Universidade de Lisboa

Faculdade de Farmácia

Safety of Gold Nanoparticles:

A preliminary *in vitro* and *in vivo* toxicity assessment

Inês Torres Pereira Silvério

Trabalho de Campo orientado pela Professora Doutora Ana Catarina Beco Pinto Reis, Professora Auxiliar e coorientado pela Doutora Maria Manuela de Jesus Guilherme Gaspar, Investigadora Auxiliar.

Mestrado Integrado em Ciências Farmacêuticas

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Abstract

Nanomedicine has brought new and improved therapeutic strategies and diagnostic techniques, and along with new challenges. Like conventional medicines, safety is a crucial parameter to thoroughly evaluate, however, the size and versatility of nanoproducts turn the assessment of their toxicity complex. Due to the novelty of this field, there is not a standardized safety assessment procedure; and so, researchers adapt existing guidelines of conventional medicines.

Our group has developed formulations with gold nanoparticles meant to be used in superficial tumours as enhancers of photothermal therapy. The obtained *in vitro* and *in vivo* results showed high potential towards several tumours. This work is intended to improve the knowledge about the safety of uncoated and coated, with polymeric-lipid material, gold nanoparticles.

A set of *in vitro* assays was performed. The biocompatibility was tested through haemolytic activity, cytotoxicity by MTT in B16F10 cells and mortality rate in *Artemia salina* bioassay. The overall results showed toxic effects in the presence of the murine melanoma cell line, B16F10, and reduced biocompatibility for the coated formulation. A preliminary *in vivo* acute toxicity test was performed in CD-1 mice using the highest concentrations tested in the *in vitro* assays, 0.179 and 0.358 mg/mL. No significant alterations were observed regarding behaviour and histopathology of the tested groups, however, one casualty happened in the group injected with coated gold nanoparticles at the equivalent concentration of 0.358 mg/mL, making dosage of 28.6 mg/kg of body weight. Bearing in mind that the histopathological analysis of all animal groups showed no significant signs of toxicity, we think that an agglomerate might have been the cause of mouse death.

Ultimately, the results obtained make us rethink the use of polymeric-lipid material coating or that some optimization is needed to ensure the design of an effective and safe formulation.

Keywords: Toxicology; Nanomedicine; Nanotoxicology; Gold nanoparticles; Cancer

Resumo

A nanomedicina trouxe novas e melhorou técnicas de diagnóstico e terapias, e, concomitantemente, novos desafios. Tal como os medicamentos ditos convencionais, a segurança é um parâmetro crucial para avaliar minuciosamente, no entanto, devido à dimensão e versatilidade dos nanoprodutos, a avaliação da sua toxicidade é complexa. Devido à novidade desta área, não existe um procedimento standard de avaliação da segurança, fazendo com que os investigadores adaptem as normas de orientação existentes da medicina convencional.

O nosso grupo desenvolveu formulações com nanopartículas de ouro destinadas a serem utilizadas em tumores superficiais como potenciadores da fototerapia térmica, tendo mostrado resultados promissores. Neste trabalho, pretende-se melhorar o conhecimento sobre a segurança das nanopartículas de ouro não revestidas e revestidas, com material polimérico-lípido.

Foi realizado um conjunto de ensaios *in vitro*; a biocompatibilidade foi testada através de actividade hemolítica, citotoxicidade por MTT em células B16F10 e taxa de mortalidade no bioensaio da *Artemia salina*. Os resultados, no geral, mostram efeitos tóxicos e biocompatibilidade reduzida na formulação com revestimento. Um teste preliminar de toxicidade aguda *in vivo* foi realizado em murganhos CD-1 utilizando as concentrações mais elevadas testadas nos ensaios *in vitro*, 0.179 e 0.358 mg/mL. Não foram observadas alterações significativas relativamente ao comportamento e a histopatologia dos grupos testados, no entanto, ocorreu uma morte no grupo injectado com nanopartículas de ouro revestidas a uma concentração equivalente a 0.358 mg/mL, fazendo uma dosagem de 28.6 mg/kg por peso corporal. Tendo em conta que a análise histopatológica de cada grupo não mostrou sinais significativos de toxicidade, pensamos que um aglomerado pode ter sido a causa da morte do murganho.

Em resumo, os resultados obtidos fazem reconsiderar o uso de revestimento de material polimérico-lípido ou que é necessária alguma optimização para assegurar uma formulação eficaz e segura.

Palavras-chave: Toxicologia; Nanomedicina; Nanotoxicologia; Nanopartículas de ouro; Cancro

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Abbreviations

- *A. salina* *Artemia salina*
- ATC Acute Toxic Category Method
- AuNPs Gold Nanoparticles
- bw Body Weight
- CTAB Cetyltrimethylammonium Bromide
- DDS Drug Delivery Systems
- DGAV Direção-Geral de Alimentação e Veterinária
- DMSO Dimethyl sulfoxide
- EFSA European Food and Safety Authority
- EPR Enhanced Permeability and Retention
- FDP Fixed Dose Procedure Method
- GLP Good Laboratory Practices
- HA Hyaluronic Acid
- ICH International Conference of Harmonisation
- IND Investigational New Drug
- MTT Assay Metabolic State and Membrane Permeability
- NIR Near-Infrared Region
- NPs Nanoparticles
- OA Oleic Acid
- *P. saccatus* *Plecantus saccatus*
- PBS Phosphate Buffered Saline
- PCL Polycaprolactone
- PdI Polydispersity Index
- PEG Polyethylene glycol
- PK Pharmacokinetics
- PLGA Poly(lactide-co-glycolide) acid
- PTT Photothermal Therapy
- RA Rosmarinic Acid
- RBC Red Blood Cells
- RES Reticuloendothelial System

- ROS Reactive Oxygen Species
- rpm Rotations per minute
- SPR Surface Plasmonic Resonance
- UDP Up-and-down Method

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1 Introduction

1.1 Toxicology in pharmaceutical research and development

Pharmaceuticals' development, whether they are drugs of synthetic or biological origin, is a very intricate, expensive and long process. It can take around 15 years for a pharmaceutical to be designed plus two years to be approved for patient use. From bench discovery to pre-clinical development, clinical trials and post-approval market surveillance, all the research and work is done with three main purposes in mind, the efficacy, quality and safety of the medicine (1,2). A new drug candidate must accomplish equally all three objectives referred. The three must be worked on in the same way and objectivity, if one does not have the same strong evidence as the others, the final product will most likely do not have the approval to be marketed.

In the pharmaceutical industry, the development of a product success rate is not the best when comparing with other industries insofar as the investment of millions of euros results in a few marketed products. The overall success rate is around 10%, both for chemical and biotech pharmaceutical companies, while this is an average, these numbers have not varied much since the beginning of the 21st century (3–5). To minimize the odds of failure throughout the development, upstream steps of this process must thoroughly think out, since this is the phase where many setbacks happen (1). Robust studies not only will give more credibility and reliability to the product being developed, as will lower the overall cost associated, with the already expensive by itself, process. Extensive and detailed non-clinical studies are in the undergirding of the successful transition into clinical trials, which is a small advance in this strenuous procedure.

Among the three backbones of the development of pharmaceuticals, safety is the one that is responsible for most setbacks in R&D and market withdrawals or suspensions. A very well-known example of market withdrawal due to toxicity problems was thalidomide. Thalidomide was marketed in 1957 as a sedative and antiemetic drug, targeted for morning sickness. It was prescribed to pregnant women and there were no apparent problems, in these individuals, related to its' use. However, soon after its' commercialization, an epidemic of severe birth defects appeared (6). Four years after commercialization, thalidomide was withdrawn from the market as a link

between its' use and birth defects was observed (7,8). After this event, regulatory authorities put their effort to determine pharmaceuticals' toxicologic profiles available for human use and made obligatory the submission of Investigational New Drug (IND) application. The IND is composed of the safety and efficacy data of the pharmaceutical before the first human exposure (9,10).

Non-clinical trials are the first step in deciding whether a potential pharmaceutical is ready for clinical trials, i.e., if it is ready to be administrated in humans. They consist of a set of studies that generate preliminary efficacy, toxicity, pharmacokinetics and safety information (11). Over the past three decades, substantial development regarding non-clinical studies has been accomplished, originating a new testing paradigm, hence improving pharmaceuticals safety noticeably (12). This development resulted due to the emergence of international guidelines, harmonized by the International Conference for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use (13), that increased the mutual acceptance of non-clinical submission documents in the European Union, Japan and the United States; and with the change of the perception of pharmaceutical toxicology (14).

1.1.1 Investigative toxicology

In the past, toxicology in the pharmaceutical industry was purely descriptive, simple 'yes' or 'no' answers to screening assays were sufficient to carry on the progress. However, when technology and knowledge evolved allowing a greater understanding of biological targets, translational biomarkers, this perspective was soon proved insufficient. Thus, investigative toxicology was born from the need to understand the underlying mechanism of toxicity, differentiate between toxic or adaptative reactions. This perspective has a problem-solving mindset and it's applied throughout the entire development process, comprising a prospective, where the screening anticipates risks; a retrospective attitude, where there is an investigation to understand the adverse effects (15).

Another driving force that contributed, along with this new mindset and advances in biological science and technology, was the concept of Animal Welfare. Investigative toxicology tries to break free from the classical toxicological approach of

assessing safety that heavily relies on animal testing. Safety assessment used to be more dependent, than nowadays, on large numbers of animals which lead to lengthier screening tests and inevitability making the process more expensive. Even though retrospective industry studies show that 70% of human toxicity is observed in experimental species (rodent and non-rodent) (16) and 48% adverse reactions can be predicted in non-clinical studies (17) These results can be misleading in two ways: firstly, they fail to capture the compounds that are ruled out due to observable toxicity in animals, therefore never tested in humans. However, if there is no proof that these adverse effects are only related to the animal's unique physiology, there is no way to know how it would behave in humans. Secondly, the translational relevance of adverse effects is sometimes difficult to assess, since they are highly dependent on the affected target organ, so what might have a big impact on animals might or not be as relevant in humans (11,14,18). So, to fill in the gaps, investigative toxicology is progressing towards more humanized *in vitro* systems to predict better human effects, and as in the last few decades, the use of *in silico* tools has been gaining ground in this field.

1.1.1.1 General non-clinical safety studies

Non-clinical studies generally investigate a battery of parameters, as safety pharmacological assessment, pharmacokinetics, acute toxicity, repeat dose toxicity, genotoxicity, reproductive toxicity and carcinogenicity; and other assays that seem reasonable or necessary to a particular drug (13). These assays are conducted following the Good Laboratory Practice (GLP), but the first exploratory studies do not require conformity with GLP principles (reproducibility, responsibility and awareness). Exploratory or preliminary studies are normally performed *in vitro* and/or with a reduced number of animals. These studies are pivotal in deciding the amount of time and budget worth investing in the new drug candidate (10,19).

Either way, similar assays are used in non-GLP and GLP studies, the methodology is the same, the conditions might differ (e.g. animal number, facilities, qualified personnel) (10). They help to characterise toxic effects regarding target organs, dose dependence, reversibility and exposure relationship. Ultimately, they complement each other, thus allowing for a robust and well-designed evaluation of the pharmaceutical candidate to clinical trials.

The first assays to be performed to assess the toxicity of a new drug candidate are *in vitro* methods. *In vitro* toxicity testing is a useful screening tool by providing data of relative toxicities the subsequently assist in the selection of a better candidate, and are extremely useful to investigate mechanisms of toxicity of effects detected *in vivo* experiments (2). One of the first assays usually performed is the Ames test. Ames test is a very common assay performed on a worldwide basis to detect genotoxicity. Besides this test, there is a myriad of other *in vitro* assays to be performed, like determine physicochemical properties, metabolic stability and pathways, assessing cell viability after exposure to a given compound (e.g., MTT); exploring the mechanisms behind observed phenomena. There is no lack of tests that help us elucidate the mechanisms of toxicity or action, so the researchers must know how to choose the ones suitable for that discovery.

After *in vitro* assays follows *in vivo* testing. A set of methodologies to assess toxicity are specified in international guidelines, some are to be employed in every situation while others are more specific to the intended drug. Generally, one of the first parameters to assess to further carry on more complex safety tests is the Maximum Tolerated Dose (MTD). Although it is not mandatory to demonstrate in every case, it is a good starting point in identifying the dose from which it is expected to show toxic effects (13).

Acute toxicity testing is conducted in a single dose study, ideally administrated to two animal species (non-rodent and rodent). It should be tested at different doses (at least three) and the effects up to 14 days after administration need to be observed. These studies allow the determination of the MTD, and/or the dose range to predict the outcomes of an overdose (13). The test can follow three approaches: the fixed procedure (FDP), acute toxic category (ATC) method and up-and-down (UDP) method. The FDP assess the nonlethal toxicity, it is administered 5, 50, 500 and 2000 mg/kg of the substance and the animal is observed for a period. The ATC method relies on the sequential use of three animals with four predetermined doses. The last method is usually recommended when using vertebrated animals as it reduces the number used. The UDP method starts with administrating the first dose to one animal and depending on its' reaction, the dose administered to the next animal may be scaled-up (no adverse reactions previously) or scaled-down (adverse reactions observed previously) (9).

After a short-term test, the assay that follows is repeated-dose toxicity studies, it lasts 28 days. As it evaluated several administrations, more complex data is generated comparatively with tests abovementioned. In this exploratory phase, the studies are generally shorter and require fewer animals but are pivotal to obtain data for the next and more complex tests that are required by regulatory toxicology.

1.2 Nanotechnology and the consequent emerging of nanotoxicology

1.2.1 Nanotechnology

Nanotechnology is not a recent subject, this term started to be used in 1974 by Norio Taniguchi, a researcher at the University of Tokyo, Japan. However, the nanotechnologies' conceptual foundations were established by Richard Feynman, a Nobel winner physicist, in 1959 (20). This term was used to illustrate the expertise of engineering materials with precision at the nanoscale, a scale ranging between 1 to 100 nm. In this scale, materials' surface atoms are rearranged and have different spacing, there is an increase in surface area and quantum effects are predominant, bestowing distinctive properties that can differ significantly from its bulk-sized equivalent (21). This type of technology is attractive to many fields as the textile industry (22), cosmetics (23), agriculture (24), electronics, renewable energy (25) and biomedicine (26).

Thus, the concept of nanomedicine soon appeared in the 1990s, depicted as a technology that takes benefits of molecular tools and knowledge of the human body with the purpose to treat it and diagnose pathologies. Nanomedicine rapidly grew in interest to researchers due to its' potentiality as a drug delivery system (DDS). Additionally, it has other applications in healthcare besides DDS (27). And it is due to DDS that this branch of nanotechnology departs from the purist definition of it since in the medical field, it is common to see NPs with diameters greater than the upper limit of the nanometre scale (100 nm). Some authors consider systems with a diameter up to 1000 nm is still considered NPs (28–30). This happens frequently in nanosystems with encapsulated compounds. The size of the nanosystem is tightly associated with the interactions between the encapsulated compound and the nanomaterial. As a representative example, Mota *et al* showed that the same compound encapsulated in

different nanomaterials like polymers such as Poly(lactide-co-glycolide) (PLGA) and Poly-E-caprolactone (PCL) and lipids, the size of each NPs is different, as well as their behaviour in its' presence. PLGA NPs showed a higher increase in size when compared to PCL NPs; yet when compared to the encapsulated lipid-based nanosystem, ethosomes, they were smaller from the get-go (31,32). Size besides being the parameter that dictates if a particle belongs to this field is one of the characteristics that have the most consequences about its efficacy, quality and safety.

