV (9%). The first, and main, component is assignable to carbon, both in LDPE and in acrylic acid, bound to C and H. The third peak corresponds to carbon in an ester or in a carboxylate group (BE for a C in a carboxylic group would be around 0.4 eV higher) [48]. The corresponding carbon bound to a single oxygen explains the existence of the second component. Hence the detection of ester and/or carboxylate group clearly indicates the grafting of AAc on the surface of LDPE films.

PEG grafted surface showed three main peaks at 285.0 (41%), 286.4 ± 0.1 (55%) and 288.5 ± 0.2 eV (4%) assigned to C–C and C–H, C–O and O–C=O ester group (Fig. 6), respectively. PEG is expected to have a single C 1s component precisely at 286.4 eV, the main component. Peaks corresponding to the O–C=O and the C–C and C–H groups are still seen but with a lower intensity than for the AAc grafted sample indicating a very thin layer or the partial coverage of surface by PEG immobilization. One of the ester components (C–O–C=O) is mixed with the PEG peak. After CHI immobilization, four peaks were fitted to the C 1s region. One centered at 285.0 eV (26%) corresponding to the CH_3 group in the acetylated chitosan moieties and also to carbons in the LDPE underneath. Anyway, as we can observe in Fig. 6, its relative importance decreases relatively to the PEG grafted film and especially relatively to the AAc grafted film, showing that the LDPE coverage increased and attesting that

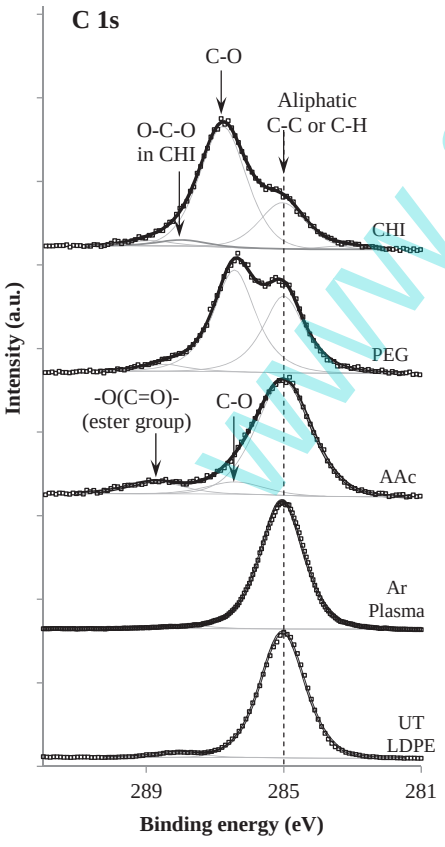


Fig. 6. The high resolution C 1s XPS spectra of untreated, Ar plasma treated, AAc grafted, PEG immobilized and CHI immobilized LDPE films.

