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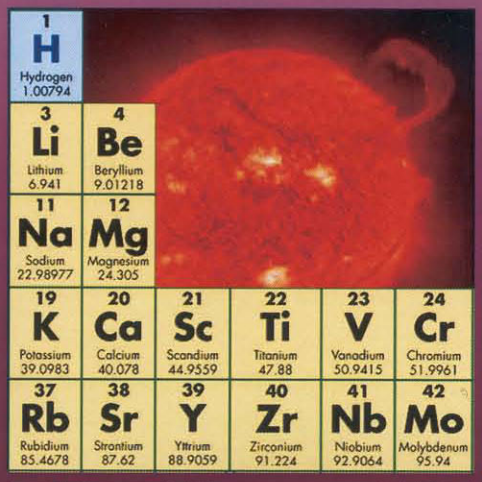

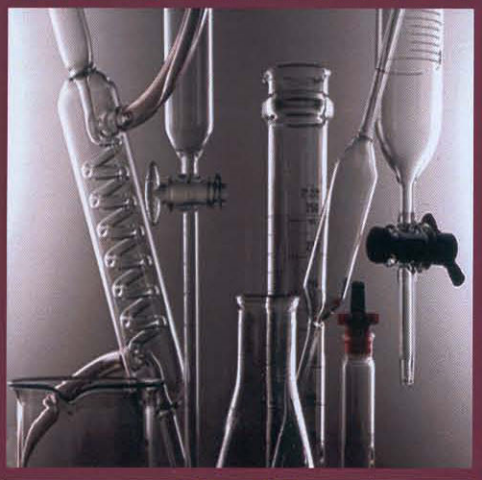

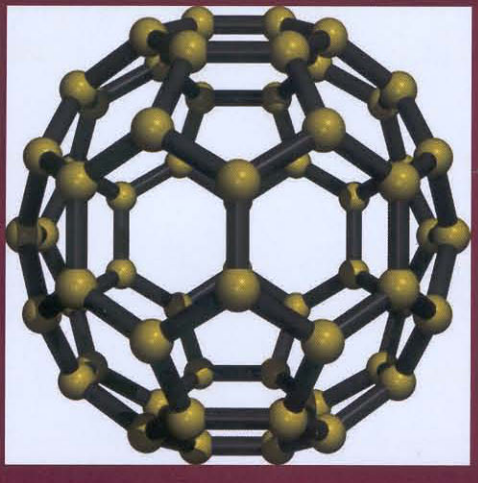

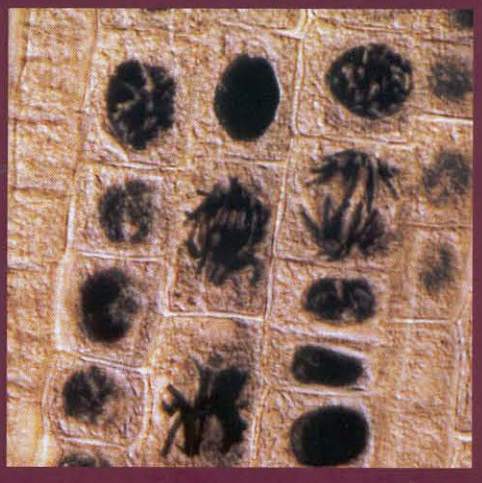

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Editorial

The most significant achievements of the modern science are joined by different sciences among them are chemistry and biology. Therefore publication of Journal, which is showing results of current investigations in the field of chemistry and biology, will allow widely exhibiting scientific problems, tasks and discoveries.

One of the basic goal of the Journal will be to promote the extensive exchange information between the scientists of all over the world. We suggest publishing original papers and materials of Chemical and Biological Conferences (after selection) holding in different countries.

The creation of special International Journal of Biology and Chemistry is of great importance because a great amount of scientists to publish their articles and it will help to wide the geography of future operations. We will be glad to publish also the papers of scientists from other continents.

The Journal will exist for the publication of experimental and theoretical investigations of chemistry, chemical technology and biology. Among the subject emphasized are: modern problems of organic synthesis technologies; scientific basis of the production of physiologically active preparations; modern problems of the processing technologies of raw materials, production of new materials and technologies; investigation of chemical and physical properties and structure of oil and coal; theoretical and practical problems of hydrocarbons processing; the modern achievement in the field of nanotechnology; results of investigations in biology, biotechnology, nano-biology, genetics and etc.

The journal is issued on the base of al-Farabi Kazakh National University. Leading scientists from different countries of the world have been agreed to be the members of an editorial board of the journal. The list of Editorial board is attached.

We are going to publish 2 numbers of Journal in 2010 and 4 numbers per year in a following years.

We shell hope to receive papers from the many laboratories which are interested in the application of the scientific principles of chemistry, chemical technology, biology and are carrying out research on the subject, whether it be in relation to production new materials, technology or ecology problems.

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Adsorption of Bacterial Lipopolysaccharides and Blood Plasma Proteins on Modified Carbonized Materials

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Abstract

Bacterial lipopolysaccharides (endotoxins) show strong biological effects at very low concentrations in human beings and many animals when entering the blood stream. These include affecting structure and function of organs and cells, changing metabolic functions, raising body temperature, triggering the coagulation cascade, modifying hemodynamics and causing septic shock. Because of this toxicity, the removal of even minute amounts is essential for safe parenteral administration of drugs and also for septic shock patients' care. The absence of a general method for endotoxin removal from liquid interfaces urgently requires finding new methods and materials to overcome this gap. Nanostructured carbonized plant parts is a promising material that showed good adsorption properties due to its vast pore network and high surface area. The aim of this study was comparative measurement of endotoxin- and blood proteins-related adsorption rate and adsorption capacity for different carboneous materials produced at different temperatures and under different surface modifications. As a main surface modificator, positively charged polymer, polyethileneimine (PEI) was used. Activated carbon materials showed good adsorption properties for LPS and some proteins used in the experiments. During the batch experiments, several techniques (dust removal, autoclaving) were used and optimized for improving the material's adsorption behavior. Also, with the results obtained it was possible to differentiate the materials according to their adsorption capacity and kinetic characteristics. Modification of the surface apparently has not affected hemoglobin binding to the adsorbent's surface. Obtained adsorption isotherms can be used as a powerful tool for designing of future column-based setups for blood purification from LPS, which is especially important for septic shock treatment.

