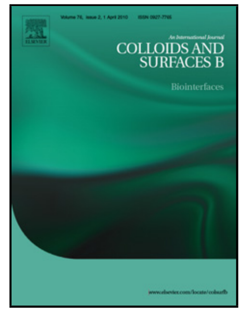

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Surface modification of polyurethane films by plasma and ultraviolet light to improve haemocompatibility for artificial heart valves

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Abstract

Prosthetic cardiac valves implantation is a common procedure used to treat heart valve diseases. Although there are different prosthesis already available in the market (either mechanical or bioprosthetic), their use presents several problems, specifically concerning thrombogenicity and structural failure. Recently, some progresses have been achieved in developing heart valves based on synthetic materials with special emphasis in polymers. Among them, polyurethanes are one of the most commonly used for the production of these devices.

Herein, Elastollan®1180A50, a thermoplastic polyurethane (TPU), was used to formulate films whose surfaces were modified by grafting 2-hydroxyethylmethacrylate (HEMA) either by Ultra-violet (UV) or by plasma treatment. All films were analyzed before and after grafting. X-ray photoelectron spectroscopy (XPS) measurements were used to evaluate TPU surfaces functionalization. HEMA grafting was confirmed by the increase of the hydroxyl (OH) groups' concentration at the surface of the films. Atomic force microscopy (AFM) analysis was done to evaluate the surface topography of the biomaterials. Results showed that the roughness of the surface decreased when HEMA was grafted, especially for plasma treated samples.

After grafting the films' hydrophilicity was improved, as well as the polar component of the surface energy, by 15 to 30%. Hydrophobic recovery studies using milli Q water or PBS were also performed to characterize the stability of the modified surface, showing that the films maintained their surface properties along time. Furthermore, blood-contact tests were performed to evaluate haemolytic and thrombogenic potential. The results obtained for HEMA grafted surfaces, using plasma treatment, confirmed biomaterials biocompatibility and low thrombogenicity.

Finally, the cytotoxicity and antibacterial activity of the materials was assessed through *in vitro* assays for both modified films. The obtained results showed enhanced bactericidal activity, especially for the films modified with plasma.

Keywords: Heart valves, plasma and UV activation, polyurethanes, surface modification, biocompatibility

1. Introduction

Valvular heart diseases (VHD) include several heart conditions that can be either congenital or acquired. Acquired VHD comprise degenerative valve diseases (which are the most common in developed countries) and rheumatic heart sickness (mostly common in developing nations) [1].

Nowadays, the number of patients diagnosed with degenerative valve disease is progressively growing with population aging [2]. In fact, it is estimated that at least one in each eight people over 75 years old will suffer from one kind of VHD, becoming a serious public healthcare problem and a significant economic burden [3].

Some patients suffering from less severe valvular lesions are able to go through their lifetime without ever needing surgical intervention. However, for others, surgery is the only viable solution. Surgical treatment may involve the repair or the replacement of the original damaged valve. The ideal choice would be to keep the original valve [4]. However, for nearly 70% of the cases this procedure is no longer viable and valve substitution must be performed [5]. Currently, nearly 280 000 heart valve substitutes are implanted each year all over the world, in an approximated proportion of 50/50 for mechanical and bioprosthetic valves [6]. Despite the improvements in the design and composition of the commercially available valve prosthesis, mechanical valves have a high associated risk of thrombogenicity, while the bioprosthetic valves may suffer from premature structural failure [7]. Moreover, after a prosthetic valve implantation, a life-threatening complication, known as prosthetic valve endocarditis (PVE), may also occur [8]. PVE is usually caused by microorganism infection, especially bacteria and fungi, and its treatment requires medical treatment. However, in advanced stages, antibiotic therapy alone may not be enough to eliminate the infection and prosthesis replacement is required [9].

In order to overcome these problems, synthetic leaflet heart valves have been investigated over the last decades, trying to combine in one material, features like durability and enhanced haemodynamics [10]. Among the different synthetic materials used so far, polyurethanes (PU) have been considered the most suitable for this purpose [11]. PU's properties such as abrasion resistance, affordable manufacturing, chemical stability, durability, elasticity and haemocompatibility are fundamental for their extended applications in the biomedical field [12]. Besides heart valves, they are also used to prepare blood oxygenators, catheters, drug delivery systems, internal lining of artificial hearts, scaffolds for tissue engineering and wound dressing membranes [13]. However, several studies have reported that heart valves produced with PU's, may suffer from premature failure, caused by their suboptimal design and low durability [7]. The production of PUs with different compositions and by applying different manufacturing techniques have resulted in materials with improved properties which have allowed to expand their potential applications [14].

Thermoplastic polyurethanes (TPU) are currently being used for several industrial uses, such as adhesives, coatings and films [15]. Furthermore, previous studies have also shown their suitability for heart valves production [16].

Hereby, a commercial pre-processed polyether-based thermoplastic polyurethane (Elastollan®1180A50), was studied in order to be applied in a near future as a base material for synthetic heart valves manufacture. Elastollan®1180A50 choice was done based on its absence of plasticizers, good heat resistance, high mechanical flexibility, and its ability to be processed by moulding. Moreover, it also exhibits excellent abrasion resistance, toughness, transparency, hydrolytic stability and fungal resistance [17].

From the literature it is known that graft copolymerization of polyurethanes with hydrophilic vinyl monomers, such as acrylic acid [18], acrylamide [19] and 2-hydroxyethylacrylate [20], is an appropriate method to enhance surface hydrophilicity, and improve its haemocompatibility [21].

Herein, 2-hydroxyethylmethacrylate (HEMA) was grafted onto the surface of Elastollan®1180A50 to increase its hydrophilicity and improve its biological properties. Plasma and UV irradiation were used to perform the modification of films' surface. Furthermore, several parameters were assessed, such as their chemical surface functionalities, roughness, antibacterial activity, blood compatibility, cytotoxicity, hydrophilicity, hydrophobic recovery, surface energy and thrombogenicity to evaluate their potential for being used in heart valve fabrication.

2. Experimental

2.1. Materials

Bacterial strain *Escherichia coli* (*E. coli*) DH5α was purchased from ATCC. Elastollan®1180A50 was obtained from BASF. Fetal bovine serum (FBS) was acquired from Biochrom AG (Berlin, Germany). Human Fibroblast Cells (Normal Human Dermal Fibroblasts adult, criopreserved cells) were bought from PromoCell (Labclinics, S.A.; Barcelona, Spain). LB agar was obtained from Pronadise. Irgacure®2959 was kindly given by CIBA (Ciba Specialty Chemicals, Basel, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium, inner salt (MTS), amphotericin B, Dimethylformamide (DMF), Dulbecco's modified Eagle's medium (DMEM-F12), ethylenediaminetetraacetic acid (EDTA), 2-hydroxyethylmethacrylate (HEMA), isopropyl alcohol, L-glutamine, penicillin G, phosphate-buffered saline solution

(PBS), streptomycin, and trypsin were acquired from Sigma-Aldrich (Sintra, Portugal).

