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Influence of unsaturated carbonic acids on hemocompatibility and cytotoxicity of poly-vinylacetate based co-polymers

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Abstract The aim of this study was to investigate hemocompatibility and cytotoxicity properties of synthetic polymer coatings containing various unsaturated carbonic acids with vinylacetate. Co-polymers of vinylacetate and crotonic acid (CA), maleic acid (MA), and itaconic acid (IA) were made. The materials were characterized in terms of their adhesion to metal supports (titanium and stainless steel) as well as hemocompatibility (% hemolysis, wettability, erythrocyte aggregation, hemoglobin content, thrombocyte count and lipid peroxidation levels) and cytotoxicity (human endothelial cell activity in vitro and chromosome aberrations, bone marrow proliferation and cell ploidy in rats). Co-polymers of unsaturated carbonic acids with vinylacetate exhibited good hemocompatibility properties, as opposed to vinylacetate homopolymer for which substantial levels of hemolysis were observed. By coating the metal supports with co-polymers the cytotoxic effects associated with the bare metal samples were markedly reduced. MA samples showed excellent hemocompatibility and no cytotoxicity, yet they lacked good adhesion properties to metal substrate, probably due to high water content. CA samples, having the highest density of carboxylic groups among the samples under investigation, showed increased bone marrow proliferation activity and cell ploidy in rats, as compared to controls. The most promising results in the present study were obtained for the samples with IA, which showed good adhesion to metal substrates, good hemocompatibility and low cytotoxicity. Thus, co-polymers of vinylacetate and IA rich in carboxylic groups are promising materials for the design of novel drug-eluting stents.

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1 Introduction

Dysfunctions of cardiovascular system are one of the primary causes of disease and mortality in humans. Therefore, development of novel materials for cardiovascular prosthetic appliances is of high relevance. Coronary stents are widely used as prosthetic appliances in heart surgery [1–6]. These are manufactured in various forms, such as bare metal stents (BMS), coated metal stents (CMS), biodegradable stents, and drug-eluting stents (DES). However, restenosis—i.e. repeated narrowing of the stent opening following the implantation—has been a significant limitation in clinical practice.

Metal stents are preferred due to their adequate mechanical properties and radiopacity/magnetic resonance imaging (MRI) compatibility [7]. Stainless steel (SS, grade 316L) is probably the most commonly used stent material due to its excellent corrosion resistance, suitable mechanical resilience, and favorable availability/price ratio [8]. However, high contents of Ni, Cr, and Mo in SS stents may give rise to allergic reactions [9] as well as local immune response and inflammation, which in turn may induce

neointimal hyperplasia and restenosis [10]. Coating of SS stents with polymers may improve their biocompatibility by preventing the release of ions as well as enable drug delivery capacity. Titanium and its alloys have been long known for their biocompatibility [11, 12] and are therefore regarded as interesting substrate materials for stents. Alternatively, biodegradable metallic stents of pure Fe received much attention since biodegradation of pure Fe stents, with oxidation and release of Fe^{2+}/Fe^{3+} ions, was found to reduce the proliferation of muscle cells in vitro, and thus may inhibit neointimal hyperplasia [13] with no local toxicity [14, 15]. Despite progress, the biocompatibility and hemocompatibility of metallic stents still remain an issue, and coating the metallic surfaces with other materials to alter their characteristics has been one rational approach to address this issue.

The biology of stent restenosis is complex and includes plaque redistribution, thrombosis and neointimal hyperplasia [16]. Usually, thrombosis of a stent occurs during the first month following the implantation, which is commonly referred to as subacute thrombosis. Whereas the usage of drug-eluting drugs has severely diminished the number of subacute thrombosis cases, the incidence of late thrombosis—i.e. several months after implantation—has markedly increased [17]. The most critical factors influencing the incidence of restenosis are the characteristics of the interface between the stent and blood, such as stent surface energy, surface tension, surface roughness, and surface net charge [18, 19]. Thus, the necessity of functionalizing the surface of stents with a permanent coating capable of minimizing the possibility of all kinds of restenosis is obvious.

Coating metallic stents with polymers not only enables one to modify the properties of the interface, but also includes the possibility of drug delivery. Among numerous synthetic polymers tested for this purpose, polyurethanes (PU) have shown promising thromboresistant and hemocompatible properties [20, 21]. Among other polymers tested, the polymers of polyethylene terphtalate (PET) [22, 23], poly-L-lactic acid (PLLA) [18, 24, 25], and poly-L-glycolic acid (PLGA) [26, 27] are often cited. Paclitaxel eluting stents with styrene maleic anhydride co-polymer was investigated previously and showed good hemocompatibility [28]. Vinylacetate co-polymers with ethylene were studied by Edelman et al. [29] as heparin delivery systems. In this respect, vinylacetate-based polymers exhibit good adhesion properties to various substrates, and generally are regarded as non-toxic [30]. However, the performance of the state-of-the-art polymers in stents is in general still far from the desirable level.

