1.3.25 Non-persistent plasmonic nanotherapeutics and 3D cancer models

Nanomaterials have attracted increasing interest for their potential to revolutionize the diagnosis and treatment of several diseases, especially neoplasms. Nowadays, despite the significant research efforts, less than 100 nanotherapeutics have progressed to the market since the acceptance of Doxil® during the 90's. Challenges in translation to the clinic are mainly due to: i) difficulties in industrialization of the synthesis processes (e.g., scalability, sterility, and homogeneity), ii) unsuitable pharmacokinetics with long retention in the body, and iii) toxicity. Actually, regulatory agencies have mostly approved soft (organic) nanomedicines designed to improve the availability of common drugs to the action site(s). In contrast, inorganic (especially plasmonic) nanomaterials, due to their size/shape-dependent optoelectronic behaviors, display advantageous properties that widen their utilization as, for instance, in combined hyperthermia, radiosensitization and medical imaging. This means that the full potential of nanomaterials in the clinical practice, and particularly in cancer management, is not yet reached. Here, we present recent advancements towards the development of non-persistent plasmonic nanomaterials for the treatment of oral malignancies and pulmonary infectious diseases, together with the progress on the composition of reliable 3D cancer models for the assessment of conventional and innovative therapeutics within the 3R's concept.

The lack of translation of noble metal nanomaterials (NPs) to clinics is mainly related to lack of industrially-compatible synthesis processes and NPs long-term retention in the body [1]. The persistence of NPs in organisms leads to possible interference with common medical diagnoses and can induce severe damages and gene expression alterations [2]. Regulatory agencies require a complete clearance from the body of the pharmaceuticals and their components in an acceptable timeframe [3]. The excretion is an essential biological process to eliminate materials from organisms, and can be accomplished by renal or hepatic pathways [1]. The renal pathway relies on glomerular filtration in kidneys, which size threshold is typically <8nm, denoting that only materials with ultrasmall hydrodynamic diameters (HD) can be efficiently eliminated through the urinary system [4]. Materials with HD >8 nm (as mostly of the NPs with intriguing behaviors for medical applications) are mainly captured by liver and spleen [5]. If the captured materials are biodegradable, the building blocks are then excreted by bile and feces, while if non-biodegradable, they result in long-term accumulation in the reticuloendothelial system (RES). As a first attempt to overcome the issue of persistence, the size of NPs has been reduced to ultrasmall range (<8nm, ultrasmall nanoparticles, USNPs) in order to enhance their renal clearance efficiency [6]. Due to this smart approach an interesting level of metal excretion was reached but commonly losing some key-features of NPs, among which physical and physiological behaviors [6].

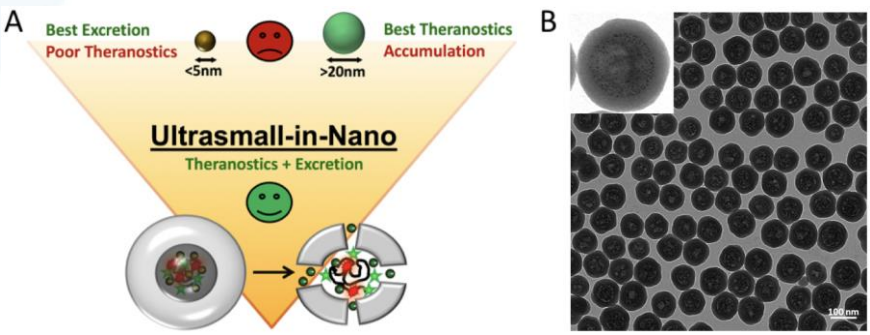

Figure 1. A) Cartoon explaining the groundbreaking nature of the ultrasmall-in-nano approach. B) Typical wide-area TEM image of NAs. Scalebar: 100 nm. Inset: zoom on one NAs.