2.2. Methods

2.2.1. Films preparation

Elastollan®1180A50 films were prepared by solvent evaporation. Elastollan®1180A50 was dissolved in DMF to a 10% (w/v) TPU solution. This solution was poured into glass Petri plates. Then, the Petri dishes were stored in an oven at 60 ºC, for 24 hours. Subsequently, films were removed from the dishes and ultrasonically cleaned with isopropyl alcohol for 15 minutes, prior to surface grafting experiments.

2.2.2. Argon plasma grafting

A laboratory and small-scale production plasma system FEMTO (low pressure plasma), manufactured by Diener Electronics, with a stainless steel plasma chamber of 100 mm diameter and 270 mm length, was used for the plasma surface modification experiments. TPU films were placed at 80 mm from the electrode and were plasma treated with Argon, in a pressure chamber of 0.6 mbar, for 3 minutes and applying 100 Watt of power to the electrodes to generate the plasma [22]. Then, the plasma-treated TPU films were dipped into a 10% (v/v) aqueous solution of HEMA and introduced in an oven at 60 ºC, for 1 hour. Finally, the modified films (TPU-Ar-HEMA) were washed abundantly with deionized water and dried until constant weight was obtained.

2.2.3. UV grafting with Irgacure®2959

For the UV grafting, films were previously activated with UV light in a 0.5% photoinitiator (Irgacure®2959) aqueous solution for 30 minutes. Afterwards, they were removed and dipped into a 10% (v/v) HEMA aqueous solution. Then, samples were irradiated with UV light during 30 minutes and the modified films were obtained (TPU-UV-HEMA).

In both steps of the modification, films were irradiated using a Mineralight® Lamp, Model UVGL-48, in the 254 nm wavelength setting. This generated a power of 6 Watt and the samples were placed at a distance of 4 cm from the light source.

2.3. Characterization techniques

2.3.1. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) measurements were made with a VGS ESCALAB 200A spectrometer with an Al Kα X-ray source. The operation conditions were set to 15 kV. The binding energy scale was fixed by assigning a binding energy of 285.0 eV to the $–CH_2–$ carbon (1s) peak. The samples were analyzed at a take-off angle of 0º relative to the normal of the surface. The C1s and O2s envelopes were analyzed and peak-fitted using a combination of Gaussian and Lorentzian peak shapes obtained from the XPS peak 4.1 software.

2.3.2. Atomic force microscopy

Atomic force microscopy (AFM) analysis of the samples was performed in a Nanoscope IVa Veeco Metrology using the tapping mode (scan size 4.0 µm, scan rate 1.0 Hz). The average roughness (Ra) was calculated directly from the AFM images.

2.3.3. Analysis of contact angle and surface free energy

The contact angle and surface energy measurements were performed at room temperature in an OCA 20 contact angle measurement unit from Dataphysics. Surface free energy (γ_S) values as well as the dispersive (γ_S^D) and polar (γ_S^P) components were obtained according to the Owens-Wendt-Rabel and Kaelbe method (OWRK) by static contact angle measurements with three liquids: water, diiodomethane and formamide. All measurements were performed on the air-facing surfaces of the films with the three liquids using the sessile drop method. Nine measurements on different points were performed on each sample from which the mean static contact angle and its standard error were determined. The surface energies were assessed for all the prepared films.

2.3.4. Hydrophobicity recovery

Samples were stored in vials containing milli Q water or PBS at 37 °C and examined after 1, 2, 7, 15 and 30 days. Samples aged in air were wrapped in aluminium foil to minimise hydrocarbon contamination, and those aged in milli Q water and PBS were thoroughly washed with milli Q water and dried before analysis. The hydrophobicity recovery was evaluated by water contact angle determination, as previously described.

2.3.5. Blood compatibility

Blood compatibility assays were performed *in vitro* accordingly to the International Standard Organization (ISO) 10993-4 [23]. Both the haemolytic potential and thrombogenicity of the prepared films were evaluated.

Haemolytic potential

The haemolysis tests were performed as described in the American Society for Testing and Materials (ASTM) F 756-00 standard [24]. Samples with 21 cm^2 were placed in polypropylene test tubes and 7 mL of PBS (10 M, pH=7.4) were added. After 72 h of incubation, at 37 ºC, the PBS was removed and the samples were left to dry. Then, 7 mL of PBS and 1 mL of diluted anticoagulated rabbit blood (ACD blood) (10 mg/mL ± 1 mg/mL) was added to each sample. Positive and negative controls were prepared by adding the same amount of ACD blood to 7 mL of water and PBS, respectively. The tubes were placed at 37 ºC, for 3 hours, and gently inverted twice every 30 minutes to maintain materials in contact with blood. After incubation, the fluid was transferred to a suitable tube and centrifuged at 700-800 *g*, for 15 minutes. The amount of haemoglobin (Hb) released by haemolysis was determined by measurement of the optical densities of the supernatants at 540 nm using a spectrophotometer UV-vis (Jasco V550). The percentages of haemolysis (HI) were calculated as described in equation 1.

$$HI = \frac{[Hb]_{test} - [Hb]_{negativecontrol}}{[Hb]_{positivecontrol} - [Hb]_{negativecontrol}} \times 100 \qquad \text{Equation 1}$$

According to the ASTM F 765-00 [24] materials can be classified as non-haemolytic when 0 > HI > 2, slightly haemolytic when 2 > HI > 5 and haemolytic when HI > 5.

Thrombogenicity

The evaluation of thrombus formation on films surfaces (n=3 for each sample) was carried out using the gravimetric method of Imai and Nose [25]. Anticoagulated rabbit blood was also used for this purpose. Before performing the tests, the films were immersed in PBS solution (pH 7.4) at 37 ºC. After 48 h of incubation, the PBS was removed and 250 μL of ACD blood were carefully placed over the surface of the films and also in an empty Petri dish, which acted as a positive control. Blood clotting tests were initiated by adding 25 μL of a 0.10 M calcium chloride solution and then stopped after 30 minutes, by adding 5 mL of water. The resultant clots were fixed with 1mL of a 36% formaldehyde solution and then dried with tissue paper and finally weighted. The percentage of thrombogenicity was determined by using equation 2.

$$\%\ thrombogenicity = \frac{m_{test} - m_{negativecontrol}}{m_{positivecontrol} - m_{negativecontrol}} \times 100 \qquad \text{Equation 2}$$

2.3.6. Evaluation of materials biocompatibility

Proliferation of human fibroblasts cells in the presence of the materials

Human Fibroblasts cells were seeded in T-flasks of 25 cm^2 with 6 mL of DMEM-F12 supplemented with heat-inactivated FBS (10% v/v) and 1% antibiotic/antimycotic solution. After cells attained confluence, they were subcultivated by a 3-5 minutes incubation in 0.18% trypsin (1:250) and 5 mM EDTA. Subsequently, cells were centrifuged, resuspended in culture medium and then seeded in T-flasks of 75 cm^2. Hereafter, cells were kept in culture at 37 °C in a 5% CO_2 humidified atmosphere, inside an incubator. To evaluate cell behavior in the presence of the materials, fibroblasts cells were seeded with materials in 96-well plates at a density of 10×10^3 cells per well, for 96 hours. Previously to cell seeding, materials were firstly sterilized using UV radiation for 30 minutes. Cell growth was monitored using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera [26].

