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# Nanoparticles in radiopharmaceuticals sciences: Review of the fundamentals, characterization techniques and futures challenges

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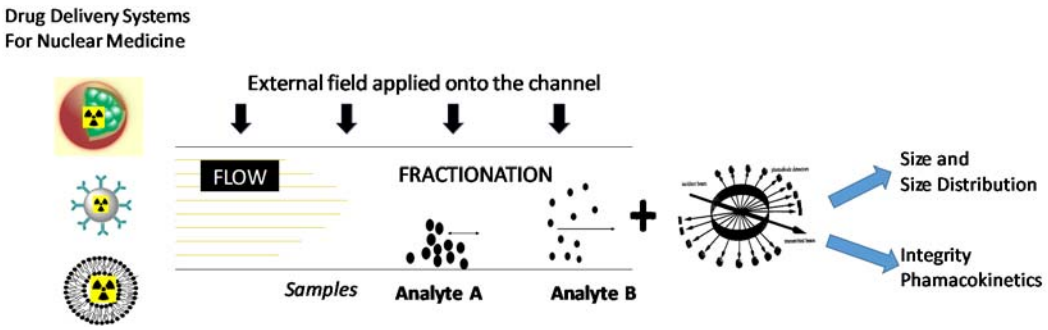
**ABSTRACT**




Nanoparticles are important players in modern medicine, with broad clinical applications ranging from contrast agents in imaging to carriers for drug and gene delivery into tumors. They combine the advantages of multiplexed analytical tools in a single support, offering new possibilities for targeting, sensing and curing pathologies, particularly in nuclear medicine. There is a trend towards integrating the diagnostic and therapeutic functions of nanoparticles, resulting in significantly improved and personalized treatment of disease. Various kinds of nanoparticles for cancer imaging and therapy were engineered since the last decade. For clinical translation, pharmacokinetics and toxicity must be evaluated to meet FDA or Eur. Pharmacopeia requirements. To this aim, a full and exhaustive characterization of these systems must be done such as morphology; chemical composition, surface charge, size and size distribution. The different analytical methods that could fully characterizing nanoparticles are reviewed; together with their advantages, drawbacks and limitations.

*Keywords: Nanoparticles, Drug-Delivery, Targeting, Imaging, Characterization, Field-Flow Fractionation*

## INTRODUCTION

The development of new advanced technologies is increasing and notably due to the development of nanomaterials and more specifically nanoparticles. They are involved in many different fields, i.e. in environmental remediation, energy generation and storage, food industry and applications in bioscience. One of the most prevalent area they have been developed, is the medical field, especially in oncology. Nanoparticles combine the advantages of multiplexed analytical tools in a single scaffold, offering new possibilities for targeting, sensing and curing pathologies. Since more than 30 years ago, therapeutic compounds based on nanoparticles have steadily increased on the market, as Zhang et al[1] pointed out. In 2006, the European Observatory for Science and Technology revealed that more than 150 companies are already developing nanoscale therapy. Nevertheless, by then, very few of them have been licensed for medical use (24 out of over 150). From a commercial perspective, this sector already served > $5.4 billion per year in 2006.

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More than 80 percent of these products are made up of liposomes and drug conjugates. Nanoparticles may consist of formulations that are polymeric or inorganic or a combination of both. Nanoparticles are usually formulated ranging from inorganic or polymeric nanoparticles, micelles, dendrimers, metallic nanoparticles and quantum dots, but also includes block ionomer complexes, engineered viral nanoparticles, albumin-based nanoparticles, polysaccharide-based nanoparticles, nano-shells, ceramic nanoparticles, and nano-rods.

Compared to traditional therapeutic and diagnostic agents, their shape, biocompatibility and selectivity can overcome some of the limitations encountered due to the unique physicochemical properties of nanomaterials (i.e., ultra-small size ranging from 1-100 nm, large surface area to mass ratio, and high reactivity, which vary from bulk materials of the same composition). More sensitive and more selective imaging agents of cancer and other diseased tissues could be reached using nanoparticles. They could be ideal carriers for cancer drug delivery and other therapeutics to diseased sites with minimal toxicity to normal tissues.

The mode of conjugating the drug to the nano-carrier and the strategy of its targeting is of importance for targeted therapy, but will not be discussed here. Briefly, cell-specific targeting with nano-carriers may be accomplished by using either active or passive mechanisms. The first strategy relies on the attraction of a drug – the nano-carrier conjugate to the affected site by using recognition ligands, attached to the surface of conjugates such as antibodies, low molecular ligands, e.g., folic acids, peptides, etc. The active strategy can also be achieved through a manipulation of physical stimuli (e.g., temperature, pH, magnetism). Passive targeting is a result of enhanced vascular permeability and retention (EPR) which is characteristic of leaky tissues of tumors 3. Once the drug-nano-carrier conjugates reach the diseased tissues, the therapeutic agents are released. A controlled release of drugs from nano-carriers can be achieved through changes in physiological environment such as temperature, pH, osmolality, or via an enzymatic activity.

Nano-carriers used for medical applications have to be biocompatible (administered without provoking an immune response or negative side effects). Undesirable effects of nanoparticles are dependent on their hydrodynamic size, shape, amount, surface chemistry, route of administration, the reaction of the immune system (especially the macrophages and granulocytes uptake) and residence time in the bloodstream. Due to the number of factors which may affect the toxicity of nanoparticles, their assessment is rather difficult and, thus, toxicological studies of each new Drug Delivery Systems (DDS) formulation are needed. However, with respect to their size, one can make some generalities – smaller particles have a greater surface area, thus, they are more reactive and, in consequence, more toxic.[2] It is generally accepted that nanoparticles with a hydrodynamic diameter of 10–100 nm have optimal pharmacokinetic properties for *in vivo* applications. 5 Smaller nanoparticles (< 10 nm) are subject to tissue extravasations and renal clearance whereas larger nanoparticles are quickly opsonized and removed from the bloodstream through the reticuloendothelial network macrophages.[3]

Cells can phagocyte nanocarrier-drug conjugates whereas cytotoxic effects may be caused by their intracellular degradation. Small size and large surface area can result in the aggregation of the targeting system based on nanoparticles, leading to handling difficulties. Scientists face other drawbacks for drug delivery systems such as low drug loading capability, low loading performance, or poor ability to control the size distribution of nanoparticles. However, no technical approach has been proved suitable for making such carriers of good quality.

It is still an ongoing research challenge that will take a useful drug delivery system to clinics if scientists can make progress on toxicity assessment (protocol setting), biocompatibility enhancement, drug charging, targeting, transportation and release along with better understanding and regulation of biological barriers interactions. Another challenge of importance is to control, detect and monitor the exposure level as well as the evaluation of the environmental impact of these DDS. The complexity is because nanoparticles are engineered specifically to combine the correct physicochemical and biological properties with the desired targeting functions.

To this aim, several analytical methods can be applied to fully characterize nanoparticles (stability, integrity, size, surface charge …). To help scientists, a "Guidance on physico-chemical characterization for manufactured nano-objects submitted for toxicological testing" (ISO/TR 13014) was published (2012).

The present paper first ambitions to take stock of the different nanoparticles that have been developed in Nuclear Medicine and what are the characterizations that have been performed and what are the lacks. The physico-chemical parameters influencing the colloidal stability (surface charge…) and thus the *in vivo* behavior will be briefly reviewed. In a second part, a review of the different analytical methods to fully characterizing nanoparticles will be given, with the ISO guidance as background, together with their advantages, drawbacks and limitations. But Nuclear Medicine involves radiation or radioactivity to diagnose, treat and prevent disease and thus imposes a certain number of constraints on the techniques envisaged. An emphasis will be given on the size and size distribution using Field-Flow Fractionation techniques.

## 1. NANOPARTICULES USED IN NUCLEAR MEDICINE: A BRIEF OVERVIEW

The majority of radionuclides having nuclear properties suitable for imaging and therapy are metals (e.g., $^{99m}$Tc, $^{111}$In, $^{90}$Y, $^{177}$Lu) and require the coordination of chelates to form complexes with the appropriate biological targeting properties. The NPs bind or encapsulate the metallic element. In this section, we will differentiate radionuclides (RN) used for imaging and RN used for therapy. The use of radionuclides in medicine is based largely on the discoveries of two critical concepts; the "tracer principle" and the "Magic Bullet". George De Hevesy (1913) developed the tracer approach and recognized first that radionuclides could be used as tracers to follow how the native element or compounds containing the element were distributed either in plants or animals.[4] He based his discovery on the principle that radioactivity has the advantage of being easily detected at very low quantities, allowing for the introduction of minuscule quantities (nano- to picomoles) that will not disturb the system. Thus the radiolabelled tracer allows for noninvasive measurement of distribution and function in a biological system. Later, C. Regnaud and A. Lacassagne (1927)

predicted that the ideal agent for cancer therapy would be composed of heavy elements capable of emitting radiation at molecular levels which selectively bind the cells that one seeks to destroy 99,[5] Furthermore, the "Magic Bullet" concept was proposed in the 19th century (principally) Paul Ehrlich, by selectively staining tissues for histological investigation, and in particular, selectively staining bacteria. Ehrlich reasoned that if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. Hence, a "Magic Bullet" would be created that killed only the targeted organism. A problem with the use of the Magic Bullet concept as it emerged from its histological roots was that people confused the dye with the agent of tissue selectivity and antibiotic activity. The name "Magic Bullet" was used in the 1940 movie Dr. Ehrlich's Magic Bullet, which depicted his life and focused on Salvarsan (arsphenamine, "compound 606"), his cure for syphilis. This last concept has been extended to biomolecules principally antibodies utilized as targeting molecules to transport toxins such as radionuclides selectively to receptors that are over expressed on certain diseased cells such as tumor cells. This concept has been extended to include a host of nano-carriers from small molecules such as folic acid to peptides and proteins, microspheres and most recently nanoparticles. Nowadays, there is extensive interest in utilizing nanoparticle constructs to stably complex metals for diagnostic and therapeutic applications. Nanoparticles are developed as an alternative due to their size and ability to circumvent some of the hurdles encountered with traditional agents. These nanoparticles are further undergoing modification by attachment of biomolecules such as peptides and antibodies to act as targeted delivery systems. Part of what is driving the use of nanoparticles is that conventional agents have resulted in low therapeutic indices due to suboptimal biodistribution where only a minute portion of the intravenously administered drug reaches the target but large doses are delivered to normal tissues. Nanoparticles have unique properties that can be optimized to allow for higher penetration and retention in tumor cells. Furthermore, unlike conventional agents where only one radionuclide can be delivered per carrier, nanoparticles offer the advantage of delivering several radionuclides per carrier, increasing the dose and thus effectiveness of the drug. This is extremely important when few receptors are present leading thus to few delivered targeting molecules.

Radiopharmaceuticals are drugs that consist of two parts: a radionuclide that reports the mechanism of action through its decay, attached to a targeting biomolecule or organic ligand that carries or determines the localization of the radiopharmaceutical. They can be used either for diagnostics for the noninvasive imaging of disease or as therapeutics to deliver a toxic payload selectively to a tumor site (a radionuclide emitting non-penetrating radiations: electrons, alpha-particles, for instance).

There are two main ways to label a polymeric or nano-dimensional system. The label could be linked to the whole polymer / particle, so called pre-radiolabeling (the label has been synthesized beforehand). In another approach, a compound is first labeled (i.e. by bifunctional chelate for metals) and then the polymer/particle is formed. It is referred to as post-radiolabeling. There are several factors that influence on both pre- and post-radiolabeling, especially the half-life of the selected radionuclide. We describe here mostly post-radiolabeling processes, meaning that the polymeric or nano-particular system is baked first, then decorated with a chelator and finally a radiolabel is introduced. This practice has been applied to various nano-dimensional structures.

In this section, a classification will be given accordingly to the type of the emitting radionuclide and by radionuclide, which is more relevant for nuclear medicine purpose. We will focus only on nanoparticles carriers. Several reviews have been done for the other vectors.[6]

The quality, safety and efficacy of nanoparticles enabled medicinal products in complex biological environments are key attributes to assess. But what to measure is a key question in nanoscience, and it is not straightforward to address as different physicochemical properties define a nanoparticle sample. Most prominent among these properties are size, size distribution, surface charge, stability and porosity. For each type, since the decay energy is specific, we will describe briefly what type of characterization has been led.

## 1.1. FOR THERAPY

Radiotherapy involves the administration of a radioactive drug that is selectively accumulated in cancerous or diseased tissue versus normal tissue and either ablates or damages the diseased tissue through the emission of an energetic particle. This particle emission can be a $\beta^-$ particle, an $\alpha$ particle, or an Auger electron ($e^-$ ). Because these particle emissions result in damage to tissue, it is imperative that the drug accumulates selectively in the diseased tissue, as any uptake in normal tissue will result in unwanted dose to the patient and injury to normal tissue. The choice of type and energy of the particle emission is largely determined by the size of the lesion or tumor being treated, site of delivery, whether the tumor is homogeneous, and whether the dose can be delivered uniformly to each cell. For example, smaller tumors may respond better to lower $\beta^-$ energies, such as for $^{177}$Lu, whereas the higher energy $\beta^-$ emitter $^{166}$Ho may be required for larger tumors. In certain cases, the elimination or minimization of toxic side effects determines which energy is optimal.

