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ORIGINAL PAPER

Hybrid sol–gel bio-films: influence of synthetic parameters on behaviour and performance of entrapped His_6-tagged organophosphorus hydrolase

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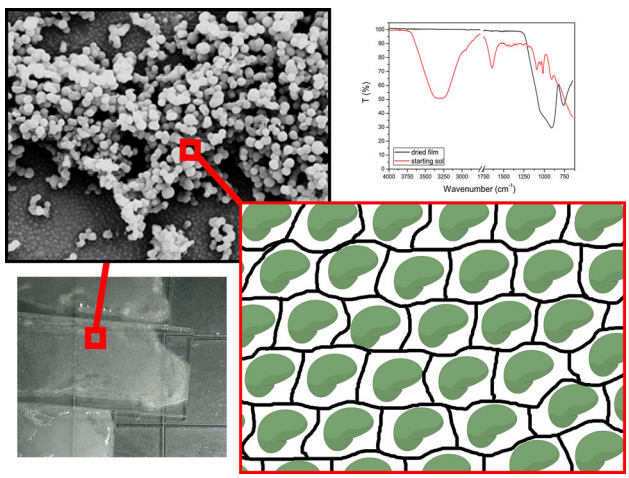
Abstract The presented work describes the optimising of sol–gel process parameters including the aging time of the sol, the water/silane ratios, type of precursor [tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS)], and the precursor ratios using organically modified silanes [methyltrimethoxysilane (MTMOS) and 3-glycidoxypropyltrimethoxysilane (GPTMS)], for the successful entrapment of hexahistidine-tagged organophosphorus hydrolase (His_6-OPH) and its applicability during organophosphate detoxification processes. The prepared bio-composite films retained enzyme activity by up to 90 % with expected differences between the starting sols' compositions. The obtained values of K_M and V_{max} for TMOS/MTMOS and TEOS/GPTMS bio-composites equalled 1.5 ± 0.1, 1.4 ± 0.3 mM, 0.40 ± 0.02 and 1.8 ± 0.2 μM/s for paraoxon, respectively, thus assuming effective application of such biocatalysts during the detoxifications of organophosphorus compounds. Furthermore, the bio-composite films showed enhanced pH and temperature stability with respect to the free enzymes as they can be easily separated from the reactive media, as well as having multiple reusages.

Graphical Abstract Bio-composite materials based on hexahistidine-tagged organophosphorus hydrolase were developed by enzyme encapsulation into silica sol–gel matrixes (tetramethoxysilane/methyltrimethoxysilane, tetraethoxysilane/3-glycidoxypropyltrimethoxysilane). The process of biomaterial production was optimised in several pathways and biomaterials were characterised via IR-spectroscopy, SEM-imaging, so as their catalytic efficiency in organophosphate hydrolysis.



Keywords Sol–gel · Silica thin film · Enzyme encapsulation · His_6-tagged organophosphorus hydrolase · Organophosphate detoxification

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1 Introduction

Nanocomposite materials of silica, prepared using the sol–gel technique within aqueous solutions, have attracted considerable attention due to their propensity for entrapping enzymes without any covalent bonding to the matrix. It is also significant that the immobilised biomolecules

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retain or even enhance their bioactivity and stability. The incorporation of enzymes into silica matrices has proved to be a good strategy for improving the catalytic efficiencies of enzymes, which is achieved by using the sol–gel technology [1].

The aqueous character of sol–gel processing and the fact that the synthesis of the bio-composite materials can be conducted at room temperature ensure that sufficiently mild conditions prevail, as is required if biomolecules are to retain their native structure and function. This process also allows for high yields of enzyme immobilisation. The framework rigidity of the sol–gel polymers prevents any leaching of the entrapped enzyme, whilst also stabilising their structures. Entrapped enzymes usually exhibit longer lifetimes than free enzymes under harsh conditions (pH, T, etc.) [2]. Furthermore, the porous silica matrix provides enzyme accessibility, thus allowing the diffusion of low-to-medium-molecular-weight species and their free interactions with the enzyme [1, 3]. During the development of bio-catalysts and biosensors for biotechnological and medical usages, the immobilisation of enzymes has been an active research topic within enzyme technology regarding the enhancements of their activities, thermal and operational stabilities, as well as reusabilities [4].

There is considerable interest in the enzymatic detection and detoxification of organophosphorus compounds (OPCs), being derivates of orthophosphoric and alkylphosphonic acids. These include those pesticides widely used in agriculture that accumulate within soil and water sources, as well as neurotoxic chemical warfare agents. In this respect, organophosphorus hydrolase, OPH (EC 3.1.8.1), a metalloenzyme capable of hydrolysing a broad spectrum of OPCs, including pesticides (e.g. paraoxon, parathion, diazinon, and malathion) and nerve agents (e.g. sarin, soman, VX), is an ideal candidate for their rapid degradation and has been extensively discussed in the past [5, 6]. Hexahistidine-tagged organophosphorus hydrolase enzyme (His_6-OPH) is a genetically modified OPH enzyme with its improved catalytic activity, especially towards P–S-containing substrates and stability under alkaline hydrolysis conditions [7]. Therefore, biocatalysts based on His_6-OPH appear to have promising applications and have been actively researched over recent years [8, 9].

The significance of this paper lies in the study of the optimal conditions for the enzyme encapsulation and the suitability of TMOS-based sol–gel matrixes for the bio-catalyst in comparison to the TEOS-based ones, to develop new bio-composites for the detoxification and possibly detection of OPCs. Our main focus was devoted to optimising and comparing the sol–gel process parameters when preparing different bio-catalysts/bio-composites and their comparisons with our previously published results [10]. The strong dependence between the silica precursor used for the encapsulation and the resulting enzymatic activity has already been reported [11–14]. For these purposes investigations of process parameters affecting final sol–gel structures were performed including the aging times of the sols, water/silane ratio (R), and precursor/Ormosil ratio (P). The influences of water and organosilane precursors on the chemical characteristics of the starting sol solutions were also studied. An alcohol-free sol–gel method employing silane precursors, tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS), combined with co-precursors; organosilane derivatives (ORMOSILs), methyltrimethoxysilane (MTMOS) and 3-glycidoxypropyltrimethoxysilane (GPTMS) was developed and used to immobilise (entrap) His_6-OPH. The ORMOSILs were used due to their possessions of those unhydrolysable hydrophobic groups that are retained during the sol–gel process, giving the final sol–gel matrix a hydrophobic character favoured by used substrate—paraoxon (POX). Furthermore, the resulting gel has better structure, porosity, and regular distribution of immobilised biomaterial than the conventional one which uses only TEOS or TMOS [3]. The goal of the optimisation of sol–gel process parameters was to develop final bio-catalysts with successfully entrapped His_6-OPH with retained catalytic activities. The bio-composite layers with encapsulated His_6-OPH of various structures (water/silane, precursor ratios) were prepared followed by the activity (U/glass slide), the residual activity (%), as well as the immobilisation efficiency (%) determinations.