Characterization of the cytotoxic profile of the films

Human fibroblasts cells were seeded in the presence of materials, in 96-well plate, with 100 µl of DMEM-F12 and following incubated at 37 °C, in a 5% CO_2 humidified atmosphere. After an incubation period (24, 48, 72 and 96 hours), cell viability was assessed through the reduction of the MTS into a water-soluble formazan product. Briefly, the medium of each well was removed and replaced with a mixture of 100 µL of fresh culture medium and 20 µL of MTS/PMS reagent solution. Then, cells were incubated for 4 hours at 37 °C, under a 5% CO_2 humidified atmosphere. The absorbance was measured at 492 nm using a microplate reader (Sanofi, Diagnostics Pauster). Wells containing cells in the culture medium without materials were used as negative controls (K^-). Ethanol (96%) was added to wells that contained cells, as a positive control (K^+) [27].

2.3.7. Characterization of the antibacterial activity of PU materials

Resazurin metabolic assay

The resazurin assay was performed to evaluate bacterial growth in the presence of the samples. Resazurin is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells [28]. Briefly, bacterial cultures (*E.coli*) were grown overnight in culture medium without antibiotics. The following day bacteria were seeded in 96 well plates at density of 5 x 10^6 colony-forming unit (CFU)/mL under aseptic conditions. Then, 10 µL of 0.1% resazurin solution were added to each well and the plate was incubated at 37 °C during 1 to 4 hours. After, digital images of the plate were acquired to evaluate bacterial growth using a Nikon digital camera (Nikon D50, Ayuthaya, Thailand).

2.3.8. Statistical analysis

The obtained results were expressed as the mean ± the standard error of the mean (n=4). Statistical significance was calculated using a one-way analysis of variance (one-way ANOVA) and differences between groups were tested by a one-way ANOVA with Dunnets post hoc test [26].

3. Results and Discussion

3.1. X-ray photoelectron spectroscopy

To evaluate the existing functionalities on TPU surfaces (before and after the grafting), wide scan and high-resolution XPS spectra were recorded. This analysis was performed 24 hours after the grafting procedures. The elemental composition of the surfaces was calculated from the XPS spectra.

As expected, the oxygen content of the grafted surface increased due to the grafting of HEMA's hydroxyl groups (OH). Figure 1 shows the different spectra obtained for the unmodified and the HEMA grafted TPU. Here, the C1s peak can also be resolved in three components: the hydrocarbon (C-C and C-H) peak at 284.5eV, the ether peak (C-O-C) at 285.9 eV and the urethane peak (NH-COO) at 288.0 eV. The ether peak increases after the grafting and due to the addition of more ether peaks and to the addition of OH groups present in the HEMA monomer structure. The increase of this peak is slightly higher when the UV grafting method was used. All these changes suggest that the surfaces were successfully grafted. The same conclusion can be inferred by looking at the O1s peak, which is resolved in two peaks: the C=O peak at 532 eV (which was already present in the untreated TPU) and the C-OH peak at 534 eV due to the hydroxyl groups [29]. From the differences observed in the peak areas and the relative composition ratio based on the area of each peak presented in Figure 1 can be easily concluded that the plasma grafting method is more efficient than the UV. Also, it can be implied that the graft density is higher for the plasma treated surface since more OH groups are present on the surface as a consequence of the grafting of HEMA.

3.2. Atomic force microscopy (AFM)

Materials surface topography was evaluated by AFM analysis. Figure 2 shows the 3-dimensional AFM images of unmodified TPU and grafted TPU.

It is known that a surface becomes smoother after grafting, when compared with its original state, depending on monomer bounded to the surface [30]. Therefore, as expected from the results obtained in XPS analysis, distinct surface topographies were observed depending on the employed grafting method. The micrographs from Figure 2 show that when plasma is used, the resulting surface is smoother than when using UV grafting treatment, which indicates that a higher graft density was obtained when plasma was used for the grafting reaction. The higher graft density results in a smoother surface. This difference between both grafting methods has already been observed for the grafting of other monomers in previous works [31].

The roughness of the materials surfaces was determined to better quantify the differences between them. The average roughness (Ra) was calculated directly from the AFM images in a 700 × 700 nm surface region. The obtained Ra results, presented in Figure 2, confirm that the roughness of the surface decreases when HEMA is grafted, especially when the plasma method is used.

3.3. Water contact angle and surface energy measurements

It is widely recognised that surface energy is an important parameter affecting polymers adhesion, material wettability and even biocompatibility [32]. The measurement of contact angles is considered as the most convenient method for determining the surface free energy of solid samples. This technique relies on the determination of the interactions between the solid sample of interest and liquids with well determined surface tensions.

According to Owens, Wendt, Rabel and Kaelble the interfacial tension can be divided in two components: dispersive interactions and polar interactions [33]. Polar interactions comprise Coulomb interactions between permanent dipoles and the ones between permanent and induced dipoles. The interactions caused by time fluctuations of the charge distribution within the molecules are called dispersive interactions.

Table 1 summarizes the obtained results for water contact angles and surface energies as well as the percentages of the polar components of the surface energies for the unmodified and modified TPU films.

The presence of polar functional groups such as OH or NH_2 increases the hydrogen bounding interactions [34] and therefore decreases water contact angle and increases the polar component of the surface energy. For this reason, in this study, a decrease in the water contact angle could be observed after HEMA grafting. The water contact angle of the surface decreases from 82.7º for the unmodified surface, to 75.0º and 60.8º after grafting HEMA by UV and plasma, respectively. Such results are ascribed to the grafting of HEMA's OH groups, and this variation is even higher when the plasma grafting method is used. Moreover, the obtained results for the surface energy presented in Table 1 show an increase in the polar component of the surface energy when films surfaces are grafted with HEMA. While the original TPU film presents a polar component of 7.1%, this value changed to 15.5% and 38.7% when HEMA was grafted onto the surface by UV irradiation and plasma treatment, respectively. The results obtained for films treated with plasma suggest that the efficiency of grafting is higher when this method is used. Furthermore, these results are in accordance with those obtained by XPS, AFM and were further validated by the thrombogenicity studies.

3.4. Hydrophobic recovery analysis

Hydrophobic recovery was evaluated by static water contact angle measurements for a period of 30 days. The water contact angles were determined before and after each modification and along time by incubating samples in different mediums (air, milli Q water and PBS). The obtained results are presented in Figure 3. TPU has a water contact angle of 82.7º ± 1.8; after the grafting procedure this value decreases to 75.0º ± 0.4 and 60.8º ± 1.7 when UV and plasma methods were used, respectively. Plasma method led to lower values, suggesting once again, that this method is more efficient for grafting HEMA than the UV method. The decrease in the water contact angle is ascribed to the HEMA polar groups (OH groups) present on TPU surface after grafting. These groups increase dipole–dipole interactions and therefore increase hydrophilicity. For this reason, the observed decrease in the water contact angles may represent an indirect measure of the extent of modification.

