Short Thesis for the Degree of Doctor of Philosophy (PhD)

Preparation and *in vitro, in vivo* investigation of nanoparticles for tumor specific targeted delivery of MR contrast agents

by **István Hajdu**

Supervisors:
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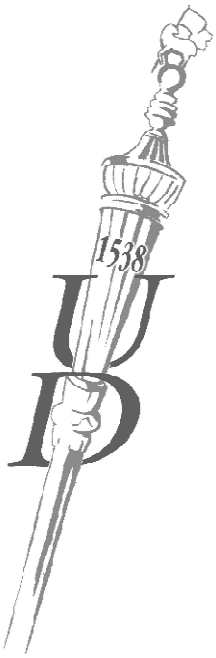

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

Debrecen
2014

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The Examination takes place at the Discussion Room (LSB 3.016) Department of Medical Chemistry, Faculty of Medicine, University of Debrecen
at 11:00 AM on September 8, 2014

Head of the **Defense Committee:** Pál Gergely PhD, DSc, member of the HAS
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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1:00 PM on September 8, 2014

Introduction

Recent advances in the understanding of biopolymer chemistry made it possible for me to choose an interdisciplinary topic for my PhD thesis. Spanning the fields of nanotechnology and molecular imaging, my thesis comprises a practical application of the chemistry of nanotechnology for a medical diagnosis, i.e., the development of MR contrast agents. Although MR contrast agents are widely used to increase the sensitivity of MR imaging, the need for more efficacious and safer MR imaging agents exists. The need for improved MR contrast agents is increasing as a result of the growth in the use of MR for earlier medical intervention.

The biomedical application of biocompatible, biodegradable macromolecules has recently attracted increased interest due to recent advances in the understanding of the chemistry of these materials combined with the need for materials that can safely be incorporated for chronic human use such as tissue regeneration and implantation as well as for drug delivery. The development of better, safer MR contrast agents falls neatly into this category as well.

In the field of cancer, earlier diagnosis has been proven to result in higher cure rates. Current MR contrast agents improve imaging but further improvements can be envisioned by the development of T1 and T2 MR contrast agents that are targeted to tumor cells. The ability to target tumors while protecting healthy cells would provide improved imaging and earlier detection while minimizing side effects due to exposure of healthy cells to the toxic effects of contrast agents.

In addition to molecular targeting, nanoparticles are hypothesized to contribute to the protection of healthy cells from the non-specific toxic effects of contrast agents by increased penetration of the “leaky” vasculature of tumor cells compared with healthy tissue.

With this end in view the aim of my research work was to develop biopolymer-based nanosystems that can be used as tumor specific MR contrast agents. The biopolymers retain their favorable biological properties after formation of nanoparticles, which as nanocarriers are suitable for tumor specific delivery of paramagnetic or superparamagnetic ligands.

Background

Biopolymers

The research topic of my thesis spans several scientific disciplines, all of which deal with biopolymers. In the narrow sense, biopolymers are the macromolecules which are biodegradable and are produced by living organisms.

Chitosan (CH) is one such biopolymer. It is a linear polysaccharide, a deacetylated derivative of chitin, the second most abundant biopolymer in nature.

CH has several favorable biological properties: it is biocompatible, biodegradable, non-toxic, renewable and has fungicidal and antibacterial properties.

Poly-gamma-glutamic acid (PGA) also corresponds to definition of a biopolymer in a narrower sense. It is an anionic polypeptide, consists of repetitive D- and L-glutamic acid units connected by amide linkages between α-amino and γ-carboxylic acid functional groups. PGA is a water soluble, biocompatible, biodegradable, non-toxic biopolymer. PGA can be produced by microorganisms using bacterial fermentation.

Biopolymer-based nanosystems

Several branches of research can be distinguished for the development of nanosystems and nanoparticles containing biopolymers. These systems can be sorted into two groups according to the role of the biopolymer: (i) nanoparticles formed from a biopolymer with other polymers/ biopolymers/ ingredients, or (ii) inorganic nanoparticles (for example metal, metal oxide or carbon) covered by biopolymers. There is increasing interest in development of both types of nanosystems.

Many recent attempts have been made to prepare biopolymer-based nanosystems developed for biomedical and pharmaceutical applications, which are biocompatible, biodegradable and suitable for specific applications, such as drug delivery.

CH and PGA are water soluble biopolymers with reactive functional groups. Under specific conditions they can behave as polyelectrolytes and can form colloid systems such as particles, films, gels, or composites by ion-ion interactions.

Preparation and investigation of several self-assembled CH/PGA nanoparticles have been published in the literature. These nanosystems were developed primarily for delivery of active substances. Numerous scientific article describe gene-, contrast agent- or other drug delivery by these nanosystems.

Targeted nanosystems

Recently there has been increasing scientific interest the possibility of the application of nanosystems as contrast agents in medical imaging.

The use of the nanosystems in medical imaging offers the possibility of improved sensitivity and safety as well as the combination of different imaging techniques to increase early stage tumor diagnosis leading to effective tumor therapy.

Targeted delivery is an emerging platform in nanomedicine, therefore many studies have focused on the development of efficient targeted delivery systems and their applications in medicine.

It has been demonstrated in the literature that tumor targeting can be achieved by coupling targeting ligands to a drug delivery system. Targeting can be conferred by ligand-receptor, antibody-cognate or other molecular interactions.

Contrast agents

In radiology, contrast means the difference between the darkest and brightest points of an image. The detectable difference between signal intensities induces an optical stimulus that makes diagnosis possible. Enhancement of this contrast is often necessary during the imaging processes, which can be obtained by the use of contrast agents.

“Contrast agents are the materials which are used for imaging processes and are drugs in the legal sense as well as altering the detectable signs to improve the diagnostic efficacy.”

Contrast agents can be sorted into three groups: X-ray, ultrasound and MR contrast agents. These agents possess different physical and chemical properties, meet the requirements of these 3 imaging techniques and enhance their sensitivity.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) have gained ground in nuclear medicine and are effective anatomical imaging techniques. These imaging techniques show the metabolism of tissues using radioactively labeled substrates which can differentiate transformed from healthy tissue and therefore suggest morphological changes.

“Hybrid” or “fusion” imaging techniques have recently been introduced to combine the most effective attributes of each technique for more effective early diagnosis. For example SPECT/CT, PET/CT, PET/MR are being increasing combined for this purpose. In cancer staging, these fusion imaging techniques provide metabolic and anatomic images at the same time to facilitate early stage tumor diagnosis.

MR contrast agents

In addition to the need for increased sensitivity in diagnostic images of solid tumors, reduction of side effects is a desired goal for the next generation of MR contrast agents.

Nanoparticles offer particular promise for MR contrast agent safety due to the well understood “leakiness” of tumor agent vasculature, which allows passive targeting of nanoparticles compared with small or large molecules such as standard MR contrast agents.

