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Correlation of the surface chemistries of polymer bioactive coatings, with their biological performances

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Surface analysis techniques have been used to characterize heparin-containing bioactive coatings. The relationship between uncoated polymer, intermediate coupling and final layer surface chemistry upon the overall quality of the coating system has been investigated. The results present data from ToFSIMS, XPS and bioactivity in terms of thrombin deactivation as measured using chromogenic assay technique on heparin-based coatings on polymer surfaces including LDPE and PVC. The effect of pretreatment of uncoated polymer surfaces has been investigated where a number of effects critical to coating performance have been identified. Studies of the intermediate coupling regime show how the first and final stages of coupling are the most critical. Finally it is shown how the integrity, mean thickness and chemical state of the heparin final layer can be measured using a combination of ToFSIMS and XPS.

1. Introduction

Polymers have been used widely as biomaterials in medical devices for several generations and they have been selected on the basis of their inherent bulk properties, such as elasticity, electrical insulation, toughness, mechanical strength and general processability. There are very few situations where a base polymer has been selected or optimized specifically for its physiological response *in vivo*. For example, no artificial polymer surfaces can mimic the blood compatability of the vascular endothelial layer with which it contacts the body's circulatory system. Exposure of blood to artificial materials provokes an activation of its defence systems including:

Coagulation activation
Platelet activation/consumption
Leukocyte activation
Endotoxin responses
Kinin activation
Fibrinolysis
Oxygen free radical formation
Compliment activation

In addition there are many cross-interactions between these processes.

As a result of the interactions between blood and artificial polymers there have been intensive and extensive investigations into methods of improving the biological performance of polymers.

It is possible to optimize, by methods such as surface modification, the physiological responses of existing polymers which are known to be functional and processable. Some of the earliest methods included the treatment of surfaces with antithrombogenic additives such as surfactants and enzymes. One of the most successful early studies was made with heparin on graphite, by Gott *et al.* [1]. Significant antithrombogenic activity was achieved *in vivo* and this has led to the widespread development of heparin as the major antithrombogenic agent in blood-containing devices.

There remain several challenges to overcome in order to reach optimum biomaterial performance by surface modification. These include the long-term retention of surface-related properties for the control of biocompatibility and interactions with cells and tissue. Interactions between an implanted polymer and its host environment are known to depend on the outermost molecular layers of the biomaterial. As a result, characterization of the surface of a biomaterial is essential in understanding its immediate biological properties.

The aims of this work were to develop feasible methods to monitor the modification of biomaterial surfaces for improved physiological response to implantation. The widespread application on many implant materials of the attachment of heparin to the surface, by methods such as the Carmeda™ process, have been evaluated by modern surface analysis techniques. The methods used include X-ray photoelectron spectroscopy (XPS or ESCA) and time-of-flight secondary ion mass spectrometry (ToF SIMS), and these have been used to determine the surface and interfacial chemistries of the multi-layered coatings on a wide range of polymer substrates.

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Comparative evaluations have been made on the various alternative methods of coupling heparin to polymer surfaces and these are supported by biological data from the coatings.

Two main methods of heparin attachment to surfaces exist; ionic binding and covalent (or grafted) binding. The former method involves the deposition of a positively charged quaternary ion layer, such as tridodecylmethylammonium chloride (TDMAC), to which the negatively charged sites on the heparin chain attach. This coating system experiences some limitations on activity lifetime, possibly due to release of heparin from the surface [2, 3].

Covalently attached heparin is bound to substrate via either hydroxyl or amino side groups or by a reactive aldehyde group on the end of the heparin chain [4]. End attachment combines the benefits of strong substrate attachment and exposed free active binding sites. This work compares the surface analysis of ionically and covalent attachment heparin coatings.

2. Experimental procedure

All XPS experiments were performed using the Fisons (VG) (SSI) M-Probe XPS instrument, with the standard (35°) take-off angle (TOA) and using 200 W monochromatized AlK_α X-rays focused into an elliptical spot size of 400 μm × 1000 μm. Survey scan analysis and high resolution analysis of C 1s, O 1s and N 1s regions were recorded. All spectra are referenced to the C 1s peak at 285.0 eV binding energy. Composition tables were derived for each surface by peak area measurement followed by the use of Scofield sensitivity factors. High-resolution data were subject to linear background subtraction prior to peak synthesis using the instrument software.