From the hydrophobicity recovery profiles shown in Figure 3, it can be seen that after the grafting reactions, despite the storage medium, surfaces partially recovered their hydrophobicity along time. The hydrophobicity recovery of a surface might be explained by air contamination or even by surface rearrangements [30,35,36]. Comparing both grafting methods, this recovery is more evident for surfaces grafted by the UV method, suggesting that these surface modifications are not stable. When comparing storage mediums, the air is more prone to allow the hydrophobicity recovery, while milli Q water or PBS maintain the surface properties, meaning that storing the grafted surfaces in PBS or milli Q water prevents surface rearrangements and mainly eliminates air contaminations preventing the hydrophobicity recovery.

3.5. Blood compatibility

3.5.1. Haemolytic potential

Haemolysis is regarded as an especially significant screening test, once it provides quantification of small levels of plasma haemoglobin that may not be measurable under *in vivo* conditions [37].

The haemolysis index (HI) of the original and modified TPU films was determined according to ASTM F 756-00 [24], by using the cyanmethemoglobin method to quantify the haemoglobin (Hb) present in the supernatant after the films were incubated with blood.

Based on the results obtained, both the unmodified and the modified films are classified as non-haemolytic. In fact, HI is very similar for all samples varying from 1.85±0.11 (for the original film) to 1.88±0.05 for TPU-UV-HEMA and finally to 1.36±0.51 for TPU-Ar-HEMA. This means that surface modification with the HEMA molecule does not induce any damage in erythrocytes' membranes that could lead to their lysis. Although some literature states that it is not possible to define a universal level of acceptable or unacceptable haemolysis values [29], by definition, a blood-compatible material should not promote haemolysis. In this work this parameter is of extreme importance since the material will be contacting directly with blood during its entire lifetime. It is therefore safe to say that the modified films will not be responsible for the lysis of the erythrocytes once implanted in the human body. Such feature is fundamental for the desired biomedical application.

3.5.2. Thrombogenicity

Serum proteins adsorption onto the surface of a material is a key phenomenon for the thrombogenic process. In fact, thrombus formation is inversely proportional to blood compatibility of a given material. This parameter is of extreme importance when materials are designed to be used in direct contact with the blood stream as it is the case of heart valves.

The induction of thrombus formation on the surface of the prepared films was evaluated by gravimetry after 30 and 60 minutes of contact with blood. Both the unmodified and modified TPU films (TPU-UV-HEMA and TPU-Ar-HEMA) were tested using three samples of each set. The resultant weights of the blood clots from these tests were obtained and the percentage of thrombogenicity was calculated. In Figure 4 it is possible to verify that the surface modification methods used influence the thrombogenicity of the materials. As depicted in this Figure, both unmodified TPU as well as TPU-UV-HEMA films are highly thrombogenic after 30 and 60 minutes of being in contact with blood. On the other hand, the TPU-Ar-HEMA films presented a much lower value for thrombus formation for both incubation times (19.33 and 21.2% after 30 and 60 minutes, respectively). These results are coherent with those obtained for the surface energies values. It has been stated that thrombogenicity is directly related with the value of surface energy presented by a surface [38]. Interestingly, in this case, the surface energies of the films are not significantly different between them. However, the polar component of surface energy changes considerably. As previously described, it was verified that the polar component of the surface energy was higher for the TPU-Ar-HEMA film, suggesting that larger amounts of hydrophilic HEMA molecules were grafted on the TPU film, when plasma method was used. Hydrophilic surfaces are usually associated to low proteins adsorption since they adsorb weakly and reversibly to these types of surfaces [38]. Considering that protein adhesion constitutes the first step of coagulation cascade that ends in thrombus formation [39], it can explain the distinct thrombogenic character presented by films studied here.

3.6. Characterization of materials biocompatibility

Films cytocompatibility was evaluated through *in vitro* studies. Human fibroblasts cells with the same initial density were seeded in the 96-well plates, with or without materials to assess films cytotoxicity. Cell adhesion and proliferation in the presence of the materials was characterized through optical microscopy by using an inverted microscope. Figure 5A shows that cells adhered and proliferated in the presence of all the materials and also in the negative control. Such results demonstrate that all films are biocompatible.

In order to characterize the physiological response of cells to the presence of the TPUs, a MTS assay was also performed. The MTS assay results (Figure 5B) showed that cells maintained a similar viability to the ones cultured in the absence of films during 96 hours. Furthermore, cells presented higher viabilities in the presence of modified TPUs during the first 72 hours.

The results obtained herein show that the modifications performed on the TPU surface did not affect cell integrity or viability, a fact that is crucial for the proposed biomedical application, *i.e.,* to be used as heart valves.

3.7. Antibacterial activity

3.7.1. Resazurin assay

The antimicrobial activity of TPUs was evaluated through a resazurin reduction assay. As demonstrated in Figure 6, TPU-Ar-HEMA presented bactericidal activity, whereas unmodified TPU and TPU-UV-HEMA did not show any significant activity. Such results are of crucial importance, because it seems that HEMA grafted TPU by plasma treatment may contribute to avoid the biofilm deposition on this material once implanted inside the human body and therefore avoid common and dangerous complications such as prosthetic valve endocarditis.

4. Conclusion

The grafting of HEMA onto the surface of Elastollan®1180A50 films (TPU) by UV irradiation and Argon low pressure plasma treatment led to a modification their properties. This modification improved some of the films properties, and the resulting materials are good candidates to be used for heart valves production. Additionally, Argon plasma treatment showed to be more efficient for the grafting of HEMA when compared to the UV method. The TPU-Ar-HEMA surface showed higher content of OH groups indicating a higher HEMA density, which led to a smoother and more hydrophilic surface. Also, Argon plasma treatment showed significantly lower values of thrombogenicity in comparison with those of unmodified and UV modified TPUs. Furthermore, the modified films upkept their intrinsic biocompatibility and, more importantly, enhanced the bactericidal activity of the materials. This fact was once again more evident for TPU-Ar-HEMA.

Based on the overall results it may be concluded that TPU-Ar-HEMA is a good candidate to be used in a near future for the preparation of prosthetic heart valves.

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List of Tables

Table 1: Water contact angle (θ), surface energy (γ_s), dispersive (γ_s^D) and polar components (γ_s^P) of the surface energy and % of polar component of the unmodified and modified TPU films. Each result is the mean ± standard error of the mean of three independent experiments.

Figures captions

Figure 1: XPS spectra of C1s (a) O1s (b) for TPU (1) and the TPU-Ar-HEMA (2) and TPU-UV-HEMA (3). The relative composition ratio based on the area of each peak for the all TPU films are also indicated.

Figure 2: Atomic force microgragh of the TPU and HEMA grafted surfaces. Surface Roughness, Ra (nm) is indicated as the mean ± standard error of the mean of three independent experiments.

Figure 3: Hydrophobic recovery of TPU, TPU-Ar-HEMA and TPU-UV-HEMA stored in air, milli Q water and PBS. All samples were stored in the mediums after grafting. Each result is the mean ± standard error of the mean of three independent experiments.

Figure 4: Values of thrombus mass obtained for original and modified TPU films after 30 and 60 minutes of blood contact. Each result is the mean ± standard error of the mean of three independent experiments.

Figure 5: A) Microscopic photographs of human fibroblasts cells seeded in the presence of the different PU materials (*) for 24, 48, 72 and 96 hours of incubation, original magnification x100. B) Evaluation of the cellular activity after 24, 48, 72 and 96 hours in contact in TPU materials. Positive control (K^+); negative control (K^-). Each result is the mean ± standard error of the mean of three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnet's post hoc test (*$p < 0.001$).

