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EXPERT REVIEW

Nanotechnology for Computed Tomography: A Real Potential Recently Disclosed

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ABSTRACT In the last decade, nanomaterials have gained considerable attention and interest in the development of new and efficient molecular probes for medical diagnosis and imaging. Compared to traditional contrast agents used from the 70s, this comes from the new possibilities offered by the increased half-life of nanosystems in blood stream, as well as by the specific accumulation in organ of lesions through passive or active targeting mechanisms. Heavy metal or iodinated-loaded nanoparticles are excellent absorbers of X-rays and can offer excellent improvement in medical diagnosis and X-ray imaging. This review aims to propose an accurate state-of-the-art of the emerging applications of nanotechnology in X-ray imaging. Likewise we will discuss and compare all the solutions proposed, and the impact of their composition, formulation methods, and physicochemical properties on their applications, efficiency, and potential industrial scaling-up.

KEY WORDS computed tomography · nano-emulsion · nanoparticle · preclinical imaging · targeted imaging

INTRODUCTION

Over the past decades, medical imaging is the field of medicine which has seen the strongest development, impacting therefore on numerous others domains like advanced diagnosis and treatment of cancers. Medical imaging consists of reconstructing an image of the patient body, disclosing anatomic or pathologic structures. In most cases, using a contrast agent is necessary to provide workable images, and even more for X-ray imaging. As well, following-up the dynamic behavior of the contrast agent in living systems can give important informations regarding the efficiency of targeted contrast agents, but also on their elimination pathways and kinetics.

On the other hand, in the same line that the emergence and development of medical imaging for humans, a significant effort was dedicated to the development of imagers for preclinical research (adapted for small laboratory animals), and mainly used in oncology. However, each imaging technology is associated to several limitations that slow down their widespread and generalize their use. For instance, the price of imagers for magnetic resonance imaging (MRI) involves heavy infrastructures. Likewise, nuclear imaging (PET, SPECT) involves a particular care regarding supply, storage, managing of radioactive animals and wastes. In contrast, preclinical optical imaging has greatly emerged from the last years, up to concerns 19% of the images performed.

The price of an image done on optical modality (including price of apparatus, maintenance, contrast agent) is in general cheaper than for X-ray imaging or MRI for instance (even if some new 3D fluorescence scanners can be as expensive asthe micro-CT scanner).

However, the resolution of optical imaging is rather low compared to the other modalities. Another important limitation comes from the low signal penetration in the body, that reduces the range of possible applications like non-invasive imaging. In addition, since the signals were only provided from labeled tissues, this technology does not allow obtaining anatomic images. This underlines the complementarity of the different imaging techniques, and thus the need to associate several modalities to provide efficient solutions. In this context, after optical imaging, the second cost-effective and efficient modality is the computed tomography (CT), *i.e.* X-ray scanners, or micro-CT for small laboratory animals. Nevertheless, X-ray imaging have still today a small place with around 7% of the medical images done, which is

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actually due to the poor efficiency and toxicity of the contrast agents currently available. Overall, during the last decades, when medical imaging has seen an extensivedevelopment, the contrast agents remain very limited, notably due to their toxicity, slowing down the transposition to humans.

Actually, micro computed tomography is a good compromise between cost, efficiency, and with a good three-dimensional resolution (1) (voxel resolution between 1 and 100 μm), much higher than the one of clinical CT (2–5) since adapted for preclinical imaging of small laboratory animals (5,6). Other important advantages are the non-invasiveness of the technology, and also the use of cold markers. Commercialapparatuses are generally designed with the X-ray source and detector rotating around the animal. Temperature and atmosphere are controlled on experimental bench in order to lower the animal stress during acquisition (1,7,8). The three dimensional image is reconstructed from the projections at different angles, establishing a three-dimensional matrix made with voxels, having each one a value of the X-ray attenuation. This allows to quantify precisely the X-ray attenuation within the different organs, and as well in function of time along with the acquisitions. Thus, the strength of micro-CT lies in the resolution of the technique, for instance, with the use of the appropriate contrast agent (9), disclosing the visualization of small vascular structures like neo-vasculature or angiogenesis involved in some pathology (5). In oncological research, micro-CT is an important tool for studying for longitudinal imaging of tumor development, giving a quantification of the progression of the lesions (*e.g.* evolution of the volume of tumors), in function of the efficacy of therapeutic treatments (10).

Therefore, since they are a condition for the potential and efficiency of medical imaging, the design of efficient and non-toxic contrast agents arises today as a major issue in medical research. It is all the more the case for computed tomography since the clinical contrast agents used for humans present significant drawbacks, and even cannot be used with the micro-CT scanners. They consist of iodinated hydrosoluble molecules, inducing well-known recurrent problems, like a rapid renal clearance inducing an acute renal toxicity. They are consequently unusable for certain applications like image-guided mini-invasive or non-invasive surgery with CT, consisting in a surgical operation guided with 3D medical images. However, the main limitation of these contrast agents is that they are not compatible with micro-CT scanners, for carrying out preclinical research. Indeed, once these iodinated molecules are in the blood of small laboratory animals, their clearance is achieved extremely rapidly,in less than 20 seconds. Insofar as the micro-CT imagers need from 1 (for the fastest apparatuses) to 12 min (for the standards ones) to perform a full acquisition, and since performing a continuous administration or repeated injection with smallanimal is not conceivable because of acute renal toxicity, no solutions can be brought with such hydrosoluble iodinated low molecular weight contrast agents.

The solution for overcoming this experimental problem is found with the *nanotechnologies*, and precisely in the development of contrast agents in the form of nanoparticles or nanoemulsions exhibiting a size sufficiently high to prevent the glomerular filtration in kidneys (the pore diameters are from 50 to 100 nm (11). Moreover, high nanoparticles size will induce a rapid uptake by the mononuclear phagocytes system (MPS) and/or the reticuloendothelial system (RES) (12), and therefore their elimination from the bloodstream, not also necessary an advantage in the design of CT contrast agents. Overall, nanoparticles size with a distribution centered around 100 nm is a good compromise to preventing their early elimination.

Nanotechnology appears as the unique efficient solution for designing efficient contrast agents for preclinical X-ray imaging, but also in other CT applications for humans, like image-guided surgery. Applied to humans, this technology would be considerably advantageous for mini-invasive surgery procedures, since only one administration of the contrast agent will be sufficient for the operation, preventing the renal toxicity due to the repeated injections. In this context, for the last decade, many research effort were dedicated to the development of such long-circulating and/or targeted contrast agent. The contrasting molecules (*e.g.* iodine) are formulated within nanoparticulate carriers, for which the formulation parameters can be summarized in several critical points: ***(i)*** The conditions in size ranges as detailed above will prevent a fast blood clearance; ***(ii)*** Controlling the surface properties of the nanoparticles is fundamental for delaying their recognition by the immune system, and thus increasing the time of circulation in bloodstream. These features are called *stealth* properties and are generally obtained with hydrophilic polymers like polyethylene glycol (PEG) decorating the nanoparticles surfaces (13–16). ***(iii)*** Another condition for designing efficiency contrast agents is the global amount of contrasting materials, not only in the nanoparticles, but also in the final suspension to be administrated. Contrasting materials are simply defined by a high Z-number (17–21). Actually, optimized systems should present the higher possible concentration per nanoparticle, thus allowing to reduce the quantity administrated to reach a significant signal, and thus the toxicity. In the case of iodine, optimized concentrations were reported around 100 mg I/mL. ***(iv)*** Finally, the last and fundamental point is the toxicity of the nanoparticulate contrast agents. The suspensions must not only show a biocompatibility towards the blood and the targeted organs, but they must be neutral regarding the biological functions of the subject, in order to avoid interferences with the undertaken studies.

