PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVI and XXXVII cycles)

Name of PhD student: Adnan Khan

Title of PhD research: **Purification and Characterization of Iron Oxide Nanoparticles**

Name of PhD supervisor: Prof. Francesco Spinozzi
Research lab name: Molecular Biophysics Lab

Cycle:
[*] XXXVI
[ ] XXXVII

PhD Curriculum::
[ ] Marine biology and ecology
[*] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[*] MaSBiC
[ ] Simulation/informatics lab
[*] Other. Please, indicate: Molecular Biophysics Lab

ABSTRACT (1000 characters, including spaces):
In today's world, nanoparticles (NPs) are widely employed as drug delivery vectors in the treatment of many diseases. The process of synthesizing pure and uniform NPs is quite expensive and time-consuming. An innovative approach to overcome these problems is the use of naturally synthesized NPs, such as the one isolated from bacteria [1]. In my PhD project, we have planned to investigate iron-NPs naturally produced by Magnetotactic bacteria (MTB). These iron-NPs, also called magnetosomes, are formed by a core of iron oxide (Fe_3O_4) coated with a lipid bilayer filled with membrane proteins [2]. This protein-rich lipid bilayer could provide a large surface area for the attachment of desirable drugs as well as bioactive molecules. The purified magnetosomes extracted from this MTB will be characterized by several biophysical experiments including Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), Small-Angle X-Ray Scattering (SAXS) and Small Angle Neutron Scattering (SANS) [3].

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Nanoparticles (NPs) are broadly used in a number of *in vivo* applications, such as Magnetic Resonance Imaging (MRI), contrast improvement, tissue healing, immunological assays, purification of organic liquids and tailored drug delivery [4]. In biomedical research, size, shape and surface topology are important features of the NPs to be used as drug carriers. To overcome the disadvantages of toxicity, cost and accuracy of the chemically synthesized NPs, naturally produced NPs have got a lot of interest in the recent era. Among them, iron oxide NPs, called *magnetosomes,* produced by magnetotactic bacteria (a class of microaerophilic organisms), are considered the ones with the most suitable characteristics for biomedical applications [5]. The advantages of magnetosomes over chemically synthesized iron-NPs are the presence of a protein-rich lipid bilayer around the iron core, which provides a large surface suitable for the binding of desirable drugs or bioactive molecules, together with high purity, thermal stability and narrow size distribution (Fig. 1) [6]. Previous studies have shown that SAXS could provide useful structural information, such as the iron core dimension and the distance between individual magnetosomes in chains [7]. However, information is scarce regarding the dependency of the magnetosome structure on the parameters of the medium in which the bacteria have been grown. Among these parameters, the most relevant are the oxygen concentration, the type of iron source and the buffer [8]. In my PhD project, it is planned to derive this missing information, which will be very useful in order to obtain magnetosomes with customized dimensions for their biomedical use, by means of both synchrotron SAXS/WAXS and SANS measurements carried out on magnetosome samples that have been purified from the magnetotactic bacterial species *Magnetospirillum gryphiswaldense* (MSR-1) grown in flask standard medium (FSM) under a wide set of controlled conditions. We have started to characterize these NPs through the following preliminary methods described in work plan and research objectives. Proposals for SAXS and SANS analysis have been submitted to European Synchrotron Radiation Facility (ESRF) and Institut Laue-Langevin (ILL), respectively.

