Colloid Polym Sci 278:878–883 (2000)

SHORT COMMUNICATION

S. Slomkowski
D. Kowalczyk
M. M. Chehimi
M. Dealamar

X-ray photoelectron spectroscopy as a tool for studies of the surface layer of microspheres. The case of polystyrene and poly(styrene–acrolein) microspheres with attached human serum albumin

Received: 23 November 1999
Accepted: 16 March 2000

S. Slomkowski (✉) · D. Kowalczyk
Center of Molecular and Macromolecular Studies
Polish Academy of Sciences
Sienkiewicza 112, 90-363 Lodz, Poland
e-mail: staslomk@bilbo.cbmm.lodz.pl
Tel.: +48-42-6819815
Fax: +48-42-6847126

M. M. Chehimi · M. Dealamar
Institut de Topologie et de Dynamique des Systèmes
Université Paris 7 – CNRS (URA 34)
1 rue Guy de la Brosse, 75005 Paris
France

Abstract The application of X-ray photoelectron spectroscopy (XPS) for studies of surface layers of objects with spherical shape was investigated using as examples polystyrene and poly(styrene–acrolein) microspheres with attached human serum albumin (HSA). The amounts of immobilized protein were determined by the standard biochemical Lowry method and by XPS, using the intensity of the N1*s* signals of HSA as a basis for evaluation. The XPS data were treated by taking into account the spherical shape of the particles analyzed (variable take-off angle of ejected electrons). The best agreement between the results of the biochemical and XPS determinations was found assuming that for the average particle the takeoff angle varies from 0° to 72.7°. This reflects the fact that in the multilayer arrangement of particles, placed onto the support of the XPS apparatus, the particles from the upper layer partially screen the edges of the particles in the layer below.

Key words X-ray photoelectron spectroscopy · Variable takeoff angle · Polystyrene microspheres · Poly(styrene–acrolein) microspheres · Human serum albumin

Introduction

X-ray photoelectron spectroscopy (XPS) is often used as a method of choice for the determination of the chemical composition of surface layers (few nanometers thick) of various objects [1–3]. Essentially, in all methods used for the treatment of XPS data it has been assumed that the surface analyzed is flat and smooth; however, investigators also applied XPS for the analysis of surfaces of powders and other particulate materials, for example, microspheres [4–12], usually without making any adjustment in the methods used for the treatment of experimental data.

Probing the chemical composition at various depths of flat and smooth surfaces is usually accomplished by changing the takeoff angle of ejected electrons. For particles the real takeoff angle (relative to the surface of the particles) varies depending on the part of the surface from which the electrons escape (Fig. 1). This is true for every setting of the angle between the support of the sample and a detector.

Recently Sheng and Sutherland proposed a method for quantitative analysis of spherically shaped powders coated with an overlayer [13]; however, their analyses were based on predictions of XPS spectra of isolated spheres. In real systems particles form assemblies with various microroughnesses on the scale of the particle dimensions and in these assemblies particles usually screen each other partially.

We were interested in finding out how important errors are when XPS data are analyzed without taking into account the microroughness of surfaces of microsphere assemblies. We also wanted to check whether it would be possible to develop methods allowing the elimination of these errors. For our studies we chose as a model system polymer microspheres [polystyrene (PS)

and poly(styrene–acrolein) (PSA)] with attached human serum albumin (HSA). Our choice was based on the following reasons. The microspheres can be obtained with well-controlled diameters and with a narrow diameter distribution ($\bar{D}_w/\bar{D}_n < 1.01$) [9], yielding material with well-defined geometry. The surface concentration of HSA attached to these particles can be determined independently from XPS analysis, using a biochemical method (e.g. the modified Lowry method developed in our laboratory for determination of proteins immobilized onto microspheres [14, 15]). The system, polymer microspheres–HSA, is characterized by the presence of nitrogen atoms exclusively in the layer composed of attached protein. Thus, the intensities of the N1*s* signals in the XPS spectra of microspheres with attached protein can be conveniently used for calculation of surface concentrations of HSA based on various models for the treatment of the XPS data. Comparison of results of these calculations with data from biochemical analysis should indicate the best method of XPS analysis.