It is important to note here that the main application field of medical imaging, gathering 70% of the images done,

concerns cancer research, and it is also true for CT or micro-CT. These new nanoparticulate contrast agents are emerging tools for improving the detection of tumors (corresponding to 49% of the images) and the follow-up of the response of treatment (68% of the images). Even, such nanotechnology allows detecting metastasis up to ~300μm (22–24). To date, several nanoparticulate contrast agents for preclinical research are commercially available, mainly in the form of iodinated lipid nano-emulsions (*e.g.* Fenestra®) or polymeric nanoparticles (*e.g.* Exitron Nano®), but their use remains limited by both their toxicity and high prices.

Actually, the development of efficient, cost-effective and non-toxic contrast agents is, still today, a major public health issue in order to allow taking benefit of the full potential of the imagers, more and more powerful (*e.g.* increasingthe resolution, and/or specificity of imaging). Moreover, it also appears as an economic issue, all the more for computed tomography owing to new potential applications offered with nanoparticulate systems. In consequence, along with the development CTscanners, many research efforts were focused on the development of this new generation of contrast agents. The first nanoparticulate systems developed were based on iodine as a good compromise between X-ray attenuation properties, safety and cost. Liposomal formulations containing contrast agents were historically the first (in the 1980s) most widespread nanotechnology for these objectives, providing efficient vascular and hepatic imaging (18,25–40). Then, iodinated lipids or triglyceride-based nano-emulsions were developed fifteen years later, and constantly improved up to now, giving interesting alternatives to liposomes (41–50). In the same period, polymeric micelles as contrast agents nano-carriers were reported (21,51–54). More recently by the 2000s, iodinated dendrimers and biodegradable nanoparticles were proposed (55–59). On the other hand, very recently (from 2006), nano-contrast agents based on inorganic compounds as contrasting materials were proposed (60). By definition, the X-ray attenuation is (roughly) linearly dependent to the Z-number of elements. Thus inorganic compounds appear more contrasting than iodine. Another interesting point lies in the chemical properties of inorganic nanomaterials, giving rise to rather simple procedures for their surface functionalization (grafting polymers or with specific ligands). Weak point of these inorganic systems, compared to iodinated ones, could be the toxicity and poor degradability of the nanoparticles if their are not eliminated. The step beyond the development of efficient, non-toxic, and cost-effective nano-contrast agents lies in the specific targeting of the nano-carriers to organs or lesions like tumors. Targeting contrast agents allows the fine detection of lesions, but also, when the nano-carrier co-encapsulate a drug, controlling the release. This enable high potentials for advanced diagnosis of cancer and personalized therapies. This additional passive or active targeting technology is performed through the control of the surface properties, and potentially with the grafting of specific ligands. The first significant *in vivo* results were reported in 2010 with gold nanoparticles (60). In this chapter, we aim to present an accurate overview of the existing and emerging solutions, all based from nanotechnologies, for the formulation of CT and micro-CT contrast agents. We will discuss and compare all the solutions proposed, and the impact of their composition, formulation method, and physicochemical properties on their application, efficiency, and potential industrial scaling-up.

LIPID-BASED SYSTEMS

Liposomes

Since it is largely described in literature, the aim of this section is not to give a general description and application of liposomes, but rather to provide an overview on their formulation and application as a solution for CT long circulating imaging agent. However, to do this, we propose a description of this system in the main lines, regarding their structure and how the contrasting material is trapped or encapsulated in the vesicle, along with the results on the toxicity, pharmacokinetics, contrasting properties, advantages and drawbacks.

Due to their poor solubility in water (*i.e.* a low critical bilayer concentration) and to their cylinder-like shape, phospholipids show a self association in the form of bilayers. Several techniques (not detailed here) takes advantage of this behavior to generate spherical vesicles (liposomes), very well characterized, and ranging in size the nanometric scale (61). Likewise, due to the innocuous nature of phospholipids, liposomes have shown a biocompatibiliy. Aqueous core ofliposomes were widely used for nano-encapsulating, protecting and carrying hydrophilic drugs and/or contrast agents (38,62–64). The first application of liposomes as contrast agents was found for liver and spleen imaging in the diagnosis of solid tumors. Due to their chemical nature, they are rapidly recognized by the immune system. Then, when their surface was functionalized with PEG, long-circulating liposomes were formulated, and their range of applications were extended to advanced diagnosis (35–37,39,56), image guided surgery (32), or personalized medicine (37,62). The PEG content, up to molar 10%, induces a significant increase in the liposome circulation time (65–69).

After hydration stage of the phospholipids with aqueous phase containing contrasting hydrophilic materials, the multilamelar large vesicles (MLV) formed are extruded to decrease and control their size (70,71). As a last step, the free (*i.e.* non-encapsulated) materials are removed from the liposomes suspension by dialysis, ultracentrifugation, gel-permeation chromatography or ion-

exchange resins. As a result, the whole process is not simple to work out and proceed, and lasts several hours (1,18,64,72). Generally, the formulation of liposomal contrast agent for CT is performed by encapsulating the hydrophilic iodinated molecules used for clinical CT described above. Iodine concentration in the suspensions generally reported varies from 25 to 110-mg/mL, and their long circulation properties gives usable contrasting properties even for 25 mg/mL (73).

On the other hand, and beside the complexity of the formulation processes, compared to the other nano-carriers, the stability is the weak point of liposomes. Liposomes can be degraded through various physical and chemical processes like auto-oxidation, hydrolysis, self-aggregation and coalescence (29). Several critical points can impact on their stability, like the stability of the bilayer, the inner osmotic pressure that can induce instabilities if it is not equilibrated with the bulkphase. Likewise, the functionalization of the surface can influence the whole liposome stability (74), and finally, the storage conditions post-formulation have an impact on the suspension stability. In this context, even if liposomescould be interesting candidates for some specific applications, and even if they can give interesting results for contrast agents formulation, compared to other nano-systems for the same purpose, they appears to be very sensitive system, not easy to handle and formulate. Of course, as described above, liposomes were the first efficient system usable as blood pool contrast agent for micro-CT (*e.g.* encapsulating iopromide (75), iodixanol (35), etc.), but now,compared to the emerging solutions described below, like nano-emulsions, liposomes show their limitation notably regarding the industrial transposition. In addition, encapsulation yields of iodinated molecules in liposomes rapidly reach their limits due to osmotic leakage, thus restraining the loading of contrasting materials. As regards the encapsulating/release properties of liposomes, their intrinsic structure involves a leakage of the encapsulated materials due to the equilibration of the chemical potentials. In the case of encapsulation of iodinated materials, this induces, after i.v. injection, a strong burst release in blood of the iodinated molecules, thus eliminated by the kidneys (64). This burst effect generally presents $t_{\frac{1}{2}}$ shorter than 15 min, and it due to the nature of formulation, since it was also observed for liposomes encapsulating MRI contrast agents such as gadoteridol (38,73). Solutions were found in reducing the concentration of the encapsulated iodinated hydrophilic molecules, however, along with the reduction of the contrasting properties of the suspensions (18,35,64). As well, formulation of liposomes with iodinated phospholipids reached high iodine concentrations up to 40 wt.% (40).

