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Author: Tomasz Siódmiak Marta Ziegler-Borowska Michał Piotr Marszałł

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Highlights

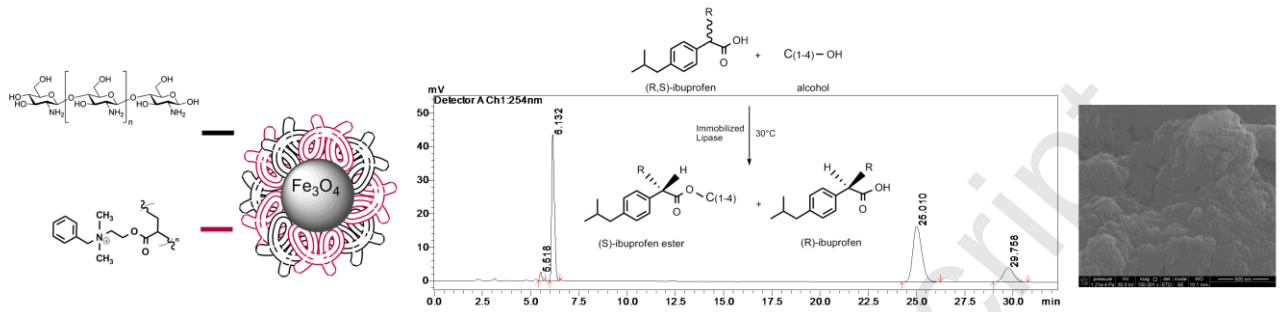
-Chitosan – poly [N-benzyl-2-(methacryloxy)-N,N -dimethyl-ethanaminium bromide] coated magnetic nanoparticles were prepared

- High enantioselectivity of kinetic resolution of (*R,S*)-ibuprofen was obtained with the use of lipase-immobilized chitosan magnetic nanoparticles

- The sulfo-NHS/EDC-activated magnetic particles provide easy recovery and reuse of lipase.

- Baseline resolution obtained for all analyzed compounds using chiral stationary phases

Graphical abstract



Lipase-immobilized magnetic chitosan nanoparticles for kinetic resolution of (*R*,*S*)-ibuprofen

Tomasz Siódmiak[a], Marta Ziegler-Borowska[b], Michał Piotr Marszałł[a*]

[a] Department of Medicinal Chemistry, Collegium Medicum in Bydgoszcz, Faculty of Pharmacy, Nicolaus Copernicus University, Dr. A. Jurasza 2, 85-089 Bydgoszcz, Poland
[b] Chair of Chemistry and Photochemistry of Polymers, Faculty of Chemistry, Nicolaus Copernicus University, Gagarina 7, 87-100 Toruń, Poland

[*]Corresponding author. Tel.: +48 52 5853540; fax: +48 525853529; e-mail address: mmars@cm.umk.pl (M.P. Marszałł); work address: Collegium Medicum in Bydgoszcz, Jurasza 2, 85-089 Bydgoszcz, Poland

Abstract

Chitosan (CS) – poly [*N*-benzyl-2-(methacryloxy)-*N*,*N* –dimethylethanaminium bromide] coated magnetic nanoparticles were prepared by co-precipitation method via epichlorohydrin CS cross-linking reaction and were used in the kinetic resolution of (*R*,*S*)-ibuprofen by enantioselective esterification. Enzyme immobilized onto the surface of the new magnetic supports with the use of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysulfo-succinimide sodium salt (sulfo-NHS) procedure demonstrated high catalytic activity that allowed to obtain (*S*)-methyl ester of ibuprofen with high enantioselectivity (E=50.6). The chiral compounds resulted from the application of magnetic nanoparticles were analyzed with the use of chiral stationary phases. It should be emphasized, that the main advantage of the support is the possibility to magnetically recovery and effective separation (even up to 5 sec.) from the reaction mixture with the use of magnet. The properties of magnetic particles allow for better optimization and may reduce the total costs of the esterification reaction of ibuprofen. Moreover, the application of lipase-immobilized magnetic supports enables to maintain high enantioselective activity after repeatedly use.

Keywords

Keywords: *Candida rugosa* lipase; chitosan magnetic nanoparticles; (*R*,*S*)-ibuprofen; immobilization; kinetic resolution.

1. Introduction

The development a new strategies for synthesis of enantiomerically pure compounds is still an open challenge in the chemical and pharmaceutical industry [1]. The biotechnology is an alternative approach offering more environmentally and economically attractive way to obtain bioactive and valuable compounds. Lipases are very suitable enzymes for organic synthesis because of their capacity of catalysing different reactions such as asymmetric esterification, asymmetric transesterifiaction and asymmetric hydrolysis [2,3]. These enzymes have been used in the resolution of racemic mixture for the preparation of optically pure compounds [4-8]. Because of their low stability the application of lipases in the industry is limited. Therefore, many lipase immobilization techniques have been employed [9-11]. The most important factors that should be taken into account in the selection of the immobilization strategy include: good catalytic activity, stability and reusability of the enzymes. Numerous

reports on the immobilization of lipase techniques onto different supports have been published so far [12-18]. The main advantage of using lipase-immobilized magnetic particles is the ability to recover them from the reaction medium and thus reduce the costs of the reaction, which might be of special importance for the chemical and pharmaceutical industry [19-21]. Most recently, the magnetic particles technology has been found to be a convenient tool for separation of any molecule or protein that has an affinity for the immobilized material onto the surface of magnetic supports [22-24].

Triiron tetraoxide magnetic nanoparticles, with their potential application for enzyme immobilization have attracted much attention because of their interesting and preferable physical and chemical properties, such as stability, biocompatibility and superparamagnetism. The special importance is that the surface properties can be modified depending on the immobilized enzyme and reaction medium [25,26]. Polymeric coatings on magnetite nanoparticles have a high potential in several areas of applications especially for organic catalysis and bio-separation. One of the most interesting polymer for magnetic nanoparticles coating is chitosan, a product obtained by partial deacetylation of chitine in alkaline conditions [27]. It is a polycationic polymer with a specific structure and properties. Its biocompatibility and the presence of readily functionalizable groups (amino and hydroxyl) allows such material to be used in biomedical and synthetic applications.

