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Surface spectroscopy of adsorbed proteins: input of data treatment by principal component analysis

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Abstract X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToF–SIMS), two surface-sensitive spectroscopic methods, are commonly used to characterize adsorbed protein layers. Principal component analysis (PCA) is a statistical method which aims at reducing the number of variables in complex sets of data while retaining most of the original information. The aim of this paper is to review work carried out in our group regarding the use of PCA with a view to facilitate and deepen the interpretation of ToF–SIMS or XPS spectra acquired on adsorbed protein layers. ToF–SIMS data acquired on polycarbonate membranes after albumin and, or insulin adsorption were treated with PCA. The results reveal the preferential exposure of particular amino acids at the outermost surface depending on the adsorption conditions (nature of the substrate and of the proteins involved, concentration in solution), giving insight into the adsorption mechanisms. PCA was applied on XPS data collected on three different substrates after albumin or fibrinogen adsorption, followed in some cases by a cleaning procedure with oxidizing agents. The results allow samples to be classified according to the nature of the substrate and to the adsorbed amount and, or the level of surface coverage by the protein. Chemical shifts of particular interest are also identified, which may facilitate further peak decomposition. It is useful to recall that the outcome of PCA strongly depends on data selection and normalisation.

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1 Introduction

When a biomaterial is placed in contact with a biological environment, its surface is immediately modified through the adsorption of biomacromolecules. In particular, proteins tend to accumulate in the interfacial layer owing to their polyamphiphilic and polyampholytic nature [1]. Adsorbed proteins provide recognition signals for microorganisms and mammalian cells. On the one hand, biofilm formation, resulting from the attachment and proliferation of bacteria on solid surfaces, is at the origin of serious difficulties to treat biomedical device-related infections. It is established that biofilm formation strongly depends on the properties of the protein layer adsorbed at the biomaterial surface [2]. On the other hand, depending on the application envisioned for a given biomaterial, adhesion of mammalian cells at its surface may be desired (e.g., bone prosthesis) or unwanted (e.g., intraocular lens). Again, it is well-known that cells interact with adsorbed proteins through cell-membrane receptors called integrins. This interaction activates multiple signalling pathways, thereby regulating cell growth and differentiation [3]. Controlling protein adsorption is thus a key issue in biomaterials science.

The development of surface-sensitive methods with a view to elucidate the nature, organization and properties of adsorbed proteins is therefore needed. X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToF–SIMS) are both commonly used for the characterization of adsorbed layers. In XPS, X-ray irradiation of the sample surface provokes the ejection of photoelectrons; their kinetic energy is measured and converted into binding energy. Moreover, chemical shifts are observed depending on the chemical environment of a given element. The spectral data give access to the elemental and functional chemical composition of the surface (probed

depth ~1–10 nm), in a quantitative manner [4]. Proteins can usually be distinguished from the substrate on which they are adsorbed owing to their nitrogen content. However, since all proteins have the same chemical backbone and are built from the same amino acids, XPS spectra of different proteins are quite similar. In ToF–SIMS, exposure of the sample surface to a beam of ions leads to the ejection of secondary ions; their mass/charge (m/z) ratio is measured. The obtained mass spectra give access to the molecular composition of the surface (probed depth ~1 nm), in a semi-quantitative manner. Given the possibility to distinguish molecular fragments originating from the different amino acids and the very low probed depth, ToF–SIMS is in principle sensitive to the nature and the orientation/conformation of adsorbed proteins [5].

