PROTEIN IMMOBILIZATION ON POLY(ETHYLENE TEREPHTHALATE) FILMS MODIFIED BY PLASMA AND CHEMICAL TREATMENTS

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The aim of this work is to improve the superficial hydrophilic properties of poly(ethyleneterephthalate) (PET) films. To achieve this object, amide and amine groups were introduced by chemical and plasma-precursor treatments with triethylenetetramine (TETA). In order to obtain information about the chemical and morphological modifications of polymers, Fourier Transform Infrared - Attenuated Total Reflectance (FTIR-ATR), X- ray diffractions (XRD), Atomic Force Microscopy (AFM), optical fluorescence and light polarization microcopy (POL) measurements were used. For surface activation, plasma-precursor treatment was more efficient than the simple chemical one. Optimal treatment parameters were determined by optical microscopy measurements after collagen immobilization.

(Received November 29, 2009; accepted January 27, 2010))

Keywords: Poly(ethyleneterephthalate), Plasma treatments, Triethylenetetramine, Collagen immobilization

1. Introduction

Although the use of medical devices is widespread, there is an increasing demand for new biomaterials used as implants or for preparing bio-artificial organs. Several surface modification techniques have been developed to improve wetting and adhesion of polymer surfaces by introducing a variety of polar groups, while little attention has been given to functional group specificity. The ideal surface modification techniques are those that introduce as close to a monolayer as possible of a desired functional group without causing irregular etching or producing significant hazardous waste.

PET due to the chemical, physical and mechanical properties, is widely use in various industrial applications [1-3]. Biaxially-oriented PET (boPET, Mylar) films are characterized by high tensile strength, chemical and dimensional stability, transparency, gas and aroma barrier properties and electrical insulation. Nitrogen containing PET films can be considered as potential biomaterial substrates for the attachment of proteins and other biologically active molecules because they allow direct attachment of bioactive molecules [4].

Reactions of semi-crystalline PET films with ester-selective reagents to perform reagent-induced functionality at polymer surface can be carried out at the film-solution. By choosing the proper experimental conditions, namely exposure time, reagents, temperature, it is possible to restrict the chemical modification of polyester to the surface of the polymer film [5].

The reaction of the ester bonds from polyesters chains with amines is one of the various attempts tested to obtain materials which can be tailored according to the demanding requirements of the specific applications. It is expected that the amide groups included through PET surface modification should generate potential sites for the creation of physical bonds of hydrogen or van der Waals type and should improve the wettability of polymer. Higher amine functionality reduces the amount of scission needed to incorporate a desired concentration of amine groups, i.e., the required change in surface properties should be achievable at a lower extent of reaction.

Furthermore, an increase in amine molecular weight should decrease diffusion into the PET film, allowing the reaction to be restricted to the film surface.

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In this work, data related to structural changes of aminated surfaces of PET film are presented. The introduction of aliphatic amine units on PET surfaces that lead to amide functionalities has been achieved by two processes: chemical treatment with triethylenetetramine (TETA) and plasma-precursor followed by chemical treatment with TETA.

2. Experimental set-up

The biaxially drawn PET film (commercial), with thickness of 30 μm was used after ultrasonic cleaning with toluene, acetone and tri-distilled water. Then the samples were dried at 40 ^{o}C and stored in a closed vessel.

2.1. Chemical treatment

The PET film samples (25 x 25 mm) were submitted to action of TETA, used as aminolysis reagent in dark, at 90° C for 10 min (C10), 20 min (C20), 30 min (C30) in an oven. The sandwich model was used: two PET films samples are separated through the medium of amine. After exposure, the treated samples were rinsed with methanol and tri-distilled water with ultrasonically cleaning to remove excess of reagent.

2.2. Plasma-precursor and chemical treatment

PET films were positioned vertically in a metal sample holder to allow treatment on both sides. Generally, the plasma reactor consists of three main parts disposed around the plasma reactor [4]: vacuum system, monomer and/or gas inlet, and discharge system. On the external walls of reactor there are placed the two semi cylindrical electrodes connected to a high frequency (HF) capacitive-coupled discharge system. High frequency generator operating at 1.2 MHz and 0 – 200 W is used. The sample to be treated is positioned into the reactor. In each run, prior to the treatment, the reactor is evacuated down to a base pressure of 0.3 Torr. The power of the glow discharge is kept at 200 W for 10 min. After plasma treatments, the samples were treated with TETA at 90 °C in oven at 10 min (PC10), 20 min (PC20) and 30 min (PC30). Samples were prepared in the sandwich form from the PET films as described above.

