PHD DISSERTATION SUMMARY

• ANDREA ANCONA

• PhD programme in PHYSICS

• Cycle: 32

• Coordinator: TRIGIANTE MARIO

• Supervisor: CAUDA VALENTINA ALICE

• Title of the dissertation:

Ultrasound-induced Reactive Oxygen Species generation by Zinc Oxide nanostructures: mechanisms and applications

Short description of the topics discussed in the dissertation

Understanding the mechanisms by which ZnO nanocrystals (NCs) generate Reactive Oxygen Species (ROS) is crucial in the path towards the applicability and optimization of this technology to solve real-world problems, such as developing new therapies for cancer or the development of methods to degrade environmental pollutants. In Chapter 2, the state-of-the-art of the use of nanotechnology for cancer therapies is described, and the Physics of ultrasound and its interaction with liquids and nanocrystals is discussed. In particular, since its crucial role for the generation of ROS, the physics of acoustic cavitation is described in great detail. Subsequently, Chapter 3 is dedicated to the study of the ROS generation by ZnO NCs. Section 3.1 will be devoted to the study of the generation of ROS by lipid-coated ZnO NCs exposed to UV irradiation, and the application of this technology for the treatment of cancer. Then, in Section 3.2, the ability of ZnO NCs to induce generation of ROS once exposed to ultrasound will be demonstrated, and the mechanisms leading to this phenomenon elucidated. Notably, the induction of acoustic cavitation by ZnO NCs when exposed to ultrasound emerged as the main mechanism leading to ROS generation. In Section 3.3, exploiting the treatment of polluted waters from industrial dyes as model system, the ultrasound-induced generation of ROS was further demonstrated. Eventually, in Chapter 4, since acoustic cavitation has been identified as main mechanism leading to ROS generation, the ZnO NCs' ability of inducing acoustic cavitation once exposed to ultrasound will be further studied, elucidating in greater details the mechanisms and characteristics of this phenomenon. The last part, Chapter 5, is devoted to a final conclusion and outlook of the presented work.

Description of the specific issues tackled in the PhD Thesis

During the three years of PhD, I have been primarily involved in the ERC Starting Grant project "TrojaNanoHorse" of Prof. Valentina Cauda. This project aims at the development of a new nanotechnology-based therapy for treating cancers. The therapy is based on the delivering of nanosized devices based on ZnO nanocrystals (ZnO NCs) functionalized with patient-derived lipid vesicles and their subsequent activation by ultrasonic irradiation in the tumor region, leading to cancer-cells killing. My role in the project was to study and optimize the ultrasound (US)-induced activation of ZnO nanocrystals to finally generate Reactive Oxygen Species (ROS) inside cancer cells and to develop the ultrasound apparatus to stimulate the nanodevice. As exposed in *Section 3.1* of the dissertation, during the first year I studied the ability of lipid-coated ZnO NCs to generate ROS upon UV light absorption in aqueous media, as first model system to start probing the generation of ROS by ZnO NCs. Using Electron Paramagnetic Resonance (EPR) coupled with the spin-trapping technique, we demonstrated and characterized the ROS generation ability of ZnO NCs in water, and we showed that the surface chemistry of the NCs greatly influences the type of photo-generated ROS. Moreover, thanks the collaboration with Dr. Hanna Engelke at the University of Munich (LMU), we showed that these NCs are effectively internalized inside cancer cells and that they are able to generate ROS intracellularly for cancer therapy. During the second year I completed the literature review about the ultrasound-based therapies for cancer treatment, with a particular focus on the mechanisms leading to the therapeutic effects (*Chapter 2*). Then I focused my work on the ultrasound-activated ROS generation by ZnO nanocrystals. I worked on developing a setup to test the ROS-generation by ZnO NCs exposed to ultrasound. Together with an ultrasound commercial device for biomedical application, I started studying the ROS and Cavitation generation ability of ZnO NCs by Electron Paramagnetic Resonance (EPR) coupled with the spin-trapping technique, B-Mode ultrasonography and Passive cavitation detection. With these techniques I showed that ZnO NCs are indeed able to generate cavitation and ROS by high frequency ultrasound, and that the main mechanism behind the ROS generation is the induction of acoustic cavitation by ZnO NCs (*Section 3.2*). Then, exploiting the treatment of polluted waters from industrial dyes as model system, we focused on the physical-chemical mechanisms leading to both ROS generation and ultimate dye degradation (*Section 3.3*). During the third year, subsequent research activity focused on the understanding of the mechanism leading to the induction of acoustic cavitation by ZnO NCs once exposed to ultrasound. Experiments have been designed to further prove the ability of ZnO NCs to induce inertial cavitation and to reveal its mechanisms. Passive Cavitation detection coupled with specific ultrasound pulse sequences have been employed to demonstrate that gas pockets adsorbed on the surface of ZnO NCs were able to induce inertial cavitation, and that gas pockets could be re-adsorbed on the surface of ZnO to induce cavitation multiple times (*Section 4.1*). Eventually, during the research period spent at the Imperial College of London in the Laboratory of Prof. James Choi, an improved ultrasound exposure setup was designed and used for acoustic characterization of the NCs. This was comprised of sub-millimiter channels within a tissue-mimicking phantom, and it allowed the minimization of the acoustic reflections from sample holder and water-air interfaces. When coupled with a high-speed camera, it revealed that ZnO NCs were able to generate micron-size bubbles, suggesting that ZnO NCs were able to induce stable inertial cavitation during the ultrasound exposure (*Section 4.2*).

