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Degradation of $\text{ZnGa}_2\text{O}_4:\text{Cr}^{3+}$ Luminescent Nanoparticles in *lysosomal-* like medium

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Abstract

The ultimate goal of *in vivo* imaging is to provide safe tools to probe the inside of a body in order to obtain pathological information, monitor activities, and examine disease progression or regression. In this context zinc gallate doped with chromium III (ZGO) nanoparticles with persistent luminescence properties have been previously developed, their biodistribution was evaluated, as well as their *in vitro* toxicity. However, to date, nothing is known about their potential transformations in biological media, which may hinder their biomedical applications. In order to know if these nanoparticles could degrade, the present work consist in studying their fate over time depending on both their coating and the aqueous media in which they are dispersed. ZGO nanoparticles have been dispersed in three different aqueous solutions and characterized by numerous technics, up to 90 days. Among the evaluated dispersion media, the *Artificial Lysosomal Fluid* (ALF) mimicking intracellular lysosome environment elicited significant degradation of ZGO nanoparticles. The chelating agents present in ALF have proven to play a major role in the degradation of the ZGO, by stabilizing the nanoparticles and increasing the contact. An important time decrease of the luminescence properties has also been observed, that correlated with the release of ions from ZGO nanoparticles as well as their decreasing size. This information is valuable since it indicates, for the first time, the long-term degradation of persistent luminescent nanoprobes in an *in vivo* like model medium. Therefore, a possible elimination of the imaging probes after *in vivo* preclinical applications could be foreseen.

Introduction

In vivo imaging requires safe tools to probe the inside of a body in order to obtain pathological information, monitor activities, examine disease progression or regression, track drug distribution, or to evaluate drug efficacy.^{1,2} Among the different existing imaging modalities, optical imaging has matured rapidly during the last years, and is expected to keep maturing. Optical imaging is highly complementary to other imaging methods, such as X-rays or magnetic resonance imaging, in particular due to its potential for acquiring data at high speed. For that purpose, various fluorescent probes enabling the study of biological processes have been developed.³ Among these probes, semiconductor quantum dots (QDs) exhibiting fluorescence optical properties have emerged as a class of nanoparticles for bioimaging and diagnostics. These nanocrystals are brightly fluorescent because of their high quantum yield, enabling their use as imaging probes both *in vitro* and *in vivo*.⁴ However, this technique is limited *in vivo* by tissue autofluorescence when exciting the fluorescent probe inside the animal body.⁵ The low probe signal to background ratio greatly limits the sensitivity of the detection. For this reason, the search for probes with improved properties is still under investigation. A couple of years ago, our lab pioneered the use of a particular optical phenomenon, called persistent luminescence, for the conception of new imaging probes.⁶ Persistent luminescence nanoparticles (PLNPs) corresponds to luminescent materials that keep emitting light for minutes to hours after the excitation has stopped.⁷ The development of nanoparticles (NPs) with this property, allows obtaining imaging information with a complete avoidance of the autofluorescence. Indeed, in that case, due to the long-lasting luminescence, the acquisition of the signal is possible after endogenous chromophores have finished emitting their luminescence. The past ten years have witnessed several advances to establish near-infrared (NIR) emitting PLNPs as highly sensitive technology for real-time optical imaging in small animals.^{8,9} The first generation of PLNPs was based on silicate matrix doped with rare earth element, such as europium, dysprosium and manganese.¹⁰ Since then, various materials have been developed with their own specificity such as *in vitro* assays,¹¹ photostimulation,¹²⁻¹⁴ multimodal imaging,¹⁵⁻¹⁸ and for therapy.¹⁹ In this trend, we recently developed $\text{ZnGa}_{1.995}\text{O}_4\text{Cr}_{0.005}$ NPs or ZGO,²⁰ that were found attractive due to their bright near-infrared persistent luminescence and the possibility to be activated *in vivo* with visible irradiation. Therefore the ZGO can emit a persistent luminescence signal through the tissue transparency window for dozen of minutes allowing performing *in vivo* imaging without any

background with a high signal to noise ratio, thus constituting a promising alternative to the actual commercially available optical near infrared probes,²⁰ More than these unique optical properties, ZGO has advantages over molecular-based imaging probes, such as tunability of size and shape²¹ and versatile functionalization capabilities for both active targeting²² and improvement of the circulation time into the blood stream.²³ Finally the absence of *in vitro* and *in vivo* toxicity²⁴⁻²⁷ of ZGO has recently been shown.

