УДК 541.64; 541.183; 541.184; 678.0

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СИНТЕЗ ПОЛІМЕРНИХ МІКРОКАПСУЛ З ІНКАПСУЛЬОВАНИМИ МАГНІТНИМИ НАНОЧАСТИНКАМИ ДЛЯ ІМОБІЛІЗАЦІЇ ФЕРМЕНТУ α-АМІЛАЗИ

Полімерні мікрокапсули з ядром на основі парафіну, які містять капсульовані наночастинки магнетиту, модифіковані олеїновою кислотою та функціоналізованою полімерною оболонкою були отримані методом "екстракційно-коацерваційного" мікроінкапсулювання. Досліджено вплив параметрів процесу на колоїдно-хімічні властивості (розмір, коефіцієнт полідисперсності, пористість поверхні) синтезованих мікрокапсул. Показано, що використання гетерофункціонального кополімеру як оболонки мікрокапсул забезпечує можливість незворотної іммобілізації ферменту а**-амілази та підтверджена її участь у реакції каталітичного розщеплення крохмалю.**

Ключові слова: гетерофункціональний кополімер, магнітні наночастинки, інкапсулювання, мікрокапсули, іммобілізація ферментів.

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SYNTHESIS OF POLYMER MICROCAPSULES WITH ENCAPSULATED MAGNETIC NANOPARTICLES FOR α-AMYLASE ENZYME IMMOBILIZATION

Polymeric microcapsules with the core based on paraffin containing magnetite nanoparticles modified by oleic acid and functional polymeric shell were synthesized using the technique of "extraction-coacervation" microencapsulation. The influence of process parameters onto colloidal chemical properties (size, polydispersity index, surface porosity) of synthesized microcapsules was studied. It was shown that the use of heterofunctional copolymer as microcapsule shell allows the possibility of irreversible immobilization of a**-amylase enzyme and proved its participation in the reaction of starch catalytic decomposition.**

Key words: heterofunctional copolymer, magnetic nanoparticles, encapsulation, microcapsules, enzyme immobilization.

Problem definition. In past decade, magnetic nanoparticles (MN) with definite size distribution and structure are of a wide attention due their unique physical and chemical properties [1]. They can be used in a wide range of industry branches such as biomolecular separation, chemical sensors, energy storage, catalysis, microwave absorption, biomedicine or biotechnology etc [2-4]. Their use as the carriers of diverse bioactive moieties is of special interest due to their unique properties such as superparamagnetism, high surface area, large surface-to-volume ratio, easy separation under external magnetic fields [5]. Compared to porous carriers, such non-porous nanoparticles have no external diffusion problems, making them more competitive especially for large scale industrial usage in solid–liquid systems (e.g., precipitated

protein) [6]. Among all magnetic materials, magnetite is the most suitable and widely used because they are characterized by low toxicity, good biocompatibility. Various modification methods have been developed to get the biocompatible iron magnetic nanoparticles for protein immobilization [7]. But bare iron magnetic nanoparticles often have high reactivity and easily undergo degradation upon direct exposing to certain environment, leading to poor stability. Hence, the elaboration of the method of obtaining polymeric microcapsules with functional shell and encapsulated MN is the important scientific and practical problem.

State of the art. Fe_3O_4 nanoparticles can be prepared by various physical, chemical and even biological methods, including co-precipitation of ions Fe^{2+} and Fe^{3+}, thermal decomposition and/or hydrothermal decomposition methods, microbial methods [8]. Co-precipitation method has some advantages because the reaction can be performed in a mild conditions using water as a solvent.

Unmodified iron magnetic nanoparticles are unstable in acidic solutions and undergo leaching, that strongly limits the reusability and reduces the lifetime of such materials. Besides, iron oxide nanoparticles have sufficiently large surface to volume ratio, that causes a high surface energy, and in order to minimize surface energy MN are prone to agglomeration. Therefore, the proper passivation of particle surface and the development of other effective methods of protection to preserve the stability of iron oxide NP is a very important issue. These methods include grafting or coating diverse organic molecules such as organic molecules or surfactants, polymers and biomolecules. Oleic acid (OLK) is a surfactant that is widely used to stabilize the magnetic nanoparticles due to the formation of strong chemical bonds between the carboxylic acid and amorphous nanoparticles of iron oxide [9]. It has been proved experimentally that in many cases the protective shells not only stabilize iron oxide nanoparticles, but can also provide their colloidal stability during the process of encapsulation.

