NEW APPROACH FOR CHARACTERIZATION AND STUDY ON REACTIVITY OF BIOMATERIALS

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Abstract

New method for study on reactivity and transport ability of biomaterials based on combination of innovative diffusion techniques and advantages of hydrogels was developed. Stationary penetration experiments (diffusion cells) were applied for biomaterials (anionic humic acids and cationic chitosan) in supported hydrogel matrix. Linear polysaccharide – agarose – was used as non-reactive matrix, which was characterized from mechanical and structural point of view. The reactivity and transport properties of chosen biomaterials were studied by the interactions with simple organic dyes (Methylene Blue C.I. 52015 and Chicago Sky Blue 6B C.I. 24410). The rate of interaction of organic dyes with biomaterials in the case of stationary experiments (break-through technique) was compared by fundamental diffusion parameters (effective diffusion coefficient, lag time and binding capacity).

Results obtained from stationary diffusion technique clearly illustrate the immobilizing effect of chitosan (humic acids, respectively) on the transport of organic dye in hydrogel. The influence of concentration of biomaterials on the transport of organic dye and the reactivity of biomaterials was studied as well.

Presented diffusion technique (together non-stationary diffusion techniques) in combination with excellent transport properties of hydrogels (diffusion is not disturbed by convection, characterization can be done very easily, etc.) seem to be suitable universal preliminary reactivity-mapping tool for wide range of biomaterials at changing experimental conditions.

Keywords

Biomaterials, hydrogel, reactivity, transport, polyelectrolytes

1. INTRODUCTION

1.1. Humic acids

Humic acids (HA) are fundamental components of natural organic matter (NOM) in the soil and water as well as sediments, peats or coal. HA are complex and heterogeneous mixture of polydispersed materials; they were formed during decades as a result of microorganism activity [1]. HA represent highly reactive material, which plays crucial environmental role in natural ecosystems, such as soils, waters and sediments. They have outstanding affinity to interact with nearly any substances [2]. HA are one of the fractions of humic substances, which are soluble at alkali conditions, but insoluble in acids unlike fulvic acids, which are soluble in both solutions. HA are very important component of soil; this valuable biomaterial is responsible for complexation of pollutants (e.g. heavy metal ions or pesticides) [3], [4]. The positive effect of HA on the on the soil fertility is known very well for decades [5].

Lots of scientific groups are interested in the chemistry of HA. Nevertheless, simple universal method for study on reactivity and transport ability of this biocompatible material is still missing. The reactivity and binding capacity of HA is mostly studied by classical sorption experiments [6]. Sorption experiments are very useful for determination of fundamental sorption parameters such as binding capacity of HA towards to cationic natural compounds. However this classical approach does not illustrate the real environment of HA (soil or water). Because of this fact, we are able determined sorption kinetics and equilibrium, but the

information about the nature of interactions between HA and pollutants in natural environment are limited. The universal reactivity-mapping tool for natural organic matter is needed because of lacks of classical sorption experiments which were mentioned above. Progressive methods aimed on the study on reactivity and binding capacity of natural HA are mentioned in our recent works [7], [8].

1.2. Chitosan

Chitosan is the most important derivative of chitin. It can be obtained by deacetylation of chitin, which it is a natural component of shrimp or crab shells [9]. Chitosan is linear polysaccharide with unique properties. It is one of the cationic natural polysaccharide and thus, chitosan finds many applications. Chitosan is insoluble in aqueous solutions, but it is soluble in acetic aqueous solutions (e.g. mineral acids). The solubility of chitosan is given by the degree of deacetylation. Chitosan consists $–NH_2$ functional groups, which can be protonated in acetic aqueous solutions and after that the chitosan becomes soluble. The cationic nature of chitosan is primarily responsible for electrostatic interactions with anionic charged molecules such as proteoglycans [10] or organic dyes [11].

Chitosan is composed of glucosamine and N-acetyl glucosamine units linked by (1-4) glycosides' bonds. The content of glucosamine units is called the degree of deacetylation [12]. Chitosan is appreciated mainly because of presence of amino groups and the biocompatibility, biodegradability and bioactivity. Nowadays, chitosan is mostly used in tissue engineering [13], [14]. Materials based on chitosan are commonly used for removal of food dyes from aquatic solutions by sorption processes. The positive affinity of chitosan towards to anionic organic dyes is known very well. [15], [16]. In spite of the fact, that the interaction of chitosan with organic dyes is well proofed, the mechanism and the determination of the rate interaction is often difficult.

