Interventional molecular imaging, a hybrid approach

(Non-targeted) radioactive/fluorescent nanoparticles and their potential in combined pre- and intraoperative imaging during sentinel lymph node resection

Chapter 2

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Buckle T, van Leeuwen AC, Chin PT, Janssen H, Muller SH, Jonkers J, van Leeuwen FWB.
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Abstract

A clear clinical precedent for the use of nano-sized imaging agents is localization of the tumor draining sentinel lymph nodes (SLNs). Specific removal of the SLN during breast cancer surgery presents physicians with the opportunity to detect early metastatic disease. In this application, radiocolloids such as ^{99m}Tc-nanocolloid are commonly used to plan the surgical procedure and to provide acoustic gamma guidance to the SLN during the intervention. An additional injection of a (visible) dye is used to provide optical surgical guidance. Hybrid nanoparticles, which are contain both a radioactive and fluorescent label, provide the potential to combine both radioactivity based surgical planning and intraoperative fluorescence guidance. In this chapter an overview is provided of the radioactive, fluorescent, and size properties of such (non-targeted) hybrid nanoparticles, and their (potential) value in SLN detection.

Introduction

In oncology, the presence of (lymphatic) metastases is considered an important predictor of distant tumor spread and consequently the life expectancy of the patient. The sentinel lymph node ((S)LN) is the first tumor draining lymph node and is generally the first LN affected by metastasizing tumor cells draining from a primary tumor. Based on the presence of tumor cells within the SLN, the likelihood of metastatic spread throughout the lymphatic system can be predicted and further treatment can be adapted accordingly.[1] Evaluation of the tumor presence in SLNs is a standard procedure during the clinical management of breast cancer patients and is also being investigated for many other tumor types e.g. melanoma and prostate cancer.[1-3] Accurate pathological analysis of the SLN depends on their accurate removal during surgical resection, which in turn relies heavily on the guidance provided during the surgical procedure.

Clinically, for SLN mapping lymphoscintigraphy is most frequently performed using radioactive colloidal particles.[4-6] In Europe, ^{99m}Tc-nanocolloid is the current clinical standard, whereas in the US ^{99m}Tc-sulfurcolloids are most frequently used.[7] Peritumoral/subdermal injection of these non-targeted colloidal particles results in transport via the lymphatic system, followed by accumulation in the SLN.[8]

Surgical resection of the SLN requires combined pre- and intraoperative imaging.[9-11] Commonly, the surgical procedure is planned according to preoperatively acquired lymphoscintigraphy using the radiocolloid.[12] Intraoperative surgical guidance is then obtained using an acoustic gamma probe and/or via co-injected dyes.[13-15] Such dyes offer the potential to add (superficial) optical intraoperative guidance. Unfortunately, the small organic dyes, such as methylene blue[15], patent blue[14], fluorescein (λ_{em}= 530 nm)[15], and the near-infrared (NIR) dye indocyanine green (ICG; λ_{em}= 808 nm)[16] do not appear to accumulate in the SLNs, limiting their use in real-time lymphatic mapping studies.[17] Differences in lymphatic migration translate to a different effective time window for both the radioactivity based and optical procedures. Hence in clinical practice pre- and intraoperative imaging consists out of two separate diagnostic approaches (radiocolloid and dye based), rather than a single integrated approach. Nano-sized hybrid imaging agents can, however, be used to combine radioactive and fluorescence properties in a single particle.[18] In such particle the radiolabel will enable surgical planning and guidance during the excision in the dm - cm range, while fluorescence will enable accurate intraoperative localization (cm - mm range; Figure 1).

