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Characterization of Eukaryotic Cell Surfaces Prior to and After Serum Protein Adsorption by X-Ray Photoelectron Spectroscopy

Fibroblasts, HELA Epithelial, and Smooth Muscle Cells

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ABSTRACT

Elemental surface concentration ratios N/C, O/C, and P/C of fibroblasts, HELA epithelial cells, and smooth muscle cells, prior to and after washing in the absence or presence of serum proteins, were determined by X-ray photoelectron spectroscopy. Cell surfaces appeared to adsorb hardly any serum proteins, and the relatively high P/C, as compared to N/C and O/C, elemental surface concentration ratio indicated that the cell surfaces consisted mainly of the phospholipid bilayer, with little or no proteins present. The lack of adsorption of serum proteins to the cell surfaces seems at odds with the common notion that

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cells require adhesive proteins in order to adhere and spread. However, the adsorption behavior of cellularly produced proteins may be completely different, particularly since they seem to be able to displace adsorbed serum proteins from biomaterials surfaces. Interestingly, only HELA epithelial cells (a tumor cell line) appeared to adsorb a very small amount of proteins.

Index Entries: X-ray photoelectron spectroscopy; eukaryotic cells; surface composition; serum proteins; adsorption.

INTRODUCTION

Adhesion and spreading of eukaryotic cells is determined, among other factors, by the surface free energies and charges of the interacting surfaces *(1–5)*, both corollaries of the chemical composition of the cell surface. Cellular adhesion and spreading are generally better on high surface free energy materials *(4)* and on surfaces with a small negative or positive charge *(6,7)*.

The presence of adsorbed serum proteins, in particular fibronectin, is essential for optimal adhesion and spreading of cells on biomaterials surfaces *(8–12)*. Whereas adsorption of serum proteins to biomaterials has been extensively studied *(13–15)*, little work has been done on the influence of serum protein adsorption onto cell surfaces.

X-ray photoelectron spectroscopy (XPS) is a technique known in physical chemistry to quantitate the elemental composition of surfaces over their outermost 5–10 nm. XPS is often applied not only to characterize biomaterials surfaces *(16,17)*, but also for the characterization of microbial cell surfaces *(18–22)* and to detect adsorbed proteins on these types of surfaces *(23–25)*. Several years ago, Millard and Bartholomew *(26)* determined the elemental surface composition of mouse fibroblasts, grown as confluent monolayers on polystyrene tissue-culture dishes, by XPS in an attempt to monitor malignant changes of the cells. It is the aim of this article to determine changes in the elemental surface composition of a number of eukaryotic cell types (fibroblasts, HELA epithelial cells, and smooth muscle cells) on adsorption of serum proteins by XPS, in order to advance our understanding of the mechanism by which these cells adhere and spread on biomaterials.

MATERIALS AND METHODS

Cell Cultures and Sample Preparation

Fibroblasts from human skin, smooth muscle cells from rat aorta, and epithelial cells from a HELA tumor line were cultured in Greiner (Alphen aande Ryn, Netherlands) flasks (75 cm^2), using RPMI 1640 medium (Gibco, Breda, Netherlands), supplemented with 100 IU/mL penicillin/streptomycin

(Gibco), 200 m*M* glutamine, and 15% fetal calf serum (Gibco) at 37°C and 5% CO_2 in humidified air. Every 2 d, cultures were subdivided (1:2) by trypsinization (0.15 w/w% trypsin 1:250, Difco, Amsterdam, Netherlands) in Ca/Mg-free Hank's balanced salt solution.

Harvested cells were either washed three times in RPMI 1640 without serum proteins or in RPMI 1640 with serum proteins (15% fetal calf serum) added. The latter procedure was included in order to let serum proteins adsorb to the cell surfaces. After the last centrifugation step, the cells were suspended in 4 mL saline (0.9% NaCl) and centrifuged. The cellular pellet was frozen in a Petri dish in solid nitrogen (–210°C) and lyophilized at –80°C (Leybold Heraeus combitron CM 30, de Bilt, Netherlands). Then the freeze-dried powder was placed in stainless-steel troughs, and cells were slightly pressed in order to produce a smooth surface. Great care was taken not to damage the integrity of the cell surface, which was checked regularly by means of Scanning Electron Microscopy.

X-Ray Photoelectron Spectroscopy (XPS)

X-Ray Photoelectron Spectroscopy (XPS) measurements were performed according to the procedures described by Amory et al. *(18)*. Briefly, six troughs with freeze-dried cells were inserted simultaneously in the chamber of the spectrometer (Vacuum Generators ESCA 3 Mk II, equipped with a Tracor Northern TN 1710 signal averager). The residual pressure in the spectrometer was approx 10^{-9} torr. A magnesium anode (Mg-K_α, 1253.6 eV) was used for X-ray production (14 kV, 20 mA). After a scan of the overall spectrum, peaks were recorded in the following order: C_{1s}, O_{1s} N_{1s}, and P_{2p}. Subsequently, the C_{1s}, peak was recorded again as a control. After linear background subtraction, the area under each peak was employed for calculation of the peak intensities. Elemental surface concentration ratios were calculated with respect to C_{1s}, using the Wagner sensitivity factors (27).

