JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 839–843

In situ ATR/FTIR studies of protein adsorption on polymeric materials: effectiveness of surface heparinization

A. MAGNANI, E. BUSI, R. BARBUCCI
C.R.I.S.M.A. and Dipartimento di Chimica-Università di Siena Via E. Bastianini, 12 and Piano dei Mantellini, 44-53100 Siena-Italy

The adsorption of two proteins from human plasma (human serum albumin (HSA) and human fibrinogen (HFG)) onto six different polymeric surfaces (two of which are heparinized), has been studied by *in situ* ATR/FTIR spectroscopy. The different surface characteristics are reflected by different interfacial behaviours of the two proteins, but while both proteins unfold upon adsorption on all the different non-heparinized materials, they maintain the native conformation once adsorbed on the heparinized surfaces. These findings emphasize the effectiveness of surface heparinization.

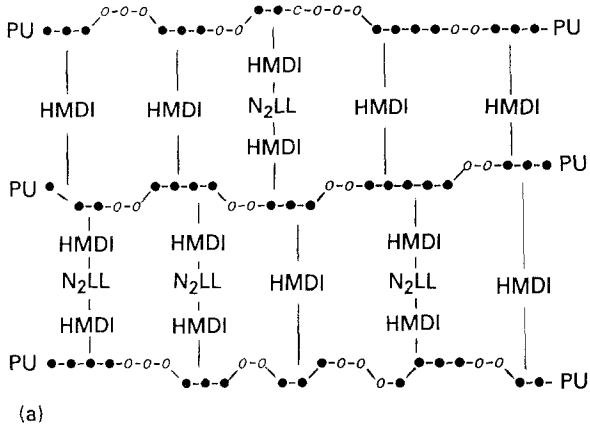
1. Introduction

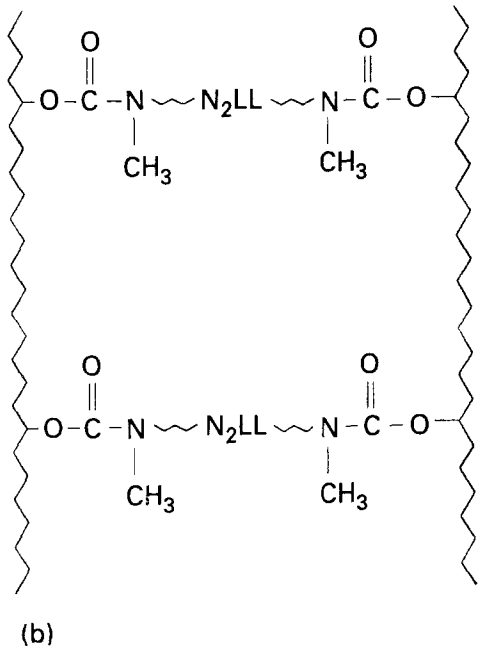
Since the major complication in the use of blood-contacting materials is the formation of a thrombus at the solid–liquid interface, current research is concerned with investigating surface modifications to improve the "performance" of these devices. Of the different approaches for optimizing surface biocompatibility, surface heparinization is a well-accepted strategy. Heparinized materials appear in fact to be highly compatible with both platelets and the plasma coagulation system [1]. Research has concentrated on methods for binding heparin to biomaterial surfaces and on understanding the reasons for the enhanced antithrombogenicity of materials after heparinization [2–10].

The work presented here deals with the study of the interaction of two plasma proteins (human serum albumin (HSA) and human fibrinogen (HFG)) with polymeric surfaces, some of which contain ionically bound heparin, in an attempt to understand the role of bound heparin in the enhancement of the material thromboresistance.

The polymeric materials analysed here are a commercial polyurethane (Pellethane® 2363-80AE (PU)), a commercial, partially hydrolized polyethyl-vinyl acetate (EVALVA), two network materials (PUPA and EVAPA) (see Scheme 1) obtained by crosslinking poly(amido-amine) chains to those of PU and EVALVA respectively [11–13], and the heparinized PUPA and EVAPA.

Both PUPA and EVAPA are able to bind heparin because they are protonated at physiological pH [14] and the heparin binding to the surface occurs through an electrostatic interaction between the negatively charged groups of heparin and the protonated aminic nitrogens of the materials [15]. The effectiveness of the surface heparinization is well documented [16].





Scheme 1(a) Structure of PUPA; (b) structure of EVAPA.

