NanoTBTech

Nanoparticles-based 2D thermal bioimaging technologies

H2020-FETOPEN-1-2016-2017

Grant Agreement: 801305

Deliverable number D4.1 (D.21)

Bio-functionalization of nanoparticles

Final Version

Project Deliverable Information Sheet

NanoTBTech Project	Project Ref. No. 801305
	Project Title: *Nanoparticles-based 2D thermal bioimaging technologies*
	Project Website: http://www.nanotbtech.eu/
	Deliverable No.: D4.1
	Deliverable Type: Report
	Dissemination Level: Public
	Contractual Delivery Date: 31 August 2019
	Actual Delivery Date: 30 August 2019
	EC Project Officer: Barbara GERRATANA

Document Control Sheet

Document	Title: Bio-functionalization of nanoparticles
	Version: Final
	Available at: Participant's Portal
Authorship	Written by: CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE CNRS (CNRS)
	Contributed by: - CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE CNRS (CNRS) - AGENCIA ESTATAL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS (CSIC) - UNIVERSITY OF BELGRADE, VINCA INSTITUTE OF NUCLEAR SCIENCES (UB) - WROCLAW POLISH ACADEMY OF SCIENCE (WPAS) - NANOIMMUNOTECH (NIT)
	Approved by: all partners

History of Changes

Version	Date	Description	Reviewer
V0	12.08.2019.	Version 0	CNRS
V1	26.08.2019.	Version 1	UAVR
Final Version	30.08.2019	Final Version	All partners

*This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No **801305.***

Abbreviations and Acronyms

Ag_2S	Silver sulfide
APTES	(3-Aminopropyl)triethoxysilane
APTMS	(3-Aminopropyl)trimethoxysilane
CNRS	Centre National de la Recherche Scientifique
CSIC	Agencia Estatal Consejo Superior de Investigaciones Cientificas
DCM	Dichloromethane
DDT	Dodecanthiol
DLS	Dynamic light scattering
DMF	Dimethylformamide
EtOH	Ethanol
GIG	Gadolinium iron garnet
NPs	Nanoparticles
PEG	Polyethylene glycol
rpm	Round per minute
UB	University of Belgrade
WP	Work package(s)
WPAS	Instytut Niskich Temperatur I Badan Strukturalnych Im. Wlodzimierza Trzebiatowskiego Polskiej Akademii Nauk
YAG	Yttrium aluminum garnet
YIG	Yttrium iron garnet

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D4.1 Bio-functionalization of nanoparticles

Work package 4 (WP4) of the project is titled: "Functionalization and usability of the NPs & heater-thermometers nanostructures". The report is mainly given by a lead beneficiary of the WP4: Centre National de la Recherche Scientifique (CNRS); along with other partners:

- Agencia Estatal Consejo Superior de Investigaciones Cientificas (CSIC)
- University of Belgrade, Vinca Institute of Nuclear Sciences (-VINCA)
- Wroclaw Polish Academy of Science (WPAS)
- Nanoimmunotech (NIT)

This report identifies the functionalization's procedures that satisfy the minimum requirements for *in vivo* use based on their outcome (optical and magnetic properties, biocompatibility, NPs size, stability in aqueous media). Additionally, it provides the calibration datasheets corresponding to the nanostructures.

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1. Nanoparticles from VINCA partner

Following the reception of several samples from the UB team, work has begun on yttrium iron garnet (YIG) nanoparticles (NPs).

First, the protocol described by the VINCA team has been used in order to obtain stable YIG NPs. The procedure was described as followed:

"Mix 400 mg of nanopowder with 76.8 ml H_2O and 3.2 ml of aqueous solution of citric acid, CA, (c_{CA} = 0.5 g/ml) in a glass beaker and measure pH value. Under slow stirring add conc. NH_3 dropwise up to pH value of 5.1.

Transfer the mixture to a glass flask (with stirrer inside) connected to a reflux condenser and immersed in oil bath. Adjust temperature to 80ºC and keep at the temperature under magnetic stirring for 90 minutes. Cool down solution to the room temperature and measure pH value. Again, adjust pH to 10.1 by adding conc. NH_3 dropwise under slow stirring.

Transfer the obtained solution to centrifuge tubes and centrifuge at 3000 rpm speed for 2 minutes. Remove supernatant. Mix obtained sediment with 50 ml H_2O and treat it for 1 minute with ultrasonic horn at 40% power. Centrifuge obtained mixture at 5000 rpm speed for 3 minutes. Now, supernatant, i.e. nanoparticles dispersed in aqueous media, are ready for further characterization and measurements, while sediment should be removed.

