Choosing the right chromatographic support in making a new acetylcholinesterase-micro-immobilised enzyme reactor for drug discovery

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Abstract

The aim of the present study was to optimize the preparation of an immobilized acetylcholinesterase (AChE)-based micro-immobilized enzyme reactor (IMER) for inhibition studies. For this purpose two polymeric monolithic disks (CIM, 3 mm × 12 mm i.d.) with different reactive groups (epoxy and ethylendiamino) and a packed silica column (3 mm × 5 mm i.d.; Glutaraldehyde-P, 40 μm) were selected as solid chromatographic supports. All these reactors were characterized in terms of rate of immobilization, stability, conditioning time for HPLC analyses, optimum mobile phase and peak shape, aspecific interactions and costs. Advantages and disadvantages were defined for each system. Immobilization through Schiff base linkage gave more stable reactors without any significant change in the enzyme behaviour; monolithic matrices showed very short conditioning time and fast recovery of the enzymatic activity that could represent very important features in high throughput analysis and satisfactory reproducibility of immobilization yield. Unpacked silica material allowed off-line low costs studies for the optimization of the immobilization step.
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1. Introduction

The drive to bring innovative drugs to market faster, without negatively impacting quality and safety, has induced to look for new strategies and associated methodologies. In fact, key aspects of the discovery process include the development of rapid methodologies (high throughput screening) for the assessment of biological activity, the analysis of drug-biomolecules interactions and the determination of the physico-chemical properties of drug candidates as predictors of administration, distribution, metabolism, excretion (ADME) characteristics. In particular, affinity chromatography on HPLC-immobilized enzyme reactors (IMERs) proved to be a promising high throughput screening methodology for the selection of active candidates (lead compounds) [1]. These reactors have proven to be useful alternatives to conventional methods in the field of drug discovery and analysis. In particular, IMERs have been applied to drug metabolism studies [2–6], enantioselective analyses [7–12] and for the identification of substrates and inhibitors as potential drugs [1]. Attractive features of immobilized enzyme reactors are the increased enzyme stability (i.e. in the case of the immobilization of glyceraldehyde-3-phosphate dehydrogenase [13]) and the reusability coupled to accuracy, automation and potential high throughput when they are inserted in a HPLC system. Considering the high cost and difficulty in over-expression, isolation and purification of recombinant enzymes, this analytical technique represents an extremely useful approach to preserve the activity of the small amount of enzyme available, to perform kinetic studies and to rapidly screen for potential drug candidates.

The immobilization procedure is always a process which may affect enzyme stability due to the "not natural" microenvironment in which it is placed. Therefore, in the design of a new bioreactor, the specific choice of the used solid matrix

for enzyme immobilization constitutes a critical step to ensure high yields, low non specific interactions with substrate, products and inhibitors and still represents object of intense research. Moreover, the insertion of the bioreactor in a HPLC system to obtain a flow through IMER adds friction stress, diffusion restrictions and non-specific interactions that may affect the catalytic behaviour. In this view, the evaluation of the kinetic parameters and the response to well-known inhibitors can give information about the altered or unaltered behavior of the immobilized enzyme. The aim of the present study was to prepare and compare the performances of immobilized acetylcholinesterase (AChE)-based micro-IMERs (containing micrograms of immobilized enzyme) for fast inhibition studies. AChE catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline and acetic acid and represents a widely studied target in Alzheimer's disease drug discovery. AChE possesses a remarkably high specific activity, functioning under biomolecular conditions at a rate approaching the diffusion controlled limit [14]. Due to the rapidity by which the enzyme is able to hydrolyse the specific substrate and the high sensitivity of the HPLC system in which the IMERs will be inserted, few micrograms of AChE should be suitable for high signal and fast analyses. Taking into account these considerations, a new IMER for AChE inhibitors screening was previously developed by us using a monolithic support [15]. In order to lower costs and optimize several parameters such as the yield of the reaction in terms of the active units retained onto the stationary phase, peaks shape and the matrix/ligand aspecific interactions we compared some commercially available chromatographic supports suitable, in our opinion, to provide high rate of immobilization, limited perturbation of enzyme properties and fast analyses. In particular two polymeric monolithic disks (CIM, 3 mm × 12 mm i.d.) characterized by different chemistries (epoxy and ethylenediamino) and a packed silica column (3 mm × 5 mm i.d.) based on a wide-pore affinity matrix (Glutaraldehyde-P, 40 μm) were selected (Table 1). Monolithic columns were chosen because they represent a suitable support for the immobilization of enzymes and fast conversion of substrates due to the almost complete lack of diffusion resistance during mass transfer. As comparison a particle silica chromatographic support available as bulk material was taken into consideration. These reactors were characterized in terms of rate of immobilization, immobilized enzyme stability, conditioning time for HPLC analyses, optimum mobile phase and peaks shape, aspecific interactions and costs. Advantages and disadvantages were defined for each system.

2. Experimental

2.1. Materials

EDA-CIM Disks (3 mm × 12 mm i.d.) and Epoxy-CIM Disks (3 mm × 12 mm i.d.) were purchased from BIA Separations (Ljubljana, Slovenia), BakerBond Wide-Pore Glutaraldehyde-P 40 μm affinity packing (Glut-P) was obtained from J.T. Baker (Phillipsburg, NJ, USA). (*S*)-Acetylthiocholine iodide (ACTh), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), glutaraldehyde 70% aqueous solution, propidium iodide and human recombinant acetylcholinesterase (AChE, EC 3.1.1.7) lyophilized powder were purchased from Sigma (Milan, Italy). Tacrine (9-amino-1,2,3,4-tetrahydro acridine hydrochloride), edrophonium chloride and monoethanolamine were obtained from Aldrich Italia (Milan, Italy). Donepezil was a kind gift from Pfizer. Ambenonium chloride pentahydrate and galathamine hydrobromide were purchased from Tocris Cookson (UK). Potassium chlorate and sodium cyanoborohydride were obtained from Fluka (Milan, Italy) and magnesium sulfate from Merck (Darmstadt, Germany).

