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Immobilization of matrix metalloproteinase 8 (MMP-8) for online drug screening

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ABSTRACT

Matrix metalloproteinase 8 (MMP-8) has been reported to have a key role in several pathologic conditions, like heart diseases, osteoarthritis, multiple sclerosis, and various other inflammatory conditions. Therefore, there is a great interest regarding the development of MMP-8 selective inhibitors. In the recent years, immobilized enzyme reactors (IMERs) proved to be an efficient alternative to solution-based assays. Besides the recycling of the enzyme, IMER approach allows a simple way to determine affinity data and thus the ranking of inhibiting potency of the compounds under study, especially when coupled to MS. In this study, the immobilization of MMP-8 was investigated in terms of type of support, kinetic parameters, storage and pH stability. Epoxy activated silica resulted the best matrix for the preparation of an immobilized enzyme reactor (IMER) containing human MMP-8. The IMER was successfully used for the online screening of known MMP-8 inhibitors in zonal chromatography and inhibition experiments.

1. Introduction

Matrix metalloproteinases (MMPs) are a group of structurally related zinc-dependent endopeptidases able to degrade the different protein components of the extracellular matrix (ECM) and basement membranes, such as collagens, glycoproteins, proteoglycans, and fibrins. These endopeptidases play an important role in physiological processes such as development, morphogenesis, bone remodeling, wound healing, and angiogenesis [1]. Otherwise, some pathological situations are associated with imbalance between MMPs and their endogenous regulators (namely, TIMPs or tissue inhibitors of MMPs), leading to an overexpression of these enzymes, which may contribute to cancer progression, degenerative and inflammatory diseases. Therefore, MMPs have been considered for long as promising therapeutic targets [2].

So far, 23 human MMPs have been identified and divided into subgroups based on their structure and substrate specificity: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (MT-MMPs). Among these, MMP-8 (neutrophil collagenase-2) represents the most effective collagenase for type 1 collagen and the most active on neutrophils, suggesting a central role in the infiltration of neutrophils. Although MMP-8 is considered as an antitarget for cancer therapy [3], it has been claimed to have a key role in heart disease [4], osteoarthritis [5], multiple sclerosis [6] and various inflammatory conditions such as hepatitis and ulcerative colitis [7]. Those reports stimulate the development of truly selective inhibitors capable of blocking the unwanted activities of this enzyme in such diseases.

Solution based assays are the most common method to screen MMPs inhibitors [8], generally including the monitoring of the cleavage activity of purified MMPs by a specific synthetic substrate, comprising a fluorophore, a quencher and a peptide portion. When intact, the quencher part adsorbs the emitting light from the fluorophore by resonance energy transfer. Once the peptide moiety is recognized by MMPs, the subsequent cleavage of the compound separates the fluorophore from the quencher, with a large increase in fluorescence, allowing the measurement of MMPs activity.

In the recent years, immobilized enzyme reactors (IMERs) proved to be an efficient alternative to batch assays for drug screening [9–12]. In this approach, the enzyme is immobilized on a suitable chromatographic support and packed into a column. The latter can then be used for online screening of potential inhibitors evaluating the reduction of the enzyme activity or their affinity for the enzyme, in particular determining the relative binding constants. By using this technique, the enzyme can be reused several times, and the IMER can be coupled to chromatographic systems with selective detectors (i.e. MS [13–16]) that allow the screening of inhibitors mixtures in just one run, with high sensitivity and the possibility of high-throughput determinations, thus greatly reducing time and cost of analysis.

Considering the advantages of the IMER approach and the great interest regarding MMPs, we were surprised to find only one very recent paper [17] describing the use of such method referred to MMPs. Therefore, we carried out a comprehensive study on the

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immobilization of MMP-8 and on the preparation of a MMP-8 IMER, evaluating the potential applications for inhibitors screening and binding affinity investigations. The results of this study are reported herein.

2. Materials and methods

2.1. Materials

HPLC grade silica gel (GROM-SIL 500 Si NP-1, 5 μm, 500 Å pore size) were purchased from Alltech Italia (Milan). *N*,*N*′-disuccinimidyl carbonate (DSC), TRIS base, 3-aminopropyltrimethoxysilane, (3-glycidyloxypropyl)trimethoxysilane, tetramethyl ortosilicate (TMOS), lysozyme from chicken egg white, poly-L-lysine hydrochloride (mol wt >30,000), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were from Sigma–Aldrich. FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) and Mca-Pro-Leu-OH (MPL) were purchased from Calbiochem. Full length E. coli-expressed human pro-MMP-8 was a kind gift of Prof. Gillian Murphy (Department of Oncology, University of Cambridge, UK). It was activated prior to use with p-aminophenylmercuric acetate 2 mM for 1 h at 37 °C. FAB (fluorometric assay buffer): Tris 50 mM, pH 7.5, NaCl 150 mM, CaCl2 10 mM, Brij 35 0.05%, DMSO 1% and 0.02% NaN_3. FABdil stands for FAB solution diluted 1:4 with water. Inhibitors **1–4** [18] and **5** [19] were prepared as previously described. Epoxy-activated Sepharose 6B and EAH Sepharose 4B were purchased from GE Healthcare and prepared for coupling according to manufacturer's instructions. IAM was a generous gift of Prof. Wainer (Gerontology Research Center, National Institutes in Aging, NIH Baltimore, USA).