However, because of their size (hydrodynamic diameter around 100 nm), the renal clearance of ZGO is not observed.^{28,29} Therefore, the majority of ZGO injected to mice are more or less rapidly cleared from the bloodstream by cells of the mononuclear phagocyte system (MPS),³⁰ principally located in the liver or the spleen and internalized into the lysosomes.³¹ While other inorganic NPs have been shown to degrade into the acidic environment of lysosomes,³²⁻³⁴ nothing is known on the long term behavior of ZGO once internalized by cells. In order to evaluate the fate of NPs, both *in vitro* and *in vivo* studies can be performed. However *in vitro* studies are often limited in time due to rapid cell division or cell senescence in culture and *in vivo* studies are restricted by ethical issues, high cost and difficulties to follow the NPs properties and integrity in animals. At first, we chose to use the Artificial Lysosomal Fluid (ALF) in order to mimic the chemical environment of lysosomes in term of pH,³⁵ ionic strength, and chelation capacity,^{36,37} milliQ water and acetate buffer³⁸ at pH 4.5 as control suspension media. The evolution of ZGO with various coating agents has been monitored up to three months by combining a set of physico-chemical analyses to determine their fate in this lysosomal-like environment. Demonstrating significant degradation of ZGO in ALF, our study is a key step for the development and characterization of bioimaging probes while reducing animals use according to the 3R rule (Reduce, Refinement, and Replacement).

Material & methods

Chemicals

(3-Aminopropyl)-triethoxysilane (99%) was obtained from Sigma-Aldrich. Zinc nitrate hexahydrate (>99%) was purchased from Fluka. Gallium oxide (99.999%) and chromium (III) nitrate nonahydrate (99.99%) were purchased from Alfa Aesar. Dimethylformamide (>99.9%) was purchased from SDS. Alpha-methoxy-omega-N-hydroxysuccinimide poly(ethylene glycol) PEG MW 5.000 Dalton was bought from Iris Biotech GmbH.

ZGO synthesis

ZnGa₂O₄:Cr³⁺ NPs were synthesized by the hydrothermal method developed in our lab²⁰. First, gallium nitrate was formed by reacting 8.94 mmol of gallium oxide with 20 mL of concentrated nitric acid (35 wt%) under hydrothermal condition at 150 °C for 24 hours. Then, a mixture of 0.04 mmol of chromium nitrate and 8.97 mmol of zinc nitrate in 10 mL of water was added to the previous solution of gallium nitrate under vigorous stirring. The resulting solution was adjusted to pH 7.5 with an ammonia solution (30 wt%), stirred for 3 hours at room temperature, and transferred into a 45 mL Teflon-lined stainless-steel autoclave for 24 h at 120 °C. The resulting compound was washed several times with water and ethanol before drying at 60 °C for 2 hours. The dry white powder was finally sintered in air at 750 °C for 5 hours. Hydroxylation was performed by basic wet grinding of the powder (500 mg) for 15 minutes, with a mortar and pestle in 2 mL of 5 mM HCl solution, and overnight vigorously stirring at room temperature at 10 mg/mL in 5 mM HCl. The hydroxylated ZGO obtained, or ZGOOH, NPs with a diameter of 80 nm were first selected from the whole polydisperse colloidal suspension by centrifugation on a SANYO MSE Mistral 1000 at 4500 rpm for 10 minutes. ZGOOH were located in the supernatant (assessed by Dynamic Light Scattering), which were gathered and concentrated.

ZGO functionalization

ZGONH₂ NPs were obtained by adding 1 wt% of 3-aminopropyl-triethoxysilane (APTES) to a suspension of ZGOOH NPs at 2.5 mg/mL in dimethylformamide (DMF). The reaction mixture was sonicated for the first 2 minutes using a Branson Ultrasonic Cleaner 1210 and kept under vigorous stirring during 6 hours at room temperature. Particles were washed from the unreacted APTES by three centrifugation and redispersion steps in DMF. ZGOPEG NPs were obtained by

reacting N-hydrosuccinimide activated polyethylene glycol terminated with methoxy group (MeO-PEG_{5kDa}-NHS) (50 mg) with ZGONH₂ NPs in a 10/1 weight ratio at 2.5 mg/mL in DMF. To ensure a maximum PEG density, the last functionalization step was performed overnight, under vigorous stirring at 90 °C. For this study ZGOPEG NPs were prepared per 10 mg batches and dried (60 °C, 10 mPa) for storage.