1.2.1.1 Nanoparticles

Nanoparticles can be found in every shape, size, material, thus having multiple applications as, for example, DDS, *in vivo* imaging, *in vitro* diagnostics, biomaterials, active implants (27). NPs can be made of a great variety of materials such as lipids, metals, silicon and silica, titanium, aluminium, polymers, proteins and carbon; and depending on the selected constituents their behaviour as a drug-delivery system and/or diagnostic tool can be influenced (29,33,34). These structures have as general characteristics a high ratio of surface area to volume; tunable optical, electronic, magnetic, and biologic properties; and they can be designed to have different shapes, sizes, surface chemical attributes; and hollow or solid structures (35–37).

Even though this field is recent, there are more nanoproducts marketed than one might expect. Polymer-based nanosystems are one of the most marketed approved nanoproducts and the most used polymer is polyethylene glycol (PEG) and the applications vary from blood disorders, hormonal disorders, autoimmune diseases to cancer (38). Lipid-based NPs have been around before nanomedicine was conceptualized, in 1965, Abelcet® was approved. It is an amphotericin B lipid complex that treats systemic fungal infections. The lipid complex improved the pharmacokinetics (PK), a better tissue distribution, lower serum concentrations, higher half-life was obtained when compared with the drug in free form and there was a decrease in renal toxicity (38,39). Although lipidic NPs have been used mainly in one area, most of the approved nanoproducts are for various types of cancers for many years, nowadays they are still relevant as they are used to produce some of the COVID-19 vaccines (40). Among the various nanomaterials that can be used and are under research, there barely are marketed metallic NPs, there were iron NPs used for imaging purposes that ultimately were discontinued due to side effects shown. However, lately,

there is a lot of research revolving around metallic NPs, towards imaging and alternative therapies with a special focus on treating cancer (38,41).

Nanosystems have a lot of advantages many of which justify their predominant use in the field of oncology. Their use allows evading intrinsic drug problems such as solubility and possible sensibilities with the surrounding biological environment. A relatively simple way to achieve this is through encapsulation of the drug (42). Polymeric and lipidic NPs are commonly used for this purpose. Polymeric NPs are very versatile as they can be prepared by different methods and polymers which itself can influence the physicochemical properties, the administration route, ability to carry multifunctional agents, and overall PK of the drug encapsulated (38). While there is a whole range of polymers to use at our disposal, there are some that are more 'popular' among researchers like PLGA and PEG (43). Both are biocompatible materials capable of *in vivo* degradation, regarded as safe materials, and have been used by the pharmaceutical field for many years prior. PEG is often used to increase the drugs' half-life, reduces the reticuloendothelial system (RES) uptake due to the polymer's hydrophilicity (44). PLGA is widely used because of its tunable drug release profiles, ability to encapsulate hydrophilic or hydrophobic drugs, practicable surface modifications (45). They are mainly used for drug delivery, but they can also be used in gene delivery and tissue engineering. Regarding lipidic NPs, they were the first nanosystem to be developed, they are synthetic vesicles inherently biocompatible, capable of carrying big payloads and its' release can be controlled, highly useful in passive drug targeting increasing the specificity of treatment while decreasing toxicity (46). Another ability of NPs is the incorporation of diagnostic, imaging, therapeutic and targeting moieties, allowing a more personalized designed pharmaceutical by controlling how it distributes in the organism; for how long; how it reaches or targets the tumour sites (41). Surface chemistry plays a big role in these sorts of modifications, some nanomaterials are not able to incorporate on their surface these moieties and strategies like using polymers such as PEG, that allows covalent bonding with ligands, antibodies, peptides, resulting in active targeting; besides improving the PK profile (44).

Like a blank canvas, with the right materials and tools, it is possible to tailor a nanoparticle with any traits of our choosing, however, there are some basic factors to

have in consideration when formulating, especially when the intended administration route is intravenous. NPs formulating is a complex process as every parameter is intertwined and a slight change in one of them can constraint others and the outcome that was wanted might not be accomplished.

1.2.1.1.1 Size and shape

Size is a vital factor in determining the characteristics and safety of the nanosystem. It influences the *in vivo* biodistribution, elimination, cellular uptake, targeting, drug loading/release, stability and toxicity (47). NPs with a diameter smaller than 10 nm are rapidly cleared by the kidneys, therefore having a short blood circulation half-life. On the other hand, NPs with a diameter superior to 200 nm activates the complement system and are quickly removed from the bloodstream, accumulating in the liver and spleen as they are major components of the reticuloendothelial system (RES). These RES organs have fenestrated vessels that enable a synergic accumulation of the NPs in their interior (48). Nanosystems between 20 – 200 nm are more likely to have the highest potential as they are large enough to evade lymphatic and renal clearance and small enough to avoid innate immunity (47). In solid tumours, leaky blood vessels cause a high permeability area that facilitates the entrance of NPs to its' interior, alongside the lymphatic drainage impairment characteristic of these pathological areas leading to an accumulation of NPs in the tumour environment. This phenomenon is called Enhanced Permeability and Retention (EPR) effect, and it can be used to our benefit. Anti-cancer drugs or alternative therapies can be delivered via NPs designed with a size that allows a high accumulation and concentration of loaded material at tumour sites (49). Various internalization assays have been performed, mostly with gold nanoparticles (AuNPs), silica and polymeric NPs. Generally, a size-dependent uptake was observed. In most of the *in vitro* assays using different types of NPs and surface functionalization, a higher cellular uptake for NPs with a mean size of 50 nm was observed (50–53). It was further demonstrated that NPs with different sizes reach distinct sections within the cell. Indeed, NPs smaller than 15 nm may reach the nucleus, which can be both an advantage and disadvantage. This size is appropriate for gene delivery therapy or if not intended it might cause nuclear damage (53,54).

Nanosystems can present different shapes. In literature, they are described as spherical, triangular, cubic, ellipsoidal, rod and star-like NPs among others. Similarly, to size, this parameter can affect cellular uptake, biodistribution and *in vivo* behaviour as well. Champion and Mitragotri have focused much of their work on the effect of morphology in phagocytosis, which in turn might reflect how NPs of a certain shape is cleared or accumulated at certain tissues. This research team showed that ellipsoid or spherical NPs were more successfully internalized depending on their curvature (55). Furthermore, they observed that geometric shapes modulate phagocytosis. In *in vitro* tests performed with spherical and of equal volume, they found that while the spheres were highly phagocytized, a negligible internalization was exhibited for worm-like NPs (56). Similarly, although in other conditions, Arnida *et al*. observed comparable results regarding the relation between curvature and internalization. This group compared rod-shaped and spherical NPs and found out that nanorods displayed a longer circulation time as well as a higher accumulation in solid tumours as they were less phagocyted (57).

When working with NPs these are the first parameters to characterize as they influence a myriad of critical parameters concerning the effectiveness of the treatment.

1.2.1.1.2 Surface properties

As abovementioned, in addition to NPs shape and size, the surface charge is also a highly important characteristic that can affect a multitude of biological effects. When NPs are injected into the bloodstream, biomolecules adsorb at their surface forming a protein corona, that can change the NPs size and consequently the PK. as well as the stability and NPs-cell interactions (34). As most of the biomolecules circulating in the bloodstream present a negative charge, cationic NPs have shown more problems related to corona formation, namely higher protein binding, resulting in biodistribution and half-life profiles that can exert toxic effects. Negative and neutrally charged NPs have shown lower protein binding and consequently fewer complications (58). Aside from interactions with plasmatic proteins, nanosystems can interact with red blood cells (RBC). Positive charged NPs can interact with RBC negative surface and this interaction can result in haemolysis. A study with PLGA NPs was performed to assess the influence of surface charge and haematologic changes. It was observed

that negative and neutrally charged nanosystems did not cause any alteration when in contact with RBC whereas positively charged NPs caused haemolysis (58).

Hydrophilicity is another factor to have in mind when designing nanosystems. RES is unable to detect particles with hydrophilic surfaces, increasing their residence time in the bloodstream. PEG is widely used to change NPs surface hydrophobicity and escape the RES (34).

1.2.2 Nanotoxicology

Nanotoxicology is a subfield of toxicology concerned with the toxic effects of nanomaterials. It has gained more attention in the last two decades as nanotechnology has evolved and gotten more interest from a wide range of researchers. Nanomedicine and nanotoxicology grew up together, not only because medicine is highly regulated concerning the safe use in patients, along with the fact that they explore the same mechanisms and affect similar metabolic pathways (59). Nanomedicine holds immense potential, and it already is employed in the real world, despite a large number of basic and preclinical studies reach clinical studies (60).

NPs can be made up of numerous materials, some of which the bulk material has been used for many years and with known toxicity profiles. However, nanosized material can reveal other properties not shown previously and the toxicity cannot be predicted from the bulk material analysis, and further studies are needed to characterize the nanomaterial thoroughly (33). These properties (high surface area, biodistribution, EPR effect, surface chemistry, *etc*.) that make NPs so attractive as a therapeutic or carrier are, paradoxically, the same ones responsible for toxic effects hence nanomedicine and nanotoxicology being so intertwined (61). Nanotoxicology addresses fundamentally structure-activity relationships, how specific properties (or combinations of properties, as they may be interconnected) interact in a biological system. Interactions nanosystem-biological medium is an obstacle as they are difficult to predict. A lot of factors are involved in these interactions' outcomes, while the correlation between the properties of the nanomaterial and the toxicity profile is extremely complex some affect every type of NPs, like particle size, surface properties, stability, route of exposure and duration. Size as aforementioned can affect almost

everything, it can be responsible for accumulation in undesirable organs and eventually cause damage, occlude small capillaries, if small enough, trespass biological barriers (e.g., brain blood barrier) (61). Surface properties are linked with the formation of protein corona which in turn can suppress or stimulate an immune response, surface charge is important to avoid haemolysis and for stability. Excessively positively charged NPs tend to aggregate, increasing size causing the problems mentioned. Surface charge is important for the formulation's stability, as it can avoid the agglomeration of the NPs. Suspensions with zeta potential values higher than +30 mV or lower than –30 mV are reported to be stable (47).

Like in regular toxicology, the same tests are used in nanotoxicology. The first approach when screening a new substance is through *in vitro* testing. Unlike animal studies, there are fewer ethical issues, they are cheaper, easier to control and reproduce. *In vitro* assessment encompasses haemolytic and aggregation tests, cell-based assays, ROS quantification/detection, immune system activation, genotoxicity. Nevertheless, the data obtained, although important for future decision-making, is simpler and cannot predict at 100% how nanosystems behave *in vivo.* Animal studies are crucial and they are used for assessing the biodistribution, clearance, serum chemistry and histopathology (47)

1.3 Gold nanoparticles - a light in biomedicine

Gold has accompanied humankind since ancient times in various forms (62). The use of gold in therapeutics is described back in old Egypt, hieroglyphs disclose that it was believed that this metal could cure physical, mental and spiritual diseases (63).

1.3.1 Old but gold treatments

Throughout the ages, gold continued to be used in the health field, in the 20th century it was used in dentistry as implants or filling and to treat rheumatoid arthritis (64). For decades, gold salts were used in rheumatology, auranofin, allochrysin, sanochrysin, myochrysin and solganol are gold salt-based drugs. Generally, they were intramuscularly administrated, except auranofin that is *per os*. The mechanism of action of these drugs never was completely understood, it was thought that an anti-inflammatory effect was originated from gold itself due to its redox properties. Gold

can reduce the oxidative stress found inflamed sites by scavenging ROS; interferes with white blood cells like monocytes as it limits their infiltrations in the synovium, T cells' proliferation is restricted and the interaction between both cell types is hindered. All combined decreases the inflammatory environment of the joint. Regarding some PK aspects, all salts peak concentration was 2 h after administration, whereas auranofin's peak is less intense due to its' administration route. When in the bloodstream they are bound mainly to albumin, gold's half-life time is around 6 days but can be increased with repeated administrations These salts are mainly excreted via urine and faeces; patients that received multiple treatments had traces of gold in various tissues wherein the synovium, lymph nodes, liver, adrenal glands and kidneys; in some cases, organs of difficult access like brain and gonads showed some traces of the metal (65). Gold is considered rather inert, usually, individuals that handle it rarely suffer adverse reactions, this metal even is used in the food industry as an additive, a recent report from EFSA (European Food Safety Authority) deemed it as safe, without systemic effects to be expected concerning its' use despite the low toxicity data of this metal (66). Nevertheless, gold therapy showed some adverse effects, some reversible that disappear with the discontinuation of the treatment most of the adverse reactions fall in this category, and others irreversible. Like many drugs, frequent side effects are GI track related, and the most frequent are rash and dermatitis. Irreversible reactions only happen with high doses of gold accumulate, one of these effects is chrysiasis, a blue to grey discolouration on the patient's skin, intimately related to exposure to light. While it is painless and does not cause other skin damage, it can be unsettling to the patient because of the aesthetics. Chrysiasis can be ocular, this present has a golden ring around the cornea and like the dermal presentation is harmless, it does not cause pain and neither affects vision (65).

1.3.2 Methods of preparation, properties and applications of AuNPs

In the last decades, there has been an increasing interest in repurposing old medicines and nanotechnology takes a big part in the 'recycling' of compounds used in the past. Gold has been used in the biomedical field for quite some time, mainly as components in sensors and for labelling and imaging techniques. Due to nanotechnology, this metal was structured in the form of AuNPs, gaining great interest

in biomedicine as they have shown promising results in a multitude of applications like DDS, gene therapy, targeting, imaging, adjuvant, photodynamic and photothermal therapy due to their physicochemical properties (67–69). This versatility is linked to the shape, size, surface chemistry and functionalization of the AuNPs, and the starting point of this diversity is the method of their synthesis. They are many approaches used to prepare AuNPs, being most of them variations from the conventional methods.

In 1951, Turkevitch *et al* (70), developed a method of production that relied on the mild reduction of Au^{+3} to Au^{0} (aqueous solution) using citrate as reduction and stabilizing agent, in this technique the pH of the solution, reaction temperature affect the NPs' size (67,71). The resulting NPs are spherical and have a negative surface which is less likely to cause complications when circulating in the bloodstream (58).

Forty-three years later, Brust-Schiffrin (72), developed a biphasic synthesis, that generates small thiolate-stabilized AuNPs (2- 5 nm) with a cubic octahedron and icosahedron shape. The stabilizing and reduction agent used is sodium borohydride, which is more reductant than citrate, the synthesis process is rather simple under normal environmental settings; the NPs obtained have high thermal, air and physical stability; it is easy to get a monodisperse population and the functionalization is easy to do by ligand substitution (71).

Another widely used method is Seed-mediated Growth Synthesis, this procedure encompasses two stages. Firstly, small size AuNPs 'seed' is prepared, then the second phase is the 'growth' of the seed in a solution of gold (III) chloride trihydrate, stabilizers and reducing agents. Generally, the AuNPs are spheres and bigger comparing to the other methods (71).