Experimental

The microspheres (PS and PSA) were synthesized in the emulsifier-free emulsion homopolymerization of styrene and/or in the emulsifier-free emulsion–precipitation copolymerization of styrene and acrolein, carried out as described in detail in an earlier report [9]. The monomers (styrene and acrolein, Aldrich) were purified from 4-*tert*-butylcatechol and hydrochinone, respectively, by distillation under reduced pressure. Potassium persulfate ($K_2S_2O_8$) was used as an initiator. Triple-distilled water was a polymerization medium. A few crystals of sodium carbonate were added to the water to adjust the pH to 6.5. The compositions of the polymerization mixtures are given in Table 1.

The polymerizations were carried out under argon at 65 °C for 28 h. Thereafter, the microspheres were purified by filtration from some particle aggregates, by stream-stripping from traces of unreacted monomers, and, finally, by centrifugation and resuspension in fresh water. The diameters of the microspheres were determined by scanning electron microscopy. For each type of microsphere, the diameters of about 1000 particles were measured and the values of the number-average diameter ($\bar{D}_n = \sum n_i D_i / \sum n_i$) and the polydispersity parameter ($\bar{D}_w/\bar{D}_n$, where $\bar{D}_w = \sum n_i D_i^4 / \sum n_i D_i^3$)) were calculated. Assuming that the density of PS and PSA microspheres is 1.05 g/cm^3 (density of PS) the specific surface per gram of microspheres (S) was calculated. The surface concentrations of aldehyde and $-SO_4$ (from the initiator) groups were determined using the specific reaction of aldehydes with 2,4–dinitrophenylhydrazine and by conductometric titration, respectively. The procedures used for these determinations are described in our earlier articles [9, 14–19]. The values of the parameters characterizing PS and PSA microspheres are given in Table 2.

HSA (Sigma, Cohn fraction V) was used without further purification.

XPS spectra were recorded with a VG Scientific Escalab MKI apparatus equipped with a Mg Kα X-ray source (200 W at 10 kV). The pressure in the analytical chamber was about 5×10^{-8} mbar. The correction of electrostatic charging was made by adjusting the C1*s* signal of the aliphatic carbon atoms to 284.8 eV (position characteristic for PS [20]).

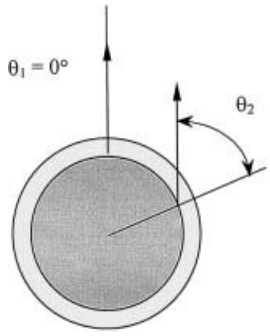

Fig. 1 Dependence of the takeoff angle, θ, from various elements of the surface of a microsphere covered with an adsorbed substance

Table 1 Compositions of polymerizing mixtures in the synthesis of polystyrene (*PS*) and poly(styrene–acrolein) (*PSA*) microspheres

Microspheres	Composition of polymerizing mixtures			
	Styrene (ml)	Acrolein (ml)	$K_2S_2O_8$ (g)	Water (ml)
PS	10	0	4.4×10^{-2}	100
PSA	10	0.6	4.4×10^{-2}	100

Table 2 Values of parameters characterizing PS and PSA microspheres

Microspheres	$\bar{D}$ (μm)	$\bar{D}_w/\bar{D}_n$	[-OSO$_3^-$] (mol/m^2)	[-CHO] (mol/m^2)	S (m^2/g)
PS	0.591	1.007	2.45×10^{-6}	0	9.66
PSA	0.492	1.002	1.38×10^{-6}	2.28×10^{-6}	12.61