Pharmacokinetics and *in vivo* becoming of liposomes have been well characterized, and notably on models like rodents, rabbits or primates (18,31,35,39,62,63,72,74). These studies disclosed that the main mechanism driving the liposomes blood clearance is the RES uptake (36). Even, some authors observed that at high injection dose of liposomes, a saturation of the RES system occurs, resulting in an increase of the half-lifes in blood (36,76–79). Globally, the residence time of liposomes can vary from 40 min to 3 days, in function of such the experimental parameters. Several other parameters can play a significant role in the *in vivo* behavior of liposomes, such as their size: large liposomes,*e.g.* 400 nm, rapidly go to liver, when smaller, *e.g.* 200 nm, present a longer circulation time (99,109); their chemical composition: *e.g.* DPPC or DSPE-PEG induces an elimination by liver (38,72), while SPG or SPC orientate the elimination towards the kidneys (31,33); finally, the presence of encapsulated materials into liposomes also impact on the metabolisation of the phospholipids (80). Nevertheless, even if saturating the RES system with high doses can appear at first sight as a solution for increasing the circulation time, it involves serious toxicity problems. Indeed, dosages beyond 100 mg/kg body weight in mice will induce hepatomegaly, granulomas, and splenomegaly (81–84). However, the encapsulation of hydrophilic iodinated contrast agents (*e.g.* iopromide) significantly reduce the lethal dose 50 (LD_{50}), from 11 g I/kg (85) for non-encapsulated iopromide, to 4.5 g I/kg for the liposomal formulation (33). Several other factor influence as well the LD_{50} of liposomes, such as surface potential (25). Likewise, lipid-related toxicity results in milky serum, affected glutamic-oxaloacetic transaminase, gamma GT, blood urea nitrogen and glutamic pyruvique transaminase (33).

Liposomal formulation provided specific preclinical imaging tools with all the advantages and drawbacks described above. As an illustration, the works of Kweon and co-workers (86) present a formulation for imaging liver and spleen, taking benefit of the fast RES recognition of liposomes. The specificity of this example lies in the iodine concentration improved thanks to the co-nanoencapsulation of hydrophilic iodinated contrast agent (Iopamidol®) in the liposome core, and of iodinated oil (Lipiodol®) in the bilayer. In order to still more increasing the iodine concentration, these authors used iodinated phospholipids in the same formulation. The final iodine concentration reaches 49.2 mg I/mL. Micro-CT scans were reported in Fig. 1a, comparing the co-loaded liposomal formulation (Fig. 1a$_1$) with Iopamidol® injected alone (Fig. 1a$_2$), in rats (i.v. administration in tail vein). A schematic representation of the liposome structure is shown in Fig. 1a$_3$. Six minutes after injection, iodo-liposomes gives a significant contrast enhancement in liver and spleen (see a$_1$), whereas the kidneys appears oversaturated immediately already after the Iopamidol® injection (see a$_2$).

On the other hand, when the stealth properties are controlled notably by grafting or post-inserting PEG onto their surface, very different behavior arise, like the one disclosed by Mukundan and co-workers (35) illustrated in Fig. 1b. Their example present a formulation of PEGylated liposomes with

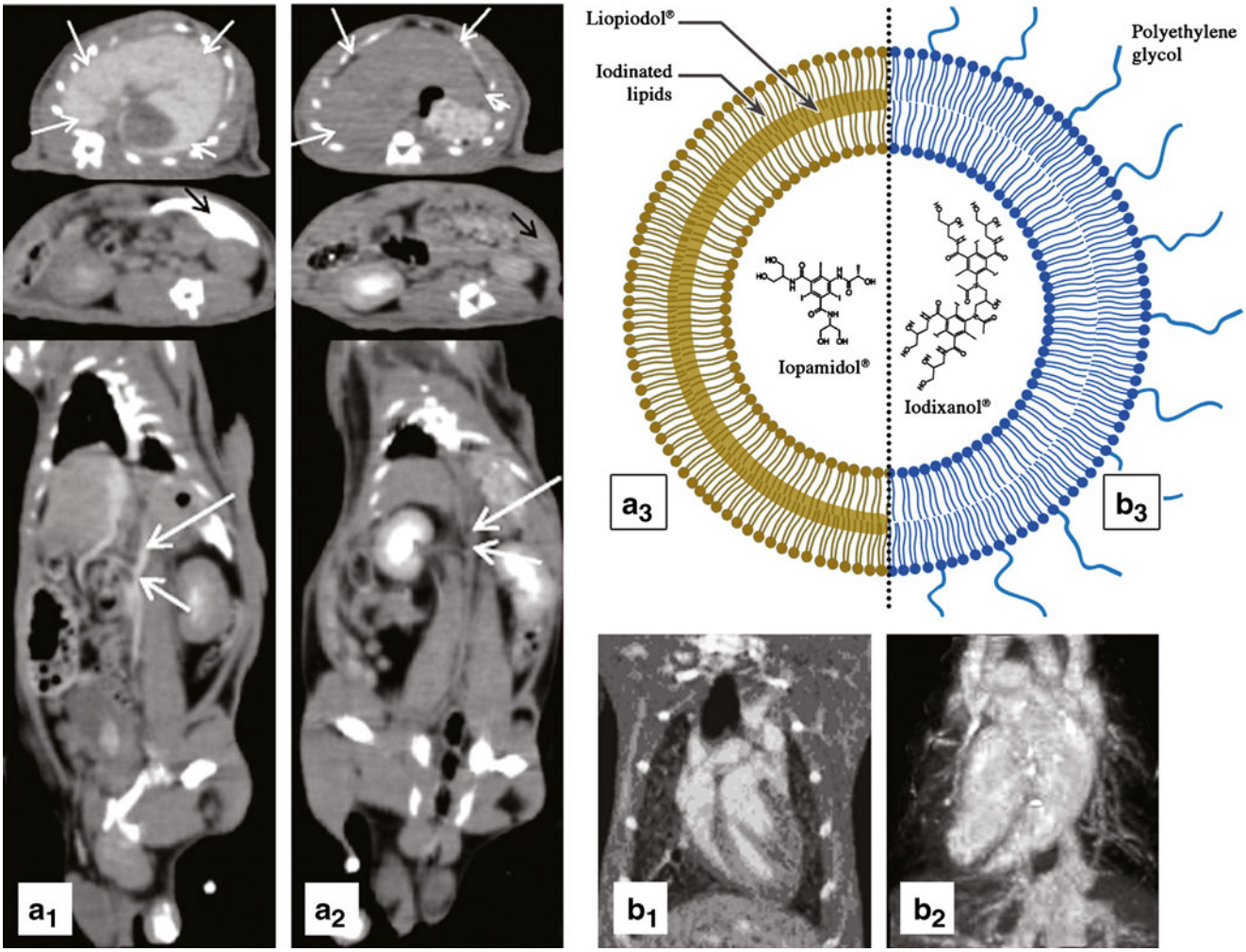

Fig. 1 Micro-CT images presenting the contrast enhancement induces by iodinated liposomal formulations. (**a**) From Ref. (86): (a_1) co-loaded liposomes built from iodinated phospholipids, encapsulating Iopamidol®, and solubilizing Lipiodol® in the bilayer. (*bottom*) Coronal cross section of the rat 1 min post injection, arrows indicates aorta; (*middle*) transverse section including spleen 90 min post injection, arrow shows the spleen; (*top*) transverse section including liver 210 min post injection, arrow shows the liver. (a_2) Control with Iopamidol® injected alone in the same conditions and concentration. (*bottom*) Coronal cross section of the rat immediately after injection, arrows indicates aorta; (*middle*) transverse section including spleen immediately after injection, arrow shows the spleen; (top) transverse section including liver 10 min post injection, arrow shows the liver. (a_3) Schematic representation of co-loaded liposomes. (**b**) From Ref. (35): Vascular constrast enhancement induces by iodixanol-loaded liposomes 1 h after i.v. administration in mice. (b_1) Coronal section showing the heart, (b_2) maximal intensity projection of cardiac and pulmonary vascular trees. (b_3) Schematic these stealth iodinated liposomes.

