MULTIFUNCTIONALIZED MATERIALS FOR PERFORMING SEPARATIONS

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The functionalization of cellulosic sorbents with reactive dyes is a way to obtain new supports for the separation of biological media. The supports have a different affinity for proteins depending on the reactive dye used. The modified support has a higher capacity for enzymes retention than pure cellulose. Affinity adsorbents based on immobilized triazine dyes offer important advantages circumventing many of the problems associated with biological ligands. Rational attempts to improve the selectivity for proteins are realized through the biomimetic dye concept according to which the biomimetic dyes are designed to mimic natural ligands. Biomimetic dyes are expected to exhibit increased affinity and purifying ability for the targeted proteins.

Key words: Multifunctionalized cellulosic sorbents, biomimetic reactive dyes, enzymes, sorption, affinity chromatography.

INTRODUCTION

Separation of biochemical products, particularly proteins and enzymes from biotechnological processes, ask major problems related to chemical composition complexity and contaminants features similarity with target product.

Immobilized chlorotriazine polysulfonated dyes have been used as affinity chromatography tools for protein and enzyme purification for about 30 years. The azo-triazine employed dyes were molecules, which are readily immobilized on polymers bearing hydroxyl groups. The low cost of these dyes, their ease of immobilization and resistance to biological and chemical degradation, and the high protein-binding capacity of the corresponding adsorbents, has led to affinity chromatography materials that are much less expensive and more stable than those based on natural biological ligands. Such dyes interact with proteins with remarkable degrees of specificity [1]. One way to improve the specificity of immobilized dyes is to use specific eluents, which allow eluting specifically the
target protein. This approach is better known as *affinity elution*, and it has been used in numerous affinity purifications with immobilized dye-ligands. If the dye interacts with the active site of the target enzyme, then a ligand of the enzyme can be used as a specific eluent of the enzyme. Another strategy is to design new dye-ligands of improved affinity and specificity for the target protein. In principle this can be achieved by designing synthetic dyes which mimic the structure and binding of natural biological ligands of the targeted protein. This new type of ligand is termed *biomimetic dye-ligand*, and it should not only display high specificity for the target protein [1].

Because of high resolution, affinity chromatography is able to overcome the major restrictions concerning the assurance of high selectivity in protein purification processes [2].

Due to the ligand property of reactive dyes to a wide range of proteins, capitalization of sorbents functionalized with dyes in affinity chromatography for separation of proteins, enzymes, polinucleotides, antibodies, antigens or biotechnological processes by-products is of exceptional importance.

Use of cellulosic sorbents modified with dyes in biological systems study is based on necessity for improvement of untreated materials affinity toward proteins.

Sorbents based on cellulose (microcrystalline, beaded) functionalized with reactive dyes are new sorbents of high selectivity in separation of nucleic acids, proteins, enzymes or some biosynthesis drugs [3].

The purpose of this work is to present a review concerning the opportunities of cellulosic sorbents modified with reactive dyes use in retention of biochemical compounds. The process is estimated on basis of Langmuir or Freundlich isotherms, and Gibbs free energy. A thorough going study is based on a series of kinetic determinations and ir analysis.

![Fig. 1. – Reactive Red ME-BA.](image1)

![Fig. 2. – Reactive Violet ME-RN.](image2)
A comparative study regarding the biochemical compounds binding capacity of two reactive dyes (Reactive Red ME-BA – Fig. 1 and Reactive Violet ME-RN – Fig. 2) as well as the untreated or dyes functionalized cellulosic sorbents has also been made.

**EXPERIMENTAL**

The synthesis of reactive dyes were carried out using classical methods of diazotization of an aromatic primary amine, coupling with activated substrates and condensation with cyanuril chloride [4].

The synthesis of reactive dye Red ME-BA is presented in (Fig. 3):

![Synthesis of Reactive Red ME-BA](image)

Fig. 3. – Synthesis of the reactive dye Red ME-BA.