- SCIENTIFIC AIMS

- Culturing of the bacterial specie *Magnetospirillum gryphiswaldense* to purify magnetosomes
- Characterization of magnetosomes through SAXS, DLS, AFM, SEM, XRD and SANS.
- Identification of surface proteins and functional groups through proteomic analysis
- Identification of active sites within the magnetosome proteins for attachment of bioactive molecules

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

Extraction and purification of magnetosomes from MSR-1

Methods

Magnetospirillum gryphiswaldense MSR-1 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) and used for all experiments [9]. MSR-1 was grown in Flask Standard Medium (FSM) in microaerobic conditions at 28 °C in a shaking incubator at 120 rpm for 6 days. Composition of FSM is shown in table 1. Cell growth was determined by OD565 and cells were harvested when the OD reached to 1.6. The culture was centrifuged at 8000 rpm for 30 minutes and the supernatant was removed. Approx. 5 grams of biomass was washed with buffer A (20 mM HEPES, 5 Mm EDTA, PF 7.4) and centrifuged again at 8000 rpm for 10 minutes. This step was repeated twice. The cells were suspended in buffer B (50 mM HEPES, 1 mM EDTA, PH 7.4) until further step.

Table 1.

FSM (Flask Standard Medium)	
Reagent	**Final Concentration**
Yeast Extract	0.1g/l
Soybean Peptone	3g/l
KH_2PO4	0.1g/l
$NaNO_3$	0.34g/l
$MgSO_4 \cdot 7H_2O$	0.15g/l
HEPES	2.38g/l
k-Lactate (60%)	3.5g/l
Iron Citrate ($C_6H_5FeO_7$)	100µM
EDTA-Chelant TES	5ml/l

Cells were disrupted by passing the biomass 3-5 times through a high pressure homogenizer at MASBIC laboratory. The sonication process was repeated until the colour of the culture changed to a black one. The lysate was transferred to 50 ml falcon tubes and re-suspended in buffer C (10 mM HEPES, 1 mM EDTA, PH 7.4). The lysate was then passed through a magnetic separation column (MACS column) placed between a strong neodymium magnet. Magnetosomes remained attached to the magnetic column after the magnet was removed and washed the column with milli-Q water 2-3 time and magnetosomes were collected.

Expected/Obtained Results.

- Collection of magnetosomes for further characterization
- Confirmation of the presence of magnetosomes through Dynamic Light Scattering

WP 2. Objective.

Characterization of magnetosomes through SAXS, AFM, SEM, XRD and SANS

Methods.

Small Angle X-Ray Scattering: Small Angle X-ray Scattering (SAXS) is a method in which the elastic diffusion of X-rays by particles, with dimensions in the order of 1-100 nm, dispersed in solution is measured at very small angles (typically less than 1°). This angular interval contains information about particle shape, size particle-particle interaction. We have planned to perform experiments by using our purified magnetosomes in phosphate buffer solution (PBS). Preliminary SAXS curves have been recorded at the beam-line ID02 ESRF, The European Synchrotron (Grenoble, France). The curves were recorded at 25° C. SAXS data analysis in progress.

Atomic Force Microscopy: The AFM can be used to observe the topography of soft biological materials in supported in a flat surface. It can also be used to probe the mechanical properties of cells and extracellular matrices, including their intrinsic elastic modulus and receptor-ligand interactions. We measured diluted solutions of purified magnetosomes deposited on a freshly cleaved mica surface and subsequently dried by nitrogen blow down. AFM measurements were carried out on an AIST-NT's Scanning Probe Microscopy, (Horiba Scientific) available at DISVA. Images were generated in non-contact mode, with a pyramidal silicon tip with radius of 8 nm.

All images were acquired at resolution of 512×512 pixels, with a scan rate of 1 Hz and were analyzed with Gwyddion and ImageJ (version 1.8.0) software. A statistical analysis of the images to get the average particle diameter was performed on a large enough number of particles (>80).

Dynamic Light Scattering: DLS is a well-established non-invasive technique for measuring the size of molecules, nanoparticles or colloids with a sub-micron size. Purified magnetosomes without dilution were measured in the Molecular Biophysics lab by using a Malvern Zetasizer PRO instrument operating with a fixed angle of 173°. The temperature was 25° C.

Scanning Electron Microscopy: SEM analysis was carried out on a FESEM ZEISS SUPRA 40 instrument available at UNIVPM, operating with an acceleration voltage of 5 kV. It exploits either in-lens secondary electron detector or an Everhart-Thornley detector at a working distance (WD) less than 4.0 mm. The instrumentation allows the observation of the three-dimensional topography of the sample with the possibility of obtaining compositional information. Our magnetosomes samples were dried on a copper grid and then plated with gold to prevent charging of the surface.

