Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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Ratan Kumar Dey[ab]; Alok R. Ray[abc]
[a] Department of Applied Chemistry, Birla Institute of Technology (Deemed University), Mesra, Ranchi, India [b] Center for Biomedical Engineering, Indian Institute of Technology, New Delhi, India [c] Center for Biomedical Engineering, All India Institute of Medical Sciences, New Delhi, India

To cite this Article Dey, Ratan Kumar and Ray, Alok R.(2005) 'Synthesis, Characterization and Blood Compatibility of Copolymers Derived from Polyamidoamines and Vinyl Acetate', Journal of Macromolecular Science, Part A, 42: 3, 351 — 364
To link to this Article: DOI: 10.1081/MA-200050470
URL: http://dx.doi.org/10.1081/MA-200050470

Journal of Macromolecular Science®, Part A: Pure and Applied Chemistry, 42:351–364, 2005

ISSN 1060-1325 print/1520-5738 online
DOI: 10.1081/MA-200050470

Synthesis, Characterization and Blood Compatibility of Copolymers Derived from Polyamidoamines and Vinyl Acetate

RATAN KUMAR DEY[1,2] AND ALOK R. RAY[1,2,3]

[1]Department of Applied Chemistry, Birla Institute of Technology (Deemed University), Mesra, Ranchi, India
[2]Center for Biomedical Engineering, Indian Institute of Technology, New Delhi, India
[3]Center for Biomedical Engineering, All India Institute of Medical Sciences, New Delhi, India

This work reports the synthesis of two different polyamidoamines (PAAs), by the reaction of piperazine (Pip) and cyclohexylamine (CHA) with N,N′-methylene bis acrylamide (MBA), which subsequently copolymerized with vinyl acetate (VAC) to improve the mechanical properties of the materials. The materials were characterized by spectroscopic techniques like IR, ^{1}H NMR, and ^{13}C NMR. Contact angle measurement data established the relative hydrophilic properties of the synthesized copolymers. From the weight of the thrombus formation with time and hemolysis assay, a conclusion could be drawn for the subsequent development of the materials for in vivo application.

Keywords polyamidoamine, polymer, synthesis

Introduction

Materials design and surface modification for blood contacting applications has been receiving considerable research attention and literature production for the last 25 years. Synthetic polymers have been increasingly used in the fabrication of the medical devices that make direct contact with human blood. Such investigations include the use of polymers with different properties like hydrophilic, hydrophobic, Zwitter ionic and charged (anionic and cationic) species (1, 2). The long-term use of polymers in blood is limited in part by surface induced thrombosis initiated by the adsorption of plasma proteins and activated platelets. Since thrombus formation occurs at the biomaterials–blood interface, appropriate surface modification of the materials in contact with blood has been adopted as a method for improving the blood compatibility of the materials.

Blood-contacting materials requires administration of certain doses of anti-coagulants like heparin, coumarine, etc., to prevent thrombus formation and embolization.

Received January 2004; Accepted August 2004
Address correspondence to Alok R. Ray, Center for Biomedical Engineering, Indian Institute of Technology, New Delhi 110 016, India. E-mail: alokray@cbme.iitd.ernet.in

However, it is often associated with the problem of bleeding in the patient. Therefore, attachment of biological molecules like heparin, albumin on the surface of the materials have also been adopted as one of the most suitable methods for making blood-compatible materials by researchers (3, 4).

Heparin is often used as an anticoagulant. It is anionic in nature, and known as highly sulfated mucopolysaccharide. Many researchers tried to develop methods of attaching and binding heparin on materials surface in order to counteract thrombogenicity and bleeding. Some recent examples, like heparin-immobilized polyurethane (PU) was prepared using the coupling reaction of polyurethane containing carboxyl group with polyethylene oxide (PEO) followed by the reaction of grafted PEO with heparin (5). Initially, within some hours, that material showed a loss of 3% of heparin from originally heparin bound material. Thereafter, almost no heparin was released when the heparin immobilized PUs were immersed in a physiological solution for 100 h, indicating the covalent immobilization of heparin onto the substrate. Similarly, Tsai et al. (6) characterized the surface properties of a genipin-fixed biological tissue immobilized with heparin using the methods of ionic bonding or covalent bonding via multipoint attachment or end-point attachment. Duncan et al. (7) also immobilized heparin onto poly(2-hydroxyethyl methacrylate) hydrogel using glutaraldehyde as a coupling agent in a heterogeneous and homogeneous phase and the significant residual heparin like activity was attributed to the immobilization of heparin in bioactive form. Morimoto et al. (8) modified the surfaces of segmented polyurethane (SPU) with the help of 2-methacryloyloxyethylphosphonylchlorine (MPC).

