Application of electron beam plasma for biopolymers modification

T M Vasilieva
Moscow Institute of Physics and Technology, Dolgoprudny, Moscow region, 141700, Russia
E-mail: tmvasilieva@gmail.com

Abstract. The effects of the Electron Beam Plasma treatment on natural polysaccharide chitosan were studied experimentally. Low molecular water-soluble products of chitosan and chitooligosaccharides were obtained by treating the original polymers in the Electron Beam Plasma of oxygen and water vapor. The molecular mass of the products varied from 18 kDa to monomeric fragments. The degradation of the original polymers was due to the action of active oxygen particles (atomic and singlet oxygen) and the particles of the water plasmolysis (hydroxyl radicals, hydrogen peroxides). The 95% yield of low molecular weight chitosans was attained by optimizing the treatment conditions. The studies of the antimicrobial activity of low molecular products showed that they strongly inhibit the multiplication of colon bacillus, aurococcus and yeast-like fungi. The EBP-stimulated degradation of polysaccharides and proteins were found to result from breaking $\beta$-1,4 glycosidic bounds and peptide bonds, respectively.

1. Introduction

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 [1, 2]. Chitosan, linear heteropolymers of $\beta$-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, biodegradability, 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 [1, 2].

To produce the low molecular weight chitosans (LMWC) several techniques, including chemical, enzymatic, and radical treatment have been suggested [3]. 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 novel approach to the water-soluble low molecular weight chitosan production which is based on the Electron Beam Plasma (EBP) application is considered in the present paper.
The EBP is generated by injecting an electron beam (EB) into a gaseous medium. Under typical conditions of the EBP generation (medium pressure $0.1 < P_m < 10$ kPa and moderate EB power $N_b < 1$ kW) the plasma is strongly non-equilibrium and cold. Being injected into the gas the EB ionizes the gas, excites the gas molecules and is able to cause molecule dissociation. The generated EBP has a complex composition and contains a lot of chemically active particles that do not exist under equilibrium conditions. With respect to non-equilibrium plasmas generated in conventional ways (for instance, with respect to gas discharge plasmas) 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 thin 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.

The aims of the present study were as follows:
1) to experimentally prove the possibility of the EBP-stimulated hydrolysis of native chitosans and formation of water-soluble low molecular weight products as a result of the chitosans plasma chemical processing;
2) to obtain the high yield of the low molecular weight products by optimizing the treatment conditions;
3) to characterize both the structure of the low molecular weight products of the plasma chemical treatment and their bioactivity;
4) to compare the effects of the EBP-treatment on polysaccharides and some other natural biopolymers, e.g. proteins.

2. Materials and methods

2.1. Materials

Crab shell chitosan with the degree of deacetylation and molecular weight of 95% and 500 kDa, respectively, was used as the original substance for the further EBP-treatment. The high molecular weight blood protein fibrin-monomer ($M_r \sim 340$ kDa) was chosen as reference substance since its EBP-modification has been studied in detail previously [4].

2.2. Characterization of EBP-treated biopolymers

2.2.1. Solubility measurements. 100±0.1 mg of the preliminary dried sample ($m_i$) 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 centrifuged 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 ($m_{dr}/m_i$)•100% ratio.

2.2.2. 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% NaN₃; the elution rate - 1 ml/min; temperature - 30°C; UV-detector with the wavelength 280 nm.
The effects of the EBP-treatment on fibrin-monomer molecular mass and structure were detected by means of the IR-spectroscopy, ion-exchange chromatography, horizontal electrophoresis in agar gel, immunoelectrophoresis and PAGE-electrophoresis as well [4].

2.2.3. Biological activity of the EBP-produced LMWC. The inhibition of the bacteria growth in vitro was measured to quantitatively characterize the bioactivity of LMWC 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.

2.2.4. Biological activity of the EBP-treated fibrin-monomer. The EBP-modification products of fibrin-monomer were tested as the platelet aggregation inhibitors [5].

3. Treatment procedure
For the controllable chitosan modification and LMWC production the special Electron Beam Plasma chemical Reactor (EBPR) was designed. The EBPR, its operation modes and optimization of the biomaterial treatment regimes were described in detail in [4].

Figure 1 illustrates the treatment procedure. The focused continuous electron beam (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 injection window 4 [6]. 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).

