Original Research Paper

Cytotoxicity of chlorambucil immobilized on magnetic iron oxide nanoparticles Fe₃O₄

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1 INTRODUCTION

Chlorambucil (4-[p-[bis[2-chloroethyl]amino]phenyl]-butanoic acid (CHB) is a nitrogen mustard alkylating agent used for the treatment of various malignant and non-malignant cancers such as chronic lymphatic leukaemia [1], Hodgkin’s and non-Hodgkin’s lymphoma [1, 2], advanced ovarian and breast cancer [3, 4] and certain autoimmune diseases [5]. Alkylating agents stop tumour growth by cross-linking guanine or adenine bases in DNA double-helix strands, which makes them unable to uncoil and separate thereby disrupting DNA replication in proliferating cells [6, 7]. Metronomic chemotherapy using nitrogen mustards has been reported and shows promising results [8–10]. However, the instability of chemotherapeutic due to the hydrolysis of the chloroethyl group is a major limitation in achieving the optimum therapeutic effect [11]. Furthermore, chlorambucil use is limited by its toxic side effects such as neurotoxicity and myelotoxicity [12, 13]. This is the reason for the limited use of optimal doses of this chemotherapeutic agent. It minimizes toxicity and patient suffering, but often results in inefficient treatment [14]. To prevent adverse side effects and to increase drug bioavailability, various polymers drug delivery systems are used. In recent years, researchers have focused especially on the poly(DL-lactide-co-glycolide) (PLGA) study [15–17]. However, other known chemotherapeutic carriers should be kept in mind. It is generally accepted that magnetic iron oxide nanoparticles Fe₃O₄ (MNPs) play an important role in the drug delivery processes [18, 19]. MNPs possess a relatively large surface which enables binding and carrying other compounds. The great advantage of such drug delivery systems is the ability to direct them using an external magnetic field only to specific places in the body. Additionally, nanoparticles depending on their size are able to penetrate almost all tissues. For medical applications, functionalization of the MNPs surface with other chemicals is necessary. The coating of the Fe₃O₄ nanoparticles is associated with the latter applications of these structures [20–21]. MNPs should have a reactive surface, thus providing the ability to be further functionalized. It also reduces their toxicity and improve stability in fluids. Magnetic nanoparticles can be, for instance, silanized with (3-aminopropyl) triethoxysilane (APTES) to render the particles with amine groups, which can next participate in various kinds

Abstract

Chlorambucil is a nitrogen mustard alkylating agent used for the treatment of various malignant and non-malignant cancers. The use of the chemotherapeutic is limited by its toxic side effects which precludes the use of optimal doses. It was decided to investigate if the immobilization of chlorambucil on the magnetic nanoparticles Fe₃O₄ would increase its cytotoxicity toward chosen cancerous cell lines: ‘Jurkat’ and ‘Hut 78’. The drug was attached to the nanoparticles coated with (3-aminopropyl)triethoxysilane. The obtained nanostructures were characterized using IR spectroscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM), transmission electron microscopy (TEM), vibrating sample magnetometer (VSM) and dynamic light scattering (DLS) technic. Zeta-potential measurements at various pH were also carried out. The drug release studies showed that chlorambucil is released from the nanoparticles surface very effectively. The cytotoxicity studies demonstrated that chlorambucil immobilized on the magnetic nanoparticles Fe₃O₄ displays significantly higher cytotoxicity in comparison to its unbound form.

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of chemical bonds, thus enabling the immobilization of organic compounds and biomolecules [22–24]. APTES also provides biocompatibility and stability to the nanoparticles [25].

The goal of our study was to immobilize chlorambucil (CHB) on the magnetic Fe₃O₄ nanoparticles functionalized using APTES, and next to investigate the cytotoxicity of the immobilized CHB as well as the cytotoxicity of the free, non-bound chemotherapeutic towards the chosen cancerous cell lines.

Owing to the fact that CHB is a weak acid and it contains in its molecular structure a carboxyl group, it was decided to immobilize this drug on the MNPs functionalized with APTES (MNPs[APTES]) by forming a salt with amine group according to the scheme shown in Figure 1. This method of the attachment to the MNPs is useful for in vitro studies as the drug can be easily and thoroughly released from the nanoparticles by decreasing the pH to highly acidic.

2 SYNTHESIS AND CHARACTERIZATION OF NANOSTRUCTURES

The chemicals were analytical grade and used as received without further purification. Chlorambucil and other reagents were purchased from Sigma-Aldrich (Poland), organic solvents from Avanter Performance Materials Poland S. A.