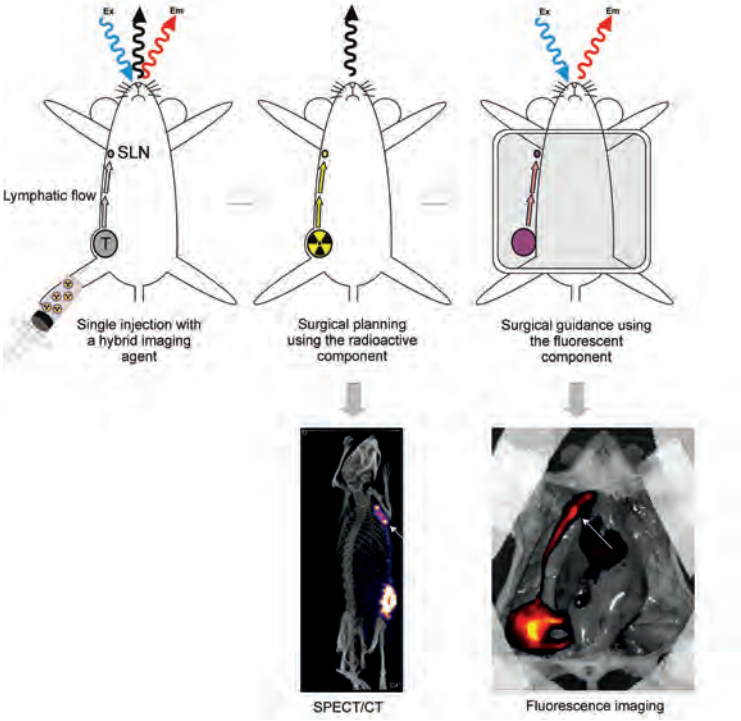

Figure 1. Schematic presentation of combined pre- and intraoperative imaging of the SLN using hybrid imaging agents that contain both a radioactive and fluorescent label (Figure adapted from Buckle et al.[24]) Both the SPECT/CT and the fluorescence image show the injection site (tumor), the draining lymphatic vessel (arrows) and the SLN.

Pre- and intraoperative imaging can be combined using non-targeted radioactive/fluorescent nano-sized materials. The physical requirements of these particles are considered to be a good guideline for the future development of nanotechnology-based imaging agents and their implementation in SLN imaging. Several reviews have been written discussing hybrid imaging agents.[18-20] Moreover, there are a number of reviews that discuss the use of nanoparticles for SLN imaging that also include MRI/optical hybrid particles.[21,22,23] As the standard clinical SLN procedure is based on radioactivity-based 3D imaging, rather than MRI, this Chapter is focused on the combination of radioactive and fluorescent antennae. We discuss the chemical properties of a variety of non-targeted hybrid nanoparticles and discuss their (potential) value in combined pre- and intraoperative SLN imaging applications.

Radioactive/fluorescent nanoparticles

Protein based

In Europe, the most widely applied protein-based nanoparticle in nuclear medicine is ^{99m}Technetium (^{99m}Tc; 140 keV; SPECT) labeled colloidal human serum albumin (nanocolloid; average diameter 14 nm).[24] Although it is not accurately described, the ^{99m}Tc-radiolabeling randomly occurs at the many available nitrogen- and/or sulfur-groups. We have recently shown that self-assembly can be used to generate a hybrid derivative of this compound, namely ICG-^{99m}Tc-nanocolloid (Figure 2a).[24,25] Due to the large number of non-covalent binding sites,[26] a single albumin particle can contain a number of non-covalently bound guest molecules.[27] As nanocolloid consists out of multiple albumin particles, this results in a further concentration of dye molecules.

