Cytometric analysis of Zn-based nanoparticles for biomedical applications

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Nanoparticles (NPs), unlike bulk or ionic forms of the same material, exhibit different pharmacodynamics and pharmacokinetics, including the ability to cross physiological barriers in the living organism. The potential of NPs for biological applications is currently widely discussed. However, the discrepancies in the literature data concerning NPs biodistribution patterns and nanotoxicity hinder and question their practical applications. On an example of widely-used Zn-based NPs, we will discuss contradictions present in the subject literature. Available reports on the biodistribution patterns of Zn-based NPs, suggest their dynamic absorption from gastrointestinal tract and subsequent distribution mainly to the liver, kidneys and spleen. However, the dynamics and accumulation patterns of NPs in the organism are difficult to interpret based on the literature data. Furthermore, available spectroscopy-based methods used in the majority of studies do not reflect the actual formula of analysed materials (ionic Zn vs. NPs). In current chapter we will discuss the use of innovative, microscopy-based quantitative and qualitative studies in the analyses of biocirculation of ZnO NPs based on the fluorescent ZnO doped with Eu.

**Keywords:** Zn-based nanoparticles; biodistribution; bioelimination; cytometry

1. Introduction

1.1 Phenomenon of nanostructures

Nowadays, nanotechnology become very promising and rapidly developing field of science in many areas related with human live. Nanostructures have different properties from bulk materials, which is greatly caused by considerable reduce of size of used materials down to manometer scale. This mechanism reflect an interesting effect of high surface area to volume ratio of nanostructures with following changes of chemical and physical properties. Consequently, these materials reveal higher diffusivity, chemical reactivity and mechanical strength compare to analogic bulk materials [1]. Above features become especially useful in the industry, where adding nanocomposites (ex. Carbon nanotubes) in the process of production of building materials, reflect their increasing strength, resistance to extreme weather conditions and reduce the porosity [2]. Rare-earth doped nanoparticles seem to be perspective as luminophores in modern optoelectronic devices, for more effective source of light [3]. Nanotechnology may be also very useful in the field of science related with animal breeding, where the proper and efficient supplementation of elements plays a key role. It has been shown that selenium, as a trace element added to the feed, leads to stimulation of the immune system of animals and has a positive effect on the health and development of the poultry. Selenium in the nanoparticle form exhibits higher bioavailability for living organisms, comparing with a traditional, inorganic form. Similar results were obtained for calcium carbonate [4]. Zinc and titanium oxide nanoparticles have been very widely using as an ingredient of sunscreens, due to their UV-blocking properties. Additionally, the antibacterial properties of zinc oxide nanoparticles become promising for dental applications, as the component of material filling the teeth [5].

1.2 Zin Oxide nanoparticles for biomedical applications

From the biological point of view, the most attractive feature of nanostructures is their small size, exceptionally important for overcoming the physiological barriers [6-9] as well as great flexibility of modification their composition and surface properties. Frequently, adjustment of chemical structure of nanoparticles can lead to obtain strictly desirable characteristics like particle stabilization, an amphiphilic, hydrophilic or hydrophobic surface properties and anti-aggregation effect [10].

The potential of nanoparticles as drug carriers, dietary supplements and diagnostic/therapeutic agents is currently widely studied. In one of the experiment researches conjugated the cytostatic drug used in anti-cancer therapies - L-asparaginase with zinc oxide nanoparticles (ZnO), for testing an improved form of drug delivery to areas of cancer. The effectiveness was tested in vitro to breast cancer cells and results showed the increased anti-tumour effect of the drug with ZnO nanoparticles [11]. Similar, in vitro study was conducted with ZnO nanoparticles combined with curcumin, as a substance with anti-cancer properties. Researches highlight the beneficial features of biological and physic-chemical
compound produced with ZnO nanoparticles, especially for the future use in cancer therapies (e.g. possibility of designed size, particle surface charge). A significant, promising conclusion obtained from the study was possibility of slower and easily-controlled drug releasing from the nanoparticles with curcumin [12]. Likewise, interesting results were obtained from the study with the anticancer effect of ZnO nanoparticles combined with doxorubicin (chemotherapeutic agents used in anti-cancer therapies) against the tumour cells. Effects, from both in vitro and in vivo studies, indicate a reduction of doxorubicine dose in the combination with ZnO nanoparticles, comparing to standard procedure, for obtaining the anti-cancer effect [13]. The anti-inflammatory effect of ZnO nanoparticles compared to ZnO in bulk form cells have been also tested in vitro on human mast cell line. Results indicated that nanoparticles inhibited the release of the pro-inflammatory cytokines and the effect was more effective comparing with ZnO in bulk form. Further research confirmed above result in vivo with application of ZnO nanoparticles orally or directly on the skin in mice with induced passive cutaneous anaphylaxis (PCA – passive cutaneous anaphylaxis [14].

