MULTIFUNCTIONAL SYSTEM

FIELD OF THE INVENTION

The present invention is related to a technological platform for diagnostic, therapeutic or theragnostic applications. The invention is related to a multifunctional system bearing a ligand capable of recognizing tumor cells or tumor-associated or inflammation-associated component and linked to a metal nanoparticle. More specifically, the multifunctional system of the invention comprises a nucleotide aptamer or a variant thereof that binds to the alpha 5 subunit of integrin and a metal nanoparticle, wherein said aptamer optionally comprises at least one modification capable of enhancing nuclease resistance thus providing a molecule with high stability for example in human urine.

BACKGROUND OF THE INVENTION

Bladder cancer (BC) is the tenth most common cancer worldwide, with more than half million new diagnoses and 200,000 death every year[1]. Approximately 75% of patients who are first diagnosed with BC have tumors confined in the urothelium (in situ carcinoma-Cis, and Ta) or in the lamina propria (T1), and are classified as non-muscle invasive bladder cancer (NMIBC)[2]. Guidelines for the clinical management of NMIBC involve endoscopic transurethral resection of bladder tumor (TURBT) followed by intravesical instillation of adjuvant therapy[3]. Unfortunately, BC patients with NMIBC often have very high tumor recurrence rate. Even the use of photodynamic diagnosis (performed using blue light after preoperative intravesical instillation of hexaminolaevulinic acid), followed by intravesical instillation of Mytomicin C could not reduce the frequency of NMIBC relapse, which stands at 60% 3 years post TURBT[4]. Hence, more than 200,000 BC patients each year are expected to have NMIBC relapse. The relapses often require multiple intervention sessions/cycles and follow-ups in order to eradicate the tumor. This, coupled with the high cost involved in TURBT[5], results in BC becoming the most expensive malignancy to treat over the lifetime of the patients, with an annual expenditure of USD 10 billion[6,7]. Consequently, reducing NMIBC relapse rate is viewed as a priority to improve the quality of life and reduce the economic burden of patients and their immediate caregivers.

To improve the success of the clinical management of NMIBC, two limitations need to be overcome. The first is technological limitation. Photodynamic diagnosis is ineffective for the detection of tumor smaller than 1 mm. This problem becomes more severe when diagnosing high-grade bladder C is due to its velvet-like appearance and with few neoplastic cells in a reddish area that is indistinguishable

from inflammation. For lesions <1 mm in size, Cis cannot be diagnosed using only imaging methods, such as CT urography, IVU, US, mpMRI and the recently described high-frequency microultrasound, which although capable of discriminating between NMIBC and MIBC, can only detect tumors larger than 5 mm[8,9]. Even cystoscopy, which includes photodynamic diagnosis, has limited diagnostic utility when it comes to Cis detection; in fact, during cystoscopy and TURBT, several biopsies from suspicious urothelium are usually performed to detect and diagnose Cis on surgical tissue specimens[10]. The technological limitation preventing the recognition of small lesions results in residual high-grade disease in 40% of patients after their first TURBT[11]. The second limitation is due to the chemoresistance of residual NMIBC, with the intravesical instillation of adjuvant therapy failing in about 50% of patients[3].

The present inventors have recently characterized a preclinical model of orthotopic BC whose neoplastic cells express integrin α5β1, an integrin that is also expressed in 75% of human bladder Cis[12]. Using this model, they delivered a solution that allows the detection of bladder cancer lesions smaller than half millimetre[12]. The solution comprised the intravesical instillation of urine-stable targeted gold nanorods (GNRs) against the integrin α5β1, and the diagnosis of BC lesions smaller than 0.5 mm expressing the integrin α5β1. This was delivered by exploiting the photoacoustic properties of GNRs using photoacoustic imaging; a diagnostic imaging modality that provides spatial resolution in the range of 50-100 microns[13]. This solution comprised the use of GNRs coated with thiolated chitosan (GNRs@Chit) and functionalized with the peptide *Iso4* (head-to-tail cyclized c(Cphg*iso*DGRG), selective for the integrin α5β1[12]. This approach demonstrated the feasibility of using GNRs@Chit-Iso4 as a urine-stable nanosystem able to maintain its chemical-physical properties and binding activity for bladder tumor cells α5β1 integrin-positive. The GNRs@Chit-Iso4 is instilled in the bladder under ultrasound-mediated shaking to avoid sedimentation in the lower part of the organ[12].

SUMMARY OF THE INVENTION

The present invention provides a novel multifunctional system that maintains chemical-physical properties in the presence of urine and binding capacity to bladder tumor cells positive for the integrin α5β1. In the multifunctional system of the invention GNRs coated with thiolated chitosan (GNRs@Chit) have been engineered with a novel stable aptamer resistant to nucleases, highly present in the urine[14]. The aptamer has been obtained through optimization of a previously generated

unmodified RNA aptamer targeting integrin α5β1[15] by eliminating dispensable nucleotides of its sequence and replacing all the pyrimidines with 2'Fluoropyrimidines (2'F-Py) to improve its stability. The shorter aptamer, named Apt-Itg, maintains the targeting efficacy of the parental aptamer.

The novel nanosystem GNRs@Chit-Apt-Itg is stable in the urinary environment, does not require ultrasound-assisted shaking because has high polydispersity index and uniform particle size distribution. In addition, the synthesis of the Apt-Itg is highly reproducible with no racemization issues, it is cheaper than Iso4 or other form of ligands as antibodies, because the quality control of the production requires a limited number of tests (i.e. polymerase chain reaction) and does not involve the use of animals that makes the production expensive and time consuming[16]. The chemical synthesis of the aptamer is more cost-effective than cyclic peptides or antibodies (low cost/high yield and small batch-to-batch variation) for industrial scale up, and allows easier conjugation to nanovectors[17], including GNRs@Chit.

It is an object of the invention a multifunctional system comprising a nucleotide aptamer or a variant thereof that binds to the alpha 5 subunit of integrin and a metal nanoparticle, wherein said aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or functional fragment thereof and optionally comprises at least one modification capable of enhancing nuclease resistance.

In a preferred embodiment said multifunctional system comprises a nucleotide aptamer or a variant thereof that binds to the alpha 5 subunit of integrin and a metal nanoparticle, wherein said aptamer comprises or consists of a nucleotide sequence that is at least 90% identical, preferably 100% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or functional fragment thereof and optionally comprises at least one modification capable of enhancing nuclease resistance.

Preferably, the at least one modification capable of enhancing nuclease resistance is fluorination in the 2'-position of a pyrimidine nucleotide.

More preferably the aptamer or variant thereof or functional fragments thereof comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2).

Even more preferably the aptamer or variant thereof or functional fragments thereof comprises or consists of a nucleotide sequence that is at least 90% identical, preferably 100% identical of: 5'-G(2'F-

C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2).

Even more preferably, the aptamer or variant thereof or functional fragments thereof is functionalized at its 5' end with a thiol group, preferably with a C6-C12 aliphatic-thio molecule, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical, preferably at least 90% identical, more preferably 100% identical of SEQ ID No: 2 or variant thereof or functional fragments thereof and is functionalized with a C6-thiol group at its 5'end.

In a preferred embodiment of the invention the metal nanoparticle is made of gold, silver, or hybrid gold/silver, preferably gold and the metal nanoparticle has photoacoustic properties, preferably wherein the metal nanoparticle is chosen among nanosphere, nanorod, nanostar, nanocage, nanoprism or nanoshell or nanowire, nanoplate, hallow shell, preferably the metal nanoparticle is gold nanorod with a size ranging from 10 to 200 nm in length, and 2 to 50 nm in width, more preferably ranging from 20 to 100 nm in length and from 10 to 25 nm in width and the preferred aspect ratio ranges between 1.2 and 15, more preferably between from 3 and 7.

In a further preferred embodiment the metal nanoparticle is coated with a polymer functionalized with thiol groups and -NH groups, said polymer preferably being selected among thiolated chitosan, thiolated and aminated alginic acid, thiolated and aminated hyaluronic acid, or a protein preferably selected from the group consisting of albumin and gelatin, or a synthetic thiolated and aminated polymer, preferably α-thio-ω-amino polyethylene glycols.

Preferably the polymer functionalized with thiol groups and -NH groups is thiolated chitosan preferably having an average molecular weight ranging from 0.5 kDa to 1000 kDa, more preferably ranging from 50 kDa to 200 kDa, and an average deacetylation degree ranging from 75% to 100%, more preferably ranging from 85% to 95%.

Preferably, in the multifunctional system said thiol groups and -NH groups of the polymer are linked via a crosslinker to the aptamer that binds to the alpha 5 subunit of integrin and wherein:

- the crosslinker bears at least one functional group able to bind to an amino group, said functional group being preferably selected from the group consisting of N-hydroxysuccinimidyl ester group (NHS ester), an isocyanate group (-NCO), an isothiocyanate group (-NCS), a sulfo-N-hydroxysuccinimidyl ester group (sulfo-NHS ester), a maleimide group or a carboxylic acid; and/or

- the cross linker bears at least one functional group able to bind a thiol group, said functional group being preferably selected from the group consisting of: a maleimide group, a terminal vinyl group or a terminal alkyne group; and/or
- the cross linker bears at least one functional group able to bind an alkyne such as an azide; and/or
- the cross linker bear at least one functional group able to bind an azide, such as an alkyne.

Preferably the crosslinker is maleimido-PEG_{12}-NHS ester.

Preferably in the multifunctional system of the invention:

- the aptamer has SEQ ID No:2 and is preferably functionalized at its 5'end with a C6-thiol group;
- the metal nanoparticle is gold nanorod;
- the functionalized polymer is thiolated chitosan;
- the crosslinker is maleimido-PEG_{12}-NHS ester.

It is a further object of the invention a composition comprising the multifunctional system as defined above or a kit comprising single use vials containing the multifunctional system as defined above, said composition and kit further comprising at least one of the following solvents: water; physiologic solution; Dulbecco's Modified Eagle Medium (DMEM); Dulbecco's phosphate-buffered saline (DPBS), HEPES buffer, TRIS buffer, PIPES buffer each containing metal divalent ions such as Ca^{2+}, Mg^{2+}.

Preferably said composition or said kit further comprises at least one or more antitumoral agents preferably chosen among a chemotherapeutic agent, an immunomodulator, an immune cell.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for medical use, preferably for use in a method of diagnosis and/or treatment in vivo, more preferably for use in the diagnosis and/or treatment of solid tumors.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for use in the treatment of a tumor selected among urothelial, bladder, gastroesophageal, colorectal, pancreatic, ovarian, lung, cervix, breast and renal cancer, brain tumors, hollow organs cancer and hepatocellular carcinoma.

It is a further object of the invention the multifunctional system or composition or kit as defined above for use in the detection of bladder cancer lesions preferably during the trans urethral resection of bladder tumor.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for use in the photothermal therapy of solid tumors, preferentially bladder cancer.

It is a further object of the invention an in-vitro diagnostic method for detecting cancer from a biological sample of a test subject, said method comprising the following steps:

a) contacting said biological sample with at least one multifunctional system of the invention or with the composition or kit as defined above, and
b) photoacoustic or ultrasound visualization of the test sample, and
c) detection of the presence of alpha 5 beta 1 integrin, and
d) evaluation by comparison with a reference sample.

