Possibilities of Collagen Adsorption on Some Polymeric Matrices Based on Styrene Copolymers

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ABSTRACT: Seven matrices based on polystyrene and styrene copolymers, with acrylic and methacrylic acid, acryl- and methacrylamide, and acrylonitrile and maleic anhydride, are tested in respect of their capacity of collagen adsorption. The aim of the study is to correlate the collagen coupling capacity by the nature of the monomers and the ability of functional groups to obtain biomaterials. The prepared vinylic polymers are relatively hydrophobic. These hydrophobic properties, together with the large mesh interstices, hinder smooth-cell seeding. In contrast, collagen offers the advantage of specific cell interactions and hydrophilicity. Therefore, synthetic polymers and collagen have been hybridized to combine their advantages. The biocompatibilization process consists of physical immobilization of the protein onto the synthesized matrice surfaces. The content of the adsorbed protein was correlated with the conditions of capture, solution pH, and sorption temperature, respectively. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 3554–3561, 2006

Key words: collagen; styrene polymers; biocompatibility; capture yields

INTRODUCTION

The application of polymeric materials for medical purposes is growing very fast. Polymers have found applications in diverse biomedical fields, such as tissue engineering, implants, artificial organs, prostheses, ophthalmology, dentistry, bone repair, and many other fields.[1] Polymer-based delivery systems enable controlled, retarded, or slow release of drug into the body.[2] Polymeric materials are also extensively used as biosensors, in testing devices, and for bioregulation.[3–5]

In the class of polymers, which are utilized in biomedical field polystyrene and styrene, copolymers occupied a special place. Thus, they are successfully utilized in petri boxes, hemolysis tubes, connection elements for catheters, and syringe bodies.[6–7] The advantage of these polymer employment is due to the specific macromolecular compound properties, such as dimensional stability, electrical properties, high chemical resistance to inorganic products, contraction to weak molding, the inertia in the biological environment, and stability to γ sterilization.[8]

Polymeric materials suitable for a biomedical application must be biocompatible, at least on its surface.[1] The term biocompatibility refers to the ability of a material to be in contact with living tissue and does not cause toxic or injurious effects.

The evaluation of the biocompatibility of medical and pharmaceutical materials is a complex task.[5–8] Biocompatibility testing and evaluation of the biomedical materials are performed to determine their potential toxicity, resulting from contact with the body. The medical materials should not (directly or through the release of their constituents) produce adverse local or systemic effects, be carcinogenic, or produce adverse reproductive and developmental effects. Therefore, evaluation of any new material intended for human use requires data from systematic testing to ensure that the benefits provided by the final product will not exceed any potential risks posed by device materials.[4]

To the present date, there are no known materials which totally satisfy the criteria of biocompatibility. Thus, when a foreign material is placed into a biological environment, inevitable reactions occur, which can be harmful to both host and material.[9–11]

An implanted polymeric material may be considered to be biocompatible, if its insertion into the body does not provoke an adverse reaction. Biocompatible polymer used for medical purpose should also be able to recognize and cooperate in harmony with bioassemblies and living cells, without any nonspecific interactions.[12]

Extensive research efforts have been made to design appropriately functionalized, truly biocompatible, and biodegradable polymeric systems. Various surface-treatments leading functionalization have been described in the literature.[1] Functional groups, prop-

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TABLE I
Recipe and Reaction Conditions for Obtaining Vinyl Latexes

Monomers (mol/L)	Potassium persulphate (M% with respect to monomer content)	Water (g)	Tensioactive system (M% with respect to monomer content	
			Sodium *N*-dodecyl-benzene sulfate	Poly (oxyethylene) nonyl-phenyl ether
Styrene (245×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (232.8×10^{-3})/acrylamide (10.6×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (192.03×10^{-3})/methacrylamide (58.82×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (233.1×10^{-3})/acrylic acid (10.41×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (228.36×10^{-3})/methacrylic acid (14.53×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (233.1×10^{-3})/acrylonitrile (14.15×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}
Styrene (233.1×10^{-3})/maleic anhydride (7.56×10^{-3})	1.387×10^{-3}	75	3.24×10^{-3}	1.72×10^{-3}

Reaction temperature, 70–75°C; rotation/min, 300; time of reaction, ≈540 min with ≈60 min thermal treatment at 80–85°C.

erly located on a polymer as well as its structure, are usually responsible for the biocompatibility or biodegradability or both and may bring on it either therapeutic or toxic characteristics. For example, carboxylic groups induce therapeutic activity of many drugs.