Results and discussion

Attachment of HSA onto the PS and PSA microspheres

Attachment of HSA onto the PS and PSA microspheres was carried out in phosphate buffered saline (pH 7.4) at room temperature. Suspensions of microspheres were incubated with HSA at room temperature for 20 h. The concentration of the microspheres was 3.0 g/l in all experiments. The initial concentration of HSA in solution was varied in the range 10^{-2}–5×10^{-1} g/l. After incubation, the microspheres were isolated from the suspension by centrifugation at 20 000 *g*. The amount of HSA (expressed in milligrams) attached to a given amount of microspheres (in grams) was determined by the standard and modified Lowry methods according to the detailed descriptions given in our earlier reports [14, 15]. These measurements and the knowledge of the specific surface of the microspheres (S, Table 2) allowed the surface concentration of HSA (Γ_{HSA}) to be calculated. For the Langmuir-type adsorption (physical

adsorption and/or reversible chemisorption) the relation between Γ_{HSA} and $[HSA]_e$, where $[HSA]_e$ denotes the protein concentration at equilibrium, should conform to the following equation [21, 22]:

$$\frac{1}{\Gamma_{HSA}} = \frac{1}{\Gamma_{HSA}(max)} + \frac{1}{\Gamma_{HSA}(max)K_A}\frac{1}{[HSA]_e} \quad . \qquad (1)$$

In Eq. (1), $\Gamma_{HSA}(max)$ denotes a maximal surface concentration of attached protein and K_A an equilibrium constant of adsorption. The dependence of $1/\Gamma_{HSA}$ on $1/[HSA]_e$ is shown in Fig. 2.

Values of $\Gamma_{HSA}(max)$, calculated from the plots in Fig. 2, were 1.44×10^{-3} and 1.14×10^{-3} g/m^2 and values of K_A were 3.00×10^4 and 3.40×10^4 l/g, for PS and PSA microspheres, respectively.

HSA is attached to PS microspheres exclusively by physical adsorption, mainly due to hydrophobic interactions. Attachment to PSA particles is, at least partially, due to the reversible formation of Schiff-base linkages (chemisorption) in the reaction of primary amino groups of protein and aldehyde groups of polyacrolein units in the surface layer of PSA particles [16, 17, 23]. Therefore, as one could expect, the value of K_A is larger for PSA than for PS microspheres. The higher value of the maximal surface concentration of HSA, $\Gamma_{HSA}(max)$, for PS than for PSA particles results from the following reasons. Molecules of HSA attached to microspheres undergo conformational changes (denaturation) and occupy a larger surface area [24, 25]. For PSA microspheres attachment via chemisorption freezes these conformations, whereas in the case of PS particles the physical adsorption of HSA allows conformational rearrangements of attached molecules, resulting in their denser packing [16, 23].

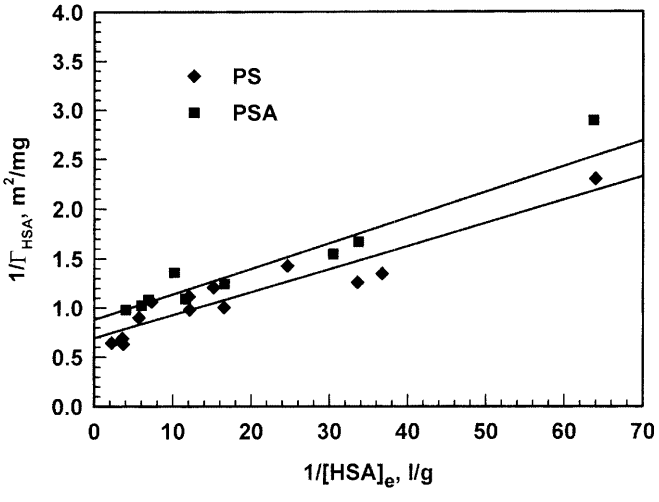

Fig. 2 Dependence of the reciprocal of the surface concentration of human serum albumin (*HSA*) attached to microspheres ($1/\Gamma_{HSA}$) on the reciprocal of the surface concentration of HSA remaining in solution ($1/[HSA]_e$)

Investigations of PS and PSA microspheres with attached HSA by XPS

A typical XPS spectrum of PS microspheres with adsorbed HSA is shown in Fig. 3.

Three signals are present in this low-resolution spectrum: at 285, 400, and 532 eV, due to electrons ejected from C1*s*, N1*s*, and O1*s* orbitals, respectively. The high-resolution signals, corresponding to these atoms, are shown in Fig. 4.