HPLC-grade methanol (Romil, UK) or bidistilled water were used to prepare the inhibitors' solutions. Purified water from a TKA ROS 300 system was used to prepare buffers and standard solutions. To prepare the buffer solutions, potassium dihydrogenphosphate, dipotassium hydrogenphosphate trihydrate, Tris–HCl (Carlo Erba, Milan, Italy) of analysis quality were used.

The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC.

2.2. Apparatus

Spectrophotometric determinations were performed using a Jasco double beam V-530 UV–vis spectrophotometer, with a slit width of 2 nm and 0.5 s data pitch.

Table 1
Characteristics of the solid matrices used for AChE immobilization

|	Epoxy-CIM disk	EDA-CIM disk	Glut-P
Reactive groups	Epoxy groups	Aldehydic groups after activation by glutaraldehyde	Aldehydic groups
Material	Poly(glycidilmathacrylate-co-etyleneglycoldimethacrylate)	Poly(glycidilmathacrylate-co-etyleneglycoldimethacrylate)	Synthetic amorphous silica gel, particle size 40 μm, pore size 300 Å
Dimension of the IMER (length × i.d.)	3 mm × 12 mm	3 mm × 12 mm	3 mm × 35 mm
Bed volume (mL)	0.34	0.34	0.06
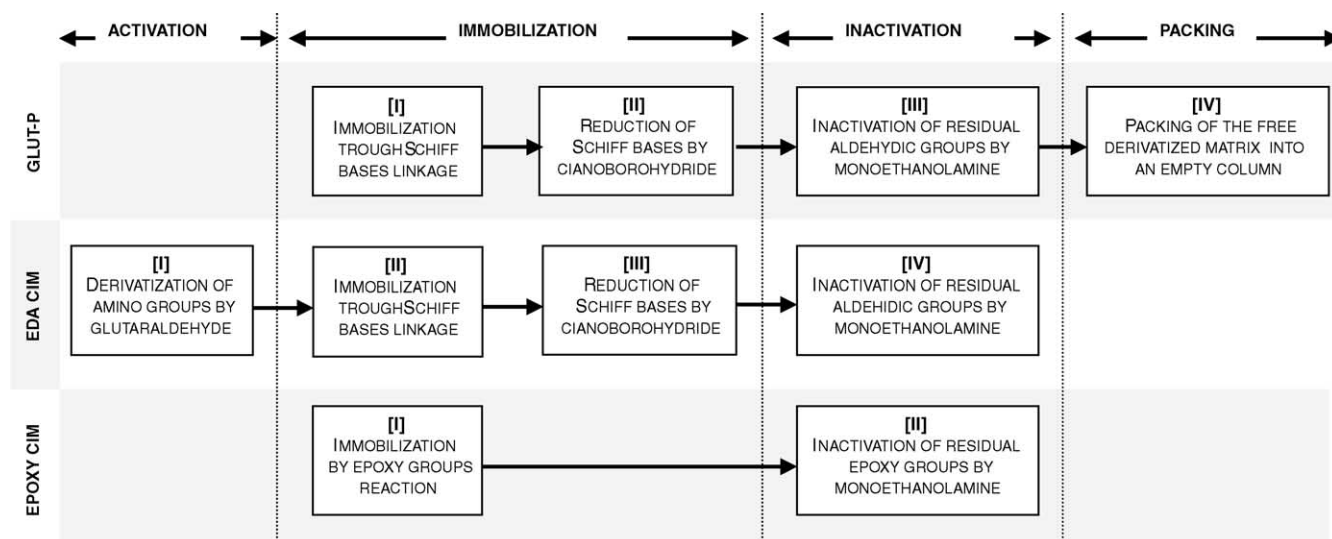
Fig. 1. Scheme of AChE immobilization procedure on chosen chromatographic supports.

The solvent delivery system was a Jasco BIP-I HPLC pump equipped with a Rheodyne Model 7125 injector with a 10 μL sample loop. The eluates were monitored by a Jasco 875-UV Intelligent UV–vis detector connected to a computer station (JCL 6000 program for chromatographic data acquisition). For routine analyses the detector wavelength was set at 450 and 480 nm.

The chromatographic analyses on AChE-IMERs were performed at 25 °C unless otherwise stated.

2.3. AChE immobilization

2.3.1. Immobilization on EDA-CIM disk

EDA-CIM-IMER was prepared as previously reported by us [15]. The followed procedure is summarized in Fig. 1.

2.3.2. Immobilization on Epoxy-CIM disk

AChE was immobilized on Epoxy-CIM disk by following the procedure proposed by the factory slightly modified. The Epoxy-CIM disk was placed in a glass beaker and washed with 10 mL phosphate buffer (100 mM, pH 8.0) followed by a washing step with phosphate buffer (100 mM, pH 8.0) containing ammonium sulfate 1.25 M. An aliquot of 7.0 μL of AChE solution in phosphate buffer (0.269 M, pH 8.0) (1.74 U/μL) was diluted to 800 μL with phosphate buffer [100 mM, pH 8.0] containing ammonium sulfate 1.25 M, was added to the matrix and left to react overnight. After immobilization, the enzyme solution was analyzed with the Ellman's assay in order to determine the unreacted enzyme units.

The matrix was then washed with phosphate buffer (20 mM, pH 8.0) and stirred for 3 h with 0.2 M monoethanolamine solution in phosphate buffer (20 mM, pH 8.0) at room temperature.

The Epoxy-CIM disk was then washed with phosphate buffer (100 mM, pH 8.0), inserted in the appropriate holder, connected to the HPLC system and conditioned with a mobile phase consisting of phosphate buffer (100 mM, pH 8.0) for 1 h at a flow rate of 0.8 mL/min and the enzymatic activity was determined as reported in Section 2.4.

