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CONSTRUCTION OF MAGNETOCARRIED NANOCOMPOSITES FOR MEDICO-BIOLOGICAL APPLICATIONS

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The research work deals with studies on interactions of nanomaterials with components of biosystems, development of new medicines based on magnetite, their application efficiency, chemical engineering of multilevel magnetosensitive nanocomposites with a hierarchical architecture and functions of biomedical nanorobots.

INTRODUCTION

Technology in the twenty first century requires the miniaturization of devices into nanometer sizes while their ultimate performance is dramatically enhanced. This raises many issues regarding to new materials for achieving specific functionality and selectivity [1]. Nanophase and nanostructured materials, a new branch of materials research are attracting a great deal of attention because of their potential applications in such areas as electronics, optics, catalysis, ceramics, magnetic data storage, and nanocomposites. The unique properties and the improved performances of nanomaterials are determined by their sizes, surface structures, and interparticle interactions. The role played by particle size is comparable, in some cases, to the particle chemical composition adding another flexible parameter for designing and controlling their behavior. Modern nanotechnologies provide tools for creation of unique agents for medicine and biology. Their practical usage is mostly based on the knowledge about interaction of nanomaterials with the components of biological environment.

Among the large amount of known materials, oxides are of a great interest for scientists. This class of compounds possesses a wide variety of properties while accumulated knowledge is used for their further optimization with respect to certain applications. This work highlights synthesis of highly-dispersive magnetic nanosized oxides for various functional purposes and, in particular, for cancer therapy.

Magnetic oxides, magnetite in particular, which possess high biocompatibility are of a great interest for medico-biological usage [2]. The purpose of our work concerns creation of the polyfunctional nanocomposite via usage of magnetite in a nanosized state as a reactive component for targeted design of multilevel nanocomposites with hierarchical architecture and functions of nanorobots which include recognition of specific microbiological objects in biological environment, targeted delivery and deposition of medicinal products into organs or cells, diagnostics and therapy of diseases at the cell level, adsorption of cell decomposition products after application of chemotherapeutic agents or hyperthermia, their removal from the organism using magnetic field. The application of polyfunctional nanocomposites of combined action, which contain monoclonal antibodies and highly efficient cytostatic compounds, in oncology may be accompanied by a synergetic effect of chemo- and immunotherapeutic drugs and result in decreased toxico-allergic response of the organism.

For any practical application, the fabrication of nanoparticles needs to be controlled in such a way that resulting nanoparticles have the following characteristics: (i) identical size of all particles, (ii) identical shape or morphology, (iii) identical chemical composition and crystal structure desired among different particles and within individual particles, such as core and surface composition must be the same, and (iv) individually dispersed or monodispersed, i.e. no agglomeration. If agglomeration does occur, nanoparticles should be readily redispersible [3–5].

In view of the increasing interest in magnetic nanoparticles in the field of medical care as described above, a facile synthetic process which allows to control the size, magnetic properties,

and surface properties of Fe_3O_4 nanoparticles, needs to be developed because those properties required for the Fe_3O_4 nanoparticles depend on the specific application. For example, the size of Fe_3O_4 nanoparticles needs to be controlled for target materials such as proteins, genes, and cells whose sizes are 5–50 nm, 2 nm wide and 10–100 nm long, and 10–100 μm, respectively. Furthermore, it is possible that particle size plays an important role in determining the state of aggregation of the nanoparticles and even the uptake property to living organisms. Additionally, magnetic nanoparticles synthesized by the facile method are expected to have surface properties which are easy to be characterized or modified with biomolecules for biomedical applications [6].

Magnetic nanoparticles are attractive for their unique properties, such as single domain structure and superparamagnetism, which are not observed for bulk materials, and are expected to be applicable in various fields including ultrahigh-density magnetic recording media, drug delivery systems, and medical imaging. Important for these applications are the techniques for stabilizing the nanoparticles in the solvent and those for functionalizing the nanoparticles surface by surface modifications. Especially for the bioapplications of magnetic nanoparticles, the surface modification with organic molecules or surfactants is essential for inhibiting the aggregation of nanoparticles and for controlling interparticle interactions and solubility in an aqueous solution. In addition, surface modification by biologically relevant substances improves the biocompatibility which enables the bioapplications of magnetic nanoparticles. Thus, surface modification of magnetic nanoparticles has become one of the most challenging issues recently.

The cell-based therapy is one of advanced treatments applied to various fields of scientific study, and it includes immunotherapy, gene therapy, and regenerative medical treatment. Recently, immunotherapy has been vigorously investigated in the field of oncology because of specific anti-tumor activity and less side-effect. Cancer treatment by immunotherapy takes advantage of having efficient effector cells which exhibit specific killing activity against cancer cells. However, local accumulation inside the tumor of these effector cells remains one of critical issues to be overcome from a point of the clinical view. Therefore, drug delivery system is a good candidate to achieve the efficient localization of drugs in the tumor. Actually, successful local delivery of drugs is reported using magnetic nanoparticles such as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) as a nanocarrier [7]. Thus, the application of magnetic drug delivery system to immunotherapy could make the development of magnetically mediated immunotherapy possible in the clinical field.

