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Polymer 43 (2002) 3541–3548

polymer

www.elsevier.com/locate/polymer

The effects of spacer arms in cross-linked hyaluronan hydrogel on Fbg and HSA adsorption and conformation

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Dedicated to Professor Imanishi on the occasion of his retirement

Received 5 December 2001; received in revised form 15 January 2002; accepted 15 January 2002

Abstract

Four hyaluronan based hydrogels with different cross-linking agents were synthesised. Compounds with an increasing hydrophobic character and length as spacer arms were chosen, i.e. *O*,*O*′-bis(2 aminopropyl)polyethylen glicol, 1.3 diaminopropane, 1.6 diaminohexane, and 1.12 diaminedodecane. The cross-linking reaction involved 50% of the carboxylate groups present in the hyaluronan macromolecule chain. Hydrogels were characterised by water-uptake measurements and SEM analysis. The adsorption of two plasma proteins (HSA and Fbg) was analysed onto the four hydrogels in flow conditions by ATR-FTIR, evaluating the adsorption kinetics and the eventual protein conformational changes. The increasing hydrophobic character of the surface imposes a clear trend to the protein adsorption kinetics. The influence of the spacer arms on the protein conformation is evident on HSA, whereas Fbg does not seem to be significantly influenced by the change of the substrate hydrophobic properties.

Keywords: Hydrogel; Plasma protein; Adsorption

1. Introduction

One of the most important goals regarding research on blood compatible materials is the knowledge of the events that occur at the material surfaces during the initial few seconds and minutes of contact with blood or plasma. It is accepted that artificial surfaces placed in contact with blood rapidly acquire a layer of adsorbed plasma proteins. It is also well known that the biocompatibility, and consequently the clinical success of the implants, is strictly dependent on this phenomenon. Upon protein adsorption, conformational changes may occur depending on the features of the solid surface. In general, it may be expected that conformational changes or denaturation of protein be more pronounced at an interface with a high interfacial free energy. When proteins undergo these conformational changes, the free energy gain increases and for this reason adsorption onto hydrophobic surfaces is usually an irreversible process [1]. The knowledge of such changes is fundamental for the development of a device because they determine the 'good' or 'bad' performance of the material used for the implant. The denaturation of the adsorbed plasma protein usually induces adhesion, aggregation and activation of platelets with the consequent thrombus formation.

Two of the most important blood proteins involved in the surface–biological fluids interaction are albumin and fibrinogen. The importance of the first protein is due to its high concentration in the plasma and the second protein presents one of the highest surface affinities. Over the last decades, several research groups studied the adsorption of these proteins on the surface of several materials for the realisation of implants, using different techniques such as circular dichroism [2], measurements of adsorption enthalpies [3], etc. These studies demonstrated that polyoxymethylene, polyaminoetherurethaneurea [4], and polyethylene [5] provoked a decrease in the α-helix content of human serum albumin and other plasma proteins, such as fibrinogen or complement factor 3 (C3) [6], indicating a loss of their conformation upon adsorption. Other researchers reported the same change of conformation on silicone and polypeptide-coated glass beads [7].

Actually, several new techniques are being developed to study this phenomenon. Lhoest et al. used time of flight-secondary ion mass spectrometry (ToF-SIMS) associated with principal component analysis (PCA) to study the adsorbed protein films [8]. We used the infrared spectroscopy coupled with the attenuated total reflection technique (ATR–FTIR), to observe the behaviour of two plasma

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0032-3861/02/$ - see front matter
PII: S0032-3861(02)00070-8

proteins (HSA and Fbg) onto polysaccharide-based hydrogels, in terms of adsorption kinetics and conformation. These materials are highly biocompatible because they absorb a large amount of water, which is the most widely diffused component of biological fluids. We synthesised four different 50% hyaluronan based hydrogels (i.e. 50% of the carboxylate groups present in the hyaluronan macromolecule chain were involved in the cross-linking reaction) using O,O'-bis(2 aminopropyl)polyethyleneglycol with a molecular weight of 800 (PEG 800), 1.3 diaminepropane (1.3 DAP), 1.6 diaminehexane (1.6 DAE) and 1.12 diaminedodecane (1.12 DAD), as cross-linking agents. The cross-linkers showed different hydrophobic characters that increased in the following order: PEG 800 $<$ 1.3 DAP $<$ 1.6 DAE $<$ 1.12 DAD. The conformation of the protein adsorbed, starting from the first layer to the final one, was analysed in order to verify whether some variations occurred during the protein accumulation upon the surface.