Future utilization of nanoparticles for biomedical application (diagnosis and treatment of diseases) seems to be very promising. However, a precise examination of mechanisms related with distribution of nanoparticles within the living organism is necessary. There are still many discrepancies in the literature data concerning nanoparticles uptake, mechanisms of circulation in the body, elimination patterns and nanotoxicity.

1.3 Contradictions of biodistribution of Zn-based nanoparticles

As mentioned above, due to the increasing use of Zn-based nanoparticles, also in the areas directly related to human, it is important to determine their exact fate in living organisms. Nowadays, many scientific reports focus on biodistribution of nanoparticles, however results are often contradictory, which prevent creation of the final model of the nanoparticles circulation in the body. Seemingly, similar studies (the same animal model, nanoparticles and route of administration of material) lead to different conclusions, which have been caused by various reasons. One of the most important factor reflecting the final effect of nanoparticles is their size and shape, as well as other chemical and physical properties (surface charge, methodological approaches etc.). Other crucial aspect of the studies in the field of biodistribution Zn-based nanoparticles is a dose and frequency of the nanomaterial application. Comparing the accumulation effects in certain organs seems to be exceptionally hard, due to multiple/large doses of nanoparticles (in many scientific reports), which led to toxic effect, not the accumulation result.

In one study 20 nm negatively charged ZnO nanoparticles were intragastrically administrated to rats in different dose: from 125 mg/kg up to 500 mg/kg of bodyweight, daily for 90 days. Results of total zinc concentration in collected organs indicated higher level of the element in the liver, small intestine, kidneys and blood serum within each experimental group. No increased zinc content was observed in the brain, reproductive systems, spleen, stomach and lungs. However, histopathologic analyses revealed pathologic changes in the spleen, stomach and eyeball [15].

Similar studies were conducted with different ZnO nanoparticles size (20 and 70 nm, negatively charged) and different dose (up to 300 mg/kg of bodyweight). Application of ZnO nanoparticles was single, with following sacrifice after various time points: from 30 min. to 96 h. Smaller nanoparticles reached blood circulation more rapidly however, level of zinc quickly returned to the control values in each experimental group. Researchers indicated kidneys, liver and lungs as organs responsible for bioaccumulation of ZnO nanoparticles. They also proposed an effective process of elimination of nanoparticle from the body, related with excreting with bile and pancreatic juice, based on high level of zinc in faeces comparing to urine of examined rats [16].

Comparative study was also performed for ZnO and TiO2 nanoparticles, given orally route, daily for 13 weeks in different dose (for ZnO nanoparticles from 134.2 mg/kg up to 536.8 mg/kg of bodyweight). Results indicated relatively low absorption level of both type of nanoparticles from the gastrointestinal tract, however increased level of zinc was observed in the blood circulation in rats from experimental group with ZnO nanoparticles. Authors interpreted the result as an effect of dissolving ZnO nanoparticles in the acid stomach environment, with following transfer of Zn ions and their absorption to the blood circulation. According to researchers, TiO2 nanoparticles were more stable in the acidic environment and consequently the level of their absorption was lower. Biodistribution results of the study revealed higher level of Zn in all tested organs, with emphasis the liver and kidneys. In the study, the concentration of Zn was increased in urine of experimental animals [17].

Other similar studies with a single, intragastrical application of 100 mg ZnO nanoparticles to mice were performed. Animals were sacrificed after different time (up to 72 h) and various organs, tissues and blood serum were analyzed for Zn concentration. Based on blood serum results, authors concluded the highest effectiveness of absorption the nanoparticles from gastrointestinal track 6 h after administration of material with following return to the control level after 48 h. Authors did not indicate any changes in zinc level in the brain, heart, testes and lungs. However, increased Zn concentration was observed in the liver, kidneys and spleen. [18].

In one of the studies, authors indicated increased level of zinc in the liver and kidneys, following by oral administration of ZnO nanoparticles for 14 consecutive days in mice (dose of 50 mg/kg or 300 mg/kg of bodyweight). Additionally, biochemical and histopathological examinations revealed liver damage after oral exposure of ZnO nanoparticles, however no changes were observed in the brain [19].

