

Synthesis of antibacterially bioinorganic composite by immobilising lysozymes in layered titanates

Jie Fu, Zhuping Han, Peng Ye, Xiaoping Dong

Department of Chemistry, School of Sciences, Zhejiang Sci-Tech University, Xiasha Higher Education Zone, Hangzhou, People's Republic of China
E-mail: ypmail2008@sohu.com

Published in Micro & Nano Letters; Received on 5th January 2013; Revised on 6th June 2013; Accepted on 24th June 2013

An antibacterially bioinorganic composite has been synthesised by intercalating lysozymes (LZs) into layered titanates with a convenient and efficient exfoliation-restacking strategy. This composite exhibits a loose slab morphology with a thickness of 5–10 layers, and the interlayer space is ~ 4.4 nm because of intercalation of enzymes. The immobilised amount of lysozymes is up to $\sim 68.3\%$ in weight because of the layer-by-layer alternate and swollen structure. This composite is stable in the neutral and weakly acidic condition, and only releases $<10\%$ of lysozymes in the $\text{pH} > 4$ solution. The immobilised LZs exhibit excellent thermal stability, which retain their initial activities of about 70% at 70°C for about 40 min. In addition, the residual activities of the immobilised enzymes are 68% after ten recycles reuse.

1. Introduction: To immobilise enzymes on a solid surface is an effective method for improving their stability and recovery for reuse [1, 2]. Therefore numerous efforts have been devoted to developing stable matrices for immobilisation of enzymes, including organic matrices [3, 4] and inorganic supporting materials [5–7]. Compared with organic matrices, inorganic materials attract more attention because of their regular structure and excellent mechanical, chemical and thermal stabilities.

Among these inorganic supports, abundant studies have focused on layered materials, which can be spontaneously adjusted to match various dimensions of guests. Up to now, a series of proteins has been intercalated into various layered structures, such as layered silicates [8], layered phosphates [9, 10], layered titanates [11, 12], layered niobates [13] and layered double hydroxides [14–16]. In addition, the electrostatic affinity of proteins to charged host layers leads to an orientated attachment and may be propitious to the accessibility of active sites [17–19], which would be partially buried because of the uncontrollable orientation of proteins in most previously reported methods [20, 21].

The immobilisation of proteins in layered materials by a conventional ion-exchange strategy provides tunable intercalation processes whereas these processes need a long term because the dimensions of the proteins is much larger than that of the inorganic ions in layered precursors. An alternative and effective approach to incorporate proteins in layered structures is to restack the exfoliated single layers with proteins by electrostatic self-assembly [11, 12]. Titanate nanosheets derived from layered precursors are ideal components for bioinorganic layered composites because of their non-toxicity and chemical stabilities [22, 23]. The exfoliated ultra-thin sheets are ~ 0.7 nm in thickness, semi-micrometre or micrometre the in lateral dimension and suitable for electrostatic assembly because of their negatively charged colloidal nature. The negatively charged nanosheets can assemble with these proteins, which have isoelectric points (IPs) above weak acid, to form bioinorganic composites. To date, some high IP proteins including haemoglobin [11] and horseradish peroxidase [24] have been incorporated in layered titanates.

Lysozyme (LZ) has been used as an antibacterial enzyme [25] in lots of applications such as food processing and the pharmaceutical industry [26, 27]. The size of the LZ is $3.0 \times 3.0 \times 4.5$ nm³ [28], and the IP is 11.4 [29]. The net charge of LZ in a neutral medium is positive, which provides an opportunity to electrostatically assemble with negatively charged titanate nanosheets. In the work

reported in this Letter, we immobilised LZ into layered titanate by the exfoliation-restacking strategy. It has been revealed that this composite possessed high enzyme amounts, and exhibited excellent thermal and reuse stabilities.

2. Materials and methods

2.1. Materials: TiO_2 ($\geq 98\%$), K_2CO_3 ($\geq 99\%$) and Li_2CO_3 ($\geq 97\%$) were used as received from the Sinopharm Chemical Reagent Co. Ltd. Tetrabutylammonium hydroxide (TBAOH) (40 wt% in water) was purchased from J&K Chemical Ltd. Hen egg-white lysozyme (E.C 3.2.1.17) and *Micrococcus lysodeikticus* were purchased from Sigma.