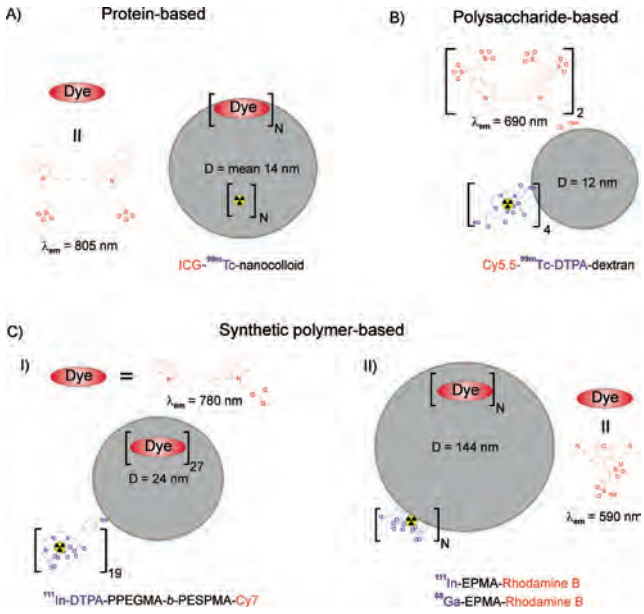

Figure 2. Schematic representation of non-targeted radioactive/fluorescent organic nanoparticles. A) Protein-based self-assembled ICG-^{99m}Tc-nanocolloid particles.[24] B) Polysaccharide-based dextran particles covalently functionalized with Cy5.5 and a ^{99m}Tc-DTPA label.[28] C) Synthetic polymer-based nanoparticles: I) hybrid co-block polymers with an ^{111}In-DTPA label and a covalently linked fluorescent label (Cy7)[31] and II) EPMA-based latex nanoparticles with non-covalently internalized dye (RhB) and surface coordinated radiolabels.[32] The radiolabels are represented in blue, the fluorescent antennae in red and the carrier molecules in gray.

Combined pre- and intraoperative imaging of the SLN with ICG-^{99m}Tc-nanocolloid has been validated in mouse models for metastatic breast and prostate cancer.[24,25] The migration properties of the hybrid derivative was found to be highly similar, if not identical to ^{99m}Tc-nanocolloid (Figure 1). Incorporation of multiple ICG molecules on the nanocolloid resulted in a huge improvement in the signal-to-background-ratio compared to free ICG.

Polysaccharide based

Polysaccharides like dextran contain a large number of reactive hydroxy groups that can be utilized for covalent functionalization. Vera et al.[28] generated 12 nm dextran particles (Mw = 70 kDa), labeled with four DTPA moieties and two Cy5.5 (λ_{em} 690 nm) dye molecules (Figure 2b). In theory any type of dye functionalized with a reactive group can be linked to this dextran backbone, allowing the generation of particles with emission wavelengths ranging from visual to the NIR. The large number of appropriate reactive groups allows introduction of increasing numbers of fluorescent antennae and radioactive labels. Unfortunately in vivo examples using these hybrid nanoparticles are not described.

Although not (commonly) used for clinical applications, dextran particles with a 7 nm size have proven their value in lymphatic (flow) imaging; both radioactive (^{99m}Tc) and fluorescent derivatives have been described.[29,30] Since the dextran scaffold is the driving factor for these properties, a dual-labeled derivative should perform equally well. Moreover, size alteration of the dextran-scaffold can be used to improve the accumulation in SLNs.

Synthetic polymer-based

Synthetic polymeric nanoparticles, such as polymer micelles[31] and latex[32] with combined radioactive/fluorescent labels have also been reported (Figure 2c). For example, PEG-coated core-crosslinked polymer micelles (24 nm) can be formed by crosslinking the block copolymer poly(PEG-methacrylate)-b-poly triethoxysilyl propylmethacrylate (PPEGMA-b-PESPMA) and 3-(triethoxysilyl)propyl-Cy7 (λ_{em} 780 nm).[31] Crosslinking resulted in incorporation of approximately 27 Cy7 molecules into the particle core. Primary amine groups available on the (cross-linked) polymer surface were subsequently functionalized with 19 chelating DTPA-moieties per particle, which in turn allowed efficient incorporation of ^{111}In at the particle surface. When linked to a 3-(triethylsilyl)propyl group other dyes can potentially also be incorporated in a similar manner. Again the number of dye molecules and radiolabels can be varied in this set-up, but no in vivo data is described.

Emulsion copolymerization of poly-2,3-epoxypropylmethacrylate (EPMA) and Rhodamine B (RhB; λ_{em} 590 nm) has been used to form latex particles with a diameter of 144 nm.[32] The dye, which does not contain a reactive end group, remains trapped within the particle structure after crosslinking of the EPMA molecules. As RhB incorporation does not appear to be based on a specific interaction between the polymer and the dye, a similar procedure could be performed using other dyes. These latex particles contain a large number of carboxyl end groups on their surface and the authors have shown that these can function as chelating moiety for radioisotopes such as ^{68}Ga and ^{111}In. However, no data on the stability of these alternative metal-complexes has been reported, nor is an in vivo application mentioned.

