FTIR microspectroscopy revealed biochemical changes in liver and kidneys as a result of exposure to low dose of iron oxide nanoparticles

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1. Introduction

Fourier transform infrared (FTIR) microspectroscopy, being a type of vibrational spectroscopy, is a physico-chemical analytical technique that allows identifying a biomolecules-characteristic functional groups on the basis of their IR absorption [1,2]. The method is a combination of optical microscopy facilitating identification of microscopic details of analysed sample and infrared spectroscopy which gives insight on chemical composition [3]. The high spatial resolution of FTIR microspectroscopy – down to single cells – enables obtaining information on the cellular content and distribution of major biological molecules such as lipids, proteins or nucleic acids in microscopic areas of the sample. Moreover FTIR microspectroscopy is also sensitive to the conformation of the biomolecules, which is a rare property among spectroscopic methods [1,3,4]. These features make FTIR imaging a powerful tool in the biochemical analysis of biological samples [2,5–13].

Nanomaterials (NMs) have been widely investigated as a potential tools in biomedical applications including medical diagnostics, therapy and tissue engineering [14,15]. They are most commonly defined as objects with one or more external dimensions size range 1–100 nm [16]. General NMs may be classified according to the chemical composition of their core material (i.e. organic, inorganic), structural features (i.e. consolidated materials, nanodispersions), shape (i.e. spheres, tubes, rods) or aggregation state (gaseous, liquid, solid) [14,17,18]. The commonly explored engineered NMs for biomedicine are based on carbon, silica and metals with different size and shape e.g. nanocrystals, fullerens, quantum dots or nanoparticles [14,18,19]. Among different NMs used in biomedical research nanoparticles (NPs), defined as the objects with two or three dimensions between 1 and 100 nm, are the most intensively investigated [20–23].

Increasing usage of nanomaterials in various fields of science and technology raises concerns about their safety, biocompatibility.
2. Materials and methods

2.1. D-mannitol coated iron(III)oxide nanoparticles

The D-mannitol coated iron(III) oxide nanoparticles were manufactured in the Institute of Macromolecular Chemistry of the Czech Academy of Sciences. The M-IONPs were originally dispersed in the 153.6 mg of d-mannitol/ml and their concentration in the solution was 4.4 mg Fe/ml. Preparation of the nanoparticles was done following procedure described by D. Horak et al. [43], where D-mannose was replaced by D-mannitol. The particle morphology and particle core size were examined by transmission electron microscopy (TEM; JEOL JEM 200 CX). An iron oxide nanoparticle dispersion in water was sprayed onto a grid with a carbon membrane. The number-average core diameter was determined by measurement of at least 10,000 particles for each sample from microphotographs using the Atlas program (Tescan, Digital Microscopy Imaging, Brno, Czech Republic). The core size of M-IONPs was evaluated for 10 nm. The hydrodynamic diameter ($D_h$, Z average) and polydispersity (PDI) as a measure of the distribution width were obtained by dynamic light scattering (DLS) with an Autosizer Lo-C (Malvern Instruments Ltd., Malvern, Great Britain). The $D_h$ of the M-IONPs was 100 nm and PDI 0.13. The DLS chart presenting M-IONPs size distribution together with TEM image were presented in Fig. 1S in Supplementary materials.

2.2. IONPs colloid

To prepare the low-dose dispersion of the M-IONPs, 0.5 ml of the stock solution was diluted with 49.5 ml of deionized water. As a result, 44 μg Fe/ml of target solution for injection was obtained. In order to exclude the presence of the agglomerates, the hydrodynamic diameter of M-IONPs was measured by DLS. The DLS measurements were conducted in the Faculty of Biochemistry, Biophysics and Biotechnology (Jagiellonian University, Krakow) with Zetasizer Nano S (Malvern). The obtained results revealed lack of the agglomerated structures, showing that only fraction with hydrodynamic diameter of 100 nm was present in the dispersion.

2.3. Experimental animals

The male Wistar rats originated from the animal husbandry of the Department of Neuroanatomy (Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow). The all animal use procedures were approved by the Local Ethical Commission in Krakow (agreement no. 121/2015) and were performed in accordance with the international standards.

