Hampered binding to blood serum albumin and antimicrobial activity of ibuprofen immobilised on magnetic nanoparticles Fe_3O_4

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Drug distribution in an organism depends largely on the binding degree of drugs with blood serum proteins like albumin. Only free fraction of the drug can induce a therapeutic effect. A different affinity of particular drugs to proteins is also one of the reasons of the occurrence of drug interactions. It was decided to investigate if the immobilisation of some model drugs on 'magnetic nanoparticles Fe_3O_4 (MNPs)' would influence their activity and protect from binding to albumin. It was proposed to attach the drugs such as ibuprofen (IB), acetylsalicylic acid and naproxen to the MNPs coated with '(3-aminopropyl)triethoxysilane' by electrostatic interaction since carboxyl groups of the drugs can form ionic bonds with amino groups of the silane. The characterisation of morphology of the obtained nanostructures was also carried out. The drug release studies showed very slow diffusion of the drugs from the MNPs surface. It has been demonstrated that IB immobilised on MNPs exhibits high antimicrobial activity even in its low concentration and in the presence of albumin – in contrast to the unattached form of the drug.

1. Introduction: Magnetic iron oxide nanoparticles Fe_3O_4 (MNPs) are of a significant interest in medical applications including magnetic resonance imaging [1], immunomagnetic separation [2] and cancer therapy (hyperthermia) [3]. MNPs are also used in a local drug delivery [4, 5] as they possess a relatively large surface which is able to bind, adsorb and carry other compounds. Specific drugs are bound on the nanoparticles surface and delivered into the affected areas of the human organism by means of magnetic field gradient. Targeting of the drug to a specific part of the body or type of cell can reduce the side effects. Moreover, nanoparticles depending on the size are able to penetrate almost all tissues. Additionally, slow release of drugs from the nanocarriers means the greater efficacy of the same quantity of a drug and eventually, a lower dose of a medical preparation. Currently, iron oxide nanoparticles are the sole magnetic nanoparticles approved for use in medical therapy [6].

For effective medical applications, it is crucial to functionalise the surface of MNPs with other chemicals. The coating of the nanoparticles is associated with the latter applications of these structures [7, 8]. Fe_3O_4 nanoparticles should have reactive surface, thus providing an ability to be further functionalised. It also reduces their toxicity and improve their stability in solution, which limits the aggregation of MNPs. The strong aggregation of MNPs in solution may hamper the effective binding of biologically active compounds to the nanoparticle surface. Magnetic nanoparticles Fe_3O_4 can be, for instance, silanised to render the particles with amine groups [9, 10]. The example of a compound belonging to the group known as aminosilanes, which is often used for the surface modification of nanoparticles, is '(3-aminopropyl)triethoxysilane (APTES)'. This aminosilane provides biocompatibility and stability to nanoparticles; additionally, the amine group of APTES can participate in various kinds of chemical bonds and thus enables the immobilisation of organic compounds and biomolecules [11, 12].

It is well known that drug distribution in the human body depends i.a. on the binding degree of drugs with blood serum proteins like albumin. The various drugs differ in the affinity to the serum albumins and the binding is more or less reversible, but undeniably this phenomenon has a great influence on the level of a free fraction of a drug in blood, i.e. the only fraction which exhibits a therapeutic effect [13]. Moreover, the different affinity of particular drugs to proteins is one of the reasons for the occurrence of drug interactions. It is also an important factor which influences the volume of distribution of a drug.

In the case of strong drug binding to the proteins, for instance 99%, only 1% of a dose of medicine is able to induce therapeutic effect. The examples of such drugs are ibuprofen (IB), acetylsalicylic acid (ASA) and naproxen (NP) – non-steroidal anti-inflammatory and analgestic agents [14]. Owing to their popularity and prevalence, it was decided to use these agents in model tests in order to answer the posed questions: firstly, we wanted to find out if the immobilisation of drugs on MNPs would influence their activity and secondly, if the immobilisation would protect them from the binding to albumin. For this purpose, we used in the studies the fact that the tested agents exhibit antimicrobial activity [15–17].