2 Materials and methods

2.1 Chemicals and enzyme

The following chemicals, all used during this study, were purchased from Sigma-Aldrich: *O,O*-diethyl *O*-(4-nitrophenyl) phosphate (paraoxon), 2-(cyclohexylamino) ethansulfonic acid (CHES), 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), and cobalt chloride hexahydrate. The tetramethoxysilane (TMOS), tetraethoxysilane (TEOS), 3-glycidoxypropyl-trimethoxysilane (GPTMS) and methyltrimethoxysilane (MTMOS) that served as the sol–gel precursors, as well as the surfactant Triton-X 100, were obtained from Fluka. All the other chemicals were of analytical grade and were purchased from Sigma-Aldrich and used as purchased. The His_6-OPH enzyme was isolated and purified as previously described [15]. The enzyme's specific activity was 6,000 U/mg.

2.2 Determination of the sol–gel aging time

In order to define an appropriate time for the enzyme encapsulation, the aging time of the prepared sol solution

was determined by visual observation of the sols, where the time t_{clear} required for the reactant mixture to progress from a two-phase emulsion to a clear, single-phase solution provided an approximate visual indication of the hydrolysis rate. Their pH and FTIR spectra were recorded for more accurate determination of the silica sols hydrolysis rates. Samples for pH measurements were taken every 18–20 h, whilst simultaneously the FTIR spectra were recorded.

2.3 Immobilisation of His_6-OPH within a sol–gel matrix

The enzyme was immobilised using the alcohol-free-route sol–gel process. Briefly, the silica-sol solutions were prepared from different molar ratios (P; P being 1:1, 1:2, 1:5, 1:10, 2:1, 5:1, 10:1, 1:0 and 0:1) of TMOS/MTMOS and TEOS/GPTMS with the final volume of approx. 2 mL. The water content during the sol preparation was carefully controlled by the molar ratio H_2O/silane (R), where an appropriate volume of 0.79 mM HCl with a selected R value was added to the precursors mixture, P (TMOS/MTMOS precursors) being 4, 26.5, 74, 148, 223, 297, and 445.5, respectively. The hydrolysis-condensation reactions were carried out under acidic catalysed conditions (HCl, 0.79 mM). The non-ionic surfactant Triton-X 100 (0.2 vol%) was also added into each solution. Preparation of the sol solutions included their 15 min sonication; afterwards they were left for an appropriate time at 4 °C, to allow hydrolysis of the precursor. The appropriate aging time was determined by pH measurements of the sol solutions accompanied by ATR–FTIR spectra recorded at selected time intervals. After a certain aging time 1.5 mg/mL of the enzyme solution (His_6-OPH in 50 mM K-phosphate buffer, pH 7.5; containing $CoCl_2 \times 6H_2O$) was mixed with the prepared sols at 1:1 volume ratios and homogenised using a vortex mixer. During the first reaction, the precursors (TMOS/MTMOS and TEOS/GPTMS) were hydrolysed in the presence of water at acidic pH (HCl, 1 mM), resulting in the formation of silanol (Si–OH) groups. The second step involves the condensation reactions between the silanol moieties resulting in the formation of siloxane (Si–O–Si) polymers, thus creating a matrix in which the enzyme was entrapped [3].

The prepared mixture (10 µL) was then spread; using an automatic pipette; over the pre-activated glass slides supports (0.8 cm × 2.0 cm) and left for 48 h at 4 °C for bio-films formation. The pre-activation step of the glass slides was carried out with a mixture of 200 mL of 2 M NaOH and 12.5 mL of 25 % H_2O_2 at 50 °C for 30 min, followed by soaking with PrOH for 30 min and finally rinsing with copious amounts of de-ionised water and EtOH. The final bio-films were stored in a sealed container at 4 °C. Prior to usage, the support surface was rinsed with phosphate buffer (50 mM, pH 7.5) to remove any excess of unbound/surface exposed enzyme. The total (theoretical) enzyme activity applied was calculated to be approx. 60 U per glass slide.

2.4 Determination of the immobilised enzyme activity and its behaviour in the bio-layers

The enzymatic activity was determined spectrophotometrically using a Perkin Elmer Lambda 35 UV/Vis Spectrometer (PerkinElmer, Waltham, USA) and Agilent 8453 UV–Visible spectroscopy system with a thermostatic cell (Agilent Technology, Waldbronn, Germany). The accumulation of the p-nitrophenolate anion was monitored ($\lambda = 405$ nm; $\varepsilon = 17{,}000$/M/cm, pH 9.0; $\varepsilon = 18{,}500$/M/cm, pH 10.5) as a hydrolysis product of paraoxon. An enzyme amount hydrolysing 1 µmol of the substrate (paraoxon) per minute at 25 °C was considered as one unit of enzymatic activity.

The enzymatic reaction rates were calculated using the initial linear sections of the kinetic curves ($v_0 = tg\ \alpha$). The maximum rate of the enzymatic reaction (V_{max}) and the Michaelis constant (K_m) were determined using the hyperbolic approximation and double reciprocal coordinates $1/v_0 - 1/[S]$ (the Lineweaver–Burk plot), in which v was the velocity, V_{max} was the maximum velocity and $[S]$ was the initial concentration of the substrate [16]. Determination of kinetic parameters was performed under controlled reactive conditions (25 °C; 50 mM CB, pH 10.5 and 9.5 for free and encapsulated enzyme, respectively).