On the 60th day of their postnatal life, 10 animals were randomly divided into 2 equinumerous groups. The first group (M-7D) was injected into the tail vein with 1 ml of M-IONPs dispersion (44 μg Fe/ml), while the second one (control group M-N) was treated with the adequate volume of physiological saline solution.

2.4. Sample preparation

On the 67th day of their postnatal life, animals from both M-N and M-7D groups were intracardially perfused with 0.9% saline solution of high analytical purity to remove the blood from their bodies. The smallest liver lobe and the right kidney were taken, deeply frozen in liquid nitrogen and sectioned with a cryomicrotome into 12-μm-thick slices. The longitudinal slices of the central parts of both organs were placed on the MیرIR low-e microscopic slides and freeze-dried.

2.5. IR data acquisition

The experiment was carried out at the SMIS beamline of SOLEIL synchrotron (Saint Aubin, France). The measurements were done in transfection mode using FTIR imaging system consisting of Agilent Cary 620 FTIR microscope equipped with a liquid nitrogen cooled 128 × 128 Focal Plane Array (FPA) MCT detector, associated with a Cary 670 spectrometer equipped with a globar IR source and a KBr beamsplitter. IR light was projected through the 4× objective
(N.A. = 0.2) providing 20.6 × 20.6 μm² pixel size with the 2640 × 2640 μm² field of view (FOV). The spectral images of the examined samples were constituted of mosaics, composite images of several sequentially collected FOVs. The spectra were collected in the mid-IR range between 800 and 3900 cm⁻¹, the spectral resolution was set at 2 cm⁻¹, and 128 scans were coadded for both sample and background spectra collection. The data acquisition was performed with Agilent Resolution Pro (version 5.3.0.1694) software.

2.6. Analysis of spectral data

The mid-IR range, covering the wavenumbers 1000–3500 cm⁻¹, was studied and the typical spectra obtained for the normal liver and kidney are presented in Fig. 1. The distributions of main biomolecules in examined tissues were obtained by chemical mapping of the main absorption bands or their ratios with use of CytoSpec (version 2.00.01), Imagej (version 1.51j8) and Surfer (version 9.0.) software. The characteristics of the absorption bands identified in the liver and kidney spectra and analysed in this study are presented in the Table 1.

Before the detailed spectral analysis, a thickness test was performed in the CytoSpec software using the criteria proposed by Lasch et al [46]. This allowed eliminating regions of the sample that were holes, or too thick or too thin and to compare only regions with similar thicknesses. Furthermore, the areas containing borders of anatomical structures were excluded from the examination in order to avoid the influence of the Mie scattering phenomenon on the examined spectra.

The chemical mapping was carried out on unprocessed spectra. The two-dimensional spectral maps were generated by displaying the integrated area of one peak or the area ratio of two peaks including trapezoidal baseline correction. Analysis of the smallest lobe of the liver, which is a fairly homogeneous organ, included the entire slice of the imaged sample (Fig. 2A). In case of kidney, due to its heterogeneity, it was necessary to identify the structures present in the organ. For the purpose of this study two areas of kidney, namely cortex with a subcortical region (K) and an outer core (R), were distinguished (Fig. 2B) [47].

In the next step average intensities (or intensity ratios) of characteristic absorption bands were calculated based on 100 pixels randomly selected from liver and each kidney region separately. The results obtained for animal groups M-N and M-7D were compared and subjected to statistical analysis in order to determine significance of the observed differences.