It is a further object of the invention an aptamer that binds to the alpha 5 subunit of integrin comprising or consisting of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or a variant thereof or a functional fragment thereof and having at least one modification capable of enhancing nuclease resistance, preferably wherein the at least one modification capable of enhancing nuclease resistance is fluorination in the 2'-position of a pyrimidine nucleotide, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2) or a variant thereof or a functional fragment thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The secondary structure of H02 full-length and Apt-Itg (nucleotides from 18 to 51, boxed). A) Sequences of the H02 full-length and Apt-Itg were predicted by using Mfold web server. The calculated change in Gibbs free energy (ΔG) values are reported. B) AlphaFold 3 molecular docking result of integrin α5β1-Apt-Itg binding complex; Apt-Itg, integrin α5 and integrin β1 predicted structures are shown in yellow, light blue and red, respectively. The predicted interaction of Apt-Itg with integrin α5, involving D154 and Y208 residues, is framed on the side.**Figure 2. The expression of integrin a5b1 by human and murine bladder cancer cell lines.** A) Western blot analysis of the α5 and β1 chain of the integrin, and of the housekeeping protein α-tubulin. B) Relative fold of expression of integrin chains against the housekeeping protein α-tubulin.

Figure 3. Binding assay of Alexa 647-Apt-Itg to T24 and RT112 cells using flow cytometry. Representative binding profiles of increasing concentrations of Alexa 647-Apt-Itg and Unrelated aptamer incubated with T24 (A) and RT112 (B) cells. Apparent dissociation curves of aptamer-cell interaction and Kd value of Apt-Itg is shown.

Figure 4. Binding assay of Alexa 647-Apt-Itg to MB49-Luc cells using flow cytometry. (A) Representative binding profiles of increasing concentrations of Alexa 647-Apt-Itg and unrelated aptamer incubated with MB49-Luc cells (left). Apparent dissociation curve of Apt-Itg-cell interaction and Kd value are shown (right). (B) Competition binding assay by flow cytometry (left) and quantification of the gMFI (right) using 1 μM Alexa 647-Apt-Itg in the absence (blue) or in the presence (red) of 30-fold excess of unlabelled unrelated aptamer.

Figure 5. Binding assay of Alexa 647-Apt-Itg to human and murine cancer cells in the presence of divalent cations. Representative binding profiles of increasing concentrations of Alexa 647-Apt-Itg incubated with human bladder cancer cell line T24 (A) and murine bladder cancer cell line MB49-Luc (B) cells in presence of 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Apparent dissociation curves of aptamer-cell interaction and Kd value of Apt-Itg is shown. Data are presented as the mean ± SD.

Figure 6. Integrin α5 silencing using siRNAs. T24, RT112 and MB49-Luc cells were left untreated (NT) or transfected with the indicated Integrin α5 siRNAs or siRNA ctrl. At 24 h post-transfection, cells were harvested, and cell lysates prepared and immunoblotted with anti-integrin α5 or anti-integrin β1 antibody. Equal loading was confirmed by immunoblot with anti-α-tubulin antibody. The histograms indicate the integrin chains/α-tubulin ratio of the densitometric signals. Values are shown relative to siRNA ctrl, arbitrarily set to 1.

Figure 7. Integrin α5 silencing results in reduced Apt-Itg binding. Binding of 1 μM Alexa647-Apt-Itg on human and murine bladder cancer cells following 24 hours-transfection with si-ITGα5 and siRNA ctrl. The histogram indicates gMFI of aptamer-treated cells normalized to the gMFI of siRNA ctrl untreated cells (black), arbitrarily set to 1.

Figure 8. Quantification of the amount of Apt-Itg and unrelated aptamer conjugated to GNRs@Chit. PCR calibration curve with unconjugated Apt-Itg (red); sample, green (A), and quantification of the Apt-Itg and unrelated aptamer loaded onto GNRs@Chitosan (B). Data shown are mean ± SD of three independent experiments.

Figure 9. Characterization of the final nanosystem, GNRs@Chit-Apt_Itg. A) Scheme representative of the aptamer Apt-Itg conjugated to the GNRs@Chit via PEG_{12} linker. B) TEM images of nanoparticles. C) UV-Vis spectra of GNRs@CTAB, GNRs@Chit and GNRs@Chit-Apt_Itg/SCR showing no significant differences in the absorption spectrum of GNRs after surface modification (SCR indicates a scrambled sequence used as control); FAAS analysis of GNRs@Chit-Apt_Itg with calibration line; thermogravimetric analysis of GNRs@Chit in N2 and air atmosphere (above 600 °C). D) Photoacoustic imaging of agar drop containing GNR@Chit-Apt-ITG (6 μg Au;

30 nmol Au, *red*), with the echogenic signal (*gray*) generated by the slime in which the agar drop is embedded. The light blue region of interest in panel A delineates the PA signal of GNRs from which the photoacoustic spectra was derived (B; normalized PA spectra of GNRs@Chit-Apt-Itg and unconjugated GNRs@Chit-*Iso4*, embedded in agar drops). The PA images were acquired and spectrally unmixed with VevoLab software to separate the contribution of the slime and GNRs (the *red signal* corresponds to the PA signal of GNRs).

Figure 10. GNRs@Chit-Apt-Itg stability in human urine. A) VIS-NIR spectra of GNRs@Chit-Apt compared to the VIS-NIR spectra of GNRs@CTAB and GNRs@Chit. B) VIS-NIR spectra and absorption intensity of GNRs@Chit-Apt in the presence of human urine over time.

Figure 11. The Apt-Itg delivers GNRs into the lysosome. Binding of GNRs@Chit-Apt-Itg to MB49 cells after 15 minutes of incubation, followed by cell internalization of GNRs and accumulation into lysosome after 4 hours.

Figure 12. The photoacoustic imaging of GNRs@chit-Apt-Itg is specific for the tumor mass. Axial frame of a murine bladder with an US recognizable tumor nine days after intravesical instillation of MB49-Luc cells, with a region of interest in which a photoacoustic spectrum has been analysed, before (A) and 15 minutes after (B) the intravesical instillation of GNRs@Chit-Apt-Itg. Axial frame of a murine bladder with a region of interest in which a photoacoustic spectrum has been analysed, before (C) and 15 minutes after (D) the intravesical instillation of GNRs@Chit-Apt-Itg. GNRs@Chit-Apt-Itg; instilled 100 ul (100 uM Au) with the "TOP" amount of aptamer.

Figure 13. The photoacoustic signalling of GNRs is lost 18 hours after intravesical instillation of GNRs@chit-Apt-Itg. Axial frame of a murine bladder with an US recognizable tumor nine days after intravesical instillation of MB49-Luc cells, with a region of interest in which a photoacoustic spectrum has been analysed, showing a PA spectra ascribed to oxygenated blood. Fifteen minutes after the intravesical instillation of GNR@Chit-Apt-Itg the correct PA spectra of the GNRs was identified on top and lateral sides of the tumor mass, but not in the surrounding non-neoplastic utrothelium (B). Eighteen hours later the PA signal of the GNRs was not detectable, and tumor was growth on the lateral sides of the mass identified the day before. The amount of intravesically instilled of GNR@Chit-Apt-Itg was 100 ul at concentration of 100 uM Au, loaded with the TOP amount of aptamer.

Figure 14. Tumor mass is recognized by GNRs@chit-Apt-Itg loaded with lower amount of Apt-Itg. Axial frame of a murine bladder with an US recognizable tumor nine days after intravesical instillation of MB49-Luc cells, with a region of interest in which a photoacoustic spectrum has been

analysed, before (A) and 15 minutes after (B) the intravesical instillation of GNRs@Chit-Apt-Itg. GNRs@Chit-Apt-Itg. GNRs@Chit-Apt-Itg; instilled 100 ul (100 uM Au) with the "1/10" amount of aptamer.

Figure 15. Absence of cytotoxicity by GNRs@Chit-Apt-Itg. A) Histological analysis of murine bladder with orthotopic tumor 24 hours after the intravesical instillation of GNR@Chit-Apt-Itg (100 µl at concentration of 100 µM Au), with a magnification of the non-neoplastic tissue (dashed box). B) MB49 cells were incubated for 15 minutes with either nanoparticles or the diluent solution, then washed with culture medium. The time point "15 minutes" was immediately tested, while the cells for the time point "24 hours" were cultivate for an additional 24 hours. The graphs show data from one out of two experiments with similar information; symbols and lines represent median and SEM of conditions tested in quadruplicate.

DETAILED DESCRIPTION OF THE INVENTION

The aim of the invention is to provide a technological platform for diagnostic, therapeutic or theragnostic applications. While the invention is susceptible to various alternative modifications, some preferred embodiments are described below in detail; said embodiments are example and shouldn't be intended as limiting of the scope of the invention to the specific alternative.

It is therefore object of the present invention the development of a multifunctional system, preferably metal nanostructures or nanoparticles bearing a ligand capable of recognizing a tumor-associated or inflammation-associated component.

It is an object of the invention a multifunctional system comprising a nucleotide aptamer or a variant thereof that binds to the alpha 5 subunit of integrin and a metal nanoparticle, wherein said aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or functional fragment thereof and optionally comprises at least one modification capable of enhancing nuclease resistance.

Preferably, the at least one modification capable of enhancing nuclease resistance is fluorination in the 2-position of a pyrimidine residue.

More preferably the aptamer or variant thereof or functional fragments thereof comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2).

Even more preferably, the aptamer or variant thereof or functional fragments thereof is functionalized at its 5' end with a thiol group, preferably with a C6-C12 aliphatic-thio molecule, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of SEQ ID No: 2 or variant thereof or functional fragments thereof and is functionalized with a C6-thiol group at its 5'end.

In a preferred embodiment of the invention the metal nanoparticle is made of gold, silver, or hybrid gold/silver, preferably gold and the metal nanoparticle has photoacoustic properties, preferably wherein the metal nanoparticle is chosen among nanosphere, nanorod, nanostar, nanocage, nanoprism or nanoshell or nanowire, nanoplate, hallow shell, preferably the metal nanoparticle is gold nanorod with a size ranging from 10 to 200 nm in length, and 2 to 50 nm in width, more preferably ranging from 20 to 100 nm in length and from 10 to 25 nm in width and the preferred aspect ratio ranges between 1.2 and 15, more preferably between from 3 and 7.

In a further preferred embodiment the metal nanoparticle is coated with a polymer functionalized with thiol groups and -NH groups, said polymer preferably being selected among thiolated chitosan, thiolated and aminated alginic acid, thiolated and aminated hyaluronic acid, or a protein preferably selected from the group consisting of albumin and gelatin, or a synthetic thiolated and aminated polymer, preferably α-thio-ω-amino polyethylene glycols.

Preferably the polymer functionalized with thiol groups and -NH groups is thiolated chitosan preferably having an average molecular weight ranging from 0.5 kDa to 1000 kDa, more preferably ranging from 50 kDa to 200 kDa, and an average deacetylation degree ranging from 75% to 100%, more preferably ranging from 85% to 95%.

Preferably, in the multifunctional system said thiol groups and -NH groups of the polymer are linked via a crosslinker to the aptamer that binds to the alpha 5 subunit of integrin and wherein:

- the crosslinker bears at least one functional group able to bind to an amino group, said functional group being preferably selected from the group consisting of N-hydroxysuccinimidyl ester group (NHS ester), an isocyanate group (-NCO), an isothiocyanate group (-NCS), a sulfo-N-hydroxysuccinimidyl ester group (sulfo-NHS ester), a maleimide group or a carboxylic acid; and/or
- the cross linker bears at least one functional group able to bind a thiol group, said functional group being preferably selected from the group consisting of: a maleimide group, a terminal vinyl group or a terminal alkyne group; and/or
- the cross linker bears at least one functional group able to bind an alkyne such as an azide; and/or

- the cross linker bear at least one functional group able to bind an azide, such as an alkyne.

