Synthesis, Morphostructure, Surface Chemistry and Preclinical Studies of Nanoporous Rice Husk-Derived Biochars for Gastrointestinal Detoxification

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Article info

Abstract

This article summarizes the methodology of synthesis, surface functionalization and structural properties of rice husk-derived nanostructured carbon enterosorbents (biochars) in connection with the preliminary *in vitro* study results of uraemic toxin adsorption in model experiments, as well as preclinical trials *in vivo*. The obtained nanostructured carbon sorbents were studied using a number of modern physicochemical methods of investigation: low-temperature nitrogen adsorption, isotherms recording and calculation of the specific surface area, pore volumes were carried out using the Autosorb-1 "Quantachrome" device. Scanning electron microscopy and EDS-analysis. Mercury intrusion porosimetry analysis of the ACs were accomplished using "Quantachrome Poremaster" data analysis software. *In vitro* adsorption results assessed by use of HPLC and UV-spectroscopy for the nanostructured carbon sorbents with respect to the investigated low-molecule toxins suggest that the rice husks-derived carbon enterosorbents modified with the functional groups are able to reduce clinically significant levels of uraemic toxins and are comparable to the commercial enterosorbents. Based on the results of the comparative analysis for biocompatibility of canine kidney epithelial cells it was determined that the samples of the modified sorbents CRH-P-450 and CRH-475-KOH-850-N do not exhibit cytotoxicity in comparison with the commercial carbon enterosorbent «Adsorbix Extra». According to the results of the *in vivo* studies, it was determined that there was a positive effect of the enterosorbent CRH-P-450 on uremia and intoxication.

1. Introduction

Rice husk (RH), a by-product of the rice milling industry, is a renewable waste with an annual world rice production over 600 mln tons [1]. Even though RH is used as a fuel in the rice producing countries, it is characterized by low caloric value and high mineral content [1, 2]. On the other hand, RH as a lignocellulosic biomass is a valuable carbonaceous precursor that can be used to obtain a carbon material with special textural properties, high specific surface area and large pore volume [3, 4]. At the same time, Chronic Kidney disease (CKD) is an increasingly prevalent and disproportionately costly condition, which also increases the risk for many other associated health conditions, including cardiovascular disease (CVD) and cancer. According to the World Health Organization, CKD and other non-communicable diseases decrease the potential annual growth rate in gross domestic product by 1–5% in developing countries experiencing rapid economic growth [5]. CKD is characterized by the accumulation of azotaemic/uraemic toxins in the plasma of patients, which include protein-bound p-cresyl sulphate (PCS) and indoxyl sulphate (IS) produced from indoles and phenols (p-cresol) by intestinal flora (via bacterial sulfotransferase enzyme sulfation pathway), followed by absorption into the

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blood and successive, yet reversible conjugation with plasma proteins to result in stable, large in size protein-bound complex formation. These processes eventually severely impede renal function/clearance, e.g.: elimination of any substance by kidney because of clogging of glomerular filtration system.

Other than that, the uraemic toxin size may vary from large inflammatory molecules such as TNF-α and IL-18 to small water-soluble molecules such as asymmetric dimethylarginine (ADMA), creatinine, uric acid and urea [6, 7].

Development and validation of novel activated carbon (AC) materials with controlled porosity for use as oral adsorbents is a topical issue, since a number of current carbons for biomedical use suffer from an unsatisfactory pore hierarchy (ratio of micro/meso/macropores volumes), which limits their ability to effectively remove uraemic toxins prominent in CKD therapy. They also have low content of surface functional groups and cannot provide both sufficient hydrophilicity and high ion-exchange capacity essential for effective removal of toxins varying in both molecular size and amphiphilicity. At the same time, with an increasing number of patients suffering from CKD worldwide, there is an increasing need for the development of a therapy using oral adsorbents, which are capable of efficiently removing azotaemic toxins. Our hypothesis is that it may be possible to limit the systemic absorption of azotaemic toxins from the gut of CKD patients by utilizing the powerful adsorptive capacity of nanostructured carbon sorbents (NCS) when administered as an oral therapy.

To this end, Kazakhstan, as well as other ODA countries has a large amount of rice husk (RH) as a waste material, which currently has restricted commercially viable use. In terms of other potential advantages, it is a renewable bioavailable material with a relatively less amount of toxic substances during its processing to high added value products, e.g.: carbons, which is in contrast with synthetic polymeric-based precursors processing.

The team at the Institute of Combustion Problems has developed technology for carbonisation and activation of carbon materials produced from rice husk [8, 9]. RH as a precursor is a unique material in terms of availability, large-scale world production (over 120 mln tons annually), it also comprises the nanoscale silica phytoliths, which serve as a template, which (upon a simple leaching) leads to creation of additional meso/macropore space within the nanoscale range. It is these nanopores that are able to optimise the adsorption

of middle molecular size azotaemic toxins. In this work the nanostructured carbon sorbents (NCS) were prepared from RH via steam-gas/chemical activation followed by surface functionalization by means of oxidation using ozonated air followed by amination with the use of ammonia; physicochemical properties and structural parameters of the obtained enterosorbents are also described in details.

The ultimate aim of this study is to develop novel carbon enterosorbents of controlled nanoporous structure and tailored surface chemistry in order to be able to validate the manufacturing technology of nanostructured carbon enterosorbents, which are capable of effectively removing azotaemic toxins in preclinical trials, to potentially serve in a therapy for Chronic kidney disease.