These methods are mainly used to obtain spheres or spheric-like NPs. All these methods rely on reducing and stabilizing agents, and the variations of them arise with using different types of these agents. Some of the variations involve using 'green' reagents. Boldeiu *et al* compared *in vitro* toxicity of two types of AuNPs obtained from the conventional Turkevitch method and its green variation where the synthesis was mediated with honey instead of citrate. Two cell lines, B16 and L929, were used to assess cytotoxicity by MTS (cell proliferation) assay, it was also evaluated, DNA replication and apoptosis metrics. Citrate stabilized AuNPs showed lower cell viability below 80%. Both NPs with lower 10 μg/mL caused slight toxicity, the L929 was the

most sensitive to their presence especially with citrate stabilized AuNPs at concentrations superior to 10 μg/mL. Cell proliferation and apoptosis also show that 10 μg/mL is the concentration from which there's less proliferation and more apoptotic cells. Honey stabilized cells barely induced apoptosis in L929 cells whereas the same can be stated in B16 cells as a higher concentration increased greatly the percentage of apoptotic cells. This result unravels the potential of using honey stabilized AuNPs as a cancer treatment approach. Whereas citrate stabilized AuNPs showed more apoptotic cells in healthy cell lines (73). Silva *et al* also studied the difference in AuNPs obtain from the different methods and different greener stabilizing agents. The three methods described earlier were tested, the Seed-mediated Growth Synthesis used hexadecyltrimethylammonium bromide (CTAB) as a stabilizer, very commonly used, and a variation of the method that uses an extract of *Plectranthus saccatus* (*P. saccatus*) with the same function as CTAB. The NPs were characterized, as expected the smallest AuNPs were the ones prepared by the Brust-Schiffrin method, followed by Turkevitch, Seed-mediated Growth Synthesis with CTAB or *P. saccatus*. The AuNPs prepared with the plant extracted were slightly bigger and the shape was modified. Its presence influenced strongly AuNPs' interesting property, surface plasmon resonance (SPR). The NPs prepared with a 'greener' stabilizer, *P. saccatus* extract, SPR band reached near-infrared (NIR) wavelength, this fact enables the use of AuNPs in light-based therapies (74).

It is possible to design AuNPs with particular properties, as mentioned, the method of synthesis enables the control of size and even shape, and like other NPs these two characteristics influence a variety of parameters and outcomes, furthermore, they influence the optical features. The AuNPs have interesting optical features that make them attractive for imaging and light-based therapies, however, the focus of these features will be directed to ones related to photothermal therapy (PTT).

The amplitude oscillation of gold's free electrons caused by the oscillating electromagnetic light field, when reaches a maximum at a given frequency is referred to as the SPR. The SPR band of AuNPs can be tuned from the visible region to NIR (75). PTT is a therapy where light energy is converted into heat causing local hyperthermia and it does not require oxygen to interact with the target tissues (76). This therapy can be enhanced if associated with NPs as they have properties that enable fast

heating and are retained in the targeted area. Local hyperthermia caused by high energy irradiation leads to cellular membrane destruction and protein denaturation whose aggregation has destructive consequences in cell dynamics and survival while avoiding damage to healthy tissues (69). Usually, in PTT, NIR is used to irradiate the targeted area, since these wavelengths can safely penetrate the tissues (77). The effectiveness of this therapy depends on the photothermal conversion efficiency, which is related to the size of the AuNPs, in general, in spherical NPs the smaller they are, the higher the conversion, however, there must be an equilibrium, as small NPs are cleared from the extracellular space quicker. Equally important, is the design of the nanoparticle to be able to absorb within the two regions of 650-850 nm or 950-1350 nm. The light source needs to be capable to work within these ranges, as non-selective heating of healthy tissues and allows deeper tissue penetration (76–78).

This therapy is being explored with the intended use in superficial tumours and the results are favourable. NIR activated AuNPs are being developed to be used as a weapon to fight cancers like melanoma (79), breast (80), anaplastic thyroid (81) and, head and neck tumours (82). Compared to conventional therapies like chemotherapy and radiotherapy that are known for developing resistance, the severe side effects and low life quality, PTT as it relies on physical phenomena to exert its' effect, thermal stress, is a good alternative for drug-resistant tumours, and with well-designed AuNPs can be an even more selective therapy with barely any adverse reactions, as they will help to concentrate the generated heat and decrease the propagation to neighbouring healthy tissues (76). Most of the AuNPs formulations for this purpose are intended to be injected, some rely on active targeting with ligands, antibodies that direct them to target, others rely on passive targeting (EPR effect, *in situ* delivery). Whatever the mechanism of targeting may be, these formulations will circulate in the bloodstream and the possibility of accumulating on an unintended tissues or problematic interactions is still there, so it is imperative to know how they behave in these conditions.

1.3.3 Potential toxicity of gold nanoparticles

Gold is generally perceived as an inert material, nonetheless, materials when reduced to the nanometric scale can behave differently. Some studies show some toxic effects of AuNPs, at the same time other show that they are very safe to use. Why does

this happen? Very different AuNPs are being tested, differences in size, shape, coating, administration, exposure, test procedures, endpoints, cell model, animal model, any and every each of these greatly influence the response obtained. Moreover, most of the toxicity studies are made in *in vitro* models, despite being a useful screening tool, the results observed do not always translate in animal models, which are the ideal system to evaluate the toxicological profile.

The methods of preparation of AuNPs must be optimized, asides influencing parameters crucial to the NPs performance, the reagents used might be a source of toxicity. CTAB is a cationic surfactant commonly used as a stabilizing agent, even though it is employed in various, efforts to find alternatives are being made, as this compound has shown toxicity. Alkilany *et al.* demonstrated that the toxicity shown in CTAB-AuNPs was not mainly due to positively charged surface, contrary to what was initially thought, but it was primarily due to free CTAB found in the suspension (83). A way to overcome this can go through the improvement of the AuNPs' isolation before administration. More and more researchers are developing alternative synthesis methods, exploring new reducing and stabilizing agents since they can influence the NPs behaviour. Citrate stabilized AuNPs are easy to obtain, they have been around for decades, but there's toxicity associated even though not completely to this factor. A comparative study was made to see differences between citrate and 11-mercaptoundecanoic acid. It was assessed the cytotoxicity, production of reactive oxygen species (ROS) and mitochondrial membrane potential. The novel stabilizer showed better results regarding cytotoxicity, at 4h incubation there was not a loss of cell viability but at 24 h, higher concentrations some toxicity is presented. The measurement of ROS at 4 h hallmark did not show any increase in both stabilized AuNPs, in fact at higher concentrations, citrate stabilized AuNPs reduced ROS. At 24 h, both AuNPs at 60 μM, increased significantly ROS, but the ones stabilized with citrate did not rose as much (84). Many innovative and 'greener' stabilizers are being studied like the honey stabilized AuNPs abovementioned. Xanthan gum is a well-known excipient used in pharmaceuticals, and a group studied its' potentiality to stabilize AuNPs. These AuNPs were incubated for 48 h with A549 (human lung cancer) cells with concentrations ranging from 0.1 to 10 μg/mL did not cause viability loss.

These NPs were loaded with doxorubicin and their efficacy was enhanced to the nano-DDS compared to the free cytotoxic drug (85).

As has been reiterated throughout the dissertation, size is pivotal in all that matters NPs. Perhaps within all features that influence NPs, size is the one with more conflicting data. Ávalos *et al.* tested different sized AuNPs, 30, 50 and 90 nm, with non-cytotoxic concentrations, based on previous studies of the group, concerning genotoxicity. All sizes showed toxicity through oxidative DNA damage induced in HepG2 (human hepatoma) and HL-60 (human leukaemia) cells. There was an increase of oxidised pyrimidines and purines in both cell lines, though HepG2 revealed more sensitivity to their presence. The oxidative damage was size-dependent, 90 d.nm AuNPs showed a ~25% increase of oxidized pyrimidines compared to control and in between cell lines. This group also studied the genotoxicity of the 30 d.nm NPs *in vivo*, and the results did correspond to what was assess *in vitro* since no mutagenic or recombinogenic activity was observed. Many researchers identified size-dependent toxicity (84,86), larger sizes showed to be more cytotoxic but other groups observed the opposite (87,88).

It is difficult to correlate with certainty a range of sizes deemed safe or unsafe as some sizes didn't show toxic effects in some studies and others caused damage in the study model. Chuen *et al* tested same sized nanorods in MTS assay in three different types of mammalian cells, PK-15 (porcine kidney), Vero (African green monkey kidney), and NIH3T3 (mouse embryonic fibroblast). All cell lines showed loss of viability in presence of the nanorods, PK-15 were the less sensitive, only high concentrations, 720 and 1000 ng/mL. In the remaining cell lines, a concentration-dependent response was noted, however, Vero cells were the most sensible as they had a marked decrease in viability (89).

AuNP' surface chemistry is easily tuned. The purpose of use of AuNPs can be determined by the chosen coating, some of the health applications are prophylaxis, using protein/peptide/carbohydrate/DNA modified AuNPs; diagnosis, with covalently or coordinated metal ions, antibodies linked; or directed for treatments and there's a myriad of ligands, coatings, molecules that can be used. However, the coatings can alter the safety compared to NPs uncoated. A classic example of coating used to increase bioavailability and biocompatibility is PEG polymer. This polymer is very widely used

however it was noticed that it can lower cellular internalization efficiency, reduces binding interactions between surfaces and protein targets, unfortunately, it isn't a solution for every type of target, therapy. Simpson *et al*. was developing a treatment directed to *Mycobacterium tuberculosis* infections using AuNPs, but PEG is unfit to be employed in lung targeted therapies; so, glutathione-coated NPs were developed. This type of coating showed low immunogenicity, clearance times, biocompatibility and efficient targeting to lung tissue (90). Alternative coatings are being explored and have shown good results regarding toxicity. For example, chitosan-coated AuNPs showed great biocompatibility, no haemolysis could detect and no loss of cell viability was detected in MTT and LDH assay (91). Biomolecules can be used, a peptide hybrid AuNPs developed by Konoeda *et al.*, and it was assessed the acute toxicity of the nanosystem. Sprague Dawley rats were injected with doses ranging from 7.5 to 120 pmol/kg of the hybrid NPs, blood was collected including heart, lung, liver, spleen, thymus, kidney, adrenal gland, muscle, skin, and injection site. A complete blood cell count and biochemistry and tissue index were performed and neither test showed signs of toxicity (92).

There are multiple studies about the toxicity of AuNPs (**Table 1**, **Table 2**) as well a myriad of gold nanosystems of every size, shape, surface functionalization and lack of standardization in the assays to perform make it very difficult to compare and reach a consensus.

Table 1 – Selected *in vitro* toxicity studies of AuNPs.

NPs coating/shape	Mean size (nm)	Cell line	Dose	Incubation period	Methodologies	Main Effects	Ref
Uncoated	400	HaCat, 8505C	20 and 80 μM	24 h	MTT	Uncoated NPs exerted the highest reduction on cell viability than the others.	(81)
HAOA-Lap/EGF/HTf-coated							
Uncoated	65	HaCat, A375, B16F10 cell	25 to 100 μM	24 h	MTT	HAOA contributed to cell viability reduction. HaCat were more sensitive to the NPs' presence, however, they are not considered toxic.	(79)
HAOA-coated	150						
HAOA- EGF-coated	160						
Ammonium-coated	5 and 20	BEAS-2B	1 to 256 μg/mL	24 and 48 h	Trypan blue, Comet and Micronucleus assay.	Neither functionalization nor size alone can be responsible for the genotoxic effects of AuNPs. Ammonium-coated AuNPs showed a high loss of cell viability.	(93)
Carboxylate-coated							
PEG-coated							

Table 1 (continued)

NPs coating/shape	Mean size (nm)	Cell line	Dose	Incubation period	Methodologies	Main Effects	Ref
Citrate-coated	45	B16 and L929	1 to 15 µg/mL	24 and 48 h	MTS, CyQuant, apoptosis metric assay	Concentrations below 10 µg/mL revealed no toxicity. B16 cells were more sensitive to citrate-coated AuNPs, while L929 proliferation was stimulated by honey-coated AuNPs.	(73)
Honey-coated	75						
Citrate-coated nanospheres	14 and 50	hCMEC/D3	1 to 60 µM	4 and 24 h	MTT, LDH and ROS assay	Larger AuNPs were more toxic (50 nm). MUA coated AuNPs were less toxic than citrate coated AuNPs.	(84)
MUA-coated nanospheres							
MUA-coated nanostars	50						
n.a.	30, 50 and 90	HL-60 and HepG2	1, 5 and 10 µg/mL	24 h	Comet assay	All three AuNPs tested sizes caused genotoxic effects; however, 90 nm was slightly more genotoxic. HepG2 were the most sensitive to AuNPs presence.	(94)

Table 1 (continued)

NPs coating/shape	Mean size (nm)	Cell line	Dose	Incubation period	Methodologies	Main Effects	Ref
Citrate-coated	10, 30 and 60	HepG2 and HT-29	10 ppb and 10 ppm	16 and 32 h	LDH, ROS, and Comet assay	Decrease cell viability 16 h after incubation, increase ROS production or generation.	(86)
n.a.	5 and 100	NPCs	4 nM to 4 mM 2 and 200 mM	36 h	MTT	Smaller AuNPs were more cytotoxic.	(87)
Citrate-coated	16	BALB/c 3T3, U937, NR8383	4.7 to 1000 µg/mL	24 h	WST-1 and NRU assay	No toxicity was observed in all cell tlines tested.	(95)
Nanorods	10 to 40	PK-15, Vero and NIH3T3	36, 72, 180, 360, 720, and 1000 ng/mL	72 h	MTS	Vero cells were the most sensitive. All cell lines showed a decrease in viability with increasing concentrations.	(89)

NPs coating/shape	Mean size (nm)	Cell line	Dose	Incubation period	Methodologies	Main Effects	Ref
Xanthan gum-coated	40	A549	0.1 to 10 µg/mL	48 h	MTT, haemolytic activity	Loaded AuNPs displayed toxicity and unloaded coated xanthan gum AuNPs were harmless.	(85)
Xanthan gum-coated and doxorubicin-loaded	15 to 20						
Citrate-coated	5 and 15	Balb/3T3	10 to 300 µM	72 h	CFE	Smaller AuNPs at higher concentrations were toxic.	(88)
Citrate-coated	14 and 20	BEAS-2B, CHO and HEK 293	1 and 5 nM	2 h and 10 min	XTT, LDH and ATP assay	Different assays displayed contradictory results. Toxicity was cell line and mean size AuNPs dependent.	(96)
Chitosan oligosaccharide-coated	28 and 300	HepG2	0.015 to 0.12 mM	24 h	MTT, LDH assay, haemolytic activity	No toxicity was observed and good hemocompatibility.	(91)

n.a. – non-available; CFE – Colony Forming Efficiency; LDH – Lactate Dehydrogenase; NRU – Neutral Red Uptake.

Table 2 – Selected *in vivo* toxicity studies of AuNPs.