2-Arylpropionic acids (profens) are known as major nonsteroidal anti-inflammatory drugs (NSAID) used in the treatment of headache, rheumatoid arthritis, cephalgia, muscular strain [28-30]. All those profen drugs have the chiral carbon atom within the propionic acid moiety. Kinetic resolution of profens is important from the pharmacological point of view because enantiomers of these drugs demonstrate different therapeutic activities. One of the most frequently used drugs within this therapeutic group is (*R,S*)- ibuprofen. The (*S*)-enantiomer of this drug is 160 times more active than its (*R*)- enantiomer in the *in vitro* inhibition of prostaglandin synthesis. Additionally, the latter contributes to increased side effects affecting to the gastrointestinal tract, normal lipids metabolism and membrane function [31,32].

In the present study, lipase-immobilized magnetic nanoparticles for the resolution of (*R,S*)-ibuprofen and its esters have been studied. The tested “enzyme magnetic supports” were assessed as potential technique to obtain (*S*)- esters of ibuprofen. Superparamagnetic triiron tetraoxide nanoparticles grafted with chitosan and amphiphilic polymer have been synthesized by co-precipitation of iron oxide in the presence of these polymers. The optimization of immobilization conditions of lipase onto EDC/sulfo-NHS-activated chitosan magnetic

nanoparticles was performed as well as the amount and activity of immobilized lipase was determined. The chiral compounds obtained as a result of the application of magnetic supports were analyzed with the use of chiral stationary phases. The optimization of chiral chromatographic conditions involved the selection of stationary phases, mobile phase composition, flow rate, volume of injected analytes and temperature of chromatographic process.

2. Material and methods

2.1. Chemicals

(*R*,*S*)-ibuprofen, (*S*)-ibuprofen, *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfo-succinimide sodium salt (sulfo-NHS), *n*-hexane, iron (II) chloride tetrahydrate, iron (III) chloride hexahydrate, chitosan (low molecular weight), acetic acid, sodium hydroxide, benzyl bromide, 2-(dimethylamino)ethylmethacrylate and Bradford reagent were purchased from Sigma-Aldrich Co. (Stainhaim, Germany). 2-propanol, cyclohexane, sodium sulphate anhydrous, sodium sulphate decahydrate, molecular sieves 4Å, sodium phosphate dibasic dehydrate, orthophosphoric acid solution, sodium, other solvents, were purchased from POCH S.A. (Gliwice, Poland). Gum Arabic and olive oil were purchased from local source. The (*R*)- and (*S*)-esters of ibuprofen were obtained by the products of standard esterification reaction of (*R*,*S*)-ibuprofen and (*S*)-ibuprofen with appropriate alcohols (methanol, ethanol, *n*-propanol, *n*-butanol) using sulphuric acid (H_2SO_4) as catalyst [33]. Lipase OF from *Candida rugosa* (activity 380,000 units/g solid) was a gift from Meito Sangyo Co., LTD. (Japan). AIBN (2,2′-Azobis(2-methylpropionitrile) was recrystallized from ethanol before use. All supernatants were separated from magnetic nanoparticles using a magnetic separator Dynal MPC-S (Invitrogen Corporation, Carlsbad, CA, USA). Water used in the study was prepared using a Milli-QWater Purification System (Millipore, Bedford, MA, USA). All incubations were performed at adjusted temperature and number of rotations (600 rpm) in Thermomixer comfort (Eppendorf Co, Germany). Experiments with air and moisture sensitive materials were carried under nitrogen atmosphere. Glassware was oven dried for several hours, assembled hot, and cooled in a stream of nitrogen.

2.2. Instrumentation

The Shimadzu HPLC system (Japan) equipped with solvent delivery pump LC-20AD combined with gradient systems, UV-VIS detector model SPD-20A, degasser model DGU-20A5, an autosampler model SIL-20ACHT and a column oven model CTO-10ASVP. Lux Cellulose-1 (LC-1) (4.6mm x 250 mm x 5µm) column with cellulose tris(3,5-dimethylphenylcarbamate) stationary phase, Lux Cellulose-2 (LC-2) (4.6mm x 250 mm x 5µm) column with cellulose tris(3-chloro-4-methylphenylcarbamate) stationary phase, Lux Cellulose-3 (LC-3) (4.6mm x 250 mm x 5µm) column with cellulose tris(4-methylbenzoate) stationary phase, Lux Amylose-2 (LA-2) (4.6mm x 250 mm x 5µm) column with amylose tris(5-chloro-2-methylphenylcarbamate) stationary phase and Guard Cartridge System model KJO-4282 were purchased from Phenomenex Co. Bradford protein assay method was used with the use of U-1800 Spectrophotometer (Hitachi, Japan).

The Fourier Transform Infrared (FTIR) absorption spectra were recorded on Spectrum 2000 Perkin Elmer spectrometer in KBr. Melting points were determined with a Büchi SMP 32 and Barnstead-Thermolyne Mel-Temp II apparatus in open capillaries and are uncorrected. Scaning electron microscope (SEM) 1430 VP LEO Electron Microscopy Ltd., was used. ^{1}H and ^{13}C NMR spectra were recorded at room temperature with Bruker Avance III 700 and 400 MHz spectrometers. Chemical shifts (in ppm) were determined relative to TMS.