Fluorescent polystyrene microspheres (20 - 200 nm) have shown potential value in lymphatic mapping procedures.[33] However, the hybrid derivatives have not yet been studied in this particular application. These polymeric hybrid particles (24 nm or 144 nm) can most likely provide synthetic alternatives to the albumin- or dextran-based nanoparticles. In addition to the possibility to chemically optimize the different polymer components of these synthetic derivatives, their size can potentially also be exploited to optimize lymphatic migratory properties even further.

Inorganic nanoparticles

In the last few decades a popular new class of inorganic dyes has been developed, referred to as quantum dots (QDs). In contrast to the earlier discussed protein-, saccharide-, and synthetic polymer-based nanoparticles, QDs do not rely on the inclusion of a dye for fluorescence. These nano-sized inorganic semiconductor crystals are inorganic fluorophores on their own. QD fluorescence induces great flexibility; alterations in size and materials allows tuning of the fluorescence from the visible part of the spectrum into the NIR.[34]

Introduction of a radioactive group on the surface of a QD is relatively easy, resulting in direct generation of a hybrid particle. This introduction commonly occurs via covalent attachment of a precoordinated reactive chelate complex to the polymer-, lipid- or surfactant coating of the QDs (Figure 3). The surface of CdSe/ZnS QDs has been radiolabeled with different isotopes, e.g. ^{99m}Tc and ^{64}Cu.[35,36,37] An advantage of surface modification is that it can easily be extrapolated to alternative QDs, e.g. InAs/ZnS.[38] A radiolabel viz. fluorine (^{18}F; PET) attached to a reactive organic molecule can be introduced onto a phospholipid coating.[39] Of course the value of such a non-covalent coating relies heavily on a good particle stability in vivo. Alternatively, a radioactive isotope can be

introduced in the crystal core during the QD synthesis. In this manner CdTe/ZnS QDs containing ^{125m}Te have been generated (Figure 3d).[40] This latter type of radiolabeling is very elegant as it requires no physical alteration to the QD. However, it appears to be less practical than a final-step introduction of a radioactive moiety on preprepared QDs.

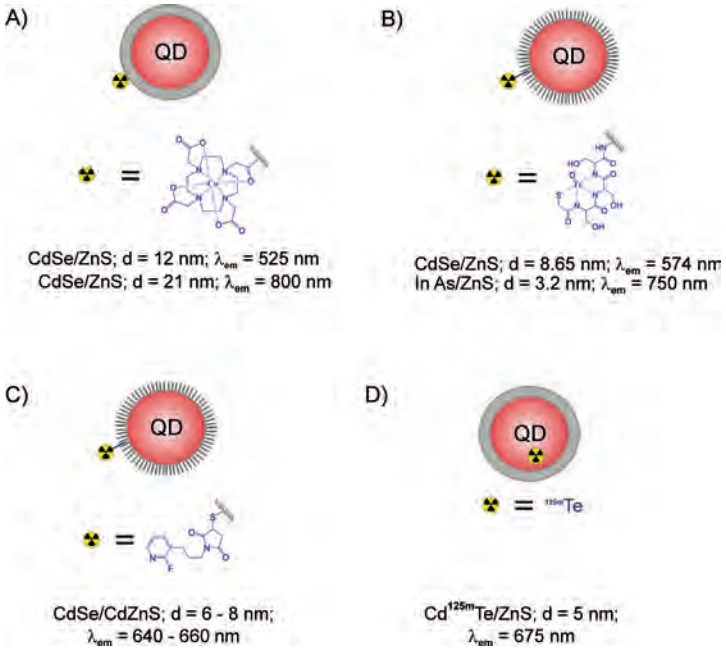

Figure 3. Schematic representation of non-targeted inorganic nanoparticles. A) Polymer coated QDs functionalized with a ^{64}Cu radiolabel[36] B) Thiol-based lipid coated QDs functionalized with a ^{99m}Tc radiolabel.[35,38] C) Lipid coated QDs functionalized with a radiolabel (^{18}F).[3] D) Core radiolabeled (^{125m}Te) QDs.[40] The radiolabels are represented in blue and the fluorescent antennae in red.