Figure 6: Evaluation of the TPU-UV-HEMA (upper line), TPU-Ar-HEMA (midlle line) and TPU (lower line) films anti-bacterial effect. Image represents the colorimetric result of a resazurin assay, performed after culturing E. coli (DH5α) with the different materials for 24 hours. For each material, several quantities were tested and bacterial culture controls were also performed, which are represented by K^+ for the negative control (blue, represents dead bacteria) and K^- for the positive control (pink, represents viable bacteria).

Table 1

	θ (º)	γ_S (mN/m)	γ_S^D (mN/m)	γ_S^P (mN/m)	% of γ_S^P
TPU	82.7 ± 1.8	35.99 ± 2.44	33.43 ± 2.33	2.56 ± 0.71	7.1
TPU-UV-HEMA	75.0 ± 0.4	36.55 ± 1.51	30.70 ± 1.27	5.65 ± 0.81	15.5
TPU-Ar-HEMA	60.8 ± 1.7	38.31 ± 2.50	23.53 ± 1.68	14.78 ± 1.86	38.7

Highlights

- PU films were modified grafting HEMA by UV irradiation and Ar low pressure plasma
- This novel functionalization of PUs improved their blood compatibility
- Modification with plasma significantly lowered values of thrombogenicity
- The modified films upkept their intrinsic biocompatibility
- Surface treatments enhanced the bactericidal activity of the materials

Graphical Abstract (for review)