NPs coating/shape	Mean size (nm)	Animal model	Dose	Administration route/duration	Methodologies	Outcomes	Ref
HAOA-coated	400	Male CD-1 mice	23 mg/kg	s.c 0.5, 1, 2, 4, 6 and 24 h	ICP-MS, histopathology	Local inflammatory response at 24 h after administration, AuNPs accumulated in liver at the highest extent.	(81)
Peptide hybrid-coated	20 nm	Male Sprague Dawley rats	7.5 to 120 pmol/kg	IV 7 days	ICP-MS, histopathology	No toxicity was observed.	(92)
n.a.	30	*Drosophila* strains	20, 25 and 30 µg/mL	n.a. 48 h	SMART test	No mutagenic or recombinogenic activity was observed.	(94)
Citrate-coated	10, 30 and 60	Male Wistar rats	0.4 mL/day	IP 9 days	ICP-MS, biochemical analysis	Smaller AuNPs caused more lipid peroxidation and accumulated to a higher extent in the liver and kidneys, with no signs of toxicity.	(86)

Table 2 (continued)

NPs coating/shape	Mean size (nm)	Animal model	Dose	Administration route/duration	Methodologies	Outcomes	Ref
Abutilon indicum-coated	1 to 25	Male Wistar rats	5 and 10 mg/kg BW	Oral 29 and 59 days	Biochemical analysis, histopathology, ICP-OES	No toxicity was observed.	(97)
Citrate-coated	14 nm	Male Sprague Dawley rats	0.9, 9 and 90 µg	IV 7 weeks (Repeated injection)	Histopathology, biochemical analysis, NAA	No observable acute or subchronic toxicity. AuNPs accumulated mainly in the liver.	(98)
Citrate-coated	16	Male Wistar rats	0.7 mg Au/kg	IV 30 min and 28 days	GFAAs, tissue index	Mild toxicity was observed particularly in the spleen.	(99)
Pentapeptide-coated							
n.a.	20 and 50	Pregnant ICR mice	n.a.	IV 24 and 48 h	ICP-MS, histopathology	AuNPs reached the placenta, but no toxic effects were observed.	(100)

Table 2 (continued)

NPs coating/shape	Mean size (nm)	Animal model	Dose	Administration route/duration	Methodologies	Outcomes	Ref
Citrate-coated	20	Male Wistar rats	15.1 µg/mL	IV 1 day, 1 week, 1 and 2 months	ICP-MS, microarray analysis	AuNPs accumulated mainly in the liver and were still detected 2 months after administration. The liver and spleen exhibited significant gene effects.	(101)
Citrate-coated	13.5	Male ICR mice	137.5 to 2200 µg/mL	Oral 14 days	Tissue index	Higher concentrations of AuNPs resulted in decreased bodyweight and increase tissue index.	(102)
n.a.	10, 50, 100 and 250	Male WU Wistar rats	1 mL	IV 24 h	ICP-MS	Smaller AuNPs were widely distributed. Higher AuNPs accumulated in the liver and spleen.	(103)
Citrate-coated	2 and 40	Female C57BL/6 mice	1 mL	IV and IP 1, 4 and 24 h	AMG	AuNPs were taken up mainly by Kupffer cells and did not cross the placenta or the BBB.	(104)

n.a. – non-available; IP- intraperitoneal; IV – intravenous; s.c. – subcutaneous; AMG – autometallography; GFAAS – Graphite Furnace Atomic Absorption Spectrometry; ICP-MS - Inductively Coupled Plasma Mass Spectrometry; ICP-OES – Inductively Coupled Plasma Optical Emission Spectrometry MUA – 11-mercaptoundecanoic acid; NAA – Neutron Activation Analysis; SMART – Somatic Mutation and Recombination.

2 Aims

Nanotoxicology has been growing in the last years, as more and more nanosystems are being developed. The same attributes that make NPs so attractive in medicine are the same ones that can cause health complications.

AuNPs have stood out in oncology, especially due to their use in PTT and imageology. A great number of AuNPs are being developed and have the most different characteristics so much that there are contradictory results regarding a multitude of aspects, especially about safety.

The formulations used in this study are intended to be used in PTT directed to superficial tumours, to be administered *in situ,* and have shown promising results. However, they have not been studied in depth regarding toxicity and dose-ranging with higher concentrations.

The purpose of this study aims to perform a preliminary general safety assessment of two AuNPs formulations: only gold core and coated with polymeric-lipid material. Toxicity evaluation is expected to be performed in successively more complex elements, starting in cell models to animal models. Both formulations will be compared to understand if the presence of the coating will have any toxic effect. The maximum gold concentration in the formulation without eliciting side effects is expected to be determined.

3 Methods and Materials

3.1 Materials

3.1.1 Reagents

Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$)silver nitrate, L-ascorbic acid (L-AA), hyaluronic acid (HA), rosmarinic acid (RA), oleic acid (OA) were purchased from Sigma-Aldrich (Steinheim, Germany) and silver nitrate from Honeywell (Seelze, Germany). The water used was purified to 18.2 MΩ·cm at 25°C through a Millipore system (Millipore, MA). All chemical products and solvents used were of analytical purity grade.

3.1.2 Cell line and culture

The cell line used to assess cytotoxicity was the murine melanoma cell line, B16F10. They were grown in Dulbecco's Modified Eagle Medium with high glucose (4500 mg/L), supplemented with 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen) hereafter mentioned as the complete medium. They were preserved at 37ºC under a 5% CO2 atmosphere and cultures were examined and maintained every 2–3 days, until achieving a confluence of about 80%.

3.1.3 Animals

Artemia salina (*A. salina*) eggs and hatching medium were purchased from JBL (Neuhofen, Germany).

Female CD1 mice were purchased from IHMT (Lisbon, Portugal). Animals were kept in polypropylene cages under standard hygiene conditions, fed commercial chow, and given acidified drinking water *ad libitum*.

All animal experiments were conducted with the approval of the Animal Welfare Organ of the Faculty of Pharmacy, University of Lisbon, permitted by the competent national authority, Direção Geral de Alimentação e Veterinária (DGAV) and in

agreement with the EU Directive (2010/63/EU), the Portuguese law (DL 113/2013, 2880/2015 and 260/2016) and all relevant legislation.

3.2 Methods

3.2.1 Synthesis of uncoated AuNPs

AuNPs uncoated was prepared following a modified Seed-mediated Growth method based on the one developed by Silva *et al* (74).

Aqueous solutions of silver nitrate, L-ascorbic acid, and rosmarinic acid were added to an aqueous solution of gold (III) chloride trihydrate. This mixture was magnetically stirred for 15 min at 800 rpm (Heidolph magnetic stirring hotplate MR 3001, Heidolph Instruments, Schwabach, Germany) at room temperature. To recover the uncoated AuNPs, the suspension was centrifuged at 1150× g for 20 minutes. The pellet of uncoated AuNPs was resuspended in phosphate-buffered saline (PBS, USP32) for the *in vivo* and *in vitro* assays; and water before being coated.

3.2.2 Synthesis of HAOA-coated AuNPs

The coating solution was prepared by adding to Milli-Q water, HA, OA and sodium hydroxide 0.1M at 60ºC and left stirring overnight at 400 rpm. When it is done and left to cool to room temperature, it is added to the aqueous suspension of uncoated AuNPs and is magnetically stirred for 30 minutes at 800 rpm, 1:1 (v/v).

To recover the HAOA coated AuNPs, the suspension was centrifuged at 7200× g for 15 minutes. The pellet is resuspended in PBS.

3.2.3 Characterization of AuNPs

Both uncoated and HAOA-coated AuNPs were characterized regarding their mean size, polydispersity index (PdI) and zeta potential. The samples were diluted in PBS at pH 7.4 (dilution factor of 1:10) Zetasizer Nano ZS (Malvern Instruments,

Malvern, UK) was used to measure the parameters referred before, using Dynamic Light Scattering. All measurements were performed in triplicate.

3.2.4 Haemolytic activity

The protocol was based on a previous paper of our research group (105). The haemolytic activity of uncoated and HAOA-coated AuNPs was determined in EDTA (ethylene-diamine tetraacetic acid)-preserved peripheral human blood, collected from a voluntary donor and was used on the same day of the experiment. To isolate the erythrocytes, the peripheral blood was centrifuged at 1000× g for 10 minutes and the supernatant, the serum, was discarded. The isolated erythrocytes were diluted in PBS and centrifuged at 1000×g for 10 min, three times. Both formulations were diluted in PBS, and the concentrations tested ranged from 0.001 to 0.170 mg/mL of AuNPs[1]. Samples were distributed in a 96-well plate, 100 µL in each well, and 100 µL of erythrocytes was added too. The positive and negative control were, respectively, samples with an equal volume of erythrocytes and distilled water (equivalent to 100% haemolysis) and samples with erythrocytes and PBS (equivalent to 0% haemolysis). The plate was incubated for 1 hour at 37ºC and centrifuged at 800x g for 10 min. Afterwards, the supernatants were collected and analysed through absorbance (Abs) at 570 nm with a reference filter of 620 nm using a BioTekTM EL ×800TM Absorbance Microplate Reader (Winooski, VT, USA). The results of this assay are calculated using the following formula:

$$Haemolytic\ Activity\ (\%) = \frac{Abs_{sample} - Abs_{negative\ control}}{Abs_{positive\ control} - Abs_{negative\ control}} \times 100 \qquad (1)$$

3.2.5 *In vitro* safety assessment

Cytotoxicity was assessed *in vitro* by MTT assay. Murine melanoma cell line B16F10 were seeded at a concentration of $5x10^4$ cells/ml (200 µL/well) and incubated with

[1] Equivalent to 2.34 to 300 µM of $HAuCl_4 \cdot 3H_2O$.

coated and uncoated AuNPs at 0.015, 0.030, 0.045, 0.060, 0.119 and 0.179 mg/mL of AuNPs[2] for 24 h. Afterwards, 50 µL of MTT reagent at a concentration of 0.5 mg/mL was added to the wells which were previously washed with PBS at pH 7.4, twice. The plate was incubated for 4 h, insoluble crystals of formazan were formed, and because of it, DMSO (dimethyl sulfoxide) was added to solubilize them, enabling the absorbance measuring (570 nm). The absorbance was measured using BioTekTM EL×800TM (Winooski, VT, USA), experiments were performed in five replicates for each concentration. Positive control of the experiment consisted of cells incubated with only a complete medium corresponding to 100% of viability. The results of this assay are calculated using the following formula:

$$Cell\ viability\ (\%) = \frac{Abs_{sample}}{Abs_{control}} \times 100 \quad (2)$$

3.2.5.1 *Artemia salina* toxicity assessment

Another *in vitro* toxicity assay was performed. This bioassay is simple to perform, does not require an aseptic technique, is quick, low cost and is indicated for ecotoxicity assessment (106). Assays performed in aquatic organisms are a good indicator of the impact nanomaterials have on the environment (59).

Dried cysts were acquired and hatched in artificial seawater in a covered-up bottle connected to a smaller bottle also covered, at a temperature of 22 ± 1ºC, with lateral illumination and they were kept in continuous suspension by using an aquarium pump. The larvae were hatched after 24 h and were separated from unhatched cysts and dead larvae by uncovering the bottle, switching the light position and turning off the pump. Then 900 µL of medium with *Artemia salina* (*A. salina*) ensuring that it contains around 10 to 15 and add it to a 24-well plate. It was added 100 µL of each formulation, uncoated and HAOA-coated AuNPs at two different concentrations, 0.179 and 0.358 mg/mL of AuNPs[3]. A higher concentration was tested because previous results showed that uncoated AuNPs at 0.179 mg/mL seemed to not have caused adverse effects in B16F10

[2] Equivalent to 25, 50, 75, 100, 200 and 300 µM of $HAuCl_4 \cdot 3H_2O$, respectively.
[3] Equivalent to 300 and 600 µM of µM of $HAuCl_4 \cdot 3H_2O$, respectively.

cell viability. Besides the wells with the different formulations and concentrations, there are positive and negative controls. Positive control consists of *A. salina* larvae with DMSO added to the medium that will cause its' death corresponding to 100% mortality; whereas negative control only has an artificial seawater medium and corresponds to low mortality. The formulations were left incubating for 24 h and then the number of dead larvae was counted. The mortality rate was determined using the following formula:

$$Mortality\ (\%) = \frac{Total\ of\ dead\ larvae\ at\ 24h}{Total\ of\ larvae} \times 100 \quad (3)$$

3.2.6 Preliminary i*n vivo* acute toxicity assessment

Female CD-1 mice were randomly distributed into four groups. Two groups (n=3) were injected intravenously with a concentration of 2.15 mg/mL of uncoated or HAOA-coated AuNPs, so that the concentration within the animal's bloodstream would be 0.179 mg/mL, corresponding to a dose of 14.3 mg/kg of body weight (bw) in terms of AuNPs. A mouse weighing 30 g was assumed to have 2.4 mL of total blood (107) The other remaining groups (n=4) were injected intravenously with the same formulations with 4.29 mg/mL concentration, so the concentration within the animal's bloodstream would be 0.358 mg/mL corresponding to a dose of 28.6 mg/kg bw in terms of AuNPs. The animals were weighed before the administration of the NPs and before the necropsy. After the injection, the mice were left in their regular housing, and after 24 h they were anaesthetized with isoflurane and sacrificed by cervical dislocation. Organs like lungs, liver, spleen and kidney were harvested for histological analysis. The samples were fixed in 10% formalin and stained with haematoxylin and eosin. Histopathological assessment was performed using an Olympus CX51 microscope (Olympus Corporation, Tokyo, Japan). Whole slide scanning was performed using the NanoZoomer-SQ Digital slide scanner—C13140-01 (Hamamtsu, Japan) and representative photos were taken using the NDP.View2 software.

The mentioned organs were weighted to determine the respective tissue indexes, according to the following formula:

$$Tissue\ index = \sqrt{\frac{organ\ weight}{animal\ weight}} \times 100 \quad (4)$$

3.2.7 Statistical analysis

All results were expressed as mean ± SD, n ≥ 3. Statistical analysis of *in vitro* assays and tissue index were performed using two-way ANOVA followed by Tukey's multiple comparisons test. *A. salina* safety assay and weight loss in the *in vivo* toxicity assay were evaluated using one-way ANOVA followed by Tukey's multiple comparisons test. All statistical analyses were performed using GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA). Results were considered significantly different when $p < 0.05$.

4 Results

4.1 Characterization of Nanoparticles

Before experiments, AuNPs were characterized in mean size, polydispersity index (PdI) and surface charge by Zeta potential measurement. The main results are shown in **Table 3**. Uncoated AuNPs were approximately 140 nm and when coated an increase in size occurred, to 200 nm. Before characterization, the samples were concentrated as a function of the gold in the formulation. Due to this, the samples used for *in vivo* testing were bigger than the ones used in *in vitro* assays, uncoated AuNPs which were ~100 nm increased to ~185 nm and HAOA-coated AuNPs from ~180 to 220 nm. Concerning the surface charge, the Zeta potential practically did not suffer changes as size did, and on average uncoated NPs are less negative, a mean -20 mV, and coated are more negative, a mean -40 mV.

Table 3 – Mean size, polydispersity index (PdI) and zeta potential uncoated and HAOA-coated AuNPs. The results are presented as mean value ± SD, n ≥ 3.

Formulation	Mean size (nm)	PdI	Zeta potential (mV)
Uncoated AuNPs	141.3± 63.2	0.260 ± 0.087	-17.6 ± 3.1
HAOA-coated AuNPs	203.1 ± 59.0	0.372 ± 0.145	-42.6 ± 6.6

4.2 Haemolytic activity

This formulation is intended for *in situ* administration at a dose below 20mg/kg of body weight in terms of the whole formulation (AuNPs and coating). Assuming that some NPs can escape into the bloodstream, a wide range of concentrations was tested, based on a previous study (81), and the results are depicted in **Figure 1**. The formulation without coating barely caused haemolysis, the highest activity was at 0.179 mg/mL, but it was below 2% which is a good predictor of biocompatibility. On the other hand, coated NPs showed high haemolytic activity predominantly at higher concentrations, and they decreased proportionally.

