Characterization and Application of High Magnetic Property Chitosan Particles

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ABSTRACT: High magnetic property chitosan particles were prepared from chitosan coated on magnetic powder, glutaraldehyde modified, and then epichlorohydrin crosslinked. The magnetic properties, the surface, and the morphology of the magnetic particles were characterized. An optimum condition of immobilization of aprotinin on magnetic particles was obtained, then these particles were used for affinity purification of trypsin, and the condition of affinity purification was discussed. The proposed method was successfully applied to the purification of trypsin. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 1175–1181, 2001

Key words: high magnetic property chitosan particles; aprotinin immobilization; affinity purification of trypsin

INTRODUCTION

Magnetic polymer particles have been of interest since they exhibit wide application in the fields of gene manipulation,[1] immunoassay,[2] cell isolation,[3] enzyme immobilization,[4] targeted delivery of drugs,[5] DNA separation,[6] and many other area.[7,8]

Magnetic properties are the important factors of magnetic polymer particles[9]; therefore, to our knowledge, there have been few investigations on the properties of magnetic polymer particles. High magnetic property chitosan particles were prepared by easy and inexpensive methods in this article. The surface of the particles was functionalized with aldehyde groups to facilitate the attachment of affinity ligands.

The trypsin is a biological medicine.[10] Gel, membrane, and resin were used for the carrier of trypsin affinity purification. The high magnetic properties of chitosan particles, combined with the aprotinin on the surface, makes them ideally suited for the preparative purification trypsin. The result of the experiment was satisfactory: high magnetic properties chitosan particles were used for a new carrier of trypsin affinity purification.

EXPERIMENTAL

Materials

Chitosan (AR), Magnetic powders were supplied from Applied Magnetic Laboratory of The State Education Commission of China. Epichlorohydrin (AR), Glutaraldehyde (AR), Di-sodium tetraborate (AR), Aprotinin, Trypsin, and Benzoyl-L-Arginine Ethyl Ester HCl were obtained from Shanghai Biochemical Research Institute. The solutions were prepared with redistilled water.

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Preparation of Magnetic Particles

To a round-bottomed flask equipped with a stirrer, magnetic powders were introduced with sodium hydroxide solution. This aqueous phase was stirred at 1000 rpm.

Chitosan solution was dropped into this aqueous phase. Using hydrogen chloride solution to adjust the pH of the aqueous, glutaraldehyde solution was added at a pH of 9–10 to react for 10 h. The sediments were filtrated and washed with water three times.

To a flask, the above sediments were introduced with sodium hydroxide solution together with epichlorohydrin as crosslinking agent at 50°C for 10 h. The sediments were filtrated and washed with water to a pH of ≈7.0, the particles were dried at 50°C, and were ground. High magnetic properties chitosan particles were produced.

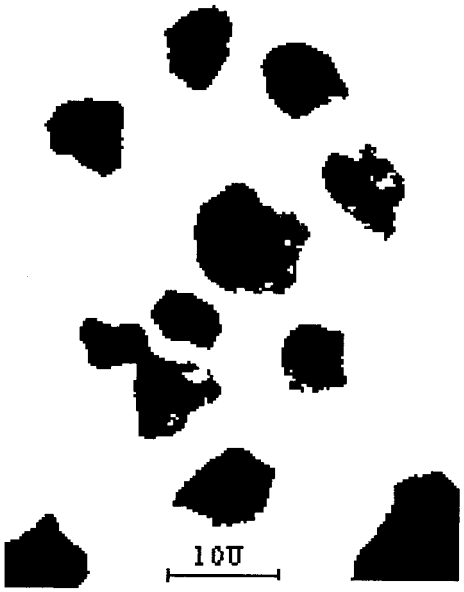

Figure 1 TEM of the magnetic chitosan particles.

Characterization of Magnetic Particles

The size and morphology of magnetic particles were investigated by H-600 transmission electron microscopy. The surface of magnetic particles was studied by S-450 scansion electron microscopy. The specific surface area of magnetic particles was measured by a specific surface area measure instrument S-3100. Magnetic properties of magnetic particles were performed by a vibrating sample magnetometer. The active groups on magnetic particles were determined by 170-SXFT-IR.