2. Experimental procedure

2.1. Proteins

HSA (non-denaturated human serum albumin, 65 000 MW, purity > 99%) and HFG (fibrinogen from human plasma, 341 000 MW, clottable proteins > 95%) have been supplied by Calbiochem, CA, USA.

HSA was used as received, and HFG was dialysed against PBS (phosphate-buffered solution) before usage.

2.2. Method

Protein adsorption experiments were carried out by surface infrared spectroscopy under flowing conditions using a dual channel ATR flow cell [17, 18].

The ATR crystals were precoated with 10–20 nm thick films of the materials to be analysed. The coatings must be much thinner than the depth of penetration of the i.r. beams (about 400–600 nm over the region of interest (1800–1200 cm^{-1})) to allow protein adsorption to be followed directly on the polymer surfaces.

After each polymer coating was equilibrated with the recirculating saline solution, the spectrum of the cell filled with saline solution was collected and stored for later use.

Adsorption experiment. The protein solution was then introduced into the flow cell and data collection was started. Spectra were collected at predetermined short time intervals. All the spectra were collected in single beam (SB) mode and the absorbance spectra were automatically calculated by the following ratios:

$$\frac{Spectrum\ of\ (protein\ +\ saline\ +\ polymer\text{-}coated\ crystal)_{SB}}{Spectrum\ of\ (polymer\text{-}coated\ crystal)_{SB}} = A_{protein} + A_{saline} \quad (1)$$

$$\frac{Spectrum\ of\ (saline\ +\ polymer\text{-}coated\ crystal)_{SB}}{Spectrum\ of\ (polymer\text{-}coated\ crystal)_{SB}} = A_{saline} \quad (2)$$

Subtraction of Equation 2 from Equation 1 gives the spectrum of the protein.

Washout experiment. A similar scanning sequence was used when the bulk protein was displaced by fresh saline.

3. Results and discussion

3.1. Kinetics

Fig. 1 shows the kinetic plots for the adsorption of HSA and HFG on all the analysed surfaces.

These kinetic curves have been obtained by plotting the absorbance at 1550 cm^{-1}, which is sensitive to the amount of the protein, against time. All the absorbance values (AU = absorbance units) have been multiplied by a factor 10.

As can be seen from the curves, the behaviour of the two proteins is quite similar, and a greater amount of both HSA and HFG is irreversibly bound to the native surfaces than to the heparinized ones. This could be interpreted as a result of the higher hydrophilicity of the heparinized surfaces with respect to the bare polymers and/or electrostatic repulsion occurring between the proteins and the heparinized surfaces at pH = 7.4 (as for HSA and HFG, the heparinized PUPA and EVAPA are in fact negatively charged at this pH).

From the kinetic curves it is also evident that the greatest amount of protein is adsorbed on the most hydrophobic surface, which is PU.

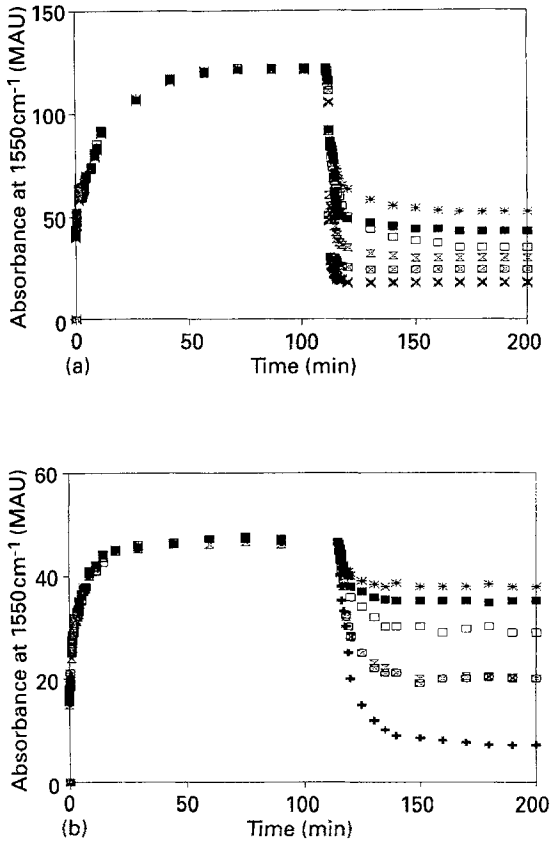


Figure 1 (a) Kinetic plots for the adsorption of HSA (*Pellethane; ⧖ PUPA; ⊠ heparinised PUPA, □ EVAPA; × heparinized EVAPA; ■ EVALVA); (b) kinetic plots for the adsorption of HFG (*Pellethane; ⧖ heparinized PUPA; ⊠ PUPA; □ EVAPA; + heparinized EVAPA; ■ EVALVA.

