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# PhD THESIS

## ALTERNATIVE, NON-INVAZIVE AND NON- TOXIC THERAPY OF BREAST CANCER BY SUPERPARAMAGNETIC HYPERTHERMIA WITH BIOCOMPATIBLE SPION–γ-CYCLODEXTRIN NANOPARTICLES

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**CONTENT**

## SUMMARY

The Ph.D. thesis, having the title "*Alternative, non-invasive and non-toxic therapy of breast cancer by superparamagnetic hyperthermia with biocompatible SPION–γ-cyclodextrins nanoparticles"*, is a research study (see table of contents) on alternative cancer therapy by superparamagnetic hyperthermia (SPMHT), using SPION magnetic nanoparticles (superparamagnetic iron oxide nanoparticles) of $Fe_3O_4$ (magnetite) biocompatible by coating with gamma-cyclodextrins (γ-CDs), as a non-invasive technique, more effective and without toxicity, compared to the conventional techniques widely used today in this field, chemo- and radiotherapy, which have a high degree of toxicity on the body and in many cases are ineffective in treating cancer. This method is an alternative to chemo- and radiotherapy and is non-invasive and non-toxic by using a low-amplitude alternating magnetic field, with a frequency in the range of hundreds of kHz, and biocompatible magnetic iron oxide nanoparticles (SPIONs), having great potential in future cancer therapy without radiation and chemotherapy, as shown by current studies in this field, both *in vitro* and *in vivo*, and even the onset of clinical trials.

For testing *in vitro* of this alternative therapy, we chose breast cancer, due to its increased incidence among the population of women and the high degree of mortality, according to the World Health Organization.

The topic addressed is very current in the field of alternative therapy of cancer, and maybe the method of the future in the treatment of cancer, according to the opinions of researchers in the field, precisely because of its non-invasive, non-toxic nature and increased effectiveness in destroying tumor cells, reaching in some *in vitro* and *in vivo* experiments even to destruction of the tumor by using a natural thermal effect in therapy: increasing the temperature in the tumor to 42-43°C under the action of the magnetic field, which leads to the irreversible destruction of cancer cells by apoptosis.

This topic is part of the current global advanced research in the field of cancer and, at the same time, the current scientific concerns of the research group in which I worked, recently embodied in a research project PN-III (2019-2022), Experimental Demonstration Project (PED). Timișoara (director, Prof. Dr. Dr. Habil. Caizer Costică) and partner University of Medicine and Pharmacy "Victor Babeș" Timișoara (partner project manager, Prof. Dr. Șoica Codruța, collaborator Prof. Dr. Dehelean Cristina).

The general objective of the doctoral thesis was the study of the possibility of using superparamagnetic hyperthermia, as an alternative and non-invasive method, in cancer therapy with increased efficiency and without toxicity, using biocompatible magnetic SPION nanoparticles of $Fe_3O_4$ bioconjugated with gamma-cyclodextrins (γ-CDs), and as operational objectives, for the implementation of the general objective, the following:

- *increasing efficiency* in superparamagnetic hyperthermia for cancer therapy, by finding the size and concentration of SPIONs magnetic nanoparticles, the external alternating magnetic field parameters, the application protocol, and the treatment duration, most suitable for this therapy;
- *reduction* or even the elimination of cellular toxicity in this therapy, by using SPIONs nanoparticles coated with gamma-cyclodextrins;
- *in vitro therapy of breast cancer*, with high efficacy and no toxicity.

