Abstract. The mechanisms of sterilization and decontamination of surfaces are compared in direct and post discharge plasma treatments in two low-pressure reactors, microwave and inductively coupled plasma. It is shown that the removal of various biomolecules, such as proteins, pyrogens or peptides, can be obtained at high rates and low temperatures in the inductively coupled plasma (ICP) by using Ar/O₂ mixtures. Similar efficiency is obtained for bacterial spores. Analysis of the discharge conditions illustrates the role of ion bombardment associated with O radicals, leading to a fast etching of organic matter. By contrast, the conditions obtained in the post discharge lead to much lower etching rates but also to a chemical modification of pyrogens, leading to their deactivation. The advantages of the two processes are discussed for the application to the practical case of decontamination of medical devices and reduction of hospital infections, illustrating the advantages and drawbacks of the two approaches.
1. Introduction

In the US, approximately 46.5 million surgical procedures and even more invasive medical procedures—including approximately 5 million gastrointestinal endoscopies—are performed each year. Each procedure involves contact between a surgical instrument and a patient’s sterile tissue or mucous membranes. These procedures present a major risk of contamination by the introduction of pathogens during the operation. Failure to properly disinfect or sterilize equipment not only carries a risk associated with the breach of host barriers but also a risk of person-to-person transmission (e.g. hepatitis B virus) and transmission of environmental pathogens (e.g. Pseudomonas aeruginosa).

Multiple studies in different countries have documented a lack of compliance with established guidelines for disinfection and sterilization. Failure to comply with scientifically based guidelines has led to numerous outbreaks. In 2002, the estimated number of Hospital Acquired Infections (HAI) in the US, was approximately 1.7 million. The estimated deaths associated with HAIs in US hospitals were about 10000 [1] at a cost of the order of 5–7 billion dollars. Similar figures were also reported for the UK, France and Italy, with 100000 HAI leading to about 5000 deaths in 2000 for the UK [2], 4000 for France [3] and 5000–7000 in Italy [4].

Although these cases cannot all be directly attributed to contamination present on surgical instruments, it is generally recognized that disinfection and sterilization are essential
for ensuring that medical and surgical instruments do not transmit infectious pathogens to patients. These operations are normally realized by placing the instrument in a mechanical washer/disinfector and subjecting them to a pre-wash at room temperature to prevent blood coagulation and adhesion of proteins. The instruments are then cleaned and sonicated with an alkaline enzymatic detergent, and finally, at high temperature, washed and rinsed. They are then visually inspected after drying and packaged for sterilization (in most cases by autoclaving, but also by use of chemical reagents such as hydrogen peroxide or EtO). These operations normally have to follow strict rules such as the ones described in the norm ISO EN 15883.

However, recent studies made in the UK have shown that visual inspection is not enough to prevent significant quantities of residues to be left on surgical instruments after complete cleaning operations [5]–[8]. In these studies made on real instruments decontaminated in a Sterile Service Unit, all items tested were showing residual contamination, sometimes severe with levels larger than 4 µg mm$^{-2}$ locally. These residues were composed of salts, proteins and undefined organic matter. This observation raises serious concerns since it has been shown that prion protein can accumulate in peripheral and skeletal tissues of patients having the different forms of Creutzfeld Jacob Disease (CJD) and could be potentially transmitted iatrogenically by contaminated medical devices.

Another concern linked to these residues is related to pyrogens coming from both gram-negative (lipopolysaccharides (LPS)) and gram-positive (peptidoglycans or lipoteichoic acids) bacteria. These pyrogens can cause sepsis when in contact with the patient bloodstream and provoke septic shock, which is a major cause of death among hospitalized patients. They are, however, extremely resistant to temperature (e.g. [9, 10]) and difficult to remove by conventional sterilization procedures (e.g. [11]).

Therefore new methods of decontamination and sterilization, which could ensure complete removal of bacteria, pyrogens and proteins, are urgently desired. As we will see, the application of non-equilibrium plasma discharges currently appears as an interesting option. This is mainly due to the capacity of plasma processes to remove organic material with high efficiency, while working at low temperature and using non-toxic gases, thus reducing both environmental impacts and safety risks. These key advantages naturally result in increased interest in the investigations of the plasma interactions with substances of biological origin and microorganisms.

Indeed, it has been shown that many pathogens can be destroyed by employing non-equilibrium plasma discharges (e.g. [12]–[14]) and the nature of plasma interactions with microorganisms or biomolecules has been extensively studied in the past few years, with aim (i) to identify the dominant processes leading to the desired effect, (ii) for treatment optimization and (iii) validation of the process. However, it has to be noted that this is a rather challenging objective: plasma discharges are capable of producing high fluxes of various neutral or ionized active species as well as energetic photons, which all interact with treated biological systems via different mechanisms that contribute to the overall efficiency of the treatment. The exact role of each of these processes naturally depends on many parameters such as e.g. process conditions (pressure, applied power, gas flow and excitation frequency), geometry of the system as well as on the kind of target to be inactivated; this considerably complicates the comparison of the results obtained in different experimental configurations and leads to controversy concerning the mechanisms at play during the process. However, as will be outlined in the following section, recent results allow us to draw certain general conclusions regarding possible strategies of plasma-based sterilization and decontamination of surfaces. Subsequently, some of the aspects
related to the proposed approaches will be discussed on the basis of experiments employing low-pressure plasma discharges and involving various kinds of biological systems, with the objective to demonstrate their advantages as well as limitations and drawbacks.

