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The use of thermal analysis in assessing the effect of bound water content and substrate rigidity on prevention of platelet adhesion

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Abstract Implant surface-induced initiation of blood coagulation leading to thrombus formation is a major problem. It is an important challenge in biomedical research to enhance thromboresistance of polymer coatings of blood-contacting implants and devices by the reduction of platelet adhesion to their surfaces. Currently, the molecular mechanisms responsible for interfacial processes related to regulation of protein adsorption and platelet adhesion in antithrombotic coatings are not yet clearly understood. We studied the role of water binding energy in chitosan molecules using differential scanning calorimetry and thermogravimetric analysis and the role of chitosan coating rigidity using dynamic mechanical thermal analysis in the prevention of platelet adhesion. It was found that the presence of loosely bound water in chitosan coatings increases platelet adhesion. The high molecular weight (HMW) chitosan coating that contains only tightly bound water prevents platelet adhesion. The high molecular weight (HMW) chitosan coating is more rigid and has higher platelet adhesion compared with more soft LMW chitosan coatings. The degree of hydration of thromboresistant coatings is significant parameter that must be taken into account in the design of blood-contacting surfaces.

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Introduction

Blood-contacting implants are extensively used in modern surgery, but their safety is often compromised by adverse foreign body reactions such as initiation of blood coagulation and thrombosis generation. Modification of implant surface by the use of a hemocompatible coating is one of the methods to enhance biocompatibility and to solve this major problem [1]. It is generally understood that surfaces control biological reactions. Proteins in blood and body fluids rapidly adsorb on implant surfaces followed by adhesion of cells. So the cells do not interact directly with implant surface, but interaction occurs through the binding sites in the layer of adsorbed interfacial proteins [2]. Cellular interaction with surfaces also depends on surface chemistry, rigidity, and wettability of the substrates [3] because these material properties affect the adsorption and surface-induced conformational changes of proteins [4, 5]. Surface-induced unfolding of fibrinogen depends on the time and determines the platelet adhesion on the surface [6] and the cell-material interaction. Keselowsky and coworkers [7] demonstrated that surface chemistry modulates adsorbed fibronectin conformation. But the mechanisms of interaction of proteins and platelets with implant surface are not fully understood so far. It is a challenge to reveal the mechanisms of adsorption of proteins from blood and tissue fluids onto the surface of implants. There is a lack of knowledge on the effect of implant polymer coating hydration on the processes at biointerface. It is generally accepted that platelet adhesion is related with fibrinogen adsorption on implant surface but it was also observed that

very hydrophilic polyurethane copolymer surfaces had very low fibrinogen adsorption and very low platelet adhesion, but some of the more hydrophobic polyurethane copolymer surfaces had relatively high fibrinogen adsorption but at the same time exhibited lower platelet adhesion [8]. So it can be suggested that the state of water molecules at interfaces is important.

It was found by combination of nuclear magnetic resonance and differential thermal analysis methods that binding energy of sorbed water molecules to polymer macromolecules varies from strongly bound water molecules with low mobility to weakly bound water molecules with intermediate mobility depending on moisture content in polymer material [9].

Bound water was found [10] to decrease the adsorption of fibronectin with increasing the amount of lattice water molecules on the crystal surface.

The behavior of water that interacts with macromolecules at interfaces and do not freeze at low temperatures has been discussed by Wolfe and co-workers [11]. Bulk water freezes at 0 °C, but freezing point depression can be observed using differential scanning calorimetry method for loosely bound water molecules in polymers. The binding energy of water molecules depends on the distance from the hydrophilic surface or hydrophilic macromolecules, and higher water freezing point depression is related to higher energy interaction with the surface or macromolecule. The tightly bound water molecules that are situated in the first and second molecular layers next to the surface have slower dynamics than the water molecules at higher distance from the surface and do not freeze at low temperature.

Tanaka and co-workers [12–17] suggested that the freezing bound water relates to the platelet compatibility of the polymers.

It was hypothesized that the nonfouling ability of materials is related with ability of tightly bound water layer to resist protein adsorption on the surface [18]. Surface modifications allow to control biological processes at interfaces and it is a challenge to prevent platelet adhesion in blood-contacting implants.

Chitosans and its derivatives have found wide applications in biomedical area due to their good biocompatibility, non-toxicity, and unique biological properties [19–25] Film-forming properties [19] in combination with antimicrobial properties [20, 21] allow to use chitosan in coatings for surgical implants and in wound dressings. Ability of cross-linked chitosan to form hydrogels was applied in controlled drug delivery systems [22]. Biocompatibility of chitosan can be related to its cationic nature and high charge density in solution at pH levels below the pKa. Anti-microbial, antioxidant [23], and anti-inflammatory [24] properties of chitosan also can be related to the cationic structure [25]. The role and joint effect of water and chitosan and its derivatives was demonstrated and analyzed in complex biopolymer food systems [26–28]. It was found that chitosan is able to affect water molecules distribution and migration in complex molecular systems.