### Table 1

| Absorption band (nature*) or ratio of absorption bands [cm⁻¹] | Characteristics                                      |
|---------------------------------------------------------------|-------------------------------------------------------|
| 3012 (=C-H st.)                                               | Distribution of unsaturated fatty acids               |
| 3012/2955                                                     | Unsaturation level of lipids                          |
| 3012/1658                                                     | Relative content of unsaturated fatty acids and proteins |
| 2800–3000                                                     | Distribution of saturated fatty acids                 |
| 2955 (CH₃ st. a.)                                             | Changes in the lipid structure including anomalies in their saturation level, length of fatty acids chain and degree of branching |
| 2924 (CH₃ st. a.)                                             | Relative content of saturated fatty acids and proteins |
| 2924/1658                                                     | Distribution of lipids, cholesterol esters, cholesterol |
| 1658 (C=N st.; C=O st.; N–H bend.)                            | Relative content of lipids, cholesterol esters, cholesterol |
| 1635/1658                                                     | Relative content of lipids, cholesterol and proteins |
| 1545/1658                                                     | Distribution of lipids, cholesterol esters, cholesterol |
| 1360–1480 cm⁻¹/1658 cm⁻¹                                      | Distribution of compounds containing phosphate group(s) including nucleic acids, phospholipids, phosphorylated carbohydrates |
| 1360–1480 cm⁻¹/2924 cm⁻¹                                      | Relative content of compounds containing phosphate group(s) and proteins |
| 1240 (-PO₂⁻ st. a.)                                           | Relative content of compounds containing phosphate group(s) and saturated fatty acids |
| 1080 (-PO₂⁻ st. b.)                                           | Relative content of compounds containing phosphate group(s) and saturated fatty acids |

* st. – stretching, a. – asymmetric, s. – symmetric, bend. – bending, sciss. – scissoring.

2.7. Statistical analysis

The Mann-Whitney 𝑈 test was applied for the statistical evaluation of the differences observed between the M-IONPs treated and normal rats. The choice of non-parametric test was dictated by the fact that the data analysed in this study did not meet the assumption about normality, homoscedasticity and linearity which are necessary for the use of its parametric alternative. In the presented study, changes in the biomolecule accumulation and structure in the organs of nanoparticle-

![Fig. 1. The typical baseline corrected IR spectra of the normal liver and kidney tissue.](Image)
treated rats relative to control group were examined at the significance level of 5% \((p\text{-value} < 0.05)\). Additionally differences at the significance level of 10% \((0.05 \leq p\text{-value} < 0.1)\), considered as biochemical trends, were analysed. The statistical analysis was performed with the STATISTICA package (version 7.1).

3. Results

Our results suggest that FTIR microspectroscopy may enable detection of the prolonged changes in composition and/or conformation of the main biomolecules within liver and kidney of animals exposed to the low dose of M-IONPs. Liver and kidney tissue sections were analysed by chemical mapping of characteristic IR absorption bands originating from compounds containing phosphate groups, cholesterol and cholesterol esters, proteins and fatty acids. The chemical maps followed by detailed spectral analysis allowed us to determine biochemical differences between examined liver and kidney regions originating from M-IONP-treated and normal animals.

3.1. The prolonged changes in accumulation and structure of main biomolecules within liver

Statistical analysis of the obtained biochemical data revealed only few anomalies within liver of animals injected with M-IONPs.
Statistically significant ($p < 0.05$) increase in the relative content (compared to proteins) of phosphate group-containing compounds ($1080 \text{ cm}^{-1}/1658 \text{ cm}^{-1}$), as well as lipids, cholesterol and cholesterol esters ($1360–1480 \text{ cm}^{-1}/1658 \text{ cm}^{-1}$), were observed in the M-7D group. What is more, unsaturation level of lipids ($3012 \text{ cm}^{-1}/2955 \text{ cm}^{-1}$) was higher in M-IONP-treated rats compared to controls. Additionally, for animals representing M-7D group, an upward trends ($0.05 \leq p < 0.1$) of the relative contents of both saturated ($2924 \text{ cm}^{-1}/1658 \text{ cm}^{-1}$) and unsaturated fatty acids ($3012 \text{ cm}^{-1}/1658 \text{ cm}^{-1}$) were found. Furthermore, in liver of rats exposed to the nanoparticles the protein content ($1658 \text{ cm}^{-1}$) decreased, which may explain the aforementioned anomalies of the relative biomolecule levels with respect to proteins. The dispersions of the described biochemical parameters in M-N and M-7D groups were presented as box-and-whiskers plots in the Fig. 3, while chemical maps of the representative liver samples are in the Fig. 4.