2. Principles of low-pressure plasma-based sterilization and decontamination

2.1. Bacterial spore sterilization

The main mechanisms of spore destruction or deactivation by plasma discharge have been the object of several reviews (e.g. [14]–[19]) that the reader should refer to for more details. In brief, two main mechanisms can be invoked for spore inactivation or destruction employing low-pressure plasma discharges:

- The first one is related to the inactivation of spores by interaction of the UV photons emitted by the discharge with the DNA (e.g. [20]–[22]). It was found that the maximum efficiency of the UV photons is typically in the wavelength range 200–300 nm [22, 23], and that there exists a minimum dose necessary for preventing DNA repair [24] that leads to sterilization.

- The second mechanism is linked to spore etching and erosion by the radicals and active species produced by the plasma discharge (e.g. atomic oxygen, atomic nitrogen and OH radicals or fluorine atoms [25]–[32]). The etching leads to the direct killing of spores by destruction of their membrane and to the removal of the material that shields the spores from exposure to UV radiation. Both effects result in an acceleration of the sterilization rate.

Nevertheless, it is clear that in common situations, both processes may act simultaneously during the treatment and thus contribute to the killing/deactivation of spores. The rates of the two mechanisms are often markedly different, which leads to the two phases of kinetics reported in the literature (e.g. [12, 15]). Moreover, the significance of these two processes is strongly dependent on the local plasma conditions, and as a consequence, on the position of treated object with respect to the plasma discharge: whereas position in the active plasma favors spore etching promoted by ion bombardment, placing samples in the discharge afterglow increases the relative role of UV radiation since ions are normally absent in these conditions.

However, it must also be emphasized that basing sterilization on the action of UV only has two serious drawbacks: the first one is connected to the distribution of the spores on the surface: in practical cases, the spores might be stacked and mixed with protein residues or biological film. With the UV photons flux decreasing exponentially with the thickness of the film they go through, the time needed for reaching the minimum dose necessary for spore inactivation increases exponentially. This makes sterilization difficult to achieve in practical cases with this approach and therefore some etching that uncovers shielded spores is normally necessary for acceptable treatment durations [14]. The second limitation is that UV photons have little or no effect on other contamination sources such as pyrogens or proteins, which results in a treatment that addresses only a part of the problem.

This is why we deliberately favor the approach where etching contributes significantly to the decontamination treatment. We will see that this approach has the advantage of sterilizing...
the surface even when significant soiling is present, and it also removes the other contaminants such as pyrogens or proteins at low temperatures and with limited treatment durations.

2.2. Destruction of pyrogens

As mentioned before, another surface contamination, overlooked most of the time, is represented by pyrogens. The outer coats of spores and bacteria contain endotoxins (e.g. LPS, peptidoglycans or lipoteichoic acids, etc), which are pyrogenic substances. They act as potent modulators of the human immune system, and their presence in the blood stream leads to physiological events such as fever, swelling or sepsis and, at higher doses, to death [33]. UV radiation in the 200–300 nm range has little effect on such substances, as we have already demonstrated [34]. Moreover, after a plasma treatment based on UV radiation only, the surface treated can have an increased pyrogenic activity due to the endotoxins liberated by the spores, and can consequently provoke an unexpected immunological response [35]. Nevertheless, it has been recently demonstrated that distinct kinds of pyrogenic substances can be inactivated readily by plasma treatment (e.g. [14, 34, 36, 37]).

2.3. Elimination of proteins

Another group of possible contaminants is constituted by protein residues, which might contain pathogens, such as the infectious prions associated with the transmission of the CJD. Unlike the case of bacterial spores, prions do not contain genetic material and UV radiation is ineffective for their destruction. Moreover, prions have been found to be extremely resistant toward the conventional sterilization and decontamination techniques (e.g. [38, 39]) because of their unique and stable secondary and ternary structure that cannot be easily altered.

Although the possibility to remove prions by means of non-equilibrium plasma discharges has already been demonstrated by Baxter et al [40], the studies focusing on their removal mechanisms are rather limited. This is primarily because of the necessity of high containment laboratories due to the biohazard connected with such proteins. Therefore, in order to understand the mechanisms of plasma–prions interactions, the effects of non-equilibrium plasma discharges on non-pathogenic models of proteins were studied (e.g. [14], [41]–[48]) using different methods for monitoring their elimination. The results of these studies reveal general important facts regarding the action of low-pressure plasma discharges on proteins, and show the possibility to remove them by oxygen-containing discharges, which induce their fragmentation and volatilization after oxidation. Nevertheless, there is still a lack of systematic analysis related to the protein removal efficiency under different experimental conditions as well as to the identification of the principal mechanism of their elimination.

The results published in the literature lead us to the conclusion that low pressure plasma processes, operated in conditions leading to high etching rates of organic matter, are the route to pursue for the development of a treatment able to both sterilize and decontaminate surface of medical devices, with a number of caveats that will be discussed at the end of this paper.

In order to get a better insight into the possibilities and limitations of low pressure plasma discharges, two types of processes (direct and post discharge) will be presented and their effect on different types of contaminations will be discussed.
3. Experimental

3.1. Plasma treatments

Two different types of plasma reactors have been used in the present work.

The first type of discharge used is a planar double coil inductively coupled plasma (ICP) source schematically depicted in figure 1. This plasma reactor allows igniting plasma discharges with a power between 100 and 500 W, in a pressure range from 0.1 to 20 Pa and gas flows from 1 to 20 sccm.

The processing chamber has a volume of approximately 5 liters and is evacuated by primary and turbomolecular pumps. The gas mixture used for the discharge is controlled by MKS mass flow controllers. The biological samples were placed 20 mm from the bottom quartz window.