The use of polystyrene and some of the styrene copolymers as matrices for bioactive composite is well known, the homopolymer being considered as a classic example.[13,14] Thus, the interest for the obtainment of macromolecular matrices is understandable, with rising capability for clutching biological active substances.[15]

Proteins, such as albumin and collagen, are very useful in the system of drug delivery, on the one hand because they realize a compatible interface between the polymeric matrix and the biologic fluid, and on the other hand their chemical structure constitutes an optimum connection between the polymer structure and the future drug. All these aspects justify the multiple preoccupations for the determination of the conditions of protein immobilization onto the polymeric matrix.[16–18]

The study pursues the possibilities for some biomaterials obtainment based on styrene polymers and copolymers, with acrylic and methacrylic acid, acryl- and methacrylamide, and acrylonitrile and maleic anhydride. The obtainment of biocompatible composites is realized through the adsorption of protein—the collagen—onto the prepared macromolecular surfaces, the processes being correlated with the procedure conditions, respectively, the influence of pH and temperature.

EXPERIMENTAL

The styrene-based matrices preparation

The procedure for the matrices syntheses, based on styrene polymers with polar comonomer as well their characterization, were previously presented.[19–21]

Materials

The used polymeric matrices were polystyrene, styrene copolymers with acrylic and methacrylic acid, acryl- and methacrylamide, acrylonitrile and maleic anhydride, respectively. The syntheses recipes as well as the characteristics of the prepared polymers, as latex or polymeric particles, are presented in Tables I and II. To obtain particles with uniform size, a multistage-seeded emulsion radical polymerization procedure has been used.

Preliminary data concerning optimum conditions to realize the biomaterials were obtained from the swelling-process study in carbon tetrachloride of the prepared macromolecular matrices. The swelling procedure was performed to obtain an enlarged and relaxed macromolecular structure to create more and facile possibilities to entrap the bioactive substance. Thus, the dried particles of the obtained macromolecular compounds have been swollen in the vapor atmosphere of CCl_4.

The biocompatibilization methods

The biocompatibilization procedure consists of covering the prepared vinylic polymeric matrices with a protein, namely collagen. The collagen was extracted from the bovine derma, by alkaline treatment, followed by extraction with acetic acid and purification by dialysis against the twice-distilled water (isoelectric pH = 4.87, $[\eta]$ = 1320 mL/g, determinate in acetic acid 0.5*M*).[22]

The coupling process of the synthetic samples with the protein was realized through a static procedure, by immersing the polymers into a 1 wt % collagen solution, for 24 h at 35°C temperature (without stirring). The ratio of macromolecular compound/protein was 1 : 1. As was already mentioned the homo- as well as the copolymers were previously swelled in CCl_4.[3,22]

In a previous study,[20] the possibility of coupling the collagen onto some polystyrene matrices was evalu-

TABLE II
Characteristics of the Polymeric Matrices

Sample	Latex characteristics		Polymer particle characteristics						
	Latex pH	Latex density for 20% concentration (g/cm^3)	N_2 content (%) Theoretic	N_2 content (%) Determined	$\eta^{25°C}$ THF (dL/g)	Particle dimension[a] (μm)	α_{2500} (%)	T_g (°C)	T_i (°C)
PSt	4	1.0207	–	–	1.23	<3	14	95	210
P(St-*co*-AA)	3.5	1.046	–	–	1.05	<3	32	104	212
P(St-*co*-MAA)	4	1.0411	–	–	2.04	<3	17	102	220
P(St-*co*-AAm)	5	1.0256	0.59	0.54	1.50	<3	24.2	98	240
P(St-*co*-MAAm)	6.5	1.0413	0.725	0.755	1.16	<3	25	96	240
P(St-*co*-AN)	4	1.0308	0.8	0.79	1.08	<3	8	98	230
P(St-*co*-AM)	2.5	1.0388	–	–	1.28	<3	21	98	220

