Blood compatible modified polycarbonate membranes

Introduction:

A biomaterial is a substance used in prosthesis or in medical devices designed for contact with the living body for the intended method of application and for the intended period. Synthetic polymers are the most diverse class of biomaterials that are widely used in both medical and pharmaceutical applications, and contribute significantly to the quality and effectiveness of health care. These applications range from a variety of implants or other supporting materials (e.g., vascular grafts, artificial hearts, intraocular lenses, joints, mammary prosthesis and sutures), to extracorporeal therapeutics and other supporting devices (e.g., hemodialysis, hemo-perfusion, blood-oxygenation, intra-veneous lines, needle catheters and blood-bags), controlled release systems (e.g., transdermal drug delivery patches, microspheres and microcapsules for targeted drug delivery devices for different routes of administration), and clinical diagnostic assays (mainly as carriers and supporting materials) [1].

Materials used in medical applications must meet certain criteria and regulatory requirements and should be biocompatible. The surfaces of biomaterials are believed to play an important role in determining their biocompatibilities. For the materials which come in contact with blood, the first event is the adsorption of blood proteins at the solid-liquid interface. Thereafter processes like the activation of intrinsic coagulation, adhesion and aggregation of platelets, and the activation of complement system may take place, depending on the composition and the conformation of the adsorbed protein layer [2]. The composition of the adsorbed protein layer usually changes as a function of exposure time. To obtain more insight in the relation between the character of the polymer surface and it's blood compatibility, protein adsorption has to be studied on series of polymers with well characterized surface structures.

Polycarbonate is a unique class of engineering thermoplastic polymer which has a wide range of applications since its properties can be readily tailored by the variation of their components. Its properties such as consistent clarity, excellent impact strength, dimensional stability, creep resistance, heat resistance, light and inert response to blood and body tissue make it useful for a variety of disposable medical devices like dialyzer, oxygenator, infusion and bypass filters, transfusion devices, etc.

Radiation sterilization of medical devices is one of the biggest areas in which radiation is effectively used and has been rapidly growing all over the world. a variety of polymer based medical devices including those made from polycarbonate are currently being sterilized by ionizing radiation such as γ-irradiation. Ethylene oxide is widely used and accepted for

industrial sterilization of medical devices. Ethylene oxide is well known to be dangerous, toxic, carcinogenic, with mutagenic effects on living organisms [3]. In comparison, occupational safety of workers at the radiation processing facilities is very well and easily controlled, and no traces of radioactivity are introduced in irradiated products. The biggest advantage of radiation over other methods is that products can be sterilized after packaging, thus avoiding problems of recontamination.

The purpose of the present research is to get an insight to the influence of γ-irradiation on the polymer surface characteristics such as hydrophilicity and interaction and adsorption of blood proteins. For this purpose, adsorption of proteins from human plasma (i.e., competitive adsorption) on the surfaces of polycarbonate membranes cast from different solutions and γ-irradiated are studied.

EXPERIMENTAL

Materials

Commercially available polycarbonate (Lexan®) granules is obtained from General Electric (USA). The solvents used in the preparation of polycarbonate membranes, chloroform, 1,2-dicholoroetane, tetrahydrofuran, 1,4-dioxane, cyclohexane are obtained from Merck (Germany). Bovine serum albumin and fibrinogen are purchased from Sigma and used as received. All other chemicals are of reagent grade and are purchased from Merck AG (Darmstadt, Germany). All water used in the experiments is purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system. The resulting purified water (deionized water) has a specific conductivity of 18 megaohm.cm^{-1}.

Methods

Preparation of Polycarbonate Membranes

Polycarbonate membranes having different surface morphology and bulk structure are prepared by dry solvent-casting method. The polymer solutions (7.0%) prepared from different solvents are poured into a round glass mould (9 cm in diameter) and placed in a temperature-controlled chamber at 25ºC until dryness. The membranes obtained are ished several times with distilled water, and cut into square pieces (0.5 cm x 0.5 cm) with a perforator. Thickness of the membranes are measured to be in the range of 45-50 μm with a precision micrometer (**Mituyoto**, Japan).

γ-Irradiation

The polycarbonate membranes are irradiated in air at room temperature (about 25°C). Irradiation is carried out in Issledovatelj self protected type ^{60}Co γ-irradiator at a dose rate of 3.5 kGy/h. Irradiation dose is changed in the range of 0-200 kGy.

Characterization of Polycarbonate Membranes

Water Content of Polycarbonate Membranes: The water uptake behavior of polycarbonate membranes are determined in distilled water. Dry membrane pieces are placed in distilled water and kept at a constant temperature of 25±0.5°C until they reach equilibrium. Swollen membranes are removed and weighed by an electronic balance (Sartorius, ± 1.10^{-4} g). The water content of the swollen membranes are calculated by using the following expression:

$$\text{Water uptake \%} = [(Ws - Wo) / Wo] \times 100 \quad (1)$$

where Wo and Ws are weights of the sample before and after swelling, respectively.

Contact Angle Measurements: Surface hydrophilicity of pristine and γ-irradiated polycarbonate membranes are characterized by captive bubble method under water contact angle measuring technique [4]. This device consists of a travelling goniometer with x15 eyepieces a variable intensity light source and a micrometer-adjustable X-Y stage vertically mounted on an optical bench. The stage contains a plexiglass container in which a teflon plate suspends. The polymer sample is held on the underside of the teflon plate by means of small teflon clips. The container is then filled with triple distilled water and the plate with sample is loared into the container until the sample immersed completely. A bubble of air with volume of about 0.5 μL is then formed at the tip of the Hamilton microsyringe detached and allowed to rise to the polymer-water interface. The air bubbles are photographed within 5 minutes after reaching equilibrium of contact with the samples. Experiments are carried out at 25°C. The equilibrium contact angles (θ_{air}) are calculated from the height (h) and the width (b) of the air bubbles at the surfaces of polycarbonate samples by using Equation 2. The mean value of ten measurements is considered. The reproducibility of contact angle measurements is ± 2%.

$$\theta_{air} = \cos^{-1} [(2h/b) - 1] \quad \text{for} \quad \theta_{air} < 90^{o} \quad (2)$$