### 1.1.1. FOR ALPHA TARGETED THERAPY

**$^{212}$Pb:** The $^{212}$Pb could be encapsulated into liposomes.[7] The formation, the characterization, the stability and *in-vivo* distribution as a function of lipid bilayer membrane were first examined by Rosenow *et al*.[8] These authors showed that liposome-associated $^{212}$Pb was rapidly taken up in large quantities by the liver and spleen. Additionally they showed liposomes could be stabilized remaining at least partially intact *in vivo* and thus in circulation in the serum; $^{212}$Pb liposomes effectively suppressed an antibody response at high doses of activity. Another study has been recently performed on such systems showing that an effective retention of $^{212}$Bi after $\beta^-$ decay of $^{212}$Pb is achievable with 100 nm liposomes. [9]

**$^{225}$Ac:** Decay of $^{225}$Ac to stable $^{209}$Bi results in the release of four $\alpha$-particles and greater than 27 MeV of energy. Attachment of $^{225}$Ac to standard targeting agents using standard chelating agents, upon decay results in the release of the daughters. A nano-carrier approach was developed for using the *in-vivo* generator $^{225}$Ac/$^{213}$Bi for disseminated metastatic cancer. Liposomes encapsulating $^{225}$Ac were formulated and shown to retain the potentially toxic daughters

at the tumour site. Sofou *et al*.[10] developed passive encapsulation of $^{225}$Ac and tested the retention of actinium and its daughters by stable pegylated phosphatidylcholine cholesterol liposomes of different sizes and charge. These authors showed that multiple $^{225}$Ac radionuclides could be entrapped per liposome but due to the large size of the liposomal structures required to contain the daughters, the approach was better suited for loco-regional therapy. Those liposomes were characterized by electron microscopy and dynamic light scattering only.

The potential utility of liposomes as carriers of $^{225}$Ac was studied by Henriksen *et al*.[11] They showed that sterically stabilized liposomes could be loaded with $^{225}$Ac with excellent stability in serum *in vitro*. Sofou *et al*.[10] proved that $^{225}$Ac was passively entrapped in multivesicular liposomes (MUVELs). PEGylated MUVELs yielded 98% $^{225}$Ac retention and 18% retention of the last daughter $^{213}$Bi for 30 days. MUVELs were then conjugated to an anti-HER2/neu antibody trastuzumab and exhibited strong binding and significant internalization (83%) by ovarian carcinoma SKOV3 cells.

Those liposomes have been characterized by electron microscopy, dynamic light scattering,[12] size–exclusion chromatography.[13] Or more recently by Asymmetric Flow Field–Flow Fractionation (AF4) has also been applied for liposome characterization, showing sizes of about 100 nm.[14,15,16]

Recently researchers have been evaluating using nanoparticles to stably complex actinides in order to sequester the daughter radionuclides, that resulted from decay such as in the case of $^{225}$Ac.[17,18] These methods often referred to as *in vivo* generators. They started with the stable complexation of a parent radionuclide that then decayed to radioactive daughters that upon decay resulted in significantly higher dose to the tumor site. Conventional chelating techniques often result in the loss of the daughters upon decay of the parent and toxicity to normal tissues that lower the overall therapeutic efficacy.

Another example consists in $^{225}$Ac that could be incorporated into a $LaPO_4$ nanoparticle matrix.[19] Studies were undertaken to evaluate lanthanum phosphate nanoparticles to which $^{225}$Ac was encapsulated to define if the parent and daughters would stay sequestered upon decay. To test if these agents could be successfully used in selective targeting the La($^{225}$Ac)$PO_4$NPs were conjugated to the monoclonal antibody mAB 201B. This antibody was chosen as the targeting occurs within minutes of injection to the thrombomodulin of lung endothelium. As mentioned previously, decay of $^{225}$Ac results in stable $^{209}$Bi, with the release of four α-particles. The studies showed a retention of ~50% of daughter nuclides within the La($^{225}$Ac)$PO_4$NPs over a period of one month. Animal experiment (i.e. biodistribution and imaging) showed ~ 30% of the La($^{225}$Ac)$PO_4$NPs accumulated in mouse lungs at 1 h post injection resulting in a greater than 200% ID/g. To further enhance the potential of sequestering multiple α particles from $^{225}$Ac, these investigators went on to design gold-coated lanthanum gadolinium phosphate nanoparticles (NPs) for the purpose of both retaining $^{225}$Ac and its daughters and providing a versatile platform for attaching targeting agents for various tumor types within the body. In order to retain $^{225}$Ac and its daughters, $^{225}$Ac was embedded into a binary mixture of $LaGdPO_4$ nanoparticles via hydrolysis of sodium tripolyphosphate in the presence of $La^{3+}$ and $Gd^{3+}$ ions. Then, citrate reduction of gold created a shell of Au on the surface of the $LaGdPO_4$ particles. Magnetic $LaGdPO_4$-AuNPs were then separated from non-magnetic AuNPs using a 0.5-T NdFeB magnet. TEM analysis of these $LaGdPO_4$-AuNPs particles showed monodisperse particles with average diameters of 4–5 nm. Radiochemical analysis indicated that $LaGdPO_4$-AuNPs without additional layers sequestered 60.2 ± 3.0% of the first decay daughter of $^{225}$Ac, $^{221}$Fr. Subsequent epitaxial growth with additional $LnPO_4$ layers increased daughter retention. The addition of two shells of LaGdPO4 and one shell of Au increased $^{221}$Fr retention to 69.2 ± 1.7%, while the addition of four shells of $GdPO_4$ and one shell of Au increased retention to 92 ± 1.0%. Retention of the first decay daughter is crucial to minimize normal tissue toxicity. Daughter sequestration in these first-generation particles was high, but retention was improved by additional layers of Au and/or $LnPO_4$. The authors went onto attach biomolecules such as peptides or antibodies such as mAb 201B and planed to evaluate this in the future.[19] The properties of low toxicity and favorable biodistribution made the $LaGdPO_4$-AuNP system a promising platform for targeted alpha therapy with $^{225}$Ac.

**$^{223}$Ra** can be obtained from a generator of $^{227}$Ac (21.8 years). It decays by 4 α-emissions and 2 β-emissions to stable $^{207}$Pb. These 4 α-emissions confer an advantage from a therapeutic point of view, but also represent a major drawback for stable radiolabelling. Furthermore, $^{219}$Rn gas emitted during the decay of $^{223}$Ra can redistribute in the body and be responsible for toxicity to healthy normal tissues. For targeting bone metastases, $^{223}$Ra is the perfect radionuclide because for this reason it is possible to use simple cationic radium (radium chloride form). After incorporation into liposomes or mAbs, efforts were made to evaluate its use. Notably, loading $^{223}$Ra into liposomes coated with folate-F(ab')$_2$ was developed by Henriksen et al. [11] The only characterization conducted on the nano-object itself was the determination of the size with a diameter of~120 nm and the polydispersity of the vesicles by dynamic light scattering.Vaidyanathan and Zalutsky gave an overview of the current status of $^{223}$Ra for targeted α-particle radiotherapy [20] and more recently by Jeon.[21]

### 1.1.2. FOR BETA TARGETED THERAPY

**$^{198}$Au** is a reactor-produced radionuclide with a half-life of 2.7 days. It emits a beta particle with a maximum energy of 0.96 MeV (99%) suitable for therapeutic applications and a 412 keV (95.6%) gamma ray that can be used for imaging and localization in biodistribution studies. Au-198 nanoparticles for tumor therapy applications. Two different synthetic methodologies have been developed, and the therapeutic efficacies of these nanoparticles in animal models have been published.[9] A major advantage of nanosized radioactive particles has been their potential to contain numerous radioactive atoms within a single nanoparticle. Delivery of a high therapeutic payload to tumors could be achieved by this method. Gold nanoparticles (AuNPs) have been around for quite some time, but their harsh production methods with toxic chemicals have prevented them from being attached easily to biomolecules for *in vivo* evaluation. New methods to produce gold nanoparticles in aqueous solutions able to be used in biomolecular applications were developed.

Balogh and coworkers used a nanocomposite device (NCD) for encapsulation of radioisotopes, providing defined size and surface properties.[22] Using this method, the number of radioactive gold atoms could be increased without destroying the targeting ability of the NCD. Gold NCDs were synthesized as monodispersed hybrid nanoparticles composed of radioactive guests immobilized by dendritic polymer hosts. In order to create nanoparticles, commercially available polymers including poly(amidoamine) PAMAM dendrimers and tecto dendrimers were used as nanocomposites. The synthesis of Au-198 nanoparticles by this method involved encapsulation of Au-198 within PAMAM dendrimers. Encapsulation was achieved by mixing dilute solutions of PAMAM dendrimer with an aqueous solution of $HAuCl_4$. Salt formation between the tetrachloroaurate anions and the dendrimer nitrogens ensured effective encapsulation of gold within the dendrimer matrix. Upon encapsulation, elemental gold was converted into Au-198 within the dendrimer matrix by direct neutron irradiation.

A different method for gold nanoparticle synthesis consists of using THPAL, a trimeric phosphinoalanine, $P(CH_2NHCH(CH_3)COOH)_3$, to reduce gold salts in aqueous solutions containing stabilizers, which coat the surface of the gold nanoparticles and form 12-15 nm sized gold nanoparticles with a hydrodynamic diameter of 60-85 nm.[23] These nanoparticles were characterized by NMR, electrospray ionization mass spectra, elemental analysis and X-ray crystallography. The methods were changed to allow for formation of both the Au-198 and Au-199 nanoparticles and also lead to formation with other metals such as palladium and silver. Gum Arabic coated gold nanoparticles (GA-Au-198-NPs) were the first and were the most studied nanoparticles to date. The reaction consists of heating water containing gum Arabic, adding gold either as the $NaAuCl_4$ salt or the $HAuCl_4$ acid along with THPAL, which changed from a pale yellow solution to a red burgundy one. Quality control showed this method resulted in 99% conversion of the radioactive gold to the nanoparticle forms. This formulation was favorably evaluated in SCID mice bearing induced human prostate tumors.

A receptor targeted approach was developed using epigallocatechin-gallate (EGCg).[24] This method proved much simpler as the nanoparticles could be formed at room temperature and EGCg not only achieved reduction of the gold but also served as a stabilizing agent, resulting in an EGCg conjugated gold nanoparticle ([$^{198}$Au] EGCg-NPs) formulation in a few minutes at room temperature in water. The characterization was done through UV-Vis. Selective targeting has also been evaluated by conjugating the 14 amino acid peptide bombesin (BBN) to the gold nanoparticle surface. Bombesin targeted the gastrin releasing peptide (GRP) receptors that were up-regulated in a variety of cancers, predominantly breast, prostate, pancreatic and lung cancers.[25] In *vitro* receptor binding studies have shown a high affinity of BBN-conjugated Au-198-NPs for the GRP receptor in PC-3 cells.[26] This conjugate (BBN-Au-198-NPs) was evaluated in SCID mice bearing human prostate PC-3 cells and in a spontaneous model of prostate cancer in the TRAMP mouse. Only the size was mentioned in this paper (12-18 nm core diameter and 85 nm hydrodynamic diameter) but without any specification on the technique used for such determination.

**$^{166}$Ho:** The lanthanide holmium-166 has recently attracted interest because of its versatile properties as a high-energy β-emitter ($E_{\beta max}$ = 1.85 MeV) it can be exploited for cancer therapy; its low-energy γ-photons (Eγ = 81 keV) make it suitable for SPECT imaging; finally, the high magnetic moment (10.6 μB) allows its use also for magnetic resonance imaging (MRI). Furthermore, its half-life of 26.8 h is advantageous for both imaging and pharmaceutical purposes. Also, the visualization by MRI is useful for medium- and long term monitoring of the progress of the treatment. Ho-based materials represent therefore an interesting opportunity to develop theranostic systems. As an example, Ho-microspheres (activity/sphere ≤ 450 Bq) designed for radioembolization have already been investigated. Their *in vivo* biocompatibility and efficacy were demonstrated through the successful completion of preclinical studies, and the systems were assessed in Phase II clinical trials. More recently, gamma shielded irradiation sites at the McMaster Nuclear Reactor (Hamilton, ON, Canada) was designed and installed that would enable it to produce clinical quality Ho-166 microspheres for North American clinical trials of this device.[27] For realistic applications, however, cancer diagnosis and treatment require efficient drug uptake and retention to the diseased site. Although the destructive effects of β particles were active even at a certain distance (maximum soft-tissue range of 8.4 mm), internalization of radioactive theranostic nanoparticles (NPs) into the target cells implied a longer residence at the site of interest, and this translated into a more effective treatment even when a lower radiation dose was applied. Therefore, smart surface functionalization was crucial to manage the behavior of the designed NPs in order to extend blood circulation time, reduce nonspecific delivery, and avoid leakage of toxic metal ions. Appropriate functionalization of NPs became even more important for diameters smaller than 40 nm, as it was known that for tinier particles the surface chemistry is the factor determining their biodistribution and *in vivo* behavior, rather than the size. Those characterization were done through DLS and UV.

When the preparation of diagnostic/therapeutic compounds involved neutron activation (as in the case of Ho), not only intrinsic properties (e.g., half-life of the radioisotope), but also practical aspects such as handling high activities during surface decoration of NPs and the effects of radiation on the integrity of conjugated organic functionalities had to be considered. For example, for the above-mentioned Ho-microspheres a qualitative deterioration of the polylactate coating was reported because of their exposure to a high neutron flux. The disadvantages of a post-irradiation functionalization were obvious, as it implied working with radioactive materials and special equipment and facilities, while the time available was limited by the decay of the isotope. Neutron activation of the ultimate product offered a much more elegant solution provided that it did not lead to disintegration of the organic components. Martinelli et al reported an investigation on the effects induced by neutron activation of Ho NPs on various conjugated organic functionalities.[28]

**$^{188}$Re:** is one of most promising generator-type therapeutic beta-emitters with the energy of positron emission of 1.96 MeV (16.7%) and 2.18 MeV (80%) and half-decay time of 17 hours. Recently, Si*NPs-PEG-$^{188}$Re conjugates have been shown to efficiently deliver $^{188}$Re through the blood stream and be retained in the tumor

region.[29] These authors synthesized Si*NPs coated with PEG and $^{88}$Re ions were complexed using the carboxyl group available on the PEG surface. DLS was also employed with an average size of 50 nm. To determine the size characteristics of these nanoparticles, high-resolution transmission electron microscopy was employed in the imaging and diffraction modes. Quite different biodistribution and pharmacokinetics between Si*NPs-PEG-$^{188}$Re conjugates and free $^{188}$Re atoms were obtained. Intratumoral administration assays showed very good retention of Si*NPs-PEG-$^{188}$Re conjugates in the tumor for more than 24 hours, while the free $^{188}$Re rapidly washed out form the tumor under similar conditions.