One procedure which was shown to remarkably reduce the possibility of thrombosis is to graft the outermost surface of the polymer coatings with chemical entities rich in carboxylic groups, via irradiation [31]. High density of carboxylic groups on the stent surface creates a large net negative charge at the interface between the stent and body fluids, which is believed to be favorable for the heparin-like activity of coatings [19, 32]. For instance, grafting of mixtures of vinylacetate and crotonic acid as well as acrylic acid and vinylpyrrolidone, via irradiation on polypropylene, has been reported in literature [31]. Further, high thromboresistance was achieved through partial neutralization of crotonic acid, and best hemocompatibility was reported for copolymers in which the content of crotonic acid was about 2% [33]. It should be mentioned that hemocompatibility of these polymers was also dependent on the nature of the counter ion, viz. Na^+ favored better hemocompatibility than Ca^{2+} or Mg^{2+}.

The grafting of polymers via irradiation has both advantages and disadvantages for development of biocompatible polymer materials. Advantages include purity of products, low process temperatures, and better control of kinetic effects during processing by modifying the dose of irradiation. However, a major shortcoming of this method is that the process is associated with high manufacturing costs, thus being of limited use for industrial scale production. It is therefore highly desirable to design novel polymer coatings for metal stents, using straightforward chemical processes which are easily adaptable for industrial scale production.

The aim of this study was, for the first time, to comprehensively investigate the hemocompatibility and cytotoxicity properties of synthetic polymer coatings containing various unsaturated carbonic acids with vinylacetate.

2 Materials and methods

2.1 Materials

2.1.1 Polymers

All reagents were purchased from Sigma Aldrich unless otherwise stated. Vinylacetate (VA) was distilled prior to use, to remove the inhibitor. Crotonic acid (CA) was re-crystallized from water solution prior to use. Itaconic acid (IA) and maleic acid (MA) were used as obtained without further purification. Benzoyl peroxide was used as an initiator and was precipitated from chloroform solution with ethanol. Oxyethylcellulose (OEC) 1% solution (viscosity of 16–20 mPa s at 20°C) was used as a protective hydrocolloid.

A 1% OEC solution was mixed with 200 ml of distilled water in a 500 ml round-bottom three-necked reaction flask. The flask was thermostated in a water bath at 65–75°C. A solution of 1 g benzoyl peroxide and 1 g of one of functional monomers—IA, CA or MA—dissolved in 99 g

of VA, was then added to the vessel under stirring. Polymerization was allowed to continue for 4–5 h until the residual monomer concentration of VA was not more than 0.5 wt%. The mixture was then allowed to chill to room temperature under stirring. The product had the appearance of translucent beads. The beads (approx. 1 mm in size) were filtered, thoroughly washed with water and dried, first in air and then under vacuum. Different samples were prepared, as detailed in Table 1. Under the employed polymerization conditions, the content of MA and IA did not exceed 1.5–2 wt% in the final product, whereas the content of CA was up to 15 wt%.

2.1.2 Metals

The following grades of stainless steel (SS) and Ti were used: SS grade X18H10T (Russian standard), which is the analogue of SS grade 316L, and Ti grade BT1-0 (Russian standard), which is the analogue of Ti grade 1–2. Pure iron (Fe, 99.9% purity) was obtained from the "Pure Iron" factory (Yerevan, Armenia).

2.1.3 Coating of metals

The surface of the metal samples was cleaned with ethanol or acetone to remove any traces of fat. The modified polyvinylacetate samples were dissolved in ethanol or acetone to obtain a 5–10 wt% solution. Metal samples were dipped in the above solutions and subsequently dried at 100–105°C. The thickness of the coating varied between 20 and 50 μm, and the coatings exhibited good water resistance.

2.2 Methods

All studies were approved by the international funding agency, viz. International Science and Technology Center (ISTC), and performed according to the "Guidelines on Animal Care and Use in ISTC Projects". The evaluation of biological action of materials was conducted according to the ISO Standard 10993.4-99 "Medical devices. Biological evaluation of medical devices. Part 4. Evaluation and selection of tests for interactions with blood".

2.2.1 Water uptake and polymer adhesion to metal substrate

The water uptake by the polymers was evaluated gravimetrically, after storing the samples in 100 ml of water for 96 h.

The strength of adhesion of polymer coatings to the metal substrate was evaluated qualitatively after incubating the samples for 3.5 months at 37°C in water, physiological solution, or phosphate buffer solution pH = 6.8. The coating was scratched by using a scalpel, and the ease of peeling of moist coating was assessed both under static and dynamic (centrifugation for 2 h at 1,500 rpm) conditions. The samples with the best adhesive properties were chosen for hemocompatibility studies.