In order to investigate the influence of pH on the enzymatic catalytic activity, various 50-mM buffers with overlapping pH values were used: K-phosphate (PB), pH 6.5–8.0 and 11.0–12.0; TRIS, pH 7.5–9.0; CHES, pH 8.5–10.0; and Na-carbonate (CB), pH 9.5–11.0. In order to determine the pH optima for the prepared bio-films, those samples with appropriate buffers and substrate (0.675 mM POX) were thermostatically controlled (25 °C); the catalytic reaction was initiated with the addition of the bio-catalyst film.

In order to determine the temperature optima of the immobilised enzyme action, those samples with a CB (50 mM, pH 9.5) and substrate (0.675 mM POX) were thermostat-controlled within a selected temperature range between 20 and 65 °C. The reaction was initiated by the addition of a bio-film.

Amongst the more important properties of the bio-catalyst for real applications is its reproducibility and reusability. Measurements of the reusability were performed with CB (50 mM, pH 9.5) and 0.675-mM substrate (POX) concentration in thermostat-controlled vessels (25 °C). Between each measurement, the samples were washed with

water three-times for 5 min in closed micro-centrifuge tubes in a vortex shaker (750 rpm).

2.5 Apparatus

The pH values of all the solutions were determined using a Mettler Toledo, Seven Easy^{TM} pH meter S20.

The ATR–FTIR spectra were recorded on a Perkin Elmer Spectrum GX spectrometer. The ATR accessory (supplied by Specac Ltd., UK) contained a diamond crystal. All the spectra (16 scans at 4 cm^{-1} resolution and rationed to the appropriate background spectrum) were recorded at room temperature.

The starting sol solutions were sonicated by ELMA Elmasonic sonicator using sonication power of 40 W and frequency of 35 kHz.

Scanning electron microscopy (SEM) was performed on the Carl Zeiss Supra 35 VP electron microscope.

3 Results and discussion

3.1 Optimisation of the synthetic parameters for the entrapment of His_6-OPH

Numerous investigations have shown that variations in synthesis conditions (e.g. the type of precursor, catalyst, and surfactants; the ratio of water to precursor, the concentrations, the medium pH, etc.) modify the structure and properties of silica sol–gels [3, 17]. Synthesis parameters influence both the hydrolysis and the condensation rates, and thereby regulate the kinetics and mechanism of the sol–gel process.

In our case, the most important optimisation step during the sol–gel entrapment of enzymes was to avoid enzyme denaturation due to alcohol release. The release of alcohol during the hydrolysis-condensation of silicon alkoxides has been considered an obstacle, due to its potential denaturing activity on the entrapped biological moiety; TMOS is therefore occasionally used instead of TEOS. This was also considered during our investigation, where the alcohol-free-route of the sol–gel process for enzyme entrapment was used, accompanied by the usages of two main precursors (TMOS, TEOS) and two organically modified precursors (MTMOS, GPTMS).

In order to determine the influences of synthetic parameters (P and R) on the chemical characteristics of the resulting sol solutions, the ATR–FTIR spectra of the starting sols with different precursors and water/silane ratios were recorded during the sols' production (Fig. 1).

The obtained FTIR spectra of silica sols based on TMOS/MTMOS and TEOS/GPTMS showed common bands as assigned to various vibrations within the network. During the reaction of silica and water, IR bands appeared at 950 and 1,090 cm^{-1}, due to the vibrations of Si–OH and Si–O–Si bonding, respectively (Fig. 1). These bands were very intense and correspond to the formation of the SiO_2 network. The absorption bands between 800 and 1,260 cm^{-1} were previously described as superimpositions of various SiO_2 peaks, Si–OH bonding and peaks due to residual organic groups [18–20]. Water demonstrated an intense characteristic absorption band between 3,000 and 3,500 cm^{-1} that was assigned to O–H stretching within the H-bonded water, and could be cross-checked through the 1,645 cm^{-1} band due to scissor bending vibration of the molecular water.

The use of silicon co-precursors (MTMOS, GPTMS) in addition to precursors (TMOS, TEOS) led to the formation of methyl- or propyl-functionalised silica sols. The corresponding FTIR spectra (Fig. 1b, d) showed several peaks within the 2,980–2,850 cm^{-1} range assigned to the C–H symmetric and asymmetric stretching vibrations of the $–CH_3$ and $–CH_2$ groups. The spectra (Fig. 1d) also showed C–H symmetric and asymmetric deformation vibrations at 1,420–1,410, 1,375–1,350 and 1,275–1,274 cm^{-1} for TMOS/MTMOS sols, respectively [18, 20]. Similarly, the C–H deformation peaks appeared between 1,470 and 1,375 cm^{-1} in TEOS/GPTMS sols (Fig. 1b). The latter bands were observed as being more pronounced in sols with higher co-precursor (MTMOS, GPTMS) ratios.

The amount of water used in the initial sol solution could significantly affect the silica framework. Any increase in the water/silane molar ratio was indicated in more pronounced peaks ascribed to water absorption bands (Fig. 1a, b). The hydrolysis rate is discussed further.

3.2 Determination of the sols aging time for the optimal entrapment of His_6-OPH

The relative rates of hydrolysis and condensation reactions that have an important effect on the final structures of the silica matrix can be very effectively influenced by pH. This behaviour was observed experimentally in the presented system, as shown in Fig. 2. Sols prepared from two different precursor mixtures, TMOS/MTMOS and TEOS/GPTMS [10], exhibited quite different behaviour. The presented experiment was performed for evaluating the aging time of TMOS/MTMOS-based sols allowing the optimal preparation of the bio-catalysts, and comparing them to previously published results [10]. According to the literature [21], it was expected that the hydrolysis-condensation reactions of TMOS-based sols would be faster than the TEOS-based ones. As expected, the pH increased in both cases over time. The plateau for TMOS/MTMOS sols was reached after approximately 24 h at a pH of 4.00. The experimental value of t_{clear} varied from 30 min to a maximum after 24 h with smaller changes in pH from 3.85

