Chapter 12

Bio-Medical Applications of the Electron-Beam Plasma

T. Vasilieva

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52576

1. Introduction

The Electron-Beam Plasma (EBP) is generated by injecting an electron beam (EB) into a gaseous medium. The EBP composition is complex: generally it contains molecules, atoms, radicals and ions in stable and excited states, plasma electrons and injected beam electrons as well. At moderate pressures (P_m <10 kPa) the EBP is strongly nonequilibrium. It means that the function of the electron energy distribution in the EBP is non-Maxwellian and heavy plasma particles mentioned above are produced in super-equilibrium concentrations, i. e. very high densities of ionized and excited particles can be reached. As a result, the EBP appears to be chemically active even at low temperature. With respect to non-equilibrium plasmas generated in conventional ways (for instance, the plasma of gas discharges) the EBP has the following advantages:

- the EB can be injected into any gases, vapors and gas-vapor mixtures;
- the EBP bulk does not contract even at very high gas pressures (P_m ~10 kPa and higher);
- the solid powders and liquid droplets injected into the gas do not prevent the EBP generation; large-size bodies can be inserted into the plasma bulk;
- both solid powders and thing films can be treated in the EBP;
- very high concentrations of chemically active particles can be obtained even at low (up to room) temperatures;
- the process of the EBP-treatment is absolutely controllable and the treatment results are replicable.

Due to its properties the EBP seems to be very promising for biomaterial treatment and especially for the modification of biopolymers.

The main objective of the study is to experimentally prove the applicability and advantages of the electron-beam plasma for actual biological, pharmacological, and medical problems.

The production of substances with novel biological and pharmacological properties on the base of EBP-modified proteins and polysaccharides is considered as example.

The products of the fibrinogen proteolytic degradation are known to inhibit the platelet aggregation [1]. Being the product of the intermediate stage of the fibrinogen-fibrin polymer conversion, fibrin-monomer strongly affects the platelet activity due to two very active sites in its molecule. These sites are formed by the proteolytic cleavage in sequence the N-temini of the fibrinogen Aα and Bβ chains and release of the fibrinopeptides A and B [2]. Low molecular weight products of the fibrin-monomer proteolytic degradation are considered to be promising compounds for the platelets inhibition. Unfortunately the industrial fibrin-monomer can not be degradated by proteolytic enzymes (such as trypsin, plasmin, thrombin, and etc.) due to its high polymerization tendency. Therefore, the alternative techniques for controllable modification of the fibrin-monomer structure should be found to produce peptides with the high antiaggregating activity and without polymerization tendency.

The natural renewable biopolymers chitin and, especially, chitosan are very promising for technological and industrial applications such as agriculture, food processing, cosmetics production and others [3, 4]. Chitosan, linear heterocopolymers of β-1,4-linked 2-amino-2-deoxy-D-glucopyranose and 2-acet-amido-2-deoxy-D-glucopyranose units, has many unique biological properties namely high biocompatibility with living tissues, biodegrability, ability to the complexation, and low toxicity. In medicine and pharmaceutics the water-soluble low molecular weight chitosans (less than 10 kDa) are usually required. These substances can be used as immune response-modulating or antibacterial agents, sorbents, radioprotectors, and for the production of microcapsules, thing films, and substrates for cell cultures [3, 4]. To produce the low molecular weight chitosans (LMWC) several techniques, including chemical, enzymatic, and radical treatment have been suggested [5]. Simple and rather low-cost chemical treatment is a conventional method, however toxic wastes and environment contamination are inherent in the chemical chitin and chitosan processing as well as in all techniques mentioned above. Besides, the chemical treatment is very time consuming and usually takes several hours. Thus, the development of the effective techniques for quick and environment friendly chitosan degradation is the burning issue of the day.

The aims of the present study were as follows:

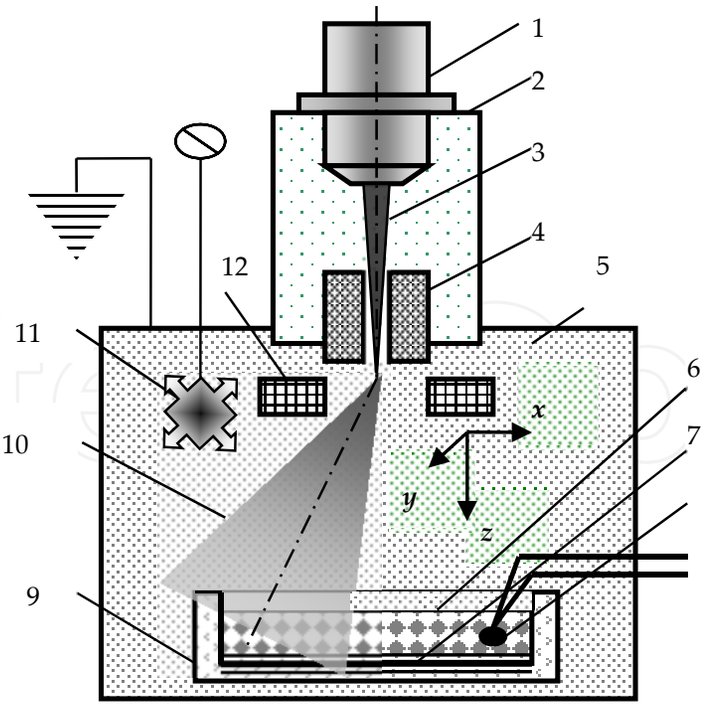
1. to experimentally prove the possibility of the EBP-stimulated hydrolysis of proteins and polysaccharides as a result of the chitosans plasmachemical processing;
2. to prove the controlablity of the EBP-treament and to develop the methods for the EBP-treament control;
3. to obtain the high yield of the low molecular weight products by optimizing the treatment conditions;
4. to characterize both the structure products of the plasmachemical treatment and their bioactivity;
5. to obtain low molecular products of fibrin-monomer and chitosan with antiaggregating and antimicrobial activity, respectively.

2. The Electron-Beam Plasmachemical reactor and the treatment procedure

For the controllable biopolymers modification and low molecular mass substances production the special Electron Beam Plasmachemical Reactor (EBPR) was designed.

Figure 1 illustrates the design and operation of the EBPR. The focused continuous EB *3* generated by the electron-beam gun 1 which is located in the high vacuum chamber *2* is injected into the working chamber *5* filled with the plasma-generating gas through the specially designed double-stage gas-dynamic injection window *4* [6]. Oxygen, nitrogen, noble gases, gaseous hydrocarbons and other atomic and molecular gases, water vapor, and vapors of some organic substances can be used for the EBP generation. The electrically heated evaporator *11* is placed inside the reaction chamber, as shown in figure 1, to add the vapor to the plasma-generating gas. Evidently, the pure vapor can be used for the EBP generation and, in this case, the reaction chamber is kept at given constant pressure by adjusting the heater power.

In passing through the gas the EB is scattered in elastic collisions and the energy of fast electrons gradually diminishes during various inelastic interactions with the medium (ionization, excitation, dissociation). As a result, the cloud *10* of the EBP is generated, all plasma parameters being functions of x, y, and z coordinates (z is the axis of the EB injection).



1 – electron beam; 2 – high vacuum chamber; 3 – EB; 4 – injection window; 5 – working chamber; 6 – mixing layer of the powder to be treated; 7 – piezoceramic plate; 8 – temperature sensor; 9 – glass container; 10 – EBP cloud; 11 – water evaporator; 12 – scanning system.

Figure 1. The design of the plasmachemical reactor and the treatment procedure

The electromagnetic scanning system *12*, which is placed inside the working chamber near the injection window is able to deflect the injected EB axis in x and y directions and, therefore, to control the spatial distribution of the plasma particles over the plasma bulk. The working chamber is preliminary evacuated to pressure ~10^{-2} Torr and then filled with plasma generating media. The samples to be treated were inserted into the EBPR reaction zone as solid powders with characteristic particle size ~ 100 mcm and as thing films. The powder of the substance to be treated partially fills the glass container *9*. Thin plate *7* made of piezoelectric ceramics is placed on the container bottom. Being fed with AC-voltage the plate vibrates, throws up the powder particles and forms the mixing layer 6 of the treated material. The miniature thermo-sensor *8* is inserted into the container to monitor the material temperature T_s during the treatment. To prevent the thermal distraction of the biological material all samples were processed at T_s<50 °C. In the case of proteins T_s was ~ 37 °C. The temperature control was carried out by selecting the EB current I_b ($1 < I_b < 100$ mA).

Special software was developed to calculate irradiation doses as functions of the electron beam characteristics, gas pressure and chemical composition, treatment duration, and beam scanning parameters [7], see section "The control of EBP-modification process".

3. The operation modes of the EBPR

The reaction zone (item *6* in figure 1) of the EBPR is the plasma of an aerosol, as it is sometimes called "dusty plasma". In general, the operation parameters of the reactor responsible for the properties of the plasma of this kind are as follows.

1. The beam parameters: the initial electron energy (E_b), that is equal to the accelerating voltage of the electron gun, and the EB power (N) injected into the reaction chamber.
2. The power N is less than the original power of the EB ($N_b = E_b I_b$, where I_b is the current of the beam generated by the gun) since the EB is partially absorbed by the injection window. The transparency coefficient of the injection window was specially measured under various conditions of the plasma generation and the data of the measurements were used to characterize the parameters of the material treatment. The values of both E_b and I_b are measured inside the high voltage power source, supplying the gun, and are displayed on the control panel of the EBP generator.
3. The plasma-generating gas parameters: the pressure (P_m) and temperature (T_m) of the gas; its chemical composition.
4. The parameters of the dust: dimensions and shape of the dust particles; the dust density, i.e. the number of the dust particles per unit volume (n_d); physical properties of the dust material (chemical composition, density, coefficients of the electron emission and some others);
5. The geometry of the plasma bulk, especially the distance (z_0) between the injection window outlet and mixing layer.