However, modifying MN is not enough to produce composite materials. At the moment, there are two main methods of obtaining composite particles that have superparamagnetic properties. The first method involves deposition of MN onto the surface of previously synthesized non-magnetic beads; and the second is based on the encapsulation of MN inside non-magnetic matrix. Both approaches of synthesis have been intensively studied and some of developed products have been used in commercial scale production.

In the case of deposition method MN can be directly deposited from colloidal suspensions [10] or obtained in *situ* from precursors [11]. While both technological methods guarantee obtaining the final product of uniform size, MN content is relatively low and often needs further modification of the product.

Encapsulation method often involves emulsion polymerization in the presence of an aqueous dispersion of MN coated with surfactant [12]. Monomers in oleo-phase mixed with an aqueous phase containing the polar solvent and MN; after which the mixture polymerized at elevated temperature. However, to achieve a uniform encapsulation of MN in each polymer capsule is a difficult task, besides weight ratio of MN in microcapsules usually does not exceed 10 %.

Usually, MN preparation for use as biocatalytic systems with magnetic release includes three stages. The first stage is preparing MN of the required size and modification of their surface by surfactant. The second one is the encapsulation of MN using the corresponding polymer with reactive functional groups. And the last, enzyme immobilization onto the microcapsule (MC) functionalized shell with the use of functional groups such as anhydride, which is capable to bind the “target” active agent – enzyme for biochemical applications.

The aim of this work is to study the processes of obtaining and properties of functionalized polymer microcapsules with paraffin core filled with MN for immobilization of α-amylase enzymes.

Experimental part. *Materials: ferric and ferrous* crystalline salts of $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$; aqueous ammonia (25%); oleic acid (OA); ethanol (EA); Polyvinyl alcohol (PVA) POVAL JP-18; Ethyl acetate (EA) grade "ch"; pharmaceutical paraffin; distilled water, alpha amylase from Sigma-Aldrich, phosphate saline buffer solution, pH=7.

Heterofunctional tetrapolymer (HFP) – copolymer of acrylonitrile (AN), butyl methacrylate (BMA), styrene (ST) and maleic anhydride (MA), the composition and properties of HFP are presented in Table 1,

IR-spectrum of synthesized HFP is presented in Fig. 1. Characteristic adsorption bands prove the presence of corresponding functional groups in HFP structure [15]: 2240 cm^{-1} – valent vibration $\nu_{C\equiv N}$ of AN nitrile group; 1856 and 1780 cm^{-1} – $\nu_{C=O}$ vibrations of MA anhydride groups; 1728 cm^{-1} – $\nu_{C=O}$ vibrations and 1220 cm^{-1} – ν_{C-O} vibrations of ester group in BMA; 3030 cm^{-1} – ν_{C-H} vibrations, 1600, 1580, 1460 cm^{-1} – skeletal vibrations of aromatic C–C bonds; 760 and 700 cm^{-1} – δ_{C-H} deformation vibrations of St phenyl groups.

Table 1

Composition and properties of HFP

Copolymer composition, % mole				[η] in acetone, $dl \cdot g^{-1}$	M_n	M_w	Polydispersity index (M_w/ M_n)	ρ, kg/m^3	T_g, K
AN	BMA	St	MA						
50,2	27,9	14,3	7,6	0,31	28602	53013	1,85	1094±3	356±1

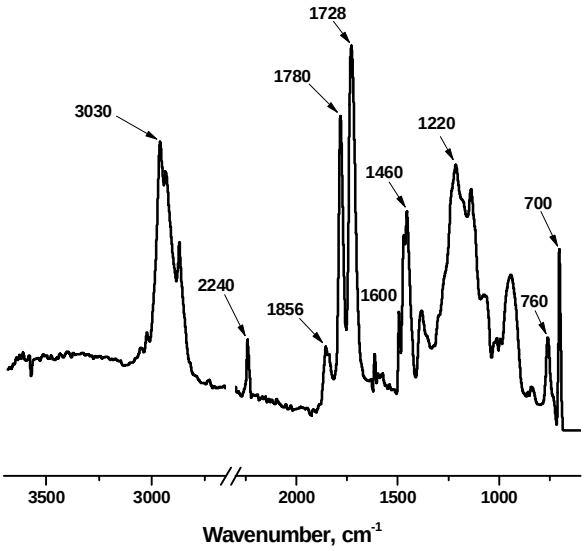


Fig. 1. IR-spectrum of synthesized HFP

The method of synthesis of magnetite nanoparticles. The synthesis of Fe_3O_4 nanoparticles modified with oleic acid was carried out using modified co-precipitation technique described in [13]. The difference of our modified method from the original technique was that in this case was used crystalline $FeSO_4 \cdot 7H_2O$, and the final product synthesis was prepared as a suspension in EA.