2. Materials

The sodium salt of the polymer was supplied by Biophil Italia S.p.A.

O,O'-bis(2 aminopropyl)polyethylen glicol, 1.3 diaminepropane, 1.6 diaminehexane, 1.12 diaminedodecane, N,N'-dimethylformamide (DMF), 2-chloro-1-methylpyridinium iodide (CMP-J), tetrabutylammonium hydroxide (TBA), triethylamine, ethanol, and all the other reagents were purchased from Fluka Chemie AG (Switzerland).

Human fibrinogen and human serum albumin were purchased from Calbiochem (Germany).

3. Methods

3.1. Synthesis

The synthesis of the hydrogels has already been described [9]. Briefly, the polysaccharide was transformed into a TBA salt by exchange column and dissolved in dimethylformamide (DMF), by stirring and nitrogen flow. Maintaining the solution at about 0 °C, the stoichiometric amount of chloromethylpyridine iodide (CMP-J) required to activate the prefixed percentage of the carboxylate groups, (50%) was added. An excess of the cross-linking agent was then added along with a small amount of triethylamine as a catalyst. The gel formed immediately, but the process was completed by stirring the mixture for approximately 4 h at room temperature. The gel was washed several times with EtOH and water, and dried in a vacuum oven at 40 °C for 24 h.

3.2. Ninhydrin test

A small quantity of the gel was suspended in 1 ml of sodium acetic/acetate (1 M) buffer pH 5 in a test tube and a point quantity of ninhydrin was added. The mixture was left in boiling water for 15 min and then 15 ml of 1:1 (v/v) ethanol/water mixture was added and left in a dark cupboard for 1 h. With free aminic groups the colour of the mixture becomes violet [10].

3.3. Potentiometric titration

Potentiometric titrations were carried out, as previously described [11], in a thermostatic glass cell at 25 °C, at a constant ionic strength of 0.1 M NaCl. The dried gels were finely dispersed and added to the 0.1 M NaCl solution with a known amount of 0.1 M HCl. Then titration was performed with 0.1 M NaOH and back titration with 0.1 M HCl. The default conditions for the experiment were a stabilisation time of 60 min for the initial system and a delay time of 18 min for every addition of the titrant.

The titration data was collected by a Crison Micro-pH-2002, equipped with a combined electrode (mod.6.0204.000), together with an automatic Crison microburette (mod. 3031), connected to a PC.

3.4. Scanning electron microscopy

Scanning electron microscopy (SEM) of cooled and lyophilised gels was performed to analyse their morphology. Water swollen gels (2.5 mg) were put in cryotubes and cooled by liquid nitrogen. After cooling, gels were lyophilised, mounted on SEM stubs and gold-sputtered with an automatic sputter coater (BAL-TEC SCD 050, Balzer). The morphology and structure of the gels were imaged using an XL 20 SEM (Philips) operated at 15 kV.

3.5. Water-uptake measurements

Established amounts (the starting weight W_d was obtained after drying the gels in a vacuum oven at 40 °C for 24 h) of cross-linked dry-polymers were enclosed in small bags of hydrophobic water-permeable nets (Nylon) and immersed at room temperature in 50 ml of buffer solution (PBS pH 7.4). At regular intervals the bags were removed from the solvent, their surfaces were pressed gently with tissue paper to remove the excess of solvent on the surface, weighed and then returned to the medium. This process of water-uptake and weighing were continued until the gels attained a constant final weight (W_s). Water-uptake was measured at intervals of 24 h, for five consecutive days, after which, no water-uptake changes were observed.

$$\text{Water-uptake} = \frac{W_s - W_d}{W_d} 100$$

3.6. FTIR–ATR analysis

The apparatus used for the ATR/FTIR experiments consisted of a dual channel ATR flow cell and a plumbing set-up, as shown in Fig. 1. The swollen hydrogels were

