Effect of plasma surface modification on the biocompatibility of UHMWPE

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Abstract

In this paper active screen plasma nitriding (ASPN) is used to chemically modify the surface of UHMWPE. This is an unexplored and new area of research. Active screen plasma nitriding allows the homogeneous treatment of any shape or surface at low temperature, therefore it was thought, that ASPN would be an effective technique to modify organic polymer surfaces. ASPN experiments were carried out at 120°C using a DC plasma nitriding unit with a 25% N₂ and 75% H₂ atmosphere at 2.5 mbar of pressure. UHMWPE samples treated for different time periods were characterized by Nanoindentation, FTIR, XPS, Interferometry and SEM. A 3T3 fibroblasts cell line was used for in vitro cell culture experiments. Nano-indentation of UHMWPE showed that hardness and elastic modulus increased with ASPN treatment compared to the untreated material. FTIR spectra did not show significant differences between the untreated and treated samples, however some changes were observed at 30 min of treatment in the range of 1500-1700 cm⁻¹ associated mainly with the presence of N-H groups. XPS studies showed that nitrogen was present on the surface and its amount increased with treatment time. Interferometry showed that no significant changes were observed on the surfaces after the treatment. Finally, cell culture experiments and SEM showed that fibroblasts attached and proliferated in a greater extend on the plasma treated surfaces leading to the conclusion, that ASPN surface treatment can potentially significantly improve the biocompatibility behaviour of polymeric materials.

Keywords: Surface modification, Plasma nitriding, UHMWPE, fibroblasts, cell seeding
1. Introduction

During the past 30 years, biomaterials evolution directed scientists to the development of a large number of different types of materials used in various biomedical applications [1]. These new materials and methods can enhance tissue engineering approaches resulting in further progress in the biomaterials field. In tissue engineering, the main interest focuses on the use of biomaterials in order to promote a new tissue formation in vitro or in vivo [2]. According to Hench et al., although the biocompatibility of a material is a very important parameter, it is not enough; the materials surface chemistry has to meet the requirements of the new tissue [3]. Consequently, surface modification often is required. It is very important to design and modify materials with the best surface properties because their biological responses are controlled by their surface chemistry and structure and therefore can become more effective in clinical applications. The surface properties play a vital role at the sequence of events happening, when cells are in contact with a surface by influencing the cellular events at the cell-materials interface, which means that the biological responses to biomaterials are associated with the chemistry and structure of surface [4, 5].

One way to modify a surface is plasma surface modification (PSM). PSM is a technique capable of modifying various properties of a material: wettability, refractive index, chemical inertness, dyability, hardness, lubricity and biocompatibility [5]. The surface changes that are possible to be introduced on a material using PSM are often increase of roughness and introduction of new functional groups (e.g. oxygen, nitrogen) depending on the gas used [6]. The parameters that influence the plasma treatment are: the type of substrate (type of material, dimensions, quantity and morphology), the type of reactor (inner wall, electrode and gas feeding), the energy input (frequency, power density and duration), ion bombardment, radiation and finally the type of reactions that take place inside the plasma furnace [7]. In general, plasma methods in the biomaterials field were introduced in 1960’s, and since then are applied to biomaterials and biomedical devices. PSM as an effective and economical technique for surface treatments has been widely used [6] but J. Georges in 1999 introduced Active Screen Plasma Nitriding (ASPN) modification, the main advantage of which is the capacity to treat homogeneously all kind of materials of any shape [8]. In the case of metallic materials the technique can enhance selectively the surface properties and biocompatibility, while the bulk properties of the materials remain unchanged [5]. However, this may not be the case when ASPN is used for the surface modification of UHMWPE. One of the concerns is that at 120°C, which is close to the melting point of UHMWPE (mp: 132°C), the mobility of chains may introduce chain rearrangements, re-crystallisation, formation of ions and consequently cross-linking or degradation of the polymer.