Atomic Force Microscopy Studies: In order to observe the surface topography of the untreated and γ-irradiated membranes, atomic force micrographs are taken with an AFM (Topometrix TMX 2000 Explorer, AFM in contact mode in air). The scanner and the tip used with this microscope are 130 micron tripod and the pyramidal type for topographic images respectively, the force exerted by probe on the surface is 0.7 nN. Four small square pieces of approximately 50x50 mm^2 area are cut from each film and fixed onto the metal sample holders by a solventless glue. In order to obtain the effect of γ-irradiation on the same region of the sample surface, a cross-like mark has been made on the sample surface by gently drawing with a surgical blade. The cross marked polycarbonate membranes with 150x150 μm^2 area with a resolution of

200x200 pixels are imaged before and after irradiation to various doses. The analyzed region are approximately 12x12 μm^2 which is directly zoomed from 150x150 μm^2 images.

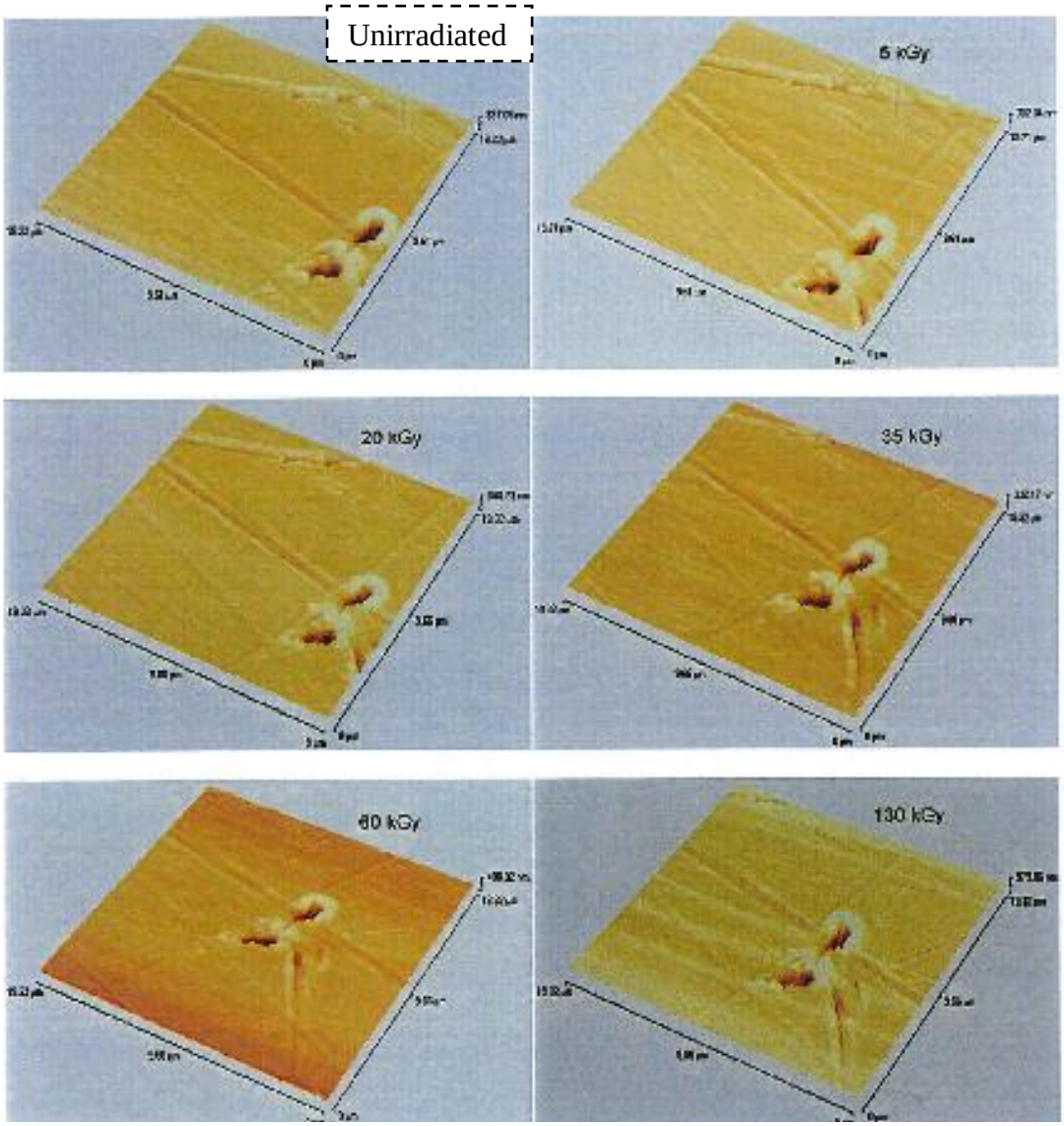

Image 1. AFM images of polycarbonate membranes prepared from chloroform with varying irradiation dose

