Adam Mickiewicz University in Poznań

The Faculty of Chemistry

DOCTORAL THESIS

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Synthesis and analysis at the nanoscale. Biomedical applications of nanotechnology on the example of virus-like particles with a magnetic core and nanoindentation.

Synteza i analiza w skali *nano*. Biomedyczne zastosowania nanotechnologii na przykładzie cząstek wirusopodobnych z magnetycznym rdzeniem oraz nanoindentacji.

In the form of a collection of published and thematically related scientific articles

Promotor: prof. UAM dr hab. inż. Jakub D. Rybka

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Streszczenie pracy w j. polskim

Głównym celem przeprowadzonych badań było zbadanie potencjału rozwiązań z obszaru syntezy i analizy w skali *nano*, do zastosowań biomedycznych. Część pracy dotycząca syntezy opiera się na otrzymywaniu i funkcjonalizacji superparamagnetycznych nanocząstek tlenku żelaza (SPION), ocenie ich właściwości biologicznych oraz wykorzystaniu do tworzenia cząstek wirusopodobnych (VLP) z magnetycznym rdzeniem. Część analityczna pracy oparta jest na nanoindentacji ludzkiej chrząstki stawu kolanowego objętej chorobą zwyrodnieniową stawów. Zaprezentowane wyniki podkreślają wysoki potencjał nanotechnologii w biomedycynie, wskazując jednocześnie pewne przeszkody na drodze do jej powszechnego zastosowania.

Pierwsza praca wchodząca w skład głównego osiągnięcia naukowego dotyczyła ewaluacji *in vitro* superparamagnetycznych nanocząstek tlenku żelaza funkcjonalizowanych diheksadecylofosoforanem (SPION-DHP). Otrzymane wyniki ukazują wysoki stopień biokompatybilności otrzymanych nanocząstek, czyniąc je obiecującym materiałem do zastosowań biomedycznych.

Druga praca wchodząca w skład głównego osiągnięcia naukowego dotyczyła tworzenia cząstek wirusopodobych z magnetycznym rdzeniem na bazie białka rdzeniowego wirusa zapalenia wątroby typu B oraz funkcjonalizowanych nanocząstek tlenku żelaza. W tym celu wykorzystano opisany w pierwszej pracy diheksadecylofosforan oraz inny związek funkcjonalizujący. Przeprowadzone badania pozwoliły na efektywne otrzymywanie cząstek wirusopodobnych z magnetycznym rdzeniem, stanowiąc istotny wkład w ten obszar nauki.

Trzecia praca wchodząca w skład głównego osiągnięcia naukowego dotyczyła analizy właściwości mechanicznych ludzkiej chrząstki stawowej pacjentów z chorobą poddanych zabiegowi całkowitej alloplastyki stawu kolanowego, oraz korelacji otrzymanych wyników ze stanem klinicznym pacjentów. Badanie zostało przeprowadzone na próbie 75 pacjentów. Otrzymane wyniki ukazują potencjał nanoindentacji do zastosowań w badaniach dotyczących progresji chorób degeneracyjnych powierzchni stawowych.

Podsumowując, prace wchodzące w skład głównego osiągnięcia naukowego dotyczą metod syntezy i analizy w skali *nano* do zastosowań w obszarach biomedycznych.

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1. General goals of the thesis

The main goal of the presented research was to investigate the potential of nanoscale synthesis and analysis for biomedical applications. The synthetic part of the thesis is based on the synthesis and functionalization of superparamagnetic iron oxide nanoparticles (SPIONs), evaluation of their biological properties, and their utilization for the creation of virus-like particles (VLPs) with magnetic core. The analytical part of the thesis is based on the nanoindentation of human osteoarthritic knee joint cartilage. The conducted research highlights the yet untapped potential of nanotechnology in biomedicine while pinpointing certain hurdles on its way to widespread adoption.

2. Specific goals of particular core scientific achievements

In the first core scientific achievement, the aim was to conduct an *in vitro* evaluation of DHP-functionalized superparamagnetic iron oxide nanoparticles (SPION-DHP). The study included synthesis and functionalization of SPION-DHP, followed by a set of biological experiments aiming to evaluate biocompatibility of the obtained nanoparticles. The study included following biological analyses: cytotoxicity and proliferation assays, reactive oxygen species assay, SPIONs uptake analysis (*via* iron staining and ICP-MS), gene expression analysis. A set of the following genes was selected for the RT-qPCR analysis: alkaline phosphatase (ALPL); ferritin light chain (FTL); serine/threonine protein phosphatase 2A (PP2A); protein tyrosine phosphatase nonreceptor type 11 (PTPN11); transferrin receptor 1 (TFRC). The experiments were conducted on SW1353 (human chondrosarcoma) and TCam-2 (human seminoma) cancer derived cell lines.

In the second core scientific achievement, the goal was to obtain virus-like particles with a magnetic core composed of Hepatitis B virus core protein (HBc) and functionalized SPIONs. As the coating compound`s length and charge are crucial for the assembly efficacy and stability of the resulting VLPs, two compounds were selected for functionalization: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy-(polyethyleneglycol)-2000] and DHP. The HBc protein was obtained *via* agroinfection of *Nicotiana benthamiana* with pEAQ-HBc plasmid. The VLP assembly was evaluated with transmission electron microscopy and functionality testing (ELISA).

In the third core scientific achievement, a nanoindentation study was conducted on cartilage samples obtained from osteoarthritic patients subjected to the total knee replacement procedure. The main objective of this work was to investigate the correlation between patients` clinical state and the mechanical properties of the resected knee cartilage. This study has been conducted on a sample of 75 patients. The cartilage samples from both high weight bearing (HWB) and low weight bearing (LWB) femoral condyles were collected and subsequently analyzed *via* nanoindentation. There was no prior scientific data regarding the optimal sample preparation methodology, what is crucial for obtaining reliable data. The sample has to remain stable during the measurement, while maintaining high level of hydration, corresponding to physiological conditions. Additionally, the fixative should not interfere with mechanical properties of the sample. The evaluated mechanical properties were correlated with patients` clinical data and subjected to statistical analysis, providing insight into the progression of osteoarthritis.