The aim of this research was to determine how molecular structure of chitosan can affect on the state of adsorbed water and relate the state of water in chitosan coatings to platelet adhesion.

Materials and methods

Preparation of chitosan coatings

Chitosan samples with different molecular masses were kindly supplied by company Primex:

Low degree of deacetylation/HMW chitosan (DDA 70 %, viscosity 2435 cP) and High degree of deacetylation/LMW chitosan (DDA 97 %, viscosity 29 cP).

Chitosan films were prepared by dissolving chitosan in aqueous solutions (2 % w/v) of acetic acid (Tachem, analytical grade, concentration 99, 9 %) to a final concentration 1.0 % w/v. Solutions were filtered, poured into mold, and dried at room temperature. Chitosan films were also treated by dipping in 1 M NaOH solution for 15 min.

Chitosan coatings were prepared on CoCr discs produced by BIOTRONIK by dipping during 15 min in chitosan solutions and drying 48 h at room temperature.

Platelet adhesion

Fresh venous blood was collected from healthy human volunteers, free from medications known to interfere with platelet function in concordance with the guidelines of the ethical committee of the P. Stradins Clinical University Hospital. Vacutainers (Vacutest KIMA, Italy) containing anticoagulant (K2EDTA 5.4 mg) were used for collection of blood. Prior to testing polymer-coated discs were sterilized by UV-irradiation (wavelength 253.7 nm) for 30 min. Each polymer-coated disc was immersed in 1 mL of blood and incubated at 37 ± 2 °C, 5 % CO_2. The control was 1 mL blood sample. Cell counter (Beckmancoulter, Coulter LH 750, USA) was used to determine platelet count in blood samples before and after incubation with the polymer-coated disc. Experiment was carried out in triplicates, and a mean value ± SD was calculated.

Thermal analysis

Differential scanning calorimetry thermograms were obtained using a differential scanning calorimeter DSC ‘‘Mettler 300’’ (Mettler-Toledo AG, Schwerzenbach,

Switzerland). Approximately, 10 mg samples were placed into the sample compartment at $T = 25$ °C and cooled to $T = -100$ °C at 10 °C min^{-1} to determine the crystallization peak of water and held at -100 °C for 5 min. Samples were then scanned from $T = -100$ °C to $T = 250$ °C with a heating rate of 10 °C min^{-1} to volatilize the water.

The moisture content was determined from mass losses measured using a UBETG thermogravimeter TG 50, MT5. The samples were heated from 25 to 200 °C at 10 °C min^{-1} to volatilize the water.

Each measurement was performed in triplicate.

Mechanical properties

Storage modulus of chitosan film samples of dimensions $4 \times 15 \times 0.1$ mm have been determined by dynamic mechanical thermal analysis (DMTA) method at frequency 1 Hz in the temperature interval from 25 to 200 °C using Mettler Toledo DMA/SDTA 861e instrument.

Results and discussion

The biocompatibility of biomaterials depends on the hydration of biopolymers. In the emerging concept of the role of water in prevention of platelet adhesion [29] to blood-contacting surfaces it is important to find out which types of water—free water, freezing bound water, or non-freezing bound water—are effective in the prevention of adhesive proteins adsorption to surfaces followed by platelet adhesion. Non-freezing water can be bound to sorption sites as well as to occur in small temporary pores [30].

An exemplary DSC curve for chitosan-containing tightly bound non-freezing water and chitosan-containing loosely bound freezing water (solid line) is shown in Fig. 1. DSC studies show that LMW chitosan films contain only tightly bound non-freezing water and do not contain loosely bound freezing water or free bulk water at all, but HMW chitosan contains small amount of loosely bound freezing water with crystallization peak at -63 °C, Table 1. Platelet adhesion has not been prevented on the HMW chitosan coating—the platelets count in the blood after the contact with LMW chitosan surface is higher than after the contact with HMW chitosan surface, Fig. 2. The presence of loosely bound water determined by the presence of crystallization peak in DSC curve at low temperature, Fig. 1, can be related to formation of several layers of water molecules over the first tightly bound layer of water near the surface of polymer coating around the hydrophilic groups. LMW chitosan with higher degree of deacetylation is more hydrophilic compared to HMW chitosan with lower degree of deacetylation. Our results show that formation of multilayer interfacial water can be linked with platelet adhesion to surface, possibly because of surface nonuniformity, and lower surface coverage by tightly bound monolayer of water molecules. Proteins can access and interact with the surface hydrophobic regions that are not covered with tightly bound water molecules. The loosely bound water molecules can be replaced by proteins.