THF, tetrahydrofuran; PS, polystyrene; P(St-*co*-AA), poly(styrene-*co*-acrylic acid); P(St-*co*-AMA), poly(styrene-*co*-methacrylic acid); (St-*co*-AAm), poly(styrene-*co*-acrylamide); P(St-*co*-MAAm), poly(styrene-*co*-methacrylamide); P(St-*co*-AN), poly(styrene-*co*-acrylonitrile); P(St-*co*-AM), poly(styrene-*co*-maleic anhydride); α, the swelling degree after the 2500 min of swelling; T_i, temperature corresponding to the beginning of thermogravimetric decomposition; T_g, glass transition temperature.

[a] By optic microscopy determination.

ated. In the present study, the reaction conditions were varied to obtain better yields of collagen coupling onto the prepared vinylic matrices. Thus, the experiments were effected for five different pH values of 2, 4, 6, 8, and 10, respectively, at temperature values, 20, 25, 30, 35, and 40°C, respectively. When the temperature was varied, the collagen solution was maintained at pH 8.

After the procedure of physical coupling, the biocomposite particles were washed three times with the twice-distilled water, and then were gently dried at 30°C for 48 h.

The content of adsorbed collagen was qualitatively evidenced by IR spectroscopy on a Perkin–Elmer 577 spectrophotometer and quantitatively through nitrogen determination by microKjeldhal method. The nitrogen quantitative determination in case of the polymeric matrix having nitrogen in the structure was established by difference between the nitrogen value after collagen immobilization and nitrogen value before immobilization.

The size of the polymer particles as well as the prepared biostructure was determined on the micrographs, obtained by means of a scanning electronic microscope BS 340 TESLA type (SEM). The films were covered with pure metallic silver. The laying down of Ag was carried out using evaporation of the metal under a high vacuum, to give a thickness of around 15–20Å. Magnification was 510× for the polymeric samples and 4200× for the prepared biomaterials.

RESULTS AND DISCUSSION

Swelling behavior

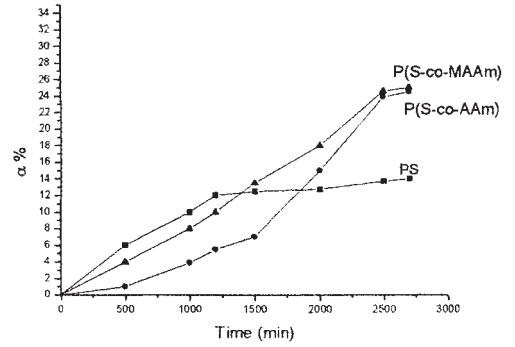
The curves of swelling in the carbon tetrachloride vapors are distinct for each sample, specifying and evidencing the existence of the differences into the polymeric structure (diverse functional groups) [Fig. 1 (a–c)]. The presence of the comonomers, except acrylonitrile case, determines the increase of swelling degree (α), but at the same time, with reduced values of α at the beginning (~1200 min) of the swelling process. Growth becomes in time, justifying the existence of physical links between chains owing to the presence of the comonomers with their functional groups.

Also, the differences of the swelling capacity registered for the studied copolymers can be justified through

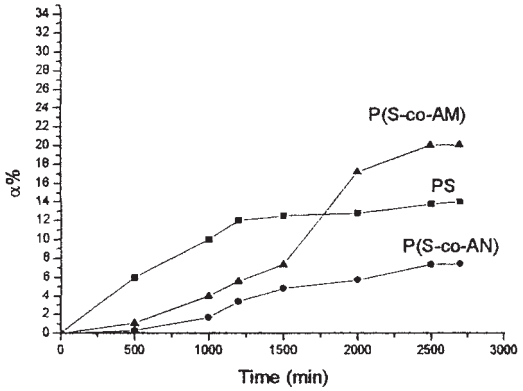
- different percentage of the functional monomer (P(St-*co*-AMA) and P(St-*co*-AA) case); higher content of functional group in copolymer with acrylic acid [Fig. 1 (c)].
- differences between the polarity of the macromolecular chains as well as differences of the realized hydrophilic/hydrophobic balance in all copolymer, evidencing a direct proportionality between hydrophilic character and swelling capacity.
- as a relationship between structure/charge density and conformation in solution of the involved molecular species.