In comparison with the plasma treatment of conventional powder materials (e.g. metals, ceramics, carbon, etc.) the biomaterials processing has at least three important peculiarities.

1. The biomaterials should be processed at low temperatures (T_s <50 °C) to prevent the material destruction caused by overheating.
2. The effect of the biomaterials modification was found to crucially depend on the dose of the biomaterial irradiation by the EBP particles. Usually, the material properties, especially its bioactivity, change abruptly when the certain irradiation dose has been accumulated, but if the dose exceeds this threshold level the bioactivity doesn't increase any more [8].
3. The direct bombardment by high energy electrons (E_b ~100 keV and higher) is known to damage or destruct bio-molecules [9, 10].

It means that biomaterials should be processed by the EBP generated by the beams of the moderate E_b and that the plasma particles densities and the local power input must be uniform over the reaction bulk, predictable and controllable.

The controlling system of the EBPR is able to vary E_b ($25 < E_b < 40$ keV) and I_b ($1 < I_b < 100$ mA). The system supports various scanning modes of the EB injected into the reaction chamber (linear in x or y directions, circle or ellipse, rectangular raster, multi-triangle or saw-tooth), and both the amplitudes and frequencies of the scanning can be varied. The controlling system also supports the intermittent modes of the EB generation that enables to the biomaterials treatment in the decaying EBP. The pulse frequency can be tuned within the range 1-1000 Hz; the shortest and longest pauses between the EB pulses are 10^{-3} s and 1.0 s respectively.

The EBPR controlling system also includes the gas-feeding unit which has a feedback with the valves of the vacuum system. The gas-feeding system supplies one or two plasma-generating gases to the reaction chamber from separate external vessels, thereby keeping the total pressure of the gas mixture (within the range $0{,}1 < P_m < 100$ Torr) and partial pressures of the mixture components constant.

4. The tests of the EBPR and optimization of the biomaterial treatment procedure

The computer simulation of the dusty EBP was carried out to preliminary estimate optimal parameters of the biomaterial treatment. The following processes were taken into account:

- The scattering of high-energy electrons by the gas molecules and dust particles;
- The gas ionization by high-energy electrons;
- The capture of high-energy electrons by dust particles;
- The gas and dust heating;
- The heat transfer between the gas and dust particles;
- The plasmachemical reactions in the gas;
- The electron-ion recombination on the dust surface;
- The electric charging of the dust.

The local EB power input $Q(x,y,z)$, densities of charged particles of the EBP in various zones of the plasma bulk (including the mixing layer), and the temperature of the dust particles

(T_s) were calculated for various modes of the EB scanning modes and various distances z_0. The results of the computer simulations were verified experimentally for some modeling plasma-generating gases at pressures $1{,}0 < P_m < 50$ Torr and dust materials ($10^3 < n_d < 10^6$ cm^{-3}). The diagnostic complex containing optical, microwave, calorimetric and some other devices was developed to measure values of $Q(x,y,z)$, T_s, and densities of the plasma particles.

Figure 2 illustrates the obtained experimental data in comparison with the calculations. It presents the radial distributions of the local power input (curves *1*-*3*) and steady-state temperature of the dust particles (curves *4*, *5*) in the air-EBP at pressure $P_m = 4$ Torr; $z_0 = 200$ mm. All quantities plotted along the axis of ordinates are normalized by their values at the point $x = 0$, $y = 0$, i.e. at the center of the given cross-section z_0 of the plasma cloud.

The best combinations of the EBPR operating parameters were found for various plasma-generating gases ($1{,}0 < P_m < 50$ Torr) and dust densities. For the experiments described they were: plasma generating gases – water vapor, oxygen, and helium, $E_b = 30$ keV, $I_b = 2$-5 mA, $P_m = 9$ Torr (water vapor), 4 Torr (oxygen) and 40 Torr (helium). The axis of the injected EB was scanned in x- and y-directions to form the square raster 13x13 cm^2 in the plane of the mixing layer. Both continuous and intermittent modes of the EBP generation were applied. The container with the powder to be treated was located at the distance $z_0 = 25$ cm from the injection window.

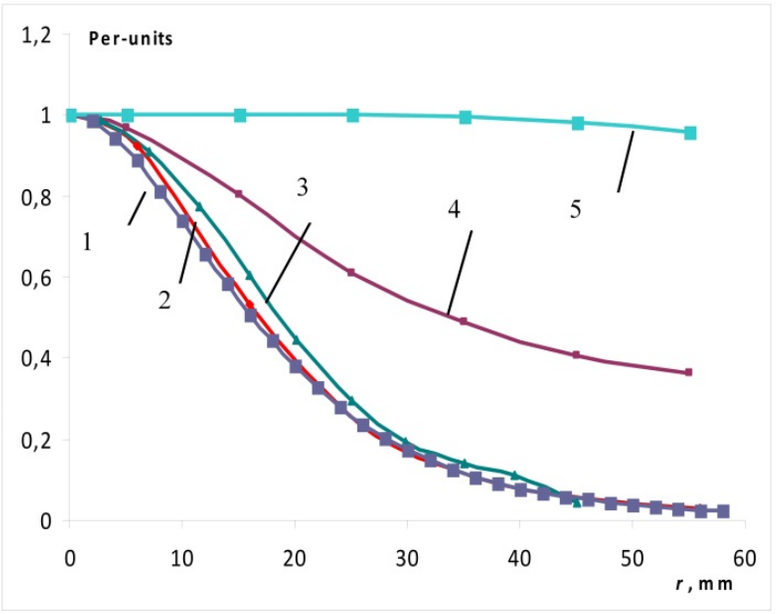


Figure 2. Radial distributions of the local power input (curves 1-3) and steady-state temperature of the dust particles (curves 4, 5): 1 – simulations, 2 – calorimetric measurements, 3 – optical measurements, 4 – thermo-sensor measurements in the non-scanning plasma cloud, 5 – thermo-sensor measurements in the plasma generated by the EB scanning along the x-axis.

Both the experiments and simulation showed that the powder is uniformly heated to the temperature T_s <50 °C and the concentrations of the chemically active plasma particles can reach 10^9-10^{11} cm^{-3} in the vicinity of the material to be treated. The concentrations of the

charged plasma particles (secondary electrons and ions) were measured by means of an open barrel-shaped microwave cavity [11] and data of the computer simulation can be used to estimate the concentrations of the neutral ones (molecules, atoms, radicals) [6]. The appropriate duration of the treatment procedure was experimentally found to achieve the required dose of the material irradiation.

5. The control of EBP-modification process

In general the following parameters are responsible for the processes (and eventually for the results) of the materials modification:

- The parameters of the EB being injected: electron energy E_b, integral current of the beam I_b and current density j_b. For the EBPR under consideration these parameters are completely predetermined by the reactor design: the designs of the electron gun and injection window, the gun accelerating voltage U.
- The medium properties: chemical composition, pressure P_m and temperature T_m of the gas.
- The geometry of the reaction bulk: location of the reaction zone inside the EBP cloud, amplitude and frequency of the EB scanning.
- Characteristic size of the powder particles and density of the aerosol in the reaction volume.
- The total dozes of the powder particle irradiation by plasma particles of various kinds; the dozes being, obviously, determined by the fluxes of these particles on the powder surface and by the treatment duration.

Special software was developed to simulate the processes of the EBP generation and plasma-powder interaction, predict the effect of the modification under various experimental conditions, and optimize the treatment procedure. The description of the numerical algorithms and physical and plasmachemical models used for the software is beyond the paper involved. Here we demonstrate only the application of the software. Figure 3 presents the incident fluxes of the plasma particles on one cm² of the container bottom (item *9* in Figure 1) calculated as a function of x- and y-distances from the container center. The EB axis scans in x- and y-directions, the amplitudes of scanning being equal to ±13 cm. Figure 3(a) illustrates the bio-material treatment in helium at pressure 40 Torr and Figure 3(b) - in water vapor at pressure 10 Torr. In the first case, four rigid peaks of the incident power appear at the raster corners and a sufficiently flat valley ≈5×5 cm^{-2} occurs between the peaks. The intensity of irradiation in the valley is about half of that on the peaks. In the EBP of water vapor a flat plateau ≈7×7 cm^{-2} with a uniform flux of plasma particles is formed. In real experiments the container with the material to be treated was placed in the zones of uniform incident fluxes.

If the container location and scanning parameters are known the values of plasma particles fluxes can be found in terms of local concentrations n (cm^{-3}) of these particles inside the container. The functions $n = n(P_m, I_b, U)$ were calculated for every plasma-generating gas used in our experiments. The variations $n = n(P_m)$ of the ion concentration

in the EBP of argon excited by the EB at two different values of the beam current I_b = 15 and I_b =7,5 mA (curves *1* and *2* respectively) are presented in Figure 4 as the example of numerical simulations. In this figure all concentration values n are divided by the maximum value of the particles concentration n_{max} for I_b = 15 mA. The Figure 3 shows that there are optimal values of P_m for given I_b and U, these optimal values being individual for each gas.