SYNTHESIS AND PROPERTIES OF MAGNETITE

Highly disperse magnetite was prepared via co-precipitation of salts [7, 8] in accord to the reaction

$$Fe^{+2}+2Fe^{+3}+8NH_4OH \rightarrow \rightarrow Fe_3O_4+4H_2O+8NH_4^{+}. \quad (1)$$

Fractionation of the prepared magnetite was carried out with magnetic field.

The method [7, 8] permits obtaining magnetite with broad size distribution of the particles (from microns to nanometres) which requires additional fractionation.

In order to achieve a better control over size distribution, we developed a cryochemical method of heterogeneous synthesis of magnetite at interface of solid (frozen iron II and III salts solution) and liquid (ammonia solution of a certain concentration) phases [3]. The ammonia solution is taken in excess while the second phase melts and releases the solution which has predetermined concentrations of the reactive components. A permanent concentration gradient is maintained at the thin interface upon melting the iron-containing solid. On the contrary to the homogeneous synthesis, growth of the nanoparticles is terminated at a certain distance from the solid phase due to absence of the iron salts. This prevents the further growth of the formed nanoparticles and preserves their initial size. The nanoparticles are collected with non-uniform magnetic field and the supernatant solution is removed. The precipitate is washed many times with water in order to dispose off the anions present in the solution.

Samples of the nanocrystalline magnetite with specific surface area of ~ 90–180 m^2/g (measured by thermal desorption of Ar) were prepared using the cryochemical method. Depending on the synthetic conditions, the

particles size comprised 6–50 nm revealing a quite narrow distribution interval. The fraction of the mostly monodomain magnetite particles of 20–50 nm in diameter was used for preparation of the magnetic carriers.

The advantages of the proposed method comprise the increased yield of the monodomain magnetite particles directly from the synthesis and their narrower size distribution.

COATING OF MAGNETITE SURFACE WITH POLYACRYLAMIDE

Stabilization and biocompatibilization of the nanosized magnetite particles were achieved via coating their surface with cross-linked poly-acrylamide (PAA). The PAA layer was prepared via co-polymerization of acryl amide and N,N'-methylene-*bis*-acryl amide in high-frequency (HF) discharge plasma at the radiator power 20 W [9–11]. The monomer and the cross-linker were coated onto the magnetite surface in a rotor evaporator at 303 K. Plasma polymerization was carried out in glowing discharge at $1 \cdot 10^{-3}$ Pa. Conversion degree of carbon-carbon double bonds was measured by the method of Kaufmann based on titration in a non-aqueous solution and the ability of the C=C bonds to combine with Br_2 [12]. The obtained samples were studied in the wavelength range 400–4000 $см^{-1}$ with a FTIR "Perkin Elmer" spectrometer (the model 1720X) [13–15]. The spectra revealed absorption bands related to the initial magnetite and the PAA coating [16].

We studied magnetic properties of the magnetite particles modified with PAA and the impact of the coating thickness. The coating weight was varied from 5 to 50% of the total weight of the composite. The specific magnetization σ_i and its function $\sigma_i = f(H)$ were calculated from the experimental data. From them, the ultimate magnetization at saturation condition σ_s, the remnant magnetization σ_r, and the coercive force H_c were determined [17].

The experimental functions of the specific magnetization on magnetic field strength for the bare magnetite and the magnetite with various PAA coating contents showed that the coating fraction up to 15 wt. % does not cause notable deviations from the initial magnetite magnetic properties. The values σ_s, H_c are located in the ranges from $61.5\ 10^{-7}$ to $62.0 \cdot 10^{-7}$ $T \cdot m^3/kg$ and from 7.20 to 3.44 kA/m, respectively, while σ_r is equal to $15.12 \cdot 10^{-7}$ $T \cdot m^3/kg$. Increase in the coating weight up to 50% leads to the lower σ_s, H_c, σ_r values: down to $51.1 \cdot 10^{-7}$ $T \cdot m^3/kg$, 6.31 kA/m, and $10.74 \cdot 10^{-7}$ $T \cdot m^3/kg$, respectively. These results show that the increase in the weight fraction of the PAA layer up to 50% makes a negligible contribution to the specific magnetic properties.

MODIFICATION OF MAGNETITE WITH γ-AMINOPROPYLSILOXANE

Surface of magnetite nanoparticles was coated with γ-aminopropylsiloxane (γ-APS) in toluene [18]. The reaction of polycondensation was carried out in accord to the scheme

$$–OH+(C_2H_5O)_3Si(CH_2)_3NH_2 \rightarrow$$

$$\rightarrow –O–Si(CH_2)_3NH_2+3C_2H_5OH\,. \qquad (2)$$

γ-Aminopropyltriethoxysilane (γ-APTES) was dried over molecular sieves and purified with distillation in vacuum. Magnetite was exposed to a solution of γ-APTES (10% vol.) in toluene for 8 h, precipitated in a centrifuge, washed with toluene, acetone, and dried at 293 K.

