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Nucleic Acid Aptamers for *In Vivo* Molecular Imaging

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1. Introduction

New drug compounds discovery has moved at an accelerated pace in recent years, with a considerable focus on the transition from *in vitro* to *in vivo* models. As a result, there has been a significant increase in the need to adapt and develop novel non-invasive, high resolution *in vivo* imaging approaches for studying disease development and quantitatively determining molecular and cellular events *in vivo*. Non-invasive imaging methods allow continuous monitoring of disease development *in vivo*. For example, real time spatial-temporal analysis of tumour growth can reveal the dynamics of cancer progression. Furthermore, the effects of therapy can be evaluated non-invasively *in vivo*. These approaches offer the ability to perform repetitive observations and interventions of the biological processes underlying disease development and progression.

Thus, the development of imaging agents with high sensitivity, high specificity, and low toxicity is necessary to obtain biochemical functional information for the early characterization of the molecular alterations and, in turn, to improve diagnosis and therapy for clinical applications.

Despite significant progress in developing rapid and accurate *ex-vivo* medical diagnostic tests, it is still essential to investigate approaches for *in vivo* non-invasive imaging because many human diseases and the outcomes of therapy not always and not easily can be determined by the analysis of blood samples or biopsies. For instance, whether a tumor shrinks during cancer therapy has to be determined by bioimaging modalities. In addition, non-invasive imaging techniques enable the examination of cellular and biomolecular events in locations that are difficult to access by any other means.

Thus, the development of imaging agents with high sensitivity, high specificity, and low toxicity is necessary to improve diagnosis and therapy for clinical applications.

In this perspective an emerging attractive class of targeting molecules is represented by short single-stranded oligonucleotides ligands, named aptamers, being able to bind with high affinity to specific protein or non-protein targets by folding into complex tertiary structures. They discriminate between closely related targets and are characterised by high

specificity and low toxicity thus representing a valid alternative to antibodies for *in vivo* cell recognition. Owing to their relatively small size in comparison to antibodies, aptamers should be better-suited for rapid target tissue penetration and blood clearance. In addition, different from antibodies, aptamers are chemically synthesized at low cost with high batch fidelity, are sufficiently stable and can be readily chemically modified to further enhance their stability, bioavailability and pharmacokinetics. Moreover, aptamer immunogenicity has never been reported. These properties indicate a great potential of aptamers as imaging agents, especially when compared to antibodies.

The present chapter will be dedicated to aptamer-based molecular imaging modalities by reviewing the recent application of aptamers as ligands for inflammation imaging, thrombus imaging and tumour imaging.

2. *In vivo* non-invasive imaging

The functional imaging techniques developed in the past decades have given raised to powerful biomedical imaging tools that permit repeated non-invasive assessment and quantification of specific biological and pharmacological processes at the molecular level in human and animal (Lyons, 2005).

By employing medical image modalities one can assess the physiological activities within a certain tissue or organ choosing among the imaging platforms available: positron-emission tomography (PET), single photon emission computed tomography (SPECT), nuclear magnetic resonance imaging (NMRI), optical imaging (OI) and computed tomography (CT).

2.1 The emission tomography

The emission tomography is based on the decay process of a specific radioactive element to detect the isotope distribution within the body as function of the time. The radionuclide concentration changes with time as consequence of the decay events as well as for the biochemical processes kinetics. Moreover the concentrations of tracer imaged the given tissue metabolic activity, in terms of the specific molecules of interest uptake (Massoud et al. 2003). Differently from radio-diagnostics methods, more directed towards a morphologic analysis, emission tomography hold an essential rule in field of diagnostic and functional analysis (heart attack, epilepsy, cardiopathy, Parkinson disease).

The compound tracer-ligand, which is of interest for its chemical binding properties to certain types of tissues, is injected into the body and depending on the nature of the radionuclide the decay product can be a photon (SPECT) or a positron (PET).

The radioactivity profiles obtained are processed by an electronic calculator by means of appropriate reconstruction algorithms for the reconstruction of the tomographic image of the studied section.

SPECT imaging is a quite new survey technique by which it is possible to reconstruct scintigraphic images related to the spatial and temporal distribution of a radioactive substance. SPECT equipment consists of one or more rotational gamma chambers which detect gamma rays, in the energy range between 80 KeV and 300 KeV, emitted from gamma-emitting radioisotopes (^{99m}Tc , ^{111}I , ^{123}I , ^{131}I , ^{125}I), previously injected into the patient. The use

of targeted radiolabeled ligands to determine the tissue distributions of receptors allows the detection of disease upon imaging with a gamma-camera. Multiple 2D image projection can be acquired at different angles resulting in the visualization of the 3D biodistribution of the radionuclides. The spatial resolution of the reconstructed image depends on the number of angular and linear samples selected besides on the collimator and the tomographic reconstruction parameters.

PET is the most extensively used technique in the field of clinical oncology, neurology and cardiology (Cantore et al., 2011). In the same way of SPECT, this technique is based on the use of radio labelled compounds which are injected into the living subject (usually into blood circulation). Especially useful to assess the degree of changes in the cellular metabolism as well as in their anatomic structure and in the dimension of the tumor mass, it plays a fundamental role in the screening, early detection, diagnosis and treatment of neoplasms (Langen et al., 2011). This powerful technique supplies the most advanced and outstanding tools on monitoring the early results of a tumor treatment plan giving the evidence on the success of the treatment itself and so avoiding useless chemotherapy cycles.

Radionuclides used in PET scanning, typically isotopes with short half-lives (^{11}C , ^{13}N , ^{15}O , ^{18}F) which undergo positron emission decay, are incorporated either into compounds normally used by the body such as, glucose, water, ammonia, or into molecules that bind to receptors. PET apparatus is made up of a detector block (scintillator crystal and photomultiplier) and a detectors ring which detects the two 511 keV gamma photons emitted in coincidence at 180 degrees to each other further to positron-electron annihilation events. By this means it is possible to localize their source along a straight line of coincidence. Following the radiotracers journey inside the body or the organs it is possible to trace its biological pathway and collect data for analysis. During the scan a record of tissue concentration is made as the tracer decays. The radiological image obtained is a fingerprint of the functionality of the examined organs. Even though PET and SPECT are similar there are remarkable differences in terms of efficiency and resolution. Due to the detection methodology, since emissions are detected "coincident" in time, PET scanner provides more radiation events and localization information, thus giving higher resolution images than SPECT, about 4-5mm against 7-8mm. In terms of efficiency only 1 to 10 photons upon 10.000 emitted can be detected with SPECT while PET efficiency is about 100 times higher (pmoles vs nanomoles).

Small animals SPECT can provide resolutions up to 200 micrometer (Acton & Kung, 2003). Sensitivity, resolution, and field of view are dependent upon the pinhole collimator of the instrument. On the other end small animals PET scanner can provide lower spatial resolution (0.6 mm). PET is best suited for small molecules and molecules with fast kinetics. Moreover PET tracers have lower specific activity, shorter half-lives and are more difficult to synthesize. Finally PET radiation dose is 10 fold higher than SPECT (Larobina et al., 2006).