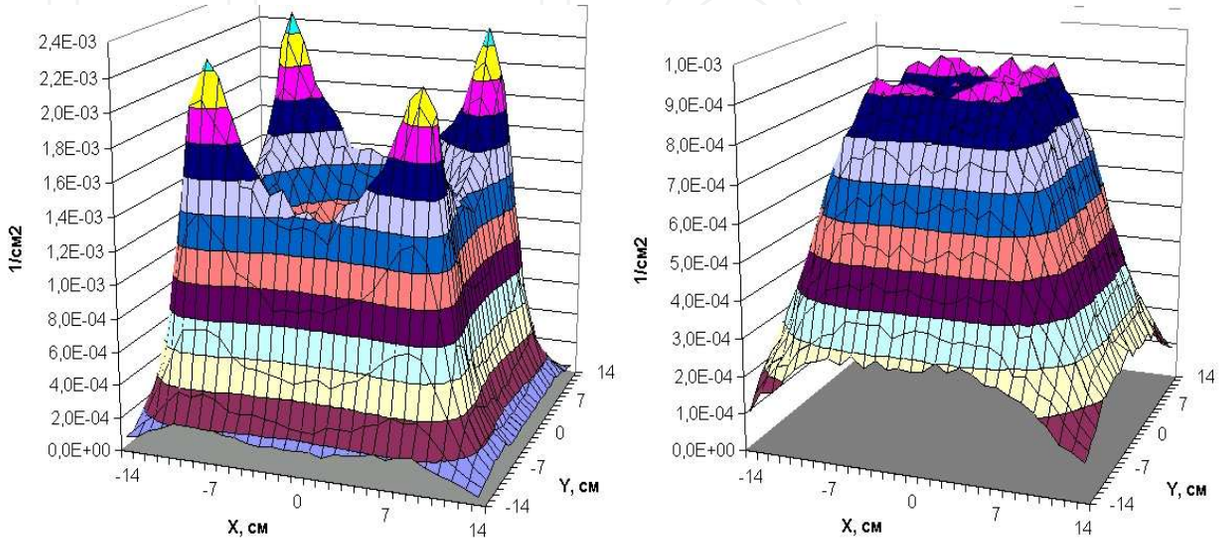


Figure 3. a.The incident radiation power of fast electrons on 1 cm² of the sample surface calculated as a function of x- and y-distances from the sample center: the treatment in the helium EBP at pressure 40 Torr. b.The incident radiation power of fast electrons on 1 cm² of the sample surface calculated as a function of x- and y-distances.

If optimal values of P_m are found the total dose of the powder particles irradiation can be calculated. In particular, the treatment durations τ required to irradiate the powder by equal dozes at variable gas pressures were calculated and relevant experiments in various gases were planned and carried out. Experiments of this kind showed the modification effect in the organic materials under consideration to appear when the accumulated doze exceeded some threshold value.

The material temperature can be controlled by varying the gas pressure value during the treatment. The lower the gas pressure - the higher the sample temperature since the scattering and absorption of the EB in gaseous media are more intensive at higher pressures than at lower ones. If higher sample temperature is required to obtain the desirable modification effect the gas pressure should be reduced. For this reason the polysaccharides were treated at lower pressures than the proteins because the higher temperature (about 400 K) was optimal for effective polysaccharides modification. On the contrary, when the proteins were treated in helium the higher pressures (P_m ≈40 Torr) were required to prevent the sample from overheating. It was because of the restrictions on the material temperature that the working pressures in the reaction chamber of the EBPR sometimes differed from their optimal values found as the maximum of the functions $n = n(P_m, I_b)$.

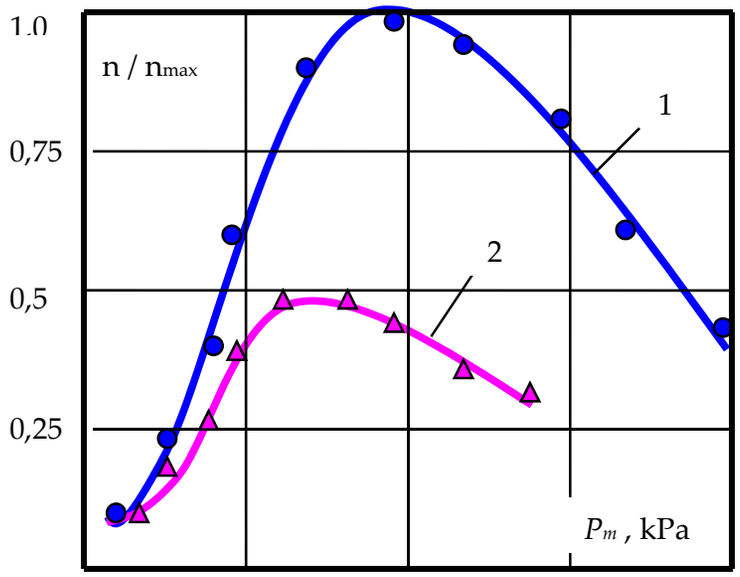


Figure 4. Concentrations of excited particles in the EBP of argon filling the reaction chamber as a function of the gas pressure, U = 25 kV, z = 130 mm: I_b = 15 mA (curve *1*), I_b = 7,5 mA (curve *2*).

6. Original substances and methods used to investigate products of their EBP-modification

The plasmachemical modification of the following natural materials was studied in detail:

- synthetic derivative of 2-aminopropanoic acid (alanine) - natural alpha-amino acid with artificially inserted pyrozolidine cycles;
- bovine serum albumin (BSA, M_r = 66 kDa)
- fibrin-monomer (FM, M_r ~340 kDa) - the natural protein which is contained in the blood of mammalians.
- cellulose materials (pure cellulose, wooden sawdust, and crushed paper);
- chitosan from crab or shrimp shells with the degree of deacetylation and molecular weight of 95% and 500 kDa, respectively;
- peat of some peat deposits in Russia.

The original powders of polysaccharides were additionally ground in the laboratory mill before treatment. Average final sizes of the powder particles were within the range 10-40 μm. The proteins were treated both in forms of solid powders (BSA and FM) and thing films (BSA). To form a film BSA was dissolved in distilled water and than water evaporation under vacuum was performed. The thickness of the formed film was ~1 μm.

The following properties of EBP-modified products were of particular interest:

- water solubility of the materials after plasma treatment;
- molecular mass;
- spectral characteristics in IR- and UV-regions;

- electrophoretic mobility;
- specific antigenic properties;
- biological activities.

Solubility measurements. 100±0,1 mg of the preliminary dried sample (m_s) were placed into a tube and 1,5 ml of distilled water were added to the sample. The resulting mixture was incubated for 24 h at room temperature under periodic mixing. After the incubation the mixture was centrifuged for 5 min and 1 ml of centrifugate was taken and dried. The mass of the dry residue (m_{dr}) was measured with an accuracy ±0,1 mg. The sample solubility was calculated as the (m_{dr}/ m_s)×100% ratio.

Molecular mass characterization. To characterize the molecular masses of the EBP-treatment products the exclusion chromatography was applied. The chromatograph *Staier* (Russia) and the chromatographic column *Phenomenex* BioSep-Sec-S-3000 (USA) with the efficiency of 30000 theoretical plates were used. The analysis conditions were as follows: the elutriating agent – 0,1 M phosphate buffer (pH 6,86) containing 0,05% NaN_3; the elution rate - 1 ml/min; temperature - 30^{o}C; UV-detector with the wavelength 280 nm.

The effects of the EBP-treatment on proteins molecular mass and structure were detected also by means of the UV- and IR-spectroscopy, ion-exchange chromatography, immunoelectrophoresis and PAGE-electrophoresis as well [12].

UV-spectroscopy. The measurements were performed with the spectrometer *Shimadzu UV-3600* (Japan). The IR-spectra were registered within wavelength λ = 226-418 nm.

IR-spectroscopy. The measurements were performed with the IR-spectrometer *Portmann Instruments AG* (Switzerland) equipped with the ZnSe crystal. The IR-spectra were registered within wave numbers ν = 500-3600 cm^{-1}. To improve the spectral resolution Fourier analysis was performed.

Ion-exachange chromatography. The ion-exchange chromatography (with the preliminary acid hydrolysis of the protein) was performed to reveal the changes in the amino acid composition of proteins due to the EBP-modification. The analyzer *AAA-339 M* (Hungary) was used. To quantitatively analyze the sulfur-containing amino acids (cystine and methionine) the biomaterial was treated with the performic acid before the hydrolysis procedure. Totally 17 basic amino acids contents were measured. The accuracy of the mesuarment was 10%.

Immunoelectrophoresis. The electrophoresis was performed in the 1,4% agar gel [13]. The commercial specific antiserum to human fibrinogen was used to characterize and compare antigenic structure of the FM before and after plasmachemical treatment.

PAGE-*electrophoresis* was performed according to Laemmli U.K. [14].

Biological activity of the EBP-produced low molecular weight chitosans (LMCW). The inhibition of the bacteria growth *in vitro* was measured to quantitatively characterize the bioactivity of LMCW obtained by the plasma treatment, gram-positive (*S. aureus*), gram-negative (*E. coli, Ps. aeruginosa*) microorganisms and yeast-like fungi (*C. albicans*) being used in these experiments.

Biological activity of the EBP-treated alanine derivative and fibrin-monomer. The EBP-modification products of fibrin-monomer were tested as the platelet aggregation inhibitors [15]. The platelet aggregation A (%) was measured by the turbodimetric method [16] and A was defined as the ratio of the light transparency of the platelet suspension after ceasing the aggregation process to the initial value of the light transparency. The aggregation was monitored by the aggregometer *Chronolog Corporation* (USA), adenosine diphosphoric acid (ADP, final concentration 1×10^{-5} M; *Boechringer Mannheim*, Germany) being used as an aggregation agent.

7. The amino-acids treatment in the Electron-Beam Plasma

Some natural amino acids with artificially inserted pirozolidine cycles into their structures were used as original substances and the products of their modification in the EBP of helium and water vapor were tested as inhibitors of the human platelet aggregation. Preliminary analysis showed the substances of this class to be promising as active agents for medical therapy of acute coronary events, and cardiovascular diseases that remain the leading cause of mortality. Their advantages are due to selectivity of the pharmacology action and limited side effects.

