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Nanoporous glass in Na_2O-B_2O_3-SiO_2 oxidic system, for potential biomedical applications

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The adsorption phenomenon of organic molecules in micro and nanoporous solids has been known for a long time but it has raised a considerable interest in the last years when different types of porous materials that are used in enzymatic catalysis, chemical biosensors, chromatographic columns or drug support, have been investigated. Because of the advantages that porous glass presents compared to other types of organic or inorganic porous materials, high mechanical resistance, thermal stability, rigid structure immune to biological and chemical degradation, etc., the glasses with a better controlled porosity are researched and used more and more. In this paper, we report on the preparation of a nanoporous glass with the pores' diameter between 10-300 nm, with interconnected pores, fabricated on the base of microphase separation in an alkaline borosilicate glass. The glass composition was chosen in the Na_2O-B_2O_3-SiO_2 system. The obtained materials have been characterized physico-chemically (chemical and thermal analysis, X-ray diffraction), microstructurally (atomic force microscopy) and from the point of view of the bioproperties that present interest (pH dynamics, noncitotoxicity, cellular proliferation, adsorption and enzymatic activity).

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1. Introduction

Along the time, numerous organic porous supports (gluten, activated carbon, etc.) and inorganic ones (alumina, kaolinite, clay, silica gel, etc.) have been used in diverse medical applications especially as a support for the enzyme immobilization [1]. The first porous glass utilized to this end was the one produced by Corning Glass Works (USA) and the person that developed this science was W. Haller [2]. Ulterior, due to the advantages that the porous glass has, compared to other enzymatic supports, these types of materials were researched and used more and more. Between the major advantages it counts: large specific surface that insures many bonds with the ligands and a high productivity, thermal stability, high mechanical resistance which insures reproducible results with constant parameters, high flow rate which saves time, rigid structure immune to biological degradation and inert to inorganic solvents and concentrated acids (excepting HF).

Glasses with nanometric porosity can be prepared in many oxide systems. The best known are the glasses in the oxide system R_2O (Na_2O, K_2O, Li_2O)-B_2O_3-SiO_2 which, subsequent to an adequate thermal treatment, undergoes a process of microphase separation through the spinodal mechanism which leads to the formation of a continuous boro-alkaline phase in the silica network. Through the solubilization of the boro-alkaline phase with inorganic acids it results a nanoporous structure whose structural properties are determined by (i) the initial glass composition, (ii) the conditions of the heat treatment and (iii) the eroding conditions [3].

Establishing the distribution and the final dimensions of the pores can be also done by taking into account a “complex” structural parameter, like the basicity percentage pB [4,5]. With the help of the phase diagrams, at a constant temperature (t), the local basicity of the formed microphases can be calculated, “the map” of the basicity changing with the conditions of thermal treatment. Thus, taking into account the basicity percentage, pB, a thermal treatment that insures certain characteristics (volume, dimensions) of the microphases and implicitly of the pores, can be thought.

Presently, abroad, glasses with controlled porosity that has uniform pore structure which allows a good spatial distribution of the ligands and excellent flow characteristics are produced and commercialized. Due to this properties, the utilization area of the porous glasses has increased, they also being used as imunosorbant, chemical/biochemical sensors, in medical applications, materials in nonlinear optical devices, solid phase synthesis and various chromatographic analysis, macromolecular sieves, etc [6,7].

In this paper, we have proposed to obtain a nanoporous glass in the Na_2O-B_2O_3-SiO_2 oxide system, with interconnected pores with the diameter between 10-300 nm, such that it can be utilized in biomedicine as support for enzymatic immobilization.

The obtained materials have been analyzed from the point of view of the physico-chemical properties of interest (chemical and thermal analysis, X-ray diffraction), of the microstructure (atomic force microscopy) as well as of the biotests such as pH dynamics, noncitotoxicity,

cellular proliferation in the presence of osteoblast cultures, absorption and enzymatic activity (glucoseoxydase).