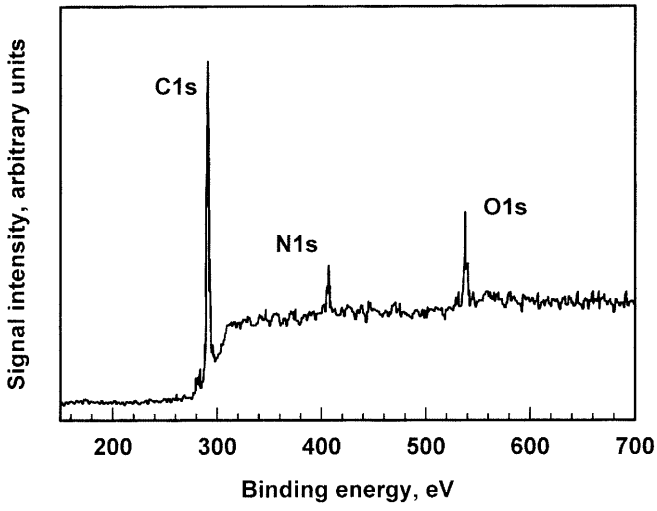

Fig. 3 Low-resolution X-ray photoelectron spectroscopy (*XPS*) spectrum of PS microspheres with adsorbed HSA

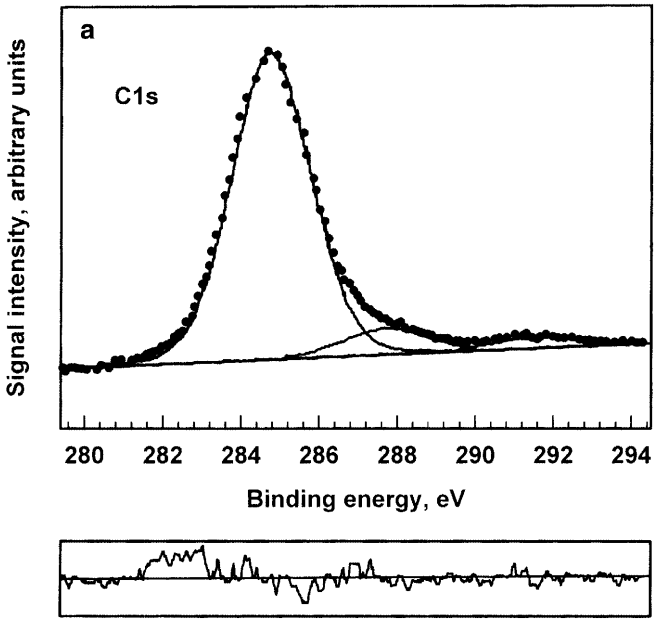

Fig. 4 Signals of **a** carbon, **b** oxygen, and **c** nitrogen atoms in high-resolution XPS spectra of PS microspheres with adsorbed HSA