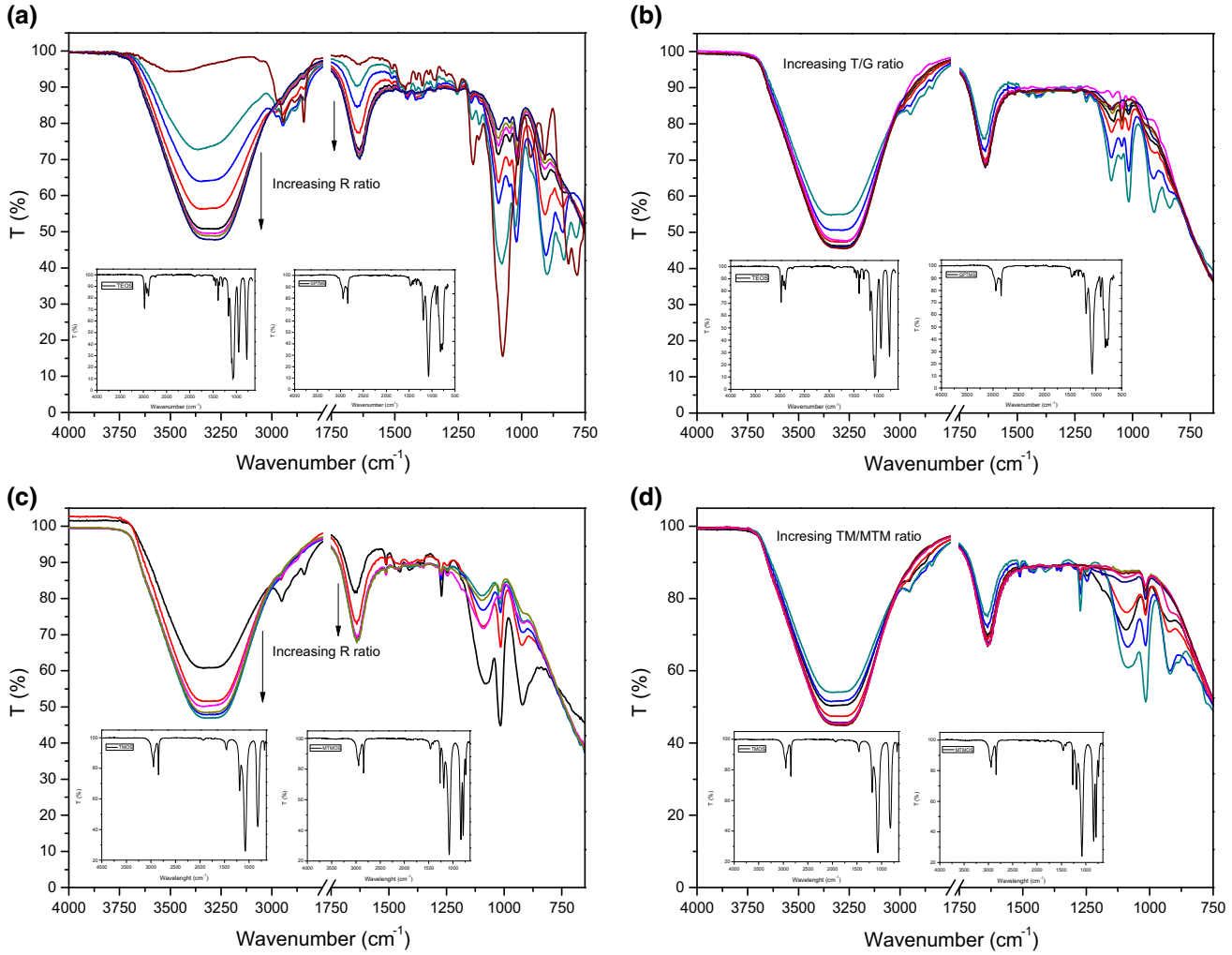

Fig. 1 The ATR–FTIR spectra of initial sols with different water/silane ratios (R) or precursors ratios (P) after 24 h of aging time **a** TEOS/GPTMS, P = 1:5; **b** TEOS/GPTMS, R = 188; **c** TMOS/MTMOS, P = 1:2; **d** TMOS/MTMOS, R = 148

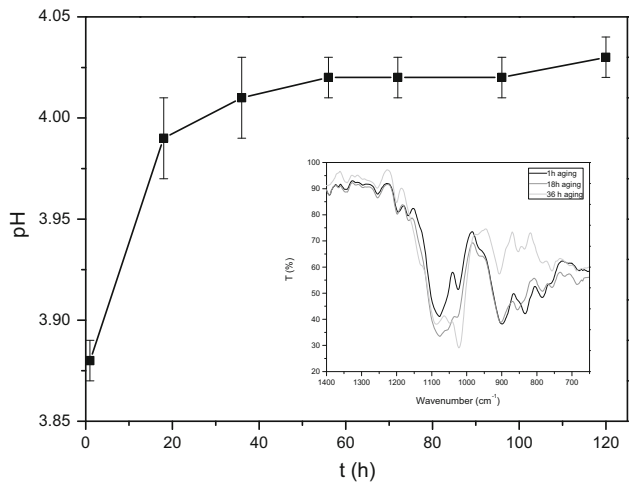

Fig. 2 pH changes of the aged sol TMOS/MTMOS over different time intervals. *Inset* ATR–FTIR spectra of aged sol recorded after 1, 18 and 36 h. Water/silane molar ratio used for sol obtaining was 145 to 4.00. In comparison to TEOS/GPTMS sols (t_{clear} 0.5–120 h) [10], t_{clear} of TMOS/MTMOS sols was reached much faster, which is consistent with the previous [21] reports. Rapid hydrolysis of the sols allows more prompt production of the final bio-active films, shortening the whole procedure. The dependence of reactive kinetics on a sol's pH suggests that pH may also strongly influence the porous structure of the dried xerogel. This effect has been observed for both polymeric and particulate silica-based sols [22, 23].

A similar conclusion supporting the results above could be withdrawn from the IR spectrum (Inset of Fig. 2), recorded during the gelation process. The ATR–FTIR spectra of the aging sol showed absorption bands arising from an asymmetric stretching vibration doublet of Si–O–Si (1,090 and 1,020 cm^{-1}), asymmetric vibration of Si–OH (~910 cm^{-1}), and symmetric vibrations of Si–O–Si (830–850 cm^{-1}). The hydrolysis rate of a silica sol was

studied by the 910 cm^{-1} band assigned to Si–OH modes, where it could be overlapped by the vibrations of the unreacted TMOS, and the 1,020 cm^{-1} band assigned to Si–O–Si modes. The first band decreased continuously within the reactive time indicating the successful hydrolysis of TMOS. The second band became more pronounced over time, further supporting our assumption about the formation of the Si–O–Si network.