The content of functional groups on the surface of magnetite was found with X-ray Photoelectron Spectroscopy (XPS) and Differential Scanning Calorimetry (DSC) combined with Differential Thermo-gravimetric Analysis (DTA). The thermal graphs were recorded in the temperature range 293–1273 K at the heating rate 0.16 deg/s on a Q-1500D thermal analyzer purchased from the company MOM (Hungary). Concentration of the –OH groups at the surface of the magnetite nanoparticles calculated from the DTA data was equal to 2.2 mmol/g or 2.4 μmol/m^2 at $S_{specific}$ = 90 m^2/g [19].

Presence of the amino-groups at the surface of the obtained nanocomposite was confirmed with XPS [20]. The XPS spectra were recorded on a spectrometer EC-2402 with an analyzer "PHOIBOS-100" SPECS using the Kα radiation of a Mg anode (EMgKα = 1253.6 eV). The spectrometer was calibrated using the line Au $4f_{7/2}$ which has the binding energy E_b = 84 eV.

Fourier Transfrom Infrared spectra were recorded on a spectrometer "Perkin Elmer" (the model 1720X) in the range 400–4000 cm^{-1}. Pronounced absorption bands at 1037 and 1130 cm^{-1} of approximately equal intensities indicates formation of a polymer layer Si–O–Si at the magnetite surface resulted from hydrolytic polycondensation of the modifier molecules [19].

SURFACE-IMMOBILIZATION OF HUMAN IMMUNOGLOBULIN

We developed procedures for immobilization of antibodies at surfaces of the magnetite-based nanocomposites coated with polyacrylamide (PAA) [16] and polysiloxane (γ-APS) [19] using a model preparation of human normal immunoglobulin (Ig) produced by Biofarma, Kyiv. The Ig was purified via dialysis in order to remove low molecular weight preserving compounds.

We measured isotherms of physical adsorption of Ig to the surfaces of the nanocomposites. Samples of the nanocomposites (100 mg) were introduced into Ig solutions (5 ml) of varying concentration. Adsorption of Ig was carried out in physiological conditions during 2 h upon shaking at ambient temperature. The amount adsorbed at the surfaces of the nanocomposites was determined from the difference between the Ig concentrations before and after adsorption. The concentrations were measured on a Lambda 35 uv/vis Spectrometer supplied by Perkin Elmer Instruments at the wavelength λ = 280 nm using a calibration curve. The results are presented in Tables 1, 2 and Fig. 1 *a, b*.

Table 1. Isotherm of non-specific adsorption of human normal immunoglobulin to the nanocomposite Fe_3O_4/ PAA

C_0, mg/ml	D(280 nm)	$C_{eq.}$, mg/ml	$A_{phys.}$, mg/g
0.35	0.485	0.33	0.84
0.45	0.629	0.43	0.90
0.55	0.776	0.53	0.85
0.71	0.966	0.66	2.32
0.86	1.153	0.79	3.40
1.40	1.762	1.21	9.48

Table 2. Isotherm of non-specific adsorption of human normal immunoglobulin to the nanocomposite Fe_3O_4/ γ-APS

C_0, mg/ml	D(280 nm)	$C_{eq.}$, mg/ml	A_{phys}, mg/g
0.15	0.249	0.18	0.00
0.25	0.305	0.22	0.00
0.45	0.607	0.44	0.64
0.55	0.745	0.54	0.66
0.71	0.964	0.69	0.80
0.86	1.178	0.85	0.57
1.00	1.361	0.98	0.97
1.40	1.911	1.38	1.18

We recorded isotherms of covalent binding of Ig to the surfaces of the nanocomposites. The chosen mechanism of the covalent binding comprises reaction of aldehyde groups created upon periodate oxidation of Ig side carbohydrate chains with amino-groups located at the grafting surfaces leading to formation of Schiff-bases (imines). An advantage of this mechanism is oriented immobilization of the antibody molecules with the Fc fragment (fragment crystallisable) facing the surface and the Fab fragment (fragment antigen binding) sticking out [21, 22].

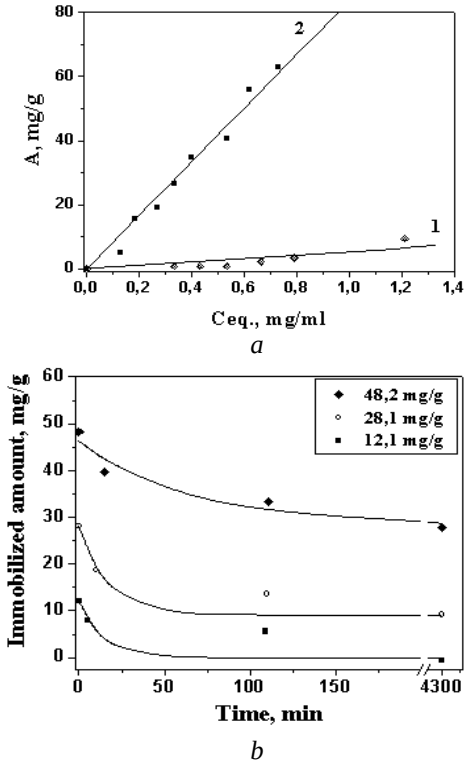

Fig. 1. Comparison of isotherms of physical adsorption of human normal Ig (*1*) and the covalent binding of the oxidized human Ig (*2*) to the nanocomposites: *a* – Fe_3O_4/PAA; *b* – Fe_3O_4/γ-APS

The nanocomposites consisting of magnetite nanoparticles coated with PAA were activated by ethylenediamine (ED) in order to form amino-groups at the surface in accord to the reaction scheme [23]

$$\begin{array}{l} \text{–PAA–CO–NH}_2\text{+H}_2\text{N–(CH}_2\text{)}_2\text{–NH}_2 \rightarrow \\ \qquad\qquad \big| \text{–PAA–CO–NH–(CH}_2\text{)}_2\text{–NH}_2. \end{array} \quad (3)$$

Since the surface of the nanocomposite Fe_3O_4 / γ-APS contains native amino-groups, no additional treatment was carried out.