Introduction

Endotoxins and septic shock

Gram-negative sepsis, a relatively rare clinical diagnosis only a few decades ago, is perhaps the most important infectious disease problem in hospitals today. Despite recent advances in our understanding of the pathophysiological mechanisms of sepsis and improved antimicrobial therapy, the mortality rate from gram-negative sepsis remains frustratingly high, particularly after the onset of shock [1, 2].

At the pathophysiological level, the development of gram-negative sepsis involves a complicated series of effects based on the composition of the bacterial cell wall. Pfeiffer first recognized the heat-stable toxic component of gram-negative bacteria at the end of the 19th century [3]. In his experiments, Pfeiffer noted that lysates of heat-inactivated *Vibrio cholerae* caused shock and death in laboratory animals. He called the toxic substance, not yet characterized, "*endotoxin*" on the assumption that it was found inside the bacterium. This also served to distinguish it from toxins secreted during bacterial growth in culture.

In the 1930s, endotoxins were isolated and characterized as lipopolysaccharide (LPS)-phospholipid-protein complexes present in the bacterial outer membrane. Subsequent efforts yielded purified and protein-free LPS that could produce all of the physiological effects of the impure substance isolated earlier. Later experiments suggested that a chemical subunit of LPS, lipid A, was the actually toxic moiety and that the 0-specific chain found on LPS was not involved in the toxic effect [4, 5].

The structure of the LPS molecule is shown schematically in Fig.1. Chemically, endotoxins are *lipopolysaccharides* (frequent synonyms of *endotoxin* are *LPS* and *pyrogen*) that consist of three biologically, chemically, genetically and serologically different parts. These are a non-polar lipid component, called lipid A, the so-called core oligosaccharide and a heteropolysaccharide representing the surface antigen (O-antigen).

The most conservative part of endotoxin is lipid A, which, apart from few exceptions, shows very narrow structural relationship in different bacterial genera. It consists of a β-1.6-linked disaccharide of glucosamine, covalently linked to 3-hydroxy-acyl substituents with 12–16 carbon atoms via amid and ester bonds; these may be

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further esterified with saturated fatty acids. This hydrophobic part of endotoxin adopts an ordered hexagonal arrangement, resulting in a more rigid structure compared with the rest of the molecule. The form of lipid A produced by *E. coli* has now been synthesized in the laboratory. Strains lacking lipid A or endotoxin are not known. The R polysaccharide or the core R antigen contains unusual short chain sugars, for example ketodeoxyoctulonate (KDO) and heptose. KDO is unique and invariably present in LPS and so it has been used as an indicator in assays for LPS (endotoxin). In *E. coli* species five different core types are known, *Salmonella* species share only one core structure [6].