Polyamidoamines possesses an ability to selectively adsorb heparin from plasma or blood giving stable complexes without any adverse effect on plasma protein and blood cells. A number of works has already been reported regarding synthesis and heparin binding characteristics of polyamidoamines (9–16). However, difficulty arises in utilizing such materials in different biomedical applications due to its poor mechanical strength. A number of attempts had also been taken by different researcher to improve the strength of polyamidoamines by grafting (17, 18), co-polymerizing PAA oligomers with other structures, thus obtaining linear elastomers or cross-linked structures (19, 20).

In this reported work, we took an attempt to copolymerize two synthesized polyamidoamines, containing vinylic group, with another monomer i.e., vinyl acetate to improve its mechanical strength. The heparinized copolymer films were tested for antithrombogenic properties and the results were compared with that of the heparinized polyvinyl acetate film, which served as a control.

Experimental

Materials

N,N′-methylene bis acrylamide (MBA) (E. Merck, Germany), Piperazine (Pip), and Cyclohexylamine (CHA) (SRL, India), were used as such. Vinyl acetate (VAC) monomer (BDH) was distilled before use and the middle fraction was collected for subsequent use. Potassium persulphate (BDH) was recrystallized from absolute methanol. Sodium salt of heparin (1000 IU) was obtained from Gland Pharma (India). All other chemicals used in the study such as acetone, diethylether, methanol, potassium dihydrogen phosphate, KOH, etc., were of laboratory grade.

Instrumentation

The FTIR spectra were recorded in a Nicolet Protege 460 spectrophotometer in the 4000–500 cm^{-1}range, in the KBr pallet. The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, (Bruker Spectrospin 300) instrument. Thermogravimetric analysis (TGA) was carried out in a Perkin-Elmer thermal analyzer at heating rate 10°C/min, under nitrogen atmosphere. Contact angle formed by a drop of distilled water on the surface of copolymer films was determined using a Rame goniometer model 100-00-230. The hemolysis study was done using a Perkin-Elmer UV/VIS spectrometer model EZ 201. Tensile properties of the materials were tested using a dumbbell-shaped specimen on a Zwick Z010 module.

Synthesis of Polyamidoamine (PAA)

A vinyl group containing polyamidoamine (PAA) was prepared by dissolving 8.0 g of N,N′-methylene bis acrylamide (MBA) and 4.19 g of Piperazine in 30 mL double distilled water. A little excess (∼10%) of N,N′-methylene bis acrylamide was used for the synthesis of the polyamidoamine. The reaction mixture was stirred under nitrogen atmosphere for 48 h at 30°C. The viscous solution thus obtained was poured into 60 mL of acetone. The polyamidoamine (Pip-MBA) was separated out as a white crystalline product, which was filtered, washed with acetone, and dried under vacuum. The yield was nearly 78%.

Similarly, another polyamidoamine was prepared using 7.9 g of MBA and 4.0 g of cyclohexylamine in 40 mL double distilled water and here we also used a little excess (∼10%) of MBA in stoichiometric proportion to get the resultant product polyamidoamine. The yield was nearly ∼74%. The solution was stirred under a nitrogen atmosphere continuously for 32 h at 30°C. Pouring the resulting viscous solution to 60 mL of acetone resulted in the separation of a white crystalline product (CHA-MBA), which was filtered, washed with acetone and dried under vacuum. Both the synthesized polyamidoamines possesses a crystalline nature having negligible mechanical strength, as well as easy solubility in water at room temperature. Hence, the use of such materials for any blood contact application purpose without suitable modification could be easily ruled out. Therefore, in a subsequent step, an attempt was taken to copolymerize it with another suitable monomer to improve its mechanical property, as well as solubility behavior of the material as a whole.

Synthesis of Copolymers

The polyamidoamines (PAAs), (Pip-MBA and CHA-MBA), obtained above were copolymerized separately with vinyl acetate (VAC). 4.0 g of PAAs were dissolved in 20 mL double distilled water and 10 mL of VAC (distilled) were added in portion to the reaction tube followed by the addition of an initiator. The initiator used was potassium persulphate [$S_2O_8^{2-}$] of concentration 0.1 mol/L. Potassium dihydrogen phosphate (0.1 M) and few mL of KOH (0.1 M) were also used to maintain the pH of the medium approximately to 7.0. The contents of the reaction tube was mixed well and then purged with nitrogen gas. The reaction was carried out keeping the sealed reaction tube in a thermally controlled water temperature bath at 65–70°C for about 90 min. Then the mixtures were poured into cold diethylether in which the resulting copolymers (Pip-MBA-VAC and CHA-MBA-VAC) were precipitated out. Homopolymer of

polyvinyl acetate (PVAC), which serves as control, was also prepared using a similar procedure for comparing the properties of synthesized copolymers with that of control. All the copolymers, as well as homopolymers. were found to be insoluble in water. The schematic diagram of the synthetic procedure is presented in Fig 1.

