Facile one-pot synthesis of silica-based lipase nanocatalysts for improving stability

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The inactivation of lipases caused by organic solvents, high temperature, extremely acidic and alkaline requires a high stability. In this work, a novel one-pot synthesis for the lipase immobilisation on silica nanoparticles was reported. The optimal amounts of (3-ammonia propyl) triethoxysilane (APTES) and ammonia were studied. The apparent K_m value of the immobilised Candida rugosa lipase (CRL) was lower than that of free enzyme, showing affinity of the immobilised CRL to its substrate had increased. The results of stability test showed that the immobilised lipase was more stable than free enzyme at different temperatures and pH values. In particular, the immobilised CRL kept 96% activity at 90°C, while the free enzyme only remained 75% activity. Immobilised lipase had high catalytic efficiency, enhanced stability and recyclable usability compared to free enzymes, because of the cross-linking between the protein and the rigid carrier. Therefore, the immobilised method would be beneficial to improving the activity and stability of enzyme universally.

1. Introduction: Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyses a wide range of bioconversions, including hydrolysis, esterification, transesterification, and aminolysis. Lipases are employed in many applications, including pharmaceutical agents, agricultural chemicals, perfumes and leather processing, as well as in the paper and detergent industries [1–5]. However, the inactivation of lipases caused by organic solvents, high temperature, extremely acidic and alkaline often becomes a problem. Therefore, stable lipases in the catalytic reactions are required [6–8]. Compared with free enzyme, the immobilised enzyme performs much better pH or temperature stability, more easy removal from the reaction mixture, higher rapid reaction rate [9–11].

To enhance stability and lifetime of lipases, various new materials and technologies have been used for immobilisation [12–16]. However, finding more practical and effective carriers and better methods to immobilise lipases has attracted many relevant researchers. Recently, much research has focused on using nanostructured materials for immobilisation [11]. The nanostructured materials are very special, which are ideal carriers for the enzyme, that can offer many advantages, such as large surface-to-volume ratio, high surface activity, and strong adsorption ability [10, 17]. Silica nanoparticles are typical representatives of nanostructured materials, and it can provide reactive groups on the surface after surface modification [18]. In comparison with the adsorption methods used in previous studies, multipoint covalent binding is more durable, stable under harsh microenvironments, and potentially provides higher loading efficiencies and better enzyme activity [19–23]. In previously reported researches, enzymes can be immobilised onto the pre-made silica nanoparticles modified by (3-aminopropyl) trimethoxysilane (APTES). The amino group of APTES reacted with the carboxyl groups on the surface of enzymes [24–26].

In this Letter, a facile route for lipases immobilisation on silica nanoparticles by a one-pot traditional reversible microemulsion tetraethylorthosilicate (TEOS) hydrolysis method under mild conditions was reported. Compared to a common method of enzyme immobilisation using nanoparticles as a support, our method was more simple and effective. Due to the long processes, multifarious operations, enzyme inactivation and suboptimal immobilised enzyme concentration result in a low enzyme activity in the common method [27]. The optimal conditions of immobilisation were studied. The enzyme activity, pH and thermo stability of immobilised lipases were also investigated. The results revealed that the immobilised lipases showed high catalytic efficiency, enhanced stability and recyclable usability. The one-pot reversible microemulsion TEOS hydrolysis method was promising for enzyme immobilisation.

2. Materials and methods

2.1. Materials: Candida rugosa lipase (CRL) (EC 3.1.1.3), TEOS, APTES and Triton X-100 were purchased from Sigma-Aldrich, USA. EDC·HCl and NHS were obtained from GL Biochem (Shanghai) Ltd. P-nitrophenyl palmitate (p-NPP) was obtained from Aladdin, Shanghai, China. Cyclohexane, n-hexanol, acetone and ammonia solution were obtained from HEOWNS Company, Tianjin, China. Bovine serum albumin (BSA) was purchased from Biotopped, China.