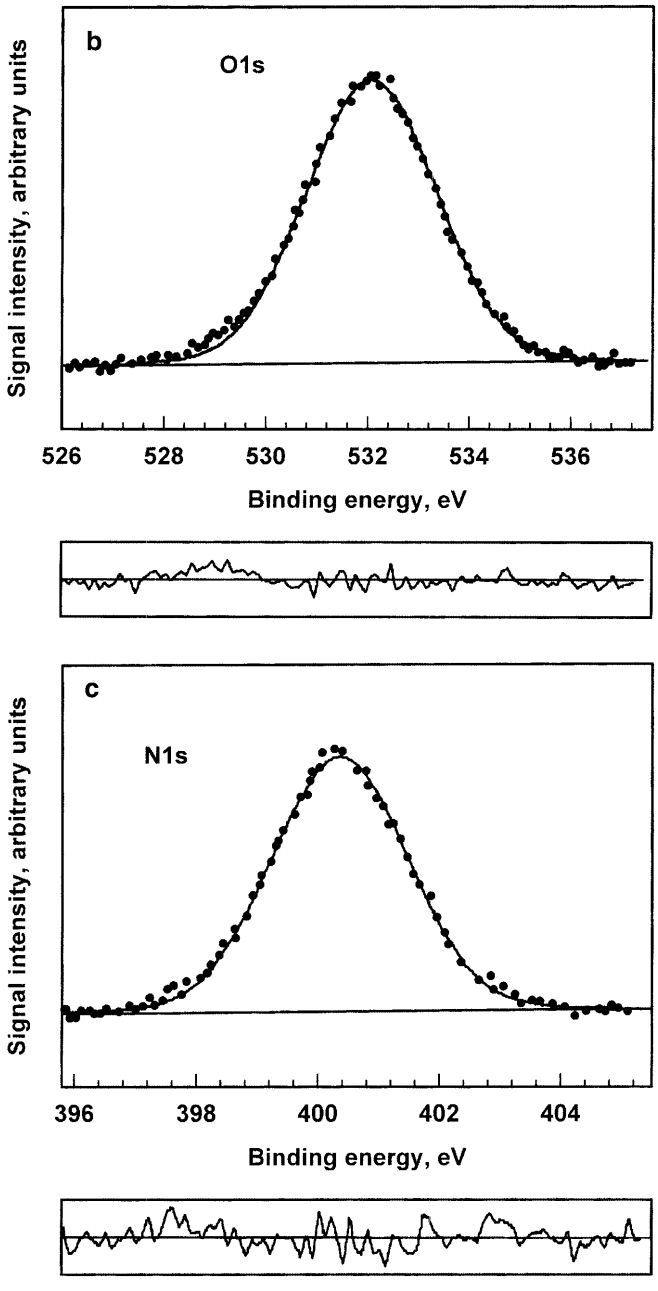

Fig. 4 (Continued)

It is worth noting that the C1*s* signal consists of three components. Besides the one centered at 284.8 eV (corresponding to aliphatic and aromatic carbon atoms) and that at 291.7 eV (a shake-up satellite due to the phenyl rings of styrene) there is also a signal at 287.9 eV due to the carbonyl carbon atoms in polyacrolein and in the amide groups of HSA. The O1*s* signal represents oxygen atoms of carbonyl groups of polyacrolein, $-OSO_3^-$ end groups due to the initiator incorporated into the macromolecules constituting the microspheres, oxygen atoms due to the often observed adventitious oxidation of the surface of the microspheres [9], and amide groups of HSA. Therefore, neither the C1*s* nor the O1*s* signal was convenient for the determination of HSA attached to the microspheres. Fortunately, the PS and PSA microspheres do not contain nitrogen atoms and their XPS spectra did not show any signals suggesting the presence of nitrogen-containing impurities. Thus, the N1*s* signal in the spectra of the microspheres with HSA at their surfaces was related exclusively to nitrogen of the protein and was used for the determination of the surface concentration of attached HSA.

The relation between the surface concentration of an analyzed substance and the intensity of the corresponding XPS signal (signal integral) is described by the Lambert–Beer law. In the case of the N1*s* signals of HSA attached to the surface, which does not contain nitrogen atoms, the equation corresponding to this law can be written in the following form:

$$\frac{I_{N1s}}{I^{\infty}_{N1s}} = 1 - \exp\left(-\frac{\Gamma_{HSA}}{\cos\theta\,\Gamma^{\infty}_{HSA}}\right) \quad . \tag{2}$$

In Eq. (2) I_{N1s} and I^{∞}_{N1s} denote the intensities of the nitrogen signals of an HSA layer at the surface of the microspheres and of an HSA layer of "infinite thickness" (layer several times thicker than the free electron path; I^{∞}_{N1s} was determined using pure HSA placed onto the sample holder of the XPS apparatus), respectively, Γ_{HSA} denotes the surface concentration of HSA at the microspheres, Γ^{∞}_{HSA} is a parameter characterizing the free electron path in HSA, and θ denotes the takeoff angle (relative to the surface) of ejected electrons. According to Seah and Dench [26] the value of Γ^{∞}_{HSA} (expressed in units of milligrams per square meter) can be calculated from the following equation:

$$\Gamma^{\infty}_{HSA} = 0.11E_K^{0.5} + 49E_K^{-2} \quad , \tag{3}$$

in which E_K denotes the kinetic energy of the N1*s* electrons (in electron-volts) ejected from HSA under the action of X-ray photons. In our system $E_K = 853.6$ eV and, thus, $\Gamma^{\infty}_{HSA} = 3.32$ mg/m^2.