Exact concentration (mg/ml) of nanoparticles in the obtained supernatant needs to be determined (by thermogravimetry for example)""

This procedure has been done two times with non-significant results. First, the pH of the suspension after suspension in water was above 5.1. Therefore, concentrated HCl was used to adjust the pH. At the end of the process, aggregation was observed by DLS.

In order to obtain stable NPs, 100 mg of YIG NPs at 10 mg/mL was dispersed in HCl 5 mM for 72h. This process is supposed to create small surface defects with the presence of -OH groups, as observed for $ZnGa_2O_4$ NPs [1]. The obtained suspension was then centrifuged to separate stable NPs from the original solution. Nevertheless, whatever the centrifugation conditions, no stable YIG NPs was obtained.

To go further in the creation of surface defects, 100 mg of YIG NPs were milled with a mortar and then hydroxylated in the same condition than before. Once again large aggregates were observed.

Then, a new approach was tried. Literature described a procedure used on YAG NPs to stabilize it in aqueous solution [2], as seen in fig. 1.

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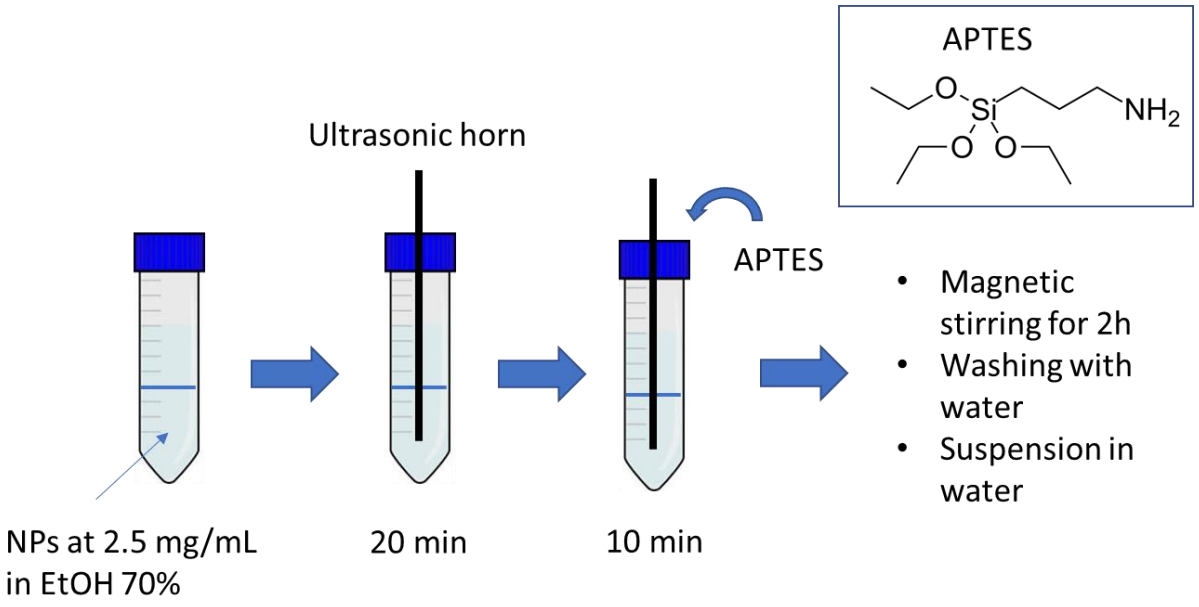

Figure 1 : schematic procedure of the ultrasonication process with APTES

It consisted in using an ultrasonic horn on YAG NPs in EtOH/H_2O 70:30, to then let it react with (3-Aminopropyl)trimethoxysilane (APTMS) in order to create a silica shell. Adaptation of this procedure has been done on both YAG and YIG NPs. 10 mg of NPs have been suspended in 70% EtOH at 2.5 mg/mL and sonicated for 20 min (Branson Sonifier 450, amplitude 5, frequency 50%). Then, 60 µL of (3-Aminopropyl)triethoxysilane (APTES, already available in our laboratory) was added dropwise, and sonicated for 10 more minutes. The suspension was then washed with 70% EtOH to eliminate excess APTES. Both samples showed good results as seen in table 1 after dynamic light scattering (DLS) measurements:

Table 1: DLS characterization of YIG and YAG NPs after ultrasonication procedure

	Before functionalization		After functionalization	
	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)
YIG	aggregates	-7.9	315 ± 25 (pdI 0.50 ± 0.01)	53.6
YAG	aggregates	-2.1	85 ± 1 (pdI 0.15 ± 0.03)	38.0

The procedure allows obtaining stable NPs, especially with YAG NPs. The change of zeta potential clearly highlights the surface changes. After these promising results, the procedure has been done one more time to insure the reproducibility. This time, 20 mg of both NPs have been functionalized with 60 µL of APTES. Furthermore, after the washes, the NPs have been functionalized with NHS-PEG(5

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kDa)-OMe (Iris Biotech) in a method described by our UTCBS partners [3], in order to insure the possibility of a complete functionalization. The procedure is shown in fig. 2:

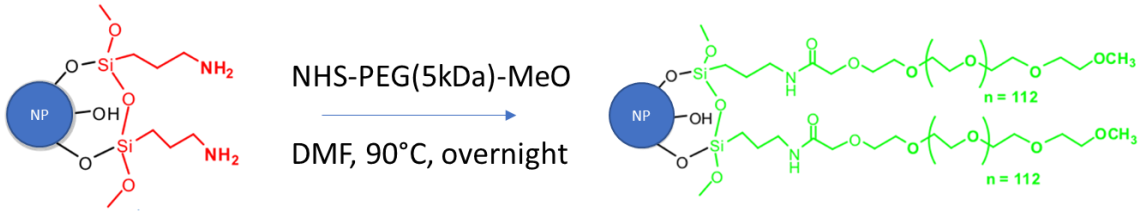

Figure 2: schematic representation of the PEGylation procedure. In a typical experiment, 10 mg of NPs-NH2 are placed in N,N-dimethylformamide (DMF) at 2.5 mg/mL. 100 mg of NHS-PEG-OMe (Iris Biotech) are added to the mixture and sonicated for 1 min. The reaction is placed in an oil bath at 90°C overnight. Finally, the NPs are recovered and washed by centrifugation (14500 rpm, 15 min) with DMF and then mQ water.

The PEGylation has been done on both YIG and YAG NPs. DLS characterization of the functionalization is shown in table 2:

Table 2: DLS characterization of YIG and YAG NPs after ultrasonication procedure and PEGylation

	Before functionalization		With APTES		With PEG	
	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)
YIG	aggregates	-1.9	aggregates	36.0	623 ± 72 (pdI 0.69 ± 0.10)	-1.3
YAG	aggregates	-2.7	105 ± 4 (pdI 0.26 ± 0.01)	34.1	129 ± 1 (pdI 0.24 ± 0.02)	-1.1

The results obtained with YAG NPs are very promising compared to the YIG NPs, with a complete elimination of aggregates as seen in fig. 3. Nevertheless, the zeta potential clearly shows a transformation in the surface state.

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To go further into the characterization, the stability of YAG-PEG NPs in water has been investigated.