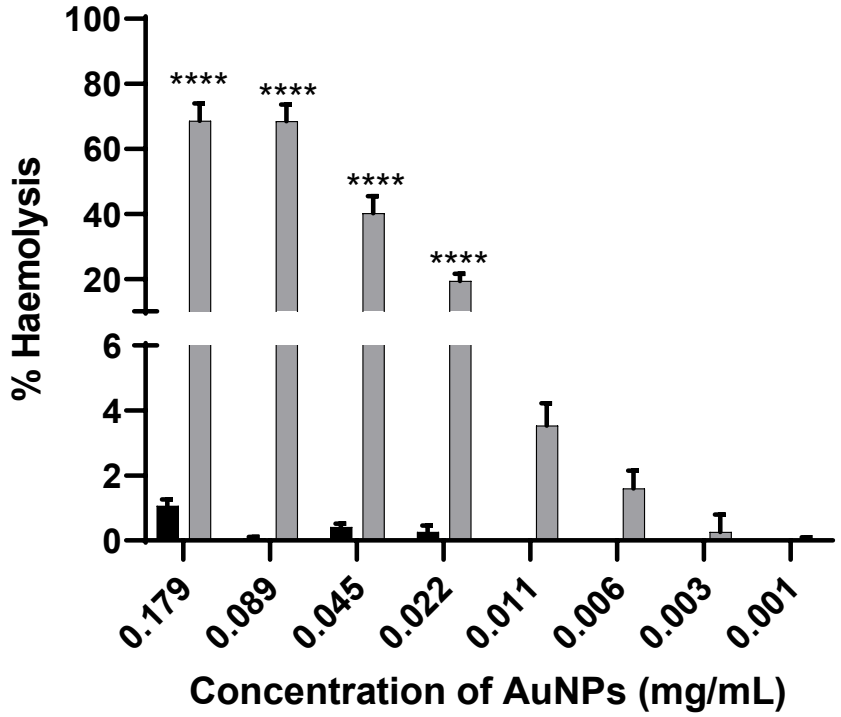

Figure 1 – Haemolytic activity of a set of concentrations of uncoated (black column) and coated AuNPs (grey column) ranging from 0.001 – 0.179 mg/mL. The results are presented as mean ± SD, n = 3 ($^{****}p < 0.0001$ vs. uncoated AuNPs).

4.3 *In vitro* safety assessment

After performing the haemolytic activity assay, a new set of concentrations were chosen (0.015 to 0.179 mg/mL) to be assessed in a more complex cell model bearing in mind the results obtained in haemolytic activity, and such high concentrations of these formulations have not been tested before. A preliminary *in vitro* toxicity assay was performed in B16F10 cells. Both formulations were incubated for 24 h with the chosen concentrations. The results are shown in **Figure 2**. A similar tendency was observed as in the haemolytic activity assay, HAOA-coated AuNPs decreased cell viability while uncoated AuNPs did cause any significant loss of viability, in fact in higher concentrations an increase was shown. A peculiar profile of cell viability is depicted in HAOA-coated AuNPs, an 'U' shape is displayed, intermediate concentrations showed the lowest viability, having an increase up and downstream.

These results show that coated AuNPs cause a reduction of viability below 70%, showing their toxic potential according to ISO 10993-5:2009(E) (108). However,

bearing in mind the cell line used, it can be interpreted as a positive result, since it caused the loss of viability of tumoral cells.

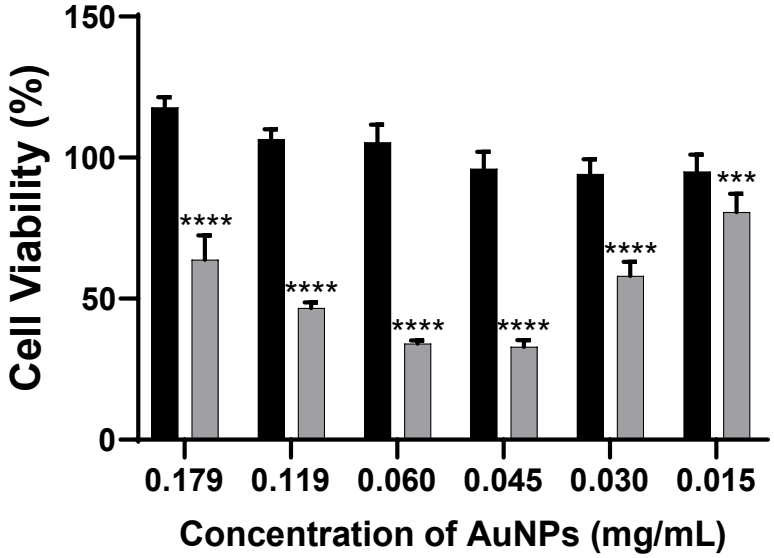

Figure 2 - Assessment of B16F10 cell viability (%), after a 24 h incubation period with uncoated (black column) and HAOA-coated AuNPs (grey column). The results are presented as mean ± SD, n = 5 ($^{***}p < 0.001$, $^{****}p < 0.0001$ vs. uncoated AuNPs).

4.4 *Artemia salina* toxicity assessment

Once again, HAOA-coated nanoparticles caused toxic effects only at 0.358 mg/mL. As shown in **Figure 3**, the remaining samples almost did not kill *A. salina* larvae or barely caused more than observed in the negative control (*A. salina* in only artificial seawater).

Uncoated and coated AuNPs at 0.179 mg/mL behaved very similarly, they had a low mortality rate under 20%, suggesting this concentration is less toxic. On the other hand, while uncoated NPs at 0.358 mg/mL did not cause any casualties, coated ones acted like the positive control killing all the specimens in the well. This data is in line with what was observed previously(79,81).

Even though this assay was supposed to enlighten regarding the toxicity of these formulations at specific concentrations, it also allowed us to see how AuNPs behave in

other media, in this case in a more extreme environment such as seawater. When in contact the *A. salina*'s growth medium quickly agglomerated, and the presence of coating turned the well murky.

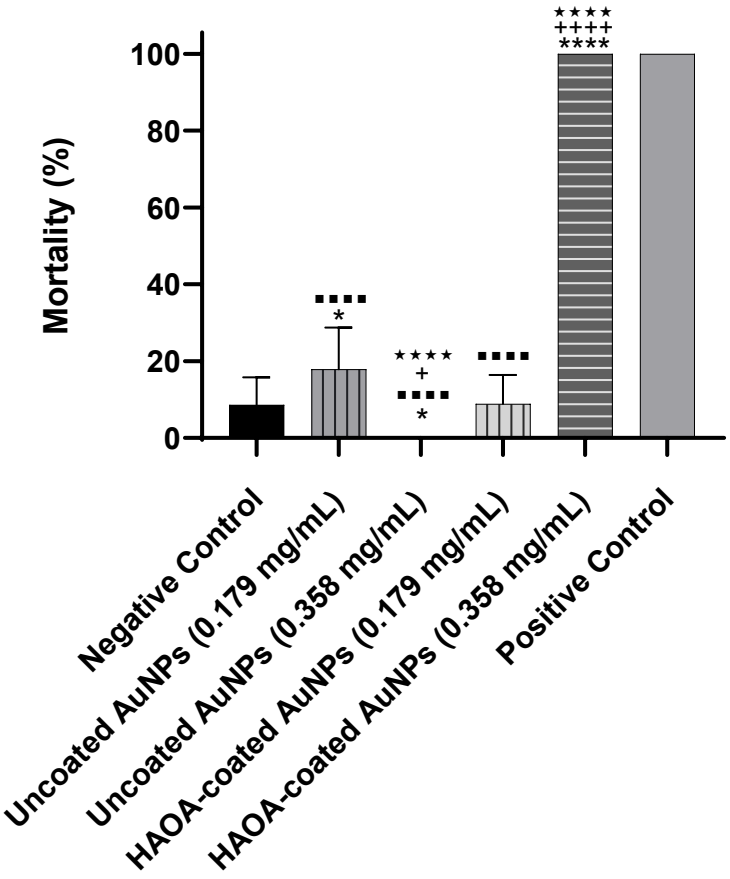

Figure 3 – Evaluation of mortality in *A. salina* after a 24h incubation period with uncoated and HAOA-coated AuNPs at 0.179 and 0.358 mg/mL. The negative control represents larvae growing in normal hatching medium, an innocuous medium, and the positive control has DMSO added which effectively kills 100% of the larvae. The results are presented as mean ± SD, n ≥ 8 ($^{*}p < 0.05$, $^{****}p < 0.0001$ vs. negative control; ▪▪▪▪ $p < 0.0001$ vs. positive control; $^{+}p < 0.05$, $^{++++}p < 0.0001$ vs. uncoated AuNPs; ★★★★ $p < 0.0001$ vs 0.179 mg/mL).

4.5 Preliminary *in vivo* acute toxicity assessment

The mice were injected with the formulations and concentrations used in the *A. salina* assay. The UPD method was used, so in the first instance, each group of mice was injected with the respective formulation at dosage 14.3 mg/kg bw. After the injection of the formulations, the animals were accommodated in their regular housing, no changes in their behaviour were observed, no signs of distress or pain and a 100% survival rate was attained. As there were no apparent major toxic effects in the first group treated with formulations at 14.3 mg/kg bw, the next step was to double this concentration and see if the same tendency observed in the *A. salina* assay was replicated in mice. Similarly, to the first set *in vivo* studies, for the new groups of mice injected with uncoated and HAOA-coated AuNPs at 28.6 mg/kg bw, no changes in animals' behaviour were observed. Nevertheless, a mouse from each group (uncoated and coated AuNPs) showed a slight blue discolouration in the tail (**Figure 4**). This colour was a sign of toxicity in patients that used Au salts and were exposed to light (65). Besides this discolouration, a mouse from the group injected with HAOA-coated AuNPs died within the 24 h duration of the assay. The mice were weighed before the injection and before the necropsy. Although a slight weight loss was observed within the 24 h period no significant differences were observed among all tested groups (**Table 4**).

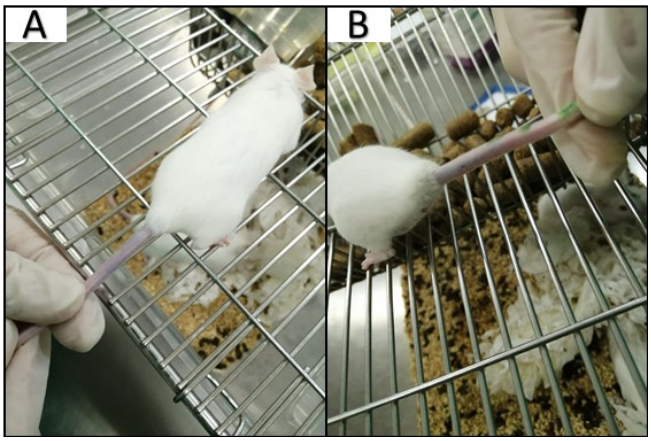

Figure 4 – Blue discolouration in the injection local of the formulations. (A) Mouse injected with 28.6 mg/kg bw of uncoated AuNPs. (B) Mouse injected with 28.6 mg/kg bw HAOA-coated AuNPs.

Table 4 – Weight loss (%) observed within 24 h after injecting the formulations. The results are presented as mean ± SD, n ≥ 3.

Formulation	Dosage (mg/kg bw)	Weight loss (%)
Uncoated AuNPs	14.3	1.7 ± 1.7
	28.6	4.7 ± 3.4
HAOA-coated AuNPs	14.3	5.4 ± 4.9
	28.6	3.5 ± 2.4

At the necropsy, the main excretion organs were collected and following a macroscopical analysis, no changes were observed. After that, the organs were weighed, and their tissue indexes were calculated. The results are shown in **Table 5**. Overall, the tissue index did not differ significantly between formulations and concentrations, except the liver. The liver tissue index of the animals injected with 28.6 mg/kg bw of uncoated AuNPs decrease compared to its counterpart at a half concentration.

Table 5 – Tissue indexes (liver, spleen, kidneys, and lungs) of mice injected with uncoated and HAOA-coated AuNPs at 14.3 and 28.6 mg/kg bw. The results are presented as mean ± SD, n ≥ 3.

Formulation	Dosage (mg/kg bw)	Tissue index			
		Liver	Spleen	Kidneys	Lungs
Uncoated AuNPs	14.3	22.9 ± 0.8	6.6 ± 0.6	11.0 ± 0.2	8.3 ± 0.7
	28.6	21.3 ± 1.0	6.3 ± 0.5	10.9 ± 0.2	8.3 ± 0.2
HAOA-coated AuNPs	14.3	22.5 ± 0.3	6.5 ± 0.1	11.2 ± 0.5	8.5 ± 0.9
	28.6	22.1 ± 0.4	6.7 ± 0.7	11.4 ± 0.4	8.0 ± 0.4

An *ex-vivo* histopathological analysis was performed on the liver, spleen, kidney and lungs. There were no significant differences between the groups. In every group, the spleen did not present any alteration. The liver and kidney had subtle infiltrations of mononuclear cells. In the liver, they were localized in the periportal space, and the kidney, in the cortex and medulla. Concerning the lungs, they could not be correctly

inflated, hindering the analysis, however, the presence of inflammatory peribronchial cuffs with mononuclear cells was seen in mice injected with 28.6 mg/kg bw of coated AuNPs.

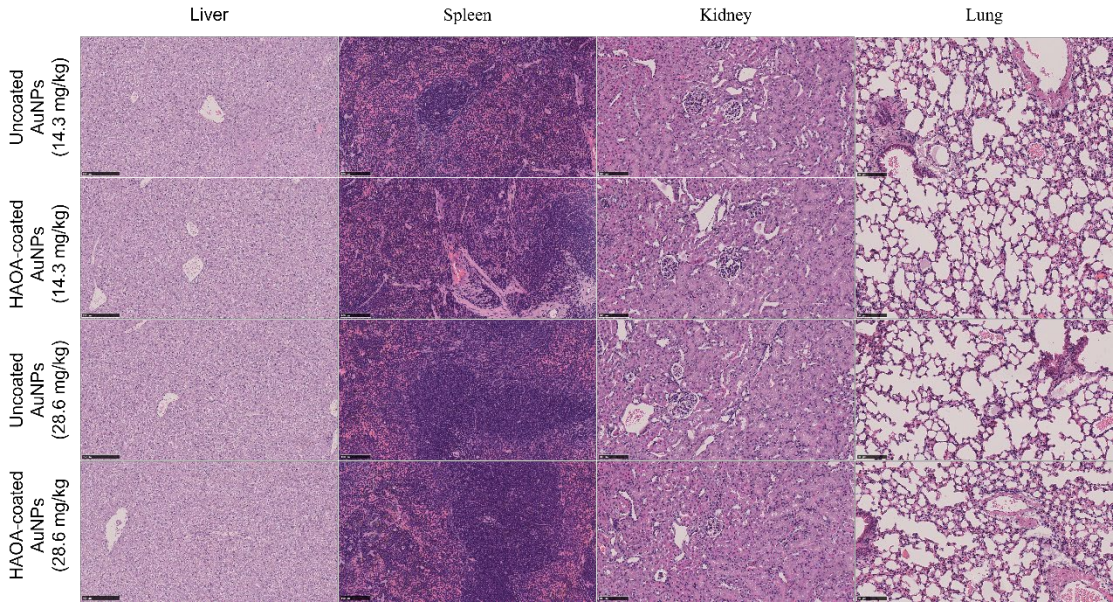

Figure 5 – Representative images of the liver, spleen, kidney and lung removed for histopathological analysis after necropsy (liver with 100× magnification; spleen, kidney and lung with 200× magnification). All groups, except the one injected with HAOA-coated AuNPs at 28.6 mg/kg bw, showed a slight mononuclear infiltration in the periportal area. In the kidney of all groups, except the one injected with uncoated AuNPs at 14.3 mg/kg bw, the cortex and medulla had slight mononuclear infiltration. Due to poor inflation, it was difficult to analyse, but it was possible to observe inflammatory with mononuclear peribronchial cuffs in the group injected with HAOA-coated AuNPs at 28.6 mg/kg bw. No histological alterations were observed in the spleen (Haematoxylin and eosin staining).