In this context, we disclosed in 2015 the ultrasmall-in-nano approach (Fig. 1) for the reliable production of a family of non-persistent noble metal nano-architectures (NAs) that jointly combine the unique behaviors of plasmonic NPs with the body excretion [7]. NAs are composed by aggregates of plasmonic USNPs comprised in silica nanocapsules (Fig. 1) that biodegrade in <48h to the building blocks in several cell lines and biological fluids (Fig. 2) [8]. Briefly, the hollow silica shell is conceived as a shielding nanocapsule that: i) preserves the encapsulated materials until its degradation, ii) enhances ultrasound echo signals and iii) offers a straightforward modifiable surface [9,10]. Meanwhile, the gold USNPs promote light-matter interactions essential for photothermal effects or for radiosensitization, while the polymer can be modified with active molecules, such as drugs and dyes [11–13]. Biocompatibility and *in vivo* biokinetics investigations prior to *in vivo* efficacy tests are of particular interest in order to significantly reduce the number of required living models. Indeed, a number of metal nanomaterials have demonstrated intriguing features for theranostic applications, albeit without possible real applications because of persistence issue [2]. The whole-body toxicity of NAs has been investigated on zebrafish during their growth (Fig. 2), evaluating different toxicity end-points such as the survival rate, hatching rate, heart beat rate, malformations and cardiac effects [14]. Our findings indicate non-toxic effects according to the normative EU law and significantly highlight the biosafety of NAs at therapeutic concentrations [15]. The data collected on zebrafish have been also confirmed by histological investigations performed on healthy murine models i.v. injected with NAs up to the maximum amount of 150 mg/kg (Fig. 2) [16]. On these models, we also assessed the biokinetics of NAs. In particular, we have confirmed an interesting metal excretion profile by the renal pathway and a significant metal reduction in excretory system organs during 10 days [15]. It is worth to mention that, in agreement with ongoing investigations on lung diseases, we have explored the biokinetics of NAs administered to the models by inhalation [17]. Beyond accumulation in the lungs, translocation to secondary organs, and an almost completely excretion of NAs from the models, we observed a transient accumulation of NAs in the central nervous system [17].

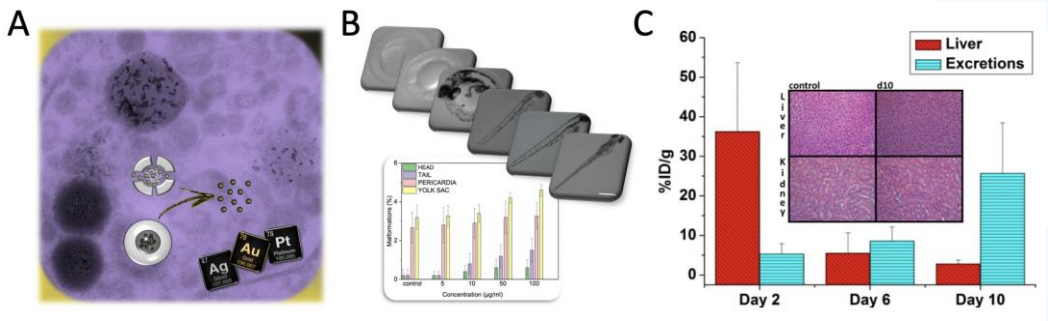

Figure 2. A) General biodegradation process of NAs in a cancer cell. The biodegradation process does not depend on the noble metal comprised in NAs. B) ICP-MS quantification of gold in liver and in excretions (cumulative) at the 3 time-points. Inset: Histological analysis of liver and kidney tissues from CD1-*Foxn1*^{nu} mice. C) Morphology of zebrafish treated with 100 µg/ml of NAs at the different time points investigated (4, 24, 48, 72, 96, 120 hpf). Scale bars= 500 µm. The graph reports the malformations rates vs NAs concentration in different larvae's parts at 96 hpf (head, tail, heart, yolk sac).

This work paves the way for the development of systemic or local pulmonary-delivered noble metal-based treatments for oncology and infectious diseases as well as for the investigation of the risks associated to the (in)voluntary inhalation of nanoparticles

During these years, some of the potential applications of NAs have been demonstrated (Fig. 3), pointing out that all the building blocks are pivotal. For example, we reported on the efficacy of NAs as a double-endogenously triggered drug delivery vehicles for a home-made cisplatin prodrug; a nanoplatform able to strongly reduce the side effects associated to cisplatin [8,13]. In 2019, we addressed two key hurdles that currently hinder the clinical translation of NPs-associated photothermal treatments: i) NIR-absorption occurs for NPs whose size is above the renal excretion threshold, and ii) anisotropic NPs undergo reshaping after PT transduction. On this regard, we demonstrate that narrow-NIR-responsive *thermo* NAs bear the optimal size for renal excretion and sustain repeated series of NIR light-to-heat transduction without losing their functionalities, avoiding metal sintering or reshaping [12]. Recently, the first multifunctional NAs for combined chemotherapy and photothermal therapy was synthesized and added to the list of NAs varieties, supporting the development of ultrasmall-in-nano multifunctional systems for combined therapeutic applications, such as photodynamic therapy-PTT and chemo-radiotherapy [18]. Beside therapeutic applications, NAs have been designed and investigated for diagnostic and imaging purposes.