2.2. Immobilisation of CRL by a one-pot method: CRL was immobilised by a one-pot traditional reversible microemulsion method. CRL aqueous solution (20 mg/ml, 0.5 ml), cyclohexane (7.5 ml), n-hexanol (1.6 ml) and Triton X-100 (1.8 ml) were mixed together and stirred about 30 min to prepare a water-in-oil microemulsion. Then 40 mg EDC·HCl and 50 mg NHS were dissolved in 0.5 ml deionised water with shaking repeatedly. Then the solution was mixed with the former water-in-oil microemulsion system and stirred for another 30 min. Then APTES (6 µl) was added and stirred for another 8 h. Then 100 µl TEOS was added to the water-in-oil microemulsion. For initiating hydrolysis and polycondensation, 100 µl of 25% ammonia was added and stirred for 15 h. Immobilised CRL nanoparticles were obtained by adding 20 ml acetone and centrifuging 30 min at 5000 rpm for precipitating silica nanoparticles. Then the nanoparticles were washed with deionised water and ethanol three times, respectively, to remove unreacted enzyme. The loading amount of enzyme protein was measured by the traditional Bradford method [28].

Scanning electron microscope (SEM, HITACHI, S4700) and transmission electron microscopy (TEM, HITACHI, H-800) were used to study the morphology of the immobilised CRL nanoparticles. For SEM, a drop of the suspension of the prepared sample was added to a cover glass pieces and dried at room temperature. For TEM, the sample was prepared by pipetting a drop of the aqueous solution of the samples onto a 230 mesh holy carbon copper grid and drying on a filter paper. Fourier transform infrared

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(FTIR) spectra were obtained by using an FTIR spectrophotometer, Nicolet1, 70SX (Hitachi, Tokyo, Japan).

2.3. Enzyme activities assay: According to the reported method, free and immobilised CRL activities were determined by using p-nitrophenyl acetate as the substrate. The activity assay was carried out at room temperature in phosphoric acid buffer solution (25 mM, pH 7.4, 3 ml), p-nitrophenyl acetate (100 mM, 50 µl), and free CRL or immobilised CRL (100 µg/ml, 50 µl). The absorbance at 410 nm after 5 min was performed on a Shimadzu UV-2450 (Kyoto, Japan) UV–visible spectrophotometer using quartz cuvettes [29]. One unit of enzymatic activity was defined as the amount of enzyme producing 1 µmol of p-nitrophenol in 1 min. The kinetic parameters, K_m and K_{cat} were determined for free and immobilised lipase using solutions of p-NPP at the following concentrations: 0.1, 0.2, 0.4, 0.8, 1.2, 1.5 mM.

2.4. Study the immobilised enzyme in different concentration of APTES and ammonia: During preparing the immobilised enzymes by the one-pot method, we changed the volume of APTES (from 0 to 10 µl). For the study of ammonia, we changed the volume of ammonia, 50, 100, 150 µl, respectively. The activity assay of immobilised enzymes was measured by the aforementioned method to find the best condition for immobilisation.

2.5. Enzyme stability test: For stability test at different pH values, the suspension of the immobilised enzyme was added to 1 ml of various pH solutions for 4 h. Then the residual overall enzymatic activity was measured by recording the absorbance at 410 nm. To test the stability at different temperatures, the suspensions of the immobilised enzyme were incubated at 30–90°C for 30 min. The activity was determined by using p-nitrophenyl acetate as the substrate. The absorbance was immediately recorded at 410 nm after 5 min. For all these assays, free CRL at the same protein concentrations were also treated and analysed using the same procedure.