2.2 Nuclear magnetic resonance imaging

NMRI is a medical imaging technique based on nuclear magnetic resonance to image nuclei of atoms inside the body and is able to distinguish pathologic tissue from normal tissue (Schellenberger, 2004). This technique is widely used in radiology as it provides good

contrast between the different soft tissues of the body in addition to a high images quality. Based on the use of strong magnetic fields and non-ionizing radiation, radio frequency range, NMRI is completely harmless to the patient.

Differences of nuclei behavior subject to a strong magnetic field gradient can be recorded to construct an image of the scanned area of the body resulting in the visualization of detailed internal structures. In the classical representation of an atom nucleus is depicted as a rotational sphere along an axis. This rotational motion is associated with a physical quantity called: angular momentum (spin). Because of the nuclear composition the so called rotational sphere is positively charged resulting in the generation of a magnetic field as a consequence of charge motion. By this means nuclei possess an intrinsic angular momentum (spin) associated to a magnetic momentum. The spin of a charged particle is associated with a magnetic dipole momentum by a proportional factor called gyromagnetic factor. The g-factor is a characteristic of each nucleus. Allowed values of spin are 0, 1/2, 1, 3/2, 2, etc., which corresponds to certain magnetic momentum values according to a specific proportional relationship. The sensitivity by which a nucleus can be detected with NMR depends on the g-factor. Nuclei with a high gyromagnetic ratio will be easily examined. Nuclei with spin value equal to zero, null magnetic momentum, are "transparent" to external magnetic field so they can not be analyzed with NMR. Therefore only atoms with an odd number of nucleons (protons and/or neutrons) resulting in a nonzero nuclear spin can be chosen for this methodic. In the presence of a magnetic field sample nuclear spins, randomly arranged, will align along the external field lines (polarization), absorbing and re-emitting electromagnetic radiation at a specific resonance frequency which depends on the strength of the magnetic field and on the magnetic properties of the sample atoms. For a set value of magnetic field applied, different nuclei give rise to distinct resonant frequencies depending on the strength of applied field. If a sample is placed in a non-uniform magnetic field then the resonance frequencies of the sample's nuclei depend on where in the field they are located. In the NMRI techniques the polarization of the magnetic nuclear spins is achieved by exposing the sample to a constant static magnetic field. The following step is the perturbation of this alignment by employing an electro-magnetic, usually radio frequency (RF), pulse. The required perturbing frequency is dependent upon the static magnetic field and the nuclei of observation. When the RF pulse is turned off nuclear spins at different locations relaxes to the equilibrium state with consequent emission of characteristic frequencies detectable by the scanner. The electromagnetic signals shifts induced by strong field gradients are recorded to construct tridimensional images of studied area. To maximize the NMR signal strength the two fields are usually chosen to be perpendicular to each other.

NMRI uses intense applied magnetic fields in order to achieve dispersion and very high stability to deliver spectral resolution. The spatial resolution of the imaging technique depends on the magnitude of magnetic field gradient. Very high resolution images, on the order of one tenth of millimeter, can be achieved with a 3 Tesla scanners.

For mouse MRI imaging can achieve 10-100 micrometer spatial resolution, with preferably at least 7.0 Tesla. To enhance the appearance of blood vessels, tumors or inflammation, contrast agents, properly modified, may be injected intravenously acting as specific molecular probe. This opens the possibility to monitor *in vivo* cellular target functions as well as get information on the spatial distribution of a specific molecular target (Percy et al., 2011).

The employment of stable isotopes as contrast agents, diversely from PET methodology, gives to this technique the great advantage of supervising metabolic pathways or receptors dynamic for infinite time.

2.3 Optical Imaging

This non-ionizing imaging technique is based on the use of electromagnetic radiation in the near-infrared and visible field to observe single-molecule in living cells. Cost-effective, rapid, easy to use, optical imaging can be readily applied to studying disease processes and biology *in vivo* (Luker & Luker, 2008). A charge-coupled device camera (CCD camera) detects light emitted allowing for real time 2D imaging and 3D reconstruction. The key feature is the different behavior of light interaction with tissues such as adsorption, emission, excitation, scattering, fluorescence and luminescence.

Molecular targets labeled with nanodiamonds, fluorescent dyes, quantum dots, as well as bioluminescent, chemiluminescent and fluorescent reporters, would enable biomolecule tracking inside cells (Rong Tong et al., 2010). Fluorescently labeled antibodies or biomarkers can target and identify tumors or other diseases in *in vivo* modalities (van der Meel et al., 2010). Molecular and biochemical events such as transgene expression, transforming phenotype and enzyme activity can be detected by means of fluorescent probes. Tumor cells, stem cells, immunological cells can be transfected to overexpress luminescent or fluorescent proteins (Pomper, 2005). Widely used in animal models trial for low costs and good sensitivity (pmol concentrations, few cells), optical labeling technology scanner has an anatomical resolution relatively poor (1mm) due to scattering of light in tissue (Hickson, 2009). OI has a minimal use in clinic owing to limited depth profiling (couple of centimeters in tissue depth) (Leblond et al., 2010).

2.4 Computed tomography

X-ray computed tomography (CT) is a radiological technique which provides high spatial resolution (less than 1 mm) of morphological imaging; three-dimensional anatomical images. Axial sections of well-defined depth are imaged upon differential x-ray absorption by different tissues. CT is based on detection of an X-ray beam by means of detectors system which converts the attenuated beam intensity into electric pulse of equivalent value.

By interaction with electrons of the material probed, photons are removed from the incident beam. The total energy of the resulting beam is then attenuated. The higher the electron density, the more interaction of X-ray with the sample material occurs and the more attenuated is the final energy. Because of the inherent high-contrast resolution of CT, differences between tissues that differ in physical density by less than 1% can be distinguished. The CT methodology is widely use in medicine as a diagnostic tool: detailed studies of bone and joint structure, bone metastasis scans, measurement of tumor size and location, and visualization of airway structure in the lungs, can be assess with high resolution. The major drawback of the technology is the high radiation dose which can be lethal or mutagenic. For imaging systems dedicated to *in vivo* studies in animal models, the optimal resolution is 50–100 micrometer. Specimen instruments have resolutions down to less than 10 micrometer and can visualize e.g. trabecular bone structure.

3. Aptamers as excellent targeting molecules for *in vivo* imaging

Aptamers are single-stranded oligonucleotides (DNA or RNA) that like peptides or monoclonal antibodies, bind to their targets by complementary shape interactions. Indeed, aptamers function by folding into unique globular three-dimensional conformation that dictates high-affinity and specificity binding to a variety of targets, each structure being unique and determined by the sequence of the nucleic acid (Figure 1 A). As discussed in this chapter, the advantages of using nucleic acid aptamers for imaging/therapy are their favourable pharmacokinetic properties (rapid renal clearance) and their excellent affinity and specificity for their targets. These properties render aptamers useful targeting molecules for *in vivo* imaging allow obtaining high-resolution images with high signal-to-noise ratios and decreasing non-specific targeting thus reducing toxicity to non-target tissues (Tavitian et al., 1998, 2009; Perkins & Missailidis, 2007).