Thus, through this study, we aimed to:

- the influence of the organic gamma-cyclodextrins (γ-CDs) layer on the maximum specific loss power in superparamagnetic hyperthermia, which leads to the heating of SPION magnetic nanoparticles to the therapy temperature (42.5-43°C), necessary for the destruction of tumor cells by apoptosis;
- finding the optimal conditions in which superparamagnetic hyperthermia with magnetite nanoparticles, biocompatible by decorating their surface with gamma-cyclodextrins ($Fe_3O_4$–γ-CDs), can be used to achieve maximum effectiveness in destroying tumor cells;
- determination of the maximum specific loss power generated in superparamagnetic hyperthermia in the case of $CoFe_2O_4$ nanoparticles covered with γ-CDs (core-shell nanostructure of $CoFe_2O_4$–γ-CDs);
- finding the conditions in which the specific loss power becomes maximum in the case of $CoFe_2O_4$ nanoparticles–γ-CDs, depending on the size of the nanoparticles, the thickness of the biocompatible layer on the surface of the nanoparticles, and the parameters of the alternating magnetic field;
- the influence of amplitudes and the frequency of the alternating magnetic field and the concentration of magnetic nanoparticles on the maximum specific loss power in superparamagnetic hyperthermia with bionanoparticles SPION-γ-CDs;
- the use of $Fe_3O_4$-PAA nanobioconjugates in superparamagnetic hyperthermia–(HP-γ-CDs) (PAA - polyacrylic acid, HP-γ-CDs - hydroxypropyl gamma-cyclodextrins) in a 'core-shell' hybrid structure ('core' - inorganic nanoparticles of $Fe_3O_4$ and 'shell' - the organic layer of PAA–(HP-γ-CDs from the surface of magnetic nanoparticles), with very good heat dissipation and biocompatibility characteristics, for maximum efficacy and minimum toxicity;
- finding suitable physicochemical characteristics for $Fe_3O_4$ nanoparticles, $Fe_3O_4$-PAA, and $Fe_3O_4$-PAA nanobioconjugates–(HP-γ-CDs) to form stable magnetic suspensions and be suitable for superparamagnetic hyperthermia, using 3D computational simulation to evaluate the maximum specific loss power (SLP) leading to heating of the magnetic nanoparticles;
- obtaining superparamagnetic hyperthermia, both in adiabatic and *in vitro* conditions, using $Fe_3O_4$-PAA nanobioconjugates–(HP-γ-CDs) as magnetic samples;
- evaluation of the biological profile of $Fe_3O_4$-PAA magnetic nanoparticles and $Fe_3O_4$-PAA nanobioconjugates–(HP-γ-CDs), both under standard conditions (37°C), as well as induced by magnetic hyperthermia (43°C), using an *in vitro* model based on healthy human cells HaCaT (human

keratinocytes) and using the most established technique for evaluating cellular toxicity *in vitro* – MTT analysis (colorimetric test for the evaluation of cellular metabolic activity);

- finding the maximum admissible biological limit for the external alternating magnetic field, without cellular damage, for which superparamagnetic hyperthermia can be safely applied in vitro;
- establishing the protocol for the safe *in vitro* application of superparamagnetic hyperthermia with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs);
- the application of superparamagnetic hyperthermia *in vitro* with nano-bioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) for effective therapy of human breast adenocarcinoma (MCF-7) cancer cells.

To achieve the objectives proposed in the thesis research, I first studied theoretically (computational), using professional software with 3D representation through simulation of real experimental conditions, the conditions under which the maximum dissipated power can be obtained in superparamagnetic hyperthermia with SPION nanoparticles of $Fe_3O_4$ coated with γ-cyclodextrins ($Fe_3O_4$-γ-CDs), which will then lead to the efficient heating of the magnetic nanoparticles at a temperature of 42.5°C, required for tumor therapy. The preliminary results obtained at this stage (chapters 3-4) were extremely useful and were later used for the experimental part (chapters 5-6), regarding the effective realization of superparamagnetic hyperthermia *in vitro* with nanobioconjugates of $Fe_3O_4$-PAA-(HP-γ-CDs) for the therapy of MCF-7 breast tumors (human breast adenocarcinoma), since through theoretical study we established the conditions that must be achieved experimentally to obtain magnetic hyperthermia with maximum effectiveness.