Polymers offer low weight, processability and inert behaviour in a biological environment and therefore are a good choice for biomedical applications. The surface properties of polymers however, do no often satisfy the requirements for biomedical applications, such as scratch resistance, wettability, biocompatibility, gas transmission, adhesion and friction [9]. The most widely used polymeric scaffolds are: PLA (polylactic acid), PGA (polyglycolic acid), copolymers of PLA and PGA (PLGA, poly (lactic-co-glycolic acid)), polyanhydrides, polyorthoesters, polycaprolactones, polycarbonates, polyimides, etc [10]. Polymeric scaffolds are often used in Tissue Engineering to repair or reconstruct damaged tissues with the main requirement to act as support for tissue regeneration [11]. It has been shown that polymers influence the viability, growth and function of attached cells controlling the cell function by the chemical, morphological and mechanical properties of the polymeric surface [12].

UHMWPE is a polymer mainly used for total joint replacement prostheses. The problem with UHMWPE is that often depending on the molecular weight, wear is present resulting in wear debris and failing to support total joint replacement, a surface modification is required [9, 13]. Studies in UHMWPE surface modification showed an improvement of the surface properties. When UHMWPE was irradiated with Ar ions, the hardness and Young’s modulus were dependent on the energy flow. Also, the hardness and Young’s modulus were both increased after irradiation and prolonged exposure to Ar atmosphere [14].
The initial sequence of events that takes place when cells interact with different surfaces in vitro is similar to in-vivo processes of cell adhesion and spreading. In fibroblast studies these initial steps can be easily observed. This explains why fibroblasts are widely used in these types of experiments. Fibroblasts are responsible for synthesizing and maintaining the ECM of most of the animal tissues. Consequently, they are the most common cells present in connective animal tissues and their importance in wound healing is crucial [15].

Silva and Luna conducted plasma modification of chitosan membranes in the presence of nitrogen and argon for 10, 20, 30 and 40 min and then seeded mouse fibroblast-like cells (L929) on the modified chitosan membranes. The surface roughness and energy were clearly increased and nitrogen and oxygen containing groups were present on the scaffolds after the plasma treatment where L929 cells proved to be more viable. Adhesion and proliferation was also significantly improved after the treatment [16].

The main aim of the paper is to investigate the effect of Active Screen Plasma Nitriding treatment on the physical and biological properties of UHMWPE surfaces. For this purpose, the experimental work focused on the measurement of hardness and elastic modulus of modified and unmodified UHMWPE surfaces by employing nanoindentation as well as on the study of surface chemistry, roughness and cells-surface interaction by XPS, interferometry and cell culture studies, respectively.

2. Materials and methods

2.1 UHMWPE

UHMWPE was supplied in the form of an A4 paper size flat sheet by Oadby Plastics Ltd. The molecular weight of the polymer was supplied by the manufacturer and was 9,200,000 g/mol. In order to prepare the UHMWPE samples for ASPN treatment, square pieces of 1.5x1.5 cm² UHMWPE were cut. Before the plasma treatment all samples were cleaned with distilled water and ethanol and were left to dry in air.

2.2 Plasma treatment

The method that was applied for the surface modification was active screen plasma nitriding (ASPN), the experimental set up of which is shown in figure 1. A conventional DC nitriding unit (Klockner, 40 kW) was used together with an active screen setup [17]. The active screen (mesh cylinder) was placed around the workload. The mesh cylinder was made of 0.7 mm thick perforated sheet steel, with a height of 130 mm and a diameter of 120 mm. A high voltage cathodic potential was applied on the screen, whereas the samples to be treated and the working table were on a floating potential and the furnace walls were on an anodic potential. The samples and the working table were insulated from the cathodic (screen) and anodic potential (furnace wall). The atmosphere in the plasma chamber was 25% N₂ and 75% H₂ and the pressure was 2.5 mbar. After the plasma treatment, all samples were placed in a desiccator under a vacuum of 10⁻² mbar. UHMWPE surfaces were modified by ASPN at a temperature of 120°C and for 10, 30 and 60 min. Table 1 shows the description of the materials used in this study.
2.3 **Cell culture experiments**

