Gel Beads Composed of Chitosan and Polyacids and Their Blood Compatibility

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ABSTRACT: Chitosan gel beads were prepared as a model for new chitosan materials and their blood compatibility was evaluated *in vitro* in human pool plasma and in human whole blood. The one-stage method for the preparation of monolithic beads consisted of performing a template polymerization of 2-acryloylamido-2-methylpropanesulfonic acid (AMPS) or copolymerization of AMPS and acrylic acid. Monolithic beads of semi-interpenetrating networks chitosan/copolymer were prepared in the presence of a cross-linking agent. The equilibrium degree of swelling of the beads was dependent on the composition. Reservoir-type beads consisting of a chitosan core and a coating of polyelectrolyte complex with anticoagulant activity and chitosan were also prepared. The reservoir-type beads and the monolithic beads showed improved compatibility with blood in comparison with the uncoated chitosan beads.

KEY WORDS: chitosan, homo- and copolymers of AMPS, gel beads, polyelectrolyte complexes, semi-IPN, anticoagulant activity, blood compatibility

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INTRODUCTION

Blood compatibility is a prerequisite for any device that is in contact with blood. The response of blood to a foreign body often led to thrombus formation on the device surface. One approach to preparing blood compatible surfaces is based on the use of the natural anticoagulant polymer heparin. There are data on the preparation of heparin-releasing coatings [1]; devices containing heparin immobilized in their bulk [2] or on their surface [3,4]. Chitosan is a natural polyaminosaccharide that was found to be a suitable material for the preparation of medical and agropharmaceutical devices because of its excellent properties such as biocompatibility, biodegradability and non-toxicity [5–10]. Chitosan, however, has been reported to cause thrombosis and embolization [6,11]. Attempts have been made to suppress the haemostatic activity of chitosan by grafting 2-hydroxyethyl-methacrylate [12], sulfonation of chitosan [13], or the formation of a polyelectrolyte complex with heparin, dextran sulfate or poly(ethylene glycol) sulfonate [14–16].

Previously we had shown that poly(2-acryloylamido-2-methyl-propanesulfonic acid) (PAMPS) and copolymers of AMPS and acrylic acid (AA) exhibited anticoagulant activity (*in vitro* for human pool plasma) [17]. The anticoagulant activity of the copolymers increased with increasing content of sulfo groups in the copolymers. The highest anticoagulant activity, equal to that of heparin, was found for PAMPS. It was expected that the blood compatibility of chitosan materials might be improved by combining them with P($AMPS_n$-*co*-$AA_{n'}$) copolymers or with PAMPS.

In the present work different types of chitosan gel beads were prepared as model chitosan materials and their blood compatibility was evaluated *in vitro* for human pool plasma and human whole blood.

MATERIALS AND METHODS

High-molecular-weight chitosan (MW 6×10^5 with 80% degree of deacetylation), commercial monomers PAMPS and AA, as well as a cross-linking agent, N,N′-methylenebisacrylamide (MBAAm), were purchased from Fluka. AA was distilled under reduced pressure in the presence of hydroquinone prior to use. All reagents used for the preparation of the copolymers were of analytical grade of purity.

Synthesis of PAMPS and P($AMPS_n$-*co*-$AA_{n'}$)

PAMPS was prepared by radical polymerization as previously described [18]. The copolymers P($AMPS_n$-*co*-$AA_{n'}$), where n and n′ are the mole fraction of AMPS- and AA-units, respectively, have been obtained with $n = 0.5, 0.8, 0.9$, and $n' = 0.5, 0.2, 0.1$, according to a previously described procedure [19,20].

Methods for Copolymers Characterization

The molecular weight characteristics of the copolymers were determined by size-exclusion chromatography (SEC) as described previously [19,20]. The number-average molecular weight ($\bar{M}_n$) of P($AMPS_{0.8}$-*co*-$AA_{0.2}$) was in the range $53 \times 10^3 - 60 \times 10^3$, and that of P($AMPS_{0.5}$-*co*-$AA_{0.5}$) and P($AMPS_{0.9}$-*co*-$AA_{0.1}$) were higher than 315×10^3. The viscometric-average molecular weight ($\bar{M}_v$) of PAMPS (9.8×10^5) was calculated from the intrinsic viscosity values, measured in 5 M aqueous NaCl with an Ubbelohde viscometer at 25 ± 0.1°C using the known Mark-Houwink constants values $K = 2.11 \times 10^{-3}$ and $\alpha = 0.80$ [21]. ^{13}C NMR spectra of copolymers were taken on a Bruker MSL 300 spectrometer operating at 75.5 MHz at 300 K in D_2O, using tetramethylsilane as a reference. The mole fraction of AMPS-units in the copolymer was determined by the ratio between the chemical shifts for C=O of AA-units (around 181 ppm) and for C=O of AMPS-units (around 178 ppm) according to the literature [19,20]. The copolymer composition, determined by ^{13}C NMR spectroscopy, was in good agreement with the data obtained by elemental analysis for carbon, hydrogen, nitrogen and sulfur.