5 Discussion

A lot of investigation is being made around NPs for a huge number of applications. Although encouraging results have been reported about their efficacy, this should not be the researchers only concern. In pharmaceutical development, there must be in mind three important aspects: efficacy, quality and safety. The safety of a medicine is a crucial parameter. Our previous findings indicate positive results.

The AuNPs used in the present work were prepared by a modified Seed-mediated Growth Synthesis. The method is following the one developed by Silva *et al.* except the plant extract was replaced by RA due to reproducibility issues (74). Before experiments, the formulations were concentrated, and their respective mean sizes were evaluated. HAOA-coated AuNPs showed a bigger size, which was expected as the coating adds another layer to the core. Lopes *et al.* prepared AuNPs using this method and the same coating solution and an increase in the mean size of coated NPs was also observed. Atomic force microscopy images showed a rough layer covering the NPs (79). The AuNPs obtained presented a mean size ranging from 140 to 205 nm. This means that they are more likely to have a higher half-life time, increasing organ accumulation (e.g., spleen) and the possibility of causing adverse effects (48). The samples were concentrated beforehand the assays, it was noticed that the NPs used in *in vivo* tests were bigger than the ones used *in vitro.* This might have happened due to the concentration process since higher concentrations in smaller final volumes were needed to inject the mice, this might have fostered clustering of NPs. In HAOA-coated AuNPs the PdI values were slightly superior to the ones observed for uncoated NPs. This parameter measures the heterogeneity of NPs in terms of mean size. Higher values mean that NPs are more polydisperse which can happen when aggregates are present in the formulation. Nonetheless, the PdIs obtained for all prepared NPs even the one from HAOA-coated AuNPs used in the *in vivo* assay, according to literature is deemed as acceptable (PdI < 0.5) (109).

The surface charge of the AuNPs was measured, and both formulations are negatively charged. The coated AuNPs are more negatively charged which was expected as the main component of the coating solution, HA is an anionic polymer (110). The coating presence approximately doubled the negative charge, and that can

be useful to improve the formulation stability. Generally NPs with Zeta potential lower than -30 mV show higher stability (47). Regarding biocompatibility, since cell membranes have a negative charge, positively charged NPs reported more adverse effects as they tend to aggregate around them or have high interactions with plasmatic proteins, causing size increase (58).

The formulations in the study are meant to be administered *in situ* and considering the sizes obtained, at first, it is not expected significant extravasation of the NPs into the bloodstream, yet tumoral environments are erratic and unique and AuNPs might reach the bloodstream. Thus, the formulations biocompatibility was assessed through the haemolytic activity. Concentrations ranging from 0.001 – 0.179 mg/mL were incubated with human RBCs. Uncoated AuNPs are considered by the literature as non-haemolytic because the percentage of haemolysis was below 10% (111). On the contrary, coated AuNPs induced a high percentage of haemolysis, particularly for the higher concentrations tested. AuNPs at 0.179 and 0.089 mg/mL had a haemolytic activity of ~70% that decreased to less than 20 and 5% for the concentrations of 0.022 and 0.011 mg/mL, respectively. These results slightly differ from the one obtained by Amaral *et al*. Amaral's AuNPs were prepared with the same method, except the ratio of RA was different from the one used in this study, obtaining higher NPs (~400 nm). All formulations tested, coated and uncoated, were non-haemolytic and despite the range of concentrations was different, some overlapped and different results were observed between the studies (81). Even though the size has some influence in haemolysis (112) this might not be the case since uncoated AuNPs did not have similar behaviour. With these first results, at first instance the formulation with HAOA coating does not seem biocompatible, however, it is important to evaluate the behaviour of AuNPs administered *in situ* and quantify their extravasation (if there's any) before assuming it is not safe for non-parenteral administration.

To assess cytotoxicity an MTT assay was performed. Coated and uncoated AuNPs with concentrations ranging from 0.015 – 0.179 mg/mL were incubated for 24 h with B16F10 cells. The results obtained were interesting; similarly, uncoated AuNPs were innocuous and coated ones caused a significant decrease in cell viability. According to ISO 10993-5:2009(E), samples that cause viability below 70% have cytotoxic potential (108). The HAOA-coated AuNPs showed an interesting cytotoxicity

profile, the relation observed was not dose-dependent like many researchers have seen in their work (73). The concentrations with higher cytotoxic potential were intermediate, 0.060 and 0.045 mg/mL, concentrations upstream were also cytotoxic but not to the same extent, the only non-cytotoxic concentration was the more diluted, 0.015 mg/mL. Lopes *et al.* tested the same type of NPs and very close concentrations, although coated samples had lower cell viability, they weren't low enough to be considered cytotoxic (79). One hypothesis came up when looking at results, HA is the main component of coating, it has been described that it promotes both proliferation of healthy and some types of cancers (113–116). While after coating core AuNPs, there is a process to remove the excess of non-reactant polymer, it might not be optimized and probably it can be present some residual HA that might mask the cytotoxic potential of the more concentrated samples. In addition, it can be stated that the presence of coating is harmful to this cell line, though it can be seen as an advantage since the formulation is intended to be used against superficial cancer such as melanoma, so there can be a synergy between light-activated AuNPs and the ones not activated.

Before the transition to *in vivo* experimentation, another general toxicity tested was performed. The *A. salina* bioassay is alternative toxicity *in vitro* test, that has a simpler procedure comparing to MTT, it is fast, convenient and cheaper. It is a preliminary toxicity screen for further experiments on mammalian animal models and is useful to assess ecotoxicity (106,117). The hatched larvae were incubated for 24 h with both formulations at 0.179 mg/mL and then at 0.358 mg/mL. The first observation noticed was the instability of both formulations in the *A. salina* growth medium (artificial seawater), instant aggregation occurred after the addition of formulations to the medium, in both concentrations tested. Both coated and uncoated AuNPs at 0.179 mg/mL displayed low mortality, HAOA-coated AuNPs at 0.358 mg/mL had a 100% mortality rate, being as effective as the negative control.

The safety assessment was also performed in CD-1 mice. To better elucidate the toxic potential, animal experimentation is needed, since it is a more complex model and better simulates how the compound/drug behaves in humans. It was injected at first, both formulations at 0.179 mg/mL, and the mice were kept for 24 h. No changes in behaviour, feeding habits were observed, the mice were weighed before and 24 h after injection. A slight reduction in the animals' weight was observed for the group injected

with coated NPs. At the moment of the organ harvesting, no macroscopic changes were noted. Since no apparent toxic effects were visible, it was decided to double the administered dose using. It proceeded in the same manner. When handling the mice, one of each group displayed a blueish discolouration at the tail and one animal of the group injected with HAOA-coated AuNPs died during the 24 h observation period. . Gold-based therapies may cause chrysiasis, a toxic effect, typically is caused by gold accumulated in the skin (65). Otherwise, the remaining mice had no alterations in behaviour. Once again, a weight loss on the tested animals was observed. Zhang *et al.* also reported a decrease in weight when animals received high doses of AuNPs (102). The weight loss was not significantly different between the groups. The harvested organs were weighted and sent to histopathological analysis. The tissue indexes calculated did not show any significant difference between the groups, and comparing to data from Charles River Laboratories, the indexes obtained do not differ from naïve animals (118). Under physiological conditions remain unchanged, when there are variations, the organs suffered some alterations. Lower values might suggest atrophy or some degenerative changes; whereas higher values indicate hypertrophy or possible congestion (81). Similarly, Fraga *et al*., with a different gold nanosystem, injected 16 nm AuNPs in rats and also observed that the spleen was atrophied and had no major toxic effects (99). The histopathological analysis revealed that no significant changes were observed between groups, it was found small mononuclear infiltrates in the liver were. Since it is a RES organ and where NPs accumulate more, the infiltrate might well be AuNPs accumulated and being cleared by phagocytes. Many researchers described this phenomenon, which is expected due to organ functionality and high blood perfusion. Another research group used different sized AuNPs some close to the size in this study, and the bigger NPs (250 nm) did accumulate in the liver and spleen (103). The kidneys also presented the same scenario which is not very typical. Usually, smaller NPs tend to accumulate in the kidneys and cause more damage (119). It was possible to observe inflammatory peribronchial cuffs with mononuclear cells in the lungs of the group injected with HAOA-coated AuNPs at 0.358 mg/mL.

This study reported some interesting results about the safety of AuNPs. Some of the results contradicted what was observed in other studies with the same NPs.

Overall, the results reveal that the uncoated formulation is rather safe and that the use of coating must be considered.

6 Conclusions and Future Perspectives

Nanomedicine is here to bring new or improved therapies, diagnostic techniques and nanoparticles are the main tools to reach that. Nanoparticles are world by themselves with a million possibilities; be it how to make them, what they are made of, where to use them, how to use them, which abilities do we want them to have. There is a lot of research on the most varied types of nanoparticles and their new applications. Oncology is the field that most takes advantage of nanosystems, some directed to imaging, tumour diagnosis as well as potential new weapons to fight these pathologies.

Gold nanoparticles present themselves as useful in oncology, as they allow diagnosing, staging, imaging and treat cancers. In this study, a formulation of gold nanoparticles is to be employed in PTT, an alternative therapy that is being used to treat some types of tumours. We have obtained great results in terms of efficacy, yet the safety of these AuNPs must be better explored.

With this study, we aimed to it was hoped to gain more knowledge about the safety of AuNPs. Since there is not a standardized methodology to evaluate and compare NPs, each study group has an even bigger responsibility to correctly assess the safety. *In vitro* and *in vivo* assays were performed, and the findings indicate that the coating used, HAOA, is responsible for the toxicity observed. HAOA-coated AuNPs showed poor biocompatibility during haemolytic activity assay. The MTT results showed that coated AuNPs reduce cell viability, but it was not in dose-dependent relation, with this, it is possible to speculate that the coating must have an optimal concentration that is non-cytotoxic. *Artemia salina* is a bioassay useful for preliminary toxicity findings, widely used to assess ecotoxicity, two concentrations were tested, and only the HAOA-coated AuNPs at 0.358 mg/mL showed a mortality rate equal to the positive control.

After the *in vitro* assessment, the next step was to evaluate the AuNPs in more complex models such as mice. *In vivo* assay was structured like *A. salina* bioassay and similarly, in the group injected with HAOA-coated AuNPs at 28.6 mg/kg bw, one mouse died. It is the unclear mechanism behind it, as the other subject's histopathological analysis showed no life-endangering alterations, we suggest that an agglomerate might have been the cause of the mouse's death.

There is still a lot of work to be done to have an extensive and well-known toxicological profile of these formulations. Regarding, *in vitro* assays much more could be done. It would be interesting to do genotoxicity tests, ROS determination since literature refers to it as the main toxic effect of gold-based therapies, MTT studies with healthy and tumoral human cells should also be conducted. Additionally, these assays could be performed with NIR-activated AuNPs.

Concerning *in vivo* safety assessment, more parameters should be evaluated such as biochemistry, biodistribution and toxicokinetics. Another animal model could be employed like it is recommended on guidelines, rats could be chosen since their size and blood volume allows multiple analysis. It would also be interesting to perform repeated-dose assays to emulate multiple treatments.

Lastly, it is important to optimize the coating solution that has been used. Its use has been demonstrated to be advantageous for murine melanoma cells due to cytotoxicity. Even though the formulations were not tested in healthy tissues, based on the results from other assays it could expect to see toxic effects. Ideally, the coating solution should be tuned so that it helps to eliminate tumour cells and don´t cause damage to healthy tissue and biocompatibility is achieved or check if the coating is indeed necessary.

Nevertheless, this experience enabled assessing the maximum tolerated concentration of the formulation and enlightened that the coating might need some adjustments for future *in vivo* studies.

References

1. Marchetti S, Schellens JHM. The impact of FDA and EMEA guidelines on drug development in relation to Phase 0 trials. Br J Cancer [Internet]. 2007;97(5):577–81. Available from: http://www.emea.europa.eu/pdfs/human/swp/259902en.pdf

2. Snodin DJ. An EU perspective on the use of in vitro methods in regulatory pharmaceutical toxicology. Toxicol Lett. 2002;127(1–3):161–8.

3. Kimmit, R. Vieira, M., Moon S. Research Synthesis: Time and Success Rates of Pharmaceutical R&D, Knowledge Portal on innovation and access to medicines. Portal Innov access to Med Grad Inst Geneva [Internet]. 2020;1–32. Available from: https://da7af2c8-d9b0-47a3-a3f6-89c3c3bfa02c.filesusr.com/ugd/356854_9dd6f18e2b114015b253736b1c666cfb.pdf.

4. BIO industry analysis. Clinical development success rates 2006-2015. Bio Ind Anal Rep [Internet]. 2016 [cited 2021 Jun 27];(June):https://www.bio.org/sites/default/files/Clinical%2. Available from: https://www.bio.org/sites/default/files/Clinical Development Success Rates 2006-2015 - BIO, Biomedtracker, Amplion 2016.pdf

5. Hingorani AD, Kuan V, Finan C, Kruger FA, Gaulton A, Chopade S, et al. Improving the odds of drug development success through human genomics: modelling study. Sci Rep. 2019;9(1):18911.

6. Vargesson N. The teratogenic effects of thalidomide on limbs [Internet]. Vol. 44, Journal of Hand Surgery: European Volume. SAGE Publications Ltd; 2019 [cited 2021 Jul 4]. p. 88–95. Available from: https://pubmed.ncbi.nlm.nih.gov/30335598/

7. Mcbride WG. THALIDOMIDE AND CONGENITAL ABNORMALITIES [Internet]. Vol. 278, The Lancet. Elsevier; 1961 [cited 2021 Jul 4]. p. 1358. Available from: http://www.thelancet.com/article/S0140673661909278/fulltext

8. Ward SP. Thalidomide and congenital abnormalities. Br Med J. 1962 Sep 8;2(5305):646–7.

9. Parasuraman S. Toxicological screening. J Pharmacol Pharmacother [Internet]. 2011 Apr [cited 2021 May 8];2(2):74–9. Available from: /pmc/articles/PMC3127354/

10. Andrade EL, Bento AF, Cavalli J, Oliveira SK, Schwanke RC, Siqueira JM, et al. Non-clinical studies in the process of new drug development - Part II: Good laboratory practice, metabolism, pharmacokinetics, safety and dose translation to clinical studies. Brazilian J Med Biol Res [Internet]. 2016 [cited 2021 Jul 3];49(12):e5646. Available from: http://dx.doi.org/10.1590/1414-431X20165646

11. Fielden MR, Kolaja KL. The role of early in vivo toxicity testing in drug discovery toxicology. Expert Opin Drug Saf [Internet]. 2008 Mar 6;7(2):107–10. Available from: http://www.tandfonline.com/doi/full/10.1517/14740338.7.2.107

12. Ahuja V, Sharma S. Drug safety testing paradigm, current progress and future challenges: An overview. J Appl Toxicol. 2014;34(6):576–94.