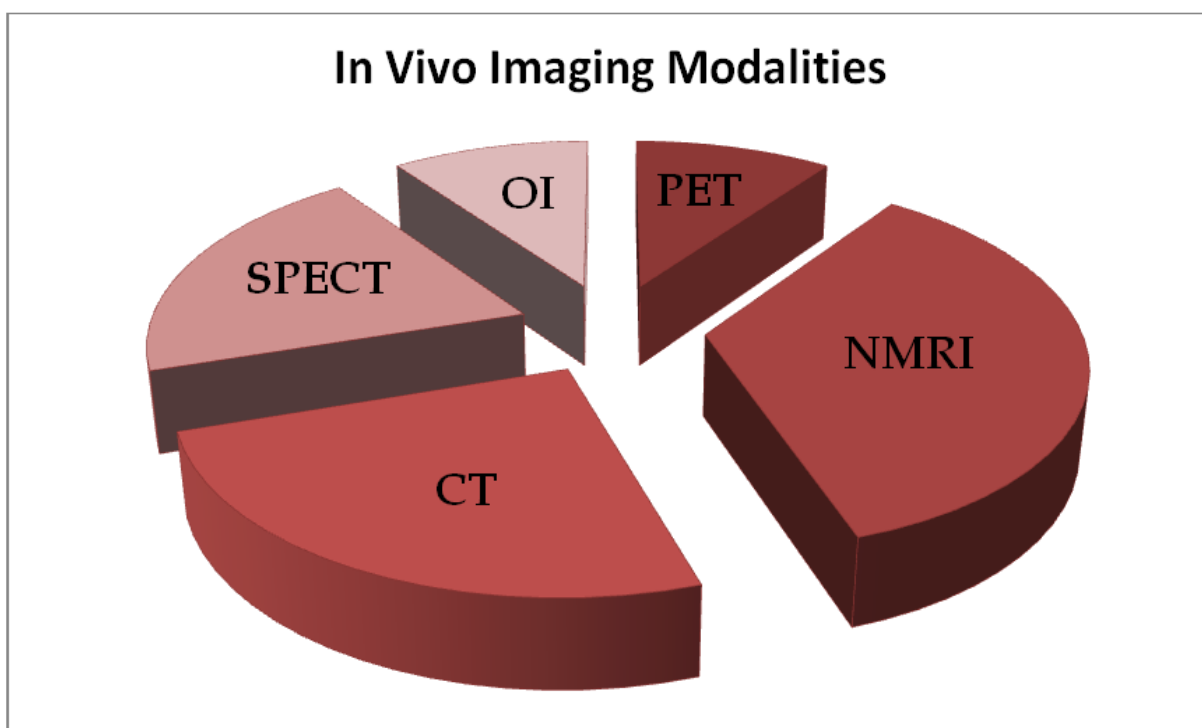


Fig. 1. The size of each slice is related to the resolution of the indicated technique; the colour scale is proportional to the cost (clockwise).

3.1 Aptamers production

Aptamers are generated by an *in vitro* evolutionary selection-amplification scheme, named SELEX (Systematic Evolution of Ligands by EXponential enrichment) from libraries of random sequence oligonucleotides of large sequence complexity (generally between 10^{13} and 10^{15} members) (Ellington & Szostak, 1990; Tuerk & Gold, 1990). As schematized in Figure 1 B, a typical SELEX experiment includes reiterated rounds of: (1) incubating the library with the target molecule; (2) partitioning nucleic acids bound specifically to the target molecule from unbound sequences; (3) dissociating the nucleic acid-protein complexes; and (4) amplifying of the nucleic acids pool enriched for specific ligands. After the final round, the PCR products are cloned and sequenced to subsequently identify the

best binding sequences. Due to the fact that the specific, three-dimensional arrangements of a small number of contact points of the aptamer mediates the target-aptamer interaction, rather than a general affinity for the sugar-phosphate backbone of the nucleic acid, aptamers can achieve high target selectivity. In addition, the binding characteristics of aptamers can be influenced by the type of the experimental system used for the selection and counter-selection (depletion of aptamers that bind to non-target molecules). As a result, aptamers bind their own target with sub-nanomolar affinity thus revealing useful for many of the applications for which antibodies are already employed because of their own definite characteristics: (i) capacity to discriminate among closely related targets (e.g., will typically not bind other proteins from the same gene family); (ii) capacity to quantify the level of expression of the protein target; (iii) through binding, may act as inhibitors to block the activity of the target product. More important, they are usable not only for *in vitro* but also for *in vivo* purposes since they are sufficiently stable and can be readily chemically modified to further enhance their stability, bioavailability and pharmacokinetics (Esposito et al., 2011; Keefe et al., 2008, 2010).

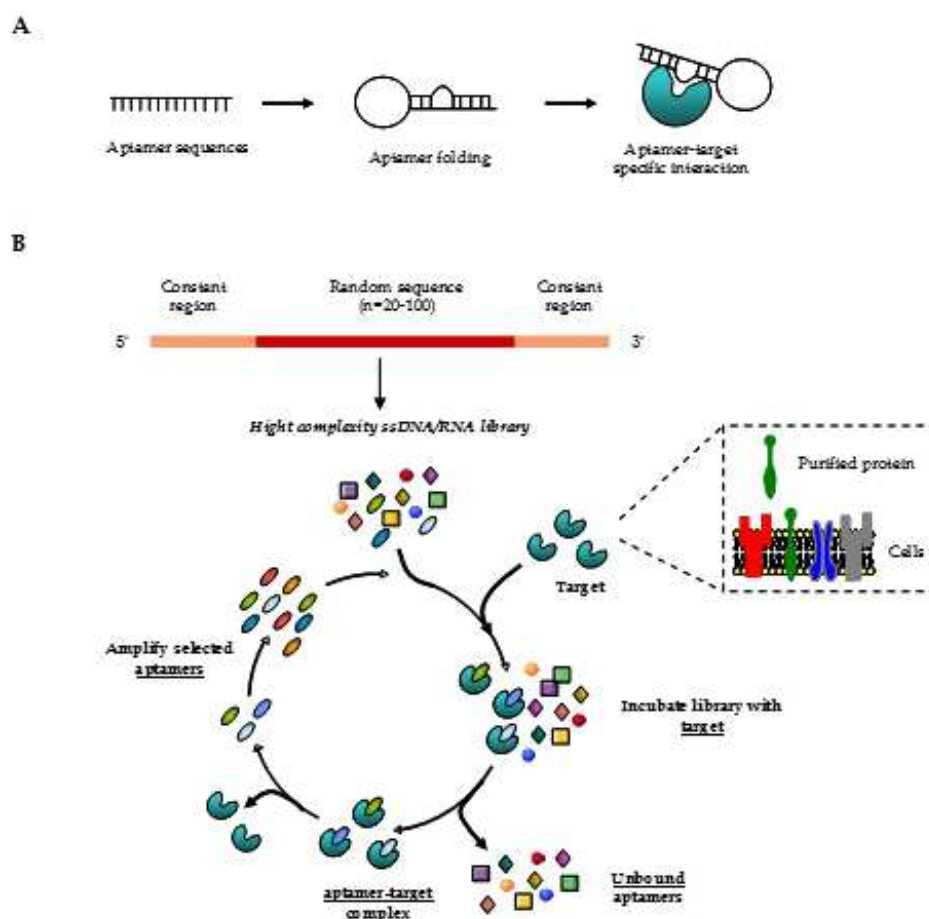


Fig. 2. Schematic representation of SELEX technology. The RNA/DNA aptamer library contains a variable region flanked by two constant regions. These constant regions include primer sites for PCR amplification and a consensus promoter for the T7-RNA polymerase (in the case of an RNA library). The library is used for the selection process. The target can be a purified recombinant protein (Protein-SELEX) or a complex target as entire living cells (cell-SELEX)

3.2 Systemic clearance of the aptamers

Furthermore, due to their relatively small size (8-15 kDa) in comparison to antibodies (150 kDa), aptamers should be better suited for rapid tissue penetration and blood clearance, two excellent characteristics for contrast agents in imaging. Indeed, when antibodies bind their target, their slow clearance may necessitate waiting for days before a significant signal emerges from the background of excess unbound ligand. This of course often results in organ toxicity and, in addition, limits the usefulness of imaging technology in diagnosing acute conditions, and precludes the use of short-lived radionuclides.