The powder samples (≈ 50 mg in mass) of the original derivative of alanine were treated in the EBP of water vapor at pressure P_m ≈ 9 Torr for variable time duration τ = 45-300 s. The typical EB power was N_b ≈ 0,1 kW, the sample temperature T_s under the treatment could be varied within the range 30-110 °C.

The untreated compound was not dissolvable in distilled water at room temperature and the water heating up to 90 °C followed by cooling to 25 °C was required to carry out the control experiments and to study its effect on human platelet aggregation *in vitro*. The treated substance became partially water-soluble at room temperature and the solution at maximum concentration was added to the platelet suspension to measure the aggregation degree.

The untreated derivative decreased human platelet aggregation only to 46±2% with respect to control (56±2%). The water-soluble products of plasma treatment reduced the aggregation degree up to ≈ 30 %, i.e. being treated by the EBP for 5 min the studied substance reduced the platelet aggregation activity by approximately 45 % (Table 1).

ADP	ADP + untreated amino acid	ADP + treated amino acid				
		τ = 45 s, T_s = 38 °C	τ = 90 s, T_s = 38 °C	τ = 180 s, T_s = 38 °C	τ = 180 s, T_s = 55 °C	τ = 300 s, T_s = 55 °C
56±2%	46±2%	41±3%	41±3%	34±3%	32±3%	31±3%

Table 1. The effect of the plasma modification in the EBP of water vapor on the anti-aggregation activity of the tested alanine derivative (*in vitro*): the aggregation degree as a function of the treatment duration τ and temperature of the substance T_s under the treatment procedure

The effect of the treatment duration on their anti-aggregation activity increased as the treatment prolonged, the anti-aggregation activity rising sharply at $90 < \tau < 180$ s. At shorter durations $\tau < \tau_0$ the plasma did not modify the original substance and the longer treatment τ

> τ_0 resulted in insignificant additional effect. Moderate sample heating amplified the treatment effect slightly, i.e. plasmachemical processes are responsible for the modification.

8. The proteins treatment in the Electron-Beam Plasma

Originally water-indissoluble native FM was found to become soluble at room temperature without bunching. Figure 5 presents the UV-spectra of absorption for original and treated fibrin-monomer. The spectral curves of the modified products differ radically from the curve representing the original substance. This supports the hypothesis of the changes in the physical-chemical properties due to the plasmachemical treatment in the EBP.

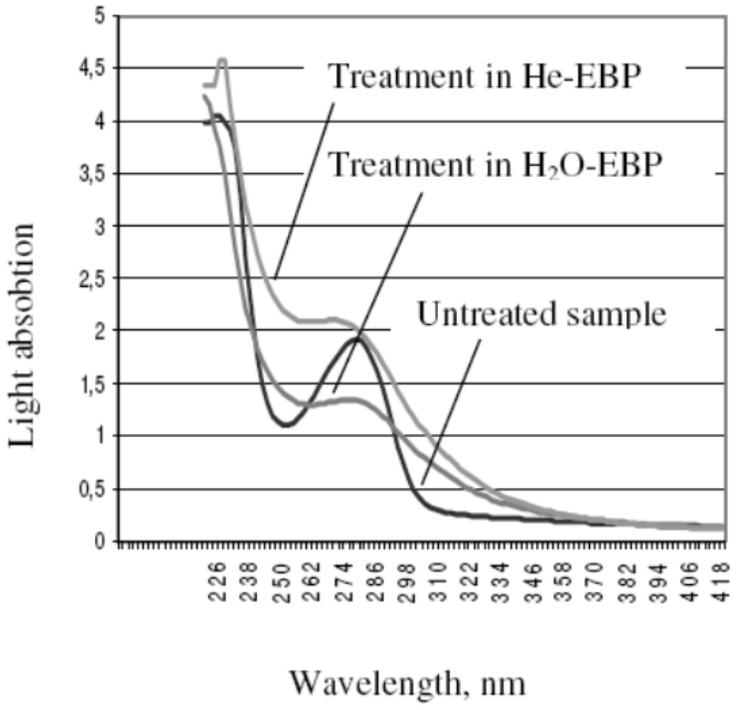


Figure 5. UV-spectra of the light absorption of fibrin-monomer solutions

Radical changes in the FM structure after the EBP-treatment were detected by means of immunoelectrophoresis. The EBP-modified products did not exhibit the specific antigenic properties of original FM and did not react with specific antibodies, while the native substance gave specific precipitation line (Figure 6).

To reveal the changes in the primary and secondary structure of the EBP-treated FM the IR-spectroscopy was used. The IR-spectra analysis showed (Figure 7 a, b):

1. The maximum of the absorbance in the band complex ν = 1711-1714 cm^{-1} (complex of amide II bands) displaced from 1711 cm^{-1} (native FM) to 1712 cm^{-1} and 1712 cm^{-1} (FM treated in the EBP of helium and water vapor, respectively). This indicates the partial destruction of peptide –CO-NH-bonds in the primary FM structure.
2. The spectral bands ν = 3354 cm^{-1} and ν = 3475 cm^{-1} characterize the valence oscillations of –N-H-bonds. The expansion of this band in the spectra of the EBP-modified FM

confirms the partial destruction of the native protein molecule and low molecular weight peptides formation.

3. The spectral bands ν = 1163 cm^{-1} and ν = 1471 cm^{-1} which characterize the oxidation of disulfide bounds are most intensive in the IR-spectra of EPB-treated FM.

These facts confirm that the EBR-treatment of the FM for τ = 5 min has caused the partial destruction of the peptide –CO-NH-bonds in the primary FM structure and the oxidation of the disulfide bonds responsible for the tertiary peptides structure.

All changes were more significant in the case of the water vapor EBP, which could result from the higher chemical activity of the water plasmolysis products (e.g. $OH^{\bullet}$).

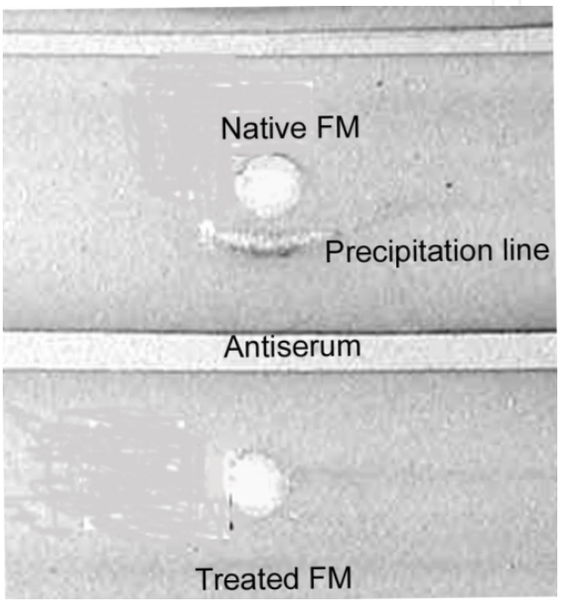


Figure 6. The immunoelectrophoresis of FM before and after the EBP-treatment

The plasma treatment reduced the amount of amino acids forming the primary protein structure (the content of 17 amino acids was studied). The percentages of lysine, threonine, glutamic acid, cystine, tyrosine and phenylalanine were found to be reduced significantly (down to 2 times with respect to the native FM, Table 2). The reduction of aspartic acid, serine, glycine, valine, methionine, leucine and isoleucine was not so sharp (only 1,3-1,5 times with respect to the native FM). The intensive peak of free NH_3 was detected in the chromatogram of the modified products whereas it was not found in the chromatogram of the original peptide.

To characterize the molecular masses of the EBP-treatment products the exclusion chromatography was applied. The peaks corresponding to 6 individual peptides with the elution times 12,30; 12,55; 13,17; 13,70; and 13,94 min were observed in the exclusion chromatograms of the FM modified by the EBP (Figure 8).

The water-soluble products of the FM appropriately treated by the EBP of both helium and water vapor were found to decrease the platelet aggregation down to ≈33-35 % *in vitro* at final concentrations 1×10^{-5}-1 mg/ml, treatment in the water vapor EBP being more effective

than that in helium (Figure 9). The effect of the treatment on the FM anti-aggregation activity increased sharply with the time within the range $90 < \tau < 180$ s. The longer treatment resulted in a negligible additional effect. The peak corresponding to the elution time 12,3 min (molecular weight ≈ 650 Da) was observed at the exclusion chromatograms of the FM modified in the EBP of helium and EBP of water vapor at a moderate irradiation dose whereas the FM treated EBP of water vapor at lower or higher irradiation doses did not produce the peak (Figure 8). This peptide is likely to be responsible for the inhibiting of the platelet aggregation.