2.3.3. Immobilization on Glutaraldehyde-P silica matrix and preparation of Glut-P-IMER

AChE was immobilized onto the Glut-P stationary phase using a modification of the procedure initially reported by Narayanan et al. [16] and optimised for our target enzyme [17]. Briefly, Glut-P stationary phase (30 mg) was placed in a 1.5 mL eppendorf tube and washed three times with 1 mL of potassium phosphate buffer (50 mM, pH 7.4). Washings were carried out by the addition of 1 mL of buffer to the packing material, the resulting mixture was vortex-mixed, centrifuged for 30 s at 7500 g and the supernatant was discarded. An aliquot of 7.0 μL of AChE solution in phosphate buffer (0.269 M, pH 8.0) (1.74 U/μL) was diluted to 600 μL with phosphate buffer (50 mM, pH 5.0), was added to the matrix and gently agitated 8 h at 4 °C. The mixture was then centrifuged and the supernatant was removed. The remaining solid support was washed three times with 1 mL aliquots of phosphate buffer (50 mM, pH 6.0). The amount of protein immobilized on Glut-P was determined by measuring the residual enzyme activity present in all supernatants by using Ellman's colorimetric assay.

The Schiff bases formed during the immobilization procedure were reduced using 1.0 mL of sodium cyanoborohydride [100 mM in buffer (50 mM, pH 6.0)] [16]. The cyanoborohydride solution was added to the matrix and allowed to react for 2.0 h at room temperature. After washing the residual aldehydic functions were inactivated by reaction with monoethanolamine 200 mM in potassium phosphate buffer (50 mM, pH 6.0) for 3.0 h at room temperature. Finally, the matrix was washed three times with 1 mL of phosphate buffer (50 mM, pH 7.4).

A HR 5/2 glass column (25 mm × 5 mm i.d.) purchased from Amersham Biosciences Europe (Cologno Monzese, Italy) was used to prepare the Glut-P-IMER for on-line studies. The column was washed with bidistilled water followed by phosphate buffer (50 mM, pH 7.4). A suspension containing 30 mg of AChE-Glut-P in phosphate buffer (50 mM, pH 7.4) was then added to the column. The top of the column was fastened and 20 mL phosphate buffer (50 mM, pH 7.4) were pumped through the packed column at a flow rate of 0.4 mL/min. The resulting Glut-P-IMER had a 3 mm × 5 mm i.d. chromatographic bed and a volume of 0.05 mL.

2.4. Determination of immobilized AChE activity

The AChE-IMERs were conditioned with the optimised mobile phases, namely Tris–HCl buffer (0.1 M, pH 8.0) containing Ellman's reagent (1.26×10^{-4} M), MgSO$_4$ (10 mM), KClO$_3$ (100 mM) (buffer A) for EDA-CIM disk based IMER, Tris–HCl buffer (0.1 M, pH 8.0) containing Ellman's reagent (1.26×10^{-4} M) (buffer B) for Epoxy-CIM-IMER; potassium phosphate buffer (0.05 M, pH 7.4) containing Ellman's reagent (2.52×10^{-4} M), MgSO$_4$ (10 mM), KClO$_3$ (100 mM) (buffer C) for Glut-P based IMER. Aliquots of 10 μL ACTh aqueous solution at increasing concentration (range comprised between 3.1 and 250 or 400 mM depending on the IMER), were injected in the HPLC system, at a flow rate of 1.0 mL/min and UV detection at 450 or 480 nm. Thiocholine, as the product of enzymatic reaction, reacted with Ellman's reagent in the mobile phase by forming a mixed disulfide and a yellow anion [15], which is stoichiometrically related to the amount of the substrate hydrolyzed.

In order to account for μmol ACTh hydrolyzed, the corresponding eluates for each substrate injection were collected in 5 mL volumetric flasks during 5 min of chromatographic elution. The absorbance at 412 nm of relative eluates were acquired by spectrophotometric analysis, using the mobile phase as blank. By dividing the absorbances for the contact time (i.e. 0.34 min for CIM disks and 0.06 min for Glut-P-IMER), the catalysis rates (ΔA/min) were derived. By plotting the catalysis rates versus the injected substrate concentrations, a Michaelis–Menten plot was obtained and K_m and V_{max} derived. As already reported [15], from the V_{max} value the immobilized active units were determined.

2.5. Optimization of the chromatographic conditions

2.5.1. Mobile phase pH and composition
Mobile phases were optimised in terms of type of buffer, salt concentration and pH. Two types of buffers were screened, i.e. phosphate buffer (generally used for the activity assay on the free enzyme) and Tris–HCl buffer. Potassium chlorate (0–200 mM) as a selective anion exchanger competitor for the protonated amine groups of EDA-IMER disk and Glut-P-IMER, magnesium sulfate (0–20 mM) as enzyme activator and Ellman's reagent concentration (0.079×10^{-4}

to 5.04×10^{-4} M) were evaluated. The peak symmetry and peak area obtained by the injection of a fixed saturating ACTh concentration were determined for each buffer type and additive concentration, by using a flow rate of 1 mL/min and UV detection at 450 or 480 nm depending on the rate of the enzymatic reaction.

The pH of the mobile phase was set on the basis of the optimum activity of the target enzyme, namely pH 8.0, and the reported stability of the solid support. The effect of the pH on the IMERs' activity was evaluated by injecting fixed ACTh concentration in triplicate onto the HPLC with a flow rate of 1.0 mL/min and UV detection at 450 nm.

2.5.2. Flow rate
A fixed acetylthiocholine concentration (250 mM) was injected in triplicate onto the HPLC with a flow rate ranging between 0.2 and 1.4 mL/min with UV detection at 450 or 480 nm. The product peak area was integrated and plotted against the used flow rate.

2.5.3. Conditioning time
Time required for the conditioning of the three IMERs was evaluated by injecting 10 μL of a fixed acetylthiocholine concentration each 5–10 min after connecting the IMER to the HPLC system. Flow rate 1.0 mL/min, $T = 25\,^{\circ}$C.

2.6. Determination of inhibitory potency (IC$_{50}$)

Stock solutions of the test compounds (1–10 mM) were prepared in water or methanol. The assay solutions were prepared by diluting the stock solutions in water together with the substrate acetylthiocholine at saturating concentration. In particular five different concentrations of each compound were mixed together with the substrate in order to obtain inhibition of AChE activity comprised between 20 and 80%.

The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (A_i/A_o \times 100)$ where A_i is the peak area calculated in the presence of inhibitor and A_o is the peak area obtained with the substrate solution only. Inhibition curves were obtained for each compound by plotting the percent inhibition versus the logarithm of injected inhibitor concentration. The linear regression parameters were determined for each curve and the IC$_{50}$ extrapolated.