2.2. Synthesis of titanate nanosheets: The method developed by Sasaki *et al.* [30] was used for the preparation of parent layered titanates. Plate-like layered potassium titanate, $\text{K}_{0.8}\text{Ti}_{1.73}\text{Li}_{0.27}\text{O}_4$ (KTLO), was prepared by solid-state calcination of a stoichiometric mixture of K_2CO_3 , Li_2CO_3 and TiO_2 at 1000°C for 20 h. K^+ and Li^+ ions were removed by treating the KTLO crystals (1.0 g) with 100 ml of a 1 M HCl solution for 3 days at room temperature. The acid solution was replaced three times by decantation to ensure complete exchange. After acid treatment, protonic titanate, $\text{H}_{1.07}\text{Ti}_{1.73}\text{O}_4 \cdot \text{H}_2\text{O}$ (HTO), was filtered, washed with distilled water several times to remove excess H^+ and finally air dried. Exfoliation of the acid-exchange phase was performed by reaction with a TBAOH solution [31]; 0.4 g of HTO powder was immersed in 100 ml of 0.0025 M TBAOH solution and agitated vigorously. After 1 week of shaking, a translucent colloidal suspension in which unilamellar crystallites of $\text{Ti}_{0.87}\text{O}_2^{0.56-}$ nanosheet were well dispersed was obtained.

2.3. Immobilisation of LZ into titanate layers: The intercalation of LZ molecules into the interlayer of titanate was carried out using the electrostatic self-assembly deposition (ESA) method [32]. The pH value of the titanate nano sheets (TNSs) solution was adjusted to 7 by dilution and the addition of 0.5 M acetic acid. The LZ powder was dissolved into distilled water and adjusted to neutral by 0.5 M acetic acid. The stock solution of the LZ (1.0 mg ml^{-1}) and the exfoliated TNSs suspension ($\sim 0.8 \text{ mg ml}^{-1}$) were mixed in equivalent volumes. The mixture was stirred for 3 h at ambient temperature and then aged overnight. The resulting precipitation was centrifuged, washed with distilled water and finally treated by lyophilising.

2.4. Characterisation: The morphologies of HTO and LZ-TNSs were observed on a Hitachi S-4800 field emission scanning electron microscope (FESEM). A high-resolution transmission electron microscope (HRTEM) image was taken on a JEOL JEM-2100 electron microscope. Fourier-transform infrared (FTIR) spectra of the samples were recorded on a Nicolet Avatar 370 FTIR spectrometer. Thermogravimetric (TG) analysis was performed on a Perkin Elmer TGA/Pyris 1 system, and samples were heated from room temperature to 800°C at a rate of 5°C min⁻¹ in ambient atmosphere.

2.5. Determination of LZ activity: The LZ activity was determined by measuring turbidity changes in *M. lysodeikticus* bacterial cell suspensions (0.5 mg ml⁻¹) in 50 mM of pH 7.0 phosphate buffer solution (PBS) [33]. The amount of proteins was measured following Bradford's method [34].

2.6. Thermal stability measurement: The thermal stabilities of free and immobilised LZ were assayed by immersing them in PBS (50 mM, pH 7.0) for 40 min at 70°C and periodically determining their activities.

3. Results and discussion

3.1. Characterisation of LZ-TNSs: A facile ESA procedure of the immobilisation of LZ into the titanate interlayer under room temperature is described in Fig. 1. Upon mixing the two stock solutions, electrostatic attraction existed between negatively charged TNSs and positively charged LZs, which would cause the exfoliated TNSs to assemble with LZ, and the LZ-intercalated TNSs (LZ-TNSs) composite was obtained.