On the contrary, aptamers not conjugated to polyethylene glycol (PEG) are cleared very quickly from blood circulation because of renal excretion and hepatobiliary clearance. A typical short, nuclease-resistant oligonucleotide administered intravenously exhibits an *in vivo* half-life of <10 min, unless filtration can be blocked by either facilitating rapid distribution out of the blood stream into tissues or by increasing the apparent molecular weight of the oligonucleotide above the effective size cut-off for the glomerulus. In addition, immunogenicity has been found to be either absent or limited when 1000-fold higher doses of an aptamer (than would be required clinically) were administered to monkeys (Foy et al., 2007).

This property depends on the fact that antibodies to synthetic oligonucleotides are not generally produced and, in addition, the innate immunity response against non-self RNAs does not hinder aptamer therapy because 2'-modified nucleotides abrogate Toll-like receptors responses (Yu et al., 2009).

3.3 Stability of the aptamers

Prerequisites for a successful *in vivo* application of aptamers as molecular target imaging agents are represented by high affinity and selectivity to their target as well by adequate stability against *in vivo* degradation. Since oligonucleotides, especially RNAs, are rapidly degraded by nucleases in whole organisms, resulting in a very short half-life in the blood, a variety of approaches have been addressed to increase the stability of natural nucleic acids in biological media (Keefe & Cload, 2008).

The most effective modifications to circumvent this limitation are the substitutions at the 2'-ribose of the pyrimidines that are mainly affected by serum nuclease degradation. Initially, attention is focused on 'post-SELEX modifications', however, due to the fact that folding rules for single-stranded oligonucleotide regions change when these modifications are introduced, the binding properties of an aptamer selected in the presence of standard nucleotides might be completely different when the same sequence is synthesized with nucleotides containing a different 2'-substituent (Blank et al., 2001; Usman & Blatt, 2000; Aurup et al., 1992). To circumvent this limitation selections can be performed directly in the presence of 2'-modified nucleotides, as long as the modified nucleotides are accepted by T7 RNA polymerase for the *in vitro* reaction steps of the selection (Aurup et al., 1992; Ruckman et al., 1998). RNA-aptamers containing 2'-fluoro and 2'-amino pyrimidine (2'-F-Py, 2'-NH₂-Py, respectively) can be generated performing the selection in the presence of 2'-modified nucleotides (Chelliserrykattil et al., 2004). Analogously, aptamers containing 2'-O-Methyl purines (2'-O-Me) have also been developed through post-SELEX modification steps or by

starting from 2'-O-Me-containing random sequence libraries during the SELEX process itself (Burmeister et al., 2004; Chelliserrykattil & Ellington, 2004).

Even if 2'-modified nucleotides-containing aptamers show considerable increase in serum stability also in the absence of other modifications, changes in the internucleotide linkages (such as the use of phosphorothioate) and in the nucleobases (for example, the substitution of 5-position of uridine) as well the capping at the oligonucleotide 3'-terminus, have been reported (Keefe & Cload, 2008).

Conformation flexibility is hypothesized to be a major factor limiting the affinity and specificity of interactions due to the entropic penalty upon binding. Furthermore, since single-stranded regions are the primary site of nuclease attack, the conformational flexibility would render the aptamer more accessible to nucleolytic degradation and thus, reduction of flexibility would be a key prerequisite for successful *in vivo* application. One way to stabilize aptamers is to increase thermal stability of double-stranded areas located within non-binding regions. In this respect, the use of locked nucleic acids (LNA), containing a methylene bridge to connect the 2'-O to the 4'-C, increases the stability of base pairing stabilizing the duplex and enhancing the resistance to nuclease. For example the introduction of LNA-modifications in the anti-tenascin C (TN-C) aptamer significantly improves its plasma stability of approximately 25% and enhances the tumor uptake (Schmidt et al., 2004).

An interesting application of the SELEX process is based on the selection of RNA aptamers binding to the mirror-image of an intended target molecule (e.g. an unnatural D-amino acid peptide), followed by the chemical synthesis of the mirror-image of the selected sequence (Eulberg & Klussmann, 2003). As a consequence of molecular symmetry, the mirror-image aptamer (made from L-ribose) binds to the natural target molecule. Because of the substitution of the natural D-ribose with L-ribose, the mirror-image aptamer (named Spiegelmer) is totally stable. NOX-A12 and NOX-E36 are two spiegelmers actually in clinical trials for the treatment of hematologic tumors and complications of type 2 diabetes, respectively (Esposito et al., 2011; Ellington, 2010).

4. Inflammation imaging with aptamers

The assessment of precise and reliable methods for the diagnosis of inflammatory processes is an important goal in medicine for the effective management of inflammation-related diseases. Acute inflammatory diseases are characterized by a significant increase in the lymphocytes and neutrophils count in the peripheral blood while chronic inflammatory diseases are characterized by infiltration of the target organ by mononuclear cells, such as lymphocytes, plasma cells, and macrophages (Weiss, 1989).

Some inflammatory processes, such as organ-specific autoimmune disorders, are difficult to diagnose because they either are asymptomatic or have non-specific symptoms. The possibility of diagnosing inflammatory processes during the early stage may allow for early therapeutic intervention and possible prevention of the disease.

During inflammation, levels of several proteolytic enzymes are significantly increased including neutrophil elastase that is released by neutrophils accumulated at the site of inflammation (Doring, 1994). Aptamers as specific irreversible inhibitors of neutrophil

elastase have been isolated by 'blended SELEX', a modification of the basic SELEX technology that allows to confer high-affinity binding to a weak, covalent inhibitor of an enzyme (Smith et al., 1995; Charlton et al., 1997).

In this process, a randomized nucleic acid library attached covalently to an inhibitory compound of elastase (a valyl diphenyl ester phosphonate) was used to select for a secondary, stabilizing contact for the target molecule. By iterative selection and amplification steps, the nucleic acid sequences that best promote the covalent reaction of the valyl phosphonate moiety with the active-site serine of elastase were identified. In addition to inhibiting elastase free in solution, these aptamers were highly effective at blocking degradation of elastin particles by activated neutrophils. Further, a truncated DNA aptamer version, named NX21909, was tested in a rat model of lung inflammation and was found to inhibit neutrophil infiltration by 53% at a dose of 40 nmol (Bless et al., 1997). Using fluorescent flow cytometry, the aptamer revealed able to bind preferentially to activated neutrophils (Charlton et al., 1997).