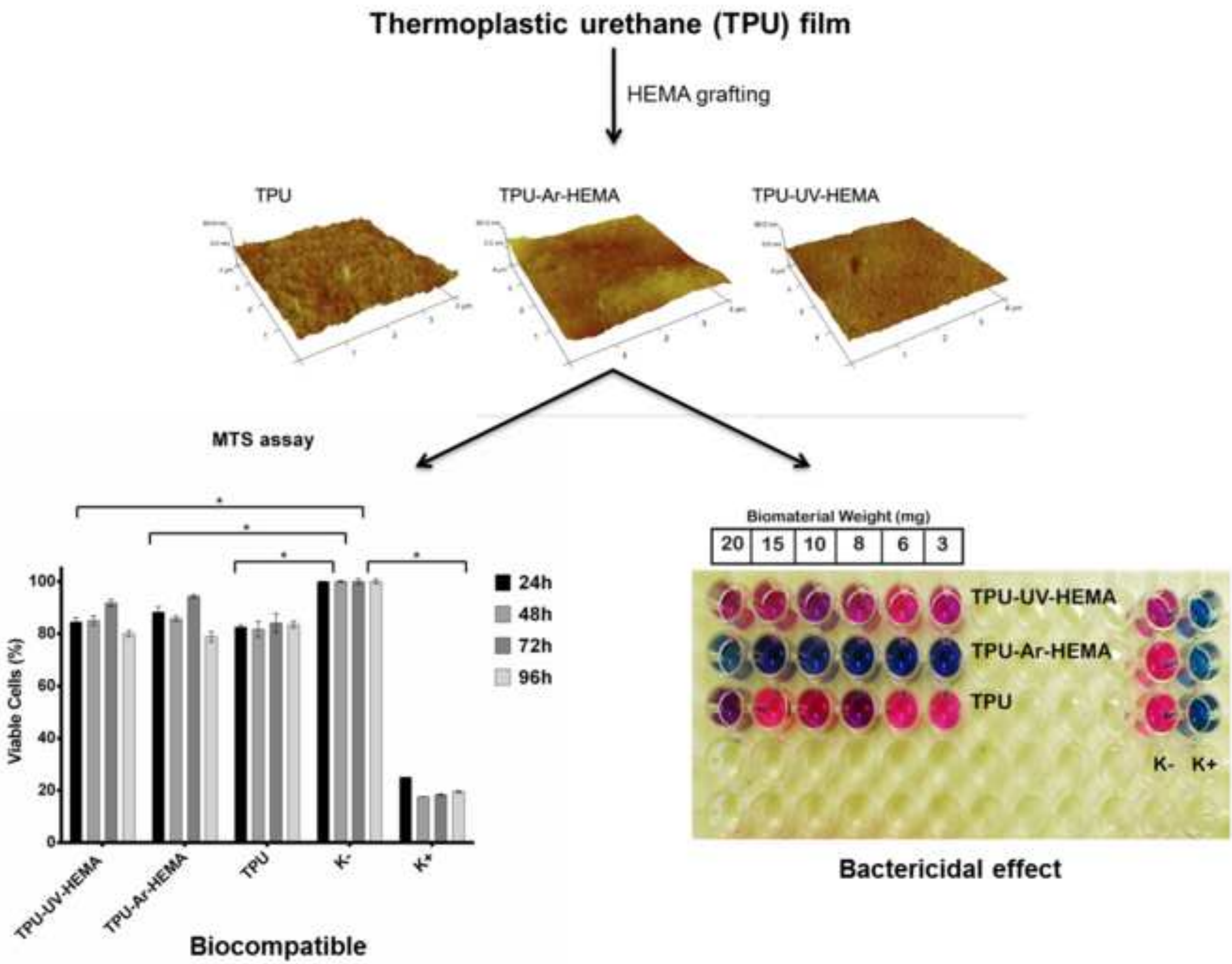


Figure 1

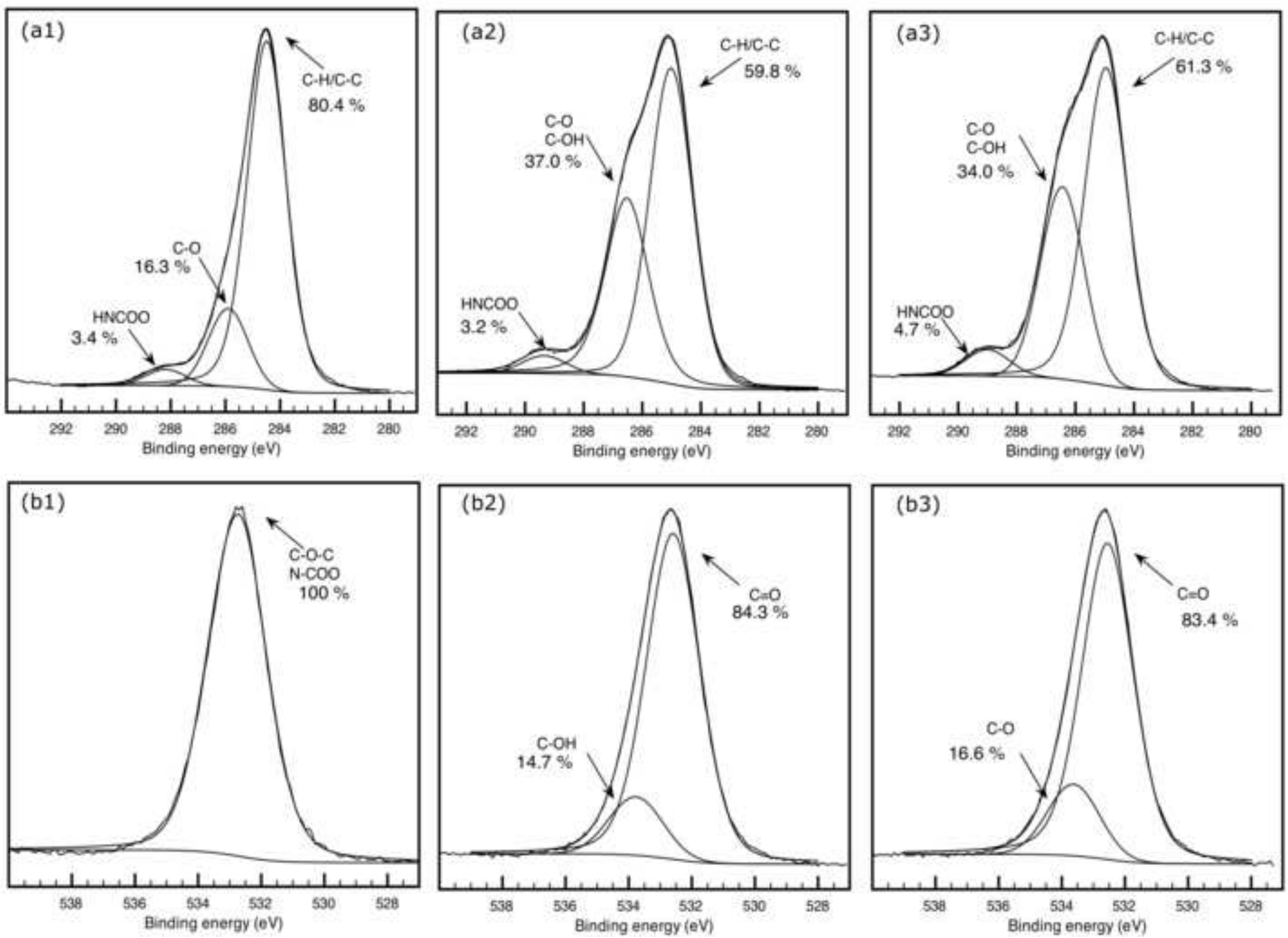

(a1)
C-H/C-C
80.4 %
C-O
16.3 %
HNCOO
3.4 %
292
290
288
286
284
282
280
Binding energy (eV)
(a2)
C-H/C-C
59.8 %
C-O
C-OH
37.0 %
HNCOO
3.2 %
Binding energy (eV)
(a3)
C-H/C-C
61.3 %
C-O
C-OH
34.0 %
HNCOO
4.7 %
Binding energy (eV)
(b1)
C-O-C
N-COO
100 %
538
536
534
532
530
528
Binding energy (eV)
(b2)
C=O
84.3 %
C-OH
14.7 %
Binding energy (eV)
(b3)
C=O
83.4 %
C-O
16.6 %
Binding energy (eV)

Figure 2

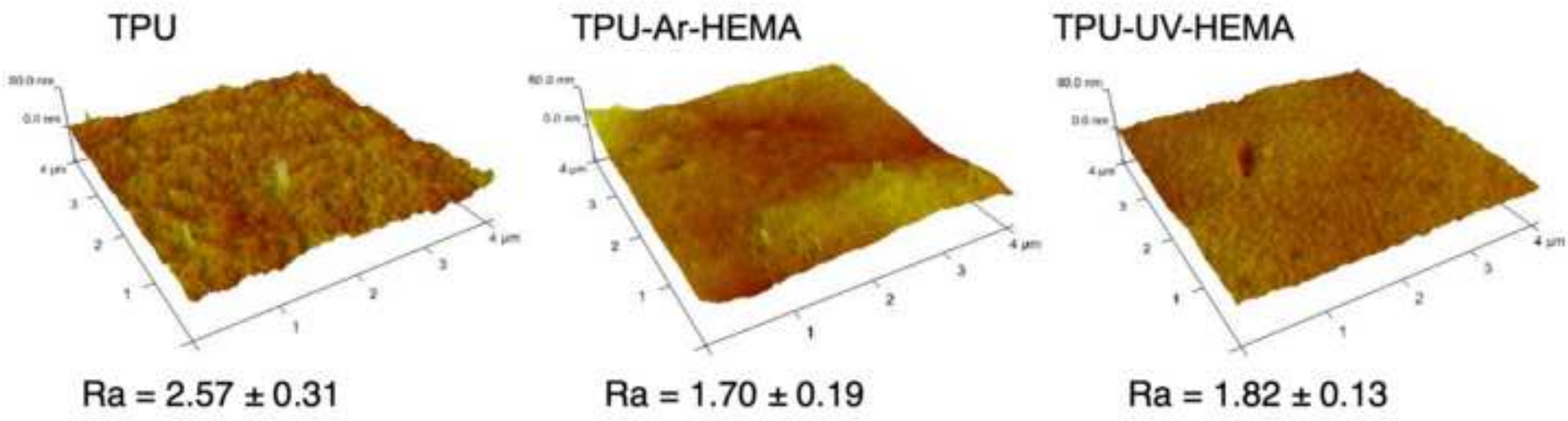
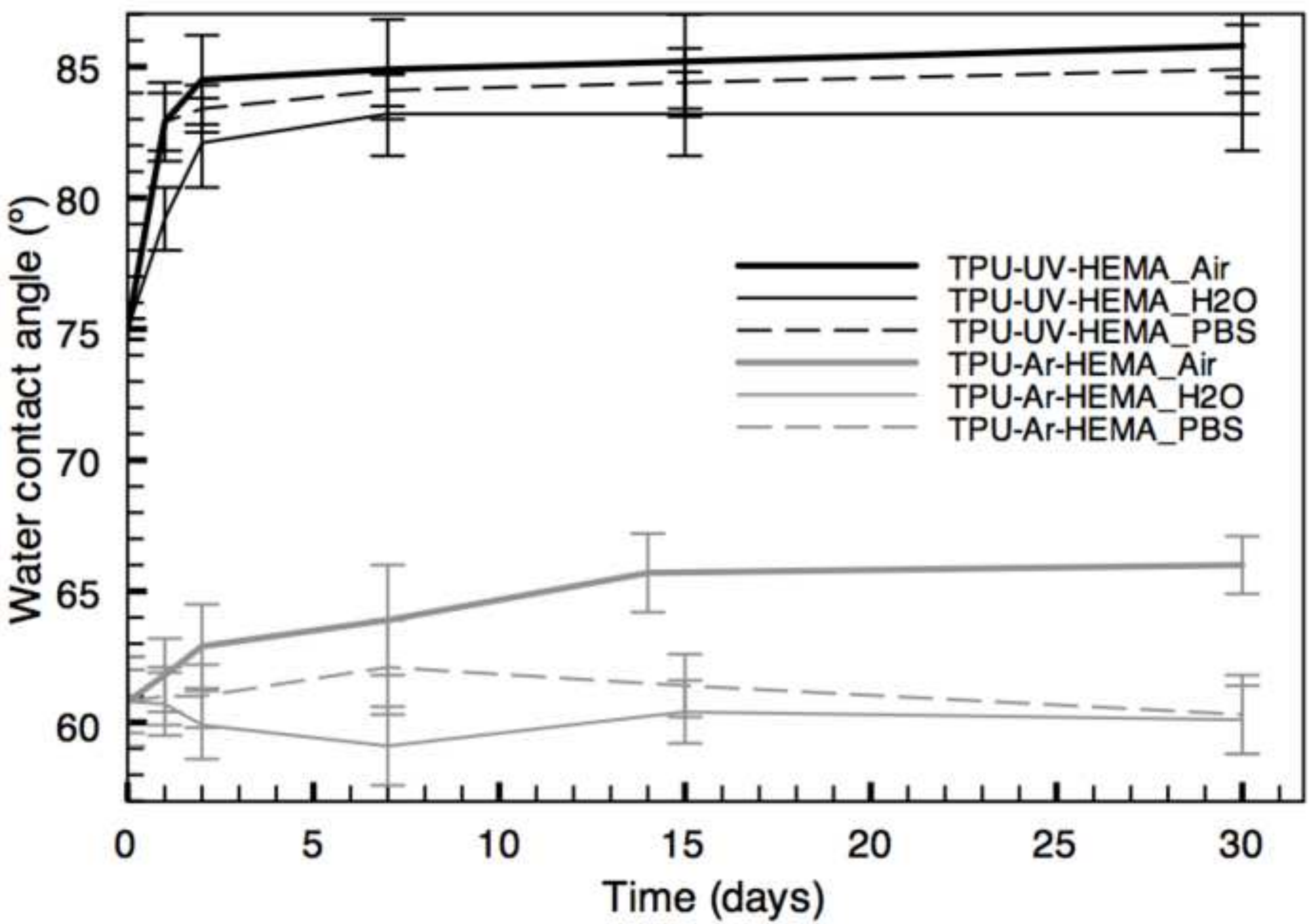


