Article

Chitosan-Collagen Coated Magnetic Nanoparticles for Lipase Immobilization – New Type of "Enzyme Friendly" Polymer Shell Crosslinking with Squaric Acid

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Abstract: The synthesis of new collagen, chitosan and chitosan-collagen coated magnetic nanoparticles have been done. Two types of cross-linkers for polymer shell stabilization were used: glutaraldehyde (Gla) as a standard cross-linker and new one – squaric acid (SqA). Structure and morphology of prepared nanoparticles were characterized by ATR-FT IR, XRD and TEM analysis. The immobilization of lipase from *Candida rugosa* was performed on the nanoparticles surface. The amount of immobilized enzyme was quantified by the Bradford method. All of lipase-biopolymers coated nanoparticles were characterized with good activity recovery. A little hyperactivation of lipase immobilized on nanoparticles with SqA was observed. All of prepared lipase-immobilized nanoparticles were characterized with very good reusability.

Keywords: immobilization, lipase, magnetic nanoparticles, chitosan, collagen, squaric acic

1. Introduction

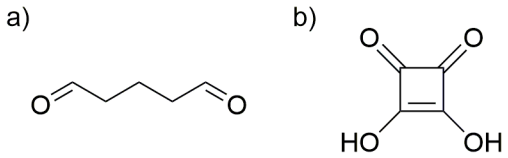
Lipases are one of the most used hydrolases applied in wide range of organic reactions as well as in analytical procedures.[1,2] However, free form of lipase has a poor stability and there is no possibility to use this enzyme in several catalytic cycles. Moreover, the separation of enzyme after reaction is somewhat complicated and needs centrifugation and precipitation. One of the most promising methods to improve the enzymes disadvantages is immobilization.[3,4] The rapidly growing field of enzymatic reactions in biotechnology needs new supports for immobilizations which guarantee simple catalytic system separation and high reusability. Usually the binding of bioligands such as enzymes is accomplished through the surface adsorption, covalent bonding and encapsulation. For enzymes there are two ways for effective immobilization: physical adsorption and chemical immobilization using the covalent bonds between reactive groups of enzyme and the support surface. Among the physical adsorption, covalent immobilization seems to be more effective for enzyme due to the stabilization of their active conformation.[5]

Magnetic nanoparticles (MNP's) are widely used as supports for physical and chemical biomolecules immobilization.[6-8] The surface of MNP's is usually specific and highly porous which makes them very good carriers capable of targeting molecules with high load of ligands.[9-12] However, pure magnetite has a poor colloidal stability particularly in neutral pH, so it needs stabilization and surface

modification.[13] Among other materials, polymers are most frequently used for magnetic nanoparticles coatings. The polymer shell coated on magnetic core needs structure stabilization by cross-linking. Glutaraldehyde (Gla)(Fig.1a) and epichlorohydrine were usually used as a cross-linkers for chitosan. For collagen materials many crossslinkers were tested eg. dialdehydes, isocyanates and carbodiimides but they are often "not friendly" for biomedical applications and can interact with bioligands reducing the effectivity of immobilization.[14,15]

Squaric acid (3,4-dihydroxy-3-cyclobutene-1,2-dione, SqA) is a planar, cyclic structure able to interact with amino groups in simple condensation reaction (Fig. 1b). Moreover, it seems to work better in biomedical applications as a nontoxic and less aggressive to biomolecules than glutaraldehyde for example.[16] In our previous study collagen/elastin hydrogels for tissue engineering have been successfully prepared with squaric acid addition.[17] The *in vitro* tests show that SqA would be a safe and effective crosslinker instead of popular glutaraldehyde or epichlorohydrine.

Figure 1. Structures of applied cross-linking agents a) glutaraldehyde (Gla), b) squaric acid (SqA)



In this study the synthesis of six types of chitosan (CS), collagen (Coll) and CS/Coll coated magnetic nanoparticles were done. Although, chitosan coated MNP's are well described, the collagen as a polymer shell for MNP's was not reported in literature. The polymer layer was crosslinked traditionally with Gla and with SqA as a new cross-linking agent. All of prepared magnetic nanomaterials have been used for covalent immobilization of lipase from *Candida rugosa* via standard procedure. Nanoparticles were characterized by analytical methods: ATR-FT IR, XRD, TEM. The activity, reusability and the amount of lipase immobilized on MNP's surface were determined. The effect of crosslinker on material stability and enzyme activity were investigated.