Owing to the fact that all the tested drugs are week acids and they contain in their molecular structure carboxyl groups, it was decided to immobilise the drugs on the MNPs by forming a salt with APTES according to the scheme shown in Fig. 1. Therefore MNPs functionalised with APTES were used (MNPs[APTES]).

This method of attachment to the MNPs is also useful for in vitro studies since the drugs can be easily and thoroughly released from the MNPs by decreasing the pH value to highly acidic.

2. Synthesis and characterisation of nanostructures: The chemicals were analytical grade and used as received without further purification. Reagents were purchased from Sigma-Aldrich (Poland), organic solvents from Avanter Performance Materials Poland S.A. (formerly POCH S.A.), drugs: IB, ASA and NP were purchased from Alfa Aesar (Poland).

The 'magnetic iron oxide nanoparticles Fe_3O_4 (MNPs)' were synthesised using the chemical co-precipitative method [18]. The black MNPs precipitate was washed several times with water and then with methanol. The black MNPs solid was then dried under vacuum in a drying chamber at 50°C for 2 h. Next 600 mg of MNPs was suspended in 200 ml of 200 mM TMAOH and sonicated for 3 h in an ultrasonic cleaner. The black MNPs precipitate was washed with distilled water and dried under vacuum in a drying chamber at 50°C for 2 h. MNPs functionalisation with APTES was carried out using Cao *et al.* method [19]. During the process a colour change of the nanoparticles suspension from black to brown was observed. After 24 h, the brown precipitate was washed several times with water and methanol and dried at 40°C

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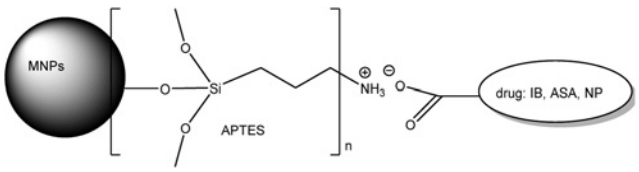

Fig. 1 *Method for binding of a drug containing a carboxyl group on MNPs coated with APTES (pH = 7.4)*

under vacuum in a drying chamber for 2 h. As a result, 600 mg of MNPs[APTES] was obtained.

Subsequently, in order to attach the tested drugs to the MNPs [APTES], 'functionalised magnetic nanoparticles (fMNPs)' were firstly sonicated for 0.5 h in an ultrasonic cleaner with a ten-fold mass excess of the drug in a mixture of PBS buffer (pH = 7.4) and methanol (1:1) in the case of IB and ASA or in a mixture of PBS buffer (pH = 7.4) and acetone (1:1) in the case of NP. Then the mixture was stirred for 24 h in room temperature. The concentrations of the MNPs[APTES] and of the drug in the starting reaction solution were 5 and 50 mg/ml, respectively. The use of organic solvents was necessary due to the poor solubility of the drugs in water. After the reaction, the precipitate was dried under vacuum in a drying chamber at 35°C for 5 h.

The amount of the attached to the MNPs[APTES] drug was determined by the method using the fact that strong acids such as HCl cause the complete removal of the weak acids (IB, ASA or NP) from their salts with APTES. Thus, 2 mg of MNPs[APTES] IB (or MNPs[APTES]ASA and MNPs[APTES]NP, respectively) was suspended in 2 ml of 0.2 M HCl and sonicated for 1 h in an ultrasonic cleaner. Next, after centrifugation (13,000 rpm, 10 min, 25°C), the concentration of the released drug in the supernatant was measured using a UV–vis spectrophotometer (2450 UV-vis Shimadzu). The results indicated that to 1 mg of MNPs[APTES] the following amounts of the drugs was attached: 450 µg of IB, 350 µg of ASA and 90 µg of NP, respectively. However, it should be noted that sometimes, despite of the same conditions of the drug loading process, the amounts of the attached drugs were slightly different. Nevertheless, it turned out to be the rule that the capacity of loading for IB was the largest, for ASA smaller and for NP the smallest.