Magnetic resonance imaging (MRI) is one of the most important diagnostic imaging techniques. It has the advantages that it is noninvasive and provides excellent soft-tissue imaging contrast. MRI has developed rapidly and has become especially useful in the diagnosis and treatment of neurological, cardiovascular and oncological diseases.

It is important that MR images are hard-contrast and sensitive, with high stereoscopic (3 dimensional) resolution. Pathological changes can be detected via physico-chemical differences, which can be visualized by varying light intensity (on the gray scale).

The resolution and sensitivity of MRI can be enhanced by using intravascular contrast agents. Superparamagnetic and paramagnetic materials can be utilized as contrast agents, since they change the homogeneity of the magnetic field and alter the relaxation time of the tissue where they reside, producing hard-contrast images.

Although Gd ions as free metal ions are toxic, they can become non-toxic when complexed. Consequently both low molecular weight and macromolecular carrier moieties have attracted considerable interest based on their ability to improve MRI signals.

Low-molecular weight Gd chelates, however, have serious shortcomings, such as a short biological half-life in the blood, rapid diffusion out of the blood and excretion through the kidney, can’t be ignored. In an effort to overcome these shortcomings, several macromolecular carriers have been developed for delivery of paramagnetic Gd ions.

Ideally, a polymer-based MRI contrast agent resides in the blood, circulates in the body for a sufficiently long time and targets the studied (cancer) cells to produce hard-contrast in MRI to allow completion of the imaging procedure; afterwards, it should be degraded and excreted through the kidneys.

MR contrast agents containing Gd ions can decrease T1 relaxation, however superparamagnetic iron oxide nanoparticles (SPION) can reduce T2 relaxation time and therefore can be used as T2 MR contrast agent.

SPION is an intensively studied research area because of its MR contrast effect and metal oxide feature. SPION containing nanosystems are suitable for several biomedical applications such as MR contrast agents, magnetic hyperthermia or targeted drug delivery.

Targeted MR contrast agents

Development of MR contrast agents has proposed the production of effective and specific systems. Their improvement proceeds on some lines: (i) production of dual contrast agents, (ii) development of contrast agents for drug delivery and for use them in "theranostics" and (iii) preparation of targeted contrast agents.

Ideally, contrast agents accumulate in the targeted tumor cells and cause detectable contrast between tumor sites and surrounding tissues based on the differing densities of tumor vs. healthy tissues and have biological half-lives that allow the necessary imaging studies to be carried out.

To achieve the tumor specificity, targeting ligand can be bound to the contrast agent. The targeting ligand should specifically guide the contrast agent to the cognate surface molecules of the targeted tumor cells.

Folic acid is a well-known targeting ligand in tumor therapy. The folic acid receptor has been shown to be present in much higher amounts on the surface of certain solid tumors than on normal tissues. Several publications in the literature describe the development of folate guided paramagnetic (Gd ion loaded) and superparamagnetic (SPION-loaded) contrast agents.

Aims

Improvement in early stage tumor diagnosis entails the intensive development of imaging techniques including improving the sensitivity of MR contrast agents by the development of targeted nanosystems for their delivery.

The present work brings together the topics introduced above: the use of biocompatible polymers to create targeted nanoparticles for the delivery of MR contrast agents to achieve earlier diagnosis of solid tumors. The present research work describes the preparation and preclinical investigation of the biodegradable, biopolymer-based self-assembled nanosystems which can recognize the targeted tumor cells due to their targeting molecules to deliver contrast ligands that are internalized selectively into these cells.

Major aims of present study were as follows:

- coupling of paramagnetic and superparamagnetic ligands to targeted nanoparticles formed by self-assembly of biopolymers to produce T1 and T2 MR contrast agents;
- performance of full physico-chemical characterization of MR contrast agents, revelation of factors influencing their properties, exploration of connections;
- study of cytotoxicity and confirmation of cell specificity of MR contrast agents using tumor cell lines overexpressing folate (MTT assay, confocal microscopy, flow cytometry, MRI);
- demonstration of MR efficacy of contrast agents *in vivo* using tumor bearing animal models: determination of optimal conditions, MR parameters and pulse sequences necessary for the measurements, examination of tumor accumulation, investigation of organ distribution;
- study of the effect of T1 and T2 contrast agents on relaxation time *in vitro* and *in vivo*.

Materials and methods

Materials

Chitosan (CH) was purchased from Sigma-Aldrich Ltd. (Budapest, Hungary), poly-gamma-glutamic acid from Vedan (Taichung, Taiwan). The biopolymers were purified before use and their molecular weight was determined: CH: M_v = 320 kDa, PGA: M_w = 282 kDa. After purification, toxicity of biopolymers was studied by MTT assay.

Materials used for experimental methods, *in vitro* and *in vivo* studies were purchased from Sigma-Aldrich Ltd. and were of analytical grade.

For determination of number of folate receptors on the surface of tumor cells Cellquant calibrator kits were purchased from Biocytex (Marseille, France) and LK26 anti-folate binding protein antibody from Abcam (Cambridge, UK).

AlexaFluor fluorescent dyes were purchased from Life Technologies Hungary Ltd. and were used for visual detection of nanosystems *in vitro*.

Experimental methods

Folic acid was conjugated to PGA using the carbodiimide technique in aqueous media to produce ***folated PGA (PGA-FA).*** The number of FA molecules per PGA was estimated by UV–VIS absorption spectroscopy (λ_{max1} = 368 nm and λ_{max2} = 283 nm).

For visual detection of nanoparticles, chitosan was fluorescently labelled with ***Alexa Fluor 546 succinimidyl ester*** and ***fluorescein isothiocyanate*** in aqueous media. The labelled chitosan was purified by dialysis against distilled water, its purity was justified by UV–VIS spectrophotometer.

Synthesis of ***superparamagnetic iron oxide nanoparticles (SPION)*** was performed in situ in presence of folated PGA: after the dropwise addition of $FeCl_3$ solution into PGA-FA solution under continuous stirring, the pH of the reaction mixture was raised to 7.0 under inert atmosphere. After that $FeCl_2x4H_2O$ in solid state was added to the reaction mixture and it was stirred under N_2 atmosphere. Temperature of reaction mixture was increased to 80 °C and the pH was raised to 10.0 with addition of ammonium hydroxide solution. Reaction time was 1 h.

Stable ***self-assembled nanoparticles*** were developed via an ionotropic gelation process between PGA/PGA-FA and CH biopolymers. Biopolymer solutions for the self-assembled nanoparticles were prepared at the same concentrations and the pH of the aqueous solutions was adjusted to the given values. Preparation of nanoparticles was performed by mixing of biopolymer solutions at the given ratios under continuous stirring at room temperature.