3. Results and discussion: In this research, we chose CRL as the guest for encapsulation, which is one of the typical lipases. The immobilised CRL nanoparticles were synthesised by a one-pot traditional reversible microemulsion TEOS hydrolysis method. The procedure of the immobilisation of CRL was shown in Fig. 1. The CRL aqueous solution was added to the organic phase to form a reversible microemulsion. Then, we added EDC, NHS and APTES into the water-in-oil microemulsion simultaneously, which was easier than common crosslinking process. EDC and NHS introduced 'zero length' amide cross-links between carboxylic groups from CRL and amino groups from APTES [30]. Finally, TEOS hydrolysis in the microemulsion formed immobilised CRL nanoparticles. The silanol groups on the silica surface play an important role in the surface modification process, which are the reactive sites for APTES [31–34].

The bioactivity of enzymes would be mostly maintained because the modification by the EDC/NHS method was usually in a mild condition [10]. However, a molecular CRL could be modified by several molecules of silanes when adding different amounts of APTES into the reaction system. It was found that the catalytic activities of immobilised CRL increased with the increase of amounts of APTES modification. According to the results in Fig. 2*a*, it was found that the relative activity of immobilised CRL attained maximum when the concentration (v/v) of APTES was 5×10^{-4}. It is the optimal content of silane modification in the immobilisation system. Because in that condition, the covalent binding to insoluble silica nanoparticles could provide a much more efficient enzyme retention with materials with open structures that present minimal mass transfer resistance to substrates [10]. Nevertheless, if the concentration of APTES was too high, more APTES was used to modify on the surface of CRL. The relative activity would decrease caused by the decrease of the active sites of enzymes. The influence of ammonia addition on immobilisation and catalytic activity of CRL was also investigated (Fig. 2*b*). The result showed that the amount of ammonia had little effect on catalytic activities of immobilised CRL. However, when the concentration of ammonia was too high, high alkaline led to low catalytic activities.

To further confirm immobilisation of the enzyme by silica nanoparticles, the immobilised CRL nanoparticles, silica nanoparticles and free CRL were analysed by FTIR. For free CRL, two obvious IR absorption bands centred at 1645 and 1552 cm^{-1} are observed (curve (1) of Fig. 3), ascribed to the typical amide I and II absorption bands, respectively [10]. Furthermore, the typical amide I and II absorption bands could also be found in the IR spectrum of immobilised CRL nanoparticles (curve (3) of Fig. 3). The peaks at 800 cm^{-1} can be attributed to the Si–O stretching

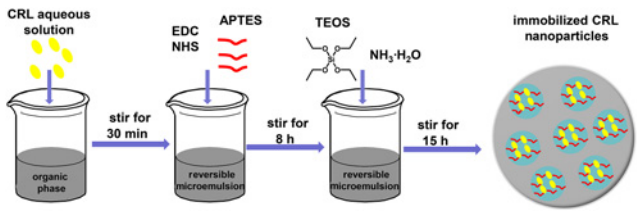

Fig. 1 *Synthesis of immobilised CRL nanoparticles by one-pot processes*

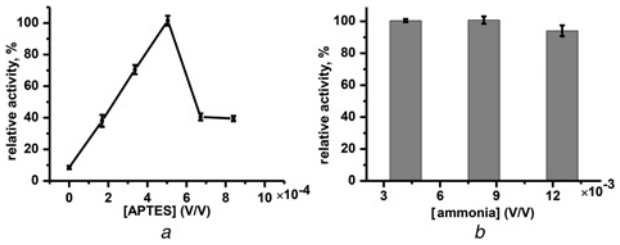

Fig. 2 *Influences on the catalytic activity of immobilised enzymes*
a APTES concentration
b Ammonia concentration
CRL activities were measured as follows: Experiments were carried out at room temperature in phosphoric acid buffer solution (25 mM, pH 7.4, 3 ml), p-nitrophenyl acetate (100 mM, 50 µl), and immobilised CRL (100 µg/ml, 50 µl)