2. Results and Discussion

2.1. Magnetic Nanoparticles Synthesis and Characterization

Six types of MNP's coated with chitosan, collagen and the blend of this two biopolymers were prepared by standard co-precipitation reaction. This route of synthesis was able due to the good solubility of used biopolymers in acidic conditions and precipitation in pH about 12. Collagen used for nanoparticles coating was isolated from the tail tendons of young rats according to literature procedure.[18] The commercially available collagen has been also used and no differences between these two types of protein were observed. The biopolymers: CS and Coll, for magnetic nanoparticles coating were used alone or in 1:1 weight ratio. Two types of crosslinkers were used for polymer layer stabilization: glutaraldehyde (Gla) and squaric acid (SqA) (Figure 1). Finally, CS/Gla(Fe_3O_4), Coll/Gla(Fe_3O_4), CS-Coll/Gla(Fe_3O_4), Coll/SqA(Fe_3O_4), CS/SqA(Fe_3O_4) and CS-Coll/SqA(Fe_3O_4) nanoparticles were obtained. Due to the presence of primary amino groups in CS and Coll structure the crosslinking reaction with carbonyl groups of Gla and SqA was possible to perform (Figure 2).[17] Although the primary amino groups in polymers reacted with crosslinkers, the surface of all prepared nanoparticles was still rich in these groups and able to bond bioligands. The amount of NH_2 groups was determined by standard ninhydrin method [19] and varied between 2.05 mM/g for CS-Coll/Gla(Fe_3O_4) and 3.73 mM/g for CS/Gla(Fe_3O_4) as it is shown in Table 1.

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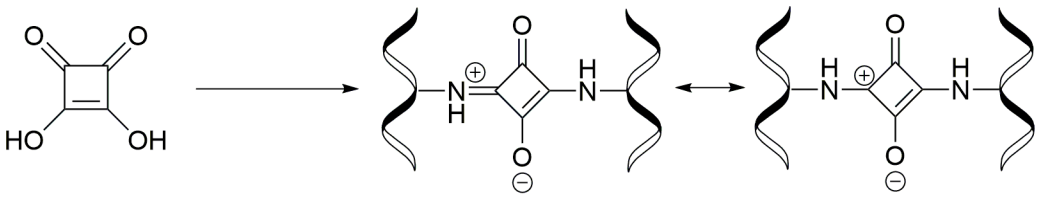

Figure 2. Scheme of collagen and chitosan cross-linking with squaric acid (SqA)

The size of prepared nanoparticles was investigated using zeta sizer (Table 1). The average size varies between 16 and 32 nanometers. The DLS analysis also demonstrated that prepared nanoparticles are rather homogenous in sizes. These observations were validated by the conventional TEM analysis which demonstrated that particles are aggregate with medium size 20-30 nm for particle (Fig.3) and in all cases little bigger when SqA was used. To determine the structure of magnetic core the electron diffraction was applied. The typical selected area diffraction pattern (SADP) for the studied group of particles was shown in Figure 3.

Table 1. Characteristic of prepared nanoparticles surface

Nanoparticles type	Size [nm]	Amount of NH_2 groups [mM/g]
CS/Gla(Fe_3O_4)	16	3.73
CS/SqA(Fe_3O_4)	20	3.31
Coll/Gla(Fe_3O_4)	21	3.51
Coll/SqA(Fe_3O_4)	29	2.17
CS-Coll/Gla(Fe_3O_4)	24	2.05
CS-Coll/SqA(Fe_3O_4)	32	2.83