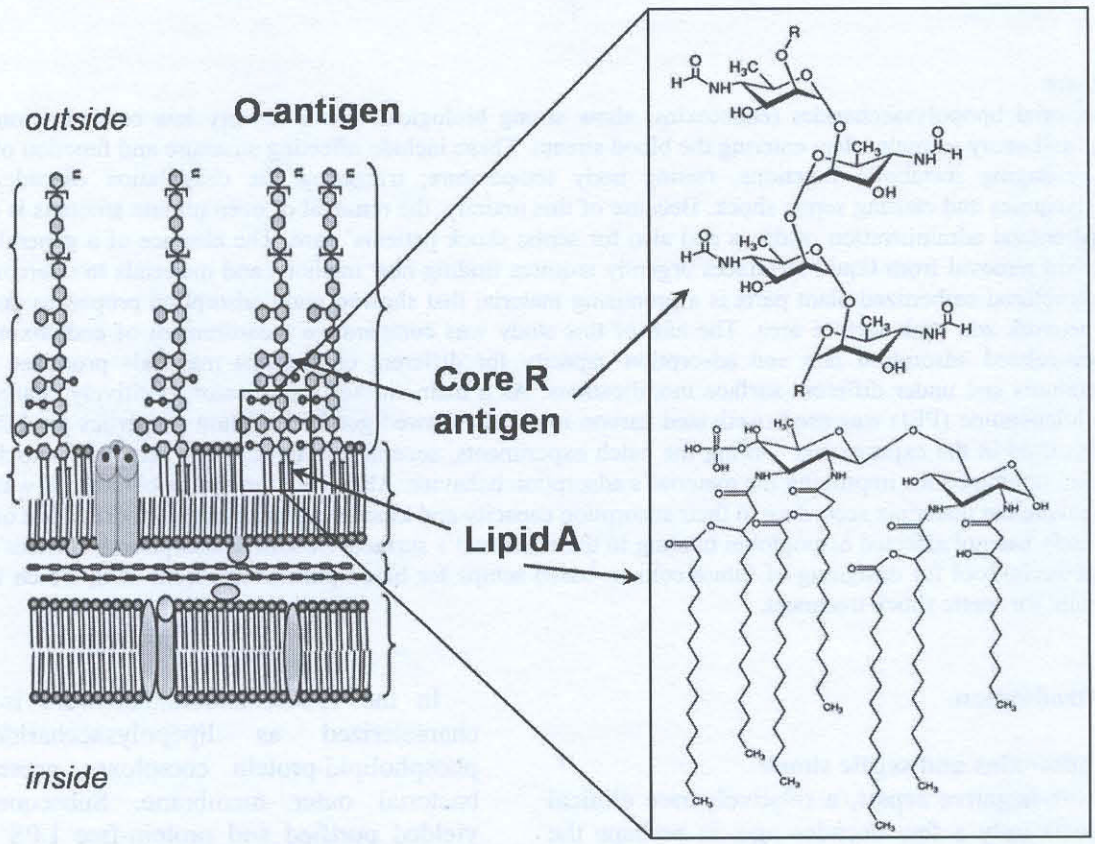

Fig. 1. LPS is largely made up of a long-chain polysaccharide (O antigen), the core, and lipid A. Each of these regions is immunogenic. The O antigen shows great diversity of structure among the various strains of gram-negative bacteria. Thus, it has a great number of epitopes and considerable potential for antigenic activity. On the other hand, lipid A is the most highly conserved subunit of the gram-negative LPS structure. Chemically, the toxic lipid A moiety has been characterized as an esterlinked glucosamine with both ester- and amide-linked pyrophosphates and fatty acids.

The O-polysaccharide is attached to the core polysaccharide and consists of repeating oligosaccharide subunits made up of 3-5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O-polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. O-side chains are easily recognized by the antibodies of the host; however the nature of the chain can easily be modified by gram-negative bacteria to avoid detection. The bacterial O-antigens are an impressive example of nature's versatility along a given chemical make-up. Some deficient strains lack the O-antigen, such as *E. coli K-12* [3, 6-7].

The physiological effects of endotoxin *in vivo* and the biochemical mechanisms underlying these effects have been extensively investigated. The administration of small doses of endotoxin to animals affects their hemodynamics, body temperature, blood clotting, cellular and humoral immunities, and other important physiologic parameters; large doses are lethal [8, 9]. In most species, the injection of LPS is associated with a rapid onset of fever, hypotension, and neutropenia. Humans are very sensitive to the pyrogenic activity of LPS and demonstrate fever at a small fraction of

the LPS dosage required to cause the febrile response in rabbits [10].

Although endotoxins are firmly anchored within the bacterial cell wall, they are continuously liberated into the surrounding medium. Endotoxin release clearly does not happen only with cell death but also during growth and division. Since bacteria can grow in nutrient poor media such aswater, saline and buffers, endotoxins are found almost everywhere. Endotoxins are very stable molecules, their biologically active part surviving extremes of temperature and pH in comparison to proteins. Routinely, temperatures of 180–250°C and acids or alkalis of at least 0.1 M must be chosen to destroy endotoxins in laboratory equipment [11, 12]. Unfortunately, many of the therapeutic methods proposed over the years for the treatment and management of sepsis and its complications have either failed to meet their initial expectations or remain unproved. Recently, however, the development of new approaches (immunotherapy, membrane-based filtration, etc.) is bringing sepsis therapy onto a new advanced level.

Specific elimination (absorption) of LPS from blood plasma represents one of the most promising methods for sepsis management. The resin-based adsorbents used for this purpose so far, still have many drawbacks, such as high costs, sophisticated manufacturing and low adsorption capacity. In this respect, activated charcoal and its derivatives possess many advantageous characteristics.

Nanostructured carbonized materials and their adsorption characteristics

The adsorption of a solute from a solution is usually determined by the porosity and the chemical nature of the adsorbent, the nature of the components of the solution, the concentration of the solution, its pH, and the mutual solubility of the components in the solution.

Activated carbons (AC) are known as excellent adsorbents. Their important applications are the adsorptive removal of color, odor, and taste, and other undesirable organic and inorganic pollutants from drinking water, in the treatment of industrial waste water; air purification in inhabited spaces, such as in restaurants, food processing, and chemical industries; for the purification of many chemical, food, and pharmaceutical products; in respirators for work under hostile environments; and in a variety of gas-phase applications. Their use in medicine and health applications to combat certain types of bacterial ailments and for the adsorptive removal of certain toxins and poisons, and for the purifications of blood, is being fast developed.

Carboneous adsorbents obtained from plant raw material are very versatile because of its extremely high surface area and micropore volume. The samples we used had been carbonized according to the procedure developed at the Laboratory of Hybrid Technologies in the Institute of Combustion Problems, Almaty, Kazakhstan. A flow set-up was used with following parameters: temperature range 250-800°C in argon flow (50-90 cm^3/min). Different temperatures would create a different pore system and therefore it would change the properties of the activated carbon [13].

Fig. 2 displays the obtained carbonized materials at different levels of magnification. Even low resolution images demonstarte characteristic surface patterning on the material's surface and its very high porosity.

Carbon surface has a unique character. It has a porous structure which determines its adsorption capacity, it has a chemical structure which influences its interaction with both polar and nonpolar molecules. Besides, it has active sites in the form of edges, dislocations and discontinuities which determine its chemical reactions with other atoms. Thus, the adsorption behavior of an activated carbon can't be interpreted on the basis of surface area and pore size distribution alone. Previous studies have proved that activated carbons having equal surface but different activation treatments show markedly different adsorption properties [14, 15].

The adsorption of a nonpolar solute will be higher on a nonpolar adsorbent. But since there is competition between the solute and the solvent, the solvent should be polar in nature for the solute to be adsorbed preferentially. The other factor that also determines the adsorption from solutions is the steric arrangement or the chemical structure of the adsorbate molecule. Lipopolysaccharides combine hydrophobic and charged groups in their structure. The core region close to lipid A and lipid A itself are partially phosphorylated; thus endotoxin molecules exhibit a net negative charge in common protein solutions. This peculiarity can be used as a starting point in optimization and improving efficacy and specificity of carbonized adsorbents. Two main strategies in surface modification can be suggested in order to stimulate LPS adsorption (Fig.3).

