Production and characterization of S100A4-targeting nanorods for an anti-cancer thermal ablation therapy

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Dissertação orientada por Jörg Klingelhöfer, Ph.D. (Danish Cancer Society) e Professora Deodália Dias (FCUL)
2012
Preface

The present master thesis is based on experimental research performed at the Danish Cancer Society, department of Tumor Microenvironment and Metastasis (TMM), and supervised by Jörg Klingelhöfer, Ph.D..

I would like to thank several people that had and important role during this process. First, I would like to thank Jörg Klingelhöfer, for agreeing to be my supervisor, and for his guidance and patience.

Also a special thank you to the head of the department, Professor Eugene Lukanidin, for allowing me to work in his department.

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I would also like to thank the staff at TMM, including all master students, for their support.

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At last, but not least important, I would like to thank my family and friends for their trust, encouragement and support that helped me through this phase.
Abstract

Cancer was responsible for 7.6 million deaths worldwide in 2008 and its high mortality rate is associated with the metastatic spread of the tumor cells to secondary sites. The tumor stroma, the microenvironment that cancer cells reside in, has been implicated in this process as well as in tumor initiation, growth and progression, leading to an increasing interest in developing new therapies, targeting the microenvironment, as it might affect the therapeutic outcome.

Nanomaterials show new means to tackle cancer by providing the tools to develop directed therapies, allowing specific tumor targeting. A stroma-targeted anticancer therapy could be achieved resorting to nanostructures as rod shape gold nanoparticles, known as nanorods, and biologically active molecules, such as antibodies, that can be coupled to the first. Nanorods are able to mediate hyperthermia, through a minimally invasive process, when irradiated with near infrared light. They absorbed light and convert it into heat conferring photothermal destruction of adjacent tumor and tumor-supporting stroma cells by disrupting their membrane integrity and denaturing their proteins. Since normal tissue minimally absorbs near infrared light, only tissue containing nanorods would be harmed. Thus, stroma-targeted nanorods have great potential for destroying the tumor stroma through thermal ablation. The microenvironment can be targeted through the coupling of monoclonal antibodies with high affinity for a stromal protein, such as S100A4, which accumulates within the tumor stroma, as the tumor progresses, making it a perfect molecular target.

The goal was to create a tumor stroma-targeted cancer therapy by using gold nanorods and anti-S100A4 monoclonal antibodies. A protocol to create these immunonoconjugated nanorods was established and optimized. The particles were characterized and subjected to several functional tests that revealed their stability, low cytotoxicity and capability to recognize and bind S100A4 in suspension. Also their thermal ablation potential was successfully tested in adherent cells and cells in suspension. In conclusion, the indicated targeting nanorods had a therapeutic potential although further testing is required.

Keywords: Cancer, microenvironment, S100A4, nanorods, thermal ablation
Resumo

O cancro foi responsável por 7.6 milhões de mortes, em 2008, em todo o mundo sendo que esta alta mortalidade está associada à disseminação metastática de células tumorais para locais secundários. O estroma do tumor tem sido implicado neste processo assim como na iniciação tumoral, crescimento e progressão, levando a um interesse crescente em desenvolver novas terapias, cujo alvo é o microambiente tumoral, uma vez que o resultado terapêutico alcançado poderia ser diferente.

Os nanomateriais apresentam novas maneiras de combater o cancro providenciando as ferramentas para desenvolver terapias direcionadas especificamente para o tumor. Uma terapia, cujo o alvo é o estroma, pode ser conseguida recorrendo a nano-estruturas como nanopartículas de ouro em forma de bastonete, denominadas de nanorods, e moléculas biologicamente ativas, como anticorpos, que podem ser acoplados aos primeiros. Os nanorods têm a capacidade de promover hipertermia, através de um processo pouco invasivo, quando irradiadas com luz na região do infravermelho. A luz é absorvida e convertida em calor, promovendo uma destruição fototérmica das células tumorais adjacentes e das células do estroma, uma vez que compromete a integridade da membrana celular e promove a desnaturação proteica. Dado que os tecidos celulares têm uma absorção mínima de luz na região do infravermelho, apenas os tecidos que contêm nanorods serão afectados. Desta modo, nanorods cujo alvo seja o estroma, têm um grande potencial para eliminar o mesmo através de termoablação. Os nanorods podem ser direcionados para o microambiente através do acoplamento de anticorpos monoclonais com uma alta afinidade para uma proteína do estroma, como a S100A4, que se acumula no mesmo, à medida que o tumor progride, tornando-a um ótimo alvo molecular.

O objectivo era criar uma terapia, cujo alvo fosse o estroma tumoral, utilizando nanorods e anticorpos monoclonais cujo alvo é a proteína S100A4. Foi desenvolvido e optimizado um protocolo para a criação de nanorods imunoconjugados. As partículas foram caracterizadas e sujeitas a vários testes funcionais que revelaram a sua estabilidade, baixa citotoxicidade e capacidade de reconhecimento da proteína em questão, em suspensão. O seu potencial de termoablação também foi testado com sucesso, em células aderentes e em suspensão. Em conclusão, os nanorods imunoconjugados apresentaram um potencial terapêutico, embora seja necessário realizar mais testes.

Palavras-chave: Cancro, microambiente, S100A4, nanorods, termoablação
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1. Introduction

1.1. Cancer

Cancer is a leading cause of death that was responsible, in 2008, for 7.6 million deaths worldwide, corresponding to 13% of all occurred\(^{1}\). This high lethality is associated with the tumor ability to metastasize, which causes 90% of cancer related death\(^{2}\). As a result, assessing the risk, preventing and developing new therapeutics are of high priority\(^{3}\).

Cancer cells are the foundation of this disease and according to Hanahan and Weinberg there are eight essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, unlimited replicative potential, evasion of immune cells, reprogrammed energy metabolism, sustained angiogenesis and tissue invasion and metastasis\(^{4}\). Cells with these hallmarks initiate tumors and drive tumor progression, liberating neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation\(^{4,5}\). Continued accumulation of mutations will eventually give rise to cells with a metastatic potential\(^{6}\).

1.2. The Tumor Microenvironment

Tumor growth and progression however, is not only dictated by genome mutations and epigenetic changes but also by interactions between cancer cells and the different surrounding cell types\(^{7}\). Among these cells are fibroblasts, pericytes, cancer stem cells, vascular and lymphovascular endothelial cells and infiltrating immune cells, which compose the tumor stroma (Figure 1)\(^{4}\). They create a microenvironment where soluble factors, such as growth factors, cytokines and proteins, act in a paracrine and autocrine way, creating permissive conditions for tumor cell growth, progression, invasion and development of metastasis\(^{3,7}\).

One protein, with metastasis promoting activity is S100A4, which is a stroma-derived factor involved in the activation of signaling pathways stimulating tumor progression and metastasis\(^{8}\).
Composition of the tumor microenvironment. The microenvironment is composed of extracellular matrix and different cell types including fibroblasts, pericytes, immune inflammatory cells, cancer stem cells, cancer cells and endothelial cells that collectively enable tumor growth and progression\textsuperscript{4}.

1.3. S100A4

The S100 protein family is composed of 24 different members that exist as homo or heterodimers within cells\textsuperscript{9,10}. They have a low molecular mass (10 to 12 kDa) and present EF-hand calcium-binding domains\textsuperscript{9,11}. Due to their nature they don’t have an enzymatic activity and exert its function by interacting and modulating other proteins\textsuperscript{9}.

S100A4, one of its members that has also be named mts1, p9ka, FSP1, CAPL, calvasculin, pEL98, metastasin, 18A2, and 42A, can be found in the cell nucleus, cytoplasm and extracellular space\textsuperscript{8,12}. It intervenes with several biological functions, such as regulation of angiogenesis, cell survival, motility and invasion\textsuperscript{8}. Given its role in cancer, S100A4 functions have been intensively studied and characterized.

1.3.1. Protein structure of S100A4

S100A4 is a 101-amino acid protein with a molecular weight of 11.5 kDa that is located on chromosome 1q21.3 in humans, 3f2 in mice and 2q34 in rats\textsuperscript{13,14}.

It usually exists as a symmetric homodimer stabilized by noncovalent interactions\textsuperscript{15}. Each subunit (monomer) is composed of two EF-hand Ca\textsuperscript{2+}-binding loops: a typical C-terminal EF-hand with 12 amino acid residues, that binds calcium with a high affinity, and a N-terminal pseudo-EF-hand with 14 amino acids residues, with a lower affinity for binding calcium\textsuperscript{14}. Structurally it consists of four \(\alpha\)-helices (H1, H2, H3 and H4) connected by short loops named L1, L2 (hinge) and L3 (Figure 2A)\textsuperscript{15}. 