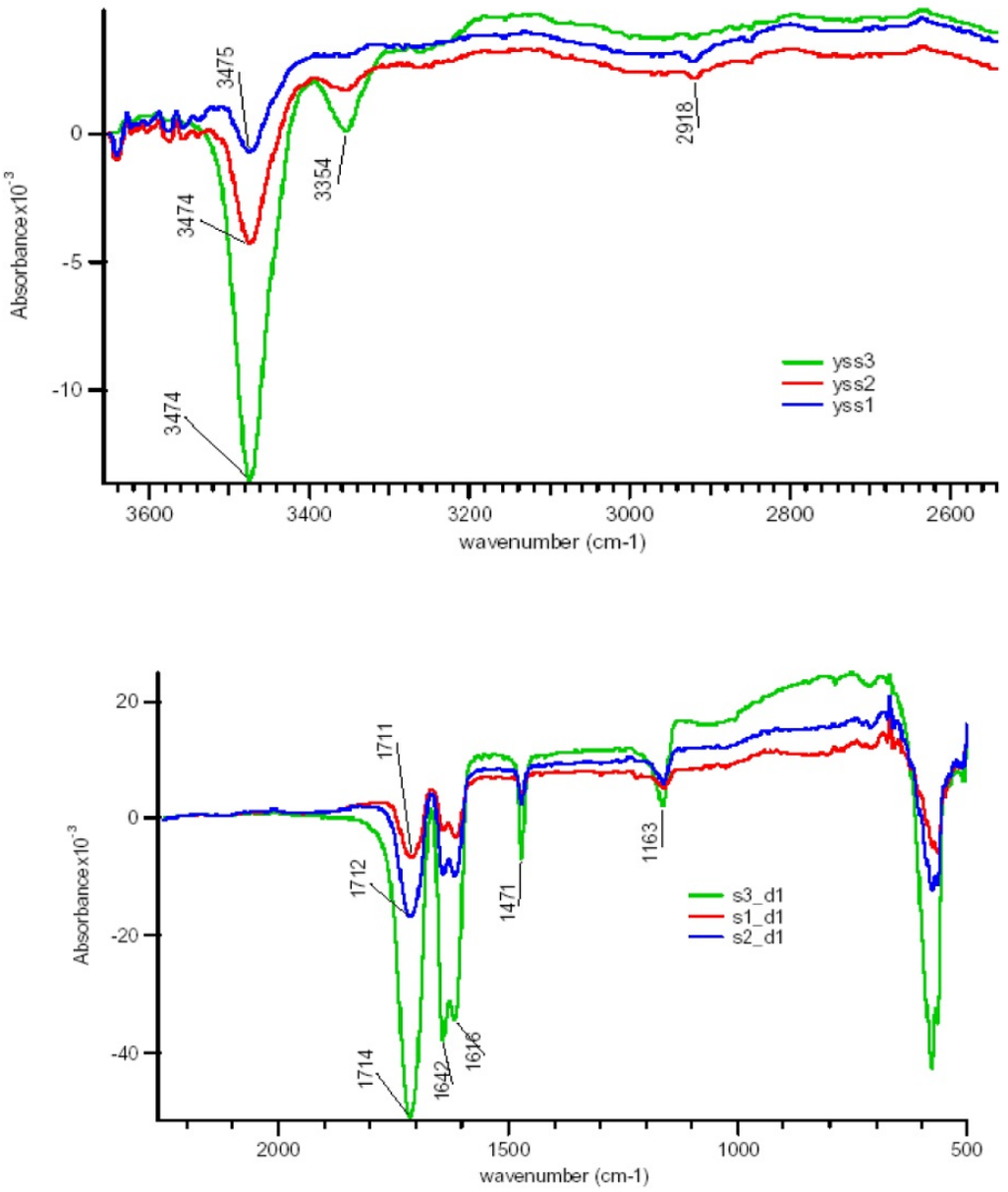


Figure 7. a. IR-spectra of the native and EBP-treated fibrin-monomer ($\tau = 5$ min): native FM – blue line, FM treated in the EBP of helium – red line, FM treated in the EBP of water vapor – green line; b.IR-spectra of the native and EBP-treated fibrin-monomer ($\tau = 5$ min): native FM – red line, FM treated in the EBP of helium – blue line, FM treated in the EBP of water vapor – green line

Amino acid	Amino acid content, %		
	Native FM	FM, treated in water vapor EPB	FM, treated in helium EPB
Lysine	0,26	0,13	0,13
Threonine	0,24	0,15	0,18
Glutamic acid	0,53	0,36	0,40
Cystine	0,068	0,039	0,051
Tyrosine	0,1	0,09	0,09
Phenylalanine	0,16	0,09	0,10
Aspartic acid	0,64	0,49	0,53
Serine	0,29	0,22	0,21
Glycine	0,24	0,22	0,22
Valine	0,15	0,12	0,13
Methionine	0,15	0,11	0,12
Leucine	0,24	0,17	0,19
Isoleucine	0,14	0,10	0,12

Table 2. The amino acid content of FM before and after EBP-treatment

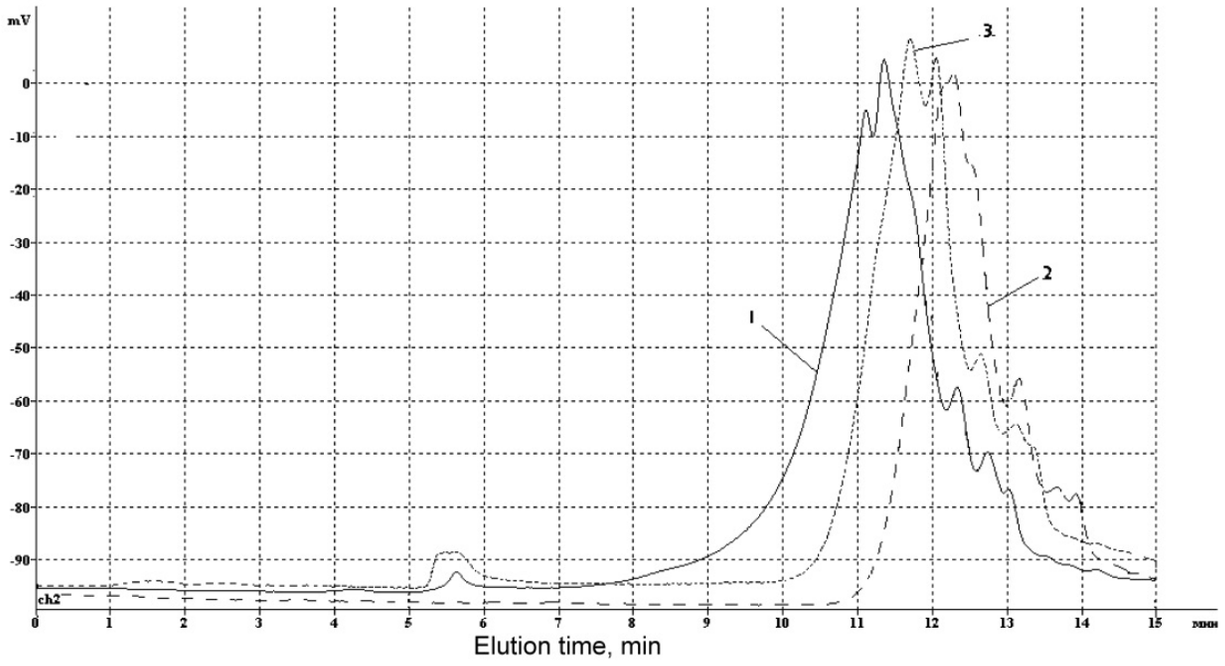


Figure 8. The chromatograms of the FM treated in the EBP of helium (curve 1), water vapor at moderate irradiation dose (curve 2) and at low irradiation dose (curve 3)

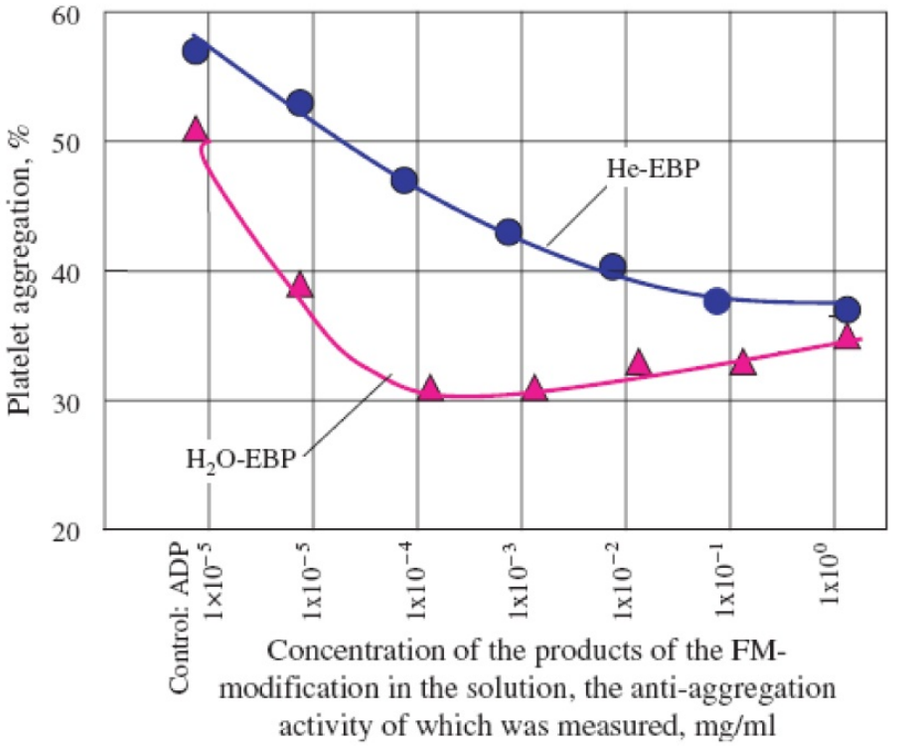


Figure 9. The reduction of the ADP-induced platelet aggregation by products of the FM modification in the EBP of water vapor and helium

The powder of globular protein BSA was treated in the EBP as well. The solubility of the native BSA was 100%. Contrary to the EPB-treated FM the products of the EBP-modification became partially indissoluble in distilled water and the solubility decrease could be observed by the unaided eye. The water solution of the treated BSA contained the of undissolved protein particles, the solution of native BSA was absolutely clear. The latter indicates that the BSA polymerization has occurred due to the EBP-treatment.

No differences in either BSA structure were found in the IR-spectrograms, PAGEs (Figure 10), changes in its amino acid composition were not observed also. Since the molecular mass of the BSA is significantly less than that of the fibrin-monomer the BSA may be more resistant to the EBP treatment and no changes in the amino acids content and their structure in the EBP-modified BSA occur. On the other hand, minor quantities of the low molecular products which could be formed due to the EBP-treatment of the BSA are undetectable by the relatively low-sensitive PAGE technique.