The magnetic nanoparticles Fe₃O₄ (MNPs) were synthesized using the chemical co-precipitative method [26]. However, the method was modified in terms of the reaction temperature as well as in terms of the concentration of the used ammonia solution. In the typical experimental procedure, 8.11 g of iron (III) chloride hexahydrate (FeCl₃·6H₂O) and 5.96 of iron (II) chloride tetrahydrate (FeCl₂·4H₂O) were added into a three-necked flask containing 200 ml of distilled water previously heated to 80°C. The system was purged with N₂ and the mixture of the iron solution was stirred followed by the slow addition of ammonia (NH₃·H₂O) to bring to pH = 9.0. The black MNPs precipitate was washed three times with distilled water and then with methanol. Next MNPs were suspended in 200 ml of 50 mM TMAOH and sonicated for 2 hours in an ultrasonic cleaner. The black MNPs precipitate was pulled away by a magnet, washed with distilled water and dried in a drying chamber at 50°C for 12 h.

MNPs[APTES] functionalization with APTES was carried out using the method used by Cao et al. [27]. Briefly, 600 mg of MNPs was dispersed into a mixture of 100 ml distilled water and 150 ml of isopropanol by ultrasonic vibration for 1 h. Then, 30 ml of concentrated (25%) NH₃ and 1.3 ml of APTES were added into the mixture under constant mechanical stirring and heating (55°C) for 20 hours. After the reaction, the brown precipitate was washed three times with water and next with methanol and dried at 35°C for 24 h in a drying chamber.

The effect of the surface functionalization of the MNPs with APTES was confirmed by FT-IR spectrum in the range of 4000–400 cm⁻¹ (Figure 2) (a Nicolet iS50 FT-IR spectrometer).

The intensive absorption band at 576 cm⁻¹ is associated with the metal–oxygen Fe–O bonds in the crystalline lattice of Fe₃O₄ (stretching vibrations). A band at 1629 cm⁻¹ and the broadband centred at 3412 cm⁻¹ derive from hydroxyl groups and are attributed to OH-bending and OH-stretching vibrations, respectively. The spectrum band at 3425 cm⁻¹ represents stretching vibrations of N–H, whereas the band at 1628 cm⁻¹ displays deformation vibrations of N–H from the amino group of APTES. The absorption bands at 2923 cm⁻¹ and 2850 cm⁻¹ derive from carbon aliphatic chains C–H (stretching vibrations). The band at 1008 cm⁻¹ comes from Si–O (stretching vibrations), whereas the band at 1440 cm⁻¹ represents stretching vibrations of C–N.

Subsequently, to attach chlorambucil to the nanostructures, 100 mg of MNPs[APTES] suspended in 10 ml of water was first sonicated for 15 minutes in an ultrasonic cleaner. Next, 100 mg of chlorambucil dissolved in 10 ml of isopropanol was added. The mixture was sonicated for 0.5 h, then stirred for 24 h in room temperature. After the reaction, the precipitate was pulled away with a magnet, washed once with water and dried in a drying chamber at 35°C for 12 h.

To determine the amount of the attached to the MNPs[APTES] chlorambucil (CHB), 5 mg of MNPs[APTES]CHB was placed in a test tube and 3 ml of the mixture 0.1 M KH₂SO₄ : Ethanol (1:1) was added. After sonification for 15 minutes in an ultrasonic cleaner, the MNPs[APTES]CHB was pulled away by a magnet, then 2 ml of the solution containing released CHB was centrifuged (13,000 rpm, 10 min, 25°C). The concentration of the released chemotherapeutic in the supernatant was measured using a UV–vis spectrophotometer at 258 nm (2450 UV–vis Shimadzu). The
results indicated that to the 1 mg of MNPs[APTES] 120 ± 7 µg of chlorambucil were attached (loading capacity of about 12%). Our method of the loading capacity quantitation turned out to be appropriate since all results were obtained with high reproducibility. Undoubtedly, the method can be used to determine the loading capacity for all drugs which are attached to the nanoparticles using electrostatic interactions on the condition that the resulted ionic bonds are formed by weak electrolytes.

The obtained nanostructures were also characterized by measurement their sizes (diameters) in methanol using the dynamic light scattering (DLS) analyzer (Malvern, Zetasizer Nano-ZS). The results indicated that the average diameter of naked MNPs was approximately 15 ± 2 nm, whereas APTES functionalized nanoparticles exhibited significantly larger sizes: 25 ± 3 nm as a consequence of multilayer coating by the silane. The size of MNPs[APES]CHB nanostructures was approximately 30 ± 3 nm. DLS results were confirmed by the scanning electron microscopy (SEM) imaging (FEI HELIOS NANOLAB 660) (Figure 3) and by atomic force microscopy (AFM) imaging (BioScope Catalyst Brucker) (Figure 4a–c). SEM and AFM images of the functionalized iron oxide nanoparticles show dense and uniformly packed structures.