Preparation of Reservoir-type Beads

Chitosan beads were prepared by capillary extrusion of chitosan solution (1.5 wt% in 0.5 wt% CH_3COOH) into 5 wt% aqueous solution of NaOH. The beads obtained were kept in the alkaline solution for 2 h, and then repeatedly washed until neutral reaction of the aqueous phase. These beads are further denoted as "unmodified chitosan beads" in order to be distinguished from the other types of beads. Chitosan beads, coated with polyelectrolyte complex chitosan/PAMPS or chitosan/P($AMPS_n$-*co*-$AA_{n'}$) were denoted as chit/PAMPS (coating) and chit/P($AMPS_n$-*co*-$AA_{n'}$) (coating), respectively. Chit/PAMPS and chit/P($AMPS_{0.5}$-*co*-$AA_{0.5}$) coatings were prepared by immersing the beads into 1.6% corresponding polyanion solution in saline for 4 h.

Chit/P($AMPS_{0.8}$-*co*-$AA_{0.2}$) and chit/P($AMPS_{0.9}$-*co*-$AA_{0.1}$) coatings were prepared by immersing the beads into 3% corresponding polyanion solution in saline for 4 h. The coated beads were repeatedly washed with saline to remove the unreacted PAMPS and P($AMPS_n$-*co*-$AA_{n'}$).

Preparation of Monolithic Beads Chit/PAMPS (Template)

Chitosan was dissolved in AMPS monomer aqueous solution at molar ratio (chitosan):(AMPS) = 1:1. Chitosan concentration was 1.5 wt%. Two of the three components of the redox initiator system necessary for the polymerization of AMPS – $Fe(NH_4)_2(SO_4)_2$ (0.05 g/L) and $(NH_4)_2S_2O_8$ (2.2 g/L) – were added. The beads were prepared by capillary extrusion of this solution into 2% aqueous solution of the third component of the initiator system – Na_2SO_3. The beads obtained were kept in this solution for 24 h and then repeatedly washed with distilled water.

Preparation of Monolithic Beads Chit/P($AMPS_{0.5}$-*co*-$AA_{0.5}$) (Template)

Chitosan was dissolved in aqueous solution of the comonomers at molar ratio (chitosan):(AMPS):(AA) = 1:0.5:0.5. Chitosan concentration was 1.5 wt%. One of the two components of the initiator system – $(NH_4)_2S_2O_8$ (4.8 g/L) – was added. The beads were prepared by capillary extrusion of the solution into 2% aqueous solution of the second component $K_2S_2O_5$ of the initiator system. The beads were kept in this solution for 24 h and then repeatedly washed with distilled water.

Preparation of Monolithic Beads *net*-PAMPS-*sipn*-chit

Chitosan was dissolved in an aqueous solution of AMPS in the presence of a cross-linking agent – MBAAm, at molar ratio (chitosan):(AMPS):(MBAAm) = 1:1:1. Chitosan concentration was 1.5 wt%. Two of the three components of the initiator system for the polymerization of AMPS – $Fe(NH_4)_2(SO_4)_2$ (0.05 g/L) and $(NH_4)_2S_2O_8$ (2.2 g/L) – were added. The beads were prepared by capillary extrusion of this solution into 2% aqueous solution of the third component of the initiator system – Na_2SO_3. The beads were kept in this solution for 24 h and then repeatedly washed with distilled water.

Preparation of Monolithic Beads *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit

Chitosan was dissolved in aqueous solution of AMPS and AA in the presence of a cross-linking agent – MBAAm, at molar ratio (chitosan):(AMPS):(AA):(MBAAm) = 1:0.5:0.5:1. Chitosan concentration was 1.5 wt%. One of the two components of the initiator system for the copolymerization of AMPS and AA – $(NH_4)_2S_2O_8$ (4.8 g/L) – was added. The beads were prepared by capillary extrusion of this solution into 2% aqueous solution of the second component of the initiator system – $K_2S_2O_5$. The beads were kept in this solution for 24 h and then repeatedly washed with distilled water.

Characterization of the Beads

The diameter of the beads (average value from ten measurements) was determined on an optical microscope (Reichert-Zetopan). The surface morphology of the beads was studied on a scanning electron microscope (Philips SEM 515). Specimens were placed on the sample holders with a double-sided adhesive tape, then vacuum coated with carbon and gold.

The equilibrium degree of swelling (α_{eq}) was determined by immersing dry beads in a buffer solution (pH = 3.8, 7 or 9, ionic strength I = 0.1). At certain time intervals the beads were taken out, the excess of the solution was carefully removed and the beads were weighed. This procedure was repeated until a constant weight of the swollen beads was reached. To estimate α_{eq}, the following equation was used: α_{eq} (%) = $[(W_e \times W_d)/W_d] \times 100$, where W_e and W_d were the weight of the sample at equilibrium swelling and in dry state, respectively. The results were an average of three measurements.

Determination of Blood Compatibility of the Gel Beads

Fresh human blood was drawn by venipuncture from healthy volunteers who had not taken any medication for at least ten days. The blood was collected in sodium citrate (3.13 g/100 mL) at a blood/citrate ratio of 9:1. The platelet number was approximately 3×10^5 per 1 mm^3 blood.