2. Experimental

2.1. Rice husk-based activated carbons preparation techniques

2.1.1. Carbonization of rice husk

Carbonization of rice husk was carried out at 475 °C in a rotary spherical steel reactor equipped with a capillary for water supply and thermocouple in an electric furnace setup. Working temperature ranges were programmed with a thermal controller: the temperature ramp rate to the required was maintained at 11 °C/min; time of carbonization process at the maximum temperature was set to 30 min. The water feed rate was set at 100 ml/h with a peristaltic pump. The yields of the obtained carbonized rice husk (CRH-475) was *ca*. 40%.

2.1.2. Activation of carbonized rice husk with potassium hydroxide

CRH-475 sample was mixed with potassium hydroxide pellets (KOH ≥85.0 wt%) at (wt/wt) CRH-475-KOH impregnation ratio of *ca*. 1:4 in a cylindrical steel reactor placed inside a vertical electric furnace and activated at 850 °C for 2 h in inert atmosphere. After the activation step the sample was thoroughly washed with hot distilled water until pH 7–8 and dried to constant weight; the yield of resulting CRH-475-KOH-850 sample is *ca*. 18%.

2.1.3. Oxidative ammonolysis of activated carbon

At the first, the ozonation of CRH-475-KOH-850 sample was carried out in a cylindrical quartz

reactor at 130 °C for 12 h. Ozonizer «TR-YCA» (TIENS Ltd, China) was used to generate ozone. To obtain gaseous ammonia and control its feed flow rate into the reactor (5–6 ml/min), immediately after ozonation 25% ammonia aqueous solution was added drop wise from the addition funnel to a Wurtz flask with solid alkali, the temperature was elevated to 350 °C and maintained for 6 h to produce CRH-475-KOH-850-N sample.

2.1.4. Activation of rice husk with phosphoric acid

A cylindrical quarts reactor of mixture of rice husk with 85% phosphoric acid ($\rho \approx 1.68$ g/cm³) at (wt/wt) RH:H_3PO_4 impregnation ratio of 1:2 was loosely covered with stopper and placed into an oven and heated at 200 °C overnight. Then the reactor was placed into a vertical cylindrical furnace equipped with chromel/alumel thermocouple and heated at the ramp rate of 5 °C /min to a temperature within range of 450 ± 100 °C and the temperature was maintained for 1h. Activation was conducted in self-generated atmosphere. Upon cooling, the carbonized material was washed a few times with hot distiller water to remove most of residual phosphoric acid. The residue was transferred into a thermo-resistant glass beaker, filled with 1M NaOH solution and boiled for 30 min to leach out silica template, then thoroughly washed with hot distilled water to remove potassium silicate until pH 7–8 (through boiling-sedimentation-decantation) and dried to constant weight. The yield of resulting CRH-P-450 sample is *ca*. 30%.

2.2. Low-temperature nitrogen adsorption studies

For low-temperature nitrogen adsorption analysis (LTNA), the ACs were degassed for 3 h at 200 °C before the analysis was carried out. The carbon adsorbents were analysed and nitrogen adsorption isotherms determined with an Autosorb-1 analyser (Quantachrome, UK) in the range of relative pressures from 0.005 to 0.991. The data was analysed using "Quantachrome" data analysis software. The specific surface area was calculated using the BET method; the pore size distributions, surface area and pore volumes of the materials were calculated using the DFT slit/cylindrical pore model recommended for activated carbon [10].

2.3. Mercury intrusion porosimetry analysis

For mercury intrusion porosimetry analysis, the

ACs were degassed overnight in a vacuum oven at 150 °C before the analysis was carried out. The carbon adsorbents were analysed and mercury intrusion/extrusion curves, "isotherms", determined with a Poremaster instrument (Quantachrome, UK). The data was analysed using "Quantachrome Poremaster" data analysis software to calculate the mesopore volume (<50 nm), as well as meso/macropore volume (<100 nm) of the materials.

2.4. Scanning electron microscopy and EDS-analysis

The morphology of activated carbons was studied by scanning electron microscopy (SEM) using QUANTA 3D 200i microscope (FEI, USA) with accelerating voltage of 30 kV. Energy-dispersive X-ray spectroscopy (EDS-analysis) was used for the elemental analysis or chemical characterization of the materials [9].

2.5. Assessment of adsorption of uraemic toxins using activated carbons

Each AC sample was weighed as 20 mg in triplicate for each incubation time (9 replicates) into Eppendorf tubes and pre-equilibrated with 4 mL phosphate buffered saline (PBS) in a shaking incubator at 37 °C for 4 h. Carbons were then centrifuged at 8000 rpm for 3 min and the supernatant removed. Freshly made PBS solution, supplemented with para-cresyl sulfate (250 μM), indoxyl sulfate (125 μM) and urea (50 mM), was incubated in a shaking incubator at 37 °C for 1 h. After the incubation 3.75 mL of spiked PBS solution (to simulate the values of the pH and ionic strength of gastrointestinal fluid media) was added to each carbon and controls and incubated in a shaking incubator at 37 °C for 5, 15, 30 and 60 min time points. At each time point the carbons were then centrifuged at 8000 rpm for 3 min and the supernatant was removed (n = 3 for each time point) into Eppendorf tubes and stored at -80 °C before analysis of the remaining concentrations [11].