In summary, the core scientific achievement is based on the application of nanotechnology in biomedicine, spanning from synthetic to analytical perspectives. The undertaken research encompasses a broad range of topics with a common denominator of "*nanotechnology in biomedicine*".

3. Core scientific achievements:

[1] **A.A. Mieloch**, M. Żurawek, M. Giersig, N. Rozwadowska, J.D. Rybka, *Bioevaluation of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with dihexadecyl phosphate (DHP).* Sci. Rep. (2020) 1–11. https://doi.org/10.1038/s41598-020-59478-2.
IF = 4.576 MNiSW = 140 pkt

[2] J.D. Rybka, **A.A. Mieloch***, A. Plis, M. Pyrski, T. Pniewski, M. Giersig, *Assembly and Characterization of HBc Derived Virus-like Particles with Magnetic Core.* Nanomater. (Basel, Switzerland). 9 (2019). https://doi.org/10.3390/nano9020155.
(* equally contributed)
IF = 4.324 MNiSW = 70 pkt

[3] **A.A. Mieloch**, M. Richter, T. Trzeciak, M. Giersig, J.D. Rybka, *Osteoarthritis Severely Decreases the Elasticity and Hardness of Knee Joint Cartilage: A Nanoindentation Study.* J. Clin. Med. 8 (2019) 1865. https://doi.org/10.3390/jcm8111865.
IF = 3.303 MNiSW = 140 pkt

Non-core scientific achievements:

[4] Szymański T, **Mieloch AA**, Richter M, Trzeciak T, Florek E, Rybka JD, Giersig M. Utilization of Carbon Nanotubes in Manufacturing of 3D Cartilage and Bone Scaffolds. Materials (Basel). 2020 Sep 11;13(18):4039. doi: 10.3390/ma13184039.
IF = 3.057 MNiSW = 100 pkt

[5] **A.A. Mieloch**, M. Kręcisz, J.D. Rybka, A. Strugała, M. Krupiński, A. Urbanowicz, M. Kozak, B. Skalski, M. Figlerowicz, M. Giersig, *The influence of ligand charge and length on the assembly of Brome mosaic virus derived virus-like particles with magnetic core.* AIP Adv. 8 (2018) 035005. https://doi.org/10.1063/1.5011138.
IF = 1.627 MNiSW = 70 pkt

[6] J.A. Semba, **A.A. Mieloch**, J.D. Rybka, *Introduction to the state-of-the-art 3D bioprinting methods, design, and applications in orthopedics.* Bioprinting. 18 (2020) e00070. https://doi.org/10.1016/j.bprint.2019.e00070.
MNiSW = 140 pkt

[7] M. Pyrski, **A.A. Mieloch**, A. Plewiński, A. Basińska-Barczak, A. Gryciuk, P. Bociag, M. Murias, J.D. Rybka, T. Pniewski, *Parenteral–oral immunization with plant-derived hbcag as a potential therapeutic vaccine against chronic hepatitis B*. Vaccines. 7 (2019). https://doi.org/10.3390/vaccines7040211.
IF = 4.086 MNiSW = 140 pkt

[8] **A.A. Mieloch**, W.M. Suchorska, *The concept of radiation-enhanced stem cell differentiation.* Radiol. Oncol. (2015). https://doi.org/10.1515/raon-2015-0022. **IF = 1.837 MNiSW = 70 pkt**

[9] Patent application: **A.A. Mieloch,** J.D. Rybka, F. Porzucek: "Hybrid nanoparticles binding antibodies, the method of their production and use for binding specific anti-SARS-CoV-2 antibodies". **[WIPO ST 10/C PL437380]**

Bibliometric data:

Total IF = 22.81 Citations: 56 Total MNiSW = 870 pt H-index = 5

Other scientific achievements:

Conferences:

CELLINK Collaborative Partnership Conference, 2019, Milan, Italy	**Oral presentation**: "Mechanical properties of human osteoarthritic articular cartilage. Nanoindentation study"
NABIOMA 2018, Toruń, Poland	**Oral presentation**: "The influence of ligand charge and length on virus-like particles assembly with magnetic core." Honorable mention for the best presentation.
Nanotechnology in Biology and Medicine 2017, Warsaw, Poland	**Oral presentation**: "Design of viral-like particles with magnetic cores."
NanoWorld Conference 2017, Boston, USA	**Poster presentation:** 1." Assembly of virus-like nanoparticles with superparamagnetic core. Multifunctional platform for biomedical applications." 2. "Superparamagnetic iron oxide nanoparticles for bioimaging of myoblasts and mesechnymal stem cells for potential use in post-infraction heart stem cells therapy"

NANOMED 2016, Warsaw, Poland	**Oral presentation**: "Assembly of virus-like nanoparticles with superparamagnetic core. Multifunctional platform for biomedical applications."
nanoFORUM 2016, Serock, Poland	**Poster presentation:** 1. "Functionalization of superparamagnetic iron oxide nanoparticles (SPIONs) with dihexadecyl phosphate (DHP). A new solution to the old issue." 2. "Virus-like particles loaded with functionalized magnetite nanoparticles as a potential biomedical application." 3. "Superparamagnetic iron oxide nanoparticles for bioimaging of myoblasts and mesenchymal stem cells for potential use in post-infraction heart stem cell therapy."
7th International Conference of Contemporary Oncology 2015, Poznań, Poland	**Poster presentation:** "Radiobiological effects of radiation on embryoid bodies."
BIO 2014 Congress, Warsaw, Poland	**Poster presentation:** "Radiobiological effects of radiation on embryoid bodies."