The second experimental set-up used is a microwave plasma reactor schematically depicted in figure 2. It consists of a cylindrical stainless steel vacuum chamber having a volume of approximately 12 liters, equipped with several diagnostics windows and one port for sample introduction. The processing chamber is fed from a gas handling system composed of mass flow controllers attached to the process gas lines and is evacuated by a primary pump and a roots blower. The pressure in the chamber during plasma operation is regulated by an MKS butterfly valve. The plasma discharge is generated at a total gas flow of 100 sccm at a pressure of 16 Pa by a microwave source (excitation frequency 2.45 GHz, applied power 1000 W) introduced into the plasma chamber through a silica window placed at the end of a circular 100 mm waveguide. In contrast to the ICP set-up, the samples to be treated were placed outside the active plasma zone at a distance of 300 mm from the quartz window, i.e. in the near post discharge, where plasma densities are low, as indicated in table 1.
In order to characterize the plasma discharges produced in both reactors, different diagnostics methods were used, namely a Langmuir probe (SmartProbe™; Scientific Systems Ltd) placed at the samples positions, optical emission spectroscopy (Avantes AVS-PC2000 monochromator equipped with a 2048-element linear CCD array) and mass spectrometry (HAL4, Hiden Analytical). Moreover, the substrate temperature during plasma treatment has been determined by IR pyrometry (Raynger MX4, Raytek).

The two reactors differ strongly by the conditions produced and the processes applied. In the microwave (MW) reactor, the samples are placed outside of the active plasma zone and submitted mostly to a flux of neutrals and radicals, while in the discharge in the ICP reactor, they are also subjected to a significant ion bombardment. In other words, although in both reactors comparable fluxes of active species reach the samples, which can be demonstrated for instance by actinometric measurements of O atom density (see figure 3), the fluxes of charged particles to the sample differ enormously, as can be seen from figure 4 and table 1, where the plasma characteristics measured at the samples’ position are given for both reactors.

Moreover, the two reactors cause markedly different heating of the treated samples. For instance, whereas temperature of Si wafer after 1 min of plasma duration in Ar was found to be 180 °C in the ICP reactor, only a slight increase of the substrate temperature was observed in the MW reactor even after 10 min of plasma duration (see figure 5). Such a difference in the heating of the samples could affect significantly the kinetics of the etching of biological
Figure 3. Emission spectra of Ar/O$_2$ 9 : 1 discharges (MW: 1000 W, 100 sccm, 16 Pa; ICP: 300 W, 20 sccm, 10 Pa).

Figure 4. Langmuir probe characteristic measured in Ar discharges at the samples’ position (MW: 1000 W, 100 sccm, 16 Pa; ICP: 300 W, 20 sccm, 10 Pa).

samples or the sterilization process in general (e.g. [49]–[51]). Therefore, in order to limit this side effect, preliminarily tests were undertaken to find out a treatment time allowing us to neglect the heating influence. Based on these experiments performed at a variable treatment duration, it has been found that the onset of the nonlinearity of the etching rate with treatment time, which can be attributed to the temperature effect, starts when the substrate is heated to the temperature of approximately 100 °C, that corresponds to the treatment duration around 30 s for ICP discharge operated at 300 W (see figure 6). Therefore, for all the etching tests performed in the ICP reactor the treatment duration was kept below 30 s (i.e. in the case of longer treatment times, the plasma treatment was performed in several consecutive steps).
Figure 5. Temporal evolution of substrate temperature in Ar and O\textsubscript{2} plasma (MW: 1000 W, 100 sccm, 16 Pa; ICP: 300 W, 20 sccm, 10 Pa).

Figure 6. Etching of BSA for plasma treatment having variable duration (ICP reactor, Ar/O\textsubscript{2} 20:1 mixture, 10 Pa, 300 W). The numbers indicate substrate temperature reached after end of each of the treatment steps.

3.2. Coating of proteins and amino acids

The samples were prepared by spotting an aqueous solutions (0.1% wt) of different selected substances, namely bovine serum albumin (BSA), lysozyme, ubiquitin as representatives of proteins and poly-L-histidine, on one-side polished, cleaned Si wafers. This substrate has been chosen to allow a precise analysis of the decontamination mechanisms, but represents an ideal case as compared with stainless steel substrates that present many surface defects.
after repeated usage. After deposition, the samples were dried overnight in a common flow hood. During the drying of the droplet, the deposit forms a thin central part with a thickness of about 100 nm, surrounded by a thicker ring (coffee ring effect) as can be seen in figure 7 for the BSA sample. Both treated and untreated samples were subsequently examined by various surface diagnostic methods: stylus profilometry (Alpha-step IQ, KLA-Tencor), atomic force microscopy (AFM type Solver P47H, NT-MDT Co.) and ToF-SIM spectroscopy (TOF-SIMS IV, IONTOF). Finally, the samples were visualized by imaging ellipsometry, which is a diagnostic method based on the determination of changes in polarized light upon its reflection from a scanned surface and allowing us to detect deposited material having thickness as low as a few nanometers. Ellipsometry measurements were performed using a variable angle multi-wavelength imaging ellipsometer (EP3, Nanofilm Surface Analysis GmbH) in air at room temperature at a wavelength of 554.3 nm at an angle of incidence of 42° and a field of view of 2000 µm × 2000 µm. A conventional PCSA (Polarizer–Compensator–Sample–Analyzer) null-ellipsometric procedure is used to obtain two-dimensional (2D) maps of ellipsometric Δ and Ψ angles [52]. In the following, the evolution of the Δ and Ψ angles are used as a semi-quantitative indication of the organic layer removal.