2.2.2 Hemocompatibility

2.2.2.1 Hemolysis Hemolysis was measured in direct contact with blood according to ISO 10993.4 Medical devices standard. The blood was obtained from a healthy volunteer (50 years old). The samples were thoroughly washed and incubated in 10 ml of physiological solution (37°C, 30 min). In each holder 200 μl of 9:1 blood mixture with citrate buffer was placed. Citrate buffer consisted of a 3.8 wt% sodium citrate mixed with 0.85 wt% sodium chloride. The samples were then incubated at 37°C for 4 h. Following the incubation, aliquots were taken and centrifuged to precipitate the erythrocytes (3,000 rpm, 15 min). Optical density of the supernatant was then measured using a photocolorimeter (КФК-2-УХЛ4.2, Russia) at 540 nm. The percentage of hemolysis was calculated according to the following formula

Table 1 Primary characteristics of vinylacetate co-polymers

	Co-monomer type	Acid number (mg NaOH/1 g of polymer)	Intrinsic viscosity (η) (g/dl) (DMF, 25°C)
Sample 118	CA	9.02	0.590
Sample 119	CA	8.95	0.695
Sample 192	CA	41.35	0.404
Sample 272	CA	53.95	0.288
Sample 264	IA	3.16	0.698
Sample 263	IA	6.32	0.642
Sample 259	MA	4.48	0.695
Sample 257	MA	7.48	0.664
Sample 256	MA	10.0	0.610
Sample 276[a]	–	–	0.796

[a] Vinylacetate homopolymer

$$\alpha_r = \frac{(E_{test} - E_c)}{E_{100}} \times 100 \quad (1)$$

where α_r is the hemolysis percentage, E_{test} is the optical density of test sample, E_c is the optical density of control, and E_{100} is the optical density of a sample exhibiting 100% hemolysis.

The samples were considered hemocompatible if the extent of hemolysis did not exceed 2%.

2.2.2.2 Wettability The contact angle of polymer films was used as an indirect measure of hemocompatibility. The contact angle was measured using a goniometer (model 100-00 115, Rame Hart, USA). Glass slides were coated with a 10 wt% acetone solution of each polymer. The coatings were dried first in air and then under vacuum. The thickness of the obtained coating was 10–15 μm, with a weight of 0.02–0.025 mg/cm^2. A droplet of distilled water was placed on the coated slide and the contact angle was measured. Results shown are means of 3 measurements per sample.

2.2.2.3 Erythrocyte aggregation The aggregation of erythrocytes was measured using an aggregometer (Solar AP 2110, Belarus) in rats in vitro. Adult white male rats from an inbred line of animals (180–185 g each) were decapitated and the blood was collected in heparinated tubes. The blood samples were then centrifuged at 3,000 rpm and the supernatant was then removed. 0.01 ml of the remaining erythrocyte mass was dissolved in 10 ml of physiological solution, in which the polymer film was subsequently immersed. The aggregation of erythrocytes was followed every 30 min for 2 h, considering that erythrocytes are active in vitro during 2–3 h. The samples were considered hemocompatible if aggregation of erythrocytes did not exceed 14%.

2.2.2.4 Hemoglobin (Hb) content and number of thrombocytes 20 μl of blood obtained from a healthy volunteer was mixed with 5 ml of transforming solution, consisting of potassium ferrocyanide (30 mmol/l), potassium bicarbonate (38 mmol/l), and potassium phosphate (50 mmol/l). The mixture was incubated for 5 min at 20–25°C. The optical density of the solution (E_s) was then measured at 520–560 nm against transforming solution used as the blank (UV-spectrophotometer CΦ-46, Russia). The Hb content was calculated according to the following formula

$$\mathrm{Hb(g/l)} = 368 \times E_s \quad (2)$$

0.5 ml of blood obtained from a healthy volunteer was mixed with a furacilin citrate solution, containing 3.5 g sodium citrate and 0.025 g furacilin in 100 ml distilled water. The solution was agitated, and the number of thrombocytes in 1 μl was subsequently counted with light microscope.

2.2.2.5 Alterations of lipid peroxides Alteration of lipid peroxide levels in erythrocytes after exposure to polymer samples was taken as an indirect measure of hemocompatibility. The sample holders, coated with a layer of the polymer, were filled with 0.04 ml of 3.8 wt% sodium citrate solution and 3 ml of blood. The blood was obtained from healthy volunteers (48 and 50 years old). After 4 h, the erythrocytes were separated via centrifugation (3,000 rpm, 15 min). 20% (wt/vol) trichloracetic acid (2.5 ml) and 1 ml of thiobarbituric acid (6.7 mg/ml) were added to 0.5 ml of plasma. The mixture was incubated in a boiling water bath for 30 min. Upon cooling, 4 ml of butanol was added to the mixture followed by thorough stirring and centrifugation at 3,000 rpm for 15 min. The extinction of malonaldehyde (MDA) in the decanted liquid was measured using a UV-spectrophotometer (CΦ-46, Russia) at 535 nm. Blood samples before exposure to co-polymers were used as controls.