Abroad internships:

01.08.2019 - 01.02.2020	**Internship at The Institute of Cancer Research, London, UK:** "The application of functionalized carbon nanotubes in near infrared photoimmunotherapy in the treatment of glioblastoma multiforme", under the supervision of dr Gabriela Kramer-Marek, Preclinical Molecular Imaging team.
15 - 19.2019	Summer School Utrecht: „3D Printing and Biofabrication."
20.07 - 02.08.2014	PLASMAG - Plasmonic and Magnetic Nanomaterials. Erasmus Intensive Programme. Freie University Berlin, Germany

Participation in research projects:

01.2021 -	**Researcher in the Single-name Hospitals (NCBiR) grant:** "Development and verification of the new COVID-19 Immunodiagnostic Tool". Dr hab. Jakub D. Rybka, prof. UAM as the principal investigator
01.2021 -	**Bioprinting Senior Researcher in the TECHMATSTRATEG (NCBiR) grant:** "Development of bioinks for 3D bioprinting based on chemically modified porcine dECM, enriched with recombinant hybrid proteins, nanomaterials, and synthetic polymers." Dr hab. Jakub D. Rybka, prof. UAM as the leader of the consortium part
11.2018 -	**Researcher in the LIDER (NCBiR) grant:** "MeniScaff 3D - 3D bioprinted, carbon nanotube-enhanced scaffolds for stimulated chondrogenic differentiation of mesenchymal stem cells for meniscus regeneration". Dr hab. Jakub D. Rybka, prof. UAM as the principal investigator
07.2017 - 09.2020	**Ph.D. student in the OPUS (NCN) grant:** "Synthesis, toxicological and functional studies on multi-walled carbon nanotubes as a scaffold for tissue engineering techniques in articular cartilage repair". Prof. dr hab. Michał Giersig as the principal investigator
07.2015 - 04.2017	**Researcher in the MAESTRO (NCN) grant:** "Targeted magnetic core viral shell particles". Prof. dr hab. Michał Giersig as the principal investigator
06.2014 - 06.2015	**The principal investigator of an internal grant** at Greater Poland Cancer Centre: "Radiobiological effects of radiation on embryoid bodies."

4. Scientific resume

After finishing education at the High School of the Insurgents of Greater Poland in Środa Wielkopolska, 09.2008, I started studying at the Medical-Vocational School in Poznań as a pharmacy technician, which I completed on 06.2010. Then, on 10.2010, I started the first degree studies at the Medical University of Karol Marcinkowski in Poznań in the field of Medical Biotechnology. I completed my BA thesis entitled "Impact of ionic liquid [CC] [MCPA] on apoptosis of A2780 cell lines derived from ovarian cancer" at the Department and Cell Biology Department under the supervision of dr Anna Szczerba in 07.2013. Then, I started my II degree studies on 10.2013 at the Faculty of Biology of the University of Adam Mickiewicz University in Poznań, majoring in Biotechnology - studies in English. I completed my master's thesis entitled "Radiobiological effects of ionizing radiation on embryoid bodies" at the Laboratory of Radiobiology, Greater Poland Cancer Centre in Poznań, under the supervision of dr hab. Wiktoria Suchorska. During my work, I received an internal grant of the same title, financed by the Greater Poland Cancer Centre. 20.08 - 02.08.2014 I took part in the intensive Erasmus program "PLASMAG - Plasmonic and Magnetic Nanomaterials. Erasmus Intensive Program Freie University Berlin, Germany." I received my master's degree in biotechnology on 09.2015. After completing my second degree studies, on 07.2015, I started working as a researcher in the MAESTRO project titled "Targeted magnetic core viral shell particles" under the supervision of prof. Michał Giersig. After the project`s completion in 04.2017, I became a Ph.D. student in the OPUS project by prof. Giersig, titled "Synthesis, toxicological and functional studies on multi-walled carbon nanotubes as a scaffold for tissue engineering techniques in articular cartilage repair". In parallel, 09.2017 I started Ph.D. studies at the Faculty of Chemistry at the University of Adam Mickiewicz in Poznań in the "ChemInter" course, implemented under the project POWR.03.02.00-00-I026/16.

I have been cooperating with prof. Jakub D. Rybka since 2015, which resulted in a jointly prepared grant application for the LIDER titled "MeniScaff 3D - 3D bioprinted carbon nanotube-enhanced scaffolds for stimulated chondrogenic differentiation of mesenchymal stem cells for meniscus regeneration", which obtained funding of 1 199 906 PLN, and in which I am employed since 11.2018 as the main co-investigator. In 2019, I took part in a summer school organized by Summer School Utrecht entitled "3D

Printing and Biofabrication". In the period from 01/08/2019 to 01/02/2020, I undertook a six-month internship at The Institute of Cancer Research, London, UK, under the supervision of prof. dr hab. Gabriela Kramer-Marek in the Preclinical Molecular Imaging team. During the internship, my research topics concerned the use of functionalized carbon nanotubes for near-infrared photoimmunotherapy in the treatment of glioblastoma multiforme.

In February 2020, together with prof. Rybka and the team, we decided to engage in the fight against the SARS-CoV-2 pandemic. As a result, we have co-authored a successful grant application titled “Development and verification of the new COVID-19 Immunodiagnostic Tool” funded by NCBiR under the “Single-name hospitals” call, acquiring 2 652 000 PLN. Since January 2021, I work in the project as a researcher. The results obtained during the first quarter allowed for patent application titled “Hybrid nanoparticles binding antibodies, the method of their production and use for binding specific anti-SARS-CoV-2 antibodies”. The ongoing work has been directed toward expanding the technology into other use cases.

The initiative has resulted in the creation of a research team composed of the Faculty of Biology at Adam Mickiewicz University (AMU), the Center for Advanced Technology at AMU, the Institute of Molecular Biology and Biotechnology at Poznań University of Medical Sciences (PUMS), and private companies: Cofactor and RobTech. The team is chaired by the Deputy Director of the AMU Center for Advanced Technologies, prof. Jakub D. Rybka.

The research initiated with the LIDER project resulted in a cooperation with the Foundation of Research and Science Development (FRSD) and the Medical University of Warsaw (MUW). Jointly with the FRSD and the MUW we have successfully applied for funding under TECHMATSTRATEG (NCBiR) call, with a grant proposal titled “Development of bioinks for 3D bioprinting based on chemically modified porcine dECM, enriched with recombinant hybrid proteins, nanomaterials and synthetic polymers”. The project acquired funding of 22 444 594 PLN. Currently, I work in the project as a Senior Bioprinting Specialist.

Adopting the term coined by the renowned French economist Frédéric Bastiat, my work can be divided into "*That Which is Seen and That Which is Not Seen*." The visible part being publications and conferences track record, while invisible being filed grant

applications. During my Ph.D. studies, I have co-authored more than fifteen grant applications, including such proposals as, e.g. LIDER, OPUS, POIR, TECHMATSTRATEG, MINIATURA, FirstTeam, from all major national agencies NCN, NCBiR, FNP, ABM, acquiring 26 296 500 PLN in total.