2.3. Collagen immobilization

Collagen is a hydrophilic, triple-helical protein with three polypeptide chains, abundantly present in skin, bone, and cartilage, acting as the stress-bearing component of connective tissue, and is often used as a biocompatible surface layer.

The aminolyzed PET films were immersed in 1 wt % glutaraldehyde (GA) solution for 3 h at room temperature, followed by rinsing with a large amount of de-ionized water for another 24 h to remove free GA. The films were then incubated in 3 mg/ml collagen /phosphate buffered solution (PBS, pH = 3.4) for 24 h at 2- 4° C. The collagen immobilized films were rinsed with 1 % acid acetic solution and then rinsed with de-ionized water for 24 h to remove free collagen. Collagen was covalent conjugated to the aminated PET surface via glutaraldehyde crosslinking on samples treated 30 min chemical (CC) and those who suffered plasma and 30 min chemical treatment (PCC).

2.4. Characterization

The exposed samples were taken out at different time intervals, and the measurement of the absorption spectra was carried out. The modification in the PET surface structure was examined by FTIR-ATR spectroscopy in the range 600-1800 cm^{-1}. Peak height measurements were performed with the spectral analysis software (Opus 5).

XRD was used to study the changes in crystallinity of the nitrogen containing PET film induced by chemical and plasma treatments. XRD patterns were recorded with a D8 Advance Bruker AXS diffractometer. X rays were generated using a CuKα source with an emission current of 36mA and a voltage of 30kV. Scans were collected over the range 2θ = 12- 35° using a step size of 0.01° and a count time of 0.5s/step.

AFM measurements were performed in air at room temperature, in the tapping mode using a Scanning Probe Microscope (Solver PRO-M, NT-MDT, Russia) with commercially available NSG10/Au Silicon cantilevers. In the tapping mode, the cantilever is oscillated at a frequency of 254.249 kHz, over a 5 x 5 μm^2 scan area, 256 x 256 scan point size images being thus obtained for each sample. The root-mean-square roughness RMS was calculated as the average value for the set of AFM frames of certain scales [4].

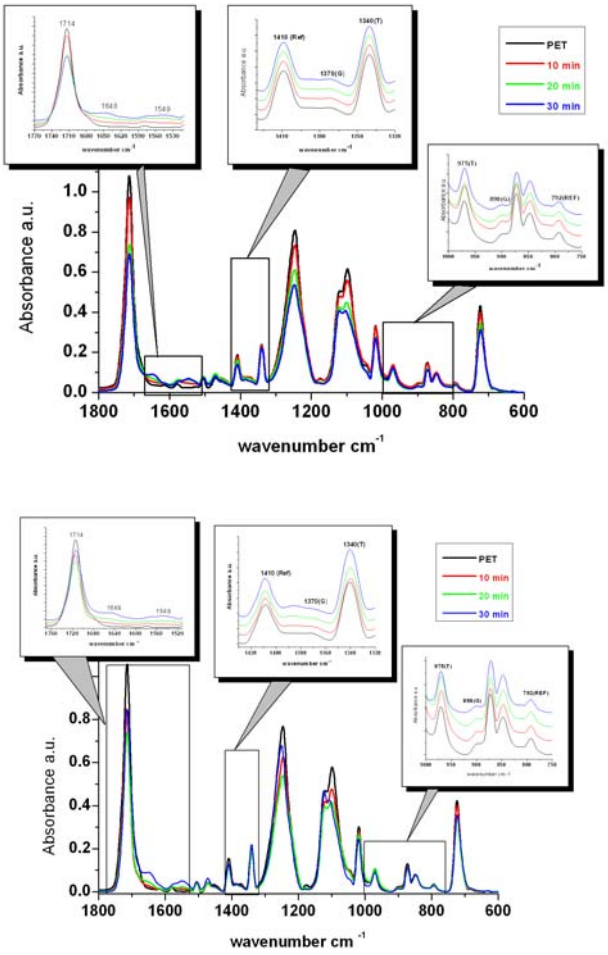
Fluorescence and light polarization measurements were carried out with an optical microscope Leica DM2500M.