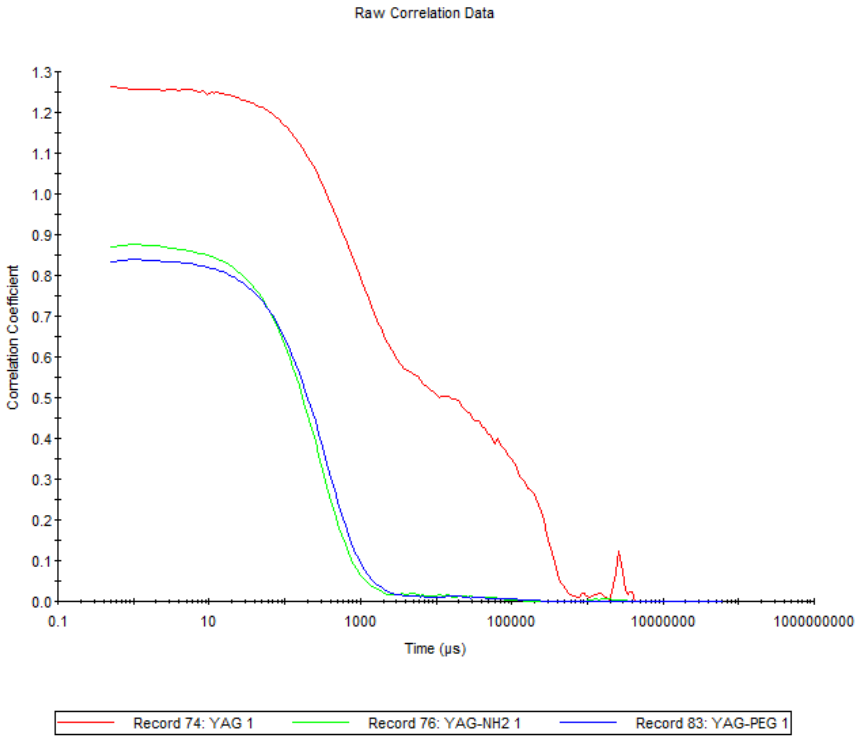

Figure 3: Correlation curves of YAG NPs (red line), YAG-NH2 NPs (green line) and YAG-PEG NPs (blue line). A strong correlation coefficient at the higher time points indicates a low mobility of the NPs, and therefore the presence of aggregates.

The hydrodynamic diameter, the zeta potential as well as the polydispersity index has been followed up to two hours. Furthermore, some of YAG-PEG NPs has been dried (1h at 60°C under vacuum, MAXI dry lyo, Heto) in order to know if a simple storage method could be used. The results are shown in fig. 4:

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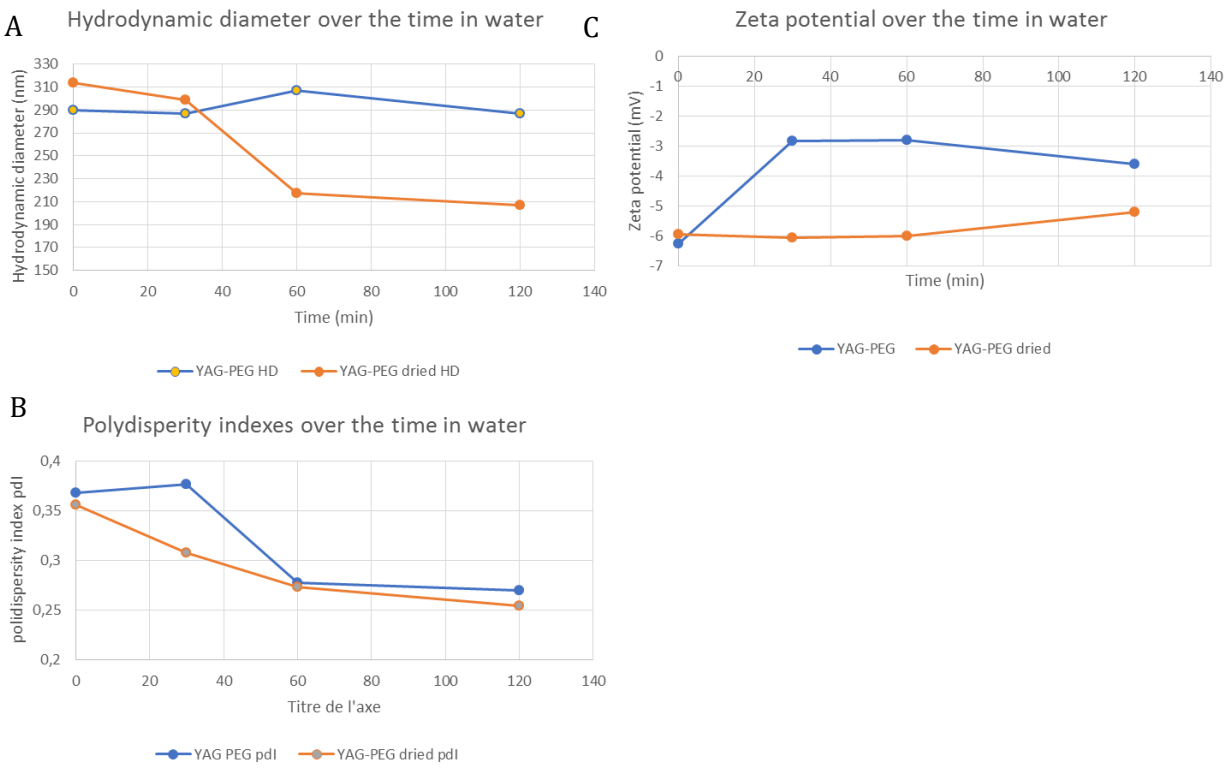

Figure 4: Stability of YAG-PEG NPs before (blue line) and after storage (orange line). (A) Evolution of the hydrodynamic diameter over time. (B) Evolution of the polydispersity index over time. (C) Evolution of the zeta potential over time.