2.6. Techniques for measurement of uraemic toxins remaining concentrations

2.6.1. High-performance liquid chromatography technique for uraemic toxins

High-performance liquid chromatography (HPLC) of indoxyl and para-cresyl sulfate samples

was performed using HPLC 1290 Infinity instrument (Agilent Technologies). First, the maximum absorption peaks were determined using Evolution 201 spectrophotometer (ThermoScientific). Next, the HPLC device was calibrated and set twice to the UV-maximum absorption peaks of PSC and IS, respectively. Two solutions (Eluents A and B) were prepared to wash the HPLC column ZORBAX Eclipse Plus C18 (2.1 × 100 mm/1.8 μm). The eluent A is 0.2% trifluoroacetic acid solution in distilled water; eluent B is 0.2% trifluoroacetic acid solution in acetonitrile.

2.6.2. UV-spectroscopy technique for determination of urea concentration

For the photocalorimetric determination of the remaining urea concentrations in solutions, Evolution 201 spectrophotometer (ThermoScientific) was used. First, an absorption maximum peak was assessed at 203.29 nm, at which the measurements of the absorption intensity (absorbance) were later determined. Then, the model aqueous solutions of urea ("Sigma" grade) in PBS with concentrations of 6.25, 12.5, 25, 50 and 100 mmol/L were prepared. Finally, a calibration curve of "concentration vs absorbance" was built in order to assess the remaining urea concentrations.

2.7. Technique for biocompatibility studies of carbon enterosorbents

2.7.1. Cultivation of canine kidney epithelial cells

The MDCK (Madin Darby canine kidney) cell line was purchased from the Russian collection of cell cultures. The cells were cultured in a DMEM (Dulbecco's Modified Eagle's medium) – a culture medium consisting of 10% fetal calf serum (FCS), 2 mM glutamax, 100 U/ml of penicillin and 100 μg/ml of streptomycin, which was conditioned at 37 °C and 5% CO_2 (all reagents were obtained from Life Technologies, USA). The medium for the cell culture was changed once in two days. The MDCK cells inhibition was rendered using a mixture of 0.25% of trypsin and 0.02% of EDTA (Sigma, USA) solutions at the intervals of 5 days. The number and viability of the cells were assessed using a Newbrow camera after staining with a 0.4% trypan blue solution. Monitoring of the growth and morphology of epithelial cells of the MDCK line was performed using an inverted Axio Observer A1 microscope (Carl Zeiss, Germany).

2.7.2. Cytotoxicity test

To assess the activated carbons toxicity the MDCK cell line was used. Cells were allocated onto 96-well flat-bottomed plates (Corning Costar, USA) at the amount of *ca.* 5×10^4 cells per well and incubated for 16 h in a CO_2 incubator at 37 °C and 5% of CO_2 concentration. The resulting medium was then removed from the wells using an aspirator and replaced with a complete DMEM nutrient medium containing the following concentrations of activated carbon: 400, 200, 100, 50, 25, 12.5 and 6.25 μg/ml. A complete DMEM nutrient medium containing 10% of FCS without activated charcoal was added to the control wells. A commercial carbon enterosorbent «Adsorbix Extra» – an activated carbon ("Norit", Netherlands) was also used as a matter of comparison at the aforementioned concentrations. After the addition/introduction of the activated carbon solutes using a set of various concentrations to the cells, incubation for 72 h was carried out in a CO_2 incubator at 37 °C at 5% CO_2 concentration level. The cell survival rate was determined using a staining technique by way of the addition of 10 μL of "Alamar Blue" dye (Invitrogen, USA) and exposition for 4 h. The absorbance was measured at a wavelength of 570 nm using a microplate spectrophotometer (Biorad 670, France). The cytotoxicity value was assessed using following Eq. 1:

$$Cytotoxicity = \frac{I_1}{I_0} \times 100\% \qquad (1)$$

where I_1 is optical density/absorbance in experimental wells and I_0 is the optical density of control wells.

2.8. Testing the efficiency of carbon enterosorbents in vivo in an animal model of Acute and Chronic kidney disease

General characteristics of experimental animals: random-bred laboratory 3-month-old rats of both sexes were selected for the study, veterinary control was carried out. The animals were divided into 3 main groups: 1st group – control, the animals of which were not subjected to any influences; 2nd group – experimental model of Acute kidney disease (AKD); 3rd group – rats, in whom AKD was experimentally caused during simultaneous intragastric administration of enterosorbent CRH-P-450. The research on the carbon enterosorbents effectiveness on the animal model of CKD

Table 1
Biochemical parameters of experimental animals blood serum at the 1st 2nd and 3rd stage of the studies

Biochemical parameters of experimental animals blood serum at the 1st stage of the study			
Value name	1st group (intact),	2nd group (model AKD), n	3rd group (AKD + enterosorbent), n
Total protein, g/l	70.1 ± 4.53	67.6 ± 5.5	69.8 ± 4.56
Urea, mmol/l	7.95 ± 0.45	113.56 ± 13.9*	106.38 ± 12.16
Creatinine, mmol/l	63.39 ± 5.38	899.67 ± 67.84*	842.0 ± 33.29
MMM, IU	29.0 ± 1.3	61.4 ± 2.69*	50.5 ± 2.01**
Biochemical parameters of experimental animals blood serum at the 2nd stage of the study			
Value name	1st group (intact), n	2nd group (model AKD), n	3rd group (AKD + enterosorbent), n
Total protein, g/l	70.1 ± 4.53	60.84 ± 4.95	66.31 ± 4.33
Urea, mmol/l	7.95 ± 0.45	90.85 ± 10.55*	85.1 ± 9.73
Creatinine, mmol/l	63.39 ± 5.38	539.8 ± 28.1*	505.2 ± 19.97
MMM, IU	29.0 ± 1.3	49.12 ± 2.15*	40.4 ± 1.61**
Biochemical parameters of experimental animals blood serum at the 3rd stage of the study			
Value name	1st group (intact), n	2nd group (model AKD), n	3rd group (AKD + enterosorbent), n
Total protein, g/l	70.1 ± 4.53	67.60 ± 5.5	72.94 ± 4.76
Urea, mmol/l	7.95 ± 0.45	56.78 ± 6.59*	25.53 ± 2.92**
Creatinine, mmol/l	63.39 ± 5.38	359.87 ± 18.73*	168.40 ± 6.66**
MMM, IU	29.0 ± 1.3	39.30 ± 1.72*	25.25 ± 1.01**
Note: * – P≤0.001 in relation to intact; ** – P≤0.001 in relation to AKD; MMM – medium-mass molecules			