3. Results and discussion

The changes in the chemical structure of the PET films were evidenced through FTIR-ATR spectroscopy analysis, which can be considered a valuable method for the characterization of polymer surface [6] and amide groups. The amide group (CO-NH) is a complex vibrational unit in infrared spectroscopy range and involves stretching and bending vibrations. A multifunctional amine known for its lower reactivity was chosen as reagent. After each scission, both amide group and primary amine functionalities are generated. The process is dependent on chain mobility and permeability of polymer film. After aminolysis, the polymer surface attain two type of additional electronegative nitrogen atoms (NH and NH_2), responsible for the increase of surface polarity.

As a result of PET plasma and chemical treatments with aliphatic amines at 90° C, for different periods of time, obvious changes in FTIR-ATR spectrum can be observed.

Figure 1 a) shows the progress of the aminolysis evidenced in the experimental IR spectra in the 600-1800 wavenumber range. Starting from the background spectrum the following spectra were recorded after different reaction time intervals up to the 30 min: C10, C20, and C30. Spectrum of PET has the characteristic absorption peaks at 1712 cm^{-1} (C=O bonds), 1410, 1018, and 872 cm^{-1} (vibration of aromatic ring), 1340 and 1177 cm^{-1} (bending vibration of $-CH_2$ groups), 1244 and 964 cm^{-1} (stretching vibration of C-O bonds), 1124 and 1100 cm^{-1} (stretching vibration of C-O bonds due to amorphous and crystalline structure of PET respectively)[2,7,8]. From the quantic chemistry theory, the COO^- bonds which are in the same plane with phenyl bond, can continuu form conjugations with p- electrons of aromatic nucleus, evidenced in IR spectrum by a displacement toward lower wavelenghts, the system being more stable. The carbonyl bonds which are out of plane don't participate to those conjugations and reaction with aliphatic amines at the polymer surface. The band at 1410 cm^{-1} resulting from phenyl ring vibrations (C-H bend coupled with ring C-C stretch) has usually been considered to be insensitive to orientation and conformation and is a reference band [9].



a) b)

Fig. 1. FTIR measurements for a) chemical treatments; b) plasma and chemical treatments

The conformational composition has been obtained from bands at 973 cm^{-1} (*trans* - extended) and 898 cm cm^{-1} (*gauche*- relaxed) [9,10], and from bands at 1340 cm^{-1} (*trans*) and 1370 cm^{-1} (*gauche*) [9].The intensity of the *trans* bands at 973 and 1340 cm^{-1} increased relative to the *gauche* bands at 898 and 1370 cm^{-1} conformers, respectively. The fraction of trans conformer (T) of the film was calculated, taking in account the intensities of the two bands at 1340 cm^{-1} (A_{1340}) and 1370 cm^{-1} (A_{1370}),respectively assigned to *trans* and *gauche* conformers, by the following equation [11]:

$$T = \frac{A_{1340}}{A_{1340} + 6.6 \times A_{1370}} \tag{1}$$

The results are shown in Table 1.

Table 1. The fraction of trans conformer T and cristallinity degree χ_C (%) for aminated PET.

Treatment	T^{FTIR}	χ_C^{XRD}
C10	29	33
C20	35	41
C30	40	43
PC10	32	37
PC20	38	40
PC30	41	43

The chain scission of the PET is reflected in the FTIR ATR spectra by decreasing of IR band corresponding to the carbonyl ester group at 1714 cm^{-1} as a function of treatment time with amine (Figure 1). At the same time a new strong absorption at about 1648 cm^{-1} assigned to the amide I band, which has a main contribution of the stretching vibration, $\nu_{C=O}$ occurred. This position of the amide I band is shifted during aminolysis. The amide II band, mainly decided by NH bending vibration (δ_{NH}) appears at about 1549 cm^{-1}. From the ATR-FTIR studies it was found that normalized peak intensity for amide I band and amide II increased significantly during treatment of PET with TETA. The position of amide I and amide II bands reveal that hydrogen bonding involving amide C=O is present. This result suggests that the carbon chains in the amine molecule is orienting toward the film surface change also during exposure to amine with increasing time of exposure the bands associated with NH stretch vibrations, both amine and temperature could contribute to variation in the structure of polymer film. Modification in chemical structure and increased mobility of the main chain in the polymer lead to generation of amide, amine and OH groups.