Ligand Attachment

The aprotinin (20 mg) was dissolved in 40 mL borax buffer solution (pH 10.5). The magnetic chitosan particles (1.0 g) were added to the aforesaid aprotinin solution, and agitation continued for 20 h at 25°C. The aprotinin modify particles (A-MP) were the particles that were magnetically sedimented. The solution was tipped, and was assayed for protein and aprotinin activity. Then A-MP wash three times with 50 mL distilled water and 50 mL adsorption buffer (pH 8, 0.1*M* borax adsorption buffer) to remove any unbound aprotinin. The particles were stored at 4°C in the adsorption buffer until further use.

Enzyme Affinity Purification

The sample contains trypsin (50 mg) were dissolved in 50 mL 0.1*M* borax adsorption buffer (pH 8). A-MP (1.0 g) was added to trypsin solution and the suspension was agitated for 30 min. The particles were sedimented on a magnetic field, the solution was tipped, and was assayed for protein and trypsin activity. The particles were washed two times with adsorption buffer for 5 min in a shaker. The trypsin was desorbed with 20 mL desorption medium containing 10 m*M* acetic acid and 0.5*M* potassium chloride. After agitation for 10 min, the particles were magnetically sedimented, the solution was tipped and was assayed for protein and trypsin activity. The solution obtained after desorption step was concentrated by ultrafiltration and freeze dried.

Determinated Method

Concentration of protein were measured by absorbance difference on 280 and 260 nm.[11]

Activity of aprotinin and trypsin were calculated with the assay method of China Pharmacopoeia.[12]

RESULTS AND DISCUSSION

Characterization

TEM photo shown in Figure 1. The magnetic particle size was calculated based on the sizes of about 100 particles on different regions of TEM photos. Weight-average diameter (D) was calculated based on eq. (1) as follows:

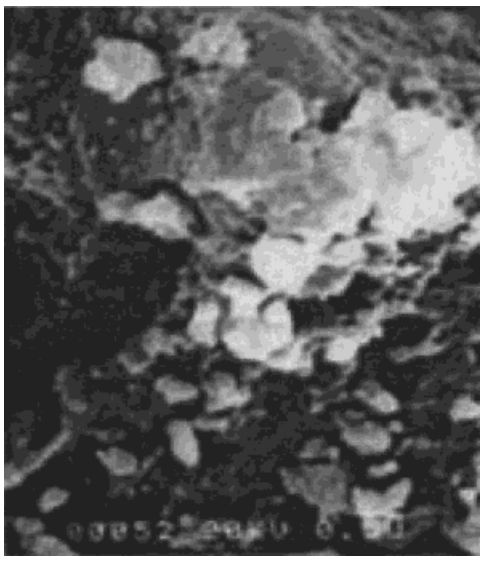

Figure 2 SEM of magnetic chitosan particles.

$$D = \sum n_i d_i^3 / \sum n_i d_i^2 \quad (1)$$

where n_i is the number of particles with diameter d_i. The diameter (D) of the magnetic chitosan particle is 10.2 μm.

Table I Magnetic Properties of Magnetic Chitosan Particles

Magnetic Properties	$H_c(O_e)$	σ_s (emu/g)[a]	σ_r (emu/g)[b]
Magnetic particles	2000	27.2	14.5

[a] σ_s is the saturated magnetization intensity.
[b] σ_r is the remanent magnetization intensity.

An SEM photo of the particles is shown in Figure 2. Figure 2 demonstrated that the surface of the particles containing cavities was not as smooth.

A magnetic hysteresis loop of the magnetic chitosan particles was given in Figure 3. Figure 3 show that coercive force (so that the remanent magnetization intensity of magnet can be decreases to zero, the reverse magnetic field intensity must be added on the magnet. The reverse magnetic field intensity is a coercive force) is large.

Magnetic properties of magnetic chitosan particles are shown in Table I. Table I demonstrated that the particles had high magnetic properties.