Time-of-flight SIMS spectra were acquired using a VG IX23S instrument based on the Poschenrieder design and equipped with a pulsed liquid metal ion source [6]. A 30 keV Ga^+ primary ion beam was used at an incident angle of 38° to the surface normal. The secondary ions were accelerated to 5 keV for the analysis by applying a sample bias. For each sample, both positive and negative secondary ion spectra were collected using a total primary ion dose that did not exceed 2×10^{11} ions cm^{-2}. Such a dose lies well below the damage threshold of 1×10^{13} ions cm^{-2} for static SIMS [7], such that the analysed surfaces were effectively undamaged as a result of the ToFSIMS studies.

Bioactivity with respect to the ability to deactivate thrombin was performed by exposing a defined surface area of coated material to a wetting solution of 0.25 M NaCl for 10 min and rinsing with DI water. The later is removed and the surface in exposed to a solution of 1 IU ml^{-1} of ATIII in a buffer solution for 15 min with gentle mixing. The ATIII solution is rinsed three times with a buffer solution and the surface gently dewetted with an air stream to assure no residual drops of loose liquid. To this surface a buffered solution of known thrombin concentration is exposed for 10 min. The thrombin solution is then collected along with two successive rinses of the surface, and analysed for active thrombin. The amount of active thrombin is measured calorimetrically as thrombin acts on the chromogenic substrate S-2238 (Kabi Diagnostica) to cleave the substrate releasing free p-nitroanilide that generates a yellow colour measurable at 405 nm. The difference between the known thrombin added in the exposure step and that measured in the collected sample is the amount of thrombin that has been deactivated by the surface.

2.1. Sample preparation

Surfaces were prepared on LDPE and PVC with the adsorbed layer process as stated in the literature by Carmeda with and without a pretreatment step. The pretreatment step for LDPE involved oxidation of the surface with an acid pretreatment solution, and the PVC was exposed to a persulfate solution capable of generating free radicals. For ionically bound heparin surfaces the commercial tubing Bentley Duraflo II™ heparin surface was used [8]. The results presented for these ionic surfaces as therefore simply on the commercial product and do not deal with the process for application to a surface, and are listed as comparisons of the properties of the final heparinized surface methods.

3. Results

Results from the surface analysis of the covalent heparin coated surfaces with and without pretreatment showed that the pretreatment greatly influenced the integrity of the subsequent coating. Intermediate layers chemistries, as determined from the changes in the topmost 3–7 nm after successive layers were applied, showed that the importance of the pretreatment in establishing an alternating amine rich upper layer. Presumably this could be due to better initial adhesion of the first cationic layer that would minimize complete dissolution into the subsequent anionic layer. Ideally alternating layers should mix and achieve enough molecular entanglement to form stable well adhered laminate, and very importantly provide an outermost layer of enhanced functionality of the last layer. Surface analysis for oxygen, nitrogen and sulphur are as follows:

Oxygen

This shows a maximum concentration for both treated and untreated on layer 4 (second dextran sulphate layer). The pretreated series contained a consistently higher level of oxygen, in different functionalities, compared to the untreated samples. The first dextran sulphate layer did not appear to deposit on the untreated LDPE.

Nitrogen

A consistent alternating of nitrogen concentration from layer to layer was observed for the pretreated series. For this set, successive PEI layers formed a surface composition slightly greater in nitrogen than the previous PEI layer. The unpretreated series did not exhibit a similar alternating nitrogen concentration pattern. The layer-by-layer build-up process seems to have defaulted between layer 3 and 7.