In addition, both the C_{1s} and the O_{1s}, peaks were decomposed into Gaussian components. The C_{1s} peak was decomposed into three components, assuming the full width at half maximum of each component to be 2.15 eV and with binding energies of 285.0, 286.6, and 288.4 eV, representative for carbon involved in C–C; C–N or C–O; and (C=O)–NH bonds, respectively. The O_{1s} peak was decomposed in two components, assuming a full width at half maximum of 2.6 eV for each component, at 531.3 and 533.2 eV, with the latter component being attributed to oxygen involved in C–OH bonds.

RESULTS

Figure 1 shows a cellular multilayer of fibroblasts as prepared for XPS. A relatively smooth surface can be obtained, and minor damage to the cells is obvious. Electron micrographs of pressed pellets of the other cell types used were similar.

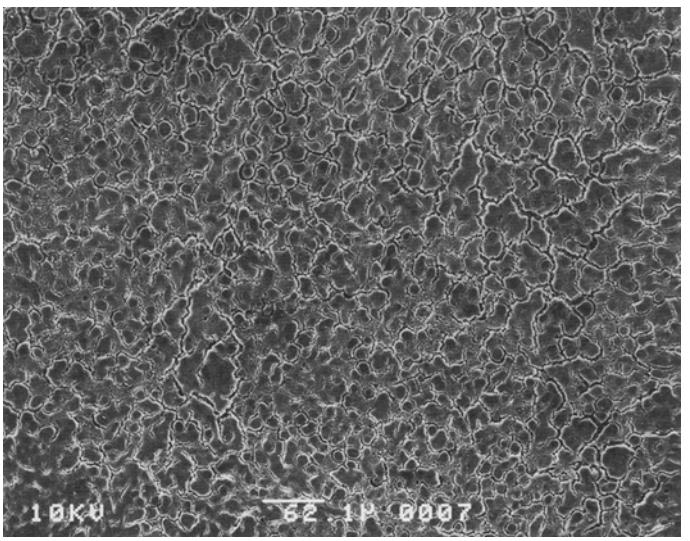

Fig. 1. Scanning electron micrograph of a pressed pellet of freeze-dried human skin fibroblasts. Note that the integrity of the cells is not affected by the sample preparation. The bar denotes 62 µm.

Overall XPS-spectra of the cells showed that carbon, oxygen, nitrogen, and phosphorus were the main elements to be quantitated. Sulfur was sometimes detectable in very small amounts. Sodium and chlorine were always present as a result of the final washing of the cells in saline.

Table 1 lists the elemental surface concentration ratios of the cells prior to and after washing in the absence or presence of serum proteins as well as the decompositions of the C_{1s} and O_{1s} peaks. An example of a C_{1s} peak and its components is shown in Fig. 2 for human skin fibroblasts.

Note (Table 1) that the presence of serum proteins in the washing medium does not yield any significant increase in the number of carbon atoms involved in (C=O)–NH bonds (the C_{1s} component set at 288.4 eV) or a systematic increase in the N/C surface concentration ratio. Also, the O/C and P/C surface concentration ratios of the cells do not demonstrate a consistent change after washing in the presence of serum proteins.

DISCUSSION

The technique used in this article to study the elemental composition of the present set of eukaryotic cells is essentially the same as that used frequently for microbial cells *(18–21)*. Elemental surface concentration ratios by XPS of a variety of freeze-dried bacterial strains were found to relate with the

Table 1
Data Derived from XPS Spectra of Cells, Obtained Using Pressed Pellets of Fibroblasts, HELA Epithelial Cells and Smooth Muscle Cells after Washing in the Absence (–) or Presence (+) of Serum Proteins[a]

	Fibroblasts		HELA epithelial		Smooth muscle	
XPS parameter	–[b]	+	–	+[c]	–	+
N/C	0.057	0.047	0.054	0.069	0.046	0.047
O/C	0.148	0.193	0.153	0.163	0.209	0.181
P/C	0.013	0.028	0.013	0.012	0.034	0.025
C_{1s}[d], 285.0 eV	0.702	0.649	0.590	0.603	0.621	0.674
286.6 eV	0.278	0.305	0.329	0.310	0.354	0.283
288.4 eV	0.041	0.044	0.080	0.086	0.025	0.043
O_{1s}[e], 531.3 eV	0.486	0.649	0.528	0.488	0.609	0.498
533.2 eV	0.514	0.351	0.472	0.512	0.391	0.502

[a]All data represent averages of two separate cultures, results of which coincided within 10–15%.