In our study, we focus on how different surface modifications, directed to increase surface hydrophobicity or positive charge, influence LPS and blood plasma protein adsorption. Surface modification by polyethyleneimine (PEI) is one of the most efficient methods of negative charge creation on a surface. Therefore, PEI was used, together with temperature treatment, as the main

surface modification agent in our study. In this paper, special attention will be given to protein-related studies since protein co-adsorption with LPS can change the composition of blood plasma. This, in turn has many consequences for the patients treated, both positive (free hemoglobin adsorption, which is advantageous for many hemolytic patients) and negative (immunoglobulin, lactoferrin, albumin adsorption). Material's behavior in respect to proteins will be, without any doubts, of great importance for future biomedical applications.

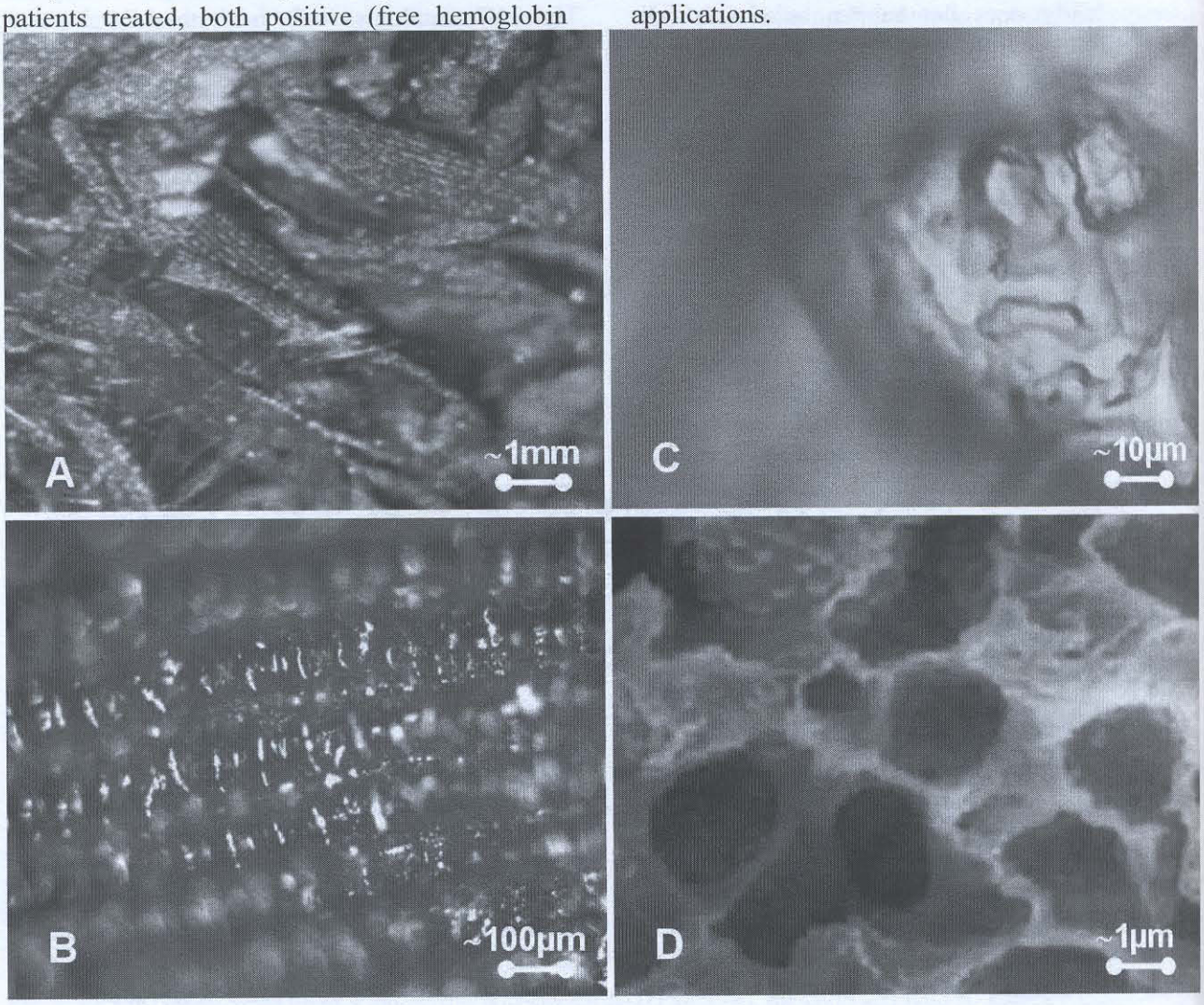

Fig. 2. Carbonized rice shell surface structure at different magnifications. Photos A and C represent light microscopy images; B was obtained by laser surface microscopy; D shows scanning electron microscopy photo. Scale bars give approximate dimensions of the structures. The topography of the sample is really diverse; we can distinguish pores and picks with different dimensions that are spread all over the surface.

Materials and Methods

Carbonized Materials and their modification

Rice shells (RS) and apricot stones (AK), activated at different temperatures served as main raw material for the adsorbent's preparation. The materials used in this study differ in surface properties because of the initial raw material used, because of temperature regime during their manufacturing and because of different concentrations of PEI used for surface modification. The materials used are listed in the Table 1.

Due to the high porosity and paramagnetic properties of the activated carbon, several steps are necessary before the carbonized materials can be used. First of all, the small-size fraction (dust) must be removed from the activated carbon sample as the measurement techniques used use light absorption, dust could interfere with the results.

After the material was weight, the samples were poured into Erlenmeyer flasks and 150 mL of distilled water was then pipetted carefully while taking the dust contents that were floating on the milieu. It is important to remove the highest amount of dust per attempt, although, on previous studies done in the laboratory, it was found that three iterations are necessary and enough to minimize the amount of high-dispersion carbon fraction (Fig. 4).