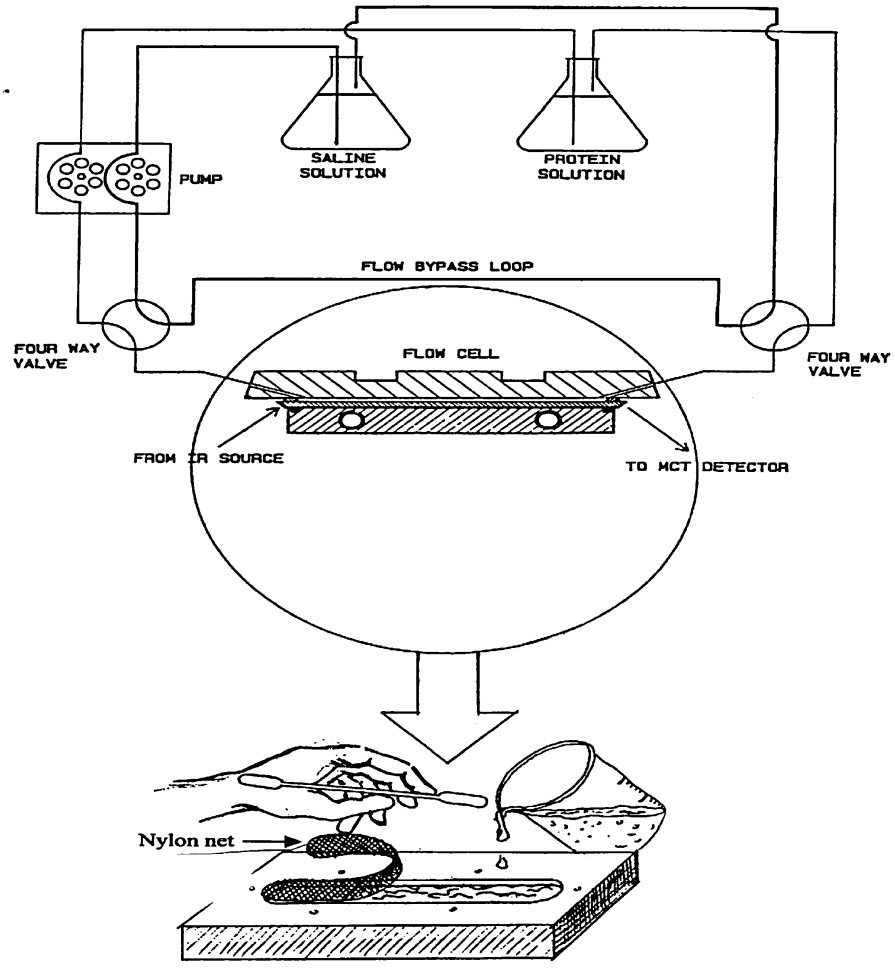

Fig. 1. Schematic representation of the apparatus for protein adsorption (ATR flow cell).

placed over the germanium ATR crystals of the flow cell to study surface interaction.

The adsorption–desorption of Fbg on the different hydrogels was evaluated starting from a solution of 2 mg ml^{-1} in PBS (pH 7.4), under laminar flow conditions (75 ml min^{-1}).

The adsorption of the HSA on four different hydrogels was studied starting from a solution of 2 mg ml^{-1} in NaCl 0.9%, under laminar flow conditions (75 ml min^{-1}).

Procedure. After the hydrogel was equilibrated with the recirculating saline (for one day), the spectrum of the cell filled with hydrogel and saline solution was collected and stored for later use.

Adsorption experiment. The two four-way valves were turned to introduce the protein solution into the flow cell, after which data collection began. Spectra were collected by the following procedure:

Seventeen 4-scan spectra were collected every 20 s, followed by 10 50-scan spectra collected every 30 s, and finally by two 300-scan spectra collected every 60 s.

All the spectra were collected in a single beam mode, and the absorbance spectra were calculated automatically by the following ratios:

$$\frac{\text{spectrum of(protein + saline + hydrogel coated crystal)}}{\text{spectrum of(hydrogel coated crystal)}} = A_{\text{protein}} + A_{\text{saline}} \quad (1)$$

$$\frac{\text{spectrum of(saline + hydrogel coated crystal)}}{\text{spectrum of(hydrogel coated crystal)}} = A_{\text{saline}} \quad (2)$$

Subtraction of Eq. (2) from Eq. (1) gave the spectrum of the protein.

Washout experiment. A similar scanning sequence was used when the bulk protein was displaced from the flow cell by fresh saline. A new adsorption-washout cycle was then performed to ensure that the protein was no longer being absorbed onto the protein-coated hydrogel, when the protein solution flowed over its surface once again.

Spectrum of native proteins. The IR spectrum of human fibrinogen was recorded in PBS solution (pH 7.4), after subtraction of PBS spectrum. The IR spectrum of HSA was obtained in saline solution (NaCl 0.9 %), after subtraction of the IR spectrum of saline solution.