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**Figure 1.** Composition of the tumor microenvironment. The microenvironment is composed of extracellular matrix and different cell types including fibroblasts, pericytes, immune inflammatory cells, cancer stem cells, cancer cells and endothelial cells that collectively enable tumor growth and progression\textsuperscript{4}.
Figure 2. S100A4 protein structure. A. Ribbon diagram of Ca^{2+} bound S100A4 monomer\cite{15}. B. Ribbon diagram of Ca^{2+} bound S100A4 dimer\cite{14}.

The inactive form of the protein (apo state), also known as the closed conformation, is a homodimer with four major helices per chain, denoted H1-H4. Calcium ions bind to the pseudo-EF and typical EF-hands at sites located between helices 1 and 2 and helices 3 and 4, respectively (Figure 2B)\cite{14}.

Upon calcium binding, S100A4 undergoes a conformational rearrangement in its three-dimensional structure, to accommodate the calcium ions, that results in the exposure of a hydrophobic binding pocket, in each monomer. These symmetrically positioned sites that are buried in the apo structure are required for recognition and binding of S100A4 various protein targets\cite{15}.

Ca^{2+}-bound S100A4 X-ray structure reveals differences in the overall shape and size of the dimers. There is a large-scale rearrangement of the hinge region L2 and helix 3 that rotates by $\sim 70^\circ$ relative to helix 4 in calcium presence, adopting a nearly antiparallel orientation. Also in the inactive form S100A4 has a more compact structure\cite{14}.

The homodimer is formed by hydrophobic interactions between the 2 monomers and exists predominantly intracellularly whereas the oligomerization of S100A4 arises through interactions between the C-terminal tail of one dimer with the hydrophobic cleft/pocket of another dimer and it’s the extracellular active form of this protein\cite{13,16,17}.

S100A4 structure allows it to bind several protein targets, participating in a wide range of biological functions, which leads to diverse biological effects.
1.3.2. S100A4 and cancer

S100A4 protein overexpression is implicated in several pathological conditions in humans, including cancer\[^{18}\]. By introduction of S100A4 gene into non metastatic lines or the suppression of its activity in metastatic ones it has been shown that S100A4 has implications in metastatic tumor progression\[^{19}\]. In genetically modified mice, which normally generate only benign tumors, the overexpression of S100A4 induced a metastatic phenotype\[^{20}\].

So far, several cancer types have demonstrated to have an elevated expression level of S100A4, when compared to the normal adjacent tissues\[^{18}\]. Altered S100A4 expression has been found in several cancer types such as gastric, pancreatic, thyroid, breast, squamous cell carcinoma, non-small cell lung, prostate and renal cancer. The data collected regarding this subject proves that there is a positive correlation in patients: they present this disease and over-express S100A4\[^{21-28}\]. Its expression has also been correlated with a poor prognosis for patients with breast, colorectal, bladder, esophageal, non-small cell lung, and gastric cancer\[^{29-34}\]. Therefore S100A4 expression was suggested to have prognostic value. According to Rudland et al, in two cohorts of breast cancer patients with S100A4 expression is the most significant predictor of patient survival, even when compared with well-established markers of disease progression. Patients with S100A4-negative tumors presented a 80% survival rate after 19 years of follow-up, whereas only 11% of those with S100A4-positive tumors were still alive\[^{34}\].

Despite all the data supporting S100A4 involvement in cancer, its precise molecular mechanism through which it enhances metastatic capability is not yet fully understood. S100A4 is able to interact with several proteins including cytoskeletal proteins, namely myosin heavy chain (NMMHC) IIA, and consequently increasing cells motility, which could contribute to an increased metastatic capacity. Other binding partners are tropomyosin, actin, p53, S100A1, septins, CCN3, liprin B1, MetAP2 and p37, however the majority of these interactions have not been confirmed in vivo. Intracellularly it also interferes with transcriptional regulation of genes of E-cadherin and matrix metalloproteinases (MMPs), which are coding for proteins involved in cell-cell adhesion and extracellular matrix (ECM) degradation, allowing cancer cells to escape the primary tumor and eventually enter the circulation.
Figure 3. S100A4 interaction mechanisms that mediate metastatic progression. A. S100A4 intracellular interactions. B. S100A4 extracellular interactions.

Extracellularly it interacts with annexin II (AII) and tissue plasminogen activator (tPA) leading to plasminogen conversion into plasmin, contributing to angiogenesis. Cell surface receptors on tumor and stromal cells, for instance RAGE, also recognize S100A4 and mediate the activation of mitogen-activated protein kinases (MAPK) and NF-KB pathway as well as the increase of the intracellular calcium concentration. These pathways will regulate target genes probably involved in metastasis stimulation[8].

In sum, S100A4 acts both intra- and extracellular affecting cell motility, invasion and angiogenesis thereby increasing the metastatic potential of cancer cells.

1.4. Stroma targeting for anti-cancer therapy

As previously mentioned cancer tumors are not only composed of cancer cells and the metastatic spread requires the cooperation of normal host cells such as endothelial cells, fibroblasts, other mesenchymal cells, and immune cells, collectively call tumor stroma cells. Despite the complexity of the tumor composition only fast growing cancer cells are the main target of chemo- and radiotherapy[35]. Regardless of their benefits, these treatments are prone to developing drug resistance and have severe side effects given their non-specificity, requiring long treatments with multiple dose regiments[36]. They exert an anti-tumor effect by selectively disrupting an aspect of tumor cell biology that gives malignant cells a relative growth advantage compared to normal cells. However recurrences are frequently experienced after apparently successful debulking of the solid tumors. This might be explained by the presence of cancer stem cells and stroma cells that have shown to be more resistant to various commonly used chemotherapeutic treatments. Cancer stem cells and cancer supporting
stroma cells may persist for years or decades regenerating a tumor once the therapy stops\cite{4,35}.

Keeping in mind the importance of tumor stromal cells in cancer development it is not surprising that the stroma is gaining more and more attention as a potential target for anticancer therapies. These cells have a lower genetic variability (compared to cancer cells) making them less susceptible to develop drug resistance\cite{4}. In particular stroma cell associated factors are considered as attractive targets for a cancer therapy. One of them is the S100A4 protein which is overexpressed and secreted by fibroblasts, macrophages, mast cells, neutrophils and activated T-lymphocytes in cancer tumors\cite{37}.

In vivo experiments showed that injection of a S100A4 expressing highly metastatic mammary carcinoma cell line (CSML-100), into S100A4-deficient mice, resulted in impaired tumorigenesis with an abnormal stroma and no metastasis formation, even though the results indicated that these cells had a high S100A4 expression \cite{38}. The altered stroma development was due to the compromised cellular recruitment and reduced blood vessel formation since S100A4 stimulates T-lymphocyte infiltration of primary tumors and cytokine release that drives tumor cells to metastasize\cite{38,39}. Furthermore, co-injection of the cells with S100A4 secreting fibroblasts restored the tumor development and metastasis formation indicating that its secretion mediates metastasis-promoting activities\cite{38}.

Since S100A4 plays an important role in tumor development and metastasis, and it is present in a great variety of cancers, accumulated within the tumor microenvironment, creating a stroma targeted therapy with S100A4 as the main molecular target could be an promising strategy disrupting the tumor-stroma interplay and avoiding further progression.

Furthermore, stroma targeted therapies can be used in combination with the traditional methods since cancer cells and tumor stroma would be targeted and therefore the therapy would be more efficient reducing relapses and associated mortality.

\section*{1.5. Nanotechnology}

Nanotechnology, an emerging science has applications in several human disease-related problems, including cancer, and it can provide tools to develop methods to evaluate the patient’s condition and provide an adequate treatment. Its name corresponds to the understanding and control of matter at a nano scale (1-100 nanometers) in at least one dimension. At this scale, materials present different and unique physical, chemical, and biological properties that differ from the properties of the same materials at a macro-scale (bulk materials, single atoms or molecules) and can present an advantage when developing new strategies of fighting cancer\cite{40}. 
Progress within the field has been made in diagnosis and therapy due to nanoscale materials unique properties. For diagnosis, nanostructures can be used for imaging and for therapeutics they can be used for drug delivery or directed therapy such as thermal ablation. Moreover, there is also the possibility to create multifunctional systems that can provide imaging and promote a therapy, using the same functionalized particles\[^{41}\].

Nanoparticles, spherical gold particles, have received increasing attention and are defined as the smallest unit (10\(^{-9}\) meters) that can still behave as a whole entity in terms of properties and transport. Nanoparticles, nanoshells or nanorods (NRs) can either improve drug bioavailability, abrogate treatment-induced drug resistance, and reduce nonspecific toxicity reducing side effects (Figure 4)\[^{40}\].

![Figure 4](image-url) Scale comparing biomacromolecules and syntetic nanomaterials. MWNT: Multiwalled carbon nanotubes; RBC: Red blood cell; SPION: Superparamagnetic iron oxide nanoparticle; SWNT: Single-walled carbon nanotubes\[^{42}\].