2.3. Chromatographic conditions

The most appropriate chromatographic conditions for (*R*)- and (*S*)-ibuprofen and their esters were optimized with *n*-hexane/2-propanol/acetic acid (99.6/0.4/0.15 v/v/v) mobile phase at a flow rate of 1 mL/min. Four types of chiral stationary phases were tested, including Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3 and Lux Amylose-2 with respect to the peak shape and the chiral resolution. The Lux Cellulose-1 (4.6mm x 250 mm x 5µm) HPLC column was chosen as an optimal one for the separation of (*R*)- and (*S*)-ibuprofen and their esters. The chromatographic process was operated at 20°C. The detection UV wavelength was set at 254 nm. **The enantiomeric excess of the substrate (ee_s) and the product (ee_p) as well as the conversion (c), enantioselectivity (E) and resolution values (Rs) were calculated using the equations described in the literature [34-36].**

2.4. Preparation of chitosan – poly [N-benzyl-2-(methacryloxy)-N,N –dimethylethanaminium bromide] coated magnetic nanoparticles

2.4.1. Synthesis of N-benzyl-2-(methacryloyloxy)-N,N-dimethylethanaminium bromide (**1**)

A 2-(dimethylamino)ethyl methacrylate (0.79g, 5mmol) was added dropwise to a solution of benzyl bromide (0.8 g, 5 mmol) in THF (20 mL) with stirring for 20 min at room temperature. A white precipitate formed almost immediately. Next, the mixture was separated by filtration, washed with acetone (3 x 10 mL) and dried under vacuum to gave white solid (mp. 139-142°C) with 95% yield (1.51g).

^{1}H NMR,$DMSO_{d\text{-}6}$, δ (ppm); 1.92 (s, 3H, CH_3), 3.06 (s, 6H, CH_3), 3.76 (t, J = 4.7 Hz, 2H, CH_2), 4.63 (m, 2H, CH_2), 4.69 (m, 2H, CH_2), 5.77 (s,1H, CH), 6.11 (s,1H, CH), 7.51- 7.61 (m, 5H, CH_{Ar}).

^{13}C NMR, $DMSO_{d\text{-}6}$, δ (ppm); 18.40, 49.99, 58.63, 62.82, 67.40, 127.12, 128.46, 129.37, 130.78, 133.59, 135.85, 166.35.

*2.4.2. Synthesis of poly [N-benzyl-2-(methacryloxy)-N,N –dimethylethanaminium] bromide (**PQ**)*

AIBN (40 mg, 0.2 mmol) was added carefully in 3 portions to a solution of **1** (1.57g, 5 mmol) in dry acetonitryle (20mL) at 60°C under nitrogen atmosphere. The mixture was stirred at 60°C under nitrogen for 120h. Insoluble polymer was separated by filtration and washed with methanol (3 x 10 mL) and dried under vacuum at 30°C for 24h to gave polymer with almost 80% yield (1.79g)

^{1}H NMR,$DMSO_{d\text{-}6}$, δ (ppm); 1.25 (m. 2H, polym. chain), 1.92 (s, 3H, CH_3), 2.20 (s, 6H, N-CH_3), 2.93 (br, 2H, CH_2), 3.40 (m, 2H, CH_2), 4.69 (br, s, 2H, CH_2), 7.51- 7.61 (m, 5H, CH_{Ar}).

2.4.3. Fe_3O_4 – CS - PQ magnetic nanoparticles

0.2 g of poly [*N*-benzyl-2-(methacryloxy)-*N*,*N* –dimethylethanaminium] bromide was added into solution of chitosan (0.2 g) in 1% acetic acid (20 mL) and mechanically stirred at room temperature for 60 min. Iron (II) chloride tetrahydrate (0.74g, 3.7 mmol), iron (III) chloride hexahydrate (2.02g, 7.5 mmol) were added (1:2 molar ratio) and the resulting solution was chemically precipitated at room temperature by adding dropwise 30% solution of NaOH (7mL). To the black mixture epichlorohydrin (0.2 g, 2.5 mmol) was added and the mixture was mechanically stirred at 50°C for 120 min. The resulting magnetic material were

recovered from the suspension by applying a magnet, washed three times with deionized water and dried under vacuum at 50°C for 24 h.

2.5. Preparation of lipase-immobilized magnetic nanoparticles

2.5.1. Covalent coupling of lipase using EDC and sulfo-NHS onto chitosan magnetic nanoparticles

The immobilization of lipase onto the surface of chitosan magnetic nanoparticles was performed by the formation of an amide bond between the carboxyl group of lipase and the primary amino group of the nanoparticle (Scheme 1). The preparation procedure was similar with slightly modifications to previously described method of the immobilization of melanin onto magnetic beads [22]. The 50 mg chitosan magnetic nanoparticles were placed into each of the four 2 mL centrifuge tubes and rinsed three times with 50 mM phosphate buffer (pH 6.4). Next, the four solutions of 36.5 mg lipase OF in 1.0 mL of 50 mM phosphate buffer (pH 6.4) were prepared. To each of the tubes with lipase the 50 μL EDC solution in phosphate buffer (2mg/50 μL) were added. The solutions were incubated at 21°C and shaken for 1 h. After that time 50 μL of sulfo-NHS solution in phosphate buffer (2.4 mg/50 μL) was added to each of the tubes containing of lipase and EDC. The solutions were also incubated at 21°C 1 h and shaken. Then, all prepared solutions were transferred into separate centrifuge tubes along with the previously rinsed chitosan magnetic nanoparticles. Next, the resulting mixtures were shaken at 600 rpm in a thermomixer for 2 h at 21°C. At last, lipase-immobilized chitosan nanoparticles were rinsed three times with 0.5 mL of water and were dried overnight at 30°C. The resulted lipase-immobilized magnetic supports were used in the esterification reaction.

The amount of immobilized lipase adsorbed onto the magnetic nanoparticles was determined by measuring the initial concentration of lipase and its final concentration in supernatant after immobilization using the Bradford protein assay method [37]. A calibration curve constructed with lipase OF solution of known concentration (0.5-2 mg/mL) was used in the calculation of protein in the initial solution and supernatant. All data used in this formula are average of triplicate of experiments.