Moreover, in our specific case of chitosan films, we show that platelet adhesion can be purely attributed to the presence of loosely bound water because at the same degree of deacetylation 97 % for LMW chitosan and LMW chitosan treated with NaOH, the surface of polymer coating can both prevent platelet adhesion and essentially promote platelet adhesion depending on the presence or absence of loosely bound water. So the content of loosely bound

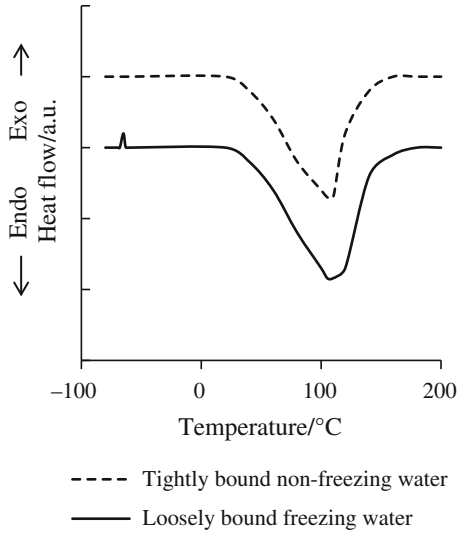

Fig. 1 An exemplary DSC scan for chitosan-containing tightly bound non-freezing water (*dashed line*) and chitosan-containing loosely bound freezing water (*solid line*)

Table 1 Rigidity of chitosan films and absorbed water crystallization and vaporization temperatures and enthalpies

Chitosan film	HMW chitosan	LMW chitosan	LMW chitosan treated with NaOH
Storage modulus/MPa	4116	865	241
Crystallization enthalpy/J g^{-1}	6.2	–	12.4
Vaporization enthalpy/J g^{-1}	333	285	285
Crystallization peak temperature/°C	−63	–	−69
Vaporization peak temperature/°C	105	109	105

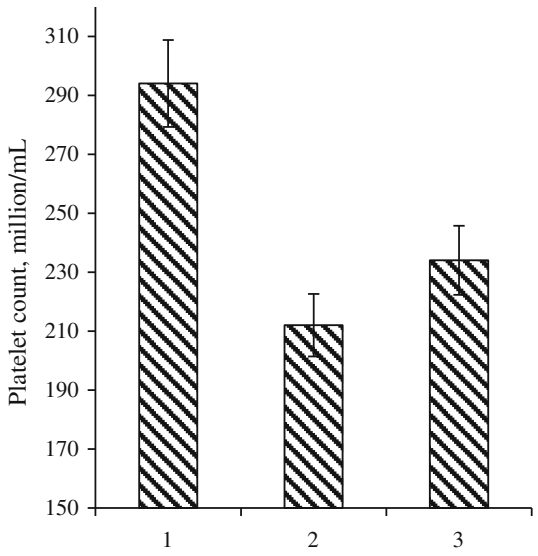

Fig. 2 Platelet count after chitosan coatings contact with blood: *1* LMW chitosan; *2* LMW chitosan treated with NaOH; *3* HMW chitosan

freezing water is more important factor than degree of deacetylation of chitosan in determining the platelet adhesion.

LMW chitosan prevents platelet adhesion to surface and platelets count in whole blood did not change after the contact with coatings. Treatment of LMW chitosan coating with NaOH results in the significant increase of platelet adhesion that can be observed by the decrease of the number of platelets in the blood that has been put into contact with treated coatings, Fig. 2.

Tanaka and co-workers [12, 31] suggested that blood compatibility of polymers and platelet adhesion on surfaces depends on the freezing loosely bound water amount at interfaces.

Herwerth and co-workers [32] suggested that the penetration of water molecules in the interior of the oligo(ethylene glycol) self-assembled monolayers is a necessary condition to develop protein resistant surfaces. So both internal hydrophilicity and terminal hydrophilicity of coating determine the protein resistance of the oligo(ethylene glycol) self-assembled monolayers.

Our data on chitosan coatings show lower platelet adhesion due to higher protein resistance for LMW chitosan coatings. It can be suggested that energy of binding of interfacial water layer plays more important role in protein resistance than internal hydrophilicity and conformational freedom of coating's macromolecules.

