HRP-LIKE AKTIVITY OF BARE AND MODIFIED MAGNETIC NANOPARTICLES

Šárka HRADILOVÁ[1], Aleš PANÁČEK[2], Libor KVÍTEK[2], Radek ZBOŘIL[2]

[1] *RCPTM, Faculty of Science, Palacky University, Olomouc, Czech Republic, EU, sarka.hradilova@upol.cz*
[2] *RCPTM, Department of Physical Chemistry, Faculty of Science, Palacky University, Olomouc, Czech Republic, EU*

Abstract

The horseradish peroxidase conjugated with antibody is used in many biochemical applications such as immunoassays or Western blots primarily for its ability to amplify a weak signal and increase detectability of a target molecules. Horseradish peroxidase is susceptible to damage due to high/low temperatures, or by changing the pH. The magnetic particles exhibit the same behavior as horseradish peroxidase, and if a suitable substrate is used, this reaction could be detectable by spectrophotometer too. They catalyze the reaction in a broader range of temperature and pH compared to peroxidase. The catalytic reaction of magnetic nanoparticles is hydrogen peroxide dependent. Magnetic particles exhibit stronger peroxidase-like activity, but for practical applications it is necessary to modify the surface to ensure the specificity of binding to the target structure and also improve biocompatibility and stability. Magnetic nanoparticles modified with chitosan were compared with particles without surface modification. We tested the effect of hydrogen peroxide concentration, the effect of magnetic nanoparticles concentration and reusability. Enzyme linked immunosorbent assay in a 96-well plate utilizing TMB substrate was used as a testing platform. Magnetic nanoparticles without surface modification exhibit a higher peroxidase - like activity in comparison with particles modified by chitosan, but modified MNPs still exhibit sufficient HRP-like activity.

Keywords: Magnetic nanoparticles, peroxidase-like, TMB, ELISA

1. INTRODUCTION

Separability of magnetic nanoparticles (MNPs) by magnetic field is widely used in many applications including target molecule separation and immunoassays [1]. Suitable surface modification of MNPs provides binding specificity, while the magnetic core allows separate the target molecule in magnetic field. For quantification is necessary to transfer the reaction signal to a measurable value - in ELISA assays is extensively used horseradish peroxidase (HRP), which upon reaction with the appropriate substrate gives a color change which is measurable on spectrophotometer. Intrinsic peroxidase activity of magnetic nanoparticles was firstly described by Gao and co-workers in 2007 [2]. This has opened up a number of possibilities how MNPs can be used with regard to their magnetic properties and the ability to mimic the HRP. The combination of capture and detection in one step is unique and provides significant advantages. MNPs offer many advantages in comparison with enzymatic labeling. HRP is an enzyme therefore is susceptible to denaturation due to high / low temperatures, changes in pH and other reaction conditions. On the other hand, MNPs are stable over a wide range of temperatures and pH [2], [3] without forgetting the economic aspect.

MNPs are further modified by surfactants, which not only affect the selectivity of binding to the target molecule, but also stability and biocompatibility [4]. One of possible method for MNPs modification is chitosan [5], [6] followed glutaraldehyde [7], [8]. Often used is also a variant with EDC/NHS [9]. Surface modification is used to expose the appropriate functional groups. Next step is binding of the antibody which recognizes a target antigen and thus ensure the specificity of the reaction. Chitosan is positively charged polysaccharide produced by deacetylation of chitin from shells of shrimps and crabs or from fungi cells. Chitosan is a carrier which contains in its molecule active amino groups which can react with functional groups of the binding partner. Glutaraldehyde (GA) serves as a crosslinking agent through which binds an

antibody or other molecule. GA has been used more frequently because of its low cost, availability, and high solubility in aqueous solution.

In this study, we monitor peroxidase-like activity of bare and modified Fe_3O_4 in presence of H_2O_2.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals including ferrous chloride, ferric chloride, NH_4OH, ferrous sulfate (Sigma-Aldrich in p.a.quality), chitosan low molecular weight (chitosan LW, Sigma-Aldrich in p.a. quality), 30 % hydrogen peroxide (p-Lab), sodium hydroxide (p-Lab), NH_4OH (p-Lab), ethanol (p-Lab), and acetic acid (p-Lab) were used without further purification. Tetramethylbenzidine - TMB, chromogenic solution, ready-to-use, (Sigma Aldrich). All solutions were prepared with deionized water.

2.2. MNPs preparation

We first prepared magnetic nanoparticles (MNPs) Fe_3O_4. Modified co-precipitation was used for preparation. Briefly: ferric chloride (FeCl3. 6 H2O, 4.86 g) and ferrous sulfate (FeSO4 . 7H2O, 3,34 g) was dissolved in 40 ml of deionized water and heated to 80°C. Next 12 ml of NH4OH was added. Solution was bubbled with nitrogen gas to prevent unwanted oxidation. After 120 minutes of stirring, the nanoparticles were removed from solution by magnetic separation, washed twice with ethanol and water until neutral pH. Nanoparticles were then dried in vacuum drier and used for experiments.