2.7. Immobilized AChE stability

AChE-IMERs stability was determined by using the chosen optimized mobile phase and injecting every day 10 μL of ACTh saturating aqueous solution under optimized flow and detection conditions. The micro-IMERs were always stored at 4 $^{\circ}$C in phosphate buffer (20 mM, pH 7.4) containing 0.1% (w/v) sodium azide when not in use.

3. Results

3.1. AChE immobilization and IMERs development

In a previous paper [15] we reported the immobilization of human recombinant AChE on a monolithic ethylenediamino disk (12 mm × 3 mm i.d.) by in situ technique after matrix activation. A buffered solution containing 15 U of active AChE (corresponding to ∼12.4 µg of protein) was used for IMER preparation. The proposed immobilization procedure gave a 3.0% yield and was found appropriate to covalently retain 0.22 ± 0.01 U of active AChE.

The same amount of enzyme was then used to prepare the two new IMERs. In particular, by following the immobilization protocol suggested by the manufacturing company, we developed a second IMER on the basis of a simpler one step reaction on an epoxy-monolithic disk, followed by inactivation of residual active groups. The pH 8.0 phosphate buffer was chosen for the immobilization because it resulted suitable for target enzyme stability and for the coupling reaction. A scheme of the AChE immobilization on Epoxy-CIM disk is reported in Fig. 1. Under the conditions reported in the Experimental section, the immobilization yield was found around 3.0% and 0.18 ± 0.01 U were retained on the epoxy matrix. A different approach was instead used for the preparation of the Glut-P-IMER. Glutharaldehyde-P is a wide pore silica support commercially available as bulk material that offers the possibility of performing a large number of parallel studies in batch-wise (off-line studies). Therefore, batch-wise experiments were previously performed in eppendorf tubes in order to optimize the immobilization conditions (i.e. optimum pH of immobilization, enzyme/matrix ratio, time of incubation). An aliquot of 30 mg of Glut-P, pH 5.0 and 8 h of incubation were found to be suitable conditions for the immobilization. The obtained derivatized matrices were characterized by off-line studies [17] and then were packed into an empty column (Amersham Biosciences) to obtain the Glut-P-IMER. The amount of active AChE immobilized units determined in flow-trough conditions after insertion into a HPLC system resulted 4.35 ± 0.01 (immobilization yield: 29%).

3.2. On-line AChE-IMER chromatographic system

As the product of the enzymatic hydrolysis, thiocholine does not present a significant chromophore for UV detection, the evaluation of enzyme activity was performed by injecting acetylthiocholine with the Ellman's reagent dissolved in the mobile phase and monitoring the amount of 5-thio-2-nitrobenzoic acid (yellow anion) which is formed by the reaction of thiocholine and Ellman's reagent [18]. The effect of the pH, composition and salts concentration of mobile phase on the catalytic activity was studied. As already reported, optimal chromatographic conditions for EDA-IMER were obtained with a mobile phase consisting of 0.1 M Tris–HCl pH 8.0 containing 100 mM $KClO_3$ as

selective competitive anion for the cationic sites on the matrix, 10 mM $MgSO_4$, 1.26×10^{-4} M Ellman's reagent [15] These conditions gave rise to a symmetric peak, completely eluted in two min. In the attempt of obtaining the same fast analysis on the two newly prepared IMERs, buffer type and salts concentration were evaluated. In particular, concerning Epoxy-IMER, two different buffers were used: potassium phosphate buffer and Tris–HCl buffer. The type of buffer did not affect either the enzymatic activity either the peak shape: Tris–HCl buffer [pH 8.0] was chosen for a direct comparison with EDA-IMER. Increasing buffer concentration (from 5 up to 200 mM) improved peak shape. A 100 mM Tris–HCl buffer was chosen in order to have a symmetric peak and to prevent salt precipitation into the HPLC system. No further salt addition was needed. The optimized mobile phase for Epoxy-IMER consisted of 100 mM Tris–HCl buffer pH 8.0 containing 1.26×10^{-4} M Ellman's reagent (buffer B).

Analogously for Glut-P packed IMER, potassium phosphate buffer and Tris–HCl buffer were compared. Moreover, the addition of potassium chlorate (0, 50, 100, 150, 200 mM) and magnesium sulfate (0, 10, 20 mM) were investigated. Finally, the Ellman's reagent concentration was increased in the mobile phase up to a stable peak area value. The wavelength of detection was fixed at 490 nm due to the higher rate of catalysis of this IMER. In general, buffer type did not affect the enzymatic activity and phosphate buffer was chosen for direct comparison with conditions employed in free enzyme assay but identical results were found by using Tris–HCl; higher buffer concentration gave sharper peak by favoring the displacement of the yellow anion from the chromatographic support. The optimized mobile phase for Glut-P-IMER consisted of 50 mM potassium phosphate buffer pH 8.0 containing 100 mM $KClO_3$, 10 mM $MgSO_4$, 2.52×10^{-4} M Ellman's reagent (buffer C).

By using the optimized mobile phases, the three IMERs gave comparable fast analyses (Fig. 2) completed in less then 5 min.