3.2. Conformation

In Fig. 2 the spectra of the proteins adsorbed on the two heparinized surfaces are compared to those of the native proteins.

It is evident that the spectra of the two proteins after surface adsorption are very similar to those of the corresponding non-adsorbed proteins, and no time-dependent changes in the amide I, II and III bands are observed even after 4 h of residence on the surface. This feature is described quantitatively in Table I by the band intensity ratio of the main peaks of the amide III region.

The great similarity between the adsorbed and native HSA and HFG spectra suggests that the heparin-

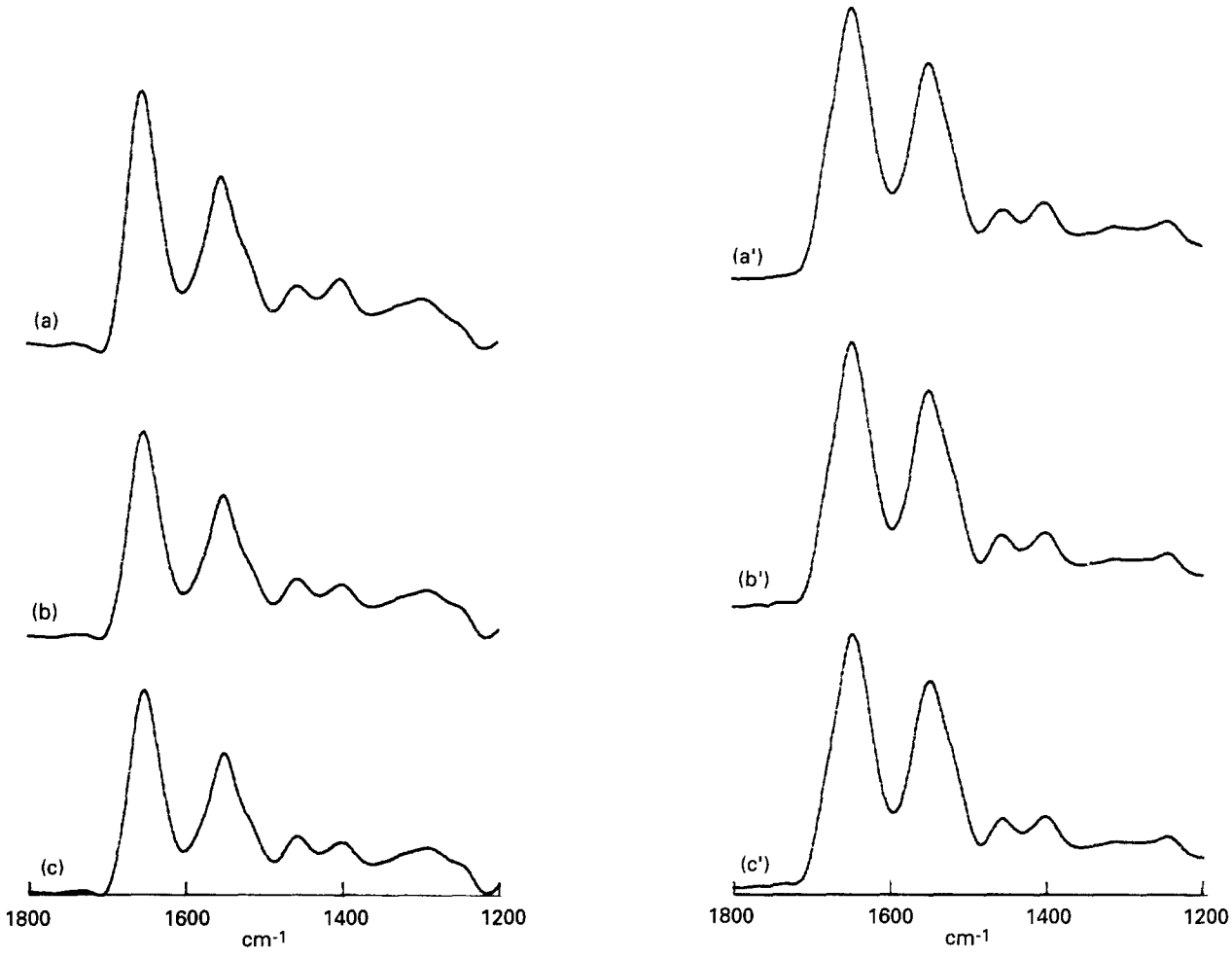


Figure 2 Spectra of: (a) native HSA; (b) HSA adsorbed on heparinized PUPA, (c) HSA adsorbed on heparinized EVAPA; (a') native HFG; (b') HFG adsorbed on heparinized PUPA, (c') HFG adsorbed on heparinised EVAPA. (Spectra collected at 1 min and 4 h residence time are identical).

TABLE I Amide III components intensity ratio

	HSA 1300 cm^{-1}/1242 cm^{-1} intensity ratio	
Native	Adsorbed on heparinized surfaces	
	after 1 min	after 4 h
1.70 ± 0.1	1.60 ± 0.3	1.60 ± 0.2