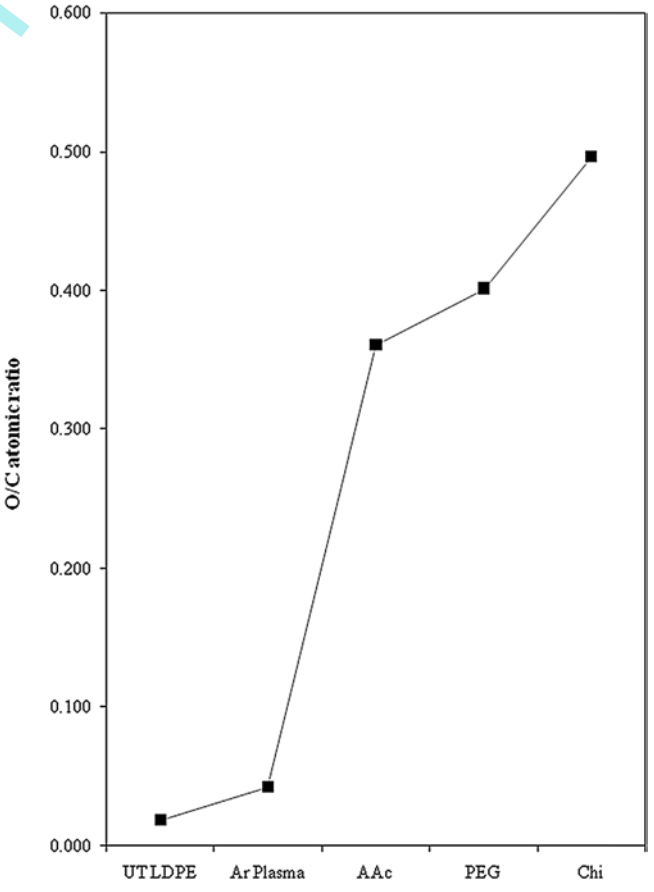


Fig. 7. Atomic ratio O/C of unmodified and surface modified LDPE films.

the chitosan immobilization was successful. The main peak is centered at 286.7 ± 0.1 eV (68%) corresponding rather well to the C 1s binding energy in polysaccharides such as cellulose and chitosan [48,49]. Nevertheless, it may also contain some carbon from the PEG layer underneath. Another peak is centered at 287.9 ± 0.2 eV (~3%) which agrees well with the C1s BE for the anomeric carbon in the glucosic-like unit [48,49]. Finally, a small component centered at 288.8 ± 0.2 eV (~3%) corresponds to the already identified ester peak due to grafting of AAc on the surface of LDPE films. The quantitative elemental analysis shows a residual amount of oxygen on the unmodified LDPE film which increased after plasma treatment. AAc grafting, PEG grafting and immobilization of CHI leads to increase in O/C ratio in the order Ar < AAc < PEG < CHI (Fig. 7).

To further check the presence of chitosan, the hetero element nitrogen would be the natural choice. However, the traces of nitrogen were observed in other cases, which may be due to the interaction of free radicals with the nitrogen present in the air when exposed to the surrounding atmosphere. However, the substantial presence of nitrogen component (N/C ratio 2.4%) was observed in the chitosan immobilized sample. In addition to this, the presence of chitosan can be demonstrated from the C 1s region analysis.

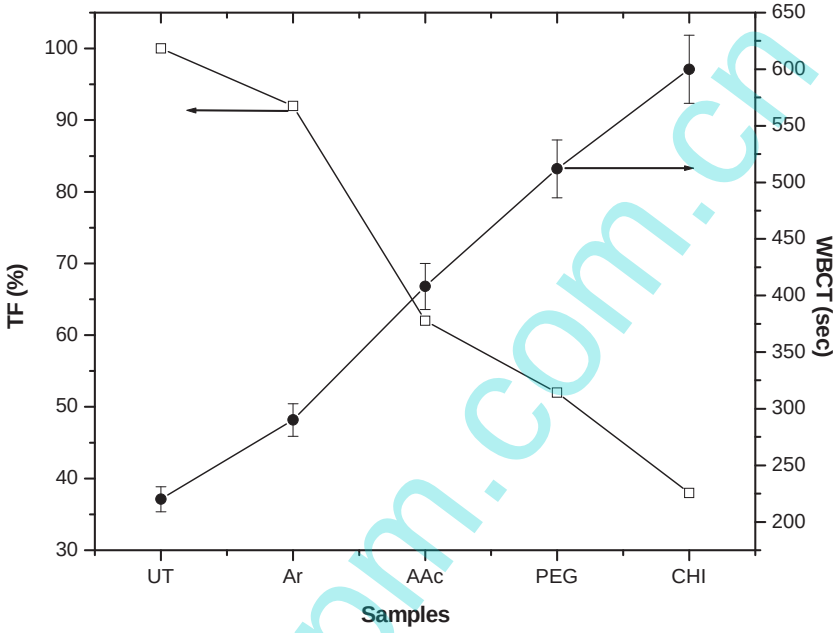
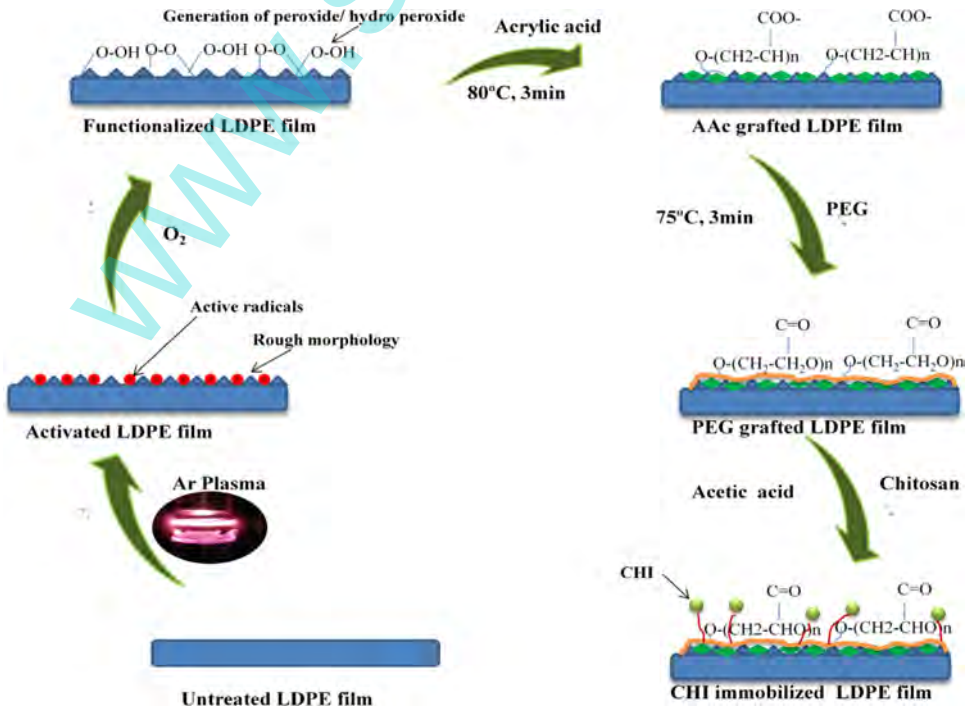