In most of the studies described above the method for determining the distribution of Zn-based nanoparticles within the body was based on a quantitative measurement of absolute Zn content in organs/tissues using spectrometry methods.
Interpreting the results, it is impossible to conclude whether measured zinc was present in the tissues as nanoparticle or in ionic form. Rapid biodegradation of administrated nanoparticles within the body, with release of Zn ions is likely possible, which cannot be determined by standard methods.

One of the example of different methodology in the study of tracking the fate of ZnO nanoparticles in the living organism is scientific report with applied neutron-activated ZnO nanoparticles. The aim of the activation was inducing radioactivity to detecting and measure Zn deriving from nanoparticles in collected tissues. Nanoparticles were administrated by gavage, in a single dose 5 mg/kg to rats. After 7 days of administration of nanoparticles, increased level of radioactive Zn was detected only in the skeletal muscle, bone and skin (despite of various collected organs and tissues). Authors concluded very efficient mechanism of elimination ZnO nanoparticles from the body, mostly with feces – where 95% of activated Zn was found up to 7 days after administration of nanoparticles [20].

Another interesting experiment was conducted using molecular imaging as a method for investigation the biodistribution of ZnO nanoparticles. In the study, ZnO nanoparticles were conjugated with fluorescent dye and orally given to rats in dose 1 mg/kg or 5 mg/kg. Results showed distribution of nanoparticles at 23 h after their administration mainly to the liver and kidneys [21].

2. Material and methods

2.1 ZnO:Eu nanoparticles synthesis

Europium(III) doped zinc oxide nanoparticles (ZnO:Eu) were prepared using microwave hydrothermal technique. This method allowed to crystallize oxide nanoparticles using wet chemistry approach only [22]. In the 200 ml of distilled water the precursors: Zn(NO₃)₂·6H₂O (99.9%, Sigma-Aldrich, Sp. z o.o. Poznań, Poland) and Eu(NO₃)₃·5H₂O (99.9%, Aldrich) were dissolved under magnetic stirring to obtain clear solution. Molar concentration of europium in relation to zinc was 0.5%. The solution was then alkalinized with aqueous ammonia solution (25%, J.T. Baker, Avantor Performance Materials Poland S.A., Gliwice) to reach pH value of 8. Resulting precipitate was triply washed with distilled water and suction filtered. Wet residue was then placed in the 100 ml teflon vessel of the Magnum II reactor (ERTEC-Poland, Wrocław, Poland) to reach filling factor of 0.8. Reactor is equipped with steel coating and 700 W microwave heating, allowing reaching pressure up to 10 MPa. The process was conducted by 20 min at pressure of 4 MPa, then the resulting product was dried at 40°C overnight. Dry powder was ground in agate mortar and stored.

2.2 Physical characterisation of ZnO:Eu nanoparticles

Luminescence spectra were taken with Horiba/Jobin-Yvon Fluorolog-3 spectrofluorimeter equipped with 450 W xenon arc lamp as a source of excitation. Signal is detected by Hamamatsu R928P photomultiplier in the UV-Vis range and cooled PbS detector in the NIR-IR range. Low temperature measurements were conducted with dedicated Janis liquid helium cryostat. Nanoparticles were further characterized with Hitachi SU-70 scanning electron microscope equipped with GATAN Mono CL3 system of cathodoluminescence measurement, EDX characteristic radiation detector and liquid helium system. Microscope was operated at accelerating voltages in the range 0.5-30 kV and signal was measured by two secondary electrons detectors or semiconducting backscattered electrons detector. Detection of luminescence signal was conducted using photomultiplier or CCD camera. To visualize single nanoparticles and their aggregates of ZnO:Eu in the tissue, samples were scanned using Leica SP8-WLL confocal system (KAWA.SKA Sp. z o.o., Poland) with excitation range 470-670 nm vs. 480-780 nm emission range and emission-excitation parameters were: 619 vs. 625-650 nm. Evaluation was conducted under 100x oil-immersion lens with 15x digital zoom, 8x frame and 8x line averages, utilizing HyD detector.