3.3. Coatings of bacterial endotoxins

The efficiency of the plasma treatment in terms of etching of pyrogenic biomolecules was studied with LPS, peptidoglycan (PGN) and lipid A, the latter constituting the major pyrogenic component of LPS. The samples were prepared following the same protocol used for deposition of proteins and poly-L-histidine, i.e. by spotting small droplets of 0.1% wt aqueous solution of these substances on polished Si wafers. The untreated samples as well as samples exposed to plasma discharges were examined by the same surface diagnostic methods used for proteins and poly-L-histidine. However, it has to be noted that in contrast to the protein samples, the deposits of bacterial endotoxins were rather spatially inhomogeneous (see figure 8), which made profilometric removal measurements unreliable.

Figure 7. 2D map of ellipsometric angle Ψ of the BSA sample.
In addition, the biological activity of the surface (i.e. its pyrogenicity) was evaluated by the ‘whole blood test’, which measures the production of interleukin IL-1β, induced by the contact between the contaminated surface and blood cells [53, 54]. To create a controlled surface contamination by pyrogens, 24 well plates were incubated with 100 µl of pyrogen solution (LPS, lipid A and PGN diluted in a range of 0.01–10 ng ml$^{-1}$ for LPS and lipid A, and 0.1–10 µg ml$^{-1}$ for PGN). The plates were dried overnight in a flow hood, exposed to the plasma discharge and afterwards tested by the whole blood incubation the next day in order to estimate remaining biological activity of the deposit. The surface biological activity was then evaluated before and after treatment by measuring the IL-1β cytokine release from human whole blood sample placed in contact with the surface treated. An enzyme-linked immunosorbent assay (ELISA) is used to measure the release of IL-1β, which is detected with commercially available antibody pairs (RD systems, Space Import-Export srl.). Detection of the biotinylated antibody is quantified by streptavidin-peroxidase (Biosource, Prodotti Gianni SpA) and its substrate TMB (3,3′,5,5′-tetramethylbenzidine; Sigma). Recombinant cytokines were used as standards (National Institute of Biological Standard and Control (NIBSC), South Mimms). Full details on the procedure can be found in [54].

3.4. Bacterial spores

The effect of plasma discharges on bacterial spores was studied using stainless steel coupons covered with a known number (about 2.5 $\times$ 10$^6$) of Geobacillus stearothermophilus spores (Raven Biological). As can be seen in figure 9, the spores on coupons are not disposed in a monolayer but are stacked and shielded by organic matter, which has a major impact on the treatment efficiency and kinetics.

Moreover, it has to be stressed that since the main aim of the present paper is to evaluate the capability of different discharges to erode or etch biological contamination, the treatment efficiency reported here refers solely to the degree of morphological changes of spores exposed to plasma, which was determined by scanning electron microscopy (SEM LEO 435VP). To the reader interested in further details concerning biological tests, we can recommend extensive literature devoted to this topic [12, 13, 15, 16, 24].
4. Results obtained with the ICP discharge

The main objective of this section is to present a comprehensive study of plasma interaction with biological systems with emphasis to their etching processes. In order to meet this objective, the section is subdivided.

First, the experiments focused on the conditions leading to the fastest reduction of deposited biological matter as well as on the identification of possible mechanisms contributing to this process are presented. In these experiments, the etching rates of BSA selected as a model substance are measured under different operational conditions (discharge mixture composition, power and pressure). These results are then compared with the properties of the discharges determined by in situ plasma diagnostics, which enables estimation of roles of different agents leading to BSA removal.

Subsequently, the operational conditions identified to be the optimal for BSA elimination are used for the plasma processing of other biological samples to test the universality of such treatment on a wider set of different biological materials.

4.1. Model case of BSA

4.1.1. Screening tests. After the plasma treatment with an Ar/O$_2$ ICP discharge, visible modifications of the protein samples were observed indicating gradual removal of the protein deposit. An example of this behavior is given in figure 10, where the ellipsometric 2D maps of the $\Delta$ angles of a BSA sample before and after plasma treatment are presented.

Moreover, the etching rate of the deposit, evaluated by surface profilometry performed on the rings of the BSA spot, was found to be strongly dependent on the gas mixture used. This is demonstrated in figure 11, where the results of screening tests performed in pure argon, and its binary and ternary discharge mixtures with hydrogen, nitrogen and oxygen are summarized. It can be clearly seen that Ar, Ar/H$_2$ and Ar/N$_2$ discharges lead to approximately the same rate of BSA elimination (around 200 nm min$^{-1}$), whereas BSA is removed markedly faster in Ar/O$_2$ plasma at otherwise identical operational conditions (i.e. pressure, power and gas flow). The removal rates obtained in this last case were higher than 600 nm min$^{-1}$, showing importance of oxygen for fast volatilization of proteins. Moreover, it can be seen as well that etching efficiency of Ar/O$_2$ discharge is not enhanced either by nitrogen or by hydrogen addition. This also indicates that other molecular radicals produced in such plasmas, like OH or NO, whose presence has been confirmed by OES (figure 12), do not contribute significantly to the process of BSA elimination.
Figure 10. 2D maps of the $\Delta$ angles of BSA (upper row) and corresponding cross-sections (lower row) before treatment (a) and after 15 s (b) and 120 s (c) of treatment in Ar/O$_2$ 20 : 1 mixture—protein dilution 1 mg ml$^{-1}$, Si wafer.