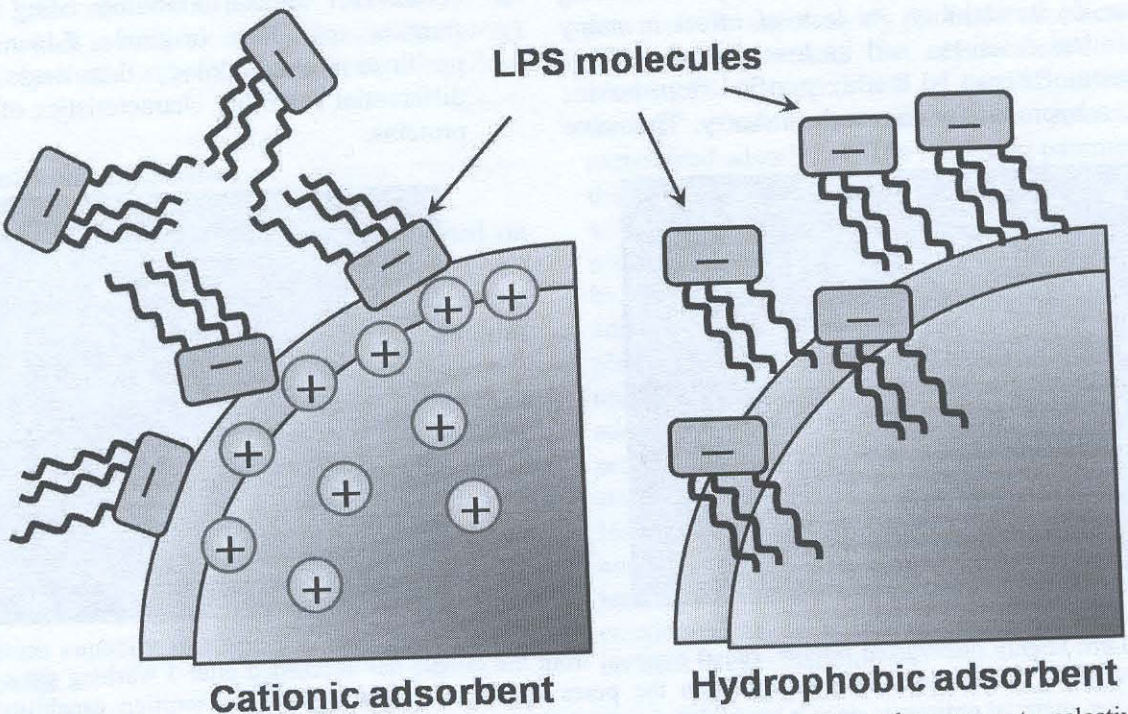

Fig. 3. Due to dualistic chemical nature of endotoxins, two approaches can be used to promote selective LPS adsorption. The first is based on administration of positively charged groups into the adsorbent's surface, which increases interaction with phosphate groups of LPS molecule. The other enhances the role of hydrophobic forces between the non-polar groups.

Table 1.
Characteristics of the materials used.

Material used	Carbonization temperature, °C	Modification agent and its concentration, (M)	Porosity, cm^3/g	Specific area, m^2/g	Hemoglobin adsorption rate, mg/min
RS-1	650	WV*	2.16	910	1,50
RS-2	650	absent	0.97	630	1,05
MRS-4	800	PEI (5.0×10^{-3})	0.97	630	1,59
MRS-5	800	PEI (2.5×10^{-3})	0.97	630	0,90
MRS-6	800	PEI (1.0×10^{-3})	0.97	630	0,77
MRS-7	800	PEI (5.0×10^{-4})	0.97	630	0,81
AK-8	800	WV*	1.84	815	1,01

WV - water vapour, PEI – polyethyleneimine

Beside of dust, air-liquid interface during the experiment must be avoided as air can occupy the sites of adsorption and therefore it must be eliminated so there won't be competition for the adsorption sites between air bubbles and the adsorbent under study. One way to do this is by autoclaving the material. The autoclaving process was done at 1 bar, 121°C for 1 hour.

Adsorbates

LPS. The lipopolysaccharide (LPS) used in this study was derived from *Escherichia coli* isolate 0111:B4. The powder samples purchased from Sigma Co. were resuspended (1μg/mL) in 30 ml of sterile 0.2 M phosphate-buffered saline (PBS) at pH 7.3 and stored at −80°C. The initial concentration used in adsorption experiments was 1.0 ng/mL.

Hemoglobin (Hb). Lyophilized Hb powder was received form Sigma Co. (Nr. H7379) and diluted with sterile 0.2 M phosphate-buffered saline (PBS) at pH 7.3 prior to experiments begin. The initial concentration used in adsorption experiments was 0.2 mg/mL.

Bovine serum albumin, bovine albumin, BSA fraction V, was purchased from Sigma Co. (Nr 85040C) as lyophilized powder and diluted to the working concentration (40 mg/mL) which corresponds to typical BSA content in human blood plasma. BSA has

numerous biochemical applications including because of its stability, its lack of effect in many biochemical reactions, and its low cost since large quantities of it can be readily purified from bovine blood, a byproduct of the cattle industry. The name "Fraction V" refers to albumin being the fifth fraction of the original Edwin Cohn purification methodology that made use of differential solubility characteristics of plasma proteins.