## 1.2. IMAGING AND THERANOSTICS AGENTS

Diagnosis of diseases such as cancer, cardiac syndrome, and neurological disorders is usually done by molecular imaging, providing information about the physiological condition of a tissue or disease level. This is also important to assist in the planning of treatments. It is based on identifying specific biomarkers or pathways with high sensitivity and selectivity, as opposed to conventional contrasting agents in the imaging. At the site of interest, the imaging agent accumulates selectively; interacts with the target site (physical, chemical, biochemical interaction) and then degrades. Among the developed contrast agents, and due to their intrinsic properties, inorganic nanoparticles (NPs) gained more attention. [30,31]

Molecular imaging samples are a distinct category of pharmaceuticals that targeting specific biochemical signatures related to disease and allowing non-invasive imaging at the molecular level. Since changes in biochemistry occur before disease reaches an advanced stage, studies of molecular imaging make it possible to classify and stage disease, select patients based on expected response, and track drug efficacy during therapy. The aim of theranostics is to develop diagnostic tests to screen a disease state directly linked to the application of specific therapies to improve efficacy and cost effectiveness. Before treatment, imaging permits the personalized diagnosis of the patient's disease by determining the specific phenotype on the molecular level. Additionally, and unlike traditional *in vitro* methods, it can assess the heterogeneity of the diseased tissue or tumor. In theranostics, molecular targeting agents are used to achieve initial low dose imaging to evaluate the biodistribution, dosimetry, dose-limiting organ or tissue, maximum tolerated dose, receptor expression and capacity and clearance.[32] The maximum tolerated dose is the highest possible dose of a drug or treatment that does not cause unacceptable side effects. Dosimetry is the calculation of the absorbed dose to tissue from the administered radioactivity. This information can then be used to pinpoint the appropriate molecular targets in diseased tissue that can be targeted with the optimal ligand and radionuclide to deliver tailored individual therapies with the most effective dose. [33] In the case of nuclear medicine, the term theranostics often refers to using a targeting vector labeled initially with a diagnostic radionuclide to assess the disease followed by personalized treatment using the same targeting vector labeled with a therapeutic radionuclide.

**Magnetic Resonance Imaging (MRI)** is an effective imaging tool without the use of ionizing radiation in clinical medicine. It makes it possible to differentiate pathological tissues from normal tissues based on fluctuations in water protons relaxation times (i.e. longitudinal ($T_1$) and transversal ($T_2$) times). Relaxation times $T_1$ and $T_2$ can be altered compared to normal tissues in pathological tissues and these changes are transformed into images. Gadolinium chelates (GdCAs) are often used as contrast agents for paramagnetic purposes. One major challenge remains: the sensitivity of MRI.

**Computed tomography (CT)** uses radiation-rays and by employing tomographic imaging techniques, creates a 3D-image. It is widely used as this offers representations of various diseases across the entire human body quickly. The image is provided by the differences in attenuation of X-rays between tissues. X-ray attenuation in bone is very effective, but less effective in soft tissue. CT needs a contrast agent for imaging soft tissues to help attenuate X-ray in the soft tissue surrounding area. Despite the ongoing controversy regarding cancer risk due to repeated X-ray exposure, CT is a reliable tool for imaging.

**Positron Emission Tomography (PET**) is a radionuclide-based nuclear imaging technique of which a positron is emitted which subsequently annihilates with an electron to produce two coincident 511 keV photons detected in a ring of detectors placed around the patient. By contrast to SPECT, PET uses electronic collimation, rather than lead collimation to remove scatter and background. The amount of radioactivity is lower for reaching a higher resolution 3D-image and sensitivity than SPECT. PET is non-invasive imaging modality that allows quantitative mapping of a drug or biomarker *in vivo* of functional processes in the body. The gold-standard PET radionuclide is $^{18}$F with a $E_{\beta+}$= 633.5 keV, $T_{1/2}$= 110 min allows for optimal resolution, a high positron branching ratio, and a half-life that both matches that of the biomarker life-span and allows for chemical synthesis and delivery of the agent *in vivo*. Other positron emitters are non-metals such as $^{11}$C, $^{15}$O, or $^{13}$N. These elements can be incorporated into biological molecules without any disturbing behavior. Despite this, the major drawback is the short half-lives that lessens their use in some molecules and complicated time consuming organic syntheses that are not always compatible with biomolecules. Other radiometals have been developed, such as $^{64}$Cu combined to 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA) chelate. Radiopharmaceuticals labeled with a variety of copper radionuclides have been developed for several applications. The review for this type of contrast agents is not given here could be found in the paper from Cutler et al.[34]

Inorganic NPs could be decorated for multiple imaging modalities purpose, like magnetic or tomography.[35,36] They could be decorated with different functional groups as well leading to selectively target the site of interest without changing their physical properties.[37] These 3 techniques (MRI, CT, PET) have been considered to be modalities for deep-tissue imaging. Other techniques existed for exploring shallow-tissue modalities. We only quote them here: i) Optical coherence tomography (OCT); ii) Photoacoustic tomography (PAT) ; iii) Two-photon microscopy; iv) Surface-Enhanced Raman Spectroscopy (SERS) imaging. Further details could be found in the review from Cho et al.[38] or in the quite recent review from Stockhofeet al. examining the radiolabeling for PET imaging of nanoparticles and polymers.[39]

**$^{99m}$Tc :** Technetium-99m has been the most prevalently used radionuclide for diagnostics based on its favorable nuclear properties, including a single 140 keV photon emission (ideal for most gamma cameras), absence of particle emission, a short half-life of 6.03 h and availability via portable $^{99}$Mo/$^{99m}$Tc generators. Technetium has a rich chemistry that has allowed for its incorporation into a plethora of formulations for a variety of drugs and *according to the International Atomic Energy Association (IAEA) and World Nuclear Association* has been currently used in over 80% of nuclear diagnostic procedures.

Carbohydrate-coated dendrimers (i.e. with mannose and lactose terminal) were synthesized and sodium pertechnetate $^{99m}TcO_4^-$ was encapsulated. Mannosylated and lactosylated poly(propylene imine) (PPI) dendrimers biodistribution studies in female Balb/c mice were found not to accumulate in liver in comparison to $^{99m}$Tc encapsulated with single PPI dendrimers.[40] These authors observed too that dextran conjugated PPI dendrimers Doxorubicin HCl Enhanced uptake by A549 cancer cell lines.

There were many methods for the characterization of dendrimers: Ultraviolet-visible spectroscopy (UV-VIS); Infrared spectroscopy (IR); Nuclear magnetic resonance (NMR); Mass spectrometry; Raman spectroscopy; Atomic force spectroscopy; X-ray photoelectron spectroscopy (XPS); High pressure liquid chromatography (HPLC).

Parrott and al synthesized aliphatic polyester dendrimer functionalized with vinyl groups at the periphery and a dipicolylamine Tc(I) chelate at the core.[41] The size of the resulting macromolecules was evaluated using DLS, and it was found that the dendrimer functionalized with $mPEG_{750}$ was molecularly dispersed in water, exhibiting a hydrodynamic diameter of 9.2 ± 2.1 nm. This PEGylated dendrimer was subsequently radiolabeled using $[^{99m}Tc(CO)_3(H_2O)_3]^+$ The reported PEGylated aliphatic polyester dendrimers represented a new platform for developing tumor-targeted molecular imaging probes for Single-Photon Emission Computed Tomography (SPECT).

Boron nitride (BN) nanoparticles, as the structural analogues of graphene, have been potential biomedicine materials because of the excellent biocompatibility, but their solubility and biosafety were the major obstacles for the clinic application. Here, the highly soluble BN nanoparticles coated by PEG (BN-PEG) were synthesized with smaller size (~10 nm), then their biodistribution *in vivo* through radioisotope ($Tc^{99m}O_4^-$) labeling studied. The results showed that BN-PEG nanoparticles mainly accumulated in the liver, lung, and spleen with the less uptake by the brain.[42]

**$^{18}$F** is the most commonly used positron emitter and its optimal positron energy ($E(\beta^+)_{max}$ = 635 keV) with high intensity ($I_{\beta+}$ =97%) makes it almost perfect for PET imaging. It was used to track in *vivo* NPs, quantum dots (QDs) or polymers as a suitable PET nuclide. Liu et al.[43] produced, $^{18}$F-rare earth with NPs. Hydrodynamic diameter distribution was determined from DLS (size of 22−30 nm). Pérez-Campaña et al. [44] developed a direct way of marking $Al_2O_3$-NPs with $^{18}$F. The radiolabeling of the NPs did not alter their surface or structural properties as demonstrated by TEM, DLS, and ζ-potential measurements. Recently, Lee et al.[45] identified a strategy for bioorthogonal labeling where they applied an *in vivo* copper-free click reaction for $^{18}$F-(pre)labeling of NPs. These NPs were characterized by TEM and ζ-potential.

**$^{199}$Au**: Interest in Au-199 was initially due to its ability form to clusters of 11 gold atoms that could then site selectively attached to monoclonal antibodies.[46] Recent interest in Au-199 was due to the wide investigation of gold nanoparticles and the ability to use Au-199 in planar and SPECT imaging to study the biodistribution and clearance of agents as well as to assess the dosimetry and maximum tolerated dose (MTD) of therapeutic gold agents.

A recent example in a pharmaceutical area showed that amyloid fibrils could exist in multiple shapes and structures, that exhibited different distinct properties which would explain why Alzheimer's and Parkinson's disease patients would show different clinical symptoms.[47] The researchers showed that gold amphiphilic anionic nanoparticles with a diameter around 3 nm, have a unique ability to efficiently label the edge of amyloid fibrils in a hydrated state. This would make the visualization of the diverse amyloid fibrils easier. It could be further envisaged to use 198Au to this aim.

**$^{64}$Cu and $^{67}$Cu** : There are two major radionuclides of Cu that are of interest for theranostic applications: $^{64}$Cu and $^{67}$Cu. $^{67}$Cu with a half-life of 2.58 days emits a maximum energy beta particle of 0.577 MeV. $^{64}$Cu with a 12.7 hours life span decays 19% by positron emission (0.653 MeV), 40% by $\beta^-$ emission (0.579 MeV), and 41% by electron capture, which can be used for both imaging and therapeutic applications $^{64}$Cu has been used to label nanoparticles that have been shown to be of interest in biomedical applications.[48,49]

Rossin et al. combined passive targeting automatically performed by NPs with active targeting by adding folic acid to their shell-cross-linked micelles. Thus, they could ratify the EPR-effect, but at the same time they could not see a clear difference between the folate-conjugated tracer and the polymer without targeting-vector.[50] TETA was used as a chelating agent and was combined with shell-cross-linked nanoparticles (SCKs) made up of an amphiphilic block copolymer. Nonetheless, no characterization of the NPs was conducted in this study.

Tu et al. coated manganese-doped QDs with dextrane and mixed as chelator a DO3A derivative.[51] They performed $^{64}$Cu-radiolabeling and characterization of the QDs was realized by DLS.

Huang *et al.* loaded near infrared (NIR)-dye onto mesoporous silica, and labeled it with two different metal ions, namely $Gd^{3+}$ ($T_1$-contrast agent in MRI) and $^{64}Cu^{2+}$ for PET imaging.[52] On the one hand, DOTA was used as a chelating agent, and on the other, the researchers exploited the fact that both copper and gadolinium were found in the pores of the substrate. Stability studies have shown that the technique offers a highly stable radiotracer that exhibited large penetration of the sentinel lymph node (SLN), which could be demonstrated in PET imaging using 4T1 tumor BALB / C mice. These authors have determined the average hydrodynamic diameter (15.1 ± 7.6 nm) by DLS.

The study of Kostarelos et al. evaluated the tissue dosimetry of liposome-radionuclide complexes toward liposome-targeted therapeutic radiopharmaceuticals, notably for $^{67}$Cu.[53] They found that ganglioside (GM1) coated-liposomes with $^{67}$Cu delivered lower doses to tumor than shorter lived radionuclides such as $^{188}$Re and $^{211}$At, but $^{67}$Cu had a more effective standardized uptake value (SUV). No characterization of these systems was realised in this work.

The pre-labeling approach to radiolabel glycol chitosan nanoparticles (CNPs) was very important.[54] For binding $^{64}$Cu-alkyne complex to azide-functionalized CNPs *in vivo*, copper-free click chemistry was applied. $^{64}$Cu-radiolabeled CNPs did not show any significant effect on the physicochemical properties, such as size, zeta potential, or spherical morphology (from TEM).

**$^{117m}$Sn** could be considered as a promising radionuclide for therapeutic applications. The use of $^{117m}$Sn-DTPA was reviewed by Lewington in the context of pathophysiology of metastatic bone pain.[55] In 2004, a report on *Radiopharmaceuticals for the Palliation of Painful Bone Metastases* pointed out that one phase I trial was performed on $^{117m}$Sn-DTPA but showed insufficient evidence to recommend this agent. The only clinical examples to-date for treating synovial inflammation using a low-energy beta-emitter was the use of $^{169}$Er colloids to treat inflammation in the small finger joints.[56] The use of appropriately-size particles labeled with $^{117m}$Sn was called for in the paper of Srivastava as agents of choice for radiation synovectomy.[57] During formation, the size of these radiolabeled particles cannot be adequately controlled, and small particles (< 10 μm) are assumed to leak out of the synovium over time. A new type of particle, made of hydroxyapatite (HA), a natural constituent of the bone, had become commercially available in different controlled sizes ranging from 1 to 80 μm. Accordingly, research attention recently centered on integrating HA particles into new radiation synovectomy agents.