2.3. Chitosan modification

Surface modification was done by chitosan based on method described by Marková (Markova et al, 2012). Final concentration of modified MNPs was determined to 10 mg/mL (concentration of Fe_3O_4).

2.4. Determining the concentration of Fe_3O_4 by AAS

Fe_3O_4 concentrations were determined by the atomic absorption spectrometry (AAS) technique with flame ionization using a ContrAA 300 (Analytik Jena AG, Germany) equipped with a high-resolution Echelle double monochromator (spectral band width of 2 pm at 200 nm) and with a continuum radiation source (xenon lamp). The absorption lines used for these analyses were 248.327 nm. The calibration standards were prepared using an Iron Standard for AAS (1001 mg /L, Fluka).

2.5. ELISA tests

All tests were performed in 96 well ELISA plates (NUNC) in room temperature. Design of tests was as follows: 50 µL MNPs (bare or modified, in water, different concentrations), 50 µL H_2O_2, different concentrations, 50 µL TMB. After 30 min reaction was stopped by 50 µL 1 M H_2SO_4 and absorbance on 450 nm was measured using a multiplate reader Infinite 200 PRO (Tecan).

Effect of reusability was done in centrifuge tube in quadruplicate volume than described above. After 30 min MNPs was magnetically collected, solution in 150 µL aliquot was transferred to well in 96 well ELISA plate, reaction was stopped by 50 µL 1 M H_2SO_4 and absorbance on 450 nm was measured. Magnetically collected MNPs was 3 times washed and reused. Activity of one used bare MNPs was determined as 100%, other values (bare and modified) were related to the 100 %.

2.6. Statistic

All measurements were repeated at least three times and the values presented are in the form: measured value ± standard deviation. The chart shows the absorbance at 450 nm or relative catalytic activity in percentage. The maximum point of each curve was set as 100% relative catalytic activity.

3. RESULTS AND DISCUSSION

3.1. TEM analysis

Prepared MNPs were analyzed by TEM (Fig. 1). Average size was 12.9 ± 2.1 nm.

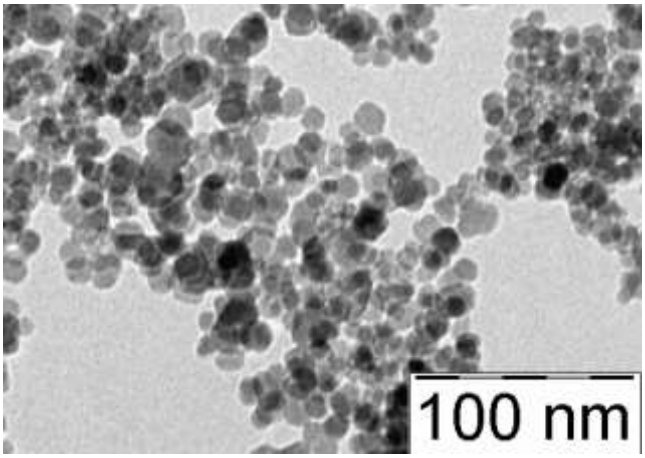

Fig. 1 TEM analysis of magnetic nanoparticles

3.2. Influence of hydrogen peroxide

The peroxidase-like behavior of bare and modified MNPs was examined by catalytic reaction of TMB in the presence of hydrogen peroxide. As was described elsewhere [2] , MNPs exhibit the same activity as HRP. Bare MNPS are although separable by magnetic field, but for real use require surface modification to provide selective interaction with target molecules. That is why chitosan and glutaradehyde as a divalent cross linker is frequently used.

Firstly behaviors of bare MNPs were tested (fig 2). Reaction with TMB is H_2O_2 dependent [2], [10], different concentration of H_2O_2 in range 5-0,125 % was used to find optimal concentration for next measurement. High concentrations of hydrogen peroxide are not suitable for use, absorbance was too high. For further experiments a 1 % concentration of hydrogen peroxide was used. It is assumed that the surface modification and lower concentrations of MNPs reduced catalytic activity and thus the measured absorbance, and therefore seems to be suitable for use as initial state of the highest possible concentration of hydrogen peroxide.