Coagulation

The anticoagulant activity of the uncoated chitosan beads, reservoir-type beads and monolithic beads was estimated from the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and reptilase time (RT) according to standard methods [22]. The values of the haemostatic parameters were calculated according to the following equations:

$$R_{PT} = \frac{PT_c}{PT_0}$$

$$R_{TT} = \frac{TT_c}{TT_0}$$

$$R_{APTT(LT)} = \frac{APTT(LT)_c}{APTT(LT)_0}$$

$$R_{APTT(kaolin)} = \frac{APTT(kaolin)_c}{APTT(kaolin)_0}$$

where PT_c, $APTT_c$, TT_c were the haemostatic parameters values at a given sample concentration, and PT_0, $APTT_0$, TT_0 were those of the control assays in absence of beads. A coagulation analyzer, STA Compact-Stago (France), was used to evaluate the anticoagulant activity of the beads. To obtain human pool plasma, the citrated whole human blood was centrifuged at 3,000 rpm for 10 min to separate the blood cells. Ten beads were immersed in saline for 48 h before the test. The beads were taken out from the saline and were immersed in 500 μL human pool plasma at the beginning of the clottability assay. The time for clot formation was determined after mixing the plasma with the corresponding reagent (STA Neoplastin® Plus, STA Thrombin, STA APTT LT, STA APTT Kaolin or STA Reptilase). The tests were repeated three times and the corresponding haemostatic parameters were expressed with standard deviations (SD). In each case a blank control assay (without added beads) was performed.

Platelet and Erythrocyte Adhesion onto the Beads

The platelet and erythrocyte adhesion was studied by direct observation of the blood cells adhering onto the surface by SEM. Twenty beads were immersed in saline for 48 h before the test. The beads were taken out from the saline and then immersed in 400 μL fresh whole citrated blood for 1 h. After that the blood was removed and the beads were washed carefully with saline to remove any nonadhered blood cells. The adhered platelets and erythrocytes were fixed by immersion in 20 mL 2.5% solution of glutaraldehyde (GA) in saline at room temperature for 20 min. The beads were freeze-dried after washing with distilled water.

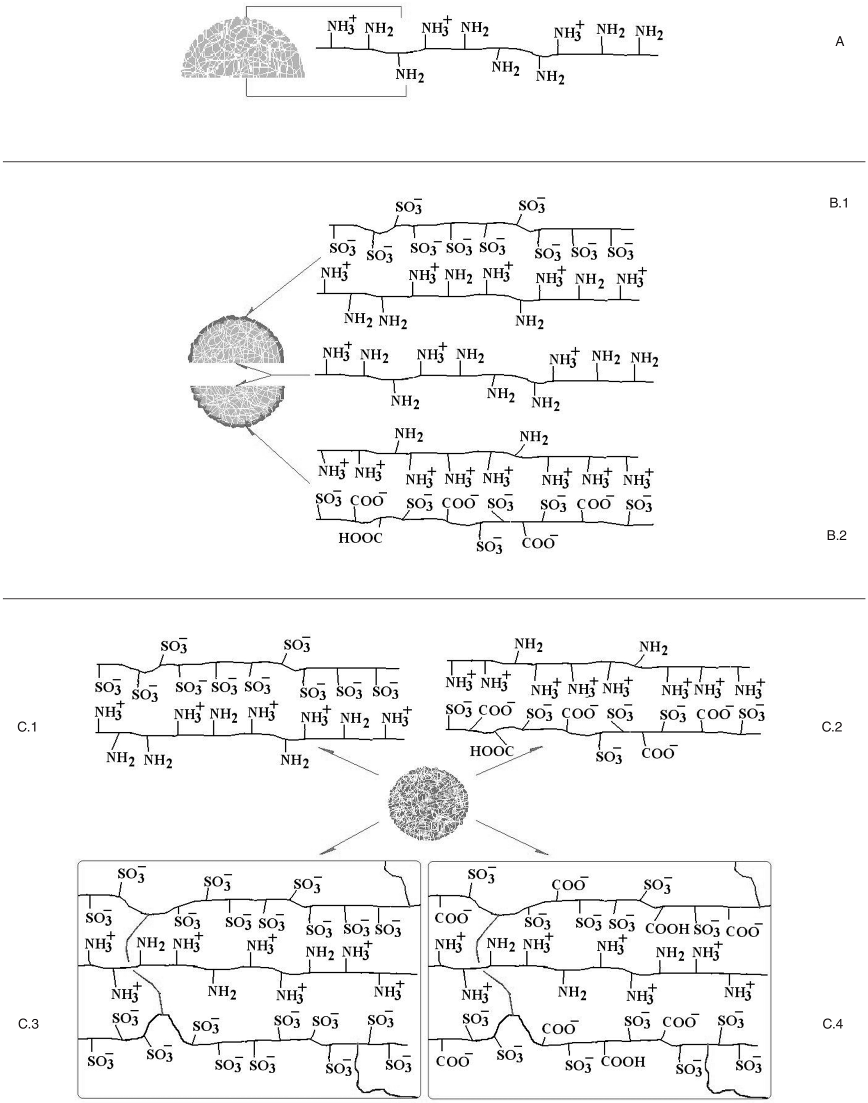
RESULTS AND DISCUSSION

Chitosan forms water-insoluble polyelectrolyte complexes with PAMPS [18] and with P(AMPS$_n$-*co*-AA$_{n'}$) copolymers [19]. In addition, chitosan beads were coated with a layer of polyelectrolyte complex chitosan/PAMPS and chitosan/P(AMPS$_n$-*co*-AA$_{n'}$), respectively. In the present work chitosan was modified by polyelectrolyte complex formation on the surface of the beads (reservoir-type beads) or in the bulk (monolithic beads). An original method was developed for preparing monolithic beads using an *in situ* template polymerization.