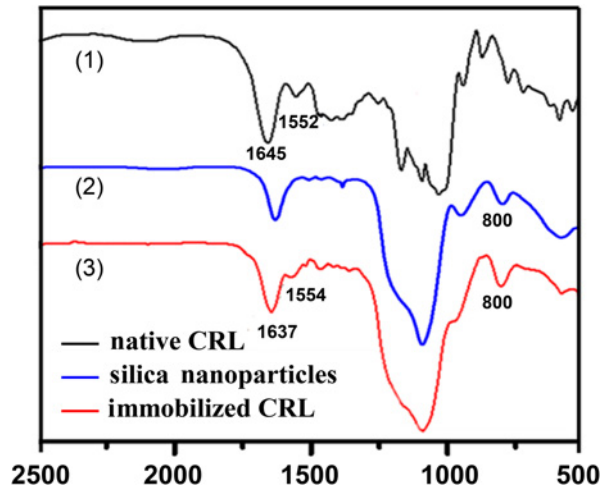

Fig. 3 *FTIR spectra of native CRL (1), silica nanoparticles (2) and immobilised CRL nanoparticles (3)*

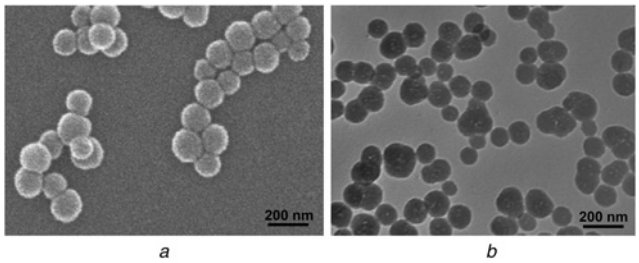

Fig. 4 *Morphology images of immobilised CRL nanoparticles*
a SEM image
b TEM image

vibrations (curves (2) and (3) of Fig. 3) [35]. Thus, it could be confirmed that CRL was successfully immobilised into silica nanoparticles.

To characterise the size and morphology of the immobilised enzyme nanoparticles, we performed SEM (Fig. 4*a*) and TEM (Fig. 4*b*). It indicated that the formed silica nanoparticles containing modified CRL have the uniform morphology, which are microspheres. The images showed that the average diameter of the immobilised CRL nanoparticles was ~80 nm. From the TEM image, it is evident that the carrier has highly porous structures, which can immobilise lots of enzymes.

Kinetic parameters (K_m and V_{max}) of free and immobilised CRL were determined by measuring initial rates of the hydrolysis of p-NPP in pH 7.4 PBS at 37°C at 410 nm [29]. The activities of free and immobilised CRL in various concentrations of the substrate were plotted in the form of Lineweaver–Burk plots. The Lineweaver–Burk equation could be written as

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\frac{1}{[S]}\right) + \frac{1}{V_{max}} \quad (1)$$

where [*S*] is the concentration of substrate and *V* and V_{max} represent the initial and maximum rates of reactions, respectively. K_m is the Michaelis–Menten constant (the substrate concentration when the rate is half of V_{max}) [36]. The K_m and V_{max} values of free and immobilised CRL were calculated from the intercepts on *x*- and *y*-axis, respectively (Table 1). In this Letter, the K_m values for free CRL and CRL immobilised onto silica nanoparticles were determined to be 2.8325 and 2.7395 mmol/l, respectively, clearly showing that the immobilisation on silica nanoparticles increase the enzyme–substrate affinity. The increase in the affinity of the CRL to its substrate was probably caused by structural changes in the enzyme introduced by the immobilisation procedure [33]. The K_{cat} value of the immobilised CRL were 17.36 s^{-1}, which was a little lower than that of free CRL. Because of immobilisation, diffusion of enzymes reduced, causing a decrease in enzyme V_{max} [29].