The first example of the use of aptamers as *in vivo* imaging probes is represented by the use of NX21909 for diagnostic imaging of inflammation in a rat model of the reverse passive Arthus reaction induced by immune complex deposition (Charlton et al., 1997). The aptamer was labelled with ^{99m}Tc and given by intravenous injection 3.5-4 h after induction of inflammation. Rats were imaged on a gamma camera in 10 min frames continuously for 40 min and additional 10 min frames were taken every hour up to 4 h after administration of imaging agent.

Remarkably, a better signal-to-noise ratio was achieved by the aptamer compared to a clinically used IgG-based inflammation imaging agent used as a positive control (for the aptamer, a ratio of 4.3 ± 0.6 was achieved at 2 h; for IgG, a ratio of 3.1 ± 0.1 was achieved at 3 h). This ratio was achieved primarily by the rapid clearance of the aptamer from the peripheral circulation, thus indicating that the aptamer technology may be successfully applied to diagnostic imaging.

5. Thrombus imaging with aptamers

Thrombin is the last enzyme in the clotting cascade functioning to cleave fibrinogen to fibrin which forms the fibrin gel of a hemostatic plug or a pathologic thrombus. In addition to its role in blood coagulation, it also triggers important anticoagulant and antifibrinolytic pathways (Petäjä, 2011). The crystal structure of thrombin has been determined (Bode et al., 1992; Stubbs & Bode, 1993) thus giving important insight into its structure-function relationship. DNA aptamers have been generated that bind to different domains of the protein. ODN1 binds to exosite 1, the thrombin substrate binding site, thus competing with fibrin for binding to the same site on thrombin (Bock et al., 1992; Wu et al., 1992). Conversely, ODN2 reacts with exosite 2, the heparin binding site on thrombin (Tasset et al., 1997). These aptamers have been extensively characterised for their binding to thrombin and ODN1, is currently being evaluated in phase II clinical trials by ARCA Biopharma/Archemix Corp. as an anticoagulant for use during acute coronary artery bypass surgery. Being unmodified and unstable *in vivo* the aptamer, designated as Nu172, has to be administered by continuous intravenous infusion (Esposito et al., 2011).

To generate thrombin-dependent images in aptamer-mediated thrombus imaging approaches, the radiolabeled aptamer must form a ternary complex with thrombin bound to

the fibrin matrix. For this reason ODN 2, but not ODN 1, has been used for thrombus imaging (Dougan et al., 2003).

In vitro experiments showed that ODN2 can form a ternary complex (with thrombin and fibrin) in clots, a necessity for thrombus imaging, thus indicating that allosteric interference from the fibrin binding site (exosite 1) did not impede ODN 2 binding at exosite 2. Thrombin-dependent uptake of ODN 2 was observed with *in situ* labeling of clots and by labeling of preformed clots. Further, the retention of [¹²⁵I]ODN 2 increased as the thrombin content of thrombi was increased whereas the rate of release of ODN 2 out of preformed thrombi decreased. Then, the aptamer was tested *in vivo*, in a rabbit jugular vein model using thrombus supplemented with human thrombin,. The labeled aptamer was introduced internally with the portion of rabbit blood, or else externally to a preformed unlabeled clot via the truncated facial vein and the thrombus area was imaged with a 2D scintigraphic camera, or excised for counting. Despite the clearly promising effect *in vitro* when ODN 2 was tested *in vivo*, the uptake was equal to the ovalbumin control and did not reflect thrombin content. A possible explanation of the failure of the *in vivo* thrombin-dependent imaging was attributed to the rapid clearance of ODN 2 from blood, combined with slow mass transfer in the thrombus. It is conceivable that nucleic acid analogs with extended lifetimes in circulation might help overcome this problem. For instance, the clearance rates of aptamers can be altered to keep them in circulation by anchoring them to liposome bilayers, by coupling them to inert large molecules such as PEG or to other hydrophobic groups (Willis et al., 1998; Healy et al., 2004).

It is noteworthy that the Food and Drug Administration (FDA)-approved aptamer, Macugen, as well as all the aptamers in clinical trials, except for Nu172, have been conjugated with PEG to enhance their half-life *in vivo* (Esposito et al., 2011).

6. Aptamers as *in vivo* imaging reagents in cancer

The aim of this section is to give a detailed overview on the aptamers developed for tumour imaging. Pointing out the attention on the structural modification as well as the bioconjugation with specific tags, we will focus on the experimental results emphasizing the essential requirements for potential application of aptamers as imaging probes for *in vivo* studies: high target specificity and affinity, rapid pharmacokinetics, exclusion of toxic side effects, tumour penetration, chemical stability, and high signal-to-noise ratio.

Five modalities (PET, SPECT, NMRI, CT and OI) are available to the scientist for oncological investigations in animals (Lewis et al., 2002; Tavitian, 2003). PET and NMRI technologies offer deep tissue penetration and high spatial resolution, but compared with non-invasive small animal optical imaging, these techniques are very costly and time consuming to implement. Aptamers have been successfully coupled to different targeted radiolabeled ligands and contrast agents to generate probes for *in vivo* medical imaging by using the five modalities previously described.

6.1 Aptamers conjugated with ^{99m}Tc for SPECT

The TTA1 aptamer against TN-C has been generated by a crossover SELEX experiment that involves crossing from cell-SELEX on TN-C-positive U251 glioblastoma cells to protein-SELEX against purified recombinant TN-C (Hicke et al., 2001).

To improve stability against plasma nucleases, therefore guarantee a good blood persistence leading to sufficient signal-to-noise ratios for imaging, aptamer backbone (2'-F-Py containing RNA, 39mer) has been modified with the addition of maximal 2'-OMe purine substitutions, a thymidine cap at the 3' end and LNA in not binding critical stem (Shimdt et al., 2004). This TTA1 derivative has been further conjugated with mercapto-acetyl diglycine (MAG2) chelate via an hexyl-aminolinker at the 5' end and labelled with ^{99m}Tc for performing single photon emission computed tomography (Hicke et al., 2006).

To assess the degree of stability, an essential feature to achieve a sufficient targeting and blood clearance, combined to low background activity in non-target organs, tumour to blood and tissue distribution of ^{99m}Tc -labeled aptamer have been examined in murine xenograft models of glioblastoma and breast cancer (Hicke et al., 2006). Images of glioblastoma and breast tumors were taken at various times after intravenous injection in mice using a γ -camera. After 3 h post injection the tumor mass was visible as well as liver and bladder. At 18h post injection the two major clearance pathways were entirely clean from any radioactivity, as predicted by biodistribution analysis (Table1), and the tumor was clearly visualized as blazing structure (Hicke et al., 2006). Data show a rapid renal and hepatobiliary clearance, 0.2 %ID/g and 1.5 %ID/g at 3h, respectively and a rapid tumor penetration (6% injected dose at 60 min). Tumor retention is durable (2.7% injected dose at 60 min) and the tumor-to-blood signal is significantly high (Hicke et al., 2006).