The potential value of QDs in SLN imaging was initially been reported by Kim et al.[41] and Ballou et al.[42] and has later underlined by a number of different groups.[43] While none of the SLN studies using QDs included the use of a radiolabel, there is no reason why radioactive/fluorescent QDs could not be equally suitable for such an application. Such hybrid QDs will also allow quantification of the kinetics in the lymphatic system, a feature that is currently lacking. QDs with a covalent coating functionalized with a radiolabel are more likely to remain stable under the desired conditions than those coated with lipid micelles.

Dendrimers

Signal enhancement can be achieved via the creation of molecules that have multiple available reactive groups. In such compounds, so called dendrimers, increasing generations contain increasing amounts of reactive end groups and an increase in size. Moreover, the number of end groups can be well documented and accurately tuned for a particular application. While a number of MRI/fluorescent dendrimers have been reported[44,45] there is, to the best of our knowledge, only one single example of dendritic structures labeled with both a radionuclide and a fluorescent dye (Figure 4).[46] After the introduction of chelating DTPA moieties, generation-6 PAMAM dendrimers containing 256 end groups were functionalized with Cy5 or one of four different Alexa dyes (Alexa660, Alexa680, Alexa700, or Alexa750). This resulted in particles of approximately 8 nm in size with a PAMAM/dye/DTPA ratio of 1:4:120. A single indium isotope (^{111}In) was introduced to one of the 120 DTPA moieties on the dendrimer. The use of five different derivatives, all containing a different dye covered an application from 670 to 780 nm. Moreover, the 120 chelating moieties present can be used to increase the radioactive signal intensity.

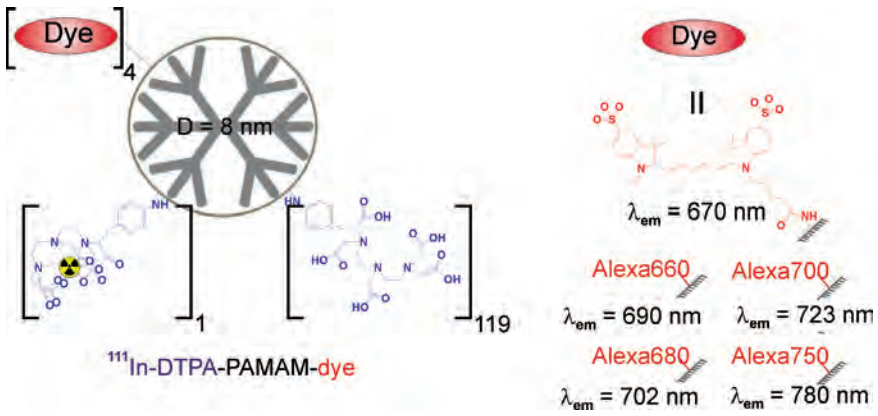

Figure 4. Schematic representation of non-targeted hybrid generation 6 PAMAM dendrimers functionalized with a radiolabel (^{111}In) and one of five different dyes; Cy5, Alexa660, Alexa680, Alexa700, or Alexa750.[46] The radiolabels are represented in blue, the NIR fluorescent antennae in red, and the linker molecule in black/gray.

After injection of five differently 'colored' dendrimers into the front paw of mice, Kobayashi et al.[44] were able to obtain accurate information with respect to the location of five separate lymphatic basins. In turn, the radiolabel provided a quantitative read out on

the lymphatic migration. When sufficient retention of these relatively small dendrimers in the SLN is obtained over time, this type of molecule is a good candidate for combined pre- and intraoperative imaging. This is strengthened by the large degree of synthetic control and the simultaneous use of differently colored dyes. The latter may be used to simultaneously identify the lymphatic draining sites of e.g. multifocal tumors.

General design guidelines that can be derived from literature examples

Based on the different radioactive/fluorescent nanostructures that have been reported, a number of general design guidelines can be derived, of which the most important are listed below.