Experimental degradation media

All the different prepared batches of NPs were dispersed into 5% glucose, pooled to obtain homogeneous populations, and then washed by centrifugation with the three different dispersion media: the artificial lysosomal fluid (ALF, see Fig. S1), an acetate solution at pH 4.5, and **milli-Q** water. The final concentration is set at 1 mg/mL and the flasks were placed in an incubator at 37 °C under a slow agitation. At time 0 (t₀), day 1 (d₁), d₈, d₁₅, d₃₀ and d₉₀ sampling has been made after homogenization of the suspension to perform further characterizations described above.

Dynamic light scattering (DLS)

20 µL of each NPs suspension was sampled in triplicate for both size and zeta potential measurements (Nano ZS, Malvern-Instruments). For the hydrodynamic diameter determination, the sample was diluted into 500 µL of milli-Q water. The presented diameters are a mean of the intensity diameter. For the zeta potential determination, the samples were diluted into 800 µL of 20 mM NaCl solution.

Thermogravimetric analysis (TGA)

TGA were performed by using a TGA/DSC 1 from Mettler–Toledo (Greifensee, Switzerland) sensitive to 1 µg and calibrated beforehand with internal standard weights. Samples of approximately 2 mg were analyzed in alumina pans with central hole of 1 mm diameter. All the experiments were performed at a 5 K/min heating rate, under a dry air flow of 70 mL/min. Coating density calculation method based on TGA is presented in supplementary information.

Record of luminescence

Dispersed suspension (1 mL) was collected and then divided into 4 samples of 250 µL. Then, 5 minutes acquisitions of the luminescence of nanoparticles was recorded on an Optima (Biospace)

camera after a 2 minutes excitation under UV light (365 nm) or under a LED lamp with a 515 nm filter.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) quantification

1 mL of each suspension has been centrifuged and 100 μ L of the supernatant has been diluted into 6 mL of 1% HNO₃. Quantification has been performed by ICP-AES (iCAP 6200 ThermoFisher). This procedure has been done in triplicate.

Transmission electron microscopy (TEM) observations

100 μ L of the suspension have been sampled, centrifuged and washed with pure ethanol. To prepare the grid, the sample has been diluted into HCl 5 mM and sonicated. Finally, 5 μ L has been dropped for 1 min and the grid has been wiped. Observations have been made at 80 kV on JEOL JEM-100S. Mass composition of single ZGO NPs has been determined by using STEM-EDX nano-analyses on a JEOL ARM 200F operated at 80 kV.

Results & discussion

In order to study the degradation of ZGO luminescent NPs, a stock of NPs was first synthesized. Starting from 1 g of raw ZGO powder, 400 mg of hydroxylated NPs (ZGOOH) could be extracted by centrifugation with a hydrodynamic diameter of about 90 nm, and among them 200 mg have been functionalized by PEG as described in Fig. 1(a). ZGOOH were mixed with the (3-aminopropyl)-triethoxysilane or APTES to obtain amine anchor groups on the surface and the obtained ZGONH₂ were then reacted with activated NHS-PEG to coat the NPs with PEG neutralize the surface. Twenty batches of 10 mg have been synthesized and then pooled together in order to obtain a homogenized population of ZGOPEG. The size and zeta potential of all batches are given in Tab.S1. The pooled batch has been characterized in term of size and surface charge as seen in Fig. 1(b)-(c) showing similar characteristics as previously described.²³ In the same manner, the luminescence properties of the starting suspension have been checked as seen in Fig. 1(d)-(e), and both the luminescence decay and the luminescence intensity have been checked to be consistent with our previous results. Thus, this batch was validated to start the degradation study. For this purpose, three aqueous media have been chosen: i) the Artificial Lysosomal Fluid (ALF) at pH 4.5 whose composition is shown in table S2, ii) acetate buffer at pH 4.5 as acidic control, and iii) milli-Q water as control. Both ZGOOH and ZGOPEG have been dispersed in these three media at 37°C in an incubator with a gentle stirring. Sampling has been performed on 7 time-points, namely t=0 and day 1, 7, 8, 15, 30 and 90, as depicted in Fig. 2. On one hand, the mean hydrodynamic diameter, the surface charge and the luminescence properties after both UV and visible light excitation have been determined on raw samples. On the other hand, samples have been centrifugated in order to separate the nanoparticles from the aqueous medium. While the pellets have been collected to perform thermogravimetric analysis (TGA) and transmission electron microscopy (TEM) imaging, the supernatant has been analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) to determine the ion release rate of gallium, zinc and chromium, constituting the ZGO.