2.2. Instrumentation

All the stirring procedures were carried out using orbital shaker at 800 rpm. Centrifugation was always done at 21,500 × *g*. Vortexing was done at 1000 rpm. HPLC affinity experiments and assays analyses were carried out on a Jasco PU-980 and Jasco PU-887 pump, respectively, with a Rheodyne sample valve (20 μL loop), equipped with a Jasco 821-FP spectrofluorometer detector (FLU). Conditions for assay analysis: Gemini C18 column (50 mm × 3 mm, 3 μm particle size, Phenomenex), eluent 0.1% trifluoroacetic acid in H_2O:MeOH 70:30, flow 0.6 mL/min, λ_{ex} = 325 nm, λ_{em} = 395 nm.

2.3. Determination of MMP-8 activity

The MMP-8 activity was determined following the proteolysis of the fluorogenic substrate FS-6 [20]. In a typical experiment, the free activated enzyme and FS-6 (final concentration 1.46 nM for MMP-8 and 2 μM for FS-6) were incubated at 37 °C in FAB for 20 min under stirring. The reaction was then stopped with an equal volume of 3% (w/v) sodium acetate pH 4.0 solution and the mixture was centrifuged. The supernatant (10 μL) was analyzed by HPLC-FLU. MPL was used as reference compound for quantitation of hydrolyzed FS-6 (Mca-Lys-Pro-Leu-Gly-OH) [20]. The calibration curve resulted linear in the range 0.05–20 μM (r^2 0.9935). To verify that MPL was a suitable substitute of hydrolyzed FS-6, a solution containing MMP-8 14.6 nM and FS-6 4 μM in FAB was incubated under stirring for 2 h to reach complete proteolysis of FS-6. Three solutions of hydrolyzed FS-6 (0.1, 0.5, 2 μM) were compared to MPL solutions at the same concentration, providing the same instrumental response.

2.4. Supports preparation

Epoxy-activated silica gel (EPSi) was obtained by toluene refluxing of silica gel and (3-glycidyloxypropyl)trimethoxysilane as described in Felix and Descorps [21]. In a similar way, aminopropylsilica (AMP) was prepared reacting 3-aminopropyltrimethoxysilane with silica gel as reported in Yu et al. [22]. AMP-SC was prepared stirring silica gel with DSC in dry acetonitrile for 1 h according to Xia et al. [23].

2.5. Immobilization of MMP-8

Recovery experiments were performed vortexing for 20 min a known amount of FS-6 or MPL together with the phase under study. In the case of MPL (that is fluorescent) supernatant or washings were directly analyzed by HPLC-FLU. In the case of FS-6, MMP-8 was added to the separated supernatant or washings or control solution, incubated and the recovery of FS-6 determined by HPLC-FLU analysis of the hydrolysis product. The comparison with a control solution of the compound at the same concentration of the initial loading provided the extent of recovery.

All the immobilization procedure shared a common protocol of washing away the uncoupled enzyme from the support, indicated as WAP: the suspension coming from the coupling of the support and the enzyme was centrifuged and the supernatant was collected to determine the immobilization yield by HPLC-FLU. The remaining support was re-dispersed in FAB (180 μL), stirred for 15 min, re-centrifuged and the supernatant (180 μL) collected for analysis. That washing process was repeated three times.

Immobilization of activated MMP-8 (by its amino groups, i.e. ε-amino group of lysine) on epoxy- and DSC-activated supports was carried out weighing the support (10 mg) in a Eppendorf tube, swelling it with FABdil (300 μL), stirring for 15 min, centrifuging and removing the supernatant (280 μL). To the resulting phase, a 16.2 nM activated MMP-8 solution (180 μL) was added, briefly vortexed and stirred for 22 h at r.t. After the WAP, treatment with 0.2 M glycine solution in FAB dil (400 μL) at r.t. for 1.5 h was carried out to block the unreacted coupling groups. In the case of EPSi, 0.2 M ethanolamine or *n*butylamine solution were also used. Once washed with FAB (400 μL each) three times as described before, the support was ready for testing.