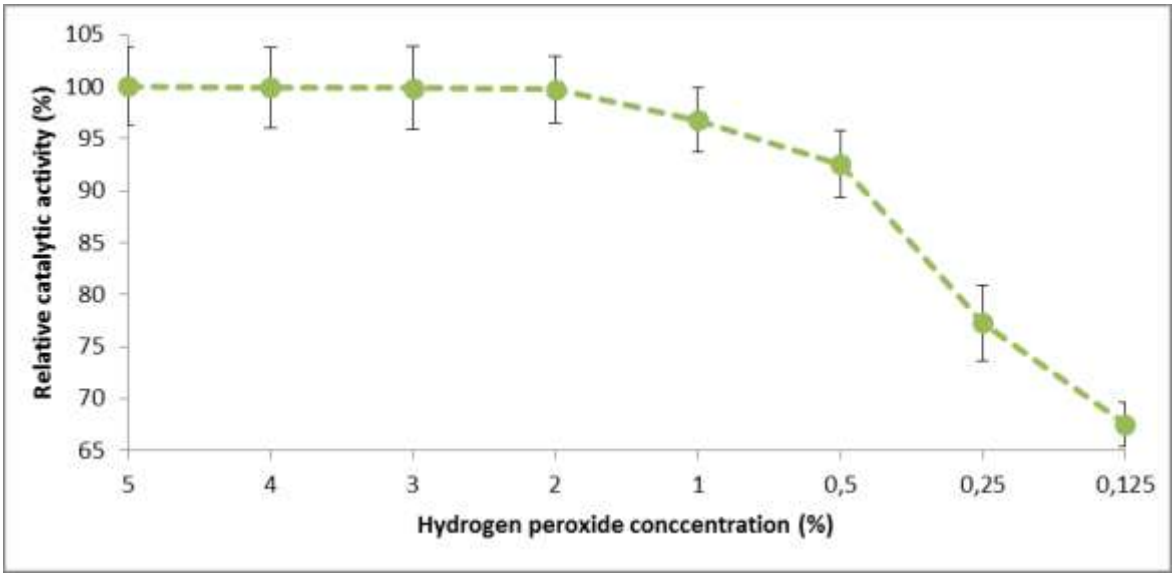

Fig. 2 Influence of H_2O_2
H_2O_2 in concentration range 5-0,125%, MNPs concentration 0,25 mM

3.3. Effect of MNPs mass concentration

Influence of mass concentration was monitored simply by dilution of MNPs from concentration 0.5 mM to 0.004 mM (binary dilution series). Other parameters were identical, changes in the relative catalytic activity corresponding to a concentration change of magnetic nanoparticles only. The figure 3 shows that the catalytic activity is proportional to the quantity of nanoparticles. If the MNPs serve as a substitute for HRP, it is necessary to monitor the linearity of absorbance or relative catalytic activity on the concentration of MNPs. For naked MNPs is linear in the range from 0.25 to 0.031 mM. The modified nanoparticles linearity is considerably weaker. Table 1 shows R-squared value for different linearity ranges.
Suitable estimate the concentration of MNPs will probably play a key role if MNPs are used for the separation and detection instead of HRP.

Table 1 R-squared value for different parts of curves from figure 3

Linearity range	R^2 for MNPs	R^2 for ChMNPs
0,25-0,031 mM	0,9964	0,9149
0,25-0,016 mM	0,9764	0,8725

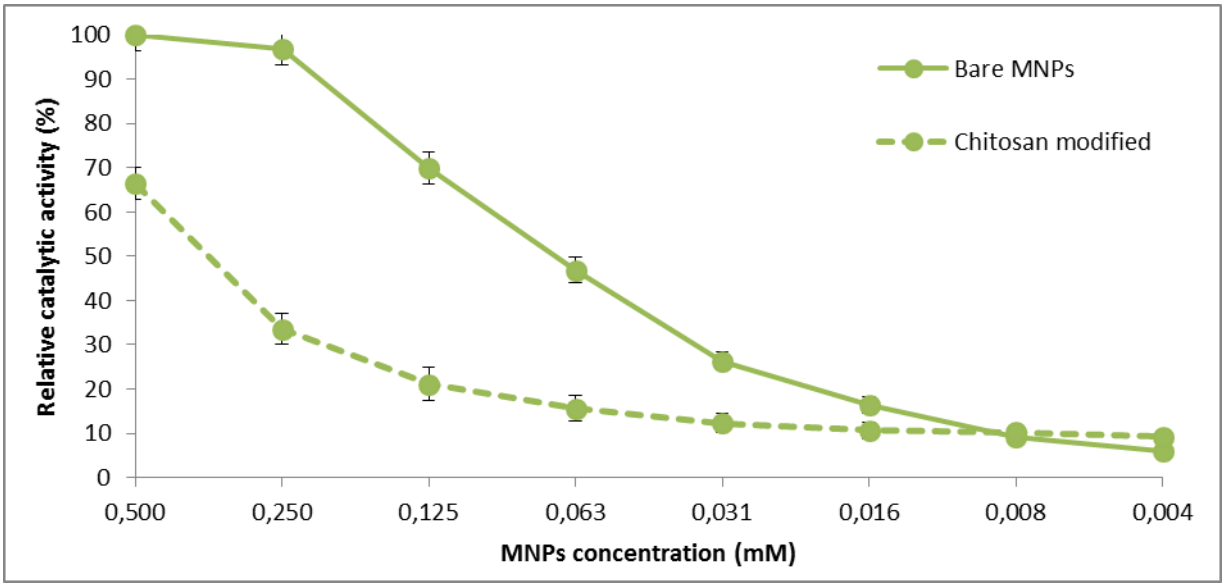

Fig. 3 Influence of MNPs mass concentration, bare and chitosan modified MNPs H_2O_2 in concentration range 1 %, MNPs concentration 0.5-0.004mM