The synthesis of reactive dye Violet ME-RN is presented in Fig. 4.

Guanidoethyl (GE) and aminooethyl (AE) cellulose modified by sorption of the reactive dyes Reactive Red ME-BA and Reactive Violet ME-RN respectively has been used.
Fig. 4. – Synthesis of the reactive dye Violet ME-RN.

The tested enzyme has been prepared as stock aqueous solution of 0.5 µg/mL to 200 µg/mL concentration. The stock solution was diluted so that the concentrations of the works solutions ranging from 1 µg/mL to 100 µg/mL (Table 1).

The retention study was carried out using the batch method, when samples of about 0.3–0.5 g sorbent (untreated or modified) were equilibrated with 50 mL protein solution containing variable amounts of active substance, at 18°C. After 1 hour, the phases were separated by filtration and the filtrate was analysed using the Lowry method of water soluble protein determination [5]. The optical density of filtered solution was determined at the wavelength corresponding to the sorption
Table 1
The characteristics of working solutions

<table>
<thead>
<tr>
<th>Biochemical compound</th>
<th>Concentration of stock solutions, [µg/mL]</th>
<th>Concentration of works solutions, [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>0.5</td>
<td>25–100</td>
</tr>
<tr>
<td>α-amylase</td>
<td>200.0</td>
<td>10–40</td>
</tr>
<tr>
<td>lysozyme</td>
<td>200.0</td>
<td>1–40</td>
</tr>
</tbody>
</table>

maximum of amine copper (II) complexes, λ = 500 nm, with respect to Lambert-Beer law, using an UV-VIS Spectrophotometer of Jenway 6105 type.

RESULTS AND DISCUSSION

Our previous studies concerning enzymes retention on functionalized – cellulose modified with reactive dyes considered the factors that determined the process and also thermodynamic and kinetic measurements are confirmed by the IR spectroscopy. The IR bands for hydrogen bonding both intramolecular and intermolecular are responsible for the efficiency of the separation – concentration processes. These interactions determine the retention rate on sorbents and even the resolution of the process. The interactions of the cellulosic sorbents with the reactive dyes determine some IR characteristic for hydrogen bondings.

Activation by different processes depends on the structure of the activating molecules, on their ionization potential, on the possibility of hydrogen bondings with OH – groups from the sorbents.

In the IR spectrum of multifunctionalized cellulose modified with reactive dye and enzyme, is observed that because of a small value for the sorbit concentration, the registered modifications are moved to higher wave numbers.

SORPTION EQUILIBRIUM

The unretained amount of protein after sorption process was calculated on basis of absorbance values from calibration curve in aqueous solution.

Results of experimental studies were expressed as µg of protein taken up per 1 g sorbent, that, in fact, is Langmuir sorption isotherm and they are shown in Fig. 5.

The quantitative evaluation of sorption process was carried out using the Langmuir isotherm model. The Langmuir expression was represented in this work by equation (1) [6].
Fig. 5. – Langmuir isotherms for lysozyme sorption on: 1) GE – cellulose modified with the reactive dye Red ME-BA. 2) GE – cellulose modified with the reactive dye Violet ME-RN.

\[ q = \frac{K \cdot c_e \cdot q_0}{1 + Kc_e} \]  

where: 
- \( K \) = sorption constant, mL/µg; 
- \( c_e \) = concentration of protein in solution at equilibrium, µg/mL 
- \( q \) = the amount of retained protein, µg/g sorbent; 
- \( q_0 \) = maximum capacity of sorption, µg/g sorbent.

The linear Langmuir plot of \( 1/q \) vs. \( 1/c_e \), shown in Fig. 6, allows to calculate the sorption capacity from the intercept and the sorption constant from the slope. The \( q_0 \) and \( K \) values thus derived are given in Table 2 [7–9].