The effect of the surface functionalisation of the MNPs was measured and confirmed using a Nicolet iS50 FT-IR spectrometer in the range of 400–4000 cm^{-1}. FT-IR spectrum of MNPs[APTES]IB is shown in Fig. 2.

The intensive absorption band in the range 500–600 cm^{-1} is characteristic for Fe_3O_4. The spectrum bands at 3430 and 1552 cm^{-1} represent, respectively, stretching and deformation

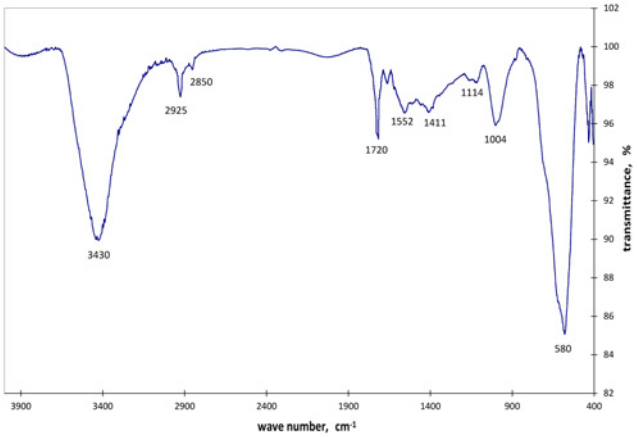

Fig. 2 *FT-IR spectrum of MNPs[APTES]IB*

vibrations of N–H from amino group of APTES. The band at 1004 cm^{-1} comes from Si–O (stretching vibrations). In respect of IB, the characteristic bands are found at 1720 cm^{-1} (stretching vibrations of carbonyl group C=O) and at 1411 cm^{-1} (–OH deformation vibrations). The area in the range 1500–1650 cm^{-1} corresponds also to stretching vibrations of aromatic C=C bonds (IB). Absorption bands in the range 2800–3000 cm^{-1} derive from carbon aliphatic chains and the band at 1114 cm^{-1} comes from C–O stretching vibrations.

The obtained nanostructures were also characterised by measurement their sizes (diameters) and the potential-zeta values using the analyser dynamic light scattering (DLS), Malvern, Zetasizer Nano-ZS. The results are presented in Table 1.

Magnetic nanoparticles Fe_3O_4 (MNPs) as well as nanoparticles functionalised with APTES (MNPs[APTES]) exhibited the strong negative zeta-potential value which provided them a stability in solution. The reason of stability is the fact that nanoparticles with a high positive or negative surface charge (potential zeta at least +30 mV or –30 mV) repel each other strongly enough to prevent agglomeration. After attachment of IB to the MNPs[APTES] the charge of the nanostructures changed to the opposite. The value of their potential zeta was +28 mV and it turned out to be sufficient in order to prove stability of the nanostructures in solution. In the case of ASA and NP – after attachment of these drugs to the MNPs[APTES], the resulting charge of the nanostructures was positive as well; however, the values of zeta-potential were lower. It lay of the bottom of a tendency of MNPs[APTES]ASA and MNPs[APTES]NP to their significant agglomeration and their slow sedimentation in solution. For that reason, the measurement of size of these nanostructures by DLS technique turned out to be unsuccessful. Nevertheless, the size in diameter of MNPs[MNPs] IB was determined. It was found that 25% of the number of the nanostructures represented diameter of 55 nm (the remaining 75% of them were within the range of 55 ± 20 nm). As was expected, the smallest size represented MNPs. Most of the MNPs (about 25%) had a diameter of 33 nm; the smallest among them had a diameter no less than 24 nm. Larger sizes exhibited APTES functionalised magnetic nanoparticles; of which almost 25% were nanostructures with a diameter of 43 nm (the remaining 75% of them were within the range of 43 ± 15 nm). Taking into consideration that aminosilanes like APTES are able to cover the nanoparticles surface with several layers [11], it can be concluded that the larger size of MNPs[APTES] in comparison to MNPs is the result of functionalisation with the silane. However, in the case of MNPs[APTES]IB, the largest size is possibly the result not only of the surface coating by the drug but also of slight agglomeration of these nanostructures (zeta-potential a little less than +30 mV).