The technique of MN microencapsulation. In this work we applied "extraction-coacervation" method of MN encapsulation described in [14].

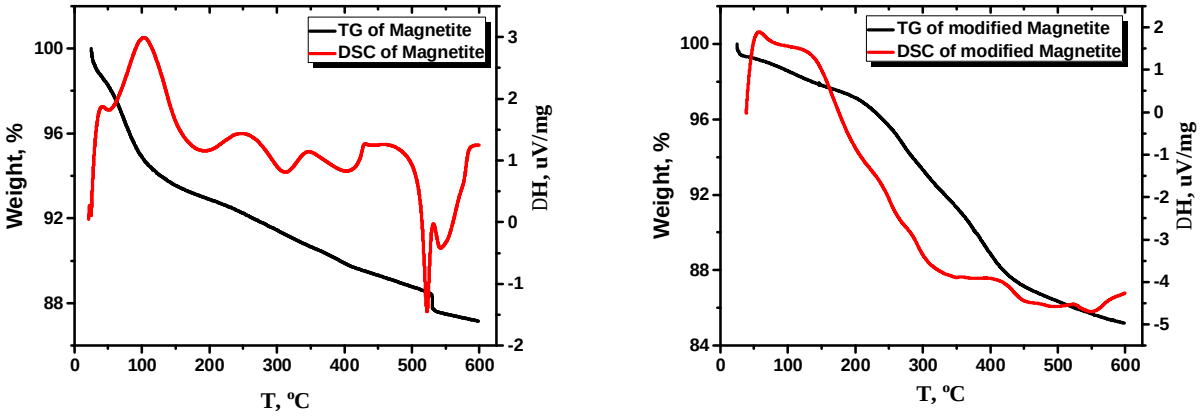
The technique of immobilization of α-amylase onto MC surface. In order to carry out the immobilization of α-amylase onto MC surface we prepared phosphate saline buffer solution (pH=6). This solution (100 ml) with 500 mg of α-amylase enzyme previously solved in it was added to the synthesized MC. The glass with obtained suspension was placed onto water bath with stirring during 3 hours. After that the enzyme solution was separated from MC containing Fe_3O_4 NP via magnetic separation. Modified MC were washed three times by 0.01 % solution of Tween 20 in water.

Methods of analysis of modified MN and MC with encapsulated MN.

The composition of synthesized HFP was proved by *IR-spectroscopy* of studied samples in the range of 3800–600 cm^{-1} using “Jasco FTIR 41000” device. *Thermogravimetric analysis* was carried out using TG

209 F1/Iris device at dynamic mode with heating rate of 10 °C/min in air atmosphere and *differential-scanning calorimetry* was performed using DSC 204 F1 Phoenix in temperature range from 25 °C to 500 °C with heating rate of 10 °C/min. Aluminium oxide was the etalon in both cases. *Optical microscopic studies* were performed "JENAVAL" Carl Zeiss microscope. Using the results of statistical treatment of the size measurements of 400-500 particles differential curves of MC size distribution were built and number-average (d_n), weight-average (d_w) sizes as well as polydispersity indexes (k_p) of synthesized MC were calculated. *Electron microscopic studies* were performed using Selmi REM-106I scanning electron microscope (SEM).

Results and discussion. ***Synthesis and modification of MN.*** As described in experimental part, at the first stage we have synthesized magnetic nanoparticles with simultaneous their modification by oleic acid. The curves of thermogravimetric analysis and differential-scanning calorimetry of synthesized Fe_3O_4 NP are presented in Fig. 2. For naked magnetite nanoparticles we observed sharp weight decrease in the range from 25 °C to 100 °C that was not observed for modified MN. This is evidently caused due to the elimination of adsorbed water because MN modified by oleic acid have hydrophobic surface and do not adsorb water molecules. Besides, for the sample of modified MN the sharp weight decrease is observed in the range 250–400 °C (close to the boiling point for oleic acid) that can be explained by elimination of unbonded oleic acid.



a *b*

Fig. 2. The curves of thermogravimetric analysis and differential-scanning calorimetry of magnetite samples: unmodified (a) and modified by oleic acid (b)

MN microencapsulation. The next step of the work was the MN encapsulation in microcapsules with paraffin core and functional polymer shell and the study of dependences of colloidal-chemical properties of synthesized MC, namely of number-average, weight-average sizes as well as polydispersity indexes on the process parameters (Table 2).