Colloidal stability and surface state

Dynamic Light Scattering (DLS) measurements have been performed to determine the evolution of both hydrodynamic diameter and zeta potential over time in the different aqueous media (Fig.3). First, hydrodynamic diameters gave us valuable information about the different behaviors of ZGO in the three media. Both ZGOOH and ZGOPEG (solid and dashed red lines) show a good stability in acetate buffer up to one month and then start to aggregate. In contrast ZGO in water (black lines) rapidly form aggregates soon after one week and increase in size up to 10-15-fold their initial diameter after three months. On the contrary, ZGO in ALF (blue lines) remain stable in size up to one month, and the diameter is only doubled at the three months' time-point. These differences of stability in the different media could be explained by both the pH and the presence of chelating agents in ALF. An acidic pH can create charges on the surface of NPs and therefore increase the electrostatic repulsions and so the colloidal stability,³⁹ as observed up to one month in acidic ALF and acetate media. However, the surface charges of NPs are opposite in ALF and in acetate buffer which could be explained by the role of the chelating agents that are able to stabilize both ZGOOH and ZGOPEG NPs in ALF. This hypothesis seems to be confirmed by the time evolution of surface charge (Fig. 3(b)). Indeed, after a marked decrease of the surface charge in ALF over one month, ZGOOH as well as ZGOPEG tends to become neutral after 3 months. This phenomenon can be attributed to a passive functionalization of NPs by chelating agents, such as citrate for example, as already described in the literature.⁴⁰ Although the charge effect is neutralized at long term, this chelating layer could stabilize the nanoparticles and prevent their aggregation. Thus, the contact surface with ZGO stabilized by ALF will be higher and therefore the degradation of the nanoparticles could be more likely to happen.

To study the impact of the degradation media on the PEG coating of ZGOPEG, TGA was performed over the three-months follow-up (Fig. 4(a)). The weight loss percentage corresponds to the mass of the organic part, and therefore the coating of NPs. Interestingly, while ZGOPEG lose half of their coating within 15 days in both water and acetate media, the weight loss percentage increases after one day incubation in ALF and seems to be stable up to one month, before slightly decreasing. A possible explanation for this increase could be the insertion of some

chelating agents present in ALF medium into the PEG coating. This hypothesis is coherent with what was observed at time 0 with the 5% difference in weight loss between ZGOPEG in ALF (15.7%) and the two other media (about 10%). One possible implication of this could be the slow replacement of PEG chains with chelating agents with lower molecular weight, such as citrate, which could explain the decrease observed between one month (24.6%) and three months (14.8%). At three months, the coating percentage correspond to 44 citrate molecules per nm^2 , which corresponds to common citrate coating density observed on nanoparticles.⁴¹ Furthermore, a carbonated shell has been detected by TEM on ZGOOH NPs after three months in ALF, as seen on Fig. 4(c). This observation could reinforce the hypothesis of a passive functionalization by the chelating agents, as this shell has not been detected on the ZGOOH stock solution, as seen on Fig. 4(b). TGA study on ZGOOH NPs could have given more information, by quantifying the density of this carbonated shell. Nevertheless, this direct observation reinforces the key role of chelating agents on the surface of the ZGO, which is consistent with what was observed with zeta potential as seen in Fig. 3(b).

Evolution of the luminescence properties

The NPs luminescence is another parameter that may reflect the degradation of their optical properties. Luminescence signal intensity was recorded after visible or UV excitation (Fig. 5) and the number of photons recorded at the end of the acquisition was normalized with the initial intensity in order to annihilate the effect of the medium on the signal, and therefore to compare one condition to another. Using this method, a significant difference could be observed between nanoparticles' luminescence in ALF and in the two other media. While the normalized luminescence remarkably changes upon the time, both controls (Ac and H₂O) show almost no significant change on the short time both after UV and visible excitation, despite some changes after 15 days. Indeed, when irradiated by UV, we can first notice a strong increase of luminescence for both ZGOOH and ZGOPEG between t₀ and day 1, followed by a slow decrease from day 8 to day 90. This increase could be explained by the suppression of surface defects quenching the luminescence of the nanoparticles, as already described in the literature.⁴² Finally, luminescence of nanoparticles in ALF reaches less than 10% of the original luminescence at time d90. After visible light excitation, the same phenomenon than previously described with the UV excitation is observed, although attenuated, and the data clearly highlight the role of ALF in the alteration of luminescence properties compared to the two other media as presented in Fig 5(a).