Fig. 4. The representative chemical maps of selected absorption bands within liver of animal exposed to M-IONPs and control rat together with the corresponding microscopic views of the samples.
3.2. The prolonged changes in accumulation and structure of main biomolecules within kidney

The two kidney regions, namely cortex with subcortex and core, were analysed separately. Obtained results revealed statistically significant changes in both examined organ areas of animals injected with low dose of M-IONPs compared to control rats.

3.3. Accumulation of compounds containing phosphate groups

Statistically significant ($p < 0.05$) increase in accumulation of compounds containing phosphate groups (1080 and 1240 cm$^{-1}$) was observed in both cortical and core kidney regions of M-7D group compared to M-N. The relative intensity of 1080 cm$^{-1}$ absorption band with respect to proteins (1080 cm$^{-1}$/1658 cm$^{-1}$) and lipids (1080 cm$^{-1}$/2955 cm$^{-1}$) was elevated only within cortex and subcortex area. In turn, the 1240 cm$^{-1}$/1658 cm$^{-1}$ and 1240 cm$^{-1}$/2924 cm$^{-1}$ absorption band ratios were significantly higher in both kidney regions of animals treated with M-IONPs. Box-and-whiskers charts presenting differences in aforementioned parameters between M-7D and M-N groups, as well as exemplary chemical maps presenting their distribution are shown in the Figs. 5 and 6.

3.4. Accumulation of lipids, cholesterol and/or cholesterol esters

Statistically relevant ($p < 0.05$) increase in the accumulation of the lipids, cholesterol and/or cholesterol esters (1360–1480 cm$^{-1}$) was observed in both core and cortex areas of the kidneys taken from animals exposed to M-IONPs. The relative intensity of the 1360–1480 cm$^{-1}$ massif comparing to the amide I band (1360–1480 cm$^{-1}$/1658 cm$^{-1}$) as well as to the band originating from saturated fatty acids (1360–1480 cm$^{-1}$/2924 cm$^{-1}$) was higher, at the assumed confidence level, within both analysed areas of kidney for M-7D group. The dispersions of the described biochemical parameters in M-N and M-7D groups were presented as box-and-whiskers plots in the Fig. 7 while chemical maps of the representative kidney samples originating from M-N and M-7D groups are presented in Fig. 8.

3.5. Anomalies in the secondary structure of proteins

The increases in both amide I (1658 cm$^{-1}$) and amide II (1545 cm$^{-1}$) band intensities, statistically significant at the assumed significance level of 0.05, were observed within the core region of the kidney originating from M-IONP-injected animals. Regarding the cortical area of the organ, only amide II level was remarkably higher in M-7D group with respect to M-N group. The amide II/amide I ratio (1545 cm$^{-1}$/1658 cm$^{-1}$) increased in both analysed regions of kidney but the changes in the relative content of β-sheet to α-helix secondary structure of proteins (1635 cm$^{-1}$/1658 cm$^{-1}$) were not noticed. The data concerning the anomalies of protein content and structure occurring in kidney are presented in the Fig. 9 and exemplary chemical maps are shown in the Fig. 10.

3.6. Accumulation and structure of lipids

Within both cortical and core region of the kidney statistically significant ($p < 0.05$) increase in the saturated fatty acids accumulation (2924 cm$^{-1}$, 2955 cm$^{-1}$ and 2800–3000 cm$^{-1}$) was observed in animals exposed to the M-IONPs compared to control group. Furthermore, both examined organ areas presented elevated relative content of saturated lipids compared to proteins (2924 cm$^{-1}$/1658 cm$^{-1}$ and 2800–3000 cm$^{-1}$/1658 cm$^{-1}$). Some structural changes of lipids within cortex and core of the kidneys of animals treated with the M-IONPs were also observed and they manifested as statistically relevant decrease in the relative intensity of 2924 cm$^{-1}$ and 2955 cm$^{-1}$ absorption bands.