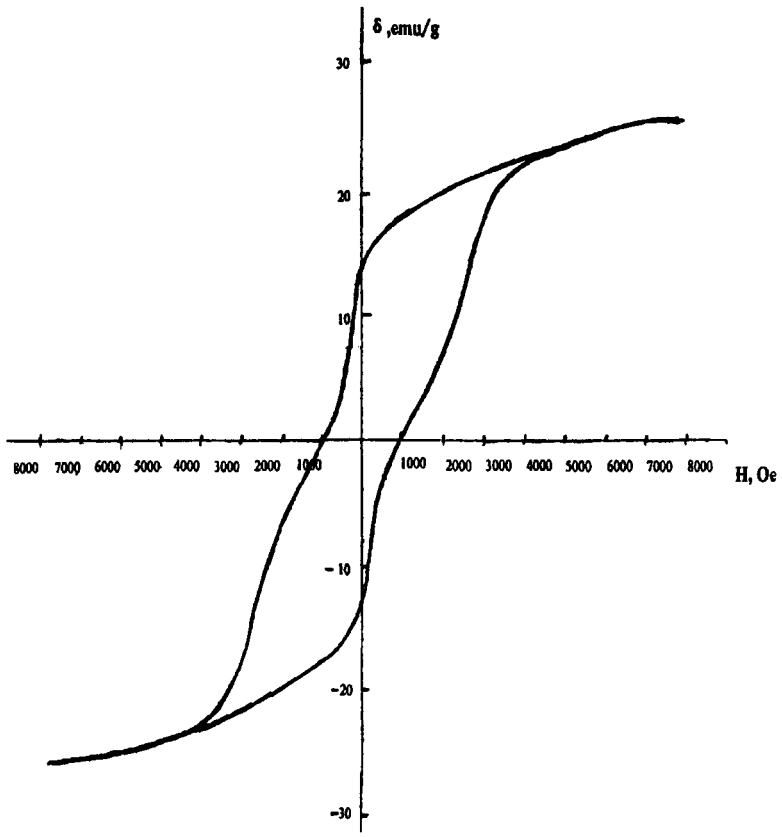

Figure 3 Magnetic hysteresis loop of the magnetic chitosan particles.

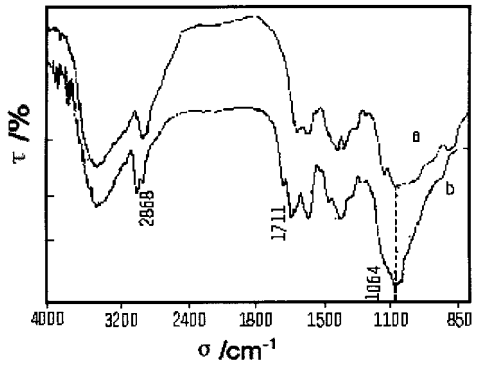

Figure 4 Infrared spectra: (a) chitosan; (b) magnetic chitosan particles.

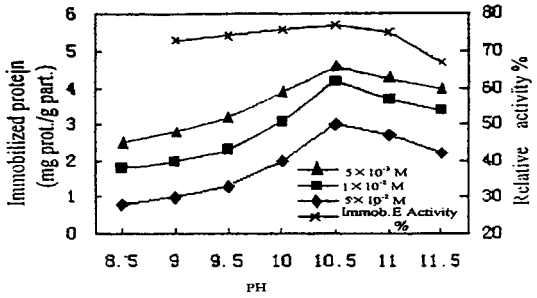

Figure 5 The effects of pH and buffer concentration on immobilized amount of aprotinin (T = 25°C), and the effect of pH on immobilized aprotinin relative activity (T = 25°C, $5 \times 10^{-3}M$ borax buff).

The specific surface areas of the particles is 16.7 m^2/g. The presence of active groups on manufactured magnetic chitosan particles were determined by the assessment of IR spectra of chitosan and magnetic chitosan particles. Strong peaks at 1711 cm^{-1} and a medium peak at 2868 cm^{-1} indicate that there are aldehyde groups on the particles (Fig. 4). Chitosan had the characteristic peak at 1064 cm^{-1}.

The magnetic materials are coated by polymer for the purpose of the polymer particles having magnetic properties. These magnetic materials are generally magnetite,[13] Fe_3O_4,[14] ironoxides,[15] gamma-Fe_2O_3,[16] strontium ferrite,[17] ferrofluids,[18] or magnetite cobalt magnetic colloid.[19] In these communications, the magnetic properties of these magnetic materials are low. For example, their coercive force (Hc) is low than 100Oe. These polymer are polystyrene,[20,21] agarose,[22] chitosan,[23] and glass.[24] The surface functionality was troublesome in refs. 20, 21, 22, and 24. The magnetic chitosan was prepped in ref. 23, but they did not for the surface functionality, only for sorption lysozyne.