As highlighted in these graphics, YAG-PEG NPs don't show any clear changes over the time and are therefore stable in solution. Furthermore, stored NPs have the same behaviour than YAG-PEG after synthesis. Overall, YAG NPs have shown to be successfully functionalized, highly stable in solution as well as storable.

The main element explaining the difference observed between YIG and YAG nanoparticles could be the magnetic properties of the YIG nanoparticles. Indeed, a homogeneous suspension can't be insured during the functionalization because YIG NPs are attracted by the magnetic stirrer. Furthermore, because of the low reaction volume (usually few mL), the use of mechanical stirrers is not possible. To overcome this issue, functionalization has been performed on hybrids YIG/YAG NPs. These NPs are based on a YAG structure with different amount of iron, from 0.05% to 50%. The main goal would be to take advantages of the functionability of the YAG while keeping the magnetic properties given by the iron loading later useful for magnetic heating.

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The ultrasonication procedure has been done on the two different samples. The results are shown below in table 3:

Table 3: DLS characterization of YAG 0.05% Fe and YAG 50% Fe NPs after ultrasonication procedure and PEGylation

	Before functionalization		With APTES		With PEG	
	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)
YAG 0.05% Fe	aggregates	-4.5	82 ± 4 (pdI 0.21 ± 0.04)	36.0	114 ± 4 (pdI 0.17 ± 0.01)	-3.8
YAG 50% Fe	aggregates	-5.1	146 ± 9 (pdI 0.33 ± 0.02)	34.1	190 ± 48 (pdI 0.37 ± 0.13)	-3.8

he results clearly highlight the successful functionalization of both NPs, even on high iron loading. The YAG 50% Fe, or YIG/YAG, could therefore be a good alternative to the YIG NPs.

Finally, work has been done on the functionalization of a third type of NPs containing gadolinium (GIG). To do so, the ultrasonication procedure with APTES has been used, followed by the PEGylation in DMF. The exact same method than with YIG and YAG has been used. Results are shown in table 4:

Tableau 4: DLS characterization of GIG NPs after ultrasonication procedure and PEGylation

	Before functionalization		With APTES		With PEG	
	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)
GIG	aggregates	-0.9	525 ± 52 (pdI 0.71 ± 0.05)	-0.6	556 ± 213 (pdI 0.70 ± 0.15)	-5.0

As seen before, GIG NPs are fully aggregated without any treatment. While the ultrasonication procedure seems to stabilize the NPs, no surface changes are seen as the zeta potential show it. The PEGylation has nonetheless been tried but it didn't show any changes. To date, no further work has been done. These primary results could indicate the same difficulties than for YIG NPs, certainly due to their magnetic properties.

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2. Nanoparticles from WPAS partners

$NaYF_4$ NPs capped with oleic acid in suspension in chloroform have been received from WPAS. In order to stabilize them in aqueous media, ligand exchange procedure has been adapted from literature [4]. The first step was to remove the chloroform. For this purpose, NPs were washed with absolute EtOH three times (centrifugation 14500 rpm, 60 min) and then dried (1 h, 60°C, under vacuum, MAXI dry lyo, Heto). Then, NPs were suspended in water and a solution of dicarboxylic acid in water at 3 mg/mL was added. The final concentration of NPs was 2.5 mg/mL. The suspension was then magnetically stirred for 1 h at room temperature. The resulting mixture was mixed with diethyl ether to remove excess of methyl oleate by extraction. Finally, the NPs were washed with water for DLS characterization. In this study, two different dicarboxylic acids have been tried: succinic acid and malonic acid. The results obtained with this procedure are presented below in table 5:

Table 4: DLS characterization of $NaYF_4$ NPs after ligand exchange procedure with malonic and succinic

	Before functionalization		After functionalization	
	Hydrodynamic diameter (nm)	Zeta potential (mV)	Hydrodynamic diameter (nm)	Zeta potential (mV)
Malonic acid	aggregates	-5.1	41 ± 0.3 (pdI 0.17 ± 0.01)	13.4
Succinic acid			75 ± 18 (pdI 0.37 ± 0.09)	23.4