Figure 3



Water contact angle (°)
85
80
75
70
65
60
0
5
10
15
20
25
30
Time (days)
TPU-UV-HEMA_Air
TPU-UV-HEMA_H2O
TPU-UV-HEMA_PBS
TPU-Ar-HEMA_Air
TPU-Ar-HEMA_H2O
TPU-Ar-HEMA_PBS

Figure 4

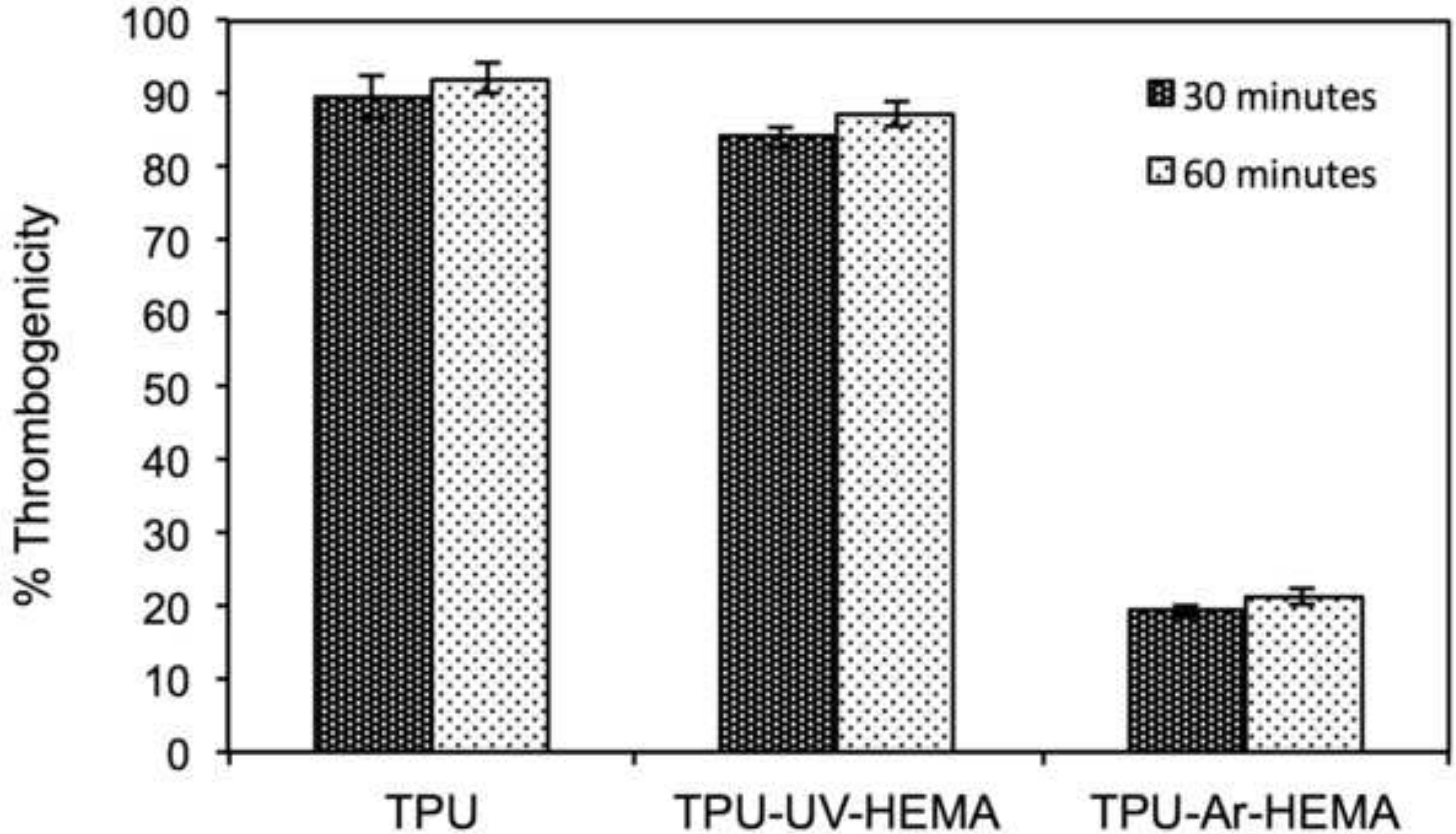

100
90
80
70
60
50
40
30
20
10
0
% Thrombogenicity
30 minutes
60 minutes
TPU
TPU-UV-HEMA
TPU-Ar-HEMA