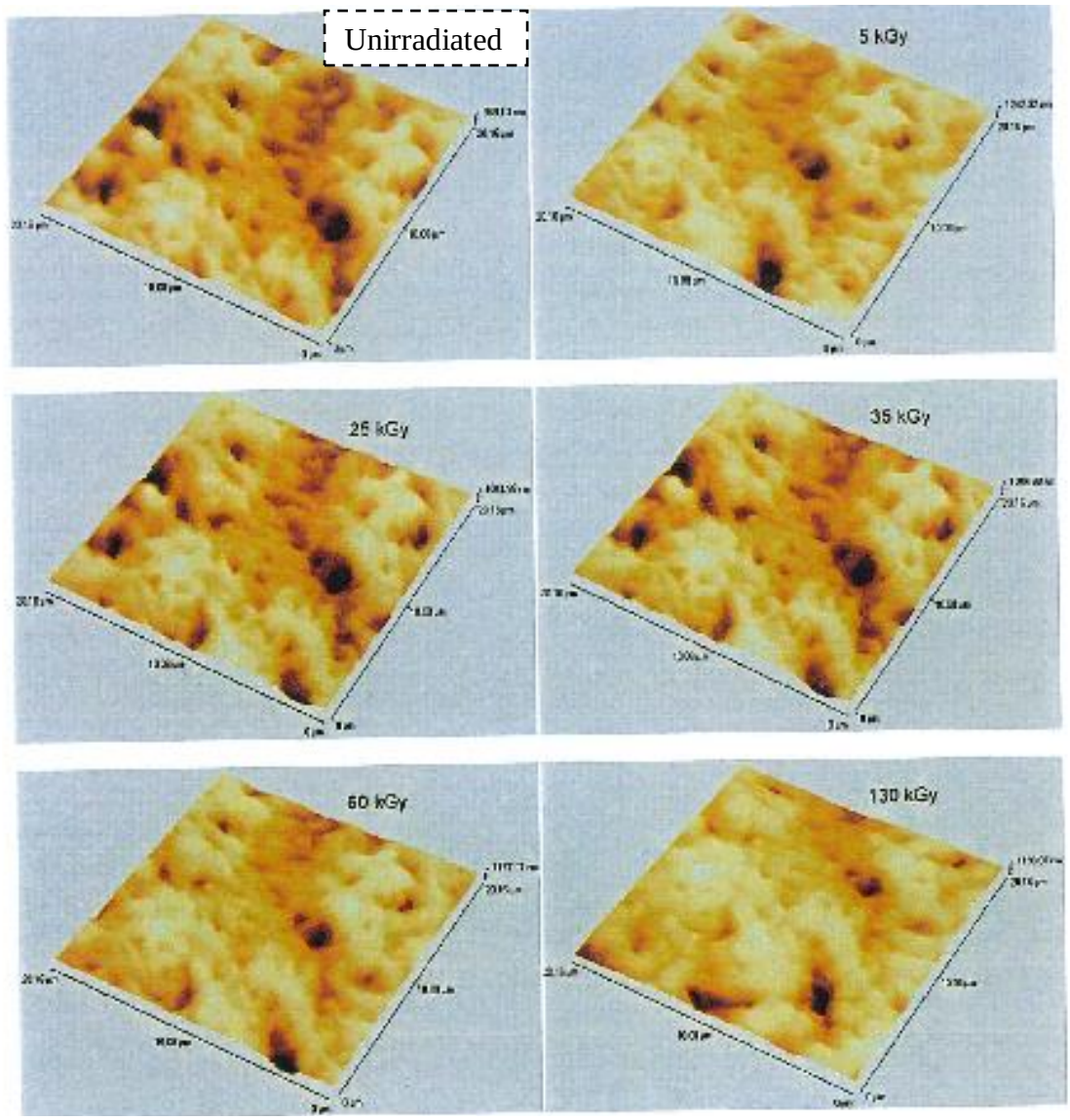

Image 2. AFM images of polycarbonate membranes prepared from dioxane with varying irradiation dose

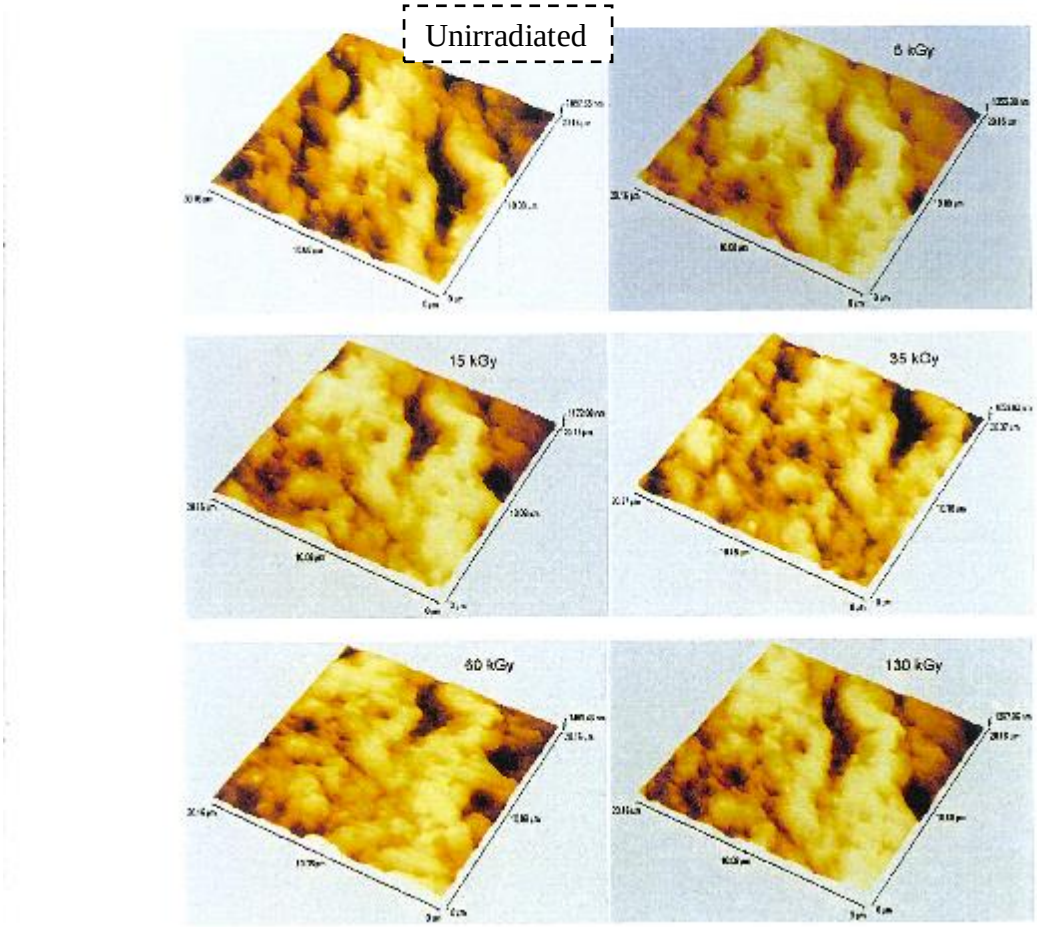

Image 3. AFM images of polycarbonate membranes prepared from dichloroethane with varying irradiation dose