The microscopic morphology and structure characteristics of LZ-TNSs were examined by FESEM and TEM analyses. In Figs. 2a and b, pristine HTO exhibits a plate-like morphology with a well-ordered layered structure, whereas the as-prepared LZ-TNSs composite is free of tabular particles even though the layered structure is maintained. The shaggy appearance of the LZ-TNSs composite is ascribed to the wrinkle and overlap of TNSs, which can be clearly noted from Fig. 2c. Furthermore, the as flocculated LZ-TNSs composite shows a disorganised microtexture and porosity, and the resultant high surface area is very helpful for adsorption and transfer of guest species. In Fig. 2c, it is revealed that the composite has an ordered multilamellar structure including 5–10 layers TNSs intercalated with LZ molecules. A slablike crystal profile and resolved particle border with evident inorganic host layers can be observed from the HRTEM image (Fig. 2d). The TNSs are parallel to each other with a basal distance of ~4.4 nm. As the thickness of the TNS monolayer is about 0.7 nm, accordingly, the gallery height is 3.7 nm. The observed interlayer distance corresponds well with the size of the LZ, which is within 3.0 × 3.0 × 4.5 nm³ [28], demonstrating a monolayer arrangement in the interlayer of TNSs. Obviously, immobilisation of proteins in an

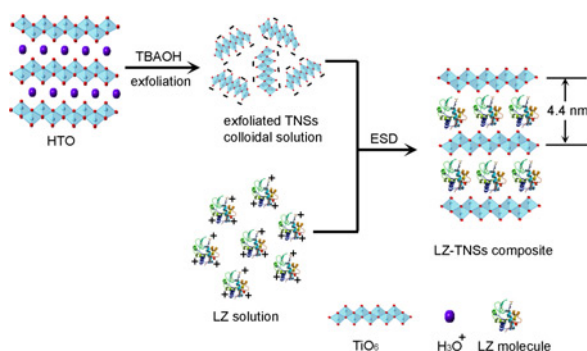


Figure 1 Schematic illustration of the formation of LZ-TNSs bioinorganic composite

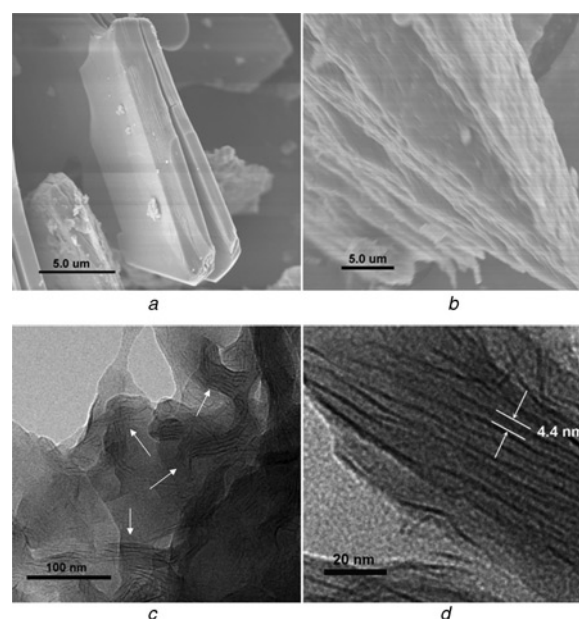


Figure 2 FESEM TEM and HRTEM images
a HTO
b LZ-TNSs composite
c TEM image of LZ-TNSs composite
d HRTEM image of LZ-TNSs composite

inorganic skeleton by the ESA method is much more feasible than direct intercalation of proteins into layered materials by ion-exchange [35], since the distance provided by charge-bearing two-dimensional (2D) nanosheets is flexible and adapts to the protein dimensions.

Fig. 3 displays the FTIR spectra of HTO, LZ and LZ-TNSs composites. The amide I and amide II bands are relative to the stretching of C=O and bending of N–H, respectively, which are sensitive to the protein secondary structure and chosen to detect LZ native structural changes. Obviously, the amide I (1653 cm⁻¹) and amide II (1540 cm⁻¹) bands of the LZ-TNSs are actually the same as those of the free LZ, suggesting that the structures of the immobilised LZ are undisturbed by intercalating them into the layered titanate. In addition, the absorption peak at 460 cm⁻¹ for the spectra of the LZ-TNSs composite is almost the same as HTO, which corresponds to the characteristic Ti–O vibration of