Gd-complex of the nanoparticles was achieved by dropwise addition of $GdCl_3$ solution to the self-assembled nanoparticles at a determined volume ratio under continuous stirring.

For ***SPION-loaded self-assembled nanoparticles***, SPION-loaded PGA-FA and CH biopolymer solutions were prepared at given concentrations and pH values. Stable nanoparticles were produced by mixing of the biopolymer solutions at defined ratios under continuous stirring at room temperature.

Material scientific investigation methods

Characterization of nanoparticles

The physico-chemical characterization of the self-assembled nanoparticles was performed as follows: hydrodynamic size and size distribution, surface charge in swollen state, morphology in dried state and transmittance of solutions containing nanoparticles were investigated.

The hydrodynamic size of particles was measured using a dynamic light scattering (DLS) technique with a Zetasizer Nano ZS (Malvern Instruments Ltd., Grovewood, Worcestershire, UK). This system is equipped with a 4 mW helium/neon laser with a wavelength of 633 nm and measures the particle size with the noninvasive backscattering technology at a detection angle of 173°. Particle size measurements were performed using a particle-sizing cell in the automatic mode. The mean hydrodynamic diameter was calculated from the autocorrelation function of the intensity of light scattered from the particles.

Electrophoretic mobility of nanoparticles was determined using a Zetasizer Nano ZS instrument. Samples were measured in the automatic mode for a minimum of 10 runs in folded capillary cells.

Size and morphology of dried nanoparticles was studied by a JEOL2000 FX-II transmission electron microscope and a Hitachi 3000N scanning electron microscope.

Sample for TEM and SEM analysis was obtained by placing a drop of the colloid dispersion containing the nanoparticles onto a G2400C carbon-coated copper grid. It was dried at room temperature and then examined without any further modification or coating. Mean diameters and the size distribution of diameters were obtained from measured particles visualized by TEM images and then analyzed using SPSS 11.0 program file. Elemental analysis of nanoparticles during the measurements was performed by electron-beam X-ray analysis using an Oxford Link-Isis energy-dispersive X-ray spectrometer equipped with a Si-Li detector. Samples for atomic force microscopy (AFM) were prepared by casting a drop of

the nanoparticle suspension (0.1 mg/ml) on a glass slide; then, it was dried under vacuum. Nanoparticles were analyzed on a WITec alpha300 A/R equipment using the tapping mode.

Transmittances of nanoparticle solutions were measured using a Hitachi U-1900 spectrophotometer at an operating wavelength of $\lambda = 500$ nm in optically homogeneous quartz cuvettes.

The Gd^{3+} content of nanoparticles was determined by a Thermo Scientific XSeries I inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer OPTIMA 3300DV). The sample introduction system consisted of a Meinhard-type nebulizer and Peltier-cooled (2 °C) spray chamber. Samples were acidified by nitric acid (c = 0.5 M) and their fivefold dilutions were prepared. The analysis was performed using an inner standard (100 ng/ml Rh) and ^{157}Gd isotope.

Magnetic resonance imaging (MRI)

MRI measurements were performed using a clinical human SIGNA LX 1,5 T MR scanner (GE Healthcare Waukesha, WI, USA) at room temperature. Results were evaluated by a LX ScanTools 2000 program file. (UD, Medical and Health Science Center, Department of Radiology)

For the measurement, the T1-weighted scans were performed with 420.0 ms of repetition time (TR) and 20.0 ms of echo time (TE); thickness was 1.5 mm and space was 0 during the measurements. For T2-weighted MR measurements, repetition time was 5520,0 ms, echo time was 90.7 ms, the thickness was 1.5 mm and the space was 0.

T1 relaxation time values were calculated from signal intensities measured by the inversion recovery spin echo method. Determination of T2 relaxation time was performed using a LX ScanTools 2000 program file.

***In vitro* investigation methods**

Cell culture

For *in vitro* measurements, human multidrug resistant ovarian carcinoma (A2780/AD2780), hepatocellular carcinoma HeDe and adherent HeLa cervical cancer cell lines were used. A2780/AD cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center), HeLa was provided by Frank Rösl (Deutsches Krebsforschungszentrum, Heidelberg, Germany), and HeDe cells were achieved from Dr. György Trencsényi (UD, Department of Nuclear Medicine). Cells were grown in a 5% (v/v) CO_2 humidified atmosphere at 37 ◦C and passaged in RPMI-1640 without phenol red supplemented with 10%

fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were passaged using standard PBS solution (Lonza) and trypsin/EDTA (0.25%/0.05%).

Determination of folate receptors

Number of folate receptors on the surface of HeLa and A2780 human tumor cells was determined using Cellquant calibrator kits by LK26 anti-folate binding protein antibody and FITC-labelled polyclonal antibody anti mouse IgG.

HeLa and A2780 tumor cells treated only with FITC-labelled polyclonal antibody anti mouse IgG were used as control. Fluorescent intensities of cells and beads as calibration were measured by flow cytometry (BD FACScan Bioanalyzer System, BD Bioscience, USA).

MTT assay

The toxicity of nanoparticles and contrast agents was investigated using MTT assay. MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow salt that is reduced to formazan in living cells by mitochondrial dehydrogenase enzyme. Cytostasis was graphed in a correlation with control cells.

Confocal microscopic and flow cytometric studies

Internalization of nanoparticles was studied by confocal microscopy and flow cytometry. Samples were imaged by an Olympus FluorView 1000 confocal microscope (Olympus, Tokyo, Japan) using a ×60 UPLSAPO oil immersion objective. Excitation was performed by using the 488 nm line of an Ar ion laser (detection: 500–530 nm) and the 543 nm line of a HeNe laser (detection: 560–610 nm) to image Alexa 488 and Alexa 546 respectively. Images were analyzed using the Olympus FluoView FV10-ASW 1.5 software package (Olympus, Tokyo, Japan).

Fluorescent intensity of centrifuged cells treated by nanoparticles was studied using flow cytometric analysis (BD FACSArray Bioanalyzer System). Excitation of Alexa Fluor 546 was performed at 532 nm wavelength by a laser and results were analyzed using a ReFlex program file.

In vivo investigation methods

Recipient animal model

In each experiment, adult male Fischer 344 rats (Charles River Mo Kft., Gödöllő, Hungary), weighting 150-200 g, and CD1 female athymic nude mice, weighting 18-22 g (Balb/c, Charles River) were used.

Nude mice were provided by National “F.J.C.” Research Institute for Radiobiology and Radiohygiene (Budapest, Hungary) and Fischer 344 rats were achieved from Department of

Nuclear Medicine, University of Debrecen. Animals were housed under pathogen free conditions in air conditioned rooms at a temperature of 23 ± 2 ◦C, with 50 ± 10% humidity and artificial lighting with a circadian cycle of 12 h. The diet and drinking water (sterilized by autoclaving) were available *ad libitum* to all the animals. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of the University of Debrecen.