High magnetic properties chitosan particles were prepared by easy and inexpensive methods in this article. The surface of the particles was functionalized with aldehyde groups to facilitate the attachment of affinity ligands.

Aprotinin Attachment

pH, time, temperature, and ionic strength are the important factors for the enzyme immobilization. In this study, the aforesaid conditions were investigated.

Effect of pH and buffer concentration are given in Figure 5.

Figure 5 shows that low buffer concentration is prepared for the aprotinin attachment. As shown in Figure 5, an optimum pH value of 10.5 is very close to the isoionic point of aprotinin (pH 10.4). The effect of pH on the activity of aprotinin was also shown in Figure 5. The activity of immobilized aprotinin was quite high at pH 10.5. Owing to a steric hindrance, the activity of immobilized aprotinin fell.

Aprotinin attachment were investigated at different temperatures (between 20 and 50°C) in Figure 6. Figure 6 shows that temperature has not effect on the amount of total bounded protein if the immobilization times is long enough (20 h).

Figure 7 shows that temperature has an effect on the activity of immobilized aprotinin. According to the date in Figure 7, the activity of immobilized aprotinin is higher at a lower temperature than at a higher temperature.

In this study, the compromise condition of aprotinin attachment is pH 10.5, $5 \times 10^{-3}M$ borax buffer concentration, 25°C and 20 h.

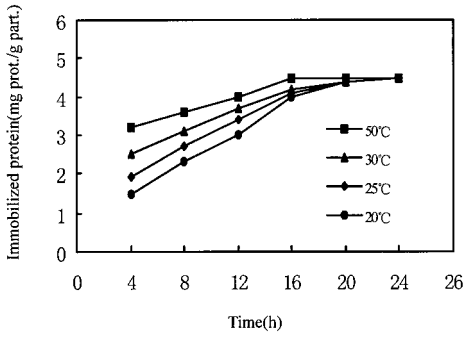

Figure 6 Change of immobilized amount of aprotinin by time (pH = 10.5).

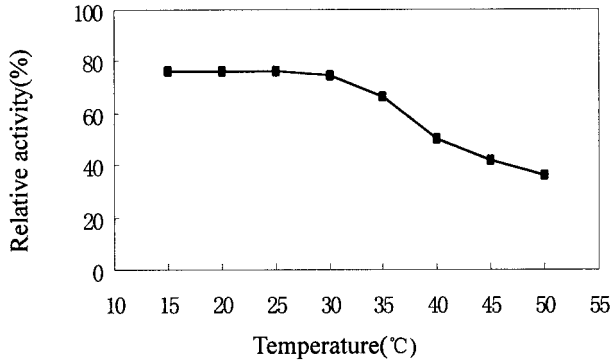

Figure 7 Effect of immobilization temperature on relative activity.

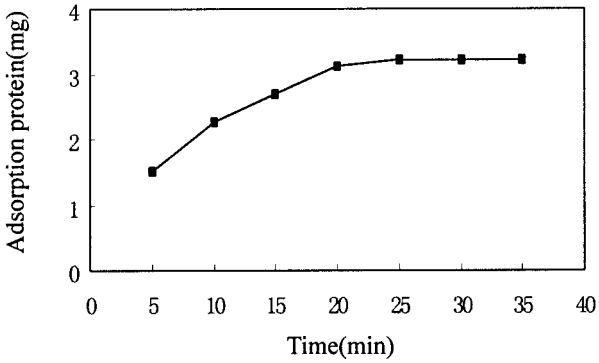

Figure 9 The effect of adsorption time on the amount of affinity adsorption protein.

Trypsin Affinity Purification

pH, ionic strength, wash times, and temperature are the important factors for affinity purification.

Because affinity between the aprotinin and trypsin is topmost at pH = 8.0, and activity of trypsin is stable at 4°C, effects of pH and temperature was omitted on trypsin affinity adsorption.

Figure 8 shows the effect of buffer concentration on the amount of affinity adsorption protein.

According to the date in Figure 8, high ion concentration was not proper for affinity adsorption.

The effects of adsorption time on the amount of affinity adsorption protein are given in Figure 9. Figure 9 shows that an optimum adsorption time is 30 min.