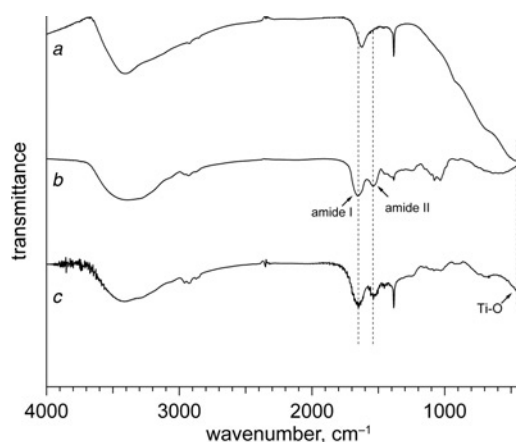


Figure 3 FTIR spectra
a HTO composite
b Free LZ composite
c LZ-TNSs composite

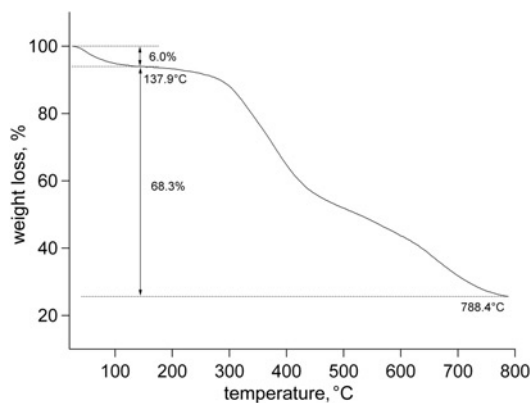


Figure 4 TG curve of LZ-TNSs composite

the TiO_6 octahedra [35], and further confirms the existence of the titanate layer in the composite.

The amount of immobilised LZ was investigated by TG analysis (Fig. 4). Weight loss in the first step (below 137.9°C) was found to be 6% which can be ascribed to the removal of absorbed water. It has been confirmed that the weight loss originating from the collapse of the titanate layers to produce an anatase phase can be ignored [36]. Thus, the second step between 137.9 and 788.4°C is assigned to the decomposition of LZ in the interlayer galleries of TNSs. The second step involves a weight loss of 68.3%, demonstrating that nearly two-thirds of the LZ-TNSs in weight is LZ, which is much higher than the immobilised value of mesoporous materials [36]. This should be attributed to the layer-to-layer alternate assembly and the titanate swollen structure [35]. Obviously, such a high immobilisation amount of LZ is of great benefit to enhance the activity of the materials.

The stability of LZ immobilisation upon pH was evaluated by immersing LZ-TNSs in PBSs with different pH (50 mM) for 30 min and then determining the amount of released protein (Fig. 5). A small amount of released protein (<10%) was found in the pH value region of 4–7, which is attributed to the strongly electrostatic interaction between LZs and titanate layers. However, as the pH value was over 4, the amount of retained protein drastically decreased, which might be explained by the replacement of protein by H_3O^+ which easily occurred under the acidic environment.

3.2. Enzyme activity and stability: After immobilisation, the enzyme always loses part of its activity. In our experiments, activity retention of the immobilised LZ is about 26%. For the

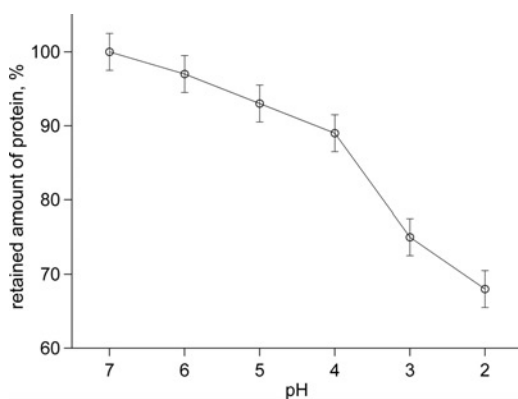


Figure 5 Effect of pH on retained amount of protein

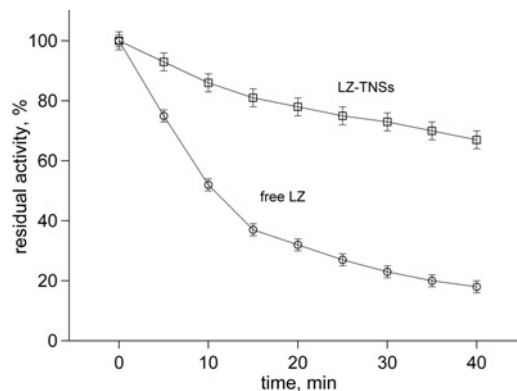


Figure 6 Thermal stabilities of the free (open circles) and immobilised (open squares) LZ

LZ-TNSs composite, the interaction between enzyme and support is electrostatic force, which belongs to physical interaction and generally favours native enzyme conformation [37, 38]. However, because of the immobilisation, it is difficult to contact LZ with bacteria which largely inhibits LZ activity. On the other hand, the LZ-TNSs composite appears as a porous structure with an irregular edge, which could make part of LZ contact bacteria readily.