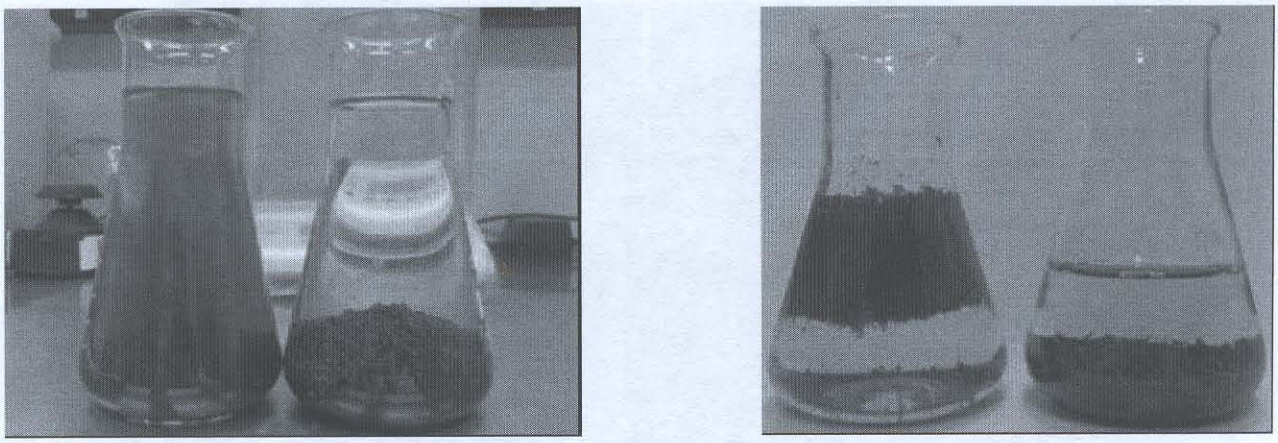

Fig. 4. Left: Highly dispergated fraction (dust) removal from the sample has succeeded after 3 washing steps. Right: Autoclaving of the material removes air from the pores and significantly enhances adsorption capability. After autoclaving, material sediments since it has higher density then water.

Adsorption experiments

Two different series of adsorption experiments have been performed to establish concentration- and time-dependent parameters of adsorption, correspondingly. Time-course batch experiments were performed to establish the dynamics of sorption for our material exposed to different adsorbates. In this set of experiments, 100 mL of adsorbate solution were added to 5 g of adsorbing material. The concentration of adsorbate was measured after certain periods of time. Afterwards, these data were used to calculate the amount of adsorbed material.

In the "concentration" experiments, we measured the amount of adsorbed material as a function of initial concentration. In this case, the adsorption was measured just once, 24 hours after the adsorbent and the adsorbate were brought in contact. The purpose of the second experimental series was to find out if it was possible to archive saturation of the material for the given concentrations (as it was stated before, those concentrations correspond to an average of those found in physiological conditions), this is important because later on, during column experiments or open systems, it's necessary to know this in order to find out the average time of use, concentrations allowed and number of uses before the material has to be changed or reactivated (the efficiency of the material depends on the free sites for adsorption, if the sites are already occupied then, its logical to expect a diminish in the adsorption process).

The experiment was carried out at room temperature using the platform shaker with a rate of 110 cycles/minutes. This was done to allow a uniform distribution of the adsorbate over the adsorbent. The rate of shaking was important as its proven later on, the rate of adsorption was vastly improved when agitation was present, it will be explained on detail once the LPS batch experiments are discussed.

Protein determination

Protein concentration was measured by the Lowry assay, as described elsewhere [15]. Before the samples were measured by the Lowry assay, the Eppendorf tubes were centrifuged using the Biofuge 15R at 8000 RPM for 1 minute, which allowed the dust content to be deposited in the bottom of the Eppendorf tube, by that it was possible to carefully take the samples out of the tube without dust content for further analysis.

Endotoxin detection: LAL test

The Limulus Amebocyte Lysate (LAL) test is a quantitative test for gram-negative endotoxin. The use of LAL for the detection of endotoxin evolved from the observation by Bang, that a gram-negative bacterial infection of *Limulus polyphemus*, the horseshoe crab, resulted in a fatal intra-vascular coagulation. Levin and Bang later demonstrated that this clotting was the result of a reaction between endotoxins and a clottable protein in the circulating amebocytes of the *Limulus*. We used a commercially available LAL-based kit QCL-1000® from Lonza Co. The method utilizes the initial part

of the LAL endotoxin reaction to activate an enzyme which in turn releases p-nitroaniline (pNA) from a synthetic substrate, producing a yellow color.

Results and Discussion

LPS Removal

The LPS adsorption experiments performed on unmodified rice shells (RS) and apricot stones (AK) showed very high efficiency of these native materials in LPS adsorption from buffer solutions (Fig.5). The time-course curve in the Figure 5 demonstrates that most of LPS was eliminated from the solution within the first 40 minutes. The adsorption rater on rice shells is relatively higher as compared to those of apricot stones. This is possibly governed by relatively higher specific area of the unmodified rice shells (see table above). The both studied materials showed also very high adsorption capacity in respect of pure solutions of endotoxins.

The obtained kinetic and adsorption capacity data are very encouraging for further application of carbonized materials in column-based setups, where LPS adsorption will be carried out in flow-through conditions, using large amounts of carbonized adsorbents. The particular parameters of the LPS-selective column (flow rate, length, etc.) will be calculated on the basis of time- and concentration-dependent kinetics for both LPS and blood plasma proteins. Concerning protein adsorption, a comparative study for different plasma proteins should be performed, in order to understand which proteins are predominantly adsorbed and how to manage and control this process. In this pioneering study, we focused our attention of hemoglobin and albumin adsorption. Hemoglobin is highly undesired protein in plasma and is typical for some organ failure and hemolytic states. Therefore, hemoglobin adsorption can be considered as an advantageous by-process during endotoxin elimination.