Preparation and Characterization of Beads

The three types of beads used in this study are represented in Scheme 1. Unmodified chitosan beads (Scheme 1A) were prepared by capillary extrusion of a chitosan solution into an alkaline medium. Reservoir-type beads were prepared in a second stage by immersing these chitosan beads into a solution of a polyacid. Thus a coating of chitosan/PAMPS (Scheme 1B.1) and chitosan/P(AMPS$_n$-*co*-AA$_{n'}$) polyelectrolyte complex (Scheme 1B.2) was formed, irrespectively, the behavior of each type of bead at different pH values was used to determine reservoir-type architecture. Chitosan dissolves in an acidic medium (pK_a of chitosan is 6.5) [23], while the polyelectrolyte complexes are stable and insoluble [18,19]. At pH values lower than 6.5 the core of the bead dissolved leaving a hollow shell of the corresponding polyelectrolyte complex. The complexes dissociate at pH values higher than 8; at these pH values chitosan is insoluble. After immersing the reservoir-type beads in an alkaline medium, the surface layer (polyelectrolyte complex) disintegrated, the insoluble core was chitosan, as determined by its solubility in acidic medium and by IR spectra. The equilibrium degree of swelling of the reservoir-type beads in saline was low and did not exceed 350–370% due to the high ionic strength of the medium.

In the present study an original one-stage method was developed for preparing monolithic beads by *in situ* template polymerization of AMPS or copolymerization of AMPS and AA taking place during the formation of the beads (Scheme 1C.1 and 1C.2). In previous studies, we showed that the chitosan/PAMPS polyelectrolyte complex could be prepared by template polymerization of AMPS in the presence of chitosan [24]. The monolithic beads, prepared by template polymerization of AMPS copolymerization of AMPS and AA, were denoted as chit/PAMPS(template) and chit/P(AMPS$_{0.5}$-*co*-AA$_{0.5}$) (template), respectively. The viscometric-average molecular weight ($\overline{M}_v$) was 9,000



Scheme 1. Schematic representation of the surface structure of: unmodified chitosan beads (A), reservoir-type beads (B) and monolithic beads (C).

Table 1. Average weight (m) and diameter (d) of different types of beads.

Beads type	Swollen beads[1]		Dry beads	
	m (mg)	d (mm)	m (mg)	d (mm)
Reservoir-type beads				
chit/P($AMPS_{0.5}$-co-$AA_{0.5}$) (coating)[2]	11	2.5	3	1
chit/P($AMPS_{0.8}$-co-$AA_{0.2}$) (coating)[3]	10	2.0	3	1
chit/P($AMPS_{0.9}$-co-$AA_{0.1}$) (coating)[3]	11	2.5	3	1
chit/PAMPS (coating)[2]	11	2.5	3	1
Monolithic beads				
chit/PAMPS (template)	1.2	1.2	0.7	$<$0.5
net-PAMPS-sipn-chit	2.2	2.0	0.9	1
net-P($AMPS_{0.5}$-co-$AA_{0.5}$)-sipn-chit unmodified chitosan beads	1.5	2.0	1.2	1
Unmodified chitosan beads[2]	12	2.8	3	1

[1]Values at equilibrium swelling in saline.
[2]Prepared using 1.5% solution of the copolymer.
[3]Prepared using 3% solution of the copolymer.

for PAMPS made under conditions for bead preparation. From the ^{13}C NMR data it was estimated that the copolymer obtained under these conditions had the composition P($AMPS_{0.5}$-*co*-$AA_{0.5}$). Its specific viscosity was 0.049. As seen in Table 1, the monolithic beads were smaller in size and were more dense. Due to the structure of the monolithic beads, entirely built by polyelectrolyte complex, the water uptake in monolithic beads was less.

A modification of the method allowed monolithic beads from semi-IPNs to be prepared by template polymerization in the presence of a cross-linking agent (MBAAm). These beads were denoted as *net*-PAMPS-*sipn*-chit and net-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit, respectively (Scheme 1C.3 and 1C.4). In contrast to the reservoir-type beads, the monolithic beads chit/PAMPS (template), chit/P($AMPS_{0.5}$-*co*-$AA_{0.5}$) (template), as well as *net*-PAMPS-*sipn*-chit and *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit swelled but did not dissolve in an acidic medium ($pH < 2$). When the monolithic beads chit/PAMPS (template) were kept in alkaline medium ($pH > 8$) for 24 h the chitosan/PAMPS polyelectrolyte complex was destroyed and the polyanion was released into the aqueous phase. The insoluble material in the alkaline medium residue was chitosan only. The monolithic beads of semi-IPNs *net*-PAMPS-*sipn*-chit and *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit swelled without releasing the polyanion in the aqueous medium thus indicating that the polyanion had been cross-linked. An additional evidence that stable semi-IPNs had been obtained was the constant weight of the samples after a prolonged stay under conditions affording solubilization of the non-cross-linked polycation (acidic medium). The high stability of the beads at low pH values is due to the fact that the polyelectrolyte complexes chitosan/PAMPS and chitosan/P(AMPS-*co*-AA), are stable at pH values up to 8 [18,19].