Temperature, pH and flow rate variations were also evaluated in order to account for matrix effect on the enzyme behavior. Concerning flow rate, as shown in Fig. 3, the acetylthiocholine catalytic conversion into its colored anion induced by the three IMERs is flow dependent in exponential decay mode. These flow dependent trends reach a stable plateau (constant product formation by increasing the flow rate) in a faster way in the case of the monolithic matrices due to the less flow resistance and substrate/product stagnation into the silica pores. No significant differences among the three IMERs were noticed for temperature and pH variations. Conditioning time for the three columns was also investigated being a very important parameter for reproducible data and high throughput analysis. The activity was assayed by injecting saturating concentration of substrate every 5–10 min for up to 200 min at 25 °C. CIM based IMERs required 5 min at 1 mL/min flow rate to be conditioned. On the contrary, the silica based IMER needed longer conditioning time to give reproducible results (Fig. 4).
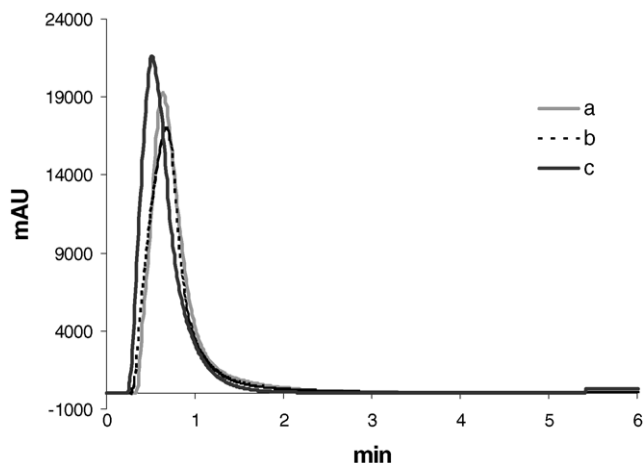
Fig. 2. Overlaid chromatograms obtained after injection of substrate at saturating concentration (150 mM) into the three IMERs (flow rate 1.0 mL/min): (a) EDA-CIM–IMER, mobile phase: buffer A, $\lambda = 450$ nm; (b) Epoxy-CIM-IMER, mobile phase: buffer B, $\lambda = 450$ nm; and (c) Glut-P-IMER, mobile phase: buffer C, $\lambda = 490$ nm.

The increasing of the temperature from 25 to 30 °C did not shorten column conditioning. A 60 min conditioning step was chosen for daily use allowing 95% of the maximal activity.

3.3. On-line determination of the retained enzymatic activity

The enzymatic activity of the three AChE-IMERs was determined by following the same method previously developed to characterize EDA-IMER [15]. Briefly, the area of the yellow anion formed by reaction between thiocholine, produced by enzymatic hydrolysis, and Ellman's reagent was correlated to the AChE activity. Michaelis–Menten plots for the IMERs were obtained by injecting 10 µL of acetylthiocholine aqueous solutions at increasing concentration and collecting eluates during the 5 min run [15]. Injected ACTh solutions ranged from 3.1 mM to 250–400 mM
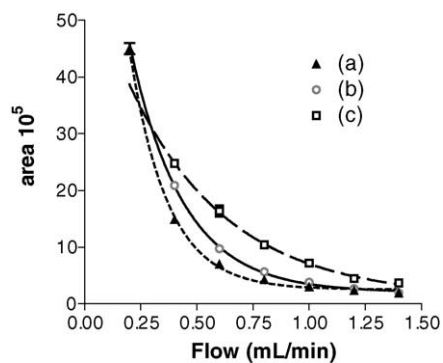

Fig. 3. Effect of mobile phase flow rate on product peak area after acetylthiocholine injection. Studies were performed in the optimized chromatographic conditions for the three IMERs: (a) EDA-IMER, mobile phase consisting in buffer A, $\lambda = 480$ nm; (b) Epoxy-CIM-IMER, mobile phase consisting in buffer B, $\lambda = 465$ nm; and (c) Glut-P-IMER, mobile phase consisting in buffer C, $\lambda = 450$ nm.
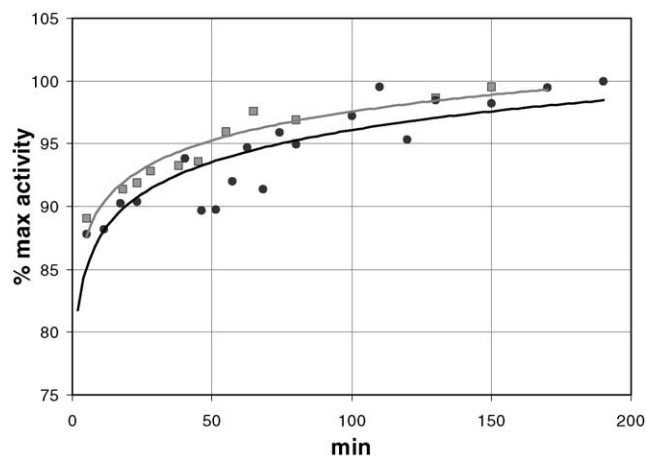

Fig. 4. Conditioning time for Glut-P-IMER. IMER's activity calculated as percent of maximal activity after 150 mM acetylthiocholine injection over time. At $t = 0$ insertion of the column into the HPLC system. Working temperature = 25 °C. The two plots are from two independent experiments.

depending on the IMER. Saturation in the Michaelis–Menten plot was reached at 150 mM acetylthiocholine concentration for EDA and Epoxy-CIM disks and 200 mM for Glut-P-IMER. These saturating concentrations were used in inhibition and kinetic studies. Lineweaver–Burk reciprocal plots of 1/activity and 1/[substrate] allowed to estimate the value of K_m and V_{max} for the substrate (Fig. 5). The apparent affinities (K_m) of ACTh for AChE in the EDA, Epoxy and Glut-P-IMER format were, respectively, 14.39 ± 2.06 mM, 9.28 ± 1.50 mM and 41.59 ± 2.42 mM. The amount of hydrolyzed substrate in steady state conditions (μmol_{max}) was also calculated from Michaelis–Menten plots and resulted
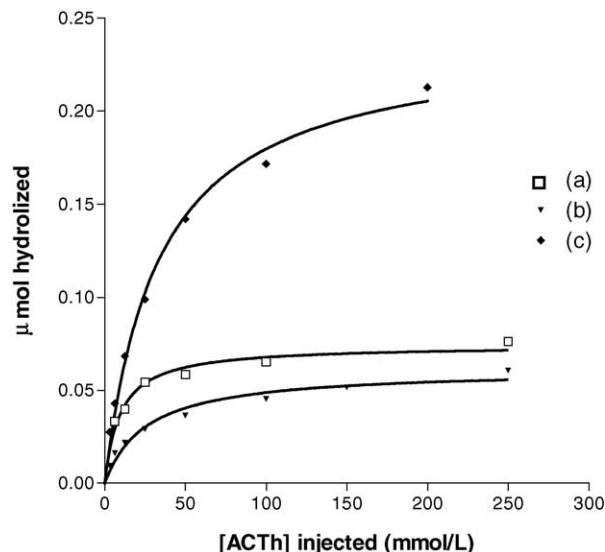

Fig. 5. Overlaid Michaelis–Menten plots for (a) EDA-IMER; (b) Epoxy-IMER; and (c) Glut-P-IMER showing the µmol of hydrolyzed substrate vs. injected ACTh concentration. Reported values are the mean of two or three independent experiments in which each injection was in duplicate.