13. ICH Guideline M3 (R2). Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals [Internet]. Vol. 3, International Conference on Harmonisation. 2009. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf%0Ahttp://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/CTD/M4E_R2_Efficacy/M4E_R2__Step_4.pdf%0Ahttp://www.ich.org/fileadmin/Publ

14. Beilmann M, Boonen H, Czich A, Dear G, Hewitt P, Mow T, et al. Optimizing drug discovery by investigative toxicology: Current and future trends. ALTEX. 2019;36(2):289–313.

15. Moggs J, Moulin P, Pognan F, Brees D, Leonard M, Busch S, et al. Investigative safety science as a competitive advantage for Pharma. Expert Opin Drug Metab Toxicol. 2012;8(9):1071–82.

16. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol. 2000;32(1):56–67.

17. Tamaki C, Nagayama T, Hashiba M, Fujiyoshi M, Hizue M, Kodaira H, et al. Potentials and limitations of nonclinical safety assessment for predicting clinical adverse drug reactions: Correlation analysis of 142 approved drugs in Japan. J Toxicol Sci [Internet]. 2013 Aug [cited 2021 Jul 4];38(4):581–98. Available from: https://pubmed.ncbi.nlm.nih.gov/23824014/

18. Waring MJ, Arrowsmith J, Leach AR, Leeson PD, Mandrell S, Owen RM, et al. An analysis of the attrition of drug candidates from four major pharmaceutical companies. Nat Rev Drug Discov [Internet]. 2015;14(7):475–86. Available from: http://dx.doi.org/10.1038/nrd4609

19. Holt JDS, Nuttall JP. Preclinical safety evaluation. Curr Top Microbiol Immunol [Internet]. 2015 [cited 2021 Jul 9];383:55–78. Available from: https://pubmed.ncbi.nlm.nih.gov/23612993/

20. Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The history of nanoscience and nanotechnology: From chemical-physical applications to nanomedicine. Molecules. 2020;25(1):1–15.

21. Nanoscience and nanotechnologies : opportunities and uncertainties. R Soc R Acad Eng. 2004;(July).

22. PATRA JK, GOUDA S. Application of nanotechnology in textile engineering: An overview. J Eng Technol Res [Internet]. 2013 Jun 30 [cited 2021 Jul 11];5(5):104–11. Available from: https://academicjournals.org/journal/JETR/article-abstract/04977A52454

23. AC S, D R, JAD S, I P, A S, D C, et al. Nanotechnological breakthroughs in the development of topical phytocompounds-based formulations. Int J Pharm [Internet]. 2019 Dec 15 [cited 2021 Jul

11];572. Available from: https://pubmed.ncbi.nlm.nih.gov/31678376/

24. Pramanik P, Krishnan P, Maity A, Mridha N, Mukherjee A, Rai V. Application of Nanotechnology in Agriculture. 2020 [cited 2021 Jul 11];317–48. Available from: https://link.springer.com/chapter/10.1007/978-3-030-26668-4_9

25. Petrovi ZL, Maguire P, Radmilovi M, Puaˇ N, Mari D, Mahony C, et al. Nanotechnology for Electronics, Photonics, and Renewable Energy. Korkin A, Krstić PS, Wells JC, editors. 2010 [cited 2021 Jul 11];211–43. Available from: http://link.springer.com/10.1007/978-1-4419-7454-9

26. Havel H, Finch G, Strode P, Wolfgang M, Zale S, Bobe I, et al. Nanomedicines: From Bench to Bedside and Beyond. AAPS J [Internet]. 2016 Nov 1;18(6):1373–8. Available from: www.fda.gov/RegulatoryInformation/Guidances

27. Wagner V, Dullaart A, Bock A, Zweck A. The emerging nanomedicine landscape. Nat Biotechnol. 2006;24(10):1211–7.

28. Mohanraj VJ, Chen Y. Nanoparticles - A review. Trop J Pharm Res [Internet]. 2007 Jul 31;5(1):561–73. Available from: http://www.tjpr.freehosting.net

29. Parveen S, Misra R, Sahoo SK. Nanoparticles: A boon to drug delivery, therapeutics, diagnostics and imaging. Nanomedicine Nanotechnology, Biol Med [Internet]. 2012;8(2):147–66. Available from: http://dx.doi.org/10.1016/j.nano.2011.05.016

30. Thorley AJ, Tetley TD. New perspectives in nanomedicine. Pharmacol Ther [Internet]. 2013;140(2):176–85. Available from: http://dx.doi.org/10.1016/j.pharmthera.2013.06.008

31. Mota AH, Duarte N, Serra AT, Ferreira A, Bronze MR, Custódio L, et al. Further evidence of possible therapeutic uses of sambucus nigra l. Extracts by the assessment of the in vitro and in vivo anti-inflammatory properties of its plga and pcl-based nanoformulations. Pharmaceutics. 2020;12(12):1–28.

32. Mota AH, Prazeres I, Mestre H, Bento-Silva A, Rodrigues MJ, Duarte N, et al. A newfangled collagenase inhibitor topical formulation based on ethosomes with sambucus nigra l. Extract. Pharmaceuticals. 2021;14(5):1–25.

33. Wolfram J, Zhu M, Yang Y, Shen J, Gentile E, Paolino D, et al. Safety of nanoparticles in medicine. Curr Drug Targets. 2015;16(14):1671–81.

34. Castillo PM, Jimenez-Ruiz A, Carnerero JM, Prado-Gotor R. Exploring Factors for the Design of Nanoparticles as Drug Delivery Vectors. ChemPhysChem. 2018;19(21):2810–28.

35. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2007 212 [Internet]. 2007 Dec [cited 2021 Jul 13];2(12):751–60. Available from: https://www.nature.com/articles/nnano.2007.387

36. Y X, Y X, B L, SE S. Shape-controlled synthesis of metal nanocrystals: simple chemistry meets complex physics? Angew Chem Int Ed Engl [Internet]. 2009 [cited 2021 Jul 13];48(1):60–103. Available from: https://pubmed.ncbi.nlm.nih.gov/19053095/

37. Kim BYS, Rutka JT, Chan WCW. Nanomedicine. N Engl J Med [Internet]. 2010 Dec 16;363(25):2434–43. Available from: http://arxiv.org/abs/1011.1669

38. Farjadian F, Ghasemi A, Gohari O, Roointan A, Karimi M, Hamblin MR. Nanopharmaceuticals and nanomedicines currently on the market: Challenges and opportunities [Internet]. Vol. 14, Nanomedicine. Future Medicine Ltd.; 2019 [cited 2021 Jul 3]. p. 93–126. Available from: /pmc/articles/PMC6391637/

39. RJ H. Amphotericin B formulations: a comparative review of efficacy and toxicity. Drugs [Internet]. 2013 Jun [cited 2021 Jul 26];73(9):919–34. Available from: https://pubmed.ncbi.nlm.nih.gov/23729001/

40. Han HJ, Nwagwu C, Anyim O, Ekweremadu C, Kim S. COVID-19 and cancer: From basic mechanisms to vaccine development using nanotechnology. Int Immunopharmacol [Internet]. 2021 [cited 2021 Jul 26];90. Available from: https://doi.org/10.1016/j.intimp.2020.107247

41. Silva CO, Pinho JO, Lopes JM, Almeida AJ, Gaspar MM, Reis C. Current trends in cancer nanotheranostics: Metallic, polymeric, and lipid-based systems. Vol. 11, Pharmaceutics. MDPI AG; 2019.

42. R G, N D. BCS class IV drugs: Highly notorious candidates for formulation development. J Control Release [Internet]. 2017 Feb 28 [cited 2021 Jul 18];248:71–95. Available from: https://pubmed.ncbi.nlm.nih.gov/28088572/

43. Li Z, Tan S, Li S, Shen Q, Wang K. Cancer drug delivery in the nano era: An overview and perspectives. Oncol Rep [Internet]. 2017 Feb 1 [cited 2021 Jul 26];38(2):611–24. Available from: https://pubmed.ncbi.nlm.nih.gov/28627697/

44. D'souza AA, Shegokar R. Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications. Expert Opin Drug Deliv [Internet]. 2016 Sep 1 [cited 2021 Jul 26];13(9):1257–75. Available from: https://pubmed.ncbi.nlm.nih.gov/27116988/

45. Mir M, Ahmed N, Rehman A ur. Recent applications of PLGA based nanostructures in drug delivery. Colloids Surfaces B Biointerfaces [Internet]. 2017;159:217–31. Available from: http://dx.doi.org/10.1016/j.colsurfb.2017.07.038

46. Selim A, Lila A, Ishida T. Liposomal Delivery Systems: Design Optimization and Current Applications. Vol. 40, Biol. Pharm. Bull. 2017.

47. A A, HA F, MA H, M E. Nanomedicine: a new paradigm to overcome drug incompatibilities. J Pharm Pharmacol [Internet]. 2020 Oct 1 [cited 2021 Jul 17];72(10):1289–305. Available from: https://pubmed.ncbi.nlm.nih.gov/32436221/

48. Hoshyar N, Gray S, Han H, Bao G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. Nanomedicine. 2016;11(6):673–92.

49. Fang J, Nakamura H, Maeda H. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev [Internet]. 2011;63(3):136–51. Available from: http://dx.doi.org/10.1016/j.addr.2010.04.009

50. Huang J, Bu L, Xie J, Chen K, Cheng Z, Li X, et al. Effects of Nanoparticle Size on Cellular Uptake and Liver MRI with Polyvinylpyrrolidone-Coated Iron Oxide Nanoparticles. ACS Nano [Internet]. 2010 Dec 28 [cited 2021 Jul 26];4(12):7151–60. Available from: https://pubs.acs.org/doi/abs/10.1021/nn101643u

51. Lu F, Wu SH, Hung Y, Mou CY. Size effect on cell uptake in well-suspended, uniform mesoporous silica nanoparticles. Small [Internet]. 2009 Jun 19 [cited 2021 Jul 26];5(12):1408–13. Available from: https://pubmed.ncbi.nlm.nih.gov/19296554/

52. Liu X, Huang N, Li H, Jin Q, Ji J. Surface and Size Effects on Cell Interaction of Gold Nanoparticles with Both Phagocytic and Nonphagocytic Cells. Langmuir [Internet]. 2013 Jul 23 [cited 2021 Jul 26];29(29):9138–48. Available from: https://pubs.acs.org/doi/abs/10.1021/la401556k

53. Cruje C, Chithrani BD. Integration of peptides for enhanced uptake of PEGylayed gold nanoparticles. J Nanosci Nanotechnol [Internet]. 2015 Mar 1 [cited 2021 Jul 26];15(3):2125–31. Available from: https://pubmed.ncbi.nlm.nih.gov/26413630/

54. Oh E, Delehanty JB, Sapsford KE, Susumu K, Goswami R, Blanco-Canosa JB, et al. Cellular uptake and fate of PEGylated gold nanoparticles is dependent on both cell-penetration peptides and particle size. ACS Nano [Internet]. 2011 Aug 23 [cited 2021 Jul 26];5(8):6434–48. Available from: https://pubmed.ncbi.nlm.nih.gov/21774456/

55. Champion JA, Mitragotri S. Role of target geometry in phagocytosis [Internet]. 2006. Available from: www.pnas.orgcgidoi10.1073pnas.0600997103

56. Champion JA, Mitragotri S. Shape induced inhibition of phagocytosis of polymer particles. Pharm Res. 2009;26(1):244–9.

57. Arnida, Janát-Amsbury MM, Ray A, Peterson CM, Ghandehari H. Geometry and surface characteristics of gold nanoparticles influence their biodistribution and uptake by macrophages. Eur J Pharm Biopharm. 2011;77(3):417–23.

58. GJ P, MM G, D M. Impact of poly(lactic-co-glycolic acid) nanoparticle surface charge on protein, cellular and haematological interactions. Colloids Surf B Biointerfaces [Internet]. 2015 Dec 1 [cited 2021 Aug 1];136:1058–66. Available from: https://pubmed.ncbi.nlm.nih.gov/26590899/

59. Zielińska A, Costa B, Ferreira M V, Miguéis D, Louros JMS, Durazzo A, et al. Nanotoxicology

and Nanosafety: Safety-by-Design and Testing at a Glance. Int J Environ Res Public Health [Internet]. 2020 Jun 28 [cited 2021 Aug 3];17(13):4657. Available from: www.mdpi.com/journal/ijerph

60. Chan WCW. Nanomedicine 2.0. Acc Chem Res [Internet]. 2017 Mar 21 [cited 2021 Aug 4];50(3):627–32. Available from: https://pubs.acs.org/doi/abs/10.1021/acs.accounts.6b00629

61. MA S, W K, C G. Cytotoxicity of Nanomaterials: Using Nanotoxicology to Address the Safety Concerns of Nanoparticles. Pharm Nanotechnol [Internet]. 2018 Apr 16 [cited 2021 Aug 3];6(1):3–16. Available from: https://pubmed.ncbi.nlm.nih.gov/29065848/

62. Sztandera K, Gorzkiewicz M, Klajnert-Maculewicz B. Gold Nanoparticles in Cancer Treatment. Mol Pharm. 2019;16(1):1–23.

63. Faa G, Gerosa C, Fanni D, Lachowicz JI, Nurchi VM. Gold - Old Drug with New Potentials. Curr Med Chem. 2017;25(1):75–84.

64. Lansdown ABG. GOLD: human exposure and update on toxic risks. Crit Rev Toxicol [Internet]. 2018;48(7):596–614. Available from: https://doi.org/10.1080/10408444.2018.1513991

65. Balfourier A, Kolosnjaj-Tabi J, Luciani N, Carn F, Gazeau F, Murphy CJ. Gold-based therapy: From past to present. Proc Natl Acad Sci U S A. 2020;117(37):22639–48.

66. Scientific Opinion on the re-evaluation of gold (E 175) as a food additive. EFSA J. 2016 May 23;14(1).

67. Boisselier E, Astruc D. Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. Chem Soc Rev. 2009;38(6):1759–82.

68. Calixto GMF, Bernegossi J, De Freitas LM, Fontana CR, Chorilli M, Grumezescu AM. Nanotechnology-based drug delivery systems for photodynamic therapy of cancer: A review. Molecules. 2016;21(3).