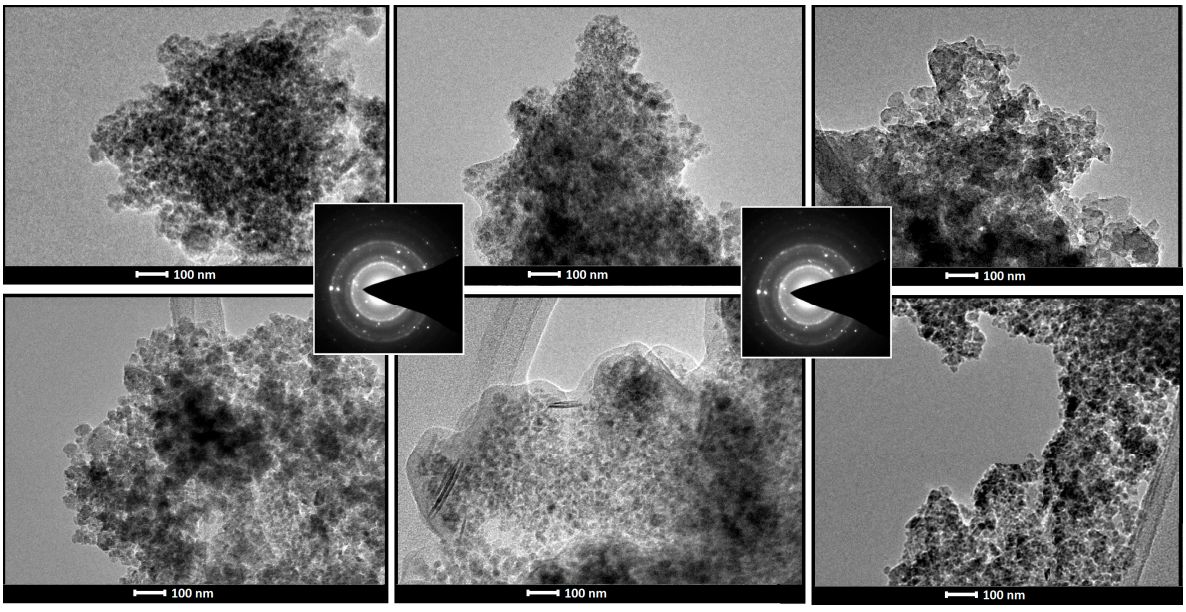

Figure 3. TEM images of (A) Coll/Gla(Fe_3O_4), (B) CS-Coll/Gla(Fe_3O_4), (C) CS/Gla(Fe_3O_4), (D) Coll/SqA(Fe_3O_4), (E) CS-Coll/SqA(Fe_3O_4), (F) CS/SqA(Fe_3O_4) nanoparticles

The structure of the magnetic nanoparticles was characterized by the ATR-FTIR spectroscopy (Fig. 4) and compared with the spectrum of pure CS and Coll. The spectrum of nanoparticles coated only with chitosan shows characteristic broad peak at 3460 – 3264 cm^{-1} from the stretching vibrations of

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OH and NH_2 groups. It should be added that the stretching vibrations of NH_2 group of chitosan were masked by the OH group peak. The vibration peak at 1647-1636 cm^{-1} increased with the C=N groups formation as a result of glutaraldehyde or squaric acid cross-linking reaction. Collagen displayed bands at 1630, 1550, and 1220 cm^{-1} characteristic for the amide I, II, and III groups. The broad absorption band was observed at 3307 cm^{-1} assigned to the stretching vibration of N-H groups from the collagen macromolecule. The spectra of the Coll/Gla(Fe_3O_4) and the Coll/SqA(Fe_3O_4) showed also the bands at 3360 cm^{-1} (N-H), 1630 cm^{-1} (amide C=O stretching vibration). Bands at 2940 and 1010 cm^{-1} indicated the aliphatic CH, and bending vibrations of CO, respectively. All of this characteristic bands were observed in CS-Coll/Gla(Fe_3O_4) and the CS-Coll/SqA(Fe_3O_4) spectra. In all spectra of nanoparticles with SqA as a crosslinker a characteristic bands at 1370 cm^{-1} (CS-Coll/SqA(Fe_3O_4)), 1356 cm^{-1} (Coll/SqA(Fe_3O_4)) and 1438 cm^{-1} (CS/SqA(Fe_3O_4)) were observed. This bands were assigned to the – OH ($-O^-$) bending vibrations from the squaric moiety. The Fe-O group of magnetite showed a signal at 595 cm^{-1} which was observed in all of nanoparticles spectra. The crystal structure and phase purity of prepared nanoparticles were also verified by XRD analysis. Six characteristic peaks for Fe_3O_4 marked by their indices ((2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0)) were observed for all samples. These peaks are consistent with the X'Pert High Score database and reveal that the resultant nanoparticles core were pure Fe_3O_4 with a spinael structure. This also confirmed that the co-precipitation coating of magnetite nanoparticles did not cause the phase change of Fe_3O_4.