Oxidation of human normal immunoglobulin was carried out in 0.1 M $NaIO_4$ solution prepared on the basis of aqueous 0.02 M acetate buffer (pH 5.0) in accord to the reaction scheme

$$Ig–gly + NaIO_4 \rightarrow Ig–COH + NaIO_3. \quad (4)$$

The oxidized Ig was purified with dialysis against 2 l of 0.02 M acetate buffer (pH 5.0). The solution obtained after the dialysis was set to pH 8–9 with 0.06 M carbonate-hydrocarbonate buffer based on 0.15 M NaCl (pH 9.5).

Covalent binding of Ig to the nanocomposites Fe_3O_4/PAA activated by ED and Fe_3O_4/γ-APS (30 mg) was carried out from 7 ml solution of 0.06 M carbonate-hydrocarbonate buffer (pH 9.0) and 0.15 M NaCl during 2 h at the ambient temperature upon shaking in accord to the reaction scheme

$$carrier–R–NH_2–Ig \rightarrow$$

$$\rightarrow carrier–R–N=CH–Ig+H_2OSchiffbase. \quad (5)$$

The bound Ig amount was determined from the difference between the initial and the final concentrations of the contact solution. The concentrations were measured by UV absorption at λ = 280 nm using a calibration curve. The data on isotherms of the covalent binding are shown in Tables 3, 4 and Fig. 1 *a, b*.

Table 3. Isotherm of the covalent binding of oxidized human normal immunoglobulin to the nanocomposite Fe_3O_4/PAA

C_0, mg/ml	D(280 nm)	$C_{eq.}$, mg/ml	A(Ig/PAA), mg/g
0.15	0.201	0.128	5.07
0.25	0.288	0.184	15.52
0.35	0.420	0.268	19.14
0.45	0.528	0.337	26.48
0.55	0.630	0.402	34.59
0.71	0.841	0.536	40.57
0.86	0.974	0.621	55.79
1.00	1.144	0.730	63.04
1.40	1.416	0.903	116.00

The obtained isotherms of non-specific adsorption (Tables 1, 2) and the covalent binding (Tables 3, 4) of Ig to the nanocomposites are linear functions with no saturation in the studied concentration range (Fig. 1 *a, b*). The experimental data were converted to the analytical form using the equation $y = E \cdot x$, and the respective distribution coefficients *E* (ml/g) were calculated. The coefficients reflect the Ig distribution between the nanocomposites surfaces and the contacting solutions. The coefficients (*E*) and the Ig amounts immobilized at the nanocomposites' surfaces at the concentration of the Ig initial solution 1.4 mg/ml (the maximal in the experiment) are summarized in Table 5. The coefficients for the covalent binding exceed the respective coefficients for non-specific adsorption by more than an order and reflect equilibrium shift towards surface immobilization of Ig.

Table 4. Isotherm of covalent binding of oxidized human normal immunoglobulin to the nanocomposite Fe_3O_4/ γ-APS

C_0, mg/ml	D(280 nm)	$C_{eq.}$, mg/ml	A(Ig/PAA), mg/g
0.15	0.282	0.140	2.42
0.25	0.352	0.224	6.14
0.35	0.468	0.298	12.07
0.45	0.594	0.379	16.67
0.55	0.723	0.461	20.70
0.71	0.924	0.590	28.08
0.86	1.025	0.653	48.19
1.00	1.207	0.770	53.71
1.40	1.742	1.111	67.41

Table 5. Values of human Ig adsorption to the nanocomposites surfaces of different nature at the concentration of the initial Ig solution C = 1.4 mg/ml

Nanocomposite	$A_{phys.}$, mg/g	$E_{phys.}$, ml/g	$A_{cov.}$, ml/g	$E_{cov.}$, ml/g
Fe_3O_4/PAA	9.48	6.1	116.00	83.53
Fe_3O_4/γ-APS	1.18	0.92	67.41	59.51

It should be noted that a significant part of Ig (64–80%) remains in the solution upon covalent binding since the reaction of Schiff base formation is reversible (Tables 3, 4).

The nature of nanocomposite surface influences the values of both the physical and the covalent immobilization of Ig. The amounts of immobilized Ig and the distribution coefficients are higher for the nanocomposite Fe_3O_4/PAA.

We studied kinetics of release of Ig to model environment (0.15 M NaCl) for the nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS which carried physically and covalently bound Ig and had been prepared upon measurements of the respective isotherms. The samples of the nanocomposites (0.030 g) carrying physically or covalently bound Ig were placed into 5 or 7 ml of 0.15 M NaCl, respectively, and the UV absorption at 280 nm of the solution was

measured after gentle shaking for certain time intervals. The concentration of the desorbed Ig was calculated using the calibration curves.