Quantification and visualization of NP degradation

In order to link the decrease of the luminescence with a degradation of the nanoparticles, ICP-AES measurements have been performed on the supernatant of each media after centrifugation (Fig. 6). Gallium, zinc and chromium have been titrated all along the study. The obtained concentrations have been compared to the initial 1 mg/mL concentration in order to evaluate the degradation rate. A clear and regular increase of the release of each element is observed. An average of 50% of degradation is obtained after one month for both ZGOOH and ZGOPEG, and 100% after three months. It is interesting to note that functionalized ZGOPEG nanoparticles show a slower release rate than ZGOOH NPs. This result seems to indicate that the PEG coating can delay ZGO degradation until PEG is replaced by chelating agents of ALF. Nevertheless, the release percentages after 3 months are equivalent between ZGOOH and ZGOPEG. Additionally, a difference of release rate is noticed between the different elements. Indeed, gallium and chromium quickly release whereas zinc does not. This could be explained by the chelating agents in the solution: while both citrate⁴³ and tartrate⁴⁴ have been described to chelate gallium and chromium, i.e. the trivalent cations, only the pyruvate has been described as a zinc chelating agent.⁴⁵ Given the differential release of zinc in comparison with gallium and chromium, a change in the crystallographic structure of NPs with the apparition of a ZnO phase could have been envisioned. STEM-EDX nano-analyses have therefore been performed locally on single NPs, in order to determine the mass percentage of each component. However, interestingly, no changes have been observed in ZGOOH composition between t0 (Ga: 52.0 ± 1.6 %, Zn: 21.9 ± 1.1 %) and three months after incubation in ALF (Ga: 53.3 ± 3.1 %, Zn: 22.3 ± 1.8 %), as seen in Fig. S1. Another possibility to the low zinc concentration detected could come from its precipitation after chelation. Despite those differences, the ALF clearly induces the release of ions from NP core in comparison to the acidic acetate buffer and water controls which show almost no release as seen in supplementary Fig. S2.

In order to fully demonstrate the degradation of the nanoparticles in the ALF, their size and shape have been studied by TEM. Both ZGOOH and ZGOPEG NPs show a decrease in size after the three-month incubation in ALF (Fig. 7). The shape also changes upon the time, the NPs becoming rounder as erosion occurred. In a view to quantify this phenomenon, pictures have

been taken upon the time and the NP surface has been measured by using an imaging software,⁴⁶ to perform statistical analysis of the different populations. Results show significant differences for ZGOOH and ZGOPEG area in ALF between t0 and d90, which is minimized for milli-Q water buffer (Fig. S3 (a-b)) and acidic acetate buffer (Fig. S3 (c-d)). These results are consistent with the ICP-AES study, and visually validate the degradation.

Conclusion

ZGOOH and ZGOPEG nanoparticles were placed in suspension in three different aqueous media up to three months in order to evaluate their possible degradation. The colloidal stability has been followed by DLS, and the PEG coating has been monitored by TGA. Hydrodynamic diameter, charge surface and remaining amount of coating seem to indicate the important role of chelating agent in stabilizing the nanoparticles and therefore maintaining the high available surface contact with the medium leading to an effective degradation of ZGO particle core. The luminescence properties in ALF changed over the time, undergoing a large increase of the signal caused by the suppression of surface defect, and then decrease due to degradation. Both ICP and TEM confirmed the degradation of ZGO in ALF showing significant differences with the neutral and acidic controls. Once again, the chelating agents seem to have a key role in the degradation process, by chelating both Ga^{3+} and Zn^{2+} on the surface of ZGO, to then release it in solution. The ALF therefore demonstrated to be a relevant medium to study the degradation of inorganic nanoparticles. According to these results, degradation and excretion of the ZGO after an *in vivo* injection and capture by RES could be a possible scenario.

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