Encapsulation and biocatalytic activity of the enzyme pepsin in fatty lipid films by selective electrostatic interactions

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The encapsulation of pepsin by electrostatically controlled diffusion from solution into thermally evaporated fatty amine films is described and the catalytic activity of the immobilized enzyme on hemoglobin is investigated.

The entrapment of proteins in different inert matrices with the aim of protecting the proteins against microbial degradation, hydrolysis, autoproteolysis, deamidation, etc.; retention of the native protein structure and accessibility of the encapsulated proteins to cofactors, substrates and redox agents is a problem of current interest especially where application in biosensors/biocatalysis is sought. Proteins have been immobilized in phospholipid bilayers,1 on self-assembled monolayers (SAMs),2 in silicate sol–gels,3 in polymer matrices,4 in Langmuir–Blodgett films,5 within the galleries of a–zirconium phosphates6 as well as polymer microspheres.7 Developing on our earlier work on the spontaneous self-organization of fatty Langmuir Bilayers,1 the QCM mass uptake curve measured ex situ from a 250 Å thick ODA film as a function of time of immersion in the pepsin solution. Details of the QCM measurement procedure may be obtained from our earlier reports.9,10 It can be seen that the diffusion of pepsin into the lipid matrix is extremely rapid with maximum protein uptake being accomplished within 10 min of immersion. The small time scale for encapsulation of the enzyme in the lipid matrix is a particularly attractive feature of this approach and considerably improves upon the days to weeks timeframe required in other techniques for synthesis of such bio-composites.3,6 At pH 3, the ODA matrix is positively charged (pKd of ODA = 10.5) while the pepsin molecules are negatively charged (pI of pepsin = 1.0) thereby leading to attractive electrostatic interactions and a rapid diffusion of the proteins into the lipid matrix (see inset of Fig. 1). The maximum mass loading in the film (Fig. 1) is measured to be ca. 3900 ng cm⁻² of the film yielding a pepsin concentration of 6.3 × 10¹³ molecules per cm² of the ODA–pepsin composite film.

It is well known that proteins spontaneously concentrate at the phase boundaries2 and simple surface adsorption of pepsin on the ODA film surface must be ruled out. Contact angle measurements of a sessile water drop (1 µl, Rame Hart 100 goniometer used) were carried out on a 250 Å thick ODA film on Si(111) substrates after immersion in 10⁻⁶ M pepsin solution at pH 3 for 60 min and careful washing of the film. The measurements carried out at different points on the film surface yielded a mean value of 90° which is very close to the contact angle of 100° recorded for the as-deposited ODA film. The contact angle recorded for the pepsin–ODA composite film is much higher than the values of 20 and 15° measured for the bare Si(111) substrate surface and a pepsin film formed on Si by evaporating a drop of the pepsin solution respectively, indicating clearly that the pepsin molecules are not adsorbed on the surface of ODA but within the lipid film.

Fig. 2 shows the FTIR spectra recorded from a 1000 Å thick as-deposited ODA film (curve 1), the ODA film after immersion in 10⁻⁶ M pepsin solution kept at pH 3 for 60 min (curve 2) and the pepsin–ODA nanocomposite film after testing the catalytic activity of the pepsin–ODA film by immersion in hemoglobin solution (curve 3, as discussed subsequently). A number of vibrational modes can be observed for the three films. The amide I band occurs at 1647 cm⁻¹ for the pepsin–ODA nanocomposite film (feature A, curve 2, Fig. 2) and the film after reaction with hemoglobin (curve 3, Fig. 2). Whereas a small feature at this wavenumber does occur in the as-deposited ODA film, the intensity of this band increases in films 2 and 3 clearly showing that it originates in the pepsin molecules.