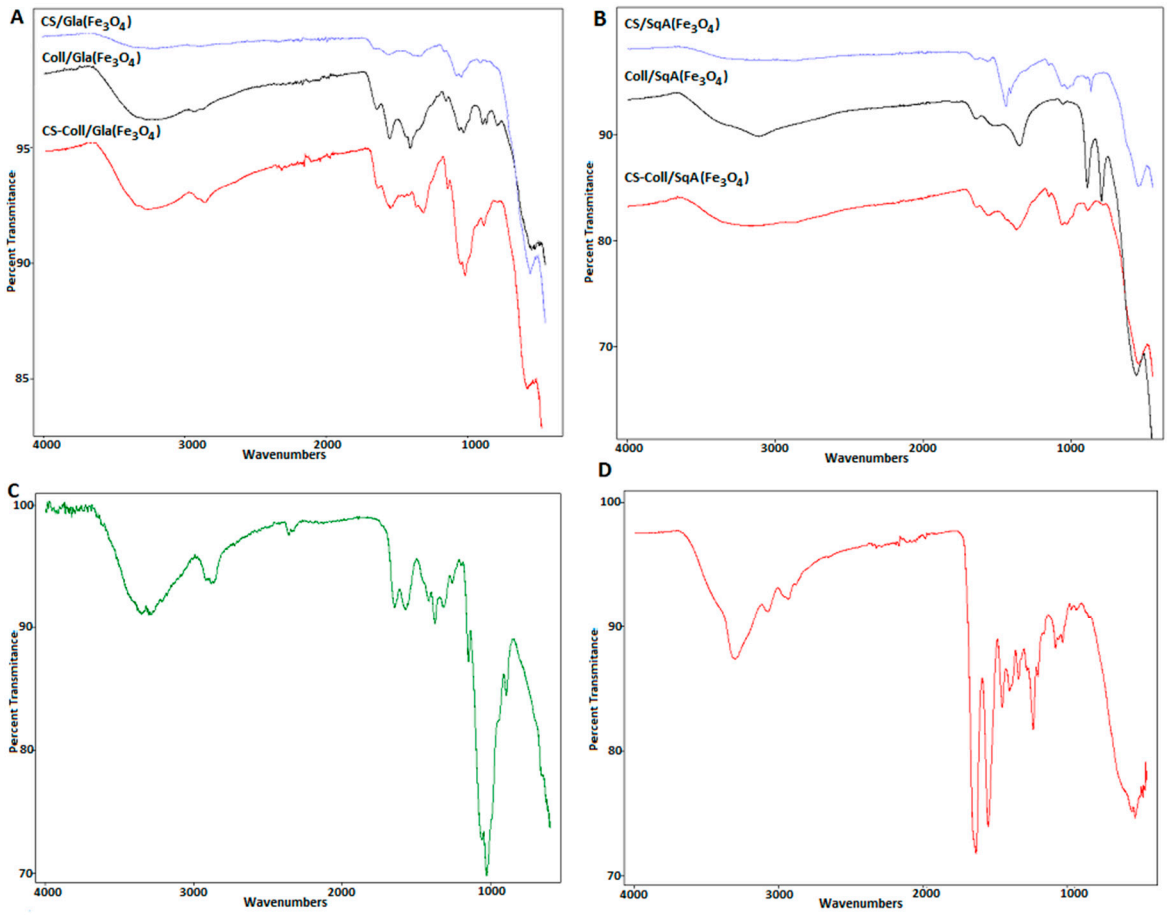

Figure 4. ATR-FTIR spectra of the obtained nanoparticles (A,B), pure chitosan (C) and collagen (D)

2.2. Lipase from Candida rugosa immobilization

Lipase from *Candida rugosa* was covalently immobilized on all of prepared nanoparticles surfaces. The immobilization took place via standard EDC/NHS procedure used NH_2 groups of support and carboxylic groups of enzyme (Fig.5).[6]

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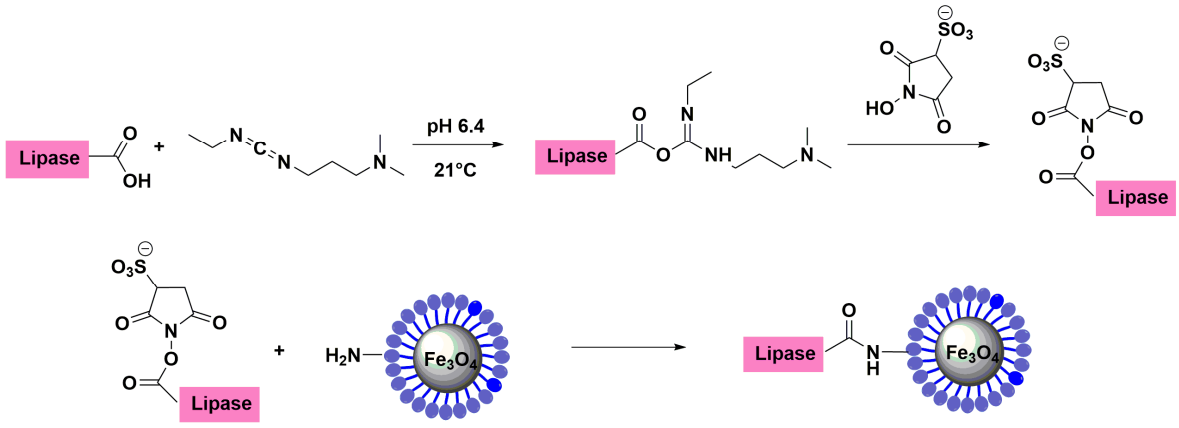

Figure 5. Scheme of lipase immobilization on MNPs

The amount of immobilized lipase bonded on the magnetic nanoparticles surface was determined by the measuring of the initial concentration of enzyme and its final concentration in a supernatant after immobilization using the Bradford protein assay method (Table 2.).[20] As it is shown the high lipase loading was observed for nanoparticles coated with the mixture of chitosan and collagen crosslinked traditionally with Gla. The cross-linked collagen coated on the nanoparticles surfaces did not distort the Bradford test quality – collagen need to be treated with 6M HCl under reflux to be detectable in this method.[21] In all cases cross-linking the polymeric shell with squaric acid (SqA) reduce the amount of immobilized lipase on support surface.