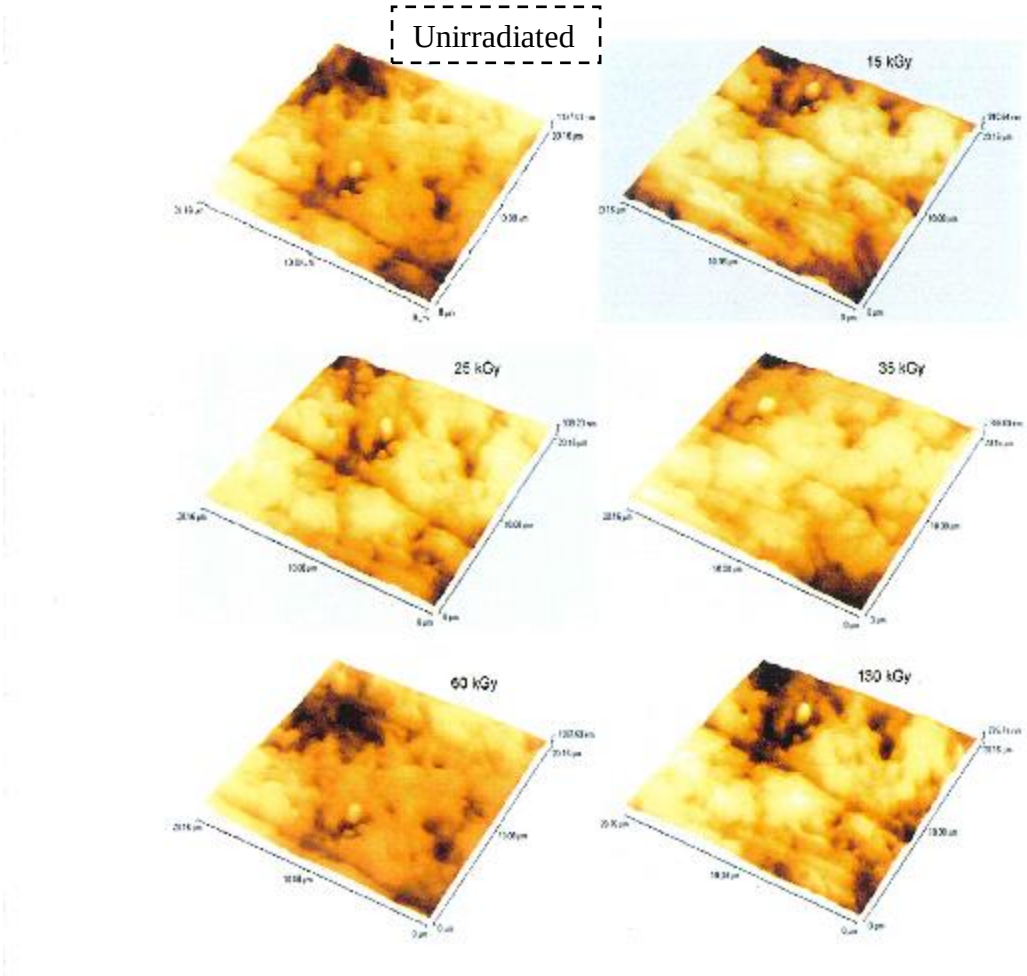

Image 4. AFM images of polycarbonate membranes prepared from cyclohexane/dioxane with varying irradiation dose