Preferably the crosslinker is maleimido-PEG_{12}-NHS ester.

Preferably in the multifunctional system of the invention:

- the aptamer has SEQ ID No:2 functionalized at its 5'end with a C6-thiol group;
- the metal nanoparticle is gold nanorod;
- the functionalized polymer is thiolated chitosan;
- the crosslinker is maleimido-PEG_{12}-NHS ester.

It is a further object of the invention a composition comprising the multifunctional system as defined above or a kit comprising single use vials containing the multifunctional system as defined above, said composition and kit further comprising at least one of following solvent: water; physiologic solution; Dulbecco's Modified Eagle Medium (DMEM); Dulbecco's phosphate-buffered saline (DPBS), HEPES buffer, TRIS buffer, PIPES buffer each containing metal divalent ions such as Ca^{2+}, Mg^{2+}.

Preferably said composition or said kit further comprises at least one or more antitumoral agents preferably chosen among a chemotherapeutic agent, an immunomodulator, an immune cell.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for medical use, preferably for use in a method of diagnosis and/or treatment in vivo, more preferably for use in the diagnosis and/or treatment of solid tumors.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for use in the treatment of a tumor selected among urothelial, bladder, gastroesophageal, colorectal, pancreatic, ovarian, lung, cervix, breast and renal cancer, brain tumors, hollow organs cancer and hepatocellular carcinoma.

It is a further object of the invention the multifunctional system or the composition or the kit as defined above for use in the photothermal therapy of solid tumors, preferentially bladder cancer.

It is a further object of the invention an in-vitro diagnostic method for detecting cancer from a biological sample of a test subject, said method comprising the following steps:

a) contacting said biological sample with at least one multifunctional system or with the composition or kit as defined above, and
b) photoacoustic or ultrasound visualization of the test sample, and
c) detection of the presence of alpha 5 beta 1 integrin, and
d) evaluation by comparison with a reference sample.

It is a further object of the invention an aptamer that binds to the alpha 5 subunit of integrin comprising or consisting of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or a variant thereof or a functional fragment thereof and at least one modification capable of enhancing nuclease resistance, preferably wherein the at least one modification capable of enhancing nuclease resistance is fluorination in the 2-position of a pyrimidine residue, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2) or a variant thereof or a functional fragment thereof.

Preferably said aptamer is for medical use, even more preferably is for use for use in a method of diagnosis and/or treatment in vivo, more preferably for use in the diagnosis and/or treatment of solid tumors, even more preferably for use in the diagnosis and/or treatment of a tumor selected among urothelial, bladder, gastroesophageal, colorectal, pancreatic, ovarian, lung, cervix, breast and renal cancer, brain tumors, hollow organs cancer and hepatocellular carcinoma.

As used herein, the term "multifunctional system" indicates the complex, functionalized system of the invention, which can be also referred as "targeted nanoparticle" or "nanovector", and includes at least the aptamer of the invention and the metal nanoparticle, preferably the aptamer, the coated metal nanoparticle and the heterobifunctional crosslinker.

In the context of the present invention, the term "variant" refers to longer or shorter polynucleotide sequences and/or polynucleotides having, for example, an identity percentage of at least 41%, 50%, 60%, 65%, 70% or 75%, more preferably of at least 85%, for example of at least 90%, and more preferably of at least 95% or 100% with the sequences described herein or with the complementary sequence thereof or with the corresponding DNA or RNA sequence thereof.

The term "variant" and the term "polynucleotide" also comprise modified synthetic oligonucleotides. These modified synthetic oligonucleotides are preferably locked nucleic acids (LNA), phosphoro-thiolated oligo, or methylated oligo, morpholines, 2'-O-methyl or 2'-O-methoxyethyl oligonucleotides and cholesterol-conjugated 2'-O-methyl modified oligonucleotides (antagomirs).

The term "variant" can also include nucleotide analogs, i.e., a ribonucleotide or a natural deoxyribonucleotide substituted by a non-natural nucleotide, and nucleic acids which can be generated by mutating one or more nucleotides or amino acids in the sequences thereof, or in the precursor sequences or equivalent. The term "variant" also comprises at least one functional fragment of the polynucleotide.

In the context of the present invention, “functional” means that it maintains the functional features of the aptamers from which they derive, e.g., the ability to bind to the alpha 5 subunit of integrin.

The term "variant" as used herein refers to compounds which have a similar but not identical chemical formula and share the same or substantially similar function as the compound with the similar chemical formula. In some embodiments, the analog is a mutant, variant, or modified sequence with respect to the unmodified or wild-type sequence on which it relies. In some embodiments, the analog is a functional fragment of any one of the nucleic acid sequences described herein. In some embodiments, the analog is a salt of any one of the nucleic acid sequences described herein. In such embodiments, the analog can retain about 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75%, 70% or less biological activity with respect to the wild or natural-type sequences on which it is based.

Preferably, the aptamer or the variant thereof according to the invention selectively binds to the alpha 5 subunit of integrin.

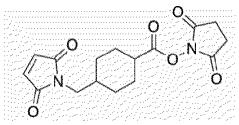
The high flexibility of the molecule also makes it suitable for further chemical modifications aimed at improving the pharmacodynamic and pharmacokinetic properties thereof, as well as for the use thereof in molecular imaging techniques. Possible chemical modifications include the functionalization at its 5’end with an aliphatic chain, preferably of 6 to 12 methylene unit, comprising a thiol group. According to the invention an aptamer thereof functionalized at its 5’ end with a thiol group, preferably with a C6-C12 aliphatic-thio molecule, is an aptamer linked at its 5’end to an aliphatic chain of the indicated length also bearing a thiol group.

According to the invention the aptamer may comprise at least one modified pyrimidine nucleotide. According to the invention the modified pyrimidine may be any modified pyrimidine known to one skilled in the art. It may be for example a chemically modified pyrimidine and/or a substituted pyrimidine, for example at the position 2’. Preferably the pyrimidine nucleotide may be modified to be a 2'-fluoro pyrimidine. Said modifications are known as enhancing nuclease resistance of the aptamer, thus increasing its stability in the in vivo setting, in particular in urine. The sequences of nucleic acids, protein or other agents of the present description can be administered, inter alia, as pharmaceutically acceptable salts, esters or amides. The term "salts" refers to inorganic and organic salts of the compounds of the present description. The salts can be prepared in situ during the final isolation and purification of a compound, or by separately reacting a purified compound in the free base or acid form thereof with a suitable organic or inorganic base or acid and isolating the salt thus formed. The representative salts include hydrobromide, hydrochloride, sulfate, bisulfate, nitrate,

acetate, oxalate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesuco, gluco, lactobionate and lauryl sulfonate salts, and the like. The salts can include cations based on alkali and alkali earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary ammonium, and amine cations including, but not limited to, ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like; see S. M. Berge, et al., J Pharm Sci, 66: 1-19 (1977), describing the saline forms of nucleic acids and incorporated by reference in the entirety thereof.

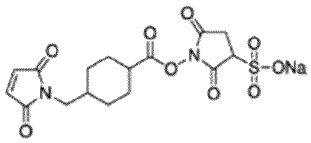
Non limitative examples of the cross-linker (also referred as crosslinker, cross linker or linker) according to the invention are reported below.

Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is a non-cleavable and membrane permeable crosslinker, with the following structure:



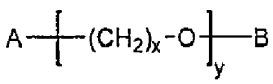
SMCC contains an amine-reactive N -hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. NHS esters react with primary amines at pH 7-9 to form stable amide bonds. Maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds.

Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) corresponds to the compound having the chemical structure indicated below:



The maleimide groups of SMCC and Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) and are unusually stable up to pH 7.5 because of the cyclohexane bridge in the spacer arm.

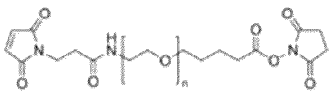
Cross-functional linker of the invention may have the general structure reported below



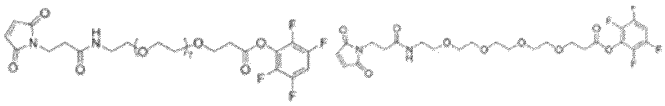
wherein A and B include different reactive groups, x is an integer from 2 to 10 (such as 2, 3 or 4), and y is an integer from 1 to 50, for example, from 2 to 30 such as from 3 to 20 or from 4 to 12.

Non limitative examples of cross-linkers of this structural class are reported below.

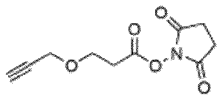
Poly(ethylene glycol) (N-hydroxysuccinimide 5-pentanoate) ether N′-(3-maleimidopropionyl)aminoethane (Cas No. 851040-94-3; MAL-PEG-NHS ester):



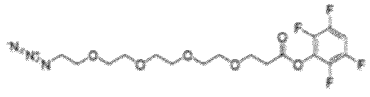
Mal-amido-PEG-TFP ester indicates a PEG linker containing a maleimide and TFP ester end group. Maleimide groups are reactive with thiols between pH 6.5 and 7.5. The TFP ester can react with primary amine groups and is also less susceptible to undergo hydrolysis compared to NHS ester. The hydrophilic PEG chains increase the water solubility of a compound in aqueous media. Longer PEG chains have improved water solubility relative to shorter PEG chains. The PEG linker has a variable number of glycol units, such as the MAL-dPEG®8-TFP ester, MAL-dPEG®4-TFP ester



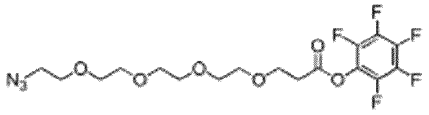
Propargyl-PEG-NHS ester is an amine reactive reagent that can be used for derivatizing peptides, antibodies, amine coated surfaces etc. The alkyne group reacts with azide-bearing compounds or biomolecules in copper catalyzed Click Chemistry reactions. It comprises for example propargyl-PEG1-NHS ester with one glycol unit and propargyl-PEG4-NHS ester which is a 4-unit amine reactive PEG linker



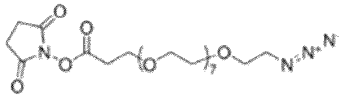
Azido-PEG-TFP ester is a click reagent containing an azide group and a TFP moiety. The azide group enables Click Chemistry. The TFP ester was used to label the primary amines (-NH2) of proteins, amine-modified oligonucleotides, and other amine-containing molecules. Chemical structure of representative compound (2,3,5,6-Tetrafluorophenyl 3-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]propanoic acid) is depicted below:



Similar PFP derivative (perfluorophenyl 1-azido-3,6,9,12-tetraoxapentadecan-15-oate; N3-PEG4-PFP) has the structure below:



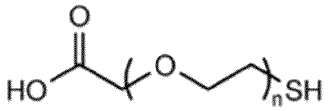
Azido-PEG-NHS ester is a PEG reagent which contain an azide group and an NHS ester. A non limitative example is the 2,5-Dioxo-1-pyrrolidinyl 3-[(23-azido-3,6,9,12,15,18,21-heptaoxatricos-1-yl)oxy]propanoate:



Thiol PEG



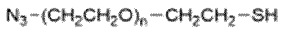
Thiol-PEG-acid



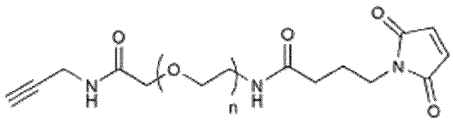
Thiol-PEG-amine



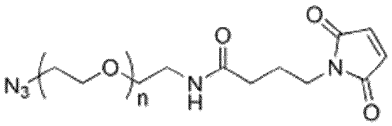
Thiol-PEG-azide



Propargyl-PEG-MAL



Azido-PEG-MAL



In the above compounds the polyethylene glycol chain (PEG) has a molecular weight comprised between 0.05-40 KDa, preferably lipoamide/lipoic acid-PEG-MAL and the PEG has a molecular weight of 5 KDa.