These two spectroscopic methods generate complex sets of data. For each analyzed sample, spectral data consist in a series of intensities recorded for each increment of binding energy in XPS or m/z ratio in ToF–SIMS. A set of data can be represented as a matrix of m rows corresponding to the individuals, i.e., the samples, for which the recorded intensities are placed in n columns corresponding to the variables, i.e., the increments of binding energies or of m/z ratio. Such a system cannot easily be represented and analyzed due to the high number of variables involved. Statistical methods such as principal component analysis (PCA) can be used to facilitate the extraction of information from the spectra. The aim of PCA is to reduce the number of variables, in order to obtain a representation of the system in a lower dimensional space, while retaining most of the information. The p new variables or new axes of the data space ($p < n$), called principal components (PC), are linear combinations of the n original ones. The weight of each original variable in the PC, which increases with the associated variance, is called "loading", while the coordinates of each individual in the new space defined by the PC are called "scores" [6]. The principle of PCA is illustrated in Fig. 1. Previous work has demonstrated the relevance of PCA to treat ToF–SIMS data [7], and in particular to treat ToF–SIMS spectra of adsorbed protein films [8]. Application of PCA to XPS data has up to now been mainly centred on noise reduction [9] and on XPS image treatment [10].

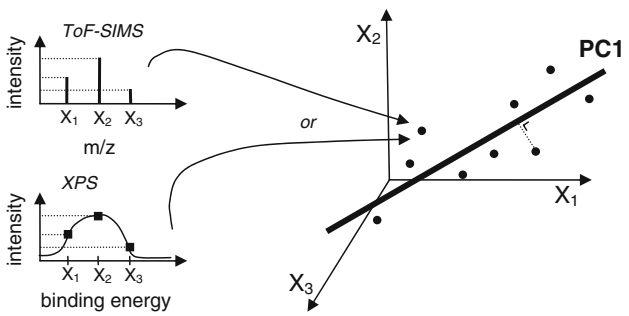

Fig. 1 Illustration of the principle of PCA applied to ToF–SIMS or XPS data. Each data point in the three-dimensional space defined by variables X_1, X_2 and X_3 represents a single ToF–SIMS or XPS spectrum (here reduced to three increments of m/z ratio or binding energy, respectively) obtained on a given sample. PC1 is defined by a linear combination of X_1, X_2 and X_3. The projection of each data point on PC1 allows the PC1 score of that sample to be obtained

The aim of this paper is to review work performed in our group, which highlights the ability of PCA to facilitate and deepen the interpretation of ToF–SIMS or XPS data, in particular regarding the characterization of adsorbed protein layers.

2 Materials and methods

Insulino-dependent diabetes affects more than 50 millions persons worldwide. The existing therapy by insulin administration fails to reproduce the physiological secretion pattern, essential to maintain the glycaemia at equilibrium. Therefore, research efforts are now concentrating on the transplantation of pancreatic islets. Strategies must be elaborated to reduce the immune response elicited by the transplanted islets. Our group has been involved in a project aiming at encapsulating Langerhans islets within artificial membranes allowing glucose and insulin diffusion but preventing the passage of immune system components. In this context, ToF–SIMS spectra were acquired on native or surface-modified polycarbonate (PCarb) membranes submitted to albumin and, or insulin adsorption (15 µm-thick membranes with 2.10^9 pores cm^{-2} and pore diameter of 30 nm (Lexan, General Electric); human serum albumin and human insulin were purchased from Sigma; more experimental details can be found in Ref. [11, 12]). Surface modification of PCarb consisted in plasma treatment in argon followed by dipping in a solution of poly(*N*-vinylpyrrolidone) (PVP; Kollidon from BASF; 1 wt%), resulting in an enhanced hydrophilicity. Albumin adsorption on PCarb and PCarb–PVP was monitored as a function of albumin concentration in solution (from 0 to 2 mg/ml) [11]. Albumin and insulin adsorption were further investigated on PCarb–PVP, as a function of protein concentration. Single-protein adsorption was performed with a concentration range of 0–2 mg/ml for albumin and 0–1 mg/ml for insulin, while adsorption in competition was examined with mixed solutions containing albumin at 1 mg/ml and an insulin concentration varying from 0 to 1 mg/ml [12]. Positive ToF–SIMS spectra were acquired with a Phi-Evans TFS-4000MMI (TRIFT 1) spectrometer equipped with a pulsed 15 keV gallium ion beam, in static analysis conditions. Selection of the data to be included in the matrix submitted to PCA was performed as follows. Based on previous work and on the knowledge of the substrates, peaks attributed to the adsorbed proteins were

identified. For each of these peaks, the intensity was computed as being the area of the peak measured between a lower and upper m/z ratio; these limits were set manually and were kept identical for all spectra in a given series. The intensities were further normalized to the sum of intensities of the selected peaks. Finally, the data were mean-centred. PCA was performed using a commercial software (Multion, Biophy Research, France).