1.6. Hyperthermia and thermal ablation

Nanoparticles can either thermal ablate or confer hyperthermia to cancer cells. Hyperthermia corresponds to a small temperature rise (40–45°C) in body tissues, global or locally. It can initiate subcellular events that lead to various forms of damage causing cell death\[^{42}\]. This is possible since the vasculature of the tumor results in regions with hypoxia and low pH due to insufficient blood perfusion, making cells more sensitive to hyperthermia\[^{43}\].

On the other hand, in thermal ablation, temperature is raised high enough to induce immediate cellular death since it causes necrosis mediated by irreparable coagulation of proteins and other biological macromolecules. However thermal ablation faces a problem: the tumor needs to be destroyed as much as possible without
harming the surrounding normal tissues. This has encouraged researchers to develop a method, which targets only the tumor, is minimally invasive and displays uniform hyperthermia. As nanoparticles are conduits for generating hyperthermia, this method might be a way of achieving those goals. One of its advantages is that the primary source of the heat – the nanoparticles – are located inside the tumor, which reduces the heat loss, diminishing damage to normal tissues while heating up the tumor from inside. Furthermore, nanoparticles are metallic and have excellent thermal conductivity that transmits the heat they generate to the surrounding tumor tissue, maximizing the energy deposited in the tumor\textsuperscript{[42]}. The particles properties depend on their size, shape and dielectric environment. Regarding hyperthermia, gold nanorods (NRs) are especially attractive. These are elongated nanoparticles whose excitation spectra exhibit two plasmon resonances, transverse and longitudinal. The transverse plasmon emerges as a result of excitation across the NR diameter and the longitudinal plasmon resonance is due to excitation along the NR length and it results in an excitation peak, which is tunable, ranging from the visible to NIR wavelengths, depending on the NR aspect ratio\textsuperscript{[44]}. Gold NRs present a high photothermal conversion efficiency, with a larger absorption cross-section at NIR frequencies per unit volume than most other nanostructures, with 96\% of the absorbed photons converted into heat by nonradiative processes\textsuperscript{[45]}. The NIR region is a spectral window which allows photons to penetrate biological tissues with relatively high transitivity since most human tissues, hemoglobin and water molecules exhibit minimal light absorption in this region of the spectrum permitting maximum excitation of the NRs within the body\textsuperscript{[46]}.

At low powers it can be verified a measurable increase in temperature resulting in a local hyperthermia whereas at high powers the repetitive absorption of photons by gold NRs leads to an extremely rapid rise in local temperature\textsuperscript{[47]}.

1.6.1 Nanorod mediated thermal ablation

The first step involves the delivery of the nanorods into the tumor. To achieve this, the nanorods could either administrated directly into it the tumor or delivered through intravenous injections. Due to their size they passively accumulate within solid tumors given the enhanced permeability and retention (EPR) effect, which is a consequence of the aberrant features of tumor vasculature (abnormal basement membrane and pericyte lining) and the poorly developed lymphatic system that limits drainage of molecules from tumor tissue (Figure 5)\textsuperscript{[48]}. The tumor vasculature is leaky
because of abnormal vessel wall formation. The fenestrated walls have pores with an average size of 400nm allowing the nanoparticles to accumulate in the tumor [49].

![Figure 5. Enhanced permeability and retention effect and active targeting. A. After intravenous injection the nanoparticles cross the leaky blood vessels endothelium that supply the tumor B. Nanoparticles accumulate in tumor stroma[50]](image)

In order for the particles to reach the tumor stroma they need to have a long circulating time, minimizing clearance by the reticuloendothelial system (RES), which is composed of phagocytic cells residing in the lymph nodes, the spleen, and the liver[51]. This is achievable by using stabilizing surfactants, particularly nonionic polymers such as polyethylene glycol (PEG)[52]. PEG coating will increase their biocompatibility therefore reducing their cytotoxicity by preventing aggregation and unspecific serum protein binding (opsonization)[53]. It also reduces non specific uptake and improves in vivo circulation after intravenous injection[52, 53].

To enhance NRs accumulation in the tumor stroma these particles can also be coupled to active biological molecules that recognize and bind tumor markers. Such molecules are small molecules like lipids, vitamins, peptides, sugars, natural polymers including proteins, enzymes, DNA and RNA that link to the particles surface trough attractive interaction, either chemisorption, electrostatic attraction or hydrophobic interaction. Various chemical functional groups possess a certain affinity to inorganic surfaces, the most famous example being thiol to gold[54].

To treat subcutaneous tumors, a NIR light is focused on the NR-enriched tumor. Given its high transmissivity of the skin with minimal absorptions of hemoglobin and water molecules, the NRs convert the energy into heat, promoting a local hyperthermia or thermal ablation[46]. In vivo experiments have demonstrated its potential since there was no evidence of recurrent tumor growth during 20 days after treatment of NR treated mice[55]. Therefore NRs show promising results and their tunable NIR excitation maximum makes them optimal to development of thermal
ablation anticancer therapies.

1.7. Stroma-targeted nanorods for anti-cancer therapy

Biological molecules coupled to NRs have demonstrated to be a promising strategy to target specific structures in tumors\textsuperscript{[56]}. One of these molecules are antibodies that with their variety and ability to combine high affinity with high specificity, have been widely used in the targeted delivery of nanoparticles\textsuperscript{[47]}. Therefore, by incorporating targeting ligands to the nanoparticles surface such as monoclonal antibodies it is possible to create a directed therapy. Its target could be an extracellular accumulated stromal protein, such as S100A4, leading to accumulation of NRs in the tumor stroma with a high potential for thermal ablation. These proteins appear to be perfect targets due to the fact that the highest hyperthermic effect is achieved with extracellular or cell surface bound NRs\textsuperscript{[57-59]}. Also, immunoconjugated NRs have a high potential for thermal ablation which can avoid tumor recurrence if the tumor stroma is eliminated\textsuperscript{[60]}. 
2. Aims of the project

The goal of the project is to produce tumor stroma targeting nanorods for thermal ablation therapy, by coupling S100A4 monoclonal antibodies to the nanorods surface. Furthermore, the produced immunoconjugated NRs should be tested in respect to functionality and biocompatibility. These nanorods will be directed to the tumor stroma, where S100A4 is accumulated, once delivered intravenously in mice. When the tumor is subjected to a near-infrared laser the nanorods will selectively absorb energy, converting the light into heat, which will thermally destroy the tumor stroma and the blood vessels supplying the tumor, without significant damage to surrounding healthy tissue. By erasing the tumor stroma further cancer progression and metastatic spread could be inhibited.
3. Materials and Methods

3.1. Cell Culture

Several cell lines, which grow in a monolayer, were cultured in an incubator at 37°C and 5% humidity (table 1). For this propose TC-flasks (Nunc) were used, with Gibco Dulbecco’s Eagle Medium (DMEM) + Glutamax™, supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 0.4% Penicillin and streptomycin (Pen/Strep) (GIBCO).

Table 1. Cultured cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSML-100</td>
<td>Cells isolated from spontaneous mammary tumors, from AS/n mice, that exhibit a high metastatic potential[^38]</td>
</tr>
<tr>
<td>S100A4^{-/-} MEF 5</td>
<td>Fibroblast isolated from AS/n mouse embryos with a S100A4 knockdown[^38]</td>
</tr>
<tr>
<td>MEF^{+/+} 2</td>
<td>Wild type fibroblast isolated from AS/n mice[^38]</td>
</tr>
<tr>
<td>MDA-MB-231 mCherry</td>
<td>Human breast cancer cells expressing the fluorescent marker mCherry</td>
</tr>
</tbody>
</table>

3.2. Immunofluorescent staining

Antibodies (Ab) 1 to 4 were purified from cell cultures and their specificity towards S100A4 was tested resorting to immunofluorescent staining. To do so, MEF^{+/+} and MEF^{+/} cell lines were grown on glass slides overnight and then fixed in 4% paraformaldehyde (Aldrich) for 20 minutes at room temperature (RT). Afterwards they were washed in 1X PBS +Ca^{2+}/+Mg^{2+} (GIBCO) and permeabilized with 1% triton X-100 (Sigma-Aldrich) diluted in 1xPBS +Ca^{2+}/+Mg^{2+}, for 5 minutes. The cells were washed in 1xPBS +Ca^{2+}/+Mg^{2+} and incubated with a solution of different primary monoclonal antibodies (antibody 1 with a concentration of 5.5 mg/ml and antibody 2,3 and 4 with a concentration of 2.2 mg/ml), in DMEM GlutaMax + 10% FBS + 0.4% Pen/Strep, using several dilutions of each. It incubated during 30 minutes at RT. The cells were again washed in 1xPBS +Ca^{2+}/+Mg^{2+}, 3 times for 3 minutes, while shaking. The secondary antibody used was AlexaFluor 488 nm (2mg/ml diluted 1:1500, Molecular probes), which was incubated together with Diamidino-2-phenylindole (DAPI)
(diluted 1:10000, Invitrogen) and phalloidin (50g/ml diluted 1:3000, Sigma) in DMEM GlutaMax + 10% FBS + 0.4% Pen/Strep, for 30 minutes at RT, protected from light. The slides were washed twice, during 3 minutes, in 1X PBS +Ca\(^{2+}\)/+Mg\(^{2+}\) and one time in demineralized water. The slides were mounted with 50µl fluoromount (Southern biotech) and stored in the dark at 4°C. The pictures were obtained through a laser scanning microscope LSM700 from Carl Zeiss.