Frozen embryonic murine mouse cells preserved in liquid nitrogen under -150°C, from the standard 3T3 fibroblast cell line were used in order to study the biocompatibility of the untreated and treated surfaces. The cells were cultured in Dulbecco’s modified Eagle’s supplemented medium (DMEM) provided by Sigma-Aldrich. The medium was supplemented with 10% foetal calf serum (FCS), 2.4% L-glutamine, 2.4% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer and 1% penicillin/streptomycin. Also, phosphate buffered saline (PBS) and trypsin were used for the cell culture following the 3T3 fibroblasts protocol. The cells were passaged the 7th day of the cell culture and were counted using a hemocytometer. When the cell culture was confluent enough, the cells were isolated and afterwards seeded on the substrates. Prior to cell seeding, all UHMWPE samples (untreated and treated for 10, 30 and 60 min) were autoclaved for 15 min at 120°C. After sterilization, the samples were placed in a disposable for cell culture. Then, the cells together with the medium were added in each sample with an additional amount of 3.5 ml of new medium. The cell culture contained 2.4x10⁶ cells / ml and each sample contained 1.2x10⁶ cells. The seeded surfaces were placed in an incubator for four days at 37°C.

2.4 **Characterisation of materials**

Nanoindentation, Fourier Transform Infrared Spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), Laser interferometry and Scanning Electron Microscopy (SEM) were employed to further characterise the plasma treated and untreated UHMWPE samples.

The nano-indentation measurements were carried out in order to understand how plasma treatment affects materials’ hardness and elastic modulus. Each sample was tested six times; the hardness and elastic modulus were calculated from the mean of the six measurements. The equipment used was a Nano Test 600 machine (Micro Materials UK).
FTIR spectroscopy was conducted in order to study the presence of possibly new chemical groups on the surface of materials caused by the treatment. A Nicolet Magna 860 spectrophotometer was used for the analysis of both treated and untreated samples. The measurements were performed in a frequency range of 400-4000 wavenumbers (cm\(^{-1}\)) and a resolution of 4 cm\(^{-1}\). 100 scans / min were taken. A background scan was conducted prior to measurements and was subtracted from each sample spectra.

Laser Interferometry was employed in order to evaluate roughness at the surface of samples caused either by the heat treatment or plasma treatment. A MicroXAM laser interferometer was used for the analysis of both treated and untreated samples. The light source was white and the scan 50 times objective.

Finally, a home built XPS was used for the analysis of both treated and untreated samples. The software used was produced by PSP Ltd, UK. The pass energy was 50 eV and the X-Ray gun run at 10 keV. The step size in order to obtain individual peaks was 0.1 eV, whereas 1 eV was used for the full spectrum of analysis. The vacuum was less than 10\(^{-8}\) mbar.

2.5 Scanning electron microscopy
The SEM used was a Jeol JSM 6060 LV (Oxford Instruments Inca, UK). The operating voltage was 20 kV, the working distance was 10 mm and the spot size was 3. Prior to testing, the cell seeded samples were chemically fixed using 2.5% glutaraldehyde for 24 hours and dehydrated with ethanol. Afterwards, the samples were washed in 70, 90 and 100% aqueous ethanol solutions for 30 min followed by 100% dried ethanol for additional 30 min. The samples were then placed in liquid CO\(_2\) at 1070 psi and 31°C for 60 minutes. Finally, the specimens were Au coated by a sputtering method using 25 mA and 1.5 kV, the thickness of spattered Au was between 10-12 nm.

3. Results and Discussion

It is important to mention here a few structural characteristics and properties of UHMWPE in order to understand possible changes in the properties of the polymer with the plasma treatment. The molecular weight of the UHMWPE that was used in this study is high and in the order of $9 \times 10^6$ g/mol. The polymer chains are linear and mostly aligned to the same direction and each chain is bonded to the other by Van der Waals secondary bonds resulting in a strong polymer structure, despite the relatively weak bonds between its molecules. It is clear that UHMWPE derives ample strength and durability from the length of each individual molecule and the preferred orientation of the chains. Due to the linear chains, UHMWPE does not have side chemical groups like esters, amides or hydroxyl groups and therefore the polymer exhibits strong resistance in chemical degradation and radiation [18]. The melting point of UHMWPE as has been measured by DSC shown in figure 2 is 132°C with an onset softening temperature of ca 88°C. The degree of crystallinity is 47.7% and was calculated from the equation below:

$$\text{Crystallinity} \% = \frac{\Delta H_{\text{sample}}}{\Delta H_{\text{UHMWPE}}} \times 100$$