Hemodialysis is used to treat patients with renal failure. Dialyzer regeneration (performed by cleaning the membranes) and reuse is widely practiced to reduce the treatment cost. It has been observed that membrane regeneration is also beneficial because it decreases complement activation, but the mechanism underlying this effect is not yet clarified. Our group has been involved in a project aiming at understanding the effect of dialysis membrane regeneration on adsorbed proteins. In this context, XPS spectra were acquired on a cellulosic membrane (Cuprophan, hereafter called Cup; purchased from AKZO), as well as on reference materials (glass—12 mm-diameter coverslips from Menzel-Gläzer; polystyrene (PS)—cut from Petri dishes, Merck-Belgolabo), submitted to adsorption of blood plasma proteins (human serum albumin (Sigma) at 35 mg/ml or human plasma fibrinogen (Sigma) at 1.8 mg/ml; these concentrations correspond roughly to those found in human blood). After adsorption and rinsing, some of the samples were further submitted to a cleaning procedure, either in 0.5% NaClO or in 3% Renalin, a commercial solution (containing 20% H_2O_2) used for dialyzer reprocessing (more experimental details can be found in Ref. [13]). XPS spectra were collected using a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminium X-ray source. For each sample, a survey spectrum as well as high resolution spectra of all elements detected on the survey spectrum were acquired. Since PCA is not commonly applied to XPS spectra, home-built routines were developed in Matlab (Mathworks). The effect of data pre-treatment on PCA output was carefully examined (see details in Ref. [14]). Correction of the binding energy scale, which is shifted in reason of the insulating character of the samples, was shown to be particularly important. Indeed, even a small binding energy shift between spectra from the same data set may hinder the identification of a given binding energy as being an important source of variance. A procedure, itself based on PCA, was developed to automatically correct the binding energy scale throughout the entire series of spectra, thereby aligning them in a very accurate manner. Data normalisation is another important issue. The overall signal intensity indeed varies a lot from sample to sample, and constitutes an important but useless source of variance. For each sample, normalisation was performed by dividing the intensity measured on each increment of binding energy by the sum of the intensities of all increments of binding energies in the survey spectrum (i.e., by the total area of the survey spectrum). Selection of the data to be included in the matrix submitted to PCA may include any combination of spectral areas from the survey or high-resolution spectra.

For both spectroscopic methods, it appears that PCA outcome will strongly depend on the choice of the spectral data to be included in the statistical analysis, as well as on the quality of data pre-treatment. A priori knowledge of the analyzed systems will help making appropriate choices with that respect.

3 Results and discussion

3.1 ToF–SIMS analysis of PCarb and PCarb–PVP after albumin and, or insulin adsorption

PCA was performed on a series of ToF–SIMS spectra acquired on PCarb and PCarb–PVP after albumin (0–2 mg/ml) adsorption. The treatment was based on the 34 most intense positive secondary ion fragments attributed to albumin. The first PC (PC1) collects 81% of the total variance. The scores of each sample for PC1 are presented in Fig. 2a, as a function of the I_{alb}/I_{total} ratio, where I_{alb} is the sum of the intensities of the 34 fragments attributed to albumin, while I_{total} is the sum of the intensities of all peaks found in the spectra after subtraction of the hydrogen and contaminants signals. The I_{alb}/I_{total} ratio should reflect the albumin surface coverage, which in turn depends on albumin concentration in solution. This ratio is higher on native PCarb (range of 0.12–0.20) compared to PCarb–PVP (range of 0.06–0.13). On native PCarb, the PC1 scores are positive and remain quite constant whatever the I_{alb}/I_{total} ratio. On PCarb–PVP, PC1 scores are negative at low I_{alb}/I_{total} ratio, and increase to a value close to zero with increasing I_{alb}/I_{total} ratio.

