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Targeted non-covalent self-assembled nanoparticles based on human serum albumin

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Abstract

Human serum albumin (HSA) is a biological nanocarrier that forms non-covalent complexes with a number of synthetic and biomolecules. Previously we demonstrated radiolabeled HSA-based nanoparticles can form non-covalent complexes with fluorescent cyanine dyes yielding imaging agents for surgical guidance towards tumor draining lymph nodes. Here the self-assembly approach enabled rapid clinical translation. Based on this experience we reasoned it would be interesting to expand this non-covalent technology to a targeted approach. The ability of HSA to form non-covalent self-assembled complexes with peptides via near-infrared (NIR) cyanine dyes was explored. Förster resonance energy transfer (FRET) quenching interactions between HSA-Cy5 and the non-covalently bound fluorescent molecules indocyanine green (ICG), IR783-CO₂H and three IR783-labeled targeting peptides were used to monitor complex assembly and disassembly. The host-guest interactions between HSA and IR783-labeled peptides enabled the formation of (bio)nanoparticles that are coated with peptides that may target $\alpha_v\beta_3$ -integrins, the chemokine receptor 4 (CXCR4), and somatostatin receptors. The potential of CXCR4-targeted (bio)nanoparticles in sentinel lymph node procedures is demonstrated. By non-covalently binding NIR-dye labeled peptides to an already clinically approved HSA-scaffold, we have readily formed targeted bionanoparticles.

Keywords

Albumin; Nanoparticle; Self assembly

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Introduction

Human serum albumin (HSA; 66.5 kD) is a multi-purpose protein present in ~42 g/L in the bloodstream with an average half-life of 19 days. Among its important functions are maintaining the colloid osmotic pressure of blood, the transportation of various compounds and detoxification by sequestering toxic (waste) compounds.[1,2] Supramolecular complexes wherein HSA functions as nanocarrier ($d = 7$ nm) are used for the transportation of hydrophobic, aromatic and charged compounds in the venous system, examples of such molecules are long chain fatty acids, bilirubin, and steroids.[1] The ability of HSA to form non-covalent host-guest complexes with a great variety of molecules is considered an important factor in the development of drugs; binding to HSA can seriously alter the biodistribution, bioactivity and metabolism of these compounds.[3,4]

Non-covalent HSA complexes, both *in situ* as *in vivo* formed, are also increasingly applied in the (molecular) imaging field. Several blood pool MR-imaging agents have specifically been developed to form non-covalent complexes with HSA after injection, e.g. Eovist, MultiHance and Vasovist.[5–7] In a similar manner the intravenous binding of the clinically approved near infrared dye indocyanine green (ICG) to native HSA (Fig. 1A) is a vital component in the success of fluorescence angiography.[8] In line with this, different cyanine dyes have been selected to control the rate of extravasation of targeting peptides from blood vessels into tissue by their affinity for HSA.[9]

Preformed non-covalent HSA/ICG complexes have been shown to remain stable in the lymphatic system of both mice and human, and can be used for surgical guidance towards tumor draining lymph nodes.[10–12] Using non-covalent complexes of ICG with already clinically approved HSA-aggregates, which act as nanocarriers, resulted in a rapid clinical translation of the newly formed imaging agents.[12] For accurate staging, it is important to know the tumor-status of the lymph nodes on a direct drainage pathway from the tumor (sentinel lymph nodes), but the non-targeted (radioactive) HSA-derivatives commonly used often migrate through the sentinel node to lodge in higher-echelon nodes downstream. [11,13] Imaging agents that improve the specificity of sentinel lymph node staining may, therefore, improve the accuracy of surgical sentinel lymph node biopsy procedures. Chemically the challenge now lies in using similar non-covalent host-guest interactions to those between ICG and HSA-derivatives to introduce targeting moieties that increase accumulation in the sentinel lymph nodes (Fig. 1D).

Next to non-covalent functionalization it is relatively straightforward to covalently functionalize large proteins such as albumin. For example HSA and NanoColl functionalized with ^{99m}Tc are clinically used for respective blood pool imaging and for lymphoscintigraphy.[14,15] Synthetically combining covalent and non-covalent interactions on the HSA platform allows for the introduction of different functionalities that can result in complementary features. One example is the joint radioactive and fluorescent labeling on ^{99m}Tc -NanoColl/ICG enabling visualization of identical features with two different modalities.[10,11] Alternatively, interaction between non-covalent and covalently attached dyes may also enable monitoring of the non-covalent complex formation process using Förster resonance energy transfer (FRET) interactions (Fig. 1C). Quenching of the donor dye

or increased fluorescence of the acceptor dye can be used as a means to study complex formation.