[b]Sulfur was detected with a concentration ratio S/C equal to 0.043.

[c]A small trace of sulfur was detected, with an approximate concentration ratio S/C equal to 0.003.

[d]The C_{1s} peak was decomposed in three components set at 285.0, 286.6, and 288.4 representative for C–C; C–O and C–N, and (C=O)–NH bonds, respectively. Data are expressed as a fraction of the C_{1s} peak area.

[e]The oxygen peak was decomposed in two components set at 531.3 and 533.2 eV, the latter being representative for C–OH bonds. Data are expressed as a fraction of the O_{1s} peak area.

hydrophobicities and zeta potentials of the bacteria *(20–22)*. Zeta potentials are measured on bacterial strains in their physiological state, whereas XPS measurements are done on freeze-dried bacteria. The relationship between XPS data and zeta potentials is particularly important, because it shows that physiologically relevant information can be obtained by XPS, despite the fact that cells are in a freeze dried state.

The sample preparation technique used in this article, based on pressing freeze-dried, trypsinized cells to a pellet, is completely different from the one used by Millard and Bartholomew *(26)*. They grew cells to confluent layers on polystyrene tissue-culture dishes and measured them after air-drying. Table 2 summarizes some of their data on mouse fibroblasts, treated in various ways. From a comparison of their data in Table 2 and our results on trypsinized fibroblasts washed in the absence of serum proteins (Table 1), it is obvious that the N/C and O/C elemental surface concentration ratios are similar for both sample preparation techniques. We have no rigorous explanation for our relatively high P/C elemental surface concentration ratios as compared to those reported by Millard and Bartholomew *(26)*, although possibly trypsinization, as done in our study, may expose more of the phospholipid

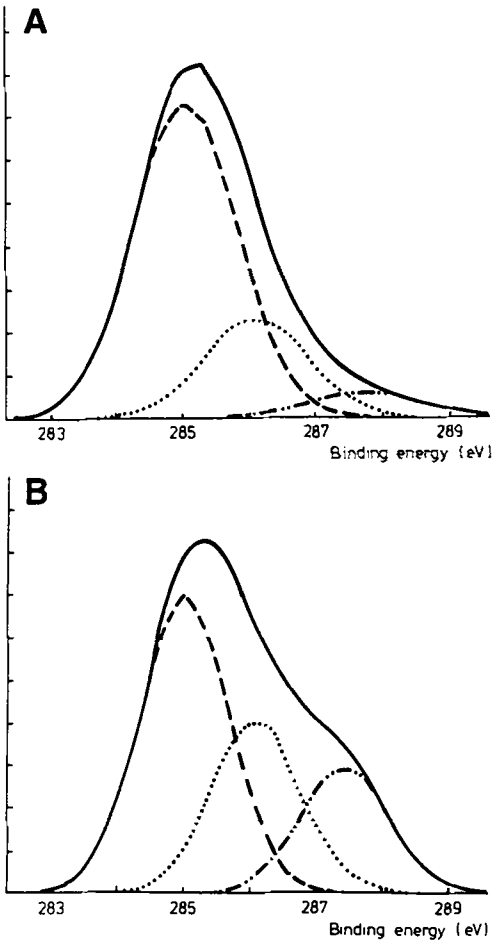

Fig. 2. A comparison of the C_{1s} peaks with their components for (A) trypsinized human skin fibroblasts (washed in the absence of serum proteins) and (B) freeze-dried bovine serum albumin. Note that the third component of the C_{1s} peak (C=O) is much greater for the pure protein than for the fibroblast cells. – – – C–C; ····· C–O and C–N; –·– (C=O)–NH.

bilayer of the cell membrane than is exposed by cells that are not trypsinized. Nevertheless, we conclude that both different sample preparation techniques yield comparable results.

XPS data have become available over recent years on a number of biological substances, including eukaryotic cells, prokaryotic cells, and proteins (*see* Table 3). Normal eukaryotic cell walls consist of the phospholipid bilayer and contain a variable amount of protein (*30*). Particularly after trypsinization, cells are usually devoid of surface proteins during approx 20–30 min. The absence of large amounts of proteins on eukaryotic cells is evidenced from their low N/C surface concentration ratios as determined by XPS (*see* Table

Table 2

Elemental Surface Concentration Ratios of Mouse Fibroblast Cells Grown to a Confluent Layer on Polystyrene Tissue-Culture Dishes as Calculated from XPS Peak Intensities Reported by Millard and Bartholomew *(26)* Using Wagner *(27)* Sensitivity Factors

Cell treatment[a]	N/C	O/C	P/C
Medium decanted	0.056	0.147	0.009
Washed with isotonic saline	0.044	0.067	0.002
Washed with distilled water	0.059	0.131	0.004

[a]Calculation of the N/C, O/C, and P/C elemental surface concentration ratios averaged over all treatments listed, yields: N/C = 0.053 ± 0.008, O/C = 0.115 ± 0.042, and P/C = 0.005 ± 0.004.