4. Results and discussion

4.1. Characterisation of the hydrogels

None of the hydrogels showed free aminic groups as obtained by the ninhydrin test. The absence of NH_2 groups guaranteed the determination of the cross-linking degree (the cross-linkers act as a bridge between two COOH belonging to different monomeric units). The cross-linking degree of the different hyaluronan based hydrogels, determined by potentiometric titration, was in good agreement with that calculated from the amount of the activating agent (CMP-J) added [12]. The experimental values were found to be 48 ± 2%, independent of the cross-linking agent used.

IR spectra of the free and cross-linked hyaluronans were shown in Fig. 2. An increase in the intensity of both the bands at 1635 cm^{-1} (stretching of amidic C=O) and the shoulder at 1570 cm^{-1} (bending N–H) is evident, due to the higher amount of amidic groups derived from the cross-linking reaction. The four hydrogels showed very similar IR spectra.

SEM analysis showed that the hydrogels exhibited different morphologies (Fig. 3(a)–(d)) according to the different cross-linking agents, after 24 h of permanence in bidistilled water (see later). Hyal 1.3 DAP was characterised by a morphology with large, laminar plates but with wide fissures between the laminae. Hyal 1.6 DAE showed an open and fragmented structure with large holes and small laminae. The morphology of Hyal gel, using 1.12 DAD as a cross-linking agent, consisted of collapsed plates, while Hyal cross-linked with PEG 800, showed a structure characterised by extended laminae with a wide presence of holes. The different cross-linking agents influenced the morphology of the hydrogels, and in particular, the increased length of the arm determined a more compact structure.

SEM analysis performed after five days i.e. at the equilibrium value of swelling (see later) pointed out that all the four hydrogels showed the same open morphology (Fig. 3(a)).

By looking at the water-uptake measurements as well

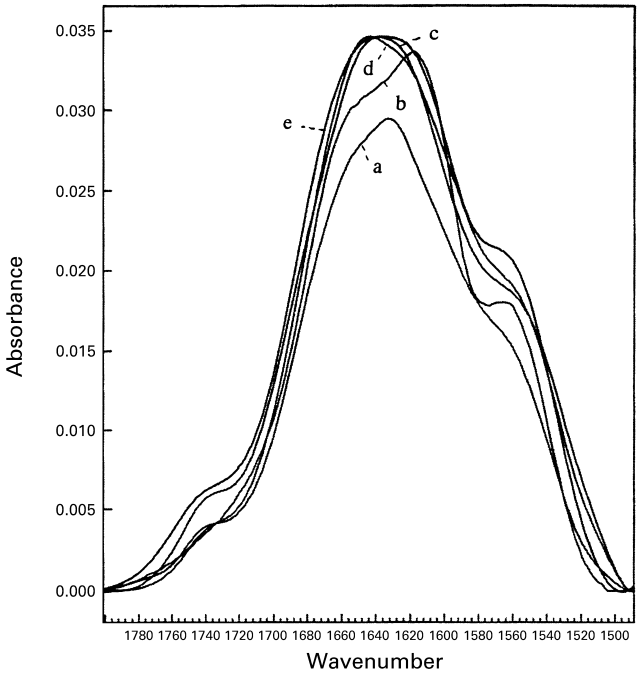

Fig. 2. IR spectra of free polymer (a), Hyal 1.3 DAP (b), Hyal 1.6 DAE (c), Hyal1.12 DAD (d) and Hyal PEG 800 (e) recorded at RT in PBS (pH 7.4).