is divided into three stages, including: 1st stage – study of biomedical measurements of intoxication and kidney function on day 3; 2nd stage – study of biomedical measurements of intoxication and kidney function on day 14; 3rd stage 3 – study of biomedical measurements of intoxication and kidney function on day 21.

At the 1st stage of the study, the AKD was simulated, for which the rats were deprived from food for 24 h, then a 50% aqueous solution of glycerol was injected intramuscularly at a dose of 10 ml/kg of the animal's body weight [12]. In the 3rd group of animals enterosorbent was administered prior to AKD simulation.
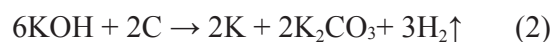
In the course of the experiment, the 3rd group of animals was injected intragastrically daily with an enterosorbent at a dosage of 650 mg/kg of body weight. Biochemical parameters of experimental animals blood serum at the 1st 2nd and 3rd stage of the study results are summarized in Table 1.

3. Results and discussion

3.1. Synthesis of functional carbon rice husk-based enterosorbents from via carbonization and/or acid-base activation and oxidative ammonolysis

According to thermodynamic calculations [13],

it is shown that the prevailing process of carbothermic potassium reduction is the reaction, expressed by the following summary Eq. (2):

$$6KOH + 2C \rightarrow 2K + 2K_2CO_3 + 3H_2\uparrow \qquad (2)$$

Nevertheless, the resulting potassium carbonate is capable of leaching silica by reaction:

$$2K_2CO_3 + SiO_2 \rightarrow K_2SiO_3 + CO_2 \qquad (3)$$

The sample CRH-475-KOH-850, obtained by carbonization of RH at 475 °C and subsequent KOH activation at 850 °C, possesses the high values of the specific surface area (up to 3020 m^2/g).

In our previous works, we have shown that the acceptable conditions for the activation of the RH with H$_3$PO$_4$ to produce NCS with a high specific surface area are: the RH/H$_3$PO$_4$ (wt/wt) impregnation ratio is 1:2, the activation time is 1 h and the temperature range – 300–600 °C. Also, to maximize yield of NCS, leaching of the silica matrix should be carried out after H$_3$PO$_4$ activation [14]. In this work, to determine the optimal activation temperature, a series of samples was obtained within the temperature range of 350–550 °C. Upon silicon dioxide removal (desilication, see the Eq. 3), the yield of the carbon material CRH-P-450 was 29.9%.
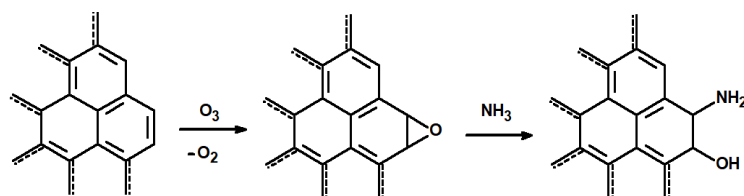
Fig. 1. Reaction scheme of oxidative ammonolysis of carbon material surface.

At the first stage, for the formation of oxygen-containing species, including epoxy groups, the developed and highly reactive surface of KOH-activated CRH sample was preliminarily treated with ozone-air mixture. A possible scheme of the chemical reactions of ammoxidation of NCS, proceeding through the 2nd stage, e.g.: ring opening of the epoxy compounds formed during the reaction of ozone with the unsaturated carbon double bond, is shown in Fig. 1.

In this case, a strong covalent bond of carbon with nitrogen is formed, i.e.: the carbon sorbent surface is functionalized with hydrophilic anion-exchange amino groups. On the other hand, in the ozonation process, carbonyl (aldehyde, ketone), as well as carboxylic (-COOH) cation-exchange groups are formed on the carbon surface, the latter also increase the hydrophilicity of the resulting carbon sorbents.

In the course of these reaction the surface of the obtained carbon materials samples was functionalized with nitrogen-containing groups via oxidative ammonolysis.

It was found that as a result of oxidative ammonolysis of CRH-475-KOH-850 sample, the specific surface area was reduced by 30%, while the specific surface area of the modified enterosorbents varied within range of 1580–2690 m²/g, while the sorption capacity (q_{max}) for the marker molecule methylene blue (MB) increased from 1350 to 1570 mg/g.

3.2. Structural morphology of carbon rice husk-based enterosorbents

Information on the micro-mesoporous texture of carbon sorbent samples was obtained by low-temperature nitrogen adsorption (LTNA) on an «Autosorb-1» instrument (Quantachrome, USA). The adsorption/desorption isotherms recorded at -196 °C are shown in Fig. 2.