The thermal stabilities of free and immobilised LZs are given in Fig. 6. It can be observed that the free LZ loses 80% of its initial activity within 40 min at 70°C, whereas the immobilised one retains its initial activity of 70% under the same condition. These results indicate that the thermal stability of the immobilised LZ is much better than that of the free one since the interaction between the enzyme and the support could prevent LZ conformation transition at high temperature.

One of the most important aims of enzyme immobilisation is to enhance enzyme reuse stability [39, 40]. To evaluate this stability, the LZ-TNSs composite was washed with PBS (50 mM, pH 7.0) after every run and reintroduced into a fresh solution, this being repeated for ten cycles. Fig. 7 shows the effect of reuse on the activities of these immobilised enzymes. After ten reuses, the residual activity of the immobilised enzyme is 68%. Inactivation of the enzyme might be caused by denaturation of protein and the leakage of protein from the supports upon use. As the special layer-by-layer structure of this composite, a large number of LZs are immobilised in the internal part of this composite and these enzymes could replace the enzymes released during the reuse cycle, so that the activity of the LZ-TNSs composite could keep a relatively high value for a long period.

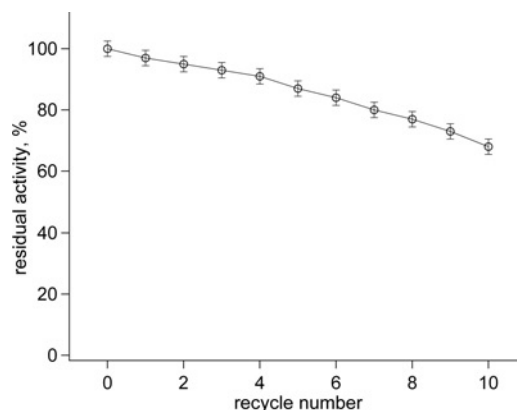


Figure 7 Reuse-stability of the immobilised LZ

4. Conclusion: In this Letter, one high IP enzyme, LZ, was intercalated into layered titanates to fabricate the bioinorganic composite, with a convenient and efficient exfoliation-restacking strategy. This bioinorganic composite exhibits a loose slab morphology with a thickness of 5–10 layers, which is very helpful for the adsorption and transfer of guests. The immobilised amount of LZ is up to ~68.3 wt%, because of the alternate protein layers and titanate host layers and the swollen structure, and is much higher than that by the common adsorption method, which cannot form such a layer-by-layer nanostructure. This composite is stable in the neutral and weakly acidic condition, and only releases <10% LZ in the pH >4 solution. The immobilised LZs exhibit excellent thermal and reuse stabilities. For example, the residual activities of the immobilised enzymes are 68% after ten recycles. These results demonstrated that the synthesis of this bioinorganic composite with high enzyme loading and excellent stability provides a potential strategy in enzyme immobilisation technology for industrial applications.

5. Acknowledgment: The authors gratefully acknowledge financial support from the National Natural Science Foundation of China (21001093, 50803059), the Zhejiang Provincial Natural Science Foundation of China (Y4090285, Y407275), and the Science Foundation of Zhejiang Sci-Tech University (0913840-Y).