In vivo toxicity

Experiments were carried out on three groups of nude mice (four animals per group). After systemic injection of physiological saline, non-targeted nanoparticles and targeted nanoparticles everyday within 1 week. Mice body weight was measured after the injection.

Magnetic resonance imaging in vivo

Experimental surgery of rats

For transplantation 10^6 HeDe cells were placed on GelasponR disc (Germed, Rudolstadt, Germany) and the tumor cell containing gelatin disc was placed under the renal capsule of left kidney of Fischer 344 rat models. Stitches closed the wound and *in vivo* experiments followed 10 days later.

Tumor injection of mice

The *in vivo* HeLa tumor xenografts were induced by subcutaneous (s.c.) injection of HeLa cell suspensions (4×10^6 cells) in the both thighs of nude mice. For the injection, experimental animals were anesthetized by intraperitoneal administration of Ketamin-Xylazin mixture.

After incubation time following the nanoparticle administration, MR investigations were carried out under anesthesia.

Each animal were used for only one investigation. Euthanasia of animals was performed by i.v. injection of sodium barbiturate or by inhalation of diethyl ether.

Statistical analysis

Data are presented as mean ± SD of at least three independent experiments. The significance was calculated by Student's t test (two-tailed). The level of significance was set at $p \leq 0.05$ unless otherwise indicated.

Results

Biopolymer-based nanocarrier

In the course of our pre-study, the self-assembly processes of chitosan and poly-gamma-glutamic acid biopolymers were investigated. Hydrophilic (nano)systems as polyelectrolyte complexes can be formed from these biopolymers by ion-ion interaction between their oppositely charged functional groups. One of the main advantages of ion-ion interaction is that the biopolymers can keep their original favorable biological properties after formation of colloid systems.

Self-assembly of two biopolymers was investigated in aqueous media at pH = 3.0. It was established that formation of individual particles or aggregates created by self-assembly at pH = 3.0 can be considerably influenced by concentration and ratio of biopolymers and order of addition.

Smaller and more and more stable particles were produced by decreasing of the concentration of biopolymers. This statement was supported by particle size and transmittance measurements. Nevertheless it was established that low soluble chitosan plays an important role in the stability of nanosystems formed at pH = 3.0.

Stability of the nanoparticles was investigated as a function of pH. Increasing the pH caused the particle size and opalescence in aqueous systems to increase and surface charge to decrease. At pH higher than 6.0 the nanoparticles precipitated due to the low solubility of chitosan. These results demonstrated that nanoparticle size and surface charge could be manipulated by pH. Due to the importance of size and surface charge in the physiological and biochemical function of MR contrast agent nanoparticles, these characteristics were of critical importance to the research program.

The next step of our research, required the extension of the above results to the production of the self-assembled nanoparticles stable at pH ≈ 7.4 for the purpose of intravenous administration.

The pH of biopolymers was carefully adjusted to different values and it wasn’t changed after self-assembly. In this way stable systems were successfully prepared at isotonic condition. The pH of PGA was adjusted to 9-9.5-re and it was done to 4.0 for CH solution. Formation of nanoparticles was performed at the same polymer ratios and order of additions used previously.

The nanoparticles were stable and their pH varied between 6.3 and 8.1 depending on the ratio of mixing.

We attempted to establish a clear relationship between transmittance and hydrodynamic size, particle size, and/or order of addition of biopolymers, however we were not successful in this endeavor.

Nevertheless it was recognized that smaller particles were formed from PGA and CH at mixing ratio of 1:1. This result could correlate with molecular weight of biopolymers, their orientation inside of the nanoparticle and being chitosan a polysaccharide.

Based on these results investigation of nanoparticles formed by self-assembly of PGA and CH at a ratio of 1:1 became the subject of further study.

Targeted nanoparticles

Targeted self-assembled nanosystems, which are stable at pH ≈ 7.4, were successfully prepared. These nanoparticles have the capability to accumulate in the targeted tumor cells and are therefore attractive candidates as tumor specific nanocarriers.

Folic acid molecules were coupled to the PGA biopolymer via its carboxyl groups. Folic acid as targeting ligand can drive the nanoparticles to tumor cells overexpressing folate receptors. The number of FA molecules per PGA was estimated by spectrophotometry to be approximately 7:1 FA:PGA.

Size of self-assembled particles was between 30 nm and 160 nm in dried state, however their hydrodynamic size varied between 80 nm and 180 nm due to their swollen state in aqueous media. Surface charge as electrokinetic mobility of nanoparticles was $u = -2.09 \pm 0.07$ (m/s)(V/cm), which overall value is due to the −1:+0.67 ratio of deprotonated COO^- and protonated NH_4^+ functional groups at given pH.

In vitro internalization of folate-targeted self-assembled nanoparticles was tested using confocal microscopy on A2780/AD ovarian carcinoma human tumor cell line overexpressing folate receptors.

It was recognized that folate-targeted nanoparticles penetrated the cell membrane within 60 min and filled up the total volume of the cytoplasm. In contrast, nanoparticles lacking folate did not readily penetrate the cell and even after 60 min, there was very limited uptake in the cytoplasm.

A quantitative estimate of the accumulation of FITC-labeled nanoparticles was determined by calculation of total fluorescent intensity inside the cells. The quantitative data obtained showed that folate-targeted nanoparticles internalized into the cells significantly faster and the total accumulation of these particles was substantially higher in the cancer cells when compared with non-targeted particles.

Based on these results it can be stated that the nanosystem as nanocarrier can accumulate effectively in targeted tumor cells overexpressing folate receptors, which can be achieved by coupling folic acid to the nanoparticles.

To assess the toxicity of the nanosystems *in vitro* cytotoxicity and *in vivo* toxicity studies were performed. *In vitro* cytotoxicity tests were carried out using MTT assay on A2780 cell line. *In vivo* toxicity tests were executed using healthy immunocompromised nude mice. It was observed that both folate-targeted and non-folated nanoparticles are non-toxic. No considerable difference between the survival of control cells and cells treated by nanoparticles was observed by cytoxicity assay. *In vivo* toxicity tests demonstrated no notable differences in body weight of mice in control vs experimental groups of animals and this fact was independent of targeted or non-targeted nanoparticles. In summary, nanoparticles prepared by self-assembly of biopolymers were observed to be non-toxic in *in vitro* and *in vivo* preclinical assays.

Gd-complexes of self-assembled nanoparticles

To develop nanocarriers as MR contrast agents Gd ions were complexed to the self-assembled nanoparticles to produce a MR T1 paramagnetic tumor specific nano-sized contrast agent.

Stable nanoparticulate systems were formed after addition of Gd ions to the self-assembled nanoparticles. The nanoparticles prepared from PGA and CH at a ratio of 2:1 had the smallest hydrodynamic size, and reducing effect of Gd ions on size of nanoparticles was the most considerable at this reaction condition too.