iodine concentrations as high as 105 mg I/mL, which allow performing very accurate images of the heart vasculature (as seen in Fig. 1b). Such tools allows the visualization of different organs, such as aorta, pulmonary vasculature, heart, and then, they are gradual uptaken by the RES giving rise to a contrast enhancement in liver and spleen (17,35,37,87). They were shown as efficient contrast agents to study the pulmonary emboli in rabbits (37), giving good and uniform opacification of the pulmonary artery (blood pool), until 4 h after administration.

The passive accumulation of liposomes in organs like liver or spleen is generally considered as a passive targeting as the contrast agent specifically accumulate in the aimed sites. In the presence of tumors, similar passive accumulation mechanisms can be observed towards these lesion sites. This phenomenon, called enhanced permeation and retention (EPR) effect, takes benefit of the affinity of the cancer cells for lipids. A gradual accumulation of such phospholipid-based long-circulating carriersoccurs into the cells. Even if the literature documents this phenomenon for several different sorts of lipid nano-carriers, in the case of micro-CT contrast agents, it is essentially illustrated with liposomal formulations (17,87–89). A representative illustration is reported in Fig. 2a, a coronal three-dimensional volume rendered image showing the gradual accumulation of contrast agents in the tumor site (arrow on image). Immediatelyafter injection, the tumor irrigation is contrasted along with the whole vasculature. 48-post administration, the liposomal contrast agent has clearly been accumulated in the tumor thanks to the EPR

effect, as well as in the spleen and liver due tothe classical passive accumulation pathway discussed above.

To finish, examples of tumor targeting with CT contrast agent, mediated by ligand/receptor interactions (*i.e.* active targeting), are rather anecdotal with liposome formulations. One illustration, reported in Fig. 2b, shows the preferential accumulation in the tumor site due to the iodo-liposome decoration with E-selectin-binding peptides. Such E-selectin receptors are expressed on activated endothelial cells, in leukocyte rolling during inflammatory processes or angiogenesis (91,92). In Fig. 7b, the targeted formulation show an accumulation into small subcutaneous tumors (b_1) compared to non-decorated iodo-liposomes (b_2). Significant and persistent contrast in the tumor rapidly arises, and lasts visible during 314 min, proving the efficiency of the peptide/receptor interactions.

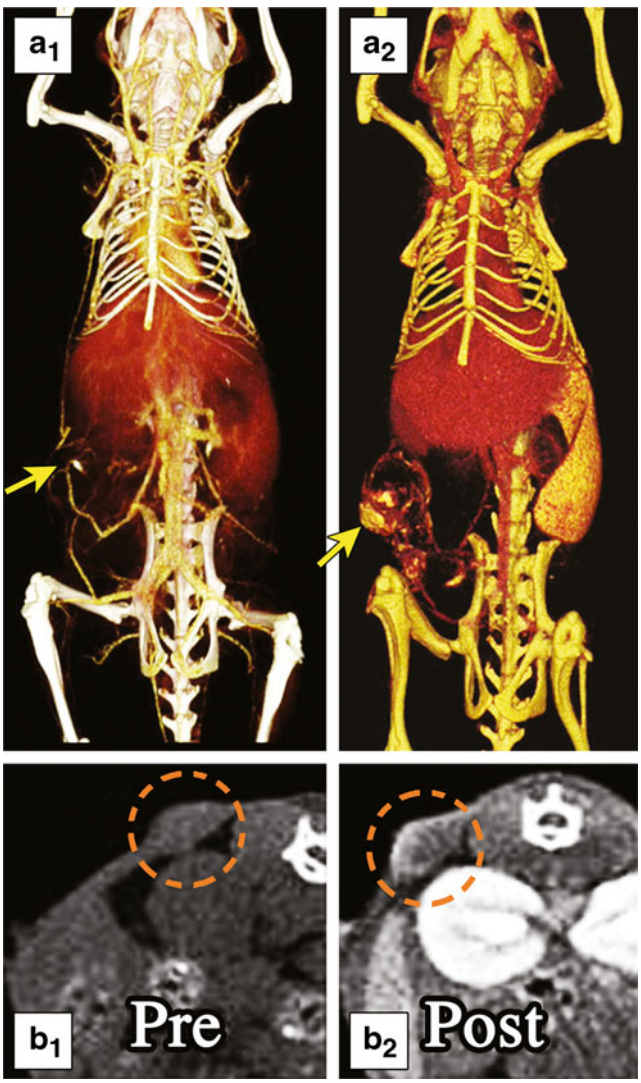

Fig. 2 Illustration of passive and active accumulation of iodinated liposomes in tumors. (**a**) From Ref. (17): Coronal 3D volume rendered images illustrating the passive accumulation of liposomes within the tumor (yellow arrow). (a_1) Immediately after the contrast agent injection, the tumor vasculature is nicely demonstrated. (a_2) Clear contrast in tumor sites 48 h post administration. (**b**) From Ref. (90): Active targeting of tumor with iodinated liposome decorated with E-selectin-binding peptide. (b_1) Before and (b_2) post injection.

Nano-emulsions

After liposomes, the most important efforts in the development of micro-CT contrast agents were done with iodinated nano-emulsions. They consist of iodinated lipids formulated in the form of nano-droplets ranging in size from around 20 to 200 nm (93–98). Nano-emulsions are fundamentally different from liposomes from many points, actually presenting much more advantages, summarized as follows and illustrated below (99–102). The first strength of nano-emulsions is their stability, and the robustness of the suspension against changes of thermodynamic environment. For example, they are stable against a parenteral administration that involves dilution and heating. The second point is the simplicity of their fabrication and formulation, for both chemical syntheses of iodinated oil and the generation process of the nano-emulsion droplets. Nano-emulsions are particularly adapted for the fabrication of CT contrast agents, since they allow the nano-encapsulation of high amount of contrasting materials like iodine (*e.g.* grafted on lipophilic molecules). Finally, the droplets surface can be easily decorated (*e.g.* with PEG or specific ligands) that can confer them strong stealth properties (16,99).

The historical iodinated contrast agents in the form of nano-emulsions, still commercially available (called Fenestra®), consist of nano-emulsions formulated from poly-iodinated triglyceride (ITG) and stabilized with phospholipids and cholesterol (45,99,103), with different surface properties adapted either for the blood pool or liver/spleen preclinical imaging (41,42,46,104,105). These formulations present an iodine concentration around 55 mg I/mL (45,99,103). This iodine concentration is high still but not enough since it involves volume of injection around 10% of the blood volume to reach exploitable contrasts. This is a significant drawback specific to this formulation, resulting in a non-negligible toxicity of the product. Nevertheless, these iodinated nano-emulsions found an important place in the market of preclinical CT contrast agents for the users of micro-CT scanners, owing to the real needs in such products, and due to the lack of other solutions compatible with industrial productions.