2.2.1. Lipase activity and recovery

The effect of cross-linking on lipase activity and stability was investigated. As it was shown the immobilization efficiency decreased for nanoparticles prepared with squaric acid as a cross-linked agent but quite different results were observed for lipase activity (Table 2).

Table 2. Characteristic of prepared nanoparticles surface

Nanoparticles type	Amount of immobilized lipase [mg/g]	Activity recovery [%]
CS/Gla(Fe_3O_4)	67	77
CS/SqA(Fe_3O_4)	47	104
Coll/Gla(Fe_3O_4)	63	85
Coll/SqA(Fe_3O_4)	18	97
CS-Coll/Gla(Fe_3O_4)	74	94
CS-Coll/SqA(Fe_3O_4)	27	112

Although the amount of immobilized lipase was rather low for nanosupports with squaric acid addition as a crosslinker, the activity recovery of immobilized enzyme increased for this materials. When glutaraldehyde as crosslinker was applied the activity recovery of immobilized lipase was about 77 to 85 % for nanoparticles coated with virgin CS or Coll and a little higher – about 94% for nanoparticles coated with mixture of these two biopolymers. Lipases immobilized onto materials crosslinked with squaric acid were characterized with better activity recovery in all types of support. The activity recovery was about 97% for nanoparticles coated with Coll and higher than 100% for two other materials: CS/SqA(Fe_3O_4) and CS-Coll/SqA(Fe_3O_4). It is known in literature that air bubbles and other protein could stabilize the soluble enzymes and consequently improved their catalytic

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activity.[22] In this case the small hyperactivation of immobilized enzyme was observed. Moreover, the crosslinking of collagen or chitosan with squaric acid in condensation reaction lead to ionic structure as it is shown in Figure 2. It was reported that very low concentration of ionic compounds made lipases in solution dramatically hyperactivated (above 300- fold).[23,24] The ionic form of squaric moiety in crosslinked polymer shell may promote activation and stabilization of open form of lipase and finally the hyperactivity was observed.

2.2.2. Immobilized lipase stability – optimal pH and temperature

To find the optimal pH value and temperature with maximum hydrolytic activity of the immobilized lipase the influence of different pH and temperatures were applied and the activity recovery of free and immobilized lipase was investigated.

The pH effect of the free and immobilized lipase was investigated by enzyme incubation in the mixture of emulsion (gum arabic and olive oil) and the phosphate buffer solution (100mM, pH 4-10). The analysis using standard activity assay procedure as well as determination of the relative activity were performed (relative activity [%] is the ratio between activity of every sample and the maximum sample activity). As it is shown in Figure 6 the optimal pH of free and immobilized lipase of all of prepared supports is 7. For pH values lower than optimal 7 the lipase immobilized on CS-Coll/SqA(Fe_3O_4) nanoparticles was more stable (about 75% of relative activity) compared to the native form of enzyme (60%). The differences were more significant in pH values between 9 to 10. As it is shown the native form of lipase remained only 20% of activity while the relative activity of lipase immobilized on prepared nanoparticles is about 60%.

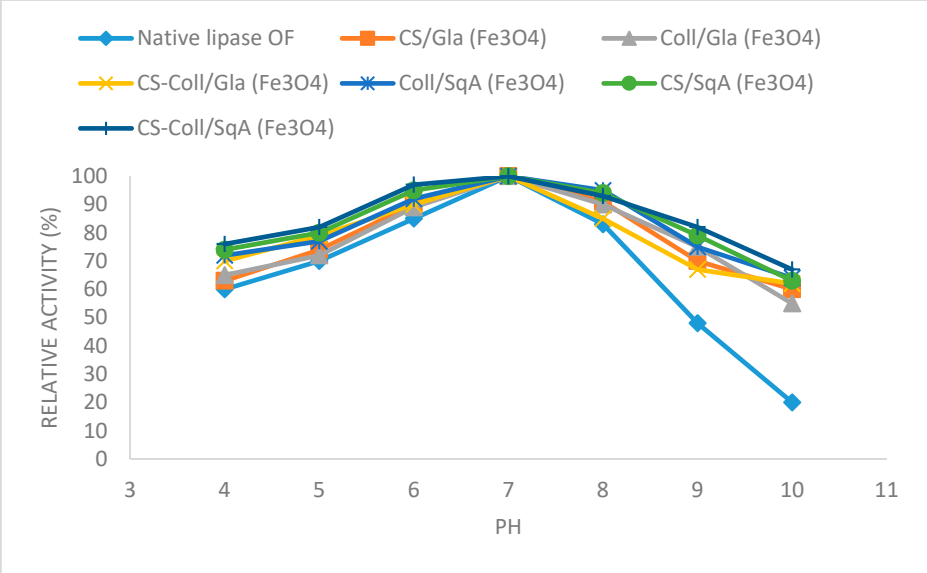

Figure 6. Effect of pH value on lipase activity

The effect of temperature on lipase activity and stability was performed in phosphate buffer pH 7.4 and temperature range from 25 to 65 °C for 30 min with the use of emulsion of gum arabic and olive oil (standard activity assay). The relative activities were determined and presented in Fig.7.