Physically immobilized Ig desorbed during 1–2 min, the desorption curves are not shown. The data on desorption of covalently bound Ig are summarized in Tables 6, 7 and Fig. 2 *a, b*. Parameters of the release kinetic curves were calculated by the method of least squares using the equation $y(x)=y_0+Ae^{-x/t}$, where y_0 is the amount of bound Ig which is not released at the given conditions, A is the amount of bound Ig which is being released, 1/t characterizes release dynamics and curvature of the kinetic function.

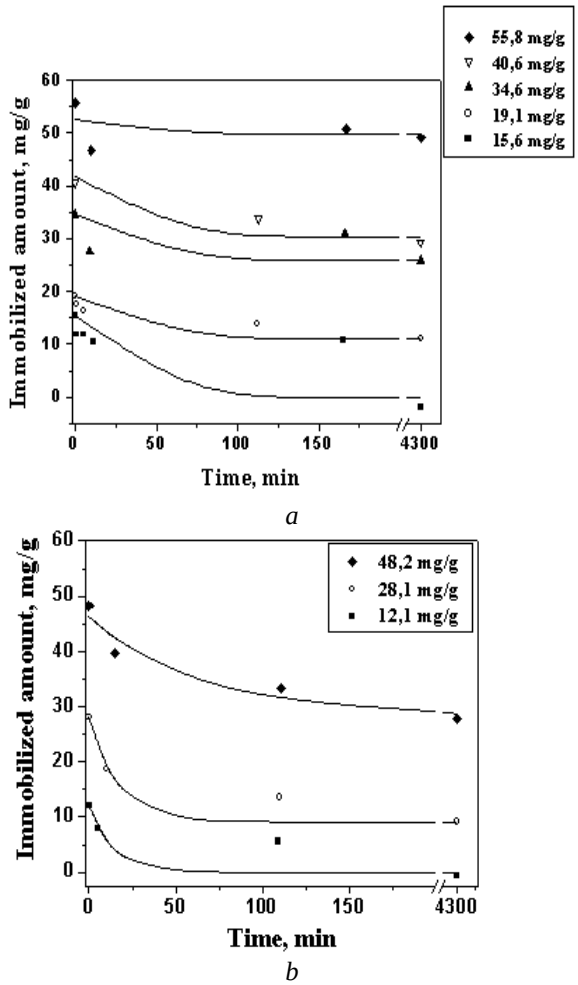

Fig. 2. Kinetics of release of covalently bound human immunoglobulin from the surface of the nanocomposites *a* – Fe_3O_4/PAA, *b* – Fe_3O_4/γ-APS. The initial bound amounts of immunoglobulin are shown in the right panels. The curves $y=y(0)+Ae^{-x/t}$ were calculated from the experimental data by the method of least squares

Table 6. Parameters of the equation $y=y(0)+Ae^{-x/t}$ describing release kinetics of covalently bound human immunoglobulin from the nanocomposite Fe_3O_4/PAA

	y(0)=55.8 mg/g	y(0)=40.6 mg/g	y(0)=34.6 mg/g	y(0)=19.1 mg/g	y(0)=15.6 mg/g
y_0	49.77±3.25	30.40±41.22	25.94±0	11.09±0	0±0
A	2.76±5.62	11.35±0	8.65±0	8.05±0	15.52±0
t	0.27±1.45	1.80±19.54	2.64±1.24	4.37±1.35	6.51±2.45

Table 7. Parameters of the equation $y=y(0)+Ae^{-x/t}$ describing release kinetic of covalently bound human immunoglobulin from the nanocomposite Fe_3O_4/γ-APS

	y(0)=48.2 mg/g	y(0)=28.1 mg/g	y(0)=12.1 mg/g
y_0	28.69±3.89	9.13±0	0±0
A	17.60±4.89	18.93±3.11	12.06±3.97
t	60.15±55.12	14.93±8.03	12.18±17.68

The obtained kinetic data show that the released Ig amount decreases upon increase of the amount initially immobilized at the surfaces of both nanocomposites. For small amounts of immobilized Ig 15.5 mg/g (Fe_3O_4/PAA) and 12.0 mg/g (Fe_3O_4/γ-APS), up to 30% of the Ig is released during the first 5–10 min. For higher immobilized Ig amounts 55.8 mg/g (Fe_3O_4/PAA) and 48.2 mg/g (Fe_3O_4/γ-APS), 16–18% of the Ig is released during the first 10–15 min. Release of surface-immobilized Ig bound via Schiff bases occurs slower and at lower extent than of physically bound Ig.

IMMOBILIZATION OF THE CD 95 ANTIBODY

We prepared nanocomposites carrying anti-tumour drug *cisplatin* and monoclonal mouse antibody CD 95 against the human Fas-antigen of the isotype IgG1, kappa, the clone DX2 produced by DakoCytomation (Denmark). The concentration of the initial solution of the antibody was 20 μg/ml.