According to the present invention the metal nanoparticles coated with a polymer bearing thiol groups and -NH groups are coupled via the aforementioned heterobifunctional crosslinker to a ligand capable of recognizing a tumor-associated or inflammation-associated component.

Any of the agents of the described embodiment can be prepared in solution or can be prepared in a lyophilized form. With the term "lyophilized" is meant also dried, freeze-dried or precipitated by means of water-miscible organic solvents such as acetone.

It also object of the present invention a composition comprising the described theragnostic agents; the composition according to the invention comprises at least one of water, physiologic solution/saline, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), HEPES buffer, TRIS buffer, PIPES buffer each containing metal divalent ions such as Ca^{2+}, Mg^{2+}, a pharmaceutical acceptable excipient, a pH regulator.

In particular, for use in treatment, the composition can be prepared comprising, in addition to the above-described components, one or more medicament, in particular at least one of a chemotherapeutic agent, an immunomodulator, an immune cell.

In a preferred embodiment chemotherapeutic agent is selected from the group of: mitomycin-C, Bacillus Calmette Guerin (BCG), doxorubicin, melphalan, gemcitabine, taxol, cisplatin, vincristine, or vinorelbine; more preferably the immunomodulator is an anticancer vaccine and/or an immune check point blocker, such as anti-PD1 or anti-PDL1 or anti-CTLA4 antibodies, and more preferably the immune cell is a lymphocyte or a genetically modified T-lymphocyte, such as CAR-T cells, or TCR redirected T-cells or NK cells.

In a preferred embodiment the invention encompasses a composition comprising gold nanoparticles, preferably nanorods according to the invention, coated with thiolated chitosan, wherein the heterobifunctional linker is maleimido-PEG_{12}-NHS ester and the targeting moiety is an aptamer that binds to the alpha 5 subunit of integrin, preferably the Apt-Itg as herein defined.

It is also object of the present invention a kit comprising single use vials containing the multifunctional system as described above, a solvent, preferably physiologic solution/saline, Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), HEPES buffer, TRIS buffer, PIPES buffer each containing metal divalent ions such as Ca^{2+}, Mg^{2+}, for resuspending the nanoparticles, optionally syringes and instruction for use.

It is also object of the present invention the application of the described multifunctional system, compositions and kits in the medical and diagnostic field.

In particular the nanoparticles object of the present invention with photoacoustic properties are developed for use in the diagnosis of solid tumors or inflammation and can be used in particular for the early detection of small cancer lesion, in particular urothelial, bladder gastroesophageal, colorectal, pancreatic, ovarian, lung, cervix, breast and renal cancer, brain tumors, hollow organ tumors and hepatocellular carcinoma. The nanoparticles, as disclosed by the present invention, are particularly suitable to detect urothelial and bladder cancer and actinic cystitis chronic by photoacoustic imaging.

In a preferred embodiment nanoparticles according to the present inventions can be used in the early diagnosis of bladder lesions, in particular of bladder cancer. Are therefore object of the present invention the disclosed nanoparticles for use in a method of in vivo diagnosis of bladder cancer in particular of small and flat urothelial lesions of high-grade bladder carcinoma in situ (CIS).

It is in fact possible to proceed with intravesical instillation of urine-stable nanoparticles designed according to the invention, having photoacoustic properties, handling urine containing the said nanoparticles in the bladder to avoid sedimentation and then use a multimodal imaging of the targeted lesions with PAI.

In a preferred embodiment the targeted area can be subject to thermal ablation; the delivery of the continuous light will irradiate the targeted area with the bound nanoparticles. Assisted photothermal therapy is generated by the excitation of particles at a chosen wavelength.

It is therefore object of the present invention the application of the described multifunctional system, composition and kits in the medical and diagnostic field, in particular for theragnostic application.

In a preferred embodiment the functionalized nanoparticles or compositions according to the present inventions can be used in combination with a medicament, in particular chemotherapeutic agent, a immunomodulator, an immune cell in a method of combination therapy wherein the administration of the nanoparticles and of the medicament can be simultaneous, contemporaneous or sequential.

In a preferred embodiment chemotherapeutic agent is selected from the group of: mitomycin-C, Bacillus Calmette Guerin (BCG), doxorubicin, melphalan, gemcitabine, taxol, cisplatin, vincristine, or vinorelbine; more preferably the immunomodulator is an anticancer vaccine and/or an immune check point blocker, such as anti-PD1 or anti-PDL1 or anti-CTLA4 antibodies, and more preferably the immune cell is a lymphocyte or a genetically modified T-lymphocyte, such as CAR-T cells, or TCR redirected T-cells or NK cells.

In order to demonstrate the effectiveness of the targeted multifunctional system according to the invention and in particular of nanoparticles object of the present invention the invention provides Gold nanorods (GNRs) that have been chemically engineered with a thiol-modified chitosan (Chit-SH), a bifunctional linker and an aptamer selective for the $\alpha 5\beta 1$ integrin to enable tumor targeting; said particles are developed for use in a method of in vivo diagnosis and treatment of bladder cancer based on the intravesical instillation of said urine-stable targeted GNRs and, the multimodal imaging of cancer lesions with PAI.

The inventors developed a combination of strategies that allow for the detection of this tumor with an unprecedented sensibility and stability. The results show that the combination of PAI and intravesical instilled GNRs@Chit-Apt-Itg in an orthotopic model of bladder cancer can reveal the presence of lesions undetectable with US imaging and bioluminescence. The technological platform, according to the invention, could detect neoplastic lesions smaller than a half millimeter, with a sensitivity that far exceeds that of the US and CT urography for bladder carcinoma. The inventors were able to realize GNRs that can be used to detect orthotopic murine bladder cancer lesions <0.5 mm, undetectable by US imaging and bioluminescence. The inventors were able to detect up to 250 µm neoplastic lesion in the upper part of the bladder of experimental mice. According to a preferred embodiment the targeted nanoparticles of the invention are gold nanorods designed with a diameter of 10 nm and aspect ratio of 3.6 in order to have a peak light absorption at 808 nm, to leverage the optical window that allows for deeper tissue penetration and to overcome the different endogenous contrast molecules present in tissues. It was investigated and established the PA dynamic range of the above nanoparticles and identified the maximum fluence and energy of the pulsed laser light to obtain diagnostic imaging using targeted nanoparticles avoiding reshaping of the nanostructure.

The gold nanoparticles according to the invention maintain more than 90% of the original light absorbance in the presence of urine in a time frame of 2 hours.

The urinary bladder environment in particular offers the possibility to exploit the intravesical instillation of the targeted nanoparticles, which is characterized by pro and cons compared to systemic delivery. Intravesical instillation allows for the avoidance of off target effects and off-target accumulations, such as the accumulation of gold in the liver, spleen, kidney, testis and brain, as has been observed in cases of systemic instillation. On the other hand, one of the problem to be solved is that intravesical delivery of the treatment i) must content with urine, which contains a broad variety of byproducts from the metabolism of endogenous and exogenous substances, bacteria, bacteria-derived mucus and floating urothelial cells, ii) is characterized by temporary retention, and iii) cannot

exploit the enhanced permeability of the tumor vasculature and retention effects of the neoplastic vasculature to accumulate the intravenously injected target nanoparticles in the neoplastic environment.

Another application of the product is for urinary cytology to screen patient's urine for cancer cells. The GNRs@Chit-Apt-Itg are used for the isolation of exfoliated cancer cells present in the urine.

MATERIALS AND METHODS

Ethics approval. All procedures and studies involving mice were approved by the Institutional Animal Care and Use Committee of San Raffaele Scientific Institute and performed according to the prescribed guidelines (IACUC, approval number 942).

Tumor cell lines. All human bladder cancer cell lines were from ATCC and cultured according instructions. RT112 cell line was from Merck (catalog number 85061106). The murine bladder cancer cell line MB49-Luc were kindly provided by Prof. Carla Molthoff (VU University Medical Center, The Netherlands) as used as recently reported[12].

Murine orthotopic bladder tumor model. The orthotopic bladder tumor in mice develops after the intravesical instillation of murine bioluminescent MB49-Luc bladder cancer cells, as recently reported[12]. Briefly, female albino C57BL/6 J mice (9 weeks old, weighing about 20 g, Charles River Laboratories, Italy) were anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) and kept in dorsal position. Using a 24-gauge catheter, the bladder of each mouse was emptied and instilled with or without MB49-Luciferated (MB49-Luc) cells (10^5 cells/100 µl in DPBS). Thirty minutes later the catheter was removed, and mice were allowed to recover and returned to their cage.

Aptamers. The SH-terminated 2'F-Py RNA Apt-Itg aptamer (5'-Thiol-C6-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3') and unrelated aptamer (5'-Thiol-C6-(2'F-U)(2'F-U)(2'F-C)G(2'F-U)A(2'F-C)(2'F-C)GGG(2'F-U)AGG(2'F-U)(2'F-U)GG(2'F-C)(2'F-U)(2'F-U)G(2'F-C)A(2'F-C)A(2'F-U)AGAA(2'F-C)G(2'F-U)G(2'F-U)(2'F-C)A-3') were purchased from Metabion (Martinsried, Germany).

Protein extraction and immunoblot. Cells were lysed in a buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 5 mM EGTA, supplemented with 1 mM Na3VO4 and protease inhibitors (Roche Diagnostics, Indianapolis, USA). After incubation on ice for 20 min, the extracts were clarified by centrifugation at 13,200 rpm for 10 min at 4°C. Protein concentration was determined by the Bradford colorimetric assay (Bio-Rad, Hercules,

CA) using bovine serum albumin as the standard. SDS-PAGE was carried out according to Laemmli. Gels were electroblotted into polyvinylidene difluoride membranes (Amersham BioSciences, UK) and filters were probed with anti-Integrin beta 1 (clone EPR16895, Abcam, Cambridge, UK), anti-Integrin alpha 5 (clone EPR7854, Abcam) or anti-α-tubulin (DM1A, Cell Signaling Technology, Danvers, MA, USA) primary antibodies, followed by the HRP-conjugated secondary antibodies. Densitometric analysis was performed on at least two different expositions to assure the linearity of each acquisition using ImageJ (v1.46r).

Integrin α5 silencing. Human T24 and RT112, and murine MB49-Luc cells (1.8×10^5) were seeded in 6-well plates and after 24 hours were overlaid with the transfection mixtures containing 60 nM small interfering RNAs (siRNAs) targeting murine and human integrin α5, respectively, and Lipofectamine RNAiMAX Reagent (ThermoFisher Scientific) in Opti-MEM I reduced serum medium (Invitrogen), according to the manufacturer's instructions of transfection reagent. siRNAs (Qiagen, Hilden, Germany) tested were: HS_ITGA5_7, HS_ITGA5_5, HS_ITGA5_4 and HS_ITGA5_2 (T24 cells); HS_ITGA5_7 and HS_ITGA5_5 (RT112 cells); MM_ITGA5_1, MM_ITGA5_2, MM_ITGA5_3 and MM_ITGA5_4 (MB49-Luc cells). Scrambled (SCR) non-targeting siRNA (siRNA ctrl, Qiagen, Hilden, Germany) was used as a negative control. After 5-hour incubation, complete culture medium was added to the cells and incubation was prolonged up to 24 hours. Silencing efficiency was assessed by immunoblotting.