Change of hydrodynamic size of PGA-CH 2:1 nanoparticles as a function of quantity of Gd ions was studied. It was observed that particle size decreased by increasing of added $GdCl_3$ reaching a minimum at volume ratio of 0.4.

Summarizing these results, it was established that PGA-CH 2:1+0,4Gd system would be used for further biological studies.

Paramagnetic MR contrast agent

Tumor specific paramagnetic MR contrast agent was prepared by self-assembly of PGA and CH biopolymers: folic acid as targeting ligand was covalently coupled to the PGA and after self-assembly Gd ions were complexed to the nanoparticles. Due to the FA conjugated to the nanoparticles tumor specific targeting was achieved and Gd ions as paramagnetic MR T1 ligands affected the MR signal intensity.

Size of PGA-FA–CH 2:1+0,4Gd self-assembled nanoparticles was studied in swollen and dried states. Average hydrodynamic size of nanoparticles was = 130±4 nm with narrow size distribution between 70 and 280 nm. The size of the dried nanoparticles ranged between 50 and 150 nm based on the SEM measurements. Mobility of particles was μ = -3.8±0.3 μmcm/Vs meaning negative surface charge at pH ≈7.4. Aqueous colloid system containing nanoparticles was opalescent with transmittance value of 85%.

Investigation of paramagnetic MR contrast agent nanoparticles was started with phantom MR measurements. T1-weighted MR images showed a bright enhancement of nanoparticles, compared with dark appearance of distilled water. Nevertheless in the MR images the light intensity on the gray scale varied in a function of nanoparticle concentration.

The measured values supporting the visual MR observation showed that the signal intensities decreased monotonically by decreasing of the concentration of nanoparticles and their Gd ion content: the signal intensity of aqueous solution of paramagnetic nanoparticular contrast agent was 1783 on the densitometer scale, compared with the value of 287 belonging to distilled water. On the other hand, the T1 relaxation time decreased from 2980 ms (characteristic for distilled water) to 244 ms as the paramagnetic nanoparticle concentration was raised, which appears as a bright image.

Cytotoxicity of tumor specific contrast agent and its ingredients was tested by MTT assay. Flow cytometry was used to determine the average number of folate receptors bound to the surface of tumor cells. These values were 1876±13 for HeLa cells and 790±9 for A2780 cells.

Cytotoxicity results revealed that HeLa cells appeared to be more sensitive to the presence of nanoparticles and their ingredients than A2780 cells. Cell survival was usually above 95% compared to 100% of control cells, only 88% of the HeLa cells treated with CH-AF modified biopolymer survived. Summarizing, MTT results confirmed that neither the nanoparticles nor their ingredients are toxic on the studied cells lines.

Cytotoxicity measurements were confirmed by cell counting, which supported the MTT results.

In the next step of our research work tumor specific internalization of developed paramagnetic nanoparticles was confirmed on tumor cell lines overexpressing folate receptors using confocal microscopic and flow cytometric measurements.

Confocal microscopic images confirmed the adsorption of folated nanoparticles to the cell membrane and their internalization into the targeted tumor cells.

To confirm the tumor specificity two complementary investigations were performed: (i) cells were treated by non-folated non-targeted nanoparticles, and (ii) cells were saturated with free folic acid before treating with folate-targeted nanoparticles.

In case of both independent investigations minimal internalization of nanoparticles into the targeted tumor cells was established and additionally their adsorption on the cell membranes was also negligible. Therefore, targeting of the nanocarrier systems appeared to be folate dependent.

These internalization studies were supported by flow cytometric results, where only the live cells were counted based on the intensity of their forward scatter signal. This analysis showed higher fluorescence intensity for both studied HeLa and HeDe cells treated with the folated nanoparticulate contrast agent than those treated with non-folated nanoparticles or for control cells. Significant shift of fluorescence intensity of cells incubated with folate-targeted nanoparticles confirmed the tumor specific uptake of the contrast agent.

In the next step of our research work was to verify that paramagnetic Gd ions transported by the nanoparticles also accumulate in the targeted tumor cells. HeLa and HeDe cells were treated by nanoparticles containing Gd ions after which the treated cell suspensions were studied by MRI.

Bright enhancement appeared in the MR images and measured signal intensity values verified that folate-targeted nanoparticles internalized and accumulated in the targeted tumor cells, therefore they have the ability to cause signal enhancement due to the delivered paramagnetic ligand, providing the necessary hard contrast with the surrounding tissue. Nevertheless the cell suspensions treated with non-folated nanoparticles resulted in a gray area in the MR images, similar to the control cell suspensions, supporting the tumor specific internalization of nanoparticles by the folic acid targeting moiety.

In the MR images, the signal intensity of HeLa cells treated by folate-targeted nanoparticles was 1351, and it was 869 for HeDe cells, which appeared in bright enhancement. The signal intensity of control HeLa cells was 480 on the densitometer scale and it was 523 in case of HeLa cells incubated with non-targeted nanoparticles. These values were 441 and 511 for HeDe cells. The low level of internalization of non-targeted nanoparticles results a gray area in the MR image, similar to the control cell suspension.

For *in vivo* investigation of nanoparticles female athymic nude mice bearing HeLa tumor xenografts induced by subcutaneous and Fischer rats bearing HeDe tumor placed under their renal capsule of left kidney were used. *In vivo* experiments were performed 10 days following tumor transplantation.

In vivo accumulation tests of folate-targeted 2PGA-FA:1CH–Gd paramagnetic contrast agent nanoparticles administrated by i.v. were executed by T1-weighted MR investigations. (Control animal were treated with 5% glucose solution.)

Based on the MR images it was observed that tumor signal intensity was considerably higher in treated animals compared to control animals. Tumor signal intensity was 396.8±7.3 for control HeDe tumors and it was 595.2±3.4 for treated animals. These data were 218 ± 8 and 293 ± 14 a.u. for HeLa tumors, respectively.

In vivo MR measurements were complemented by PET imaging after intravenous administration of ^{18}F-FDG radiopharmacon. Anatomy and metabolism can be combined by these fusion image slices of MRI and PET images to exhibit clearly the presence of the tumor and to provide its accurate localization.

Superparamagnetic MR contrast agent

SPION was synthesized in situ in presence of folated PGA (PFS). The superparamagnetic T2 MR contrast agent nanoparticles were prepared by self-assembly of this SPION-loaded folated PGA and chitosan biopolymers.

Hydrodynamic size, size distribution, surface charge and stability of 2PFS:1CH and 3PFS:1CH nanoparticles was studied. It was found that the nanoparticles were stable for several weeks. The hydrodynamic size by intensity of 2PFS:1CH nanoparticles was d = 110 nm with narrow size distribution, their surface charge was $u = -1.99$ (m/s)(V/cm); however these data were d = 90 nm and $u = -2.07$ (m/s)(V/cm) respectively.