Coupling of MMP-8 (by its carboxylic groups) to EAH Sepharose 4B (that has free amino groups) and AMP phases was obtained swelling the support (10 mg) with HCl 10 μM (180 μL). To the resulting suspension, a 10 mg/mL EDC solution in HCl 10 μM (15 μL) and a 525 nM activated MMP-8 solution (5 μL) were added, briefly vortexed and stirred for 6 h at r.t. After the WAP, the support was ready for testing. Immobilization on IAM phase was carried out as described for epoxy-activated supports without the final treatement with glycine solution. Biosilica [24] immobilization experiments were performed by a common protocol using PLL or lysozyme as silica precipitating agent and different amounts of silicic acid, and MMP-8. The freshly prepared 1 M stock solution of silicic acid was obtained by the addition of 1 mM HCl (170 μL) to TMOS (30 μL). We obtained the best result (retained activity 22%) mixing a 14.6 nM solution of activated MMP-8 (200 μL) with 10 mg/mL aqueous lysozyme solution (30 μL). After stirring for 10 min, a 100 mM solution of silicic acid in TRIS buffer pH 7.5 (230 μL) was added and stirred for further 30 min. A silicate precipitate was formed within few minutes. After the WAP, the support was ready for testing.

Immobilization yield was determined by assaying the proteolytic activity of supernatants and washings samples coming from immobilization experiments and comparing their residual activity with that of a control solution at the same concentration and volume used in the immobilization. In the same way, immobilized enzyme activity was assessed mixing MMP-8 supported phase with FS-6 (final concentration 2 μM) in FAB and incubating the suspension at 37 °C in FAB for 20 min under stirring. Then, the mixture was centrifuged and the supernatant was removed, quenched with an equal volume of 3% (w/v) sodium acetate pH 4.0 solution and

analyzed by HPLC-FLU (10 μL). The retained activity is then given by comparison with the response provided by a MMP-8 control solution matching the concentration of the enzyme loaded on the support.

2.6. Michaelis Menten of free and immobilized MMP-8

The initial velocity region of MMP-8 reaction was determined from reaction progress curve obtained analyzing the hydrolysis of a 2 μM solution of FS-6 in FAB in the presence of 1.5 nM of MMP-8 over time (2.5–90 min) by HPLC-FLU. A 20 min incubation time resulted appropriate for all the activity assays. The kinetic parameters of free and EPSi MMP-8 were determined from initial velocity measurement (expressed as nmol of FS-6 hydrolysis product/nmol enzyme per min) varying substrate concentration (2–300 μM). Initial velocity data were obtained by quantitation of the formation of FS-6 hydrolysis product by HPLC-FLU analysis. The amount of free enzyme used was equal to the one employed in the immobilization on EPSi. K_m and V_{max} parameters were obtained from the so generated Michaelis-Menten saturation curve. Kinetic parameters values were obtained using GraphPad Prism Software 5.0.

2.7. Storage stability and pH dependence of activity

Free and EPSi immobilized MMP-8 were stored at 4 °C and r.t. in FAB for 40 days. The storage stability was obtained from the ratio of free or immobilized enzyme activity after storage to their initial activity. The pH dependence of activity was evaluated carrying out the activity assay in FAB at various pHs both with free and immobilized enzyme, considering activity at pH 7.5 as reference. The latter was incubated with the buffer at the proper pH for 1 h before the assay.

2.8. Batch inhibition assay

For assay measurements, the 10 mM in DMSO inhibitors stock solutions were further diluted in FAB at the required concentration. In the inhibition assay, the enzyme (5 nM for free MMP-8, 5 nM for the immobilized MMP-8, calculated based on the residual activity showed by the phase) and the inhibitor at the proper concentration were stirred together at r.t. for 2 h in FAB. Then, FS-6 (final concentration 2 μM) was added and the mixture incubated at 37 °C in FAB for 20 min. The reaction was finally quenched and analyzed as described for MMP-8 activity determination. The percentage of inhibition was calculated by comparison with a control reaction without the inhibitor. IC_{50} values were obtained using GraphPad Prism Software 5.0.

2.9. Affinity and inhibition experiments on EPSi-MMP8 column

EPSi-MMP-8 IMER was prepared immobilizing activated MMP-8 on EPSi according to the procedure described above. This support (210 mg) was packed as a slurry in FAB in a 4.6 mm × 30 mm steel column (Phenomenex) and FAB was delivered through the column at 0.5 mL/min for 3 h. A similar column was prepared in the same way packing 210 mg of EPSi treated with 0.2 M glycine solution in FAB dil for 2 h. The latter was used as a control column (EPSi-GLY) to assess non-specific interactions. Inhibition and affinity experiments were carried out using the experimental and control column on a HPLC-FLU chromatographic system. For all the assays the eluent was FAB at 0.15 mL/min. In a set of inhibitory experiments, FS-6 0.5 μM was injected into MMP-8 IMER alone and in the presence of the inhibitor (**1**, **2**, **4**, **5**) at 500 μM concentration. The peak areas of hydrolyzed FS-6 with or without inhibitor were recorded and compared as reported in Ma and Chan [17]. In the same way, inhibition activity of compound **5** was assayed at different concentrations (300, 500, 600 μM). Experimental column was washed with FAB for 1 h at 0.3 mL/min and the activity checked by FS-6 injection after each experiment.