The pH stability of free and immobilised CRL was examined at six different pH values. The result of Fig. 5*a* shows that the immobilised CRL exhibited higher activity than that of the free enzyme in all extreme pH values. It means that the pH ability of immobilised enzyme was enhanced, due to the protecting effect of the nanoparticles in acidic and alkaline conditions. For thermal stability experiment, free and immobilised CRL were investigated in the different temperatures (from 30 to 90°C) for 30 min. The immobilised CRL retained the better relative activity, compared with that of free enzyme (Fig. 5*b*). Especially at 90°C, the immobilised CRL

Table 1 Kinetic parameters of free and immobilised CRL

	V_{max}, μmol/min	K_{max}, mmol/l	K_{cat}, 1/s
free	0.0306	2.8325	18.972
immobilised	0.028	2.7395	17.36

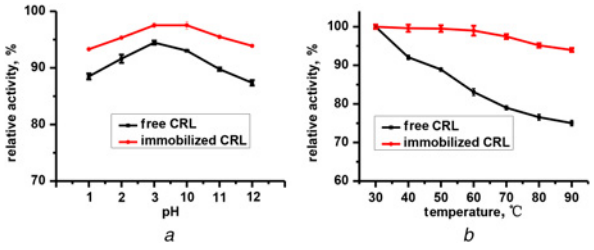

Fig. 5 *The stabilities of immobilised CRL nanoparticles and free CRL*
a pH stability and
b Thermo-stability
The activity assay was carried out in phosphoric acid buffer solution (25 mM, pH 7.4, 3 ml) by using pnitrophenyl acetate (100 mM, 50 μl) as the substrate

kept 96% activity, while the free enzyme only remained 75% activity. It indicated that the immobilised CRL showed a high thermostability. Therefore, the enzyme stability could be improved by the microemulsion against various environmental factors, because the cross-linking between the protein and the rigid carrier has a big effect on the properties of enzyme [10].

4. Conclusions: In this Letter, we reported a novel one-pot method for immobilisation of enzymes. CRL was immobilised into silica nanoparticles successfully and formed uniform microspheres. Kinetic constants indicated the affinity of the immobilised enzyme for the substrate increased, because of the structural changes in the enzyme introduced by the immobilisation procedure. The results showed that the immobilised CRL was more stable than free enzyme in different temperatures and pH values. In particular, the immobilised CRL nanoparticles showed a high thermo-stability, which could still remain 96% activity at 90°C, due to the cross-linking between the protein and the rigid carrier had a big effect on these properties. This method would also mostly reduce the diffusion and decomposition of the intermediates. These results point to the significant potential for the reuse of CRL when immobilised onto the silica nanoparticles. Furthermore, the proposed protocol is expected to find wide applications in the immobilisation of other biomacromolecules. It can universally be beneficial to improving the activity and stability of enzymes.

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6 References

[1] Choudhury P.: 'Industrial application of lipase: a review', *Biopharm*, 2015, **1**, (2), pp. 41–47
[2] Maharana A., Ray P.: 'A novel cold-active lipase from psychrotolerant pseudomonas sp. Akm-L5 showed organic solvent resistant and suitable for detergent formulation', *J. Mol. Catal. B Enzymatic*, 2015, **120**, (10), pp. 173–178
[3] Mathew S., Jeong S.S., Chung T., *ET AL.*: 'Asymmetric synthesis of aromatic B-amino acids using Ω-transaminase: optimizing the lipase concentration to obtain thermodynamically unstable B-Keto acids', *Biotechnol. J.*, 2015, **11**, (1), pp. 185–190
[4] Pereira M.G., Facchini F.D.A., Polizeli A.M., *ET AL.*: 'Stabilization of the lipase of hypocrea pseudokoningii by multipoint covalent immobilization after chemical modification and application of the biocatalyst in oil hydrolysis', *J. Mol. Catal. B Enzymatic*, 2015, **121**, pp. 82–89

doi: 10.1049/mnl.2017.0402

[5] Raita M., Arnthong J., Champreda V., *ET AL.*: 'Modification of magnetic nanoparticle lipase designs for biodiesel production from palm oil', *Fuel Process. Technol.*, 2015, **134**, pp. 189–197