The exclusion chromatography, that has higher sensitivity, was used to detect the products of the BSA treatment. The peaks corresponding to the individual peptides with molecular weights >800 kDa, 350 kDa, 150 kDa, 66 kDa and less (elution times 5,6; 7,2; 8,0; 8,7 min and more than 8,7 min, respectively) were observed in the exclusion chromatograms of the BSA modified by the EBP of both oxygen and water vapor (Figure 11).The treatment of the BSA for longer time (up to 20 min) did not result in the additional changes of its properties and composition.

Whereas the products of the EBP-induced polymerization and very small concentration of low molecular peptides are detected the most significant peak (>2400 mV) corresponds to protein with molecular mass 66 kDa i.e. to the unmodified BSA. This confirms that though the EBP-treatment of the BSA causes some changes in its structure they are not as pronounced as in the case of the fibrin-monomer treatment. Therefore the modification effect of the plasma treatment depends on the size and structure of the original protein molecule.

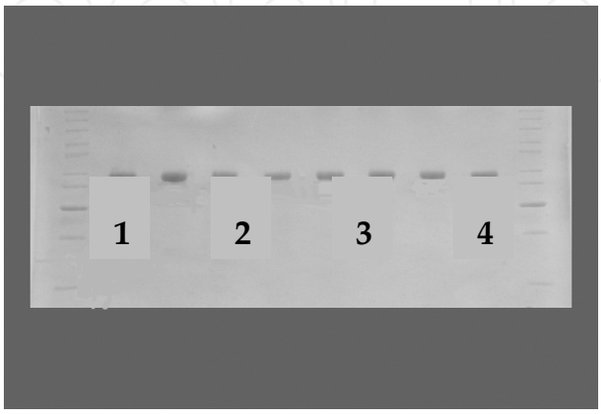


Figure 10. PAGE-electrophoresis of four BSA samples: 1 – control BSA; 2, 3 and 4 – BSA treated in the EBP of water vapor for 2,5, 5 and 10 min, respectively

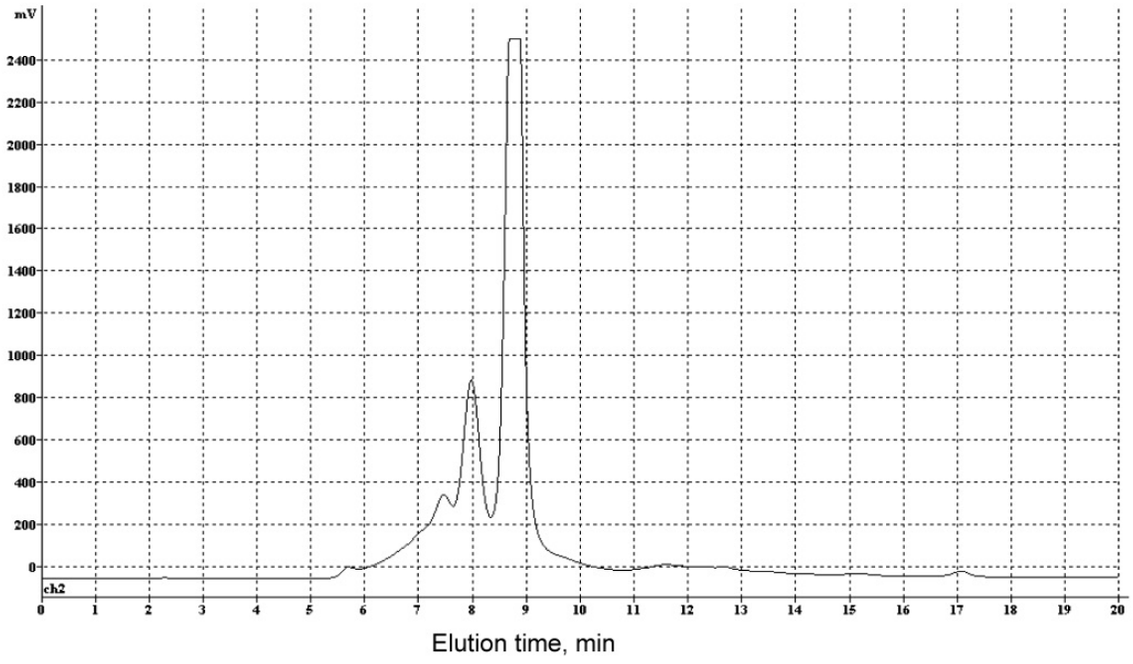


Figure 11. The exclusion chromatogram of BSA powder treated in EBP of oxygen (τ = 10 min)

On the other hand the small concentration of low molecular weight peptides is probably due to a small depth (a few micrometers) of a powder particle involved in the plasmachemical treatment. To enhance the homogeneity of the EBP-modification, the samples of globular protein were treated in the form of thin films. Such treatment arrangement can considerably

increase the yield of low molecular weight products with the comparison to the powdered samples.

The PAGE of the BSA samples modified as films in EBP revealed a dramatic decrease in the intensity of the albumin band and the formation of a number of low molecular peptides, of which most have a molecular mass below 14 kDa. The exclusion chromatograms also displayed numerous peaks corresponding to low molecular mass products with elution times of 11,8; 15,1; and 17 min (Figure 12). The concentration of the low molecular mass compounds depended on the plasma treatment time and the nature of the plasma gas: as the treatment time increased, their concentration grew, and BSA degradation being more significant in the water vapor EBP. A decrease in the content of almost all amino acids was observed. The most significant decrease (by 2–3,5 times with respect to untreated BSA) was in the case of lysine, aspartic and glutamic acids, tyrosine, and cystine (Table 3).

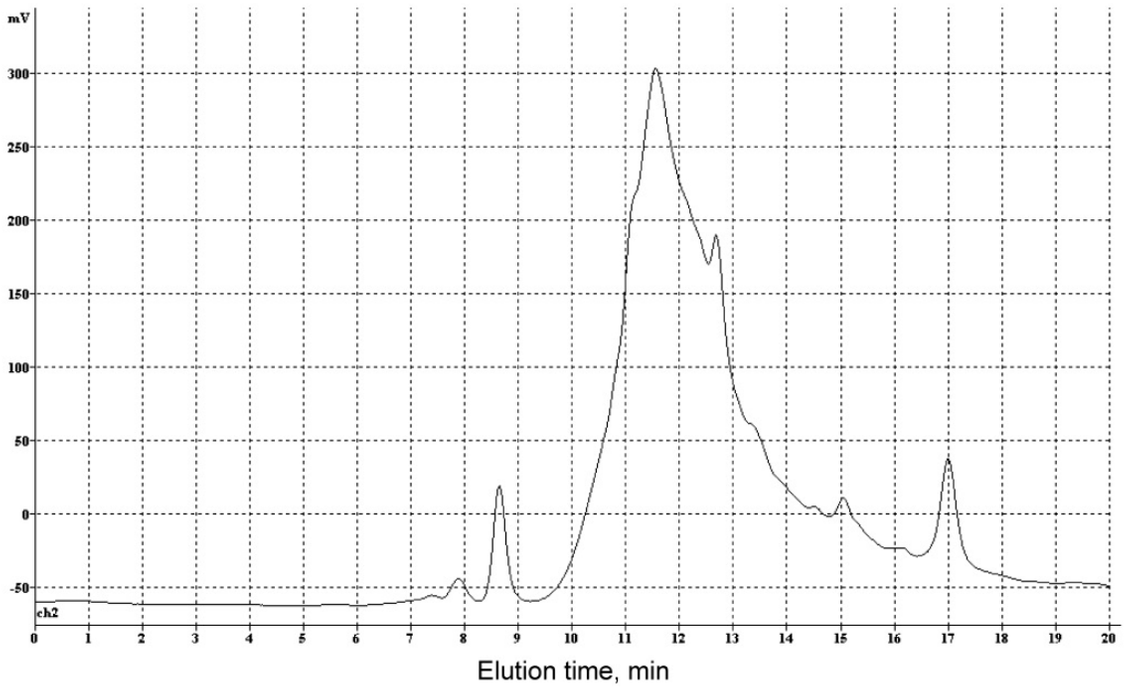


Figure 12. The exclusion chromatogram of BSA thing film treated in EBP of oxygen (τ = 10 min)

Amino acid	Amino acid content, %			
	Native BSA	BSA, treated in water vapor EPB τ = 5 min	BSA, treated in oxygen EPB τ = 5 min	BSA, treated in oxygen EPB τ = 10 min
Lysine	10,25	3,68	2,73	2,45
Threonine	5,02	2,65	2,63	2,27
Glutamic acid	16,78	5,78	6,64	5,90
Cystine	4,73	1,78	2,07	1,75
Tyrosine	4,30	1,70	1,55	1,79
Phenylalanine	5,01	3,43	3,37	2,90
Aspartic acid	9,17	3,86	5,12	5,14
Serine	3,75	2,03	2,11	1,89
Glycine	1,45	2,13	2,48	2,54
Valine	4,45	3,70	3,25	3,51
Methionine	2,02	0,76	0,89	0,63
Leucine	9,61	6,55	6,05	5,47
Isoleucine	1,85	1,34	3,37	2,90

Table 3. The amino acid content of BSA before and after EBP-treatment (BSA was treared as thing film)

9. Discussions and conclusions

1. Our experiments show that the powders of various proteins can be effectively modified in the EBP and that the plasmachemical processes are responsible for the modification. With the use of special procedures of sample preparation for the EBP-treatment, it is possible not only to increase the yield of plasma modification products but also to effectively control their composition. It was found that unlike powdered samples, the materials in the form of a freeze dried film of ~1 μm thickness can be homogeneously treated to an extent sufficient for the practical use of the compounds produced.
2. Plasmachemical reactions in thin films occur throughout the entire volume of the sample, leading to the formation of final low molecular mass products. The powdered samples are characterized by a thin surface layer (of at most 10 μm in thickness) in which the effective modification of the protein takes place. The protein remains intact in deep layers of such samples, and polymerization of protein molecules is likely to occur in intermediate layers. Our results are consisted with the data obtained in some other studies [17, 18]. The destruction of BSA thing films was also observed in the experiments using the atmospheric pressure glow discharge. Nevertheless the effective BSA degradation in the atmospheric pressure glow discharge was observed when the very small amounts of the protein sample (~20 μl) were treated [18]. Thus, the EBP-treatment seems to be more powerful technique for the protein degradation and low molecular peptides formation with respect to the gas-discharge plasmas.