Comparison of the binding affinity of compound **1**, **2**, **4**, and **5** with respect to **3** was carried out by zonal chromatography displacement experiments. Compound **3** was used as marker exploiting its fluorescence (λ_{ex} = 290 nm, λ_{em} = 420 nm). In those experiments, the marker (0.5 μM) was injected (10 μL) into the IMER equilibrated with a mobile phase containing a fixed concentration of the competing agent (6 μM). The retention time change of the marker is a measure of the relative binding affinity of the competing agents studied. The IMER was washed with FAB for 2 h at 0.4 mL/min and its activity checked by injection of FS-6 before starting a new experiment.

3. Results and discussion

3.1. MMP-8 immobilization

The properties of the support and the techniques used for the immobilization of enzymes are of critical importance in the development of efficient IMERs. The ideal support has physical resistance to compression, hydrophilicity, inertness and ease of coupling toward enzymes, high surface area, biocompatibility, resistance to microbial attack, low cost and it has to retain as much as possible the enzyme biological properties. Typical matrices are based on derivatized silica or polymers (agarose, sepharose, synthetic polymers) and the embedding of the enzyme on the support can be accomplished by chemical or physical mechanisms. Chemical immobilization entails the formation of stable covalent bonds between the enzyme and reactive groups present on the matrix surface (i.e. amino, thiol, epoxy, carboxylic groups). Physical methods usually involve the adsorption or the entrapment of enzyme molecules on suitable supports.

Considering that there was only one report [17] regarding the immobilization of MMP-9, we carried out a screening of different matrices to find out the most efficient method for anchoring MMP-8 to a support. Recovery experiments of FS-6 and MPL (representative of hydrolyzed FS-6) from the phases under study were performed at first before the immobilization. MPL was fully recovered from all the supports tested, while FS-6 resulted strongly retained only by immobilized artificial membrane (IAM), a chromatographic phase containing monolayers of phospholipid ligands that was successfully employed in the entrapment of other enzymes [25]. All the investigated matrices gave quantitative embedding of MMP-8, excepting epoxy-activated sepharose 6B (Table 1). The activity assays on MMP-8 trapped on IAM provided no evidence of FS-6 hydrolysis, even increasing the amount of FS-6, just like the peptide never reached the active site of the enzyme. IAM phase was then abandoned.

Table 1
Immobilization of MMP-8 onto different supports.

Support	Immobilization yield (%)[a]	Retention of activity (%)[a]
IAM[b]	100	n.d.
Biosilica	100	22
EPSi[b]	100	45
EPSep[b]	9	n.d.
AMP-SC[b]	100	8
EAH[b]	100	12
AMP[b]	100	4

[a] See Section 2.5 for calculations.
[b] Immobilized artificial membrane (IAM), N-hydroxysuccinimide activated silica (AMP-SC), epoxy activated silica (EPSi), epoxy activated Sepharose 6B (EPSep), aminopropyl silica (AMP), EAH Sepharose 4B (EAH, has free amino groups at the end of 11-atom spacer arms), n.d., not detected.

Recently, biomimetic silicification was reported as an efficient method for enzyme immobilization [24]. In the so called "bioinspired" formation of the silica material, condensation of silicic acid is triggered at neutral pH and room temperature by specific polypeptides or polyamines, acting also as a template for the forming silica network. When the enzyme is added to the condensation catalyst and the silicic acid solution, co-precipitation and encapsulation of the protein occurs into the silica particles in a one pot procedure. In that way, it is rather simple to obtain immobilized enzymes on a support with enhanced mechanical stability and high loading capacity. We then carried out several experiments of biosilica entrapment varying the MMP-8 to silicic acid ratio and the precipitating agent (polylysine or lysozyme). The best result in terms of retained activity was 22% (Table 1, see Section 2.5 for details), but the procedure was not highly reproducible in our hands.

We then turned our attention to MMP-8 immobilization through covalent binding. Agarose and silica materials derivatized with epoxy and amino functions as reactive groups for covalent attachment have been widely used for the preparation of IMERs [10,26]. In our screening, we used commercially available derivatized Sepharose, while silica based supports were prepared in our laboratory from HPLC silica gel, according to well assessed and reported procedures [21,23]. Classic coupling protocols were employed as described in Section 2.5. The data regarding the immobilization efficiency and percentage of retained activity of the various phases are reported in Table 1. While we were unable to improve the low immobilization yield of MMP-8 on EPSep even working at pH 9, EPSi matrix showed the highest retained activity among the tested supports. Therefore, all further experiments were carried out with this material.