Figure 11. BSA etching rates—protein dilution 1 mg ml$^{-1}$, Si wafer, plasma treatment 10 Pa, 200 W, 22 sccm.
4.1.2. Ar/O<sub>2</sub> plasma discharge—process optimization. As demonstrated above, the BSA removal rate has been found to be the highest in Ar/O<sub>2</sub> mixture as compared with the discharges sustained in other gas mixtures. In order to identify the optimal treatment conditions, the etching rate was measured as a function of argon/oxygen ratio, pressure and applied RF power.

Regarding the dependence of removal rate on discharge mixture composition, it has been found that the addition of oxygen into argon leads initially to a strong enhancement of the BSA removal rate. Nevertheless, further increase of oxygen fraction in the Ar/O<sub>2</sub> mixture above approximately 10–15% results in a gradual decrease of removal efficiency as depicted in figure 13. Moreover, it can be seen that the pressure dependence of BSA etching rate changes with discharge mixture composition: whereas in argon-dominated mixtures increase of pressure causes increase of etching rate, the opposite trend has been observed in oxygen-rich mixtures.

Finally, the etching rate has been found to rise substantially with the applied RF power. For instance, a power increase from 200 to 300 W resulted in almost double the BSA removal rate in Ar/O<sub>2</sub> 20 : 1.

4.1.3. Ar/O<sub>2</sub> ICP plasma discharge—study of etching mechanism

4.1.3.1. Influence of O atoms. As mentioned above, the capability of ICP discharges to eliminate BSA protein is strongly dependent on the presence of oxygen in the discharge mixture. Therefore, the main mechanism leading to the protein removal could be chemical etching. In order to test this hypothesis, O atom density in Ar/O<sub>2</sub> discharges has been measured by mass
spectroscopy and the obtained results were compared with BSA etching rates. It can be seen in figure 14 that the density of O atoms increases monotonically with rising O₂ content in the discharge mixture in contrast to the etching rate, which shows a well-defined maximum for Ar/O₂ ratio around 9 : 1. This finding therefore suggests that pure chemical etching is not dominant, or at least is not the only mechanism that causes BSA removal.

However, changing the composition of Ar/O₂ discharge mixture not only causes changes in the O atom density, but also in the density of positive ions as will be discussed later in the text. Therefore, to exclude the possible effect of ions, additional experiments were performed at fixed plasma density and variable O atom density, i.e. conditions achieved by simultaneously decreasing the argon over oxygen ratio and increasing the applied RF power. The results of

Figure 13. Etching rate of BSA as a function of Ar/O₂ discharge mixture composition (total gas flow 20 sccm).

Figure 14. O atom density and BSA removal rate in Ar/O₂ (300 W, 10 Pa, 20 sccm).
these experiments revealed that the rate of BSA removal follows concentration of O atoms in the discharge (figure 15), which clearly shows that there exists a correlation between these two quantities.

4.1.3.2. Influence of charged particles. The removal of biological material from surfaces could be attributed to sputtering of the deposit by charged particles. However, this mechanism does not seem to be the major cause of the observed effects: the density of charged particles dramatically decreases with increasing O\textsubscript{2} fraction in the discharge mixture (see figure 16), which is in contrast to the evolution of the etching results. Furthermore, the energies of ions, measured by ion mass spectrometry, are too low to enable effective sputtering of BSA protein (figure 17): based on the preliminary experiments using ion beams with variable ions energy, effective organic material sputtering requires ion energy beyond 150–200 eV [55, 59].

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Analogous to the case of evaluating the effect of O atoms, further experiments were performed at constant O atom density and variable plasma density. Such conditions were achieved by increasing applied RF power at fixed Ar/O₂ mixture composition and pressure, as verified both by mass spectrometry and OES. The results obtained in this set of experiments are summarized in figure 18, which shows that the rate of BSA removal follows plasma density. Since O atom density variations in these experiments are negligible, this result indicates the important contribution of charged particles to the process of protein elimination.

4.1.3.3. Combined effect of O atoms and ions. As demonstrated in the previous sections, the variation of the BSA removal rate with composition of the Ar/O₂ discharge mixture cannot be fully explained either by pure chemical etching connected with atomic oxygen or by sputtering
of the protein by energetic ions: it has been found that atomic oxygen alone does not contribute significantly to the etching of the films, while Ar sputtering is observed only at energies much higher than those measured in our experiments. Nevertheless, it was found that O atoms and ions together contribute to the process and therefore it can be assumed that their synergetic action is behind the observed behavior of the BSA removal rate. One possible reaction scheme combining the effects of O etching and impact of ions is the process of chemical sputtering. In this reaction scheme, the role of ions is a cleavage of bonds in the protein structure. This occurs by the displacement of atoms caused by impact of energetic ions, which leads to the creation of dangling bonds and thus to the rise of radical sites close to the protein surface. Such created sites react with impinging oxygen atoms leading to the formation of volatile products (such as CO, NO and OH) that are indeed observed in the emission spectra in the initial stage of plasma treatment (figure 19). These two sequences have to occur simultaneously because the passivation of an open bond by O species needs to happen faster than recombination in the cascade volume. In other words, cleavage of bonds in the protein structure by ions facilitate surface reaction of O atoms and enhance their natural capability to volatilize organic substances.

Obviously, the efficiency of this process is governed by fluxes of both ionic species and oxygen atoms to the protein surface, and the ratio of ion/atom fluxes need to be high to observe this effect. Whereas O atom density, and thus the flux of O atoms, increases with increasing O$_2$ in the discharge mixture (figure 14), leading to an enhancement of the removal of proteins, the density of charged particles steeply decreases at the same time (figure 16). The latter reduces the creation of defects in the protein film and consequently lowers the number of active reaction sites, thereby limiting the etching rate of the film. These two opposing effects can explain the appearance of a well-defined maximum of the etching rate at the conditions representing an optimal trade-off between the radicals’ production and ion density.