2.6. Assay of lipase activity

The enzymatic activities of free and immobilized lipase were measured by titration of the fatty acid which came from the hydrolysis of olive oil [38, 39]. A 100 mL of olive oil emulsion was prepared by mixing of olive oil (50 mL) and gum Arabic solution (50 mL, 7%

w/v). The assay mixture consisted of emulsion (5 mL), phosphate buffer (2 mL, 100 mM, pH 7.4) and free enzyme (1 mL, 6.47 mg/mL) or immobilized lipase (50 mg nanoparticles in 1 mL buffer). Oil hydrolysis was carried out at 37ºC for 30 min. in a shaking water bath at 150 rpm. The reaction was stopped by the addition of 10 mL of ethanol-acetone solution (1:1). The liberated fatty acid in the medium was determined by titration with 50 mM NaOH solution using phenolphthalein indicator. One unit of lipase activity (U) was defined as the amount of enzyme that hydrolyzed olive oil liberating 1 μmol fatty acid per minute under the assay condition. **Activity recovery (%) remaining after immobilization was the ratio between the activity of immobilized lipase and the activity of the same amount of free lipase in solution that has been immobilized onto magnetic nanoparticles.**

2.7. Lipase-immobilized chitosan magnetic nanoparticles in the (R,S)-ibuprofen resolution

The reaction mixture was composed of cyclohexane (700 μL), racemic ibuprofen (8.25 mg, 0.04 mM) and one of the alcohols: methanol (4.88 μL), ethanol (7.04 μL), *n*-propanol (9.02 μL), *n*-buthanol (11.04 μL) as an acyl acceptors. The reaction was started by adding this solution to the magnetic chitosan nanoparticles with immobilized lipase (6.47 mg of lipase) in a 1.5 mL tube (substrate/enzyme ratio – 1.27). The effect of water activity on the esterification reaction was controlled through direct addition of a salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (35 mg in total, with molar ratio of 1:1) and the molecular sieves 4Å. The suspension was incubated at 30°C, shaken (600 rpm) for 140 h in a thermomixer. The samples were withdrawn after 50 h, 100 h and 140 h. The collected supernatant was removed by evaporation at room temperature and the residue was dissolved in 0.7 mL mobile phase and injected (25 μL) into HPLC. The esterification reactions of racemic ibuprofen with alcohols are shown in Scheme 2.

To investigate the reusability of immobilized lipase, after each catalytic cycle lipase-immobilized magnetic nanoparticles were washed three times with cyclohexane and then were air dried overnight to remove the organic solvent. Next, magnetic supports were placed into a fresh medium containing a mixture of methanol (4.88 μL), (*R,S*)-ibuprofen (8.25 mg), salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (35 mg in total) and molecular sieves 4Å in cyclohexane (700 μL).

3. Results and discussion

3.1. *Characterization of chitosan – poly [N-benzyl-2-(methacryloxy)-N,N -dimethyl-ethanaminium bromide] coated magnetic nanoparticles*

Amphiphilic polymer - (poly [*N*-benzyl-2-(methacryloxy)-*N*,*N*-dimethylethan-aminium bromide) (PQ) has been prepared from *N*-benzyl-2-(methacryloyloxy)-*N*,*N*-dimethylethanaminium bromide (**1**) as a substrate via free radical polymerization with AIBN as an initiator. Polymerization was performed in nitrogen atmosphere and poly [*N*-benzyl-2-(methacryloxy)-*N*,*N*-dimethylethanaminium] was synthesized with 80% yield after 120 h reaction.

Magnetite (Fe_3O_4)-chitosan (CS) - poly [*N*-benzyl-2-(methacryloxy)-*N*,*N* – dimethylethanaminium bromide] (PQ) nanoparticles with polymers weight ratio 1:1 were prepared by co-precipitation method via epichlorohydrin CS cross-linking reaction (Figure 1). Because of the surface of magnetite with a negative charge has an affinity toward chitosan, protonated chitosan and quaternized amphiphilic polymer can coat the magnetic Fe_3O_4 nanoparticles via electrostatic interactions [40]. The cross-linking of chitosan via epichlorohydrin made polymeric coating more stable without dilution of amino group content onto the surface of nanoparticles.

The chemical structure of prepared magnetic nanoparticles has been characterized by FTIR spectroscopy (Figure 2). The spectrum of nanoparticles shows peaks at 3439, 2936 and 1078 cm^{-1} and indicates the stretching vibrations of NH_2 and partially OH group, aliphatic CH and bending vibrations of CO respectively. The characteristic peak for the Fe-O group of magnetite at 598 cm^{-1} was observed which indicated the successful generation of Fe_3O_4 – CS – PQ particles. Peaks at 1720 cm^{-1} and at 1330 cm^{-1} increasing the stretching vibrations of C=O groups and quaternized ammonium groups of polymer PQ. Peak at 1155 cm^{-1} increase the C-O-C groups from cross-linked chitosan.

The surface morphology of prepared nanoparticles is shown in Figure 3. The average size was 25-30 nm and the magnetic nanoparticles were physically aggregated.

3.2. *Application of lipase-immobilized chitosan magnetic nanoparticles in the esterification of (R,S)- ibuprofen with primary alcohols*

The problem of the stability and recovery of commonly used and novel catalysts in chemistry still exists. A new magnetic nanoparticle-based products were recently introduced for immobilization of different enzymes. But up to date all studies have focused mainly on the

optimization of the immobilization process to characterize the size, structure, magnetic activity and amount of the immobilized enzyme using the Bradford method.