Taking into consideration that the fusion enthalpy of the fully crystalline UHMWPE is $\Delta H_{\text{UHMWPE}} = 290 \text{J/g}$ as reported by Reggiani et al [19]. On cooling crystallisation occurs at $T_c = 118$°C as shown in figure 2 below. It is important to note, that the ASPN treatment was conducted at 120°C, which is a temperature at which softening of the polymer chains may occur. However, crystallisation on heating was not observed in the DSC curve and therefore it is thought that the treatment at 120°C would not result in crystallisation of the amorphous part of the polymer.
3.1 Nanoindentation measurements

The mean values of hardness and elastic modulus of PE-0, PE-PT1, PE-PT2 and PE-PT3 are shown in table 2, whereas the change in hardness and elastic modulus of all UHMWPE samples with treatment time are shown in figure 3. As it can be observed, the untreated sample (PE-0) exhibits lower hardness and modulus compared to the treated samples. The time of treatment does not seem to affect significantly the hardness and modulus of treated samples although a slight increase in modulus was observed for PE-PT3 which was treated for 60 min. It is well known, that the degree of crystallinity can affect significantly the mechanical properties of UHMWPE. Specifically, re-crystallisation on heating could result in a profound increase of both tensile strength and modulus of elasticity of UHMWPE [20, 21]. In our case recrystallization on heating does not occur and therefore it is clear, that the increase in hardness and modulus of the treated UHMWPE surfaces is solely due to the ASPN treatment. It is therefore necessary to understand what is happening on the surface of UHMWPE during the ASPN treatment considering the temperature and the atmosphere under which the treatment was conducted. For this reason the FTIR and XPS spectra of treated and untreated samples were analysed.
Figure 3: Hardness and Elastic modulus of UHMWPE samples.

Table 2: Nanoindentation results for UHMWPE.

<table>
<thead>
<tr>
<th>Material</th>
<th>Hardness (GPa)</th>
<th>Elastic modulus (GPa)</th>
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<tbody>
<tr>
<td>PE-0</td>
<td>0.085104 (±0.016227)</td>
<td>1.544146 (±0.274932)</td>
</tr>
<tr>
<td>PE-PT1</td>
<td>0.143618 (±0.074972)</td>
<td>2.258981 (±0.8971)</td>
</tr>
<tr>
<td>PE-PT2</td>
<td>0.14204 (±0.036)</td>
<td>2.22783 (±0.339)</td>
</tr>
<tr>
<td>PE-PT3</td>
<td>0.137621 (±0.023881)</td>
<td>2.452585 (±0.375956)</td>
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3.2 Fourier Transform Infrared Spectroscopy – FT-IR

Figure 4 represents the FT-IR spectra of plasma treated and untreated samples. The description of the main FTIR peaks is given in table 3. Generally, only some small differences were observed in the FTIR spectra between the treated and untreated samples and the description of peaks is in good agreement with the literature [22-24]. The FTIR spectra for the plasma treated samples (figure 4) showed that untreated and 10 min treated samples had very similar peaks indicating that the surface chemistry did not change significantly due to the ASPN treatment. The situation however is different in the case of PE-PT2. Clearly, nitrogen was present on the materials surface shown by the presence of peaks at 829 cm\(^{-1}\) associated with N-H stretching vibrations and at 1288 cm\(^{-1}\) associated with C-N stretching vibrations leading to the conclusion that new bonds were formed on the surface after the treatment. The surface of PE-PT3 appeared to be also affected by the plasma treatment but it was not possible to resolve all the peaks observed in the case of PE-PT2. This could not be explained as it was expected that the effect of treatment on the surface chemistry would be stronger after 60 min.
Figure 4: FT-IR spectrum of all plasma treated and untreated samples.