Viscosity Measurement

Viscosity measurements of polymer solutions were carried out with a Ubbelohde viscometer, using acetone as solvent at $29 \pm 0.5^{\circ}C$. The flow time was measured for solutions at six different concentrations. The intrinsic viscosity was calculated by plotting specific viscosity $[\eta]_{sp}$ at various concentrations versus concentrations (C) of solutions and then extra-plotting to zero concentrations.

Preparation of Heparinized Copolymer Films

Thin films of copolymers were prepared by dissolving copolymers in acetone at room temperature and subsequently, spreading the solution over a glass plate. The solvent was then evaporated using a vacuum evaporator, and the resulting thin film was carefully removed from the glass plate. Films were then equilibrated with saline solution (0.5% NaCl solution) for 10 h. Sodium salt of the heparin (1000 IU) was

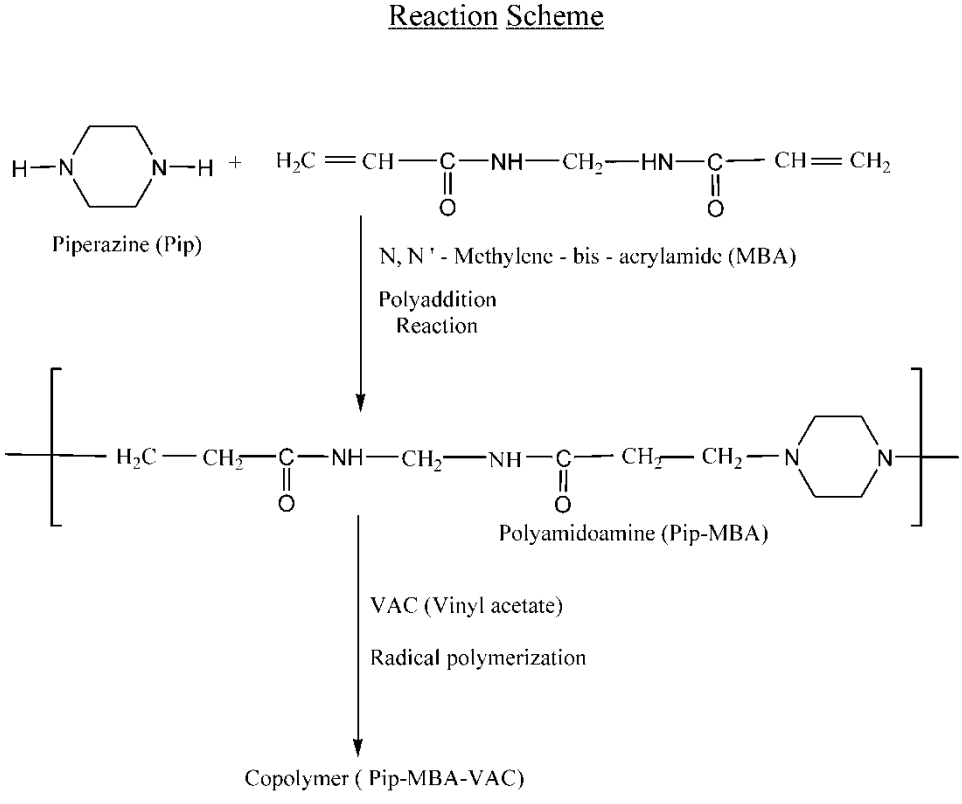

Figure 1. Reaction scheme of synthesis of polyamidoamine and copolymer.

diluted 20 times with 1 : 1 : water : ethanol mixture. Films were treated with dilute heparin sodium solution at room temperature for 12 h. Treated films were immersed with a diluted (2%) aqueous solution of glutaraldehyde solution for 2 min, washed with water, followed by ethanol and dried at room temperature (30°C) in a vacuum desiccator.

Thrombogenicity Assay

Thrombus formation studies were carried out using a *in vitro* "kinetic method" developed by Imai and Nose (21). ACD human blood was used for this purpose. ACD blood was prepared by adding a 1 mL of acid citrate dextrose (ACD) solution to 9 mL of fresh human blood. The ACD solution was prepared by mixing 0.544 g of anhydrous citric acid, 1.65 g of trisodium citrate dihydrate. and 1.84 g of dextrose monohydrate, to 75 mL of distilled water. ACD human blood (0.4 mL) was placed onto each film. Blood clotting was initiated by adding a drop of M/10 calcium chloride solution and mixing it properly with a teflon stick. The clotting process was stopped by adding a drop of distilled water (5 mL) after 15, 30, 45, and 60 min. The formed clot was fixed in 36% (v/v) formaldehyde solution (5 mL) for 2–3 min. A fixed clot was blotted between tissue papers and weighed.