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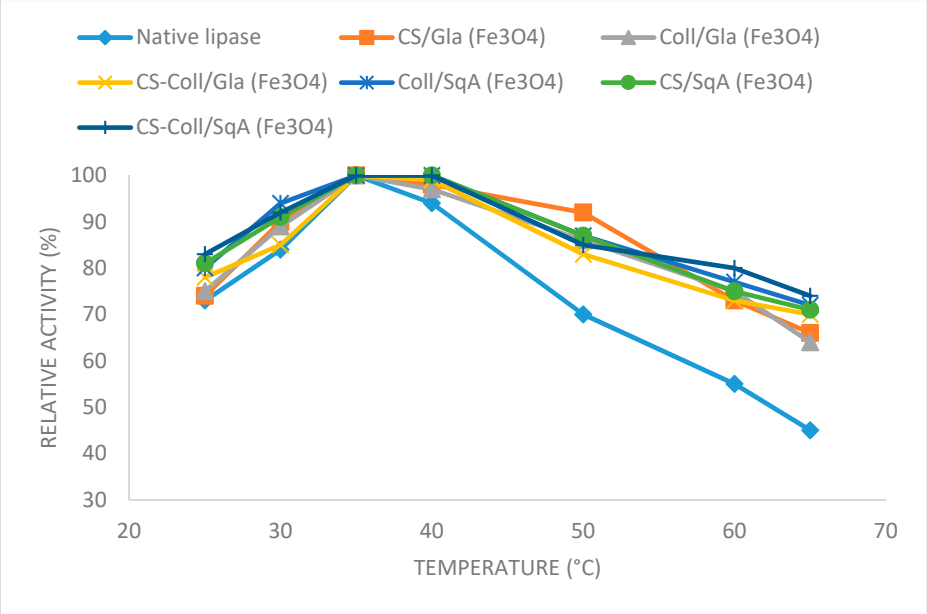

Figure 7. Effect of temperature on lipase activity

The optimal temperature of lipase immobilized on all supports was about 35 to 41°C and it is similar to optimal temperature for native lipase (35°C). The lipase immobilized on MNP's with polymer shell crosslinked with squaric acid was the most stable (the relative activity was 100% in the range of 34-41°C). The relative activity of immobilized lipase at higher temperature 50-65°C was about 75% while the activity of free lipase decreased to 50-45%. In this case the most stable was lipase immobilized on CS-Coll/SqA(Fe_3O_4) and Coll/SqA(Fe_3O_4) nanoparticles. What should be stressed, the immobilized enzyme is more resistant for temperature changes, compared to the native form of lipase. The relative activity was higher for bounded biocatalyst compared to free form at all range of tested temperatures (T = 25-65°C).

2.2.2. Reusability of immobilized lipase

The reusability of lipase-immobilized onto magnetic nanoparticles in catalytic cycles was tested in the olive oil hydrolysis reactions. The operational stability of the immobilized lipase was investigated for 10 consecutive cycles. As it is shown in Fig.8 after 5th using, the residual activities for all studied magnetic nanoparticles were more than 80% and at the end of the 10th use, lipases still retained almost 80% of their activities, depending on the support applied.

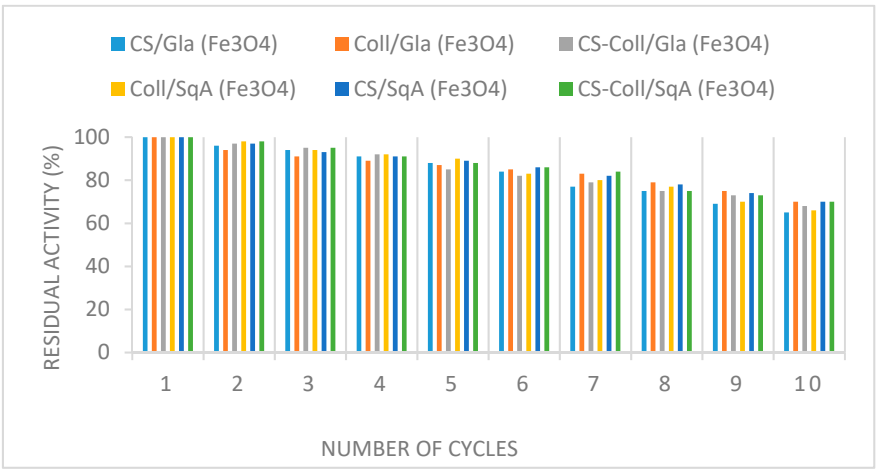

Figure 8. Reusability of lipase immobilized on magnetic nanoparticles

4. Materials and Methods

4.1. Isolation of collagen from rats tail tendons

After washing with distilled water, the rat tendons were dipped in acetic acid solution (0.1 M) for 4 days at 4°C. Collagen dissolved in acetic acid was freeze-dried (48h, -20C, 100 Pa) to obtain pure protein.