	HFG 1282 cm^{-1}/1240 cm^{-1} intensity ratio	
Native	Adsorbed on heparinized surfaces	
	after 1 min	after 4 h
0.48 ± 0.2	0.50 ± 0.2	0.50 ± 0.3

ized surface does not induce significant conformational changes on the two plasma proteins either at the initial stage of adsorption or with increase of the residence time of the proteins on the surface.

However, a peculiar behaviour of the interaction of the heparinized EVAPA surface with the first layer of adsorbed HSA must be addressed. The initially adsorbed protein seems to interact very strongly with the heparin bound to the surface, leading to the formation of a "complex", as revealed by the i.r. spectra collected during the first few seconds of adsorption (Fig. 3). The protein which later adsorbs, behaves instead, as observed for the heparinised PUPA.

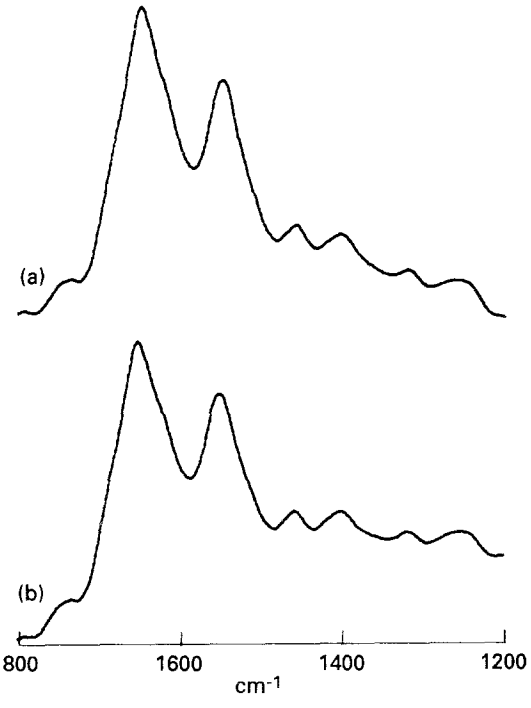


Figure 3 (a) Spectrum of HSA adsorbed on heparinized EVAPA at 2 s adsorption time; (b) spectrum of HSA-heparin system in water.

HSA and HFG change their conformation once adsorbed on all the other surfaces. The spectra of both HSA and HFG adsorbed on PUPA and EVAPA surfaces (Fig. 4) show a wider amide I band, due to the