Nitrogen adsorption data for phosphoric acid activated RH and potassium hydroxide activated CRH clearly anticipate the presence of a highly developed porous structure with micropore filling at low relative pressures and the presence of a highly developed mesoporous structure (2–50 nm). The
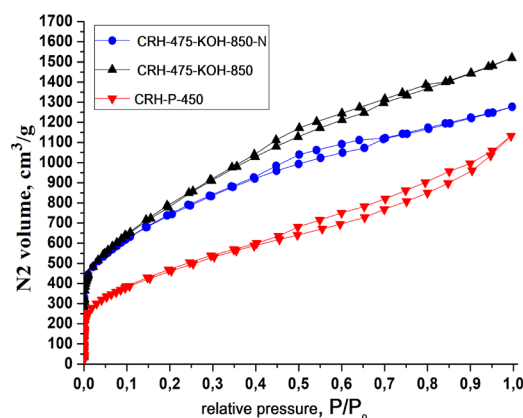

Fig. 2. Nitrogen adsorption/desorption isotherms for adsorbent samples.

presence of mesoporosity in these samples is also reflected by the appearance of a hysteresis loop formed by the adsorption and desorption branches of the isotherms.

The textural characteristics of samples obtained from RH by H_3PO_4 activation and subsequent desilication are presented in Table 2, while the characteristics of samples activated with KOH CRH-475-KOH-850 and CRH-475-KOH-850-N are shown in Table 2: S_{BET} − specific surface area calculated using Brunauer-Emmett-Teller theory; S_{DFT}, V_{DFT} and D_{DFT} are the specific surface area, the total pore volume and the average pore diameter calculated by Quenched Solid State Functional Theory, respectively; V_{BJH} is the mesopore volume calculated by the Barrett-Joyner-Halenda model; V_{DR} is the volume of micropores calculated using Dubinin-Radushkevich's method, and also V_{MIP} − volumes of mesopores calculated from mercury intrusion porosimetry data in the ranges of 6–50 nm and 6–100 nm.

From the data in Table 2, it can be seen that a correlation is observed between the activation temperature of the RH with H_3PO_4, followed by the leaching of silica, and the textural characteristics of the resulting materials. The values of S_{DFT} are somewhat lower, but on average correspond to S_{BET} values. As expected, the specific surface area and pore volumes of the samples obtained at tem-

Table 2

Textural characteristics of the samples obtained by activation of RH with phosphoric acid and subsequent desilication

Sample code	S_{BET}, m²/g	S_{DFT}, m²/g	V_{DFT}, cm³/g	V_{BJH}, cm³/g	V_{DR}, cm³/g	V_{MIP} <50 nm, cm³/g	V_{MIP} <100 nm, m³/g	D_{DFT}, nm
CRH-P-350	1280	1125	1.24	0.95	0.38	0.60	0.81	1.1
CRH-P-450	1700	1400	1.56	1.15	0.48	0.68	0.84	1.1
CRH-P-550	1320	1130	1.26	0.95	0.35	0.56	0.66	1.2

Table 3

Textural characteristics of the samples CRH-475-KOH-850 and CRH-475-KOH-850-N

Sample code	S_{BET}, m²/g	S_{DFT}, m²/g	V_{DFT}, cm³/g	V_{BJH}, cm³/g	V_{DR}, cm³/g	V_{MIP} <50 nm, cm³/g	V_{MIP} <100 nm, cm³/g	D_{DFT}, nm
CRH-475-KOH-850	2938	2356	2.17	1.15	1.07	0.77	0.88	0.93
CRH-475-KOH-850-N	2695	2330	1.83	0.85	1.01	0.71	0.82	0.89

peratures of 350 and 550 °C are lower than for the CRH-P-450 sample, since the carbon matrix first undergoes expansion, starting from an activation temperature above 400 °C, and then at temperatures above 450 °C, its contraction occurs [15]. Thus, the CRH-P-450 sample has the "optimal" textural characteristics. The values of the specific surface area calculated by BET and QSDFT theories were 1700 and 1400 m²/g, the values of mesopore volumes calculated using mercury intrusion porosimetry data and low-temperature nitrogen adsorption data using BJH-method were 0.68 and 1.15 cm³/g, and the micropore volume was 0.48 cm³/g, respectively.

From the data given in Table 3 it follows that as a result of oxidative ammonolysis there is an insignificant decrease in the specific surface area, pore volumes and average pore diameter. According to calculations of low-temperature nitrogen adsorption data using BET and QSDFT methods, it was determined that the specific surface areas and QSDFT total pore volume of the samples were: CRH-475-KOH-850 – 2938 m²/g (BET); 2356 m²/g (DFT) and 2.17 cm³/g; CRH-475-KOH-850-N – 2695 m²/g (BET); 2330 m²/g (DFT) and 1.83 cm³/g, respectively. The values of mesopore volumes calculated using mercury porosimetry data and low-temperature nitrogen adsorption data using BJH-calculation method were as follows: for CRH-475-KOH-850 – 0.77 cm³/g and 1.15 cm³/g; for CRH-475-KOH-850-N – 0.71 cm³/g and 0.85 cm³/g, respectively, and the volume of micropores is *ca.* 1 cm³/g, i.e.: about half of the total pore volume of the resulting micro-mesoporous carbon adsorbents.