Atomic force microscopy (AFM) images were obtained on a Bioscope Catalyst Brucker machine and are shown in Figs. 3 and 4.

AFM images display the surface morphology of the APTES functionalised iron oxide nanoparticles coated by IB (Fig. 3) and ASA (Fig. 4) formed in methanol. The values are consistent with DLS results. The AFM image of MNPs[APTES]IB shows dense and uniformly packed structures; however, the AFM image of MNPs[APTES]ASA presents very high agglomeration of the nanostructures what disenabled determination of their size by the DLS technique (see Table 1).

3. Investigation of drug release: It stands to reason that for the proper activity of the immobilised drug, its effective release from nanoparticles is essential. Thus the investigation of the drug release degree from MNPs[APTES] for IB, ASA and NP was carried out.

Each sample contained 20 mg of MNPs[APTES]DRUG (DRUG = IB, ASA or NP, respectively) suspended in 2 ml of PBS buffer (pH = 7.4). The samples were incubated at 37°C for 168 h (7 days). At specified time intervals, the tested sample was centrifugated (13,000 rpm, 10 min, 25°C) and the concentration

Table 1 Zeta-potential values and size of the nanostructures in methanol

	Zeta potential, mV	Zeta deviation, mV	Size (diameter), nm	Size deviation, nm	Significant agglomeration
MNPs	−44.0	4.9	33.0	8.7	no
MNPs[APTES]	−37.0	7.3	43.0	14.9	no
MNPs[APTES]IB	+28.0	8.1	55.0	19.9	no
MNPs[APTES]ASA	+17.5	5.8	–[a]	—	yes
MNPs[APTES]NP	−3.0	4.7	–[a]	—	yes

[a]Agglomeration of nanostructures disenables determination of the size by the DLS technique.