1.3. Theory of diffusion

Proposal article presented two diffusion techniques which are used for study on reactivity of biomaterials. Diffusion cell technique (break-through method) is related with 1. Fick's law [17] (stationary diffusion). The effective diffusion coefficient, as the fundamental diffusion parameter can be calculated from changes of concentration of diffusion probe in acceptor chamber of diffusion cell with time according equation 1 [7].

$$\ln \frac{\left(c_D - c_A\right)_t}{\left(c_D - c_A\right)_0} = -\beta \cdot D \cdot t \tag{1}$$

where c_D is the concentration of organic dye in source chamber, c_A is the concentration in acceptor compartment, β symbolized the geometrical parameter (diffusion cell constant) determined experimentally [7] and t is the time of diffusion. The values of steady-state diffusion flux and the lag time were determined from linear region of the break-through curve (the concentration of organic dye as the function of time). Typical break-through curve has two regions. In first few hours (depend on amount of biomaterials and agarose concentration), the changes of concentration of organic dye as the time function is equal to zero. During this transition step, the sorption stage and penetration of organic dye through hydrogel occurs. Therefore, the concentration of organic dye in acceptor compartment is equal to zero. After some time (lag time or break through time), the concentration of organic dye increases linearly in acceptor chamber. The slope of this increase is proportional to effective diffusion coefficient (the main important diffusion parameter).

2. EXPERIMENTAL

2.1. Determination of pore size

Pore size of agarose hydrogels was determined by ultraviolet-visible spectroscopy. Utilization of this spectroscopic method for determination of agarose pore size is clearly described in [18]. Agarose hydrogels with exact concentration were prepared in PMMA cuvettes. The ultraviolet-visible spectra were of these agarose hydrogels were collected on double beam spectrophotometer (Hitachi U3900) at given range of

wavelength (700 – 800 nm). Average absorbance was calculated in whole range of wavelengths. Logarithm of turbidity was plotted as a function logarithm of wavelength. Turbidity was calculated according equation 2.

$$\tau(\lambda) = \frac{2.3 \cdot A(\lambda)}{L} \quad (2)$$

where A is the absorbance and L is the optical length (1 cm in this case). Wavelength exponent was obtained from linear regression of logarithm of turbidity as a function of logarithm of wavelength. According theoretical assumptions (Aymard et al. [19]), the wavelength exponent is proportional of correlation length, pore size respectively.

Pore size of 1 wt. % agarose hydrogel was determined by unconventional method (UV-VIS spectroscopy). The main aim of this determination was choosing of the appropriate concentration of agarose in the hydrogel. Higher concentration of agarose causes the reduction of pore size. On the other hand, if the concentration of agarose in hydrogel is not sufficient, the mechanical properties of these hydrogels will not be satisfying. Optimal pore size of agarose hydrogel is very important parameter, because biomaterials, which are studied, must be immobilized in the structure of hydrogel, which can be done only, when the pore size is sufficiently small. Because of these facts, the determination of pore size was realized for 4 samples (0.5 wt. %, 1 wt. %, 2 % wt. and 4 wt. %) agarose hydrogels.

2.2. Rheology

Differences in mechanical properties of agarose hydrogels were compared by oscillatory measurements on Rheometer AR-G2 TA Instruments. Mechanical properties of agarose hydrogels with/without addition of biomaterials were determined by frequency sweep (constant strain) and strain sweep (constant frequency of oscillation) oscillatory tests. First of all, the strain sweep of each agarose hydrogel was realized at constant frequency of oscillation (1 Hz). Mechanical response of hydrogels was measured in given range of strain (0.1 % − 100 %). Normal force used during compression of sample was maintained under 2 N.

Second oscillatory test – frequency sweep was done at these conditions: constant strain (0.1 %), range of oscillation frequencies (0.1 – 20 Hz), measured with downward trend, maximal normal force during compression was 2 N. Both oscillation tests were realized at constant temperature (25 °C) by using flow thermostat (Haake DC5). The sensor used for determination of mechanical properties of agarose gels was plate/plate 40 mm (steel). Layer thickness of measured sample was 1 000 μm in all cases. Measurements were repeated three times.