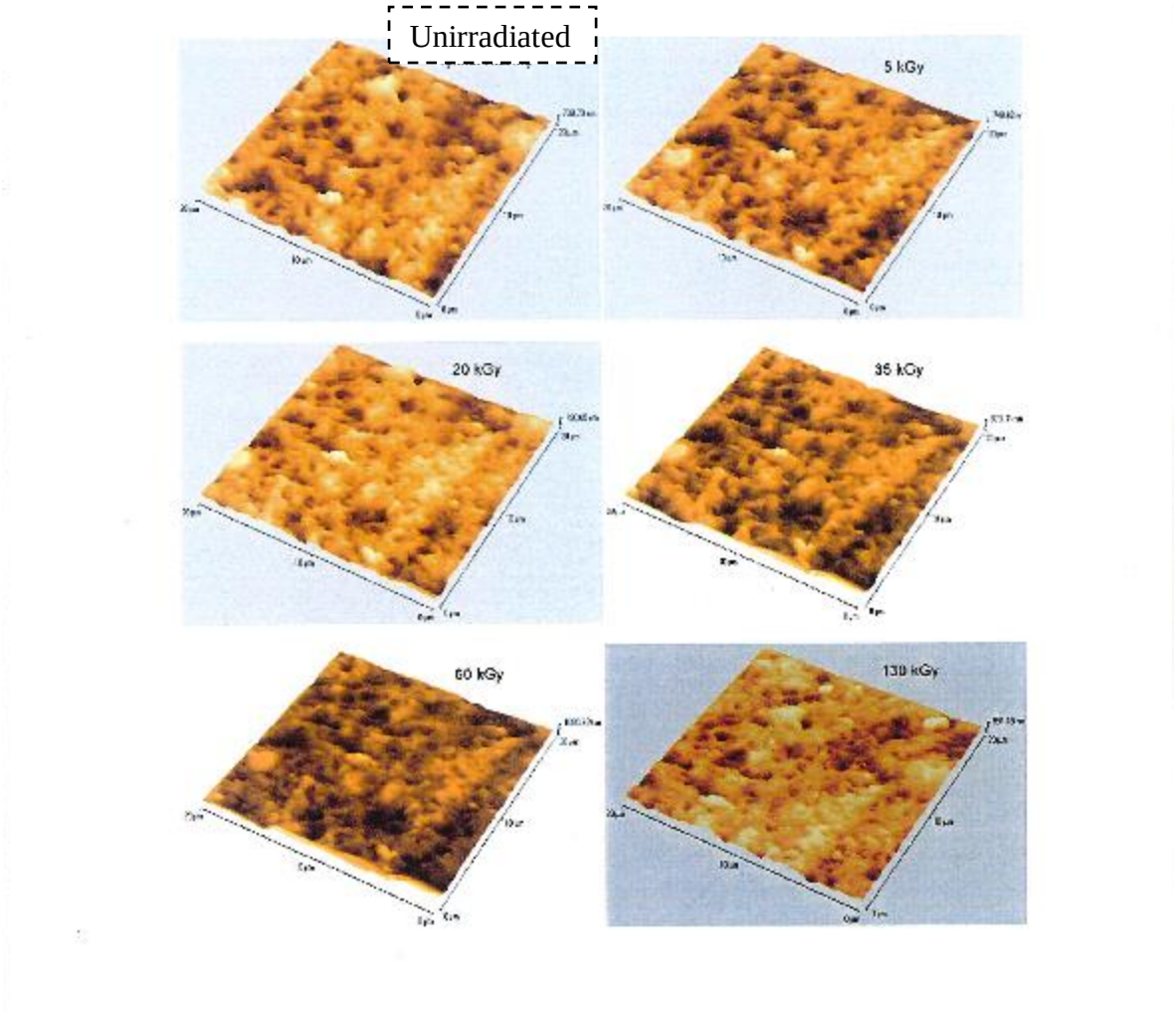

Image 5. AFM images of polycarbonate membranes prepared from tetrahydrofuran with varying irradiation dose

Competitive Adsorption of Blood Proteins from Human Plasma

Adsorption of blood proteins (i.e., human serum albumin, γ-globulin and fibrinogen) from human plasma on the untreated and γ-irradiated polycarbonate membranes is studied in batch-wise reactor. The blood samples are obtained from healthy human donors and centrifuged at 500 g for 30 min at room temperature to separate the plasma. 10 mL of the freshly separated human plasma containing albumin (38.2 mg/mL), fibrinogen (2.5 mg/mL) and γ-globulin (17.9 mg/mL) is incubated with 3 circular pieces (1 cm in diameter) of the untreated or γ-irradiated polycarbonate membranes for 1 h. Total protein concentration is measured by using the total protein reagent (Ciba Corning Diagnostics Ltd, Halstead, Essex, England; Catalog Ref. No: 712076) at 540 nm, based on Biuret reaction [5]. Chronometric determination of fibrinogen is performed using Fibrinogene-Kit (Ref No: 68452 and 68582, bioMerieux Laboratory Reagents and Instruments, Marcy-l'Etoile, France) [6]. Human serum albumin concentration is determined using Ciba Corning Albumin Reagent (Ciba Corning Diagnostics Ltd, Halstead,

Essex, England; Catalog Ref. No: 229241), based on bromocresol method [6]. γ-globulin concentration is determined from the difference.

Blood-Compatibility Studies

Coagulation Time (CT): Untreated and γ-irradiated polycarbonate membranes are incubated in 0.1 M phosphate buffer solution (pH 7.4) for 24 h at room temperature and ished on a glass filter with 0.5 M NaCl solution and distilled water. Fresh frozen pooled human plasma (1 ml) is preheated to 37°C for 2 min and then a piece of film is placed into this medium and mixed immediately. The clotting time is measured by using fibrometer method [7].

Activated Partial Thromboplastin Time (APTT): Untreated and γ-irradiated polycarbonate membranes are incubated in 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature and ished on a glass filter with 0.5 M NaCl solution and distilled water. Fresh frozen pooled human plasma (1 ml) is preheated to 37°C for 2 min. The partial thromboplastin (3 ml, bioMerieux, Marcy-l'Etoile, France) is also preheated to 37°C for 2 min and is introduced to preheated human plasma. Then, a piece of film is introduced into this medium. Thirty seconds later $CaCl_2$ (1 ml, 0.025 M) is added, then, the activated partial thromboplastin time (APTT) is determined by using the fibrometer method [8].

Prothrombin Time (PT): In order to determine prothrombin time (PT), one-stage prothrombin method is used [9]. Untreated and γ-irradiated polycarbonate membranes are incubated in 0.1 M phosphate buffer solution (pH 7.4) for 24 h at room temperature. Fresh frozen pooled human plasma (1 ml) is

preheated to 37°C for 2 min. The thromboplastin (2 ml, bioMerieux, Marcy-l'Etoile, France) is also preheated to 37°C for 2 min and is added to preheated human plasma. Then a piece of film is placed into this medium. Thirty seconds later $CaCl_2$ (1 ml, 0.025 M) is transferred into the medium. After these operations, the prothrombin time is measured by using fibrometer method.

Cell Adhesion Studies: Untreated and γ-irradiated polycarbonate membranes are incubated in 0.1 M phosphate buffer solution (pH 7.4) for 24 h at room temperature. Then polycarbonate membranes are ished with distilled water and NaCl solution (0.5 M). Fresh frozen pooled human plasma (1 ml) is preheated to 37°C for 2 min. Then a piece of film is placed into blood and interaction is carried out for 1 h. Blood samples are withdrawn from the medium, and the platelet and leukocyte count of samples are determined.

RESULTS AND DISCUSSIONS

Surface Properties of Polycarbonate Membranes

The changes in the surface properties of polycarbonate membranes prepared from different solvents and irradiated up to 200 kGy dose are evaluated by taking the AFM micrographs, and measuring the water content angles by captive-bubble method. In order to follow the physical changes brought about by irradiation first the topography of the surfaces are determined by AFM measurements. By using the software of the AFM system, the surface roughness values are determined for every sample investigated. The results collected in Table 1 show that, the mean surface roughness of polycarbonate surfaces are smaller than that of γ-irradiated membranes for all samples which are prepared in this study. It is also apparent that the γ-irradiated polycarbonate membranes had larger mean surface roughness for all solvent types. It should also be mentioned that the mean surface roughness of the samples are increased with increasing irradiation dose most probably due to the formation and evolution of gaseous radiolysis products and subsequent increase in the roughness/surface porosity. The original roughness of the membranes are found to be strongly dependent on the type of solvent from which they are prepared. While surface of the film samples prepared from chloroform showed the minimum roughness, those obtained from dichloroethane had relatively high roughnesses. The mean surface roughness increased in the following order: dichloroethane > dioxane > cyclohexane-dioxane > THF > Chloroform.