The level of unsaturated fatty acids (3012 cm$^{-1}$) was also significantly higher within the analysed areas of the organ for M-7D group compared to M-N group. Moreover, in both cortical and core region of kidney the elevated relative content of unsaturated lipids compared to proteins (3012 cm$^{-1}$/1658 cm$^{-1}$) was noticed. Additionally, increased lipid unsaturation level (3012 cm$^{-1}$/2955 cm$^{-1}$) was observed in the cortical/subcortical area.

The dispersions of the average values of aforementioned biochemical parameters in examined animal groups are presented in the Fig. 11 while distributions of intensities of absorption bands (or their ratios) in the scanned tissue areas in the Fig. 12.
4. Discussion

In the present paper the prolonged biochemical anomalies induced by the intravenous administration of low dose of α-mannitol-coated iron(III) oxide nanoparticles within rat liver and kidneys were analysed by the means of FTIR microspectroscopy. For this purpose two-dimensional chemical maps presenting distributions of absorption bands (or absorption band ratios) of the main biomolecules were done for the organs taken from control and M-IONP-treated animals 7 days after nanoparticle injection. The choice of organs being examined was dictated by their particular roles in the detoxification of the blood circulation system from the foreign bodies such as nanoparticles [27,36]. The comparison of the results obtained for both analysed animal groups allowed us to detect changes that M-IONPs introduced in the content and/or structure of compounds containing phosphate groups, cholesterol...
and cholesterol esters, proteins and fatty acids within liver and kidneys.

In our previous study the elemental changes occurring within main rat organs as a consequence of the PEG-IONP administration were discussed [49,50]. The results obtained showed significant changes of Fe, Ca, Cu and Zn levels in liver and kidney suggesting the nanoparticles accumulation and/or the presence of oxidative stress together with inflammatory processes after 7 days from the exposure to the particles [49,50]. Based on this observation, we hypothesized that injection of the IONPs may also affect the biomolecular composition of the organs. Current FTIR microspectroscopy study revealed the prolonged abnormalities in the accumulation and/or structure of selected biological macromolecules in both liver and kidneys of rats treated with M-IONPs. The observed anomalies are commonly considered as biological markers of some pathological processes taking place in the injured tissue [6,9,45,51–55].

The biochemical analysis of the liver revealed a few differences between rats exposed to the M-IONPs and normal animals. The decreasing trend \((p < 0.1)\) of protein accumulation \((1658 \text{ cm}^{-1})\) was observed in the organ of animals treated with the nanoparticles after 7 days from their administration. The region of amide I, present in IR spectra of biological samples, reflects the secondary structure of proteins and hence the reduction of the intensity of this particular absorption band may indicate progressive changes in the proteins conformation [51,52]. However, no statistically significant change in the relative \(\beta\)-sheet content compared to the \(\alpha\)-helix content was noticed, which suggests the lack of conformational anomalies in proteins for the liver tissue of animals exposed to M-IONPs [45,53,54]. The decrease of the amide I band intensity was most likely the cause of the observed statistically significant increases in the relative contents of phosphate groups \((1080 \text{ cm}^{-1}/1658 \text{ cm}^{-1})\), lipids, cholesterol and cholesterol esters \((1360–1480 \text{ cm}^{-1}/1658 \text{ cm}^{-1})\) as well as saturated \((2924 \text{ cm}^{-1}/...
1658 cm$^{-1}$) and unsaturated fatty acids (3012 cm$^{-1}$/1658 cm$^{-1}$). The absolute intensities of the aforementioned absorption bands in the liver tissue remained at the level of the control group. Furthermore, statistically relevant elevation of the unsaturated lipids, manifested by increase in the relative content of unsaturated compared to saturated fatty acids (3012 cm$^{-1}$/2924 cm$^{-1}$), was found in the organ [6]. The M-IONPs induced biochemical anomalies were much more intense within kidneys, both in core and cortex regions of the organ. The cores of examined nanoparticles had the diameter of 10 nm and thus it was expected that, in case of d-mannitol coating decomposition in blood circulation system, they would be easily removed from the organism by the renal clearance [27,56,57]. However, the biochemical changes present within the organ tissue after 7 days from the exposure to nanoparticles [6,60,62]. Additionally, in the cortex area of the kidneys originating from rats exposed to M-IONPs, the increase of relative intensity of 3012 cm$^{-1}$ band compared to the band of 2955 cm$^{-1}$ was detected. This effect is associated with increase in the unsaturated lipid level [6,60,63]. In turn the elevated relative content of saturated and unsaturated fatty acids compared to proteins (2924 cm$^{-1}$/1658 cm$^{-1}$, 2800–3000 cm$^{-1}$/1658 cm$^{-1}$ and 3012 cm$^{-1}$/1658 cm$^{-1}$), recorded in both analysed kidney regions, suggests that changes in the lipids levels are more intensive compared to those occurring for proteins.