Substitution of DTPA- ^{111}In or insertion of a PEG_{3,400} linker have also been tested for biodistribution analysis showing a dramatic alteration of tissue uptake and clearance patterns, indicating that simple changes to chelator can have significant effects on tissue uptake and clearance patterns.

DNA-aptamers that target cell-surface mucin 1 glycoprotein (MUC1), generated by Protein-SELEX against purified MUC1 peptides (Ferreira et al., 2006), have also been labeled with ^{99m}Tc (Ferreira et al., 2006). The aptamers, named AptA and AptB, have been selected against the protein core or the glycosylated protein, respectively. Various chelating agents have been conjugated to the aptamers and multi aptamer complexes have shown increased retention of the complex in circulation without increasing the immunogenicity of the complex or adversely altering its tumor penetration properties. The biodistribution data of the labeled products have shown differences that depend on the size of the conjugate and the type of ligand (Borbas et al., 2007). Four types of chelators have been coupled to anti-MUC1 aptamers to generate a novel complex for diagnostic imaging with improved properties: MetCyc, MAG₃, DOTA and 4 (Figure 3).

Differently from the monomeric Tc-MetCyc-Apt and Tc-MAG3-Apt, the tetrameric compounds, DOTA and 4, conjugate with ^{99m}Tc are able to grab four aptamer molecules modified at 5' by insertion of amine groups to facilitate coupling with the ligands carboxylic terminations and 3' ends to protect against nuclease degradation. Both tetrameric and monomeric aptamers were tested in nude mice bearing xenografted MCF7 breast cancer tumor. Compared to the monomeric biomarker composites, DOTA and 4 showed an increased retention in the circulation by means of their largest molecular weight. Despite the tetrameric complexes showed improved tumor retention and pharmacokinetic properties compared to the monomeric compounds, biodistribution studies have exhibited presence of free technetium in the stomach and large intestine highlighting some kind of lack in the Tc-aptamer binding (Borbas et al., 2007).

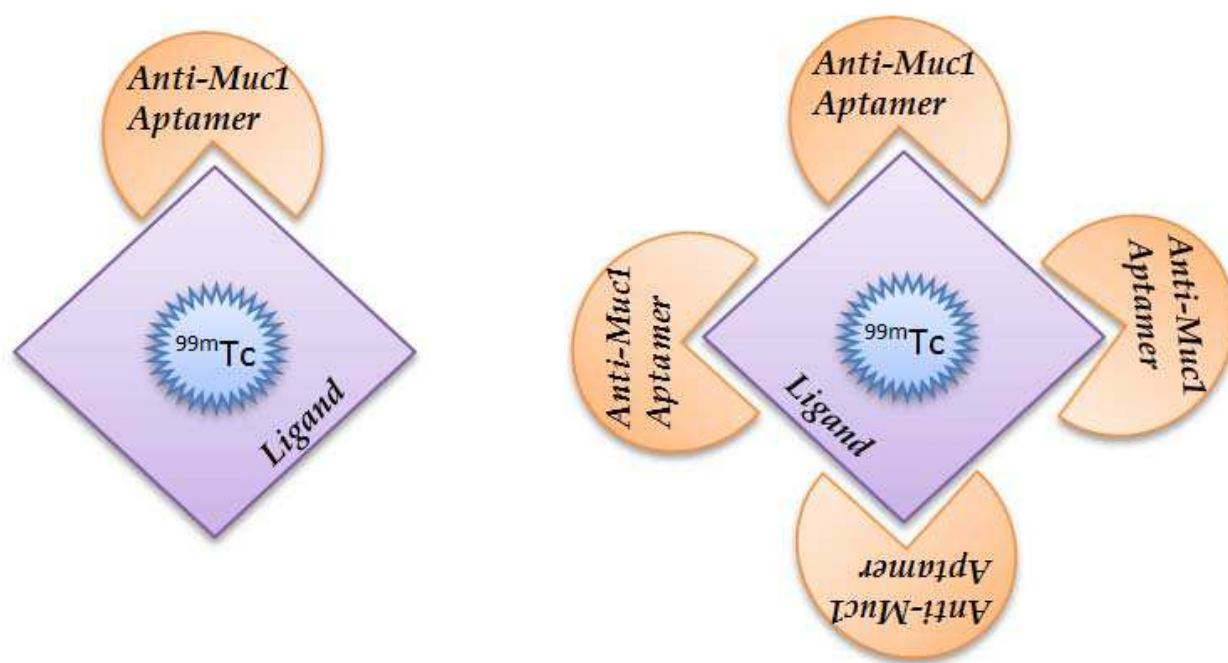


Fig. 3. Schematic representation of monomeric and tetrameric aptamer bioconjugates (see text for details).

Anti-MUC1 aptamers have also been successfully conjugated to MAG2chelator and the complexes have been labeled with ^{99m}Tc (Pieve et al., 2009). Analysis of two aptamer-radionuclide conjugates, AptA and AptB, biodistribution properties in MCF7 xenograft-bearing nude mice shows strong ^{99m}Tc binding properties (70-80% for AptA and $\geq 80\%$ for AptB) and high stability *in vivo*, in terms of nuclease degradation and leaking of the metal (Table 1). Both aptamers show a maximum tumor uptake after 5h post injection, even though Apt B has higher accumulation rate, 0.12 %ID/g (for AptA) and 0.14 %ID/g (for AptB), followed by a decrease at 16h, 0.033 %ID/g (for AptA) and 0.016 %ID/g (for AptB) and 22h, 0.008 %ID/g (for AptA) and 0.013 %ID/g (for AptB). As pointed out by these data AptB releases quicker from the target keeping his activity constant between 16h and 22h, whilst AptA activity decreases constantly in time. The activity discrepancy is consequence of a different kinetic behaviour upon binding with the molecular target. AptB, in fact, internalized in the cancer cell quicker than AptA. Table 1 shows tumour-to-blood ratios values, taken at different times post injection, of both oligonucleotide carriers, confirming the assumption of Missailidis's group. The maximum levels of tumour uptake and clearance has reached at 5h post injection for AptA and AptB (table 1) but whiles the tumour-to-blood ratio trend of AptB, after 5h, is constant in time, AptA decreases drastically at 16 h, reaching his minimum value, and increases again at 22h, suggesting that the bound aptamer was not internalized.

Even if the radiolabeled aptamers demonstrate good tumor uptake and clearance, they require further optimization before diagnostic use.

Target	TNC	MUC1	
Aptamer	TTA1/2'Fy-RNA	AptA/DNA	AptB/DNA
Length(nt)	39	25	25
K _d (nM)	5	5	5
Tag	^{99m} Tc	^{99m} Tc	^{99m} Tc
Tumour (%ID/g)			
10 min	5.9±0.6		
1h	2.69±0.30		
3h	1.88±0.10	0.051±0.022	0.044±0.008
5h		0.12±0.046	0.14±0.040
16h		0.033±0.034	0.016±0.005
22h		0.008±0.001	0.013±0.005
Blood (%ID/g)			
10 min	2.27±0.25		
1h	0.11±0.00		
3h	0.03±0.00	0.027±0.002	0.024±0.001
5h		0.14±0.04	0.19±0.04
16h		0.005±0.001	0.009±0.001
22h		0.004±0.001	0.007±0.004
T/Blood			
10 min	2.6		
1h	24.0		
3h	62.6	1.8	1.8
5h		0.8	0.7
16h		6.6	1.8
22h		2	1.8

Table 1. *In vitro* characteristics and pharmacokinetics in mice for anti-TNC and anti- MUC1 aptamers.