The doctoral thesis is structured into 6 Chapters, an introduction, and conclusions (another distinct chapter, which also contains the personal contributions of the doctoral student).

The first two Chapters, having the titles "*Current methods and techniques for alternative cancer therapy"* and "*Superparamagnetic hyperthermia with SPMNP magnetic nanoparticles as an alternative and non-invasive method in cancer therapy: in vitro, in vivo results and clinical trials"*, contain the general part of the thesis, being a documented synthesis regarding the issue of research in the field of magnetic/superparamagnetic hyperthermia, as an alternative method for *in vitro* and *in vivo* cancer therapy, as well as the current state in this field. The following conclusions emerged from these studies, very important for future research.

The field of alternative cancer therapy, especially nanomedicine with magnetic nanoparticles, is one in continuous development and is gaining more and more interest compared to classical methods, radio- and chemotherapy, which have a high level of toxicity. Among the alternative methods for cancer therapy, superparamagnetic hyperthermia (SPMHT) has been established as the most suitable for this type of therapy, due to its increased efficiency regarding the dissipated power and heating of magnetic nanoparticles, and the reduced toxicity, as a result of the use of nanoparticles very small magnetic fields (<15-20 nm) and harmonic alternating magnetic fields of low amplitude (1-30 kA/m), with frequency in the range of hundreds of kHz (100-500 kHz). However, although this method has

evolved a lot in the last two decades, there are still issues that need to be clarified through research, before it is a feasible therapy for clinical trials, and then successfully applied in human cancer therapy. These problems would be:

(i) finding the most suitable magnetic nanoparticles, as type (chemical composition), size, shape, and magnetic behavior, in terms of efficiency in superparamagnetic hyperthermia and toxicity;
(ii) the best biocompatibility offered by the organic layer on the surface of magnetic nanoparticles, and the stability of nanoparticles in colloidal suspension;
(iii) suitable biofunctionalization of magnetic nanoparticles to have an affinity towards tumor cells;
(iv) finding suitable parameters for the magnetic field in SPMHT;
(v) finding the optimal conditions for the effective implementation of SPMHT, the doses and times of exposure to the magnetic field for this type of therapy;
(vi) the clinical implementation of SPMHT, through preliminary studies *in vitro*, *in vivo*, and finally in clinical trials, with maximum efficacy on tumors and minimal toxicity, or even without toxicity.

Conformable studies and results obtained in vitro, *in vivo*, and in the early stage of clinical trials, superparamagnetic hyperthermia has proven to be a feasible method as an alternative and non-invasive therapy in the treatment of cancer, compared to conventional techniques (radio- and chemotherapy), which have a degree high toxicity. But, at the same time, additional research is needed to standardize the method for its application and with maximum effectiveness in destroying tumor cells, especially in the case of human subjects (in clinical trials), so that a protocol can be developed to apply them in clinical therapies.

The next four Chapters (Chapters 3–6) contain the methodology and research results obtained within the thesis, both theoretical (Chapters 3-4) and experimental (Chapters 5-6). Each of the four chapters with results begins with a documented introduction to the issue addressed in the context of current international research in the field, at the same time establishing the motivation and purpose/objectives of the study done.

Chapter 3, entitled "*Contributions on the maximum specific loss power in superparamagnetic hyperthermia with biocompatible SPION nanoparticles of $Fe_3O_4$ decorated with γ-cyclodextrins for alternative cancer therapy*", contains a theoretical study (computational 3D) referring to maximum loss power in superparamagnetic hyperthermia with biocompatible $Fe_3O_4$ SPION nanoparticles decorated with γ-cyclodextrins (γ-CDs), for effective and non-toxic alternative cancer therapy [223]. This study establishes the optimal conditions under which superparamagnetic hyperthermia can be achieved with such nanoparticles, by studying the maximum specific loss power, to be able to achieve efficient heating of the nanoparticles at the temperature of 42.5°C necessary in cancer hyperthermia (irreversible destruction of tumor cells by apoptosis due to high temperature). The following points emerged from this study.