Cell Binding Assay. Binding of Apt-Itg to human T24 and RT112, and murine MB49-Luc cells was assessed by flow cytometry. Apt-Itg and unrelated aptamer were internally labelled with Alexa Fluor 647 fluorescent probe by using the Ulysis Nucleic Acid Labeling Kit (Invitrogen), which provides a non-enzymatic method for chemically labeling purine bases in the entire sequence with the fluorescent dye.

Cells were detached from culture plates with 0.02% EDTA and washed twice with 500 µl Dulbecco's phosphate-buffered saline (DPBS). Cells (2.0×10^5) were left untreated or incubated with increasing concentrations of Alexa Fluor 647-Apt-Itg or unrelated aptamer in binding buffer (DPBS supplemented with 1 mg/ml yeast tRNA and 1 mg/ml ultrapure salmon sperm DNA, as nonspecific competitors), with or without 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. After 10 min at room temperature (RT), cells were washed three times with 100 µL DPBS, suspended in 100 µl DPBS and analysed by flow cytometry (BD Accuri™ C6). Data analysis was performed using FlowJo software (version 10.0.7). The geometric mean fluorescence intensity (gMFI) of aptamer-treated samples was normalized to the gMFI of the cells alone and the apparent dissociation constant (Kd) of the aptamer-

cell interaction was obtained by fitting the dependence of relative gMFI on the concentration of aptamer to the one-site saturation equation Y = Bmax × X/(Kd + X), using GraphPad Prism version 6.00, as previously described[18,19]. Binding curves represent data from at least two independent experiments.

Binding of 1 μM Alexa 647-Apt-Itg to human and murine bladder cancer cell lines transfected with HS_ITGA5_7 and MM_ITGA5_4 (both referred to as si-ITGα5), respectively, was performed after 24 hours from transfection using flow cytometry, as described above.

Intravesical instillation of GNRs@chit-Apt-Itg. GNRs@Chit-Apt-Itg were produced and instilled into the bladder through a catheter, as reported[12]. The only reported protocol modification is that, instead of using ultrasound-mediated shaking, GNRs instilled into the bladder lumen were kept in suspension by flushing the bladder 3 times through a catheter that was maintained in the urethra for 15 min, a procedure that is in agreement with the current operations made in the surgery room where the surgeon flushes the bladder with saline solution.

In vivo ultrasound (US) and photoacoustic imaging (PAI) of mice instilled with GNRs@Chit-Apt-Itg. Photoacoustic (PA) and ultrasound (US) imaging was carried out on female albino C57BL/6 J mice bearing orthotopic bladder tumour following intravesical instillation with GNRs@Chit-Apt-Itg. High-resolution US and PA imaging have been acquired using the Vevo LAZR-X platform (FUJIFILM VisualSonics, Inc.,Toronto, ON, Canada). The imaging platform includes a high frequency US system (Vevo 3100) combined with an Nd:YAG nano-second pulsed laser with repetition rate of 20 Hz. The linear US transducer array Mx 550D consists of 256 elements with a nominal center frequency of 40 MHz (25–55 MHz bandwidth) and a spatial resolution of 40 μm with a maximum imaging depth of 15 mm. Light from the laser is delivered to the tissue through optical fibers mounted on either side of the transducer; since the GNRs are susceptible to change in shape at the higher laser threshold, light attenuators were prepared to reduce the laser fluence to avoid GNRs reshaping, as recently reported[12]. During volumetric US-PA acquisitions, a stepper motor is used for the linear translation of the US transducer and optical fibers along the sample. The linear stepper motor moves in steps of a minimum of 0:1 mm while capturing 2-D parallel images, for a maximum 3D range distance of 6.4 cm.

3D B mode scan was carried out for mouse bladder. The photoacoustic spectra between 680 nm and 970 nm were scanned with a step size of 5 nm; for the in vivo studies (murine bladder) the 3D multispectral PA scans were acquired selecting PA spectral curve of tissue components melanin, deoxy- and oxy-genated blood, and GNRs; the processed wavelengths (680; 722; 764; 810; 924;

970 nm) were automatically selected from the spectral curve used to spectrally unmix the GNRs signal from other endogenous tissue chromophore signals such as oxy, deoxy hemoglobin. The algorithm reported by Luke et al.[20] was used to select these wavelengths which is ideal for separating the signal from GNRs from other endogenous absorbers. Data analyses were conducted using the Vevo®Lab software; volumes of interest were obtained by manually drawing Volumes of Interest (VOIs) on 3D B-mode images. GNRs, melanin, oxy- and deoxy-hemoglobin content were estimated through spectral unmixing analyses of the spectroscopic data.

***In vitro* PAI of GNRs.** The PA spectra of GNRs@Chit and GNRs@Cit-Apt-Itg was evaluated in vitro, using agar drop, as recently reported[12]. Briefly, GNRs (30 µl in DPBS with calcium and magnesium) were mixed with a 1% agar solution (30 µl), the mixture was then poured on Parafilm® M (Sigma) and left to solidify in a humidified chamber. The solidified product (called "agar drop") was then placed on an ultrasound gel pad (Aquaflex, Parker), embedded in slime made of polyvinyl alcohol polymer crosslinked with sodium tetraborate (Barrel of slime) and PAI was performed using the two optical fiber bearing light attenuators. The transducer was perpendicularly placed on the object under investigation previously covered with ultrasound transmission gel (Aquasonic 100, Parker). Axial sections were acquired using the following settings for B-Mode: 2D Power 100%; 2D Gain 13 dB; for PA-mode: Pa Power 100%; PA gain *44 dB*; TGC and depth were maintained identical for all drops. PA and US data have been analyzed using VevoLab 3.2.5 software.

Synthesis of GNRs@CTAB with maximal absorption at 800 nm. Cetyltrimethylammonium bromide (CTAB)-coated GNRs (GNRs@CTAB) with a maximum absorption of 800 nm, were prepared according to a previously published procedure with minor modifications [1] and scaled to a volume of 2 liters. The initial solution for the growth of GNRs was prepared by dissolving CTAB (31.14 g, 85.4 mmol) and sodium oleate (4.28 g, 14.0 mmol) in 1.8 L of warm water (~ 50°C) in a thermostatically controlled 2-L jacketed reactor, equipped with mechanical stirring. When the solution reached 30°C, 810 µL of $AgNO_3$ solution (0.4 M in ultrapure water) were added and the mixture was then incubated for 15 min without stirring. Then, under continuous stirring (700 rpm), 8.652 mL of $HAuCl_4$ solution (0.1 M in ultrapure water) were added. Au(III) was reduced to Au(I) thanks to sodium oleate for 90 min, after which 3.56 mL of hydrochloric acid (37 %) and 3.6 mL of ascorbic acid (0.079 M) were added to adjust the pH to 1.0 and to ensure the complete reduction of the gold precursor. Separately, a seed solution was prepared by dissolving 364 mg (1.0 mmol) of CTAB in 10 mL of warm water in a 50 mL round-bottomed flask. After cooling down to room temperature 25 µL of $HAuCl_4$ solution (0.1 M) were added while stirring. The seeds were formed by

quickly injecting 600 µL of ice-cold sodium borohydride (0.01 M) into the solution, causing the color of the solution to change from yellow to brown, indicating the formation of ultra-small gold seeds. Finally, after ageing the seed solution for 30 minutes at room temperature, 690 µL of it were added to the growth solution, which was vigorously stirred for 30 s then left undisturbed overnight at 30°C to allow for GNRs growth. Purification of GNRs@CTAB was performed by i) centrifugation in 50-mL Falcon tubes (6000 rpm for 100 min), ii) removal of 40 mL of the supernatant and iii) re-dispersion in 40 mL of ultrapure water; the process was repeated 3 times. The final product was then collected in 200 ml of ultrapure water. The gold concentration in GNRs@CTAB was 2.09 mM, as determined by atomic absorption spectroscopy.

GNRs were designed for displaying peak absorption in the NIR biological window, placed around 800 nm. The synthesis of GNRs@CTAB performed in a 2-L jacketed reactor is able to produce 200 mL of 2.09 mM GNRs@CTAB (Yield = 49%), determined by Flame Atomic Absorption Spectroscopy (FAAS), and a total dry matter of 0.8 mg/mL, measured by gravimetric analysis. This means that the gold content is approximately 60% of the nanosystem mass, in agreement with the fact that the CTAB completely covers the gold core and an excess of surfactant in solution is required to keep the nanosystem stable. The surface zeta potential is + 29.8 mV, as expected for the model of CTAB-capped gold nanosystems.

Coating of GNRs with chitosan (GNRs@Chit). For this system, chitosan was used for coating the nanostructures: this biopolymer is nowadays extensively used in biomedical applications thanks to its biocompatibility and biodegradability; chitosan is also employed in the field of nanoparticles due to its capacity to stabilize GNRs during the synthesis process, not including any toxic reagent. Moreover, chitosan is full of NH_2 groups that allows the functionalization with amine-reactive linkers in order to perform the conjugation with several drugs, antibodies or peptides. GNRs@CTAB were covered with chitosan by means of a thiolated-chitosan conjugate. This thiol insertion happens on the single chitosan monomer and is performed through carbodiimide-assisted coupling between amino groups of chitosan and the carboxylic acid moiety of thioglycolic acid. The effective modification of chitosan with thiol groups was confirmed by the mean of 1H-NMR.

Medical grade chitosan (500 mg, 3.1 mmol) was dissolved in 50 mL of 1 vol. % acetic acid and mixed with 0.5 mL (7.2 mmol) of thioglycolic acid under moderate stirring. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (500 mg, 2.6 mmol) was then added to activate the carboxylic group of thioglycolic acid and to promote the coupling to the amino groups of chitosan. The reaction was left to incubate for 6 h at room temperature under continuous stirring. The product was then

dialyzed using a 3.5 kDa cut-off dialysis tube overnight against ultrapure water and the resulting thiolated-chitosan was diluted to 500 mL with water. At this point, 30 mL of GNRs@CTAB were added dropwise under mild stirring and the resulting solution was incubated (48 h, room temperature) to allow for the coupling of thiolated-chitosan and GNRs. The product was subsequently concentrated using an Amicon® stirred cell equipped with PES membranes (100 kDa cut-off, using 4 bar nitrogen pressure) to remove CTAB, and the final product (60 mL), called GNRs@Chit, was then split in aliquots, freeze dryed and stored at +4 °C until the subsequent step.

The replacement of CTAB with chitosan first and the binding does not modify the shape, morphology, or optical properties of the GNRs, as they result perfectly preserved. The whole replacement of CTAB was assessed by 1H-NMR and by mean of surface zeta potential measurement of purified GNRs that ranged from + 35 mV to + 45 mV. This increased zeta potential suggests that the exchange CTAB/Chit happened, being that the residual non-functionalized amino groups of chitosan preserve their cationic nature in water.