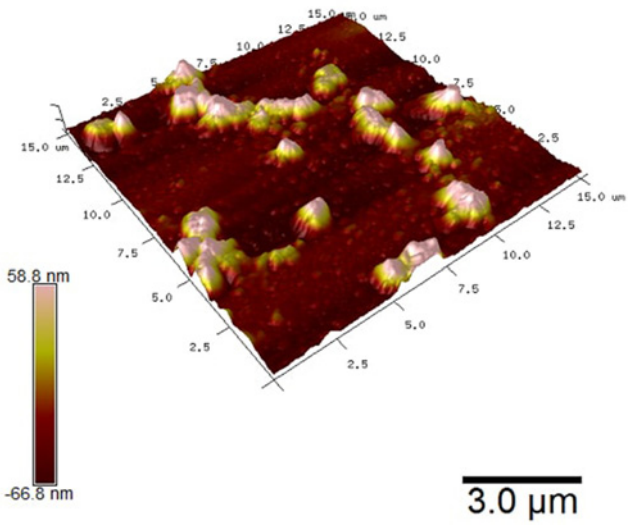

Fig. 3 *Atomic force microscopy image of MNPs[APTES]IB*

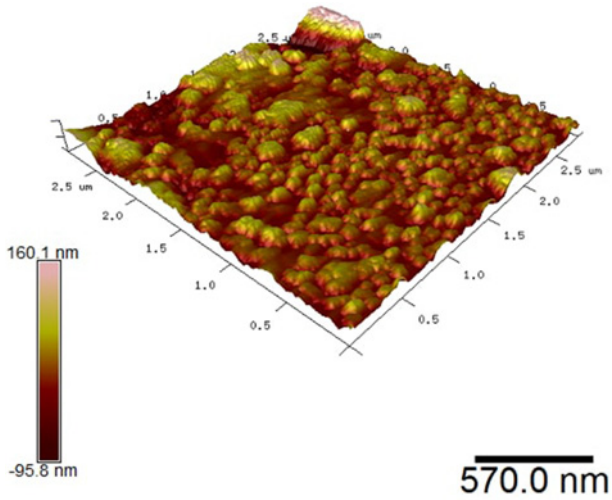

Fig. 4 *Atomic force microscopy image of MNPs[APTES]ASA*

of the released drug was read on a UV–vis spectrophotometer. To the remaining after centrifugation precipitate of the nanostructures 2 ml of fresh PBS buffer was added and MNPs[APTES]DRUG was carefully suspended (sonication in an ultrasonic cleaner, 1 min). Incubation at 37°C of the sample was continued until the next measurement. Continuous removing of the released drug was necessary. Otherwise, the equilibrium state of dissociation of the salt $APTES^{+}DRUG^{-}$ was reached.

The experiments were performed three times with satisfying reproducibility of results.

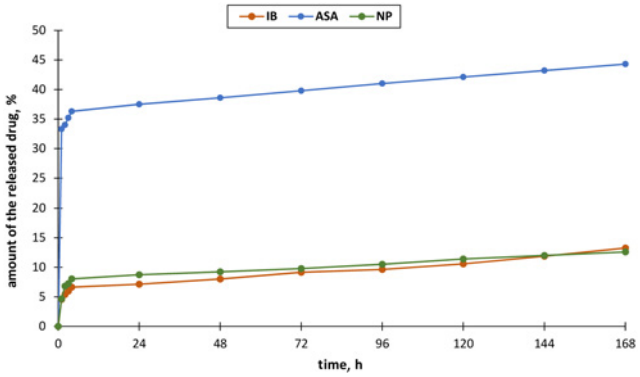

Fig. 5 *Release degree of IB, ASA and NP from MNPs[APTES] in PBS buffer pH = 7.4 during seven days*

The results of the amount (in percent) of the released drugs from the MNPs[APTES] surface according to the time of releasing are shown in Fig. 5.

In the first hour of the process, the drugs were released rapidly. After this time, in the tested samples ∼4.5% of free IB and a similar amount of free NP were detected relative to the total initial amount of the immobilised drugs. The largest release degree in the first hour was observed for ASA (∼33%). It is believed that the root of this fact is higher acidity of ASA ($pK_a = 3.48$) in comparison to acidity of IB and NP ($pK_a = 4.45$ and 4.19, respectively) and also much higher solubility of ASA in water (4.6 g/L) in comparison to solubility of IB and NP (21 and 15.9 mg/L, respectively). The release rate significantly decreased after the first hour and as the consequence after 7 days of the process the following amounts of the drug were determined: ∼13% of IB and NP and 44% of ASA.

Figures 6*a* and *b* show the increase in concentrations of the released drugs during the process.

Fig. 6*a* shows clearly that the rate of increase in concentration of the drugs was not constant in the first hour of the process but then a steady change in concentration per unit of time was observed. After the fourth hour of process (Fig. 6*b*), the rate of increase in concentration of the drugs was noticeably constant; it means that the release process occurred according to zero-order kinetics with the following release constants: $k_0 = 1.219$ µg/ml for IB (correlation coefficient of the line in Fig. 4*b*: 0.986), $k_0 = 1.234$ µg/ml for ASA (correlation coefficient: 0.999) and 0.229 µg/ml for NP (correlation coefficient: 0.996).