The following factors influence the biomaterial placed into the plasma cloud:

- the fast electrons of the partially degraded EB that bombard the sample;
- the secondary electrons of moderate energy produced in the EBP can also act on the powder;
- the EBP-radiation, especially UV one and X-ray (bremsstrahlung);
- chemically active heavy particles of the EBP (excited molecules and atoms, ions, radicals). This factor is likely plays the pivotal role for the low molecular peptides formation and for the production of bioactive products [19]. Due to the action of the reactive oxygen species (ROS) the destruction of the peptide bonds occures. The Figure 13 illustrates the possible mechanism of peptide bonds cleavage [20]. The formation of intermolecular cross-links can also occure under the ROS influence and the production of some high molecular weight substances was detected by the exclusion chromatography after the EBP-treament of fibrin-monomer powder (see Figure 8).
  Some new chemical groups might be formed in the molecule structure during the treatment in the EBP. For instance, the ROS generated in the EBP of the water vapor or oxygen can react with the amino acid residues resulting in their oxidation and the formation of various keto-products. The formation of such oxidized substances (e.g. pyruvic acid, kynurenine, glutamic semialdehyde, disulfides, cysteic acid [21]) is possible reason for the changes in the UV-spectra and amino acid composition (see Figure 5 and Tables 2 and 3).

3. the EBPR with the aerosol reaction zone was successfully used to produce compounds with new pharmacological activity and therapeutic effect, e.g. for the production of effective platelet aggregation antagonists. Also the destruction of peptides in the EBP can be used for the effective sterelization of medical instruments, inactivation of pathogenic bateria and prion proteins.

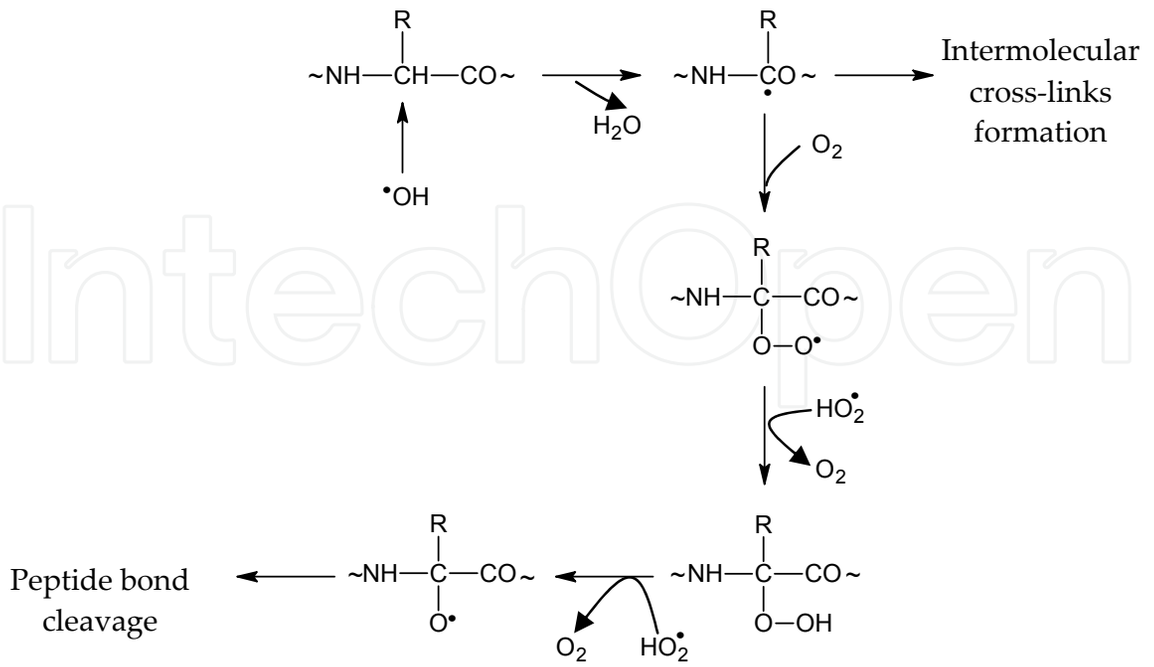


Figure 13. The scheme of peptide bound degradation and cross-links formation under ROS action in the EBP of the water vapor or oxygen

10. The polysaccharides treatment in the Electron-Beam Plasma

The plasmachemical modifications of cellulose materials, peat, and chitosan in EBP of O_2, He, and H_2O, are studied. The yield (S, %) and the average molecular mass of water-soluble products extracted from the solution were taken as the quantitative criteria for the modification. Note, typical content of water-soluble substances in untreated cellulose materials does not exceed 1–2%.The molecular mass of the cellulose may reach several million. The water-soluble products yield depends upon the processing conditions and has a maximum value S_{max} for each plasma-generating gas: for oxygen plasma $S_{max} \approx 45\%$, for plasma of water vapor $S_{max} \approx 56\%$, which is reached during certain time periods of the treatment τ_0. At first the dependence $S(\tau)$ increases smoothly, then - steeply in the vicinity of τ_0 after which the yield of the water-soluble products does not change whatever the time of the treatment could be (Figure 14). The NMR- and IR-spectroscopy analyses showed the final water-soluble products of the cellulose modification to be β-(C1→C4)-tetrasaccharide, the molecule structure and chemical bonds of the products being identified. In particular, low-molecular products (M ≈250–800 Da) have attached carbonyl and/or carboxyl groups on their ends if the original materials have been treated in O_2 or H_2O plasmas.

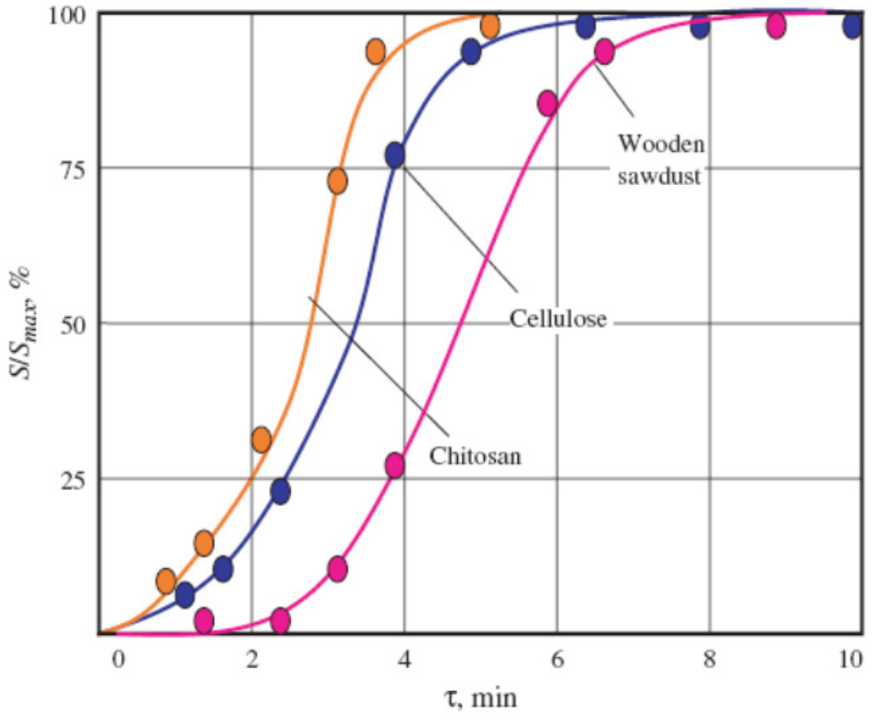


Figure 14. The modification effect in studied polysaccharides as a function of the treatment duration in the EBP of water vapor

The products of the EBP modification of all the substances mentioned above turned out to be bioactive. For instance, modified cellulose and peat are effective substratesfor microorganisms and fungi: the yield of the yeastrel on the plasmachemically treated peat reached 0,14 kg per 1 kg of the peat, whereas the productivity of the untreated substrate was only 0,053 kg.

The original chitosan was not water-soluble while its EBP-treament products became soluble and the effect increased with the prolongation of τ. The maximum yield of water-soluble substances was obtained after 10 min and the solubility of these products was up to 95% up at room temperature.

The exclusion chromatography of the EBP-treated chitosan revealed the formation of a number of LMWC with molecular weight varied from 18 kDa to monomeric fragments (Figure 15). The majority of products formed with the EBP-treatment were oligosaccharides with the molecular mass 1 kDa (the elution time 11,3 min).