During my scientific journey, I have often undertaken new research topics, which always has resulted in broadening my scientific horizons. The wide range of subjects is reflected in the scope of my scientific achievements and ongoing research endeavors. Multidisciplinarity of research is one of the main currents of modern science, promoted by the most prestigious academic centers in the world. Presumably, quoting Shakespeare's Hamlet: "*there is a method in my madness,*" and the scientific path I choose to tread will lead to a holistic understanding of the Nature, rather than a chaotic and superficial experience of many subjects.

Time will tell.

5. Introduction

Nanotechnology is a rapidly developing field of multidisciplinary science, combining such fields as, e.g., chemistry, physics, biology. This work is focused on the synthesis and analysis at the nanoscale, on the example of virus-like particles (VLPs) with magnetic core and nanoindentation.

The biomedical applications of superparamagnetic iron oxide nanoparticles (SPIONs) is a topic that gained wide attention. Exceptional magnetic properties, high biocompatibility, precise size/shape control, and flexible surface modifications have led to many suitable solutions in magnetic hyperthermia, targeted drug delivery, magnetic resonance imaging (MRI) contrast, bioseparation, antimicrobial properties. Magnetic particle imaging (MPI) has recently emerged as a non-invasive imaging technique, based on SPIONs, which provides quantitative data rather than acting as an MRI contrasting agent. MPI can be utilized for cell tracking, tissue perfusion, and MPI-guided hyperthermia [1]. Iron oxide nanoparticles can also be effectively used as theranostic agents, combining targeted drug delivery, magnetic imaging, and local hyperthermia or thermal ablation [2]. SPIONs were also successfully used as photocatalyst and adsorbent in wastewater treatment [3]. In general, SPIONs can be viewed as multi-purpose particles, with

exceptional biocompatibility and outstanding functional design flexibility. The synthetic routes for SPIONs can be divided into two broad categories: top-down approach, and bottom-up approach.

The top-down approach focuses on a mechanical disruption of the bulk material through grinding and milling techniques. The milling can be divided into dry, and wet milling methods. A significant shortcoming of dry milling stems from a phenomenon known as “cold-welding”, in which the particles fuse together, forming larger aggregates. In this method, only sub-micrometer particles can be obtained [4]. Interestingly, an improved method of mechanical disruption called high-energy ball milling (HEBM) was shown to produce iron particles in 2-4 nm range [5]. To overcome the cold-welding effect without the need for increased kinetic energy, a wet milling method can be used. In this method, a water with or without surfactant is added to the milling jar, decreasing the surface energy of the particles, thus inhibiting the cold-welding. Using this method, 30 nm iron oxide nanoparticles were obtained [6]. Despite being environmentally friendly due to the lack of required solvents and chemicals, top-down approach of nanoparticles synthesis suffers from an inherent drawback of high polydispersity. Unfortunately, high levels of polydispersity disqualify nanoparticles from *in vivo* usage, as it directly affects various aspects of ADME (Adsorption, Metabolism, Elimination), making its` physiological faith unpredictable [7].

In turn, the bottom-up approach provides more controllable size distribution and also enables design of the surface properties *via* functionalization. There are several approaches to bottom-up synthesis including, but not limited to: co-precipitation, sol-gel reaction, sonochemical synthesis, hydrothermal synthesis, microemulsion reaction, thermal decomposition.

The thermal decomposition is not the most simplistic one, however, provides the highest degree of monodisperisty, colloidal stability, and scalability. In brief, the reaction of thermal decomposition of iron (III) acetylacetonate is performed under inert gas conditions (N_2, Ar), in an organic solvent (e.g. 1-octadecene), in the presence of surfactant(s) (e.g. oleic acid, oleylamine). The molar ratio between the $Fe(acac)_3$ and the surfactants is used to determine the size and shape of the resulting particles. Other factors such as time-at-temperature, solvent type, or gas flow rate may also affect the morphology of the SPIONs and have to be taken into consideration [8]. The main

drawback of this approach is the necessity for further functionalization as the resulted SPIONs are water insoluble. However, in my opinion the pros heavily outweigh cones, and therefore thermal decomposition was selected for the SPIONs synthesis.

As described above, SPIONs are an excellent choice for biomedical applications, and under this assumption, were selected for the core magnetic material for VLPs creation. The requirement of water solubility, highly negative surface charge, low production cost, and a straightforward functionalization route pinpointed several candidate coating compounds, from which dihexadecyl phosphate (DHP) was selected for SPIONs functionalization. Our previously published [**NSA 5**], successful utilization of DHP-coated SPIONs for VLPs creation has sparked our interest in detailing its biocompatibility, which has led to the publication listed as the first core scientific achievement [**CSA 1**].

Virus-like particles are constructs composed of viral proteins with an ordered structure (**Fig. 1**). Due to their native ability to self-assemble, structural proteins of the capsid are most commonly used for the VLPs creation. Importantly, VLPs are devoid of viral genetic material and, therefore, do not pose the risk of infection. Homo-multimeric construction of a capsid's subunits allows for relative ease of modification through standard techniques of protein engineering. A high degree of epitope ordering translates into high immunogenicity, which is particularly important for vaccines. Currently, there are several vaccines available on the market, developed based on VLP technology: Cervarix, Gardasil against HPV (human papillomavirus), Sci-B-Vac against HBV (hepatitis B virus) or Mosquirix against malaria [9]. Additionally, there is an ongoing development focused on multivalent vaccines, capable of presenting several antigens, characteristic of different viruses. For example, a vaccine prototype has been developed against Zika, Chikungunya, Yellow Fever, and Japanese Encephalitis, which share a common vector of infection – a mosquito [10]. Due to a high tissue specificity characteristic for viruses, VLPs can also be applied in targeted drug delivery [11]. For VLPs production, standard protein expression systems can be utilized, e.g., *e.coli*, yeasts, insects, plants, or mammalian cell lines [12].