In this manuscript we describe the development of HSA coated with targeting peptides functionalized with the NIR-dye IR783,[16] which is a derivative of the clinically applied ICG (Fig 1B and D).[17,18] These bionanoparticles are formed via non-covalent self-assembly based on the host-guest interaction of HSA and near infrared cyanine dyes. A covalently attached Cy5-dye is applied to follow the complex assembly and disassembly by FRET under various conditions. The stability and targeting of the CXCR4-targeted HSA nanoparticles is studied *in vitro* and *in vivo* to evaluate the applicability of these complexes in a more receptor-specific sentinel lymph node procedure.

Material and Methods

Chemicals

For preparation of dyes, dye-labeled peptides and Cy5-labeled HSA, see supporting information.

Quenching of Cy5-fluorescence on HSA with ICG and IR783-CO₂H

For studying the FRET interaction of non-covalently bound ICG or IR783-CO₂H with covalently attached Cy5 on HSA, a quartz cuvette was filled with 3 mL ICG or IR783-CO₂H (2 μ M) to which HSA-Cy5 was added (1 μ M [HSA], 0.7 Cy5/HSA). Fluorescence spectra were recorded using a 627 nm LED lamp (Luxeon III Star, red, Quadica Developments Inc., Canada) and a VS140-VISNIR-CCD spectrometer (Horiba Jobin Yvon, Japan).

The quenching of the Cy5-fluorescence was studied by titrating an ICG solution (75 μ M in water) to a quartz cuvette filled with 3 mL HSA-Cy5 (0.5 μ M [HSA]; 0.7 Cy5/HSA), ranging from 0.1 equiv. to 15 equiv. ICG (2–300 μ L) compared to HSA. Absorption spectra were recorded on a Lambda Bio 20 spectrometer (Perkin Elmer, USA) and fluorescence spectra were recorded using a 627 nm LED lamp and a VS140-VISNIR-CCD spectrometer after each titration point.

The same experiment was performed in a 96 wells plate. A range of solutions was prepared in triplo, containing HSA-Cy5 (0.5 μ M [HSA]; 0.7 Cy5/HSA) and either ICG or IR783-CO₂H (0–50 equiv. compared to HSA) in 200 μ L water. A same range of solutions without Cy5-HSA was used as control. Fluorescence was imaged with an IVIS 200 (Caliper LifeSciences, USA) at the standard Cy5.5-settings (exc: 615–665 nm, em: 695–770 nm) and quantified with Living Image 3D software package (Caliper LifeSciences, USA). The Stern Volmer constants (K_{sv}) were determined by applying linear regression on a plot of $(F_0/F)-1$ against [ligand].

Quenching of Cy5-fluorescence on HSA with IR783-labeled targeting peptides

Quenching of Cy5 fluorescence on HSA was performed with IR783 coupled to (tumor) targeting peptides c[RGDfK]-IR783 (RGD, integrin $\alpha_v\beta_3$ targeting),[19] Ac-TZ14011-IR783 (CXCR4 targeting)[20] and octreotate-IR783 (somatostatin receptor targeting) (Fig.

3).[21] A dedicated 96 well plate was filled with a range of solutions, each containing HSA-Cy5 (0.5 μ M [HSA]; 0.7 Cy5/HSA) and either c[RGDfK]-IR783, Ac-TZ14011-IR783 or octreotate-IR783 (0–50 equiv. compared to HSA) in 200 μ L water. The experiment was performed in triplicate. Fluorescence was imaged with an IVIS 200 at the standard Cy5.5-settings and quantified with Living Image 3D software package. The Stern Volmer constants (K_{sv}) were determined by applying linear regression on a plot of $(F_0/F)-1$ against [ligand].

Competition between HSA-Cy5 and HSA for the non-covalent binding of NIR dyes and NIR dye-labeled targeting peptides

Samples of 200 μ L, containing HSA-Cy5 (0.5 μ M [HSA]; 0.7 Cy5/HSA) and ICG, IR783- CO_2H or IR783-labeled peptide (5 μ M) and different concentrations HSA (0–50 equiv. compared to HSA-Cy5), were prepared in a 96 wells plate. Fluorescence was imaged with an IVIS 200 at the standard Cy5.5-settings and quantified with Living Image 3D software package.