Treatments PC10, PC20 and PC30 (Figure 1 b) show in FTIR spectra an increasing of new functional groups at the PET surface, compared with simple chemical treatment. The origin of forming carboxyl groups by air-plasma treatments has been proposed by Mas et al.[12] to be due to residual oxygen and adsorbed water vapor in the plasma reactor, while Nitschke et al.[13] suggested the polymer degradation by chains sccindation. PET is an aromatic hydrocarbon with ring-shaped structures that contain delocalized electron systems, for instance phenyl rings. After breaking of an inner-molecular bond, the chains of unsatured aliphatic and aromatic polymers have a higher propensity to crosslinking then aliphatic ones [14].

From Figure 1b it can be seen that as the reaction time increases, the absorbance of 1340 cm^{-1} band grows, while the band at 1370 cm^{-1} changes considerably. For PC10 treatment the maximum of the *gauche* CH_2 wagging band is at 1370 cm^{-1}. For PC20 this band shifts to the high-wavenumber region and become visible as shoulder at band positioned at 1386 cm-1, corresponding to crystalline phase. On further PC30 treatment, two distinct bands, one at 1370 cm^{-1} and other at 1386 cm^{-1} appear. The absorbance changes of the 1340 cm^{-1}, 1370 cm^{-1} and 1386 cm^{-1} band indicate the occurrence of reorientation of the PET chains and increasing of crystallinity. After 30 min, the degradation process is higher and with the increasing of time, N will be present in regions deeper and deeper in PET film, so an increasing in the surface N content cannot be achieved through longer reaction times. The PC30 treatment can be considered as threshold value: before this value the efficiency of treatment is lower, after this value the degradation process become higher.

Changes in the crystallinity, as a result of aminolysis, were measured using XRD. The diffraction pattern was obtained over the range $2\theta = 12 - 35°$. Gaussian fit of all data demonstrates the presence of three peaks in the diffraction pattern referring to the (010), (110) and (100) atomic planes of PET [14], respectively. As Table 1 shows, the crystallinity degree (χ_C) is the highest for the film aminated for PC30, indicating that the sample is most crystalline. These results are in good concordance with FTIR measurements. The increasing of crystallinity degree is explained by the model of Prevorsek et al. [15] for structural constitution of polyesters. This model postulates the existence, alongside the crystalline zones of the intra fibrillar amorphous phase (located between the crystallites within the micro-fibrils) and the interfibrillar amorphous phase (located between adjacent micro-fibrils). The content of interconnecting tie molecules which determines the sample elastic properties is decreased, attributed to the decay of crystal network structures caused by the degradation of amorphous regions.

Grafting was also evidenced by using tapping-mode AFM experiments. The surface topography of films as observed by AFM undergoes significant changes as a result of PET treatments. The height histogram of topographical images shows the statistical distribution of z-values (heights) within the image. The native films (Figure 2a) are homogeneous with an RMS value equal to 1.39 nm. Height histogram (Figure 2a') is symmetric with well-behaved tails and height between 5 and 8 nm. Due to the abrasive effect of the air-plasma treatment on the surface, a nano-patternation of the surface appears (Figure 2b) with RMS being equal to 1.78 nm. The plasma treatment gives a characteristic hill-valley structure in agreement with results obtained by

others on PET fibers [16, 17]. The surface is homogenous and "valleys" are predominant. From height histograms (Figure 3b'), the distance peak-to valley is evaluated to be 10 nm.