The best signal was obtained for antibody 1 was with the dilution 1:500 in MEF\(^{+/+}\) cells, whereas the best signal was found in the dilution 1:2000 for antibody 2 to 4 in MEF\(^{+/+}\). Also for MEF\(^{-/-}\) the best signal was obtained with a 1:2000 dilution.

3.3. Extracellular detection of S100A4

MEF\(^{-/-}\) cells were added to glass slides and grown overnight at 37°C with 5% humidity. The cells were incubated with three different S100A4 dilutions (4 µg/ml, 2 µg/ml and 1 µg/ml) in DMEM GlutaMax + 10% FBS + 0.4% Pen/Strep for 30 minutes at RT. The solution was removed and the cells were washed once with 1X PBS +Ca\(^{2+}\)/+Mg\(^{2+}\) (GIBCO) and then subjected to the immunofluorescence staining protocol described above with a single difference: only Ab 4 (2 mg/ml, diluted 1:2000) was used as primary monoclonal antibody.

3.4. Mouse tumor interstitial fluids (TIFs)

8x10\(^5\) CSML-100 cells were injected into AS/n mice with 19 to 24 weeks. The tumor grew for 6 weeks meanwhile the mice were treded with IgG. After being sacrificed, the tumors were removed, cut in pieces and weighted. 1X PBS +Ca\(^{2+}\)/+Mg\(^{2+}\) (GIBCO) was added to each tumor piece (about 100 mg) in a ratio of 1:20 (w/v), and it was incubated for 2 hours, at 37°C. Afterwards the PBS containing the secreted proteins was collected, the liquid sterilefiltrated (filter with 0.45 µm pores) and stored at -80°C.

3.5. Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Monoclonal antibody 4 (2mg/ml) was diluted 1:1000 in Coating buffer (Carbonat pH p 9,5: 1,6 g Na\(_2\)CO\(_3\) + 3 g NaHCO\(_3\)). 75 µl of this solution was added to each well in a 96 well plate and incubated overnight at 4°C. The plate was washed once with washing buffer (1X PBS -Ca\(^{2+}\)/Mg\(^{2+}\), 0,1% Tween 20), rinsed twice with superblock solution (super block solution from Pierce #37515, diluted 1:2 in 1X PBS -
Ca\textsuperscript{2+}/Mg\textsuperscript{2+}) and washed again with washing buffer. 75 µl of each sample was added to the plate (as show in table 1). After one hour of incubation at RT with 30° rotation, it was washed 4 times with washing buffer. A solution of a polyclonal S100A4 antibody (4mg/ml) was made, diluting it 1:4000 in dilution buffer (SuperBlock from Pierce #37515, diluted 1:5 in 1X PBS -Ca\textsuperscript{2+}/Mg\textsuperscript{2+}). 75 µl was added to each well and the plate was incubated for 1 hour at RT with 30° rotation. After this period it was washed with washing buffer and 75 µl of a solution of secondary antibody, an anti-rabbit horseradish peroxidase (Dako p0448) diluted 1:2000 in dilution buffer, was added to it. This was also incubated for 1 hour at RT with 30° rotation, washed 4x in washing buffer and developed with tetramethylbenzidine (TMB) reagent (Sigma-Aldrich) by adding 75µl per well of it and neutralizing it with 75µl per well of sulfuric acid (1M) after a few minutes. The plate was read at 450nm using a microplate reader (Molecular devices). The results were analyzed using GraphPad Prism linear regression to determine the S100A4 standard curve.

Table 2. Samples used in sandwich ELISA

<table>
<thead>
<tr>
<th>Samples</th>
<th>Replicates</th>
<th>Dilutions</th>
<th>Tumor size [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100A4</td>
<td>3</td>
<td>5,2.5,1.25,0.625,0.312,0.156,0.078 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>TIF Nº 1681</td>
<td>2</td>
<td>40x</td>
<td>7x7</td>
</tr>
<tr>
<td>TIF Nº 1688</td>
<td>2</td>
<td>40x</td>
<td>5x5</td>
</tr>
<tr>
<td>TIF Nº 1694</td>
<td>2</td>
<td>40x</td>
<td>6x7</td>
</tr>
</tbody>
</table>

3.6. Production of immunoconjugated NRs

3.6.1. Coupling antibody 4 to the Orthopyridyl disulfide functionalized polyethylene glycol (OPSS-PEG-NHS) linker

Antibody 4 was previously dialyzed with 10 mM sodium bicarbonate (NaHCO\textsubscript{3}), pH 8.47 and concentrated to 5.56mg/ml. 89 µl antibody 4 (5.56mg/ml) was mixed with 0.0166 mg OPSS-PEG-NHS linker (Creative PEGWorks) that had been previously dissolved in 12.5 µl 100mM NaHCO\textsubscript{3}. The solution was vortexed and incubated for two hours at 4°C and then stored at -80°C. The final concentration of antibody-linker was 32.5 µM.
3.6.2. Removal of Cetyltrimethylammonium bromide (CTAB) from NRs

500 μL of gold NR solution ([Au]= 0.25mM) was centrifuged at 15000 g for 10 minutes at RT. The supernatant was then decanted and the pellet resuspended in 500 μL milli Q H$_2$O or milli Q H$_2$O with 5% of sodium bicarbonate (NaHCO$_3$, Sigma-Aldrich).

3.6.3. Coupling of Ab-linker to NR

After removal of CTAB, 0.4 μl (25 Ab/NR), 0.8 μl (50 Ab/NR), 1.6 μl (100 Ab/NR), 3.2 μl (200 Ab/NR) or 6.4 μl (400 Ab/NR) of 32.5 μM antibody-linker (OPSS-PEG-NHS) was added to 500 μL of NR. The NRs were incubated for 24 hours at RT in the dark while rotating. The tubes were centrifuged at 12000 g RT for 10 minutes, or 8000 g RT for 10 minutes. If they were centrifuged at 8000 g RT for 10 minutes, the procedure was repeated 3 times. The supernatants were discard and the pallets collected in one tube. Milli Q water or milli Q water with 5% of sodium bicarbonate was added up to a volume of 500 μL. The purpose of the centrifugation and wash was to remove unbound antibody-linker molecules.

3.6.4. Coupling of PEG to NR

500 μL of naked NR ([Au]= 0.25mM) or immunoconjugated NRs were incubated with 100 μl of 5 mM PEG-SH 5000 Da for 24 hours while rotating. The NRs were then centrifuged like the Ab-coupled NR (12,000 g or 8000 g) and resuspended in 500 μL milli Q water, 1X PBS -Ca$^{2+}$/-Mg$^{2+}$ (GIBCO) or DMEM GlutaMax + 10% FBS + 0.4% Pen/Strep (GIBCO). The mentioned centrifugation and wash was conducted to remove unbound PEG chains.

3.7. NRs Functional testing

3.7.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were mixed with 1x or 5x SDS loading buffer, boiled for 10 minutes and vortexed. Different pore size gels were produced (8-15%), depending on
the protein of interest, with a 5% stacking gel. The molecular marker used was Seeblue® Plus2 Pre-Stained Standard (Invitrogen) and loaded together with the samples, and electrophoreses was performed for two hours at 120 V using a tris-glycine running buffer (25 mM Tris, 25 mM glycine, 0.1% SDS, pH 8.6 – all from Sigma).

3.7.2. Immunoblotting analysis (Western blot)

The proteins were transferred onto a polyvinylidene fluoride membrane (Immobilion™-P Millipore) using a semi-dry system with a constant current of 2.33 mA/cm²/h and conducted in 1x blotting buffer (48 mM Tris, 39 mM glycine, 0.375% SDS and 20% ethanol – all from Sigma). Afterwards the membrane was blocked with a blocking solution (1x PBS – Ca²⁺/Mg²⁺, 5% nonfat Milk Powder (Arla foods ingredients) and 5% FBS) for one hour at RT, with agitation. Secondly it was incubated with the primary antibody diluted in blocking buffer for either one hour at RT or overnight at 4°C. The membrane was then washed 4 times, during 5 minutes in 1x tris buffered saline (TBS) with 0.1% Tween-20 (Sigma). The secondary antibody was also diluted in blocking buffer and incubated for 40 minutes at RT while shaking. Before developing the membrane it was washed again four times, 5 minutes and developed using 1 ml ECL Supersignal detection reagent (Amersham), a Kodak BioMax Cassette, an auto X-ray developer (Kodak), and X-ray films (CL-eXpose™, Pierce).