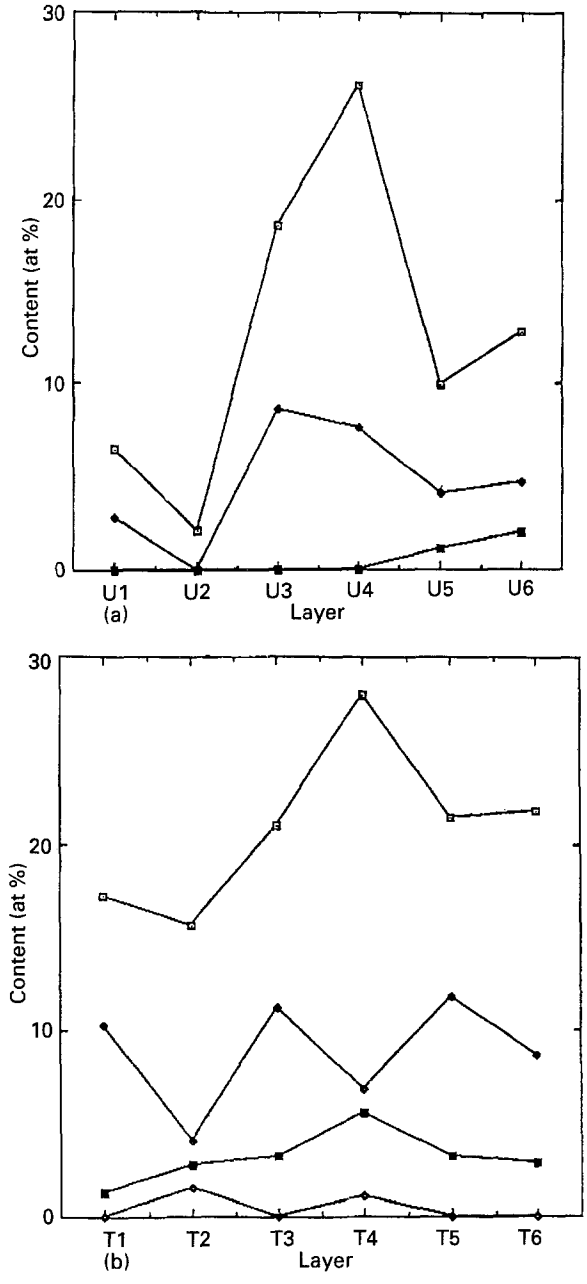

Figure 1 Comparison of layer-by-layer surfaces compositors; (a) untreated LDPE (□ %O; ◆ %N; ■ %S) and (b) acid treated LDPE (□ %O; ◆ %N; ■ %S; ◇ %Na).

Sulphur

The sulphur concentration marks the level of dextran sulphate deposited and detectable in the analysis depth of XPS (3–5 nm). On the pretreated samples there is a reasonable inverse relationship between the sulphur and nitrogen concentrations, as expected, however, no such effect can be observed for the untreated LDPE. Sulphur is detected only in the layers 4, 5 and 6, and always at lower levels than the equivalent from the pretreated series.

Failure in forming one of the intermediate layers successfully was not so common and had less effect on the quality of the final coating. Some substrates, such as PVC were inherently more difficult to couple layers to than other substrates, such as polyolefins (Fig. 2). High levels of mobile additive molecules, such as plasticizers, were identified as the main cause of initial layer bonding failure and suitable pre-treatments were devised to solve this specific problem.

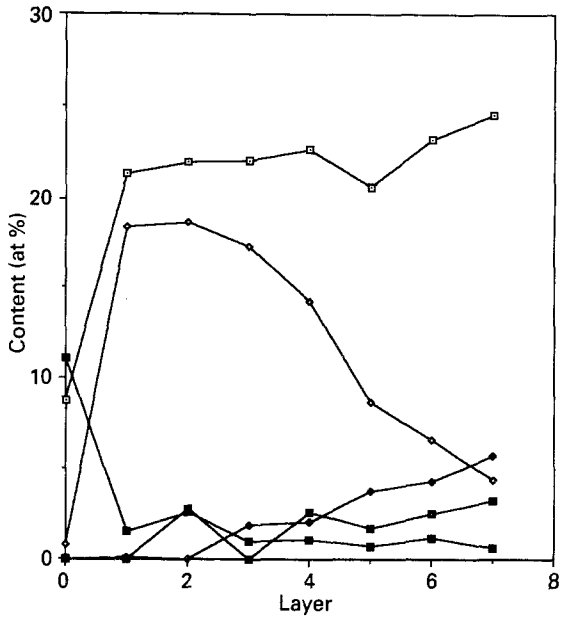

Figure 2 Layer-by-layer surface compositions PVC (□ oxygen; ◆ nitrogen; ■ sulphur; ◇ silicon; ■ chlorine).