Figure 1. *Schematic representation of a virus-like particle with magnetic core.*

The primary mechanism driving viral self-assembly is an electrostatic interaction between positively charged inward part of capsid subunits and negatively charged nucleic acid. This property allows for a substitution of the genetic material with other negatively charged functional constituents while maintaining the capacity for self-assembly. *In silico* simulations revealed a high complexity of the kinetics involved in this process. It was demonstrated that the introduction of a core in size commensurate with the inner diameter of a capsid, may facilitate the assembly and improve the overall stability of VLPs [13,14]. Additionally, the introduction of a core particle may provide extended functionality. In the second core scientific achievement [**CSA 2**], Hepatitis B virus core protein (HBc), and DHP functionalized iron oxide nanoparticles were used to create virus-like particles with a magnetic core.

Nanotechnology in biomedicine extends beyond the search of novel nanomaterials and synthetic routes, encompassing an equally promising field of analyses at the *nano* scale. The analytical aspect of this work is focused on the nanoindentation of human cartilage tissue. In general, indentation tests are performed to assess the mechanical properties of a sample and can be conducted at different scales (*macro, micro, nano*). Regardless of the scale, they are based on the same principle. A hard tip with defined mechanical properties is pushed into the sample. The load applied on the tip is gradually increased until reaching a previously specified value. The probe penetrates the sample to a specified depth and is subsequently removed from the material. During the whole process, the load applied and the displacement of the probe are recorded, resulting in a load-displacement curve (**Fig. 2**).

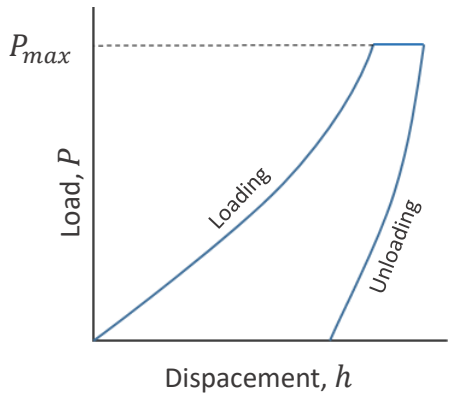

Figure 2. *Load-displacement curve.*

From the load-displacement curve, the mechanical properties of a sample can be derived. The hardness of a sample can be defined as relative resistance of the material's surface to penetration by a harder body. In order to calculate the hardness (H), an exact geometry of the probe has to be specified as it is defined as the maximum load (P_{max}), divided by the residual indentation area (A_r), according to the formula:

$$H = \frac{P_{max}}{A_r}$$

H – hardness
P_{max} – maximum load
A_r – residual indentation area

Another mechanical property that can be derived from the indentation technique is the Young's modulus, describing the stiffness of a sample. A solid material subjected to uniaxial loading (i.e., compression or extension) undergoes elastic deformation. As a result, the stress-strain curve can be obtained. The relationship between stress and strain in a linear elasticity regime describes the Young's modulus, summarized by the formula:

$$E = \frac{\sigma}{\varepsilon}$$

E – Young's modulus
σ – the uniaxial stress, or force per unit surface
ε – the strain or proportional deformation
The SI unit for the Young's modulus is the pascal (Pa), Pa = kg · m^{-1} · s^{-2}.

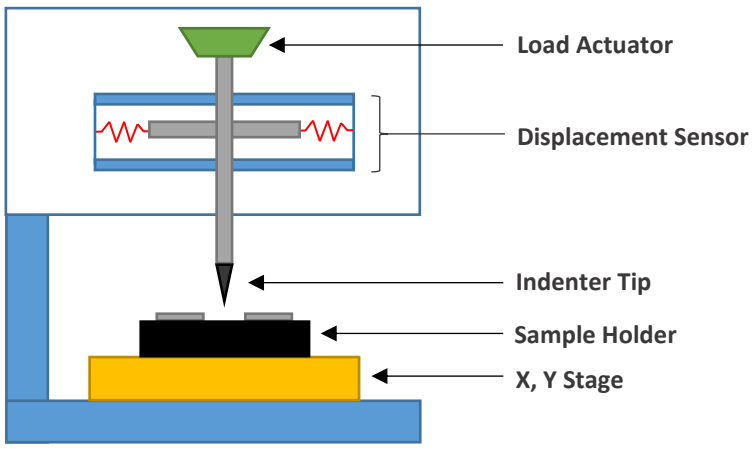

Figure 3. *Schematic representation of a nanoindenter.*

In nanoindentation, a modified version of the equation is used – the reduced Young's modulus, which includes the mechanical properties of the tip. The modulus is derived from the slope of the unloading phase of the load-displacement curve. Nanoindentation was developed to measure the hardness of materials at low volumes (**Fig. 3**). Most commonly, load actuators are based on magnetic coils, electrostatic force generators, or piezoelectric elements [15]. The displacement sensor can be capacitance or inductance based. An increasingly growing body of research indicates the relevance of tissue mechanics in disease progression, tissue remodeling, and regeneration. The main challenge in applying nanoindentation for testing biological materials stems from their complex and often hierarchical structure. In the case of layered materials, indentation depth is crucial for assessing the mechanical properties of a specific layer. Additionally, hydration of a sample has been shown to have a significant effect on its mechanical properties [16].

Biological samples are inherently non-homogenous, and therefore, establishing an exact thickness of a layer is a significant limitation of this method. Nanoindentation has been successfully used in the determination of the mechanical properties of such tissues as cortical bone, enamel, thoracic aorta, brain, or eye [17–21]. While hard tissues are relatively easy to analyze, soft tissues pose a significant challenge, due to viscoelastic properties and adhesion. The nanoindentation can be performed in quasi-static mode (slow loading phase, allowing for the system to retain internal equilibrium) or continuous stiffness measurement (CSM) mode. In CSM mode, an additional oscillatory load signal with a small amplitude and high frequency is applied throughout the whole loading phase. The main advantage of CSM over quasi-static mode is the ability to obtain hardness and elastic modulus throughout the whole loading phase, which is especially important for inhomogeneous and layered samples. In the third core scientific achievement [**CSA 3**], nanoindentation has been utilized to determine the mechanical properties of human cartilage, harvested from femoral condyles of patients diagnosed with osteoarthritis. The study was performed to define the relations between mechanical properties of the diseased cartilage and a clinical image of the patients after total knee replacement procedure.

6. Core achievements commentaries

Bioevaluation of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with dihexadecyl phosphate (DHP).