Description of most relevant results obtained in the PhD Thesis

(Section 3.1) ZnO has been synthetized as nanocrystals having round shape and 15 nm in diameter. To induce the generation of ROS, the NCs have been exposed to UV light. The semiconducting

properties of ZnO played the greatest role in the mechanism of ROS generation: once a photon was absorbed, an electron was excited from the valence band to the conduction band, generating an electron-hole pair that could further react with the environment, in this case water. Electron Paramagnetic Resonance technique coupled with spin trapping enabled the detection of the generation of ROS from ZnO. Toward a biomedical application of this phenomenon, ZnO NCs were coated with a lipid bilayer to improve their stability in biological environments. Interestingly, this coating, while not inhibiting the generation of ROS, changed the type of photo-generated ROS. Eventually, as proof of concept, the possibility to exploit the ROS-generation ability of ZnO to induce cancer cell killing was tested in in-vitro conditions. These experiments revealed that UV light and ZnO NCs show a synergistic effect towards the damage of cancer cells, implying the potential of lipid-coated NCs as innovative ROS-generators for therapeutic activity against cancer.

(Section 3.2) Since UV light has numerous disadvantages in the context of biomedical applications, such as carcinogenic properties and limited penetration depth within the human body, in the subsequent experiments a different external stimulus has been investigated to generate ROS in combination with nanostructured ZnO, i.e. ultrasound. This has the fundamental advantages of both being safe for the human body (within a certain range of parameters) and having a larger penetration depth in the body than light irradiation. In the following research activity, the ROS generation ability of ultrasound irradiated ZnO NCs has been demonstrated. Indeed, when exposed to 1 MHz ultrasound at pressures of 1.5 MPa, OH radicals were generated in water solutions, as detected by the EPR technique. Importantly, ROS generation was directly correlated with the induction of inertial acoustic cavitation in the sample, as shown by both the passive cavitation detection and echographic contrasts experiments. Thus, to generate ROS, ZnO NCs had to induce inertial cavitation that, during the violent collapse phase, generate the homolysis of water leading to ROS. Therefore, by these experiments, the crucial role played by acoustic cavitation in the ultrasound-induced ROS generation ability of ZnO NCs was revealed.

(Section 3.3) Once demonstrated that the combination of ultrasound and ZnO NCs could lead to the generation of ROS, the possibility to exploit this mechanism for the degradation of an industrial water pollutant was explored. Rhodamine B was chosen as model pollutant due to its large use both in the scientific literature and in several industries like textiles, leather and food. The synergistic effect of low-frequency ultrasound (40 KHz) and ZnO NCs resulted in high degradation efficiency compared to ultrasound and ZnO NCs alone and control experiments with anti-oxidant confirmed the key role of ROS-generation in the dye degradation mechanism. Moreover, different ZnO morphologies have been synthetized and tested to evaluate the effects of size and morphology on the degradation efficiencies. Micron-size desert roses resulted both in the highest dye degradation efficiencies and ROS generation: this might be explained by the fact that, despite the lower surface area than ZnO NCs, their bigger size and morphology could help the trapping of more gas bubbles on their surface, thus lowering the cavitation threshold and enabling higher ROS generation. Eventually, in the attempt to further increase the degradation efficiency of ZnO desert roses, visible light irradiation was coupled with ultrasound during the degradation process and this resulted in a marked improvement of degradation performances.