Table 1. Effect of γ-irradiation dose on the mean surface roughness of polycarbonate membranes.

Dose (kGy)	Roughness (nm)				
	Chloroform	THF	Dioxane	Cyclohexan /Dioxane	dichloroethane
0	28.81	80.16	126.02	121.05	185.44
5	29.11	80.23	125.28	125.29	181.22
15	-	-	127.01	124.46	177.58
25	-	-	129.14	125.47	191.20
35	29.15	81.52	129.14	122.78	196.76
60	31.97	83.13	127.91	131.88	195.17
130	42.75	94.37	138.68	141.55	196.42

Contact angle values of untreated and γ-irradiated polycarbonate membranes are given in Table 2. It is observed that the γ-irradiation of the polycarbonate membranes decreased the contact angles for all surfaces which prepared different solvents due to the increase of surface porosity and roughness. It is worth to note that the contact angles are measured through the water phase. Relatively small contact angles indicate a relatively more hydrophilic structures. Although contact angle values of the polycarbonate membranes decreased with irradiation dose, it must

be considered that hydrophobic character of the polycarbonate membranes did not change significantly with γ-irradiation.

Table 2. Effect of γ-irradiation dose on contact angle values of polycarbonate membranes.

Solvent Type	Contact Angle* (°)	
	unirradiated membranes	Irradiated Membranes (200 kGy)
Chloroform	88.7 ± 3.5	81.7 ± 4.5
Tetrahydrofuran	77.6 ± 5.5	66.5 ± 2.5
Cyclohexan/Dioxane	78.8 ± 5.5	73.4 ± 3.5
Dichloroethane	86.0 ± 2.2	80.0 ± 1.0
Dioxane	83.6 ± 3.9	82.8 ± 3.0

* Each data point is the average of three parallel studies.

To define the effect of γ-irradiation on water-uptake properties, the water-uptake of the untreated and γ-irradiated are determined in bidistilled water (Table 3). The equilibrium water-uptake of untreated polycarbonate membranes are low for all solvent types which used for preparation. But after γ-irradiation, the equilibrium water-uptake increased due to the formation of radiolysis products and increase in the roughness/surface porosity.

Table 3. Effect of γ-irradiation dose on water uptake values of polycarbonate membranes.

Solvent Type	Water Uptake* (%)		
	Unirradiated	Irradiated, 25 kGy	Irradiated, 200 kGy
Chloroform	0.00	0.01	0.84
Tetrahydrofuran	0.39	0.69	0.96
Dichloroethane	0.48	0.52	1.02
Dioxane	0.31	0.50	0.51
Cyclohexan/Dioxane	0.10	0.38	0.47

* Each data point is the average of three parallel studies.

Competitive Adsorption of Blood Proteins from Human Plasma

It has been shown that the adsorption of plasma proteins to polymer materials profoundly affects the interaction of blood cells with polymer materials and consequently thrombus formation on the materials. In particular, fibrinogen is known to be a protein of high surface activity [12]. It plays an important role in the clotting system in both the plasma phase ("intrinsic") and the cellular phase (platelet aggregation) in normal hemostasis and also been implicated in thrombosis on foreign surfaces [13]. Fibrinogen adsorption is a well-known contributer to surface-induced thrombosis. Platelets contain a receptor site specific for fibrinogen which is active only when platelets are activated. Meanwhile, thrombus formation decreases if the first adsorbed protein is albumin.

When blood is placed in contact with any foreign surface, a spontaneous competitive adsorption of proteins and glycoproteins occurs at the surface and forms a complex protein coating on the surface. These adsorptions greatly depend on the surface characteristics of polymers, which affect their blood-compatibility. Some of these adsorption processes are partially or completely reversible [14].

Blood plasma and γ-irradiated polycarbonate membranes are incubated together and the competitive adsorption behaviour of blood-proteins are obtained as a function of solvent used in casting the membranes and irradiation dose. The results are given in Figures 1-3. When the adsorption capacities of blood-proteins are compared, the adsorption of albumin (HSA) is higher than γ-globulin and fibrinogen (Fgn). This shows the strong affinity between albumin and the surface. From these results the order of the adsorption capacity is given in the following order: albumin > γ-globulin > fibrinogen.

Interesting results are obtained in the competitive protein adsorption studies. Figure 1-3 show that the adsorbed amounts of blood proteins increased with increase in irradiation dose, and reached to saturation for the irradiation doses of 100 kGy.

Effects of solvent type and irradiation dose on adsorption of blood proteins (i.e., albumin, fibrinogen and g-globulin) are given. As shown in these figures, maximum protein adsorption is observed for polycarbonate membranes prepared from dichloroethane whereas the lowest protein adsorption is obtained for polycarbonate membranes prepared from cyclohexane-dioxane solvent mixture. It is well known that surface roughness affects protein adsorption. Polycarbonate membranes prepared with dichloroethane had very porous structure. The mean surface roughness of that is also larger than the other surfaces. In this case high amount of protein adsorption is observed. When cyclohexane-dioxane solvent mixture is used for preparation of polycarbonate membranes, it is observed that these surfaces has relatively non-porous and smooth surfaces. This leads to obtain low protein adsorption.

On the other hand surface hydrophobicity is another important factor in protein adsorption. Hydrophobic surfaces show higher affinity for proteins. Increase in surface heterogenity also

contributes to high adsorption due to increasing of hydrophobicity [12]. It should be also noted that polycarbonate membranes prepared from dichloroethane has the most hydrophobic surface as shown in Table 2 & 3. With small variations, very similar trend is observed for γ-globulin and fibrinogen adsortion (Figure 2 & 3).