Functionalization of GNRs@Chit with aptamers. GNRs@Chit was functionalized with Apt-Itg and unrelated aptamer. 10 ml of Maleimide-PEG12-NHS (1 mg/ml in ultrapure water, 11.55 µmol = 10 mg) were mixed with 10 ml of GNR@Chit (1 mM of Au, 10 µmol = 1.969 mg Au) under stirring to achieve a final weight ratio of Maleimide-PEG12-NHS:Au = 5:1. The mixture was then left to incubate overnight at RT and then dialyzed against ultrapure water using a 3.5 kDa cut-off dialysis tube for 24 h, at RT, to remove the excess of the crosslinker. The product (25 ml), consisting of activated GNRs@Chit-PEG12-maleimide, was then mixed with pre-established appropriate amount of aptamer (Apt-Itg or Unrelated), previously dissolved in 1 ml of water and activated by three cycles at 85°-0°-37°C. The mixture was left to react for 24 h. The day after, the solutions of GNRs@Chit-Apt-Itg were washed and concentrated by using Slide-A-Lyzer Dialysis Cassettes (3.5 kDa MWCO), freeze dried in sterile conditions and stored at +4 °C until the shipping. Each vial, at the end of the fabrication, contains 98.48 µg Au and has to be redissolved in 500 µL of water or saline for reaching 1 mM [Au] final concentration. The dry content for the freeze-dried vial is about 4.2 mg each.

The inventors exploited a heterobifunctional crosslinker reagent, composed of an ethylene oxide spacer (PEG12) bearing a N-hydroxysuccinimidyl (NHS) ester to its extremities and a maleimide functional group (NHS-PEG12-maleimide). The NHS ester terminus reacts with the free amino groups on chitosan ensuring the binding of the linker to the chitosan, while the maleimide group is available to react in a further step with the sulfhydryl group of the aptamer, expressly designed with the thiol at the 5' end. Apt-Itg or Unrelated was then coupled with activated GNRs@Chit, prior a

short denaturation-renaturation step (85 °C for 5 minutes, snap-cooled on ice for 2 minutes, and allowed to warm up to 37 °C).

Quantification of the Apt-Itg bound to the GNRs@Chit-Apt-Itg. To determine the amount of aptamers bound to GNRs@Chit-Apt-Itg, 5 µl of nanoparticles were incubated with aptamer specific 3′ primers, heated at 65°C for 5 min and annealed at 22°C for 5 min. RNA was reverse transcribed (RT) using Tetro Reverse Transcriptase (Bioline, London, UK) at 42°C for 15 min followed by an extension at 50°C for 30 min and enzyme inactivation at 95°C for 5 min. The product from the RT reaction was PCR amplified with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in the presence of the Apt-Itg or unrelated aptamer 5′ and 3′ primers. The qPCR protocol was as follows: the RT product was heated at 95°C for 2 min, followed by 40 cycles of heating at 95°C for 30 sec, annealing at 55°C for 30 sec, and extending at 60°C for 30 sec. A melt curve stage by heating at 60-95°C was performed. Reactions were all done in 25 µL volume in triplicate.

The primers used were:

Apt-Itg: 5′ primer, 5′- AGCGGACGGACAGAG-3′ (SEQ ID No: 3); 3′ primer, 5′-GCGGCACGGCAGGTT-3' (SEQ ID No: 4).

Unrelated aptamer: 5′ primer, 5'-TAATACGACTCACTATAGGGTTCGTACCGGGT-3' (SEQ ID No: 5); 3′ primer, 5'- TGACACGTTCTATGTGCA-3' (SEQ ID No: 6).

The quantity of the amplified product was extrapolated from a standard amplification curve obtained with unconjugated aptamers. Three independent experiments were performed.

Analysis of GNRs@Chit-Apt-Itg stability in human urine. Half mL of water was added to a lyophilized vial of functionalized gold-nanorods (GNRs@Chit-Apt-Itg), obtaining a concentration of 1 mM of gold.

0.1 mL of GNRs@Chit-Apt-Itg were added to 3 mL of human urine from a healthy volunteer and subjected to VIS-NIR spectroscopy (Cary 3500 UV–VIS–NIR modular spectrometer, Agilent Technologies, Santa Clara, USA) using a 1 cm path-length plastic cuvette, collecting absorbance values between 500 and 1100 nm at various time points at 25°C. Undiluted human urine solution was used as reference for the analysis.

Transmission Electron Microscopy (TEM). MB49-Luc cells incubated with 100 µM GNRs@Chit-Apt-Itg were prepared for TEM imaging as follows. After exposure to nanoparticles for 15 minutes, 2 hours, and 4 hours, the cells were fixed in a monolayer using 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min at room temperature, then scraped and pelleted. The pellets were subsequently fixed for 24 hours at 4°C and post-fixed for 1 hour in a reduced osmium solution

(1.5% potassium ferricyanide and 1% aqueous osmium tetroxide in 0.1 M cacodylate buffer). Following the initial incubation with the heavy metal-based solutions, the pellets were washed with bi-distilled water at room temperature, then immersed in a 0.5% uranyl acetate solution and left overnight at 4°C. The samples were dehydrated using a graded ethanol series (70%, 80%, 90%, 100%) and acetone for 10 min each, before being embedded in Epon resin. After curing at 60°C for 48 hours, thin sections were cut using a Leica UC7 ultramicrotome (Leica Microsystems, Vienna, Austria). The sections were mounted onto 300-mesh copper grids and imaged using a Talos L120C G2 transmission electron microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) at an acceleration voltage of 120 kV.

Cytotoxicity assay. MB49-Luc cells were plated in 96 well culture plates (30.000 cells in 200 µl of culture medium each well) and incubated in CO_2 incubator at 37°C. The day after the culture supernatant was removed and cells were treated with culture medium added of GNR@Chit-Apt-Itg or the diluent (GNRs@Chit-Apt-Itg are suspended with 1% acetic acid /PBS to obtain a stock of 1 mM Au). Fifteen minutes after the treatment was removed, the cells were washed twice with culture medium and assayed for cytotoxicity or returned to culture for an additional 24 hours. MTT assay was carried out as detailed by the producer (SIGMA-Aldrich) and the absorbance was read at 570 nm on a Multimode microplate reader (Mithras LB 940, Berthold Technologies).

Results.

Aptamers. New 34-mer Apt-Itg aptamer was derived from the reported 68-mer H02 unmodified RNA sequence[15] by removing nucleotides from 1 to 17 and 52 to 68 in the fixed flanks of the full-length molecule. The secondary structure and free energy change (ΔG) of the new and shorter aptamer (Apt-Itg) was predicted by using Mfold web server[21] (Figure 1A); the ΔG value of -14.4 Kcal/mol indicates the high structural stability of the Apt-Itg.

In the present invention, all of the pyrimidine residues in Apt-Itg sequence are modified to 2'F-Py to enhance RNA stability, and the 5'-end is modified by adding a Thiol-C6 group for functionalization of GNRs@Chit.

The sequence of the 2'F-Py RNA Apt-Itg (SEQ ID. No: 2) is the following:

5'-Thiol-C6-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3'

In the invention, the sequence of the 2'F-Py RNA Apt-Itg is modified at the 5'-end by adding a Thiol-C6 group for functionalization of GNRs@Chit.

The sequence of the 2'F-Py RNA Apt-Itg modified with Thiol-C6 group is the following:

5'-Thiol-C6-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No: 7)The SH-terminated 2'F-Py RNA with no affinity for integrin α5β1, consisting in the scrambled sequence of a previously generated anti-EGFR aptamer[22] was here used as negative control, and is indicated as "Unrelated".

The sequence of the 2'F-Py RNA unrelated aptamer is the following:

5'-(2'F-U)(2'F-U)(2'F-C)G(2'F-U)A(2'F-C)(2'F-C)GGG(2'F-U)AGG(2'F-U)(2'F-U)GG(2'F-C)(2'F-U)(2'F-U)G(2'F-C)A(2'F-C)A(2'F-U)AGAA(2'F-C)G(2'F-U)G(2'F-U)(2'F-C)A-3' (SEQ ID No: 8).

The sequence of the 2'F-Py RNA unrelated aptamer modified with Thiol-C6 group is the following:

5'-Thiol-C6-(2'F-U)(2'F-U)(2'F-C)G(2'F-U)A(2'F-C)(2'F-C)GGG(2'F-U)AGG(2'F-U)(2'F-U)GG(2'F-C)(2'F-U)(2'F-U)G(2'F-C)A(2'F-C)A(2'F-U)AGAA(2'F-C)G(2'F-U)G(2'F-U)(2'F-C)A-3' (SEQ ID No:9).

In order to confer active targeting abilities to GNRs@Chit toward integrin α5β1-positive MB49 cells, the nuclease-resistant 2'F-Py RNA Apt-Itg aptamer was used as the targeting agent.

Apt-Itg has the following advantages over the original 68-mer H02 longer sequence: 1) easier chemical synthesis at lower costs and higher yield; 2) easier conjugation to GNRs@Chit nanosystem; 3) increased resistance toward nucleases due to the modified pyrimidine residues[23].

To have a better understanding of the interaction between Apt-Itg and integrin α5β1, the inventors led molecular docking analysis using AlphaFold 3 Server prediction[24]. AlphaFold 3 is an artificial intelligence-based system that can model protein–nucleic acid interactions at high accuracy[25]. The potential binding region of Apt-Itg on integrin α5 within the integrin α5β1 heterodimer is shown in Figure 1B. Interestingly, D154 and Y208, which are key residues for the interaction of integrin α5 with the synergy site of its natural ligand fibronectin, are predicted to be involved in the interaction with Apt-Itg (Figure 1B, frame on the side)[26].

Identification of the integrin α5β1 on bladder cancer cell lines. Murine and human bladder cancer cell lines were searched for the expression of the integrin α5β1. Recently published data showing that human bladder cancer cell lines express the integrin α5β1[12] were confirmed, and here it was further demonstrated that the integrin α5β1 is mainly expressed by bladder cancer cells of grade 2/3 (RT4=G1; T24=G3; 5637=G2; HT-1376=G3; RT112=G2), as the human in situ carcinoma that

always appears as high grade/grade 3[3]. The murine bladder cancer cell line MB49-Luc also expressed the integrin α5β1, similarly to the human bladder cancer cell line of grade 2/3 (Figure 2).

Apt-Itg binds to human and murine bladder cancer cell lines with nanomolar affinity. Since H02 was selected to bind to the human integrin α5[15], the inventors first checked whether Apt-Itg contains the H02 active site and binds to human integrin α5β1-positive T24 and RT112 bladder cancer cell lines, expressing high and moderate levels of integrin α5, respectively, and comparable levels of integrin β1 (Figure 2). Binding was assessed through flow cytometric assays using internally Alexa 647-labeled aptamer. As shown (Figure 3), Apt-Itg binds to T24 cells with a Kd value of 222.2 ± 38.1 nM which is somewhat comparable to that observed for the full-length H02 aptamer on human glioblastoma U87MG cells genetically modified to overexpress integrin α5 (277.8 ± 51.8 nM). A higher Kd value (450.6 ± 95.6 nM) was calculated on RT112 cells, thus reflecting the lower expression level of α5 on these cells compared to T24 cells. By contrast, the unrelated control aptamer gave no appreciable binding on the two cell lines, even at concentrations as high as 4 μM.

Next, in order to test GNRs@Chit conjugated to Apt-Itg in mice bearing MB49-Luc-derived tumors, the capability of Apt-Itg to bind to murine MB49-Luc cells was checked. Even if some species specificity has been shown in aptamers recognition ability[27], inventors hypothesized a high affinity binding for Apt-Itg to murine integrin α5, which shares about 90% sequence identity, according to BLAST analysis, in the extracellular region with the human receptor. As expected, Apt-Itg efficiently binds to MB49-Luc cells, with a Kd value of 247.5 ± 72.5 nM, even in the presence of pre-treatment of cells with a 30-fold excess of unrelated aptamer that shows almost undetectable nonspecific binding on cells (Figure 4).