2. Materials and methods

The research was based on the (%mol.): 65 SiO_2; 25 B_2O_3; 10 Na_2O oxide compositions, situated in the nemiscibility domain of the system. Base glass synthesis (NBS) was achieved through the classical procedure of melting using an electric oven of superkanthal and raw materials of analytic purity (quartz, H_3BO_3, Na_2CO_3). To over compensate the volatilization losses supplements were used for H_3BO_3 and Na_2CO_3, the melting taking place at 1480 ºC in alumina crucibles. The obtained glass was annealed for 3 hours in the proximity of the vitreous transition temperature, T_g, and cut into prismatic samples in order to make controlled thermal treatments at 650ºC and 700 ºC between 1-3 hours. The obtaining of the nanopores was made through the solubilization of the boro-sodic microphase (subsequent to the spinodal separation) with HCl, 1M, for periods between 30 min. and 3 hours. After the HCl attack, a neutralization of the samples was realized with NaOH 1M, followed by a washing with distilled water which also has the purpose of eliminating the silica particles remained inside the formed pores. The real chemical composition resulted after the melting was determined through a complete chemical and spectrographic analysis and the identification of the possible crystalline formations, appeared after the thermal treatment, was made with the help of the x-ray diffraction utilizing a Shimadzu XRD6000 difractometer. Using a dilatometer of the LINSEIS type with an amplification factor A=1000, the structural transformation temperatures, the inferior annealed temperature, T_{ir}, the vitreous transition temperature, T_g, the superior annealed temperature, T_{sr}, the dilatometer softening temperature, T_D, and the thermal dilation coefficient were determined for the obtained base glass, the sample being heated with 4 ºC/min.

With the help of an atomic force microscope Nanosurf (AFM), the morphology at the nanometric level of the glass samples after the thermal treatment and the solubilization of the boro-alkaline phase, which were effectuated in the conditions presented above, could be visualized.

From the biological point of view the pH modifications that were produced in a solution which imitates the human blood plasma (SBF) were tested in the presence of the synthesized base glass. The SBF solution was prepared using the recipe proposed by T. Kokubo [8], which has the ionic concentration approximately equal to that of human plasma and the pH=7.4. The measurement of the pH was made through the potentiometric method.

Biologically, MTT noncitotoxicity tests (the 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazoliu bromide) and of cellular proliferation were effectuated using osteoblast cell cultures [9]. The osteoblasts were trypsinized, washed and inseminated on Petri plates, at a density of $4\cdot10^5$cellules/ml, simultaneously with the inseration of the NBS glass pieces in the culture. The MTT method of monitorization of cellular viability was based on the measurement of the activity of the mitocondrial dehydrogenazes existent in the viable cellules. The osteoblast evidenciation on the glass pieces was realized by marking the latter with hypericine for 6 hours, at 37ºC and by the ulterior analysis at the fluorescence microscope, at distance, at close as well as on each of the glass samples analysed.

The enzymatic apsorption was also determined by decreasing, with time, the proteic concentration of a glucoseoxydase sollution, utilizing the Lowry method, with the fixation on the porous support. The evidenciation of the enzymatic activity of the imobilized glucoseoxydase was made by decreasing the concentration, in time, of a glucose sollution in which the porous glass samples with the imobilized glucoseoxydase were immersed.

3. Results and discussion

Following the chemical analysis, a real composition of the base synthetised glass of (%mol): 63.6 SiO_2; 27.7 B_2O_3; 8.7 Na_2O resulted. The spectrographic analysis also evidenciated a neglectable content of Al_2O_3, of 5×10^{-3} - 1×10^{-2} %, due to the Al_2O_3 diffusion from the crucible used at melting.

In Table 1 are presented the data obtained from the glass sample's thermogram.

Table 1. The lower annealed temperature, T_{ir}, the vitreous transition temperature, T_g, the upper annealed temperature, T_{sr}, the dilatometer softening temperature, T_D, and the thermal dilation coefficient, for synthetised NBS glass.

Sample	T_{ir} (°C	T_g(°C)	T_{sr}(°C)	T_D(°C)	$\alpha_{27}^{307}\times10^{-7}$(°C^{-1})
1	468.5	491.9	504.1	602.0	57

The thermal treatments, realized on the obtained borosilicatic sodium glass samples, at the temperature of 650 °C and 700 °C for periods between 1-3 hours, led to the appearance of the microphase separation phenomenon through the spinodal mechanism with the formation of a continuous, borosilicate phase in a prevalently silicatic network. This fact is also confirmed by the atomic force microscopy images realized on the obtained samples (Fig. 2-5) and even by the aspect of the samples which, subsequent to the thermal treatment, become opaque.

