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Photoemission analysis of chemical differences between the membrane and cytoplasm of neuronal cells

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Abstract. We demonstrate the possibility of using x-ray photoemission spectroscopy (XPS) to distinguish between the chemical properties of the neuron membrane and of the cytoplasm in brain cells. This was possible by analysing cells de-capped by using a newly developed technique. The XPS spectra clearly show chemical differences between the cell cytoplasm and the membrane, for example between nitrogen in $–NH_2$ and $–NH_3$, which are characteristic of the proteins and membrane phospholipids, respectively. A preliminary (and not conclusive) interpretation of all the observed spectroscopic features is given.

1. Introduction

Experimental methods of surface physics have recently been applied with increasing success in biophysics [1–5]. In particular, the spatial resolution of x-ray photoemission and absorption has reached the sub-micrometre range required to investigate the local chemistry of biological samples [1, 2]. This improvement, therefore, has expanded the application domain of conventional spectroscopic techniques, including for example the observation of toxic and physiological elements in biological systems [3–5].

However, photoemission-based spectromicroscopies in biology are still adversely affected by their surface sensitivities. X-ray absorption spectromicroscopy can, in fact, probe the first 100–200 Å of the sample surface, whereas photoemission spectromicroscopy only probes a depth of 5–10 Å. A cell is typically a few micrometres thick. These techniques, therefore, could not previously study the cell cytoplasm and were confined to the study of the cell surface (the membrane, less than 100 Å thick, and the proteins embedded in it).

We recently succeeded in overcoming this handicap by de-capping the cells and removing only a controlled portion of the cell membrane and/or cytoplasm. The quantitative calibration of this de-capping procedure was performed by atomic force microscopy (AFM), as described in [6]. After de-capping of cells, it becomes possible to investigate the cytoplasm and its structures by surface-sensitive techniques like XPS.

We present a successful test of this approach; namely an XPS chemical analysis of the brain cell cytoplasm. In section 2 we describe the sample preparation and de-capping techniques and the XPS apparatus. The chemical differences between the cytoplasm and the membrane are discussed in detail in section 3. Section 4 presents concluding remarks.

2. Materials and methods

2.1. The method of sample preparation

The primary cultures of rat cerebellar granule cells were obtained by enzymatic and mechanical dissociation of eight-day-old rat cerebella. Cerebellar tissue was sliced, suspended in Krebs–Ringer solution and incubated with trypsin, centrifuged, re-suspended and finely triturated. Dissociated cells were recovered by centrifugation and re-suspended in basal Eagle's medium with Eagle's salt containing 10% foetal calf serum. The cells were plated and allowed to grow on poly-L-lysine-treated gold-coated

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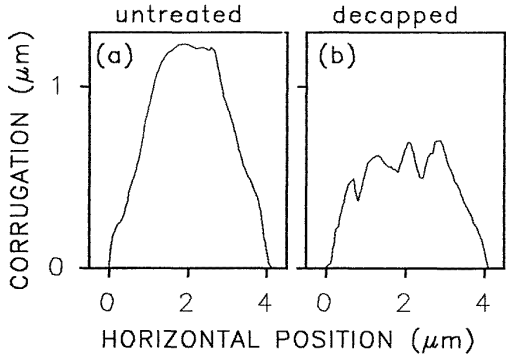

Figure 1. Corrugation profiles topography obtained by atomic force microscopy for: (a) an untreated sample and (b) a de-capped sample.

stainless steel substrates in an incubator at 37 °C in a 5% CO_2 humidified atmosphere. After eight days *in vitro*, the granule network was fully developed. At the end of the growth period all the cultures were washed and pre-fixed for 30 s with 0.4% glutaraldehyde in phosphate-buffered saline solution (PBS). Further details on the specimen preparation can be found in [3–5].

The cells were de-capped as described in [3, 4]. In short, the excess uptake buffer was drawn and each specimen was covered with a filter membrane (Millipore HAWP 0.45 μm pore size) soaked with CS buffer (100 mM piperazine-*N*, *N′-bis*-(2-ethanesulphonic acid) from Sigma, 5 mM ethylene glycol-*bis*-(β-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid from Sigma and 2 mM $MgCl_2$ from Carlo Erba, pH 6.8). After 2 min the filter membrane was removed, thus peeling off the portion of cell membrane adhering to the filter membrane. Immediately after that the membrane portion had been peeled off, the specimens were fixed with 4% paraformaldehyde (from Merk) in PBS and dehydrated. We also left some of the cell cultures not de-capped (untreated) for comparison.