In order to investigate the influence of the polarity of the compounds used for the epoxy groups encapping on the activity of the supported enzyme, three different MMP-8 immobilizations were performed on EPSi using 0.2 M glycine, ethanolamine or *n*butylamine solution, that provided a carboxylic, hydroxy, alkyl group as terminal end, respectively. The residual activity decreased going from the polar glycine (45%) to the less polar ethanolamine (26%) to the substantially apolar butylamine (10%). Those data indicate that the presence of a hydrophobic moiety close to the enzyme surface is detrimental in our case, and thus a more hydrophilic reagent like glycine has to be used in the blocking step [27].

3.2. Kinetic parameters, storage stability and pH dependence of free and immobilized MMP-8

The determination of the kinetic parameters of the free human MMP-8 was carried out in order to get reference data to be compared with those of the immobilized enzyme. We used the fluorogenic peptide FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) to monitor the enzymatic proteolytic activity, which comprises the cleavage of FS-6 into the quenching (NH_2-Leu-Dpa-Ala-Arg-NH_2) and the fluorescent portion (Mca-Lys-Pro-Leu-Gly-OH) by MMP-8 [20]. The extent of FS-6 hydrolysis was followed by RP-HPLC coupled to fluorometric detection (FLU) by using Mca-Pro-Leu-OH (MPL) as reference compound for quantitative determinations, as hydrolyzed FS-6 is not commercially available.

A reaction progress curve for the enzymatic hydrolysis was obtained mixing MMP-8 with FS-6 in FAB and measuring the subsequent product that was generated over 1.5 h. From the analysis of the curve, we chose a 20 min period of reaction time to carry on the subsequent kinetics experiments with a linear initial velocity. A saturation curve exhibiting the classic Michaelis-Menten kinetics was then obtained varying FS-6 concentration and measuring the initial rate of the hydrolysis product formation. The K_m and V_{max} were determined to be $80 \pm 16\,\mu M$ and 510 ± 41 nmol/nmol enz * min respectively from non-linear regression of Michaelis-Menten data (Fig. 1).

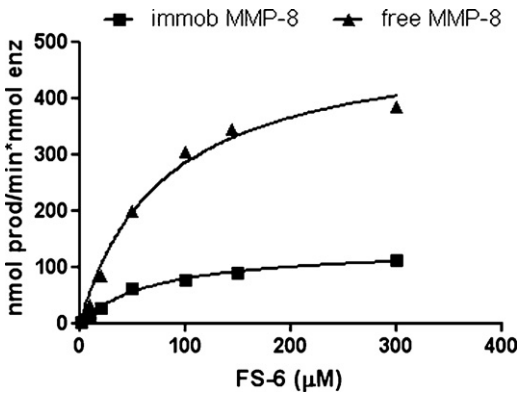

Fig. 1. Determination of kinetic parameters K_m and V_{max} for the hydrolysis of FS-6 in FAB by free (triangles) and immobilized (squares) MMP-8. Data were fitted to the Michaelis-Menten equation.

In the same way, the kinetic parameters of immobilized EPSi-MMP-8 phase were obtained. The curve showed classic Michaelis-Menten kinetics (Fig. 1), K_m and V_{max} being $71 \pm 9\,\mu M$ and 135 ± 6 nmol/nmol enz * min, respectively. Hence, the K_m values of free and immobilized enzyme resulted pretty close, indicating that the immobilization process had not affected the affinity characteristics of the native MMP-8 for FS-6. Conversely, V_{max} proved to be higher for the free protein with respect to the embedded MMP-8, probably because of diffusional restrictions.

Storage stability tests were performed at r.t. and 4 °C on both free and EPSi-MMP8 for 40 days (Fig. 2). The enzymatic activity of both systems decreased very slowly and no significant differences were observed. Those data suggested that the stability of MMP-8 was not affected by the immobilization process.

The effect of pH on the activity of the free and immobilized enzyme was also investigated, carrying out a set of experiments in the 6.5–9 pH range. The optimal pH value of 7.5 for the highest MMP-8 activity was unchanged after immobilization. A similar profile of activity loss was observed for both free and EPSi-MMP-8 moving to the ends of the pH range assayed, but it was more marked for the immobilized enzyme, with a difference in activity up to 12% at pH 6.5 with respect to the native counterpart (Fig. 3). This difference can possibly be attributed to the interactions of the glycine groups on the surface of the support that change their polarity with the pH.