Imaging of the obtained nanostructures using TEM microscopy was also performed. The sample was imaged using Tecnai G2 20 X-TWIN transmission electron microscope (from FEI), equipped with, among others, an electron gun with LaB6 cathode, a HAADF detector and an EDS (Energy Dispersive X-Ray Spectroscopy) spectrometer from EDAX with Si(Li) detector. Two preparations A and B were prepared. Preparation A: The sample was rubbed in an agate mortar in the presence of 99.8% ethanol (POCH) into a fine powder. The resulting powder was poured with 99.8% ethanol (POCH) to form a suspension and inserted into the homogenizer for 30 s. A small amount of the suspension was then applied to a copper mesh (300 mesh) coated with lacy formvar stabilized with carbon and left to evaporate ethanol. In addition, to depict Fe3O4 shells without interaction with the solvent and without the influence of ultrasound, preparation B was prepared by rubbing the sample in an agate mortar without ethanol, and then the powder was applied to identical mesh as in the case of preparation A, and the excess was blown with compressed air.

A majority of structures (core + shell) with sizes from over a dozen to about 20 nm were observed (Figures 5a, b). The sizes of the shells vary (from 1 nm to several nm), which is probably the effect of differences in the number of layers of polymerizing APTES. Additionally made EDS spectra confirmed the presence of elements of both the core (Fe, O) and the shell consisting of APTES and CHB (O, C, Cl, Si) (Figure 5c). The Cu signal present on the spectrum comes from the mesh. Nanostructures merge into larger aggregates.
Zeta-potential measurements were carried out depending on the pH value (citrate-phosphate buffer pH = 2.0, 5.0, 7.5 and borate buffer pH = 9.0), which is shown in Figure 6. MNPs displayed a negative surface charge across the whole pH range. MNPs coated with APTES showed a high positive Zeta-potential value (approximately +35 mV) at low pHs due to protonation of an amino group; however, at pH = 5.0, the change of positive charge to negative was observed. The surface charge for MNPs[APTES]CHB in the acidic range of pH was slightly positive. Above the pH = 5.0 the charge began to change towards negative values (approximately –13 ± 0.2 mV at pH = 7.5 and –25 ± 2 mV at pH = 9.0), which was undoubtedly a consequence of a high ionization degree of a carboxyl group (CHB).

To characterize the magnetic properties of MNPs and related nanostructures, a vibrating sample magnetometer (7407 LakeShore) was used to record hysteresis loops of the samples (Figure 7). According to this data, the saturation magnetization of pure MNPs was found to be approx. 60 emu/g, while the saturation magnetization of MNPs[APTES] and MNPs[APTES]CHB was approx. 54 and 49 emu/g, respectively. The coating substances diminish the force exerted by an external magnetic field on MNPs owing to the difficult alignment of magnetic domains in the material. Therefore, the magnetization of coated particles is lower than that of the uncoated ones.

3 | INVESTIGATION OF DRUG RELEASE

Since the effective release from the nanoparticles of the immobilized drug is essential for its proper activity, the investigation of CHB release degree from the MNPs[APTES] was investigated. 20 mg of MNPs[APTES]CHB was suspended in 2 ml of PBS buffer (pH = 7.45) and the sample was incubated at 37°C for 144 hours (6 days). At specified time intervals, the tested sample was centrifuged (5000 rpm, 10 min, 25°C) and the concentration of the released CHB was read on a UV–vis spectrophotometer (258 nm). To the remaining after centrifugation precipitate of the nanostructures 2 ml of fresh PBS buffer was added and the MNPs[APTES]CHB was carefully suspended (sonication in an ultrasonic cleaner, 20 seconds). Incubation at 37°C of the sample was continued until the next measurement. The experiments were performed three times with satisfactory reproducibility of results.

The results of the amount (in per cent) of CHB released from the MNPs[APTES] surface depending on time are shown.
in Figure 8. In the first 2 hours of the process the drug was released rapidly. After that time, approximately $62 \pm 3\%$ of the total amount of the immobilized CHB was detected in the buffer. Subsequently, the process was significantly slower as it was found that after 6 days, the amount of the drug released from MNPs[APES] increased by only about 4%.