The observed anomalies in the overall lipid content and structure together with elevated IR absorption at wavenumbers specific for lipids, cholesterol and its derived products (1360–1480 cm$^{-1}$) as well as phosphate groups (1080 cm$^{-1}$ and 1240 cm$^{-1}$) may indicate M-IONP-induced accumulation of lipid droplets (LDs) in the renal tissue [64]. LDs are assembling of neutral lipids, such as triacylglycerols, diacylglycerols, cholesterol esters and cholesterol and surrounded by single layer of a phospholipids [64,65]. The formation of LDs occurs when free fatty acids are available in the cells [66,67]. Under physiological conditions such macromolecular structures are found mostly in the tissue directly involved in energy metabolism, e.g., adipocytes, liver and muscle [65]. However, the LDs can be dynamically synthesized and decomposed in response to cellular demand and environmental signals in all types of tissues [68,69]. This protective mechanism, which may be induced by oxidative stress or energetic and redox imbalance, is directed by bioenergetic homeostasis in cell [68,70]. Thus, the elevated level of lipids, compounds containing phosphate groups and cholesterol/cholesterol...
esters may indicate occurrence of redox and/or energy balance disruption within the kidneys.

The observed biochemical changes suggesting the presence of redox imbalance within kidneys might be connected with the mechanical injuries caused by M-IONPs during removal through renal way [27,71]. Due to their hydrodynamic size of 100 nm, M-IONPs cannot be simply eliminated by kidneys that are able to filter out particles with size up to 15 nm but trapped in their fenestrae [71,72]. Such trapped nanoparticles may be the source of mechanical and following oxidative stress triggering the LDs formation as the defence mechanism [68,73]. On the other hand, as the in vivo stability of the d-mannitol coated IONPs is not well known, the occurrence of the M-IONPs degradation after 7 days from the injection also cannot be excluded [49,50]. After administration to the blood circulation, IONPs are sooner or later recognised, internalised and decomposed to free Fe ions by macromolecular phagocytic system represented by the liver Kupfer cells as well as by the intraglomerular mesangial cells present in kidneys [27,47,74]. Thus, the redox imbalance underlying the assumed LDs formation may be caused by redundant Fe ions present in the renal tissue [75,76].

5. Conclusions

The results confirmed usefulness of the FTIR microspectroscopy in the analysis of prolonged biochemical anomalies induced within liver and kidneys by intravenously injected M-IONPs. Conducted research revealed changes in both content and structure of main biomolecules, occurring 7 days after exposure to M-IONPs. Furthermore, abnormalities in the biochemical composition were much more intensified within kidneys than in liver. Even though low dose of the M-IONPs was administered to experimental animals, the observed changes in the content and/or structure of lipids, phosphate groups as well as cholesterol and cholesterol esters in the renal tissue may indirectly indicate at the occurrence of oxidative stress within the organ. Such disturbance in the redox homeostasis the most probably triggered formation of LDs, which is the defence mechanism directed to suppress oxidative stress.

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CRediT authorship contribution statement

Agnieszka Drozdz: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Writing - original draft. Katarzyna Matusiak: Investigation, Writing - review & editing. Zuzanna Setkowicz: Conceptualization, Methodology, Resources, Investigation, Writing - review & editing. Malgorzata Ciarcia: Resources. Krzysztof Janeczko: Conceptualization, Writing - review & editing. Christophe Sandt: Investigation, Writing - review & editing. Ferenc Borondics: Investigation. Daniel Horak: Resources, Writing - review.
**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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