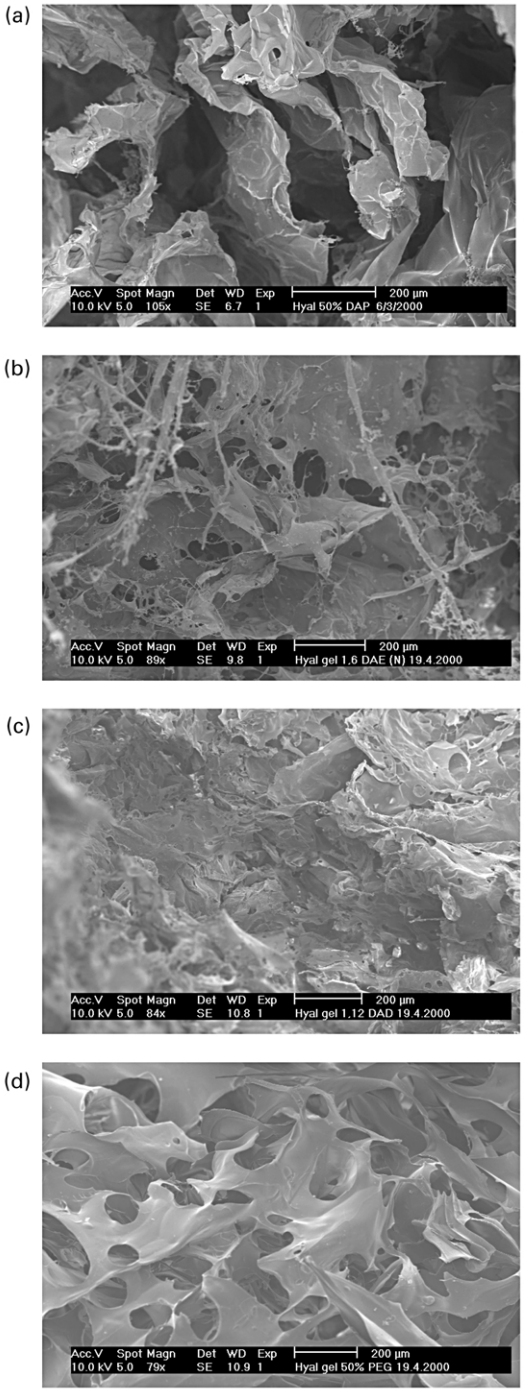

Fig. 3. SEM micrographs of the four hydrogels: (a) Hyal 1.3 DAP; (b) Hyal 1.6 DAE; (c) Hyal 1.12 DAD, and (d) Hyal PEG 800.

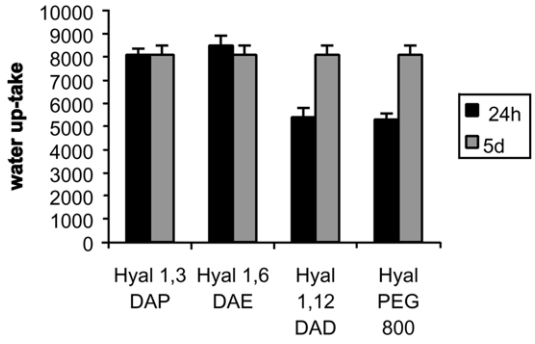

Fig. 4. Water uptake measurements performed at RT in PBS (pH 7.4).

(Fig. 4), the different lengths of the cross-linking agent affected the swelling kinetics, without altering the water-uptake values (8500 ± 1300), which remained constant for all hydrogels. The equilibrium was reached after five days with 1.12 DAD and PEG 800 Hyal hydrogels, instead of 24 h for hydrogels cross-linked by 1.3 DAP and 1.6 DAE (Fig. 4), meaning that the first two arms render the gels more hindered to absorb water. This is probably due, in the case of 1.12 DAD, to the hydrophobic interactions occurring among the aliphatic components, in the case of PEG 800 to the chain–chain interactions [13,14], while on the contrary with 1.3 diaminepropane (1.3 DAP) and 1.6 diaminehexane (1.6 DAE), as cross-linking agents, the hydrogels presented an open structure which favoured permeation, and as a result the water-uptake. As a matter of fact, Hyal 1.12 DAD and Hyal PEG 800 showed a compact structure with extended laminae which slowed down the water absorption.

4.2. Protein adsorption

The IR spectrum of proteins is characterised by bands relative to the different combinations of vibrational modes of the peptidic group. The spectral regions of these modes are known as Amides I, II, and III. From the analysis of Amide I, (stretching of peptidic C=O) and Amide III (stretching of C–N 'plus' bending of N–H), it is possible to obtain information on the conformation of the protein molecule.

The relative amount of protein adsorbed on the four hydrogel surfaces, either reversibly or irreversibly, can be obtained, even if not quantitatively determined, by the intensity of the 1550–1550 cm^{-1} band, within the Amide II spectral region (1580–1500 cm^{-1}), which comes from the 1550 cm^{-1} band, within the Amide II spectral region (1580–1500 cm^{-1}), which comes from the contribution of stretching of C–N plus the bending of N–H, sensitive to the quantity of protein present within the evanescent field of the IR radiation.

4.3. Fbg adsorption

4.3.1. Kinetics analysis

The kinetics curves relative to the four different hydrogels, reported in Fig. 5, showed the same trend. The absorbance values at 1550 cm^{-1} of the Amide II increased rapidly at the beginning of the process and the plateau was reached after 8 min for each hydrogel. During the flow of the protein solution, no desorption of protein was observed. Even during the washout experiments no protein desorption was detected from any of the hydrogels.