In order to interpret the XPS spectra of cells, we prepared two reference samples, one containing a protein, one containing phospholipids. The protein sample was a dried droplet of 1 μg ml^{-1} bovine serum albumine (BSA) in PBS cross linked with paraformaldehyde (4% in PBS). The phospholipid sample was obtained by extracting the membrane phospholipids from the cerebellar cell cultures of same type with chloroform and methanol.

AFM was used to characterize the portion of the cell that had been removed and to test the effectiveness of the de-capping procedure. This technique has recently been applied to study cultured neurons [6], by imaging granule cells and their neurites with a lateral resolution of a few tens of nanometres. The AFM technique is extremely sensitive to surface topography, it does not alter the investigated specimens and it does not require conductive samples. The AFM apparatus was described in detail elsewhere [7]. Constant-force AFM images were obtained by working in the repulsive mode with a force smaller than 1 nN from zero cantilever deflection. No degradation of the specimen was observed and the AFM results were reproducible over several days.

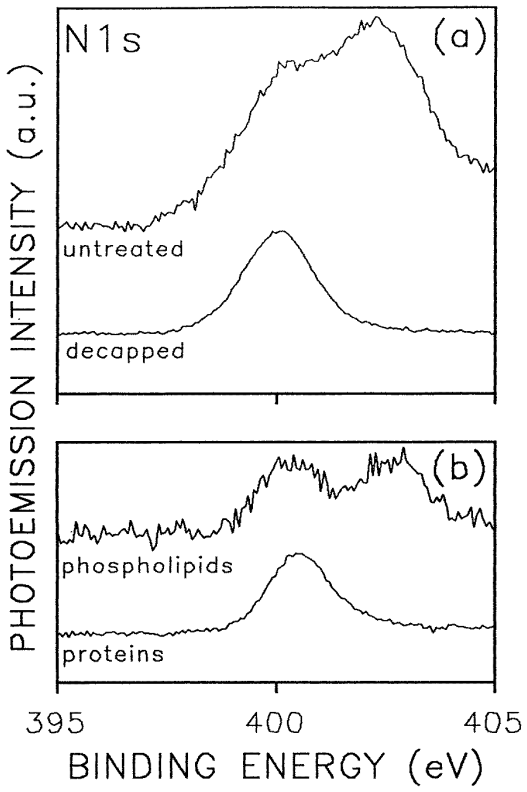

Figure 2. (a) N 1s XPS spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. The peak at 402.3 eV is related to $-NH_3^+$ in phospholipids on the cell membrane, whereas the feature at 400.1 eV is related to $-NH_2$ in proteins.

2.2. X-ray photoemission spectroscopy techniques

Our ESCA-300 (Scienta) system was described in detail in [8]. The operating pressure was less than 3×10^{-10} mbar, with an energy resolution of $\simeq$0.8 eV. The monochromatized x-ray beam was the Kα emission line of aluminium (1486 eV), produced by a $\simeq$3 kW rotating anode.

No charging-compensation devices (such as a flood gun) were used, since our neuron networks grown on gold substrates were not affected by charging problems. In fact, the aliphatic carbon C 1s peak was always observed at a binding energy of 285.0 eV.

The unsmoothed spectra were fitted using Voight functions and linear background subtraction [9]. All spectra were recorded under the same experimental conditions and with the same acquisition time. Preliminary tests demonstrated that the spectral features did not change from place to place on the specimens on our lateral resolution scale, indicating good homogeneity. The reported experimental results are representative of a much larger body of consistent data.

3. Results and discussion

Figures 1(a) and (b) show the AFM results for untreated and de-capped samples, respectively. The de-capped sample

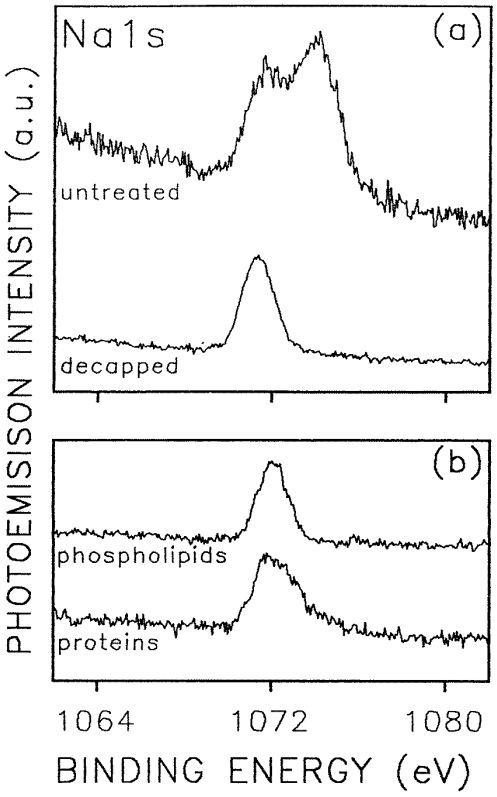

Figure 3. (a) Na 1s spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. The low-energy peak is related to proteins and phospholipids. The feature at higher energies could be caused by oxidized sodium localized in the sodium channels on the cell membrane.