2.3. Diffusion cell technique

Agarose hydrogels were prepared via thermo reversible gelation of aqueous solution of agarose (routine use class, < 10 wt. % moisture content). The concentration was chosen as 1 wt. %. Four hydrogels samples with different concentration of biomaterial were prepared. The concentration of biomaterial in the final hydrogel was 0.002 wt. %, 0.005 wt. % and 0.010 wt. %. The fourth sample was 1 wt. % agarose hydrogel without addition of biomaterial, as reference. Accurately weighted amount of agarose powder was dissolved in distillated water or in aqueous solution of HA, chitosan respectively. This mixture was heated to 85 °C; the transparent solution of agarose was degassed in ultrasonic bath for 1 minute at 85 °C. The hot solution after degassing was poured into the PTFE mold (40 mm in diameter and 5 mm thickness). Upon the cooling to the laboratory temperature liquid mixture gradually solidified into the cylindrical hydrogel sample. The hydrogel in the mold was placed between two chambers of diffusion cell. Source chamber was filled up by basic organic dye (Methylene Blue C.I. 52015 and Chicago Sky Blue 6B C.I. 24410) and on the other side (acceptor chamber) was distillated water. The change of concentration of organic dye was determined by UV-VIS fiber spectrometer USB 2000+ (Ocean Optics, Inc.) in the acceptor chamber of diffusion cell. The UV-VIS spectra were collected continuously at given time intervals. The water-jacketed side-by-side diffusion cell was purchased by Permegear Inc. The diffusion cell volume of each solution was 60 cm^3. The stirring of solutions in source and acceptor chambers of diffusion cell was realized on magnetic stirrer (250 RPM).

3. RESULTS AND DISCUSSION

3.1. Pore size determination

Pore size of 1 wt. % agarose hydrogel was determined by unconventional method (UV-VIS spectroscopy). Results obtained from this analysis clearly illustrate exponential decrease of pore size distribution with increasing concentration of agarose in hydrogel.

Table 1 Pore size of agarose hydrogels with different concentration determined by UV-VIS spectroscopy

Concentration of agarose (wt. %)	Wavelength exponent	Pore size (nm)
0.5	-2.40	800 ± 12
1.0	-2.59	380 ± 10
2.0	-3.06	160 ± 8
4.0	-3,82	90 ± 7

1 wt. % agarose hydrogel was chosen as optimal concentration of agarose in final hydrogel with respect to pore size distribution and mechanical properties. Average particle size of HA determined by dynamic light scattering was 459 ± 8 nm [20], Because of this fact, the pore size of agarose hydrogels must be lower than average particle size of HA.

3.2. Oscillatory measurements

The mechanical properties of agarose hydrogels with/without addition of HA/chitosan were compared by basic rheological parameters (storage modules (G') and loss modules (G'')). First of all, the strain sweep test was done. The main aim of this test is the determination of linear viscoelastic region (LVR). In this region, both modules (storage and loss) are independent on the amplitude of strain, the structure of hydrogel is able to resist to applied stress (reversible deformation). After overcome of LVR, hydrogels nodes are irreversible destroyed. The end of LVR is noticeable, when the storage module (G') rapidly decrease and loss module (G'') also decrease after slight increasing. The end of LVR is characterized by critical value of strain amplitude (last point when modules are independent on strain amplitude). Critical strain amplitude is similar for 1 wt. % agarose hydrogel in comparison with 1 wt. % agarose hydrogel with addition of HA. Enormous difference in mechanical properties of agarose hydrogels are seen after addition of chitosan.

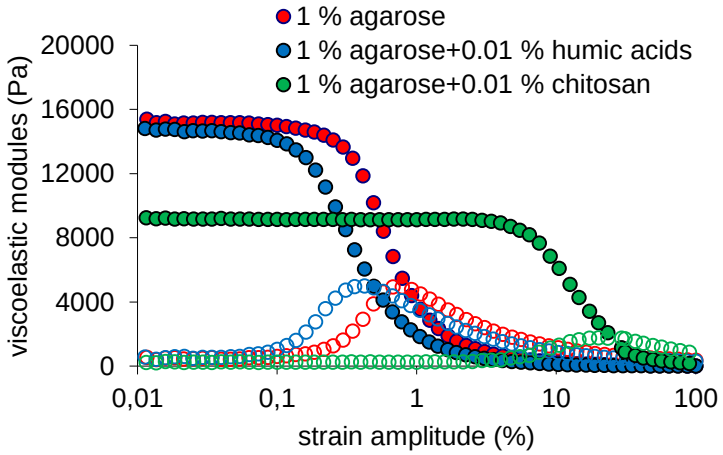

Fig 1 Strain sweep test and of 1 wt. % agarose hydrogel with addition of HA respectively chitosan. Full symbols represent storage modules (G') and empty symbols represent loss modules (G'').