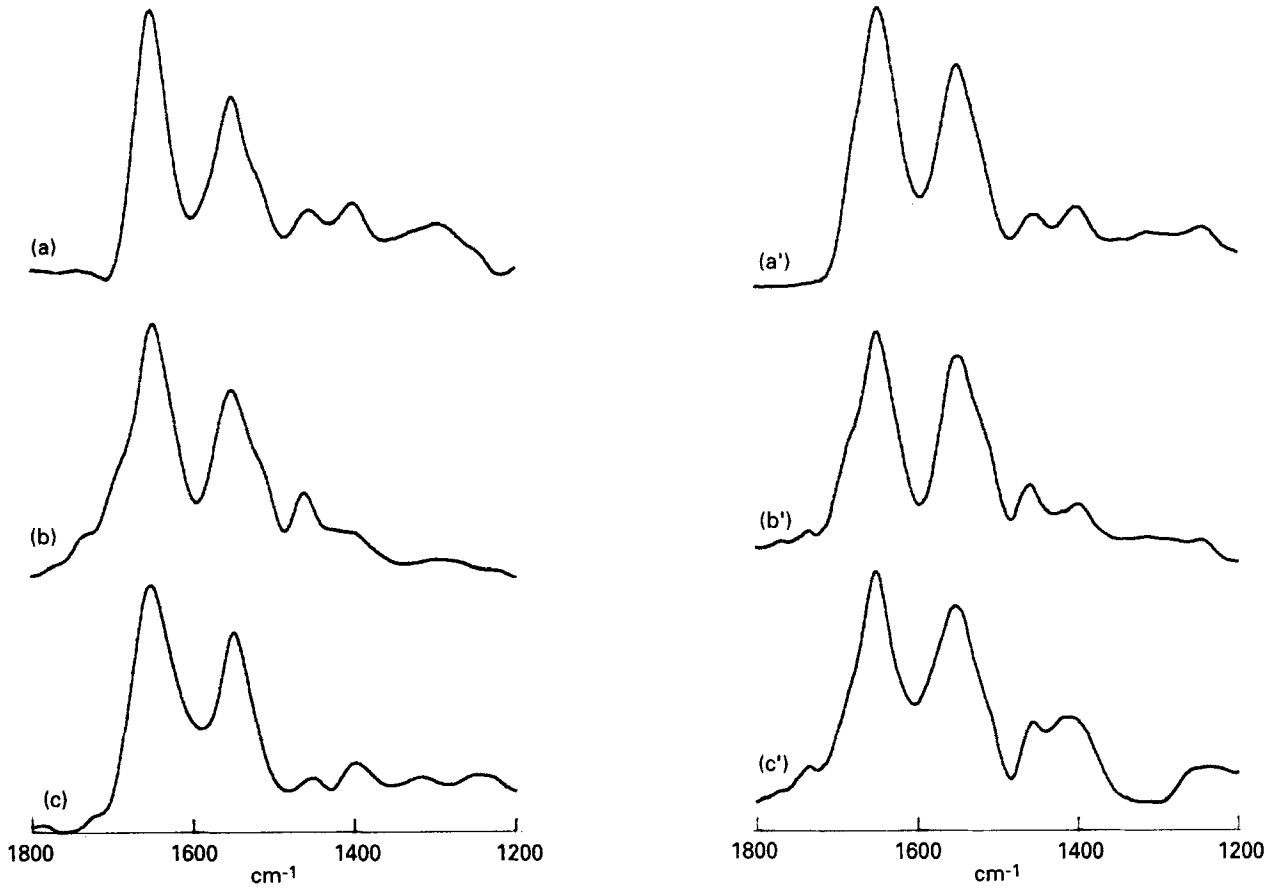


Figure 4 Spectra of: (a) native HSA; (b) HSA adsorbed on PUPA; (c) HSA adsorbed on EVAPA; (a') native HFG, (b') HFG adsorbed on PUPA, (c') HFG adsorbed on EVAPA. (Spectra collected at 1 min and 4 h residence time are identical).

presence of a wider number of microstructures. A loss of symmetry of the amide II band and a variation of the relative intensity of the amide III component are in accordance with a loss of α-helix and a gain of β structures [19]. The increase of the residence time of the adsorbed proteins on the surfaces does not cause further changes in their secondary structure.

In the case of PU and EVALVA surfaces the conformational changes of the adsorbed proteins are time dependent. The spectra of the two proteins initially adsorbed are not very different from those of the corresponding proteins in solution (Figs 5 and 6). This suggests that the structure of the initially adsorbed protein is the same as that in solution. Greater changes are evident with increasing residence time, suggesting that the unfolding and denaturation of the two proteins adsorbed on these surfaces is time dependent.

By considering the "three domains" model for human albumin [20, 21] (which includes the three major domains and the disulfide-bonded α-helical subdomains) it is known that domains I and II are negatively charged (net charge = − 9 and − 8, respectively), whereas domain III is weakly positive (net charge = + 2) at pH = 7 [21]. Domains I and II are known to be less stable than domain III [20].

When human albumin adsorbs at the negatively charged heparinized surfaces, domain III would tend to adsorb, thus little conformational change is expected due to the stability of domain III as it contains bound fatty acid. The infrared spectra prove this hypothesis.