2.2.3 Cytotoxicity

2.2.3.1 Cell viability assay The effects of polymers on cell viability were investigated by using human epithelial cell line (A549 lung adenocarcinoma cells) as a target system. Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-activated fetal calf serum and 2 mM L-glutamine, under a water-saturated 5% CO_2/95% air atmosphere, at 37°C. Cells were plated at 3,000 cells/well in 96-well plates (Sarstedt) and used 24 h later. The polymers were coated on T-shaped metal supports (size: approx. 9 × 10 mm). The following metals were used as supports: Ti plates 0.5 mm in thickness and 1.7 mm in width, SS double-bundled wire (0.5–0.6 mm each). Prior to this, the T-shaped supports were sterilized by γ-irradiation (for details see below 2.2.2.4. *Bacterial proliferation*). For incubation with cells, T-shaped polymer-coated supports were applied to each well of the 96-well plate. Care was taken in order to avoid direct contact of the polymer coating with the cell layer at the bottom of the well. Cells were allowed to grow for additional 48 h. The coated supports were then removed, and cell viability was assayed by means of the WST-1 assay kit (Roche) according to the manufacturer's protocol. The concentration of formazan produced in the reaction was measured at 450 nm using a multilabel counter (Wallac 1420-Victor 3, Perkin Elmer). The results were expressed as percentage of values observed in untreated controls.

2.2.3.2 Cell proliferation, chromosome aberrations, and cell ploidy in rats Cytotoxic effects of polymers were

monitored as cell proliferation, frequency of chromosome aberrations, analysis of cell ploidy, in rats. Six animals per sample type were tested. Adult white male rats from an inbred line of animals (180–185 g each) were used. The polymers were implanted subcutaneously under dorsal skin. The biological activity of the polymers was monitored on bone marrow cells of rats. The exposure time was up to 75 days.

75 min prior to decapitation, 2 ml 0.04% colchicine solution was injected intraperitonially. Following decapitation, the hip bones were removed, rinsed in hypotonic 0.075 wt% KCl solution, and stored in 5 ml of the above-mentioned solution at 37°C for 10 min in a thermostat. The sample was then centrifuged for 5 min at 750 rpm. The supernatant was separated and then treated with a mixture of 6–7 ml methanol + concentrated acetic acid (3:1) by storing for 30 min at 4°C. The sample was then centrifuged again for 3 min at 1,000 rpm. The liquid portion was decanted and placed drop by drop on a preparation glass slide (0.5 ml). The samples were stained with 3% Giemsa dye (Sigma Aldrich) for 5–10 min. 100 metaphase discs containing 39–42 chromosomes per animal were analyzed using a light microscope, and the results were compared with controls. All kinds of aberrations were accounted, i.e. singular and pair-wise fragments, dicentric aberrations, and polyploidy cells.

2.2.3.3 Extract cytotoxicity As it will be further motivated in 3. Results and Discussion section below, sample 263 was additionally tested for verify its safety. A dozen of adult white male rats from an inbred line of animals (180–185 g each) were split into two groups of 6 animals each. 2 ml of the extract from sample 263 were injected intraperitoneally. The extract from sample 263 was prepared by soaking the co-polymer in distilled water for 10 days at 37°C. 2 ml of physiological solution was injected in animal controls. The bone marrow from animals was sampled 24 h after injections, in order to investigate chromosome aberrations, proliferation activity, and cell ploidy.

2.2.3.4 Bacterial proliferation The samples were sterilized by γ-irradiation using PYM-17 apparatus (Russia). The irradiation was performed at 183 R/min power dose. The total irradiation dose was 2 kGy. Following sterilization, the stents were immersed in a nutritional medium for bacterial control. The following media were used: THIO, glucose broth (35–36°C), and Saburo broth (20–22°C). Two vessels of specific nutritional medium were used per each sample. The incubation time was 10 days.

2.2.3.5 Acute systemic toxicity Acute system toxicity was estimated from morbidity data (e.g. external signs of intoxication, anxiety, death) in rats. Adult white male inbred rats having an average weight of 180–185 g were used. The polymer samples were comminuted and fed to animals with 5 ml vegetable oil orally in 100–120 mg/kg doses. The status of animals was monitored for 15 days.

2.2.3.6 Statistical analysis The statistical analysis was performed according to the Student's paired *t*-test and the confidence intervals were derived ($n = 6$).

3 Results and discussion

The chemical composition and primary characteristics of the studied materials are summarized in Table 1. It is seen from this table that the intrinsic viscosity of the co-polymer samples and, thus, their molecular weight decreases with the increase in acid number (i.e. amount of unsaturated carbonic acid monomers). The latter result is in full accordance with previous results that high molecular weights of poly-VA-CA co-polymers are unobtainable due to the chain transfer activity of CA [30, 33].