Transport properties of final materials are significantly influenced by mechanical properties of agarose hydrogels. The transport properties of agarose hydrogel with addition of HA are not influenced by changes of mechanical properties. Changes in fundamental diffusion parameters at diffusion experiments are given only

by interactions of HA with organic dyes. However, transport properties of agarose hydrogels with addition of chitosan can be significantly influenced by changes in mechanical properties. The end of LVR in the case of chitosan is almost 10 times higher in comparison with agarose hydrogels with/without addition of HA.

3.3. Stationary diffusion experiments

Our previous publication [7] clearly illustrate the effect of interactions between HA and Methylene Blue on the transport of this ionic dye in model aqueous environments provided by agarose hydrogel. The data presented in this paper continues with the same experimental approach. The reactivity of IHSS (International Humic Substances Society) standard HA (1S104H) is studied by interactions with Methylene Blue.

First of all, the influence of concentration of IHSS HA was studied by diffusion cell technique. It is obvious that the small increase of concentration of HA in 1 wt. % agarose hydrogel slows down the rate of diffusion processes of Methylene Blue. Figure 2A summarized the effective diffusion coefficients for IHSS HA and for chitosan. In the case of chitosan, the different organic dye was used (Chicago Sky Blue 6B). This dye has higher molecular weight, therefore effective diffusion coefficient for pure agarose without addition of chitosan is lower in comparison with effective diffusion coefficient of Methylene Blue. The decrease of effective diffusion coefficients for Chicago Blue in the case of chitosan is not so significant in comparison with HA. Interaction of anionic compounds (organic dyes) with cationic chitosan is given mainly through amino groups in dissociated state. The presence of these functional groups is responsible for the fact, that chitosan is cationic biopolymer, which is able to immobilize and interact with different compounds, especially anionic.

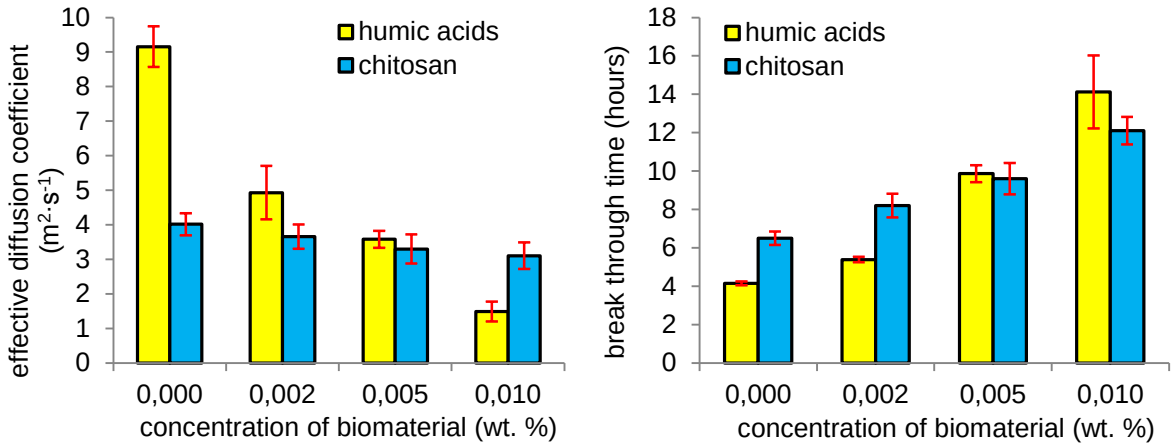

Fig 2 Effective diffusion coefficients of organic dye as function of biomaterial concentration (A). Lag time of diffusion process as the function of biomaterial concentration (B) for chitosan and HA in both cases.

Increasing content of HA in the hydrogel samples led to a considerable increase in absorbed amount of chosen dye in the hydrogel and to the decrease in steady-state diffusion flux. The total content of HA significantly affected also the value of lag time (Figure 2B) which indicates extensive physic-chemical interactions between diffusing organic dyes and HA contained in the hydrogel. When the binding capacity is depleted all of binding sites are occupied by molecules of organic dye. Lag time is indirectly connected with ability of samples to retain active compounds.

4. CONCLUSIONS

The presented diffusion approach to study on reactivity of biomaterials provided comprehensive illustration of the influence of interaction between HA and cationic organic dyes (chitosan and anionic organic dyes) on barrier properties of these biomaterials and represent valuable approach in order to better understanding the behavior of these valuable compounds in their natural environment. Presented diffusion techniques should also become an universal method applicable for wide range of ionic compounds especially biomaterials at changing experimental conditions (temperature, ionic strength, modification of biomaterials etc.).

5. ACKNOWLEDGEMENT

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