The primary antibody was Ab 4 (2mg/ml diluted 1:8000) to target S100A4 protein. The secondary antibodies were DAKO P0260 (diluted 1:2000) or DAKO P0488 (diluted 1:2000).

3.7.3. S100A4 pull down assay

The ability of the NRs to bind S100A4 was tested through an S100A4 pull down where 500 ng of recombinant tetrameric mouse S100A4 was added to 300 µl of naked, PEG coated, or antibody-linker coupled and PEG coated nanorods. The samples were incubated for 30 minutes at RT under rotation. The tubes were then centrifuged for 10 minutes at RT at 12000 g. The supernatant was discard and the nanorods were resuspended in 40 mM HEPES (Sigma) pH 8.6 with 0.5% NP40 (Fluka). This was repeated three times. The final time, the pellet was mixed with 5x SDS loading buffer and Milli Q water to a final volume of ~30 µl. The samples were boiled for 10 minutes at 95°C, briefly vortexed, and subjected to SDS-PAGE and western blot along with appropriate controls.
3.7.4. Semi-quantification of Western blot

Western blot quantification was conducted by measuring the pixel density of protein bands with the program ImageJ (version 1.45s). As the exact amount of protein in the control band was known, the other protein bands’ pixel density were measured and compared in percentage to the control’s pixel density. The correlation was based on the assumption that there is a linear relationship between pixel density and protein concentration.

3.7.5. NRs Sonication

Eppendorf tubes containing PEGylated immunoconjugated nanorods and PEG-coated nanorods were inserted in to an ultrasonic bath for 1 minute, before making a S100A4 pull-down and after it. The particles were then used to perform a western blot.

3.7.6. Protocol efficiency

CTAB covered nanorods, immunoconjugated nanorods, PEG-coated nanorods and immunoconjugated and PEGylated NRs were used to determine the absorbance spectrum and all results obtained were plotted in to graphs and analyzed using Graphpad Prism software.

3.7.7. Immunoconjugated NRs stability

PEGylated, immunoconjugated and immunoconjugated and PEGylated NRs were prepared according to previously described and in its final step, diluted in milli Q water. Its absorbance spectrum was measured and the nanorods were stored at 4°C. After 30 days its absorption spectrum was measured again and both spectrums compared, using GraphPad Prism software.

3.7.8. Temperature curves

CTAB covered nanorods were centrifuged at 15000g and resuspendend in 1x PBS – Ca²⁺/Mg²⁺. Three solutions were prepared with a final concentration of [Au]= 50,
25 and 12 µg/ml. The different solutions were subjected to a NIR laser at 1.5 Ampere. The temperatures were recorded every 30 seconds for 4 minutes.

3.7.9. Cytotoxicity determination

CSML-100 cells were seeded in two 96-well plates (Nunc) at density of 1x10⁴ and maintained overnight at 37 ºC. Each plate had four replicates of untreated cells, cells treated with [Au]=100, 50, 25 and 12 µg/ml of CTAB coated NR and immunoconjugated and PEGylated NRs. Both were diluted in DMEM GlutaMax + 10% FBS + 0.4% Penicillin and streptomycin, and were added to each well in a final volume of 50 µL. One plate was incubated for 24 hours and the other for 40 hours. After the incubation period the plates were gently inverted and blotted on to paper towels to remove the medium and the plates were frozen at -80 ºC. To unfreeze they were left at RT and 200 µL of CyQUANT® reagent mixture (Invitrogen), was added to each well. The plates were incubated during 5 minutes at RT, protected from light and afterwards were read in a fluorescence microplate reader (Molecular devices) with excitation at 480nm and emission at 520nm. The background absorbance values (pure medium) were subtracted from all other measured absorbance values. A two-way analysis of variance (ANOVA) with not repeated measures was carried out. Bonferroni post-tests were run to compare replicate means by row.

3.8. Thermal ablation

3.8.1. Thermal ablation of cells in suspension and LDH assay

Adherent MEF+/+ and CSML-100 cells were tripsinized and added to a 96 well plate in a density of 4x10⁵ in a volume of 50 µL. There were 6 replicates of each sample and to some wells were added PEGylated and immunoconjugated nanorods, others triton X-100 and only some were submitted to a NIR light (2 Ampere) as described in table 3.

After the experiment was conducted, the plate was centrifuged at 250 g and 50 µl of cell-cleared medium was aspirated from each sample and transferred to another 96 well plate. To each well, 50 µl of reaction mixture LDH cytotoxicity detection kit (Roche) was added and incubated for 30 minutes at RT, in the dark. The LDH assay quantifies, indirectly, the amount of cell death by monitoring the conversion of tetrazolium salt into formazan salts. By this test the lactate dehydrogenase (LDH)
activity is quantified, an enzyme that is released from the cytoplasm of dying cells, into
the culture medium. Afterwards the plate was read in a microplate reader (Molecular
devices) at 490 nm with 650 nm as a reference wavelength. The background absorbance
values (pure medium) were subtracted from all other measured absorbance values.
The cytotoxicity was calculated by saying = (mean sample absorbance/mean Triton X-
100 absorbance)*100 values. The cytotoxicity values were plotted and a two-way
ANOVA with not repeated measures was carried out. Bonferroni post-tests were run to
compare replicate means by row.

**Table 3.** Samples used for thermal ablation and LDH assay

<table>
<thead>
<tr>
<th>Samples</th>
<th>Added solutions</th>
<th>NIR exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>100 µL</td>
<td>-</td>
</tr>
<tr>
<td>MEF&lt;sup&gt;+/−&lt;/sup&gt; cells</td>
<td>Medium (50 µL)</td>
<td>-</td>
</tr>
<tr>
<td>(50 µL)</td>
<td>Medium (50 µL)</td>
<td>2 minutes</td>
</tr>
<tr>
<td></td>
<td>NRs (50 µL of [Au]= 50µg/ml)</td>
<td>2 minutes</td>
</tr>
<tr>
<td></td>
<td>2% Triton X-100 (50 µL)</td>
<td>-</td>
</tr>
<tr>
<td>CSML-100</td>
<td>Medium (50 µL)</td>
<td>-</td>
</tr>
<tr>
<td>(50 µL)</td>
<td>Medium (50 µL)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NRs (50 µL of [Au]= 50µg/ml)</td>
<td>2 minutes</td>
</tr>
<tr>
<td></td>
<td>2% Triton X-100 (50 µL)</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>

**3.8.2. Thermal ablation of adhering cells**

2x10<sup>4</sup> MDA-MB-231 cells were seeded in a 96 well plate. The cells were grown
overnight and rinsed with 1xPBS +Ca<sup>2+</sup>/Mg<sup>2+</sup>, at RT, to remove floating cells. A solution
of DMEM GlutaMax + 10% FBS + 0.4% Pen/Strep and PEGylated and immunoconjugated and PEGylated NRs ([Au]=0.25 mM) was prepared. One other
sample was first incubated with 2000ng of S100A4 for 30 minutes at RT. Afterwards it
was washed twice with 1xPBS +Ca<sup>2+</sup>/Mg<sup>2+</sup> and 50 µl of it the prepared NRs solution
was added to each well along with DAPI (1:10000). The samples were subjected to a
NIR light, with continuous wave irradiation, during 180 seconds, at 2 or 3.4 Ampere
(~97.56 W/ cm<sup>2</sup>). One other sample was first incubated with 2000ng of S100A4 for 30
minutes at RT.

The pictures were acquired using the Zeiss Axiovert S100TV microscope (25x
objective) and the Metamorph software.
4. Results and Discussion

4.1. Production and characterization of anti-S100A4 antibodies

For the production of S100A4 targeting nanorods, the nanorods needed to be coupled with antibodies that recognize the S100A4 protein. The TMM laboratory, has produced four different monoclonal antibodies and purified them from hybridoma culture supernatant through affinity chromatography. Their specificities were characterized by a Western blot analysis. Results revealed that all four antibodies were specific for S100A4 recognition and no cross reactivity against other S100 family members was detected. All four antibodies recognize mouse S100A4 protein. The antibodies (Ab) 1, 2 and 4 cross-react with human S100A4 additionally. All four antibodies belong to the IgG 1 kappa isotype (data not shown).

To further characterize the antibodies and to analyze whether they bind endogenous S100A4, an indirect immunofluorescence staining was carried out. S100A4 expressing fibroblasts (MEF\(^{+/+}\)) and S100A4-knockout cells (MEF\(^{-/-}\)), which served as a control, were used. The cells were stained with DAPI and phalloidin to visualize the nucleus and the cytoskeleton, respectively. The staining pattern observed is similar with all four antibodies reveling a staining in the cytoplasm with increasing concentration towards the perinuclear area of the cells (Figure 6). This staining pattern is typical for S100A4. The best detectable signal was obtained with Ab4, whereas Ab1 shows the weakest signal. MEF\(^{-/-}\) cells showed no S100A4 staining, which indicated no cross reactivity, underlining the antibodies specificity towards S100A4. These data associated with the fact that antibody 4 has the highest affinity for S100A4 in sandwich ELISA experiments (conducted by Inge Skibshøj, data not shown) and the experimental results that showed its efficacy in blocking metastasis in vivo (data not shown), lead to its choice to create immunoconjugated nanorods.