Hemolysis Assay

Polymer films were cut into small pieces and the weight of each film was taken. ACD human blood (0.2 mL) was added to each sample. After a predetermined time period, 1 mL of saline water was added to each sample to stop hemolysis and those samples were kept at a constant temperature (35°C) for 1 h. Positive and negative controls were produced by adding 0.2 mL of human blood to 4 mL of distilled water and saline water, respectively. All the samples were centrifuged. Optical density of the supernatant was measured at 545 nm. The percent of hemolysis was calculated as follows: % Hemolysis = [OD of test sample − OD(−) control/OD(+) control − OD(−) control] × 100

Results and Discussion

Intrinsic Vscosity

The values of intrinsic viscosity have been found to be 0.204 and 0.164 dL/g, respectively, for Pip-MBA-VAC and CHA-MBA-VAC copolymers using acetone as a solvent.

Analysis of FTIR Spectra

Analysis of the FTIR spectrum (Fig. 2) of the polyamidoamine (Pip-MBA) showed the characteristic amide I, and amide II linkage at 1624 and 1537 cm^{-1}, respectively. The two amide bands resulted from the electronic coupling of $\mu_{C=O}$ and μ_{C-N} modes and mechanical coupling of μ_{C-N} and in plane δ_{NH} mode. The peak intensities appear at 1906, 1624, and 951 cm^{-1} assigned to the presence of a vinyl group unit in the polyamidoamine, were found to either vanish or diminished in intensities in the copolymer (Pip-MBA-VAC) suggesting that the copolymerization had taken place through the vinyl group units. The Pip-MBA-VAC copolymer showed absorption at 1740, 1223 cm^{-1}, assignable to carbonyl group stretching frequency of C(O)O– unit. The peak at 1598 cm^{-1} impluied

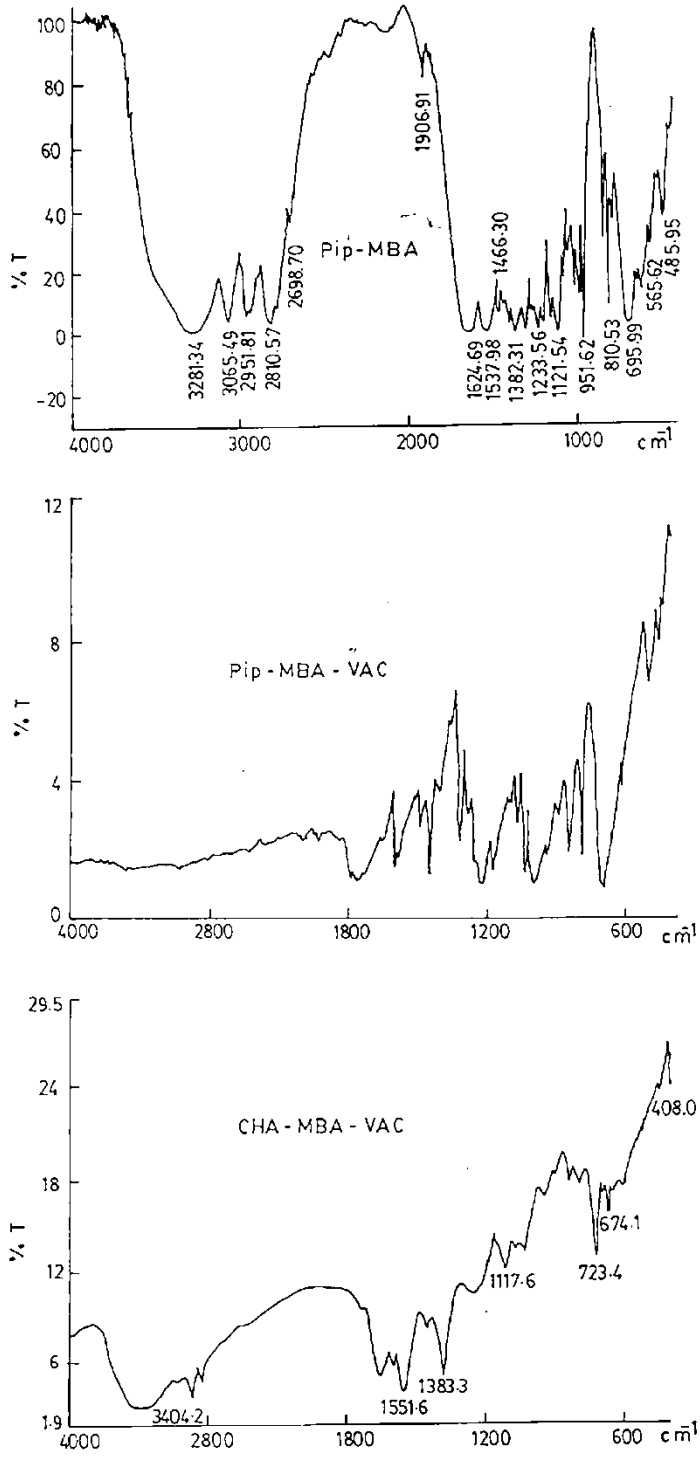

Figure 2. Infrared spectra of polyamidoamine: Pip-MBA; copolymers: Pip-MBA-VAC, CHA-MBA-VAC.