We studied both non-specific (physical) adsorption and the covalent binding of the monoclonal antibody CD 95 to the nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS. We prepared 4 samples (0.03 g). Each sample was introduced into 1.0 ml solution of the antibody or 1.7 ml solution of the oxidized antibody, respectively:

1. Fe_3O_4 / PAA + CD 95;
2. Fe_3O_4 / γ-APS + CD 95;
3. Fe_3O_4 / PAA + CD $95_{oxidized}$;
4. Fe_3O_4 / γ-APS + CD $95_{oxidized}$.

Physical adsorption of the monoclonal antibody CD 95 (20 μg/ml) to the nanocomposites (the samples 1 and 2) was carried out in 0.15 M NaCl (1.0 ml) during 2 h upon shaking at the ambient temperature.

Oxidation of the monoclonal antibody CD 95 was carried out in acetate buffer-based solution of 0.1 M $NaIO_4$ (pH 5.0). The oxidized CD 95 was purified by dialysis against 2 l of 0.02 M acetate buffer (pH 5.0). The solution of the oxidized antibody was set to pH 8–9 after dialysis against a solution of 0.06 M carbonate-hydrocarbonate buffer (pH 9.5) and 0.15 M NaCl. The concentration of the antibody CD 95 after the dialysis was equal to 13 μg/ml.

Covalent bonding of the oxidized and purified monoclonal antibody to the nanocomposites Fe_3O_4/PAA (activated with ED) and Fe_3O_4/γ-APS (the samples 3 and 4) was carried out in a solution of 0.06 M carbonate-hydrocarbonate buffer (pH 9.0) and 0.15 M NaCl during 2 h upon shaking at the ambient temperature. Then the nanocomposites were separated using magnetic field and the antibody concentrations in the contact solution were measured using a combined reader for a microplate Synergy HT, Model SIAFRTD, Serial Number 202993 (Bio Tek).

Quantitative measurements of protein contents in the solutions were carried out by the method of Bradford [24]. The method is based on recording light absorption of a complex between Coomassie Blue G-250 dye and protein which has a maximum at 595 nm. The antibody concentration was determined from a calibration curve. The adsorbed amount of the antibody was calculated from the difference between its concentrations in the contact solution prior and after adsorption (Table 8).

The obtained results show that the covalent binding via Schiff bases has the following advantages with respect to non-specific adsorption: higher thermodynamic stability of the immobilized layer originating from the covalent bonding and better kinetic stability due to hampered release arising from slow hydrolysis of the Schiff bases.

Table 8. Immobilization of the monoclonal antibody CD 95 at the surfaces of magnetosensitive nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS

Nanocomposite	C_0, μg/ml	D	C_{eq}, μg/ml	A(CD 95), μg/g
Fe_3O_4/PAA + CD 95	20.00	0.44	19.93	2.3
Fe_3O_4/γ-APS + CD 95	20.00	0.42	19.96	1.2
Fe_3O_4/PAA + CD $95_{oxidized}$	3.88	0.73	1.00	163.2
Fe_3O_4/γ-APS + CD $95_{oxidized}$	3.88	0.72	1.45	137.7

where C_0 is the initial antibody concentration;
D is the optical density;
C_{eq}, is the equilibrium concentration of the antibody upon adsorption;
A is adsorbed amount of CD 95 at the surfaces of the nanocomposites

IMMOBILIZATION OF CISPLATIN AT THE SURFACES OF THE NANOCOMPOSITES

Cisplatin (CP) is an anti-tumour platinum-containing drug supplied as aqueous solution. Mechanism of the anti-tumour activity of platinum derivatives comprises DNA chains bifunctional alkylating which suppresses biosynthesis of nucleic acids and induces cells' apoptosis.

CP passes poorly through the hematoencephalic barrier and is quickly transformed into inactive metabolites. Binding to proteins in the state of the metabolites reaches 90%.

The period $\tau_{1/2}$ of half-excretion of the drug from blood is equal to 20–49 min at the initial stage, 58–73 h at the final stage assuming normal excretion kidney function, and 240 h upon anuria. The drug is excreted by kidneys in the amount of 27–43% in 5 days while platinum can still be found in tissues during 4 months after introduction.

We studied stability of CP via measuring its cytotoxic activity after 10, 20, and 30 days and found that it remains constant within a month.

Adsorption kinetics of CP at surfaces of the nanocomposites [25] was measured upon shaking an aqueous solution of CP (50 ml) with magnetic particles of the nanocomposites (200 mg) during 18 h at the ambient temperature. Probes (5 ml) were taken from the solutions every 2 h. The adsorbed amounts were determined as the difference between the initial and current concentrations of Pt^{2+} ions in the contact solutions. The measurements were carried out with a single beam two-channel atomic-

absorbance spectrophotometer C-115 M1 with flame atomizer, deuterium background corrector, and digital registration. A hollow cathode lamp for platinum (the analytical line 265.9 nm) and the fuel-oxidizer system acetylene-air were used.

The adsorption kinetic curves are shown in Fig. 3 *a*, *b*.