For the hypothermal therapy the immunoconjugated nanorods should be able to detect S100A4 protein at the surface of living cells. To test the ability of S100A4 to immobilize to the surface of cells S100A4 negative MEF\(^{-}\), were incubated with three different concentrations of S100A4 (1 µg/ml, 2 µg/ml and 4 µg/ml) and afterwards subjected to immunofluorescence staining. Ab4 was used as the primary antibody to detect S100A4 (Figure 7). The results show that S100A4 strongly adhere to the glass. In addition S100A4 is also immobilized to the cells surface (Figure 7 A-C).
**Figure 6.** Detection of endogenous S100A4. S100A4 knock-out and wild type MEFs were incubated with the different S100A4 monoclonal antibodies (Ab1, Ab2, Ab3, Ab4) and stained with DAPI (nuclear staining), Phalloidin (actin cytoskeleton) and Alexa 488 rabbit anti mouse polyclonal antibody. All monoclonal antibodies recognize and bind endogenous S100A4 present in the MEF+/+, while the MEF−/− cells which were used as control showed no staining. Scale bar: 20µm
Figure 7. Extraneous added S100A4 bind to the surface of MEF<sup>−/−</sup> cells. A. Cells incubated with 1 µg/ml, B. 2 µg/ml and C. 4 µg/ml solution of S100A4 and stained with DAPI (nuclear staining), Phalloidin (actin cytoskeleton) and Alexa 488 rabbit anti mouse polyclonal antibody. Scale bar: 20µm

4.2. S100A4 is released in the tumors environment

In order to use S100A4 to target the tumor stroma, it needs to be released into the tumor environment. To determine whether S100A4 is present in tumors, mouse tumor interstitial fluids (TIF), developed from subcutaneous injected breast cancer cells, were prepared (as described in methods). TIFs samples were used to perform a sandwich ELISA to detect S100A4. The standard curve used, was based on serial dilutions of recombinant S100A4 protein, and used to quantify the amount of S100A4 present in the TIF samples (supplementary data, Figure 21 and table 4).

According to the results, the three samples show different amounts of S100A4 (Figure 8). The sample that shows the highest amount of protein levels is also from the biggest tumor (#1681) and the lowest is from the smallest tumor (#1688), which could indicate that its expression and/or secretion is dependent on the tumor size. However the number of samples and replicates used is not sufficient to correlate the size to the amount of secreted S100A4. Also, the amount of extracellular S100A4 has been demonstrated to fluctuate as the tumor progresses, in a spontaneous tumor model, showing an increase of its concentration in TIFs of 12-week-old mice with tumors when compared to TIFs obtained in an earlier and latter stage. These alterations have not been correlated to tumor size but with the abundance of S100A4 secreting cells in the stroma at distinct stages of development<sup>[39]</sup>. Other results have reveled S100A4 secretion into the tumor environment in breast cancer patients, making this protein a good therapeutic target in humans<sup>[37]</sup>.

In summary, this experiment confirmed the presence of secreted S100A4 in the tumor environment, which can be targeted by the immunoconjugated NRs. However it is still unknown if the detected S100A4 is present in a sufficient amount, which allows
the particles recognition and binding, *in vivo*. Further *in vitro* experiments are required to determine the minimum amount that a determined concentration of NRs can recognize and bind S100A4 before being certain that the amount present in the tumors is enough to target the nanorods.

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**Figure 8.** Detection of S100A4 in TIFs. The S100A4 concentration present in TIF samples were estimated by sandwich ELISA. 1681: TIF obtained from a 49 mm$^2$ tumor 1688: a 25 mm$^2$ and 1694: a 42 mm$^2$ tumor secreted S100A4 in concentration between 24 and 102 ng/ml.

### 4.3. Production of immunoconjugated nanorods

#### 4.3.1. Coupling Ab 4 to a OPSS-PEG-NHS linker

The first step to create immunoconjugated nanorods is to determine how to link the biological molecules to the particles. In the case of antibodies, it has been shown that they can be adsorbed to the gold surface of nanoparticles by electrostatic interactions. However these interactions are not strong and there is a risk of being replaced with other molecules. Also, high concentrations of antibody are required for this process. Fortunately these shortcomings can be overcome by resorting to a linker like an OPSS-PEG-NHS linker. The NHS extremity in the linker, attaches to the antibody primary amines, present in the lysine residues or the N-terminus.

To determine whether the linker would effectively react with the Ab 4 creating an antibody-linker complex, both samples of Ab4 and Ab4 subjected to the linking protocol previously described (Ab-L) were loaded to a SDS gel and a Western blot was performed. The results revealed that the antibody was successfully coupled to the OPSS-PEG-NHS linker as the coupling to the linker (~ 5 kDa) can be visualized by a shift in mobility of the heavy chain band from 59 kDa to 64 kDa (Hc-L) (Figure 9).
The S100A4 antibody was successfully coupled to the OPSS-PEG-NHS via an amid bond. Ab 4 was incubated with the linker during 24 hours under rotation, protected from light resulting in a successful coupling. Ab 4: Antibody 4 sample. Ab-L: Antibody 4 subjected to the OPSS-PEG-NHS linking protocol. Hc-L: Antibodies heavy chain coupled to the linker. Hc: Antibodies heavy chain. Lc: Antibodies light chain.

Amine modifications can affect the antibodies biological function since they can interfere with the antigen-binding site or in the Fc-region functionality. For instance, via the Fc region immune system responses are mediated by interactions with complement proteins and cell surface receptors. A linker attachment in the region could compromise immune system activation and reduce immune responses. Interference with the antigen-binding site could reduce the ability of the antibody to inhibit metastasis formation in mice. Further testing will be necessary to determine the functionality of antibody after coupling to the linker.

4.3.2. CTAB removal

The nanorods used to develop this project were provided by Filip Novotný. The particles were stabilized by cetyltrimethylammonium bromide (CTAB), a cationic surfactant, which avoids aggregation by forming bilayers or multilayers around the NRs\cite{63}. Nevertheless, free CTAB molecules and the ones adsorbed to the gold surface, have demonstrated to be cytotoxic. This makes the CTAB removal and replacement, by other molecules, necessary to avoid cytotoxic effects on the cells\cite{64}.

Taken this into consideration the excess of CTAB had to be removed before starting the immunoconjugation or PEGylation process. In order to remove excess of CTAB without inducing aggregation of the NRs, we developed a protocol where the NR solution is only centrifuged once before the immunoconjugation or PEGylation steps. This was accomplished by subjecting the nanorods to a centrifugation step at 15,000 g for 10 minutes at RT, and subsequent discarding the supernatant. A too
rigorous washing protocol with several centrifugation steps would lead to an effective CTAB removal from the nanorods surface. This way, a higher fraction of the gold would be exposed and unspecific protein interactions occur, leading to aggregation\[65\].

4.3.3. Coupling the antibody-linker complex to the nanorods

After removing the CTAB, the next step in producing immunoconjugated nanorods, is to proceed to the coupling of the antibody-linker complex to their gold surface. The linker used (OPSS-PEG-NHS) has an orthopyridyldisulfide extremity (OPSS) that allows it to chemically bind the NRs gold surface through the S-S terminal group. Also, the PEG molecules create a space between the probe and the antibody which leads to a favorable position of the antibody improving the efficiency of antibody-antigen interaction\[66\].

To determine whether or not the coupling was successful, the nanorods, after incubated with antibody-linker, were subjected to a SDS-PAGE and a Western blot analysis. As in figure 10 shown, the nanorods were not able to move through the resolving Tris-HCL gel, and stayed in the loading wells. All samples, except the control, present 3 bands which correspond to the antibodies heavy chain coupled to the linker (Hc-L) and without linker (Hc) as well as the antibodies light chain (Lc). This result clearly shows that the antibody-linker complex was successfully coupled to the nanorods.