Table 2

MC formation conditions and characteristics
(MN to paraffin ratio is 1:22 weight parts, dispergation rate – 400 rpm)

№ Sample	PVA content in stabilizing solution, %	T, K	MC number-average size (d_n), μm	MC weight-average size (d_w), μm	k_p
MC1	1,0	328	47,9	78, 1	1,63
MC2	2,0	328	37,5	69,6	1,86
MC3	3,0	328	28,3	63,5	2,24

According to obtained data (Table 2) one can conclude that the increase of PVA concentration in stabilizing solution causes the decrease of number-average and weight-average size of MCs. At the same time the value of polydispersity index increases.

One can see (Fig. 3), that as a result of encapsulation of magnetic nanoparticles of magnetite microcapsules with regular spherical shape were obtained and agglomeration of particles was not observed.

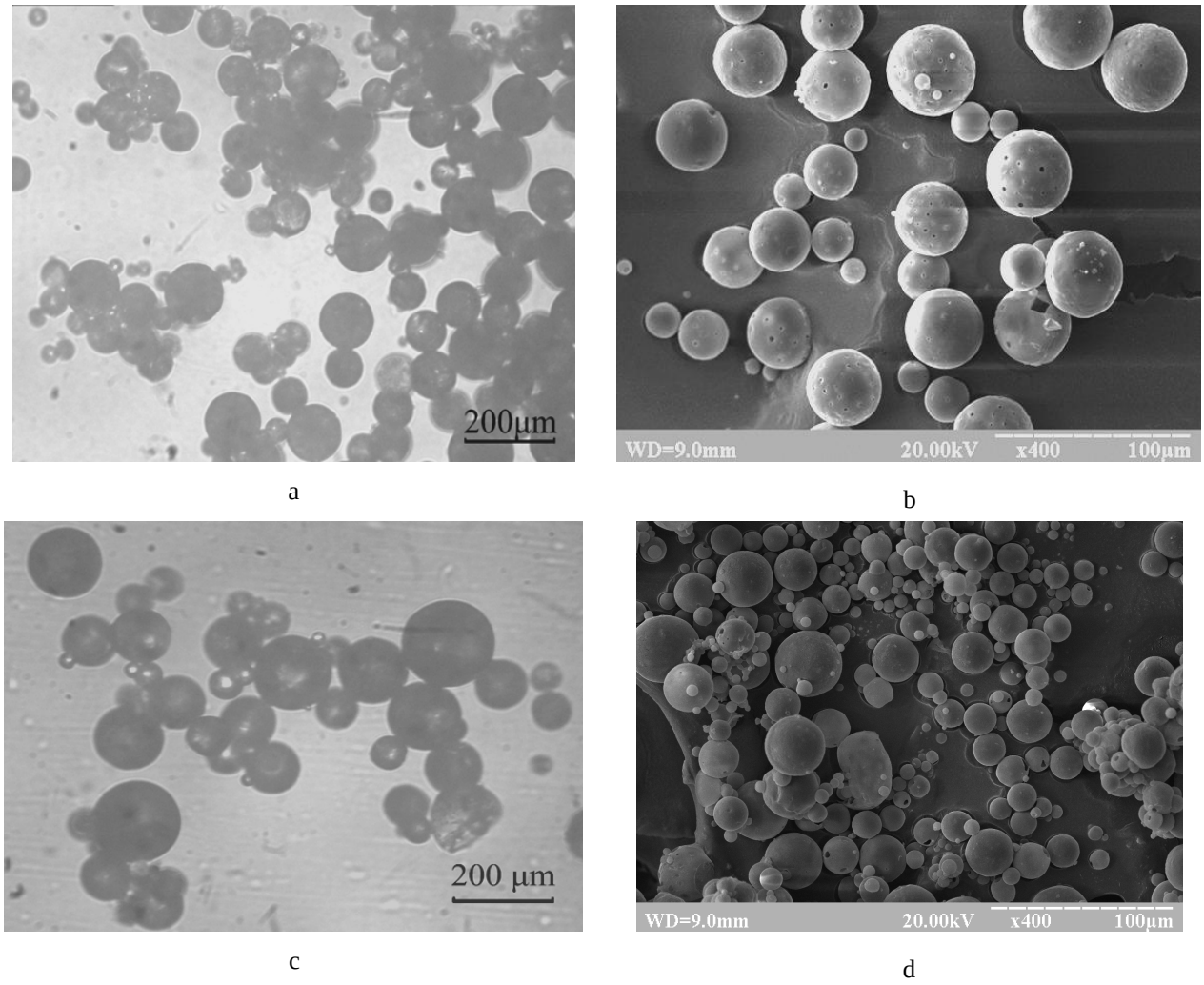


Fig 3. Microcapsules with encapsulated MN: MC 1 (a, b), MC 3 (c, d). Optical microscopy (a, c), scanning electron microscopy (b, d)

As one can see the porosity of the surface of microcapsules obtained with 3% of PVA in stabilizing solution is less as compare with microcapsules synthesized at the PVA concentration of 1%. It is caused by the fact that greater concentration of PVA leads to the increase of solution viscosity. As a result, during the stage of water addition extraction of ethyl acetate into water phase proceeds slower and the microcapsule surface is formed smoother with less amount of pores.