The characteristics of Langmuir isotherm can be expressed by a dimensionless constant separation factor or equilibrium parameter “\( a \)” which is defined by Equation (2), and the “\( a \)” values are shown in Table 2 [5].

\[ a = \frac{1}{1 + K \cdot c_0} \]  

where \( c_0 \) is initial protein concentration, µg/mL; \( K \) is sorption constant, determined graphically from the linear Langmuir plots.

The parameter indicates the shape of the isotherms as follow [6]:
Fig. 6. – Linear Langmuir isotherms for α-amylase sorption on: 1) GE – cellulose modified with the reactive dye Red ME-BA; 2) GE – cellulose modified with the reactive dye Violet ME-RN.

VALUES OF “α”

<table>
<thead>
<tr>
<th>α</th>
<th>Type of isotherm</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 1</td>
<td>Unfavourable</td>
</tr>
<tr>
<td>1</td>
<td>Linear</td>
</tr>
<tr>
<td>0 &lt; α &lt; 1</td>
<td>Favourable</td>
</tr>
<tr>
<td>0</td>
<td>Irreversible</td>
</tr>
</tbody>
</table>

Table 2

The parameters of sorption processes

<table>
<thead>
<tr>
<th>Biochimic compound</th>
<th>Support</th>
<th>$K$ [mL/µg]</th>
<th>$q_0$ [µg/g]</th>
<th>α</th>
<th>$C_0$ [µg/mL]</th>
<th>$\Delta G$ [J/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>AE-Red ME-BA</td>
<td>$11.2 \times 10^{-3}$</td>
<td>2000.00</td>
<td>0.151</td>
<td>500</td>
<td>$-10.7 \times 10^3$</td>
</tr>
<tr>
<td>α-amylase</td>
<td>GE-Red ME-BA</td>
<td>0.4</td>
<td>1538.46</td>
<td>0.012</td>
<td>200</td>
<td>$-22.17 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>GE-Violet ME-RN</td>
<td>0.227</td>
<td>4000.00</td>
<td>0.021</td>
<td>200</td>
<td>$-3.59 \times 10^3$</td>
</tr>
<tr>
<td>lysosime</td>
<td>GE-Red ME-BA</td>
<td>1.111</td>
<td>2222.22</td>
<td>0.005</td>
<td>200</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>GE-Violet ME-RN</td>
<td>$3.33 \times 10^{-4}$</td>
<td>25000.00</td>
<td>0.937</td>
<td>200</td>
<td>$-19.37 \times 10^3$</td>
</tr>
</tbody>
</table>

Referring to Table 2 it is obvious that the sorption of biochemical compounds is favourable on both tested sorbents.

Interpreting the lysosime sorption on GE cellulose modified with reactive dye Red ME-BA using the Freundlich isotherm, equation 3 [10].
Hence a plot of $\log q_e$ vs. $\log c_e$ as shown in Fig. 7 is linear, with intercept and the slope related to Freundlich constants ($K_F$ and $n$ respectively).

![Fig. 7. – Linear Freundlich isotherm for lysozyme sorption on GE-cellulose modified with the reactive dye Red ME-BA.](image)

The values of Freundlich constants $K_F = 0.67$ and $n = 6.8$ respectively have been derived.

$$\log q_e = \log K_F + \frac{1}{n} \log c_e$$  \hspace{1cm} (3)

where:  
$q_e$ = the amount of retained dye at equilibrium, µg/g;  
$K_F$ = sorption constant, ml/µg;  
$c_e$ = enzyme concentration at equilibrium, µg/ml;  
$1/n$ = constant

The $K_F$ and $n$ values derived from the Freundlich isotherm are given in Table 2. The $n$ value compared to the condition of $n > 1$ required in literature suggest a favourable sorption in the cellulosics modified – enzyme system.