4. Transport through the membrane and antibacterial activity studies: The main aim of the research was to investigate how the immobilisation of the drug on the magnetic nanoparticles would influence the degree of the drug binding to blood serum proteins like albumin and what consequences it would have in respect of the biological activity of the drug. Therefore, the further studies were carried out only with the use of IB immobilised on MNPs [APTES], because these nanostructures had the features which

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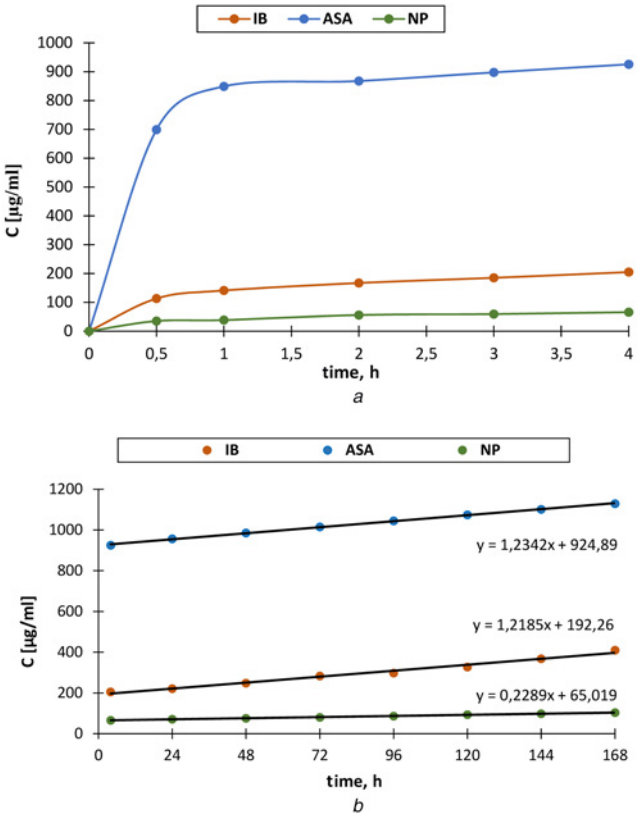

Fig. 6 *Increase of the concentration of IB, ASA and NP in PBS buffer (pH = 7.4) as a consequence of releasing from MNPs[APTES]*
a During the first four hours of the process
b After the fourth hour of the process

seemed to be desirable and propitious in the planned research. Firstly, IB represented the largest capacity to the loading on the MNPs[APTES] surface, secondly the release process of IB from the nanoparticles occurred slowly. Furthermore, IB shows high antibacterial activity (e.g., *E. aureus* MIC = 952 µg/ml, *P. aeruginosa* MIC = 1292 µg/ml, *P. mirabilis* MIC = 1465 µg/ml) [15–17] and this fact was useful and thereby employed in the reported research.

The transport studies of MNPs[APTES]IB through the membrane were carried out using dialysis membranes Spectra/Por Biotech CE, MWCO 100–500 D (Spectrumlabs.com).

1.2 mg of MNPs[APES]IB was suspended in 0.4 ml of PBS buffer (pH = 7.4) (1 min sonication in an ultrasonic cleaner) and the suspension was put into a small space surrounded by the dialysis membrane. A such prepared sample was placed in a beaker filled with 15 ml of PBS buffer (pH = 7.4) and incubated at 37°C for 7 days. The absorbance of the buffer in the beaker was read on a UV–vis spectrophotometer at 225 nm at specified time intervals. The results are shown in Fig. 7.

The penetration degree of the tested nanostructures was moderate. The highest transport rates were observed within the first 72 h of the process – after the 3 days ~28% of the total amount of MNPs[APTES]IB were transported. After the next 4 days (168 h of the process), the amount of the transported via membrane nanostructures increased only to the value of ~35%. It is supposed that the crucial factor affecting the transport rates was the size of MNPs[APTES]IB (55 ± 20 nm).

The results of transport studies were relevant information for the interpretation of the antibacterial activity tests.