Immobilization of commercially available lipase OF from *Candida rugosa* onto the chitosan magnetic nanoparticles was performed by procedure via EDC/sulfo-NHS cross-linking reaction. In order to address the issue of advantage of the magnetic nanoparticles support for the biocatalyst, the optical purity of products, enantioselectivity, separation ability of used magnetic tools, as well as amount of immobilized enzyme and lipolytic activity of lipase were determined. Lipase-immobilized chitosan magnetic nanoparticles were applied in the esterification reaction of (*R*,*S*)-ibuprofen with four primary alcohols: methanol, ethanol, *n*-propanol and *n*-buthanol. Enantioselective activity of the used enzyme demonstrates the ability to catalyzing the esterification of the *S*-enantiomer of ibuprofen. Depending on the applied alcohols different effects on the enantioselctivity and conversion of the reactions were observed. It is assumed that the accessibility of the alcohol to the acyl-enzyme intermediate has significant impact on the final reaction yield. Therefore, nature of the alcohol moiety play an important role in the development of the enantioselective esterification reaction catalyzed by lipase. The influence of the alcohol moiety on the lipase activity were reported several times in the literature [41-45]. Because of the fact, that active site of enzyme has hydrophobic character, it is believed that the hydrophobic alcohols are the most appropriate for esterification catalyzed by lipase. Furthermore, the polar alcohols, like methanol or ethanol cause the low conversion, because they are able to dehydrate the enzyme [46]. Based on the results (Table 1), it can be seen, that ethanol has detrimental impact on the enzyme activity, contributing to decrease the enantioselectivity (E=6.2). However, what should be emphasized, application of methanol allowed to obtained very high enantioselectivity (enantiomeric excess of product (ee_p = 93.5%) and value of enantioselectivity (E=50.6) - higher than with *n*-butanol (E=18). The comparison of the enantionselectivity of non- and immobilized forms of lipase OF in similar reaction conditions demonstrates the lower enantioselectivity of lipase in native form for all tested alcohols than for immobilized enzyme. These facts suggest that, immobilization of lipase onto magnetic support affects on enzyme conformation, enabling effective binding fast-reacting enantiomer to the active site of lipase with formation acyl-enzyme intermediate.

The addition of a salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (35 mg in total, with molar ratio of 1:1) and the molecular sieves 4Å in to the mixture containing (*R*,*S*)-ibuprofen, alcohol and lipase-immobilized chitosan magnetic nanoparticles allowed to control the water activity. Lipase acts at the interface between hydrophobic and hydrophilic regions, therefore water

content is one of the most important factors affecting the enantioselectivity of this enzyme. The small amount of water is needed to retain their active three-dimensional conformation state, stability and active site polarity. During reaction, salt hydrates adsorb water generated as a by-product and simultaneously provide crystallization water to the components maintaining appropriate water activity. Additional, the use of molecular sieves in the reaction improve the adsorption of water.

The amount of lipase immobilized onto the support was found to be 129.4 mg/g magnetic nanoparticles with a recovery activity yield of 78%. The high value of activity recovery indicates, that much of the protein was immobilized in an active form. As reported in the literature, the preference for catalyzing by lipase wide range organic acid chain length can be modified by the application of different immobilization procedures and carriers exhibiting different polarity [47-49]. Based on the results, it can be assumed, that application of quaternary amphiphilic polymer in the synthesis of magnetic nanoparticles increases the affinity between lipase and hydrophobic substrates and thereby affects on the lipolytic activity of the immobilized lipase.

However, the main advantage of the proposed lipase-immobilized chitosan magnetic nanoparticles is that the immobilized lipase is recoverable magnetically and can be effectively separated from the reaction mixture with the use of magnet, faster than other classical methods (Figure 4). From economic point of view, the reusability of immobilized lipase is an important aspect in industrial applications. Moreover, the fast separation of catalysts (even up to 5 sec.) from mixture of products allows to precisely optimize the esterification reaction of ibuprofen. It should be also noted, that application of lipase-immobilized chitosan magnetic nanoparticles enabled to obtain products with high enantioselectivity for selected alcohols. What is important the "magnetic biocatalyst" has maintained enantioselective activity in repeatedly use (Figure 5). Enantioselective activity of lipase expressed as an enantiomeric excess of products (ee_p) demonstrated high value (ee_p= 90%) after the 5^{th} cycle. The slight decrease in enantioselective activity could be caused by the denaturation or leak of lipase from magnetic nanoparticles. Summarizing the results, it could be concluded that immobilized lipase has good durability and reusability.

3.3.Analysis of (R,S)-ibuprofen and its esters with the use of chiral stationary phases

After optimization of chromatographic parameters of the four commercial polysaccharide-based CSPs: Lux Cellulose-1 (LC-1), Lux Cellulose-2 (LC-2), Lux Cellulose-3 (LC-3), Lux Amylose-2 (LA-2) and based on the previously proposed method by Matthijs et al., the final

chromatographic conditions for enantioselective separation of (*R*,*S*)-ibuprofen and its esters with the use of Lux Cellulose-1 column in NPLC was selected (Figure 6) [49,50]. The main aim of the optimization strategy for resolution of these compounds was the acceptable baseline resolution (Rs > 1.5), time of analysis and peak shape. Only LC-1 stationary phase allowed to obtain acceptable parameters of enantioseparation. The last three columns (LC-2, LC-3, LA-2) demonstrated lower ability for resolution of (*R*,*S*)-ibuprofen and its esters (Rs<1.5) or no resolution (Rs=0), long time of the elution and inappropriate peak shapes. Optimized mobile phase for LC-1 was composed with *n*-hexane/2-propanol/ acetic acid (99.6/0.4/0.15 v/v/v) at a flow rate of 1 mL/min. The NPLC analyses were performed at temperature of 20°C. Tested compounds ((*R*,*S*)-ibuprofen and its esters) were eluted within 32 min. and showed appropriate peaks shapes and baseline resolution (Rs >1.5).

4. Conclusions

In this study the new synthesized chitosan – poly [*N*-benzyl-2-(methacryloxy)-*N*,*N* -dimethyl-ethanaminium bromide] magnetic particles were used in the kinetic resolution of (*R*,*S*)-ibuprofen by enantioselective esterification. Enzyme immobilized onto the new magnetic support demonstrated high catalytic activity and allowed to obtain products of the esterification of (*R*,*S*)-ibuprofen with high enantioselectivity. Additionally, **lipolytic activity assay proved high recovery activity (78%) of the immobilized lipase.** What is crucial, the presented "recovering" technique enables the magnetically separation of biocatalyst attached to the nanoparticles from the reaction media and effectively reuse in other reaction (up to 5 catalytic cycles). From the commercial point of view the use of lipase-immobilized chitosan magnetic nanoparticles is very important, because of the cost reduction of the reaction, what might be of special importance for the industrial application. It should be noted, that optimized of chromatographic conditions with the used of chiral stationary phases allowed for a baseline resolution (Rs>1.5) of both substrates and products during one chromatographic analysis.