(Section 4.1) As previously discussed, acoustic cavitation emerged as the key phenomenon leading to ROS generation. Following experiments were dedicated to the investigation of the mechanisms leading to the induction of acoustic cavitation by ZnO NCs. Experiments have been designed to prove the ability of ZnO NCs to induce inertial cavitation and to reveal its mechanisms. Passive Cavitation detection coupled with specific ultrasound pulse sequences have been employed to demonstrate that gas pockets adsorbed on the surface of ZnO NCs were able to induce inertial cavitation. By removing gas pockets by either ethanol addition in solution or degassing of the solution, ZnO NCs lost their cavitation-induction ability and changing the type of gas dissolved in solution modified the inertial cavitation behavior. Moreover, once gas pockets were detached from the NC surface and collapsed during cavitation, they could re-adsorb on the ZnO NCs surface multiple times, thus allowing for multiple inertial cavitation inductions. This could be crucial for biomedical applications, where a limited dose of NCs could deliver therapeutic effects multiple times. As a proof of concept, the possibility to exploit ZnO NCs as ultrasound contrast enhancer was investigated, revealing that that they could generate up to 30 minutes of continuous enhanced ultrasound contrast.

(Section 4.2) Eventually, during the period spent at the Imperial College of London in the Laboratory of non-invasive surgery and biopsy, an improved ultrasound exposure setup was designed and used for acoustic characterization of the NCs. Exposure of NCs to 1 MHz ultrasound confirmed the inertial cavitation threshold values obtained in previous experiments and when coupled with a high-speed camera, revealed that during the ultrasound exposure micron-size bubbles were generated, suggesting that ZnO NCs were able to induce stable inertial cavitation.

Publications

- **Ancona A**., Dumontel B., Garino N., Demarco B., Charzitheodoridou D., Fazzini W., Engelke H. , Cauda V., "Lipid-Coated Zinc Oxide Nanocrystals as Innovative ROS-Generators for Photodynamic Therapy in Cancer Cells", *Nanomaterials* 2018, *8*(3), 143
- Dumontel B., Canta M., Engelke H., Chiodoni A., Racca L., **Ancona A**., Limongi T., Canavese G., Cauda V. (2017) "Enhanced Biostability and Cellular Uptake of Zinc Oxide Nanocrystals Shielded with Phospholipid Bilayer*". J. Mater. Chem***. B**, 2017,**5**, 8799-8813
- Canavese G., **Ancona A.**, Racca L., Canta M., Dumontel B., Barbaresco F., Limongi T., Cauda V. "Nanocrystal-assisted ultrasound: A special focus on sonodynamic therapy against cancer". *Chemical Engineering Journal* 340 (2018) 155–172
- Lops C., **Ancona A.**, Di Cesare K., Dumontel B., Garino N., Canavese G., Hernandez S., Cauda V. "In-depth study of the sonophotocathalitic degradation mechanisms of pollutant dye via radicals generation by micro and nano zinc oxide particles" *Applied Catalysis B: Environmental,* 2019 April ; 243: 629–640.*,*
- Battaglini M., Tapeinos C., Cavaliere I., Marino A., **Ancona A.**, Garino N., Cauda V., Prato M., Debellis D., Ciofani G., "Design, fabrication and in vitro evaluation of CeO2 nanocrystals-loaded nanostructured lipid carriers for the treatment of neurological diseases", ACS Biomater. Sci. Eng.2019, 5, 2, 670-682)
- Limongi, Tania; Canta, Marta; Racca, Luisa; **Ancona, A**; Tritta, Stefania; Vighetto, Veronica; Cauda, Valentina , "Improving dispersal of therapeutic nanocrystals in the human body", *Nanomedicine*, VOL. 14, no. 7 2019

- Veronica Vighetto, **A. Ancona**, Luisa Racca, Tania Limongi, Adriano Troia, Giancarlo Canavese, and Valentina Alice Cauda "The synergistic effect of nanocrystals combined with ultrasound in the generation of reactive oxygen species for biomedical applications" *Frontiers in Bioengineering and Biotechnology, section Nanobiotechnology*, 2019, Accepted

Under review in International journals:

- **A. Ancona,** Dr Adriano Troia, Dr. Nadia Garino, B. Dumontel, Dr. Giancarlo Canavese and V. Cauda "Leveraging re-chargeable nanobubbles on ZnO nanocrystals for sustained ultrasound cavitation towards ecographic imaging", *Ultrasonic Sonochemistry,* 2019

Patents:

"Biomimetic Non-Immunogenic Nanoassembly for the Antitumor Therapy" (Original title:"Nanocostrutto Biomimetico Non Immunogenico per la Terapia Antitumorale") Italian Patent N. 102017000129243 of 13th Nov. 2017, Inventors: Cauda V., Canavese G., Limongi T., Garino N., Laurenti M., Dumontel B., Canta M., Racca L., Ancona A., filed with Research Report.

"Sonosensitizing agent and its method of activation" (Original title:"Agente sonosensibilizzante e suo metodo di attivazione") application n.P3008IT00, Inventors: Cauda V., Cicero G., Canavese G., Limongi T., Garino N., Racca L., Ancona A., Dumontel B., Canta M., Serpe L., Canaparo R., Foglietta F., Francovich A., Durando G., waiting for research report