Resulting data from the swelling study of the synthesized polymers allow us to exploit them for the next step, the protein coupling. Thus, all polymeric samples were swelled for 2550 min, which correspond to the maximum degree of swelling to have relaxed macromolecular structure ready to retain the bioactive substances.

After swelling, the polymeric matrices were washed many times in twice-distilled water, and then were gently dried, and the aforementioned protein adsorption procedure was applied.



a



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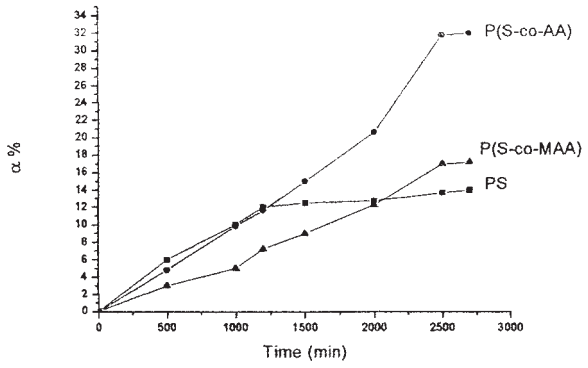


c

Figure 1 Swelling behavior in CCl_4 of the prepared polymeric samples.

The tests of biocompatibilization of the macromolecular matrices

The procedure of covering of the polymeric particles with the protein layers has two purposes: first, to make the polymers biocompatible and second, to introduce the protein functional groups for further link bridges. Covering the macromolecular particles with protein layers makes the structure biocompatible and also introduces supplementary functional groups of the protein capable to participate in a potential drug immobilization process.

Figures 2 and 3 present IR spectra, which evidence the protein immobilization onto the polymeric substrate. The complexity of the protein structure is manifested in IR spectra through large, intense, and undifferentiated bands. The cause of this shape emerged from the protein composition, respectively, to the constitutive amino-acid's diversity. Thus, the large band is in reality the spectra of the individual aminoacids, which totally or partially overlap. Only the characteristic vibrations of the peptidic group —NH—CO— could be identified, and thus to recognize the proteic structure.[23]

The specific adsorptions of protein appear in three distinguished domains as follows: 3050–3550 cm^{-1}, 1470–1700 cm^{-1}, and 1300–1400 cm^{-1}, as can be seen from the spectra. The 3000 cm^{-1} domain bands correspond to valence vibrations of —NH bonds from $—NH_2$ and —NHR groups. The presence of these bands into the polymeric matrix spectra is due to the relative remanent humidity. A superposition of the band in 1500–1600 cm^{-1} domains due to amine characteristic bands as well as of aromatic ring vibration can also be observed. Visual inspection of the spectra confirms the corresponding peaks of the polymeric

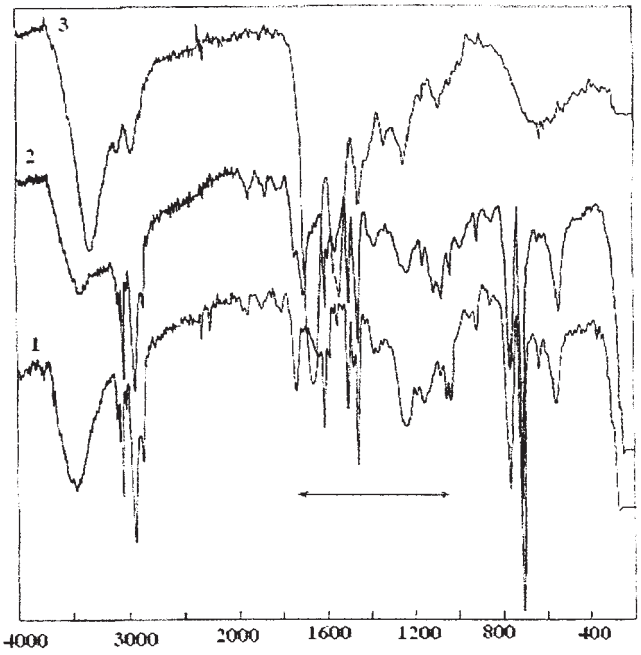


Figure 2 IR spectra of the collagen/poly(styrene-*co*-acrylonitrile) composite (2) compared with collagen (3) and poly(styrene-*co*-acrylonitrile) (1).