The effect of borax buffer concentration and wash times desorbed amount of nonaffinity adsorption protein was given in Figure 10.

Figure 10 shows that 0.1*M* borax buffer concentration is proper for desorbtion of nonaffinity adsorption protein; however, the nonaffinity adsorption protein was not completely eliminated with 0.1*M* borax buffer concentration in the first wash. The reason is that a few amounts of protein are adsorbed by hydrophobic forces. In this study, we chosen to wash two times to eliminate nonaffinity adsorption protein.

For the sake of the activity of trypsin, the pH of the desorption buff was chosen at pH = 2.7 and the temperature of the desorption was chosen at 4°C.

The effect of the potassium chloride solution concentration and wash times on the desorbed amount of trypsin (20 mL desorption medium is used) is given in Figure 11.

Figure 11 shows that the rate of desorption protein is 95%, when the trypsin was desorbed by 20 mL desorption medium containing 10 m*M* acetic acid and 0.5*M* potassium chloride on one wash time. In this study, we chosen one wash time.

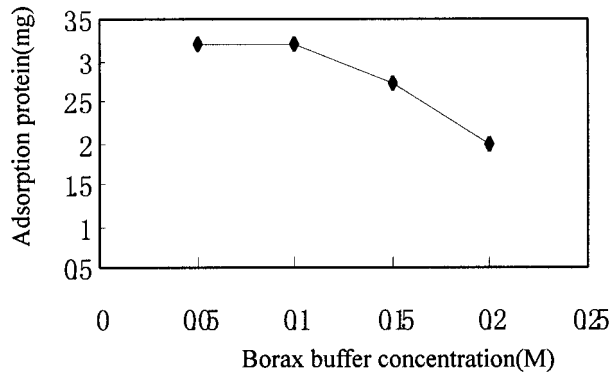

Figure 8 The effect of borax buffer concentration on the amount of affinity adsorption protein.

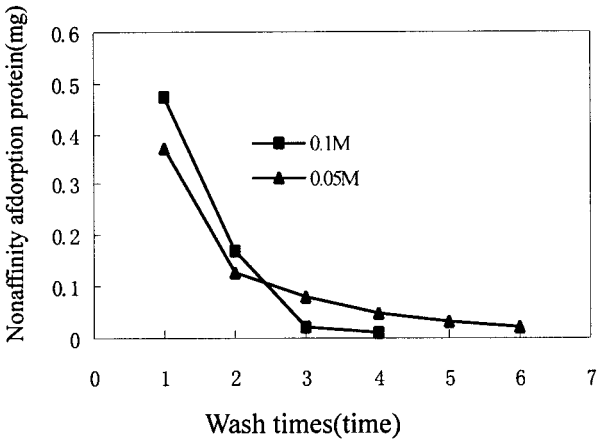

Figure 10 The effect of borax buffer concentration and wash times on desorbed amount of nonaffinity adsorption protein.

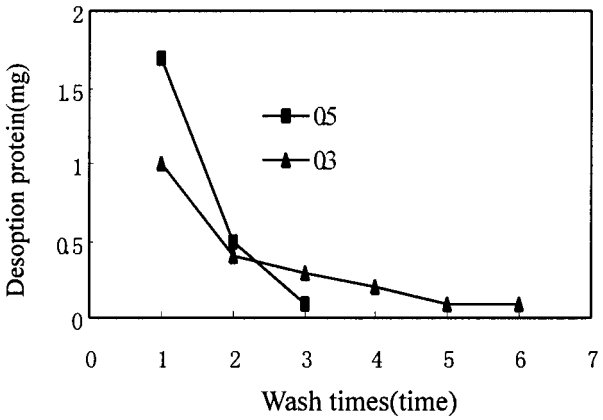

Figure 11 The effect of potassium chloride solution concentration and wash times on desorbed amount of trypsin.

The amount of trypsin purification per gram particle on different initial protein concentrations were studied; the results are given in Table II.

Table II shows that initial protein concentration effects the activity of trypsin, so that 10 mL protein solution (initial concentration: 1.0 mg/mL) is proper for the trypsin affinity purification.

APPLICATION

High magnetic properties magnetic chitosan particles bound aprotinin on the surface; this makes them applied to the affinity purification of trypsin. The results are given in Table III.