the presence of N–H group vibration from CONH groups suggesting the presence of polyamidoamine moiety in the copolymer unit. Peaks at 1450, 1380, 793 cm^{-1} were characteristics of various methyl and methylene group vibrations.

The spectra of (CHA-MBA) showed the peaks at 3389, 1575, and 1478 cm^{-1}, which were the characteristic peaks for the secondary amide group. The amide I, and amide II linkages were observed at 1615 and 1545 cm^{-1}, respectively. The presence of a vinyl group was characterized by the presence of peaks at 1630, 966, and 925 cm^{-1}. Upon copolymerization, the resulting polymer (CHA-MBA-VAC) showed peaks at 1710 cm^{-1} assignable to carbonyl group frequency of –C(O)O unit, along with the amide II bands at 1551 cm^{-1}. Peaks at 2900 and 2850 cm^{-1} were assigned to the C–H stretching frequency. The peak at 1455 cm^{-1} assigned to the methylene group vibration of the cyclohexyl unit. The C–N stretching frequency was observed at 1117 cm^{-1}. The absence of vinyl group frequencies at 990 and 910 cm^{-1} indicated the copolymerization through vinyl group unit.

Analysis of ^{1}H NMR spectra

The ^{1}H NMR spectrum (Fig. 3) of Pip-MBA showed the peak at 7.26 ppm (d) ascribed to secondary amide group in the structure. A characteristic higher order pattern was observed for vinylic group protons within the range of 5.6–6.3 ppm. The $-CH_2-$ group bonded to nitrogen observed in the range 4.64–4.78 ppm. Peaks in the range of 2.53–2.65 ppm were peaks of the $-CH_2-$ bonded to carbonyl group.

The ^{1}H NMR spectrum of Pip-MBA-VAC showed a reduction in intensity of amide proton peaks in addition to the reduction of intensities of vinyl group protons. Peak 4.7 ppm referred to the methylene protons of vinyl acetate moiety. Peak at 3.52 ppm represents the chain methine and ring methylene groups (of piperazine), which are bonded to the nitrogen atom. The peaks in the 2.49–1.74 ppm range were ascribed to the presence of methylene groups in the vinyl acetate and the polyamidoamine moiety, which appeared as broad overlapping multiplets.

Similarly, the ^{1}H NMR spectrum of CHA-MBA showed the presence of secondary amide group proton at 7.2 ppm. The presence of methylene groups alpha to nitrogen atom were shown as triplet in the 2.64–2.58 ppm range. At a high field, the methylene group alpha or beta to carbonyl group overlap to form a complex set of multiplet in the 1.83–1.48 ppm range. The cyclohexyl group methylene protons showed a complex set of multiplet in the 1.8–1.01 ppm range.

On the other hand, in the copolymer CHA-MBA-VAC, the secondary amide group protons observed in the 7.05–7.14 ppm region. The reduction of intensities of vinyl groups' protons was observed in the 4.47–4.37 ppm region. The peak at 4.8 ppm assigned to the methylene group of the vinyl acetate moiety. Peaks in the 3.2–3.0 ppm region represents the chain methine and methylene groups, which are bonded to the nitrogen atom. Other characteristic peaks of VAC and cyclohexyl ring protons appeared in the 2.90–1.40 ppm range as a complex set of multiplets.

Analysis of ^{13}C NMR Spectra

The ^{13}C NMR spectra (Fig. 4) of Pip-MBA showed a peak at 165.1 ppm ascribed to the CONH carbon atom. The vinyl group protons present in the polyamidoamines moiety were observed at 130.9 and 125.2 ppm as sharp peaks. The peak at 78.0 ppm corresponds to the carbon of the deuterated chloroform used for dissolving the sample. The peak at 51.7 ppm corresponds to