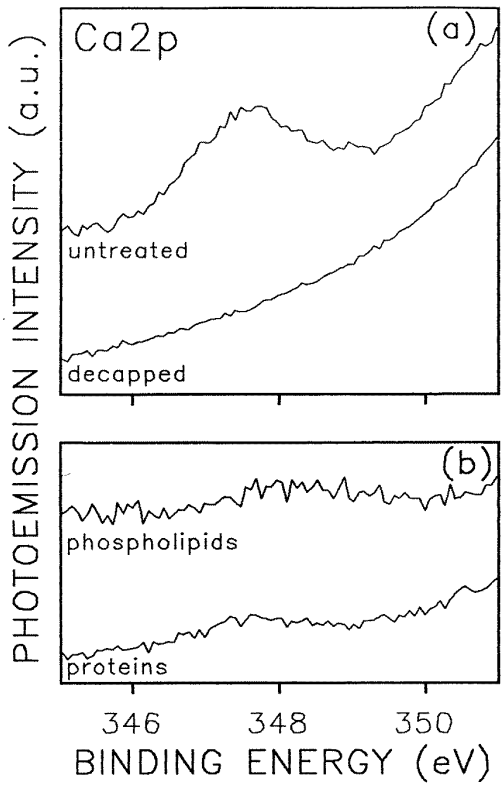

Figure 4. (a) Ca 2p spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. Just like in the case of sodium, the experimental results suggest that there is a greater degree of confinement of the calcium on the cell membrane, which is presumably related to the calcium channels.

was much thinner than the untreated one and showed irregular features related to the peeling-off procedure. The results of figure 1 clearly show that the de-capping procedure allowed us to remove the upper portion of the cell body in a controlled way. This effect can be attributed to the hydrophobic character of the fatty acid chains in the cell membrane; that is, the cell membranes preferentially adhere to the filter membrane rather than being immersed in the buffer. The calibration of the de-capping procedure was reported elsewhere [6].

In figure 2(a) we present the N 1s XPS core-level spectra recorded for unde-capped and de-capped cells. These spectra were acquired on 4 mm^2 samples as were all the following ones; therefore, they correspond to an average over several thousand cells. These spectra clearly show two resolved peaks at 400.1 and 402.3 eV. Nitrogen chemical states such as –CN, $–NH_2$, –OCONH– and $–CONH_2$ are known to give an N 1s binding energy (BE) in the range 399–401 eV [10]. We attribute the 400.1 eV peak to the amino group $–NH_2$ present in basic amino acids and to the –CONHC– group in peptide bonds.

On the other hand, on going from $–NH_2$ to $–NH_3^+$ (ternary to quaternary amines the N 1s BE increases by $\simeq$1.5 eV [10]. Therefore, the peak at 402.3 eV can be attributed to the $–NH_3^+$ group present in the polar head of the phospholipids of the cell membrane [11]. We did not observe oxidized nitrogen states that would have BEs of 408 eV ($–ONO_2$), 407 eV (NO_2) and 405 eV (–ONO) [10]. This is due to the fact that these components are not present in cells. Also, in this case, the peak at about 400 eV could be related to the amino group because of the procedure of extraction we used.

Notice that, although the proteins are present everywhere in the cell, the phospholipids are mainly localized in the cell membrane [11]. Therefore our results are entirely plausible: as the membrane is removed, the peak at 402.3 eV ($–NH_3^+$ in phospholipids) disappears, whereas the effect of de-capping on the 400.1 eV peak ($–NH_2$ in proteins) is negligible. Furthermore, we observe a total loss of the membrane signal in figure 2(a), despite the fact that a significant amount of membrane was probably still present. This fact is a consequence of the de-capping technique, which will remove mainly the membrane orthogonal to the detector, namely the most detectable portion.

The N 1s XPS spectra of the reference samples reported in figure 2(b) confirm our interpretation: the peak at 400.1 eV is related to the protein in BSA, whereas the peak at 402.3 eV is related to the $–NH_3^+$ present in the membrane phospholipids.