For the last two years, new perspectives, much more efficient than Fenestra®, were proposed. They consist of formulating nano-emulsion with new iodinated oils with very high iodine weight content, up to 50% of the oil molecule. In that way, the amount to inject, needed to reach a significant contrast, will be decreased, reducing the toxicity. The second point to favor the innocuousness of the product relies in the choice of native oil and surfactant compatible with the

parenteral administration. The first example of this new generation of nano-emulsions was given by de Vries *et al.* (106), where iodinated oils were based on 2-octanol, 3,7-dimethyl-1-octanol or 2-methyl heptanoic acid, and the surfactants were phospholipids (DSPC, *i.e.* 1,2-distearoyl-sn-glycero-3-phosphocholine), Pluronic F68®

(PEG-b-poly(propylene oxide)-b-PEG), or PBD-PEO (poly(butadiene)-b-PEG). Compared with the commercial formulations discussed above (Fenestra®), these new nano-emulsions showed significantly better toxicity results, notably quantified with cell viability assays. Once injected in mice, such nano-emulsions showed a good contrast enhancement (contrast enhancement of 220 Hounsfield Units (HU) was measured after intravenous administration of 520 mg I/kg), without signs of toxicity, and circulation times higher than 3 h.

Furthermore, as illustrated in Fig. 3 and Fig. 4, in the same line and more optimized, we recently proposed (100–102) new kinds of nano-emulsion formulations based on the spontaneous emulsification of lipids containing high content of iodine (up to around 50% of the molecule). In order to even more decrease the potential toxicity of the contrast agents, the oily compound (oily core of droplets) on which is grafted the iodine(*i.e.* triiodobenzenic acid) were molecules naturally present in the body, *e.g.* α-tocopherol for the example presented in Fig. 3. In this formulation, iodine proportion into the oil molecules reached 41.7enhancement ΔHU around 240 HU, and $t_{1/2}$=9.0 h. Fig. 3a shows a schematic representation of the nano-emulsion droplets, disclosing their very simple structure only composed of the oily iodinated core and the PEGylated surfactant. They are spontaneously generated by a mixing of two phases (94,96,102). Fig. 3b, c show the main results regarding their physicochemical characterization, respectively, size distribution (assessed bydynamic light scattering) and morphology (obtained by transmission electron microscopy).

In vivo evaluation were reported in Fig. 4, the suspension of nano-droplets were injected intravenously in mice, with injection volume around 8.5% of the blood volume (102). Fig. 4 (left) presents coronal and transverse section including heart and liver, before, just after injection, and 48 h post injection. From these X-ray attenuation results, three-dimensional volume rendering images were built and reported in Fig. 4 (right) showing the vascular network, heart, main arteries and veins, just after injection, and the accumulation of the contrast agent in the liver 48 h after administration. It is clear that such nanotechnology allows reaching the objectives aimed for structural preclinical CT imaging. Beside their efficiency for providing accurate contrasts enhancement, the main advantage of these nano-emulsions is the simplicity of the syntheses and formulations.

On the other hand, lipid iodinated nano-emulsions have been shown as efficient contrast agents for imaging of tumors, taking benefit of the passive accumulation of iodinated lipids in liver and/or spleen. Actually, in function of their chemical composition, the blood clearance of the lipid droplets is generally achieved either uptaken by the RES or internalized by hepatocytes. Actually, primary and secondary liver tumor cells do not internalize iodinated lipids like do normal liver cells. This results in a contrast difference between the tumor area and healthy liver tissue. This is assumed to be due to the deficiency in hepatic lipase of tumor cells, preventing the internalization of iodinated lipids (22–24,45). As a result, tumors are discloses by negative staining of these organs, allowing to detect lesions as small as 300 μm, with a detectability superior to 80% (23,46). Detection of hepatic tumors with ITG nano-emulsions isillustrated in Fig. 5a, from Ref. (19), with Fenestra LC® in nude mice. The difference in contrast enhancement let appear (negatively contrasted) the spacial repartition in the liver. Moreover, since the liver vasculature also appears

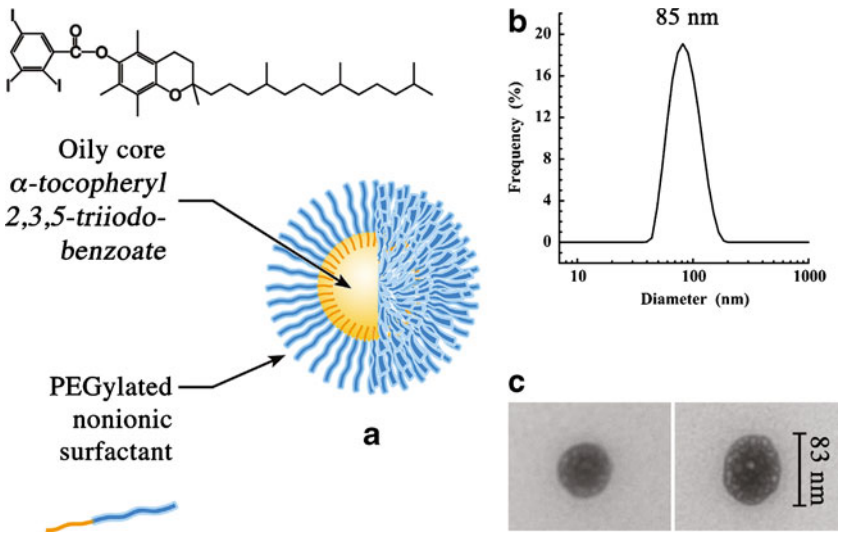

Fig. 3 Iodinated stealth nano-emulsions. (**a**) Schematic representation of the nano-emulsion droplets structure (chemical structure of the iodinated oil, α-tocopheryl-2,3,5-triiodobenzoate). (**b**) Size distribution obtained by DLS measurements. (**c**) TEM micrographs of iodinated nano-emulsion droplets.