Further interpretation is made possible by examination of the loading plot, presented in Fig. 2b. The higher the absolute value of the loading for a given m/z ratio, the stronger the influence of that given secondary ion fragment in the definition of PC1. Fragments showing a high positive loading value are correlated with samples associated to a high positive value on the score plot (Fig. 2a). Conversely, high negative values on the loading plot and the score plot can be correlated. Fragments with a loading close to zero do not contribute much to the definition of PC1, i.e., they are not a significant source of variance within the dataset. Each fragment can be associated to one or a few corresponding amino acids [11]. On the native PCarb membrane, the positive score recorded for PC1 may be related to the

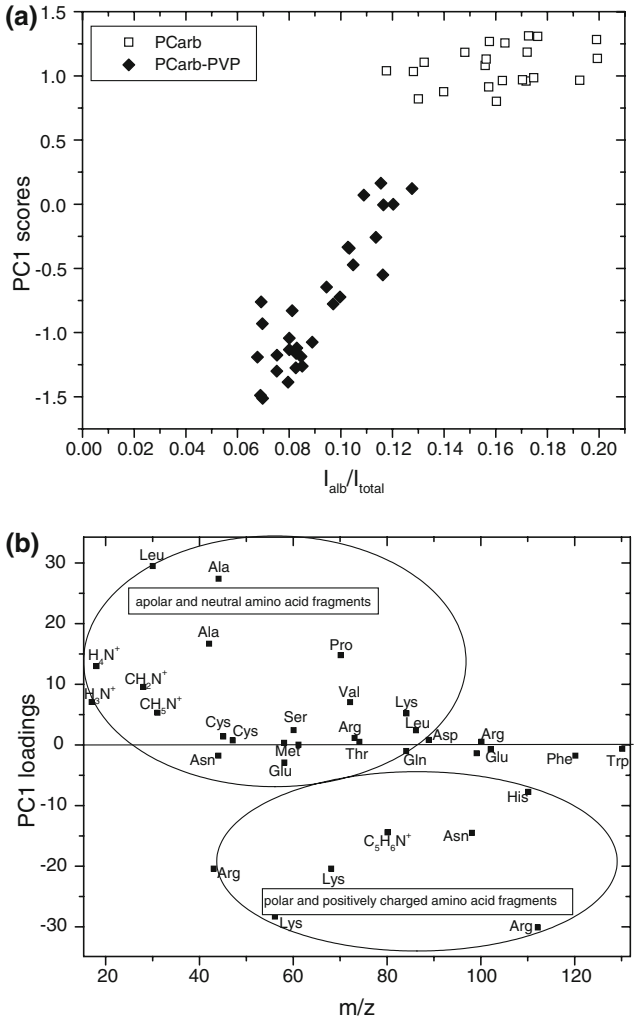

Fig. 2 Results from PCA performed on ToF–SIMS spectra acquired on PCarb and PCarb–PVP membranes after albumin adsorption from solutions at different concentrations (0–2 mg/ml): **a** PC1 scores as a function of I_{alb}/I_{total} (see text for details); **b** PC1 loadings as a function of the m/z ratio of the positive ion fragments. The label near each symbol denotes the amino acid associated with the fragment, or the structure of the fragment when this latter is common to all amino acids. Adapted with permission from Ref. [11]. Copyright 2003 American Chemical Society

exposure of amino acids with apolar side chains (leucine, alanine, proline) at the outermost surface. On PCarb–PVP, at low albumin surface coverage, the negative score recorded for PC1 may be related to the presence at the extreme surface of amino acids with positively charged polar side chains (arginine, lysine). The increase of PC1 scores with I_{alb}/I_{total} may be attributed to a progressively lower exposure of amino acids with positively charged side chains, compensated by a progressively more pronounced exposure of amino acids with apolar side chains.

It must be recalled here that ToF–SIMS analysis are performed in ultra high vacuum. This could lead to significant changes of protein orientation and, or conformation compared to physiological conditions. In particular, preferential exposure of apolar functions is expected.