2.3 In-vivo experiments

Adult, healthy Balb-c mice (3-6 months, n=35) were kept in the individual cages, in the standard and controlled conditions: 25°C, humidity 30% and 12 h day-night cycle (UniProtect Air Flow Cabinet, Merazet SA, Poland). Feed and water were provided ad libitum. All procedures were approved by the Local Ethical Committee (agreement No 44/2012) and conducted according to the EU and local directives. Experimental procedures started after seven days of acclimatization. After immobilization, 0.3 ml suspension of nanoparticles (10 mg/ml) in water was administrated via gastric gavage (IG) to mice (Herberholz HAUPTNER 31011, Merazet). After 0 h, 24 h, 7 d, 14 d or 1 m mice were sacrificed (with CO2 Box, Bioscape, Merazet) and internal organs/tissues were collected for evaluation (including duodenum, liver and brain). Collected tissues were embedded in paraffin, cut into 6 μm-thin sections (Leica-PM2255, KAWA.SKA) and mounted on the microscope slides (Equimed Sp.J. Warsaw, Poland). Following rehydration procedure, cell nuclei of the samples were counterstained with Hoechst 33342 (Sigma-Aldrich) and mounted with Fluoromount (Sigma-Aldrich) under the coverslips.

SCAN^R scanning cytometry (Olympus Polska Sp. z o.o., Warsaw, Poland) was used for identification of ZnO:Eu nanoparticles within the tissues, based on Eu-related red fluorescence. In the analysed organs identification of cells was carried out based on the fluorescence of cell nuclei (labelled with Hoechst 33342). Index of positive cells (relative mean
fluorescence from the cell above 25) was quantitatively evaluated in the whole tissue sample and tissue maps showing distribution of ZnO:Eu positive cells were prepared. Map of the distribution of ZnO:Eu nanoparticles with index of cells positive for the ZnO:Eu fluorescence was performed for collected organs in every time point. In the control samples the false-positive signals did not exceed 2.5% for the liver and 0.5% for the rest of organs. Representative distribution maps were presented.

3. Results

3.1 Structure of ZnO:Eu nanoparticles

Microwave hydrothermally grown europium (III) doped zinc oxide (ZnO:Eu) crystallized in the hexagonal wurtzite-type structure as was found earlier [8, 23]. The nanoparticles with mean size of 48 nm [24] were bound in the bluntly terminated, elongated hexagonal prism shaped agglomerates (Fig. 1). The size distribution of nanoparticle aggregates ranged wide from 100 nm up to 1 μm.

3.2 Spectroscopy analyses

ZnO:Eu nanoparticles luminescence excitation spectrum (Fig. 2) shown many different emission mechanisms present in the material. The excitation spectrum was taken by monitoring luminescence line at 616 nm in the function of changing excitation wavelength in the range 250-550 nm. The line at red spectral range was chosen, as it is most intense luminescence of Eu(III) ions in ZnO matrix, basically not sensitive to the chemical surroundings. Luminescence spectra at three excitation wavelengths are shown to visualize spectral behavior of material. First excitation in middle ultraviolet region (260 nm, Fig. 2a) resulted in activation of broad signals peaking around 400 and 650 nm as well as number of sharp lines, where the most prominent are placed at 594, 617 and 693 nm. The broad ones are related to the fundamental absorption edge (marked as NBE on the chart) and defects (marked as DLE) of ZnO matrix. Slender lines result from the intrashell 4f transitions of Eu(III) ions. Second excitation in near ultraviolet region (385 nm) resulted in significant activation of the defect related luminescence (Fig. 2b), almost covering Eu(III) luminescence. Last excitation is placed in the visible range (544 nm) and resulted in activation nearly solely europium related red luminescence.
3.3 Tissue distribution of ZnO:Eu nanoparticles

Representative maps of the distribution of cells positive for ZnO:Eu nanoparticles and index of cells positive for NPs in analysed organs are presented on Fig. 3. In duodenum and liver the index of cells positive for NPs was the highest as soon as 3 h after IG with respectively 73.73% and 53.06% cells showing fluorescence related to nanoparticles. In subsequent time-points (with exception to 14 d) a change in the pattern of distribution of ZnO:Eu was observed with positive cells predominantly located in the tips of the villi. Alongside, a drop in the index of positive cells was observed. In the liver the gradual decrease of index of ZnO:Eu-positive cells was observed until 7 d, with following gradual increase of ZnO:Eu-positive cell number. In the brain the index of ZnO:Eu-positive cells was the highest at 24 h after IG. At 3 h, a number of randomly-scattered positive cells was observed, with more pronounced regionalisation of ZnO:Eu nanoparticles in following time-points. The areas of predominant allocation of nanoparticles at 24 h and later time-points were associated with loci of high concentration of neurons in the limbic system and cerebellum.