Water crystallization peak was not observed for LMW chitosan film, Table 1. Treatment of LMW chitosan coating with NaOH results in generation of the freezing water crystallization peak at −68 °C with the enthalpy of crystallization 12.4 J g^{-1}. The enthalpy of vaporization of LMW chitosan film is 285 J g^{-1} and did not change after the treatment with NaOH but vaporization peak temperature shifts from 109 to 105 °C.

Freezing loosely bound water crystallization peak at −63 °C with enthalpy 6.2 J g^{-1} was observed for HMW chitosan film. The enthalpy of vaporization of HMW chitosan film is 333 J g^{-1} and vaporization peak temperature 105 °C. Enthalpy of vaporization is higher but enthalpy of crystallization is lower for HMW chitosan compared with LMW chitosan treated with NaOH, Table 1. So the platelet adhesion is lower for HMW chitosan compared with LMW chitosan treated with NaOH, Fig. 2, because HMW chitosan contains higher amount of tightly bound water and lower amount of loosely bound water. It can be suggested that higher enthalpy and lower vaporization peak temperature for HMW chitosan compared to LMW chitosan, combined with the presence of crystallization peak at low temperatures, can be related with higher platelet adhesion. It means that in the LMW chitosan the amount of water is lower but this water is more tightly bound due to formation of hydrogen bonds compared with the water in the HMW chitosan.

These experimental data are consistent with the opinion of Morisaku and co-workers [33] in the study of hydration of poly(2-methacryloyloxyethyl phosphorylcholine) chains that the degree of protein adsorption resistance depends on the amount of nonfreezable tightly bound water and not consistent with another proposed mechanism that only amount of loosely bound freezing water determines platelet adhesion and do not support the hypothesis that "the freezing-bound water layer between free water and non-freezing water was an important factor for the excellent blood compatibility" used to explain blood compatibility of poly(2-methoxyethyl acrylate) [16]. We consider that a more accurate formulation of the hypothesis "freezing-bound water, which prevents the biocomponents from directly contacting the polymer surface or non-freezing water on the polymer surface, plays an important role in the excellent blood compatibility of PMEA" has been stated in more recent paper related to blood compatibility mechanism at the blood-poly(meth)acrylate interface [13].

According to Vogler "ignoring the role of water in protein adsorption has been a fatal flaw in much of the existing literature." Water prevents protein adsorption to foreign body surface due to competition with adsorbing protein molecules that cannot displace interphase water especially in vicinity of hydrophilic surfaces [29].

It was suggested that proteins maintain their native conformation if contacted with the coatings containing freezing bound water and low platelet adhesion and spreading can be attributed to the low degree of the denaturation of the protein adsorbed onto such coating surface [17].