The results obtained on PCarb could then be explained by such a mechanism. However, the results clearly show that the nature of the amino acids detected at the extreme surface depends on the chemical composition of the substrate (PCarb versus PCarb–PVP) and on the level of albumin surface coverage (represented by I_{alb}/I_{total}). It thus appears that some insight into the adsorption mechanisms may be gained. It can be speculated that, at low surface coverage on PCarb–PVP, interactions between negatively charged albumin residues and the substrate are maximized, leading to a relatively more pronounced exposure of positively charged amino acid side chains at the extreme surface. Positive charges at the PCarb–PVP surface, arising from the plasma treatment, may be responsible for this behaviour. When the concentration of albumin in solution is raised, the surface occupancy increases faster, leaving less time to the adsorbed albumin molecules for such rearrangements.

Albumin adsorption on PCarb–PVP was then investigated in competition with insulin, and compared to the adsorption of each of these proteins alone. PCA was based on a selection of 43 positive secondary ion fragments attributed to proteins. The score plot for PC1 and PC2, which, respectively, collect 43.5 and 19.3% of the total variance, is presented in Fig. 3a. There is a clear separation of the data along PC2 into three groups of samples: negative values are found for single albumin adsorption, positive values are found for single insulin adsorption, and values close to zero are found for adsorption from mixed albumin/insulin solutions. Samples are separated along PC1 in a way which can be correlated with the level of surface coverage by the protein. This surface coverage, which can be estimated from the I_{prot}/I_{total} ratio (I_{prot} is the sum of the intensities of all fragments attributed to albumin and, or insulin), depends on the protein concentration in solution, as shown in Fig. 3b. For insulin, it passes through a maximum around 50 μg/ml. For albumin, it increases with the concentration, in a pronounced manner for low concentrations, then in a more progressive manner for higher concentrations. For mixed albumin/insulin solutions, it remains constant and at a level close to that found for 1000 μg/ml of albumin whatever the insulin concentration. The same trends are observed regarding sample distribution along PC1 in Fig. 3a. Negative and positive PC1 values may then be associated with, respectively, low and high surface coverage levels.

Again, it appears clearly that the combination of ToF–SIMS and PCA brings about information regarding the nature and the orientation and, or conformation of adsorbed proteins. The separation along PC2 of samples with adsorbed albumin, on the one hand, and insulin, on the