Internalization of folate-targeted superparamagnetic nanoparticles into HeLa cells was tested by confocal microscopy and flow cytometry. Confocal microscopic images confirmed that the folate-targeted superparamagnetic nanoparticles accumulated in the targeted tumor cells, which results were supported by flow cytometric measurements.

Tumor cells incubated with folate-targeted nanoparticles presented considerable shifts of fluorescence intensity compared to control cells. Nevertheless shifts of two types of SPION-loaded nanoparticles were similar and showed minimal difference, which implied their similar internalization. The numerical data demonstrated higher relative FI of HeLa cells treated with nanoparticles compared to control cells. The relative intensity was 44 times higher for PFS:CH=2:1 and 47 for PFS:CH=3:1 nanoparticles than that of control cells.

Phantom MR investigation was performed to confirm the superparamagnetic effect of SPION-loaded self-assembled nanoparticles. It was found that both nanoparticles

considerably reduced T2 relaxation time and caused MR contrast enhancement, which appeared as a dark enhancement in the MR images.

There was no substantial difference between the superparamagnetic effect of two nanoparticles: 3PFS:1CH nanoparticles contain more SPION, but its effect was not measurable.

MR measurements following *in vitro* internalization were performed to confirm the superparamagnetic effect of nanoparticles. HeLa cell suspensions were imaged after treatment with SPION-loaded nanoparticles. Treated HeLa cell suspensions appeared as dark enhancement with reduced T2 relaxation time in the T2-weighted MR images compared to the bright appearance of control untreated cell suspension. This result visually verified the applicability of tumor specific superparamagnetic nanoparticles as T2 MR contrast agent.

T2 relaxation time of cell suspensions treated by 2PFS:1CH and 3PFS:1CH nanoparticles was 424 ms ad 388 ms, however it was 923 ms for untreated control cells. The difference between relaxation time of treated and untreated tumor cells is significant.

In vivo tests of superparamagnetic nanoparticles were performed using female athymic nude mice bearing HeLa tumor xenografts. Untreated nude mice bearing HeLa tumors were used as control animals.

Accumulated contrast agent nanoparticles considerably decreased the signal intensity and relaxation time of tumor tissue, which appeared unequivocally in the T2-weighted MR images. Dark tumor tissue of treated animal was observed compared to the brighter tumor of control animal. Signal intensity of tumor of treated animal was 582±13 au. with relaxation time of 602±15 ms, however these values were 793±19 au. and 1018±23 ms respectively for control animal.

Discussion

My research work was performed in an interdisciplinary cross section of science, where nanotechnology and imaging diagnostics overlap. This field of science has become to the forefront of biomedical applications of biopolymers, development of MR contrast agents and nanotechnology.

Nanocarrier

As a result of advances in materials science and our understanding of the chemistry of nanoparticles, nanosystems formed by biocompatible, biodegradable macromolecules have attracted considerable interest. An increasing number of publications dealing with biopolymer-based nanosystems developed for biomedical and pharmaceutical uses.

We chose to combine chitosan and poly-gamma-glutamic acid biopolymers, which have several favorable biological properties such as biocompatibility, biodegradability and non-toxicity.

CH and PGA biopolymers can behave as polyelectrolytes in aqueous media due their functional groups and can form polyelectrolyte complex particles by self-assembly via ion-ion interactions. Due to their lack of covalent bonds polyelectrolyte complexes offer many advantages in the field of nanoparticle formation and nanoparticular drug delivery.

Preparation and investigation of several self-assembled CH/PGA nanoparticles can be found in the literature. These nanosystems were generally developed for complexing and delivery of drugs without any targeting.

The properties of polyelectrolyte nanocarriers formed by self-assembly of PGA and CH without any drugs was initially studied. In order to build the platform for product development, the critical factors required for formation of the nanosystem needed to be understood. It was established that opalescent colloid systems containing nanoparticles were formed by mixing of aqueous solutions of PGA and CH biopolymers at pH = 3.0. The self-assembled nanoparticles were stable under acidic conditions. The size of nanoparticles was influenced by concentration and ratio of biopolymers. However, these particles were sensitive to the change of pH of environment and precipitated at pH above 6.0.

Although stability at acidic pH is required for oral drug delivery due to the acidic pH of the stomach, most cancer diagnostics are delivered intravenously and thus must be stable at physiologic pH. Accordingly to this we wanted to produce the nanosystems by self-assembly

of PGA and CH, which were stable at pH ≈ 7.4, because we intended to study them *in vitro* and *in vivo* by intravenous administration.

To solve this problem reaction conditions had to be changed to generate stable nanoparticles at pH close to the neutral. Consequently, the pH of PGA was adjusted to 9-9.5 and of CH to 4.0. Formation of nanoparticles was studied at these reaction conditions as a function of the ratio of biopolymers and order of addition.

It was established that opalescent aqueous colloid systems containing nanoparticles were formed, but an unequivocal relationship between transmittance and hydrodynamic size results could not be established, nor could any other clear relationship between reaction conditions and biophysical properties of the nanoparticles.

In order to achieve specific targeting to tumor cells, the folic acid moiety was chosen based on its small size and reactive functional groups. Folic acid is a recognized targeting ligand, based on the observation that certain tumor cells overexpress folate the cognate receptor on their surface in high numbers relative to healthy cells.

Many attempts have recently been made to develop folate-targeted nanosystems including biopolymer-based nanosystems, but the literature shows a paucity of folate-targeted polyelectrolyte complexes. Thus the combination of folate targeting and polyelectrolyte-based nanoparticles provides a novel approach to the problem of targeted contrast agent delivery.

Folic acid as targeting ligand was covalently coupled to PGA biopolymer followed by self-assembly of folated PGA and CH biopolymers into stable nanoparticles.

The size of these nanoparticles was demonstrated to be 80-180 nm with a net negative surface charge. Targeting efficacy of folic acid was tested on A2780 tumor cell line overexpressing folate receptor. Confocal microscopic images confirmed that folate-targeted nanoparticles penetrated the cell membrane within 60 min and filled the total volume of the cytoplasm. In contrast, nanoparticles lacking folate did not readily penetrate the cell even after 60 min, and there was very limited uptake in the cytoplasm.

To confirm the non-toxic properties of nanoparticles, i*n vitro* cytotoxicity and *in vivo* toxicity tests were performed. The lack of toxicity suggested by these results support our theory that these nanoparticles may be suitable for targeted delivery of several types of drugs and imaging agents.