**THERMODYNAMIC STUDY**

The equilibrium constant $K$ in the Langmuir equation can be used to determined the Gibbs free energy of sorption ($\Delta G$) using equation 4:

$$\Delta G = -RT \ln K$$ \hspace{1cm} (4)

where:  
$R$ = constant ($R = 8.314$ J/mol)  
$T$ = absolute temperature.
The $\Delta G$ values shown in Table 2, point out a spontaneous sorption ($\Delta G < 0$) for enzymes on celluloses modified with reactive dyes tested [6–9].

**KINETIC STUDY**

The experiments have been conducted by mixing 0.2 g of modified sorbent (AE cellulose) with 200 mL of casein solution of 65.8 $\mu$g/mL concentration. At settled periods of time (1, 3, 5, 7, 10, 15 20, 35, 45, 60 minutes) have been taken of samples of 0.5 mL of solution which they have been diluted to the requisite volume of 25 mL.

The adsorption rate constant of casein on AE – cellulose modified with reactive dye Red ME-BA was obtained using rate equation of Langergren (5) [10]:

$$\log(q_e - q) = \log q_e - \frac{k'}{2.303} t$$

(5)

where: $q_e$ and $q$ are the amount of casein sorbed (µg/g) at equilibrium and respectively at time $t$; $k'$ is the rate constant.

The $k'$ values were calculated from the slopes of the linear plots of the log($q_e - q$) vs. $t$, shown in Fig. 8, and the $q_e$ values were calculated from the Langmuir equation [5].

The $k'$ values from the sorption of casein on cellulose modified with reactive dye are $6.14 \times 10^{-2}$ minute$^{-1}$.

According to the literature data, if the rate limiting step is intraparticle diffusion, a plot of casein adsorbed ($q$) against square root of contact time ($t^{1/2}$) should yield a straight line passing the origin.

![Fig. 8. – Langergren plot for the sorption of casein by AE celluloses modified with reactive dye Red ME-BA.](image)
These plots (Fig. 9) give straight line at these concentration pass through the origin showing that the intraparticle diffusion is the only rate controlling step.

![Graph](image)

The rate constant of intraparticle diffusion \((k_p)\) were calculated from slopes of the linear portion of the plots \(q\) vs. \(t\) (Fig. 9) at these concentration, and are \(k_p = 6.25 \mu g/g/\text{minute}^{1/2}\).

On the other hand, the new biomimetic dyes are able to discriminate between different enzymes, since each dye recognizes and binds selectively one of the two targeted enzymes.

The bound enzyme can be desorbed from the respective affinity column biospecifically in high purity and yield [9].

Biomimetic dyes and ligands in general, are expected to continue to play an important role as affinity chromatography tools in protein purification.

Biocomputing offers a powerful approach to biomimetic dye ligand design. The exploitation of computational techniques in molecular design requires the knowledge of the three-dimensional structure of the target protein, or at least, the amino acid sequence of the target protein and the three-dimensional structure of a highly homologous protein. There are several examples of enzyme purifications (trypsin, urokinase, kallikrein, alkaline phosphatase, malate dehydrogenase, formate dehydrogenase, oxaloacetate decarboxylase and lactate dehydrogenase) where synthetic biomimetic dyes have been used as affinity chromatography tools [11, 12].

CONCLUSIONS

The cellulose modified with the bifunctional chlortriazine Red ME-BA and Violet ME-RN proved to be an effective sorbent in removal of proteins traces from biological liquids.
The modified support has a higher capacity for protein retention than that of the pure cellulose.

As follows from the data, all the values found for rate constants, ΔG, Langmuir and Freundlich constants, makes it possible for cellulose modified with rective dye Red ME-BA and Violet ME-RN as an effective sorbents for the proteins water soluble removal.

The reactive dyes can be viewed as an example of a group – specific ligand that can bind more than one protein.

The multifunctionalized cellulosic sorbents modified with reactive dyes are very actual in the separation and / or concentration processes of some protein substances, and also in the extraction process of some active principles in biotechnological processes.

REFERENCES