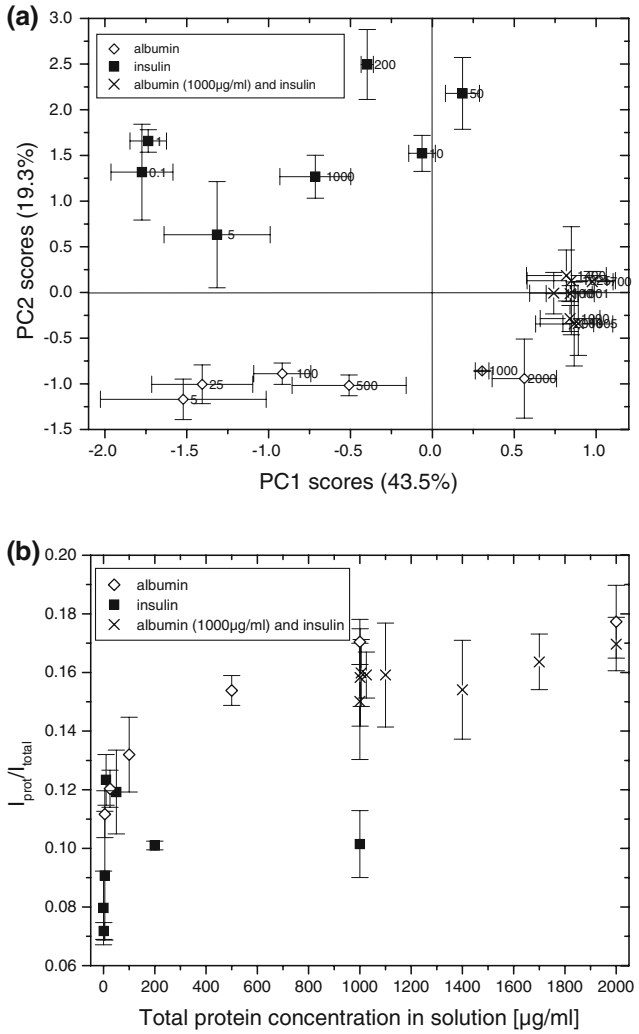

Fig. 3 **a** Score plot (PC1 versus PC2) obtained after PCA performed on ToF–SIMS spectra acquired on PCarb–PVP membranes after adsorption of albumin (◇), insulin (■) or from a mixed albumin/insulin solution (×). The label near each symbol gives the concentration of protein in solution (in µg/ml). **b** Evolution of the I_{prot}/I_{total} ratio (see text for details) as a function of the total protein concentration in solution for the same systems. The error bars show the standard deviation ($n = 6$ for single protein adsorption; $n = 12$ for adsorption in competition). Adapted with permission from Ref. [12]. Copyright 2008 American Chemical Society

other hand, shows that the "fingerprint" (i.e., the most exposed amino acids) of each of these proteins is different. The intermediate position of samples submitted to a mixed albumin/insulin solution shows that both proteins are found in the resulting adsorbed layer. The separation of samples along PC1, which is related to the level of surface coverage by the proteins, cannot be attributed to a variation of the detected protein amount, since the data were normalized by the sum of the intensities of all selected protein peaks before PCA. Therefore, the observed differences are due to the preferential exposure of different amino acids at the extreme surface depending on the surface occupancy by the proteins. This can be related to the fact that relaxation phenomena, inducing conformation changes, depend on the available space at the interface and on the adsorption kinetics.

3.2 XPS analysis of Cup, glass and PS after albumin or fibrinogen adsorption

PCA was performed on a set of XPS spectra acquired on three substrates (Cup, glass and PS) after adsorption of albumin or fibrinogen, followed in some cases by cleaning with either NaClO or Renalin. The statistical treatment was based on a combination of the individual C 1s, O 1s and N 1s peaks. The obtained score plot is shown in Fig. 4a; PC1 and PC2, respectively, collect 68.9 and 20.3% of the total variance. The samples can be separated into three clusters corresponding to the three substrates. Regarding PC1, PS samples show a positive score while values close to zero or negative are observed for Cup and glass samples. Regarding PC2 scores, negative values are found for glass samples, values close to zero or positive are found for Cup, and PS samples are spread from negative to positive values.

Further interpretation may be guided by the loading plots, which are presented on Fig. 4b for each spectral area (C 1s, O 1s, N 1s). The main positive input into PC1 is associated to the C 1s peak component at ~284.8 eV, attributed to carbon atoms bound to carbon or hydrogen (C–(C,H)), as found in PS. The main negative input into PC1 is related to O 1s peak component at ~533 eV, which can be attributed to oxygen involved in an alcohol or acetal function, typical of Cup, as well as to oxygen in SiO_2 (i.e., typical of glass). It thus appears that sample classification along PC1 is driven by the signal originating from the substrates, even when an adsorbed layer is present. On the basis of PC1, glass and Cup samples cannot be distinguished since the chemical shift of oxygen is similar for these two materials.

Three peak components show a significant positive loading for PC2: the C 1s peak components at ~284.8 eV and at ~286.6 eV can be attributed to C–(C,H) and to C–O in Cup, respectively, while the O 1s peak component located at ~533 eV is attributed to either Cup or glass (see here above). One peak component bears a significant negative loading; it is located at ~531.4 eV. This latter component is attributed to oxygen atoms involved in the amide bond (O=C–N) typical of proteins. Note that the loadings corresponding to the N 1s peak are very low and thus not significant, for both PC1 and PC2. Sample classification along PC2 (see score plot on Fig. 4a) could then be explained as follows. On the one hand, more negative values would correspond to samples with a high signal originating from adsorbed proteins. Accordingly, within