Confocal imaging of CXCR4 targeted complexes

MDA-MB-231 cells, transfected with human CXCR4-GFP,[22] were cultured under standard conditions in MEM medium enriched with 10% fetal bovine serum, MEM vitamins, l-glutamin, non-essential amino acids, sodium/pyruvate and penicillin/streptomycin solution (all BD Biosciences, USA). Cells were seeded onto coverslips ($\varnothing$ 24mm; Karl Hecht GmbH & Co, Germany) and incubated overnight. The next day, cells were washed with MEM medium without additions and incubated with 15 μ M Ac-TZ14011-IR783, 3 μ M HSA-Cy5 or both for 1 h at 4°C. Incubation with 15 μ M Ac-TZ14011-Cy5 under the same conditions was included to visualize binding of Ac-TZ14011, because the IR783 dye could not be detected by the used confocal microscope. After washing with ice cold PBS, confocal microscopy images were taken at 37°C on a Leica TCS-SP2-AOBS Live confocal microscope (Leica Microsystems, Germany).

Sentinel lymph node imaging

HSA (Vascuosis, Iba, Belgium; 150 nmole) was radiolabeled with pertechnetate (^{99m}Tc) (2 mL saline containing 500 MBq). Ac-TZ14011-IR783 (18 nmole) in 3 μ L ethanol was mixed with 50 μ L of the ^{99m}Tc -HSA solution. A sentinel lymph node procedure was performed as described previously.[11] Balb/c nude mice (n=5) were anesthetized with a mixture of hypnorm (Vetapharma, UK), dormicum (Roche, Switzerland), and water (1:1:2; 5 μ L/g intraperitoneal). An incision of approximately 1 cm was made in the lower abdomen and the mice were injected in the prostate with Ac-TZ14011-IR783 (1 mg/mL; 50 μ L)(n=2) or with the Ac-TZ14011-IR783/ ^{99m}Tc -HSA mixture (50 μ L)(n=3). The site was massaged for 5 min to stimulate lymphatic migration, after which the incision was closed. The animals were sacrificed 60 min post injection and the caudal, lumbar and renal lymph nodes were dissected. All dissected lymph nodes were scanned *ex vivo* with an IVIS 200 at standard ICG-settings and the fluorescent signal was quantified by standard Living Image Analysis software. Subsequently, the lymph nodes from the mice injected with ^{99m}Tc -HSA were measured with a gamma counter (Wizard 3" 1480 automatic gamma counter, Perkin Elmer, USA; 126–159keV; 60sec) to determine the amount of ^{99m}Tc -HSA present in each lymph

node. Linear regression was used to determine the correlation between the fluorescence and radioactive signals.

Results

To study the potential of supramolecular fluorescent HSA complexes we investigated a number of steps: 1) FRET interaction between HSA-Cy5 and non-covalently bound ICG and IR783-CO₂H, 2) binding of IR783-labeled peptides on HSA-Cy5, 3) reversibility of complex formation, 4) validation of the complex stability and targeting ability *in vitro* and 5) *in vivo* application of ^{99m}Tc-HSA/Ac-TZ14011-IR783 in sentinel lymph node imaging. The results of these experiments are described below.

FRET interaction between HSA-Cy5 and non-covalently bound ICG and IR783-CO₂H

The fluorescent emission of Cy5 ($\lambda_{em,max} = 672$ nm) and the excitation of the NIR dyes ICG ($\lambda_{em,max} = 820$ nm) and IR783-CO₂H ($\lambda_{em,max} = 811$ nm) are complementary (Fig. S1A). This allows FRET between covalently attached Cy5 (0.7 Cy5/HSA, see supporting info) and non-covalently bound ICG (Fig. 1C). Because of the very low absorbance of the NIR-dyes at 627 nm, the excitation wavelength for Cy5 ($\lambda_{em,max} = 642$ nm), excitation of a solution of 2 μ M ICG or IR783-CO₂H resulted in a negligible fluorescent signal >800 nm (<50 counts/s). Excitation of a 0.5 μ M solution of HSA-Cy5 with the same intensity 627 nm light resulted in a strong fluorescence maximum at 672 nm (17750 counts/s). Combining HSA-Cy5 with the NIR dyes resulted in a large decrease of Cy5 fluorescence (672 nm, 4500 counts/s) and an increase in ICG and IR783 emission (>800 nm, 186 and 1163 counts/s respectively)(Fig. 2A). Quenching of the Cy5-fluorescence is the largest effect and can most easily be used for the analysis of the complex formation.