The dependence of the equilibrium degree of swelling (α_{eq}) of the monolithic beads chit/PAMPS (template), *net*-PAMPS-*sipn*-chit and *net*-P(AMPS-*co*-AA)-*sipn*-chit on the pH of the medium is presented in Figure 1. The highest α_{eq} value was determined in acidic medium for the beads chit/PAMPS (template). The lowest α_{eq} value for all monolithic beads was at pH 7 due to the low degree of ionization of chitosan amino groups (24% at pH 7) and of the stability of the complexes at this pH value. The monolithic chit/PAMPS beads (template) swelled significantly at pH 9; the α_{eq} value exceeded 1,000% at the seventeenth hour. As a result of the disintegration of the chitosan/PAMPS polyelectrolyte complex and the diffusion of the polyanion in the buffer solution the weight of the beads began to decrease after the seventeenth hour. This did not allow determination of α_{eq} value at pH 9. After 48 h at pH = 9 an insoluble residue remained. The solubility test and IR spectroscopy analysis data showed that this residue was chitosan.

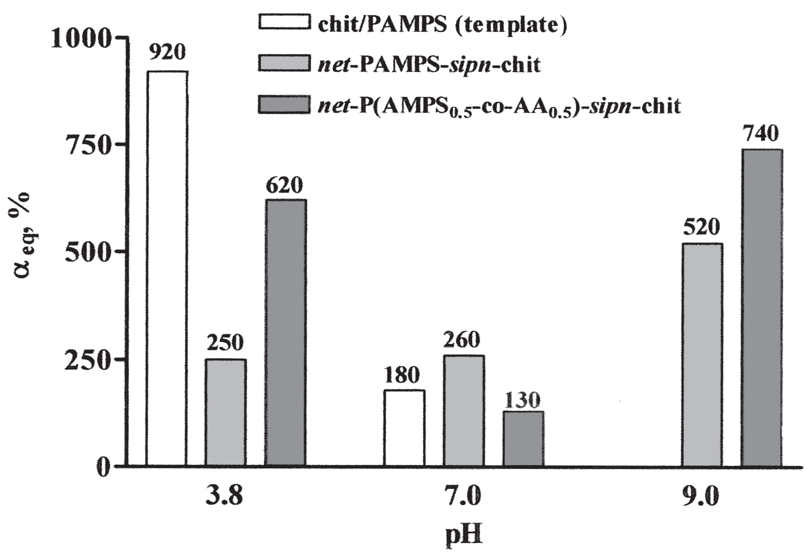


Figure 1. Dependence of equilibrium degree of swelling (α_{eq}) of monolithic beads chit/PAMPS (template), *net*-PAMPS-*sipn*-chit and *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit on the pH of the medium (I = 0.1; 25ºC).

The monolithic beads made of *net*-PAMPS-*sipn*-chit swelled only slightly at pH 3.8 and pH 7.0; the α_{eq} values were 250 and 260%, respectively (Figure 1). At pH 9 the α_{eq} value was 520%. This value was considerably higher than the determined α_{eq} value of unmodified chitosan beads at the same pH value ($\alpha_{eq} = 180\%$). The higher α_{eq} value of the *net*-PAMPS-*sipn*-chit monolithic beads at pH 9 may be explained with the increased number of available free ionized sulfogroups resulting from the destruction of the polyelectrolyte complex chitosan/PAMPS due to the conversion of the protonated amino groups of chitosan into NH_2. In the case of the *net*-P($AMPS_{0.5}$-co-$AA_{0.5}$)-*sipn*-chit monolithic beads, the lowest α_{eq} value was measured at pH 7 – only 130%. This pH value provides conditions for the most compact and hydrophobized structure as compared with the structure at pH 3.8 or pH 9. This is due to the different possibilities for complex formation provided by the different number of ionized groups at the pH values studied. At pH 7 all of the carboxylic and sulfo groups in the copolymer are ionized and are able to participate in complexation. At the same pH value, ~24% of the chitosan amino groups are ionized. At pH 3.8, the chitosan amino groups are almost 100% ionized, however, only 9% of the carboxylic groups are in their (ionized) complexable form (pK_a value of PAA is 4.8) [25]. Hence, at pH 3.8 only a small part of the protonated chitosan amino groups interact with the carboxylate anions and the complex formation proceeds mainly by ionic interaction between the $—NH_3^+$ groups of chitosan and SO_3^- groups of the copolymer. This lead to the formation of loops from the copolymer that were hydrophilic and thus increased water uptake in the beads. The high α_{eq} value (740%) at pH 9 is due only to the swelling of the copolymer network; the swelling is unrestricted, due to the dissociation of the complex at this pH value. Interconnected channels were observed by scanning electron microscopy (SEM) of freeze-dried monolithic beads made of chit/PAMPS (template) (Figure 2). The average diameter of the pores was 2.8 μm. The surface of the reservoir-type beads was smoother than that of the monolithic beads [19].

Blood Compatibility Studies

In vitro studies on the clottability and on platelet and erythrocytes adhesion onto the surface of the beads were performed in order to evaluate the blood compatibility of the prepared beads.