6 References

- [1] Bornscheuer U.T.: 'Immobilized enzymes: how to create more suitable biocatalyst', *Angew. Chem. Int. Ed.*, 2003, **42**, pp. 3336–3337
- [2] Klibanov A.M.: 'Immobilized enzymes and cells as practical catalysts', *Science*, 1983, **219**, pp. 722–727
- [3] Hansen J.A., Wang J., Kawde A.N., Xiang Y., Gothelf K.V., Collins G.: 'Quantum-dot/aptamer-based ultrasensitive multi-analyte electrochemical biosensor', *J. Am. Chem. Soc.*, 2006, **128**, pp. 2228–2229
- [4] Wang J., Musameh M., Lin Y.H.: 'Solubilization of carbon nanotubes by nafion toward the preparation of amperometric biosensors', *J. Am. Chem. Soc.*, 2003, **125**, pp. 2408–2409
- [5] Kalska-Szostko B., Rogowska M., Dubis A., Szymański K.: 'Enzymes immobilization on Fe₃O₄-gold nanoparticles', *Appl. Surf. Sci.*, 2012, **258**, pp. 2783–2787
- [6] Zhou Y.L., Hu N.F., Zeng Y.H., Rusling J.F.: 'Heme protein-clay films: direct electrochemistry and electrochemical catalysis', *Langmuir*, 2002, **18**, pp. 211–219
- [7] Kawai A., Urabe Y., Itoh Mizukami T.F.: 'Immobilization of lysozyme on the layered silicate RUB-15', *Mater. Chem. Phys.*, 2010, **122**, pp. 269–272
- [8] Peng S.G., Gao Q.M., Wang Q.G., Shi J.L.: 'Layered structural heme protein magadiite nanocomposites with high enzyme-like peroxidase activity', *Chem. Mater.*, 2004, **16**, pp. 2675–2684
- [9] Ding Y., Jones D.J., Maireles-Torres P., Roziers J.: 'Two-dimensional nanocomposites: alternating inorganic-organic polymer layers in zirconium phosphate', *Chem. Mater.*, 1995, **7**, pp. 562–571
- [10] Kumar C.V., McLendon C.L.: 'Nanoencapsulation of cytochrome c and horseradish peroxidase at the galleries of α -zirconium phosphate', *Chem. Mater.*, 1997, **9**, pp. 863–870
- [11] Wang Q.G., Gao Q.M., Shi J.L.: 'Enhanced catalytic activity of hemoglobin in organic solvents by layered titanate immobilization', *J. Am. Chem. Soc.*, 2004, **126**, pp. 14346–14367
- [12] Han Z.P., Fu J., Ye P., Dong X.P.: 'A general strategy for protein immobilization in layered titanates: polyelectrolyte-assisted self-assembly', *Enzyme Microb. Tech.*, 2013, **53**, pp. 79–84
- [13] Gao L., Gao Q.M., Shi J.L.: 'Immobilization of hemoglobin at the galleries of layered niobate HCa₂Nb₃O₁₀', *Biomaterials*, 2005, **26**, pp. 5267–5275
- [14] Vial S., Ghanbaja J., Forano C.: 'Precipitation of Zn₂Al LDH by urease enzyme', *Chem. Commun.*, 2006, **290**, pp. 290–292
- [15] Shan D., Cosnier S., Mousty C.: 'Layered double hydroxides: an attractive material for electrochemical biosensor design', *Anal. Chem.*, 2003, **75**, pp. 3872–3879
- [16] An Z., Lu S., He J., Wang Y.: 'Colloidal assembly of proteins with delaminated lamellas of layered metal hydroxide', *Langmuir*, 2009, **25**, pp. 10704–10710
- [17] Wang X., Zhou D., Sinniah K., *ET AL.*: 'Electrostatic orientation of enzymes on surfaces for ligand screening probed by force spectroscopy', *Langmuir*, 2006, **22**, pp. 887–892
- [18] MacBeath G., Schreiber S.L.: 'Printing proteins as microarrays for high-throughput function determination', *Science*, 2000, **289**, pp. 1760–1763
- [19] An Z., He J., Lu S., Yang L.: 'Electrostatic-induced interfacial assembly of enzymes with nanosheets: controlled orientation and optimized activity', *AIChE J.*, 2010, **56**, pp. 2677–2686
- [20] Mao H., Yang T., Cremer P.S.: 'Design and characterization of immobilized enzymes in microfluidic systems', *Anal. Chem.*, 2002, **74**, pp. 379–385
- [21] DeLouise L.A., Miller B.L.: 'Quantitative assessment of enzyme immobilization capacity in porous silicon', *Anal. Chem.*, 2004, **76**, pp. 6915–6920