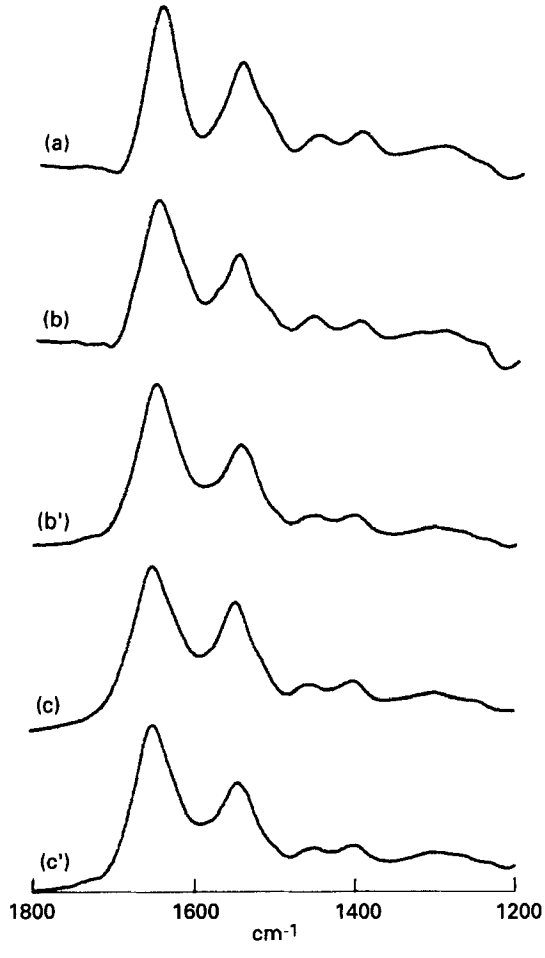


Figure 5 Spectra of: (a) native HSA; (b) HSA adsorbed on PU after 1 min and (b') after 4 h of residence time, (c) HSA adsorbed on EVALVA after 1 min and (c') after 4 h of residence time.

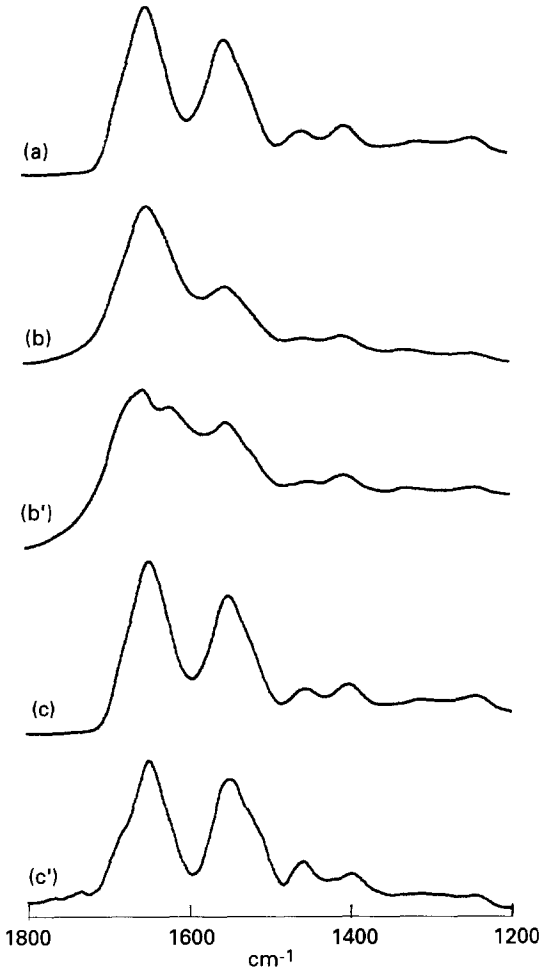


Figure 6 Spectra of: (a) native HFG; (b) HFG adsorbed on PU after 1 min, and (b') after 4 h of residence time, (c) HFG adsorbed on EVALVA after 1 min, and (c') after 4 h of residence time.

In contrast, when the protein adsorbs on the positively charged PUPA and EVAPA surfaces, domains I and II should be preferentially bound; since these domains are less stable than domain III [20] the adsorbed protein is more denaturated, as shown by infrared spectra.

The adsorption of the protein on the hydrophobic PU and EVALVA surfaces is not governed by electrostatic interactions. In this case the first loop in domain I, which is the most hydrophobic, would tend to bind. As this loop is less stable than other loops, a slow time-dependent denaturation would be expected. This is confirmed by the time-dependent conformational changes observed in the spectra of the surface adsorbed protein.