Fig. 4 presents the IR-spectrum of microcapsules formed at different PVA concentration. This spectrum is almost identical to the spectrum of heterofunctional copolymer (Fig. 1) that witnesses that the shell of MC consists of HFP and its composition does not depend on MC formation conditions. Besides, the absence of the adsorption band in the range of 3400 cm^{-1}, that corresponds to stretching vibrations of alcohol hydroxyls witness about PVA molecule absence at the MC surface.

It is necessary to note that obtained MCs are rather stable even in acidic medium. After their deposition into 1.5% solution of HCl during one month they keep magnetic properties, that witnesses in favor of dense encapsulation of modified magnetic nanoparticles in the paraffin core.

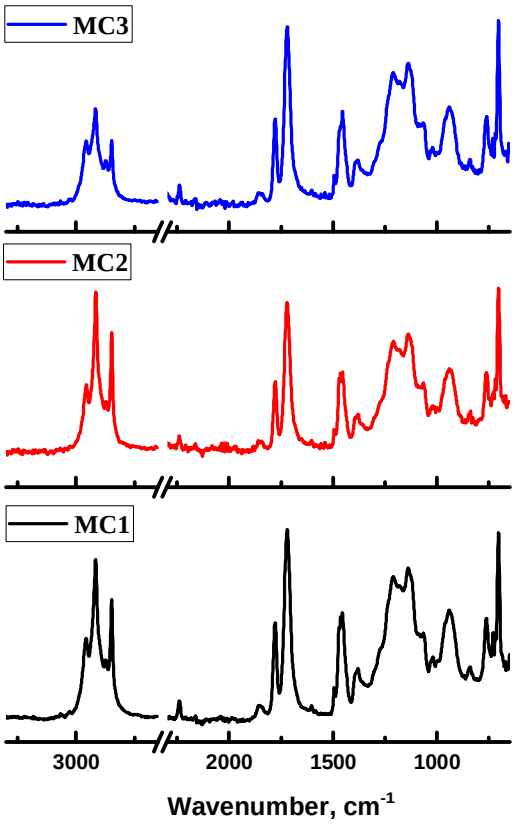


Fig. 4. IR-spectra of synthesized MC (see Table 2)

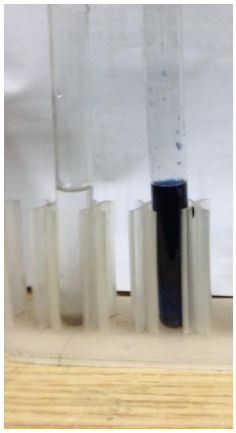


Fig. 5. The vials with MC containing immobilized α-amylase (left) and without MC (right)

The next step of the work was the immobilization of α-amylase enzyme onto MC surface using the technique described above. To prove α-amylase immobilization the obtained MC were placed into vial. 5% starch solution (1 ml) and phosphate buffer solution (5ml) were added to the vial. To another vial the same solutions were added but without MC. These vials were deposited into thermostat at 40°C during 20 hours. After that 1 drop of iodine were added to the each vial. In the vial that did not contain MC the appearance of intensive color was observed while the solution in the vial containing MC remained colorless (Fig. 5). The test was repeated a few times. Obtained result proves the catalytic action of immobilized α-amylase in the reaction of starch decomposition.

Conclusions. The results of performed studies witness that the method proposed allows to obtain microcapsules with paraffin core containing magnetic nanoparticles and functionalized polymeric shell. The influence of process parameters onto colloidal-chemical properties of synthesized microcapsules has been studied. It was shown that MC size and polymeric shell porosity decreases with the increase of polyvinyl alcohol concentration in the stabilizing solution. IR-spectra of obtained MCs prove that heterofunctional copolymer forms their shell while PVA does not participate in shell formation. The presence of functional groups in MC shell structure allows to irreversibly immobilized enzymes that can be used as biocatalysts for industrial application.

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