One of the aims of my research work was to develop MR contrast agents based on these self-assembled targeted nanoparticles. To realize this Gd ions were complexed to the basic nanocarrier to produce a tumor specific nano-sized paramagnetic MR T1 contrast agent. Based on our concept PGA may be capable of complexing and transporting positively charged

ions via its carboxyl functional groups. To produce paramagnetic MR contrast agent $GdCl_3$ was finally added to the self-assembled nanoparticles, so the Gd ions could bound to the free carboxyl groups inside the nanoparticles.

The sub-molecular complexing process of paramagnetic metal ions was not studied. Based on the compounds and preparation processes used, the nanoparticles were believed to contain numerous amino and carboxyl functional groups. The literature states that metal ions can be complexed to nanoparticles via the carboxyl groups of glutamate-peptide. Therefore the paramagnetic nanoparticles containing Gd ions in which the ratio of PGA was higher than the ratio of CH were chosen as the most promising candidates for further study and development. These nanoparticles contained free carboxyl groups and therefore could complex and deliver paramagnetic ions.

The effect of increasing the PGA ratio and quantity of Gd ions on the size of the contrast agent nanoparticles was studied. Relationships minimum points were found in both cases. Summarizing the results it was established that nanoparticles with 2PGA:1CH+0,4Gd compound showed optimal properties for further development.

Paramagnetic MR contrast agent

Gd ions are paramagnetic, and can reduce T1 relaxation time. Therefore they may be useful as MR contrast ligands. However free Gd ions are toxic, so they must be sequestered in order to be useful in a clinical setting. Several small molecular Gd-complexes are currently approved and marketed for this purpose. Use of these contrast agents is safe, because Gd ions are complexed and thus sequestered. These low-molecular weight Gd chelated have serious shortcomings, which can’t be ignored. To overcome these shortcomings, several low-molecular and macromolecular carriers have been developed to improve MR contrast enhancement.

Many attempts have recently been made to create macromolecular MR contrast agents including paramagnetic polyelectrolyte complexes, but development of targeting and tumor specific accumulation of these systems has not yet been achieved.

Targeted Gd ion delivery by biopolymer-based self-assembled polyelectrolyte complexes can be found in the literature. Extending this line of research, tumor specific paramagnetic MR contrast agent was prepared by self-assembly of PGA and CH in our research work. Folic acid as targeting ligand was covalently coupled to the PGA and Gd ion were complexed to the nanoparticles after self-assembly. We successfully demonstrated tumor

specific targeting by means of folic acid with Gd ions as paramagnetic MR T1 ligands influencing the MR signal intensity.

Nanoparticles containing Gd ions were successfully prepared. These nanoparticles had 130±4 nm average hydrodynamic size, negative surface charge and 85% transmittance at biopolymer concentration of c = 0.3 mg/ml.

Paramagnetic properties, Gd containing, non-toxic effect and tumor specific accumulation of contrast agent nanoparticles were confirmed. Based on the results it could be established that the developed T1 MR contrast agent nanoparticles is an attractive development candidate as a paramagnetic contrast agent.

The non-toxic effect of nanoparticles and their ingredients was verified by MTT assay, therefore they are suitable for *in vitro* and *in vivo* efficacy tests.

Tumor specific internalization of nanoparticles was studied by confocal microscopic and flow cytometric measurements. The results met the following requirements: folic acid as targeting ligand was capable of tumor specific targeted delivery of nanoparticles and folate-targeted nanoparticles selectively accumulated in targeted tumor cells overexpressing folate receptors.

The next part of our research work verified that the nanoparticle is suitable for delivery of Gd ions and can target them specifically into the tumor cells. In these studies the Gd ions were complexed directly to the nanoparticles, by the carboxyl groups of PGA without any complexing agents.

To verify the stability of complexed Gd ions delivered *in vitro* HeLa and HeDe cells were treated with nanoparticles containing Gd ions followed by MR imaging. T1-weighted MR images confirmed that folate-targeted paramagnetic nanoparticles accumulated specifically in the targeted tumor cells and due to the transported Gd ions signal intensity changed considerably. Based on these results the nanoparticles appear to be candidates for further development as a paramagnetic MR contrast agent.

The paramagnetic contrast agent nanoparticles were tested *in vivo* using HeDe tumor bearing rat and human HeLa tumor bearing mouse models. T1-weighted MR images showed notable tumor accumulation after treatment. The investigations were performed using incubation times (2 h, 24 h) longer than current clinical practice. These studies confirmed that the nanoparticular MR contrast agent resides in the blood, circulates in the body for a sufficiently long time and its tumor accumulation is long-draw.

In summary it can be concluded that tumor specific stable paramagnetic nanoparticles were prepared successfully by self-assembly of CH and PGA biopolymers. These

nanoparticles have optimal physico-chemical parameters, are non-toxic and are internalized specifically into targeted tumor cells *in vitro* and *in vivo*. Due to the delivered paramagnetic ligands these nanoparticles can induce considerable changes in signal intensity in the T1-weighted MR images and therefore may facilitate the early stage tumor diagnosis.

Superparamagnetic MR contrast agent

In order build upon my earlier body of results, I sought to reveal opportunities for development of MR T2 contrast agents from these polyelectrolyte complex nanocarriers. Therefore the study of folate-targeted SPION-loaded self-assembled nanoparticles as superparamagnetic contrast agents was undertaken.

SPION is known as MR active ligand influencing T2 relaxation time. But SPION is not stable in aqueous media, so stabilization of iron oxide nanoparticles is necessary before their use. This stabilization is usually provided by a covering comprised of biopolymers. In our research work SPION was synthesized *in situ* in presence of folated PGA. The superparamagnetic T2 MR contrast agent nanoparticles were prepared by self-assembly of this SPION-loaded folated PGA and chitosan biopolymers.

Studies of folate-targeted SPIONs on KB cell lines can be found in the literature, but these systems consist of folate-targeted lipophilic SPIONs or polyacrylic acid-coated targeted SPIONs in which folic acid was coupled via PEG linkers to the system. Examples of SPIONs coated by polyelectrolyte complexes or dispersed in them as well as their targeted delivery could appear to be novel as they currently cannot be found in the literature.

In this part of my research work folate-targeted nanoparticles were prepared by self-assembly of folated PGA-coated SPION and CH. These nanoparticles were studied as T2 MR contrast agent.

It was previously confirmed that folic acid is able to target the paramagnetic self-assembled nanoparticles, however verification of this result became necessary again for superparamagnetic nanoparticles because the SPION synthesis in the presence of folated PGA is performed at high temperature.

Confocal microscopic images and flow cytometric results confirmed unequivocally that folate-targeted self-assembled superparamagnetic nanoparticles accumulated in the targeted HeLa tumor cells overexpressing folate receptors.

To verify the superparamagnetic effect of nanoparticles phantom and *in vitro* MR measurements were performed. Based on these results it was established that the superparamagnetic nanoparticles were able to reduce considerably the T2 relaxation time and

to cause MR contrast enhancement. During the *in vitro* T2 MR measurements HeLa cell suspensions treated by targeted superparamagnetic nanoparticles were studied and compared to the control cells. T2 relaxation time results of cell suspensions showed that the nanoparticles internalized into the targeted tumor cells, transported the SPIONs and therefore caused considerable relaxation time reduction compared to control cells.