[6] Hasan F., Shah A.A., Hameed A.: 'Industrial applications of microbial lipases. Enzyme microbiol technol', *Enzyme Microb. Technol.*, 2006, **39**, (2), pp. 235–251

[7] Kajiwara S., Yamada R., Mori H., *ET AL.*: 'Development of sucrose-complexed lipase to improve its transesterification activity and stability in organic solvents', *Biochem. Eng. J.*, 2017, **121**, pp. 83–87

[8] Ogino H., Ishikawa H.: 'Enzymes which are stable in the presence of organic solvents', *J. Biosci. Bioeng.*, 2001, **91**, (2), pp. 109–116

[9] Ren X., Dong C., Meng X., *ET AL.*: 'Zinc oxide nanoparticles/glucose oxidase photoelectrochemical system for the fabrication of biosensor', *J. Colloid Interface Sci.*, 2009, **334**, (2), pp. 183–187

[10] Tian F., Guo Y., Lin F., *ET AL.*: 'Rational surface silane modification for immobilizing glucose oxidase', *Int. J. Biol. Macromol.*, 2016, **87**, pp. 191–194

[11] Zhou X., Liu Y., Yuan Q., *ET AL.*: 'Preparation of multi-enzyme co-immobilized nanoparticles by bis-aryl hydrazone bond conjugation', *Biotechnol. Appl. Biochem.*, 2016, **63**, pp. 214–219

[12] de Lathouder K.M., Marques Fló T., Kapteijn F., *ET AL.*: 'A novel structured bioreactor: development of a monolithic stirrer reactor with immobilized lipase', *Catal. Today*, 2005, **105**, (3–4), pp. 443–447

[13] Kartal F., Janssen M.H.A., Hollmann F., *ET AL.*: 'Improved esterification activity of *Candida rugosa* lipase in organic solvent by immobilization as cross-linked enzyme aggregates (CLEAs)', *J. Mol. Catal. B, Enzymatic*, 2011, **71**, (3–4), pp. 85–89

[14] Li P., Wang T., Lei F., *ET AL.*: 'Lipase immobilized on rosin-based functional polymers as a biocatalyst for the synthesis of ethyl dodecanoate', *J. Mol. Catal. B, Enzymatic*, 2016, **133**, pp. S106–S113

[15] Pellis A., Herrero Acero E., Ferrario V., *ET AL.*: 'The closure of the cycle: enzymatic synthesis and functionalization of bio-based polyesters', *Trends Biotechnol.*, 2016, **34**, (4), pp. 316–328

[16] Zhao K., Cao X., Di Q., *ET AL.*: 'Synthesis, characterization and optimization of a two-step immobilized lipase', *Renew. Energy*, 2017, **103**, pp. 383–387

[17] Liang H., Jiang S., Yuan Q., *ET AL.*: 'Co-immobilization of multiple enzymes by metal coordinated nucleotide hydrogel nanofibers: improved stability and an enzyme cascade for glucose detection', *Nanoscale*, 2016, **8**, (11), p. 6071

[18] Zhang Y., Gao F., Zhang S.P., *ET AL.*: 'Simultaneous production of 1,3-dihydroxyacetone and xylitol from glycerol and xylose using a nanoparticle-supported multi-enzyme system with in situ cofactor regeneration', *Bioresour. Technol.*, 2011, **102**, (2), pp. 1837–1843

[19] Gray J.J.: 'The interaction of proteins with solid surfaces', *Curr. Opin. Struct. Biol.*, 2004, **35**, (26), pp. 110–115

[20] Johnson A.K., Zawadzka A.M., Deobald L.A., *ET AL.*: 'Novel method for immobilization of enzymes to magnetic nanoparticles', *J. Nanoparticle Res.*, 2008, **10**, (6), pp. 1009–1025

[21] Pandey P., Singh S.P., Arya S.K., *ET AL.*: 'Application of thiolated gold nanoparticles for the enhancement of glucose oxidase activity', *Langmuir*, 2007, **23**, (6), pp. 3333–3337