This reaction scheme has also been observed in experiments performed on bacterial spores [56]–[58] and proteins [59] with Ar ions and O neutral beams applied for mimicking plasma effects. These experiments demonstrated the synergetic effect of O radicals and ions on
decontamination of surfaces, further supporting the role of chemical sputtering in the removal of biological films by low pressure plasma discharges.

4.1.4. Detailed characterization of treated samples. In order to better characterize changes of chemical composition and morphology of protein samples, further experiments were performed using atomic force microscopy (AFM) and time-of-flight secondary ion mass spectroscopy (ToF-SIMS) on protein films before and after treatment.

It has been found that the etching of the protein films is not homogenous but accompanied by a strong increase of their roughness (see figure 20) indicating a spatial inhomogeneity of the etching process.

Moreover, a detailed investigation of temporal evolution of root mean square (rms) roughness of BSA samples exposed to plasma discharges revealed three distinct phases (see figure 21):

- Initially the rms roughness increases relatively slightly with the amount of removed material (denoted as phase I).
- After removal of a sufficient quantity of the protein film, a second phase starts, which is characterized by an enhanced increase of the rms roughness with increasing amount of removed BSA.
- Finally, as soon as the amount of removed material reaches a value close to the initial thickness of protein deposit, the surface roughness starts to decrease with further protein removal.

Whereas the transition between phases II and III, observed also in previous study [60], can be easily identified as the point at which underlying Si substrate is locally reached by plasma treatment, the transition between phases I and II remains unclear. This phenomenon has already been observed in other studies, for instance, during the plasma etching of polymer resist, where it was attributed to an amplification of initially random defects created at the surface, related to a local preferential sputtering due to the change of angle of incidence of ions impinging the defects’ surface when the surface topography evolves.

Another possibility can be related to the presence of inorganic compounds or impurities in the protein native structure or preparation (sulphur, sodium, calcium, etc). Plasma discharge in the first phase volatilizes carbon-containing species, but due to the presence of inorganic...
compounds that are distributed in the deposit, certain parts of protein surface will be enriched in such compounds, which are much more difficult to remove. Subsequent plasma action will therefore remove preferably the parts not covered by these inorganic residuals. The delayed onset of the phase II can be—under this hypothesis—caused by time needed for the formation of local sites with high density of non-volatile residuals.

Although another series of dedicated experiments are still necessary for identification of process leading to the observed time evolution of surface roughness of treated samples, the presence and densification of non-organic compounds with treatment time has been confirmed by means of ToF-SIMS analysis. As is demonstrated in figure 22, the organic compounds
4.2. Application of Ar/O\textsubscript{2} plasma for elimination of other biological species

In order to demonstrate the capability of Ar/O\textsubscript{2} ICP plasma discharge optimized for BSA to remove other biological deposits, further experiments were performed using different proteins, as well as poly-L-histidine, lipid A and bacterial spores. To limit the heating of the samples during their plasma treatment, the applied RF power was decreased to 200 W.

Regarding the plasma etching efficiency of different proteins and poly-L-histidine, i.e. one elemental building blocks of proteins, we observe only relatively small differences in terms of their etching rates as depicted in figure 23. However, these differences can be caused by variable presence of inorganic compounds—for instance poly-L-histidine solution was prepared from HCl-containing powders—whose presence could influence the removal efficiency. Moreover, it should be noted that a recent study performed on different homopolymers of amino acids revealed that their etching rates are very similar in spite of their distinct chemical properties [61]. This finding, i.e. similarity of the etching rates of various biomolecules, supports the proposed mechanism of chemical sputtering. For the initiation of the destruction of biomolecules, active sites have to be formed on their surfaces. In the case of pure chemical etching in oxygen containing mixtures, this is achieved by hydrogen abstraction from the polymeric structure, which is a process highly dependent on the exact chemical structure of treated substance. In contrast, in the case of chemical sputtering, the active sites are generated by bond cleavage caused by impact of energetic ions, which is rather insensitive to the chemical composition of treated biomolecules in this energy range. This in turn makes the exact chemical structure of treated substance irrelevant for resulting etch rate as observed in our experiments.

As mentioned above, the samples of bacterial endotoxins were highly spatially inhomogeneous and therefore it was not possible to estimate their removal rate directly.
However, imaging ellipsometry showed that similarly to the previous cases, lipid A and PGN can be readily removed from the surface by Ar/O₂ plasma as can be seen in figures 24 and 25, which show 2D maps of ellipsometric angles Δ of untreated and plasma treated samples together with their typical cross-sections.

Finally, it has been found that bacterial spores can also be significantly eroded by Ar/O₂ plasma discharge as demonstrated in figure 26. This finding clearly demonstrates, contrary to the statements made previously [62], that etching can significantly contribute to the sterilization process; etching can explain fast inactivation of bacterial spores in those plasma discharges where only limited intensity of UV radiation is produced.

Nevertheless, our results also revealed a significant difference between plasma treatment of biomolecules and spores: namely temporal nonlinearity of the etching rate and the much lower values obtained in the case of spores (see figure 27). This is most likely due to much higher fraction of inorganic compounds in the spores’ outer walls than in the protein samples, which reduces the efficiency of the process.