Figure 9 shows the increase in concentration of the drug released. The kinetic process in the first 2 hours can be described by a first-order model and the equation can be
and Experimental Therapy, Polish Academy of Sciences) were
cultured at 37°C, in 5% CO2 and in a humidified atmosphere
in RPMI 1640 medium supplemented with 10% FBS, 2 mM
glutamine and 100 µg/ml penicillin and streptomycin (Sigma
Aldrich, USA).

In cytotoxicity studies, the MTT (3-(4,5-dimethylthiazol-2-
yl)-2,5-diphenyl tetrazolium bromide) assay was used, which
is one of the most popular tests to assess the activity of poten-
tial anticancer compounds, and it is also the most popular assay
for examining compound interactions. It was created and first
described by Mosmann in 1983 [28]. MTT is an indirect test to
determine cell viability by utilizing the ability of living cells to
catalyze reactions, yielding measurable product [29]. As a result
of succinate dehydrogenase activity, a mitochondrial enzyme
present in living cells, yellow, water-soluble, tetrazole salt (tri-
avazole blue formazan – MTT) is transformed into purple, water-
insoluble formazan crystals. The amount of crystals produced is
proportional to the number of living cells.

Tests were carried out in sterile 24-well plates. Cells in the log-
arithmetic growth phase were added on the plate in the amount
of 1 × 10^4/100 µl RPMI 1640 medium with 10% FCS/well.
Then the plates with cells were incubated for 24 h at 37°C,
5% CO2. After that time, MNPs[APTES], MNPs[APTES]CHB
and non-bound CHB respectively, were added to the cells and
incubated for 24 h (37°C, 5% CO2). Next, cell viability was
examined by adding MTT substrate (4 mg/ml of PBS) in the
amount of 200 µg/well. After 3 hours, the plates were cen-
trifuged (10 min, 30,000 rpm, room temperature). The super-
natant was removed, while the water-insoluble formazan crys-
tals were dissolved in 150 µl of DMSO. The amount of for-
mazan reduced by living cells was determined colourimetrically
at 570 nm. The experiments were performed four times with
satisfactory reproducibility of results.

Statistical analysis: Statistical analysis for the determination of
statistically significant differences between cytotoxicity of the
free and immobilized drug was performed using a Student’s t-
test (MS Excel, Microsoft Office 365), as the variable distribu-
tions in the case of the cytotoxicity results were normal. P value
below 0.05 was considered as statistically significant.

The results of the MNPs[APTES] cytotoxicity are shown in
Figure 10.

According to the Figure 10, magnetic nanoparticles coated
with APTES have no significant effect on the cells viability.
This allows to conclude that in the subsequent tests with the
use of MNPs[APTES]CHB and CHB, only the drug
causes cell’s death. The results of the cytotoxicity of the
MNPs[APTES]CHB and CHB are shown in Figures 11 and 12.

Cytotoxicity of CHB towards Jurkat cell line is significantly
lower in comparison to the immobilized drug. It is particularly
noticeable for the concentration C = 50 µg/ml of CHB: Viab-
ility of the cells was approx. 40% (CHB in unbound form),
whereas in the case of the immobilized chemotherapeutic only
approx. 10%. CHB at the concentration of 100 µg/ml caused
the death of about 85% of the cells, while in the case of
MNPs[APTES]CHB their viability declined virtually to zero.
IC_{50} for MNPs[APTES]CHB was found to be 18,4 µg/ml,
whereas for unbound CHB IC_{50} was found to be 42,6 µg/ml.

4 | CYTOTOXICITY STUDIES

The first step was to investigate whether magnetic nanoparticles
coated with APTES (MNPs[APTES]) have any effect on the
tested cell lines viability. Next, the effect of chlorambucil immo-
bilized on MNPs[APTES] and also the effect of non-bound
chlorambucil on cells viability was studied.

Cell cultures: The Jurkat T-cell line (German Collection
of Microorganisms and Cell Cultures, ATCC® Number TIB-152)
and HuT 78 (a gift from A. Miazek – Institute of Immunology

integrated into: ln(C) = −kt + ln C_0, where k is a rate constant
[1/h] and C_0 is related to the total amount of CHB immobilized
on MNPs[APTES] (t = 0). The experimental data indicated that
in the first hour (Δt = 0–1 h) the process occurred at the rate
constant k_1 = 0.848 [1/h], whereas in the next hour (Δt = 1–2 h)
at the rate constant k_2 = 0.105 [1/h]. After the third hour, the
kinetic process can be described by a zero-order model with the
rate constant k = 0.0761 [µg/ml]. Correlations coefficients of
the linear plots (R^2) were higher than 0.9.