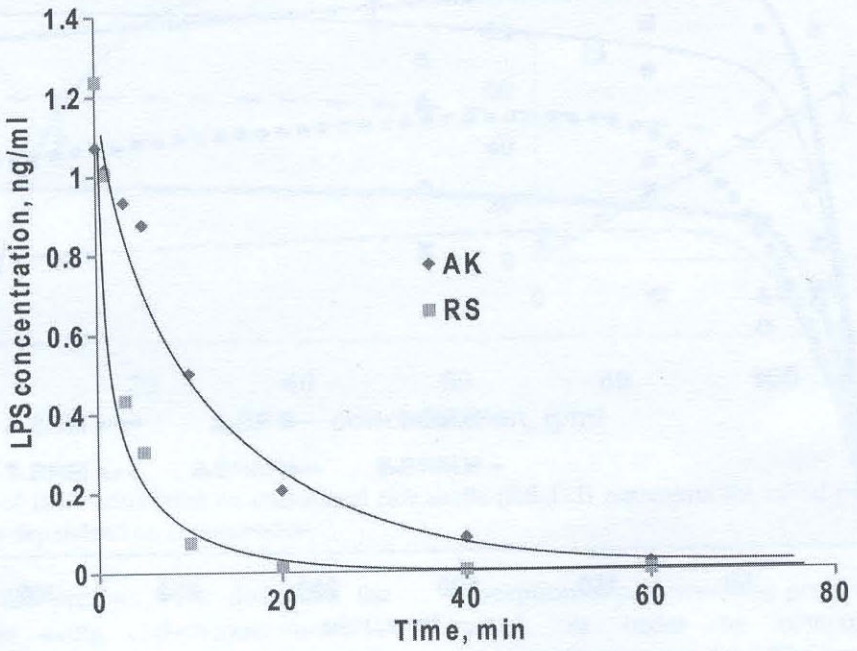

Fig. 5. Endotoxin adsorption from 1 ng/mL solution in PBS buffer as a function of time. Obviously, rice shells (RS) demonstrate faster adsorption kinetics as compared with apricot stones (AK).

Hemoglobin removal

In our studies, hemoglobin adsorption was studied as a time-dependent process by using different materials with various surface modifications (**Fig. 6**). As follows from the Figure, the hemoglobin adsorption occurred within first 50 minutes for all the materials used. After that, an adsorption equilibrium is achieved. The further shape of the curves, for the data points taken for later periods of time, implies rather desorption behavior.

The direct comparison the percentage of hemoglobin removal shown in the Figure 6, allows drawing conclusions about the adsorption characteristics of modified and native materials. Among the adsorbents used, the highest adsorption capacity accompanied by the highest adsorption rate was observed for unmodified rice shells (RS-1

group) and MRS-4 group modified by the highest concentration of PEI (5.0×10^{-3} M).

The collation of modified materials within MRS group suggests the direct relationship between general adsorption ability and the amount of PEI on the surface. In other words, only relatively high PEI concentration (5.0×10^{-3} M) on the surface stimulates its interaction with LPS, whereas using all other concentrations rather interferes with LPS adsorption. We can speculate that in case of lower PEI concentrations, the attractive role of hydrophobic interactions is weakened, but electrostatic interactions are yet not strong enough and only use of highest PEI concentrations allows electrostatic forces compensate the lack of hydrophobic attraction.

Together with surface optimization, the studies on adsorption capacity and concentration dependence are of critical importance for future column-based applications. Batch tests can be used to measure the constant of adsorption equilibrium, **K**. The determination of adsorption constants is very important value because it can be used for calculation of many thermodynamic characteristics of the adsorption processes'and plays significant role in optimal column design. Thus, the following series of experiments was devoted to finding out how the initial concentration of the adsorbate influences the amount of adsorbed material. Since the measurements are performed at fixed temperature (25°C) and for a fixed time interval, the obtained curve represents an isotherm.

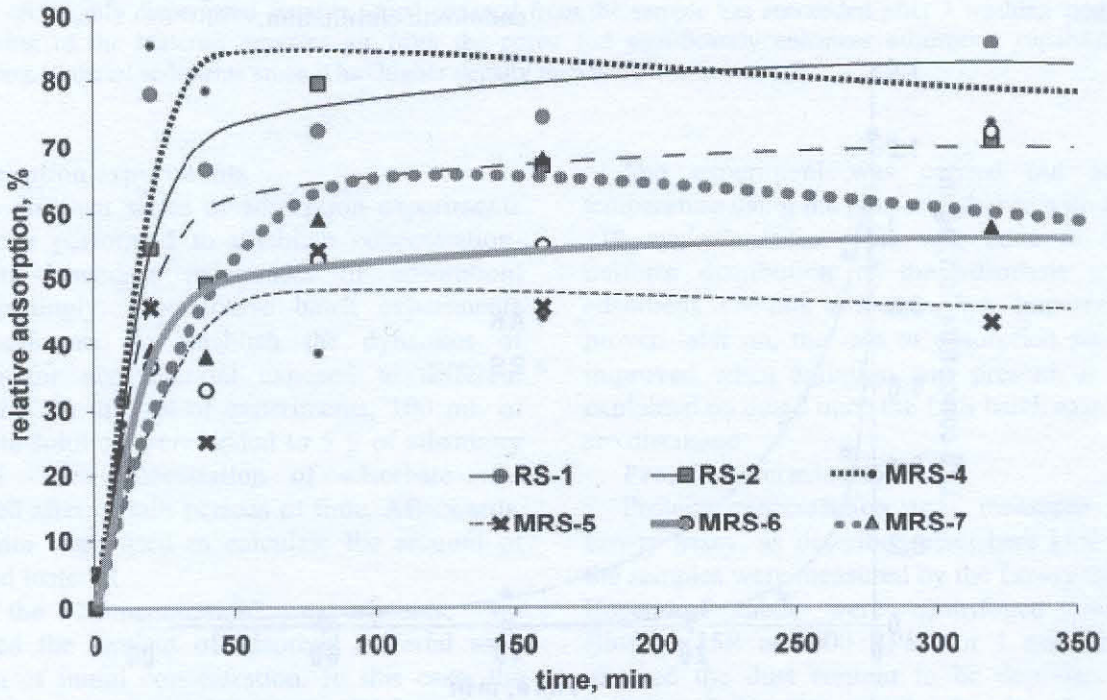

Fig. 6. Kinetics of hemoglobin adsorprion on different carbonized materials. For legends, refer the Table 1.