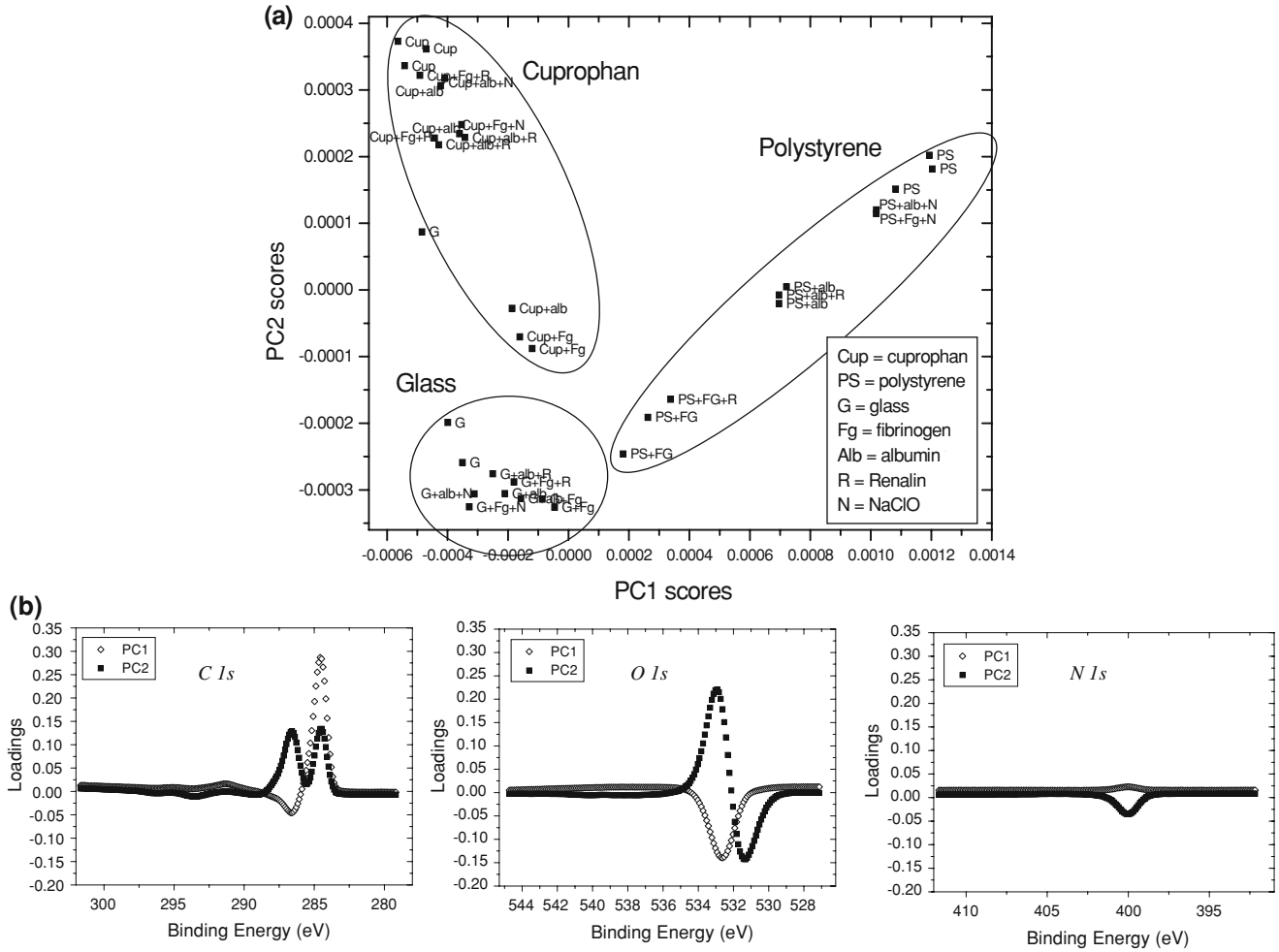

Fig. 4 Results from PCA performed on XPS spectra obtained on three substrates (Cup, PS and glass) as such or after adsorption of albumin or fibrinogen, followed in some cases by cleaning with either Renalin or NaClO. **a** Score plot (PC1 versus PC2). The label near each symbol summarizes the treatment to which the sample was submitted (see abbreviation list on the Figure). **b** Loading plots related to PC1 (◇) and PC2 (■) for different spectral areas: C 1s (*left*), O 1s (*center*), N 1s (*right*)