Consistent with t_{clear} the end of the hydrolysis step was reached after 24 h for the TMOS/MTMOS sols. From the practical point of view of the experimental conditions, we chose 24 h (TMOS/MTMOS) and 48 h (TEOS/GPTMS) hydrolysis of the prepared sols before their further usages, even though longer aging times are usually preferable for stronger network conformation. Thus, when comparing TEOS/GPTSM [10] and TMOS/MTMOS, the latter sol composition offered an advantage in terms of the rapidity of sol "readiness" for further applicability, namely enzyme encapsulation.

3.3 Bio-composite layer characterisation

After optimisation of the synthesis conditions we prepared bio-films with the addition of the His_6-OPH.

Based on ATR–FTIR spectra recorded for aged bio-films (Fig. 3), after 4 days of drying at 4 °C the characteristic absorption band for H-bound water disappeared (3,500–3,000 cm^{-1}), as well as its corresponding band at 1,645 cm^{-1}. The existing absorption bands within the mid-infrared spectrum from 800 to 1,300 cm^{-1} could be ascribed to the silica. The more dominant band was assigned to the asymmetric stretching vibrations of Si–O–Si within the range of 1,130–1,060 cm^{-1}. A second weaker band related to silanol vibrations could be observed within the range of 900–880 cm^{-1}; this band was assigned to the bending vibration of the silanol group. The existing band at 760 cm^{-1} was characteristic for Si–C modes, which could possibly indicate covalent interaction between amino acids of the His_6-OPH and the solid support that appeared during the enzyme encapsulation. According to FTIR spectra we produced dry silica based bio-composite films.

The compositions of the various starting sols resulted in films with very different appearances, as can be seen from the SEM micrographs (Fig. 3a–e). Some of the bio-composite layers had a thin and glassy appearance, whereas some of the bio-composite layers had lower optical purity or were even tending to crack (Fig. 3c). It could be concluded that different bio-composites can be produced for various purposes by varying their components, namely: solid multilayer bio-composite for biodegradable application, single-point OPC's detection using isolated micro- and submicron-particles and as micro-particle agglomerates for "strip" OPC analysis. Gel glasses of high optical quality were obtained from different precursor (TMOS/MTMOS) and water/silane ratios (Table 1); nevertheless the enzymatic performance of the layer was quite distinct.

The particles started to aggregate after adding the enzyme solution (pH 7.5) to the starting sol solution (approx. pH 4.0). For both starting sols TMOS/MTMOS and TEOS/GPTMS [10] the aggregation was more intense in the case of lower water-to-precursor (R) ratio. The appearance of prepared bio-composite films was further strongly influenced by the P ratio, where the films based on TEOS/GPTMS with higher P = 5:1 (Fig. 3d) contained deposited spherical microparticles with median diameters 1 ± 0.5 μm and the layers with P = 1:2 were more uniform, containing only separate particles with the same size deposited on the top of the layer (Fig. 3b). Bio-films based on TMOS/MTMOS were of good optical purity and the depositings of agglomerated particles (1.5 ± 0.5 μm) were on the tops of the films (R1 and R2 in Table 1; Fig. 3a, e). Bio-composite films with higher P ratios were impossible to prepare due to fast particle formation, their aggregation and gelation. For both the starting compositions, bio-composite films of low P molar ratios resulted in poor optical clarity and cracking. These phenomena can be ascribed as a potential disadvantage of ORMOSILs where the alkyl group reduces the degree of cross-linking, leading to less robust materials.

3.4 Performance, stability and reusability of the bio-composite layer

The effect of starting the sol–gel synthetic parameters e.g. water/silane ratio (R) and precursor's ratio (P) had a major impact on the performances of the dried biocatalyst films. The results consistent with those of our previous publication [10] were also obtained for TMOS/MTMOS-based biocatalyst films. The velocity of the reaction was accelerated due to the higher H_2O amount (higher R value), and consequently the activities of the biocatalyst films increased (Table 1). The release of alcohol during condensation is potentially harmful to the enzymes. Thus, the ratio of water to sol during gel formation must be high enough to avoid the denaturation of the enzyme by methanol or ethanol during immobilisation, in order to diminish the diffusion resistance of the gel layer. In addition, the sol–gel polymerisation under a low R stimulated the enzyme aggregation producing lower activities that were in agreement with previous reports on enzyme immobilisation within a sol–gel matrix [24, 25].

The influences of the precursors' molar ratio (P) for two types of biocatalyst films were quite distinct, whilst in the cases of TEOS/GPTMS-based films reaching higher relative activity favouring the lower P ratio, the TMOS/MTMOS-based films reached higher relative activities using higher P ratio (Fig. 4). Based on our previous data