Detection sensitivity: Radioactivity vs. fluorescence

Recent developments in sensitive surgical fluorescence cameras,[47] and the ability to optimize both the color and intensity of the fluorescence signal, make fluorescence imaging an attractive optical supplement to acoustic (intraoperative) radioactivity based detection. Despite the similar contrast sensitivity,[48] the signal penetration through tissue of radioactivity- and fluorescence-based imaging techniques differs quite dramatically.[49] The 25 - 511 keV gamma emission used in gamma camera imaging, single photon emission computed tomography (SPECT), and positron emission tomography (PET) provides total body penetration. In fluorescence imaging, the penetration of emitted photons remains confined within the cm - mm range, even those with NIR wavelengths. This difference influences the (clinical) application of these modalities. Whereas gamma camera imaging, SPECT, and PET are extremely suitable for non-invasive total body imaging (e.g. 3D surgical planning), fluorescence imaging is predominately suitable for superficial detection (e.g. intraoperative imaging).

In SLN applications dye concentrations used are generally much higher than the concentration of radiocolloids. The hybrid imaging agent ICG-^{99m}Tc-nanocolloid, enables detection of the SLNs using both radioactivity- and fluorescence-based imaging without changing the administered nanocolloid dose. While in ICG-^{99m}Tc-nanocolloid multiple dye molecules are present per particle, a 1:1 ratio between the radioactive and fluorescent imaging label was also proven sufficient in a recent review on peptide based tumor specific hybrid imaging agents.[20]

Chemical properties and SLN retention

The optimal particle size for SLN imaging applications is estimated to be approximately 40 nm. Smaller particles are expected to migrate throughout the complete lymphatic trajectory while migration speed of larger particles will possibly be too slow.[50] Nunezet et al. underlined that nanometer sized particles are more preferable than microsized particles.[51] Nanocolloid is said to have a favorable hydrodynamic diameter for SLN imaging[51]; via TEM analysis a mean particle size of 14 nm was found.[24] The diagnostic value of nanocolloid particles suggests that perhaps there is more to the accumulation in the SLN than size alone. Recognition of nanoparticles by the immune system may play a role in the accumulation of particles in the SLN.[8] The administered concentration of nanocolloid was also shown to be of influence on the visualization of SLNs.[10] Combined, size, recognition by the immune system, and injected concentration, seem to be the most dominant features in the feasibility of visualization of SLNs.

Radiolabel

The choice of a radiolabel is largely dictated by the radioactive half-life of the isotope. Due to the time needed for lymphatic migration and to bridge the timespan between pre- and intraoperative imaging, isotopes that can still be detected 4 - 6 hrs after administration, e.g. ^{99m}Tc and ^{111}In, are preferred. Several of the chelating moieties can complex different isotopes, rendering the compound suitable for a use in either SPECT or PET imaging, depending on the demand of the user. In general a single radiolabel is sufficient for accurate detection.

Dyes

Different to radioactivity based imaging procedures that directly detect the emitted gamma photons from the imaging agent, fluorescence imaging requires an external excitation light source (λ_{ex}) to obtain a fluorescent signal (λ_{em}; Figure 1). In the body significant tissue absorption and a strong autofluorescence is observed between 400 and 650 nm. As a consequence, NIR fluorescent dyes (λ_{em} > 700 nm) are considered most suitable for 'deep' tissue imaging (cm range) in vivo, explaining the mainstream focus on NIR dyes for surgical image guidance.[53]

In combined radioactive and fluorescent imaging agents, the radioactive component already allows for initial guidance towards the lesion. The fluorescent component is only required for superficial visualization of the SLN (Figure 5).[44]

An interesting expansion of the radioactive/fluorescent labeling technology was suggested by Liu et al., who demonstrated that ^{131}I-isotopes can also be used to excite QDs.[54] Although not performed on the same molecule, such a hybrid cocktail application can be of use in, for example, tumor marker seeds,[55] which in turn can be used to mark lymph nodes (LNs).[56]