3.4. Effect of chitosan surface modification

Other parameter we tested was surface modification. Frequently used MNPs surface modification by chitosan was tested. The next step is glutaraldehyde and antibody, which selectively bind target antigen. This specific targeting was not monitored in our study, because for different antibodies is necessary to optimize conditions such as concentrations, reaction time and many other factors and our goal was at all times monitor the impact of one parameter only.

Figure 3 shows changes in relative catalytic activity of chitosan modified MNPs vs naked MNPs. As expected, the catalytic activity of the modified particles is low in comparison with unmodified. The explanation may be that the surface modification leads to a reduction of the surface / volume ratio.

3.5. Effect of reusability

Repeated usage after washing was tested. The same MNPs was regenerated simply by collecting in magnetic field and repeatedly washed with deionized water. As is apparent from figure 4, repeated usage

the same nanoparticles for catalytic reaction leads to decrease in activity. But nine times used particles are still able to sufficiently react with the substrate and catalytic activity is 44,6 % and 22,32 % respectively.

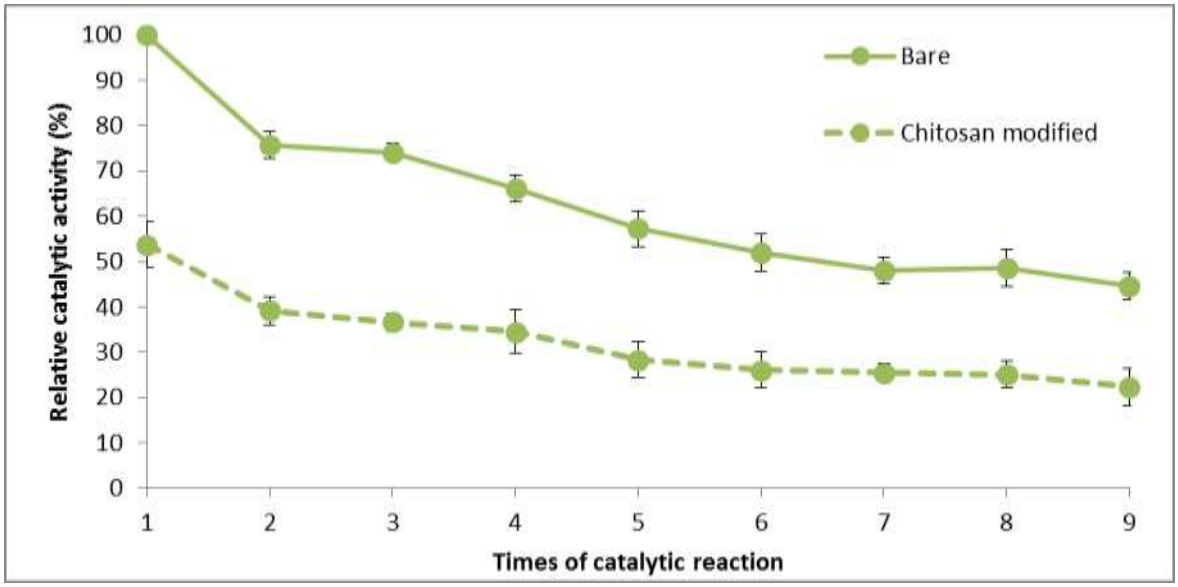

Fig. 4 Effect of reusability, bare and chitosan modified MNPs
H_2O_2 in concentration 1 %, MNPs concentration 0,5 mM

4. CONCLUSION

In summary, we examined the ability of non-modified and chitosan modified magnetic nanoparticles to catalyze the reaction with TMB in the presence of hydrogen peroxide. Bare MNPs and chitosan modified MNPs exhibit the same catalytic properties as HRP. The effect of hydrogen peroxide concentration, the concentration of nanoparticles, surface modification and reusability was tested. The process of preparation and surface modification can affect the catalytic properties of MNPs. Surface modification with chitosan leads to a reduction in catalytic activity, but surface modification is necessary to ensure specific interaction with the target structure, and increases stability and biocompatibility. Test in ELISA plates showed that this system is applicable and can replace conventional enzymatic systems.

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