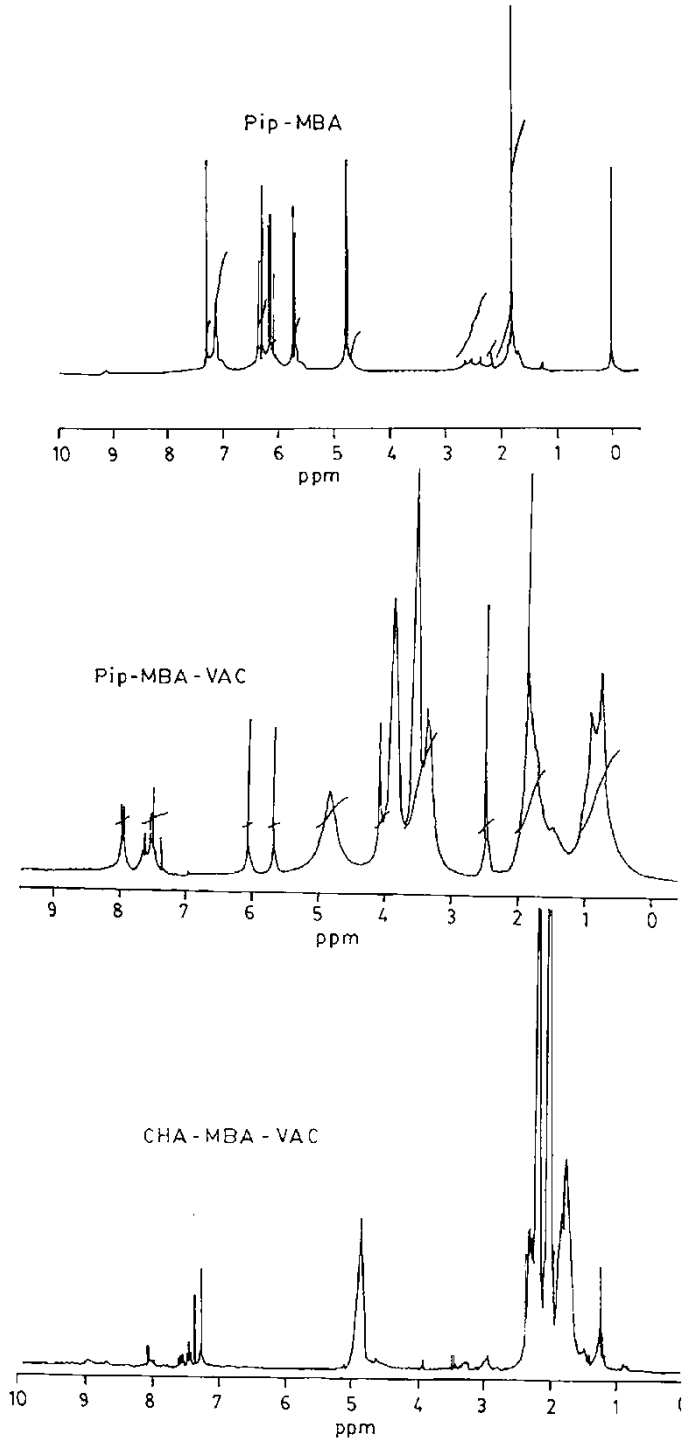

Figure 3. ^{1}H NMR spectra of polyamidoamine: Pip-MBA; copolymers: Pip-MBA-VAC, CHA-MBA-VAC.