**$^{68}$Ga:** It is a generator-produced nuclide. Germanium-68 is his father with a half-life of 270.8 d that decays into gallium-68 through electron capture. $^{68}$Ga ($T_{1/2}$ = 67.71 min) is a $\beta^+$-emitter decaying into stable zinc-68. The positron branching is 89%, with 3.22% of γ-emission. Its mean positron energy is 740 keV is suitable for PET imaging, resulting in a high spatial resolution. No pre-radiolabeling method has been published yet in gallium-chemistry as in copper-chemistry. Of note, the gallium-68's much shorter half-life plays the dominant role. The volume of articles is much lower than for $^{64}$Cu for $^{68}$Ga-labeled polymers and NPs. Sing *et al.* almost quantitatively had $^{68}$Ga radiolabeling yields on nanogels.*58* The resulting nanogels exhibited a well-defined spherical shape with a diameter of 290 ± 50 nm, determined by DLS. Locatelli and coworkers[59] obtained higher yields in different conditions but they used inorganic NPs composed of γ-$Fe_3O_4$ together with an ammonium nitrate salt of cerium, whereas Singh *et al.* investigated (organic) polymeric nanogels.[56] The size and size distribution (44–55 nm) were performed by the means of TEM and DLS; and a negative ζ potential was determined.

Several other positron emitters have been introduced for radiolabeling of NPs, such as $^{13}$N, $^{11}$C, $^{86}$Y, $^{89}$Zr or $^{124}$I. No further discussion on these other radionuclides is provided in the present paper.

### 1.3. MULTIMODALITY IMAGING

Some poorly explored scientific landscapes remain and the multimodality in imaging is one of those. Gathering the advantages of different analytical tools on a single nanometric scaffold allows combining and possibly enhancing the performances of each in terms of medicinal, biological and chemical capabilities, offering new possibilities of multiplexed tools for targeting, sensing and curing pathologies.

Several multifunctional NPs were also developed as multimodal imaging contrast agents. These included gold-coated QDs for optical and fluorescence imaging,[60] gold-coated iron oxide NPs for optical imaging and MRI,[61] and gold NPs combined with Gd-chelate compounds for X-ray imaging and MRI (*in vivo*),[62] and QDs combined with fluorescence imaging and MRI Gd-chelate compounds.[63] For both MRI and fluorescence imaging, silica particles filled with QDs and iron oxide NPs were also developed.[64]

For instance, a multimodal nanoparticle called AGuIX® for multimodal imaging and for radiotherapy guided by MRI has been developed including gadolinium-bound chelates and free chelates that can be labelled with radioactive isotopes. Gadolinium-based nanoparticles have been developed which could be detected by imaging modalities used in routine clinical practice, and thus be used as radiosensitizing agents in cancer therapy.[65] NPs were composed of a core of rare-earth atoms (gadolinium), surrounded by a polysiloxane shell (4 silica /one gadolinium in this study) that can be used to label specific targeting molecules. These NPs were composed of a polysiloxane network around which DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or NODA (1,4,7-triazacyclononane-1,4-diacetate) molecules were arranged. In addition, DOTA was bound by the amino functional groups present on SRP surface. 2-4.5 DOTA were free to bind a radioisotope and the remaining DOTA were bound with Gd. Mignot et al.[66] showed that the NPs size was approximately 4.5 nm, that ensured rapid renal clearance.[67] These NPs were promising multimodal imaging agents since they could be detected by four complementary imaging techniques, *i.e.* MRI, SPECT, CT and fluorescence imaging. On the other hand, silica nanoparticles could be functionalized with different linkers as amine, thiol, carboxylic or methacrylate. Then, a targeting molecule could be conjugated through the selected linker.[68] In addition, these GBN exhibited sensitizing properties which rendered them hopeful therapeutic agents for radiotherapy.[69,70] These NPs exhibited long circulation time in the bloodstream and displayed significant accumulation in tumour tissues thus suggesting the existence of an Enhanced Permeability and Retention (EPR) effect.[71] These NPs had advantages in terms of drug-delivery and imaging. The delivery of radionuclides could be increased per targeting biorecognition compared to simple immunotargeted drugs, reducing the side effects associated with this type of cancer therapies. They were also designed to provide better protection against enzymatic or environmental degradation, and to avoid obstacles like the blood-brain barrier or the vascular endothelium.[72] The essential disadvantage of these NPs was eventual toxicity. From above, there was a huge variety of NPs, which was the consequence of their composition, structure, size, …., depending on the application they were envisaged for.

## 2. DRUG LOADING

An effective nano-delivery system, as tested by Singh and Lillard,[73] should have a high drug-loading efficiency, thereby reducing the amount of matrix materials for administration. Two methods could be used to load drugs. The method of incorporation required the drug to be incorporated when formulating nanoparticles. The methods of adsorption / absorption allowed the drug to be absorbed after the formation of nanoparticles; this was

accomplished by incubating a concentrated drug solution to the nano-carrier. Drug loading and trapping efficiency depend on drug solubility in the excipient matrix material (solid polymer or liquid dispersion agent) related to matrix structure, molecular weight, drug-polymer interactions, and the presence of end functional groups (i.e., ester or carboxyl) in either the drug or matrix.[74]. PEG is a polymer of choice for certain formulations of nanoparticles, which has little or no effect on drug loading and interactions.[75] Moreover, when loaded at or near their isoelectric point (pI), the macromolecules, drugs or proteins encapsulated in nanoparticles showed the greatest loading efficiency.[76] Studies showed that the use of ionic interaction between drug materials and matrix materials could be very efficient in rising drug charging for small molecules.[77]

## 3. DRUG RELEASE

When designing a nanoparticulate delivery system, it is important to consider both the release of drugs and the biodegradation of polymer. The rate of release of drugs generally depends on: (1) drug solubility; (2) surface-bound or adsorbed drug desorption; (3) drug diffusion through the matrix of nanoparticles; (4) erosion or degradation of the matrix of nanoparticles; and (5) the combination of erosion and diffusion. The release process is therefore regulated by solubility, diffusion, and biodegradation of the particle matrix.

In the case of nanospheres, where the drug is distributed uniformly, the release of drugs occurs through diffusion or matrix erosion. If the diffusion of the product is slower than the degradation of the matrix, then a cycle of diffusion essentially regulates the release mechanism. The rapid, initial release or ' burst' is primarily attributed to the relatively large surface of nanoparticles, which are weakly bound or adsorbed.[78] It is clear that the integration approach has an impact on the profile of the launch. If the drug is charged using the integration process, the device will have a relatively small burst effect and sustained release properties.[79] If the nanoparticle is polymer-coated, then the release is controlled by the polymer membrane diffusion of the drug.

Membrane coating acts as a barrier to drug release, making drug solubility and diffusion within or across the polymer membrane a determining factor in drug release. In addition, ionic interactions between the drug and the auxiliary ingredients can also influence the release speed. When the trapped drug interacts with auxiliary ingredients, a less water-soluble complex may develop that may slow the release of the drug – with almost no burst release effect.[80] Whereas if the addition of auxiliary ingredients such as ethylene oxide-propylene oxide block copolymer (PEO-PPO) to chitosan decreases the drug's interaction with the matrix material due to PEO-PPO's efficient electrostatic interaction with chitosan, an improvement in drug release could be achieved. [81].

Different methods were used to study drug release from nanoparticles: (1) side-by-side diffusion cells with artificial or biological membranes; (2) diffusion of the dialysis bag; (3) diffusion of the reverse dialysis bag; (4) agitation followed by ultracentrifugation / centrifugation; or (5) ultrafiltration. The release analysis has been normally performed by managed agitation accompanied by centrifugation. The dialysis technique has been generally preferred due to the time-consuming nature and technical difficulties encountered in separating nanoparticles from release media. Such processes, however, have been proven difficult for commercial use to reproduce and scale up.

Differentiating between ' free ' and ' set ' nano particles was critical. The formers posed a direct threat to safety as they were harder to control because of airborne and could be inhaled. Nanoparticles reached the human body in several ways : through the lungs where rapid translocation to vital organ through the bloodstream was possible, including through the Blood Brain Barrier (BBB) and through the intestinal tract or through the skin.[82]

Metal ions, produced as a result of nano-object test sample dissolution, could contribute to test cell toxicity. The concentration of metal ions in the working suspension shall be measured after separation of particulate matter. Particulate matter could be separated from the ionic fraction by ultrafiltration (U/F), ultra-filtration assisted by centrifugation (C-U/F) or tangential flow filtration (TFF). The measurement shall be made for all metallic elements that were included in the nano-object sample. An appropriate method shall be selected to measure the metal ion concentrations from among inductively coupled plasma-atomic emission spectrometry (ICP-AES), ICP-MS, atomic absorption spectrometry (AAS) and the colorimetric method. Measurement results of concentrations shall be expressed in the unit of molarity, mass/mass or mass/volume. The measurements could be omitted when a toxic effect was not observed to the cells in the working suspensions.

Calibration curves for the metal ions of interest were necessary using standard solutions of the metal ion. Measurement of ICP-AES should follow the standard of ISO 11885. Pre-treatment of the working suspension and generation of the calibration curves were conducted. The measurements must follow the relevant standards of ISO 17294-1 and ISO 17294-2.

## 4. TOXICITY ISSUES

Nanotoxicology has emerged as the discipline that aimed to investigate the safety of nanotechnology. Specifically, nanotoxicology has been intended to assess the risks associated with exposure to nanomaterials, to explore the routes of entry of nanoparticles into the organism and to study the molecular mechanisms of nanoparticles toxicity.[83,84]

Recent studies have shown that the same properties that made nanoparticles so unique, such as their small size, large surface area, chemical composition, solubility and geometry, could also be the reason for their potential hazard to human health. For instance there was an inverse relationship between quantum dot size and concentration and their adverse effects; the smaller sizes and higher concentrations were more cytotoxic.[85]

Ideally, it would be advantageous if the nanoparticles could be secreted or degraded without toxic side effects after they had exerted their function(s). One approach to combating this problem was coating the nanoparticles. The coating was comprised of biodegradable polymeric materials that were already in use in biomedicine or to design novel nanoparticulate systems with biodegradable polymers. These polymers may be either polyethylene glycol (PEG), poly(vinylpyrrolidone) (PVP), natural polymers like dextran, chitosan, pullulan or surfactants like sodium oleate, dodecylamine. In addition, coating nanoparticles could be

used to prevent agglomeration and keeping the particles in colloidal suspension. [86]

It is well known that cationic macromolecules interact with negative biological membranes that destabilize them and induce lysis of the cells.[87,88.] Nanosized particles such as dendrimers may interact with cellular nanometric components such as cell membranes, cell organelles and proteins.[89] Dendrimers with cationic surface groups tend to associate with lipid bilayer, increase permeability, and decrease biological membrane integrity, similar to macromolecules. The mechanism involved is the leakage of cytosolic proteins such as luciferase and lactate dehydrogenase, on dendrimer interaction with the cell membrane which ultimately causes its disruption and cell lysis.

Jevprasesphant et al. investigated the cytotoxicity of PAMAM dendrimers using Caco-2 cells and concluded that anionic or half-generation dendrimers exhibited significantly low toxicity compared to their respective cationic family.[90] In addition, the *in vitro* cytotoxicity of cationic melamine dendrimers with surface groups such as amine, guanidine, carboxylate, sulfonate or phosphonate has been documented and cationic dendrimers have been found to be much more cytototoxic than anionic or PEGylated dendrimers.[91] In order to assess the toxicity of various altered or native dendrimers on different cell lines, comprehensive *in vitro* studies have been carried out. There were also few scientists reporting the dendrimers ' critical *in vivo* toxicity. More recently, Jones et al. performed nano-toxicological experiments on amine-terminated PAMAM dendrimers which revealed that intravenous administration was lethal to mice and triggered coagulation-like disseminated intravascular syndrome.[92] Using flow cytometry and microscopic examination, it was shown that cationic isothiocyanate fluorescein labeled G7 PAMAM dendrimers induced platelet disruption, while neutral (hydroxyl terminated) and anionic (carboxyl terminated) PAMAM dendrimers did not alter platelet morphology or function.

As described in previous paragraphs, many *in vitro* studies were based on the use of conventional 2D culture models, such as cancer cell lines. These models enabled drastic progresses in the understanding of many cellular functionalities.[93,94] However, 2D cultures presented many issues such as the induction of stress conditions, or the lack of cells/cells, cells/extra cell matrix (MEC) interactions which were essential in regulating many cellular functions, especially concerning interactions with the NPs such as NP distribution, uptake and effects.[95,96] These models appeared far from *in-vivo* organization, skewing the accuracy and representativeness of biological studies, particularly the NPs impact evaluation.[97, 98, 99,100.]