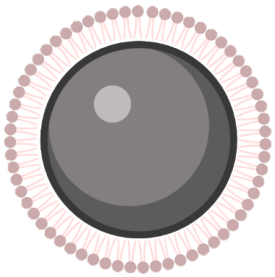

Figure 4. *Schematic representation of DHP-coated SPIONs.*

The aim of this work was to evaluate the biological properties of DHP-coated SPIONs. DHP functionalization provides SPIONs with negative surface charge and hydrophilic properties, which we have successfully utilized in our previous research regarding VLPs with magnetic core [**NSA 5**] (**Fig. 4**). My role in this work was multifaceted and included co-conceptualization of the experiments, synthesis, and functionalization of superparamagnetic iron oxide nanoparticles, sample preparation for inductively coupled plasma mass spectrometry analysis (ICP-MS), cytotoxicity study, data analysis, and manuscript preparation. The SPIONs were synthesized *via* thermal decomposition of iron (III) acetylacetonate $Fe(acac)_3$ with oleic acid as the surfactant. This method provides monodisperse SPIONs coated with oleic acid residues. The functionalization step with DHP is performed *via* phase transition approach. Briefly, DHP and SPIONs suspended in chloroform are mixed with hexane (organic phase). Subsequently, water is added, and the whole mixture is placed in a sonicating bath for several hours. As a result, DHP-coated SPIONs migrate into the water phase and can be easily extracted and purified for further use (**Fig. 5**). To obtain the precise measurement of the nanoparticles concentration, thermogravimetric analysis (TGA) was utilized. In short, a 20 µl sample was heated in the range of 20-150°C under nitrogen flow, to the point of the lowest mass. The density was calculated according to the formula $p = \frac{m}{V}$. The obtained SPION-DHP nanoparticles were administered to TCam-2 (human testicular seminoma) and SW1353 (human chondrosarcoma) cell lines. Proliferation and cytotoxicity assay was performed with commercially available CellTiter-Glo 2.0 assay (Promega). The assay determines the number of viable cells in culture by quantifying ATP, which indicates the presence of metabolically active cells. Luminescence readout is directly proportional to the number of viable cells in culture. The proliferation and

cytotoxicity were assessed 24h after SPION-DHP administration. Due to the inability to assess the cellular iron concentration *via* Prussian blue reaction, ICP-MS analysis was implemented. The method allowed for the quantitative determination of the nanoparticles uptake. The sample preparation requires freezing at -80°C, thawing and lysis with 10% SDS (sodium dodecyl sulfate), secondary freeze-thawing, sonication bath, and finally, dissolution in 65% nitric acid at 80°C. The samples were subsequently diluted ten-fold in DI water and used for measurement. Iron content was determined based on the standard curve prepared with a multielement standard solution for ICP-MS in the 1, 10, 100, 1000 ppb range.

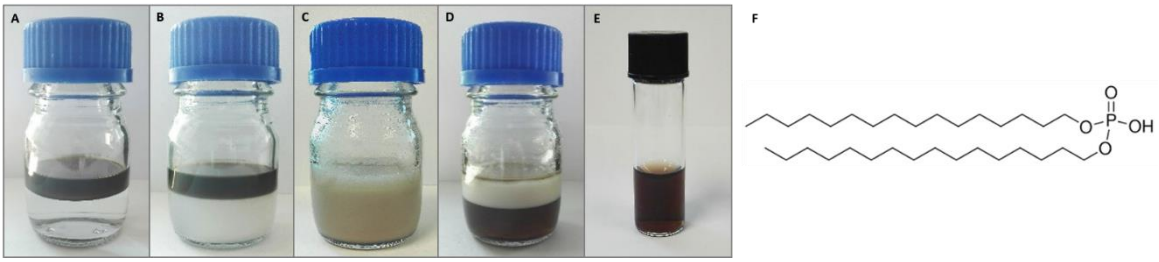

Figure 5. ***A-E*** *- sequential steps of SPIONs functionalization with DHP.* ***F*** *- structure of DHP*

In summary, this work was first to provide a detailed description of SPIONs functionalization with DHP and delve into the biological properties of the obtained nanoparticles. SPION-DHP nanoparticles did not reveal significant cytotoxicity in the range of tested concentrations, in selected cell lines. This preliminary investigation indicated that DHP-coated SPIONs may be safely utilized for biomedical applications.

Assembly and Characterization of HBc Derived Virus-like Particles with Magnetic Core.

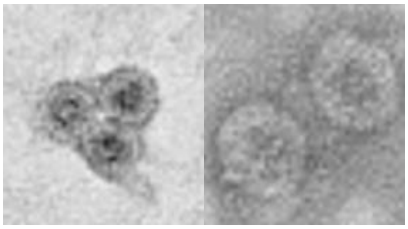

Figure 6. *HBc-derived VLPs with functionalizaed SPIONs as the magnetic core.*

The aim of this work was to utilize the hepatitis B virus core protein (HBc) to create virus-like particles (VLPs) with a magnetic core. My role in this project was co-conceptualization of the experiments, synthesis, and functionalization of superparamagnetic iron oxide nanoparticles, data analysis, and manuscript preparation. In this work, SPIONs were synthesized in the same way as described above. For the functionalization purposes, two compounds were used: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy-(polyethyleneglycol)-2000] (ammonium salt) (PL-PEG-COOH) and dihexadecyl phosphate (DHP). The mechanism of functionalization in both cases relies on hydrophobic interactions between alkyl chains of oleic acid residues present on the surface of SPIONs and alkyl chains of the compounds used. The DHP functionalization methodology is presented above. PL-PEG-COOH was performed as follows. PL-PEG-COOH was dissolved in chloroform and mixed with chloroform suspension of SPIONs. The mix was placed briefly in the sonicating bath, and the chloroform was subsequently evaporated. The obtained waxy solid was heated for 1 min in an 80°C water bath, and DI water was added, forming SPION-PL-PEG-COOH (SPION-PEG) nanoparticles. The solution was washed with chloroform to remove unbound PL-PEG-COOH, filtered, and subjected to further analysis. The concentration of both functionalized nanoparticles was assessed with TGA accordingly to the procedure described above. Both functionalizations resulted in negatively-charged nanoparticles, which is crucial for electrostatically-driven assembly of VLPs.