Fig. 8. Amount of thrombus formed and WBCT on surface of modified LDPE film.



Scheme 1. Mechanism of plasma polymerization and immobilization of chitosan on LDPE film.

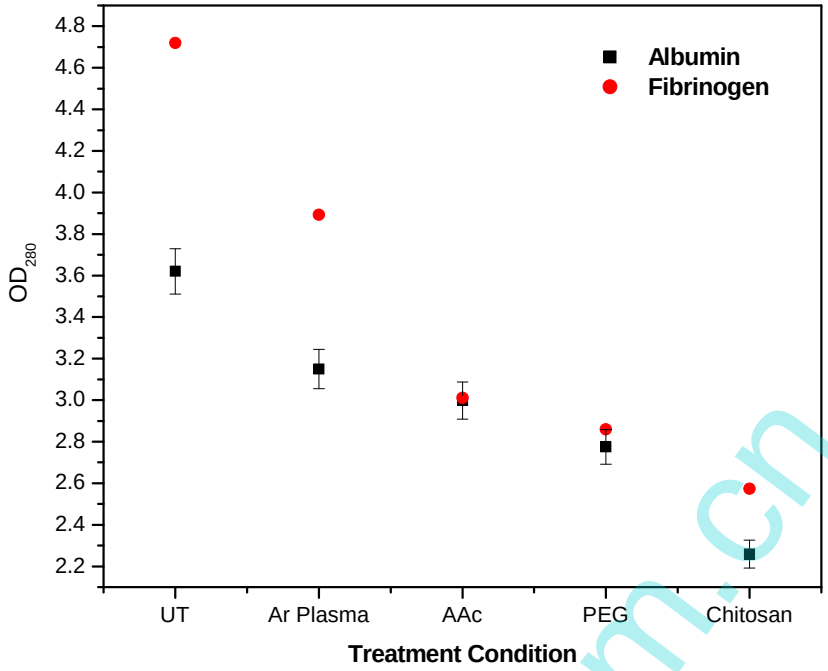


Fig. 9. Adsorption of plasma proteins on the surface modified LDPE films.