[22] Williams R.A., Blanch H.W.: 'Covalent immobilization of protein monolayers for biosensor applications', *Biosens. Bioelectron.*, 1994, **9**, (2), pp. 159–226

[23] Matharu Z., Sumana G., Arya S.K., *ET AL.*: 'Polyaniline Langmuir–Blodgett film based cholesterol biosensor', *Langmuir*, 2007, **23**, (26), pp. 13188–13192

[24] Jang E., Park S., Park S., *ET AL.*: 'Fabrication of poly(ethylene glycol)-based hydrogels entrapping enzyme-immobilized silica nanoparticles', *Polym. Adv. Technol.*, 2010, **21**, (7), pp. 476–482

[25] Popat A., Hartono S.B., Stahr F., *ET AL.*: 'Mesoporous silica nanoparticles for bioadsorption, enzyme immobilisation, and delivery carriers', *Nanoscale*, 2011, **3**, (7), pp. 2801–2818

[26] Qiu J., Peng H., Liang R.: 'Ferrocene-modified Fe_3O_4 @Sio_2 magnetic nanoparticles as building blocks for construction of reagentless enzyme-based biosensors', *Electrochem. Commun.*, 2007, **9**, (11), pp. 2734–2738

[27] Lin J., Siddiqui J.A., Ottenbrite R.M.: 'Surface modification of inorganic oxide particles with silane coupling agent and organic dyes', *Polym. Adv. Technol.*, 2001, **12**, (5), pp. 285–292

[28] Bradford M.M.: 'A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding', *Anal. Biochem.*, 2015, **72**, pp. 248–254

[29] Živković L.T.I., Živković L.S., Babić B.M., *ET AL.*: 'Immobilization of *Candida rugosa* lipase by adsorption onto biosafe meso/macroporous silica and zirconia', *Biochem. Eng. J.*, 2015, **93**, pp. 73–83

[30] Yue T.W., Chien W.C., Tseng S.J., *ET AL.*: 'EDC/NHS-mediated heparinization of small intestinal submucosa for recombinant adeno-associated virus serotype 2 binding and transduction', *Biomaterials*, 2007, **28**, (14), pp. 2350–2357

[31] Ahmed N., Konduru N.K., Praveen A.K., *ET AL.*: 'Silica supported-double metal cyanides (DMCs): a green and highly efficient catalytic protocol for isomerisation of 2′-hydroxychalcones to flavanones', *J. Mol. Catal. A Chem.*, 2013, **373**, pp. 135–141

[32] Aygar G., Kaya M., Özkan N., *ET AL.*: 'Preparation of silica coated cobalt ferrite magnetic nanoparticles for the purification of histidine-tagged proteins', *J. Phys. Chem. Solids*, 2015, **87**, pp. 64–71

[33] Lei L., Cao Z., Xie Q., *ET AL.*: 'One-pot electrodeposition of 3-aminopropyltriethoxysilane–chitosan hybrid gel film to immobilize glucose oxidase for biosensing', *Sens. Actuators B, Chem.*, 2011, **157**, (1), pp. 282–289

[34] Sun L., Hao L., Yuan Q., *ET AL.*: 'Study on a carboxyl-activated carrier and its properties for papain immobilization', *J. Chem. Technol. Biotechnol.*, 2012, **87**, (8), pp. 1083–1088

[35] Xiang G.-Q., Ren Y., Xia Y., *ET AL.*: 'Carbon-dot-based dual-emission silica nanoparticles as a ratiometric fluorescent probe for Bisphenol A', *Spectrochim. Acta A, Mol. Biomol. Spectrosc.*, 2017, **177**, pp. 153–157

[36] Zhang B., Li P., Zhang H., *ET AL.*: 'Papain/Zn3(Po4)2 hybrid nanoflower: preparation, characterization and its enhanced catalytic activity as an immobilized enzyme', *RSC Adv.*, 2016, **6**, (52), pp. 46702–46710