Cy5 quenching via the non-covalent interactions between ICG and HSA was spectroscopically studied in detail by titrating ICG to HSA-Cy5 (0.5 μ M). Addition of aliquots of ICG (up to a ratio of 15:1, ICG:HSA), yielded a steady decrease of Cy5 fluorescence (Fig. 2B). Similar results were obtained when experiments were carried out in a 96 well plate and the fluorescence intensity was imaged using an IVIS 200 fluorescence imaging system. Practically, this imaging-approach allows the measurement of multiple samples at the same time (Fig. 2C and D).

ICG HSA-Cy5 quenching experiments showed that the Cy5-fluorescence was quenched >95% at a ratio of 20:1 (ICG:HSA). As reference, no quenching was observed up to an ICG to Cy5 ratio of 20:1 when ICG was titrated to unconjugated Cy5 (Fig. 2C). Evidently, quenching only occurs when the two dyes reside on the same particle, one via covalent attachment and the other via non-covalent self-assembly. A pH dependence of Cy5-quenching by ICG was observed in a similar experiment at various pH-values, which indicated that complex formation was most efficient at pH 7 (see Fig. S2). Similar to ICG, IR783-CO₂H also demonstrated strong quenching of the fluorescence of HSA-Cy5, resulting in >95 % quenching at a ratio of 20:1 (IR783-CO₂H:HSA), while no quenching was observed when IR783-CO₂H was mixed with Cy5 up to a ratio of 20:1 (Fig. 2C). Clearly both ICG and IR783-CO₂H yield a comparable concentration dependant quenching of the HSA-Cy5 fluorescence.

Binding of IR783-labeled peptides on HSA-Cy5

Applying IR783-CO₂H in a host-guest interaction with HSA-Cy5 provides a reactive group in the guest molecule that is suitable for conjugation with targeting peptides and subsequently forms non-covalent targeted fluorescent nanocarriers (Fig. 1D). To study this complex formation, quenching studies were performed with three different IR783-labeled (targeted) peptides on Cy5-labeled HSA (Fig. 3). Binding of these IR783-labeled peptides to HSA resulted in a similar quenching of the Cy5-fluorescence of HSA-Cy5 as was observed with the unconjugated dyes (Fig. 2C and 4A). However, higher concentrations of the IR783-labeled peptides were required to reach the same level of quenching. Complete quenching was reached with RGD-IR783, Ac-TZ14011-IR783 and octreotate-IR783 reached 88–89% quenching.

To quantify and compare the quenching capacity of ICG, IR783-CO₂H and the IR783-labeled peptides, the quenching data were analyzed according to the Stern-Volmer equation: $F_0/F = 1 + K_{sv}[\text{ligand}]$. Analysis of the Cy5-quenching data by linear regression yielded a Stern-Volmer quenching constant (K_{sv}) for all five ligands, providing a measure for their individual quenching capacity (Fig. 4B and C). A higher quenching capacity translates into a stronger binding, so these values indicate that ICG and IR783-CO₂H have similar affinities for HSA and that the addition of a peptide reduces the affinity of IR783 for HSA. Moreover, the affinity varies between peptides. The RGD-based peptide has the highest affinity ($K_{sv} = 1.00 \cdot 10^6 \text{ M}^{-1}$) while the Ac-TZ14011 and octreotate peptides have lower affinities ($K_{sv} = 0.34$ and $0.29 \cdot 10^6 \text{ M}^{-1}$ respectively) (Fig. 3 and 4). Quenching with Ac-TZ14011-IR783 and octreotate-IR783 did not reach the maximum quenching found with the dyes alone, indicating that not all potential binding sites for IR783 could be occupied with the larger peptides attached. The “smaller” RGD peptide seems to have much less influence on the affinity.

In the case of static quenching of a 1:1 complex of a FRET pair, the Stern-Volmer constant can be regarded as the association constant (K_a) of the complex. However, our quenching experiments and separation of the formed complexes by size exclusion chromatography (SEC; see supporting information) suggest that the formation of the HSA/ICG complex is not a 1:1 complex, but contains up to a maximum of 17–18 ICG molecules bound per HSA.