In contrast, 3D culture systems, in particular those using a 3D scaffold structure, produced an optimal microenvironment for all the cell functionalities by conservation of the 3D morphology; and the cell interactions by the emission and reception of signals of regulation; leading to the development of a complex 3D network close to the *in vivo* models.[101,102,103] The 3D models were a reliable alternative to the use of animals in order to meet the ethical rule of the 3Rs (Reduce, Replace, Refine) limiting the use of animal experimentation.[99-101] In that way, there was a growing number of studies concerning the development of 3D model used as bio-indicators for the measurement of the toxicity of the NPs.[104,105]

Murthy reviewed the toxicity assessment of various NPs and the reader could find more details in this paper.[106] Briefly, exposure of the inorganic core by deterioration of the organic layer was the main toxicological risk associated with the use of QD *in vivo*. To mention only a few, QDs could be made from a wide variety of inorganic metal complexes such as CdSe, ZnS, CdTe, InP, InAs, GaAs. Each of these compounds had different chemical properties that could have a profound impact on its toxicology. Although there was not comprehensive literature on the toxicity of such *in vivo* substances, there were articles outlining major concerns and suggesting the need for more research. A detailed review of the QDs toxicology was published by Hardman.[107.]Derfus et al. used an *in vitro* model consisting of primary rat hepatocytes to examine the toxicity of a range of cadmium-based QDs.[108] Choosing this type of cell was motivated by the fact that the liver is the main target for exposure to Cd. The acute cytotoxicity of QDs with CdSe cores capped with mercaptoacetic acid (MAA) and TOPO was determined. Ballou et al. investigated the *in vivo* toxicity of either amphiphilic poly(acrylic acid) or PEG-coated CdSe / ZnS QDs in mice.[109] There was no necrosis in the liver, spleen, or bone marrow where the QDs were found to be stored, and the animals remained viable for 133 days when tissue analysis was performed.

The toxicity of superparamagnetic iron oxide nanoparticles used in rats as MRI contrast agents was examined by Muldoon et al.[110] The nanoparticles were either intracerebral or intra-arterially delivered to the brain. Although the intensity of the MRI signal decreased over time (weeks to months), healthy rats did not present any abnormal changes in brain tissue. Such findings were consistent with a toxicity analysis carried out by Weissleder et al on iron oxide nanoparticles in mice and dogs.[111] The safety of various nanoparticles based on iron oxide used in clinical use as contrast agents is now well known.

In their use as X-ray contrast agents, Hainfeld et al. investigated the toxicity of gold nanoparticles.[112] Accumulation was observed in the kidneys and tumors when injected intravenously into mice (retention was low in the liver and spleen). Histology of organs and blood testing showed no toxicity sign up to 30 days after injection.

Polymeric and liposomal nanoparticles are probably the least dangerous class of nanoparticles in terms of toxicity because the particles are usually made from or coated with natural or highly biocompatible polymers (such as PEG). Such particles also beared cytotoxic drugs in drug delivery applications (to kill cancer cells), but were prevented from reaching other parts of the body by the targeted targeting as described earlier in Murthy's review.[104] The use of natural polymers such as chitosan or natural lipids in the assembly of nanoparticles based on polymer or liposome was advantageous as these polymers were not recognized by the body as alien and were readily metabolized. Depending on the polymer form and structure, nanoparticles made from synthetic polymers could vary widely in the rate of clearance from the blood stream and accumulation in mononuclear phagocytic system (MPS) organs (such as liver and spleen).[113] As discussed above, the introduction of PEG into the structure of nanoparticles could delay the removal of nanoparticles from the blood stream. Therefore, PEG-coated particles were considered less harmful than non-coated particles as they were less likely to saturate MPS.[99]

Furthermore, *in vitro* toxicity assays using cultured cells were often used as a method for testing hazardous materials. This experiment offered important information to understand the mechanisms of the materials-induced biological effects. Nano-objects, however, required specific considerations about *in vitro* toxicity assays, since their activities were different from water soluble chemicals. For example, immediately after inserting nano-object samples into the culture medium, the nano-objects underwent changes such as (1) the dissolution of nano-objects into their ionic counterparts, (2) the formation of corona, which was the adsorption of the components of the crop medium to the nano-object surface, or (3) changes in the aggregation / agglomeration state, leading to changes in particle size and sedimentation. Therefore, in clarifying whether the observed effects were relevant to the studied nano-object itself or from other unregulated sources, it was important to consider the aforementioned anomalies and to prevent misinterpretation of the test results. To exclude experimental artifacts *in vitro*, it was important to rigorously define the research suspension prior to and during in vitro toxicity assays. For example, corona formation, the release of metal ions from nano-objects and impurities (residual from the process of nano-object synthesis) could interfere with some *in vitro* assays, resulting in inaccurate results. In addition, agglomerates and aggregates could alter a suspension's toxicity. Therefore, it was critical that the suspension characteristics of nano-objects being tested were carefully evaluated and defined. To reach these aims, several working groups set within the ISO committee in order to clearly delineate these methods (issue on particle characterization should be carried out in TC 24/SC 4.)

## 5. NEEDS OF NPS CHARACTERIZATION

NP surfaces have been continuously modified to provide stability, biocompatibility, selectivity, and functionality for biological applications by binding functional groups or molecules.

*In vitro* and *in vivo* studies have shown that the interactions of nanoparticles with cells are correlated with particle size, shape, and surface characteristics[114,115,116,117,118,119,120] It has been demonstrated that the cellular uptake of nanoparticles is size-dependent, with smaller particles being taken up more easily than larger particles. Nanoparticles with dimensions between 250 nm and 3 μm could be internalized within cells *in vitro* via phagocytosis and micropinocytosis. Nanoparticles smaller than 200 nm, on the other hand, were more likely to involve other cellular uptake routes, such as clathrin- or caveolin-mediated endocytosis, independent endocytosis mechanisms, or passive transport.[111,117] Nevertheless, the internalization pathway would not adhere to these typical size guidelines if there were specific ligands on the nanoparticle surface.[111,121] The enhanced permeability and retention effect could be observed with nanoparticles with diameters ranging from 40–400 nm.[111] Particle size has been shown to affect the circulation time of liposomes.[122] Likewise, the circulation time of dendrimers depended on their size, as only dendrimers with a low generation number and a hydrodynamic radius less than 3.5 nm were likely to be eliminated into the urine.[123]

Nanoparticle shape could influence intracellular nanomaterial trafficking. Hexagonal shapes were shown to be retained in the cytoplasm, whereas rod-like particles could move towards the nucleus by microtubules.[115] The characterization of drug-loaded nanoparticles circulation time of elliptical discs has been shown to be longer than that of spherical particles, and differences in cellular internalization have been observed for cylinders, cubes, and particles of varying rigidity.[124,114,115,117,125] However, the particles would shrink during the drying step, causing an under-estimation of actual particle diameters.[126] The individual sizes of a large number of nanoparticles must be tallied to determine average size and polydispersity index (PDI)

Besides these regularly classified properties, it was necessary to know the concentrations of NPs in biological samples to assess their toxicity and stability and to evaluate their binding in biological samples or cellular media with target materials. Therefore, the classification of NPs required a faster and more effective quantitative analytical process. Pal et al.[127] reviewed the identification, preparation methods, characterization, usage, nanoparticles benefits and health outlook.

Surface charge could also affect nanoparticle biodistribution, opsonization, and toxicity.[116] Typically, larger and negatively charged nanoparticles exhibited less toxicity compared to smaller and positively charged polymeric particles

Precise characterization of nano-object populations was a significant and important measurement task. The following section has listed the different parameters that needed to be characterized and the techniques available. Some discussion would be given regarding the advantages, drawbacks and limitations of each technique.

## 6. PHYSICO-CHEMICAL CHARACTERIZATION AND TOXICITY ASSAYS OF NPS: REVIEW OF THE AVAILABLE TECHNIQUES

As recently highlighted by Modena et al[128]. What to measure ? has been a key question in nanoscience, and it has not been straightforward to address as different physicochemical properties define a nanoparticle sample.

There were various techniques for detecting, measuring and characterizing nanoparticles. Different analytical techniques to analyze the physicochemical parameters of dendrimers have been documented in the literature. This involved spectroscopic, dynamic light scattering (DLS), microscopic, chromatographic, rheological, calorimetric and electrophoretic characterization: Nuclear magnetic resonance (NMR), infrared, ultraviolet (UV)-visible, fluorescence and mass spectroscopy; small angle X-ray scattering, small angle neutron scattering, laser light scattering; atomic force microscopy (AFM), transmission electron microscopy (TEM); size exclusion chromatography, high performance liquid chromatography (HPLC); DSC, temperature modulated calorimetry and dielectric spectroscopy; Polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis. These methods, however, were very time-consuming and complex.[129]

There was not a method that could be selected that is the “best” method but rather a method was chosen to balance the restriction of the type of sample, the information required, time constraints and the cost of the analysis. As highlighted by Mourdikoudis et al.[130] the choice of the most suitable method was complicated by different strengths and limitations of each technique, while often a combinatorial approach to characterization was needed. A straight forward technique would simply detect the presence of

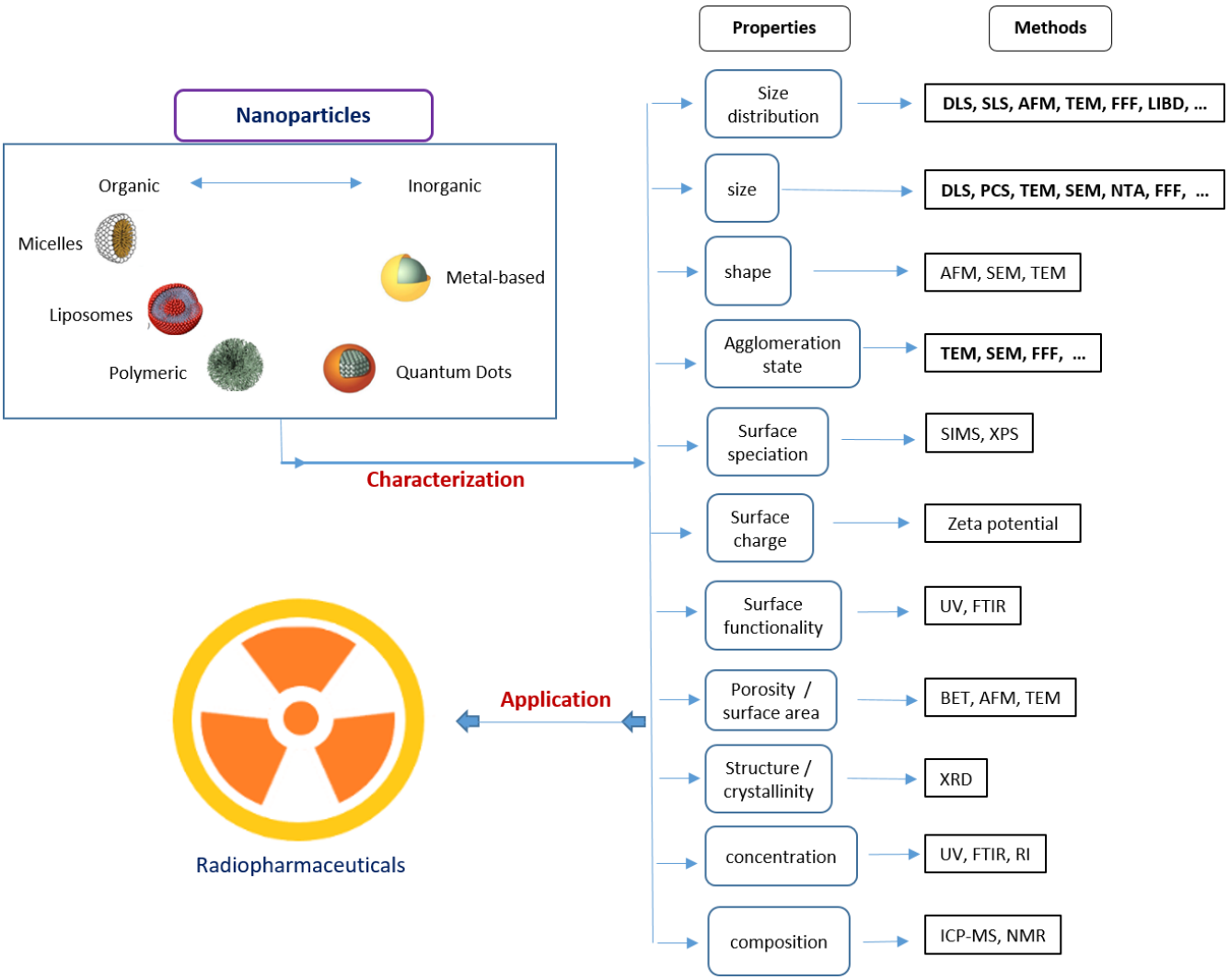


**Figure 1**. Physico-chemical characterization of manufactured nano-objects subjected to toxicological tests for evaluation (from ISO/TR 13014: 2012)

nanoparticles; others would give the quantity, the size distribution or the surface area of the nanoparticles. These measurement techniques differed from characterization techniques for assessing the chemical content of a nanoparticle sample, the reactions on the surface of the nanoparticles or for the interactions with other chemical species present. There was also a divide between techniques that gave information on an amount of nanoparticulate material and those that could look at the individual nanoparticle within the sample. Sometimes measurement techniques would be combined to provide more information from one sample. Different techniques would suit different types of sample.

Stability of working suspension was a key characteristic as it directly influenced the *in vitro* assay conditions in terms of the dose of the nano-objects to the cells. Aggregation/agglomeration and gravitational settling of the nano-objects were major issues that would affect the stability of the suspended nano-objects. The stability should be evaluated for the two characteristics, i.e. the relative change of representative size of secondary particles of nano-objects and the relative change of the concentration of nano-objects in the working suspension resulting from gravitational settling during an *in vitro* toxicity assay by considering experimental duration required for the *in vitro* toxicity assay. Evaluation results of the stability should be expressed in the unit of percent (%) over the time scale for *in vitro* toxicity assay.

There were techniques for *in situ* measurements of samples and others that required treatment of the sample before analysis. Sometimes samples would not be able to withstand the required treatment and decompose or react. The amount of sample required could also vary and restricted choice of technique. Since different techniques provided different information and accuracy, efforts have and would be made to standardize the way nanoparticles were measured to assess occupational exposure, health risks from products and environment risk. All the techniques had related costs whether they were provided by an analysis company or if the equipment was purchased. These would also be a restriction on the choice of technique as with some techniques the ongoing costs of calibration and maintenance, essential to maintaining accuracy, could be substantial. Measurement techniques were continuously evolving as they were stretched and improved by research.