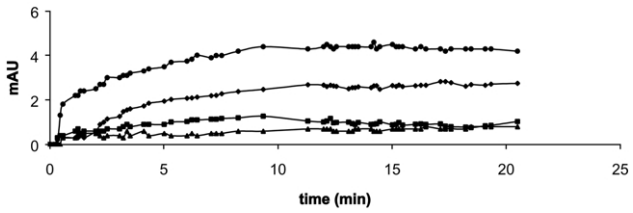

Fig. 5. Kinetics analysis of Fbg adsorption on the four hydrogels (▲:Hyal 1,3 DAP; ◆: Hyal 1,6 DA; ● Hyal 1,12 DAD; ; ■: Hyal PEG 800).

The relative quantity of Fbg adsorbed on the four different hydrogels changed with the hydrophobic character of the intermolecular chain. The 1550 cm^{-1} band intensity of Fbg adsorbed on Hyal hydrogel was in the order of 1 mAU with PEG 800 and 1.3 DAP, whereas on Hyal 1.6 DAE, the absorbance reached the value of 2.7 mAU, increasing consequently on Hyal 1.12 DAD, which reached the highest value of 4.0 mAU (see Table 1). From these data, it is evident how the relative amount of Fbg adsorbed on the hydrogel was strictly related to the hydrophobic properties of the intermolecular chain. The same experiment performed on the hydrophobic PE material showed an absorbance of 50 mAU (not reported data). So it was possible to conclude that the hydrophobic character of the spacer arm affected the amount of adsorbed Fbg without influencing the absorption kinetics.

4.3.2. Conformational analysis

The conformation of the protein adsorbed on hydrogels after 1 and 30 min from the beginning of the process was obtained from the Amide I and III bands. The results for the four hydrogels are as follows:

- *Fbg adsorbed on Hyal 1.3 DAP*. The Fbg initially adsorbed on the hydrogel was very similar to the native protein, whereas with the lengthening of the residence time, the Fbg secondary structure underwent a negligible

Table 1
Variation of 1550 cm^{-1} band intensity and conformational changes relative to Fbg adsorption on four hydrogels

Hydrogels	1550 cm^{-1} band intensity	Conformational change
Hyal 1.3 DAP	1.0	Slight increase in β-sheet component
Hyal 1.6 DAE	2.7	Slight increase in random-coil component
Hyal 1.12 DAD	4.0	No conformational change
Hyal PEG 800	1.0	Slight increase in random-coil component

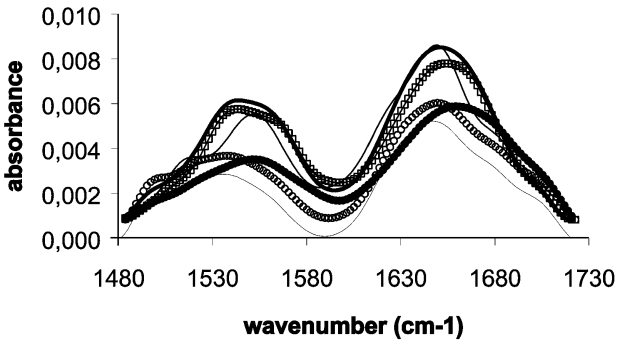

Fig. 6. IR spectra of Fbg in solution (—) and Fbg adsorbed on Hyal 1.3 DAP (○), Hyal 1.6 DAE (—), Hyal 1.12 DAD (□), and Hyal PEG 800 (●). All the reported spectra were collected 30 min after the beginning of the experiment.

progressive modification, resulting in an increase of the β-sheet structure and a decrease of the α-helix component, (see, for example, the spectrum collected after 30 min, shown in Fig. 6).

- *Fbg adsorbed on Hyal 1.6 DAE*. The Fbg adsorbed on the Hyal 1.6 DAE suffered for only an initial negligible conformational change as shown by the increase in the intensity of the band at 1670 cm^{-1} due to the increase in random-coil structure. The protein structure did not change any longer with residence time.
- *Fbg adsorbed on Hyal 1.12 DAD*. The adsorption on the hydrogel surface did not provoke conformational changes. Both the spectrum of Fbg after 1 min and after 30 min were similar to that of the native protein.
- *Fbg adsorbed on Hyal PEG 800*. from the analysis of the bands in the Amide I region, no significant changes between the free protein and the absorbed one were detected. The initial (within 1 min of adsorption) protein–hydrogel interaction provoked a negligible conformational change consisting of an increase in the contribution of random-coil structure and a consequent decrease in α-helix and β-sheet structures. The permanence of Fbg on hydrogel surface did not induce greater conformational variations on the protein, as shown by the spectrum recorded after 30 min, which was very similar to the previous ones (Fig. 6).