The studies on structure and morphology, e.g.: the porosity and texture of the resulting enterosorbents were assessed using scanning electron microscopy (SEM), together with energy-dispersive X-ray microanalysis (EDX-analysis) using the «QUANTA 3D 200i» electron microscope (FEI, USA). Figures 3 and 4 show SEM-images and EDS-spectrograms of CRH-P-450 and CRH-475- KOH-850-N samples, according to the SEM/EDX analysis of their surface.
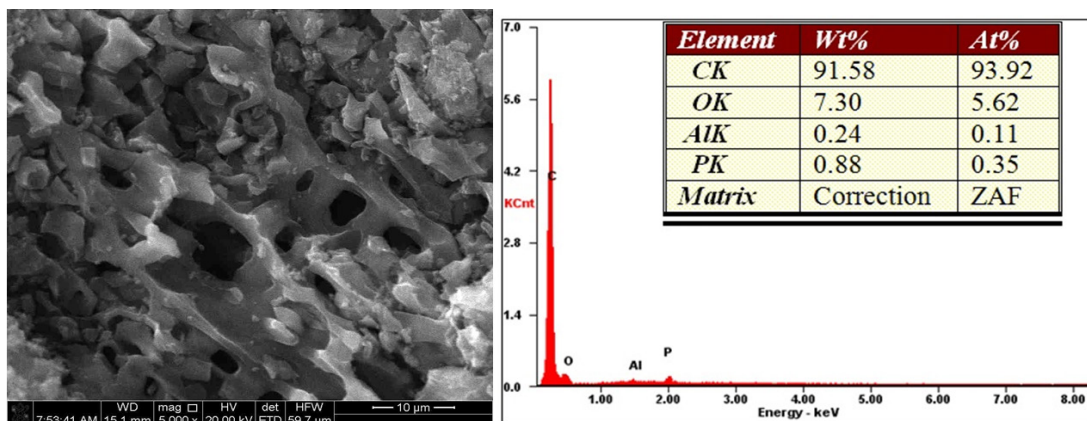

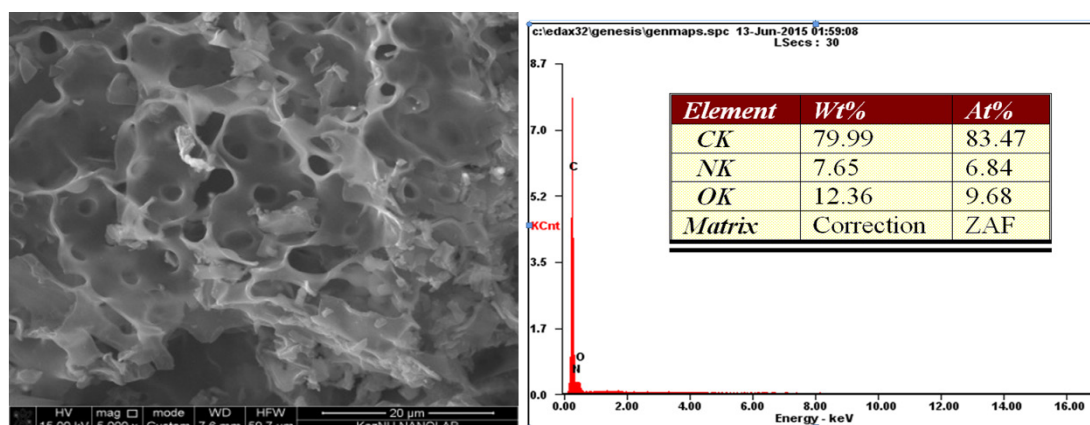Fig. 3. SEM-image and EDS-spectrogram of the CRH-P-450 sample.

Fig. 4. SEM-image and EDS-spectrogram of CRH-475-KOH-850-N sample.

Based on the results of surface morphology studies on the obtained carbon enterosorbents using SEM and their textural properties using LTNA, it was revealed that the samples retain cellular macrostructure consisting of channels (transport macropores), which is characteristic of carbon adsorbents derived from rice husk. Nevertheless, during the chemical activation of the samples, a micro-mesoporous structure was developed and, thus, the surface of the resulting carbon enterosorbents is characterized by the presence of a large amount of both macro- and nanopores. According to the data of energy-dispersive X-ray microanalysis, it can be concluded that after the activation of RH with phosphoric acid, subsequent alkaline treatment and washing, the CRH-P-450 sorbent mainly consists of carbon (91.6%), oxygen (7.3%); there is no silicon present in the sample, but there is a large amount of oxygen and up to 1% of phosphorus, which indirectly indicates the presence of phosphate groups on the surface of the sorbent. It has been established that upon oxidative ammonolysis in order to modify the sorbent surface, the semiquantitative content of carbon, nitrogen and oxygen in the sorbent sample CRH-475-KOH-850- N is about 80, 7 and 12%, which indirectly indicates the presence of oxygen- and nitrogen-containing groups (including, probably, in the form of carboxyl and amino groups).

3.3. In vitro adsorption studies of the enterosorbents towards the uraemic toxins: indoxyl and p-cresyl sulfate, as well as urea

Adsorption studies were carried out in kinetic conditions to test the efficiency to remove the marker molecules by the obtained samples of enterosorbents from model solutions: indoxyl and para-cresyl sulfates, urea. Residual concentrations were determined using HPLC and UV-spectroscopy. Data on the sorption capacity of the resulting enterosorbents with respect to medium- and low-molecular azotaemic toxins: indoxyl sulfate, para-cresyl sulfate and urea are presented in Table 4.