In general, there is a drastic lack of extensive characterization of nanoparticles used in radiopharmaceuticals sciences. In some works, these characterizations have not even been indicated. Some techniques were more appropriate for certain type of Drug Delivery Systems but the goal here has been to give a glimpse of the existing techniques for characterizing nano-drugs in nuclear medicine. The figure 1 summarizes the physico-chemical characterization of manufactured nano-objects subjected to toxicological tests for evaluation (ISO/TR 13014: 2012).

An appropriate method should be selected to measure the concentration change of nano-objects suspended in the biological media from among the light scattering, plasma mass spectrometry inductively coupled (ICP-MS), ultraviolet-visible (UV-Vis) absorption, X-ray transmission and the total organic carbon analysis.

In a recent book, a list of the different techniques has been established to characaterize NPs.[131] In the following, we have drawn a special attention to the size and size distribution determination and how FFF-based techniques could bring a wise and a more complete characterization of nano-drugs. The Table 1 provides a summary of some techniques available as well as a size range for each. The nanoradiopharmaceuticals presented in this paper were ranging from 3nm to 120 nm, with the exception of synovectomy NPs with a size of 80µm.

**Table 1**. Analytical tools to characterize nano-objects and size range

| Type | Technique | | Information | Size range (nm) |
|---|---|---|---|---|
| Batch | Centrifugation Filtration | | Size distribution | 20 – 500<br>3- 500 |
| On-line separation | Chromatography | Size Exclusion (SEC) | Size distribution | 3 -20,000 |
| | | Hydrodynamic (HDC) | | Few nm – few µm |
| | Capillary Electrophoresis (CE) | | | 1 – 10,000 |
| | Field-Flow Fractionation (FFF) | Flow (Fl-FFF) | Size distribution | 1 – 1,000 |
| | | Sedimental (Sd-FFF) | Size distribution + density | 1,000-100,000 |
| Imaging | Electronic Microscopies | Scanning Electron Microscopies (SEM) | Size distribution number of particles shape + structure + chemical composition | 50 -> 1000 |
| | | Transmission Electron Microscopies (TEM) | | 1 - > 1,000 |
| | | Atomic Force Microscopies (AFM) | | 0.5 - > 1,000 |
| Spectroscopies | Raman | | Size | |
| | Light Scattering (LS) | Dynamic (DLS) / Static (SLS) / Multi-Angle (MALS) | Size<br>Size Distribution | DLS : 1 – 500<br>MALS : 20 - 500 |

### 6.1. Morphologies

Calvaresi has recently reviewed the problem of nanoparticles shapes metrology.[132] Many of the new nanoparticles, either synthesized in the laboratory or naturally discovered, were reported without any form of systematic strategy for naming them. The inevitable confusion created by the lack of method could be solved only by categorizing and cross-referencing to previous structures and by adopting a straightforward, consistent and generally agreed nomenclature to define the shape of nanoparticles.

#### 6.1.1. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) provided direct visualization for morphological examination. In morphological and sizing analysis, the techniques based on electron microscopy offered several advantages; however, they provided limited information about size distribution and true population average. Nanoparticles solution should first be processed into a dry powder for SEM characterization, which was then placed on a sample holder and coated with a conductive metal like gold using a sputter coater. The sample was scanned with a focused fine beam of electrons[133]. The sample's surface characteristics were derived from the sample surface's secondary electrons released. The nanoparticles would be able to withstand the vacuum, and the polymer could be damaged by the electron beam. SEM's mean size was comparable to dynamic light scattering results. In addition, these techniques were time consuming, expensive and often required additional information on the distribution of sizes.[134] This technique has been extensively used for characterization of NPs in radiopharmaceutical sciences.

#### 6.1.2. Transmission electron microscopy (TEM)

TEM worked on a different concept than SEM, but often offered the same data sort. It has been employed as SEM for characterization of nano-objects dedicated to radiopharmaceuticals. TEM sample preparation was complex and time consuming due to its need to be ultra-thin for electron transmittance. The dispersion of nanoparticles was deposited on grids or films for support. To order to avoid the instrument vacuum and allow handling, nanoparticles were fixed using either a negative staining substance such as phosphotungstic acid and derivatives, uranyl acetate, etc., or plastic embedding. Upon embedding in vitreous ice, the alternative method was to expose the sample to liquid nitrogen temperatures. The surface characteristics of the sample were obtained by transmitting a beam of electrons via an ultra-thin tube, interacting with the sample as it passed through.[127] This was potentially unrepresentative of the real environment and this could involve a bias in what was interpreted from the picture. “It is only seen what you are looking at”.

#### 6.1.3. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) provided ultra-high resolution in particle size measurement and was based on a physical scanning of samples at sub-micron level using a probe tip of atomic scale.[135] The instrument offered a topographic map based on forces between the tip and the surface of the sample. Depending on their properties, samples were usually screened in contact or non-contact mode. The

topographical map was created in contact mode by tapping the probe onto the surface across the sample, and the probe hovers in non-contact mode over the conducting surface. AFM's primary advantage was its ability to image non-conductive specimens without any specific treatment, enabling the imaging of sensitive biological and polymeric nano and microstructures.[136] AFM provided the most accurate size and volume distribution description and did not require computational consideration. In addition, the particle size obtained through the AFM technique provided a real image that helped to understand the effects of different biological conditions. [137] This technique has been employed notably for characterizing NPs for multimodal imaging when stable metals were in the NPs.

#### 6.1.4. Thermal Field Flow Fractionation (Th-FFF)

Thermal FFF (ThFFF) was one of the first FFF method and device to be developed.[138,139,140] The fractogram showed the signal for detecting analytes over time. In ThFFF the field applied consisted in a high temperature gradient ($\approx 10^4$°K $cm^{-1}$) generated by clamping the channel spacer between two heat conducting blocks. One was a hot block constituting the depletion wall, for which the temperature was controlled by heating element. The second, the cold block, constitutes the accumulation wall for which the temperature was regulated by water or coolant circulation. In ThFFF, the field, which depended on difference in the temperature between the two blocks (≈ 80-100°K), was proportional to the Soret coefficient *St* = Dt/D, where *Dt* was the thermal diffusion coefficient, *D* the diffusion coefficient, and dT/dx the applied field. By measuring the retention ratio ($t_0/t_r$), it was possible to extract from the fractogram the value of *St*, for which *D* depended on the molar mass of the species, while *Dt* depended on the chemical composition of the sample as well as the mobile phase nature and composition.[131,,141,142]

Then, if *Dt* was known for the particles or the studied polymers, *D* could be easily obtained from the UV fractogram by calculating *St*, and could be in turn used to calculate the $M_W$, as $D = A.M_W^{-sm}$, where A was obtained from the calibration plot, and –Sm (mass based selectivity) was around 0.6, depending on the nature of mobile phase.[137,143] The hyphenation of ThFFF with MALLS and DLS could be very helpful to directly measured $M_W$, without any calibration and then to calculate *Dt* from *St*.[137,144]

Concerning *Dt*, it specifically depended on the polymers composition and microstructure which was an important value of ThFFF methods that have been used to characterize a great variety of polymers (homopolymers, polymers blends, copolymers, microgels…) nanoparticles (interfacial composition) colloids and aggregates. [137,145,146] Some of these polymers were then employed for the synthesis of NPs designed for radiopharmaceutical purposes. But, to our knowledge, thermal FFF has not been used so for a direct characterization of radiopharmaceutical NPs. For NPs, *Dt* more exactly corresponded to a thermophoresis, then retention ratio depended on the NPs size for having homogeneous surface composition, but also depended on the nature of coating, adsorbed or uptake material. [134] Then by coupling ThFFF with MALLS, DLS, viscosimeter, SEM, MRI, it would be easy to characterize the size and the surface composition of NPs,[137-140] that could be helpful for characterization of multilayer NPs along their synthesis process, further use or interactions with environment, such as differential SdFFF. [147]

### 6.2. Surface properties of nanoparticles

#### 6.2.1. Why and how surface functionalization is required

Surface functionalization of NPs was required for several reasons:

1-Colloidal stability: the addition of functional groups at the surface of NPs played a role on electrostatic and/or steric repulsive forces balance as well as on Van der Waals attractive forces.

2-Active targeting: it lead to anchoring of recognition vectors of the diseased cells like antibodies.

3-Circulation time: it lead to anchoring of anti-biofouling polymers (PEG) to avoid uptake by the macrophages.

The surface functionalization has been realized through:

- Organic ligands, with very high affinity for the surface, were needed to avoid *in vivo* desorption
- Organic polymers: they were like a set of ligands at the surface but all linked together thus really harder to desorb
- Inorganic polymers: The nanoparticle was encapsulated in an inorganic shell, like silica.

In addition, nanoparticles had different structures and shapes besides being organic or inorganic. Nanoparticles could be of various forms and shapes such as spherical, tubular, irregularly shaped. Also, they could be separated based on if the nanoparticles could exist in fused aggregates or agglomerated forms.[79]

The association of a drug with conventional carriers lead to changes in the profile of drug biodistribution, as it was mainly delivered to the mononuclear phagocyte system (MPS) such as liver, spleen, lungs and bone marrow. The host immune system could identify nanoparticles when administered intravenously and removed from the bloodstream by phagocytes. Nanoparticle hydrophobicity determined, apart from the size of nanoparticles, the level of blood components (e.g., opsonins) binding this surface. Hydrophobicity thus affected the *in vivo* fate of nanoparticles.[78] Nonetheless, unmodified surface nanoparticles (conventional nanoparticles) were easily opsonized and massively cleared by the MPS once in the blood stream.[148]

It was important to reduce the opsonization and extend the distribution of nanoparticles *in vivo* to increase the likelihood of success in drug targeting. This could be achieved through coating nanoparticles with hydrophilic polymers / surfactants or by formulating biodegradable copolymers with hydrophilic properties such as polyethylene glycol (PEG), polyethylene oxide, polyoxamer, poloxamine, and polysorbate 80 (Tween 80).

Studies showed that PEG on surfaces with nanoparticles prevented complement and other serum factors from opsonizing. PEG molecules with brush-like and intermediate structures decreased phagocytosis and complemented activation, while PEG-like structure surfaces were strong complementary activators and favored phagocytosis. [149]

Differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) were the main methods used to characterize and quantify the amount of free and bound water in hydrogels. The proton NMR provided information on the interchange between the

so-called free and bound states of water molecules [150] The usage of DSC was based on the premise that only the free water could be frozen, and it was presumed that the endotherm determined when the frozen gel was heated, reflected the free water melting and that value would produce the amount of free water tested in the hydrogel sample. The bound water was then obtained by the difference in the total water content of the hydrogel sample and the free water content measured.[151] thermo-gravimetric analysis, [152] [153154] X-ray diffraction, [155] sol-gel analysis, [156] etc... were also used to confirm the formation of hydrogel cross-linked network gel structures.

Gel permeation chromatography coupled on line with a multi angle laser light scattering (GPC-MALLS) was a commonly used technique for determining a polymer system's molecular distribution. This method could be used to quantify hydrogel in a polymeric process.[157] This approach was widely used to measure specific hydrocolloids such as gum arabic, gelatin and pullulan hydrogels. How the mass recovery data obtained from GPC-MALLS compared with the actual amount of hydrogel collected in solid state for dextran radiation couldbe demonstrated.[158]

### 6.2.2. Zeta potential

Nanoparticles zeta potential was widely used to describe nanoparticles surface charging properties.[159] This represented the particle's electrical potential and was determined by the particle structure and the medium it was distributed through. The type and strength of nanoparticles surface charge was very important because it determined both their interaction with the biological environment and their electrostatic interaction with bioactive compounds. The colloidal stability was analyzed using nanoparticles zeta potential. This potential was an indirect measure of the surface charge. It refered to the potential difference between the shear surface and the outer Helmholtz plane. Measuring the zeta potential allowed predictions of colloidal dispersion storage stability. To ensure stability and avoid aggregation of the particles, high zeta potential values, either positive or negative, should be achieved. Nanoparticles with a zeta potential above ± 30 mV were shown to be stable in suspension because the surface charge prevents particle aggregation. It was then possible to predict the extent of surface hydrophobicity from the zeta potential values. The zeta potential could also provide information about the nature of nanocapsulated or surface-coated material.[160]

Surface charge, expressed as zeta potential, critically influenced the interaction of an NP with the environment, but it has not been systematically determined for NPs aimed to radiopharmaceuticals. [161] There were two liquid layers surrounding an NP: strongly bound inner part (Stern layer) and weakly bound outer layer. Zeta potential was commonly measured by laser Doppler electrophoresis, which evaluated electrophoretic mobility of suspended NPs in the medium, thus measuring the potential at the boundary of the outer layer. One limitation was that in bimodal samples the zeta potential value of larger particles dominated the scattering signal of smaller particles, similar to DLS size measurements.[162] The zeta potential measurement depended on the strength and valency of ions contained in the NP suspension. High ionic strength and high valency ions compressed the electric double layer, resulting in reduction of the zeta potential.[163,164] The pH, the concentration of hydrogen ions in the medium, greatly influenced the zeta potential as well. When the suspension was acidic, the NPs acquired more positive charge, and vice versa. Therefore, a zeta potential value without indication of solution pH was a virtually meaningless number. It was recommended that information of the NP suspension was precisely described in reporting the zeta potential, including the ionic strength, composition of the medium, and the pH. [165,166] For comparison of results across different studies, it was conceivable to normalize the zeta potential by pC.

### 6.3. PARTICLE SIZE

The most important characteristics of nanoparticles were the particle size and distribution of the sizes. They assessed the ability of these delivery systems for *in vivo* transmission, biological fate, toxicity, and targeting. They would also affect drug loading, drug release, and nanoparticles stability. In addition, some good laboratory practices and harmonized methods needed to be developed. It was usually more convenient to measure the particle size of nanoparticles in a liquid suspension by dynamic light scattering (also known as photon correlation spectroscopy) because, it was fast and easy. Characterization of particle size was incomplete without the polydispersity index (PDI), which described the width of the particle size distribution.