The sample contains trypsin (1500 USP trypsin units in each mg) was purified by the magnetic particles; activity of the trypsin has risen 0.73 times, and achieved the standard of China Pharmacopoeia[10] (2500 USP trypsin units in each mg).

Table II Effects of Initial Protein Concentration at Optimum Conditions

Vo (mL)	Initial Protein (mg/mL)	Adsorption Protein (mg)	Desorption Protein (mg)	Activity of Trypsin (USP Trypsin Units in Each mg)
10	0.5	3.0	2.0	2578
10	1.0	3.4	2.4	2598
10	1.5	3.8	2.6	2493
15	1.0	3.4	2.4	2506

Table III The Results of Trypsin Affinity Purification (USP Trypsin Units in Each mg)

Activity of Sample (USP/mg)	Activity of Purification Trypsin (USP/mg)	Rate of Recovery Activity (%)	Purification Time
1500	2598	41.57	1.73

CONCLUSION

One of the important points of this study is that the high magnetic property chitosan particles were used for affinity purification of trypsin. The particles prepared in this study present some advantage. First, preparation of the particles is very easy. Second, the magnetic particles having high magnetic properties can easily and gently are separated by using a magnetic field. Third, the magnetic particles have active groups for the enzyme immobilization without any particle activation process. On the other hand, the method of trypsin affinity purification in this study is easy, inexpensive, highly efficient, and successful.

REFERENCES

1. Tagle, D. A.; Swaroop, M.; Lovett, M.; Collins, F. S. Nature 1993, 361, 751.
2. Deaver, D. R. Nature 1995, 377, 758.
3. Alaifac, M. J.; Jia, M. H. J Invest Dermatol 1992, 99, 237.
4. Nyren, P. J Biolumin Chemilumin 1994, 9, 29.
5. Lubbe, A. S.; Bergemann, C.; Riess, H. Cancer Res 1996, 56, 4686.
6. Uhlen, M. Nature 1989, 340, 733.
7. Benkirane, R. M.; Gaillot, E.; Mouton, C. J Clin Mirobiol 1995, 33, 2908.
8. Mosbach, K.; Kriz, D.; Ansell, R. J PCT WO 97/22366, 1997.
9. Hirschein, B. L. Chem Technol 1982, Pharmacopoeia Committee of People's Republic of China, March, 170.
10. Republic of China. China Pharmacopoeia, Chemistry Industry Press: Beijing, 1995; p. 256, 2nd ed.

11. Cai, W. C.; Yuan, H. J. General Chemical Analysis Methods for Biomolecule; Science Press: Beijing, 1982; p. 98.
12. Pharmacopoeia Committee of People's Republic of China, China Pharmacopoeia, Chemistry Industry Press: Beijing, 1995; pp. 256, 274, 2nd ed.
13. Tanyolac, D.; Ozdural, A. R. React Funct Polym 2000, 45, 235.
14. Mullerschulte, D.; Brunner, H. J Chromtogr A 1995, 711, 53.
15. Horak, D.; Bohacek, J.; Subrt, M. J. Polym Sci Part A Polym Chem 2000, 38, 1161.
16. Wan, M. X.; Fan, J. H. J Polym Sci Part A Polym Chem 1998, 36, 2749.
17. Matutes-Aquino, J.; Rios-Jara, D.; Ayala-Valenzuela, O.; Gallardo, P. S.; De Valle, L. F. R.; Fernandez, O. S. R. Polym Compos 2000, 21, 734.
18. Hwee, P. K.; David, K.; Stephen, D.; Nigel, A. T.; Evgeny, N. Y. Biotechnol Bioeng 1998, 60, 419.
19. Christopher, H.; Christopher, L. U.S. Pat. 4,920,061 (1990).
20. Daniel, J. C.; Schyooiser, J. L.; Tricot, M. U.S. Pat. 4358388 (1982).
21. Manchium, C.; Michael, S. C. U.S. Pat. 4873102 (1989).
22. Davies, M. J.; Smethurst, D. E.; Howard, K. M.; Todd, M.; Higgins, L. M.; Bruce, J. Appl Biochem Biotechnol 1997, 68, 95.
23. Safarik, I.; Safarikova, M.; J Biochem Biophys Methods 1993, 27, 327.
24. Bartl, K.; Wenzig, P.; Kleiber, J. Clin Chem Lab Med 1998, 36, 557.