The position of this band is close to that reported for the amide I band of peptide bonds in proteins. The amide I band occurs at 1647 cm⁻¹ for the pepsin–ODA nanocomposite film (feature A, curve 2, Fig. 2) and the film after reaction with hemoglobin (curve 3, Fig. 2). Whereas a small feature at this wavenumber does occur in the as-deposited ODA film, the intensity of this band increases in films 2 and 3 clearly showing that it originates in the pepsin molecules.
FTIR spectrum recorded from a 1000 Å thick ODA film before (curve 1) and after immersion for 60 min in pepsin solution at pH 3 (curve 2). The spectrum measured from the pepsin–ODA film after assay with hemoglobin is also shown (curve 3). Features A–C are discussed in the text.

secondary structure of the enzyme in the ODA environment is unperturbed. The amide II band, which occurs at 1521 cm⁻¹ (feature C, Fig. 2), can also clearly be seen for the pepsin–ODA composite film as well as the film after the activity test with hemoglobin (curves 2 and 3 respectively). This band also indicates that the secondary structure of the protein is maintained in the encapsulated form. Small differences are observed in the amide II bands for the pepsin–ODA films before and after reaction with hemoglobin. This may be due to a contribution of hemoglobin molecules in the ODA matrix in the digested/undigested form and suggests a possible mechanism for the action of encapsulated pepsin on the heme-protein. However, further work is required before an unequivocal statement can be made on this observation. The origin of the band at 1564 cm⁻¹ (feature B, Fig. 2) is as yet not fully understood but it clearly arises from the ODA matrix and its intensity is reduced on complexation of the ODA molecules with pepsin.

The biochemical activity of the enzyme encapsulated in the lipid matrix was determined by reaction with a solution of hemoglobin (5 mg ml⁻¹) prepared in glycine–HCl buffer (0.05 M, pH 3.0) and by incubating the reaction mixture at 37 °C for 30 min. Pepsin digests hemoglobin and yields acid soluble products which are readily detected by their strong UV-visible absorbance. The reaction mixture and the precipitate removed by centrifugation.

The supernatant containing the acid soluble tyrosine and tryptophan residues was analysed using UV–VIS spectroscopy and the optical absorbance at 280 nm determined (Shimadzu-6201 PC spectrophotometer operated at a resolution of 1 nm). For comparison, the enzymatic activity of pepsin in solution was determined in a similar fashion. The values obtained from these activity measurements are given in Table 1. It is observed that the activity of pepsin in the ODA matrix is slightly less than that of the enzyme in solution. This may be a consequence of the orientation of the enzyme in the lipid matrix limiting the accessibility of the substrate hemoglobin to the active enzyme sites. Another possibility is that all the enzyme molecules do not participate in the biocatalysis in the first run. That this is so is indicated by the fact that one of the films showed enzymatic activity during three successive runs, albeit considerably reduced each time (Table 1).

The different measurements on the pepsin–ODA composite films clearly establish the following. The electrostatically controlled diffusion of the enzyme molecules from the aqueous phase into the lipid matrix may be accomplished under conditions close to that where the enzyme shows maximum activity by suitable choice of the lipid (either cationic or anionic). The enzyme molecules are encapsulated within the ODA matrix without significant distortion to the native structure. The elasticity of the bilayers may be primarily responsible for this and enables the matrix to adopt the contours of the enzyme molecule (Schematic, inset of Fig. 1). The reasonably fast time-scales for the synthesis of the enzyme–lipid composites and the enzyme–friendly encapsulation conditions are a major improvement over other techniques currently being investigated.¹ ³ ⁴ ⁶

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### Table 1 Comparison of enzymatic activity of pepsin in solution and ODA entrapped pepsin using hemoglobin as the substrate

<table>
<thead>
<tr>
<th>System</th>
<th>Amount of pepsin/μg</th>
<th>Activity/μg</th>
<th>Activity/μg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin</td>
<td>2.0</td>
<td>7.5</td>
<td>3.75</td>
</tr>
<tr>
<td>Pepsin–ODA (film 1)</td>
<td>2.7</td>
<td>9.5</td>
<td>3.33</td>
</tr>
<tr>
<td>Pepsin–ODA (film 2, run 1)</td>
<td>3.6</td>
<td>10.0</td>
<td>2.77</td>
</tr>
<tr>
<td>Pepsin–ODA (film 2, run 2)</td>
<td>3.6</td>
<td>12.0</td>
<td>0.333</td>
</tr>
<tr>
<td>Pepsin–ODA (film 2, run 3)</td>
<td>3.6</td>
<td>0.5</td>
<td>0.136</td>
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</tbody>
</table>

¹ One unit of enzyme will produce a change in absorbance at 280 nm of 0.001 min⁻¹ at pH 3.0 and 37 °C measured as PCA soluble products using hemoglobin as the substrate (ref. 14).

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**Notes and references**