69. Bagheri S, Yasemi M, Safaie-Qamsari E, Rashidiani J, Abkar M, Hassani M, et al. Using gold nanoparticles in diagnosis and treatment of melanoma cancer. Artif Cells, Nanomedicine Biotechnol [Internet]. 2018;46(sup1):462–71. Available from: https://doi.org/10.1080/21691401.2018.1430585

70. Turkevich J, Stevenson PC, Hillier J. A study of the nucleation and growth processes in the synthesis of colloidal gold. Discuss Faraday Soc [Internet]. 1951 Jan 1 [cited 2021 Aug 14];11(0):55–75. Available from: https://pubs.rsc.org/en/content/articlehtml/1951/df/df9511100055

71. J F, Y C, M S. Functionalized Gold Nanoparticles: Synthesis, Properties and Biomedical Applications. Chem Rec [Internet]. 2020 Dec 1 [cited 2021 Aug 14];20(12):1474–504. Available from: https://pubmed.ncbi.nlm.nih.gov/32970383/

72. Brust M, Walker M, Bethell D, Schiffrin DJ, Whyman R. Synthesis of thiol-derivatised gold nanoparticles in a two-phase Liquid–Liquid system. J Chem Soc Chem Commun [Internet]. 1994 Jan 1 [cited 2021 Aug 14];(7):801–2. Available from: https://pubs.rsc.org/en/content/articlehtml/1994/c3/c39940000801

73. Boldeiu A, Simion M, Mihalache I, Radoi A, Banu M, Varasteanu P, et al. Comparative analysis of honey and citrate stabilized gold nanoparticles: In vitro interaction with proteins and toxicity studies. J Photochem Photobiol B Biol [Internet]. 2019;197(October 2018):111519. Available from: https://doi.org/10.1016/j.jphotobiol.2019.111519

74. Silva CO, Rijo P, Molpeceres J, Ascensão L, Roberto A, Fernandes AS, et al. Bioproduction of gold nanoparticles for photothermal therapy. Ther Deliv. 2016;7(5):287–304.

75. Guo J, Rahme K, He Y, Li LL, Holmes JD, O'Driscoll CM. Gold nanoparticles enlighten the future of cancer theranostics. Int J Nanomedicine. 2017;12:6131–52.

76. Doughty ACV, Hoover AR, Layton E, Murray CK, Howard EW, Chen WR. Nanomaterial applications in photothermal therapy for cancer. Materials (Basel). 2019;12(5).

77. Riley RS, Day ES. Gold nanoparticle-mediated photothermal therapy: applications and opportunities for multimodal cancer treatment. Wiley Interdiscip Rev Nanomedicine Nanobiotechnology [Internet]. 2017 Jul;9(4):e1449. Available from: http://doi.wiley.com/10.1002/wnan.1449

78. Clement M, Daniel G, Trelles M. Optimising the design of a broad-band light source for the treatment of skin. J Cosmet Laser Ther. 2005;7(3–4):177–89.

79. Lopes J, Ferreira-Gonçalves T, Figueiredo I V., Rodrigues CMP, Ferreira H, Ferreira D, et al. Proof-of-Concept Study of Multifunctional Hybrid Nanoparticle System Combined with NIR Laser Irradiation for the Treatment of Melanoma. Biomolecules [Internet]. 2021 Mar 30 [cited 2021 Jul 3];11(4):511. Available from: https://www.mdpi.com/2218-273X/11/4/511

80. Costa E, Ferreira-Gonçalves T, Cardoso M, Coelho JMP, Gaspar MM, Faísca P, et al. A Step Forward in Breast Cancer Research: From a Natural-Like Experimental Model to a Preliminary Photothermal Approach. Int J Mol Sci [Internet]. 2020 Dec 18 [cited 2021 Jul 3];21(24):9681. Available from: https://www.mdpi.com/1422-0067/21/24/9681

81. Amaral M, Charmier AJ, Afonso RA, Catarino J, Faísca P, Carvalho L, et al. Gold-based nanoplataform for the treatment of anaplastic thyroid carcinoma: A step forward. Cancers (Basel). 2021;13(6):1–24.

82. EB D, EC D, X H, IH E-S, H C, S P, et al. Gold nanorod assisted near-infrared plasmonic photothermal therapy (PPTT) of squamous cell carcinoma in mice. Cancer Lett [Internet]. 2008 Sep 28 [cited 2021 Aug 15];269(1):57–66. Available from: https://pubmed.ncbi.nlm.nih.gov/18541363/

83. AM A, SE L, CJ M. The gold standard: gold nanoparticle libraries to understand the nano-bio interface. Acc Chem Res [Internet]. 2013 Mar 19 [cited 2021 Aug 15];46(3):650–61. Available from: https://pubmed.ncbi.nlm.nih.gov/22732239/

84. Enea M, Peixoto de Almeida M, Eaton P, Dias da Silva D, Pereira E, Soares ME, et al. A multiparametric study of gold nanoparticles cytotoxicity, internalization and permeability using an in vitro model of blood–brain barrier. Influence of size, shape and capping agent. Nanotoxicology [Internet]. 2019;13(7):990–1004. Available from: https://doi.org/10.1080/17435390.2019.1621398

85. Pooja D, Panyaram S, Kulhari H, Rachamalla SS, Sistla R. Xanthan gum stabilized gold nanoparticles: Characterization, biocompatibility, stability and cytotoxicity. Carbohydr Polym [Internet]. 2014;110:1–9. Available from: http://dx.doi.org/10.1016/j.carbpol.2014.03.041

86. Lopez-Chaves C, Soto-Alvaredo J, Montes-Bayon M, Bettmer J, Llopis J, Sanchez-Gonzalez C. Gold nanoparticles: Distribution, bioaccumulation and toxicity. In vitro and in vivo studies. Nanomedicine Nanotechnology, Biol Med [Internet]. 2018 Jan;14(1):1–12. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1549963417301582

87. Lee U, Yoo CJ, Kim YJ, Yoo YM. Cytotoxicity of gold nanoparticles in human neural precursor cells and rat cerebral cortex. J Biosci Bioeng [Internet]. 2016;121(3):341–4. Available from: http://dx.doi.org/10.1016/j.jbiosc.2015.07.004

88. Coradeghini R, Gioria S, García CP, Nativo P, Franchini F, Gilliland D, et al. Size-dependent toxicity and cell interaction mechanisms of gold nanoparticles on mouse fibroblasts. Toxicol Lett. 2013 Mar 13;217(3):205–16.

89. Chueh PJ, Liang RY, Lee YH, Zeng ZM, Chuang SM. Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines. J Hazard Mater [Internet]. 2014;264(2014):303–12. Available from: http://dx.doi.org/10.1016/j.jhazmat.2013.11.031

90. Simpson CA, Salleng KJ, Cliffel DE, Feldheim DL. In vivo toxicity, biodistribution, and clearance of glutathione-coated gold nanoparticles. Nanomedicine Nanotechnology, Biol Med [Internet]. 2013;9(2):257–63. Available from: http://dx.doi.org/10.1016/j.nano.2012.06.002

91. Liu X, Huang H, Liu G, Zhou W, Chen Y, Jin Q, et al. Multidentate zwitterionic chitosan oligosaccharide modified gold nanoparticles: Stability, biocompatibility and cell interactions. Nanoscale [Internet]. 2013 Apr 19 [cited 2021 Aug 10];5(9):3982–91. Available from: https://pubs.rsc.org/en/content/articlehtml/2013/nr/c3nr00284e

92. Konoeda H, Takizawa H, Gower A, Zhao M, Adeyi OA, Liu M. Pharmacokinetics, tissue distribution and safety of gold nanoparticle/PKC Delta inhibitor peptide hybrid in rats. Nanotoxicology [Internet]. 2020;14(3):341–54. Available from: https://doi.org/10.1080/17435390.2019.1702731

93. Vales G, Suhonen S, Siivola KM, Savolainen KM, Catalán J, Norppa H. Genotoxicity and Cytotoxicity of Gold Nanoparticles In Vitro: Role of Surface Functionalization and Particle Size. Nanomaterials [Internet]. 2020 Feb 6 [cited 2021 Jul 3];10(2):271. Available from: https://pubmed.ncbi.nlm.nih.gov/32041143/

94. Ávalos A, Haza AI, Mateo D, Morales P. In vitro and in vivo genotoxicity assessment of gold nanoparticles of different sizes comet and SMART assays. Food Chem Toxicol [Internet]. 2018;120(June):81–8. Available from: https://doi.org/10.1016/j.fct.2018.06.061

95. Mannerström M, Zou J, Toimela T, Pyykkö I, Heinonen T. The applicability of conventional cytotoxicity assays to predict safety/toxicity of mesoporous silica nanoparticles, silver and gold nanoparticles and multi-walled carbon nanotubes. Toxicol Vitr [Internet]. 2016;37:113–20. Available from: http://dx.doi.org/10.1016/j.tiv.2016.09.012

96. Vetten MA, Tlotleng N, Tanner Rascher D, Skepu A, Keter FK, Boodhia K, et al. Label-free in vitro toxicity and uptake assessment of citrate stabilised gold nanoparticles in three cell lines. Part Fibre Toxicol. 2013;10(1):1–15.

97. Mata R, Nakkala JR, Chandra VK, Raja K, Sadras SR. In vivo bio-distribution, clearance and toxicity assessment of biogenic silver and gold nanoparticles synthesized from Abutilon indicum in Wistar rats. J Trace Elem Med Biol. 2018 Jul 1;48:157–65.

98. Rambanapasi C, Zeevaart J, Buntting H, Bester C, Kotze D, Hayeshi R, et al. Bioaccumulation and Subchronic Toxicity of 14 nm Gold Nanoparticles in Rats. Molecules [Internet]. 2016 Jun 10;21(6):763. Available from: http://www.mdpi.com/1420-3049/21/6/763

99. Fraga S, Brandão A, Soares ME, Morais T, Duarte JA, Pereira L, et al. Short- and long-term distribution and toxicity of gold nanoparticles in the rat after a single-dose intravenous administration. Nanomedicine Nanotechnology, Biol Med [Internet]. 2014;10(8):1757–66. Available from: http://dx.doi.org/10.1016/j.nano.2014.06.005

100. Rattanapinyopituk K, Shimada A, Morita T, Sakurai M, Asano A, Hasegawa T, et al. Demonstration of the clathrin- and caveolin-mediated endocytosis at the maternal-fetal barrier in mouse placenta after intravenous administration of gold nanoparticles. J Vet Med Sci. 2014;76(3):377–87.

101. Balasubramanian SK, Jittiwat J, Manikandan J, Ong C-N, Yu LE, Ong W-Y. Biodistribution of gold nanoparticles and gene expression changes in the liver and spleen after intravenous administration in rats. Biomaterials [Internet]. 2010 Mar [cited 2021 Aug 10];31(8):2034–42. Available from: https://pubmed.ncbi.nlm.nih.gov/20044133/

102. Zhang XD, Wu HY, Wu D, Wang YY, Chang JH, Zhai Z Bin, et al. Toxicologic effects of gold nanoparticles in vivo by different administration routes. Int J Nanomedicine. 2010;5(1):771–81.

103. De Jong WH, Hagens WI, Krystek P, Burger MC, Sips AJAM, Geertsma RE. Particle size-

dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials [Internet]. 2008 Apr 1 [cited 2021 Aug 8];29(12):1912–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S014296120701071X

104. Sadauskas E, Wallin H, Stoltenberg M, Vogel U, Doering P, Larsen A, et al. Kupffer cells are central in the removal of nanoparticles from the organism. Part Fibre Toxicol [Internet]. 2007 [cited 2021 Aug 10];4. Available from: http://www.particleandfibretoxicology.com/content/4/1/10

105. Nave M, Castro RE, Rodrigues CMP, Casini A, Soveral G, Gaspar MM. Nanoformulations of a potent copper-based aquaporin inhibitor with cytotoxic effect against cancer cells. Nanomedicine. 2016;11(14):1817–30.

106. Rajabi S, Ramazani A, Hamidi M, Naji T. Artemia salina as a model organism in toxicity assessment of nanoparticles. DARU, J Pharm Sci. 2015;23(1):1–6.

107. Wolfensohn S, Lloyd M. Handbook of Laboratory Animal Management and Welfare [Internet]. Handbook of Laboratory Animal Management and Welfare. Blackwell Publishing Ltd; 2003 [cited 2021 Jul 4]. Available from: www.blackwellpublishing.com

108. Standardization t IO for. ISO 10993-5:2009(E) Biological Devices—Part 5: Tests for In Vitro Cytotoxicity. 2009.

109. Öztürk AA, Yenilmez E, Güçlü Özarda M. Clarithromycin-Loaded Poly (Lactic-co-glycolic Acid) (PLGA) Nanoparticles for Oral Administration: Effect of Polymer Molecular Weight and Surface Modification with Chitosan on Formulation, Nanoparticle Characterization and Antibacterial Effects. Available from: www.mdpi.com/journal/polymers

110. Kundu B, Reis RL, Kundu SC. Metastasis in three-dimensional biomaterials. In: Biomaterials for 3D Tumor Modeling. Elsevier; 2020. p. 191–216.

111. Amin K, Dannenfelser R-M. In vitro hemolysis: Guidance for the pharmaceutical scientist. J Pharm Sci [Internet]. 2006 Jun 1 [cited 2021 Aug 17];95(6):1173–6. Available from: http://jpharmsci.org/article/S0022354916320354/fulltext

112. Aseichev A V., Azizova OA, Beckman EM, Skotnikova OI, Dudnik LB, Shcheglovitova ON, et al. Effects of gold nanoparticles on erythrocyte hemolysis. Bull Exp Biol Med. 2014;156(4):495–8.

113. Khetan S, Corey O. Maintenance of stem cell viability and differentiation potential following cryopreservation within 3-dimensional hyaluronic acid hydrogels. Cryobiology [Internet]. 2019 Oct 1 [cited 2021 Apr 13];90:83–8. Available from: https://pubmed.ncbi.nlm.nih.gov/31381877/

114. Kim BS, Choi JS, Kim JD, Yeo TY, Cho YW. Improvement of stem cell viability in hyaluronic acid hydrogels using dextran microspheres. J Biomater Sci Polym Ed [Internet]. 2010 Sep 1 [cited 2021 Apr 13];21(13):1701–11. Available from:

https://pubmed.ncbi.nlm.nih.gov/20537250/

115. Osti L, Berardocco M, Di Giacomo V, Di Bernardo G, Oliva F, Berardi AC. Hyaluronic acid increases tendon derived cell viability and collagen type i expression in vitro: Comparative study of four different Hyaluronic acid preparations by molecular weight. BMC Musculoskelet Disord [Internet]. 2015 Oct 6 [cited 2021 Apr 13];16(1). Available from: https://pubmed.ncbi.nlm.nih.gov/26444018/

116. Zamboni F, Keays M, Hayes S, Albadarin AB, Walker GM, Kiely PA, et al. Enhanced cell viability in hyaluronic acid coated poly(lactic-co-glycolic acid) porous scaffolds within microfluidic channels. Int J Pharm [Internet]. 2017 Oct 30 [cited 2021 Apr 13];532(1):595–602. Available from: https://pubmed.ncbi.nlm.nih.gov/28939462/

117. Wu DC. An important player in brine shrimp lethality bioassay: The solvent. J Adv Pharm Technol Res [Internet]. 2014 [cited 2021 Aug 17];5(1):57. Available from: /pmc/articles/PMC3960796/

118. Diehl L, Morse Charles River M, Chemistry C. A Comparison of Selected Organ Weights and Clinical Pathology Parameters in Male and Female CD-1 and CByB6F1 Hybrid Mice 12-14 Weeks in Age.

119. Zhang XD, Wu D, Shen X, Liu PX, Fan FY, Fan SJ. In vivo renal clearance, biodistribution, toxicity of gold nanoclusters. Biomaterials. 2012;33(18):4628–38.