- [22] Sasaki T., Watanabe M., Hashizume H., Yamada H., Nakazawa H.: 'Macromolecule-like aspects for a colloidal suspension of an exfoliated titanate. Pairwise association of nanosheets and dynamic reassembling process initiated from it', *J. Am. Chem. Soc.*, 1996, **118**, pp. 8329–8335
- [23] Sasaki T., Watanabe M.: 'Osmotic swelling to exfoliation. Exceptionally high degrees of hydration of a layered titanate', *J. Am. Chem. Soc.*, 1998, **120**, pp. 4682–4689
- [24] Kamada K., Nakamura T., Tsukahara S.: 'Photoswitching of enzyme activity of horseradish peroxidase intercalated into semiconducting layers', *Chem. Mater.*, 2011, **23**, pp. 2968–2972
- [25] Pellegrini A., Thomas U., Fellenberg R., Wild P.: 'Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character', *J. Appl. Bacteriol.*, 1992, **72**, pp. 180–187
- [26] Lei J., Fan J., Yu C., *ET AL.*: 'Immobilization of enzymes in mesoporous materials: controlling the entrance to nanospace', *Micropor. Mesopor. Mater.*, 2004, **73**, pp. 121–128
- [27] Ghosh R., Cui Z.F.: 'Purification of lysozyme using ultrafiltration', *Biotechnol. Bioeng.*, 2000, **68**, pp. 191–203
- [28] Jackler G., Steitz R., Czeslik C.: 'Effect of temperature on the adsorption of lysozyme at the silica/water interface studied by optical and neutron reflectometry', *Langmuir*, 2002, **18**, pp. 6565–6570
- [29] Kisler J.M., Stevens G.W., O'Connor A.J.: 'Adsorption of proteins on mesoporous molecular sieves', *Mater. Phys. Mech.*, 2001, **4**, pp. 89–93
- [30] Sasaki T., Kooli F., Iida M., *ET AL.*: 'A mixed alkali metal titanate with the lepidocrocite-like layered structure. Preparation, crystal structure, protonic form, and acid-base intercalation properties', *Chem. Mater.*, 1998, **10**, pp. 4123–4128
- [31] Tanaka T., Ebina Y., Takada K., Kurashima K., Sasaki T.: 'Oversized titania nanosheet crystallites derived from flux-grown layered titanate single crystals', *Chem. Mater.*, 2003, **15**, pp. 3564–3568
- [32] Unal U., Matsumoto Y., Tanaka N., Kimura Y., Tamoto N.: 'Electrostatic self-assembly deposition of titanate (IV) layered oxides intercalated with transition metal complexes and their electrochemical properties', *J. Phys. Chem. B*, 2003, **107**, pp. 12680–12689
- [33] Shugar D.: 'The measurement of lysozyme activity and the ultraviolet inactivation of lysozyme', *Biochem. Biophys. Acta*, 1952, **8**, pp. 302–309
- [34] Bradford M.M.: 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Anal. Biochem.*, 1976, **72**, pp. 248–254
- [35] Wang Q.G., Gao Q.M., Shi J.L.: 'Gold nanoparticle embedded, self-sustained chitosan films as substrates for surface-enhanced Raman scattering', *Langmuir*, 2004, **20**, pp. 10231–10237
- [36] Guo T., Wang L.S., Evans D.G., Yang W.S.: 'Synthesis and photocatalytic properties of a polyaniline-intercalated layered protonic titanate nanocomposite with a p-n heterojunction structure', *J. Phys. Chem. C*, 2010, **114**, pp. 4765–4772
- [37] Watanabe H., Matsuyama T., Yamamoto H.: 'Preparation of immobilized enzyme gel particles using an electrostatic atomization technique', *Biochem. Eng. J.*, 2001, **8**, pp. 171–174
- [38] Dreyer S., Salim P., Kragl U.: 'Driving forces of protein partitioning in an ionic liquid-based aqueous two-phase system', *Biochem. Eng. J.*, 2009, **46**, pp. 176–185
- [39] Murakami-Nitta T., Kirimura K., Kino K.: 'Degradation of dimethyl sulfoxide by the immobilized cells of *Hyphomicrobium denitrificans* WU-K217', *Biochem. Eng. J.*, 2003, **15**, pp. 199–204
- [40] Chen B., Hu J., Miller E.M., Xie W., Cai M., Gross R.A.: 'Candida antarctica lipase B chemically immobilized on epoxy-activated micro- and nanobeads: catalysts for polyester synthesis', *Biomacromolecules*, 2008, **9**, pp. 463–471