Table 2
Kinetic and chromatographic parameters determined for the three AChE-IMERs

	K_m (mM)	t_r (min); peak width	Bed volume (mL)	μmol_{max} ACTh hydrolyzed	Units (μmol_{max}/CT)	K_m/U (mM/U)
EDA-CIM IMER	14.39 ± 2.06	0.67; 1.8	0.34	0.074 ± 0.008	0.22 ± 0.01	65.4
Epoxy-CIM IMER	9.28 ± 1.50	0.69; 2.0	0.34	0.062 ± 0.007	0.18 ± 0.01	51.5
Glut-P IMER	41.59 ± 2.42	0.53; 2.0	0.06	0.257 ± 0.001	4.35 ± 0.01	9.56

0.074 ± 007, 0.062 ± 008, 0.257 ± 0.001 for EDA-, Epoxy- and Glut-P-IMER, respectively (Table 2).

By general definition, the units of active immobilized enzyme would be defined by μmol of substrate hydrolyzed per unit of time in steady state conditions. In the ideal case of no stagnation, mass transfer limitation phenomena or matrix/substrate interactions, the contact time (CT) between enzyme and substrate will be related only to flow rate (FR) and bed volume (BV) by the simple equation CT = BV/FR. Therefore, theoretical CT was calculated for the three IMERs and resulted 0.34 min for CIM disks and 0.06 min for Glut-P-IMER (Table 2). The amount of active units retained after immobilization was therefore obtained by dividing μmol_{max} by CT and resulted to be 0.22 ± 0.01 U; 0.18 ± 0.01 U and 4.35 ± 0.01 U on EDA-, Epoxy- and Glut-P-IMER, respectively (Table 2).

3.4. Stability of immobilized enzyme

The stability of the AChE-IMERs was investigated by injecting 10 μL of saturating substrate solution every day. The enzymatic activity was preserved by using phosphate buffer [pH 7.4] containing 0.1% (w/v) sodium azide as storage buffer and keeping the IMERs at 4 °C when not in use. These conditions previously showed to be optimal in the storage of the EDA-IMER [15] and were chosen to store the two newly prepared IMERs. Under these storage conditions, over 80% of the initial activity was retained on EDA and Glut-P IMERs up to two months. Epoxy-CIM IMER showed an higher instability and just 30% of the initial activity was retained after 40 days of continuous use (Fig. 6). This storage buffer showed to be also suitable for long term storage, i.e.
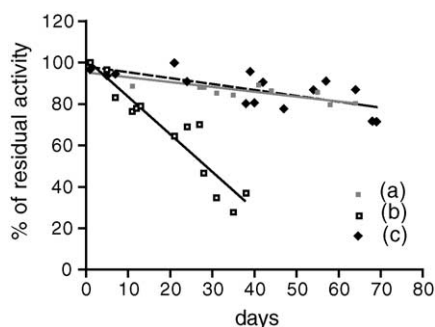

Fig. 6. Stability study on AChE-micro-IMERs: (a) EDA-CIM-IMER; (b) Epoxy-CIM-IMER; and (c) Glut-P IMER. Residual activity calculated as percent initial activity (yellow anion peak area) after injection of saturating acetylthiocholine concentrations over time.

Glut-P IMER showed an unmodified activity after 3 months of not use (data not shown).

3.5. Determination of inhibitory potency (IC_{50})

The sensitivity of the immobilized AChE in the AChE-micro-IMERs format to well-known inhibitors (tacrine, edrophonium, ambenonium, donepezil, galanthamine and propidium) (Fig. 7) was examined. The IC_{50} estimation was assessed by injecting simultaneously both substrate at a fixed saturating concentration and inhibitors at increasing concentration. Increasing reduction of the yellow anion peak area (i.e. inhibition of enzyme rate of hydrolysis), when compared to the area obtained by the sole substrate, was observed for increasing inhibitors concentration. The percent inhibition was plotted against the logarithm of injected inhibitor concentration to obtain the inhibition curves from which IC_{50} values were extrapolated.

The pIC_{50} values obtained on the three IMERs were compared with the values obtained for the free enzyme (Fig. 8) and the correlation values were calculated. Only four up to six inhibitors were tested on Epoxy-CIM disk because of the stronger interactions between the tested compounds (i.e. donepezil and propidium) and the chromatographic support. Correlation factors for EDA-IMER and Glut-P-IMER were, respectively, 0.9756 and 0.8848 (both $n = 6$).

4. Discussion

The physical properties of immobilized enzymes usually differ from those displayed by native enzyme primarily because of (a) the influence of the matrix dependent microenvironment, (b) the restraining influence of the covalent bond and (c) the steric constraints caused by the proximity of the solid support. The degree in which each of these factors influences the enzyme will depend on the type of matrix and on the method of immobilization employed.