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, thereby, 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 the plasma generating media (oxygen or water vapor). The water vapor was produced by means of the electrically heated evaporator 11 placed inside the working chamber. The samples to be treated were inserted into the EBPR reaction zone as solid powders with characteristic particle size ~ 100 mc. The powder partially fills the glass container 9 over the thin plate 7 made of piezoelectric ceramics which is at 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 inside the container. The miniature thermo-sensor 8 is inserted into the...
container to monitor the material temperature \( T_s \) during the treatment. To prevent the thermal destruction of the biological material all samples were processed at \( T_s < 50 \, ^\circ C \). In the case of proteins \( T_s \) was \( \sim 37 \, ^\circ C \). The temperature control was carried out by selecting the EB current \( I_b \) \((1 < I_b < 100 \, mA)\).

The experimental conditions were as follows:

- the pressure of the plasma generating gas was 4 Torr, 9 Torr, and 40 Torr for oxygen, water vapor, and helium, respectively;
- the distance between the injection window and sample surface was 250 mm;
- the EB scanning - square raster with 130 mm side length.

The optimal treatment time \( \tau \) to achieve the required dose of the material irradiation was experimentally found by varying \( \tau \) from 5 (for proteins) to 10 min (for chitosan).

4. **Chitosan treatment in the electron-beam plasma. Production of LMWC**

The original chitosan was not water-soluble while its EBP-treatment products became soluble and the effect increased with the prolongation of \( \tau \). 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 2). The majority of products formed with the EBP-treatment were oligosaccharides with the molecular mass 1 kDa (the elution time 11.3 min).

![Figure 2. The exclusion chromatogram of chitosan treated in the EBP of water vapor for 10 min.](image)

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 plasma chemical processes and the products of the water plasmolysis (e.g. OH′) are likely to be of the most importance. These chemically active particles break the \( \beta-1,4 \) glycosidic bound and decrease the chitosan molecular weight Figure 3 illustrates the possible degradation mechanism [7].

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The LMWC with molecular weight 30-180 kDa and chitooligosaccharide mixtures are known to possess antimicrobial properties [8, 9]. To quantitatively characterize the bioactivity of the EBP-produced LMCW the bacteria growth in vitro was studied (Table 1). The LMCW produced by the EBP-treatment in water vapor at concentration 1000 mcg/ml were found to completely suppress the multiplication of colon bacillus, aurocoecus and yeast-like fungi. At lower doses the EBP-treatment products were also active and strongly inhibited the microorganism multiplication. 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 [10].

### Table 1. The microorganism growth under EBP-treated chitosan.

| Test microorganism | EBP-treated chitosan concentration, mcg/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

**Figure 3.** The scheme of chitosan degradation under hydroxyl radical action in the EBP of the water vapor [6].
5. The EBP-treatment of fibrin-monomer

The EBP-treatment of protein FM changed its structure radically due to the action of the chemically active plasma particles. Originally water-indissoluble native FM was found to become soluble at room temperature without bunching. The EBP-modified products did not exhibit the specific antigenic properties of the original FM and did not react with specific antibodies, while the native substance gave a specific precipitation line.

To reveal the changes in the primary and secondary structure of the EBP-treated FM the IR-spectroscopy was used. The EBR-treatment of the FM for $\tau = 5$ min 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. These resulted in the FM destruction and low molecular weight peptides formation.

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 4).

The water-soluble products of the FM treated by EBP under specially adjusted conditions were found to decrease the platelet aggregation down to $\approx 33-35 \%$ in vitro at concentrations $1 \times 10^{-4}$-1 mg/ml [5]. The peak corresponding to the elution time 12.3 min (molecular weight $\approx 650$ Da) was observed at the exclusion chromatograms of the FM modified in the EBP of helium and in the EBP of water vapor. This peptide is likely to be responsible for inhibiting the platelet aggregation.

![Exclusion Chromatogram](image-url)

**Figure 4.** The exclusion chromatogram of fibrin-monomer treated in the EBP: 1- EBP of helium, $\tau = 10$ min; 2- EBP of water vapor, $\tau = 5$ min; 3- EBP of water vapor, $\tau = 15$ min.
6. Conclusions

- The possibility of the EBP-stimulated hydrolysis of native chitosan and formation of water-soluble low molecular weight products was proved experimentally.
- 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 EBP-stimulated degradation mechanisms occurring both in polysaccharides and high molecular weight proteins are similar. The active oxygen species produced in plasma chemical reactions and the products of water plasmolysis are responsible for the LMWC, chitooligosaccharides, and peptides formation.

Thus, our experiments have demonstrated that the EBP can be used for the effective and controllable modification of some natural biopolymers. The technique involved is likely to be promising to engineer the compounds with unique pharmacological activities and the EBPRs seem to be competitive with technologies conventionally used in the pharmaceutical industry.

Acknowledgments

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