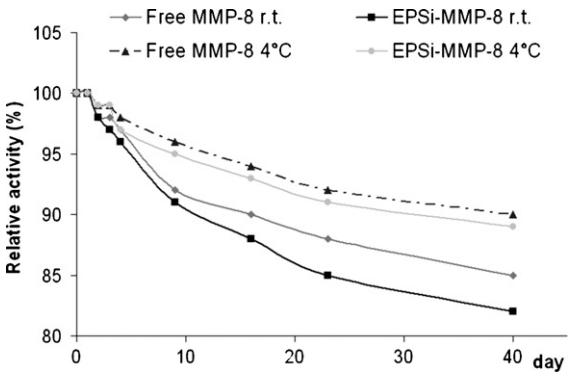

Fig. 2. Stability of free and immobilized enzyme during storage at r.t. and 4 °C.

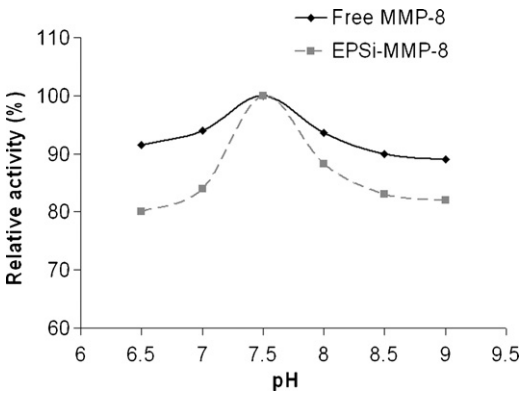

Fig. 3. Effect of pH on the activity of free and immobilized MMP-8.

3.3. Inhibition and affinity study

After the characterization of the supported system in terms of kinetic parameters, stability and optimal pH, we came to the actual goal of the study, the use of the immobilized MMP-8 for the screening of low molecular weight synthetic inhibitors. Hence, the determination of the IC_{50} values for five MMP-8 inhibitors (**1–5**, Fig. 4) was carried out both on free and EPSi-MMP-8 (Table 2). The MMP-8 inhibitory activity of those compounds had been previously determined by plate reader fluorometric assay [18,19]. The ranking of the inhibitors potency observed with solubilized and immobilized enzyme resulted the same (**5** > **4** > **3**–**2** > **1**), showing that the

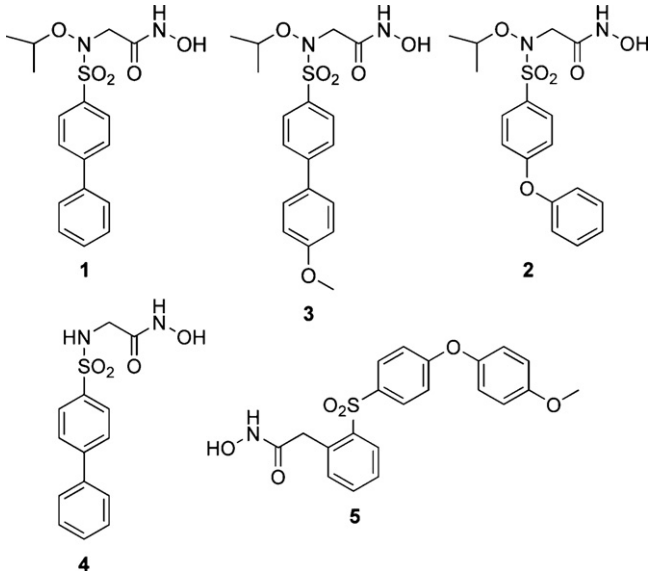

Fig. 4. Structure of inhibitors **1–5**.

Table 2
Effect of inhibitors **1–5** on the activity of free and immobilized MMP-8 determined by fluorometric plates reader and HPLC-FLU.[a]

Compound	IC50 (nM) Free MMP-8 FLU plates read	IC50 (nM) Free MMP-8 HPLC-FLU	IC50 (nM) EPSi-MMP-8 HPLC-FLU
1	260	497	435
2	65	230	330
3	66	250	346
4	7	30	63
5	5	10	34

[a] In the free MMP-8 FLU plates reader [MMP-8] = 1.5 nM, while in the free MMP-8 HPLC-FLU [MMP-8] = 5 nM. [FS-6] = 2 μM in both cases.

interactions with the catalytic domain of MMP-8 were not affected by the immobilization. The differences in the IC_{50} values between the free and supported enzyme can be ascribed again to diffusional limitations and, possibly, to non specific interactions between the compounds and the support.

All these data confirmed the possibility of using EPSiMMP-8 for drug screening studies in a batchwise manner. Therefore, we turned our attention on the preparation of an IMER for online affinity investigations.