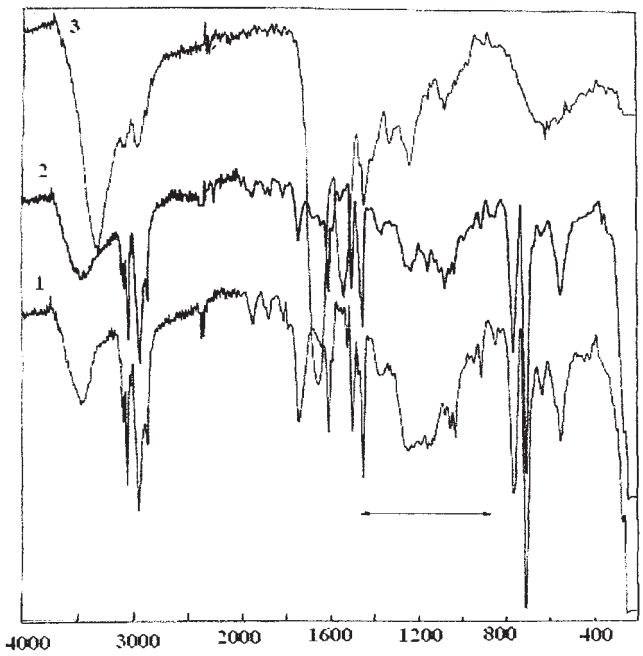


Figure 3 IR spectra of the collagen/poly(styrene-*co*-maleic anhydride) biomaterial (2) compared with collagen (3) and poly(styrene-*co*-maleic anhydride) (1).

matrix as well as of the collagen. The characteristic bands 3050–3550 cm^{-1}, 1470–1700 cm^{-1}, and 1300–1400 cm^{-1} evidence the immobilization of the protein on the surface of the matrix.

The protein's presence evidenced by IR spectra was quantitatively confirmed by analysis of nitrogen. The results are given in Figures 4 and 5.

As it is well known, the replacement of neutral aqueous collagen solutions with other aqueous solutions, having acid or alkaline pH, determines the modification of swelling degree as well as the viscosity of collagen solution, as a result of the concentration and of the chemical structure of the bioactive compound The two types of substances involved in solutions allow the establishment of ionic links. The new associations are modifying the content of the ionized and unionized zwitterionic groups (Scheme 1), and in consequence, increase significantly the quantity of the immobilized water through dipolar bonds.[22,24]

Literature data offer information concerning the collagen behavior in solutions with different pH, generated by acid or base presence.[22]

Figure 4 illustrates the collagen coupling behavior onto prepared polymeric samples in correlation with the solution pH. On studying polymeric variants at pH 6, which corresponds to the isoelectric point of the collagen, were registered the smallest quantities of coupled protein. For other pH values, the coupling behavior was correlated with collagen charge and the availability of the functional groups designed for coupling.

The explanation for the coupling manner is on the basis of the ionic strength of the solution. As it is well known, collagen is water insoluble, but it swells when solvent occupies the inter- or intrafibrillar spaces. Also, the swelling of collagen depends on two factors: osmotic and lyotropic ones. Osmotic swelling occurs due to a high concentration of bound, nondiffusing ions, located inside the structure, and it takes place when pH of the solution is off the isoelectric point. This behavior corresponds also with the data resulted in our study of collagen coupling process onto the prepared vinylic polymeric samples. Thus, based on the net charge of the collagen[25] (Table III), determined according to the values available from the Lehninger's Biochemistry book, it is evident that around the collagen isoelectric pH values registered the smallest quantity of coupled collagen onto polymeric matrices.