Nanoparticles had a number of advantages over microparticles in many experiments.[167] Nanoparticles usually had relatively high cell absorption compared to microparticles, and due to their small size and mobility, they were accessible to a wider range of cell and intracellular targets. After the opening of endothelium tight junctions with hyperosmotic mannitol, nanoparticles could cross the blood-brain barrier, which would provide a sustained supply of therapeutic agents for difficult-to-treat diseases such as brain tumors.[168] They have also been shown to cross the blood-brain barrier for Tween 80-coated nanoparticles.[169] The majority of cell types took up submicron nanoparticles, but not larger microparticles.[170] In fact, 100 nm nanoparticles had a 2.5-fold higher absorption rate than 1 μm microparticles and a 6-fold higher absorption by Caco-2 cells than 10 μm microparticles.[171] A similar study found that nanoparticles penetrated throughout the submucosal layers of a rat intestinal loop system, while microparticles were primarily located in the epithelial lining. [172] This indicated that by controlling particle size, at least in part, the distribution of particles could be tuned. Particle size also influenced the release of medications. Smaller particles had a greater surface-to-volume ratio; therefore, most of the drug associated with small particles would be on or near the surface of the particles, resulting in a faster release of drugs. In addition, larger particles had wider cores that made it possible to encapsulate more drugs per particle and to release more slowly.[164] Thus, particle size control provided a means to adjust the rates of drug release.

Different particle size analysis methods also yielded different equivalent sized, that was important to consider when comparing size values obtained using different methods. Different techniques gave different size averages depending on if they fundamentally relied on an instrument response to particle numbers, volume, mass or optical property. The averages were the same for spherical, monodisperse particles. It had to be taken into account that there would be part of the nanoparticle (or nanoparticle aggregate) size

distribution that was “hidden” for the applied method.[173] For instance, it was indicated that DLS induced a minimum perturbation of the sample for size analysis whereas it was put at a low level for FFF techniques and was great for microscopy techniques (SEM/TEM). In a review from Hasselov,[170] fractionation methods were already highlighted. Nanoparticles were a hot topic and the question of their characterization was already of great important couples years ago. This fact has not changed.

As highlighted by Singh and Lillard,[174] photon-correlation spectroscopy or dynamic light scattering were the fastest and most routine methods for determining nanoparticle size. Photon-correlation spectroscopy required the medium's viscosity to be known and determined the particle's diameter by Brownian motion and properties of light dispersion.[175]

This technique provided information of particle size, size distribution and a real time view of the nanoparticles in the sample. The sample would be a suspension for which a wide range of solvents could be used. It was placed on an optically opaque background and a laser light used so that the nanoparticles could be directly visualized through an optical microscope. A digital camera was also used to record the observed particles. Software could then produce a frequency size distribution graph with a range of sizing from 10 to 1000 nm.

### 6.3.1. Photon Correlation Spectroscopy (PCS)

PCS measured the scattering pattern produced when light was shown through a sample. It combined this with calculations of the diffusion caused by Brownian Motion in the sample in a relationship described in the Stokes-Einstein equation. This would give the radius of a particle and therefore an estimation of the average particle size and distribution of particles through the sample. The size was based on a spherical pattern that could be a problem for some NPs. The sample would be a liquid, solution or suspension. It would also be very dilute or the scattering of light could be unclear. This high dilution could also alter the properties of the suspension. The technique was sensitive to impurities and the viscosity of the sample should be known. The range of particle sizes that could be measured, has been quoted between 1nm - 10µm. To our knowledge, this technique has not been employed with nanoradiopharmaceuticals.

### 6.3.2. New insights from FFF-based techniques

#### 6.3.2.1. Sample preparation

The capacity to isolate and analyze diverse populations of nano-objects and their agglomerates, often suspended in, or extracted from, complex matrices, is critical for applications ranging from materials discovery and nano-manufacturing to regulatory oversight and environmental risk assessment. Now, there is a growing interest in the very last decade for nanoparticles for medicinal purpose and for the evaluation of their impact and toxicology. For environmental purpose, samples contained complex mixtures of particles and it was mentioned that pre-fractionation was often required. Microfiltration was one of the most commonly used technique but with many artifacts. Fractionation by ultrafiltration, nanofiltration and dialysis were described.[170] Membrane fractionation could either be performed by applying pressure to overcome the pressure drop across a membrane that sieved molecules or particles depending on their size as in ultrafiltration, or it could be performed by allowing solutes to balance across the membrane as in dialysis. The microfiltration artifacts mentioned above became greater as the pore size of the filter decreased (ultrafiltration and nanofiltration). This was especially critical where membranes were used as macromolecular sieves.

Ultrafiltration was a preparative size fractionation method that could be scaled to process large sample volumes and produced large quantities of isolated nanomaterials. Although it was limited to two fractions (above and below the membrane pore size), multi stage filtrations could allow for a crude size fractionation, however, this was extremely labor and time intensive.

Dialysis was an ultra- or nanofiltration method that operated on diffusion of solutes across a membrane that arose from concentration gradients and osmotic pressure instead of pressure driven filtration. Dialysis was a very mild fractionation method and it could be used to separate truly dissolved components (ions and small molecules) from their nanoparticle counterparts.

Furthermore, the ability to characterize these analytes with minimal perturbation of their natural or native state was highly desirable. The list of available techniques capable of achieving such objectives was relatively short, and while all techniques had advantages and disadvantages, and no single technique was solely adequate or appropriate for all possible applications and materials, There was a rapidly growing interest in the analytical approach frequently referred to as ' hyphenation ' among the many different particle measurement tools currently available, either commercially or as prototype designs. Upstream particle fractionation was coupled with one or more downstream online detectors in the hyphened approach. Hyphenation enabled size-resolved physico-chemical characterization of complex NP populations under in situ conditions that minimally disrupted the native dispersed environment. For such applications, a variety of upstream fractionation methods were explored, including size exclusion chromatography, electrospray–differential mobility analysis, capillary electrophoresis and various forms of field flow fractionation.

#### 6.3.2.2. FFF Techniques

Flow field-flow fractionation (fl-FFF) is perhaps the most widely adaptable method of fractionation for nanoscale-to-microscale particles among commercially available methods. A group of related separation techniques known collectively as field-flow fractionation (FFF), conceptually proposed by J. Calvin Giddings in 1966 offered many advantages for nanotechnology applications.[176] In FFF, the analyte, suspended in a liquid medium, was fractionated by the application of a cross-field (e.g., flow, centrifugal, electric, thermal-gradient, magnetic) perpendicular to the direction of flow of the analyte and mobile phase eluting through a thin defined channel. Separation occured when the analyte responded to the applied field, such that populations with different response sensitivities reached equilibrium positions (i.e., in equilibrium with diffusional forces) higher or lower in the

laminar flow streamlines, thus eluting differentially. There was no solid phase that could degrade NPs (especially in the case of organic polymers).The limitations of the techniques were related to the high dilution of the sample that would not reflect the suspension and the possible interaction of NPs with the membrane, but that was usually quantified. Among the FFF variants, asymmetric flow FFF (AFFFF or A4F or AF4) and centrifugal FFF (CFFF), were available commercially and have been most widely adopted in the nanotechnology field.[177,178] AFFFF was arguably the most versatile technique with respect to the wide range of applications, materials and particle sizes to which it has been applied. Symmetric flow FFF (fFFF), the original "flow" based technique as first described in 1976,[179] has been supplanted commercially by AFFFF, introduced in 1987,[180] due to several advantages, including a simpler channel design, the ability to visualize the sample through a transparent upper wall, and reduced analyte band width. The theory and application of CFFF (also called sedimentation FFF or SdFFF) as it is presently applied was described by Giddings and coworkers in 1974,[173] although a centrifugal field based FFF system was first developed and tested independently by Berg and Purcell in 1967.[181] There were different elution modes possible in FFF, but for particles in the nanoscale size range the principal elution mode was referred to as normal (or Brownian). In normal mode elution, Brownian dynamics dominated such that particle concentration diffusion balances the flux induced by the perpendicular field (a cross-flow in the case of fl-FFF) that would otherwise drive the analytes toward the accumulation wall. In this case, translational diffusion was the key parameter that controlled the concentration profile of the analyte. Other FFF variants, such as thermal, electrical and magnetic, provided unique capabilities, but have been limited in the scope of their applications vis-a-vis nanotechnology or commercial availability.

Where FFF was once predominantly the domain of specialists, these instruments are now commonly and increasingly utilized by non-specialists in government, industry and academic laboratories as part of the nano-characterization toolbox. Two factors are driving this increase in nanotechnology utilization: maturation of commercial instrumentation and versatility with respect to coupling a wide range of detectors to FFF systems. In the latter case, recent developments have led to the use of highly sensitive elemental detectors (e.g., an inductively coupled plasma mass spectrometer or ICP-MS), which offered enhanced characterization and quantification for many materials. Additionally, traditional concentration or sizing detectors, such as ultraviolet visible (UV-Vis) absorbance, fluorescence, multi-angle light scattering (MALS) and dynamic light scattering (DLS), yield on-line data for eluting populations, and theoretically provide more accurate information then obtainable using off-line measurements of unfractionated mixtures. Depending on theoretical relationships or comparison with a defined size norm, the measured retention time of an eluting peak could also be used to assess the hydrodynamic size by AFFF. On the other hand, AFFF had the unique capacity to rapidly separate species of the same size but differing in density.

Although developed primarily for polymer analysis, combined with the advent of commercially available equipment, the dynamic range had greatly increased the prominence of fl-FFF over the past decade, extending its application to NPs and colloidal particulate matter.[170,171,182,183,184,185,186] The main advantage of fl-FFF was its ability to provide accurate size information and fractionation in complex populations with minimal contact between the analyte particles and the channel of separation, depending on hydrodynamic forces to achieve diffusion-based separation (length and form). The main drawback of fl-FFF has been the lack of defined protocols and guidelines for its implementation, hence the persistent burden of repeated production of sample-specific methods. Fl-FFF has been employed with liposomes and inorganic NPs for radiotherapy, with $^{212}Pb/^{212}Bi$ or with $^{166}Ho$ notably (see corresponding sections above).

In order to develop and validate methods for application of FFF to the analysis of nano-objects and their agglomerates, and to properly report experimental results and conditions in order to enable reproducibility across laboratories, it was critical to specify key parameters that should be controlled and reported. These parameters defined all aspects of FFF methodology, including sample/analyte, instrumentation, fractionation, calibration, qualification, performance specifications, and data analysis.
The review by Gigault et al. proposed a detailed and logical measurement strategy that offers specific guidance for applying asymmetrical-flow field flow fractionation (A4F) to the size-dependent separation and characterization of dispersed in aqueous media nanoscale particles (NPs).[187]

### 6.3.3. Sedimentation field-flow fractionation (SdFFF)

Among the FFF family, Sedimentation Field-Flow Fractionation (SdFFF) is a technique that could serve to characterize the surface functionalization of NPs. Sd-FFF corresponds to methods utilizing a sedimentation external field. Regarding to the nature of the field, two sub-techniques could be distinguished, the gravitational FFF (Gr/GFFF) using the simple earth gravity, and the centrifugal FFF, classically defined as SdFFF, using a multi-gravitational field.[170,188] GrFFF was based on the use of a weak, poorly modulate field which led to long elution time to reach an efficient separation. Nevertheless, GrFFF was achieved in one of the simplest and cheapest FFF device that could be built in any lab by the hermetical sandwiching of a Mylar spacer, in which the separation channel was cut, between two plastic plates constituting the depletion and accumulation walls.

In contrast, SdFFF needed the development of specific devices which were ones of the most complexes and expensive between FFF family. It required the insertion of the channel (Mylar band) in a centrifuge device in which the rotating speed define the intensity and the variation of the multi-gravitational external field strength. The second important parts were the rotating seals allowing the mobile phase and sample flowing from the injection port to the detectors, through the rotating channel without leakage. Commercial apparatus (Postnova) were available, or some have been developed by FFF groups.[189,190] The advantage of this complex instrumentation was the ability to produce a strong, variable and programmable external field allowing fast and efficient separation of a broad range of particles (20 nm-100 µm) in the range of nano-particles, colloids, polymers, microbiology (virus, yeast, bacteria), or eukaryote cells sorting. The development of device reaching high rotation speed, associated with the high size selectivity, defined SdFFF as a very promising device for NPs analysis.[191]

An important contribution for characterization of NPs used in the biomedical field was due to K. Caldwell and co-workers. One of the first study [192] concerned the surface modification of polystyrene (PS) nanospheres by amphiphilic surfactants polyethyleneoxide/polypropyleneoxide (PEO/PPO) pluronic (F108) and tetronic (F908) acids block copolymers, in order to produce injectable therapeutic or diagnostic agents with an increased body diffusion and life time, by reducing macrophages capture. SdFFF and Photon Correlation Spectroscopy were used to study the adsorption mechanism of surfactant by measuring the thickness of adsorbed materials, as well as the percentage of the surface coverage[10]. The 75nm PS NPs coated by F108 pluronic acid presented an increased half-life without side effect linked to a low plasma proteins attachment. Fromell et al. showed that SdFFF could be very useful to characterize multilayers NPs all along their synthesis steps.[193]