<table>
<thead>
<tr>
<th>Characteristic peaks cm$^{-1}$</th>
<th>Description</th>
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<tbody>
<tr>
<td>717</td>
<td>-CH$_2$- In plane vibration</td>
</tr>
<tr>
<td>719</td>
<td>-CH$_2$- In plane vibration</td>
</tr>
<tr>
<td>829</td>
<td>-NO$_3$-</td>
</tr>
<tr>
<td>1147</td>
<td>-C=O- Stretching vibration</td>
</tr>
<tr>
<td>1286</td>
<td>-C-N- Stretching vibration</td>
</tr>
<tr>
<td>1288</td>
<td>-C-N- Stretching vibration</td>
</tr>
<tr>
<td>1461</td>
<td>-CH$_3$- Non-symmetric stretching vibration</td>
</tr>
<tr>
<td>1462</td>
<td>-CH$_3$- Non-symmetric stretching vibration</td>
</tr>
<tr>
<td>1596</td>
<td>-C=C- Stretching vibration</td>
</tr>
<tr>
<td>2846</td>
<td>-CH$_3$- Symmetric stretching vibration</td>
</tr>
<tr>
<td>2913</td>
<td>-CH$_3$- Non-symmetric stretching vibration</td>
</tr>
<tr>
<td>2914</td>
<td>-CH$_3$- Non-symmetric stretching vibration</td>
</tr>
</tbody>
</table>
It has been reported by Teodoru et al [25] on the other hand, that N\textsubscript{2} plasma treatments of polymer surfaces induce the formation of olefinic hydrocarbons and the surface becomes more dense due to the increase in C=C that was observed in our case with the presence of a peak at 1596 cm\textsuperscript{-1} associated with C=C stretching vibration in the case of PE-PT2 (figure 4). In addition, due to sample exposure in air during sample transfer possible oxidation could occur that would result to surface crosslinking due to the presence of C=C bonds resulting in an increase in hardness and modulus of the surface [25].

3.3 X-Ray photoelectron spectroscopy

Figure 5 shows the XPS spectra of untreated and treated UHMWPE samples. All materials showed the presence of carbon and oxygen whereas only the treated surfaces exhibited also the presence of nitrogen. All samples experienced physisorption and chemisorption of oxygen as a result of exposure to air during sample transfer. The amount of each element present is given in figure 6. Generally, the amount of nitrogen present in all treated samples did not increase significantly with the treatment time. The peak around 288 eV is associated with C1s photoelectrons on the surface of the polymer and is present in all samples. Specifically, linkages such as C-O-C, C=O and O-C=O may be associated with this peak. According to Kurtz [26] UHMWPE might contain peroxides used for cross linking during the processing of the polymer to rods or sheets. These peroxides during plasma treatment may lead to oxidation of the surface resulting in the presence of ketones, alcohols, esters and carboxylic acids. This is in good agreement with the present results as a peak at 534 eV associated also with the presence of C-O or C=O appeared in the spectra of all samples that became more intense in the spectra of all treated surfaces. Also, since the functionality of the surfaces increased significantly with the ASPN treatment any exposure in air would also result in surface oxidation and would contribute to the intensity of the above peak. Similar observations have been also reported by Rhodes et al and Teodoru et al. [25, 27]. The peak at 402 eV is associated with C-N linkages suggesting that during the plasma treatment new covalent bonds between carbon and nitrogen were formed in all treated surfaces. This is also in good agreement with the FTIR spectra discussed above where the presence of C-N linkages on the surface of treated UHMWPE samples was suggested with the presence of a small peak at 1288 cm\textsuperscript{-1}.
3.4 Surface roughness
Figure 7 shows the surface topography and the 3D image of both treated and untreated samples. Figure 8 shows the numerical values of both $S_a$ (average roughness) and $S_q$ (root-mean-square roughness). It is important to report both of these values as there is no significant difference between these two terms. Generally, the plasma surface modification resulted in an increase in surface roughness [5]. Silva et al. reported that chitosan membranes treated by plasma modification in the presence of nitrogen and oxygen, exhibited an increase in the surface roughness and energy. The increase in surface roughness was thought to be due to plasma etching effects [16]. Teodoru et al. reported AFM data of $N_2$ plasma treatment of UHMWPE surfaces and suggested that the treatment resulted in the formation of micropits as well as ridges [25]. A similar observation was reported by Rhodes et al., who suggested that $N_2$ plasma treatment of UHMWPE resulted in light etching evidenced by an increase in the roughness and surface area measured by AFM [27]. In our case however the surface roughness did not change significantly due to the treatment. A possible explanation could be that the surface of UHMWPE exhibited already a degree of roughness ($S_a=834\text{nm}$) and it is possible that main difference between the treated and the untreated samples is in the surface area that was not possible to be measured by the laser interferometer. In order to
examine in depth the effect of active screen plasma nitriding on the roughness of UHMWPE surface, further measurements using AFM should be conducted where the surface area could be accurately measured. Also, AFM studies of ASPN treated UHMWPE surfaces with very small roughness could also provide important information.