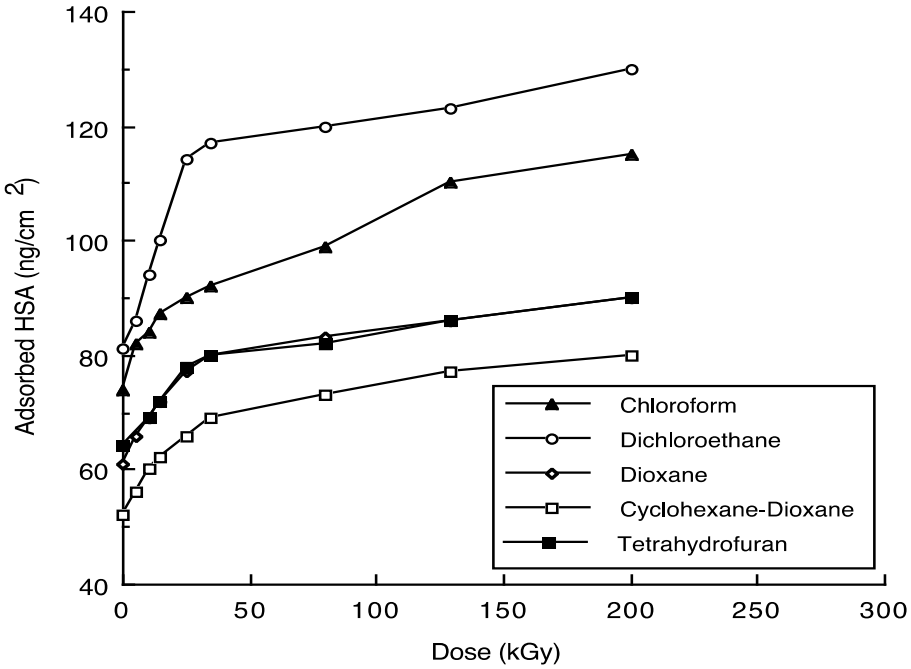

Figure 1. Effects of irradiation dose on competitive adsorption of HSA onto polycarbonate membranes: HSA concentration: 38.2 mg/mL. Each data point is the average of three parallel studies.

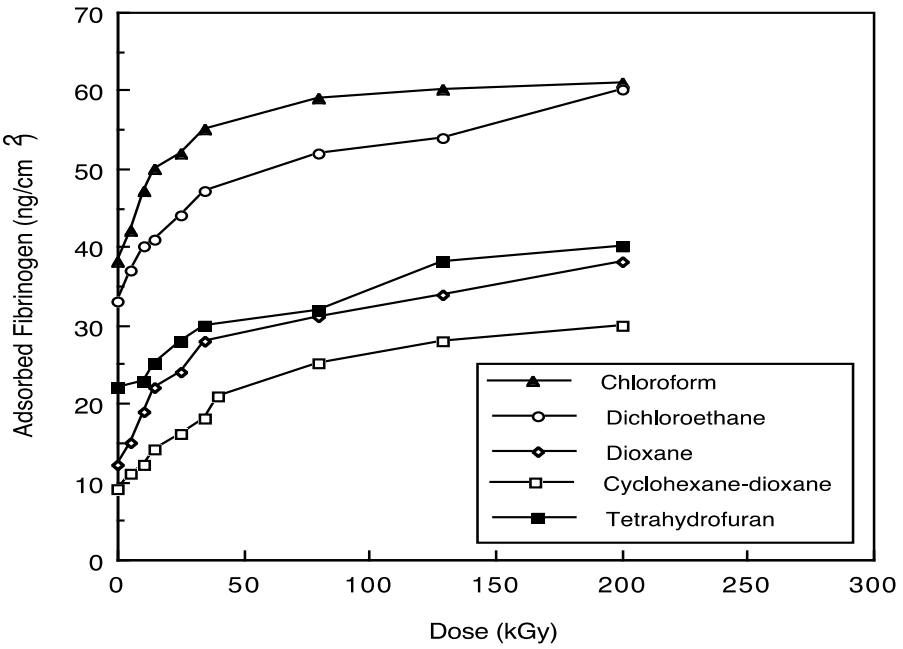

Figure 2. Effects of irradiation dose on competitive adsorption of fibrinogen onto polycarbonate membranes: Fibrinogen concentration: 2.4 mg/mL. Each data point is the average of three parallel studies.

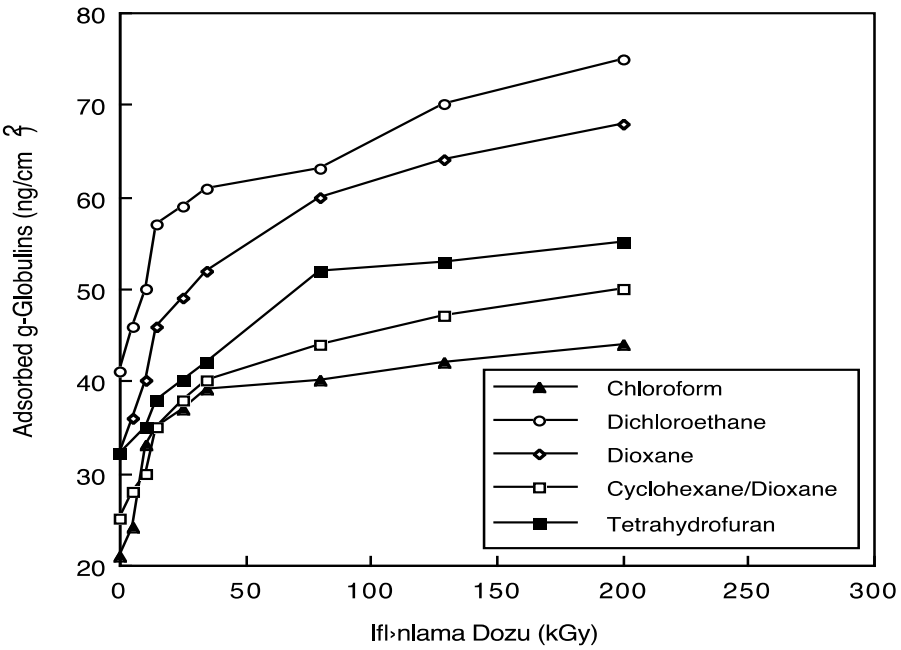

Figure 3. Effects of irradiation dose on competitive adsorption of γ-globulin onto polycarbonate membranes: γ-globulin concentration: 17.9 mg/mL. Each data point is the average of three parallel studies.