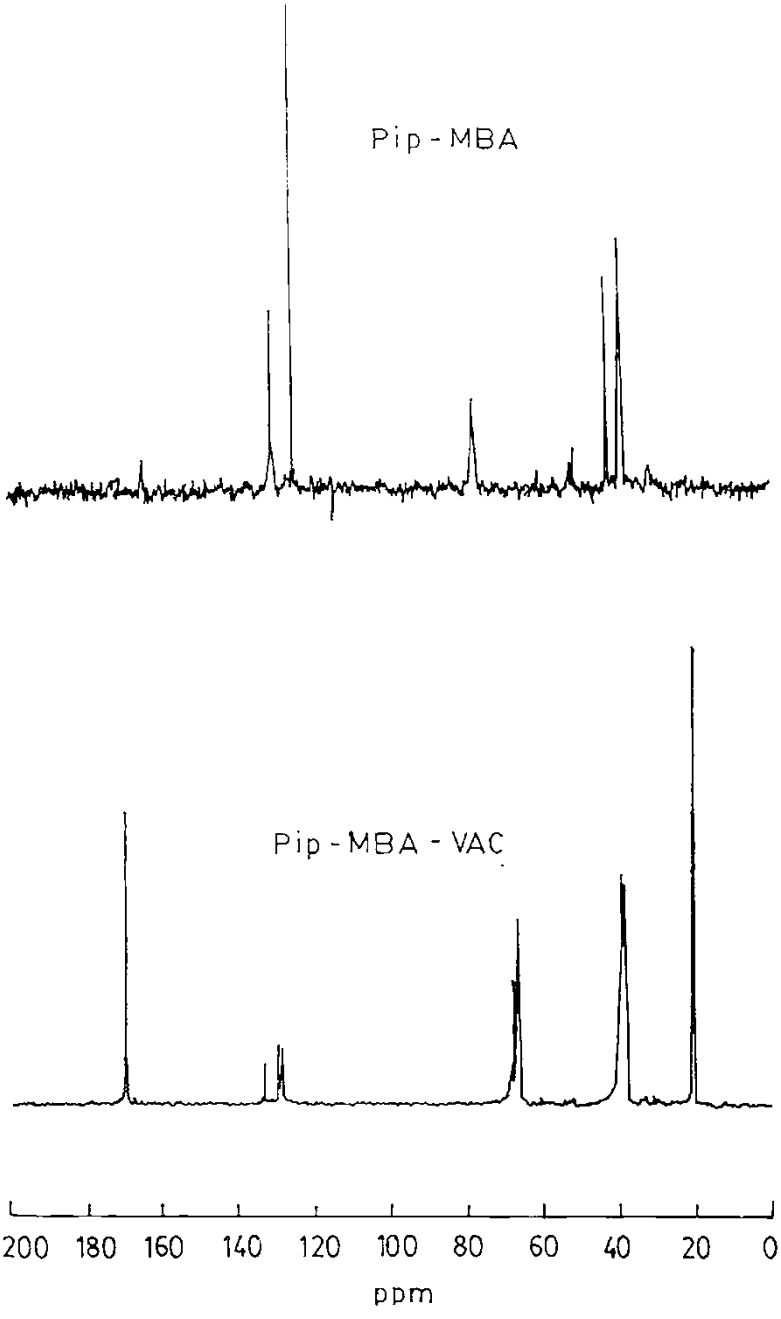

Figure 4. ^{13}C NMR spectra of polyamidoamine: Pip-MBA; copolymers: Pip-MBA-VAC.

the carbons of the piperazine ring. Other peaks within the 43.1–32.2 ppm range referred to various aliphatic group carbons present in the polyamidoamine moiety.

On the other hand, the ^{13}C NMR spectra of Pip-MBA-VAC showed peaks at 169 ppm and corresponds to the presence of carbonyl carbon of C(O)O– and –CONH group.

The intensities of vinyl group carbons were reduced within the 135–125 ppm range, indicating the fact of participation of vinyl group in the copolymerization process. The peak at 20.8 ppm corresponds to the $-CH_3$ group carbon of vinyl acetate moiety. The presence of methine carbon at 38 ppm, as well as the presence of various aliphatic group carbons, were observed within the 38.5–40.0 ppm range. Hill et al., while studying the copolymerization of methylmethacrylate and diethylene glycol bis (allyl carbonate), observed the –CH peak at around 30 ppm (22).

Evaluation of Surface Properties

Interaction of implant materials with tissues and physiological fluids stimulates the body's defense mechanism. The surface of implant material plays a key role in determining the nature of immunological reaction.

Measurement of Contact Angle

The contact angle measurement data used to evaluate the relative hydrophilicity and hydrophobicity of the synthesized polymers in contact with water. The contact angle was measured by putting a sessile drop of water on the surface of the polymer. Measurements were made after an equilibration time of 2 min. The contact angle measurement data was summarized in Table 1. The data showed that copolymers of Pip-MBA-VAC and CHA-MBA-VAC possess relative hydrophilic characteristics with respect to polyvinyl acetate film. This hydrophilic characteristic may be helpful in facilitating the blood-compatible characteristics of the copolymer film.

In addition, the contact angle measurement data could also serve as evidence for the presence of polyamidoamines (PAA) in the matrix of vinyl acetate (VAC) through the covalent bonding process. The water contact angle measurement was done in control polyvinyl acetate (PVAC), copolymer film of Pip-MBA-VAC, as well as in a mixture of PAA (Pip-MBA) and PVAC matrix. The PVAC and the physical mixture of PVAC and PAA retained almost the same water contact angle in clear contrast to the copolymer film Pip-MBA-VAC. Characteristics of these findings indicated that PAA chains were covalently bound to the VAC matrix through reacting double bond backbone.

Table 1
Measurement of contact angle

Material surface	Contact angle (°)
Smooth glass	50
PVAC	57
Pip-MBA-VAC	54
Pip-MBA-VAC (Heparinized)	62
CHA-MBA-VAC	56
CHA-MBA-VAC (Heparinized)	65
PVAC+Pip-MBA (Physical mixture)	56

PVAC: Polyvinyl acetate; Pip: Piperazine; MBA: N,N′-Methylene bis acrylamide; CHA: Cyclohexylamine; VAC: vinyl acetate.