Results from the final coating, containing heparin, include its mean thickness and integrity. The final layer evaluation of ionic versus covalently coupled heparin on PVC show that specific ratios for sulphur, nitrogen, carbon and oxygen were related to the quantity of the heparin present. The surface compositions, from random 1.0 mm × 0.4 mm areas, were as follows:

Sample binding	%C	%O	%N	%S	%Cl	%Na
Ionic	76.3	17.4	2.4	2.5	0.8	0.6
Covalent	65.1	23.4	8.3	3.1	–	–
Heparin (theoretical)	34	54	3	9	–	–

The covalent coating appears to be higher in heparin concentration than the ionic as judged from the levels of oxygen, nitrogen and sulphur. The excess nitrogen on the covalent surface may be linked to the polyimine surface just below the heparin. Sulphur concentration is likely to be a good indication of heparin coverage on these surfaces. Excess carbon on both surfaces is due to plasticizer, migrating from the bulk PVC. The chlorine present on the ionic surface is at a binding energy equivalent to that of PVC. This indicates that the PVC tubing is not totally covered by this coating. Sodium on the ionic surface is ionic and not associated with the chlorine.

Also, the presence from XPS of specific bonding states, including carbon and oxygen as OC–C–O; with nitrogen sulphur and oxygen as $-HNSO_3^-$ groups, has been used to quantify the nature of the heparin coating.

High resolution scans of the carbon peak identified the following states:

Sample	%C–C	%C–O	%O–C–C	%O = C–O
Ionic	78	18	2	2
Covalent	54	35	7	4
Heparin	8	62	15	15

This confirms that the covalently bound surface exhibits more heparin-like states that the ionic surface. The excess of C–C bonding is linked to the migration of plasticizer to the outer surface and is more excessive on the ionic surface.

Both surfaces exhibited identical sulphur 2p peak positions of:

Sulphur 2p(3/2) = 168.4 eV

Sulphur 2p(1/2) = 169.6 eV

These peaks positions are identified as those of sulphur in heparin.

On the covalent sample, peak splitting can be identified, as expected, and is ascribed to the two states of sulphur in heparin. At higher peak resolution the two similar chemical states of sulphur in heparin can be separated thus:

NSO_3: Sulphur 2p(3/2) = 167.7 eV (21%)

OSO_3: Sulphur 2p(3/2) = 168.5 eV (79%)

There is a higher level of OSO_3 than expected (67%) and this may be due to extra sulphate groups on the heparin chain or dextran sulphate exposed from a lower layer.

Two chemical states of nitrogen were found on both coatings, but the equivalent peak positions on the two coatings are not the same:

Peak position (eV)	399.4	399.7	401.8	402.1
Chemical state	C–NH–S	C–NH–S	R_4N^+	R_4N^+
Ionic bound	31	–	–	69
Covalent bound	–	61	39	–

These ratios indicate that there is about twice as much heparin at the surface of the covalently attached coating than on the ionic binding surface. The difference in equivalent peak positions may be due to the different bonding environments present in the two coatings.

Complementary surface characterization by ToF SIMS has resulted in an accurate determination of the relative intensities of mass spectral peaks due to sulphite, protonated amine and characteristic molecular fragments from heparin itself. For each sample, ToFSIMS spectra were collected from an area 0.6 mm × 0.6 mm. Both samples displayed signals characteristic of heparin. For the ionic-bound heparin, signals were also observed corresponding to the counter ion (i.e. cation):

$$Ph–CH_2.(CH_3)_2N^+(C_{18}H_{37})$$

For both samples, intense signals characteristic of di-octyl-phthalate plasticizer for PVC were evident (Fig. 3). On a comparative basis the results suggest higher surface levels of di-octyl-phthalate in the case of the ionic-bound heparin.