FIGURE 8 Release degree of CHB from MNPs[APTES] in PBS buffer
(pH = 7.45) during six days. Data are presented as mean ± SD, n = 3

FIGURE 9 Increase of the concentration of CHB in PBS buffer
(pH = 7.45) as a consequence of releasing from MNPs[APTES]. Data are
presented as mean ± SD, n = 3
Cytotoxicity of CHB in its unbound form towards Hut 78 cell line (Figure 12) is rather low – up to the concentration equal 50 µg/ml, the drug caused the death of only approx. 10% of the cells. Only above this concentration, cell viability began to decline. On the contrary, in the case of MNPs[APTES]CHB, merely at the drug concentration of 10 µg/ml, cell viability was below 70%, and at the concentration $C = 100 \mu g/ml$ approx. 40%. IC$_{50}$ for immobilized CHB was found to be 46 µg/ml, while even the highest dose of free CHB used (100 µg/ml) did not lead to the death of at least 50% of the cells.

5 | CONCLUSION

In summary, we have successfully fabricated MNPs[APTES]CHB nanostructures and presented their characteristics. The process of the drug release from the nanoparticles proved to be very effective; after 2 hours approx. 60% of the total amount of the immobilized CHB was detected in buffer. The results of the cytotoxicity studies showed that in the case of the use of the immobilized chlorambucil on MNPs[APTES], the viability of the tested cancerous cell lines was significantly lower than when CHB in its unbound form was used. We believe that the reason for differences in the cytotoxicity of unbound and immobilized CHB is the difference in solubility of both forms of the drug. Solubility in water of CHB in its unbound form (powder) is low ($< 0.1 g/l$), while, as we recently reported [30], acidic (containing carboxyl group) drugs immobilized on MNPs[APTES] have much higher solubility while diffusing from the nanoparticles surface into the aqueous environment. Consequently, this will reduce the dose of the drug needed to achieve a therapeutic effect, and thus reduce side effects. In addition, drugs immobilized on magnetic nanoparticles can be transported to their targets quickly and selectively by using an external magnetic field [31–37]. In this way, the immobilized chemotherapeutic agent can be guided to the sites of metastases. Alternatively, MNPs[APTES]CHB can be used similarly to the unbound anticancer drugs in traditional chemotherapy – when administered intravenously, they act on the entire body. The advantage of the former is significantly higher, which would reduce the dose of the drug.

Unfortunately, our studies have shown that APTES functionalized nanoparticles with CHB attached exhibited a zeta potential value of about $-13 \text{ mV}$ at physiological pH, while the full stability of nanoparticles is provided by their at least $-30 \text{ mV}$ charge. Consequently, the stability of the obtained nanostructures is rather moderate. One of the ways to increase their stability is to stow them inside liposomes. The liposomes are stable at physiological pH, while they are degraded at the low pH of cancer cells to release drugs in the tumour area [38–39]. Another important issue to remember when using nanoparticles in vivo is that functionalized MNPs are subject to opsonization when entering the bloodstream. This process is the non-specific adhesion of plasma proteins on the surface of the MNPs and then internalization by the RES [40]. To prevent MNPs from opsonization and to increase their ability to escape the RES, MNPs size distribution must be optimized. Indeed MNPs suspensions with mean hydrodynamic size in the 10–100 nm range is optimal for a good biodistribution and in vivo delivery,
while the smaller (< 10 nm) are rapidly cleared by the renal system, and the larger (> 200 nm) are rapidly sequestered by the RES [41, 42]. Ergo designed by us nanostructures with a diameter of about 30 nm are in the optimal range presented above. Endothelial fenestrae in various types of blood vessels is also an important point in this topic. In the area of the nervous system endothelial cells of the vessels adhere tightly together, whereas, in the area of muscles, lungs and skin, the fenestrae are about 6 nm. Larger fenestrae (50-60 nm) are found around the kidneys, intestines and some glands [43]. The greatest fenestrae in the endothelial tissues occur around the bone marrow, liver and spleen [44]. These latter parts of the body are also rich in macrophages, which answer for clearing the body. This is why most nanoparticles eventually reach the liver and spleen, where they are accumulated and in the case of iron oxide nanoparticles are further broken down into free iron and excreted from the body [41]. However, the above data pertain to a healthy organism. In the case of neoplastic diseases, MNPs can flow throughout the blood for a long time enough to allow them to passively penetrate through the fenestrations, which are typically in the range of 200-600 nm, of leaky vasculature in correspondence to the tumour tissue [45]. The selectivity of targeting is essentially due to the absence of such fenestration in healthy tissues. In conclusion, this work will benefit the development of novel drug delivery systems.

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