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Figure legends

Scheme 1. Immobilization of lipase using EDC and sulfo-NHS onto the surface of chitosan magnetic nanoparticles.

Scheme 2. The enantioselective esterification of racemic ibuprofen with alcohols (methanol, ethanol, *n*-propanol, *n*-butanol) with the use of lipase-immobilized superparamagnetic triiron tetraoxide nanoparticles.

Figure 1. Superparamagnetic triiron tetraoxide nanoparticle with chitosan and amphiphilic polymer cross-linked by epichlorohydrin.

Figure 2. FTIR spectrum of the Fe_3O_4 – CS – PQ nanoparticles.

Figure 3. SEM image of magnetic nanoparticles: chitosan and amphiphilic polymer in weight ratio 1:1 cross-linked by epichlorohydrin.

Figure 4. Magnetic nanoparticles with immobilized lipase dispersed in the reaction medium: a) without magnet; b) attracted by magnet.

Figure 5. Effect of the reuse of lipase (OF)-immobilized chitosan magnetic nanoparticles on the enantioselectivity of the esterification of (*R*,*S*)-ibuprofen. Reaction conditions: (*R*,*S*)-ibuprofen (8.25 mg), methanol (4.88 μL), immobilized lipase OF (50 mg of magnetic nanoparticles), salt hydrate pair $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (totally 35mg, with molar ratio of 1:1), molecular sieves 4Å, cyclohexane (700 μL), temp. 30°C, after 140 h.

Figure 6. HPLC chromatograms of ibuprofen and its esters: A) *R*-enantiomer of methyl ester (t_R =5.518), *S*-enantiomer of methyl ester (t_R =6.132), *R*-ibuprofen (t_R =25.010) and *S*-ibuprofen (t_R =29.758); B) *R*-enantiomer of ethyl ester (t_R =5.184), *S*-enantiomer of ethyl ester (t_R =5.600), *R*-ibuprofen (t_R =25.026) and *S*-ibuprofen (t_R =29.831); C) *R*-enantiomer of *n*-propyl ester (t_R =4.902), *S*-enantiomer of *n*-propyl ester (t_R =5.281), *R*-ibuprofen (t_R =25.026) and *S*-ibuprofen (t_R =29.920); D) *R*-enantiomer of *n*-butyl ester (t_R =4.798), *S*-enantiomer of *n*-butyl ester (t_R =5.166), *R*-ibuprofen (t_R =24.992) and *S*-ibuprofen (t_R =29.984);

Resolution values (Rs): (*R*,*S*)- ibuprofen (Rs=5.45), (*R*,*S*)-ibuprofen methyl ester (Rs=2.60), (*R*,*S*)-ibuprofen ethyl ester (Rs=1.94), (*R*,*S*)-ibuprofen *n*-propyl ester (Rs=1.86), (*R*,*S*)-ibuprofen *n*-buthyl ester (Rs=1.78);

Chromatographic conditions: Lux Cellulose-1 (4.6mm x 250 mm x 5μm) column, mobile phase: *n*-hexane/2-propanol/ acetic acid (99.6/0.4/0.15 v/v/v), F=1 mL/min., t = 20°C, UV= 254 nm.

Table 1. The influence of alcohols on the conversion and the enantioselectivty of the esterification reaction of (*R,S*)-ibuprofen with the application of lipase OF-immobilized superparamagnetic triiron tetraoxide nanoparticles.

Alcohol	ee_p (%)	ee_s (%)	C (%)	E
Methanol	93.5	54.0	36.7	50.6
Ethanol	61.7	40.0	39.3	6.2
n-Propanol	68.3	54.1	44.2	9.1
n-Butanol	76.5	79.1	50.8	18.0

Reaction conditions: racemic ibuprofen (8.25 mg, 0.04 mM), one of the alcohols: methanol (4.88 μL), ethanol (7.04 μL), *n*-propanol (9.02 μL), *n*-buthanol (11.04 μL), lipase OF-immobilized superparamagnetic triiron tetraoxide nanoparticles, cyclohexane (700 μL), $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (35 mg in total, with molar ratio of 1:1), molecular sieves 4Å; temp. 30°C, shaking 600 rpm.; after 140 h; C-conversion, ee_s - enantiomeric excess of the substrate, ee_p – enantiomeric excess of the product, E – enantioselectivity.

Figure 1.

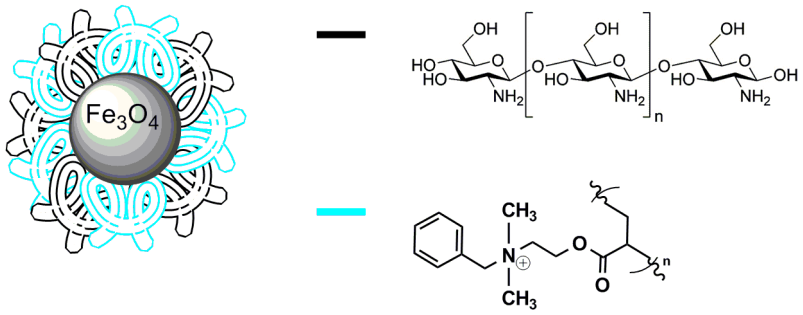


Figure 2.