The values of I_{N1s} and I^{∞}_{N1s} could be determined experimentally and, thus, for flat and smooth surfaces covered with HSA the simple combination of Eqs. (2) and (3) yields Eq. (4), allowing Γ_{HSA} to be calculated.

$$\Gamma_{HSA} = \Gamma^{\infty}_{HSA}\cos\theta\,\ln\left(\frac{I_{HSA}}{I^{\infty}_{HSA}}\right) \quad . \tag{4}$$

For the nonoverlapping spherical particles with diameters significantly greater than the electron free path the measured intensity is averaged over angles, θ, in the range from 0° to 90° (I_{N1s}) (Fig. 5a).

Therefore, Eq. (2) has to be replaced with the following:

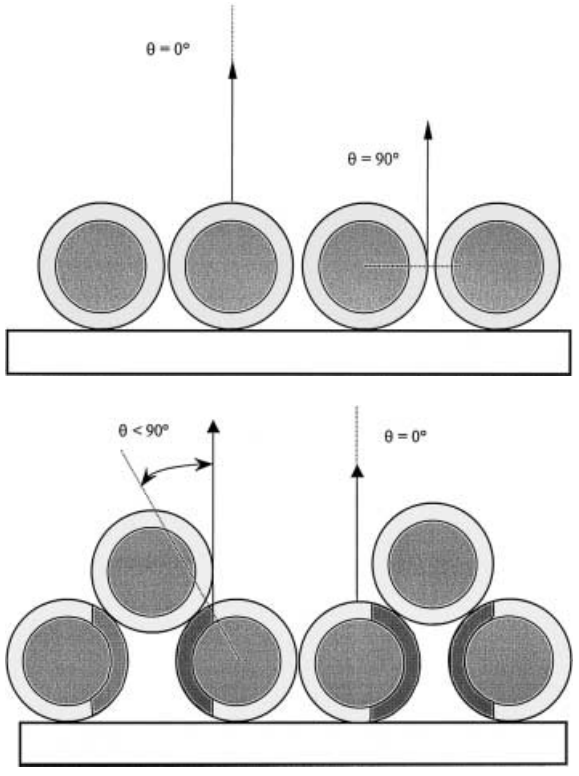

Fig. 5 **a** Range of the photoelectron takeoff angles from surfaces of microspheres forming a monolayer. **b** Schematic illustration of reduced range of the photoelectron takeoff angles from surfaces of microspheres in multilayers in which edges of some particles are screened by microspheres in the upper layer

$$\frac{I_{N1s}}{I^{\infty}_{N1s}} = 1 - \frac{2}{\pi}\int_0^{\pi/2} \exp\left(-\frac{\Gamma_{HSA}}{\cos\theta\,\Gamma^{\infty}_{HSA}}\right)d\theta \ . \qquad (5)$$

Numerical integration allowed the determination of the relation between $\Gamma_{HSA}/\Gamma^{\infty}_{HSA}$ and I_{N1s}/I^{∞}_{N1s}.

This relation is shown in Fig. 6 for a system of nonoverlapping spherical particles (calculations based on Eq. 5) and for a flat and smooth surface with $\theta = 0°$ (calculations based on Eq. 4).

From Fig. 6 it follows that for the values of I_{N1s}/I^{∞}_{N1s} in the range 0–0.4 the corresponding values of Γ_{HSA} can differ significantly for smooth surfaces and for surfaces covered with spherical particles.