Several examples are reported in literature in which biospecific interactions were efficiently characterized by high-performance affinity chromatography (HPAC) [28]. HPAC is a method in which a biologically related ligand is used as a stationary phase in an HPLC system. This approach can be used to quantitate or separate compounds in complex samples, but it can also be employed to study the interactions of biological systems, like ligand-enzyme or receptor interactions. In that regard, one way to perform a HPAC experiment is to examine the elution time or volume of an analyte injected onto a column containing the protein of interest immobilized on a suitable HPLC support. Thus, it is possible to obtain information regarding the equilibrium constants describing the analyte binding affinity for the ligand. Moreover, the addition of other agents to the mobile phase allows the study of the effect of these agents on the analyte-ligand interactions, such as inhibition or competition studies. In general, HPAC techniques provide higher precision, automation speed and better correlation over traditional solution-based methods [28].

The two most used approaches in HPAC in order to examine substrate-protein affinity are zonal elution (ZC) and frontal affinity chromatography (FAC). In ZC, generally the mobile phase containing a known concentration of a competing agent is continuously applied to the immobilized ligand column while a small amount of analyte is injected (displacement studies). Information regarding the binding constant between the analyte and the ligand are then calculated from the retention changes of the analyte [28]. In that way, an affinity order among a set of compounds can be obtained comparing the extent of the retention time shift of a reference analyte following the injection of the substrates under analysis. In FAC, the mobile phase containing a known concentration of the analyte is continuously applied through a column containing a fixed amount of immobilized ligand. As the amount of bound solute in the column increases, it begins to saturate the active sites of the embedded ligand, and the initial flat portion of the chromatographic trace changes into a vertical rise which ends in a plateau when protein saturation is reached, providing the so called breakthrough curve [25]. The midpoint of this curve can be related to the concentration of applied solute, the amount of immobilized ligand and the association constant for the solute-ligand binding affinity.

In our study, to carry out the affinity experiments two 4.6 mm × 30 mm steel columns were prepared to differentiate between specific and non-specific interactions, one containing EPSi-MMP-8 (experimental column) and the other one packed with EPSi quenched with 0.2 M glycine solution, thus without the protein (control column). In this experiments the assumption is that the non-specific interactions between the support and the compound under study are the same for the control and experimental column, and they are evaluated by the retention time on the control column. If a retardation in the elution time on the experimental column is observed, the difference provides a measure of the extent of specific interactions between the injected compound and the immobilized MMP-8.

Compound **3** was used as reference compound in the displacement ZC experiments, as its signal can be easily detected by fluorescence without interferences from the other compounds assayed. We did not observe noticeable changes in the capacity factors for **3** when using slightly higher or lower sample con-

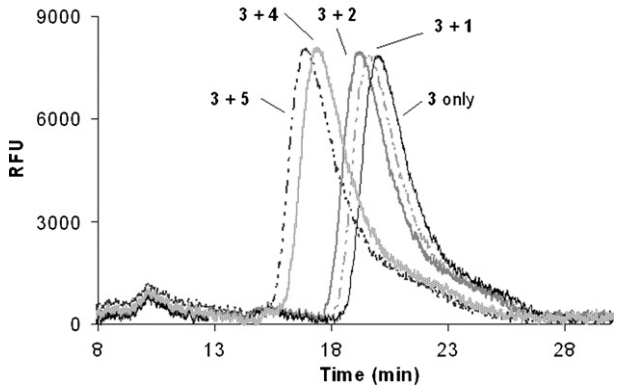

Fig. 5. Displacement ZC experiments using 0.5 μM 3 as analyte and 6 μM **1**, **2**, **4**, **5** as competing agents.

centrations or different flow-rates, indicating that linear elution conditions assumed in ZC studies were present during ZC experiments. Mobile phase containing **1**, **2**, **4** and **5** at 6 μM was passed through the experimental column while injecting **3** at 0.5 μM. The same solution of **3** injected on the control column provided a retention time 10 min shorter than elution on the experimental column, indicating that specific interactions occurred on the latter column. As it can be observed from Fig. 5, the affinity order obtained from the shift in the **3** capacity factor resulted **5** > **4** > **2** > **1** (lower k' of **3**, higher affinity of the competing agent), and it parallels the inhibition activity obtained using the free enzyme. Moreover, taking advantage of the fluorescent properties of compound **3**, we were able to observe specific interactions also in a set of very preliminary FAC experiments, infusing **3** in the IMER at different concentrations (data not shown). That outcome was promising for the ongoing development of the IMER coupling to LC–MS to carry out FAC–MS affinity experiments [13,29].

The reported results demonstrated that the IMER can be successfully employed to obtain affinity data for MMP-8 drug screening.