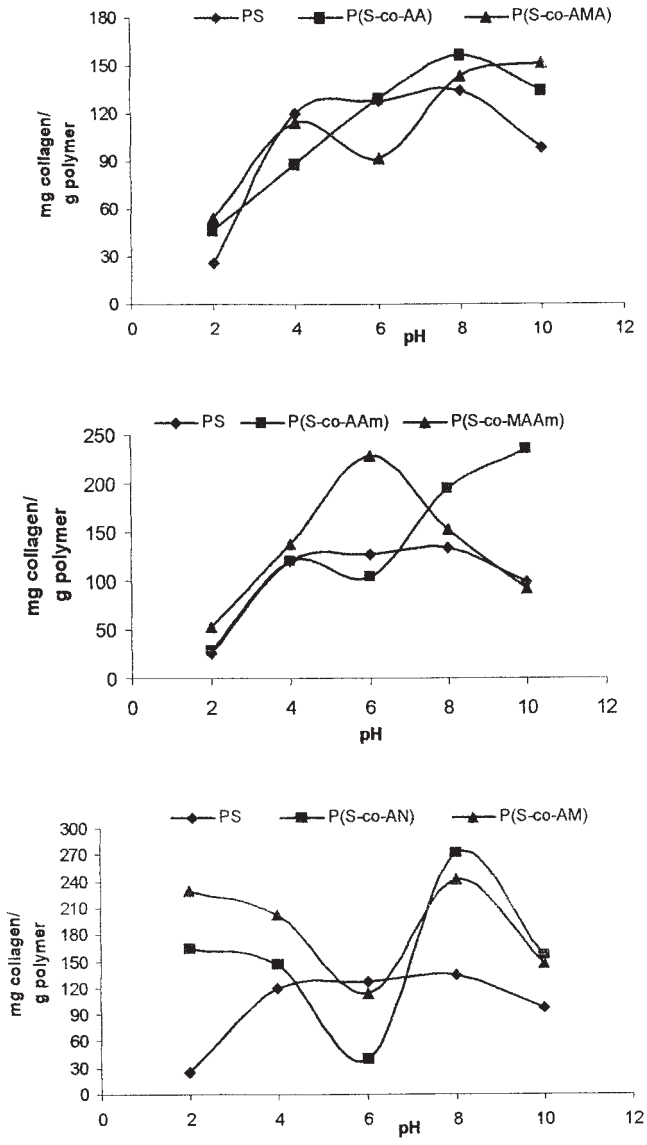


Figure 4 Amount of immobilized collagen onto polymeric samples—dependence on solution pH.

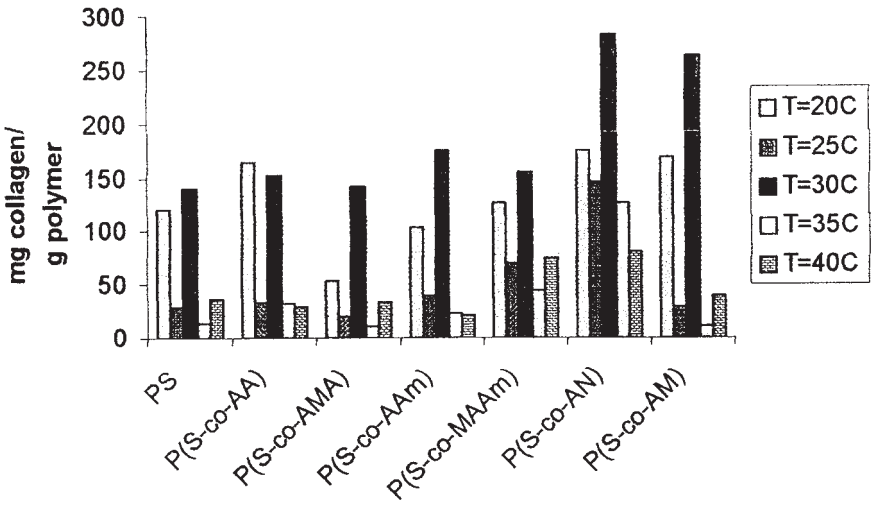


Figure 5 The collagen immobilization yield onto prepared polymeric matrix—dependence on temperature conditions.