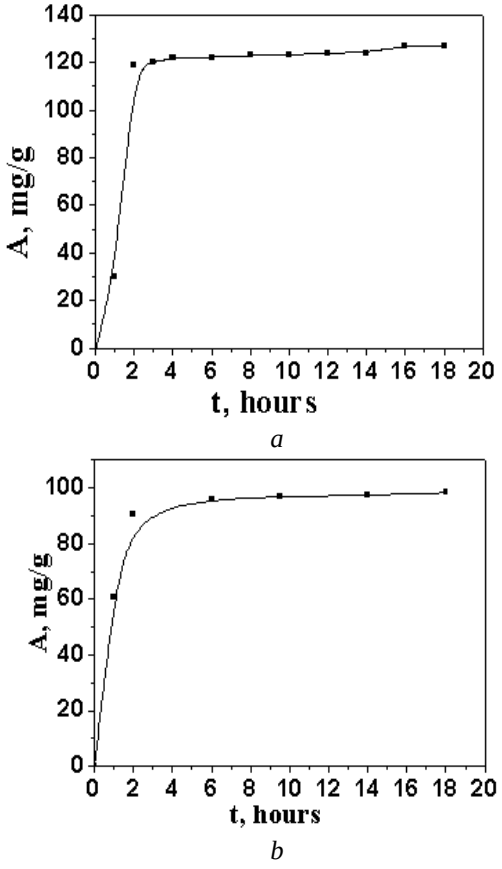

Fig. 3. Kinetic curves of adsorption of cisplatin at the surfaces of the magnetosensitive nanocomposites Fe_3O_4/PAA (*a*) and Fe_3O_4/γ-APS (*b*)

The adsorbed amounts of cisplatin (calculated for Pt^{2+}) at the surfaces of the nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS comprise 128 mg/g and 98.3 mg/g, respectively. The major part of the drug is adsorbed during the first 2–3 hours.

PREPARATION AND CYTOTOXIC PROPERTIES OF MAGNETICALLY DRIVEN POLYFUNCTIONAL NANOCOMPOSITES (MODELS OF NANOROBOTS)

We prepared the following samples for studies of impact of the magnetically driven nanocomposites carrying the cytostatic drug and the monoclonal antibody on vital activity of cancer cells:

1. Fe_3O_4 / PAA + CD 95;
2. Fe_3O_4 / PAA + CP;
3. Fe_3O_4 / PAA + CD 95 + CP;
4. Fe_3O_4 / γ-APS + CD 95;
5. Fe_3O_4 / γ-APS + CP;
6. Fe_3O_4 / γ-APS + CD 95 + CP.

The antibody CD 95 was bound to the nanocomposites via formation of Schiff bases (the samples 1, 3, 4, 6). The samples 3 and 6 were prepared in two steps: firstly, the oxidized monoclonal antibody CD 95 was conjugated with the nanocomposites Fe_3O_4/PAA (activated with ED) and Fe_3O_4/γ-APS, then the cytostatic drug was adsorbed.

Oxidation of the monoclonal antibody CD 95 was carried out in accord to the reaction scheme (4).

Covalent binding of the monoclonal antibody CD 95 (V = 1.7 ml, C = 3.88 μg/ml) to the surfaces of the nanocomposites Fe_3O_4/PAA (activated with ED) and Fe_3O_4/γ-APS was carried out during 1.5 hours upon shaking at the ambient temperature in accord to the reaction scheme (5). The obtained magnetic samples were collected in magnetic field of a permanent magnet.

The nanocomposites containing the covalently bound monoclonal antibody (the immobilized amounts of CD 95 were 163.2 mg/g for Fe_3O_4/PAA and 137.7 mg/g for Fe_3O_4/γ-APS) were introduced into 10 ml of CP aqueous solution (1 mg/ml). Adsorption was carried out for 4 hours upon shaking. The precipitate was collected in magnetic field of a permanent magnet. The adsorbed amounts of CP were 128 mg/g for Fe_3O_4/PAA and 98.3 mg/g for Fe_3O_4/γ-APS.

Cytotoxic impact of the nanocomposites carrying immobilized the monoclonal antibody and the cytostatic drug on cancer cells was studied *in vitro*. The nanocomposites were taken in the amounts which contained the quantity of CP equal to the biological equivalent of efficiency IC_{25}, i.e. 25% of the IC concentration which 100% suppresses the cells. Our earlier experiments showed that IC_{50} = 5 μg/ml, therefore IC_{25} = 2.5 μg/ml. At that concentration of the nanocomposites, the concentration of the monoclonal antibody CD 95 was equal to 0.2 μg/ml (the doze used for clinical treatment is equal to 10–30 μg/ml). The studies of cytotoxicity were carried out at P.E. Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the National Academy of

Sciences of Ukraine [26]. The cytotoxic impact of the nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS carrying immobilized the monoclonal antibody CD 95 and CP was measured for the human mammary gland cancer cell line MCF-7. The cytotoxic activity of the respective nanocomposites carrying only CP or only the monoclonal antibody was also measured for comparison. The following solutions were used as the control samples: pure nutrient medium, CP (2.5 μg/ml), and the monoclonal antibody CD 95 solution (0.2 μg/ml). We studied also the cytotoxic effect of the bare magnetite nanoparticles and the bare nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS.