In real systems the surface analyzed is usually composed of a multilayer of randomly arranged microspheres. For these systems the edges of some microspheres are screened by other particles (see schematic illustration in Fig. 5b); therefore, the takeoff angle should be averaged in a narrower range ($0° \le \theta \le \alpha$, where α denotes the average value of an angle characterizing the screening of the edges of the microspheres) than in the case of the nonoverlapping microspheres. Therefore, for the real systems, with surfaces covered with multilayers of microspheres, the following equation holds:

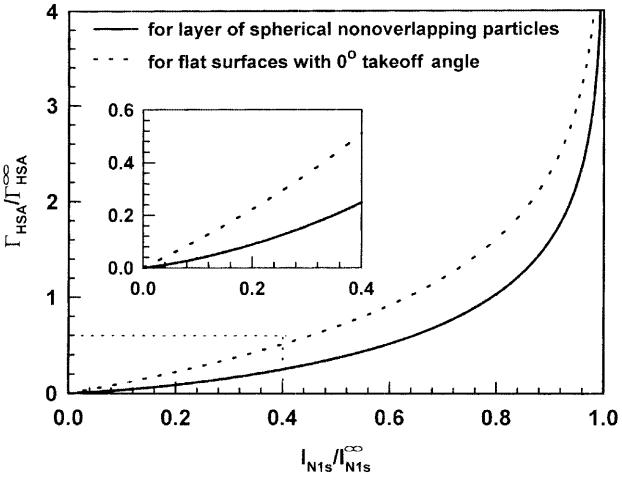

Fig. 6 Relations between the ratios $\Gamma_{HSA}/\Gamma_{HSA}$ and I_{N1s}/I^{∞}_{N1s} calculated assuming that the surface analyzed is flat and/or composed of nonoverlapping microspheres

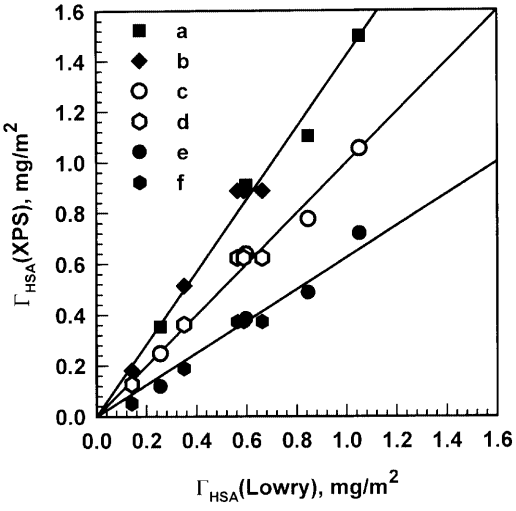

Fig. 7 Correlation between surface concentrations of HSA attached to PS and PSA microspheres determined by XPS and surface concentration of attached HSA determined by the Lowry method. The XPS data were analyzed with the assumption that a surface with adsorbed protein is flat and smooth (*a* data for PS, *b* for PSA microspheres), is composed of nonoverlapping microspheres (*e* PS, *f* PSA particles), and is composed of a multilayer of microspheres (*c* PS, *d* PSA), in which, due to the edge screening, the average maximal photoelectron takeoff angle is 72.7°

$$\frac{I_{N1s}}{I^{\infty}_{N1s}} = 1 - \frac{1}{\alpha}\int_0^{\alpha} \exp\left(-\frac{\Gamma_{HSA}}{\cos\theta\,\Gamma^{\infty}_{HSA}}\right)d\theta \ . \qquad (6)$$

The correlation between the values of the surface concentrations of HSA determined by XPS [Γ_{HSA}(XPS)] and independently by the Lowry method [Γ_{HSA}(Lowry)] is illustrated in Fig. 7.

Calculations of Γ_{HSA}(XPS) were performed for three models: assuming that the surface analyzed is flat

(takeoff angle = 0°), assuming that the support is covered with nonoverlapping microspheres, and assuming that the support is covered with overlapping particles. For the first model the slope of the plot of Γ_{HSA}(XPS) versus Γ_{HSA}(Lowry) was 1.42, indicating that the analysis of the XPS data using Eq. (4) yields results which are overestimated by 42%. For the second model the slope of a similar plot was 0.625, which indicates that the values of Γ_{HSA}(XPS) calculated using Eq. (4) are underestimated by 37.5%. The best result was obtained for the third model, taking into account partial overlapping of the microspheres. When $\alpha = 72.7°$ the slope is 0.988 and the regression coefficient is 0.978, indicating good agreement between the surface concentrations of adsorbed HSA determined by XPS and by the Lowry method. It is worth noting that for both types of microspheres (for the PS particles onto which HSA is adsorbed and for the PSA microspheres onto which some fraction of HSA is immobilized covalently [16, 17, 23]), the best results were obtained using the same procedure for the treatment of the XPS data.