In superparamagnetic hyperthermia with $Fe_3O_4$ coated SPION nanoparticles γ-CDs specific loss power is determined, both by Néel magnetic relaxation processes and by Brown relaxation processes, as a result of the organic layer on the surface of the nanoparticles which determines a hydrodynamic diameter. Néel relaxation processes are dominant at higher frequencies, towards 400–500 kHz, and at lower frequencies, <150 kHz, both Néel and Brown relaxation processes are present in approximately equal proportions.

As a result of the presence of the layer of γ-CDs on the surface of $Fe_3O_4$ nanoparticles, which contribute significantly to the maximum of Brown relaxation at lower frequencies, the maximum of the specific loss power obtained under optimal conditions for the admissible limit frequency in superparamagnetic hyperthermia with bionanoparticles of $Fe_3O_4$-γ-CDs move in the frequency range 100-1000 kHz, from the nanoparticle diameter of 18.1 nm at 100 kHz to the diameter of 15.5 nm at 1000 kHz, a shift that is not influenced by the size of the magnetic field.

Maximum specific loss power in superparamagnetic hyperthermia with $Fe_3O_4$- nanoparticles γ-CDs increases approximately linearly, both with increasing magnetic field in the range 5-50 kA/m and with increasing frequency in the range 100-500 kHz.

At frequencies lower than 250 kHz, due to the presence of Brownian relaxation, magnetic hyperthermia can be obtained in a wider range of values for nanoparticle diameters, extending from 16.9 nm to 25-30 nm. Although the maximum specific loss power decreases at these larger diameter values, it is still sufficient to heat the nanoparticles to the optimal temperature of 42.5–43°C required for the destruction of tumor cells by apoptosis.

For diameters greater than 18.5-19 nm, magnetic hyperthermia is obtained exclusively through Brown relaxation processes, Néel relaxation processes being negligible in this nanoparticle size range. This is a major advantage in practice, in the real case of the existence of nanoparticle size distributions, which is usually obtained by the preparation methods of the nanoparticles, since the diameter of the nanoparticles, in this case, is no longer a critical parameter and, therefore, no longer has to have a strict value (e.g. the value of 17.1 nm at the frequency of 200 kHz to obtain the maximum dissipated power). Thus, at low frequencies, wide distributions of nanoparticle sizes can practically be used to obtain magnetic hyperthermia through Brownian relaxation.

Under optimal conditions and for the permissible biological limit for field and frequency, the maximum specific loss power in superparamagnetic hyperthermia with bionanoparticles $Fe_3O_4$-γ-CDs is obtained for the magnetic field in the range of 10-25 kA/m, and the frequency in the range of 200-500 kHz, with nanoparticle diameters in the range of 16.2-17.1 nm.

This study [223] allows the practical implementation of superparamagnetic hyperthermia under optimal conditions, using biocompatible SPION nanoparticles of $Fe_3O_4$ coated with γ-CDs ($Fe_3O_4$-γ-CDs), to achieve maximum efficiency and lack of cellular toxicity.

Aiming to achieve intracellular (inside the cells) hyperthermic therapy, which is more effective in destroying tumor cells, in chapter 4, entitled "*Contributions regarding superparamagnetic hyperthermia with biocompatible SPION nanoparticles of $CoFe_2O_4$–γ-CDs for application in alternative cancer therapy*", present the achievement of superparamagnetic hyperthermia with $CoFe_2O_4$ nanoparticles coated with γ-CDs, obtained by substituting the $Fe^{2+}$ ion in the structure of the magnetite molecule ($Fe_3O_4$) with the $Co^{2+}$ ion. The study done on the specific loss power that leads to the heating of nanoparticles, and which is the basic indicator in establishing the efficiency in magnetic hyperthermia, led to special and different results compared to those obtained in the case of $Fe_3O_4$–γ-CDs nanoparticles, with high potential in effective cancer therapy, and without toxicity. The conclusions of this study are reproduced below.