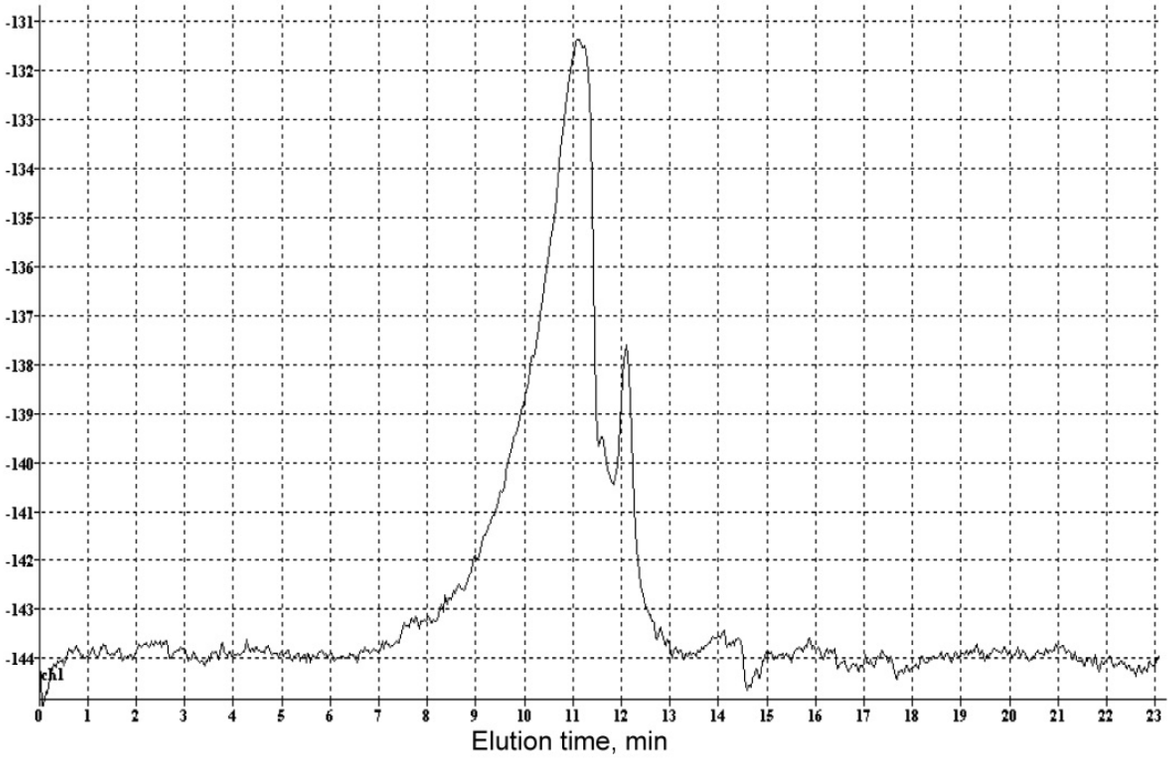


Figure 15. The exclusion chromatogram of chitosan treated in the EBP of water vapor (τ = 10 min)

The degradation of the original polymer is due to the action of free radicals formed in the EBP. Active oxygen particles (O, O•, singlet oxygen) that are produced in plasmachemical processes and the products of the water plasmolisys (e.g. OH•) are likely to be of the most importance. These chemically active particles break the β-1,4 glycosidic bound and decrease the chitosan molecular weight Figure 16 illustrates the possible degradation mechanism [22].

To quantitatively characterize the bioactivity of the EBPproduced LMCW the bacteria growth *in vitro* was studied. The LMCW produced by the EBP-treatment in water vapor at concentration 1000 μg/ml were found to completely suppress the multiplication of colon bacillus, aurococcus and yeast-like fungi. At lower doses the EBP-treatment products were also active and strongly inhibited the microorganism multiplication (Table 4).

Test microorganism	EBP-treated chitosan concentration, μg/ml				Control
	1000	500	250	125	
E. coli	----	±	±	+	+
Ps. aeruginosa	+	+	+	+	+
S. aureus	----	±	+	+	+
C. albicans	----	±	+	+	+

--- the absence of microorganism growth; ± weak microorganism growth; + microorganism growth comparable with reference sample

Table 4. The microorganism growth under EBP-treated chitosan

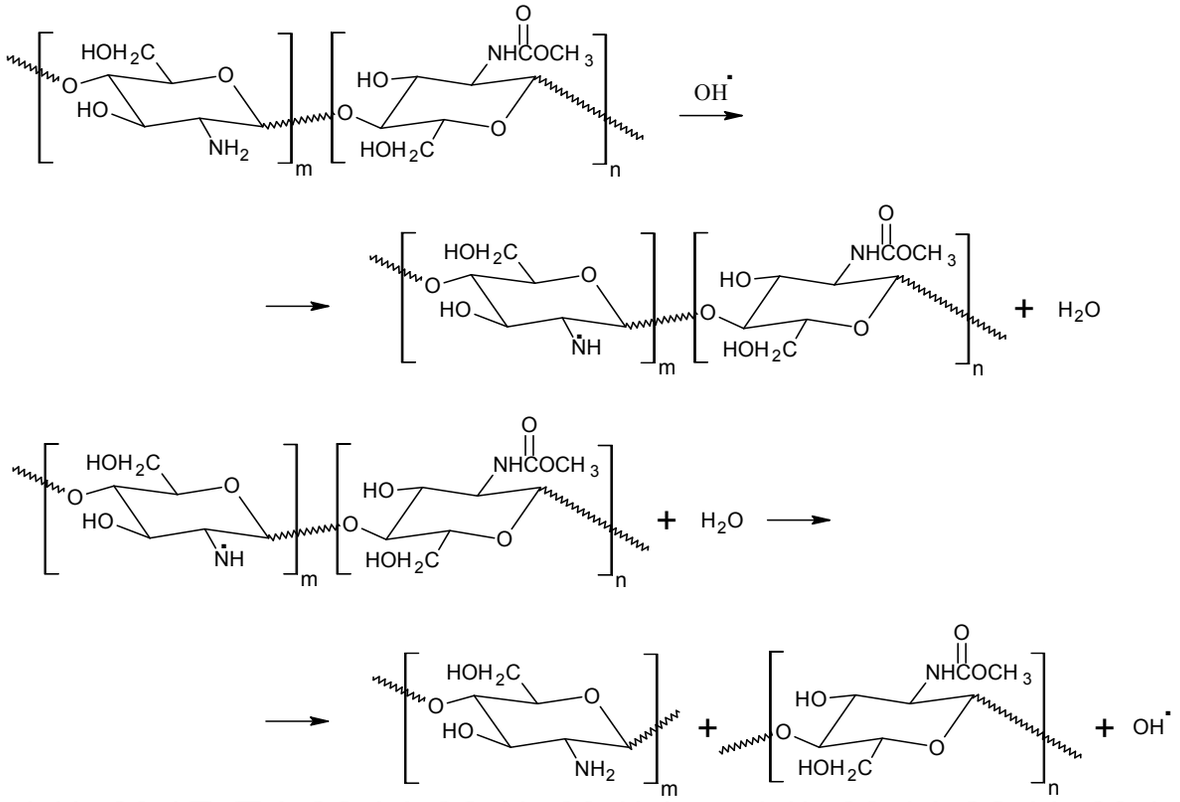


Figure 16. The scheme of chitosan degradation under hydroxyl radical action in the EBP of the water vapor [22]

11. Discussions and conclusions

- The possibility of the EBP-stimulated hydrolysis of native polysaccharides and formation of water-soluble low molecular weight bioactive products was proved experimentally. The yield of the water-soluble products depends on the doze of the irradiation by the plasma particles. Other things being equal, the yield of the bioactive products began to increase abruptly at particular duration of treatment τ_0. At shorter duration $\tau < \tau_0$ the plasma didn't modify the original substance and the longer treatment $\tau > \tau_0$ resulted in an insignificant additional effect.

- The 95% yield of low molecular weight EBP-treatment products was attained by optimizing the treatment procedure. The high yields of low molecular weight water soluble products are obtained at treatment time ~10 min whereas the traditional chitosan hydrolysis usually takes several days. The hazardous by-products and toxic wastes are not generated during the EBP-treatment.
- The low molecular water-soluble forms of the chitosan obtained by its treatment in the EBP of oxygen and water vapor were found to inhibit the multiplication of colon bacillus, aurococcus and yeast-like fungi. The LMWC with molecular weight 30-180 kDa and chitooligosaccharide mixtures are known to possess antimicrobial properties [22, 23]. We suppose that the antibacterial activity of the EBP-produced LMCW results from the LMCW interaction with the cell walls of microorganisms. This mechanism was considered in detail in [24].
- The EBP-stimulated degradation mechanisms occurring both in polysaccharides and high molecular weight proteins are similar and occur due to the plasmachemical processes, whereas the attendant of fast electrons and X-ray irradiations are minor factors only.The active oxygen species produced in plasmachemical reactions and the products of water plasmolisys are responsible for the LMWC and chitooligosaccharides formation. Parameters of the treatment process can be adjusted by the control system of the EBPR and the results of the modification are predictable.

12. Future developments

1. Our experiments have demonstrated that the EBP can be used for the effective and controllable modification of some natural biopolymers. Using various plasma-generating gases the functional groups containing sulfur, nitrogen, phosphorus can be introduced into the biopolymer structure to obtain substances with unique pharmacological and biological activities (anticoagulant, hemostatic, antibacterial, etc.);
2. On the basis of present study the novel strategies of plasma-stimulated synthesis and plasma-assisted bioactive coating deposition can be developed. Two different ways of the plasma-assisted formation of bioactive coatings by means of the deposition of organic and inorganic vapors activated by the electron beam are possible: 1) on a preliminary prepared plane substrate; 2) on a surface of powder particles levitating in the plasma trap. The following materials can be synthesized:
    - supramolecular complexes “protein-drug agent” or “polysaccharide- drug agent” for the addressed drug delivery;
    - medical hybrid materials and coatings with high bio- and hemo-compatibility and/or combined pharmacological action (e.g. haemostatics with antimicrobial or regenerative properties);
3. The technique is potentionally interesting for:
    - increasing the wettability of the original organic and inorganic materials to obtain effective sorbents, membranes for hemodialysis, and microbiological substrates;
    - improving membranes selectivity and stability and production more sensitive biosensors.

Author details

T. Vasilieva

Department of General Chemistry, Moscow Institute of Physics and Technology, Dolgoprudny, Moscow region, Russia

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