Figure 5

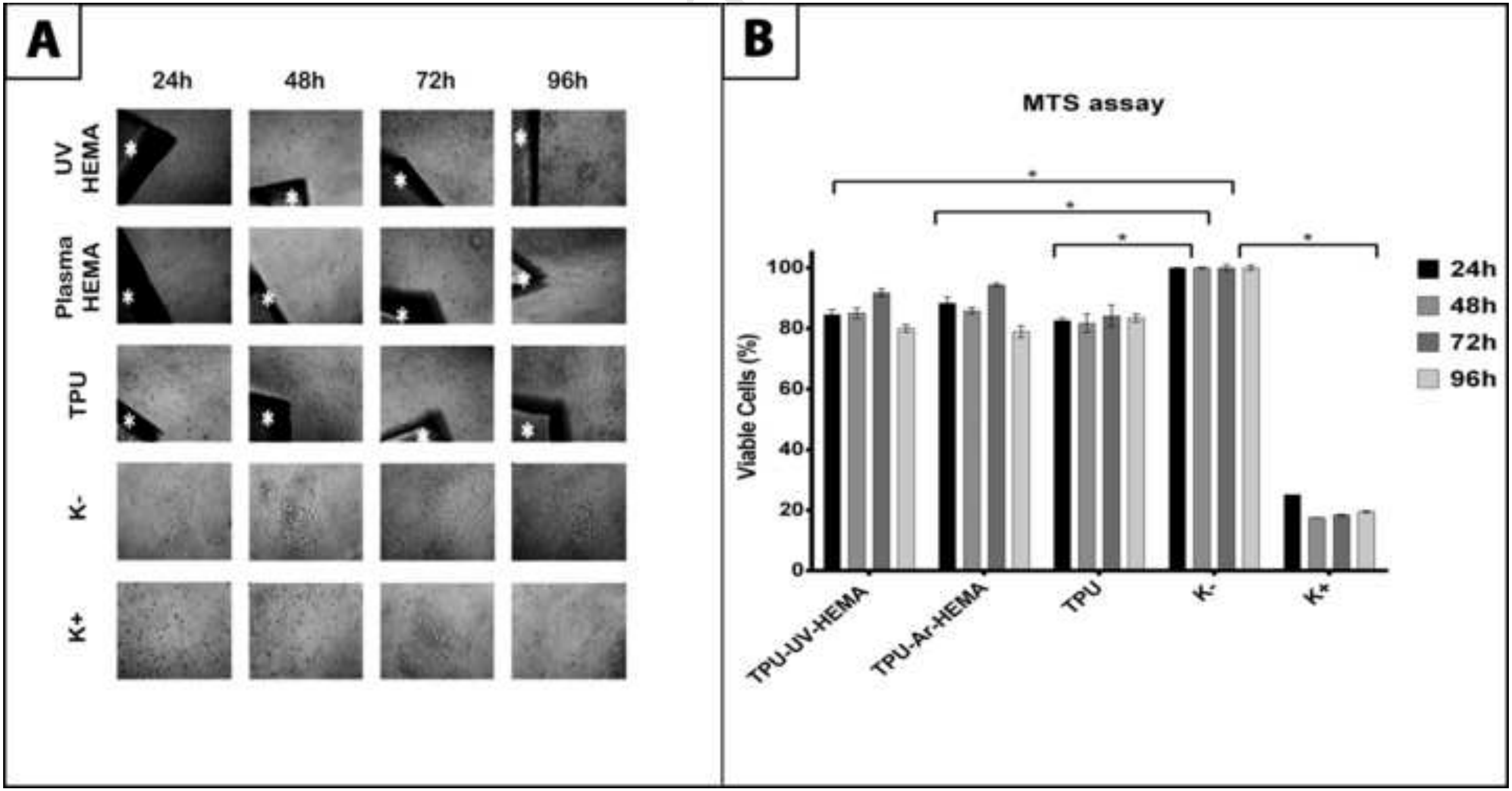
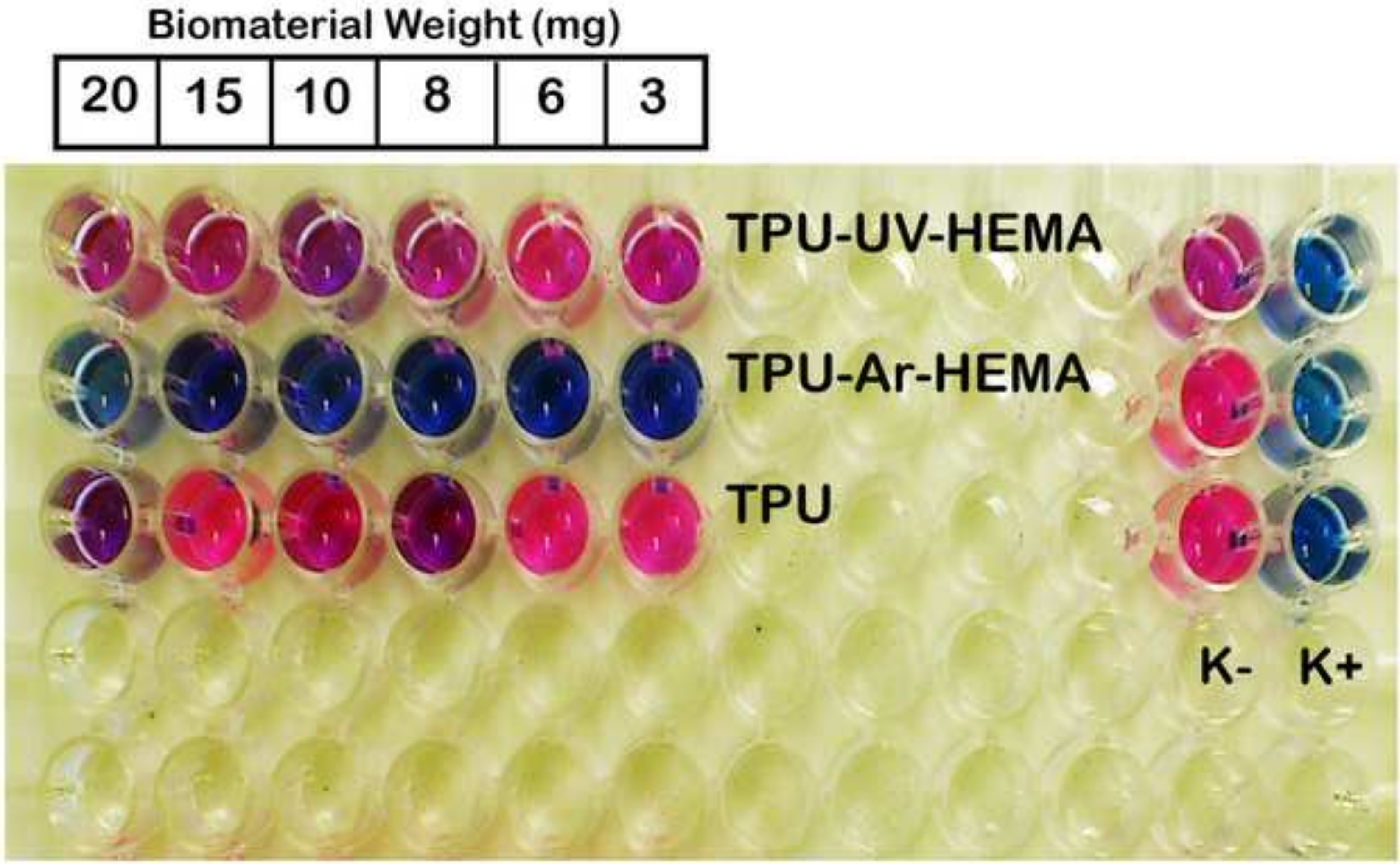

A
24h
48h
72h
96h
UV HEMA
Plasma HEMA
TPU
K-
K+
B
MTS assay
Viable Cells (%)
0
20
40
60
80
100
TPU-UV-HEMA
TPU-Ar-HEMA
TPU
K-
K+
24h
48h
72h
96h

Figure 6


Biomaterial Weight (mg)
20
15
10
8
6
3
TPU-UV-HEMA
TPU-Ar-HEMA
TPU
K-
K+