Reversibility of complex formation

The reversible nature of the self-assembled complexes between HSA and the NIR-dyes and IR783-labeled peptides was studied in a competition experiment with unlabeled HSA. When increasing amounts of unlabeled HSA were added to a quenched mixture of HSA-Cy5 and NIR-ligands (10:1; ligand:HSA), the fluorescent emission of Cy5 was recovered and approached the levels of unquenched HSA-Cy5, indicating the non-covalently bound dyes were stripped of the HSA scaffold (Fig. 4D). Only the complex with the positively charged Ac-TZ14011-IR783 peptide deviated from the expected recovery of Cy5 fluorescence.

Validation of the complex stability and targeting ability in vitro

The non-covalent quenched complexes of HSA-Cy5 and IR783-labeled peptides form efficiently in aqueous solution as shown above. However, the biggest challenge is their

stability in biologically relevant conditions, starting *in vitro*. As HSA-Cy5/Ac-TZ14011-IR783 had the highest complex stability in the albumin competition experiment and the CXCR4-receptor is expressed in lymph nodes (see below), we used this complex for further experiments. Ac-TZ14011 is a potent binder of the chemokine receptor 4(CXCR4)[20] and the Cy5-labeled version of this peptide shows a clear membrane staining of CXCR4-GFP expressing MDA-MB-231 cells (Fig. 5B), which corresponds to the CXCR4-GFP expression and is similar as previously observed with FITC-labeled derivatives (Fig. 5A and B).[23] HSA-Cy5 alone also binds to the cell membranes of the MDA-MB-231 cells, probably via the albumin binding protein or via non-specific interactions (Fig. 5C).[24] However, when the non-covalent complex of HSA-Cy5 and Ac-TZ14011-IR783 (not visible at the used confocal laser and filter settings) was added to the MDA-MB-231 CXCR4-GFP cells, the Cy5 signal at the membrane decreased considerably (Fig. 5D). Based on the above, this decrease/quenching of the fluorescent signal at the location where both individual components reside (Fig 5B and C) indicates that both the peptide and HSA-Cy5 retain at this position. Because the two dyes Cy5 and IR783 need to be on the HSA-scaffold to allow FRET interactions (see above; Fig. 2C), quenching of the Cy5 emission demonstrates that both HSA-Cy5 and Ac-TZ14011-IR783 are still bound to each other under these *in vitro* conditions.

In vivo application of ^{99m}Tc -HSA/Ac-TZ14011-IR783 in sentinel lymph node imaging

A hybrid radioactive/fluorescent HSA-based complex that drains through the lymphatic system and concentrates mainly in the lymph nodes on which the tumor drains directly (sentinel nodes) has a clinical applicability in the identification of sentinel lymph nodes. [10,12] Based on our previous results, we studied the lymphatic drainage of ^{99m}Tc -HSA/Ac-TZ14011-IR783 and its parental compounds ^{99m}Tc -HSA and Ac-TZ14011-IR783 from the prostates of mice (Fig. 6A).[11,25] The CXCR4-targeting nanoparticle (Fig. 6B) was used for this application as several lymph node residing cells, e.g. lymphocytes and macrophages, express the CXCR4-receptor on their membrane.[26,27] A good correlation was found between the fluorescent signal from Ac-TZ14011-IR783 and the radioactive signal from ^{99m}Tc -HSA, as measured in the dissected lymph nodes (Fig. 6D). The average correlation of the individual fluorescent and radioactive signal intensities in the lymph nodes, which is an indicator for the stability of the complex, obtained with the ^{99m}Tc -HSA/Ac-TZ14011-IR783 complex ($R^2 = 0.75$; Fig 6D) is higher than previously reported for the ^{99m}Tc -HSA/ICG complex ($R^2 = 0.51$).[11] Moreover, an increased accumulation of ^{99m}Tc -HSA/Ac-TZ14011-IR783 was found in the caudal and lumbar lymph nodes, compared to ^{99m}Tc -HSA (see Fig 6E).[11] Ac-TZ14011-IR783 alone, also concentrated in the caudal and lumbar lymph nodes (Fig. 6C), indicating this improved retention is driven by the interactions between the peptide and its target (CXCR4).