6.2 Nanoparticle-aptamer conjugates for OI and NMRI

Aptamers were also used to functionalize nanoparticles surface for a potential *in vivo* cell imaging applications (Farokhzad et al., 2004; Javier et al., 2008; Chu et al., 2006; Rong et al., 2010; Savla et al., 2011).

Much work has been made by using the two 2'fluoro-pyrimidine (2'Fy)-RNA aptamers (A9 and A10) that have been generated against the extracellular domain of PSMA (prostate-specific membrane antigen), (Lupold et al., 2002). These aptamers bind with high affinity to the acinar epithelial cells of prostate cancer tissue. They have been used to deliver nanoparticles, quantum dots (QDs), toxin, or siRNA to prostate cancer cells (Cerchia & de Franciscis, 2010).

Poly(D,L-lactic acid) (PLA)-PEG-COOH nanoparticles and microparticles, conjugated with the A10 RNA aptamer have been developed to target the (PSMA) cells *in vivo* (Farokhzad et al., 2004).

PLA derivatives exhibit a desirable characteristic by means of their neutral to slightly negative surface charge, preserving the aptamers binding characteristic by specific interaction with them. On the opposite, particles with a positive surface charge may interact non specifically with the negatively charged aptamers affecting their binding properties.

Moreover the incorporation of PEG containing a terminal hydroxyl and carboxylic acid functional groups results essential to maximize nanoparticle circulating half-life and to allow the covalent coupling with 3'-NH₂-modified aptamer.

To examine the presence of the aptamer-nanoparticle conjugates on the surface of PSMA-positive cells, the A10 aptamer has been labeled at 5' end with FITC and uptake by LNCaP cells has been evaluated by fluorescent microscopy. Images taken after 2 h and 16 h of incubation, showed a massive internalization just after 2h of nanoparticle-aptamer compound versus control group (aptamer-treatment of PSMA-negative PC3 cells).

These aptamer-nanoparticles conjugates have been loaded with docetaxel, anti-cancer therapeutic and successfully used for targeted delivery to PSMA-positive cells (Farokhzad et al., 2006).

PSMA has been also labelled using aptamer-quantum dots conjugates (Chu et al., 2006). A different anti-PSMA aptamer, A9, has been selected and conjugated to luminescent CdSe and CdTe nanocrystals for cell labelling.

Quantum dots (QD) represent the most promising fluorescent markers and delivery vehicles. These semiconductor particles have nanometre size and offer revolutionary fluorescence performance over traditional fluorescence dyes such as long-term photostability for live-cell imaging and dynamics studies, brilliant colors for simple, single-excitation multicolor analysis, fixability for follow-up immunofluorescence after *in vivo* studies, narrow and symmetrical emission spectra for low interchannel crosstalk.

Before conjugation the synthesized aptamer was biotinylated at its 3'-end by periodate oxidation and conjugation to biotin hydrazide (Qin & Pyle, 1999).

Labeling experiments were carried out for fixed and live cells and live cells embedded in a collagen matrix. The A9 aptamer-QD conjugates showed specific *in vitro* labeling for the selected target exhibited a good capacity to penetrate deep into tissues.

QD has also been conjugated with MUC1 aptamer and doxorubicin (QD-MUC1-DOX), via acid labile hydrazine bond, for *in vivo* imaging and drug delivery (Salva et al., 2011). *In vitro* and *in vivo* studies confirmed that the complex QD-MUC1-DOX accumulate predominately in the tumor and effectively release inside cancer cells (Salva et al., 2011).

A novel, multifunctional, thermally cross-linked superparamagnetic iron oxide nanoparticle (TCL-SPION) which can detect prostate cancer (PCa) cells and deliver targeted chemotherapeutic drugs has been developed (Wang et al., 2008). TCL-SPION nanoparticle has been conjugated with A10 aptamer and the complex so obtained has been tested for PCa imaging, using a single-sided NMR probe, and therapy, loading the bioconjugate with DOX.

NMR studies confirm the potential of the TLC-SPION-Apt complex as a targeted MR contrast showing that the bioconjugate can detect PSMA-expressing PCa cells with high sensitivity. The Dox-loaded TLC-SPION-Apt compound has been tested for antiproliferative activity against both the LNCaP and PC3 cell lines. Results showed selective delivery to PSMA-expressing cells without loss in cytotoxicity (Wang et al., 2008).

Further, PLA nanoparticles containing stably incorporated cyanine dyes have been tested for *in vitro* and *in vivo* imaging applications (Tong et al., 2010). Cy5-PLA/A10 aptamer nanoparticles have been found to only bind to and get internalized by LNCaP and canine prostate adenocarcinoma cells (PSMA-positive), but not to PC3 cells (PSMA-negative). Then, conjugates have been intravenously administered to balb/c mice and *in vivo* biodistribution evaluated by using the LI-COR Odyssey scanner. Accumulation of Cy5-PLA NP within visceral organs allows for greater than >10-fold increase in fluorescent intensity in comparison with background autofluorescence. Splenic accumulation of Cy5-PLA NP is the greatest for all visceral organs examined (spleen, liver, kidney, lung, and heart).

Another application of the anti-PSMA A9 aptamer as molecular-specific contrast agents for prostate cancer imaging has been developed by Javier et al (Javier et al., 2008). The aptamer has been conjugated by using an extended aptamer design where the extension is complementary to an oligonucleotide sequence attached to the surface of gold nanoparticles. Binding of nanoparticle-aptamer bioconjugates to prostate LNCaP cells has been valued by reflectance imaging of the labeled cells with confocal microscopy in reflectance mode.

More recently, *in vivo* imaging of tumor has been performed by using the DNA aptamer sgc8, generated by cell-SELEX on human acute lymphoblastic leukaemia cells and identified to interact with protein tyrosine kinase-7 (PTK7) (Shi et al., 2011). The aptamer has been modified in order to generate an activatable probe consisting of the binding site of the aptamer (A-strand), a poly-T linker and a short DNA sequence complementary to a part of the A-strand, with a fluorophore and a quencher attached at either terminus. In the absence of binding, the aptamer is in a hairpin structure resulting in a quenched fluorescence. When the probe binds to cell-surface receptors, it changes conformation resulting in an activated fluorescence signal. This molecule was intravenously injected into tumor-bearing mice and fluorescence images were collected. Compared to always-on aptamer probes, the activatable aptamer showed significantly enhanced image contrast and shortened diagnosis time to 15 min.