The X-ray diffraction for the thermally treated sample at 700 °C for an hour (Fig. 1) shows that peaks, corresponding to crystalline compounds, do not appear, the sample being completely vitreous.

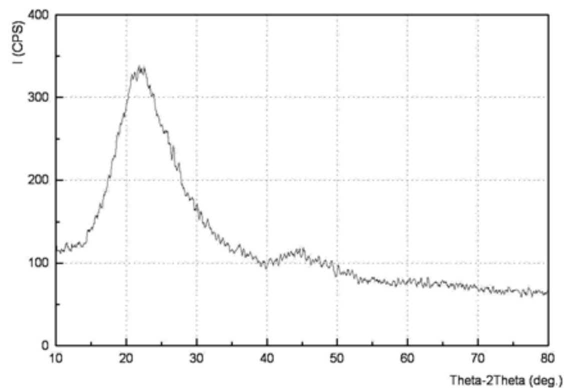
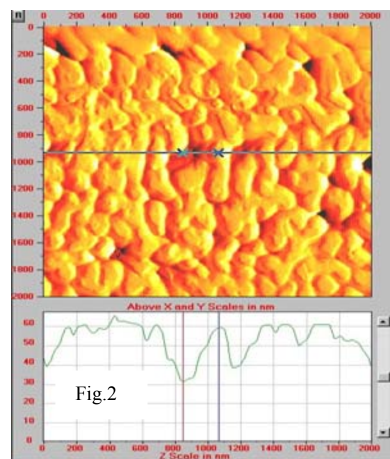


Fig. 1. The diffractogram for the sodium borosilicate glass thermally treated at 700 ºC for 1 hour.

The solubilization of the boro-sodic phase with HCl, 1M, led to the obtaining of a porous structure, prevalently silicate, with the pore's dimensions between 10-300 nm. The atomic force microscopy images realized on a series of NBS glass samples, thermally treated and differently solubilized, show that with the increase of temperature and time of the thermal treatment, the microphase separation is more powerful and, implicitly, the pores' dimensions are bigger, Fig. 2-4. A greater erosion of the sample can also be observed in the case of the boric phase solubilization with HCl (1M) for a longer period of time (Fig. 5), the resulted surface of the sample being more rugged and the pores' dimension increasing with the increase of the solubilization time. Yet, the atomic force microscopy images also show a change in the silicate structure, due to a too strong acid attack, which can have undesired results because of the breaking of pieces from the SiO_2 network and the appearance of the silica particles in the pores.



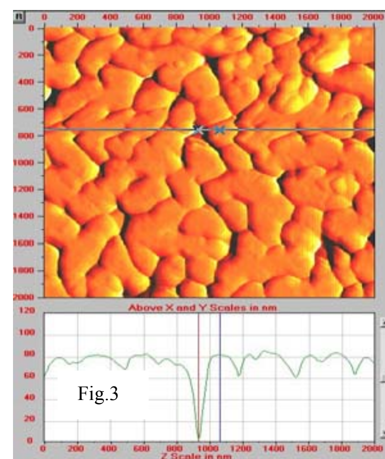
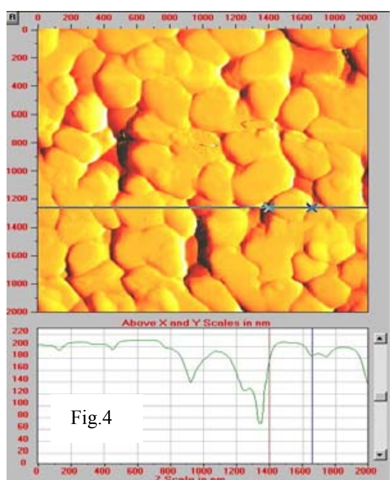


Fig. 2-3. Atomic force microscopy (AFM) images for the obtained porous glass, after a thermal treatment at 650 °C (Fig. 2), respectively 700 ºC (Fig. 3) for 1 hour and the solubilization of the boro-alkaline phase with HCl, 1M, for 30 min., at room temperature.