This work demonstrated the effects of ligands on the assembly of HBV derived virus-like particles with a magnetic core. In both cases, the successful creation of the VLPs was achieved (**Fig. 6**). The article expands on the magnetic core parameters governing the process of electrostatic self-assembly and furthers the knowledge on HBV based VLP systems. Considering the growing interest in utilizing VLP platforms for vaccine development, this research is relevant not only from the standpoint of basic but also applied science.

Osteoarthritis Severely Decreases the Elasticity and Hardness of Knee Joint Cartilage: A Nanoindentation Study.

The aim of this work was to evaluate the elasticity and hardness of the knee joint cartilage derived from patients diagnosed with osteoarthritis and subjected to a total knee replacement procedure. My role in this project was co-conceptualization of the experiments, developing a methodology for sample preparation for nanoindentation, conducting nanoindentation experiments, data analysis, and manuscript preparation. Nanoindentation is a highly precise measurement. Therefore sample preparation is essential for obtaining reliable data. Harvesting tissue samples from osteoarthritic cartilage for nanoindentation study is not a trivial matter. The damage may span from several osteophytes and slightly worn-out cartilage to a complete lack of cartilage tissue.

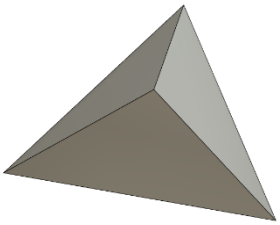

Figure 7. *Berkovich indentation tip model.*

Additionally, the variation between knee joint sizes hinders the selection of the same regions. A sample has to be horizontally and firmly placed in a holder. In the case of articular cartilage, which is highly hydrated tissue, ensuring water conditions for the whole measurement (ca. 3h) is a prerequisite. Taking all of the above into consideration,

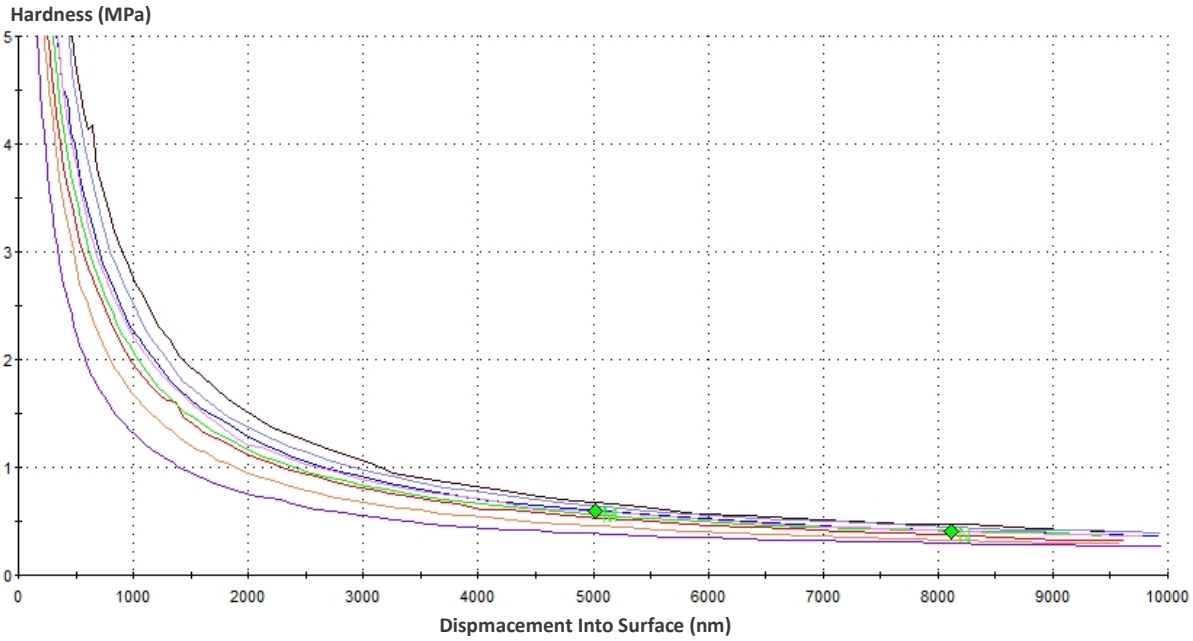

Figure 8. *An example of hardenss measurement. Colors represent separate indentations of one sample.*

the acrylic resin was chosen for sample preparation. After fixation, samples were rehydrated

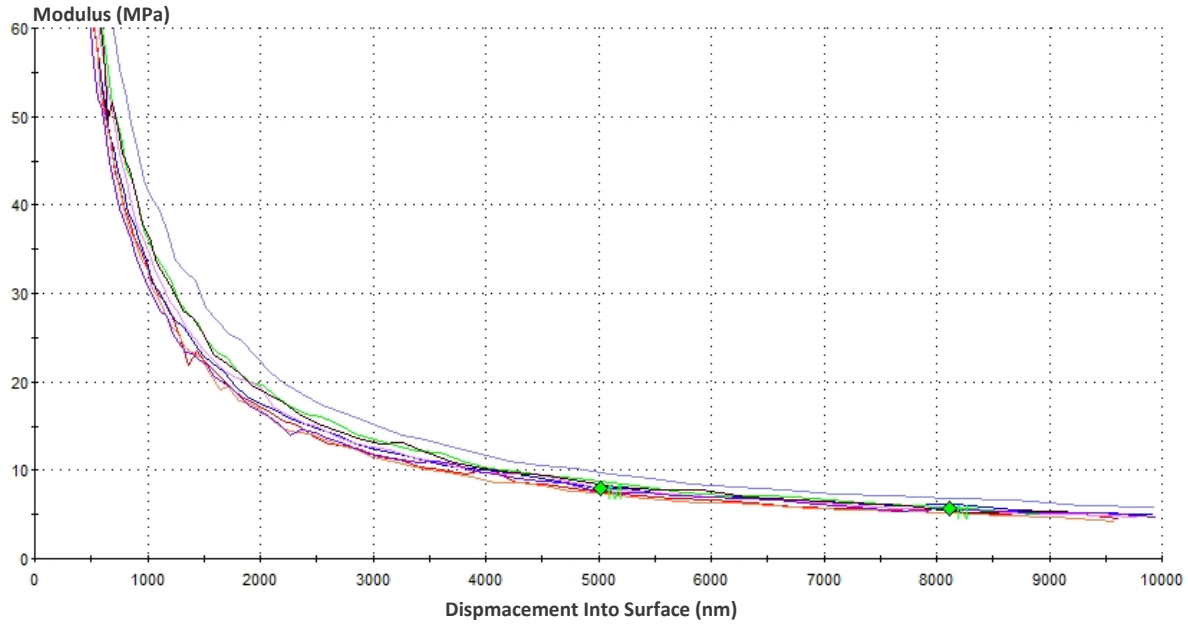

Figure 9. *An example of modulus measurement. Colors represent separate indentations of one sample.*