6.3 Nanoparticle-aptamer conjugates for *in vivo* multimodal imaging

Recently, a multimodal cancer-targeted imaging system capable of simultaneous *in vivo* fluorescence imaging, radionuclide imaging and magnetic resonance imaging in mice, has been reported by using the AS1411 aptamer as targeting molecule (Hwang et al., 2010). AS1411 aptamer is a 26-mer guanine-rich oligonucleotide (GRO), which in solution folds into quadruplex structures, that make it very stable and resistant to degradation by serum enzymes. This aptamer that binds to nucleolin, a protein that is often over-expressed on the surface of cancer cells, is currently in Phase II of clinical development (Bates et al., 1999, 2009; Mongelard & Bouvet, 2010). In this study, the aptamer was conjugated with a cobalt-ferrite nanoparticle surrounded by fluorescent rhodamine, and the resulting particle was bound with 2-(p-isothio-cyanatobenzyl)-1,4,7-triazacyclonane-1,4,7-triacetic acid (p-SCN-bn-NOTA) chelating agent and further labeled with radionuclide gallium citrate Ga-67.

The purified nanoparticles, designated MFR-AS1411, were intravenously injected into a mice xenografted with nucleolin-expressing C6 rat glioma cells and radionuclide images were acquired at 1, 6, and 24 h after injection. Scintigraphic images of C6 tumors in mice that received MFRAS1411 showed that tumors had accumulated MFR-AS1411 at 24 h after injection whereas the mutant, not functional, MFR-AS1411 administration revealed rapid clearance via the bloodstream.

Further, bioluminescence images analysed using IVIS200 system in the C6 cells, stably expressing the luciferase gene, illustrated the *in vivo* distribution. MR images of the same mice injected with MFR-AS1411 showed dark T2 signals inside the tumor region, compared with the MRI signal of the tumor region injected with mutant MFR-AS1411 particles.

Taken together, the above results by means of multifunctional imaging modality platforms demonstrate that MFR-AS1411 specifically targeted cancer cells.

7. Future perspectives

To date only few aptamers have been developed as targeting agents in imaging modalities, as discussed in the present chapter and schematized in figure 4, nevertheless, their low immunogenicity, good tumor penetration, rapid uptake and fast systemic clearance, indicate a great potential of these molecules as imaging agents especially when compared to antibodies. Given the nature of the aptamers and their discrimination and targeting capacities, the majority of the aptamers not bound to the target tissues is cleared rapidly from the system. In comparison, intact radiolabelled antibodies remain in circulation for a very long time, often resulting in organ toxicity.

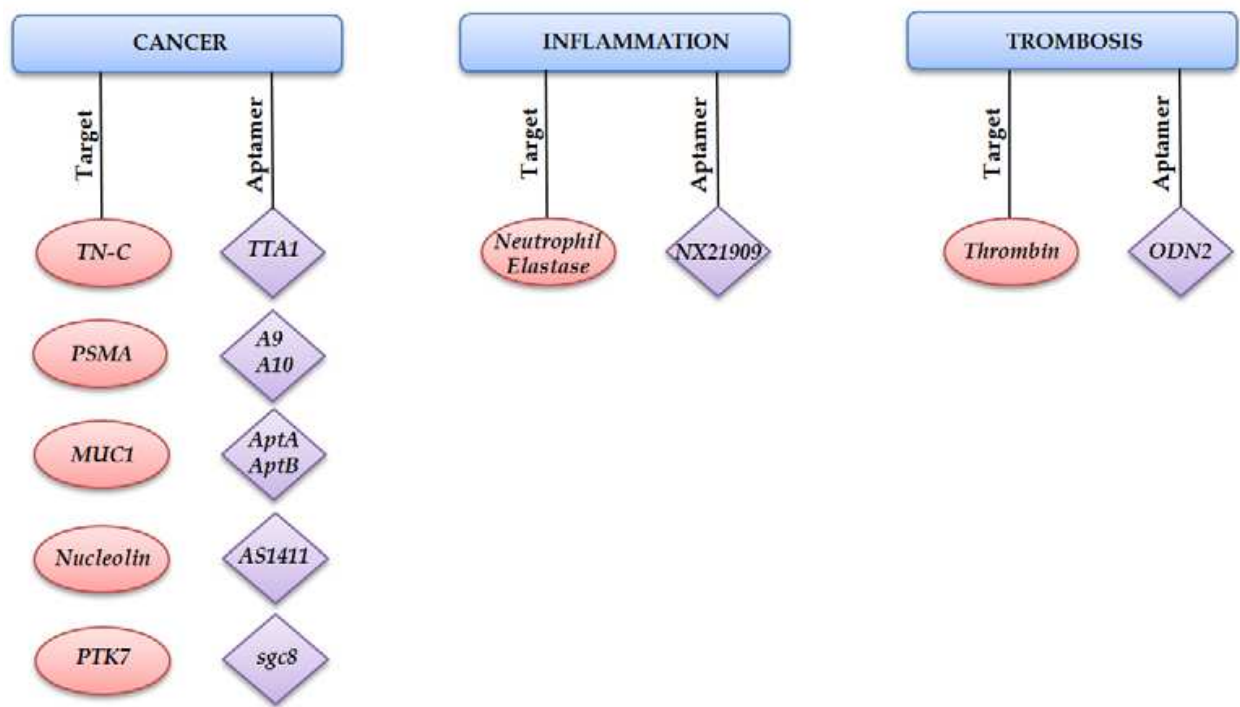


Fig. 4. Aptamers to date developed as ligands for tumour imaging, inflammation imaging and thrombus imaging

Further, the continuous advances in SELEX technology allow now to easily generate aptamers against virtually any protein and it is plausible speculate that the global market demand for nucleic acid aptamers will increase in the next few years opening the aptamer field for development for therapy, diagnosis and drug development imaging.

8. Take-home-message

Some molecule, most notably those found in nature, have the ability to reach out and grab other molecules and bound to them. Aptamers appear to fulfill this criterion and have been identified as excellent candidates for targeting specific epitopes in clinical diagnosis and therapy of different diseases. Further, because of their high specificity and low toxicity aptamers may reveal as the compound of choice for *in vivo* cell recognition as delivery agents for nanoparticles, small interfering RNAs bioconjugates, chemotherapeutic cargos and molecular imaging probes (Cerchia & de Franciscis, 2010).

There have been significant technological advances in cell-SELEX and *in vivo*-SELEX strategies. Although aptamers are relatively new to the clinic, the fact that they can act as specific recognition ligands to target cells *in vivo* suggests that these molecules will have a considerable impact in patient care in the near future.

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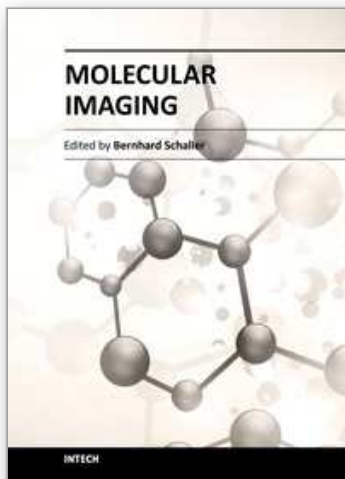
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The present book gives an exceptional overview of molecular imaging. Practical approach represents the red thread through the whole book, covering at the same time detailed background information that goes very deep into molecular as well as cellular level. Ideas how molecular imaging will develop in the near future present a special delicacy. This should be of special interest as the contributors are members of leading research groups from all over the world.

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