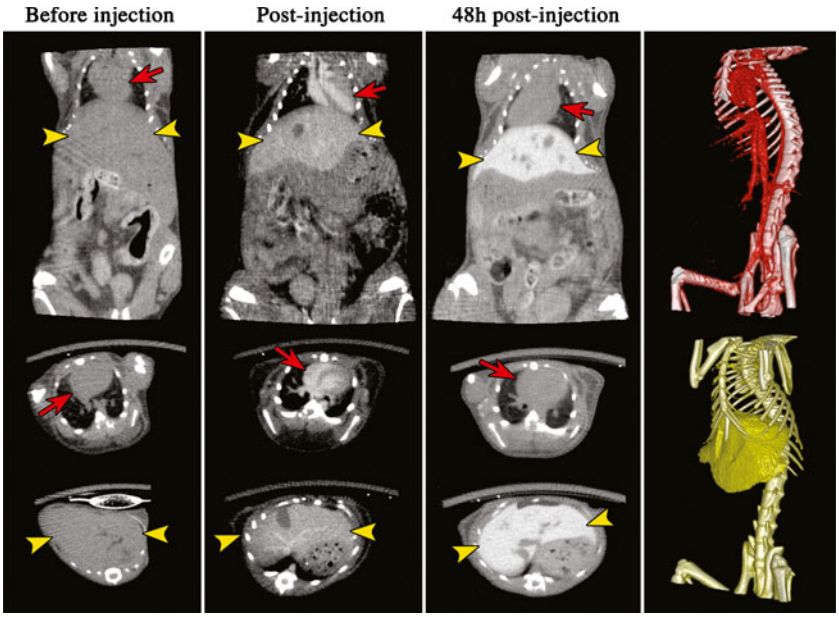

Fig. 4 From Ref. (102): *In vivo* evaluation of iodinated nano-emulsions in mice. Micro-CT scans: maximal intensity projections, coronal sections (*top*), transverse slices through the heart (*middle*), and transverse slices through the liver (*bottom*). (Right) 3D volume rendering, post-injection (*right-top*), and 48 after injection (*right-bottom*). *Red arrows* show the heart and *yellow arrow* head the liver.

negatively contrasted (the lipid droplets are eliminated from blood to accumulate in liver) a common pitfall is a misinterpretation of the images with a confusion between tumor sites and liver irrigation. The solution is found in the co-administration of liver contrast agent and blood pool contrast agent: only the tumors present in liver will finally appear negatively contrasted (45). This last case is illustrated in Fig. 5b, from Ref. (107), and allowed following very accurately the evolution of a hepatic tumor.

POLYMERIC NANO-CARRIERS

Up to now, we have presented the potentials of the lipid-based systems for the formulation of nano-carriers of X-ray contrast agents. On the other hand, the polymer-based nanoparticulate systems constitute another fundamental family used for the development of drug delivery systems. In the present section, we will present an overview of the compatibility of all the sorts of polymer-based nano-carriers

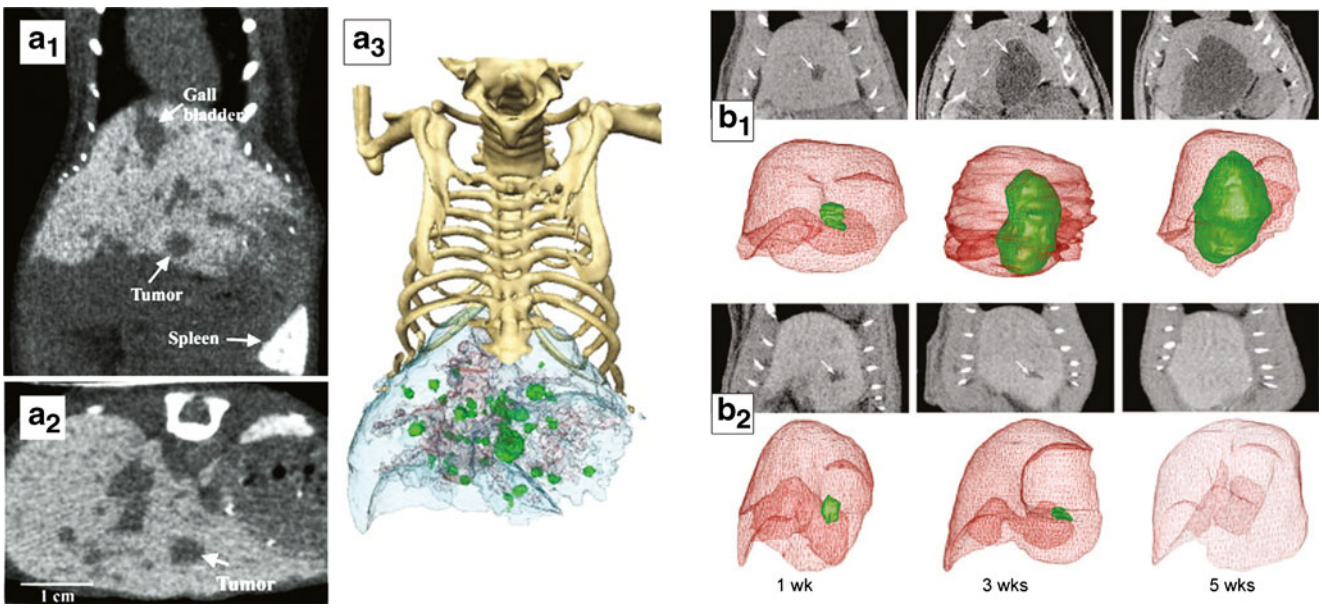

Fig. 5 Tumors imaging by micro-CT, with iodinated nano-emulsions as contrast agent. (**a**) From Ref. (19): micro-CT scans, 3 h post injection in nude mice with liver tumors, using Fenestra LC®. (a_1) Coronal and (a_2) transverse sections. Arrows indicate the same tumoral site in all views. (a_3) 3D volume rendered image showing tumors (*green*), liver lobes (*blue*) and liver vessels (*red*). (**b**) From Ref. (107): negative staining of livertumors with the co-use of hepotocyte-selective contrast agent (Fenestra LC®) and blood pool contrast agent (Fenestra VC®). (b_1) Untreated rats and (b_2) rats treated with Myo-inositol trispyrophosphate.

for the encapsulation of X-ray contrasting material and their application as preclinical X-ray contrast agents.

Several types of polymeric nano-carriers have shown interesting results as micro-CT contrast agents: we can find first the dendrimers on which are grafted the iodinated compounds (55,57), but also recently reported, combined with inorganic materials like gold nanoparticles directly synthetized in the dendrimer core (108–111). Then, some other systems like polymeric micelles have shown interesting results (21,51). Finally, since increasing the iodine concentration on such systems could be a limitation for increasing the X-ray attenuation properties, another efficient solution was found in including inorganic material (having higher X-ray attenuation properties than iodine) in polymeric nanosphere (60). This can be performed through a specific polymer coating of the inorganic nanoparticles (112–114). Such methods significantly reduced the toxicity of inorganic nanoparticles.

Dendrimers

A dendrimer is a highly branched macromolecule with well-defined structures (115,116). Dendrimer-based X-ray contrast agents were first developed by grafting the hydrosoluble iodinated molecules (like Iobitridol®) onto the external corona of the molecule. Several illustration are reported in literature, for instance in Ref. (57) illustrated in Fig. 6a, though a condensation between dendritic polylysine terminal free amino groups, or in Ref. (129) by grafting of triiodo amino acid (DMAA-IPA) onto the surface of stardust polyamidoamine (PAMAM) dendrimers (G-4-(DMAA-IPA)$_{37}$).

The second strategy undertaken lies in the *in situ* synthesis of gold nanoparticle within the dendrimer core, drastically enhancing their X-ray attenuation properties, *e.g.* reduction of $HAuCl_4$ (108–110). Several approaches aimed to optimize the contrasting properties by combining different compounds, like iodine and gold as described (without *in vivo* evaluation) in Ref. (117), giving an iodine equivalent concentration twice higher thancommercial hydrophilic molecules like Omnipaque®. Even though of the dendrimers are claimed to have high potential for the formulation of efficient contrast agent, and despite the research efforts led in that field, and the X-ray attenuation properties presented higher than classical contrast agents, any *in vivo* results appears very convincing compared to the lipid-based systems. This could be due to their toxicity, limiting the injectable dose. Another weak point is their residence time in blood pool typically around 30 min (57,109), even if the surface PEGylation allows improving this aspect extending the blood half-life up to 5 h (111). Examples of blood contrast enhancement, after i.v. administration in rats, are reported in Fig. 6. Fig. 6a shows transverse section of liver induced by iodinated dendrimers, and Fig. 6b illustrated the contrast enhancement obtained with gold nanoparticle-containing dendrimers. The toxicity of small dendrimer, below 50 nm (57,111), likely meets the one of the iodinated hydrophilic contrast agents due to their extensive renal excretion. However, as regards larger dendrimers, the toxicity is rather relied to their surface properties, *i.e.* surface potentials and number of generations (118–121). On th eother hand, compared to lipid systems (reminded in Fig. 6c with iodinated nano-emulsions), the contrasting properties of dendrimers appear relative and remain rather poor.