3.1 Hemocompatibility

One of the routes to develop hemocompatible polymeric materials is the synthesis of polymers which are rich in ionizable groups. Hydrogels produced by cross-linking of water-soluble polymers are often hemocompatible, but lack the mechanical stability and adhesivity to the substrate due to their high water content. Coatings of poly-vinylacetate modified with unsaturated carbonic acids, such as MA, CA, or IA, showed good adhesion to metal substrates in the present study. When tested in static conditions, the coatings of modified polyvinylacetate were in general more durable than those of the homopolymer, i.e. Sample 276. Thus, by functionalizing the polymers with unsaturated carbonic acids, the adhesion to metal substrate was markedly improved. Among the carbonic acid modified samples, the lowest durability of coatings was observed for the samples functionalized with MA. Under dynamic conditions simulating blood flow, the highest durability was observed for the samples functionalized with IA (both on a Ti and SS substrate). The water uptake capacity of the samples modified with different unsaturated carbonic acids varied depending on the chemical composition. The water uptake capacity of Sample 192 was estimated to be 0.039 wt%, whereas that of samples 263 and 257 was 0.043 and 0.26 wt%, respectively. Thus, it can be concluded that the most hydrophilic polymers in the series were those functionalized with MA, whereas those functionalized with CA, featuring the highest number of carboxyl groups, exhibited lowest hydrophilicity in the series.

Table 2 summarizes the hemocompatibility results as investigated by erythrocyte aggregation, wettability, and hemolysis in vitro. From these data it can be concluded that none of the modified vinylacetate co-polymers produces significant hemolysis and/or aggregation of erythrocytes, unlike the VA homopolymer which showed substantial hemolytic activity. Both the extent of hemolysis (viz. 8%) and degree of erythrocyte aggregation (viz. 21.3%) for VA homopolymer were well above acceptable levels (2% and 14%, respectively). The latter clearly indicates that introduction of unsaturated carbonic acid monomers improves the hemocompatibility of VA co-polymers in general. It should further be noted that relatively higher degrees of erythrocytes aggregation were observed for samples with CA (118 and 192).

In the past, thromboresistive materials with both high and low contact angle values were reported in literature [34]. No direct correlation between the wettability of polymer coatings and hemocompatibility was observed in this study. Thus, it can be concluded that hemocompatibility is not only dependent on the wetting properties of the material, but it is a complex biospecific response determined by interactions with various blood components.

MDA is the final product of peroxide oxidation of lipids, and increased levels of MDA are generally associated with increased propensity to erythrocyte membrane lysis. Table 3 summarizes lipid peroxidation results in erythrocytes before and 4 h after exposure to co-polymers. The results indicate decreased levels of lipid peroxidation for all samples, as compared to MDA levels detectable before experiments. It seems therefore that the hemolytic activity of the polymers under investigation is unrelated to lipid peroxidation.

Table 4 summarizes the results of changes in Hb content and number of thrombocytes in contact with the polymer coatings on SS substrates. Increased concentrations of Hb along with reduced numbers of thrombocytes indicate a hemolytic response to polymer coatings. As can be seen in Table 3, with CA co-polymers (samples 118 and 119) the changes in Hb content and number of thrombocytes were markedly altered, indicating a higher propensity of these samples to induce hemolysis. The number of carboxylic groups in samples 118 and 119 with CA is comparable to that of polymers with other functional groups in this study. Thus the relatively high Hb contents and low number of thrombocytes following the exposure to these co-polymers appears to be a specific response to CA moieties.

Table 3 Lipid peroxidation in erythrocytes following exposure to co-polymer coatings

	Co-monomer type	MDA[a] content (nmole/ml blood)	
		Before	After
Sample 118	CA	1.51 (0.042)	1.43 (0.036)
Sample 192	CA	0.60 (0.009)	0.56 (0.020)
Sample 272	CA	1.51 (0.042)	1.44 (0.016)
Sample 263	IA	0.71 (0.013)	0.64 (0.051)
Sample 257	MA	1.51 (0.042)	1.48 (0.020)
Sample 276[b]	–	1.51 (0.042)	1.45 (0.060)

Values shown are means (standard deviations in parenthesis; $n = 3$)

[a] MDA is malonic dialdehyde

[b] Vinylacetate homopolymer

3.2 Cytotoxicity

Incubation of samples in various nutritive media did not show any bacterial growth after 10 days of storage. Based on these results, the samples were further characterized for their cytotoxicity properties in cell cultures in vitro as well as in rats.

In order to enable drug delivery from the coatings, the rate of polymer resorption is crucial, wherein the release of the drug substance is either limited by erosion of the polymer coating or by diffusion of the drug from polymer matrix. The results of polymer implantation in rats showed that the sample 256 functionalized with MA was completely resorbed within 30 days. The weight decrease for the sample 192 functionalized with CA was 1.075, 6.0 and 7.462% after 7, 15, and 30 days, respectively. The samples

Table 2 Hemocompatibility following the exposure to co-polymer coatings in vitro

	Co-monomer type	Contact angle[a] (°)	Erythrocyte aggregation (%)		Hemolysis[a] (%)
			SS[a]	Film	
Sample 118	CA	56	10.5	–	1.7
Sample 192	CA	90	9.5	10.7	1.1
Sample 272	CA	51	–	–	–
Sample 263	IA	58	4.6	5.8	0
Sample 264	IA	65	–	8.3	–
Sample 256	MA	61	–	4.7	0.3
Sample 257	MA	61	1.3	–	0
Sample 276[b]	–	53	21.3	27.8	8.0