Time-dependence profile/diagrams of the adsorption degree of uraemic toxins (urea, indoxyl and para-cresyl sulfates) for enterosorbent samples are shown in Fig. 5.
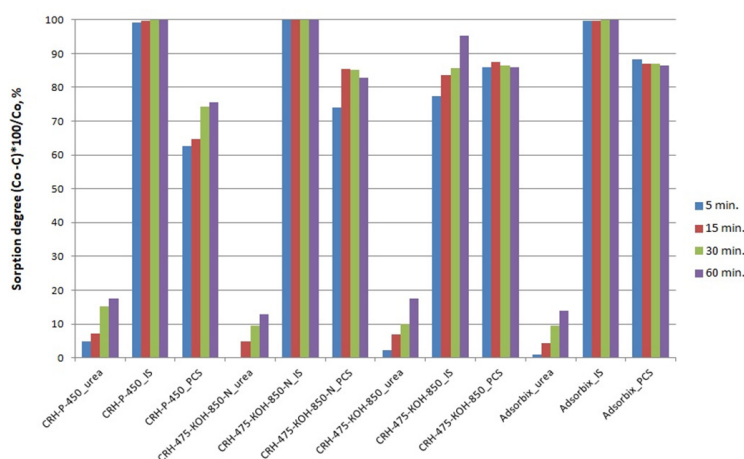

Fig. 5. Diagram of time-dependence of sorption degree profiles for indoxyl sulfate, para-cresyl sulfate and urea by enterosorbents.

Table 4
Values of sorption degree $[(C-C_o)*100/C_o]$ for enterosorbents samples obtained
with respect to indoxyl sulfate, para-cresyl sulfate and urea

The name of a sample	Para-cresyl sulfate (%)				Indoxyl sulfate (%)				Urea (%)			
Time (min)	5	15	30	60	5	15	30	60	5	15	30	60
CRH-475-KOH-850-N	74.1	85.5	85.3	82.9	90.8	90.8	99.9	99.9	0.1	4.8	9.5	12.8
CRH-475-KOH-850	85.8	87.5	86.5	86.0	77.3	82.7	85.6	95.2	2.3	7	9.9	17.4
CRH-P-450	62.7	64.8	74.3	75.5	99.0	99.7	99.9	99.9	4.9	7.1	15.1	17.4
«Adsorbix Extra»	88.2	87.0	87.0	86.4	99.7	99.7	99.9	99.9	1.0	4.4	9.4	13.9

All of the toxins in use of this study are considered to be low molecular weight molecules, e.g.: urea – 60, PCS – 188 and IS – 213 DA, respectively. However, urea is highly soluble and that is the reason why all of the ACs exhibit poor adsorption profiles with the maximal removal rate (sorption degree) of up to *ca.* 17% for the sample CRH-P-450. On a contrary, PCS and IS adsorption profiles exhibit good dynamics and the sorption degree values lie within 75–99%. The different values of sorption degree between PCS and IS can therefore be explained by twofold initial concentration, e.g.: PCS – 250 μM vs IS –125 μM; their chemical structures are similar to one another both having planar aromatic ring systems, e.g.: methoxy substituted benzene ring for para-cresyl sulfate and bicyclic aromatic conjugated system of benzene/pyrrole rings fused in case of indoxyl sulfate. The enterosorbents adsorption appears to be influenced by a combination of textural paramenters: the size of the mesopores, their pore volume in addition to the overall surface area. Based on the results of *in vitro* adsorption studies of the NCS with respect to the investigated low-molecule toxins (PCS, IS and urea), it was found that the rice husks-derived carbon enterosorbents modified with the functional groups are able to reduce clinically significant levels of uraemic toxins and are comparable to the commercial enterosorbents.

3.4. Investigation of biocompatibility of the enterosorbents

One of the purposes of this work was to study the effect of various enterosorbents samples obtained on the survival rate of epithelial cells *in vitro*.

To assess the cytotoxicity of enterosorbent samples with respect to the epithelial cell culture of dog kidney – the MDCK, the cell survival test was applied using «Alamar Blue» dye. The obtained enterosorbents samples were analyzed: CRH-475- KOH-850, CRH-475-KOH-850-N and CRH-P-450 in comparison with the commercial enterosorbent «Adsorbix Extra» – micronized activated carbon used as a control.

After successive dilution of the sorbent samples in the complete nutrient mixture of DMEM (Dulbecco's Modified Eagle's medium) at concentrations of 400 to 6.25 μg/ml, a suspension of the samples was added to a monolayer of MDCK cells in a volume of 100 μl per well. After 72 h of incubation, the media were analyzed in order to assess the survival rate of cells by dyeing with Alamar Blue. The cytotoxicity of the enterosorbents samples and «Adsorbix Extra» was measured individually for each concentration in 6 replicates. The results of the study are shown in Fig. 6.
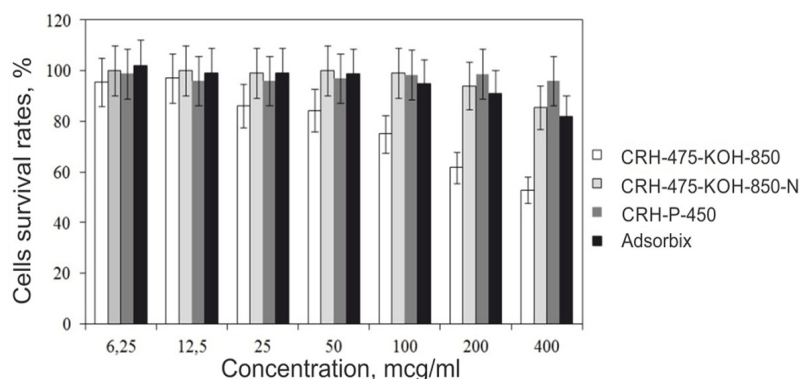

Fig. 6. Comparative analysis of MDCK epithelial cells survival rate in the presence of the obtained sorbents and «Adsorbix Extra».