Sodium and chlorine were detected for both specimens. On a comparative basis the results suggest higher surface levels of both sodium and chlorine in the case of ionic-bound heparin. XPS has already shown that the presence of chlorine in the case of the ionic bound surface corresponds to exposed PVC substrate.

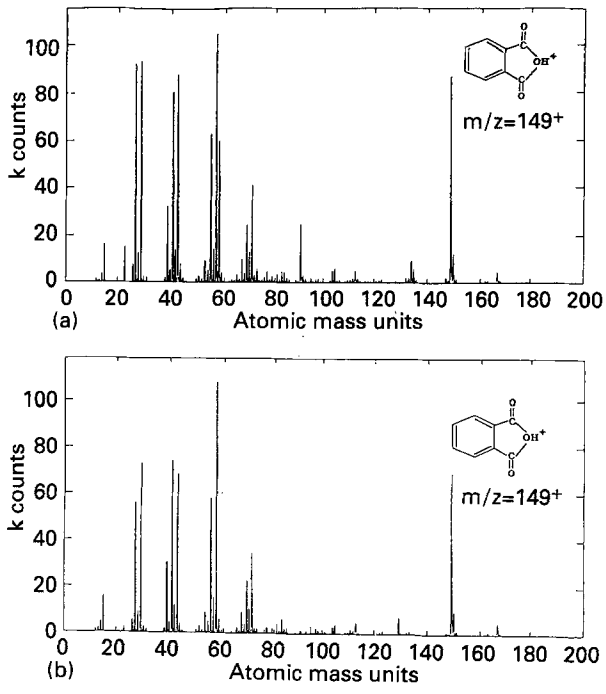

Figure 3 Positive ion ToF SIMS spectra: ionic-bound heparin (a) and covalent-bound heparin (b) on PVC.

A quantitative assessment of the quality of the final heparin coating has thus been obtained from the combined analysis using XPS and ToF SIMS.

4. Bioactivity

Determinations of the heparinized surface's ability to deactivate thrombin with respect to the method of heparin attachment (ionic or covalent) are as follows:

Surface	IU Th deact cm^{-2}*
Ionic	< 0.01
Covalent**	0.21
Covalent***	0.12

* International units of thrombin deactivated per square centimeter of sample surface

** Pretreated surfaces on PVC

*** Non pretreated PVC

5. Conclusions

The work described here has investigated bioactive coatings containing heparin on polymer substrates using surface analysis techniques. The precoating surface chemistry, the integrity of the intermediate coupling chemistry and the uniformity of the final heparin layer are all important factors that govern the antithromobogenic character of the coated surface. Using techniques such as ToF SIMS and XPS an understanding was obtained as to how a specific adsorbed layer laminate with covalent heparin attached to the upper most layer (Carmeda process) could optimally be produced with respect to its antithrombogenic properties.

The presence of heparin on a surface does not automatically render a surface antithrombogenic. As

can be seen by the lack of bioactivity on the commercial ionic heparin surface there is no activity even though heparin is present. In addition to having almost twice as much heparin 'at' the surface, the covalent heparin did not have the associated quaternary ammonium cation (benzalkonium-Ph–CH_2–$(CH_3)_2N^+(C_{18}H_{37})$). This ionic heparin as well as other commercially available ionic heparins are complexes with an abundance of ionic bonding sights between the heparin and the quaternary ammonium cation. These interactions render the heparin incapable of interacting with ATIII and thrombin unless the heparin releases. Gott's initial work was to adsorb heparin to a cationic surface with the ability of the heparin to release and have some impact. The commercial ionic surface in this study releases only traces of heparin, and thus has no measurable activity at the surface. The covalent heparin has demonstrated in this study that it can deactivate thrombin without releasing heparin indicating that the method of attachment allows the heparin to interact with ATIII and thrombin in the classical reaction mechanism.

Acknowledgements

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