4.2. Synthesis of collagen coated nanoparticles cross-linked with glutaraldehyde Coll/Gla(Fe_3O_4)

Collagen from rats tail (0.2 g, local source) was added into 1% acetic acid solution (20 mL) and mechanically stirred at room temperature until solution become homogenous. Iron(II) chloride tetrahydrate (3.7 mmol), iron(III)chloride hexahydrate (7.5 mmol) were added and the resulting solution was precipitated at room temperature by adding dropwise 30% solution of NaOH (15 mL). The black mixture was separated by filtration and washed by deionized water for five times. Then 20 mL of 6,5% glutaraldehyde water solution was added and the mixture was mechanically stirred at room temperature for 30 min. The resulting magnetic material was recovered from the suspension by applying a magnet, washed with deionized water and dried under vacuum at 50°C for 24 h.

4.3. Synthesis of collagen coated nanoparticles cross-linked with squaric acid Coll/SqA(Fe_3O_4)

Collagen from rat tails (0.2 g, local source) was added into 1% acetic acid solution (20 mL) and mechanically stirred at room temperature until solution become homogenous. Iron(II) chloride tetrahydrate (3.7 mmol), iron(III)chloride hexahydrate (7.5 mmol) were added and the resulting solution was precipitated at room temperature by adding dropwise 30% solution of NaOH (15 mL). The black mixture was separated by filtration and washed by deionized water for five times. Then (0.022 g, 0.2 mmol) of squaric acid in etanol (100 mL) was added and the mixture was magnetically stirred at 30°C for 2 h. The resulting magnetic material was recovered from the suspension by applying a magnet, washed with deionized water and dried under vacuum at 50°C for 24 h.

4.4. Synthesis of chitosan coated nanoparticles cross-linked with glutaraldehyde CS/Gla(Fe_3O_4)

The procedure for Coll/Gla(Fe_3O_4) was used with 0.2 g of chitosan.

4.5. Synthesis of chitosan coated nanoparticles cross-linked with squaric acid CS/SqA(Fe_3O_4)

The procedure for Coll/SqA(Fe_3O_4) was used with 0.2 g of chitosan.

4.6. Synthesis of chitosan-collagen (1:1) coated nanoparticles cross-linked with glutaraldehyde CS-Coll/Gla(Fe_3O_4)

Collagen from rat tail (0.1 g, local source) and chitosan (0.1g) were added into 1% acetic acid solution (20 mL) and mechanically stirred at room temperature until solution become homogenous. Iron(II) chloride tetrahydrate (3.7 mmol), iron(III)chloride hexahydrate (7.5 mmol) were added and the resulting solution was precipitated at room temperature by adding dropwise 30% solution of NaOH (15 mL). The black mixture was separated by filtration and washed by deionized water for five times. Then 20 mL of 6,5% glutaraldehyde water solution was added and the mixture was mechanically stirred at room temperature for 30 min. The resulting magnetic material was recovered from the suspension by applying a magnet, washed with deionized water and dried under vacuum at 50°C for 24 h.

4.7. Synthesis of chitosan-collagen (1:1) coated nanoparticles cross-linked with squaric acid CS-Coll/SqA(Fe_3O_4)

Collagen from rat tail (0.1 g, local source) and chitosan (0.1g) were added into 1% acetic acid solution (20 mL) and mechanically stirred at room temperature until solution become homogenous. Iron(II)

chloride tetrahydrate (3.7 mmol), iron(III)chloride hexahydrate (7.5 mmol) were added and the resulting solution was precipitated at room temperature by adding dropwise 30% solution of NaOH (15 mL). The black mixture was separated by filtration and washed by deionized water for five times. Then (0.022 g, 0.2 mmol) of squaric acid in etanol (100 mL) was added and the mixture was magnetically stirred at 30°C for 2 h. The resulting magnetic material was recovered from the suspension by applying a magnet, washed with deionized water and dried under vacuum at 50°C for 24 h.