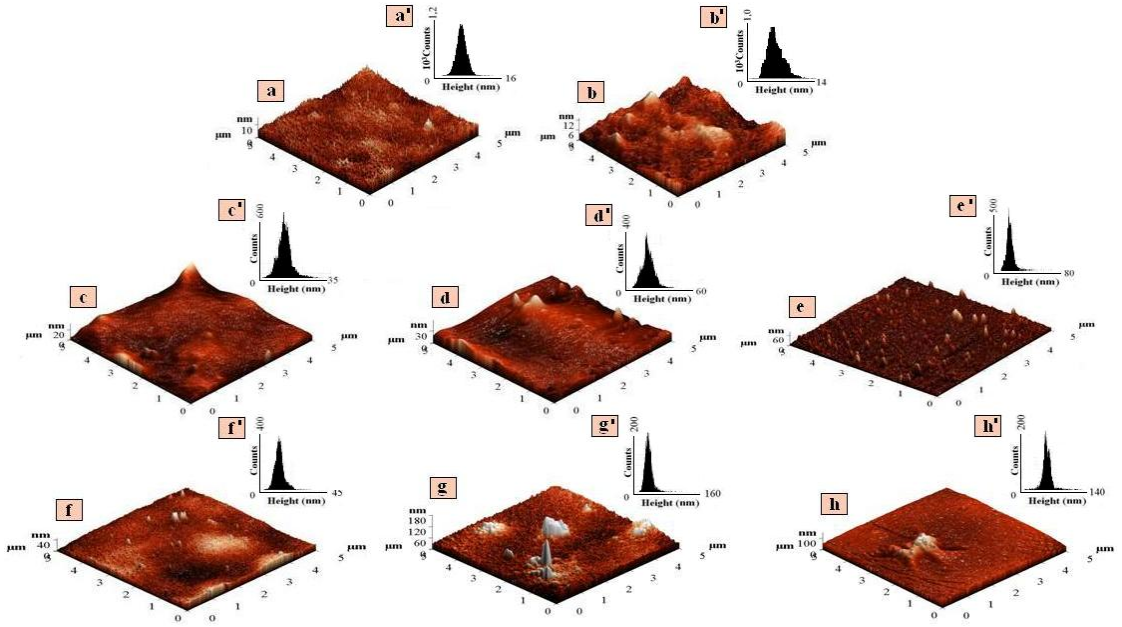


Fig. 2. Tappig mode of AFM images and histograms of a) untreated PET; b) 10 min plasma treatment; c) C10; d) C20; e) C30; f) PC10; g) PC20; h) PC30

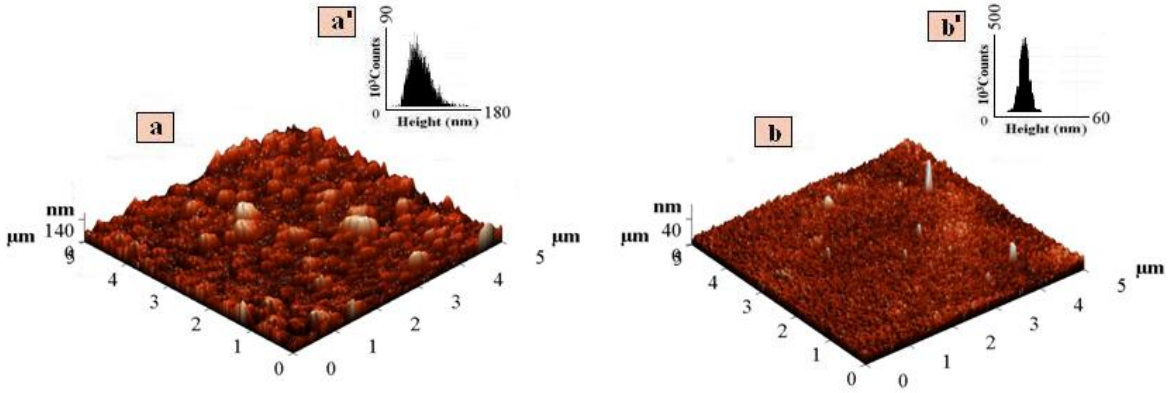


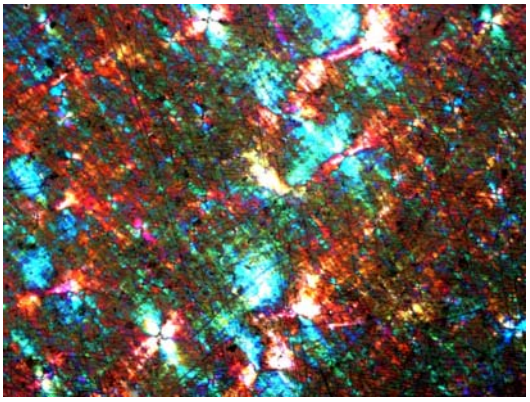
Fig. 3. Tappig mode of AFM images and histograms of immobilized collagen surfaces a) after C30; b) after PC30