As seen above, the behaviour of $NaYF_4$ NPs in aqueous medium drastically changes after the procedure. Promising results have been obtained with malonic acid, with small hydrodynamic diameters and a narrow size distribution. The succinic acid also shows great results, but with a larger size distribution. The comparison between both ligands is shown in fig.5:

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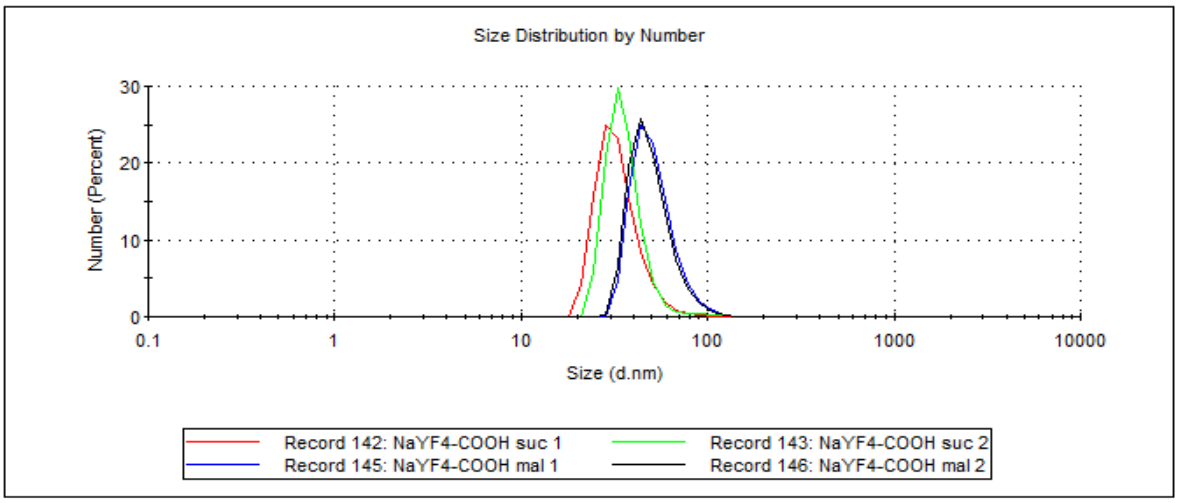

Figure 5: Size distribution of $NaYF_4$-malonic (blue and black lines) and $NaYF_4$-succinic (red and green lines) NPs. The superposition of the curves shows the stability of the sample.

We can conclude that this ligand exchange procedure was successful to stabilize $NaYF_4$ NPs in aqueous medium. Following these results, the same approach was tried in order to PEGylate the NPs. The ligand exchange is based on the simple fact that carboxylate groups will bind to the surface, removing the existing oleic acid ligand. Therefore, MeO-PEG(10 kDa)-COOH (Iris Biotech) polymer was used instead of dicarboxylic acid in the exact same conditions. Unfortunately, the stability didn't improve, and $NaYF_4$ NPs formed large aggregates. One possible explanation could come from the size of the polymer chain and therefore the low probability of carboxylic group interacting with the NP surface, compared to the dicarboxylic compounds. Increase the amount of polymer could be a possible solution to overcome this issue. In order to functionalize the $NaYF_4$ NPs, further work will be done using the malonic acid as a starting group for PEGylation.

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3. Nanoparticles from CNRS group

Ag_2S NPs capped with dodecanethiol (DDT) in chloroform were received from LCMCP partners. In order to stabilize them in aqueous media, a ligand exchange procedure was adapted from the literature [5]. In a typical procedure, 5 mg of NPs were washed three times with dichloromethane (DCM) by centrifugation (14500 rpm, 15 min) and suspended in 2mL of fresh DCM. Then, MeO-PEG(5 kDa)-SH (Iris Biotech) polymer was added to the suspension in a 1/10 NP to polymer mass ratio. The mixture is then sonicated in an ultrasonic bath for 1h. The resulting suspension was then centrifuged (14500 rpm, 15 min) and the supernatant was discarded. The pellet was then dried under air flux for 1h. The dried NPs were then washed three times with water (145000 rpm, 15 min) and suspended at 5 mg/mL. The results are shown in table 6:

Table 5: DLS characterization of Ag_2S NPs after ligand exchange procedure with SH-PEG in comparison with commercial NPs

	Ag_2S-DDT	Ag_2S-PEG	Commercial Ag_2S-PEG
Zeta potential (mV)	-2.0	-2.1	-1.3
Hydrodynamic diameter (nm)	aggregates	369 ± 41 (pdI 0.43 ± 0.05)	22 ± 4 (pdI 0.43 ± 0.10)

In term of hydrodynamic diameter, the ligand exchange procedure was successful, avoiding any aggregation phenomena. In term of surface charge, it is not possible to distinguish the two surface states. Compared to the commercial Ag2S-PEG used as reference (Suzhou NIR-Optics Technologies Co. Ltd, China), the size is largely higher, but an important stability was nevertheless observed. These results are promising and could be better if the procedure parameter (drying time and method, concentrations, NP to polymer ratio) were optimised.