Apt-Itg binds human and murine cancer cells also in the presence of divalent cations. Integrins contain binding sites for divalent cations (such as calcium and magnesium) that regulate their ligand-binding affinity. We tested the binding capacity of the aptamenr that recognized the a5b1 integrin, and we report no significant changes in Kd values of Apt-Itg, on either human and murine cancer cell lines in the presence of physiological concentrations of Ca^{2+} and Mg^{2+} cations (Figure 5). These data show that the integrin α5 recognition by the aptamer is not affected by cation-triggered conformational changes of the receptor.

Silencing of the integrin α5 reduced the binding of the Apt-Itg. In order to confirm the specificity of Apt-Itg aptamer for integrin α5, siRNA was used to reduce integrin α5 expression in human T24 and RT112 cells, and murine MB49-Luc. Compared with siRNA ctrl, the tested anti-α5 siRNAs specifically reduced the expression of α5 (Figure 6). Importantly, Apt-Itg binding to either human

and murine cells transfected with HS_ITGA5_7 and MM_ ITGA5_4, respectively, was significantly reduced compared to binding to siRNA ctrl treated cells (Figure 7). Overall, these results indicate Apt-Itg as a viable integrin α5-targeting candidate for nanovectors functionalization.

Quantification of Apt-Itg bound to GNRs@Chit by Real-time PCR. To evaluate the efficiency and reproducibility of the conjugation of the Apt-Itg to the nanoparticles, GNRs@Chit were left reacting with three different amount of Apt-Itg, such as 100, 50 and 10 pmol. The resulting product was called GNRs@Chit-Apt-Itg "TOP", 1/2 sample and 1/10 sample. For the unrelated aptamer only the 'TOP' concentration was tested. From the three preparation of GNRs@Chit-Apt-Itg the inventors quantified by RT-qPCR analysis 4.0, 1.9 and 0.7 pmol of Apt-Itg-SH aptamer for the 'TOP', 1/2 and 1/10 preparation, respectively (Figure 8). This data shows that the recovery of the bound Apt-Itg was 4% for the TOP and 1/2 batches of nanoparticles, and 7% for the 1/10 preparation. For the "TOP" preparation a conjugation of 1.14 pmol Itg-SH per mg of dry content was estimated, which means 45 ng of aptamer for 0.1 mg of gold. Overall, the efficiency of conjugation of the Apt-Itg ranged between 4% and 7% with a mean±SEM equal to 5±1%.

Overall, this information shows that the use of Apt-Itg allows a reproducible preparation of targeted GNRs, with low batch-to-batch variation.

The conjugation of the Itg-aptamer does not modify the chemical-physical properties of the GNRs@Chit. A heterobifunctional crosslinker reagent was exploited, composed of an ethylene oxide spacer (PEG12) bearing a N-hydroxysuccinimidyl (NHS) ester to its extremities and a maleimide functional group (NHS-PEG12-maleimide). The NHS ester terminus reacts with the free amino groups on chitosan ensuring the binding of the linker to the chitosan, while the maleimide group is available to react in a further step with the sulfhydryl group of the aptamer, expressly designed with the thiol at the 5' end (Figure 9A).

Transmission electron microscopy (TEM) confirmed effective synthesis of monocrystalline GNRs with the appropriate aspect ratio (3.43 ± 0.52) showing the presence of cylindrical gold nanostructures with width (25.7 ± 2.0 nm) and length (88.2 ± 6.4 nm), compatible with the NIR optical behavior of the nanosystem (Figure 9B).

Next, the physical-chemical properties of the metal core of GNRs@Chit-Apt-Itg/Unrelated were assessed, determining the longitudinal-LSPR to peak at 802 nm, shape and dispersion, and aspect ratio of 3.62 ± 0.67 (length 90.2 ± 7.2 nm, width 24.9 ± 2.6 nm. These findings show that conjugation of aptamers does not modify the physical-chemical properties of GNRs, with only small and gradual

variation of the longitudinal-LSPR peak vs GNRs@Chit (800 nm) and GNR@CTAB (798 nm) that is representative of the variation of the chemical environment surrounding the GNRs (Figure 9C).

Flame Atomic Absorption Spectroscopy (FAAS) and thermogravimetric analysis (TGA) were then used to establish the relative amount of gold and chitosan in the final nanosystem (Figure 9C). TGA estimated a total dry matter of 8.4 mg/mL. FAAS estimated a gold amount of 0.2 mg/mL of gold. The rheology in terms of viscosity of the solution was estimated by varying the temperature from +4°C to +40°C: the result is a constant decrease of the viscosity with increasing temperature. These findings indicate that GNRs@Chit were made of 2% Au and 98% chitosan, and that this solution is so suitable for intravesical instillation.

Both the Apt-Itg and the unrelated-conjugated nanosystems displayed a reduction in the Z-potential (+ 10.8 mV and + 10.3 mV respectively, vs. + 40.0 mV measured for GNRs@Chit), according to the reduced amount of free amino groups on chitosan due to the presence of the PEG linker.

Moreover, the crystallinity and the composition of the GNRs@Chit-Apt-Itg/Unrelated was proven to be conserved after the consecutive conjugation steps, additionally revealing no detectable Br atoms from CTAB. GNRs@Chit-Apt-Itg/Unrelated was therefore aliquoted in vials, freeze-dried and vacuum sealed to achieve sterile single use vials containing the lyophilized product.

The freeze-dried GNRs@Chit-Apt-Itg/ Unrelated were then dissolved in 500 µl of 1% acetic acid in water to obtain 1 mM solution of gold. The PA properties of the GNRs were investigated using agar drops containing GNRs@Chit-Apt-Itg or GNRs@Chit. The results show that the PA spectra of GNRs@Chit-Apt-Itg is superimposable to that of GNRs@Chit, thus demonstrating that the functionalization of GNRs@Chit with the Apt-Itg does not modify the PA spectra of the GNRs@Chit (Figure 9D).

GNRs@Chit-Apt-Itg is stable in human urine. Urine represents a harsh environment, characterized by a complex mixture of metabolites and bacteria that could interact with the nanoparticles. Therefore, we estimated the potential impact of urine on the light absorption of the three formulations of GNRs (GNRs@CTAB, GNRs@Chit, GNRs@Chit-Apt-Itg), and the stability of the GNRs@Chit-Apt-Itg in urine in a time frame of 2 hours.

The longitudinal LSPR peak of GNRs@Chit-Apt-Itg was observed at 834 nm, consistently with the LSPR peak of the unconjugated GNRs@Chit that was at 836 nm (Figure 10A, Table 1).

The urine stability of GNRs@Chit-Apt-Itg over time was also investigated, showing only a 7% decrease of light absorbance after 2 hours (Figure 10B, Table 2).

Table 1: Wavelength and absorbance values for GNRs with different functionalization.

GNRs formulation	λ_{max} (nm)	A_{max}	A_{800nm}
GNRs@CTAB	818	0,573	0,534
GNRs@Chit	836	0,493	0,377
GNRs@Chit-Apt-Itg	834	0,418	0,336

Table 2: Wavelength and absorbance values of GNRs@Chit-Apt-Itg in human urine over time.

Time (min)	A max	λ (nm)	A%
0	0,391	851	100%
5	0,386	853	99%
10	0,386	850	99%
15	0,378	852	97%
30	0,375	854	96%
45	0,376	854	96%
60	0,376	849	96%
120	0,363	854	93%

The Apt-Itg delivers the GNRs into lysosomes. The integrin α5β1 is targeting the lysosomal compartment after binding to its natural ligands.[28] By transmission electron microscopy the inventors followed the pathway of GNRs delivered by the Apt-Itg, by incubating integrin α5β1+ MB49 cells with 100 μM GNRs@Chit-Apt-Itg. Fifteen minutes after treatment the nanoparticles were located on the cell membrane, were internalized within 2 hours and accumulated in lysosomes after 4 hours (Figure 11). Noteworthy, despite being in the lysosomal acidic compartment the GNRs still maintained their characteristic shape, in agreement with in vitro data that supports the stability of GNRs in low pH conditions.

Feasibility and specificity of the use of GNRs@chit-Apt-Itg for the recognition of the orthotopic bladder cancer. Before the intravesical instillation of targeted GNRs the periphery of the tumor mass was negative for the spectra of GNRs, but positive for the PA spectra of oxygenated blood (Figure 12A). After the intravesical instillation of the targeted GNRs the PA spectra of GNRs@chit-Apt-Itg was located only on the surface of the tumor mass but not on the non-neoplastic urothelium (Figure 12B). The same protocol was used for control animals in the absence of tumor, showing vascularization of the bladder wall (PA spectra of oxygenated blood) before (Figure 12C) and after the intravesical instillation of GNRs@chit-Apt-Itg (Figure 12D). These information show that

GNRs@chit-Apt-Itg binds only the tumor cells, but not the non-neoplastic tissue in tumor environment nor the normal urothelium.

The photoacoustic signal of GNRs@chit-Apt-Itg identifies tumor areas that develops in the following day. It was next assessed whether the tissue areas identified through the PA signal of the GNRs@Chit-Apt-Itg were predictive of tumor growth, using animals with detectable tumor (Figure 13A). In the tumor bearing animals the GNRs@Chit-Apt-Itg correctly identified the tumor mass (red ROI in Figure 10B) and the surrounding tumor margins (blue and orange ROI in Figure 13B). In the following 18 hours the PA signal was lost, but the tumor was growth on the lateral sides of the mass identified the day before (Figure 13C).

Lowering the amount of Apt-Itg loaded onto the GNRs@Chit does not impact on the recognition of the tumor mass. It was next evaluated the effectiveness of tumor recognition using nanoparticles loaded with 1/10 of Apt-Itg previously used in Figure 9 and 10. Using the same amount of gold but loaded with 1/10 of Apt-Itg the tumor mass, but not the surrounding non-neoplastic urothelium, was identified (Figure 14). This data owns twofold information, such as that i) lowering the amount of Apt-Itg loaded onto the GNRs@Chit does not impact on the efficacy to recognize the tumor, and ii) the sites of the linker that have not been conjugated with the Apt-Itg do not deliver an unspecific binding, thus do not provide an unspecific PA signal of the GNRs.

Absence of cytotoxicity by GNRs@Chit-Apt-Itg. In vivo and in vitro tests were carried out to assess the safety of the treatment with GNRs@Chit-Apt-Itg. The day after intravesical instillation of targeted GNRs in mouse bladders containing the tumor the histological analysis showed the presence of an intact bladder wall with no signs of inflammation, and the urothelium composed by 2-3 layers of urothelial cells with no signs of cell proliferation (Figure 15A). Cytotoxicity test was also carried out on the integrin $\alpha5\beta1$+ target cells that are the murine bladder cancer cell line MB49-Luc, used to induce the orthotopic tumor, and treated with GNRs@Chit-Apt-Itg up to the concentration of 100 μM Au, or the diluent. Cytotoxicity was evaluated 15 minutes and 24 hours after treatment, and both the nanoparticles or the diluent did not have impact on cellular metabolic activity (Figure 15B).

DISCUSSION

The present invention provides an aptamer instead of the Iso4 peptide for the recognition of tumor expressing the integrin $\alpha5\beta1$. A new 34-mer aptamer against the integrin $\alpha5\beta1$ (Apt-Itg) was delivered, which was stable and active in the urinary environment. The urine-stable Atp-Itg was

further modified at the 5'-end by adding a Thiol-C6 group to allow for efficient binding to and functionalization of GNRs@Chit. Indeed, dose-dependent amount of Apt-Itg conjugated to the GNRs@Chit was obtained when three different concentrations of ligand were used during the process of synthesis of the GNRs@Chit-Apt-Itg. Using three different concentrations of Apt-Itg during the synthesis process, the invention demonstrated an average conjugation efficiency of 5%, in agreement with previous report[32], and a reduced batch-to-batch variation.