[a] Film coatings on stainless steel

[b] Vinylacetate homopolymer

Table 4 Changes in hemoglobin (Hb) content and number of thrombocytes in blood incubated contact with co-polymer coatings on SS substrates in vitro

	Co-monomer type	Hb content (g/l)		Δ Hb content (%)	No of thrombocytes × 1000 in 1 µl		Δ No of thrombocytes (%)
		Before	After 4 h		Before	After 4 h	
Sample 118	CA	168	294	42.9	275	215	21.8
Sample 119	CA	138	177	21.8	310	280	9.7
Sample 192	CA	134	169	20.8	285	280	1.8
Sample 263	IA	151	147	−2.6	250	235	6.0
Sample 264	IA	147	177	16.9	230	220	4.3
Sample 257	MA	103	110	6.4	240	234	2.5
Sample 276[a]	–	150	154	2.6	270	265	1.8

[a] Vinylacetate homopolymer

192 and 272 functionalized with CA were resorbed slower than sample 256 functionalized with MA. The slowest rate of resorption was observed for sample 263 functionalized with IA, which was not completely resorbed even after 75 days. IR spectral analysis of samples 192 functionalized with CA and 263 functionalized with IA, which was performed in order to investigate the possible chemical changes in the polymers during implantation, did not detect any chemical changes in these materials during the period examined. Because the sample 263 was not completely resorbed even after 75 days, the implant was removed and the cytotoxicity of its extract was additionally tested as will be shown below.

Acute systemic toxicity was evaluated as observed following 15 days of feeding rats with co-polymers. On the second day of the experiment, the animals showed signs of anxiety (adynamia, loss of appetite) which was over in 2–3 days. No external signs of intoxication were observed since then, and no deaths of animals were recorded throughout the course of the study. The LD_{50} of the studied co-polymers was found to be above 100 mg/kg. The results for the samples modified with CA are in accordance with previous results suggesting that co-polymers of VA-CA are generally non-toxic [30].

Figure 1 summarizes the cytotoxicity results in pulmonary A549 epithelial cells. Cell viability was not suppressed in the presence of co-polymers, except for sample 272 functionalized with CA, featuring a high density of carboxylic groups. The samples coated with MA-modified polyvinylacetate partially leaked into solution during cytotoxicity studies, which is in accordance with the coating adhesion results, indicating that these samples are least durable. The sample 263 functionalized with IA did not influence cell viability, whereas sample 192 functionalized with CA somewhat even increased cell proliferation. The bare metal substrates (both Ti and SS) without coating depressed cell viability by nearly 35%. It can be concluded from these results that unlike the bare metal samples, the coating of metal supports with polymers in most cases did not impose any negative effects on cell viability. The results on A549 lung adenocarcinoma cells indicate that the sample 263 functionalized with IA shows most favorable properties since it does not have adverse effects on cell viability and shows good adhesion to metal substrate.

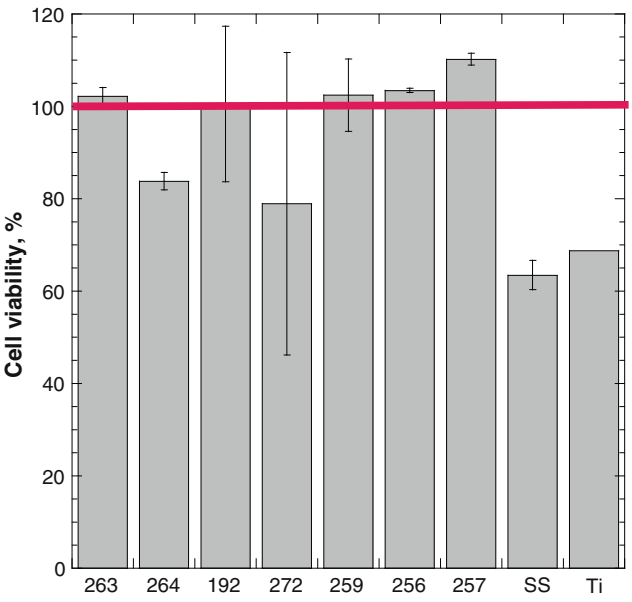

Fig. 1 Effects of different stent materials on the viability of a human epithelial cell line (A549 adenocarcinoma cells). *Bars* represent viability of cells exposed to the stent materials. The *horizontal* band shows levels measured in unexposed controls

The chromosome aberration results are summarized in Fig. 2. The bare pure iron sample produced a chromosome aberration level remarkably higher than that of controls. After 45 days, the chromosome aberration level of the coated pure iron sample with 192 was slightly lower than the control, which indicates the protective nature of polymer coating. Interestingly, the 192 (CA) sample as such produced aberration levels slightly higher than in controls