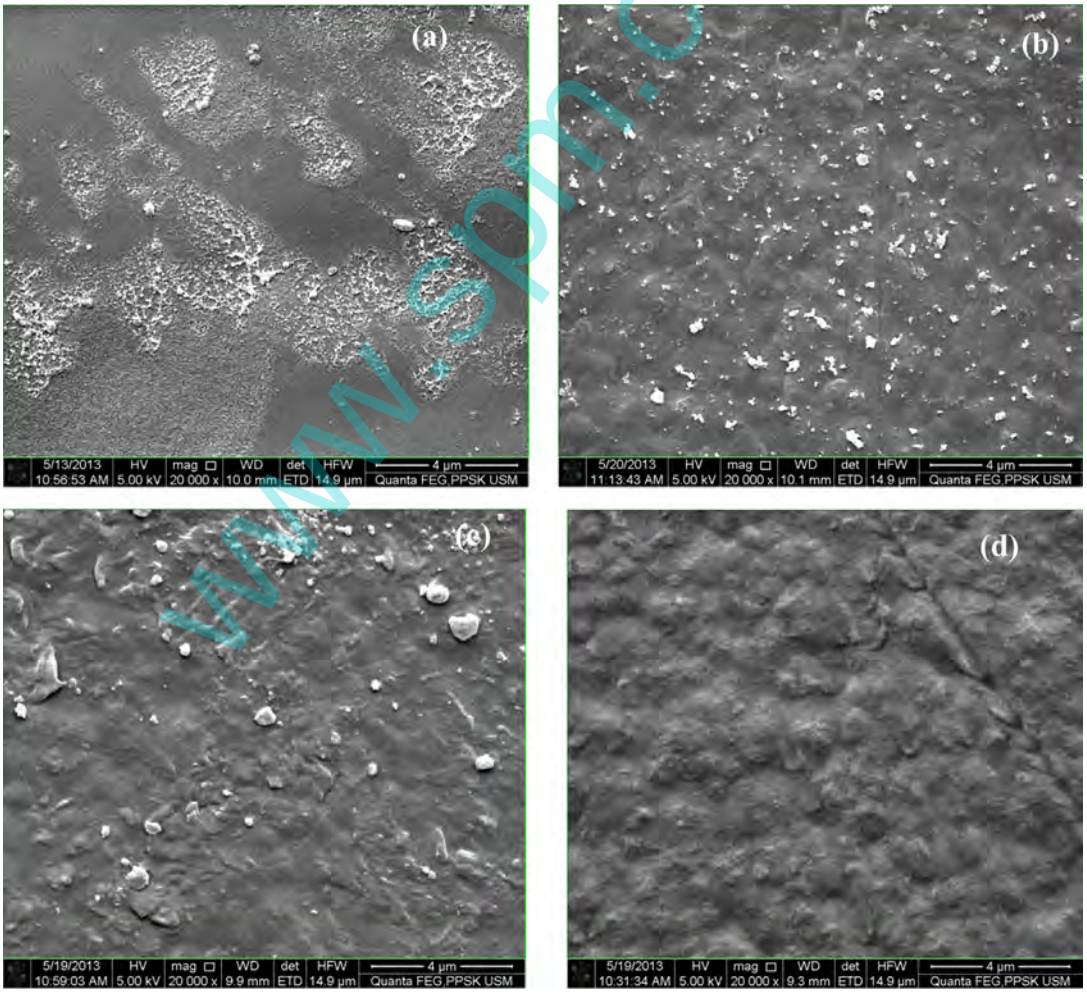


Fig. 10. SEM photograph of *in vitro* platelet adhesion tests: (a) untreated, (b) AAc grafted, (c) PEG immobilized and (d) CHI immobilized LDPE films.

The incorporation of higher concentrations of oxygen and nitrogen containing functional groups leads to improved hydrophilicity of modified LDPE films reaching the higher value for the film modified with immobilized chitosan [50,51]. The detailed mechanism of the polymerization is described in the Scheme 1.