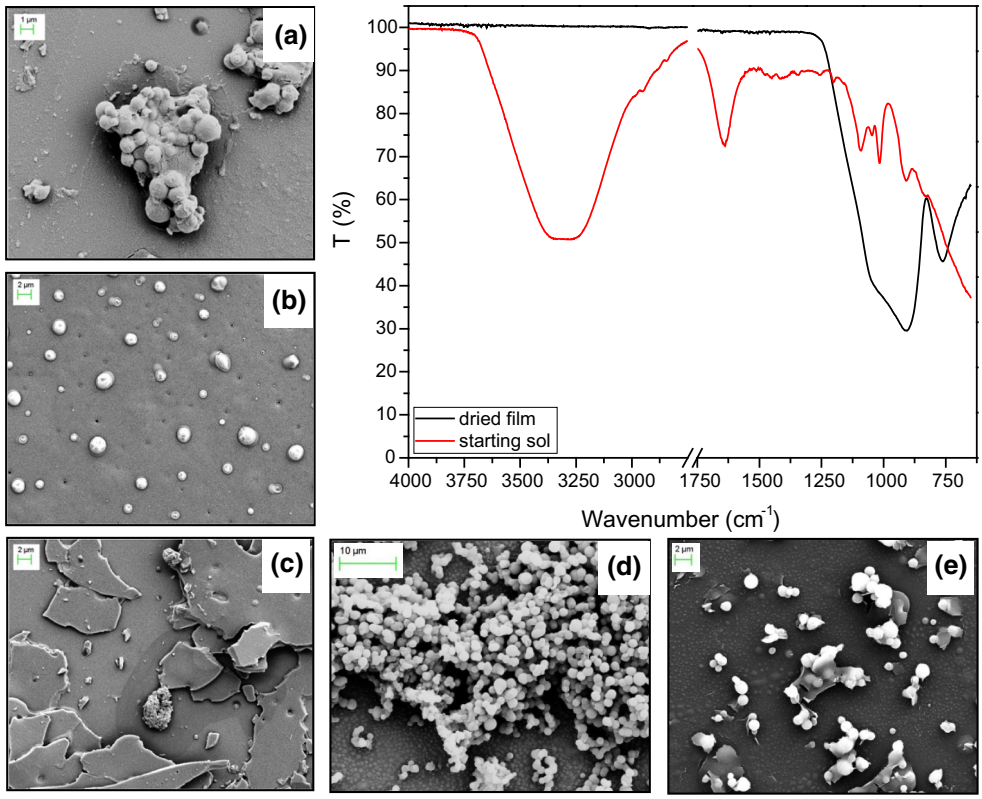

Fig. 3 The ATR–FTIR spectra and SEM micrographs of prepared bio-catalyst films. Legend for SEM micrographs: **a** TMOS/MTMOS, R = 74.2 and P = 1:2; **b** TEOS/GPTMS, R = 188 and P = 1:2; **c** TMOS/MTMOS, R = 148 and P = 1:10; **d** TEOS/GPTMS, R = 188 and P = 5:1; **e** R = 148 and P = 1:2

Table 1 Influences of water/silane (R) and precursor TMOS/MTMOS (P) molar ratios on the immobilisation efficiencies of bio-films based on His_6-OPH

Sample	TMOS:MTMOS molar ratio	Water:precursor molar ratio	Activity (U/glass slide)	Remaining activity (%)
P1	1:1	148:1	2.4 ± 0.5	4.0 ± 1
P2	1:2		38.3 ± 1.2	64 ± 3
P3	1:10		31.2 ± 0.7	52 ± 4
R1	1:2	74.2:1	22.8 ± 0.6	38 ± 2
R2		148:1	38.3 ± 1.2	64 ± 3
R3		222.7:1	44.4 ± 1.6	74 ± 6

Substrate: 0.675 mM POX; 25 °C, pH 9.5 (50 mM CB). Theoretical activity of bio-film based on His_6-OPH was calculated to be approx. 60 U/glass slide. All measurements were performed as three parallels

[16], the better performance in hydrolysis of POX was reached by those samples containing low P ratio. Although we tried to prepare bio-films with lower P ratios for the TMOS/MTMOS, mixing the starting sol with an enzyme solution led to rapid particle formation and gelation, making it non-feasible for us to prepare suitable samples for adequate characterisation. On the other hand, such a mechanism for particle formation can be easily used for single bio-composite nano- and micro-particle production in bulk solution. This opens up new perspectives for current work development.

The determined activity of the prepared bio-composite films (glass slides) based on TMOS/MTMOS varied significantly according to the water contents within the starting sol mixtures (Table 1). The lowest activity for those biocatalyst films based on TMOS/MTMOS was measured as 2.5 U (Table 1; P1), and then reached almost 44.5 U, which is almost 75 % of the theoretical activity at 60 U

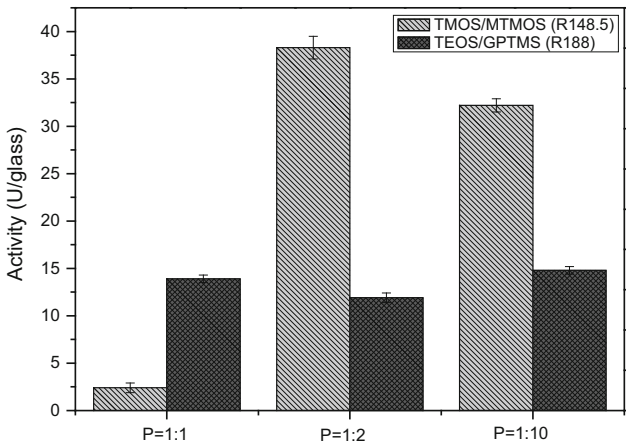

Fig. 4 Influences of precursors' molar ratios (P) for both samples (TMOS/MTMOS and TEOS/GPTMS [16]) on the activities of sol–gel encapsulated His_6-OPH, as presented in the U/glass slide

(Table 1; R3). The probability is that MTMOS carried out some protective action against lowering enzyme activity by TMOS during bio-composite layer formation. Therefore, MTMOS could be combined with TEOS to improve encapsulation efficiency further. The obtained results when compared to the TEOS/GPTMS matrix [10] indicated a preference for the latter composition, where the activities of the bio-composite films were found to be 58 U reaching 95 % of the theoretical value. Furthermore, the activities of the named bio-composite films were never lower than 15 % (9 U) of theoretical activity.

Optimal bio-composite layers of two types; TEOS/GPTMS (R = 148:1 and P = 1:2) and TMOS/MTMOS (R = 188 and P = 5:1), were chosen for further investigation involving enzyme kinetics (performance), stability, and reusability. Important issue in designing a biocatalyst for the use in detoxification/determination processes is diffusion resistance against substrate penetration. This can be evaluated by comparing apparent K_M values for free and immobilised enzymes. Typically, such differences in K_M are due to carriers' steric effects, ionic strength and diffusion limitations, as the porous material possesses an additional mass transfer resistance resulting of diffusion from bulk solution through the pores. Determination of the kinetic parameters for paraoxon hydrolysis by free and immobilised His_6-OPH demonstrated that, depending on the staring sol composition (TMOS/MTMOS vs. TEOS/GPTMS), the catalytic efficiency (k_{cat}/K_M) of an immobilised enzyme was approximately 400–1,800 times lower than the same one for the free enzyme (Table 2), primarily due to a decrease in turnover number (V_{max}/E_0). When an enzyme was immobilised, regardless of the sol–gel film composition, the K_M of the immobilised enzyme increased (approx. 140–150 times), whilst the V_{max} slightly decreased (approx. 1.4 and 6 times). The affinity changes of the enzyme towards substrate are probably a result of the structural changes of the enzyme that were introduced by the encapsulation procedure. Lower accessibility of the substrate to the active sites of the immobilised enzyme due to the surrounding sol–gel network is another possible explanation. The precursor pair TMOS/MTMOS seems to result in stronger enzyme molecular fixing within the bio-composite matrix thus lowering the catalytically important group's mobility. In comparison with TMOS (Table 1), the previously used TEOS led to a more flexible nanoenvironment surrounding the enzyme molecule. Another reason for the lower activities of TMOS-based bio-composites that might be worth considering is more effective binding of TMOS to the enzyme catalytic groups, if compared to TEOS-based films [11].