at RT with phosphate buffer saline (PBS) and subjected to nanoindentation analysis. The indentation tests were conducted on a nanoindenter Agilent G200 with a DCMII head fitted with a Berkovich-type indenter tip (**Fig. 7**). The tip was calibrated before each measurement on quartz crystal (Young's modulus E = 74 GPa).

The measurements were performed in CSM mode. The indentation depth was set at 10 µm at a strain rate of [1/s] the Poisson's ratio of 0,4. For each sample, 12 indents were performed in a 3 × 4 matrix with 200 µm X,Y indent separation. The hardness and mean elastic modulus were obtained from the 5.0–8.0 µm indentation depth range, which was established experimentally (**Fig. 8, 9**).

This work is one of the first studies of the nanomechanical properties of weight-bearing and non-weight-bearing articular cartilage at different stages of osteoarthritis conducted at this scale. The results provide insight into the mechanical behavior of the cartilage at different stages of osteoarthritis in correlation to the patients' ages, which is essential from the clinical perspective. It has also highlighted the limitations of this approach and provided practical guidelines to mitigate some of them.

7. Summary and perspectives

In regard to the core scientific achievements, to following conclusions can be drawn:

[**CSA 1**] **Bioevaluation of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with dihexadecyl phosphate (DHP).**

1. DHP-coated SPIONs did not reveal significant cytotoxicity in the range of tested concentrations.
2. SPION-DHP were successfully internalized by the cells, without eliciting significant alterations in gene expression profile of the selected genes.
3. Due to unknow reasons, iron staining is not compatible methodology for DHP-SPION detection.
4. SPION-DHP nanoparticles are a promising tool for biomedical applications.

[**CSA 2**] **Assembly and Characterization of HBc Derived Virus-like Particles with Magnetic Core.**

1. Both dihexadecyl phosphate and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy-(polyethylene glycol)-2000] can be successfully used as functionalizing agents for the creation of HBc derived VLPs with magnetic core.
2. The obtained VLPs retained its ability to bind specific antibodies.
3. SPION-DHP displayed higher effectiveness in driving VLPs self-assembly.
4. The study provided meaningful insights into design and preparation of VLPs with magnetic core.

[**CSA 3**] **Osteoarthritis Severely Decreases the Elasticity and Hardness of Knee Joint Cartilage: A Nanoindentation Study.**

1. Nanoindentation requires careful experimental design including probe`s geometry, indentation depth, region of sample collection, and sample preparation methodology to provide reliable data.
2. Age and disease progression significantly affects mechanical properties of the chondral tissue.
3. In combination with biochemical analysis, nanoindentation may provide deeper understating of degenerative processes driving the progression of osteoarthritis.

The common denominator for the works presented is nanotechnology in biomedicine. Both synthesis and analysis at the nanoscale regime require careful design and execution of experimental techniques. Despite many research initiatives devoted to nanotechnology, there is still a missing link between basic science and widespread adoption. Hopefully, my work will further the understanding of nanotechnology and facilitate its broad adoption in biomedicine.

My current research is devoted to the application of nanotechnology in 3D bioprinting, which is an exciting technology allowing for precise layer-by-layer deposition of cells and biomaterials. 3D bioprinting opens an avenue for novel tissue engineering approaches, aiming at tissue reconstruction or regeneration. It may also be utilized for the creation of spatially refined tissue or disease models. The goal of utilizing nanotechnology in 3D bioprinting is to create optimal conditions for cellular growth, proliferation, and differentiation. My yet unpublished results focus on creating polycaprolactone reinforced with carbon nanotubes, which can be utilized as a strengthening scaffold for tissue constructs. Preliminary data indicate that at a particular concentration of carbon nanotubes, cell adhesion is significantly facilitated while maintaining excellent printability.

Regarding VLPs, my current work is focused on utilizing SARS-CoV-2 epitopes for specific binding of anty-SARS-CoV-2 antibodies for immunodiagnostic purposes. As mentioned previously, It has resulted in patent application titled “Hybrid nanoparticles

binding antibodies, the method of their production and use for binding specific anti-SARS-CoV-2 antibodies" [**NCA 9]**.

My current involvement in thematically different research projects allows me to continue the exploration of various STEM field related topics, while utilizing and building upon the knowledge I have gathered so far.

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9. List of core publications with links

[1] **A.A. Mieloch**, M. Żurawek, M. Giersig, N. Rozwadowska, J.D. Rybka, *Bioevaluation of superparamagnetic iron oxide nanoparticles (SPIONs) functionalized with dihexadecyl phosphate (DHP).* Sci. Rep. (2020) 1–11. https://doi.org/10.1038/s41598-020-59478-2
https://www.nature.com/articles/s41598-020-59478-2

[2] J.D. Rybka, **A.A. Mieloch***, A. Plis, M. Pyrski, T. Pniewski, M. Giersig, *Assembly and Characterization of HBc Derived Virus-like Particles with Magnetic Core.* Nanomater. (Basel, Switzerland). 9 (2019). https://doi.org/10.3390/nano9020155
(* equally contributed)
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[3] **A.A. Mieloch**, M. Richter, T. Trzeciak, M. Giersig, J.D. Rybka, *Osteoarthritis Severely Decreases the Elasticity and Hardness of Knee Joint Cartilage: A Nanoindentation Study.* J. Clin. Med. 8 (2019) 1865. https://doi.org/10.3390/jcm8111865
https://www.mdpi.com/2077-0383/8/11/1865