Coagulation Assay

It has been reported that chitosan does not influence the intrinsic

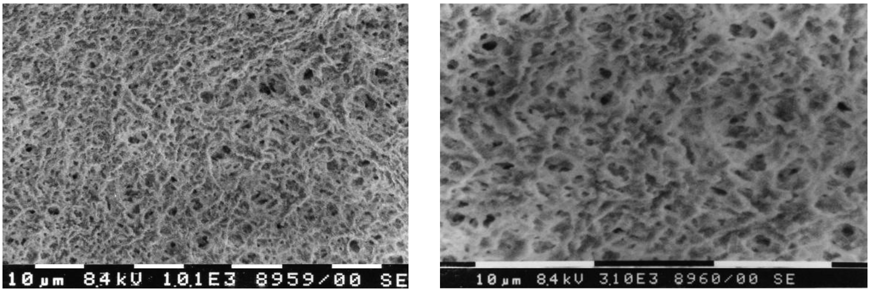


Figure 2. SEM micrographs of freeze-dried monolithic beads chit/PAMPS (template) (magnification ×1010 (1) and ×3100 (2)).

and extrinsic coagulation pathway [6] and that chitosan does not lead to significant change in the haemostatic parameters PT, APTT and TT. Previously, we found that solutions of PAMPS-*co*-AA copolymers increased PT, APTT and TT and thus showing anticoagulant activity [17]. In the present study, the same haemostatic parameters were used to estimate the *in vitro* anticoagulant activity of unmodified chitosan beads, reservoir-type beads and monolithic beads using human pool plasma. As seen from Figure 3, PT was only slightly prolonged in the case of both reservoir-type beads and monolithic beads. The presence of PAMPS in polyelectrolyte complex chitosan/PAMPS on the beads surface (reservoir-type beads) or in the bulk (monolithic beads) led to slightly higher prolongation of PT. This is in accordance with our previous findings that the copolymer composition does not significantly affect the R_{PT} value of PAMPS or P($AMPS_n$-*co*-$AA_{n'}$) in solution in the concentration range from 10 to 40 μg/mL.

The APTT(LT) and APTT(kaolin) values obtained for different types of beads are presented in Figures 4 and 5. The changes of R_{TT} occurring when the beads are immersed into human pool plasma, are presented in Figure 6. As seen, the type of the beads had a considerable effect on the R_{APTT} and R_{TT} values. The R_{APTT} and R_{TT} for unmodified chitosan beads were close to that of the control assay. The R_{APTT} and R_{TT} value for the reservoir-type beads showed that on increasing the amount of AMPS units in the copolymer the APTT and TT value increased, although these values remained close to that of the control. The highest APTT and TT values were obtained with chit/PAMPS coating ~3 and 8 times higher than the control, respectively. This prolongation of APTT and TT may be due to the presence of the free sulfo groups on the surface of the chit/PAMPS coating that led to an increase of the anticoagulant activity. The APTT and TT values for the

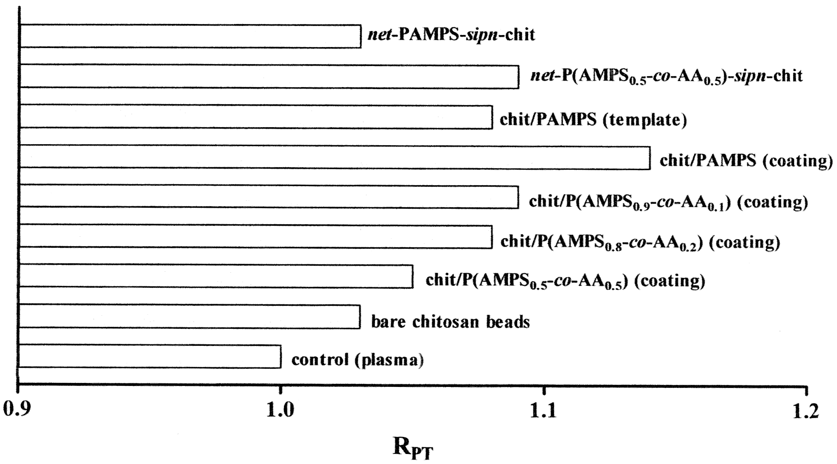


Figure 3. Prothrombin time ratio R_{PT} (calculated from the equation: $R_{PT} = PT_c/PT_0$; SD in determining the PT_c and PT_0 was in the range ±0.01–0.02) for different type of beads.

chit/PAMPS (template), *net*-PAMPS-*sipn*-chit and *net*-P(AMPS-*co*-AA)-*sipn*-chit monolithic beads were close to that of the control assay.