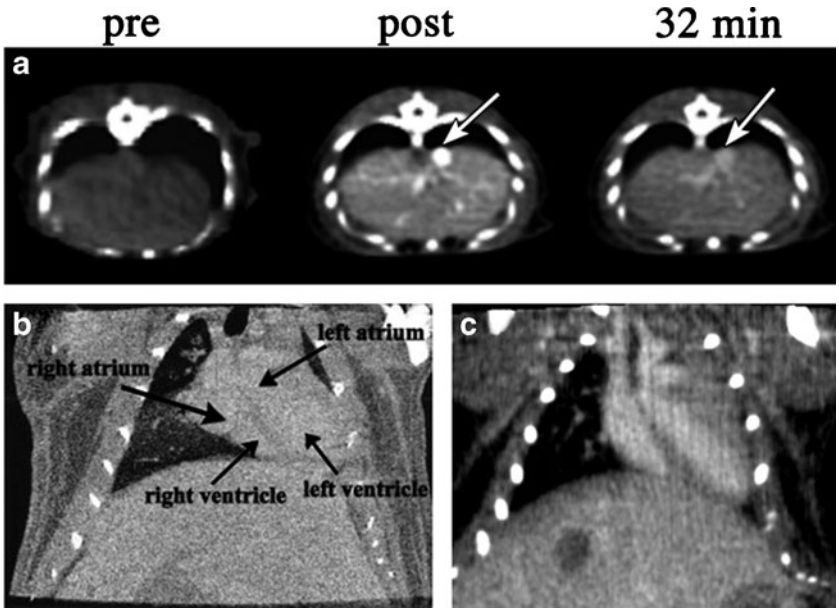

Fig. 6 (**a**, **b**) contrast enhancement obtained after i.v. injection of radiopaque dendrimers in rats. (**a**) From Ref. (57): iodinated Dendrimers, transverse section including liver, the arrow indicates the inferior vena cava. (**b**) From Ref. (111): gold nanoparticle entrapped in dendrimers. (**c**) Comparison with similar images obtained with iodinated nano-emulsions, form Fig. 4 above.

Polymeric Micelles

In the period in which the radiopaque lipid nano-systems were developed, the first polymer based examples emerged as well, they were polymeric micelles. They consist of nanoparticulate carrier (below 100 nm) generally composed of amphiphilic block polymers containing iodine, and formed spontaneously for thermodynamic reasons (52,122). An critical parameter conditioning imaging applications is their stability towards fluctuations of the thermodynamic environment. Actually by definition, micelles can be affected by dilution or temperature changes, happening with the parenteral administration. This is actually overridden by synthesis of polymers having a very low critical micelle concentration (CMC). Their inner hydrophobic core allows the possible incorporation of drugs or contrast agents solubilized or chemically grafted, stabilizing compounds, as well as the hydrophilic corona can be built with hydrophilic polymers (like PEG) to play on the blood clearance kinetics (99,116,123).

For instance, in Ref. (21,51) the authors published the synthesis of iodinated micelles along with their *in vivo* evaluation as contrast agent. Iodine-containing amphiphilic bockcopolymer (MPEG-iodolysine) micelles,sizing around 80 nm in water, and having a iodine content around 30 wt.% of the polymer molecular weight. After i.v. administration in rat and rabbits, they observed a significant contrast enhancement in heart, liver, aorta, kidney, spleen, and up to 24 h in function of the examples (52,53). However, even if several other examples were published, their efficiency remains to date, still below the nano-emulsions or liposomes. This is actually due to the limitation of the iodine concentration in the suspensions.

Hybrid Inorganic/Polymeric Nanoparticles

Besides iodine, numerous other examples of X-ray contrasting materials, like heavy metal, are also interesting candidates for being used as CT contrast agents. However, this is only recently that inorganic compounds were formulated in nanoparticulate systems for micro-CT applications, overall in the form fo inorganic/polymeric nanoparticles. Basically, heavy metals present high X-ray absorption properties and good chemical stability (60). The great interest in these compounds is due to the fact that heavy metal have X-ray adsorption coefficients significantly higher than the one of iodine, therefore, for the same concentration they give a higher contrast enhancement. On the other hand, their formulation in the form of nanoparticles, their stabilization and protection in aqueous media, or their surface functionalization, is generally achieved with their association with polymeric materials.

Different inorganic compounds has been used for the design of hybrid nanoparticulate CT contrast agent. Most significant examples regarding heavy metals concern the gold nanoparticles (60), owing to their good X-ray attenuation properties, 2.7 times higher than iodine (absorption coefficient at 100 keV are respectively 5.16 and 1.94 cm^2g^{-1}), and also since they are nontoxic *in vivo* (113,124–130). Stabilization of gold nanoparticles in aqueous media is achieved through their surface modification with polymer and potentially with targeting agents. Globally, this is only from the last five years that efficient *in vivo* imaging and/or targeted imaging using hybrid gold/polymer nanoparticles were published (128,131–136). Another advantage is the simple chemistry involved in the surface functionalization of these gold nanoparticles. It allowed reaching different imaging applications like the vascular imaging with a simple decoration with PEG (128,131) (using PEG-SH ou PEG-sulfhydryl) as illustrated in Fig. 7a, or the hepatic imaging at 2 h post injection in mice, with heparin coated gold nanoparticles (132). Furthermore, coating the nanoparticles with functional polymer (PEG-COOH) connected with a specific ligands like antibodies (respectively monoclonal anti mouse CD-4 and anti-Her2) induce an active targeting of lymph nodes or breast tumors (133,136).

Potentially, included in polymer nanoparticles, high Z-number element are potentially interesting candidates for the design of CT-contrast agents. Even if nude inorganic particles can induce a significant cytotoxicity, it can be totally inhibited once embedded into the polymeric matrix or polymeric envelop. The case of bismuth sulphide (Bi_2S_3) nanoparticles coated with polyvinylpyrrolidone (PVP) is a typical illustration, improving the LD50 of around one order of magnitude compared to bismuth salts (20). Bismuth sulphide nanoparticles induce a blood opacification until 140 min post-injection (illustrated in Fig. 7b), and are recognized by immune system to finally accumulate in the spleen.