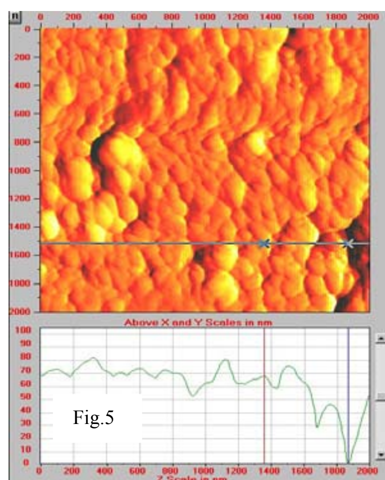


Fig. 4-5. Atomic force microscopy (AFM) images for the obtained porous glass, after a thermal treatment at 700 °C for 3 hours (Fig. 4), respectively 650 ºC for 1 hour (Fig. 5) and the solubilization of the boro-alkaline phase with HCl, 1M, for 30 min., respectively 3 hours, at room temperature.

From the biological point of view, for the synthetized sodium boro-silicatic glass (NBS) that was not thermally treated, the shifting of the solution's pH to more basic values with the increase of the immersion time in SBF was observed, this phenomenon having a longer rate of evolution during the beginning stages, up to 24 hours of immersion, followed by a deceleration of the process, with saturation tendences (Fig. 6). The pH variations can be charged on the obtained material's capacity to biointeract with live tissue, and it can be a measure of the associated bioactivity degree.

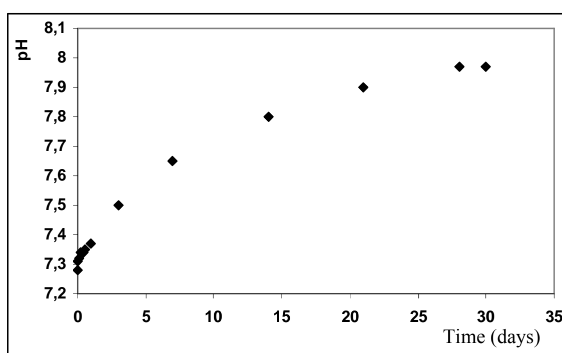


Fig. 6. Variation in time of the SBF solution's pH, in contact with the synthesized sodium boro-silicatic glass.

The cellular viability tests (citotoxicity and cellular proliferation) have indicated that the presence of the NBS glass in the osteoblasts culture does not exert citotoxic effects at cellular level in fact stimulating the multiplication of the osteoblasts, these ones maintaining their normal phenotypical characteristics. The number of surviving cells was obtained by reducing the MTT-formazan colour agent and by determining the optical density at 570 nm compared to izopropanol.

The procentual viability of human embrionary osteoblasts in the presence of the synthetized NBS glass is presented in Table 2 compared to the witness sample.

Table 2. The procentual viability of human embrionary osteoblasts in the presence of the synthetized NBS glass, compared to the witness sample.

Sample	DO_{570nm}	*% viability compared to the witness*
Witness	0.130	100
NBS	0.353	271

In Figs. 7-8 the witness osteoblast cellules and those cultivated in the presence of the NBS glass piece are presented, after 72 hours of incubation, the emphasis of the adhered and multiplied osteoblasts cells on the surface of the sample being made with hypericine for 6 hours at 37 ºC (Fig. 9).

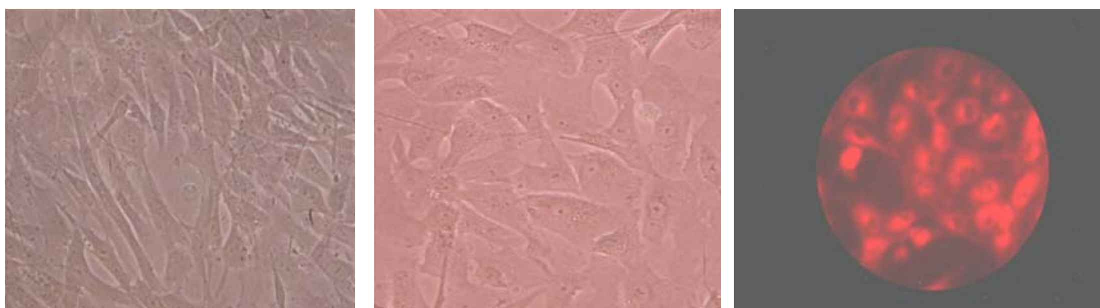
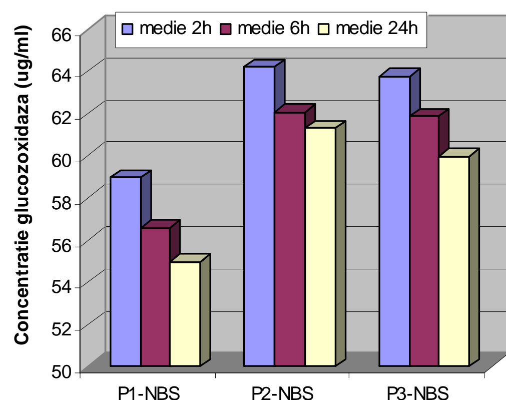