Conclusions

Surface microroughness is very important in quantitative analysis of the XPS data. For substances composed of spherical micron-sized particles the surface concentrations of adsorbed and/or covalently immobilized compounds can be determined using Eq. (6) with $\alpha = 72.7°$.

Acknowledgements This work was realized with the support of KBN Grant 3 T09 033 11 and the Program Polonium supporting collaboration of Polish and French research groups.

References

1. Watts JF (1990) An introduction to surface analysis by electron spectroscopy. Oxford University Press and Royal Microscopical Society, Oxford
2. Briggs D (1990) In: Briggs D, Seah MP (eds) Practical surface analysis. Vol 1 Auger and X-ray photoelectron spectroscopy. Wiley, New York, p 437
3. Lewis KB, Ratner BD (1993) J Colloid Interface Sci 159:77
4. Okubo M, Yamamoto Y, Kamei S (1989) Colloid Polym Sci 267:861
5. Brindley A, Davies MC, Lynn RAP, Davis SS, Hearn J, Watts JF (1992) Polymer 33:1112
6. Davies MC, Lynn RAP, Davis SS, Hearn J, Watts JF, Vickerman JC, Johnson D (1993) J Colloid Interface Sci 156:229
7. Stone-Masui JH, Stone WEE (1993) Polym Int 30:169
8. Kobayashi S, Uyama H, Choi JH, Matsumoto Y (1993) Polym Int 30:265
9. Basinska T, Slomkowski S, Delamar M (1993) J Bioact Compat Polym 8:205
10. Maeda S, Corradi R, Armes SP (1995) Macromolecules 28:2905
11. Davies MC, Lynn RAP, Hearn J, Paul AJ, Vickerman JC, Watts JF (1995) Langmuir 11:4313
12. Shakesheff KM, Evora C, Soriano I, Langer R (1997) J Colloid Interface Sci 185:538
13. Sheng E, Southerland I (1994) Surf Sci 314:325
14. Basinska T, Slomkowski S (1991) J Biomater Sci Polym Ed 3:115
15. Slomkowski S, Basinska T (1992) In: Daniels ES, Sudol ED, El-Assse MS (eds) Polymer latexes, preparation, characterization, and applications. ACS Symposium Series 492. American Chemical Society, Washington, DC, p 328
16. Basinska T, Slomkowski S (1993) In: Guilbault G, Mascini M (eds) Uses of immobilized biological compounds. Kluwer, Dordrecht, p 453
17. Basinska T, Kowalczyk D, Miksa B, Slomkowski S (1995) Polym Adv Technol 6:526
18. Miksa B, Slomkowski S (1995) J Biomater Sci Polym Ed 7:77
19. Slomkowski S (1998) Prog Polym Sci 23:815
20. Beamson G, Briggs D (1992) High-resolution XPS of organic polymers. The Sienta ESCA 300 Database. Wiley, p 72
21. Moelvyn-Hughes EA (1961) Physical chemistry, 2nd revised edn. Pergamon, Oxford, pp 952
22. Andrade JD, Hlady V (1986) Adv Polym Sci 79:1
23. Basinska T, Kowalczyk D, Slomkowski S, Wang FW (1994) In: Schmitz KS (ed) Macro-ion characterization from dilute solutions to complex fluids. ACS Symposium Series 548, American Chemical Society, Washington, DC, p 449
24. Kowalczyk D, Slomkowski S, Wang FW (1994) J Bioact Compat Polym 9:282
25. Kowalczyk D, Marsault J-P, Slomkowski S (1996) Colloid Polym Sci 274:513
26. Seah MP, Dench WA (1979) Surf Interface Anal 1:2