The bacteria growth inhibition tests used as a model system a *Lactobacillus acidophilus* ATCC 4356 strain (Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and

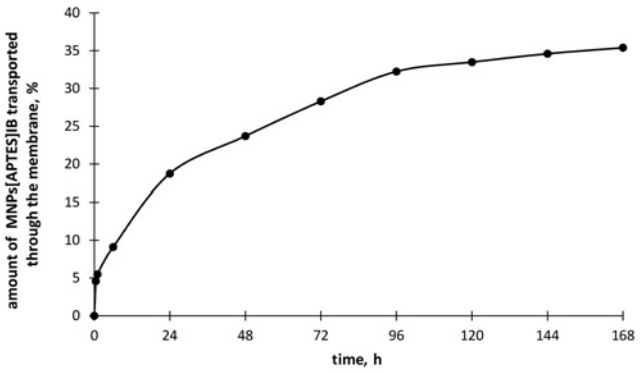

Fig. 7 *Penetration degree of MNPs[APTES]IB through the membrane*

Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland). Albumin from bovine pancreas was purchased from Sigma-Aldrich (Poland)

Bacteria were grown at 37°C in 200 mL of MRS medium (commercially available: BTL Ltd, Poland) to a final optical density at 660 nm (OD_{660}) of 0.25. Then the cell suspension was divided into five portions in new flasks. To each of them (15 ml of the cell suspension), the following supplements were sequentially added: (i) 5 ml of PBS buffer (control of bacteria growth), (ii) 5 ml of PBS buffer containing 11 mg of MNPs[APTES] (control of bacteria growth in the presence of magnetic nanoparticles without a drug), (iii) 5 ml of PBS buffer containing 16 mg of MNPs[APTES]IB (the amount of the nanostructures containing 5 mg of immobilised IB), (iv) 5 ml of PBS buffer containing 16 mg of MNPs[APTES]IB and 200 mg of albumin, (v) 5 ml of PBS buffer containing 5 mg of IB (suspension), (vi) 5 ml of PBS buffer containing 30 mg of IB (suspension), (vii) 5 ml of PBS buffer containing 30 mg of IB (suspension) and 200 mg of albumin. Bacteria were grown at 37°C with shaking for 6 h. The optical density (OD_{660}) was measured every hour.

The experiments were performed three times with satisfying reproducibility of results.

The results are shown in Fig. 8.

fMNPs[APTES] had only a little effect on the bacteria growth – slight inhibition was observed. The inhibition on the same level was

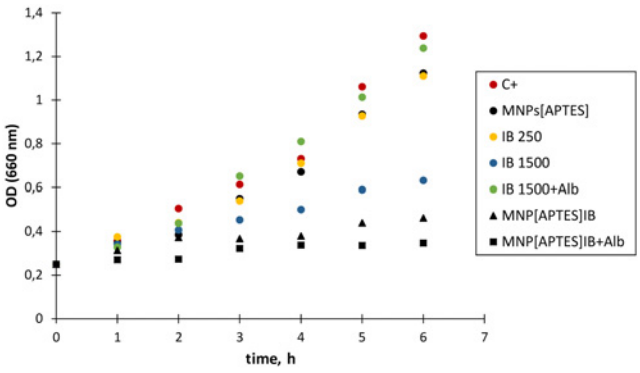

Fig. 8 *Growth of Lactobacillus acidophilus ATCC 4356 in the presence of specific supplements*
C+: no supplements (growth control)
MNPs[APTES]: functionalised nanoparticles without IB
IB 250: IB with the final concentration 250 µg/ml
IB 1500: IB with the final concentration 1500 µg/ml
IB 1500 + Alb: IB with the final concentration 1500 µg/ml and addition of albumin
MNPs[APTES]IB: functionalised nanoparticles with IB attached
MNPs[APTES]IB + Alb: functionalised nanoparticles with IB attached and addition of albumin