For C10, C20 and C30 the influence of monomer concentration in surface topography has been revealed. For low monomer concentration (C10), Figure 2c and 2c' show an amplification of the nanopatternation with RMS being equal to 3.30 nm. Figure 2f shows a relatively flat surface, with nanopatterns of 10-20 nm height and distance of 35-40 nm between them. With increasing monomer concentration, the surface becomes homogenous with high features of about 100 nm (Figure 2g). For PC30 the RMS is the highest, but from Figure 2h and 2h', two kinds of nanopatterns are evidenced. The first one has about 50 nm height forming a lower smooth surface, and the second one has a maximum of 140 nm heights.

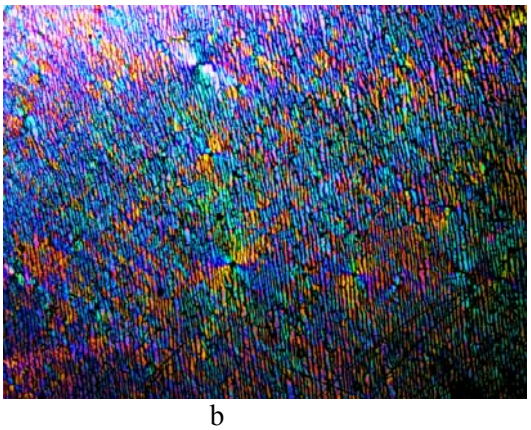
Following collagen immobilization onto PET surface, the AFM images highlight the distribution of collagen molecules, evidencing an irregular sphere-like appearance, due to the occasional overlapping of some collagen molecules to each other (Figure 3a). For smooth hydrophilic substrates, collagen forms homogeneous layers with small surface features (Figure 3b), while elongated structures attributed to collagen aggregates are found on hydrophobic ones

(figure 3a). The AFM images for collagen immobilization indicate a surface structure with more randomly distributed large grains (with average diameter of 98nm, height of 14nm and a density of 197 grains in 5x5 μm^2 area) in CC case (Figure 3a), and small grains (with average diameter of 39nm, height of 1.8 nm and a density of 1573 grains in 5x5 μm^2 area) for PCC treatments (Figure 3b).

PET surfaces at the microscale (5x5 μm) as a result of monomer action is further confirmed by optical florescence and polarizing microscopy (figure 4). It is critical to elucidate the effect of treatment on the collagen immobilization because cell adhesion and migration on biomaterial surfaces are dependent on this [18]. The fibers from figure 4 consist of many self-assembled collagen molecules with intra- and intermolecular cross-links stabilizing the structure. The cross-links are believed to be responsible for the observed strong auto-fluorescence of collagen.



a



b

Fig. 4. Microscopic images of immobilized collagen surfaces, 50x light polarization: a) after C30; (b) after PC30

4. Conclusions

The spectral changes observed in the FTIR-ATR spectra of aminated PET film seems to be due to the combined effects of molecular orientation, conformational changes, initial degradation restricted to the amorphous regions, the chain scission of tie segments between crystallites and reaction of ester group with TETA. From the ATR-FTIR studies it was found that normalized peak intensity for amide I band and amide II increased significantly during treatment of PET with TETA. The exposure to amine favored crystallization, formation of amide group and degradation of PET simultaneously. The presence of amine groups in the structure of treated PET film is favorable for further derivatization of polymer with biomolecules.

XRD measurements demonstrated the influence of amination conditions (time of exposure, type of amination: simple chemical or plasma and chemical treatments) on crystallinity.

AFM images at nanoscale resolution suggest that the surfaces are fully populated with polymer chains. The increase in monomer concentration leads to a heterogeneous and rough surface. These arguments are in good agreement with the results of FTIR-ATR measurements, which indicate the increase in grafting density with increasing monomer concentration (treatment time) for both types of treatments.

For surface activation and collagen immobilization, AFM and microscopic measurements demonstrated that plasma-precursor and chemical treatment was more efficient than simple chemical treatment.

Acknowlegments

The authors thanks to dr. Iulia Stoica for AFM data aquisition.

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