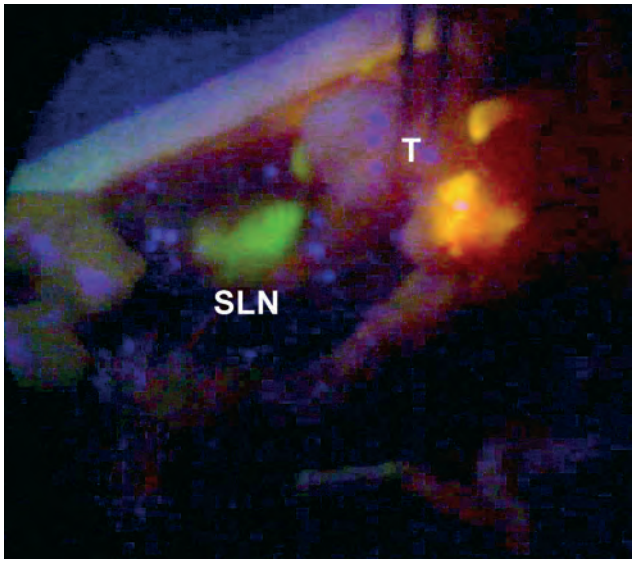

Figure 5. Example of superficial visual fluorescence guidance towards the SLN using InP/ZnS QDs (Figure adapted from Chin et al.).[43]

In general, the type of organic dye used on the nanoparticles is not expected to alter the lymphatic distribution. This is mainly due to the dominance of the parental scaffold molecule. However, an important feature to keep in mind is the loading rate of the dye. When dyes are not physically separated in the hybrid particles, (self-)quenching of the fluorescence signal may occur. For the inorganic QD-based systems, the size related properties are used to tune the fluorescence to the desired wavelength. The fluorescence of QDs can be further tuned by inclusion of a dopant[57] in to the QD core or by the creations of QD hetero structures, which are often referred to as type II QDs.[58]

Particle stability

Especially for the (non-covalent) self-assembled systems, the type of linkage between the radiolabel and the fluorophore may be instrumental for final in vivo stability of the complex. For example, in self-assembled amphiphilic lipids, the most investigated (hybrid) nano-platforms for biomedical purposes[59], dissociation may occur during SLN applications via exchange with fatty tissue leading to particle aggregation.[39] Unfortunately in vivo monitoring of the stability of non-covalent assembled particles can be difficult. To validate the value of non-covalent complexes in SLN applications, it is important that the distribution of all the individual components of these imaging agents can be monitored (Figure 1).[24]

Toxicity

This Chapter is predominantly focused on the fluorescent, radioactive, and size properties of hybrid nanoparticles. However, the clearance and resulting toxicity profile will be decisive for clinical implementation of these nanoparticles. The synthetic structures that are not based on endogenous proteins, e.g. dendrimers and (inorganic) nanoparticles, may suffer from toxic side effects. The highly toxic cadmium in many of the commercial available QDs is a prime example of a property that currently stands in the way of clinical translation. Significant effort is required to generate hybrid nanoparticles with advantageous properties but with a readily translatable toxicity profile. For example the combination of clinically applied radiocolloids and a clinically applied NIR dye (ICG) yielded a good translational character; currently the hybrid imaging agent ICG-^{99m}Tc-nanocolloid is being clinically evaluated for use in SLN procedures in patients with melanoma in the head and neck area or on the trunk, penile or prostate carcinoma, and oral cavity cancer.[60-64]

Conclusion

Non-targeted hybrid nanoparticles that contain both a radioactive and fluorescent label represent a relatively unexploited area of chemical/nanotechnological development, with only a minor amount of compounds being produced. Despite the limited examples in literature, the potential to initiate a novel route for medical diagnostics, namely combined (preoperative) 3D radioactive imaging and (intraoperative) 2D fluorescence imaging is eminent. Diagnostics of the SLN provides a logical clinical application where size matters and nano actually performs better than sub nanometer sized molecules. As long as the size of the parental scaffold particle induces sufficient lymphatic retention, a variety of dyes and radiolabels can be introduced. This allows finetuning to the specific demands of a user.

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