4.3. Concluding remarks

It has been demonstrated that the ICP plasma discharge is capable of effectively removing or eroding a wide range of biomolecules having distinctly different chemical structures as well as bacterial spores representing highly resistant types of micro-organisms. Regarding the rates of
these processes, their highest values can be achieved in an Ar/O\textsubscript{2} discharge mixture. It has been shown that this is a result of not only high fluxes of O atoms reaching the treated samples but also by the action of ions bombarding their surfaces and thus promoting creation of surface active sites. Moreover, it should be noted that this discharge mixture was already found to inactivate effectively bacterial spores (e.g. 6 log reduction of surviving spores was reported to be achieved within 40 s of plasma treatment under similar operational conditions [61]). Therefore, the plasma treatment based on the Ar/O\textsubscript{2} discharge mixture has all the pre-conditions to be a highly universal sterilization and decontamination method.

In spite of these highly encouraging results, certain drawbacks of the process based solely on extensive etching of pathogens have to be mentioned.

Firstly, it has been demonstrated that Ar/O\textsubscript{2} plasma is not well suited for volatilization of inorganic compounds. This results in severe implications for application of this approach in a real situation, when the pathogens are for instance mixed with salts originating from body fluids or contain such compounds per se, as in the case for bacterial spores. The markedly lower etching rates connected with presence of inorganic compounds implies the necessity of longer treatment duration that consequently represents risk of degradation of treated objects by their extensive heating. The effect of heating can be significantly reduced either by pulsing the discharge, or—as suggested recently for the case of bacterial spores—by application of ternary Ar/O\textsubscript{2}/N\textsubscript{2} [63, 64], or Ar/NO [65] mixtures providing not only high etching rates but also intense UV radiation capable of inactivating spores effectively. However, the persistence of

Figure 25. 2D maps of ellipsometric angle $\Delta$ and corresponding cross-sections of PGN sample (a) untreated (b) treated 10 s and (c) sample treated 270 s in Ar/O\textsubscript{2} 20 : 1 plasma (10 Pa, 200 W).
Figure 26. SEM images of (a) untreated *G. stearothermophilus* spores and spores treated for (b) 30 s (c) 60 s and (d) 120 s (Ar/O₂ 20 : 1, 10 Pa, 200 W).

Figure 27. Comparison of etching of BSA and *G. stearothermophilus* spores (Ar/O₂ 20 : 1, 10 Pa, 200 W).
remaining inorganic material, whose relevance for the safety of medical instruments still needs to be investigated, seems to be the limiting factor of the process based on oxygen-containing mixtures.

Secondly, the conditions suitable for effective etching of biological samples can cause etching also of surfaces of polymer-based instruments, or at least could induce significant alterations of their chemical and physical properties. For instance, recent comparison of the effect of different sterilization methods on polyethylene glycol coatings revealed that their exposure to plasma leads to a much faster decrease of their non-fouling character as compared with traditional sterilization techniques [66]. Therefore, other approaches have to be developed to limit these undesirable effects.

5. Results obtained with the MW discharge

As mentioned above, basing sterilization and decontamination of surfaces solely of etching has certain drawbacks, connected mainly with undesirable etching of polymeric substrates. Moreover, it has been demonstrated that for fast etching of biomolecules, high flux of ions on their surfaces is necessary. Therefore in order to reduce the possible impact of plasma processes on the treated objects, further experiments were performed using a MW plasma reactor with samples placed downstream of the active discharge.

5.1. Etching of proteins

As expected, placing samples outside the active plasma zone has a strong impact on the etching of biomolecules: whereas in the case of the ICP reactor the BSA etching rates were typically higher than 200 nm min$^{-1}$, the values obtained in the case of a post discharge were not greater than several nanometers per minute as can be seen in figure 28, which compares BSA etching rates in different gas mixtures.
Moreover, the optimal discharge mixture in terms of BSA removal differs for the two employed plasma discharges: whereas in the case of ICP discharge, the fastest BSA removal was achieved in Ar/O₂ mixture having low fraction of oxygen, the discharge offering highest efficiency of removal of BSA in the MW reactor is the one sustained in an O₂/H₂ mixture. However, unlike the case of ICP discharge, the increased etching rate after hydrogen addition into oxygen discharge cannot be explained by chemical sputtering, since both O atom density and ion density decrease with increasing amounts of hydrogen as can be seen in figure 29. Therefore, the observed tendency has to be explained purely by action of chemically active radicals produced under such conditions. In order to volatilize organic compounds, active sites have to be created on the deposit surface first, which can be done either by impact of ions as described in the previous section, or by abstraction of hydrogen from the polymeric structure of treated material. This can proceed by chemical reaction with atomic oxygen, but also by reaction with H atoms or highly reactive OH radicals, both effectively produced in O₂/H₂ plasma discharge (see figure 30), which can in turn explain the observed results.

5.2. Bacterial endotoxins

Similarly to BSA protein, the fastest removal rate of bacterial endotoxins can be achieved in the MW reactor in O₂/H₂ mixture as reported previously [14]. However, the rate of this process is again markedly lower than that obtained in the ICP reactor. Nevertheless, biological tests performed on untreated and treated samples showed significant decrease of their bio-activity after their exposure to the plasma discharge. Concretely, as can be seen in figure 31, the bioactivity of 1 ng of coated LPS decreased after 5 min of the treatment to the value lower than that corresponding to the pyrogenicity of 0.001 ng of the initially deposited material. In other words, 5 min of plasma treatment is sufficient to cause decrease of LPS bioactivity corresponding to more than 3 log lower than initial contamination.

Similar depyrogenation efficiency was observed for other hydrogen containing discharge mixtures (see figure 32) that offers even markedly lower etching rates of pyrogens than that
obtained in O$_2$/H$_2$ mixture [14]. This is a finding of high importance, since it clearly shows that depyrogenation of surfaces can be reached not only by physico-chemical elimination of pyrogenic substances from surfaces as in the case of ICP discharge but also by alteration of their chemical structure.