Fig. 7-9. Witness osteoblast cells celule (Fig. 7) and osteoblast cells cultivated in the presence of the NBS glass piece (Fig. 8), after 72 hours of incubation. Osteoblast cells adhered to the surface of the sample, marked with hypericine (Fig. 9).

For the enzymatic imobilization on porous support, 3 samples of porous glass with nanopores < 300 nm were used, which were obtained from the NBS base glass thermally treated at 700 ºC for 1 hour and solubilized with HCl – 1M, 30min. (P1-NBS sample), thermally treated at 650 ºC for 1 hour and solubilized with HCl – 1M, 3 hours (P2-NBS) and thermally treated at 650 ºC for 1 hour and solubilized with HCl – 1M, 30 minutes (P3-NBS sample). The samples were incubated in a glucoseoxydase sollution 1mg/ml different time intervals under continuous stirring, the proteic concentration of the sollution being determined by the Lowry method. In Fig. 10, the proteic variation of the sollution in which the porous glass samples were incubated is presented with the evidenciation of a decrease of the proteic concentration in the sollution proportional to the incubation interval, decrease due to the fixation of the protein on the porous support.



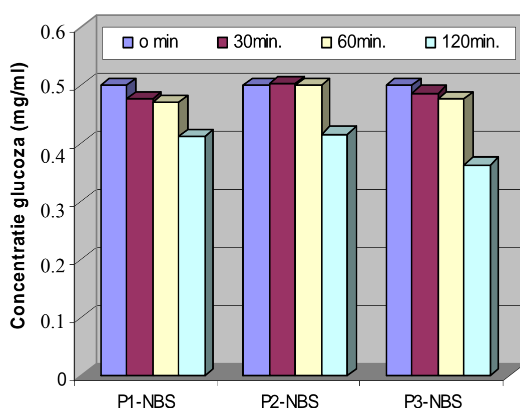


Fig. 10-11. The glucoseoxydase *(µg/ml)(Fig. 10) and glucose (mg/ml)(Fig. 11) sollution proteic concentration variation, after different incubation periods, in the presence of the glass samples P1-NBS; P2-NBS; P3-NBS.*

The enzymatic activity of the imobilized glucoseoxydase was evaluated through the immersion of the porous samples with the imobilized glucoseoxydase in a glucose sollution 50 mg% for different time intervals, at 37 °C under constant stirring. After 30, 60 and 120 minutes of incubation the glucose concentration existent in the sollution in which the porous glass samples were immersed was determined. The glucose dosage was realized using the o-toluidyne dosage method. In Fig. 11, the decrease of the glucose concentration existent in the sollution in which the porous glass (P1 NBS; P2 NBS; P3 NBS) samples were immersed can be observed as a result of the enzymatic activity of the glucoxydaze, the enzyme that was imobilized on the glass support and which mantained its activity. The glucose degradation takes place in time, the biggest decrease compared to the initial concentration (0.5 mg/ml) being registered after 120 minutes of incubation in the presence of the glass samples.

4. Conclusions

In conclusion, in the Na_2O-B_2O_3-SiO_2 oxidic system, a nanoporous (nanopores < 300 nm) vitreous material was obtained that was analysed from the point of view of the physico-chemical properties of interest as well as of the biotests (pH dynamics, non-citotoxicity, cellular proliferation, enzymatic absorption and activity). It was emphasized that the nanoporous vitreous material obtained permits a good cellular viability and proliferation as well as the fact that the enzyme (glucoseoxydase) fixed on the surface of the porous support presents enzymatic activity.

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