The aim of this study was to analyze the performances of three different matrices for the immobilization of AChE by evaluating their use as high throughput IMERs for Alzheimer's disease drug discovery. For this purpose three different matrices characterized by different chemistries (epoxy or aldehydic active groups), structural features (polymeric or silica based material) and format (loose material or monolithic column) were selected (for structural characteristics of the chosen matrices see Table 1). A key feature that drove our choice was the small dimensions of the final IMER.
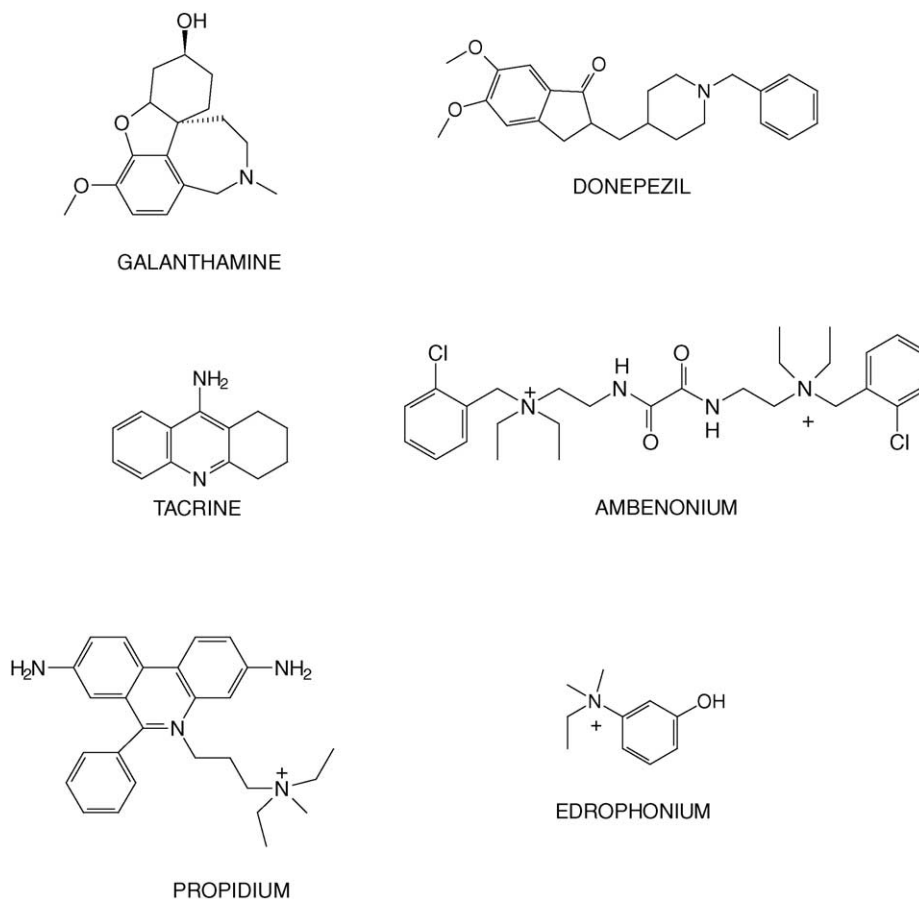
Fig. 7. Chemical structures of tested acetylcholinesterase inhibitors.

In particular, the development and characterization of the AChE-micro-IMER on EDA-CIM disk (EDA-IMER) was already reported by us in a previous paper [15]. In brief, the immobilization proceeded trough Schiff bases linkage and
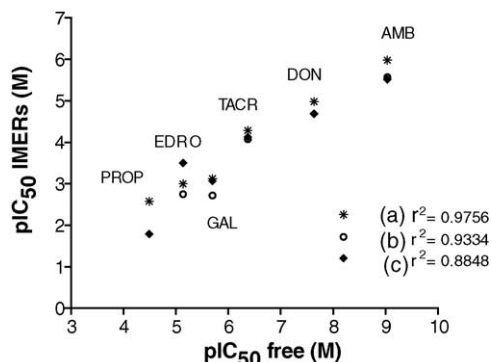

Fig. 8. Correlation plot for inhibitory potency (pIC$_{50}$) of six well kwon inhibitors obtained on immobilized-enzyme reactors and with the enzyme in solution. pIC$_{50}$ values [$-\log$ IC$_{50}$ (M)] represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements: (a) EDA-CIM-IMER, $n = 6$; (b) Epoxy-CIM-IMER, $n = 4$; (c) Glut-P-IMER, $n = 6$. PROP: propidium, EDRO: edrophonium, GAL: galanthamine, TACR: tacrine, DON: donepezil, and AMB: ambenonium.

required, as first step, the activation of the primary amino groups by reaction with a 10% glutaraldehyde solution.

In this work, attention was driven on a simpler immobilization procedure which occurs in one step by using the Epoxy-CIM disk. This immobilization is thought to proceed via the initial enzyme physical adsorption on the supports followed by a covalent linkage between the nucleophilic groups of the adsorbed enzyme (e.g. amino, thiol or hydroxy groups) and the dense layer of epoxy groups on the matrix [19]. Incubation at pH 8 was found suitable for coupling proteins via NH$_2$-groups. After the reaction, the remaining oxirane groups have to be inactivated by adding ethanolamine in order to prevent further uncontrolled reactions between the support and the enzyme that could decrease its stability.

Considering that the immobilization of small biomolecules results easier because small proteins can be directly immobilized on the support, in the case of large molecules, the active center of the enzyme could be no longer accessible at all or only to a limited degree. In these cases, an improvement can be achieved by introducing a spacer, which allows maximum enzymatic conversion. The spacer was inserted in the amino matrix monolithic EDA-CIM disk, whereas it is already contained in the wide pore silica based Glut-P. The former matrix is originally a weak ion exchange polymeric column that can be used for coupling proteins,

Table 3
Comparison of the three AChE-IMERs

	Epoxy-CIM disk	EDA-CIM disk	Glut-P
Reactive groups	Epoxy groups	Aldehydic groups after activation by glutaraldehyde, needs to be activated	Aldehydic groups
Method of immobilization	In situ	In situ	Batch-wise
Total time for immobilization (h)	27	18	14
Immobilization yield	3.0% (0.18 U)	3.0% (0.22 U)	29% (4.35 U)
Conditioning time (min)	5	5	60
Stability (residual activity after 2 months) (%)	<30	∼80	∼80
Back pressure	•	•	•
Salt concentration in the mobile phase	••	•••	•••
Time of analysis (min)	5	5	5
Possibility of working continuously at the optimum pH for AChE activity	•	•	–
Aspecific interaction with tested compounds	•••	•	•
Optimisation of the immobilization procedure	–	–	•
Possibility of vary the amount of matrix and column size	–	–	•
Potential high-throughput screening	••	•••	•
Best fit to our purpose	•	•••	••
Costs	•••	•••	•

(–) null; (•) low; (••) medium; and (•••) high.

peptides or other ligands after crosslinking reaction with a suitable bifunctional reagent, i.e. glutaric dialdehyde [15,20], while the latter is commercially available with the reactive aldehydic groups and does not need to be activated. Therefore, in both matrices the same chemistry is at the basis of the immobilization process: a Schiff base reaction is involved in the coupling of the ligand to the affinity mediums and requires one additional step to reduce and therefore stabilize the imine groups (Fig. 1). At this level, one of the advantages is that Glut-P is a cheaper chromatographic support which allows a more detailed study by the performance of a high number of trials for the optimization of the immobilization conditions. Moreover, being an unpacked silica material, Glut-P allowed simple off-line studies that could be performed in parallel using eppendorf tubes [17], and offered the possibility of vary the amount of matrix, i.e. the enzyme/matrix ratio, and therefore the final IMER dimension.