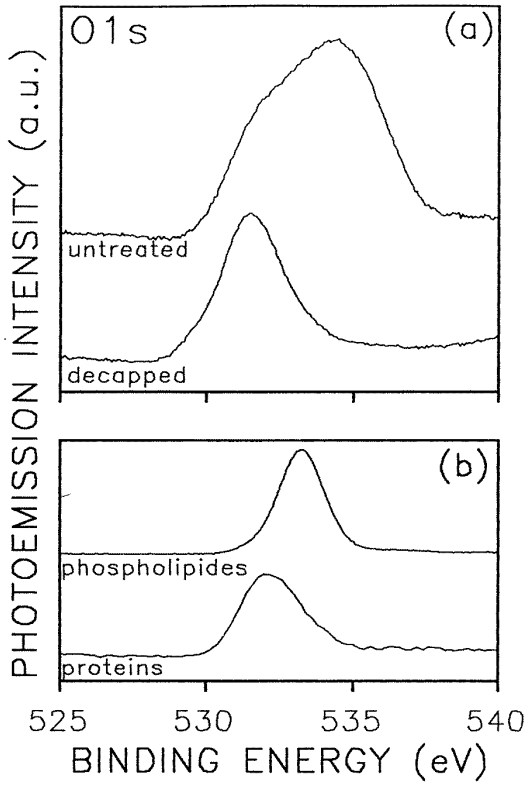

Figure 5. (a) O 1s spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. The peak at lower energy is caused by proteins, whereas the feature at higher energy is related to oxygen in phospholipids and/or peroxides and hyperoxides in the cell membrane.

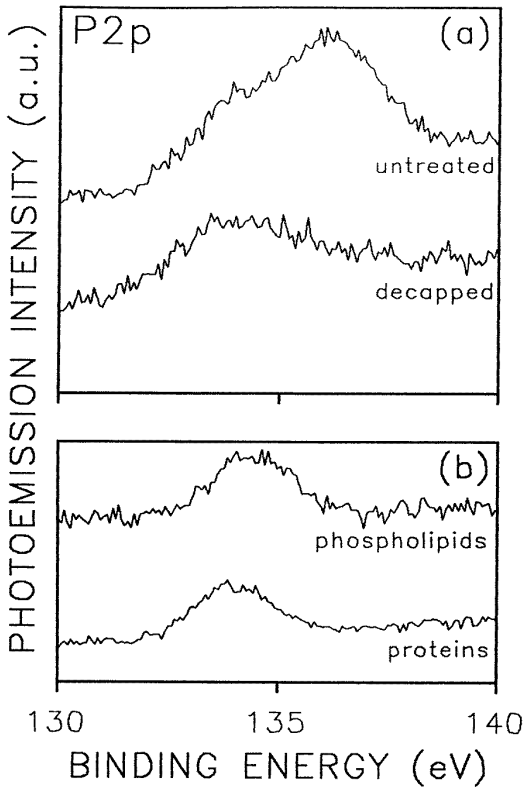

Figure 6. (a) P 2p spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. The high-energy feature is related to chemical species localized on the cell membrane, but it does not seem to be associated with the phosphorus in phospholipids or proteins.

In figure 3, we present Na 1s spectra for untreated and de-capped samples. Two peaks are again evident, of which the one at higher energy disappears as the de-capping increases, whereas the one at lower energy remains unchanged.

The lower energy feature could in principle be due to one of the many sodium chemical states [10] which give Na 1s BEs in the narrow region 1071–1072 eV. These are 1071.8 eV for metallic sodium (evidently not present in cells), 1071.6 eV for $NaNO_2$ and 1071.4 eV for $NaNO_3$ [10]. These salts are very unlikely to be present in our samples because they are soluble and hence would have been removed by cell washing. Note that there is no evidence of $–NO_2$ or $–NO_3$ groups (figure 2). Other possibilities [10] are NaOOCH (Na 1s BE of 1071.1 eV), which is widely present in proteins and therefore throughout cells, NaH_2PO_4 and $NaPO_3$ (see below).

As a possible interpretation of the results in figure 3, the high-energy peak could be attributed to an oxidized compound originating from the ionic sodium in the sodium channels of the cell membrane. It was detectable only when the cell membrane was present. The reference spectra seem to confirm our interpretation (figure 3(b)): no shift to higher energy was observed, indicating that the sodium, although localized on the cell membrane, is not related to phospholipids. However, we remark that the confirmation of this hypothesis would require a detailed analysis, involving XPS sensitivity and the use of sodium-channel toxins, which was well beyond the purpose of the present work.