Discussion

Based on the non-covalent host-guest interactions between NIR cyanine dyes and HSA, we were able to form peptide coated (bio)nanoparticles that can be used for (interventional) molecular imaging purposes. The rationale behind this imaging agent design is that self-assembled complexes using simple and biocompatible components have an increased chance

to be of clinical value in comparison with fully synthetic nanoparticles. We have previously demonstrated a similar approach using ^{99m}Tc -NanoColl/ICG.[12]

The non-covalent complex formation between HSA-Cy5 (0.7 Cy5/HSA) and ICG or IR783- CO_2H demonstrated both characteristics of FRET, namely quenching of the donor dye (Cy5) and increased fluorescence of the acceptor dye (ICG or IR783- CO_2H) (Fig 2A). FRET interactions indicate that both dyes are in close proximity to each other at a distance around the Förster radius (typically 20–60 Å), which, at these low concentrations ($< 1 \mu\text{M}$), will most likely occur when the NIR-dyes bind to the Cy5-labeled HSA-scaffold ($d = 70 \text{ Å}$). Because the Cy5-fluorescence is effectively reduced up to 2 % of the original signal (Fig. 2C), it can be concluded that the energy transfer between the donor and acceptor dye is effective. Therefore, the low increase in fluorescence of the acceptor NIR dyes is most likely caused by the low quantum yield of the NIR-dyes ($\phi=0.04$ and 0.02).[16,28] When using these dye pairs, FRET quenching of the Cy5 emission provides the most sensitive way to monitor the complex assembly and/or disassembly. The pH is shown to be of influence on the complex formation between HSA and ICG with an optimum pH 7 (Fig. S2) indicating the involvement of protonated side chains like histidine or the influence of the pH-dependent conformations of HSA.[29]

As FRET interactions are dependent on the distance and orientation of the FRET pair, the similarity in the quenching of the Cy5 fluorescence by ICG and IR783- CO_2H (Fig. 2C) suggests that they occupy most likely the same binding sites on HSA. This assumption is underlined by the fact that these two dyes have a large structural similarity (Fig. 1B), which suggests they can initiate similar host-guest interactions with HSA.

Previously, quenching of the fluorescence of tryptophan residues, present in HSA, has been applied to follow albumin binding ($\lambda_{\text{ex}} = 279 \text{ nm}$; $\lambda_{\text{em}} = 345 \text{ nm}$).[9,30] However, one bound ligand can cause complete quenching of tryptophan fluorescence.[9] This effect impairs finding multiple ligands bound to HSA as we found with NIR dyes and NIR dye labeled peptides on HSA-Cy5. Loading-rate dependent quenching of covalently attached Cy5, as used here, can in theory complement the more traditional methods used for identifying complex formation on albumin.

HSA can non-covalently bind NIR-dyes like ICG and IR783 based on their structure comprising anionic, lipophilic and aromatic characteristics. The number and type of binding sites on HSA for cyanine dyes like ICG, but also for other drug-like compounds and fatty acids, is an ongoing discussion in literature. In theory, HSA has two sudlow binding sites which mainly bind aromatic and heterocyclic compounds,[31] at least 5 additional binding sites for long anionic molecules and, on top of that, HSA can also bind additional molecules in a less specific manner.[2,32–35] In agreement with this, we previously found an average 6:1 (ICG:HSA) loading ratio for HSA colloids with ICG.[10,11] Our SEC data (Fig. S3) suggests that there are up to eighteen binding sites available for cyanine dyes like ICG when an excess of dye is used. This, however, does not contradict the presence of a limited number of high affinity binding sites next to multiple low affinity binding sites.

The reduced quenching capacity observed when IR783 is conjugated to peptides seems to suggest that the affinity of IR783 for HSA decreases and the number of guest molecules bound by the HSA host are reduced. It is often observed that labeling a targeting peptide lowers its affinity for its target.[36] Our study suggests this influence is also true the other way around, the affinity of the NIR dye for HSA is lowered by the targeting peptide. This is in contrast to what was previously suggested by Berezin et al.[9] In disassembly/competition studies the unconjugated dyes, RGD-IR783 and octreotate-IR783 revealed an expected recovery of the Cy5 fluorescence of HSA-Cy5. Ac-TZ14011, however, showed an almost linear relationship. This might indicate that the 5+ charged Ac-TZ14011 peptide itself is also interacting with HSA, which apparently results in Ac-TZ14011-IR783 being reluctant to leave its original HSA molecule and an increased stability of the complex.