The volumes 100 μl of the MCF-7 line cells ($1 \cdot 10^5$ cells/ml) were deposited into 96-cavity microplates. The cells were cultivated in a modified medium Dulbecco – ISCOV (Sigma, Germany) with addition of 10% embryonic calve serum and antibiotic gentamycin at 40 μg/ml in standard conditions at 37°C and air saturation with 5% CO_2. The samples being studied were added to the cells after a 24 hour period of the cells adapting to the cultivating conditions. Each sample was added in 3 parallels and incubated in the same conditions. Cytotoxicity was measured after 24 hours.

The impact was evaluated with the MTT-colorimetric test, The method is based on the ability of mitochondrion ferments of living cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (yellow salt) into crystalline MTT-formazan (lilac) [26]. The MTT solution (Sigma, 20 μl, 5 mg/ml in phosphate-saline buffer) was added to the cavities of the plastic plate and incubated for 3 hours. Then the plate was centrifuged at 1500 rev/min for 5 min and the supernatant was removed with an automated suction. Dimethylsulfoxide (Serva, 100 μl) was added to each cavity to dissolve the formazan crystals. The optical absorbance was measured with a multi-cavity spectrophotometer at the wavelength 540 nm. The results of the study are summarized in the Table 9.

In accord to the obtained data, the magnetosensitive nanocomposites carrying adsorbed CP in quantity twice below the therapeutic range and the amount of the monoclonal antibody CD 95 almost by one order lower cause death of 46–57% of the tumour cells which exceeds the impact of the control solution (CP+CD 95) by up to 50%. This synergy effect can be explained as follows. Firstly, the targeted delivery of the complex cytostatic drug–antibody to the tumour cells was accomplished. The cytotoxic effect of CP is achieved through formation of covalent bonds between the drug and DNA. Traumatic effect of the nanocomposite on the cell membrane facilitates the process and improves transport of the therapeutic preparation through the membrane barrier. Bifuctional products of the interaction, the so-called DNA-adducts, block replication, transcription, and, as a consequence, cell proliferation. Secondly, the system ligand/receptor plays an important role in apoptosis of malignant cells. The antibody binding its receptor launches a system of signal transmission which leads to apoptosis. There are also reports [6] that this system may cause death of tumour cells upon influence of cytotoxic drugs.

Table 9. Impact of the magnetosensitive nanocomposites Fe_3O_4/PAA and Fe_3O_4/γ-APS carrying adsorbed cisplatin (CP) and the conjugated monoclonal antibody CD 95 on the vital activity of the MCF-7 line cells

	Fe_3O_4/ γ-APS + CP	**Fe_3O_4/ γ-APS + CD 95**	**Fe_3O_4/ γ-APS + CP + CD 95**	**Fe_3O_4/ PAA + CP**	**Fe_3O_4/ PAA + CD 95**	**Fe_3O_4/ PAA + CP + CD 95**
Suppressed cells, %	31	20	46	38	21	57

	CP, 2,5 μg/ml	**CD 95, 0,2 μg/ml**	**CP, 2,5 μg/ml + CD 95, 0,2 μg/ml**
Suppressed cells, %	25	10	38

Consequently, the impact of the magnetically driven nanocomposites (models of nanorobots) carrying the anti-tumour drug and the monoclonal antibody CD 95 on the cancer cells MCF-7 exerts a synergic effect and provides the desired cytotoxicity at lower concentrations. Thus the toxic effect of the medical chemotherapeutic preparation on a whole organism can be decreased. Magnetic properties of the nanorobot models, peculiarities of their transport in the vascular system, their use for creation of hyperthermia zones, desorption kinetics of the cytostatic drug and efficiency of its influence on cell lines are discussed elsewhere [3].

CONCLUSIONS

A method for preparation of magnetosensitive nanocomposites on basis of surface-modified magnetite carrying immobilized cisplatin and monoclonal antibody CD 95 (a medico-biological nanorobot model) was developed.

Adsorption and covalent immobilization of monoclonal antibody CD 95 and human normal immunoglobulin on nanocomposites comprising magnetite coated with polyacrylamide and γ-aminopropylsiloxane. Isotherms of covalent attachment of oxidized immunoglobulin via formation of Schiff bases and non-specific (physical) adsorption of the normal immunoglobulin were compared. Kinetics of release of the immunoglobulin to a model environment was studied.

Interaction of the prepared models of nanorobots with the cell line MCF-7 was studied. It was shown that use of magnetically driven nanocomposites carrying the anti-tumour drug and the monoclonal antibody CD 95 causes a synergic cytotoxic effect which exceeds the influence of the control dozes up to 50%.

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Конструювання магнітокерованих нанокомпозитів медико-біологічного призначення

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Дослідження спрямовані на вивчення взаємодій наноматеріалів з компонентами біосистем, розробку новітніх терапевтичних засобів на основі магнетиту, аналіз ефективності їх використання, хімічне конструювання багаторівневих магніточутливих нанокомпозитів з ієрархічною архітектурою та функціями медико-біологічних нанороботів.

Конструирование магнитоуправляемых нанокомпозитов медико-биологического назначения

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Исследования направлены на изучение взаимодействий наноматериалов с компонентами биосистем, разработку новых терапевтических средств на основе магнетита, анализ эффективности их применения, химическое конструирование многоуровневых магниточувствительных нанокомпозитов с иерархической архитектурой и функциями медико-биологических нанороботов.