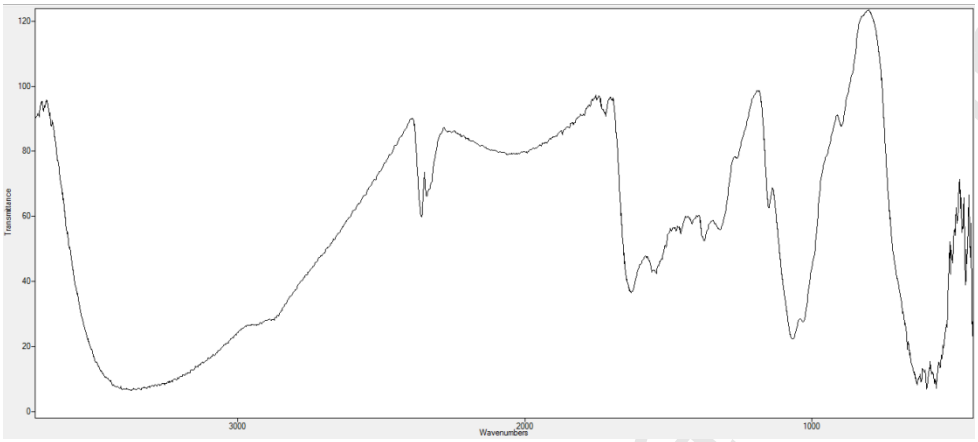


Figure 3.

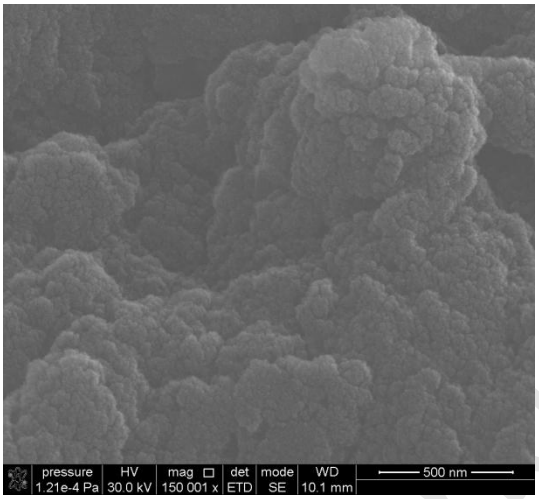
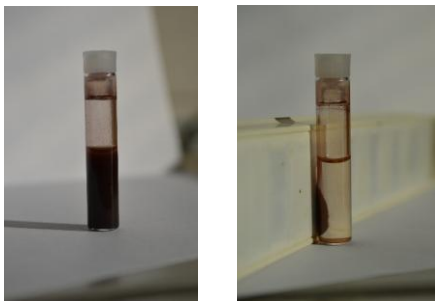
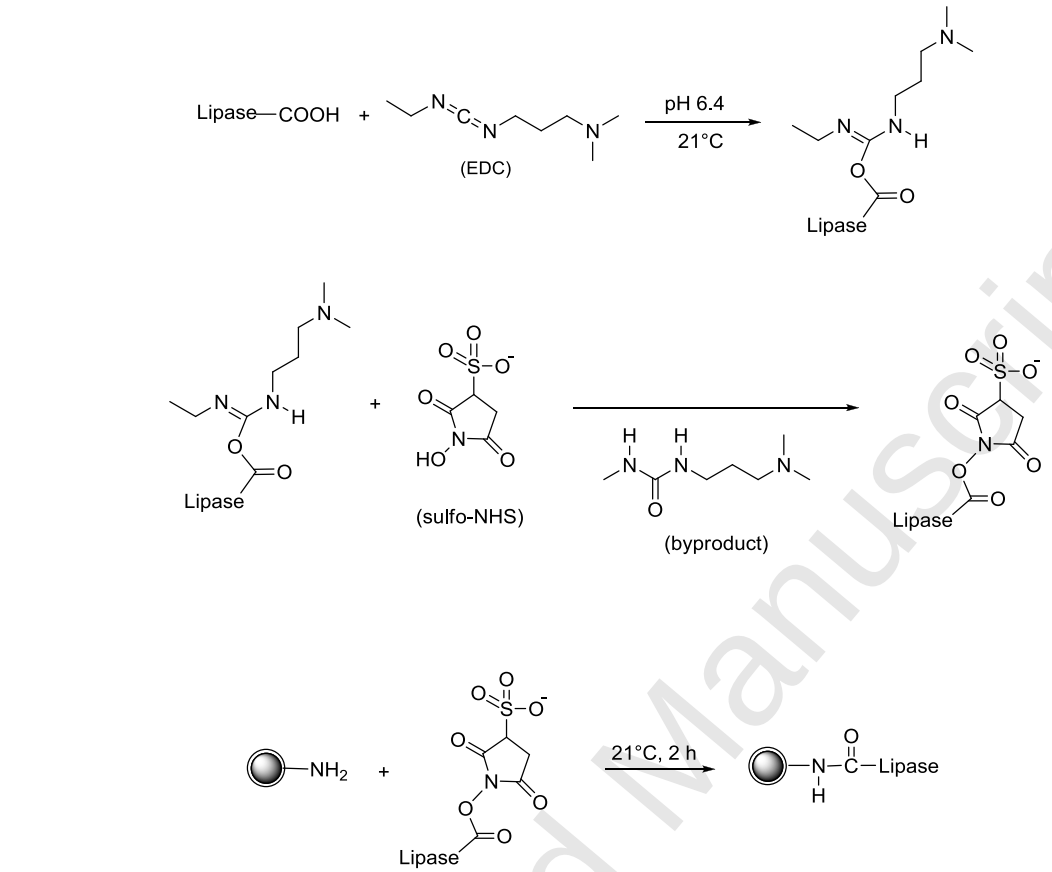


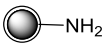
Figure 4.



a) b)

Scheme 1.





Amine-terminated chitosan magnetic nanoparticles

Figure 5.