Expected/Obtained Results.

SAXS

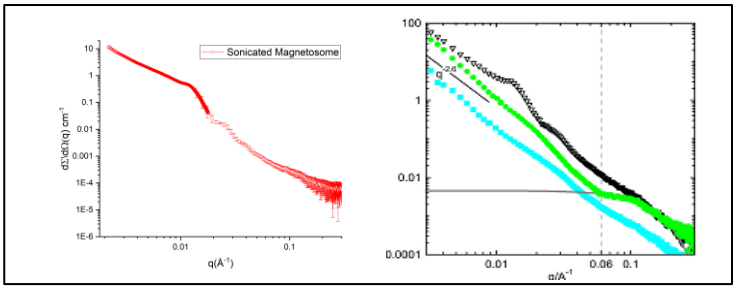

Fig 1 Fig 2

Comparison of the preliminary SAXS curve recorded at ID2 of our magnetosomes sample (Figure 1) with the ones published by Sabine et al. [3] (Figure 2). The low q curve alongside "0" shows a structural similarity between the two samples as can be seen in red and black curves.

AFM

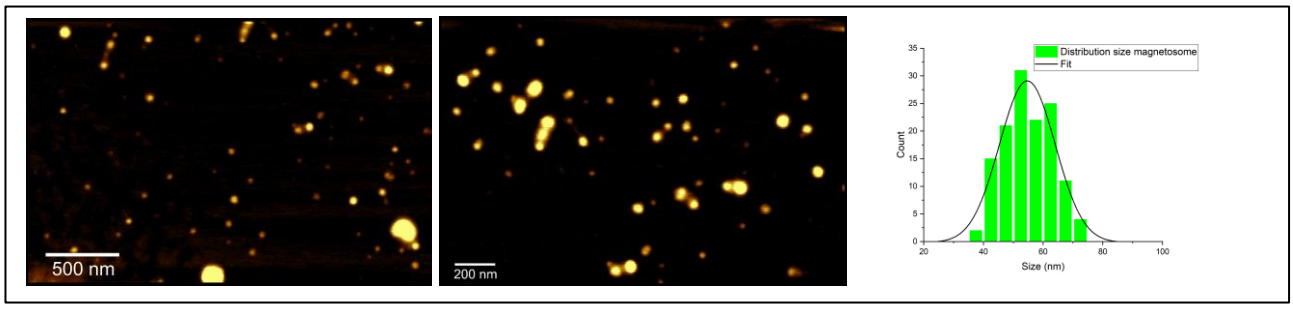

Fig 3 Fig 4 Fig 5

Fig 5 shows the diameter distribution function of the magnetosomes seen in Figs. 3 and 4. The average diameter, corresponding to the maximum of the Gaussian distribution, is 54.7 nm with a poly-dispersed index (PDI) 18.6%.

DLS

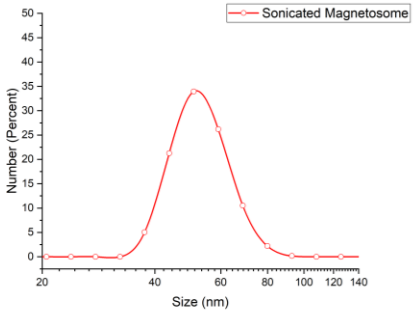

Fig 6

Dynamic Light Scattering spectrum of magnetosomes shows the diameter around 35-80 nm, with a poly dispersion index (PDI) of 20%. Particles with a size range of about 50 nm was at high percentage of 35 as shown in the fig 6.