The proteins have the most compact conformation at the isoelectric point, because they have the least intramolecular electrorepulsive force at the isoelectric point, and the least intra- and intermolecular repulsive force result in the smallest amount of adsorption around this point.

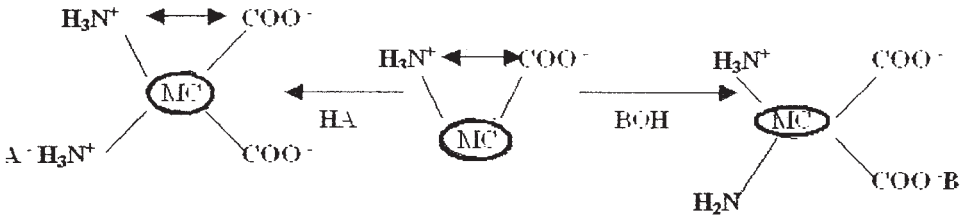
The exception constitutes the P(St-*co*-MAAm) and P(St-*co*-AA) matrix. Thus, P(St-*co*-MAAm) matrix presents a maximum coupling yield at this pH value. Also, P(St-*co*-AA) offers a different behavior at this pH value, having more possibilities of protein immobilization. This behavior can be explained knowing that at low pH the polyelectrolytes have a hypercoiled sphere conformation. At pH ~6, the polymer coils begin to open, while at higher pH (*ca.* 8) the chains are stretched out due to the electrostatic repulsion of the anionic groups. A further increase in the pH leads to a recoiling of the polyelectrolytes attributed to an increase in the ionic strength of the solution. These change of conformation disturb the coupling process. There are necessary supplementary data concerning the behavior of polymeric matrix during the collagen coupling process in acid or base media.

The coupling manner is allowed to the conformation of protein that occupies the least surface area for adsorption, which corresponds to the densest adsorption and a corresponding charge, the conformation capable of coupling with the polymeric matrices, but also to changes of both the shape and the size of the macromolecules with solution pH. Thus, at low pH the macromolecules with carboxylic groups adopt a hypercoiled form to minimize the hydrophobic interactions, whereas at higher pH, the chain stretches to a more extended, rod-like form.

It also remarks a decrease of the immobilization quantity at the extreme values of pH when the collagen structure is denatured. At the same time, marked differences of the collagen immobilization are registered between styrene homopolymer and their corresponding copolymer substrates, respectively. Thus, higher capacity of coupling corresponds to the copolymer matrix compared with the homopolymer (Fig. 4).

The highest capacity of the collagen immobilization at pH 8 corresponds to the polymeric matrices based on P(St-*co*-AN) and P(S-*co*-AM). The fact is ascribed to the functional groups presence as well as to the polymeric network.

A decrease of yield of protein immobilization is observed in case of polymeric substrates with a better capacity of swelling (see Fig. 1): P(St-*co*-AA), P(St-*co*-AMA). This concludes that swelling degree is not a decisive factor in obtaining good yields of protein immobilization, as for example, pH values designate



Scheme 1 The zwitterionic group formation.

TABLE III
The Net Charge of Collagen at Corresponding pH Values

pH	Charge
2	0.68
2.5	0.4
3	0.17
3.5	0.06
4	0.02
4.5	$6e^{-3}$
5	$2e^{-3}$
5.5	$6e^{-4}$
6[a]	$1.45e^{-5}$
6.5	$-5e^{-4}$
7	$-2e^{-3}$
7.5	$-6e^{-3}$
8	−0.02
8.5	−0.06
9	−0.16
9.5	−0.39
10	−0.67

[a] The isoelectric point.

the optimum conditions to make and induce new electrostatic forces.