The binding of IR783-labeled peptides on HSA resulted in supramolecular bionanoparticles with targeting moieties on their surface. The Ac-TZ14011 coated particles allow for CXCR4-targeting in vitro and in vivo (see Fig. 5 and 6), both experiments indicate that the non-covalently formed complex remained stable. The hybrid ^{99m}Tc -HSA/Ac-TZ14011-IR783 complex demonstrated the influence targeting moieties may have in a pelvic sentinel lymph node procedure.[11] Here, targeting the CXCR4 receptors present on several lymph node residing cell populations resulted in superior retention in the lymph nodes on a direct drainage pathway from the tumor compared to the parental ^{99m}Tc -HSA particle.

Although these studies are of a more fundamental nature than our earlier work with ^{99m}Tc -NanoColl/ICG,[10–12] they illustrate how the current clinical studies with hybrid tracers in the lymphatic system may be expanded towards more specific targeting. Currently, several NIR-fluorescent peptides are being developed for molecular imaging applications by a variety of research groups.[37–40] Depending on the NIR dye used, these compounds may also be used in a host-guest complex with albumin, possibly expanding their application and enriching the field of lymphatic imaging.

Conclusions

In this manuscript we report the formation of non-covalent complexes based on the host-guest interaction HSA with NIR cyanine dyes. IR783-labeled peptides and HSA were applied in the formation of non-covalent targeted bionanoparticles that remained stable *in vitro* and in the lymphatic system *in vivo*. These bionanoparticles were based on the clinically approved ^{99m}Tc -HSA-nanocarrier and can be used to introduce targeted applications in e.g. sentinel lymph node procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Formation of a fluorescent non-covalent complex of HSA/ICG, B) chemical structures of ICG, IR783-CO₂H and Cy5, C) covalent labeling of HSA with Cy5 and subsequent formation of a non-covalent quenched complex of HSA-Cy5 with the NIR-dyes ICG or IR783-CO₂H, D) formation of targeted bionanoparticles based upon the interaction of IR783-labeled peptides with HSA.

Figure 2.

A) Fluorescence spectra of 1 μ M HSA-Cy5, 2 μ M ICG or IR783-CO₂H and mixtures of both after excitation with 627 nm light (Cy5 excitation), B) Quenching of Cy5-fluorescence of HSA-Cy5 (0.5 μ M) by ICG (0–7.5 μ M), C) Quenching of Cy5-fluorescence of Cy5 (0.5 μ M) and HSA-Cy5 (0.5 μ M) by ICG and IR783-CO₂H (0–25 μ M), D) Image depicting the fluorescence decrease during a typical quenching experiment. Each well contains 200 μ L 0.5 M HSA-Cy5 and increasing concentrations of ICG.

Figure 3. Chemical structure of the IR783-labeled peptides applied in the formation of targeted bionanoparticles based upon the HSA-scaffold.

Figure 4.

A) Quenching of Cy5-fluorescence of HSA-Cy5 (0.5 μ M; 0.7 Cy5/HSA) by IR783-labeled targeting peptides, B) Stern-Volmer plot of Cy5-fluorescence quenching by ICG, IR783-CO₂H and IR783 labeled targeting peptides, C) Stern-Volmer constants (K_{sv}), D) Competition between HSA-Cy5 and HSA for the binding of ICG, IR783-CO₂H and IR783-labeled peptides.

Figure 5. MDA-MB-231 cells expressing CXCR4-GFP excited with 488 nm (GFP, I) or 633 nm (Cy5, III) incubated with: A) no incubation, B) Ac-TZ14011-Cy5 (3 μM), C) HSA-Cy5 (3 μM), D) HSA-Cy5 (3 μM) and Ac-TZ14011-IR783 (15 μM).

Figure 6. A) Potential prostate draining lymph nodes indicated in a schematic drawing of a mouse, B) Schematic formation of a complex using ^{99m}Tc-HSA and Ac-TZ14011-IR783, yielding a targeted hybrid nanoparticle that is both radioactive and NIR fluorescent, C) Fluorescence image of the dissected lymph nodes of a mouse injected with Ac-TZ14011-IR783, D) Correlation between the radioactive and fluorescent signal obtained from the dissected lymph nodes ($R^2=0.75$), E) Comparison of the radioactive signal from the dissected lymph nodes from mice injected with the CXCR4-targeted ^{99m}Tc-HSA/Ac-TZ14011-IR783 and ^{99m}Tc-HSA/ICG.[11]