Hybrid polymeric nanoparticles encapsulating alkaline rare earth were shown as efficient contrasting materials for micro-CT contrast agent applications. They are commercially available (as ExiTron nano®) and consist of polymeric nanoparticles sizing around 110 nm, decorated or not with PEG in function of the aimed applications, and lasting in liver up to 6 months (137). However to date, no data regarding the toxicology of these products, nor the ways of eliminations from the body if any, were published. Nevertheless, i.v. injection in mice gives rise to a strong blood contrast enhancement illustrated in Fig. 7c, with half life in blood around 3 h. On the other hand, these authors show thatsuch contrasting nanoparticles undergo a RES uptake resulting in a specific accumulation in lymph nodes, adrenal glands, and liver (with a maximum intensity in liver at 4 h post-injection). In that way, similarly to lipid systems, they are not uptaken by tumor cells and allow a negative staining of the tumor regions. This is illustrated in Fig. 7d showing the development of metastases

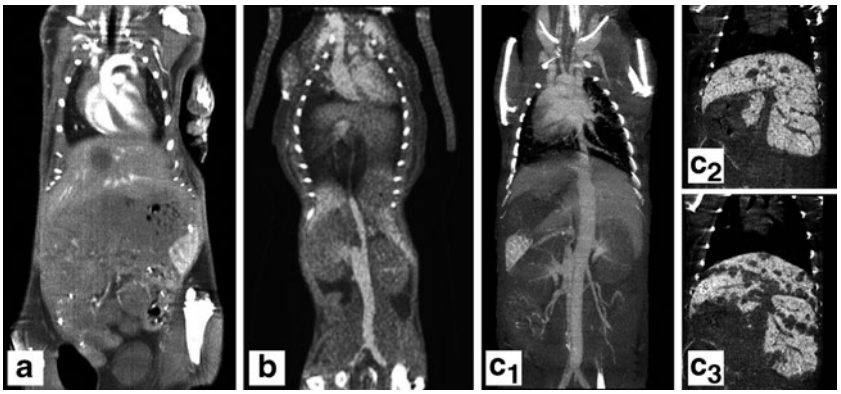

Fig. 7 Micro-CT scans showing contrast enhancement obtained with hybrid inorganic/polymer nanoparticles. (**a**) From Ref. (131): coronal section 6 h after injection of PEGylated gold nanoparticles in mice. (**b**) From Ref. (20): Coronal section after injection of polyvinylpyrrolidone-coated bismuth sulphide nanoparticles in mouse. (**c**) From Ref. (137): (c_1) Coronal section obtained 30 min after injection of ExiTron nano 12,000® in mouse. (c_2) and (c_3) liver tumors imaging by negative contrast after injection of ExiTron nano 6,000® in mouse, 9 days (c_2) and 14 days (c_3) after intrasplenic injection of MC38 colon tumor cells.

in liver 9 days (d_1) and 14 days (d_2) after intrasplenic injection of cancer cells (MC38 colon tumor cells).

INDUSTRIAL POTENTIAL OF NANOPARTICULATE MICRO-CT CONTRAST AGENTS

The industrial potential of polymeric and inorganic contrast agents is strongly linked to both their toxicity and their biodegradability after *in vivo* administration. As described above, a significant contrast enhancement withthe CT modality is linked to the high concentration of contrasting molecules (iodine, gold) compared to MRI or fluorescence needing much less contrasting compounds. Therefore, when using, *e.g.*, inorganic iron oxide nanoparticles in MRI, the quantity of contrast agent necessary for obtaining the aimed contrast is significantly lower (of a few order of magnitudes) and inhibits their potential toxicity. On the other hand, the high concentration of materials necessary for X-ray imaging gives rise to in some limitations regarding the potential industrial scaling-up or use for human.

Thus, the main limitation to industrial application in the formulation of CT contrast agent lies in their *in vivo* becoming after administration. We can note that globally, the contrast agents for CT imaging commercially available are based on biodegradable or nontoxic compounds like lipids, simply due to their compatibility with the high encapsulation of X-ray contrasting materials (iodine) as well as to their total elimination from the body. Inorganic or hybrid polymeric/inorganic nanoparticulate systems are however very studied at a fundamental research level because of their X-ray attenuation properties are better than iodine, but also due to the fact that their surface functionalization is easier. However, inorganic or hybrid nanoparticles are not degradable and show an accumulation in the body, limiting their use (to date) to research purposes. As regards pure polymeric nanoparticles, solutions are imaginable by using biodegradable polymers as template (*e.g.* poly(D,L-lactide-*co*-glycolide (PLGA), poly lactic acid (PLA), poly-∈-caprolactone (PCL), etc.) but to date, no examples were reported in that sense maybe due to the difficulty in increasing the iodine content in the molecular weight of the polymer (similarly to the limitation reached with dendrimers or micelles).

Finally, when iodine is a compromise between toxicity and cost, another limitation for industrial potential of inorganic contrast agents (like gold) is their price.

FUTURE TRENDS

As we saw along this review, elaboration of new CT contrast agents involves many technical challenges that have to be filled simultaneously. The main ones are a low toxicity and a good concentration of contrasting materials, after it comes the specific *in vivo* behavior like circulation time in blood or passive or active accumulation in specific sites, in order to get images of the aimed biological compartment or lesion. We saw that in function of the chemical nature of the nano-carrier, these specifications can be filled. However, remaining challenges and the future development in the elaboration of new CT contrast agents will concerns the following aspects. Formulating blood-pool or passive accumulating contrast agents compatible with the administration to humans. That is to say with a very controlled and understood toxicology, as well as clearance from the body. Potential research ways for this purpose can be focused on formulations with still more loading of contrasting materials and based on biodegradable compounds, like it is the case with the formulation of nanoemulsion with compatible iodinated oils. The second challenging point concerns elaboration of CT contrast for performing active targeted imaging. This point actually gathers the previous one since the dosage will further be

reduced whether the nano-carriers specifically accumulate in the desired sites, *i.e.* reducing their potential toxicity. Aswe saw above, the earlier results of active targeted CT contrast agent comes from the gold-based nano-carriers, with their specific advantages and drawbacks (detailed above). Future trends of this point will be extending such technologies to lipid-based or polymeric-based contrast agents (that are, at present, only designed for blood-pool imaging or passive targeting). Once the toxicity and *in vivo* behavior is understood and finely controlled, next step will be the co-encapsulation of contrast agent and drugs. It is certain that the CT modality have an important place in the future theranostics, however, it means that the nanoparticulate contrast agent must have a reservoir structure, *e.g.* like the lipid-based or polymeric nano-carriers. Finally, a last point that will see an important development in the coming decade due to the real need of surgeons, is the development of multimodal contrast agents including the CT modality. All the advantage of CT like the 3D high resolution or the imaging of specific sites will find high benefice to be combined with less accurate but more sensitive modalities.

CONCLUSION

Micro scanner X, or computed tomography, is a very powerful imaging modality thanks to the low cost of imagers (compared to other modality like MRI), as well as to the rapidity and accuracy of the images obtained on small animal models. Besides the realadvances of the quality and performance of imagers, the use of micro-CT is strongly limited by the toxicity of the contrast agents. In this context, nanotechnology brings out realistic solutions to allow performing both structural and functional imaging. They consist of lipid or polymer based stealth nanoparticles containing high amounts of contrasting materials, like iodine or high Z-number element, stable in aqueous suspension, and having controlled surface properties. These nanoparticulate systems have been shown not only very efficient for imaging blood vessels, but also for imaging specific organs of lesion sites through passive accumulation mechanism or active targeting *via* their surface functionalization. In this chapter, we have discussed and compared all the solutions proposed, and the impact of their composition, formulation method, and physicochemical properties on the application, efficiency, and potential industrial scaling up.

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