Figure 7: Surface topography of all plasma treated and untreated samples and the 3D image of the sample’s surface.
Figure 8: Roughness results for untreated and plasma treated samples.

3.5 Scanning Electron Microscopy

SEM was used in order to examine the cell seeded surfaces. The purpose of this study was to observe the type of surfaces that cells prefer to grow upon, to investigate the plasma treatment effects on the cells behaviour compared to untreated surfaces and the effect of the treatment duration on cell spreading and proliferation. This is a preliminary biocompatibility study and it is expected that further investigation concerning protein attachment should be carried out. The emphasis of this paper has been given to the effect of ASPN on the physicochemical surface properties of the materials under study. Figure 9 shows fibroblasts seeded on PE-0, PE-PT1, PE-PT2 and PE-PT3 surfaces. The magnification range varies from x150 to x1800. A slight cell attachment was observed in the case of untreated samples (PE-0). Moreover, the cells were dispersed across the polymer surface and did not seem to be connected with each other (figure 9.a and b). Better attachment and proliferation across the polymer surface was observed in the case of PE-PT1. Despite the fact, that fibroblasts had their edges connected, there were still big gaps among the cells (figure 9.c and d). On the other hand, in the case of PE-PT2 (figure 9.e and f) the fibroblasts were better connected with each other compared to PE-0 and PE-PT1 and were attached to the surface. However, the cells did not look healthy as body cracks could be observed caused probably by the treatment required to process SEM samples. The fibroblasts on the surface of PE-PT3 looked healthier, the cell attachment was improved and the cells exhibited a larger degree of proliferation. The cells were well connected with each other forming layers of cells on the treated polymer surface. However, isolated cells appeared to be damaged due to the fixation treatment as was mentioned above. The fibroblasts preference to adhere on the treated surfaces rather than the untreated one, was clear in figures 9.g and h. It is believed, that the main reason is the presence of amine groups on the surface of treated samples as discussed in the FTIR and XPS sections. It is suggested, that the amine groups are good promoters for cell attachment in nitrogen containing plasma surfaces. This is due to the fact that glycoproteins such as fibronectin (Fn) and vitronectin (Vn), which mediate cell attachment on a substrate [28, 29], are highly influenced by the substrates properties and mainly by N-containing surfaces which have Fn and Vn adsorptive characteristics. In addition, mouse fibroblast-like cells (L929) seeded on Ar and N₂ plasma treated chitosan membranes proved to be more viable compared to cells seeded on untreated chitosan surfaces [16]. Rhodes et al also reported that generally plasma modification polymer surfaces can dramatically reduce protein and cell activation events that are involved in failure observed after implantation of various blood-contacting devices [27]. Finally, it can be concluded there is evidence to
support that UHMWPE treated surfaces can be very good substrates for 3T3 fibroblasts showing enhanced adhesion and proliferation.

Figure 9: SEM micrographs of UHMWPE seeded with cells. a,b PE-0, c,d PE-PT1, e,f PE-PT2, g,h PE-PT3.

4. Conclusions

Active Screen Plasma Nitriding (ASPN) using a gas mixture containing 25% N$_2$ and 75% H$_2$ was conducted for different periods of time on UHMWPE surfaces. The ASPN treatment resulted in an increase of hardness and elastic modulus of the treated surfaces. FTIR and XPS showed the formation of C-N and N-H groups resulting in an increase of the functionality of the treated surfaces. Possible cross-linking on the surfaces was also observed due to exposure of the treated surfaces in the air with consequent oxidation of functional groups present on the surface such as C=C. The roughness of the treated surfaces did not change significantly by the treatment although there are opposite reports in the literature. 3T3 fibroblasts cell culture studies showed that the ASPN treatment had a positive effect on the adhesion and proliferation of cells. Finally there is enough evidence to support that ASPN can be an effective treatment for polymer surfaces in order to improve not only their mechanical, physical and chemical properties but also their biocompatibility.

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