For the case of fibrinogen we cannot propose the same mechanisms, because of the lack of the needed structural information; from the infrared data we may, however, assume that the heparinized surface prevents the unfolding and denaturation of fibrinogen as much as for albumin.

4. Conclusions

It is evident from infrared data that the surface structure, hydrophobicity, and charge affect the behaviour of proteins at interfaces. The most hydrophobic surfaces adsorb the greatest amount of protein and the protein unfolding at the surface is time-dependent.

The amount of protein adsorbed on all the hydrophilic surfaces is nearly the same in the case of albumin, while some differences occur for fibrinogen. However, proteins interacting with the positively charged surfaces experience a conformational change because of their initial adsorption mechanism, while the negatively charged heparinized surfaces seem to act in a manner so as to prevent a change in conformation of the adsorbed proteins.

These behaviours emphasize the effectiveness of surface heparinization in enhancing blood-surface compatibility.

Acknowledgement

The authors thank the Italian Research Council (C.N.R.) "Progetto Speciale Biomateriali" for financial support.

References

1. Y. ITO, *J. Biomater. Applic.* **2** (1987) 235.
2. R. SHIBUTA, M. TANAKA, M. SISIDO and Y. IMANISHI, *J. Biomed. Mater. Res.* **20** (1986) 971.
3. D. BASMADJIAN and M. V. SEFTON, *ibid.* **17** (1983) 509.
4. Y. NOISHIKI, S. NAGAOKA, T. KIKUCHI and Y. MORI, *Trans. Amer. Soc. Artif. Intern. Organs* **27** (1981) 213.
5. Y. ITO, M. SISIDO and Y. IMANISHI, *J. Biomed. Mater. Res.* **20** (1986) 1017.
6. Y. MIURA, S. AOYAGI, Y. KUSADA and K. MIYAMOTO, *ibid.* **14** (1980) 619.
7. M. F. A. GOOSE and M. V. SEFTON, *ibid.* **17** (1983) 359.
8. O. LARM, R. LARSSON and P. OLSSON, *Biomat. Med. Dev. Art. Org.* **11** (1983) 161.
9. P. W. HEYMAN, C. S. CHO, J. C. McREA, D. B. OLSEN and S. W. KIM, *J. Biomed. Mater. Res.* **19** (1985) 419.
10. R. BARBUCCI, A. MAGNANI, A. ALBANESE and F. TEMPESTI, *ASAIO J.* **14** (1991) 409.
11. S. CIMMINO, E. MARTUSCELLI, C. SILVESTRE, R. BARBUCCI, A. MAGNANI and F. TEMPESTI, *J. Polym. Sci.* **47** (4) (1993) 631.
12. R. BARBUCCI, M. BENVENUTI, G. DAL MASO, M. NOCENTINI, F. TEMPESTI, M. LOSI and R. RUSSO, *Biomaterials* **10** (1989) 299.
13. R. BARBUCCI, M. BENVENUTI, A. MAGNANI and F. TEMPESTI, *Die Makromolekulare Chemie* **193** (1992) 2979.
14. R. BARBUCCI, M. CASOLARO, A. MAGNANI and C. RONCOLINI, *Polymer* **32** (1991) 897.
15. R. BARBUCCI, A. MAGNANI and C. RONCOLINI, *Clin. Mater.* **8** (1991) 17.
16. A. ALBANESE, R. BARBUCCI, J. BELLEVILLE, S. BOWRY, R. ELOY, H. D. LEMKE and L. SABATINI, *Biomaterials* **15** (1994) 129.
17. R. M. GENDREAU, in "Spectroscopy in the biomedical sciences" (Boca Raton, CRC Press, 1986) pp. 21–52.
18. A. MAGNANI and R. BARBUCCI, in "Test procedures for the blood compatibility of biomaterials" edited by S. Dawids (Kluwer Academic Publisher, The Netherlands, 1993) pp. 171–184.
19. T. J. LENK, B. D. RATNER, R. M. GENDREAU and K. K. CHITTUR, *J. Biomed. Mater. Res.* **23** (1989) 549.
20. T. Jr. PETERS, *Adv. Protein Chem.* **37** (1985) 161.
21. J. D. ANDRADE, V. HLADY, A-P. WEI, C-H. HO, A. S. LEA, S. I. JEON, Y. S. LIN and E. STROUP, *Clin. Mater.* **11** (1992) 67.