From these results we may conclude that the four hydrogels did not provoke substantial changes in the conformation of the protein upon adsorption. Hyal cross-linked with 1.3 diaminepropane induced a very slight conformational change which becomes more evident with time for a decrease in the α-helix component. Hyal cross-linked with 1.6 diaminehexane (1.6 DAE) and with PEG 800 induced a slight structural variation with an increase in the random coil component from the beginning of the adsorption. The Fbg adsorbed on Hyal 1.12 DAD did not suffer any change with respect to the native protein.

In conclusion, from the kinetics analysis it was possible to affirm that the relative amount of Fbg adsorbed, increased by increasing the hydrophobic properties of the arm; as far as conformational changes are concerned, unlike the literature data, the increase in hydrophobic character determines a lower influence on the protein conformational change.

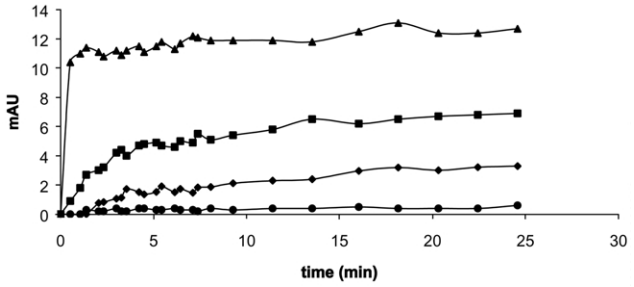

Fig. 7. Kinetics analysis of Fbg adsorption on the four hydrogels (▲) Hyal 1.3 DAP; (◆) Hyal 1.6 DAE; (●) Hyal 1.12 DAD; (■) Hyal PEG 800).

4.4. HSA adsorption

4.4.1. Kinetics analysis

All the curves showed the same trend: the absorbance increased rapidly at the beginning of the process and the plateau was reached after 10–13 min, a time longer than that for Fbg may be due to the lower surface affinity of HSA (Fig. 7).

Also, in this case during the protein solution flow, no desorption of free protein was observed. Therefore, the adsorption kinetics was not influenced by the cross-linking agent, but the relative amount of adsorbed protein changed with the hydrophobic character of the intermolecular chain. The 1550 cm^{-1} band intensity was 10 mAU on Hyal PEG 800, and decreased from 6 mAU on Hyal 1.3 DAP to 3 mAU on Hyal 1.6 DAE and to 1 mAU when 1.12 DAD was used as a cross-linking agent (see Table 2).

In conclusion, unlike Fbg, the relative quantity of HSA adsorbed on the four hydrogels decreased with the increase in the hydrophobic properties of the spacer arms.

4.4.2. Conformational analysis

- *HSA adsorbed on Hyal 1.3 DAP*. The adsorption of HSA on Hyal 1.3 DAP provoked a very light initial modification in the structure of the protein with an increase in the random-coil component (Fig. 8).
- *HSA adsorbed on Hyal 1.6 DAE*. The protein after adsorption on this hydrogel, suffered a greater conformational change with the loss of the α-helix component and the increase in the random coil and β-sheet components. These structural modifications appeared from initial adsorption of the protein and remained constant for the whole adsorption time period. Because of the very small amount of the protein adsorbed, it was not possible to also analyse the Amide III region (the signal of the Amide III region was almost undetectable) (Fig. 8).
- *HSA adsorbed on Hyal 1.12 DAD*. The IR spectra of

Table 2
Variation of 1550 cm^{-1} band intensity and conformational changes relative to HSA adsorption on four hydrogels

Hydrogels	1550 cm^{-1} band intensity	Conformational change
Hyal 1.3 DAP	6.0	Slight conformational change
Hyal 1.6 DAE	3.0	Great increase in random coil and β-sheet components
Hyal 1.12 DAD	1.0	Great increase in random coil, β-sheet and β-turns components
Hyal PEG 800	10	Negligible conformational change

adsorbed HSA compared with that of the native protein appeared quite different. Also in this case, due to the fact that the initial adsorption of the α-helix component disappeared in favour of not only random coil and β-sheet structures, but also β-turns conformation. As reported for Hyal 1.6 DAE, in this case as well, the relative amount of adsorbed protein was so small that it was impossible to detect the Amide III region (Fig. 8).