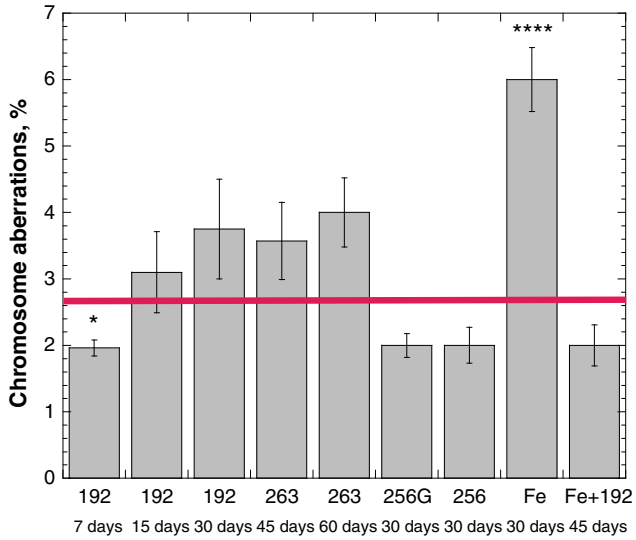

Fig. 2 Number of chromosome aberrations in rats following implantation. The results are means of six animals. The *horizontal* band shows the levels measured in controls without co-polymer implantation (2.7 ± 0.31; $P > 0.05$; $n = 6$). *256G*, granulated sample 256. * $0.02 < P < 0.05$, **** $P < 0.001$, $P > 0.05$

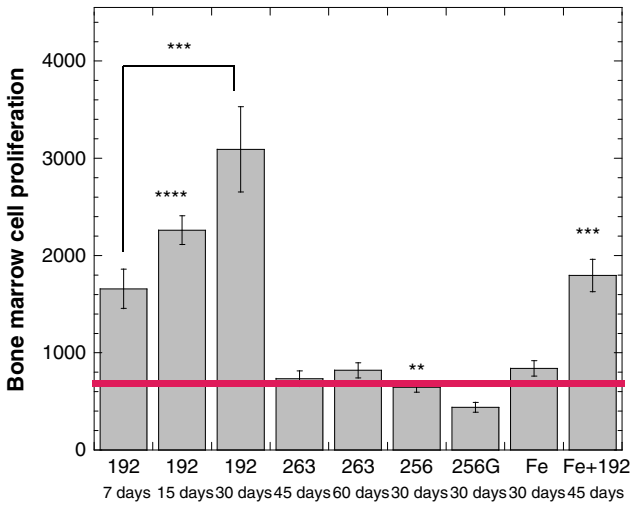

Fig. 3 Bone marrow proliferation activity in rats following the implantation of co-polymers. The results are means of six animals. The *horizontal* band shows the levels measured in controls without polymer implantation (769 ± 67.1; $P > 0.05$; $n = 6$). 256G corresponds to the granulated sample 256. ** $0.01 < P < 0.02$, *** $0.002 < P < 0.01$, **** $P < 0.001$, $P > 0.05$

after 15 and 30 days of implantation. The level of chromosome aberrations in samples 263 for 45 and 60 days (IA) was somewhat higher than in controls, whereas for samples 256 (MA) these levels did not exceed the controls.

Figure 3 shows the influence of the polymer samples on bone marrow cell proliferation activity. It is seen from Fig. 3 that the modified samples functionalized with MA (sample 256) slightly inhibit the bone marrow cell proliferation as compared to control. The modified samples functionalized with IA (sample 263) did not differ from the control, whereas the samples modified with CA (sample 192) substantially increased bone marrow cell proliferation. For comparison, samples of pure iron were implanted and the bone marrow cell proliferation levels were found to correspond to control. For pure iron samples, coated with sample 192, the proliferative activity was doubled after 45 days of implantation. Figure 4 shows the results of cell ploidy analysis under the effect of polymers. Ploidy of controls was within the range of 0.2–0.3 per 100 mitoses. Sample 192 produced cell ploidy levels above controls. It was found that type 256 and 263 samples produced ploidy levels below controls.

Neither the co-polymers as such nor their byproducts should induce toxic effects in order to be considered biocompatible. In order to investigate this aspect, an extract from sample 263 was tested. Additional cytotoxicity studies from the extract of sample 263 did not reveal cytotoxic effects. Figure 5 illustrates the typical chromosome stocks following the abdominal injection of an extract from polymer 263 in rats as compared to controls.