Another way to

![Figure 10. Efficiency of Antibody-linker coupling to GNRs at different concentrations and pH conditions. C: control sample composed of CTAB covered NRs. 50\(_H\): immunoconjugated NRs coupled in milli Q water with a solution that allowed a maximum binding of ~50 Ab-L molecules per particle. 100\(_H\): immunoconjugated NRs coupled in milli Q water with a solution that allowed a maximum binding of ~100 Ab-L molecules per particle. 50\(_N/H\): immunoconjugated NRs coupled in milli Q water with 5% of sodium bicarbonate with a solution that allowed a maximum binding of ~50 Ab-L molecules per particle and PEGylated in milli Q water. 50\(_N\): immunoconjugated NRs coupled in milli Q water with 5% of sodium bicarbonate with a solution that allowed a maximum binding of ~50 Ab-L molecules per particle. 100\(_N\): immunoconjugated NRs coupled in milli Q water with 5% of sodium bicarbonate with a solution that allowed a maximum binding of ~100 Ab-L molecules per particle. Ab-L: antibody-linker Hc-L: Antibodies heavy chain coupled to the linker. Hc: Antibodies heavy chain. Lc: Antibodies light chain.](image-url)
determine if the antibody-linker had been successfully conjugated to the nanorods is to monitor the absorption peak shift by UV-vis spectroscopy. The coupling would be successful if a shift of a few nm was detected, since it arises due to the addition of a protein layer around the nanorods, changing the local refractive index\[^{62}\].

As seen in Figure 11 A and B an 8 nm shift was detected. The CTAB NRs had a maximum absorption at 789 nm and the immunoconjugated and PEGylated NRs had the maximum absorption at 781 nm. This result corroborated the previous ones and the coupling was estimated to be successful.

Figure 11. Absorbance spectrum shift shows a successful coupling. When comparing the absorption spectrum of CTAB and PEGylated and immunoconjugated NRs a 8 nm shift was detected. Both coupling processes occurred in milli Q water. The absorbance shift was analyzed using GraphPad Prism. **CTAB NRs**: CTAB covered NRs ([Au]=0.25mM). **Ab-L/PEG NRs**: Immunoconjugated and PEGylated NRs ([Au]=0.25mM). A. Absorbance spectrum of CTAB covered nanorods and immunoconjugated and PEGylated nanorods. B. Detailed absorption spectrum and absorbance shift.
4.3.4. Coating the NRs with PEG molecules

The next step is to cover the remaining gold surface of the NRs with polyethylene glycol (PEG). This process is called PEGylation. This coverage enhances the NRs circulation abilities by reducing non-specific interactions with proteins and unwanted tissue adsorption, inhibiting the activation of the complement system and thereby increasing their biocompatibility\textsuperscript{[62, 67]}. PEG coating also reduces the nanorods cytotoxicity since it replaces the toxic CTAB bilayer.

For the PEGylation, the PEG-SH with a molecular weight of 5 kDa was chosen. This size of molecule was expected to have higher stability during blood circulation when compared to smaller PEG chains\textsuperscript{[68]}.

To determine the PEG coating efficiency, CTAB covered nanorods and PEG coated nanorods were subjected to a S100A4 pull-down assay, and successive analyzed by Western blot.

The results are shown in figure 12. The CTAB covered nanorods (Figure 12, sample C) were able to pull-down the S100A4 protein. As S100A4 contains 4 thiol moieties, it is able to interact with the NPs' gold surface and it has remained attached to it during the successive washing steps of S100A4 pull down assay.

The PEGylation of the NRs (Figure 12, sample P), prevented S100A4 binding indicating a successful surface covering by the PEG molecules to block unspecific protein interaction. Immunoconjugated NRs coupled with antibody-linker and PEG, demonstrated that the antibody-linker complex remains stable and coupled to the NRs surface, during the process. Also, the antibody when coupled to the linker and the NRs, preserved its function and it was able to recognize and bind S100A4 protein in suspension (Figure 12, Ab sample). The immunoconjugated NRs bound more protein than the control sample, which indicates that a higher specificity towards the protein is achieved by coupling the antibody.

Some batches of immunoconjugated and PEGylated NRs showed some tendency to aggregate. Therefore an ultrasonic treatment was used to promote NRs resuspension. To determine whether or not the ultrasound would promote antibody-linker and PEG molecules release, several samples were prepared and subjected to an ultrasound bath for 1 minute. The PEG-coated NRs after sonication did not bind S100A4 (Figure 12, sample P1), indicating that the PEG-shell is not affected by the ultra-sonic treatment. Similar results were obtained when immunoconjugated and PEGylated NRs were analyzed. Figure 12 shows a sample that wasn’t subjected to an ultra-sonic treatment (Ab), a sample subjected to the treatment before (Ab1) and after S100A4 incubation (Ab2). All three samples showed a similar amount of coupled antibody-linker suggesting that the ultrasound treatment doesn’t promote antibody-
linker removal. However the amount of bound S100A4 was reduced in the Ab2 sample. This could be explained by the fact that the antibody-linker and PEG molecules are attached to the nanorods through a semi-covalent bound which are stronger than the non-covalent bound linking S100A4 to the antibodies.

**Figure 12.** PEGylation prevents unspecific protein binding and ultrasound bath doesn’t promote Ab-L release. Western blot of a S100A4 pull down was carried out to determine PEGylation influence in unspecific protein binding and ultrasound bath effect on immunoconjugated and PEGylation nanorods. The Ab, Ab1 and Ab2 samples were coupled at pH 8 to ~50Abs per nanorods and PEGylated in milli Q water. P and P1 were PEGylated in milli Q water. The samples were incubated with 500ng of S100A4 and subjected to a pull-down. C: control sample composed of CTAB covered NRs. P: PEGylated nanorods. P1: PEGylated nanorods subjected to an ultrasound bath prior to S100A4 incubation. Ab: Immunoconjugated NRs. Ab1: Immunoconjugated NRs subjected to an ultrasound bath before incubation with S100A4. Ab2: Immunoconjugated NRs subjected to an ultrasound bath after S100A4 incubation. Ab-L: Antibody 4 sample subjected to the OPSS-PEG-NHS linking protocol. A4: S100A4 protein. Hc-L: Antibodies heavy chain coupled to the linker. Hc: Antibodies heavy chain. Lc: Antibodies light chain. *: Samples sonicated for 1 minute

**4.3.5. Antibody-linker coupling efficiency increment**

As some batches of immunoconjugated NRs tended to aggregate it was suspected that the coupling might not be very efficient, allowing gold surface to be partially exposed. The insufficient coupling might be caused by unsuccessful gold-thiol bond formation under low pH conditions. Therefore the coupling efficiency at different pH-values was tested. To perform this experiment, the immunoconjugation and PEGylation steps were carried out in milli Q water at about pH 5 (Figure 10, 50$_h$ and 100$_h$) or in sodium bicarbonate buffer at pH 8 (Figure 10, 50$_n$ and 100$_n$). A third sample was prepared with milli Q water with sodium bicarbonate to couple the antibody-linker and milli Q water to couple PEG (Figure 10, 50$_{N/h}$). The comparison of the
samples prepared in water (50_H and 100_H) and in sodium bicarbonate at pH 8 (50_N and 100_N), revealed that the high pH enhances the antibody-linker coupling. However, during the PEGylation process the samples tended to adhere to the polypropylene tubes reducing the concentration of NRs in the solution (data not shown). To avoid the immobilization to the polypropylene tubes, a sample was prepared using sodium bicarbonate to enhance the antibody-linker coupling and water for the PEGylation process. As a result, the immunoconjugated nanorods didn’t attach to the tubes and the amount of antibody-linker showed in the Western blot is higher in this sample, indicating an increment in the coupling efficiency. Hence this protocol was adopted.

4.3.6. High number of antibodies per NR lead to a saturation

The coupling of antibody to the NRs had the purpose to target the NRs to the tumor microenvironment. Increasing the ratio of bound antibody molecules per NR could enhance the targeting and the enrichment of the local dose of antibodies which could increase the antimetastatic effect in vivo. According to Morton et al. 0.02 antibody/nm² in nanoshells produced a good effect in targeting experiments conducted in vitro⁶⁹. To test the antibody coverage on the NRs, different concentrations of the antibody-linker solution were added to the NRs, which were then PEGylated and subjected to a S100A4 pull-down assay. The amount of antibody-linker added to the NRs was calculated to have an average of 25, 50, 100, 200 or 400 antibody molecules per NR (antibodies/NR), under the assumption that all molecules interacted with the NRs.

The semi-quantitative assessment of the coupling efficiency revealed a proportional increase between the antibody-linker concentration and the amount of antibodies coupled per nanorods up to 100 antibodies/NR (Figure 13 A and B). The 200 and 400 antibodies/NR showed a decrease in this proportion. Only 94% of the estimated 200 antibodies/NR added were coupled to the NRs and approximately 82% to the 400 antibodies/NR sample (Figure 13 B). The observed proportional decrease in coupling is most likely due to a saturation of the NRs gold surface. Still, only 100 antibodies/NR showed a 100% coupling of the antibody-linker molecules. The 25, 50, 200 and 400 antibodies/NR bound ~0.24, ~12, ~188 and ~330 antibodies/NR respectively. Analyzing the S100A4 pull-down, it showed that the amount of bounded S100A4, increased with the amount of added antibody-linker (Figure 13 C). A surface saturation by the antibody-linker can however inhibit an effective PEG coating, blocking the access to the gold surface. Since there is more gold surface exposed, the protein might bind it through unspecific interactions. Although an ineffective coverage
can result in instability leading to NR aggregation, this was not verified in these samples, but in previous experiments.