We also carried out inhibition experiments as described in Ma and Chan [17]. As reported by those authors, also our system required high concentration of inhibitors to obtain a detectable reduction of the immobilized MMP-8. FS-6 at 0.5 μM was injected into the IMER alone and in the presence of increasing concentrations of **5** (300, 500, 600 μM). A concentration-dependent

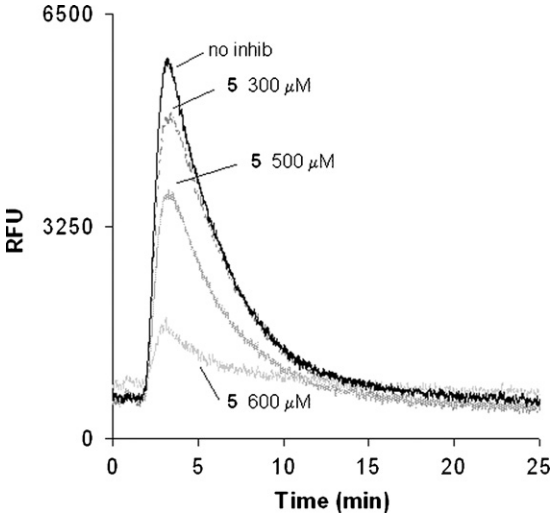

Fig. 6. Inhibition of FS-6 proteolytic activity of immobilized MMP-8 using **5** at 300, 500 and 600 μM.

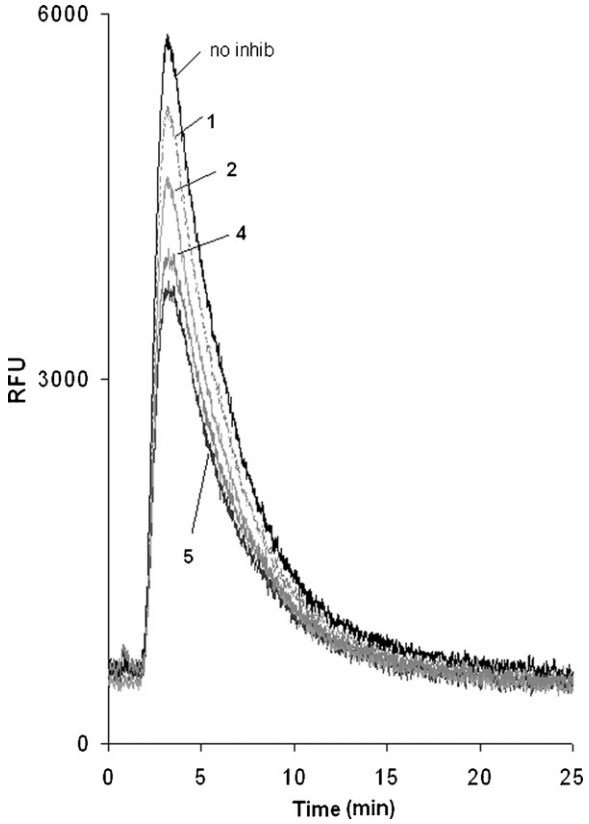

Fig. 7. Inhibition of FS-6 proteolytic activity of immobilized MMP-8 using **1**, **2**, **4**, **5** at 500 μM.

inhibition effect was observed on the FS-6 proteolytic activity of the immobilized MMP-8 (Fig. 6). A second set of inhibition experiments was performed, in which FS-6 was injected in the presence of **1**, **2**, **4**, **5** at 500 μM. Also in this case, we were pleased that the order of the inhibition activity of the compounds assayed matched the rank given by the solution-based test (Fig. 7).

The only rate-limiting step in the use of our IMER for online drug screening was the regeneration time of the binding affinity ability of the column. ZC experiments required about 2 h washing time with FAB to recover the starting affinity. Preliminary studies showed that increasing the ionic strength can improve the wash out time. The addition of organic modifiers will be also taken into consideration. All these approaches are under investigation.

4. Conclusion

In summary, a thorough study on the immobilization of MMP-8 for online drug screening was described. Several supports were investigated to find the matrix most suitable in the retention of the native proteolytic activity and affinity properties of MMP-8. In that regard, EPSi provided the best result, with a quantitative immobilization and a 45% retention of the starting activity. The immobilization process did not alter the biological properties of the native MMP-8, as showed by the kinetic parameters, the inhibition batch assays, the storage and pH stability. EPSi was then used as the support for the preparation of the IMER, that was used for HPAC online affinity studies. The zonal chromatography and the inhibition online experiments confirmed that the system obtained can be successfully used for MMP-8 drug screening. According to preliminary tests we carried out (not reported here), the IMER approach can be also applied for other MMPs. The data obtained resulted very

promising for the further development of the IMER coupling to MS to perform FAC–MS affinity studies.

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