Blood-Compatibility Studies

Coagulation Times

In order to estimate the blood compatibility of unirradiated and γ-irradiated polycarbonate membranes in-vitro CT, APTT and PT tests are carried out with the untreated and irradiated membranes to 25 and 100 kGy doses. APTT test exhibits the bioactivity of intrinsic blood coagulation factors and PT test relates to extrinsic blood coagulation factors on material surface. CT test shows in-vitro coagulation time. Table 4 shows the blood coagulation data obtained in these tests.

As it can be seen from Table 4 for all practical purposes, the clotting times (CT, APTT and PT) for the polycarbonate membranes are observed to be almost the same irrespective of the irradiation dose and method of preparation. Note that the CT, APTT and PT values are taken after ishing and drying of polymer membranes, but there are no difference that are observed

previously. Therefore we concluded that the blood compatibility is preserved during the irradiation process that we applied.

Table 4. Effect of γ-irradiation dose on the coagulation times*.

Solvent/Dose (kGy)	APTT (s)	PT (s)	CT (s)
Chloroform			
0	33.2 ± 0.4	13.3 ± 0.2	225 ± 8.6
25	35.4 ± 0.5	12.5 ± 0.4	246 ± 7.3
200	34.6 ± 0.3	12.8 ± 0.3	243 ± 6.6
Dichloroethane			
0	36.4 ± 0.4	14.5 ± 0.5	248 ± 7.9
25	35.5 ± 0.5	13.5 ± 0.3	264 ± 9.3
200	34.3 ± 0.5	12.8 ± 0.5	255 ± 8.4
Dioxane			
0	38.9 ± 0.6	12.4 ± 0.1	238 ± 6.1
25	38.9 ± 0.6	12.8 ± 0.3	229 ± 8.2
200	38.9 ± 0.7	13.1 ± 0.4	221 ± 7.5
THF			
0	39.8 ± 0.7	13.3 ± 0.4	218 ± 6.9
25	38.4 ± 0.4	13.6 ± 0.3	223±8.4
200	35.7 ± 0.5	12.7 ± 0.5	234 ± 7.3
Cyclohexan-Dioxane			
0	38.2 ± 0.2	12.6 ± 0.4	222 ± 7.5
25	40.5 ± 0.2	12.9 ± 0.7	200 ± 7.5
200	39.6 ± 0.2	13.4 ± 0.8	210 ± 6.5

* Each data point is the average of three parallel studies.

Cell Adhesion Studies

In this part, the untreated and γ-irradiated polycarbonate membranes are used in an in-vitro test system. The blood samples are incubated with these membranes and platelet, leukocyte count are taken. Table 5 shows hematological data. Loss of platelet and leukocyte with γ-irradiated membranes are around 1-5%. These observations concluded that no significant amount of blood cells adhere on the untreated and γ-irradiated polycarbonate membranes. When these data are compared with literature it can be said that these values are too low and body can tolerate this level of decrease easily [14].

Table 5. Effect of γ-irradiation dose on the cell adhesion*.

Solvent/Dose (kGy)	Platelet ($x\ 10^{-3}/mm^3$)		Leucocyte ($x\ 10^{-3}/mm^3$)	
	Initial/End	Decrease (%)	Initial/End	Decrease (%)
Chloroform				
0	360/342	5.0	4.75/4.49	5.4
25	360/358	0.6	4.75/4.55	4.2
200	360/357	0.8	4.75/4.60	3.2
THF				
0	360/359	0.3	4.75/4.66	1.9
25	360/354	1.7	4.75/4.69	1.2
200	360/357	0.8	4.75/4.65	2.1
Dichloroethane				
0	360/355	1.4	4.75/4.48	5.6
25	360/356	1.1	4.75/4.59	3.4
200	360/358	0.6	4.75/4.55	4.2
Cyclohexane/Dioxane				
0	360/349	3.1	4.75/4.51	5.1
25	360/354	1.7	4.75/4.55	4.2
200	360/355	1.3	4.75/4.64	2.3
Dioxane				
0	360/358	0.6	4.75/4.64	2.3
25	360/355	1.4	4.75/4.61	2.9
200	360/352	2.2	4.75/4.68	1.5

* Each data point is the average of three parallel studies.

CONCLUSIONS

Although a number of previous studies have examined the effects of surface morphology of membranes on the blood-surface interactions, the data obtained in this study provides the results for the effects of surface morphology and hydrophobicity of membranes on

biocompatibility of materials for the first time. In the present study, polycarbonate membranes prepared by solvent-casting technique, had different surface morphology depending on type of solvent systems. Polycarbonate membranes are irradiated in the range of 0-200 kGy. The effects of γ-irradiation on biocompatibility of polycarbonate are investigated. Characterizations of the membranes are achieved by contact angle and water-uptake studies as well as AFM images. It has been found that, irradiated membranes are more hydrophilic than unirradiated membranes. This results state that as the films became more hydrophilic with irradiation, their biocompatibility have increased. AFM images showed that surface roughness increased with radiation dose. It is feasible to say that as the roughness of polycarbonate films are increased, the films became more blood compatible.

The interaction of plasma proteins with artificial surface is known to play important roles in the thrombus formation when blood contacts artificial surfaces. It is generally accepted that the thrombogenecity of a material may be related directly to the affinity of particular proteins such as fibrinogen, IgG and thrombin for the material's surface, since the adsorption of proteins has been shown to potentiate greatly the subsequent adhesion of platelets and possibly leukocytes (Since the values of cell adhesion are too low, body can tolerate this level of cell adhesion). It is such a significant modification in the characteristics of blood compatible studies of polycarbonate membranes. It has been found that protein adsorption experiments carried out with human plasma demonstrated that protein adsorption drastically increased by increasing the applied irradiation dose. Loss of blood cells and clotting times are followed. It has been concluded that loss of blood cells in the plasma contacting with irradiated membranes is negligible (Irradiation has no side effects on usage of modified polycarbonate films.). It can be further concluded that medical devices or biomaterials made from polycarbonate can be safely sterilized by γ-rays without causing any significant changes in their surface properties as has been assessed by the results of competitive protein adsorption studies carried out in this work on polycarbonate membranes with different initial topologies. As a result, findings of this study merits further investigation of the biopolymers in medical field in terms of its use in biocompatibility.

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