In figure 4(a), we present Ca 2p spectra for untreated and de-capped cultured neurons. The reference spectra show a very weak signal related to Ca 2p, as do the spectra of de-capped cells. The spectral feature of calcium, on the other hand, was more intense for the unde-capped cells (figure 4(a)). A possible interpretation of the results is confinement of calcium on the cell membrane, which could be related to the ionic calcium channels. Also in this case, a more refined analysis would be necessary in order to confirm this assumption.

Figure 5(a) shows the O 1s spectra for untreated and de-capped cells. We see a high-energy peak related to the cell membrane and a lower energy peak related to the cell membrane and a lower energy peak not affected by de-capping. The O 1s BEs of most oxygen chemical states are known to fall within the range of 2 eV around 533 eV [10]; our peak at $\simeq$532 eV is certainly related to one of these. The 534.6 eV peak can be attributed either to peroxides and/or hyperoxides in the phospholipid fatty chain [10, 11] or to phenolic –OH [10] or C–O bonds [12].

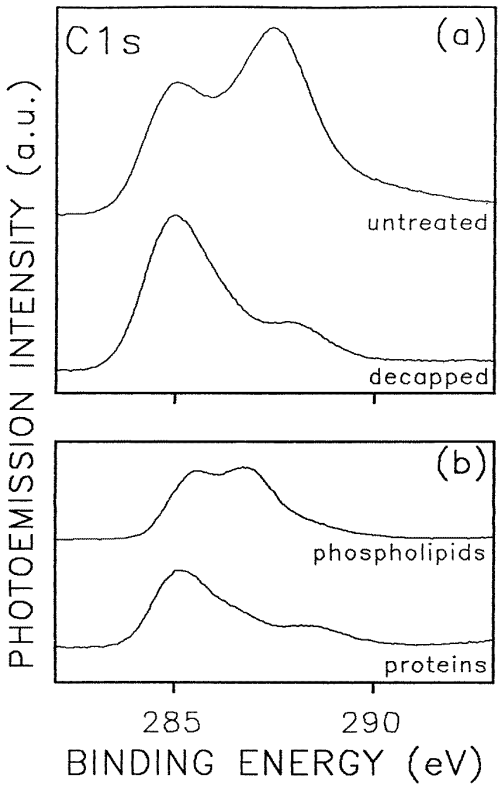

Figure 7. (a) C 1s spectra of untreated and de-capped neurons. (b) Reference spectra of phospholipids and bovine serum albumin. The higher intensity of the features related to oxidized carbon is mainly related to the membrane phospholipids.

The high-energy shift in the spectra of the control samples (figure 5(b)) can also be related to O 1s states in the lipids.

Figure 6(a) shows P 2p spectra for untreated and de-capped neutrons. The 134.0 eV peak could be due to P in phosphates (134.2 eV for NaH_2PO_4 and 134.7 eV for $NaPO_3$) [10]. The phospholipid and protein spectra do not show the higher energy (135.8 eV) feature present in the spectra of the unde-capped cells, suggesting that a chemical species is localized on the cell membrane.

We remark that we noticed that the signal-to-background ratio decreased dramatically as the de-capped portion of the cell increased. This effect, which was particularly evident in the case of the phosphorus spectra, was in fact present for all our spectra. It can be attributed to the fact that the de-capping procedure 'cleans' large regions of the substrate, therefore increasing the background signal.

The C 1s spectra are shown in figure 7(a). Carbon bound to itself and/or to hydrogen gives C 1s at 285.0 eV [10]. Oxygen bonding induced shifts to higher BEs, by 1.5 eV per C–O bond [10]. The experimental results indicate that an increase in the oxidation state of carbon occurs on the cell membranes. We remark, however, that the component with higher BEs is partially due to a multiplicity of sub-components. The spectra of figure 7(b) give a qualitative confirmation of this result, by showing that there is a more intense component at about 287 eV (oxidized carbon) in phospholipids than there is in proteins.

4. Conclusions

Our main objective was to show that the combined use of XPS and our new de-capping technique can overcome the basic handicap of photoemission in biology, namely, its high surface sensitivity. The experiment allowed us to detect both de-capping-dependent peaks and features that were not altered by de-capping. Furthermore, our preliminary and not conclusive spectroscopic identification offers a plausible explanation in terms of chemical states of various elements that prevail in the membrane or are present everywhere in the cell, in good agreement with the experimental results on the reference samples.

Our results open up new perspectives for the study of intracellular chemistry. We have demonstrated with XPS and AFM that the cell membrane was successfully removed by our de-capping technique. This makes the inner portion of the cell available to be studied using surface-sensitive techniques, including synchrotron photoelectron spectromicroscopy (scanning or imaging modes), XPS, Auger scanning microscopy and low-energy electron microscopy. With these techniques one can investigate the presence of specific elements and their chemical statuses.

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