3.5. *In vitro blood compatibility analysis*

Interaction between foreign material surface and blood are a multifarious sequence of dynamic events through adsorption of proteins, aggregation and spreading of platelets, followed by formation of thrombus on the surface. The adsorption of protein, adhesion of platelet and formation of thrombus are important factors to estimate the blood compatibility of the materials which have high potential risk during the use of artificial materials *in vivo*. Fig. 8 shows thrombus formation (TF) on unmodified and modified LDPE film surfaces; the thrombus formed on unmodified LDPE for a contact of 30 min with blood was considered as 100%. The TF on the LDPE film was slightly lower after the argon plasma treatment and decreased noticeably by the grafting of AAc and PEG. Furthermore, the CHI immobilized surface suppressed 62% of thrombus formation on the surface of LDPE films compared with unmodified surface of LDPE. Similarly, the WBCT of the unmodified LDPE is 220 s increasing with respect to treatment condition in the following order UT < Ar plasma < AAc < PEG < CHI. The above results clearly show that surface modified LDPE films exhibit noticeable anticoagulation activity. Adsorption of human blood proteins such as fibrinogen and albumin plays an important role to initiate the coagulation activity on the foreign materials surfaces when it comes in contact with blood. Hence, protein adsorption behavior of the materials can help to improve the blood compatibility of the materials used for blood contacting devices.

Fig. 9 displays the adsorption of albumin and fibrinogen onto LDPE surfaces which clearly shows that the OD_{280} (absorbance) values of albumin and fibrinogen of the unmodified LDPE film are 3.62 and 4.79, respectively. After surface modification, the OD_{280} values of albumin and fibrinogen – proportional to the adsorbed amount of protein on the surface of the film are significantly reduced. Hence, surface modified LDPE films avoid the adsorption of blood plasma protein on the surface. Generally, adsorption of albumin in high amounts would inactivate the blood-material interface, while fibrinogen adsorbed in high amounts would favor the platelet adherence and the activation of the blood coagulation system [52,53]. For surface-modified LDPE films, the interfacial energy between polymers and fibrinogen is small, which ensures low driving forces for protein deposition. The protein adsorption results indicate that modified LDPE surface exhibit excellent inhibition of protein adsorption.

The morphology of the adhered platelet on the surface of unmodified and modified LDPE film surfaces are depicted in Fig. 10a–d. It clearly shows that high dense platelets are adhered and accumulated on the surface of unmodified LDPE films. On the contrary, adhesion and accumulation of platelets on the LDPE films was further suppressed by AAc and PEG grafting, being almost absent on the CHI immobilized LDPE film surfaces. The above platelet adhesion analysis clearly revealed that the AAc, PEG and CHI immobilized surface could inhibit the adhesion and activation of platelets on the LDPE film surfaces which is attributed to the increase of the resistance to the adsorption of plasma proteins.

4. Conclusion

The dc excited low-pressure plasma induced graft polymerization of AAc is the effective method to improve the biocompatibility of the LDPE films which further provides the active sites for immobilization of PEG andCHI molecules on the surface. The morphological, chemical and hydrophilic changes of the LDPE films induced by the plasma grafting were characterized by AFM, XPS, contact angle and surface energy analysis, respectively. Furthermore, blood compatibility of the surface modified LDPE films were studied by *in vitro* analysis which includes protein adsorption, platelet adhesion, anti thrombus and WBCT tests. The contact angle and surface energy of the modified LDPE film surfaces exhibited highly hydrophilic behaviors compatible with the FTIR and XPS results that show higher concentrations of oxygen and nitrogen containing groups such as C–O, O–C–O and O–C=O as well as C–N as the modification of the surface of LDPE films proceeds. The above contact angle, surface energy and XPS and FTIR analyses confirmed that the surface of the LDPE was highly hydrophilic and also attest the successful grafting of AAc, PEG and CHI on the LDPE film surfaces. The AFM analysis of the LDPE films shows different morphologies after grafting and immobilization. Moreover, the surface modified LDPE films highly resist and inhibit the adhesion of platelets, adsorption of protein and formation of thrombus. These results indicate that surface modified LDPE films are highly biocompatible which can be considered for a wide range of applications in biomedical and functional materials fields. Finally, it was concluded that this plasma-based surface engineering technology has great potential in various biomedical industries for manufacturing blood contacting devices.

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