3). The cell wall of prokaryotics is composed of a peptidoglycan layer, covered by a wide variety of proteins and (lipo)polysaccharides *(31)*. For certain strains, N/C ratios have been measured approaching the ones of pure proteins (*see also* Table 3).

For the majority of biological substances studied so far by XPS, (N/C + O/C) showed an excellent, linear correlation with the fraction of carbon involved in C–O/C–N and (C=O)–NH bonds, i.e., the components of the C_{1s} peak set at 286.6 and 288.4 eV *(18–22,24,28,29)*. Data for the present set of eukaryotics do not follow this linear relationship as is obvious from Table 3, which probably indicates that in these cells, oxygen is bound differently than in the other biological substances studied.

Freeze-dried proteins and also thick protein films adsorbed to glass disks are characterized by high N/C surface concentration ratios and a relative large component of the C_{1s} peak at 288.4 eV. Therefore, the absence of major amounts of protein on our collection of eukaryotic cell types cannot only be inferred from the low N/C surface concentration ratios, but also from a comparison of their C_{1s} peak shapes with the one of a pure protein (*see* Table 3 and Fig. 2). Since washing the cells in the presence of serum protein, hardly affected their N/C surface concentration ratios or the components of the C_{1s} peak set at 288.4 eV, it is likely that these eukaryotic cells appear to adsorb no or very few (the HELA epithelial, tumor cells) serum proteins.

This is an important observation for understanding the mechanism of cellular adhesion and spreading, and seems somewhat at odds with the common notion that cells need proteins, viz. fibronectin, in order to adhere and spread. Obviously, proteins from serum cannot serve as adhesive proteins, since they do not adsorb to the cell surface. This conclusion is consistent with recent observations by Van Wachem et al. *(32,33)* and Dekker et al. *(34)*, showing that serum proteins preadsorbed to biomaterials did not serve as adhesive proteins for endothelial cells and that, in addition, these proteins were

Table 3

A Comparison of Data Derived from XPS Spectra of Various Biological Substances

XPS parameter	N/C	O/C	P/C	C_{1s} peak decomposition[a] ~285.0 eV	~286.6 eV	288.4 eV	O_{1s} peak decomposition[a] 531.3 eV	533.2 eV	Reference
Eukaryotic cells									
Cells	.05–.07	.15–.21	.01–.03	.6–.7	.3–.4	≤.1	.5–.7	.3–.5	this study
Yeasts	.01–.02	.28–.53	<.01	.3–.6	.3–.6	.1	0	1	*(18,28)*
Prokaryotic cells									
Gram positive oral streptococci	.05–.13	.31–.50	≤.01	.5–.6	.3–.4	.1	.2–.5	.5–.8	*(20,21,24)*
Gram positive staphylococci	.15–.20	.45–.55	.02–.04	.3–.4	.4–.5	.2–.3	.3–.4	.6–.7	*(22)*
Gram negative *E. coli*	.03–.09	.28–.43	.01–.02	.4–.6	.3–.4	.1–.2	.2–.3	.7–.8	*(29)*
Thick protein films on glass disks									
Albumin	.217	.277	–[b]	.564	.214	.222	–	–	*(25)*
γ-Globulin	.223	.357	–	.542	.217	.241	–	–	*(25)*
Fibrinogen	.173	.303	–	.475	.303	.222	–	–	*(25)*
Lipoproteins	.053	.156	–	.793	.144	.063	–	–	*(25)*
Freeze-dried proteins									
Bovine serum albumin	.240	.292	<.001	.503	.289	.207	.894	.106	Unpublished
Salivary glycoproteins	.182	.432	.003	.518	.323	. 159	.780	.220	Unpublished

[a]Expressed as a fraction of the peak area.
[b]Not reported.

displaced by fibronectin produced by the cell itself. Thus, cells most likely have a higher affinity for proteins that they have produced themselves than for noncellular proteins.

Interestingly, only the tumor cells seem possibly to adsorb a very small amount of serum proteins (not statistically significant). Tumor cells are known to behave differently from normal cells in a number of other aspects as well; for instance, they can be grown in liquid medium, and do not have to adhere and spread in order to grow. Therefore, minor adsorption of serum proteins may be another typical property of tumor cell lines uncommon to normal cells and resulting from a different cell-surface composition owing to the malignant evolution of these cells.

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