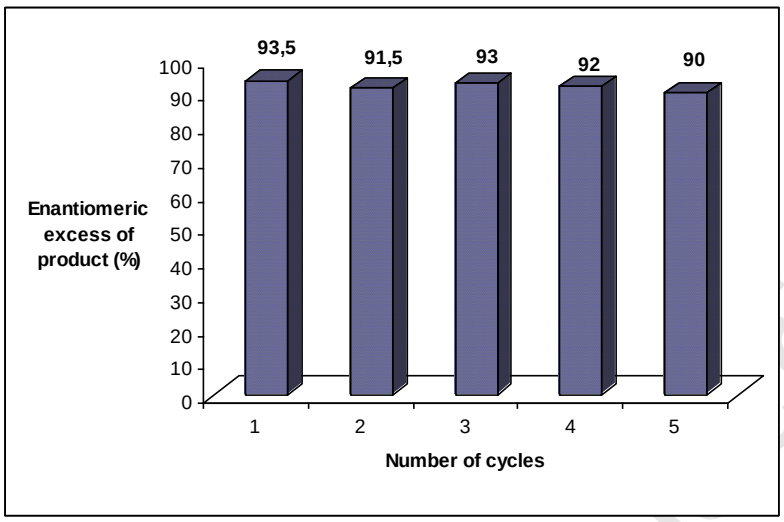
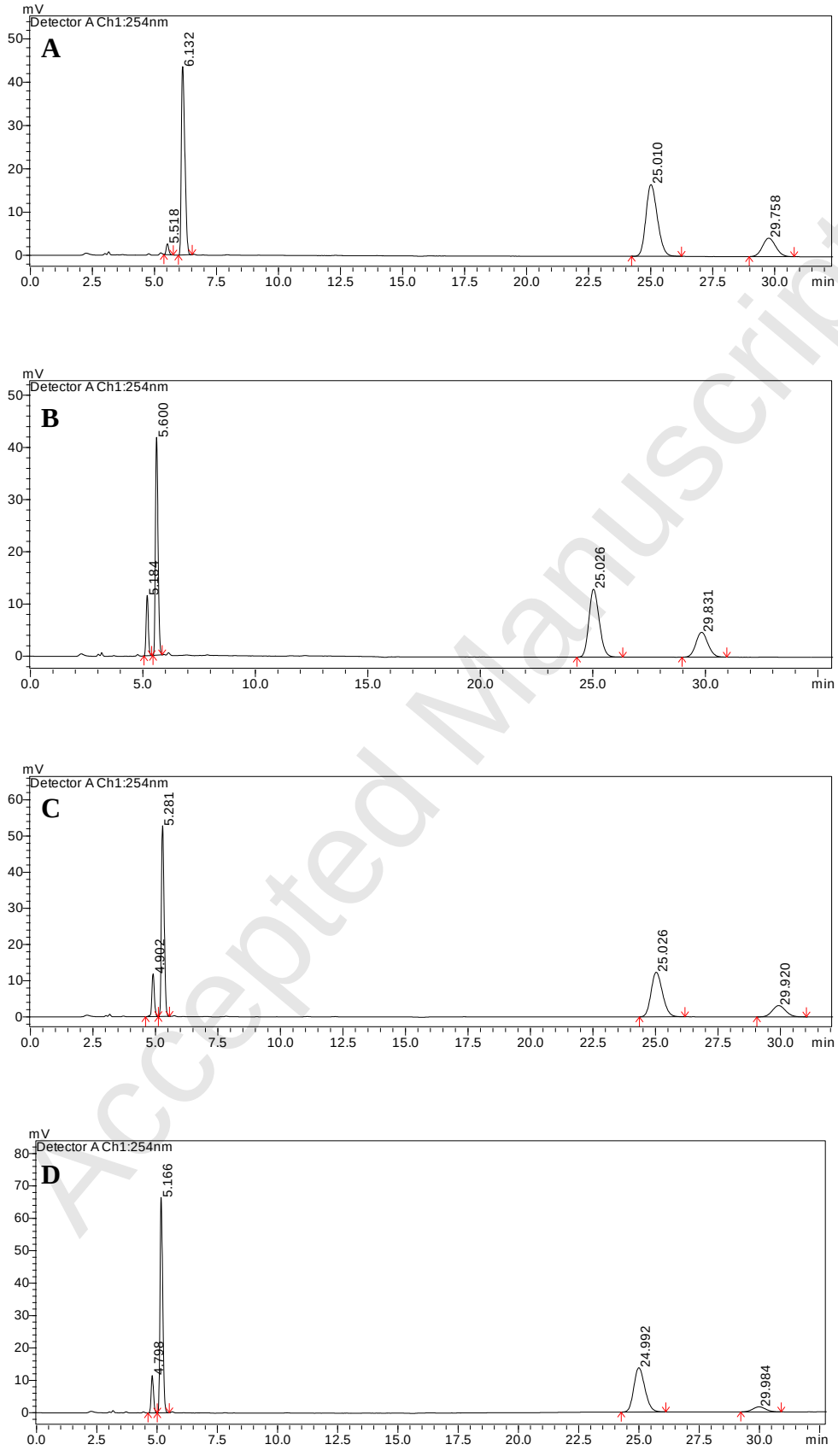
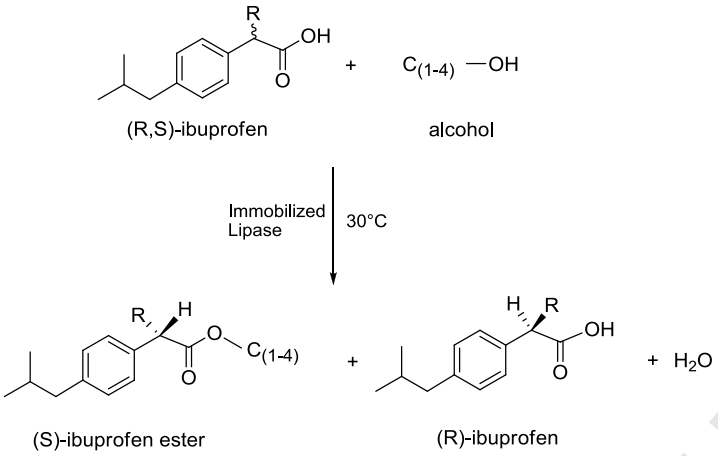


Figure 6.



Scheme 2.



C_1 - Methyl

C_2 - Ethyl

C_3 - *n*-Propyl

C_4 - *n*-Butyl