It was interesting to know at this stage if the Ag_2S-PEG would keep the same optical properties than the commercial ones. To do so, quartz cuvette usable for wavelength (excitation and emission) in range of [250nm:2500nm] was filled with 1mL of solution contained Ag_2S. Solution was excited at 735nm using a continuous LED. Solution temperature was controlled using a heating plate and monitored with a thermocouple. Emission spectra were recorded against temperature using a spectroscopy camera operating in the near-infrared region (NIRVANA, Princeton) and centred at 1200nm. As seen in fig. 6, the PEGylated Ag_2S NPs show a similar temperature response, underlying a similar surface state. These results are really promising and constitute a solid alternative to the commercial NPs used in the consortium.

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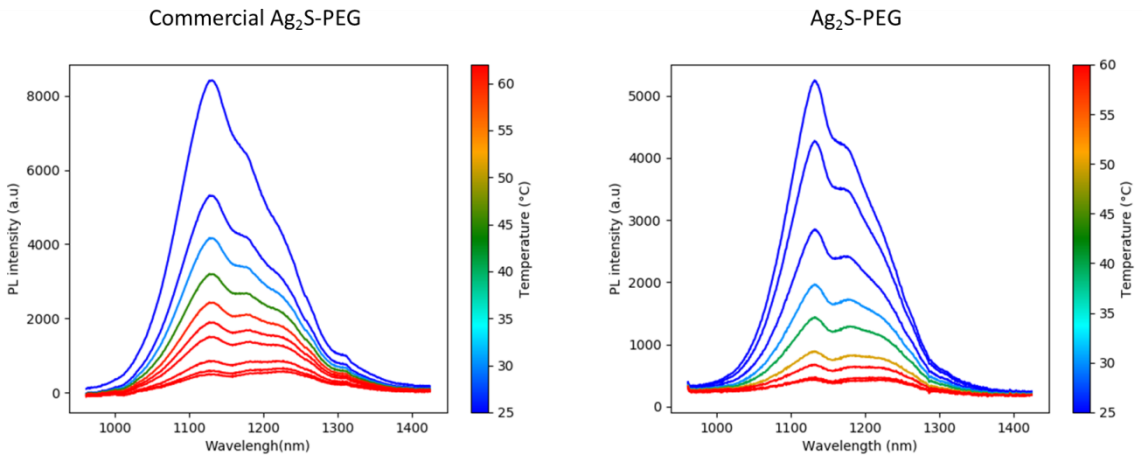

Figure 6: Emission spectra of commercially available Ag_2S-PEG (left) and CNRS made Ag_2S-PEG (right) depending on the solution temperature.

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4. General conclusions and perspectives/ further work

	Procedure used	Actual state	Next step
YIG	- UB experimental procedure - Milling and hydroxylation procedure - Ultrasonication procedure	No stable NPs for the moment	- search of new protocols - development of a new method for size distribution determination
YAG	Ultrasonication procedure	- stable NPs - successful PEGylation - storage and stability of PEGylated NPs investigated	- full characterization of the PEGylated NPs - active targeting: grafting of *cetuximab* - studying the impact of the coating on the heating properties
YIG/YAG	Ultrasonication procedure	- stable NPs - successful PEGylation	- full characterization of the PEGylated NPs - active targeting: grafting of *cetuximab* - studying the impact of the coating on the heating properties
GIG	Ultrasonication procedure	No stable NPs for the moment	- search of new protocols - development of a new method for size distribution determination
$NaYF_4$	Ligand exchange with SH-PEG	- stable NPs - non optimized PEGylation	- optimization of the PEGylation - studying the impact of the coating on the optical properties
Ag_2S	- Ligand exchange with dicarboxylic acid - Ligand exchange with COOH-PEG	- stable NPs - non optimized PEGylation	- optimization of the PEGylation - studying the impact of the coating on the optical properties

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