GNRs functionalized with an aptamer for the application in the urinary environment have never been reported. The nanosystem GNRs@Chit-Apt-Itg represents a novel contrast agent for the detection of $\alpha5\beta1+$ bladder cancer with high specificity. However, the GNRs employed here can also be used for the application of photothermal therapy (PTT)[33,34], which is obtained by using continuous laser at the same wavelength used for the photoacoustic imaging (pulsed laser) that is 810 nm and is approved for clinical practice by the American National *Standards* Institute[35]. The nanosystem GNRs@Chit-Apt-Itg offers unmet theranostic opportunities, and being unrelated to cellular metabolism, it will overcome bias in the gender medicine[36], and will be equally effective in male and female bladder cancer patients[37,38].

The feasibility of using intravesical instillation of nano- and micro-particles for the photoacoustic imaging of bladder cancer has been previously demonstrated, such as in our recent report that used GNRs@Chit-Iso4[12] and silicon dioxide microparticle[39], respectively. Indeed, both systems are biased by settling down to the bottom side of the bladder, and thus requires solutions (US-assisted shaking or magnetic field) applied to the external part of the body to keep them in suspension in the bladder. By delivering nanoparticles that have positive charge and uniform size distribution the nanosystem GNRs@Chit-Apt-Itg has overcome the above bias, and does not require external solutions to keep the nanoparticles in suspension inside the bladder. Furthermore, the system of the invention has the advantage of using an aptamer for the selective recognition of integrins. The aptamers are obtainable through a consolidated, highly reproducible, large-scale synthesis and very economical prices compared to monoclonal antibodies and certain complex peptides such as the cyclic-one. All these characteristics are very important in light of future scale-ups to obtain quantities suitable for clinical trials.

GNRs functionalized with an aptamer for the application in the urinary environment have never been reported. The nanosystem GNRs@Chit-Apt-Itg represents a novel contrast agent for the detection of $\alpha5\beta1^+$ bladder cancer with high specificity. The same GNRs of the invention can also be used for the application of photothermal therapy (PTT)[33,34,41,42], which is obtained by using continuous laser at

the same wavelength used by the pulsed laser to carry out the photoacoustic imaging. The wavelength of irradiation of GNRs@Chit-Apt-Itg is 836 nm, which is in the biological optical window (near infrared region-I at a wavelength of 680-970 nm) and is approved for clinical practice by the American National Standards Institute[35].

The intravesical delivery of the nanosystem GNRs@Chit-Apt-Itg offers unmet theranostic opportunities for bladder cancer, and being unrelated to cellular metabolism, it will overcome bias in the gender medicine[36], will be equally effective in male and female bladder cancer patients[37,38], is not impaired by the use of antibiotic,[43] and is also suitable for frail patients who may have troubles in tolerating the standard of care for high-grade NMIBC that is the intravesical instillation of Bacillus Calmette-Guerin.[44] The possibility of detecting small high-grade bladder cancer lesions that express the integrin $\alpha5\beta1$ allows to identify and treat the residual high-grade disease found after the first trans urethral resection of the bladder tumor (TURBT)[11], and is associated with high or very high risk of relapse and progression[45]. The solution provided herein is applicable during the TURBT procedure, to detect small lesions that are not recognized by TURBT. This solution can be applied to a variety of NMIBC patients, such as i) at the first TURBT to detect the smaller lesions and thus to provide a more accurate diagnosis to drive the follow-up: ii) in a second-look TURBT to identify the residual disease: iii) in relapsing patients; and iv) to investigate the presence/absence of residual disease in patients enrolled in bladder-sparing protocols[46]. The acceptability of present solution in the urological clinical scenario is supported by several facts, as it does not use ionizing agents, but uses gold as biocompatible metal that is delivered locally into the bladder through intravesical instillation and an incubation period of the contrast agent followed by intravesical washes, as it is carried out for another agent of current use that is Hexvix®[47].

The present invention provides a solution for the recognition of bladder tumors expressing the integrin $\alpha5\beta1$ that exploits the exquisite selectivity of Apt-Itg for these tumors. The use of the aptamer as functionalization agent of the nanoparticles only requires chemical synthesis of the aptamer and PCR as quality control test on the functionalized GNRs, making the production of GNRs@Chit-Apt-Itg cheaper than using other ligands. Being the GNRs@Chit-Apt-Itg used as contrast agent for photoacoustic imaging that provides spatial resolution in the range of 50-100 microns[13] this novel, cost-effective, highly urine-stable and reproducible nanosystem allows for the detection of small tumor areas that are currently undetectable. Moreover the same system can be applied to GNRs-assisted PTT.

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Claims

1. A multifunctional system comprising i) a nucleotide aptamer or a variant thereof that binds to the alpha 5 subunit of integrin and ii) a metal nanoparticle, wherein said aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or functional fragment thereof and optionally comprises at least one modification capable of enhancing nuclease resistance.

2. The multifunctional system according to claim 1 wherein the at least one modification capable of enhancing nuclease resistance is fluorination in the 2-position of a pyrimidine residue.

3. The multifunctional system according to claim 1 and 2 wherein the aptamer or variant thereof or functional fragments thereof comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2).

4. The multifunctional system according to any one of previous claims wherein the aptamer or variant thereof or functional fragments thereof is functionalized at its 5' end with a thiol group, preferably with a C6-C12 aliphatic-thio molecule, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of SEQ ID No: 2 or variant thereof or functional fragments thereof and is functionalized with a C6-thiol group at its 5'end.

5. The multifunctional system according to any one of previous claims wherein the aptamer or variant thereof or functional fragment thereof consists of a nucleotide sequence that is at least 90%, preferably 100% identical of SEQ ID No: 1 or of SEQ ID No: 2.

6. The multifunctional system according to any one of previous claims wherein the metal nanoparticle is made of gold, silver, or hybrid gold/silver, preferably gold and the metal nanoparticle has photoacoustic properties, preferably wherein the metal nanoparticle is chosen among nanosphere, nanorod, nanostar, nanocage, nanoprism or nanoshell or nanowire, nanoplate, hallow shell, preferably the metal nanoparticle is gold nanorod with a size ranging from 10 to 200 nm in length, and 2 to 50

nm in width, more preferably ranging from 20 to 100 nm in length and from 10 to 25 nm in width and the preferred aspect ratio ranges between 1.2 and 15, more preferably between from 3 and 7.

7. The multifunctional system according to any one of previous claims wherein the metal nanoparticle is coated with a polymer functionalized with thiol groups and -NH groups, said polymer preferably being selected among thiolated chitosan, thiolated chitin, thiolated and aminated alginic acid, thiolated and aminated hyaluronic acid, or a protein preferably selected from the group consisting of albumin and gelatin, or a synthetic thiolated and aminated polymer, preferably α-thio-ω-amino polyethylene glycols.

8. The multifunctional system according to claim 7 wherein the polymer functionalized with thiol groups and -NH groups is thiolated chitosan preferably having an average molecular weight ranging from 0.5 kDa to 1000 kDa, more preferably ranging from 50 kDa to 200 kDa, and an average deacetylation degree ranging from 75% to 100%, more preferably ranging from 85% to 95%.

9. The multifunctional system according to any one of claims 7 and 8, wherein said thiol groups and -NH groups of the polymer are linked via a crosslinker to the aptamer that binds to the alpha 5 subunit of integrin and wherein:

- the crosslinker bears at least one functional group able to bind to an amino group, said functional group being preferably selected from the group consisting of N-hydroxysuccinimidyl ester group (NHS ester), an isocyanate group (-NCO), an isothiocyanate group (-NCS), a sulfo-N-hydroxysuccinimidyl ester group (sulfo-NHS ester), a maleimide group or a carboxylic acid; and/or
- the cross linker bears at least one functional group able to bind a thiol group, said functional group being preferably selected from the group consisting of: a maleimide group, a terminal vinyl group or a terminal alkyne group; and/or
- the cross linker bears at least one functional group able to bind an alkyne such as an azide; and/or
- the cross linker bears at least one functional group able to bind an azide, such as an alkyne.

10. The multifunctional system according to claim 9 wherein the crosslinker is maleimido-PEG_{12}-NHS ester.

11. The multifunctional system according to any one of the preceding claims wherein:
- the aptamer has SEQ ID No:2 functionalized at its 5'end with a C6-thiol group;
- the metal nanoparticle is gold nanorod;
- the functionalized polymer is thiolated chitosan;
- the crosslinker is maleimido-PEG_{12}-NHS ester.

12. A composition comprising the multifunctional system according to any of the preceding claims or a kit comprising single use vials containing the multifunctional system according to any one of the preceding claims, said composition and kit further comprising at least one of following solvent: water; physiologic solution; Dulbecco's Modified Eagle Medium (DMEM); Dulbecco's phosphate-buffered saline (DPBS), HEPES buffer, TRIS buffer, PIPES buffer each containing metal divalent ions such as Ca^{2+}, Mg^{2+}.

13. The composition and kit according to claim 12 comprising at least one or more antitumoral agents preferably chosen among a chemotherapeutic agent, an immunomodulator, an immune cell.

14. The multifunctional system according to claims 1-11 or the composition and kit according to claim 12-13 for medical use, preferably for use in a method of diagnosis and/or treatment in vivo.

15. The multifunctional system or composition or kit according to claim 14 for use in the diagnosis and/or treatment of solid tumors.

16. The multifunctional system or composition or kit according to claim 15 wherein the tumor is selected among urothelial, bladder, gastroesophageal, colorectal, pancreatic, ovarian, lung, cervix, breast and renal cancer, brain tumors, hollow organs cancer and hepatocellular carcinoma.

17. The multifunctional system or composition or kit according to claims 15 or 16 for use in the detection of bladder cancer lesions preferably during the trans urethral resection of bladder tumor.

18. The multifunctional system or composition or kit according to any of claims 14-16 for use in the photothermal therapy of solid tumors, preferentially bladder cancer.

19. An in-vitro diagnostic method for detecting cancer from a biological sample of a test subject, said method comprising the following steps:

a) contacting said biological sample with at least one multifunctional system according to claims 1-11 or with the composition or kit according to claim 12-13, and

b) photoacoustic or ultrasound visualization of the test sample, and

c) detection of the presence of alpha 5 beta 1 integrin, and

d) evaluation by comparison with a reference sample.

20. An aptamer that binds to the alpha 5 subunit of integrin comprising or consisting of a nucleotide sequence that is at least 70% identical of 5'-GCGGACGGACAGAGAGUGCAACCUGCCGUGCCGC-3' (SEQ ID No:1) or a variant thereof or a functional fragment thereof and at least one modification capable of enhancing nuclease resistance, preferably wherein the at least one modification capable of enhancing nuclease resistance is fluorination in the 2-position of a pyrimidine residue, preferably wherein the aptamer comprises or consists of a nucleotide sequence that is at least 70% identical of: 5'-G(2'F-C)GGA(2'F-C)GGA(2'F-C)AGAGAG(2'F-U)G(2'F-C)AA(2'F-C)(2'F-C)(2'F-U)G(2'F-C)(2'F-C)G(2'F-U)G(2'F-C)(2'F-C)G(2'F-C)-3' (SEQ ID No:2) or a variant thereof or a functional fragment thereof.

21. The aptamer according to claim 20 consisting of a nucleotide sequence that is at least 90% or at least 100% identical of SEQ ID No: 1, or consisting of a nucleotide sequence that is at least 90% or at least 100% identical of SEQ ID No: 2.