$CoFe_2O_4$–γ-CDs bionanoparticles can be successfully used in superparamagnetic hyperthermia for alternative cancer therapy ([279]) *in vitro* and *in vivo* and in the future in clinical trials due to increased efficacy on tumors and non-toxicity on healthy cells.

Specific loss power in superparamagnetic hyperthermia with $CoFe_2O_4$-γ-CDs nanoparticles in suspension is obtained exclusively by Brownian relaxation processes, which leads to at least the following benefits for magnetic hyperthermia:

(i) the existence of the diameter of nanoparticles leading to the maximum specific dissipated power, in a wide range of values ~(9-14) nm (depending on the amplitude and frequency of the magnetic field), simultaneously with the significant increase of the maximum specific loss power (increasing the efficiency and effectiveness in therapy ); this diameter in the ~(9-14) nm range is more suitable for superparamagnetic hyperthermia compared to the diameter of ~(6-6.5) nm obtained in the case of $CoFe_2O_4$ nanoparticles and Néel relaxation;

(ii) the diameter of nanoparticles, in this case, is no longer a critical parameter (as in the case of Néel relaxation), this being a major advantage in the practical implementation of magnetic hyperthermia, including the use of nanoparticles with wide diameter distributions;

(iii) the possibility of using larger magnetic fields, not being limited to small magnetic fields, as in the case of Néel relaxation, which practically leads to the possibility of obtaining significantly higher specific dissipated powers in superparamagnetic hyperthermia, with beneficial effects in hyperthermia.

However, the maximum specific loss power is indicated to be obtained up to the maximum frequency of 1000 kHz, where a maximum power saturation effect occurs, and optimal for the range ~(200-500) kHz, depending mainly on the amplitude of the magnetic field.

Changing the amplitude of the external magnetic field radically changes the value of the optimal diameter, which gives the maximum of the specific loss power in superparamagnetic hyperthermia, in contrast to the cases when the maximum of the power is determined by Néel relaxation, a situation in which the optimal diameter does not change.

The present of γ-CDs on the surface of $CoFe_2O_4$ nanoparticles, in addition to ensuring biocompatibility and cellular non-toxicity in magnetic hyperthermia, and eliminating the interactions between magnetic nanoparticles, which are major benefits in magnetic hyperthermia, also allows a significant increase in the packing volume fraction of nanoparticles (increasing the concentration nanoparticles). This leads to a significant increase of the specific loss power and the heating temperature in this case (the efficiency in magnetic hyperthermia), compared to the use of other larger bionanostructures, for example, liposomes (with the size of tens – hundreds of nm), which decrease significantly effectiveness in magnetic hyperthermia.

The obtained results ([279]) allow the practical implementation under optimal conditions of superparamagnetic hyperthermia using bionanoparticles of $CoFe_2O_4$–γ-CDs, to increase efficiency and effectiveness in cancer therapy without cytotoxicity.

The theoretical studies presented in Chapters 3 and 4 constitute the basis for the subsequent experimental studies, presented in Chapters 5 and 6, regarding the achievement of superparamagnetic hyperthermia in optimal experimental conditions *in vitro* with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs), for the effective and non-toxic therapy of MCF-7 breast cancer (human breast adenocarcinoma).

Thus, in Chapter 5, with the title "*Contributions regarding the increase of effectiveness in superparamagnetic hyperthermia with biocompatible SPION nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) for alternative cancer therapy without toxicity*", the experimental results obtained after the application are presented superparamagnetic hyperthermia with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) (PAA - polyacrylic acid, HP-γ-CDs - hydroxypropyl gamma-cyclodextrins)on the healthy human keratinocyte cell line HaCaT, showing the efficiency and non-toxicity of the method on healthy cells. Through this study, the following conclusions were drawn.

Nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs), with "core-shell" structure, having the average diameter of the magnetic core (core) of the $Fe_3O_4$ nanoparticles of ~16 nm, covered with the thin organic layer (shell) of PAA–(HP-γ-CDs), ~2 nm thick, which provides very good cellular biocompatibility after testing on healthy human HaCaT cells (cell viability around 100% for concentrations up to 1 mg/mL), is the most suitable nanobiostructure to be used in superparamagnetic hyperthermia of tumors, with increased efficiency and no toxicity ([345]).

The organic layer of PAA–(HP-γ-CDs) from the surface of $Fe_3O_4$ magnetic nanoparticles, with a reduced thickness of only ~2 nm, in addition to the very good biocompatibility created by the magnetic nanoparticles, also allows a significant increase (up to an eventual acceptable upper limit of toxicity) of the packing volume fraction of nanobioconjugates (concentration), respectively of $Fe_3O_4$ nanoparticles in the injectable suspension, to obtain superparamagnetic hyperthermia. It will lead to a significant increase in the specific loss power (SLP), and implicitly in the heating temperature, while reducing the heating time to the optimal value of ~42.5°C used in tumor hyperthermia. In other words,

reducing the thickness of the biocompatible organic layer from the surface of the magnetic nanoparticles to the acceptable limit of 1.5-2 nm allows for increasing the efficiency in obtaining the hyperthermic effect in superparamagnetic hyperthermia, compared to the case of other large biostructures (e.g. liposomes or others), where efficiency is reduced.

$Fe_3O_4$ nanoparticles, having an average diameter of ~16 nm, bioconjugates with γ-CDs branched using PAA biopolymer ($Fe_3O_4$-PAA–(HP-γ-CDs)), up to a concentration of 10 mg/mL in suspension have no cellular toxicity and therefore can be successfully used in superparamagnetic hyperthermia with increased efficiency.

In the *in vitro* experimental conditions established as optimal, the temperature of 42.5°C required in the magnetic hyperthermia of tumors is reached in a relatively short time interval, i.e. only 23 min., a duration that does not affect healthy tissues in the therapy.

*In vitro* application for 30 min. of superparamagnetic hyperthermia with $Fe_3O_4$-PAA–(HP-γ-CDs) nanobioconjugates and the parameters established for the external alternating magnetic field at the permissible biological limit, do not affect healthy cells of human keratinocytes HaCaT (in suspension); superparamagnetic hyperthermia was experimentally obtained in the magnetic field of 15.92 kA/m and the frequency of 312.2 kHz, for nanoparticles with the average diameter of the magnetic core of 15.8 nm, which give the maximum hyperthermic effect, leading to the temperature of 42,5°C necessary in the therapy of tumors in a short time, so that healthy cells are not affected.

Ferrimagnetic nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) can be safely used in superparamagnetic hyperthermia (without toxicity) and with high efficiency due to the existence of PAA–(HP-γ-CDs) organic layer from the surface of magnetic nanoparticles, which is suitable for this therapeutic technique. The biological evaluation shows no cytotoxicity of $Fe_3O_4$-PAA nanoparticles and $Fe_3O_4$-PAA–(HP-γ-CDs) nanobioconjugates on HaCaT cells after an interval of 24 h, both for standard conditions (37°C), as well as in the conditions of magnetic hyperthermia (42.5°C).

The study done ([345]) establishes the conditions under which superparamagnetic hyperthermia can be optimally and safely applied *in vitro*, using $Fe_3O_4$-PAA–(HP-γ-CDs) nanobioconjugates, more effective and without cellular toxicity, to be used in future cancer therapy.