SdFFF was also used to accurately quantify the kinetics of F108 adsorption (4% F108 solution) on different PS NPs, demonstrating that an 80% surface coverage could be achieved in one hour incubation.[194] In 2003, Frommel and Caldwell studied the surface-dependent functionalization of bare and coated PS NP by proteins. [195] As the adsorption of F108 linker on PS was done by the PPO hydrophobic part,[178] the flanking hydrophilic PEO block still available for further coupling (proteins) and modifications such as the introduction of a pyridildisulfoxide group (PDS).[196] PDS could be used in turn to control immobilization of many biomolecules (proteins, oligonucleotides…) without affecting their properties, leading to a great variety of functionalized NPs. Then, SdFFF and UV-Vis spectroscopy were used to control step by step multi-shell NPs construction from the bare PS particles/ PS-F108 / PS-F108-DPS to PS-F108-oligonucleotides (decamers of guanine or cytosine dG or dC). [197] Anderson et al. measured that $4.5\times10^{-16}$ g /NP of F108 (≈ 18000 molecules/particles) and $7.9\times10^{-17}$ g /NP of dG (≈ 13000 molecules/particles) were adsorbed, demonstrated the high potential of SdFFF to characterized multi-shell NPs involved as recognition system in immunoassays as dot blotting and ELISA.[196] Anderson et al used the PS-F108-DPS system to covalently fixed a cell adhesive peptide containing the Arg-Gly-Asp (RGD) motif increasing intestinal transport of modified NPs. Here again, SdFFF was used as a high resolution technique to measure the surface concentration and the number of attached molecule/particles.[196] Fromell et al. had developed on the same base a multilectine nanoparticle array used for glycoprotein mapping.[198] In this way, they produced F108-DPS NP coated both by 15mers of dC, as array surface attachment system (to a 15mers dG), and concanavalin A (Con-A) as glycoprotein recognition system. SdFFF was then used to characterize surface modifications,[190, 191] showing that a number as low as 700 molecules of Con-A were attached per NP, with a precision of ± 55 molecules. Similar NP system was developed to produce particulate platform for bio-luminescent imaging sensor with a femtomole detection limit.[187] More recently, SdFFF allowed the characterization of PS-F108-DPS N coated with ricin antibodies in order to produce probe for ribosome inactivating protein assays.[199]

Another important contribution to biomedical NP studies was due to Contado and co-workers.[200] Leo et al. deployed the nanoprecipitation method to encapsulate a prodrug (5'-octanoyl-CPA (Oct-CPA) of the antiischemic N-6-cyclopentyladenosine (CPA, drug) in poly(lactic acid) (PLA) nanoparticles. SdFFF determined PLA PSD, helping to the best formulation and purification processes to obtain optimal NPs stability and drug release.[201] The effectiveness to characterize these NPs by A4F and SdFFF were compared, demonstrating that both techniques have a good accuracy, nevertheless Flow FFF was limited by the used of membrane and aqueous mobile phase, while SdFFF need to know the particle density to convert retention to PSD. SdFFF was also performed to test the effect of various concentration of F68 pluronic acid on PLGA (polylactic-coglycolitic acid) nanospheres sizing, stability, aging and usability for brain-drug transport.[202] Dalpiaz et al. also developed PLGA NP containing Ursodeoxycholic acid (UDCA) conjugated with AZT (prodrug UDCA-AZT) to treat AIDS and hepatis.[203] Here again, by measuring PSD, SdFFF was very helpful to optimize formulation and loading processes for optimal *in-vivo* release.

Esposito et al. worked on Mono-olein Aqueous Dispersions (MADs) and Nanostructured Lipid Carriers (NLCs) as Bromocriptine carriers (anti-parkinsonian drug).[204] SdFFF was particularly helpful to measure particle size-dependent drug encapsulation and long-term released by NLCs. NLCs was also used to carry other antiparkinsonian drug such as rimonabant or Levodopa,[205,206] or also antimicrobial molecules.[207] The bio-distribution of NLCs was assessed by incorporation of Tc-99m based tracer, allowing a tomographic image of rat body by small-animal PET scanner.[208] In these studies,[204,205] SdFFF was used in association with Cryo-TEM, Photon-Correlation-Spectroscopy and X-Ray diffraction to characterize PSD and structure of NPs. According to EC recommendations, for illustration, and even if it was not directly related to Nuclear Medicine, Contado et al. performed similar characterization techniques concerning food additive silica NPs, before examining biological and toxicological effect of these NPs presented in food sample.[209]

SdFFF was also used to study gold NP [210,211] and quantum dots,[212] particles for which the size and PSD defined the colors of emitted light, and constituting a key step of the quality control along they production and utilization processes. Unfortunately, so far, Sd-FFF has not been used for nanoradiopharmaceuticals. SdFFF displayed many advantages against Dynamic Light Scattering or TEM methods, in the case of polydispersed population characterization.[211]

Beside measurement of absorbed mass or molecule number per particles, or determination of PSD, SdFFF could also implemented in NPs detection protocols in various biological matrix as well as at cellular or tissue level.[213,214] Until now, the principal FFF method used to determine cellular uptake, distribution, elimination or toxicology was A4F.[215,216,217]

Additionally to NPs characterization goal, in the future a multi-FFF platform approach based on A4F and SdFFF could be proposed. Indeed, SdFFF was used until 80's as cell sorting method in many areas such as oncology, neurology and stem cells applications, acting as a rapid, gentle and non-invasive method based on the cell intrinsic biophysical parameters: size, density, shape and rigidity. [218219] This label-free method took advantages on FACS or MACS, when commercial labels did not exist or when they could interfere with further cell uses (culture, transplantation)

or when they could induce differentiation (stem cells).[220,221] SdFFF was used to sort enriched specific sub-populations either from cell lines, tissues or biopsies. As example SdFFF allowed neurons, astrocytes of various degrees of maturity or neural stem cells sorting. [222,223,224,225] Then, SdFFF could be used to prepared enriched neural sub-population of interest, either from normal or pathological models that could be used in turn as support to test cell uptake, distribution, elimination in order to asses NPs toxicity, or to improve NPs targeting. Finally, both Sd and A4F methods could be performed to asses NPs behavior in *in-vitro* and *ex-vivo* models. In conclusion, Sd-FFF appeared as an important tool to characterize and control NPs, and the commercial availability of devoted apparatus would certainly help to technology spreading.

### 6.3.4. Capillary electrophoresis (CE)

Capillary Electrophoresis was considered to be a useful technique for the evaluation and analysis of NPs. CE offered an alternative and rapid analytical technique that showed good efficiency in NPs research and analysis. CE was one of the major separation tools because it could be used to separate a wide variety of compounds — from small ions to biomolecules like proteins and DNA. However, the combination of CE with laser-induced fluorescence (CE – LIF), which was considered one of CE's most sensitive detection modes, offers optimum sensitivity and specificity for analyzing trace compounds in complex sample matrices. Therefore, the number of CE applications in biological research was expanding rapidly; CE was now also being used in medical and pharmaceutical sciences and environmental studies since the first CE study in the 1980s. CE had recently developed into a powerful technique that quickly provided information on the size and surface characteristics of NPs in a very simple way, allowing the study of their toxicity, stability and aggregation in biological samples. NPs could also enhance the separation of compounds in a CE process because they could be used as a pseudo-stationary step to adjust the capillary to isolate and analyze various complicated compounds. Consequently, the number of studies and implementations of NPs in CE has been increasingly growing, and several papers on NPs in CE systems, including several review articles, were published over the past decade.

CE was primarily used for the separation of many different types of molecular species (including compounds and many other biopolymers) on the basis of differences in charge and hydrophobicity (in addition to size), using various modes of separation, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary isoelectric focusing, capillary gel electrophoresis (CGE), capillary electrochromatography (CEC), and affinity CE. These different CE modes have been used for the analysis or application of NPs in medical, pharmaceutical, and biological areas of study. CZE was the simplest of these CE modes; it was most often employed for the analysis and separation of various kinds of compounds, such as biological cells, nano- and microparticles, and small ions.[226]

Recently, in the analysis of inorganic materials, CE was demonstrated to be one of the most powerful separation techniques. In particular, using CZE and CGE, water-soluble NPs that were charged in aqueous solution, were separated and analyzed under electric fields on the basis of their electrophoretic mobilities. A variety of materials of different sizes could be separated in this manner, including inorganic NPs. Size-dependent separation of particles with approximately equal surface-charge densities was possible with CE analysis.[227] Many studies have shown that CE could be used to effectively determine the size of NPs, but not with a radiopharmaceutical purpose.[228,229] CE combined with a diode-array detector or a dark-field microscope could be used to determine the chemical nature of NPs and could be used for the visual and size analysis of NPs.[230,231]

In contrast to CZE, MEKC, CGE, and CEC were developed to improve the separation efficiency of compounds using materials such as polymers or surfactants as pseudo-stationary phases within the capillary. In MEKC, the analyte was separated according to differences in the partition coefficients in the micelle (the pseudo-stationary phase) and the running buffer. MEKC was widely utilized for the separation of small molecules, both charged and neutral.[232] Moreover, CGE was effectively used to purify crude NPs with the polydispersity.[233] NPs were also used as a pseudo-stationary phase for the improvement of the separation efficiency of analytes in the CGE system.[234] Another technique for biological investigations was CEC, which combined the advantages of chromatography and CE. When a sample was injected, its separation occured on the basis of two mechanisms, which would operate separately or together: (1) partition between the mobile and stationary phases and (2) differences in charge. This technique provides improved sensitivity and selectivity, compared with HPLC, for the quantification of neutral and charged compounds with packed capillaries. CEC has been extensively applied in biological, medical, and environmental studies and in food analysis, because CEC has been a separation technique that combines the selectivity of HPLC with the efficiency of CE.

In biological and medical studies, the need for multiple analyses has emphasized the importance of achieving high throughput via automation. The development of miniaturized systems has provided improved sensitivity and high- throughput analysis. Recent advances in the integration of CE with miniaturized-chip technology have dramatically increased the potential of CE in the area of high-throughput measurements because of the simple CE instrumental configuration. [218] The development and applications of microchip-based CE have been ontinuing, because high-throughput and effective analysis of bio-molecules has been needed in various areas of research. In particular, the use of microchip-based CE for the characterization and analysis of biomolecules such as DNA, proteins, and peptides in the biological and biomedical areas has greatly increased.[236] However, the microchip CE system had limitations with regard to performing stable and reproducible separations due to the adsorption of analytes onto the channel surfaces. A complete review of the analysis of nanoparticles using CE has been done by Ban et al. with sizes ranging from few nm to couple hundreds nm.[236]

### 6.3.5. Size Exclusion Chromatography (SEC)

Size Exclusion Chromatography is a chromatographic method in which molecules in solution are separated by their size and in some cases by their molecular weight. SEC could be applicable for NPs smaller than 100 nm. Different examples for sizing NPs have been achieved for Au/Pt core/shell NPs [235] iron-based ferritin nanocores

[236] and synthesis and characterization of gold NPs for cancer imaging.[237] A porous packing material was thus required and unspecific adsorption could cause unwanted interactions which would require the addition of additives to block sorption sites. The limitation of SEC was that calibration standards were required. The method had lower resolution and particle discrimination compared to FFF methods, leading to poorer analytical accuracy. [238] Deformable particles such as emulsions and biological cells were not analyzable by SEC. SEC combined with different detection techniques such as voltammetry, ICP-MS, DLS, multi-angle light scattering (MALS) and that has been successfully applied to the characterization of QDs and carbon nano-walls.[239] To our knowledge, SEC has not been employed for a direct characterization of NPs containing a radionuclide, but could be used for further purification of radiolabeled compounds before *in-vivo* injections.

### 6.3.6. HydroDynamic Chromatography (HDC)

HDC is a size-based separation method. The column is packed with a non-porous micro-particles and separation is achieved by flow velocity and the velocity gradient across them. HDC coupled to ICP-MS was applied to characterize NPs in environment (Ag-NPs in sewage sludge for instance) but not in biomedical area.[240] We just quote the technique for being quite exhaustive.

## CONCLUSION

Nanoparticles have been appealing to medical purposes because of their essential and special characteristics, such as their much larger surface-to-mass ratio than that of other particles, their quantum properties, and their ability to adsorb and hold other substances. In addition, nanoparticles have been able to bind, adsorb and hold other compounds such as drugs, probes, and proteins due to their relatively large surface area. The development of nanotechnology in the medical field has the potential to influence the science in a positive way. Nano delivery systems have great potential to resolve some of the obstacles to reach a number of different types of cells efficiently. This has been an innovative way to overcome drug resistance issues in target cells and promote drug transport through barriers (e.g., BBB). Recent innovation in the food industry has enabled us to expect that nanomedicine would eventually be integrated into nano-food.

The objective of regulatory affairs is to protect public health. It is mandatory to control the safety and efficacy of products, which are intended to be used clinically such as pharmaceuticals, medical devices, veterinary medicines, agrochemicals and cosmetics. Regulatory affairs are particularly important in case of health-care industries like pharmaceuticals, foods, *in vitro* diagnostics, biologicals, nutritional products, cosmetics and medical devices. The continuously emerging new concepts in the field of drug delivery like nano-carriers (nanoparticles, dendrimers, carbon nano tubes etc.), diagnostic agents, bioactives etc. have increased the importance of regulations for new pharmaceutical products.

Researchers need to keep in mind that each nanoparticle property (i.e. small size, large surface area, chemical composition, solubility and geometry) determines the biological response, and careful evaluation of its effects on biological systems are critical for further development and implementation.

Among all the panel of techniques described in this paper, FFF-based techniques are one of the most powerful tool that cover wide applications in the biomedical area.

Also, toxicity of each component needs to be thoroughly analyzed since information on nanoparticle toxicity in humans is still unavailable. Nano-medicine's challenge remains the precise characterization of molecular targets and ensuring that only selected organs are impacted by these molecules. In addition, knowing the fate of drugs when delivered to the nucleus and other sensitive organelles is crucial.

Finally, social acceptance of nanotechnologies that use manufactured nanoparticles should be also studied in terms of cultural diversity, social and economic benefit, detriment and benefit, social system, morals, natural science, and social science. A vision must be formulated for the purpose of advancing industry use and realizing a safe and secure life while also maximizing the social benefits offered by using nanotechnologies.

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