4.8. Quantification of available primary amino groups on magnetic nanoparticles surface

The amount of primary amino groups on prepared magnetic MNPs was estimated by the standard ninhydrin method. The calibration curve was prepared using glycine as a standard - ranging from 0.6 mM to 2 mM in 0.1mM acetate buffer, pH 5.5 (0.5g of ninhydrin was dissolved in the solution of 30 mL of isopropanol and 20mL of acetate buffer to prepared). Freshly prepared ninhydrin reagent (2mL) was added to 2 mL solution of each concentration of glycine and well mixed. The blank reagent was composed with 2 mL of distilled water and 2 mL of prepared ninhydrin reagent. Each of synthesized nanoparticles were dispersed in 2 mL of 0.1 mL buffer acetate (pH 5.5) and then 2 mL of ninhydrin reagent was added. All standard curve solutions and suspensions of nanoparticles were capped, mixed by hand and heated in boiling water for 15 min. After cooling, 3 mL of 50 % ethanol was added to each tube. The concentration of primary amino groups was determined with the use of spectrophotometric measurements at 570 nm.

4.9. Immobilization of lipase from candida rugose onto MNPs surface

Magnetic nanoparticles (50 mg) were placed into 2 mL centrifuge tubes and rinsed three times with 50 mM phosphate buffer (pH 6.4). Next, the 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 solutions in phosphate buffer (2mg/50 μL) were added. The solutions were incubated at 21°C and shaked for 1 h. Next 50 μL of sulfo-NHS solution in phosphate buffer (2.4 mg/50 μL) was added to each of the tubes containing the lipase and EDC. The solutions were incubated at 21°C for 1 h and shaked. Further on, 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. Finally, lipase-immobilized chitosan nanoparticles were rinsed three times with 0.5 mL of water and were dried overnight at 30°C. The amount of immobilized lipase was determined by the measuring of the initial concentration of lipase and its final concentration in a supernatant after immobilization, using the Bradford protein assay method. A calibration curve constructed with lipase OF solution of known concentration (0.5-2 mg/mL) was used in the calculation of enzyme concentrations. All data used for calculation are average of triplicate of the experiments.

4.10. Assay of immobilized lipase activity

The activity of free and immobilized lipase was measured by titration of the fatty acid arising from the hydrolysis of the olive oil. A 100 mL of olive oil emulsion was prepared by mixing of olive oil (50 mL) and Arabic Gum solution (50 mL, 7% w/v). The assay mixture consisted of the emulsion (5 mL), phosphate buffer (2 mL, 100 mM, pH 7.4) and either free enzyme (1 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 amount of the liberated fatty acid in the medium was determined by the titration with 50 mM NaOH solution using phenolphthalein indicator. One unit of lipase activity (U) was defined as the amount of the enzyme that hydrolyzed olive oil releasing 1 μM of fatty acid per minute under the assay condition. Activity recovery (%) was the ratio between the activity of immobilized lipase and the activity of the same amount of the free lipase in solution.

4.11. Operational stability (reusability) of lipase immobilized onto MNPs

The operational stability of immobilized lipase in repeated use was determined by the hydrolysis of the olive oil with the use of the immobilized enzyme recovered by the magnet. Magnetic nanoparticles with lipase after catalytic cycle were washed three times with the phosphate buffer (2 mL, 100 mM, pH 7.4) and next mixed with the freshly prepared reaction mixture to start a new cycle. The immobilized enzyme was used in the olive oil hydrolysis up to 10 batch reactions. The residual activity (%) was estimated as the ratio between the activity of the immobilized lipase in *n*th cycle and the activity of immobilized lipase in the 1st cycle, where the activity of the first cycle is defined as 100%. All obtained values were expressed in percentage.

5. Conclusions

Six types of magnetic nanoparticles coated with two biopolymers: chitosan and collagen were synthesized. A squaric acid (SqA) as a nontoxic crosslinking agent was applied for polymeric shell stabilization and compared with glutaraldehyde (Gla) as a widely used cross-linker. Lipase from *Candida rugosa* was immobilized onto all prepared nanoparticles and the activity, reusability and thermal stability were investigated. Enzymes immobilized onto MNPs with polymer shell crosslinked with SqA were characterized with better activity than enzymes immobilized onto MNPS with Gla using. Moreover, the lipase hyperactivation was observed on materials contained collagen and crosslinked with SqA. The lipase thermal and pH stability were also better for materials with SqA addition.

Summary, squaric acid (SqA) is a safe, "enzyme friendly" cross-linking agent which could be successfully applied for polymers crosslinking in any type of support designed for enzyme immobilization or encapsulation.

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Author Contributions Marta Ziegler-Borowska conceived and designed the experiments, partially performed the experiments, analyzed the data, contributed reagents/materials/analysis tools and wrote the paper; D. Chelminiak and K. Wegrzynowska-Drzymalska performed the nanoparticles synthesis and IR analysis. A. Sikora, T. Siodmiak and M. P. Marszałł performed the enzyme immobilization. J. Skopinska-Wisniewska isolated the collagen from rat tails. H. Kaczmarek partially analyzed the data.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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