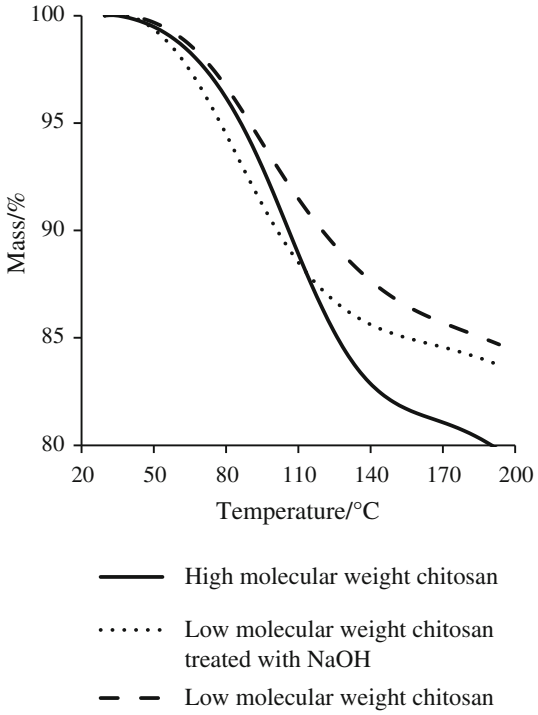

Fig. 3 TG curves of chitosan films

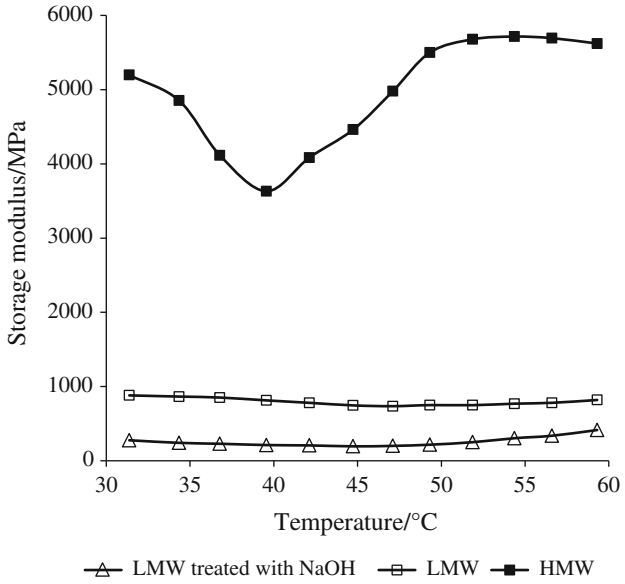

Fig. 4 Storage modulus of LMW and HMW chitosan coatings. *Filled square* HMW chitosan; *open square* LMW chitosan; *open triangle* LMW chitosan treated with NaOH

Chain hydration and chain flexibility of hydrophilic polymers can be related with surface resistance to non-specific protein adsorption. In the recent review [18] the lack of robust theoretical models for mechanisms of cell/surface interaction was recognized. The molecular simulation studies have shown that a tightly bound water layer near the coating surface results in the repulsive hydration force and the total interfacial force is generated by hydration water, with very low contribution from the surface of self-assembled monolayers.. It is very important to realize that the tightly bound water, non freezable water layer between the protein and the self-assembled monolayer coating, generates the total force acting on the protein. This repulsive force increases with the increasing number of hydrogen bonds between water molecules and hydrophilic sites on polymer coating due to the creation of energy barrier for protein adsorption and prevention the protein from direct contact with the surface [18, 34, 35]. It was also concluded that tightly bound water molecules play a dominant role in surface resistance to non-specific protein adsorption.

Protein molecules change conformation to a greater extent on hydrophobic surfaces than on hydrophilic surfaces [36]. Water molecules are stronger bound to hydrophilic surfaces and protein molecules do not change conformation in the vicinity of tightly bound water layer near surface but at hydrophobic surfaces water binding energy is low and proteins can contact with surface directly and change their conformation.

So platelet resistant surfaces do not allow proteins to change their conformations as a result of interaction with such surfaces.

HMW chitosan films contained more water than LMW chitosan films, Fig. 3. Mass losses due to water vaporization are less intensive at low temperatures for LMW chitosan film than for HMW chitosan film and for LMW chitosan film treated with NaOH. The treatment with NaOH decreases water binding energy to chitosan.

Chitosans with higher degree of deacetylation are more flexible. Reduction of rigidity of the molecular chains was observed with the increase of the degree of deacetylation of chitosan [37].

It is evident that changes of the water molecules state and structure in the vicinity of solid surfaces compared to the structure of bulk water also must change the interaction of surface water layers with protein molecules.

The rigidity of coating plays important role in regulation of platelet adhesion through integrin-mediated signaling. It was demonstrated that soft substrates do not support platelet spreading and they do not induce sufficient signaling. Platelet signaling also depends on fibrinogen adsorbtion density. Insufficient signaling and reduced force generation were related with substrat soft surfaces [38]. Our data show that rigidity of substrat must be optimal, not too rigid and not too soft in order to reduce platelet adhesion, Fig. 4 and Table 1. Our results confirm the influence of material rigidity on platelet adhesion—the higher platelet adhesion was observed on more rigid material with higher storage modulus HMW chitosan

compared with more soft material with much lower storage modulus LMW chitosan. At the same time soft material with a low storage modulus LMW chitosan containing loosely bound freezing water as a result of treatment with NaOH has even higher platelet adhesion than rigid HMW chitosan. So in the specific case of chitosan the presence of loosely bound water has more pronounced effect on the increase of platelet adhesion than the value of elastic modulus of a material. The regulation of platelet adhesion is possible by the regulation of surface hydration and by adjustment of mechanical properties of the coating material.

Conclusions

Platelet adhesion to chitosan polymer-coating surface increases with the increase of loosely bound freezing water content. Non-freezing tightly bound water at interfaces is more effective in the prevention of platelet adhesion if compared with freezing loosely bound water because tightly bound water cannot be displaced from surface by proteins. LMW chitosan films treatment with NaOH results in the increase of loosely bound freezing water content, and as a consequence in the increase of platelet adhesion. At the same time, HMW chitosan coating with more rigid surface and containing loosely bound freezing water has lower platelet adhesion compared with LMW chitosan coating containing loosely bound freezing water and has higher platelet adhesion compared with LMW chitosan coating without freezing water and containing only tightly bound non-freezing water. It is possible to regulate platelet adhesion by physical and chemical treatment of polymer coatings.

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