The catalytic activities for most enzymes are strongly dependent on the pH and temperature. The pH dependence of the enzymatic activities of both types of bio-composite layers and the enzyme in solution had quite distinct patterns (Fig. 5a). The maximum activity for the enzyme in solution was observed over a relatively wide pH range, from 9.5 to 11.5. The maximum activity for the immobilised enzyme (TMOS/MTMOS) was narrower and shifted towards a neutral range by 0.5–1.0 pH units, the optima being between 9.5 and 10.0. On the other hand, the optimal pH range for the TEOS/GPTMS bio-composite layer was broadened, being between 8.0 and 10.0. Both bio-composite layers displayed a steady catalytic activity over a wide pH range. The results revealed the greater activity of immobilised enzyme within a pH range from 6.5 to 9.5, thus allowing the His_6-OPH to operate with more than a 20–40 % higher relative activity around neutral pH when compared to the enzyme in solution. The observed trends further supported the assumption that the interactions between the His_6-OPH enzyme and the silica matrix prevented the enzyme from structural deterioration and could also produce a slight change in the pH micro-environment inside the sol–gel.

A similar trend regarding modified stability and activity was further observed for the performances of bio-films at different temperatures (Fig. 5b), where there was a tendency to shift the temperature optimas for both bio-composite layers to lower temperatures (39–43 °C) for TMOS/GPTMS, and to higher temperatures (44–59 °C) for TEOS/GPTMS. When compared to the enzyme in solution, the bio-film's activity (TEOS/GPTMS) exhibited 10–15 % higher relative activity within the whole tested temperature range, whereas the bio-layer based on TMOS/MTMOS showed increased relative activity (10–15 %) within the range between 35 and 50 °C, with a drastic drop in activity above 50 °C. From the practical point of view, it is obvious that the usage of the present bio-catalytic film

Table 2 Kinetic constants for POX hydrolysis by free and immobilised His_6-OPH

Sample	V_{max} (10^{-3} mM × s^{-1})	K_M (mM)	V_{max}/E_0 (s^{-1})	V_{max}/E_0K_M (M^{-1} × s^{-1})
Free enzyme—His_6-OPH [7]	2.5 ± 0.1	(10 ± 0.5) × 10^{-3}	5,100 ± 100	(5.1 ± 0.3) × 10^8
Bio-composite TEOS/GPTMS [16]	1.8 ± 0.2	1.5 ± 0.2	1,825 ± 75	(1.2 ± 0.1) × 10^6
Bio-composite TMOS/MTMOS	0.4 ± 0.1	1.4 ± 0.2	405 ± 50	(2.8 ± 0.2) × 10^5

T = 25 °C, pH 9.5 (50-mM CB). Bio-composite TEOS/GPTMS, R = 188, P = 5:1. Bio-composite TMOS/MTMOS, R = 148, P = 1:2

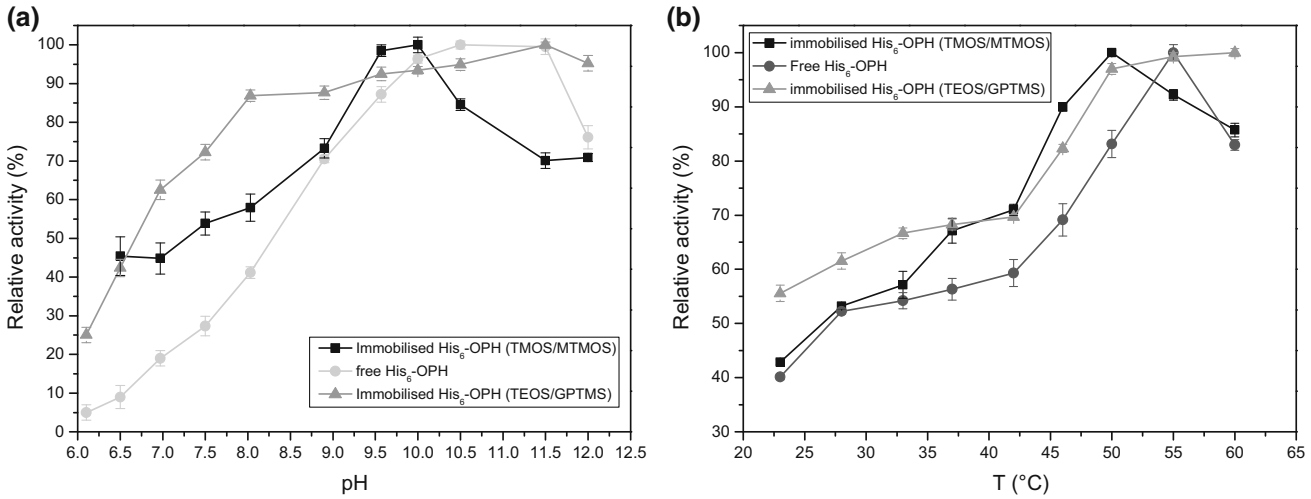

Fig. 5 Dependences of the relative enzymatic activity (%) of the immobilised His_6-OPH and His_6-OPH in solution on the pH (**a**) and temperature (**b**) of the reaction medium. The experimental conditions were set at 25 °C for the determination of pH dependence, and at fixed pH of 10.5 (50 mM CB) for temperature dependence, respectively. 0.675 mM POX was used as substrate

offers an advantage during processes based on enzyme catalysis, as it avoids the rather stringent conditions that most processes require when they are based on free enzyme catalysis.