Previously we found that PAMPS solutions in the concentration range 16 to 40 μg/mL led to TT, which was 18 times higher than that in the control assay [17]. It might be assumed that when PAMPS is in the form of a polyelectrolyte complex, its anticoagulant activity is somewhat decreased by the engagement of the sulfo groups in the formation of the complex. The bound sulfo groups cannot interact with the natural inhibitor of thrombin – antithrombin, thus explaining the eight-fold increase in R_{TT} in the case of chit/PAMPS coating, in comparison with the eighteen-fold increase in the case of PAMPS in solution. The TT values for *net*-PAMPS-*sipn*-chit and *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit were close to that of the control assay. In the case of chit/PAMPS (template) the TT value was much smaller than that of chit/PAMPS (coating). This difference may be explained by the peculiar method of performing the template polymerization. In the initial solution for the template polymerization, since AMPS is the only acid present in the system, AMPS sulfo groups are ionically bound with chitosan amino groups. Consequently, PAMPS chains formed in the course of the reaction remain ionically bound with the chitosan template. The PAMPS chains are replicas of chitosan segments built of aminoglucoside units only (the acetylated units along the chitosan chain lead to termination of PAMPS chain growth). That is why the

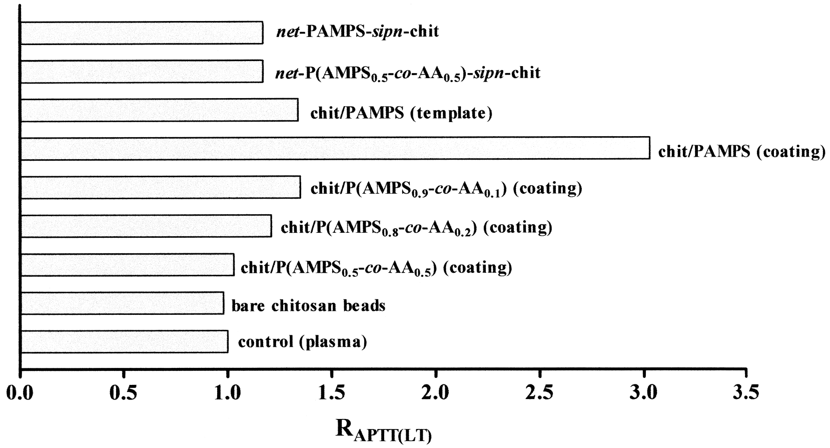


Figure 4. Activated partial thromboplastin time ratio $R_{APTT(LT)}$ (calculated from the equation: $R_{APTT(LT)} = APTT_c/APTT_0$; SD in determining the $APTT_c$ and $APTT_0$ values was in the range ±2.00–2.90) for the different beads.

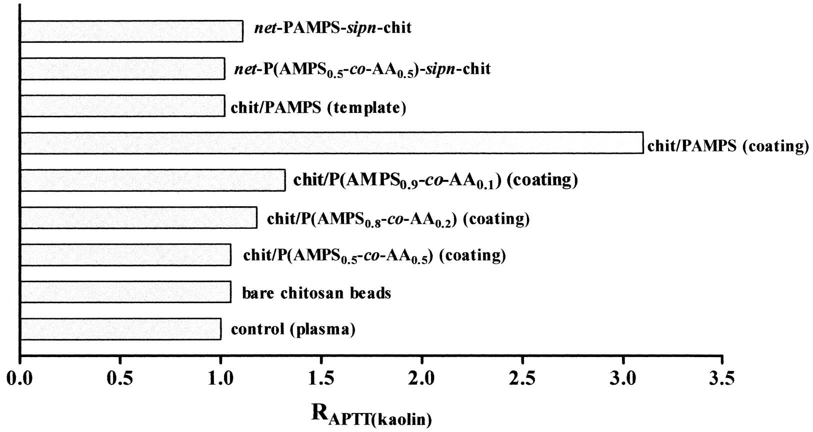


Figure 5. Activated partial thromboplastin time ratio $R_{APTT(kaolin)}$ (calculated from the equation: $R_{APTT(kaolin)} = APTT_c/APTT_0$; SD in determining the $APTT_c$ and $APTT_0$ values was in the range ±2.00–2.80) for the different beads.

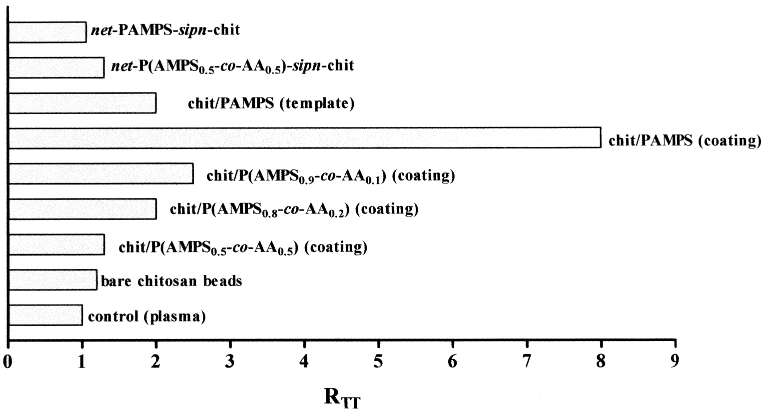


Figure 6. Thrombin time ratio R_{TT} (calculated from the equation: $R_{TT} = TT_c/TT_0$; SD in determining the TT_c and TT_0 values was in the range $\pm$1.90–2.50) for the different beads.