In order to evaluate the chemical changes leading to the suppression of the pyrogens’ bioactivity, ToF-SIMS analysis was performed on treated and untreated Lipid A, i.e. pyrogenic center of LPS. As can be seen in figure 33 and in detail in figure 34, treatment in hydrogen containing mixtures cause significant changes in the ToF-SIMS spectra, namely rapid decrease.

**Figure 30.** Optical emission spectra of O$_2$/H$_2$ 50:50 MW discharge (1000 W, 13 Pa, 100 sccm).

**Figure 31.** LPS pyrogenicity before and after its treatment in O$_2$/H$_2$ 50:50 MW discharge (1000 W, 13 Pa, 100 sccm).
of the peaks originating from the fatty acid chains (e.g. C_{12}H_{23}O_2 and C_{14}H_{27}O_2) as well as substantial alterations of phosphoryl groups (PO, PO_2, PO_3 and PH_2O_4), i.e. two parts of lipid A structure suggested to govern its bioactivity.

However, the mechanism leading to such modification is still not clear, and it should be noted that a similar effect has been observed after irradiation of lipid A samples by intense VUV radiation emitted by a deuterium lamp having the peak of radiation intensity at 162 nm (see figure 35), which shows that VUV radiation removes peaks corresponding to the whole lipid A molecule as well as its major fragments. Therefore, one possible explanation refers to the degradation of lipid A induced by intense VUV radiation emitted by hydrogen-containing plasma discharges.

5.3. Concluding remarks

As was demonstrated on the example of the MW reactor, placing samples to the plasma afterglow reduces drastically the removal rate of the biomolecules. This effect is produced predominantly by the significant decrease of ion flux reaching surface of the samples, which in turn leads to the lowering of the importance of chemical sputtering that plays a major role in the active discharge. However, this effect can be seen from two different points of view, depending on the particular application. It is obvious that lowering etching rates of organic materials slows down the efficiency of the sterilization and decontamination process and thus represents a clear disadvantage as compared with the treatment done in the active plasma zone. In contrast, this approach lowers adverse impact of the treatment on the integrity and properties of processed instruments. Moreover, it has been demonstrated as well that even under conditions leading to almost negligible physico-chemical removal of biomolecules from surfaces, it is possible to find out conditions leading to the fast decrease of their bioactivity. Nevertheless, concerning this issue, it is important to stress that optimal conditions for inactivation of biological pathogens are strongly dependent on their nature. As an example of the selectivity of plasma post-discharge,
Figure 33. ToF-SIMS spectra of untreated lipid A (a) and lipid A treated 10 s in (b) Ar/H$_2$ 50:50 and (c) O$_2$/H$_2$ 50:50 MW discharges (1000 W, 13 Pa, 100 sccm).

the inactivation of bacterial spores requires high intensity of UV radiation that cannot be achieved in hydrogen-based mixtures found to be advantageous for depyrogenation of bacterial endotoxins. Therefore, the process based on the inactivation lacks universality of the process based on the extensive etching of biological systems. Moreover, it is still not clear whether chemical inactivation is possible for all types of pathogens.

6. Conclusions

While the use of plasma discharges for the sterilization of bacterial spores has been studied in detail in the past few years, relatively fewer results have been published concerning the removal of proteins and pyrogens from surgical instruments. Recent studies revealed, however, that the limited effect of the present decontamination and sterilization procedures pose a real public health problem. Therefore, the possibility to eliminate such biomolecules by two types of low-pressure plasma treatment was addressed in this study.

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Figure 34. Part of ToF-SIMS spectra of untreated lipid A and lipid A treated 10 s in different MW discharges (1000 W, 13 Pa, 100 sccm).

Figure 35. (a) Geometry of grid placed on the lipid A sample and corresponding 2D chemical maps of samples irradiated by VUV lamp, (b) whole lipid A image, (c) C$_{12}$H$_{23}$O$_2$ peaks and (d) C$_{14}$H$_{27}$O$_2$ peak (the squares correspond to lipid A exposed to VUV and destroyed by the radiation).

It was demonstrated, using model proteins and peptides, that biomolecules having distinctly different properties can be efficiently removed by directly exposing the surface to be treated to low pressure ICP discharges. Analysis of various discharges shows that Ar/O$_2$ mixtures are the most efficient for a fast removal of these residues by a mechanism related to chemical sputtering involving both radicals and ions. This mechanism also intervenes for bacterial spores that are inactivated and destroyed by etching as well, which is advantageous.
mainly with respect to the universality of this approach. Nevertheless, the etching of biomolecules could be accompanied by etching of substrate materials.

Different conclusions can be drawn concerning treatment performed in a post discharge. Although in this case it is still possible to sterilize bacterial spores laying on a surface by the UV radiation emitted in post-discharge or inactivate pyrogens by the chemical reaction of radicals with their surfaces, the protein removal rates observed in a post discharge are much lower as compared with a direct plasma treatment. This result illustrates the problem related to the treatment of real surgical instrument surfaces: since the practical experience obtained from hospitals shows that protein residues can be found on a significant proportion of surgical tools after the decontamination, the strategy relying on a post discharge treatment can be questioned, since no assurance can be given that these residues will be harmless after treatment. These findings have a serious implication on the type of material that can be treated without serious deleterious effects.

However, our results have to be confirmed by experiments on more relevant types of contamination (involving inorganic compounds, lipids, blood, etc), since complex matrices can significantly reduce the etching rates obtained on pure proteins.

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