In Chapter 6, the experimental results obtained following the *in vitro* application of superparamagnetic hyperthermia with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) for the therapy of MCF-7 human breast adenocarcinoma (human breast cancer cells). The results demonstrated the high efficacy in breast cancer therapy (95.11%) of this alternative, non-invasive, and non-toxic method. The conclusions resulting from this study are reproduced below.

Superparamagnetic hyperthermia therapy (SPMHT) with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs), having the average diameter of the $Fe_3O_4$ nanoparticles of ~16 nm and the average hydrodynamic diameter of the nanobioconjugates of ~20 nm, leads to the destruction of MCF-7 breast cancer cells by apoptosis in a very high percentage, of 95.11 % ( cell viability of 4.89% compared to

standard conditions), after 24 h of treatment, under the following conditions: therapy duration of 30 min., therapy temperature of 42.9°C, the applied harmonic alternating magnetic field with the amplitude of 160 Gs (12.73 kA/m) and the frequency of 312.2 kHz, the concentration of 10 mg/mL of the magnetic nanoparticles from the nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) suspended in the cell culture medium.

The very good efficacy obtained in breast cancer therapy in vitro, by applying superparamagnetic hyperthermia using nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs), is due to the optimal experimental conditions we found for the realization of SPMHT therapy: the optimal size of $Fe_3O_4$ nanoparticles, the effective concentration of magnetic nanoparticles, the use of branched cyclodextrins HP-γ–CDs without toxicity, for the coating of $Fe_3O_4$ magnetic nanoparticles, the very small thickness (~2 nm) of the organic layer on the surface of $Fe_3O_4$ magnetic SPION nanoparticles, the suitable external alternating magnetic field magnitude and frequency, and the duration of therapy without cellular damage ([223, 345, 414]).

The maximum permissible biological limit for the applied magnetic field, without cellular damage, can be significantly extended, at the value of $H \times f \sim 9.5 \times 10^9$ $Am^{-1}Hz$ in the case of MCF-7 mammary cells, a limit which is double the one known so far ($5 \times 10^9$ $Am^{-1}Hz$) in magnetic hyperthermia experiments. The obtained result is very important for the practical implementation of superparamagnetic hyperthermia (SPMHT) *in vitro* and, in the future, *in vivo*, as it allows the significant increase of the magnetic field that can be used safely. At the same time, this allows for a significant reduction in the concentration of magnetic nanoparticles that will be used in superparamagnetic hyperthermia therapy, with a major beneficial effect on the elimination of cytotoxicity in this therapy.

The percentage of destruction of cancer cells decreases slightly after the application of superparamagnetic hyperthermia when the concentration of magnetic nanoparticles decreases from 10 mg/mL to 5 mg/mL and 1 mg/mL, respectively, namely: to 90.91 % (cell viability of 9.09 %) and 88.68 % (cell viability of 11.32 %), respectively, but percentages remain at rather high levels.

However, even in these cases, anticancer therapy by superparamagnetic hyperthermia can remain as effective as in the case of the concentration of 10 mg/mL, by repeating the therapy session after 24 h, or even increasing the duration of therapy beyond 30 min., as was used in the experiment. Thus, the effectiveness of destroying cancer cells could be increased even up to 100%.

The thesis ends with the conclusions that contain the significant results obtained as a result of this study done within the thesis, being also marked as the personal, original contributions of the doctoral student.

In conclusion, it can be said that superparamagnetic hyperthermia with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs is a non-invasive, viable technique for the effective and non-toxic alternative therapy of breast cancer *in vitro* ([414]).

In addition, the obtained results show the viability of superparamagnetic hyperthermia with nanobioconjugates of $Fe_3O_4$-PAA–(HP-γ-CDs) in alternative cancer therapy. At the same time, the obtained results open new directions of research, such as therapy for other types of *in vitro* cancers (with an increased incidence among the population and high degree of mortality, such as malignant melanoma, prostate cancer, glioblastoma, etc.) and then *in vivo*, in an animal model, and prospectively in human clinical trials.

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