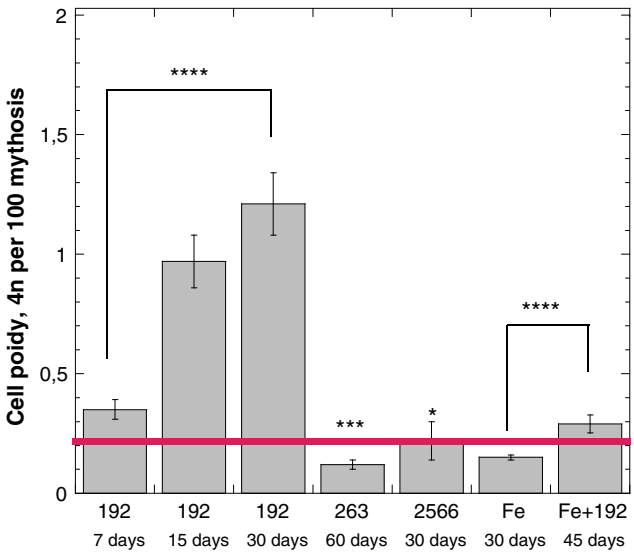

Fig. 4 Cell ploidy in rats following implantation. The results are an average from six animals. The *horizontal* band shows the level of the measured controls without polymer implantation (0.2; $P > 0.05$; $n = 6$). * $0.02 < P < 0.05$, *** $0.002 < P < 0.01$, **** $P < 0.001$

The arrows in the figure pinpoint the typically observed aberrations. The results following the injection of co-polymer extract are summarized in Table 5. It is seen from Table 5 that the values for chromosome aberrations, cell ploidy, and proliferation activity are not significantly different from the controls. It can thus be concluded that sample 263 does not present pronounced cytotoxic effects.

Among the samples studied, the most promising results were obtained with sample 263 functionalized with IA.

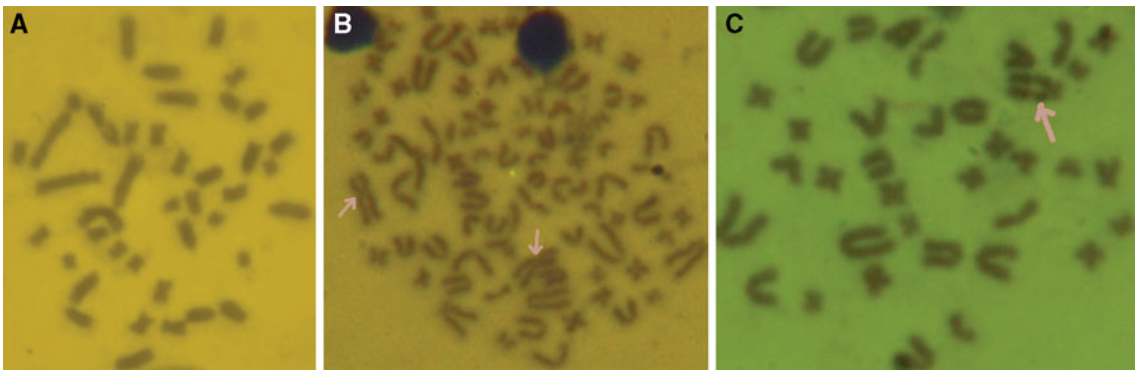

Fig. 5 Microscopic analysis of caryotype in rat cells. **a** Typical caryotype of normal cells. **b** Typical cell ploidy after injection of 2 ml of the extract from sample 263—the *arrows* pinpoint aberrant chromosomes. **c** Typical chromosome aberrations (*arrow*) after injection of 2 ml of the extract from sample 263

Table 5 Cytogenicity in rats after injection of 2 ml of extract from sample 263 (10 days of exposure)

	Control	Sample 263
Proliferation activity	884 (38.02)	825 (57.5)
Chromosome aberrations	2.00 (0.28)	2.41 (0.24)
Cell ploidy 4n	0.37 (0.04)	0.31 (0.06)

Values shown are means (standard deviations in parenthesis; $n = 6$, $P > 0.05$)

This sample showed good adhesion to metal substrates, good wettability, no significant hemolytic activity, and it could be considered generally biocompatible and non-toxic. The slightly higher level of chromosome aberrations following the implantation in rats for 60 days could be due to the larger intrinsic statistical variability and spread of values, since both controls and samples showed $P > 0.05$. Importantly, no significant differences in the number of chromosome aberrations was seen following the abdominal injection of an extract from sample 263, which otherwise could have confirmed the potential cytotoxicity of this sample. Bearing the latter in mind, sample 263 represents a promising synthetic polymeric candidate material for stent coatings. Future work will therefore be focused on investigating the in vivo effects of this material, as well as on designing drug-eluting coatings for sustained release.

4 Conclusions

In general, introduction of unsaturated carbonic acid monomers in a polyvinylcetate backbone resulted in polymers featuring good hemocompatibility as compared to vinylacetate homopolymer, which produced instead substantial levels of hemolysis. All modified samples showed low systemic toxicity and no bacterial growth was detected in nutritive media. Coating the metal substrates with the polymers under study improved the formers' biocompatibility. The samples modified with CA, which were featured with the highest density of carboxylic groups among the co-polymers studied, showed the lowest hydrophilicity (as obtained from contact angle values), and significantly increased bone marrow proliferation activity as well as cell ploidy. The samples modified with MA showed good hemocompatibility and no significant cytotoxicity; however, these samples lacked good adhesive properties on metal substrate which was probably due to high water content. The sample 263 functionalized with IA acid showed the best potential among the samples examined. It exhibited good adhesion to metal substrates, good hemocompatibility, and adequate inertness in most of the cytotoxicity studies.

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