One of the major advantages of the sol–gel immobilisation of enzymes is the possibility of using them in multiple catalytic reactions with insignificant loss of activity due to repeated usage. The immobilisation stability of an enzyme can be determined from the number of possible recycles. The obtained results showed that both bio-catalysts remained fairly active throughout the whole nine cycles (Fig. 6a). After the 1st cycle both biocatalyst layers lost some activity (10–20 %). As stated in our previous publication [10], the decrease in activity after the 1st cycle is probably due to detachment of the non-bound enzyme or incomplete recoveries of the produced bio-composite films. The TEOS/GPTMS sol–gel matrix presumably offers more convenient environment for the enzyme, resulting in 15 % higher relative activity after nine cycles, where activity of TMOS/MTMOS biocatalyst was still high enough at approx. 65 %. However, comparing both bio-catalyst films leads to the conclusion that the difference between TEOS/GPTMS and TMOS/MTMOS sol–gel matrix after nine cycles is negligible and statistically insignificant according to the paired t test ($p = 0.132$). Usually, it is considered that an enzyme can be reused until its activity decreases to less than 25 % from the initial value [26]. Therefore, it is obvious that the developed bio-composites met such conditions during at least nine recycles.

The stabilities of both types of bio-films were investigated by measuring the activities of the bio-catalyst films on various days after the bio-films' preparation. The bio-films were stored dry at 4 °C within closed vessels; the experiment being carried out over a period of 65 days. The biocatalyst films' activities on the 1st day were considered to be 100 %. Drastic drops of the activities for both bio-catalyst films were observed after 3–4 weeks, reaching about 50 % of initial level. After the 65th day, 20–25 % of the initial activity levels remained (Fig. 6b). Different compositions of starting sols had no major influence regarding the enzyme's stability and its activity over time. In both cases, to obtain optimal performance, the recommended usage of the bio-catalyst films was within the first 3 weeks after their preparation.

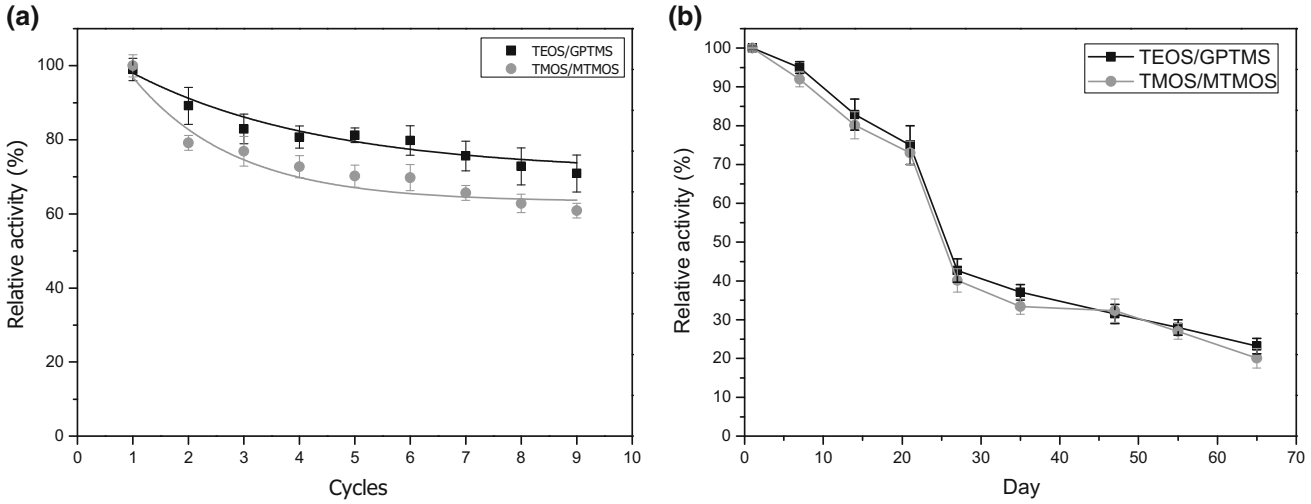

Fig. 6 Comparison between two types of biocatalyst films TEOS/GPTMS (R = 188, P = 5:1) and TMOS/MTMOS (R = 148, P = 1:2) for repeated use during the detoxification of POX. Conditions: 0.675 mM POX, T = 25 °C, 50-mM CB (pH 9.5)

4 Conclusion

The development of robust analytical devices for biocatalysis and biosensing relies on having the behaviour of the entrapped enzyme replicated or even enhanced relative to its behaviour in solution (i.e. enhanced activity or stability to denaturation). In this paper we have described and compared an effective method for the successful entrapment of the His_6-OPH enzyme within two types of sol–gel matrixes, TMOS/MTMOS and TEOS/GPTMS. The results indicated the strong influence of the sol–gel process parameters on the characteristics and final catalytic performances of the bio-composite films; TEOS-based matrix being more appropriate than TMOS-based for entrapment of His_6-OPH. The prepared silica matrixes offer a number of advantages over conventional organic polymers; such as immobilisation platforms for bio-catalysts and biosensors owing to their superior mechanical strength, chemical inertness, hydrophilic nature and, above all optical transparency. Based on the results we can conclude that the used enzyme favours the entrapment within a TEOS/GPTMS-based silica matrix. The encapsulated enzymes in both cases followed the Michaelis–Menten kinetics and maintained a good catalytic activity; the immobilisation efficiency resulted in 95 % for TEOS/GPTMS bio-film and 75 % for the TMOS/MTMOS bio-film. It was possible to use the same bio-composite thin film for nine repeated cycles. The encapsulated enzyme also possessed broadened pH and temperature working ranges; furthermore it revealed excellent activities at neutral pH in comparison with the native enzyme, thus allowing it to be more properly applied during biosensing or detoxification processes of OPCs (e.g. paraoxon in our case). Owing to its stability and reusability, this novel metalloenzyme-doped silica matrix provides a promising platform for the development of biosensors and immobilised enzyme reactors. Another interesting potential of such matrixes is the possibility of submicron-particle formation that would offer new perspectives for further development.

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