each cluster corresponding to a given substrate, control samples (i.e., virgin substrates) are found at the more positive PC2 values. On the other hand, more positive values would correspond to a combination of a high C–(C,H) content, of the occurrence of C–O functions found in Cup, and of the presence of oxygen involved in functions typical of both Cup and glass. Note that the C–(C,H) component, which is typical of PS, may as well be found in proteins (aliphatic functions of amino acid side chains), and is also the major component of the contamination layer which is always present at the surface of substrates such as glass or Cup [13]. Cup samples can then be separated from glass samples along PC2 owing to the combined influence of a higher contamination level on Cup compared to glass, as observed by Caillou et al. [13], of the C–O signal typical of Cup, and of the overall lower level of protein adsorption observed on Cup. This latter effect was confirmed by measuring the adsorbed fibrinogen amount using radiolabeling: it was of $\sim$0.8 μg/cm^2 on glass, and of $\sim$0.4 μg/cm^2 on Cup in similar conditions [13].

The spreading of PS samples along PC2 (see Fig. 4a) can be explained by the higher adsorbed amount of fibrinogen compared to albumin. This is confirmed by the higher nitrogen content found on PS after fibrinogen compared to albumin adsorption [13]. Cleaning efficiency by Renalin and NaClO can then be inferred from the position of samples submitted to cleaning in the score plot. After cleaning with NaClO, the samples are located close to virgin PS, whatever the nature of the adsorbed protein. After cleaning with Renalin, the samples remain at a position close to that of the original adsorbed fibrinogen or albumin layer. It can be deduced that NaClO cleaning is very effective and restores the surface in a state close to its native one regarding chemical composition, while the adsorbed layer is essentially not affected by cleaning with Renalin. Caillou et al. [13] actually showed that the

proteins left on the surface after such cleaning were oxidized. This was detected on the basis of the S 2p peak which was not included in the present data treatment. PS samples form a diagonal on the score plot because the C–(C,H) component is positively correlated with both PC1 and PC2. Increase of the screening of the substrate by the adsorbed layer leads to a decrease of that component, thus to decreased PC1 and PC2 scores.

This example shows that PCA may be very helpful to treat XPS data. It allows relationships between samples to be revealed, as well as chemical shifts of interest to be identified. It may therefore serve as a guide for sample classification and for further data treatment, including peak decomposition. As far as adsorbed protein layers are concerned, since XPS is in itself not sensitive to the nature of proteins, no statistical treatment of the data is expected to bring information with that respect. However, differences regarding protein adsorbed amounts and, or the spatial organisation of adsorbed layers may be highlighted, as illustrated here above. It must be recalled here that the protein signal recorded by XPS is not strictly related to the adsorbed amount, in reason of the low probed depth: if the protein molecules form aggregates or multilayers, peak components associated to the protein will be less intense compared to a smooth monolayer at equal adsorbed amount. It is interesting to note that most of the discrimination that could be made here between samples is related to differences concerning the substrates on which protein layers were adsorbed, which actually can be linked with different levels of screening of the substrate by the adsorbed molecules.

4 Conclusion

PCA was used to extract information from series of ToF–SIMS or XPS spectra collected on adsorbed protein layers. The results show that such statistical treatment of spectroscopic data may help understanding the protein layer structure and composition, as well as adsorption mechanisms. It must be emphasized that the outcome of PCA depends on all pre-treatments made on the data set, including data selection and normalisation, and that caution must be exerted when interpreting the data, due to the effect that ultra high vacuum may have on adsorbed proteins.

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