BSA Adsorption

Albumin is the most abundant protein in plasma and will be expectedly adsorbed in large amounts. This fact, in turn, means the possibility of competition between BSA and LPS for active adsorption sites and can represent a serious problem in the future. Therefore, the characterization of BSA adsorption both in concentration- and time-related terms is necessary for future interpretation and management of LPS elimination from complex protein mixtures.

Figure 7 shows how the amount of adsorbed BSA is affected by the initial concentration of BSA in solution. As one can expect, the initial part of the curve is linear, meaning the direct correlation between the amount of adsorbed material and the concentration of the compound in the solution. Interestingly the linearity persists till very high BSA concentration, which supports our previous data on very high adsorption capacity of the materials used. At even higher concentrations, in the interval 20-40 g/mL, the number of active sites on the surface is not enough to accommodate the protein molecules. This results in a plateau from the concentration 40mg/mL on.

The shape of the obtained curve completely corresponds to the Langmuir model of monolayer adsorption. The Langmuir theory is based on a

kinetic principle, that is the rate of adsorption (which is the striking rate at the surface multiplied by a sticking coefficient, sometimes called the accommodation coefficient) is equal to the rate of desorption from the surface [17, 18].

The rate of attachment to the surface should be proportional to a driving force times an area. The driving force is the concentration in the fluid, and the area is the amount of bare surface. The kinetic equations for the constants of adsorption K_a and desorption K_d can be transformed to the Langmuir isotherm:

$$\frac{\Gamma}{\Gamma_{max}} = \frac{K_L * C_e}{(1 + K_L * C_e)}$$

where Γ = Adsorbed amount of surfactant [mg/g]; Γmax = Maximum adsorbed amount of surfactant [mg/g]; C_e = Concentration of absorbate in the solution (mg/mL), q = Γ / Γmax = Fraction filled by the adsorbing solute and K_L = K_a/ K_d = Equilibrium constant for Langmuir Isotherm (how strong the pollutant is adsorbed to the material).

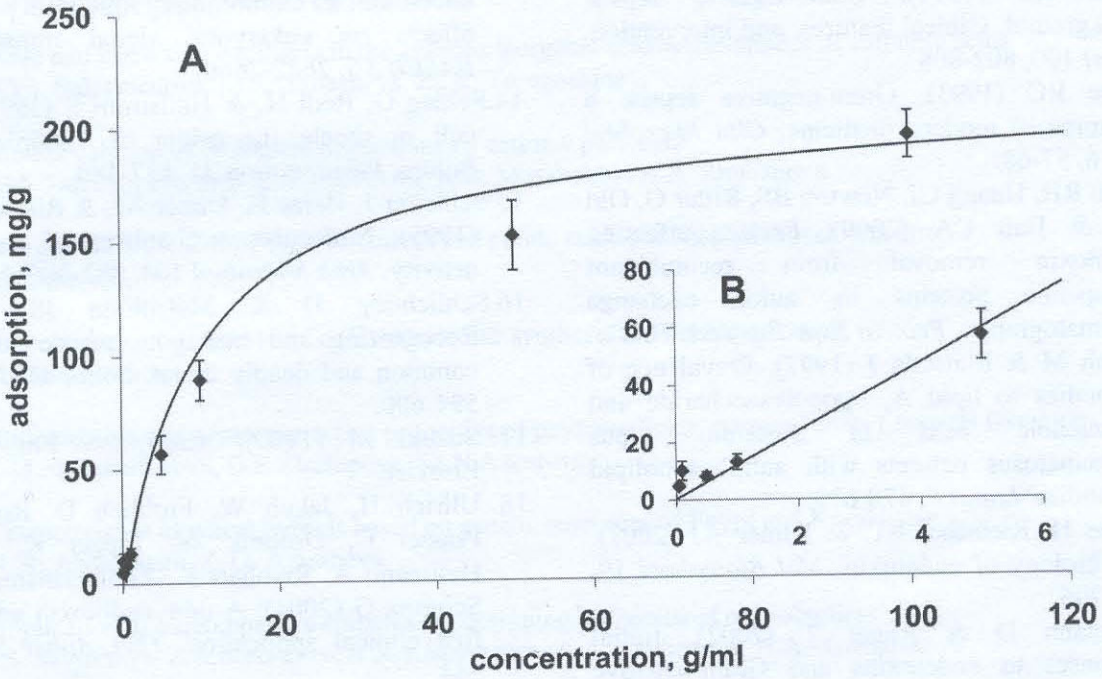

Fig. 7. A. Isotherm of BSA adsorprion on carbonized rice shells (RS-1).B represents the initial part of the curve, where the adsorption is linearly dependend on concentration.

Summarizing, the present work described the adsorption process using carboneous materials samples for the removal of endotoxins and protein adsorption from aqueous solutions. During the batch experiment setup, several techniques were used and improved for the handling of the material during the experiments; from it those the results obtained had an improvement from previous experimental work. The dust contents were significantly reduced and autoclaving allowed the elimination of the air interface in the carboneous material before interaction with the endotoxins and proteins.

From the data obtained after the different experiments were done it was possible to analyze the characteristics of each type of carboneus material and when where they had better adsorption capabilities with respect to the other. A design for adsorption experiment was presented using a batch model, in order to compare kinetic and concentration parameters. The amount of endotoxin removed from the solution in comparison to the amount of proteins gives is encouraging further studies and suggests possible ways to improve the performance of LPS-elimination setups. Together with batch experiments, it is important to proceed into direction of column-based flow-through setups, as very promising from the point of view of efficiency.

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