Based on the results of the comparative analysis for biocompatibility it was determined that the unmodified CRH-475-KOH-850 sample exhibits a toxic effect on the MDCK epithelial cells at the high concentration range of 100–400 µg/ml, whereas the samples of the modified sorbents CRH-475-KOH-850-N and CRH-P-450 do not exhibit cytotoxicity in comparaison with to the commercial carbon enterosorbent «Adsorbix Extra».

3.5. Assessment of the efficiency of carbon enterosorbents in vivo in an animal model of Chronic kidney disease

Analysis of the results of the 1st and 2nd stages of the study allowed confirming the presence of AKD in experimental animals, as well as the beginning of the formation of CKD at the 3rd stages of the study, as follows from the Table 1. Specifically, according to the results summarized for all three stages in Table 1 and shown in Fig. 7 (for the 3rd stage only), it is evident that statistically significant differences in rats with renal pathology persisted on the 21st day of the experimental study, but also differences emerged in the groups 2 or 3rd 3 (comparison from 1st to 3rd stages) which means the formation of CKD.

Specifically, in animals receiving enterosorbent (3rd group), statistically significant differences in the parameters of uremia and intoxication appeared by 21 days. Hence the urea level in the 2nd group with AKD was 56.78 ± 6.59 mmol/l, creatinine 359.87 ± 18.73 mmol/l, both blood serum values demonstrated the manifestation of renal failure. In turn, in the 3rd group of animals that received an enterosorbent daily at a dosage of 650 mg/kg body weight, these parameters were statistically significantly lower (P ≤ 0.001) and were 25.53 ± 2.92 mmol/l for urea and creatinine 168.40 ± 6.66 mmol/l. The level of medium-mass molecules (MMM), which was assessed using the spectrophotometric method according to M.Ya. Malakhova [16], in this group was also statistically significantly lower (P ≤ 0.001) and amounted to 25.25 ± 1.01 IU, which is in fact a normal physiological level.

It was established that the daily intragastric administration of enterosorbent CRH-P-450 at a dosage of 650 mg/kg of body weight for 21 days, after the formation of AKD, is able to reduce uremia (urea, creatinine) and endogenous intoxication (MMM) with statistical reliability (P ≤ 0.001). According to the results of the conducted studies, the experimental model of acute renal failure on white mongrel rats was tested and confirmed by laboratory data, normative biochemical indices of renal function and MMM were obtained, the dynamics of the transition of arterial hypertension to CKD on the background of acute intoxication, as well as the positive effect of enterosorbent on uremia and intoxication.
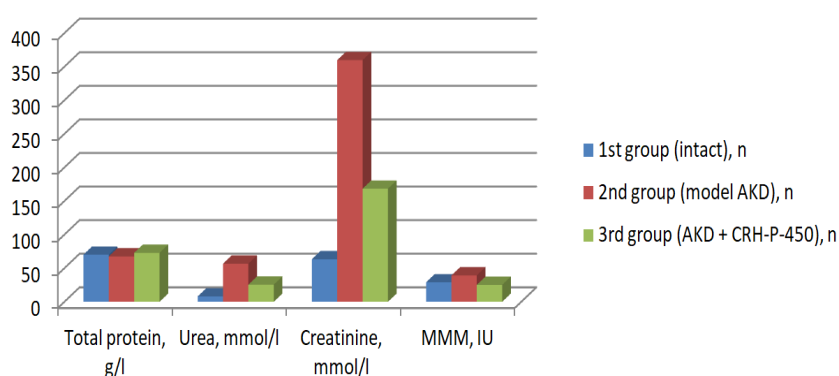

Fig. 7. Biochemical parameters of blood serum of experimental animals at the 3rd stages of the study.

4. Conclusions

The methods of synthesized modified carbon enterosorbents with a controlled nanoporous structure and functional surface chemistry were optimized by rice husk activation with phosphoric acid and potassium hydroxide of carbonized rice husk, followed by oxidative amination under controlled conditions. The obtained nanostructured carbon adsorbents were studied using a number of modern physicochemical methods of investigation: low-temperature nitrogen adsorption: isotherms recording and calculation of the specific surface area, pore volumes carried out using the Autosorb-1 "Quantachrome" device; scanning electron microscopy (SEM " Zeiss NTS ") in vitro adsorption study results for the NCS with respect to the investigated low-molecule toxins (PCS, IS and urea) suggest that the rice husks-derived carbon enterosorbents modified with the functional groups are able to

reduce clinically significant levels of uraemic toxins and are comparable to the commercial enterosorbents. Based on the results of the comparative analysis for biocompatibility it was determined that the unmodified CRH-475-KOH-850 sample exhibits a toxic effect on the MDCK epithelial cells at the high concentration range of 100–400 μg/ml, whereas the samples of the modified sorbents CRH-475-KOH-850-N and CRH-P-450 do not exhibit cytotoxicity and are comparable to the commercial carbon enterosorbent «Adsorbix Extra».

It has been established that daily intragastric introduction of the enterosorbent CRH-P-450 at a dosage of 650 mg/kg of body weight during 21 days after the formation of AKD statistically reliably (P ≤ 0.001) reduces the uremia (urea & creatinine levels) and endogenous intoxication (MMM).

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