Three IMERs were therefore obtained by following the scheme reported in Fig. 1 and the conditions described in the experimental part. In order to compare the three IMERs, these reactors were inserted into a HPLC system and were characterized in terms of rate of immobilization, stability, conditioning time for HPLC analyses, optimum mobile phase and peaks shape, matrix/ligand aspecific interactions and costs. Advantages and disadvantages were defined for each system and are summarized in Table 3. The resulting IMERs showed to partially retain their catalytic activities. As reported in Table 2 the percent of active immobilized units was higher for AChE-GlutP than for the AChE-CIMs. This result was interpreted on the basis of the wider surface contact area between the small particles of the silica matrix and the enzyme which facilitates the interaction between the active groups on the matrix and the reactive groups on the enzyme leading to the

higher ratio of enzyme/matrix. However, besides the yield of immobilized active units, the catalytic efficiency was evaluated. This parameter was determined for the three IMERs by the ratio K_m/V_{max}. In Table 2 it is reported that the monolithic IMERs showed the best values. This faster enzymatic conversion, expressed by a low ratio K_m/V_{max}, might be due to lack of diffusion resistance during mass transfer. Conversely, in the Glut-P-AChE-IMER the traditional pore geometry can produce mobile phase stagnation and low substrate diffusion, resulting in a slower catalysis rate. The same peak width values were obtained for the three IMERs, notwithstanding the different internal volume (bed volume, Table 2), and account for this phenomenon in the silica based AChE-IMER. For the same reasons long conditioning time was observed for Glut-P-IMER (Fig. 4); monolithic matrices showed instead very short conditioning time (5 min) and fast recovery of the enzymatic activity that could represent very important features in high-throughput analysis.

Regarding IMERs' stability, AChE-Epoxy-CIM was found to be the less stable (Fig. 6). While AChE-Glut-P and AChE-EDA showed almost 80% retention of activity after 2 months of continuous daily use, AChE-Epoxy-CIM was inactivated at a much faster rate (∼30% of activity retained after 2 months of use). This behavior might be due to the lack of the spacer chain which, keeping the enzyme at a suitable distance away from the matrix, preserve the enzyme from side adsorption and loss of suitable conformation leading to denaturation. On the other hand the residual hydroxyl vicinal group can give some instability to the newly formed C−N bond, by the oxygen nucleophilic attack to the carbon atom, which might cause the cleavage of the C−N bond. Immobilization trough Schiff base linkage therefore gave more stable reactors that could be used for several months, i.e. EDA-CIM-

IMER retained 12% of its initial activity after 15 months and could still be used for inhibition studies just by switching the detector wavelength from 450 to 412 nm (maximum in yellow anion spectrum).

Concerning the optimization of the chromatographic parameters, mobile phase composition, pH and flow rate were evaluated. AChE-Epoxy-CIM was used with the simpler mobile phase (Tris–HCl buffer pH 8.0 containing Ellman's reagent), because no capping of residual cationic groups on the matrix had to be performed. Moreover, the polymeric IMERs resulted stable in a wider range of pH (2–14 according to manufacturer protocol of usage [20]) and could be used continuously with a basic mobile phase.

The purpose of the development of a AChE based micro-IMER is the rapid on-line screening of new potential inhibitors for Alzheimer's disease treatment. Therefore, the sensitivity of the immobilized human recombinant AChE to three well known AChE inhibitors (edrophonium, ambenonium, and propidium) and three US Food and Drug Administration (FDA) approved drugs (tacrine, donepezil, galanthamine) [21] (Fig. 7) was investigated and the inhibitory potency (pIC_{50}), was extrapolated from the inhibition plots. The pIC_{50} values on the three IMERs are summarized in Fig. 8 versus the corresponding values obtained in solution by the classic spectrophotometric Ellman's assay [18] with the same batch of human recombinant enzyme used for the immobilization. The goodness of the correlation between data obtained with IMERs and free enzyme is a key requisite in drug discovery in order to compare data from on-line studies with data from literature. Moreover, the linear correlation between on- and off-line studies is a parameters that may account for not specific interactions between inhibitors and chromatographic support. Some tested compounds presented stronger side interactions with the Epoxy-CIM-IMER: the more hydrophobic donepezil and propidium could not be tested and the addition of 2% (v/v) n-propanol in the mobile phase was not sufficient to allow inhibition studies for these two compounds. On the other hand, both Glut-P and EDA-CIM-IMER showed to be suitable for the inhibitory potency evaluation of all the six selected inhibitors, EDA-CIM-IMER showing the best linear regression: $r^2_{EDA-IMER} = 0.9756$; $r^2_{Glut-P-IMER} = 0.8848$ ($n = 6$).

5. Conclusions

Object of the present study was to prepare and compare the performances of immobilized AChE-based micro-IMERs for inhibition studies. With this aim, two new IMERs based on a monolithic epoxy column and a silica based loose material were developed, characterized and compared with a previously reported AChE-EDA-IMER in the attempt to reduce costs and improve IMER performances. Advantages and disadvantages were defined for each reactor after insertion in a HPLC system. In particular the obtained results showed that: (a) immobilization trough Schiff base linkage (EDA and Glut-P matrices) gave more stable reactors without any significant change in the enzyme behaviour; (b) monolithic matrices showed very short conditioning time (5 min) and fast recovery of the enzymatic activity that could represent very important features in high-throughput analysis; (c) unpacked silica material allowed off-line low costs studies, along with the possibility of varying the enzyme/matrix ratio and therefore the final IMER dimensions.

As general conclusion, EDA-CIM disk showed to fulfil at best our general requirements for fast and reproducible analyses and could be used for rapidly assess the inhibitory potency of unknown inhibitors. Unpacked affinity media showed to be useful to pre-screen optimal conditions for a further immobilization step on the monolithic amino matrix with saving of money and costly material.

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