In vivo tumor specificity of superparamagnetic nanoparticles was confirmed after *in vitro* tests. HeLa tumor bearing nude mice were used to test the effect of the contrast agent nanoparticles on the relaxation time and signal intensity. The results supported unequivocally that the folate-targeted contrast agent nanoparticles accumulated *in vivo* in HeLa tumor cells and due to the SPION-containing they reduced the relaxation time and signal intensity of tumor tissues considerably, which appeared clearly in the T2-weighted MR images.

New scientific results

- Targeted nanocarriers stable at pH 7.4 were prepared by self-assembly of poly-gamma-glutamic acid and chitosan biopolymers.
- It was established that the biopolymer-based self-assembled nanoparticles are suitable for targeted delivery of paramagnetic and superparamagnetic ligands.
- It was ascertained that physico-chemical parameters of paramagnetic contrast agent nanoparticles can be considerably influenced by ratio of biopolymers, order of addition, quantity of delivered paramagnetic ligands and the pH of the environment.
- MR measurements were performed to confirm that the paramagnetic and superparamagnetic nanoparticles reduce effectively the relaxation time and therefore are useable as MR contrast agents.
- It was established that the targeted paramagnetic and superparamagnetic contrast agent nanoparticles internalize effectively into the targeted tumor cells *in vitro*.
- Effective tumor accumulation of targeted contrast agent nanoparticles was verified *in vivo* using animal and human tumor models.
- It was realized that the novel paramagnetic and superparamagnetic nanoparticles can be applicable as targeted MR contrast agents.

Summary

Stable nanocarriers were successfully prepared by self-assembly of chitosan and poly-gamma-glutamic acid biopolymers. These nanoparticles have 100-120 nm hydrodynamic size, negative surface charge, and are able to transport paramagnetic Gd ions or superparamagnetic SPIONs. Folic acid as targeting molecule was successfully coupled to the biopolymers via their functional groups to realize the preparation of targeted contrast agent nanoparticles.

Full physico-chemical characterization of paramagnetic MR contrast agent nanoparticles was performed, and factors that influence the parameters of nanoparticles were defined. It was established that concentration and ratio of biopolymers, the order of addition and the pH of the environment effect the properties of nanocarriers. The transported Gd ions changed the size of nanocarrier particles. MR T1 measurements confirmed that the nanoparticles are paramagnetic and can increase the signal intensity and reduce T1 relaxation time.

Confocal microscopic, flow cytometric and *in vitro* MR investigations were carried out to confirm that folate targeted paramagnetic and superparamagnetic nanoparticles specifically accumulate in the studied tumor cells overexpressing folate receptors, transport the MR active ligands (Gd ions, SPIONs) and therefore they can cause significant contrast in MR images.

HeDe tumor bearing Fischer 344 rat models and HeLa tumor bearing nude mice were used to test the accumulation of paramagnetic folate targeted nanoparticles *in vivo*. Based on the MR images it was confirmed that the developed nanoparticles behaved as effective paramagnetic contrast agent for both studied animal and tumor models. The nanoparticles specifically accumulated in studied tumors and caused significant contrast compared to control animals.

In vivo investigation of SPION-loaded folate targeted nanoparticles as T2 MR contrast agents was performed using HeLa tumor bearing female athymic nude mice. Based on the T2-weighted MR images it was established that folate targeted contrast agent nanoparticles accumulate in tumor and considerably reduce the relaxation time of tumor tissue.

Summarizing, biopolymer-based, tumor specific paramagnetic or superparamagnetic nanoparticulate MR contrast agents were successfully prepared, which can facilitate the early tumor diagnosis.

Publications

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Register Number: DEENKÉTK/33/2014.
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Subject: Ph.D. List of Publications

Candidate: István Hajdu
Neptun ID: FWTI7S
Doctoral School: Doctoral School of Molecular Medicine
Mtmt ID: 10035948

List of publications related to the dissertation

1. **Hajdu, I.**, Trencsényi, G., Bodnár, M., Emri, M., Bánfalvi, G., Sikula, J., Márián, T., Kollár, J., Vámosi, G., Borbély, J.: Tumor-specific localization of self-assembled nanoparticle PET/MR modalities. *Anticancer Res.* *34* (1), 49-59, 2014.
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Total IF of journals (all publications): 19.293

Total IF of journals (publications related to the dissertation): 7.228

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

18 February, 2014

List of patents connecting to the topic of the thesis:

János Borbély, Magdolna Bodnár, John F. Hartmann, István Hajdu, József Kollár, György Vámosi

Cancer Cell Diagnosis by Targeting Delivery of Nanodevices

U. S. Patent, Patent No.: US 7,976,825 B2 Date of Patent: Jul. 12, 2011

List of conference presentations connecting to the topic of the thesis:

Magdolna Bodnár, István Hajdu, Genovéva Filipcsei, John F. Hartmann, Tamara Minko, János Borbély

Formation and characterization of polyelectrolyte complexes based on self-assembly of chitosan and poly-γ-glutamic acid

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Hajdu István, Borbély János

Nanoanyagok alkalmazása in vitro kísérletekben

VIII. Téli Iskola, Balatonfüred, Február 6-8, 2008.

István Hajdu, Éva Nagy, Magdolna Bodnár, John F. Hartmann, Sándor Damjanovich, József Kollár, György Vámosi, László Nagy, Tamara Minko and János Borbély

Cancer Cell Diagnosis and Drug Delivery by Targeted Delivery of Biopolymer-Based Nanodevices

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Kontrasztanyagok fejlesztése multimodális képalkotás céljára

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István Hajdu, Magdolna Bodnár, Zsuzsanna Csikós, György Trencsényi, Miklós Emri, Imre Lajtos, András Polyák, Judit Sikula, György Vámosi, Terez Marian, Lajos Balogh, János Borbély, József Kollár

Preparation and investigation of multimodal contrast agents

World Molecular Imaging Congress September 5-8, 2012 Dublin Ireland

List of posters connecting to the topic of the thesis:

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Nanoparticles Prepared by Self-assembly of Chitosan and Poly-γ-glutamic Acid

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Új szuperparamágneses nanorendszer kontrasztanyag PET és MR vizsgálata

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Tc-99m-mel jelzett önrendeződő biopolimer-bázisú nanorészecskék receptormediált felvétele, alkalmazása tumorok diagnosztizálásához

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Első klinikai tapasztalatok spontán beteg állatok folát-receptort kifejező tumorainak SPECT/CT vizsgálataiban, Tc-99m-mel jelzett nanorészecskék használatával

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