SEM

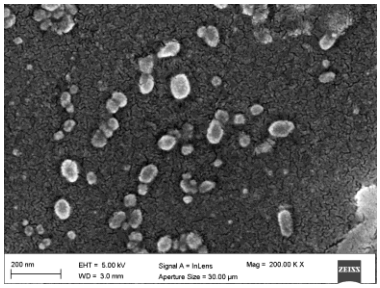

Fig 7

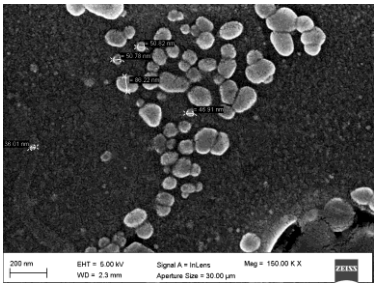

Fig 8

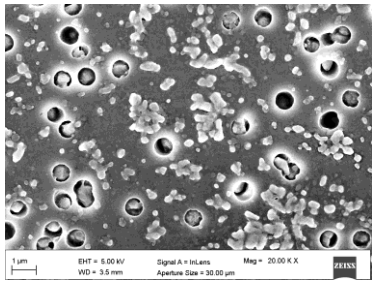

Fig 9

Images taken with Scanning Electron Microscope show the shape and aggregation state of the nanoparticles in the solution. With a resolution of 200 nm and a voltage of 5 kV, the size of the particles was from 40-120 nm.

- REFERENCES

1. D. Acosta-Avalos Abreu F. Bacteriology. London: Intech Open Ali I, Peng C, Khan Z M, Naz I. Yield cultivation of magnetotactic bacteria and magnetosomes: A review. Journal of Basic Microbiology, (2018) 57(8): 643–652

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3. S. Rosenfeldt, Riese CN, Mickoleit F, Schüler D, Schenk AS. Probing the nanostructure and arrangement of bacterial magnetosomes by small-angle X-ray scattering. Appl Environ Microbiol (2019) 85: e01513-19.

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5. T. Matsunaga, T. Suzuki, M. Tanaka and A. Arakaki. Molecular analysis of magnetotactic bacteria and development of functional bacterial magnetic particles for nano-biotechnology. *Trends Biotechnol*, (2007) 25:182-188.
6. J. Y. C. Edgar and H. Wang. Introduction for design of nanoparticle-based drug delivery systems. *Current Pharmaceutical Design*, (2017) 23: 2108-2112.

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9. A. Balows, H. Troper, M. Dworkin, W. Harder, K. Schleifer (Eds.), The prokaryotes (2nd ed.), Springer Berlin Heidelberg, New York (1992) 3352-3378

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Design of research: European Projects by Prof. Nicola Paone. 4 lessons of 3 hours each and 1 lesson of 4 hours. From 10/01/2022 to 24/01/2022

2. Technology Transfer and Innovation by Prof. Donato Iacobocci. 4 lessons of 3 hours each and 1 lesson of 2 hours. From 10/03/2022 to 07/04/2022.

3. Giornate Didattiche SISN 2022, 7-15 Settembre, Bosco Chiesonuova, Verona (VR), Italy. This include 32.5 hours of frontal lessons and 16 hours of experimental tutorials on Small Angle Neutron Scattering (SANS).

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List of periods spent abroad

1.

2.

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List of conferences/workshops attended and of contributions eventually presented

1. XXXIII Congresso Annuale SISN, Milano 14-16 Settembre 2022. Abstract and Poster presentation

2. Second DiSVA-MaSBiC Annual Symposium on PROTEIN STRUCTURE AND FUNCTION IN BIOLOGY,MEDICINE AND NANOTECHNOLOGY, 13-14 October 2022, Univpm, Italy. Poster presentation.

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Part 3. PhD student information on publications

Review "Magnetosomes as Drug Delivery Vectors" in preparation

Research article in preparation

List of publications on international journals

J1. ...

List of publications on conference proceedings

C1. ...

C2. ...

List of other publications (books, book chapters, patents)

B1. ...

B2. ...

[Date] 14/10/2022

Student signature

Supervisor signature