Thrombogenicity Attempt

The weight of the blood clot formed at 15 min intervals (up to a 60 min time period) with various copolymer films along with homopolymer VAC were shown in Table 2. It was observed that the heparinized polyamidoamine-vinyl acetate copolymer films showed a decrease in the weight of the clot. Negligible blood clotting was observed up to a 45 min time period. It is known that surface properties play an important role at a molecular level in surface-induced thrombosis. The inhibition of blood clotting properties of the copolymer may be due to the adverse interaction of blood components via protein denaturation. Ishihara et al. (23) has recently showed that an increase in hydrophilicity does not have a similar effect on blood compatibility. In our case, the improvement in blood compatibility of the polyamidoamine copolymers might be due to the presence of amine groups of the polyamidoamine moiety, which absorbs heparin, thus inhibiting the clotting process. A similar effect had not been found on heparin treatment of homopolymer i.e., polyvinyl acetate film. A very small decrease in blood clotting observed in heparin treated PVAC film can be attributed to loosely bound heparin. These results were in agreement with the findings of earlier workers for various polyamidoamines (24, 25).

Hemolysis

Hemolysis of the blood is a problem associated with bio-incompatibility (26). Red blood cells hemolyze when they come in contact with water. This problem may be aggravated in the presence of an implant material. Results obtained for hemolysis of ACD blood with heparinized copolymers were shown in Table 3. It was observed that hemolysis was less than 9.0%.

Tensile Properties of the Materials

Tensile testing was done using a dumbbell-shaped specimen on a Zwick Z010 module testing machine at room temperature according to the procedure described in ASTM D638. The gauge length used was 40 mm and the crosshead speed was 500 mm/min. The dimension at the center of the test specimen was 2.5 mm^2. The result of the

Table 2
Determination of relative thrombogenicity of the materials by measuring weight of the thrombus formed

	Weight of the thrombus formed (mg)			
Polymer samples	15 min	30 min	45 min	60 min
PVAC film	1.1	2.4	3.2	3.9
PVAC-HEP (control)	0.9	1.6	2.8	3.0
Pip-MBA-VAC	0.8	1.1	1.6	2.3
Pip-MBA-VAC-HEP	0	0	0.3	0.9
CHA-MBA-VAC	0.96	1.4	1.7	2.6
CHA-MBA-VAC-HEP	0	0	0.5	1.1

PVAC: Polyvinyl acetate; Pip: Piperazine; MBA: N,N′-Methylene bis acrylamide; CHA: Cyclohexylamine; VAC: vinyl acetate.

Table 3
Hemolysis

Sample	Optical density at 545 nm	Hemolysis (%)
Water	1.960	+ve control
Saline	0.031	−ve control
Pip-MBA-VAC	0.25	11.35
Pip-MBA-VAC-HEP	0.187	8.34
CHA-MBA-VAC	0.29	13.42
CHA-MBA-VAC-HEP	0.201	8.81

PVAC: Polyvinyl acetate; Pip: Piperazine; MBA: N,N′-Methylene bis acrylamide; CHA: Cyclohexylamine; VAC: vinyl acetate.

average three-test specimen was recorded. The maximum stress to break the copolymer film recorded for the polyvinyl acetate film and the copolymer Pip-MBA-VAC was found to be 1.02 MPa and 0.93 MPa, respectively. The corresponding percentage of elongation at the point of maximum stress was found to be 108.9 and 3.63 for polyvinyl acetate film and the copolymer Pip-MBA-VAC, respectively. Therefore, improvement in mechanical properties of the purely crystalline polyamidoamine could be marked upon copolymerization with vinyl acetate monomer. However, further improvement in the materials properties requires a suitable combination of ratio of monomers, reaction condition, etc., which is in progress for a subsequent communication.

Conclusions

Two polyamidoamines consisting of piperazine-N,N′-methylene bis acrylamide (Pip-MBA) and cyclohexylamine-N,N′-methylene bis acrylamide (CHA-MBA) having heparin binding capability were synthesized. These were subsequently copolymerized with vinyl acetate (VAC) under suitable reaction conditions to yield two copolymers, Pip-MBA-VAC and CHA-MBA-VAC. Observation of a decrease in water contact angle of the copolymers established the relative hydrophilic nature of the copolymers with respect to polyvinyl acetate film. The heparinized copolymer films had shown little thrombus formation (<2.6 mg) within a 60 min time period and the percentage of hemolysis in heparinized copolymers showed a value less than 9.0% when compared with other reported values. The maximum stress required to break the copolymer film of Pip-MBA-VAC was 0.93 MPa, which showed improvement in the mechanical properties of the synthesized polyamidoamines.

Acknowledgment

Financial support from the Life Science Research Board (LSRB), Defense Research Development Laboratory to carryout the research works is gratefully acknowledged.

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