To confirm that recorded fluorescence signal was generated in the intact ZnO:Eu nanoparticles, samples of liver and brain were immobilised on silicon wafers and evaluated under high magnification confocal microscope (Fig. 4 C).
Following confocal imaging, identified fluorescent structures were re-evaluated under SEM elemental analysis (Fig. 4 A-B). High-magnification confocal imaging of both liver and brain showed aggregates of elongated objects brightly fluorescent in the Eu(III) doped ZnO detection range of 600 to 800 nm. Structures were non-uniform and consisted of an assembly of smaller elongated objects similar in shape to ZnO:Eu nanoparticles. SEM imaging confirmed presence of ZnO:Eu aggregates of similar shapes and dimensions to observed under confocal microscope (Fig. 4 B). To confirm the presence of crystalline ZnO in analysed tissues, EDX measurements were conducted in the regions indicated by cross-hairs (ROI 1 on the structure and ROI 2 in the surrounding tissue). The results of elemental analysis are shown in Table 1.

Table 1  Molar concentrations of the key elements identified in the ROI 1 and 2 from the liver and brain samples (for reference see Fig. 4 B).

<table>
<thead>
<tr>
<th>element</th>
<th>liver ROI 1</th>
<th>liver ROI 2</th>
<th>brain ROI 1</th>
<th>brain ROI 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>80.9</td>
<td>77.9</td>
<td>29.0</td>
<td>41.3</td>
</tr>
<tr>
<td>O</td>
<td>13.0</td>
<td>14.8</td>
<td>63.4</td>
<td>34.0</td>
</tr>
<tr>
<td>Si</td>
<td>3.7</td>
<td>7.1</td>
<td>3.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Zn</td>
<td>2.0</td>
<td>0.1</td>
<td>0.8</td>
<td>0.0</td>
</tr>
</tbody>
</table>

All ROI indicated presence of high carbon and oxygen signals originating from the surrounding tissue (Fig. 4 A). Additionally, presence of silicon was also confirmed and related to the preparation of samples. However, the Zn-related signal was identified only in the ROI 1 of both liver and brain tissues. Also, the Zn:O ratio changed strongly from ROI 1 at 1:6.5 to ROI 2 at 1:148. The ratio observed in ROI 1 was much closer to the 1:1 ratio in the stoichiometric ZnO, suggesting that observed objects were indeed ZnO:Eu nanoparticles.

4. Discussion

As we mentioned in the introduction, most of the studies which focus on biodistribution of ZnO NPs in the body are based on spectroscopy methods. This method, however quantitative, gives no indication on the actual formula of analysed materials, whether detected Zn is in the nanoparticle or ionic form. Since effective degradation of Zn-based NPs in the tissues was reported [23, 24], this provides a key question in the evaluation of dynamics and kinetics of nanoparticle distribution and elimination processes in the organism. Here we propose a novel cytometric approach to the analysis of nanoparticle biodistribution based on ZnO doped with Eu with fluorescent properties.

One of the most important and, unfortunately, highly inconsistent factor of available studies conducted with the use of ZnO nanoparticles, is the dosage of administrated Zn. This inconsistence renders concluding/comparing circulation patterns of Zn-based NPs impossible. Summing up the total dosage of zinc per mouse from the groups with the highest dose of ZnO NPs in the previous studies with multiple treatments, animals received approximately: 45 g/kg [15], 48.8 g/kg [17] and 4.2 g/kg [19], a dose exceedingly over the recommended, safe dose for animals. Acute effects were observed at doses higher than 0.48 g/kg bw/day and in rats a reproductive toxicity was observed at a dose approximately 0.2 g/kg bw/day [25]. In the studies where single application of ZnO NPs was used, the dosage of zinc
was approximately 300 mg/kg [16] and 100 mg per animal [18]. Not surprisingly with such high (even extreme) doses of Zn, the toxic effects were reported in the studies [15, 19]. For comparison, in our study a dosage of zinc was approximately 0.09 g/kg (3 mg per mouse), a dose more relevant to the actual NPs concentrations for biomedical applications. In our experiments, we also performed spectroscopy-based analysis of biodistribution of administrated ZnO:Eu NPs [8], however relatively low dosage of NPs per mouse did not affect significantly the overall levels of Zn in studied organs and tissues. Effective Zn metabolism could explain, at least partially, the high Zn doses used by other authors. Methods based on the spectrometric analysis of Zn concentration seem to be effective (provided results variable from control) only where the total dosage of administered Zn was high [15-19]. On the other hand, our cytometric method provided a viable tool to detect relatively low dose of used NPs. Thus, we did not observe any toxic effects of Zn in any analysed tissue (data not shown). Furthermore, a distinct, non-uniform pattern of distribution of NPs in different tissues could be described for the first time (Fig. 3). Since Europium exhibits the fluorescent properties only when the Eu ions are locked within the matrix of ZnO, the fluorescent signal cannot be detected after a disintegration of ZnO:Eu NPs. This indicates that for a first time a tool that allows distinction between crystalline (intact) ZnO NPs and Zn in ionic form (dissolved, metabolised NPs) is available. Thus presence of intact NPs can be quantitatively assessed with scanning cytometry (Fig. 3). Finally, a presence of crystalline ZnO:Eu NPs was confirmed in the samples of liver and brain by high-magnification confocal visualization, with scanning electron microscopy and elemental analyses (Fig. 4, Table 1).