Other variables taken into consideration during coupling process of collagen was the temperature. The manner of coupling depends on temperature, and thus, because osmotic swelling of collagen takes place when pH of the solution is off the isoelectric point, the ionic strength of the solution is small and also the temperature is low.[26]

The equilibrium state of biopolymers is considered: temperature *T*, 20°C; relative humidity, 65%; and atmospheric pressure, 760 mmHg. Any temperature variation in positive or negative direction will induce modifications into energetic content of the biopolymers, with changes of the intermolecular interactions. Accordingly, with general theory of the biopolymers structure, the result of the temperature intervention is interpreted as a structural transformation of the bioactive compounds. In this context, the process of the collagen immobilization at different values of temperature onto mentioned polymeric structures was studied. Figure 5 illustrates the collagen coupling process onto polymeric matrices, with temperature variation. Thus, two optima temperatures for immobilization, at 20 and 30°C, are observed. The diminution of the temperature determines the favorable condition for coupling. The reduction of the temperature causes decreased atom mobility and a compression of the biopolymer molecular volume modification. The modifications determine the production of new intermolecular links owing to decreasing of distances between the molecules involved into interactions as well as the higher capacity of interpenetrating into the macromolecular matrix network.

The physical process of coupling determines the formation of a collagen thin coating onto the styrenic macromolecular surfaces as can be seen from Figure 6.

The film formation supposes a significant growth of biocompatibility of the synthesized copolymers. There are necessarily supplementary data concerning the stability of the collagenic film at 37°C.

Temperature rising amplifies the movement capacity of atoms as well as the expansion of the molecular volume of biopolymer. Supplementary energetic contribution provokes the molecular links breaking, and this disturbance determines the diminution of the coupling amount.

Temperature at 31.2°C corresponds to the transition of soluble collagen from helix to ball shape (thermal distortion of first degree). The temperature growth corresponds also to more processes of distortions, which are finally—at temperature higher than 50°C—finished with a damaged biopolymer.

CONCLUSIONS

The study pursued the possibilities of collagen coupling onto seven matrices, based on polystyrene and

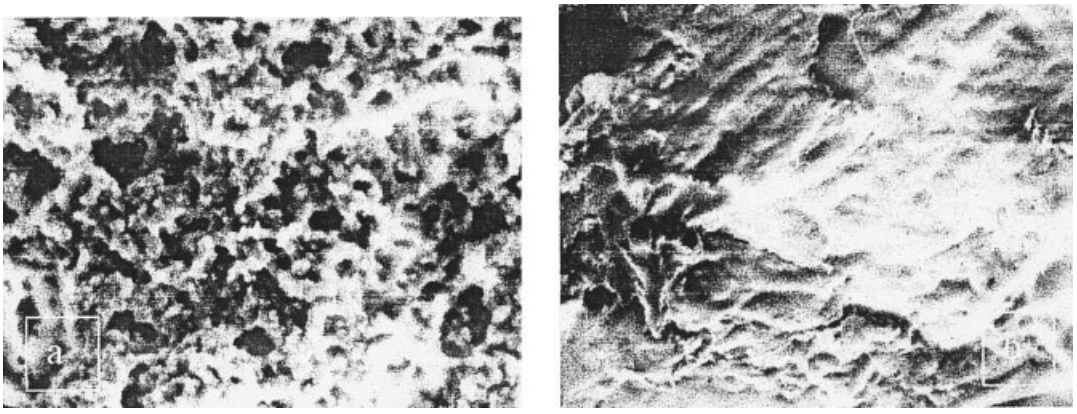


Figure 6 SEM micrographs of poly(styrene) (a) and poly(styrene) covered with collagen (b).

styrene copolymers with acrylic and methacrylic acid, acryl- and methacrylamide, and acrylonitrile and maleic anhydride. The process depends on the chemical nature of the matrix, the physical parameters and the solvent conditions, the interaction between polymers and protein varying over a broad range, not only in terms of strength, but also in terms of characteristic time scales. Thus, styrene copolymers with polar groups and controlled dimension size (<3 mμ) exhibit a better absorption capacity of proteins—collagen, and therefore, a better biocompatibility in respect with polystyrene.

The optima conditions to perform the biocompatible process covering the macromolecular matrix with collagen are at pH 8, and at temperature 30°C, respectively, and the most suitable polymeric matrices being P(St-*co*-AM) and P(St-*co*-AN) copolymers.

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