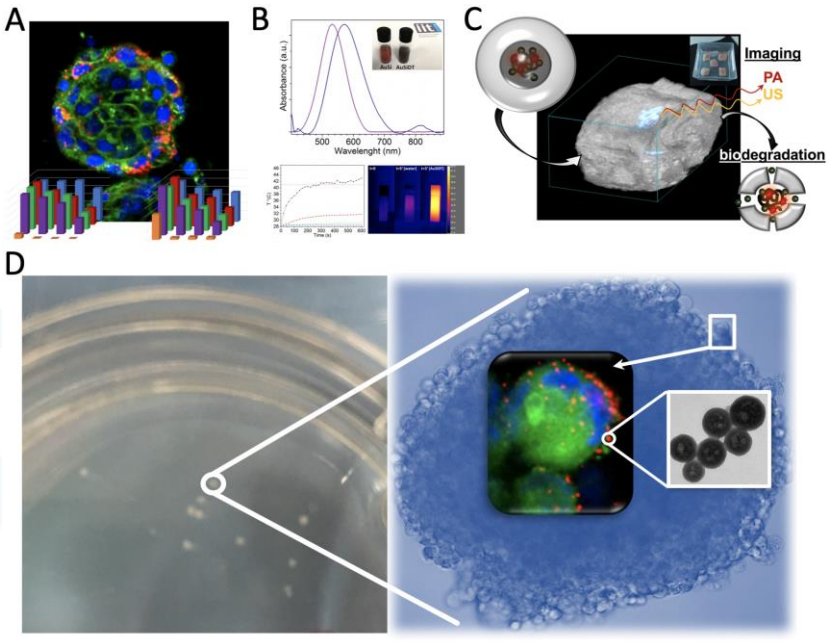

Figure 3. A) Combined image depicting the action of the double endogenous-controlled NAs platform as cisplatin prodrug carrier on cancer spheroids. B) UV/vis spectra of NAs and *thermo* NAs (upper panel) and the photothermal conversion during irradiation in the first biological window (bottom panel). C) Scheme depicting the *ex vivo* employment of NAs as dual photoacoustic/ultrasound (PAUS) contrast agents. D) Micrometastasis-like oral malignances (left panel) and optical/electronic zooms (right panel).

The design of NAs is a key-factor for their performances, among which are stability and high output signal. On this hand, we introduced a novel paradigm that exploits the synergistic interaction between commercial NIR-fluorophores and ultrasmall metal nanoparticles to produce a photoacoustic signal enhancement in the first biological window [11].

We have demonstrated that NAs are intrinsically bi-modal contrast agents for both photoacoustic and ultrasound imaging [10,11]. They can be exploited to jointly provide complementary information from both techniques, paving the way: i) for an enhanced diagnostic accuracy of neoplasms without employing ionizing radiation or ii) to follow and localize potential treatments [10].

Beyond metal persistence and bio-nano interactions, a major requirement for a successful scale-up and clinical translation of metal nanoplatforms are related to the production, which should be cost-effective and reproducible. In this direction, we have standardized and generalized the protocols for the production of NAs and the cascade characterization assay, reaching an high level of reproducibility and batch-by-batch homogeneity within samples [19,20]. Now, we are working on the semi-automatization of the procedures.

Finally, an important portion of our research at NEST is devoted to the development of 3D cancer models in order to perform investigations within the EU and local directives on animal models (Fig. 3) [21]. 3D tumor models act as a bridge between the investigations on cell monolayers and *in vivo* animal models. While both 2D cell culture and animal models cannot be completely avoided, the addition of 3D models in the research workflow provides robust and economical preclinical insights for the evaluation of conventional and nanomaterial-based treatments, as well as for the evaluation of nanomaterial features and nano-bio interactions. Moreover, they are employed to refine nanomaterials properties in order to reduce the use of animals for research. The accessibility of 3D tumor models can be extended to primary cell cultures, paving the way for personalized screenings. Ultimately, the full implementation of 3D tumor models in the pipeline of nanomaterials assessment will foster the advancement and translation of novel nanotechnology-based diagnostic and treatment systems to the clinical practice. On this hand, we employed 3D models in all our assessments and we have already standardized our procedures for their production and employment [22]. Our efforts are now aimed to the establishment of more complex 3D models that even better represent the neoplasms behaviors and environment.

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