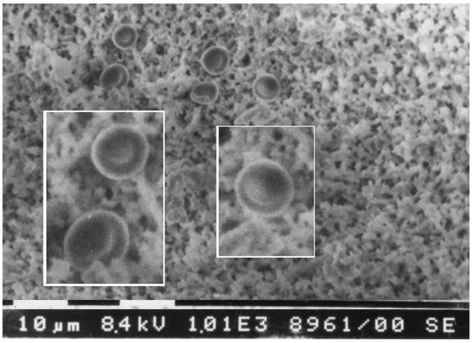
PAMPS chains formed in the beads by *in situ* template polymerization are much more engaged in the polyelectrolyte complex than in the case of the other monolithic beads and a smaller amount of free sulfo groups remain on the surface of the beads.

In order to clarify if the prolongation of TT was only due to the presence of the beads or to fibrinogen inactivation or to other factors, the change of RT was followed in the presence of the copolymers. In the presence of heparin and substances with heparin-like activity, since the plasma inhibitors (AT) of thrombin do not influence reptilase, in contrast to TT, RT is not prolonged. Experimentally, in the presence of reservoir-type beads or monolithic beads, the RT value remained constant (16–17 s) and was equal to that of the control assay. This shows that the prolongation of TT is due only to the anticoagulant activity of the beads.

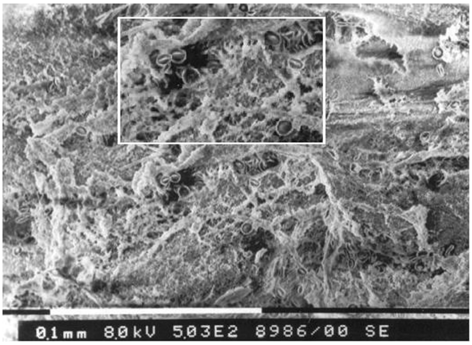
Platelet and Erythrocytes Adhesion onto the Surface of the Beads

The surface of the beads was examined by SEM, to detect any adhered blood cells on the surface of the beads after being in whole blood for 1 hour. No adhesion or aggregation of platelets and erythrocytes onto the surface of the beads was observed.

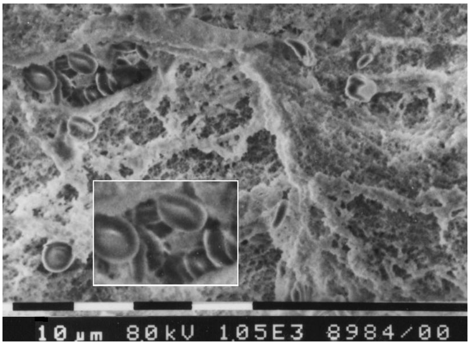
A SEM micrograph of the surface of chit/PAMPS (template) after contact with whole blood is shown in Figure 7(a). Single erythrocytes were observed in the pores; but, as seen in the micrograph, the



(a) Magnification ×1010



(b) Magnification ×503



(c) Magnification ×1050

Figure 7. SEM micrographs of beads after exposure to whole blood for 1 h: (a) chit/PAMPS (template), (b) *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit, (c) *net*-P($AMPS_{0.5}$-*co*-$AA_{0.5}$)-*sipn*-chit.

erythrocytes have kept their morphology. No deformation occurred during the contact with the polymer surface. Nor were any adhered platelets observed on the surface of the beads. SEM micrographs of *net*-P(AMPS$_{0.5}$-*co*-AA$_{0.5}$)-*sipn*-chit are shown in Figure 7(b) and (c). No platelets were observed on the surface of these beads. In contrast, however, the erythrocytes had adhered on the *net*-P(AMPS$_{0.5}$-*co*-AA$_{0.5}$)-*sipn*-chit beads and were deformed. The observed aggregation of the erythrocytes is known as haemagglutination and is typical for chitosan [6,11]. As a polycation chitosan may bind to the negatively charged cell membrane of the erythrocyte and platelet by ionic interactions. One of the reasons for the higher blood compatibility of the modified beads in comparison with unmodified chitosan beads might be the increased hydrophilicity resulting from the presence of sulfo and carboxylic groups. Another reason might be that the polyacids engage and/or shield the amino groups of chitosan, thus preventing the adhesion of the blood cells on the bead surface.

SEM analyses revealed the presence of pores on the surface of the chit/PAMPS (template) and chit/P(AMPS-*co*-AA) (template) monolithic beads in contrast to the smooth surface of reservoir-type beads. Probably, single erythrocytes adhered to the porous beads chit/PAMPS (template) and chit/P(AMPS-*co*-AA) (template) by physical attachment only and due to the lack of ionic interaction between chitosan and the blood cell membranes, the morphology of the adhered erythrocytes remained unchanged. Deformation and agglutination of the erythrocytes were observed in the case of *net*-PAMPS-*sipn*-chit and *net*-P(AMPS$_{0.5}$-*co*-AA$_{0.5}$)-*sipn*-chit monolithic beads (Figure 7(b) and (c)). This might be attributed to ionic interaction between the surface of the beads and the erythrocyte membranes The understanding of the intimate mechanism of interaction between blood and the beads requires further studies to be performed.

CONCLUSIONS

The formation of a chitosan/PAMPS or chitosan/P(AMPS-*co*-AA) polyelectrolyte complex leads to an improvement in the blood compatibility of chitosan materials. For the first time, beads have been prepared by template copolymerization in the presence of chitosan. These model chitosan materials possess unique properties and reveal good compatibility with blood cells. These materials could have applications in medicine and tissue engineering for preparation of devices that are in contact with blood.

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