The long-term presence of intact ZnO:Eu NPs and fluctuations in the index of cells positive for ZnO:Eu fluorescence in the duodenum and liver after single IG could indicate the presence of recirculation-redistribution of NPs in the organism. Basing on scanning cytometry analyses we can conclude very efficient process of NPs absorption (via the intact intestinal barrier) as soon as 3 h after IG (Fig. 3). At this time point NPs were evenly distributed throughout the duodenal mucosa and the index of cells positive for NPs exceeded 70%. Effective and fast biodistribution of ZnO:Eu NPs with blood seem to be confirmed by results obtained for the liver, where an index of cells positive for the NPs was over 50% 3 h after IG. At this time point distribution of NPs within the liver was relatively uniform. Following time points showed variations in the distribution pattern of ZnO:Eu NPs in the liver with NPs accumulation in the vicinity of portal veins at 24 h and hepatic arteries/bile ducts at 7, 14 and 30 d post IG (see Fig. 3 LIVER). In the duodenum (Fig. 3 DUO), the uniform distribution of NPs-positive cells observed at 3 h, changed to the spotted, uneven distribution pattern at 24 h indicating effective absorption of ZnO:Eu from the gut. At following time-points the allocation of NPs in duodenum was limited to the villi (7 and 14 d) and tips of the villi (30 d post IG). Observed changes in the distribution pattern of NPs in the liver and duodenum as well as the dynamic fluctuations in the index of cells positive for ZnO:Eu seem to confirm the suggested role of liver as the main elimination organ [6-9, 15-18], with bile as the main elimination medium for NPs [8]. However, the persistence of high index of NPs-positive cells in the duodenum at 7-30 d post IG indicate a highly probable recirculation of NPs in the liver-bile-enterocyte cycle.

Permeability of the blood-brain barrier by NPs was already reported for ZrO$_2$:Pr [6] and Y$_2$O$_3$:Eu NPs [26]. In present study an interesting pattern of distribution of NPs in the brain can be observed (Fig. 3 BRAIN). Initially, at 3 h after IG, the uniform low distribution of ZnO:Eu in the brain can be observed. The maximum of the NPs transfer to the brain seems to occur in under 24 h post IG with majority of NPs allocating in the areas of dense neuronal networks of limbic system (Fig. 3) and cerebellum [6, 26]. During following days, the predominant association with limbic system remains, however the distribution pattern changes to more linear. This, in accordance to the trafficking of NPs along neuronal projections described earlier [9], suggests a possible trafficking and/or elimination route of NP from the brain via neuronal transport.

5. Conclusions

Firstly, in the current study an innovative, both quantitative and qualitative microscopy-based method for analyses of biodistribution of ZnO NPs doped with Eu for fluorescence. Unlike to usual spectroscopy-based methods used in the majority of similar studies, presented technique is suitable for detection low doses of ZnO:Eu NPs in the tissues and organs. Furthermore, obtained results overcome the biggest disadvantage of mineral analyses, the question whether analysed substance is in the ionic or NPs form, since Eu atoms emit fluorescence only when locked inside the ZnO crystalline matrix.

Secondly, changes in the distribution pattern of ZnO:Eu-positive cells in the liver and duodenum may suggest recirculation mechanism of NPs in the organism. NPs eliminated with the bile are secreted to the duodenum, where a portion undergoes reabsorption to the blood and following distribution. This may indicate the need for further redevelopment of the concepts guiding pharmacodynamics and pharmacokinetics of nanomaterials.

Thirdly, the distribution pattern of ZnO:Eu-positive cells in the brain suggest a predominant allocation of ZnO-based NPs in the limbic system and possible elimination route from brain along neuronal projections.

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