found in the case of the incubation of bacteria with IB with the final drug concentration of 250 μg/ml. This concentration of free IB turned out to be too low for the effective antibacterial activity and it is believed that the main cause of this fact is very poor solubility of IB in water. The higher concentration of free IB (1500 μg/ml), which corresponded to the MIC values for bacteria tested by other researches [15–17], caused a significant inhibition of bacteria growth. Nevertheless, the same amount of IB (1500 μg/ml) but in the presence of albumin was completely ineffective. The reason was undoubtedly a strong binding of IB to albumin what impeded the activity of the drug. However, the studies showed that IB immobilised on the MNPs[APTES] exhibited high antimicrobial activity even in the presence of albumin. Moreover, it should be noted that the amount of immobilised IB corresponded to the quantity of free IB (250 μg/ml) which was insufficient for antimicrobial activity even without the presence of albumin in the analogic test. In other words, 5 mg of free IB in 20 ml of a cell culture ($C = 250$ μg/ml) did not inhibit the growth of bacteria whereas 5 mg of IB immobilised on MNPs[APTES] in the same conditions caused a strong inhibition, also in the presence of albumin. It is supposed that one of the reason of that fact is a positive charge of the MNPs[APTES]IB (see zeta-potential measurements) what brought the nanostructures close to the bacteria cells because of their negative charged wall (wall teichoic acids of Gram-positive bacteria). In a short distance from the cells IB was released effectively, i.e. as a good dissolved form of the drug and it was immediately transported inside the bacteria what undoubtedly limited to a large degree or even impeded entirely exposition to albumin.

5. Conclusion: The studies have confirmed that drugs containing in their molecular structure a carboxyl group can be attached to the surface of iron oxide nanoparticles by the electrostatic interaction with an amino group of APTES in aqueous medium at pH = 7.4, i. e. at the pH value that is typical for blood serum. In this study, IB, ASA and NP as model drugs were used, but undoubtedly this kind of interaction can be employed for various others agents containing a carboxyl group. Moreover, it is possible to predict the drug release degree of tested drugs since it surely depends on the pK_a of an agent and its solubility in water. More acidic agents and better soluble in water will be arguably released faster. It is also a useful method for the attachment of such drugs to the nanoparticles for in vitro studies, because the binding strength of a drug to MNPs as well as the release degree can be regulated by the pH value of buffer while lowering the pH below the value of 3 will cause the complete release of the agent.

The results showed that IB immobilised on MNPs had a very high antibacterial activity even in the presence of albumin. The same quantity of the tested drug but in its uncombined form was insufficient for inhibition of the bacteria growth. Admittedly the higher concentration of IB showed an antibacterial activity, but only in an environment without the addition of albumin. Therefore, it can be concluded that immobilisation of drugs on MNPs brings many advantages. Firstly, the drugs are protected from binding to albumin, what is important considering that the therapeutic effect depends on the level of the free fraction of the drug. Furthermore, the drug which is bond on the MNPs surface will not displace other agents from their binding sites with proteins what in turn may reduce or exclude drug–drug interactions.

Via appropriate magnetic field manipulation, the drug immobilised on the MNPs is able to reach pathogenic cells fast and selectively, nevertheless, the adequate charge of the nanostructures (strong positive charge in the case of MNPs[APTES]IB) can also cause agglomeration of the drug close to the cells. It seems that in such a local area, the drug diffuses into the aquatic environment as a well dissolved form, what can be the reason of its high activity even in a low concentration. Consequently, it could reduce the necessary dosage of drugs. The fact that the tested nanostructures had a quite large size (50 ± 20 nm) and were not transported into cells to a large extent (as indicated by the transport studies), seems to be an advantage since it may reduce the risk of cytotoxicity induced by the MNPs themselves.

We believe that the results of the presented Letter will be useful for the further research on the use of MNPs as drug carriers.

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