- *HSA adsorbed on Hyal PEG 800*. Comparing the IR spectra of the native HSA with those of the HSA adsorbed on the hydrogel at the beginning and at the end of the process, no conformational changes were detected. Only from the analysis of the Amide III region did the secondary structure of adsorbed HSA showed a negligible modification consisting of a little increase in the random-coil component, whereas the α-helix and β-sheet slightly decreased (Fig. 8).
- From these data we may conclude that in the case of HSA adsorption, the increase in the hydrophobic character of the cross-linking arm is reflected in both an increase in the conformational change of the adsorbed protein and a decrease in the relative amount of adsorbed HSA.

5. Conclusions

The higher biocompatibility of hydrogels in comparison with other polymeric materials is proved here by the very low amount of both albumin and fibrinogen adsorbed on Hyal hydrogel and mainly by the slight protein conformational changes upon adsorption.

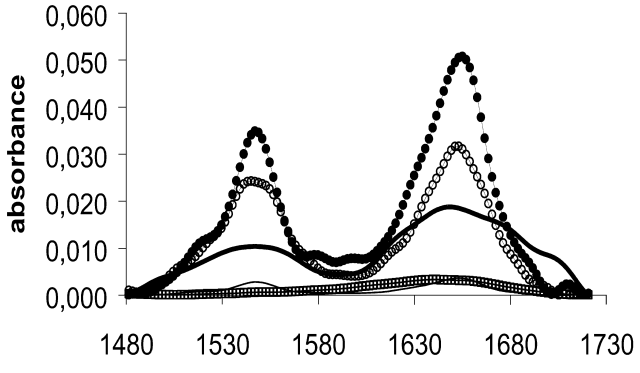

Fig. 8. IR spectra of Fbg in solution (—) and Fbg adsorbed on Hyal 1.3 DAP (○), Hyal 1.6 DAE (—), Hyal 1.12 DAD (□) and Hyal PEG 800 (●). All the reported spectra were collected 30 min after the beginning of the experiment.

Fibrinogen on the four hyaluronan based hydrogels was adsorbed to a negligible amount. The different hydrophobic properties imposed on the hydrogels by changing the spacer arms influenced the relative amount of the adsorbed fibrinogen. The adsorbed amount rises from the most hydrophilic to the most hydrophobic support, as shown by the increase in the Amide II band intensity (from 1.0 mAU for Hyal PEG to 4.0 mAU for Hyal 1.12 DAD). The most important aspect derived from these studies is that this protein did not undergo conformational changes. Indeed the role of denaturated Fbg in several pathological processes, such as the thrombogenic one, is well known. After denaturation, Fbg is in fact, able to interact with platelet receptor (GpIIb-IIIa), activating them and promoting their degranulation [15–18]. Alterated Fbg also plays an important role in the inflammation process, favouring and modulating monocytes/macrophages adhesion [19,20]. The results obtained with these hydrogels indicate that these materials are no longer thrombotic and give no inflammation.

HSA showed a different behaviour. Also, in this case the amount of adsorbed protein was negligible, compared with the values found in literature for other materials, but the protein underwent conformational changes with the increase in hydrophobic character on the surfaces. This trend was also found by Pantazaki et al. who studied the conformation of HSA after adsorption on two different hydrophilic/hydrophobic ratio supports. On the hydrophilic material, (porous silica coated with a polymer layer anion exchanger PVI) they detected only very weak structural changes with the involvement of only a small percentage of α-helix. In the hydrophobic support, (porous silica coated with C_6 alkyl chains) relatively important infrared spectral changes were observed, which reflected alterations on the secondary structure [21].

In conclusion, in spite of the negligible increase in the hydrophobic character of the hydrogels (due to the different cross-linking agents used), it nevertheless imposed a clear trend on the relative amount of adsorbed proteins, as albumin is preferably adsorbed onto hydrophilic support, while Fbg, similar to the other adhesive proteins, is absorbed onto a hydrophobic one [22,23].

The different hydrophobic character of the spacer arms influenced the two adsorbed plasma protein conformations differently. Fbg showed negligible conformational changes

independent of the cross-linking agents used, whereas HSA underwent more significant modifications with the increase in the hydrophobic character of the surface. These conformational changes are however negligible when compared with those already reported on other polymeric substrates.

Acknowledgements

The authors gratefully acknowledge the CNR (Consiglio Nazionale delle ricerche) and MIUR (Ministero dell'Istruzione dell'Università e della Ricerca) for financial support.

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