To choose the number of antibodies/NR that should be used, the “binding site barrier” effect need to be taken into consideration. This effect is caused by increased coverage of Ab on a NR and increased avidity, which is preventing the NR to penetrate deep into the tumor. This occurs due to the fact that high affinity antibodies will recognize and bind to their antigens as soon as the antibodies leave the tumor vasculature causing a heterogeneous distribution of antibodies in the tumor\[70].

Taken all these data into consideration, 50 antibody molecules per NR seemed to be a good choice. This sample demonstrated
that 24% of the antibodies bound the NRs surface (~12 antibodies) and that it can bind approximately 1/5 of the added S100A4 in the pull-down assay. The data obtained for nanoshells by Morton et al. would correspond to ~34 antibodies/NR\textsuperscript{[69]}. Yet, the 50 antibodies/NR (~12 Ab) sample bound a very similar amount of S100A4 as the 100 antibodies/NR (~100 Ab). Also, by choosing the 50 antibodies/NR, the ‘binding site barrier’ effect might be avoided since a similar result is achieved using a lower amount of antibodies.

4.4. Nanorods characterization

4.4.1. Protocol efficiency

The protocol to create immunoconjugated NRs requires successive washing steps to remove unbound antibody-linker and PEG molecules. As previously described, this is accomplished by centrifuging the samples and decanting the supernatant. Initially, the CTAB NRs were centrifuged at 14000g, for 10 minutes, and after coupling the antibody-linker and PEG, they were centrifuged at 12000g for 10 minutes. However, as the process progressed the samples would acquire a lighter color, when compared to the original solution, which might indicate loss of some NRs during the washes.

To determine if some particles were being effectively lost during these steps, several samples were prepared and their absorbance spectrum was measured (Figure 14 A). As suspected, at each washing step NRs were lost in the supernatant, as Ab-L NRs and Ab-L/PEG NRs exhibit gradually a lower maximum absorption in the UV-vis spectrum, compared to the original solution (CTAB NRs). Also, the PEGylated NRs sample (PEG NRs), show a loss of particles, even though they were only subjected to one washing step.
In order to optimize the protocol, several other samples were prepared using different centrifugation speeds and number of washes. The most satisfied result was obtained by centrifuging the samples three times at 8000g at RT after the antibody-linker coupling and the PEGylation process (Figure 14 B). Both PEGylated NRs (PEG NRs) and immunoconjugated and PEGylated NRs (Ab-L/PEG NRs) show a maximum absorbance value very similar to the original solution, demonstrating a minimum loss of particles during the protocol execution. Also, the lower centrifugation speed avoided particle aggregation and adherence to the tubes. Thus, this protocol was adopted for further immunoconjugated NR preparations.

4.4.2. Nanorod stability

CTAB is the cationic surfactant that stabilizes the NRs, preventing their aggregation in solution. Therefore, an important parameter when using
immunoconjugated NRs is their stability, since there is a very low amount or no CTAB molecules in the samples, once the coupling process is completed.

To determine the stability of the produced NRs and to determine the protocol efficiency (Figure 14 B), a sample of antibody-linker nanorods were kept in water, at 4°C, during 30 days. They were measured again and their absorbance spectrum analyzed. The PEGylated NRs (PEG NRs) and immunoconjugated and PEGylated NRs (Ab-L/PEG NRs) proved to be stable under these conditions as their absorbance values remained similar to the CTAB covered NRs (Figure 15 A and B). There was no aggregation or adherence to the tubes. The absorbance spectrum of the antibody-linker

![Figure 15. UV-Vis absorbance spectrums (0.25 mM NRs solution). These samples were prepared using milli Q water for antibody-linker and PEG-SH coupling. The washing process was a three wash at 8000g for 10 minutes at RT in between each coupling. In the end they were also resuspended in water and kept for 30 days at 4°C. A. PEGylated NRs spectrum compared to CTAB covered NRs. B. Antibody-linker coupled NRs spectrum compared to CTAB covered NRs. C. Antibody-linker coupled and PEGylated NRs spectrum compared to CTAB covered NRs.]
coupled NRs (Ab-L NRs), on the other hand, revealed a significant difference when compared to the original solution indicating that they loss their stability (Figure 15 C). The solution exhibited aggregation and precipitation of the nanorods thus the spectrum observed doesn’t show the correspondent NRs absorption peaks. This result enhances the importance of a PEG coverage in achieving NRs stability.

### 4.4.3. NIR irradiated NRs can induce hyperthermia

The gold NRs (10-40 nm) strongly absorb light at NIR wavelength due to surface plasmon resonance converting absorbed light into localized heat\(^{40}\). It has been described that a solution of 100 \(\mu g/ml\) of gold NRs can lead to a 35°C increase above the initial temperature\(^{71}\). To determine how efficiently our NRs produce heat, 3 dilutions of CTAB covered NRs were prepared in 1x PBS - Ca\(^{2+}\)/Mg\(^{2+}\). The samples were subjected to a NIR laser at 1.5 Ampere and the temperatures recorded every 30 seconds, during 4 minutes.

The control sample (PBS) had a small temperature increase with a maximum reached temperature of 7.1°C (Figure 16 A and B). The other samples demonstrated a temperature increase dependent on the NR concentration of each solution since the highest temperature reached was obtained.

![Figure 16. Nanorods dependent temperature rise. These samples were prepared using CTAB NRs which were subjected to one centrifugation step at 14000g for 10 min at RT. The CTAB excess of was discarded and the NRs resuspended in 1x PBS -Ca\(^{2+}\)/Mg\(^{2+}\). The samples were subjected to a NIR laser at 1.5 Ampere for 4 minutes while measuring the temperature. A. Temperature rise consequent of the NRs presence and dependent on the gold concentration. B. Maximum temperature increase resultant from NIR light exposure. PBS: 1x PBS -Ca\(^{2+}\)/Mg\(^{2+}\). [Au]: CTAB covered NRs with a concentration of 12 \(\mu g/ml\), 25 \(\mu g/ml\) and 50 \(\mu g/ml\).]
with a NR solution corresponding to 50 µg/ml gold (Figure 16 A and B). However these maximum reached temperatures were not measured after 4 minutes of laser exposure but between 180 (12 µg/ml sample) and 210 seconds (25 and 50 µg/ml samples). This might be explained by the fact that the interaction of the laser with the NRs can result in shape and structure change due to melting. The surface area of the NRs is larger than its equivalent volume sphere, and its dimensions are what make NRs so efficient for thermal ablation. Therefore a conformational change would result in less heat production and a faster cooling to the surrounding solution hereby decreasing its temperature, as observed in all samples. Although the temperature decrease detected was very small, a longer exposure time could reveal a higher temperature drop\[72\].

The temperature increase observed is sufficient to promote selective destruction of abnormal cells through hyperthermia (40°C–45°C). Because of their poor vascular network and reduced heat tolerance, tumors are selectively destroyed in this temperature range due to irreversible damage caused by protein denaturation and loss of membrane integrity\[42, 43\]. Thus, hyperthermia for anticancer treatment could inhibit tumor cell proliferation by destroying cancer cells or making them more sensitive to the effects of conventional antitumor therapies, such as radiation or chemotherapy. Therefore the used NRs have a therapeutic potential and are usable for preclinical studies.

### 4.4.4. Cytotoxicity

The determination of the immunoconjugated NRs cytotoxicity in vitro is an important part of their characterization since the NRs will be used for preclinical studies in mice.

To access potential cytotoxicity of the NRs a CyQUANT®-assay was conducted with CSML-100 tumor cell line, using 4 replicates. The basis of this assay is the use of a green fluorescent dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids, after cells were frozen and lysed. A two-way ANOVA test (Supplementary data, Table 5), showed that after the cells were incubated for 24 hours with the immunoconjugated and PEGylated NRs, the lower concentrated samples (6 and 12 µg/ml of gold) didn’t affect the viability of the cells, when compared with the untreated cells (UT). While at this time point the cells treated with high concentration of NRs showed significant loss of viability. At 40 hours, all immunoconjugated and PEGylated NRs solutions showed a significant difference in cell growth when compared to the untreated cells pointing to toxic effects after long exposure even at low NR concentrations (Figure 17 B). The CTAB covered NRs exhibited at all
concentrations very high toxicity when compared to immunoconjugated NRs Figure 17 C). This is in accordance with the literature that demonstrates that CTAB is known to have a cytotoxic effect on cultured cells[64]. Taken together immunoconjugated and PEGylated NRs showed a dose-dependent loss in viability. Whether this effect is due to inducing of cell death or cytostatic effect need to be tested in future experiments.

For an in vivo experiment, an intravenous injection of 100 µl of 0.5 mM ([Au]= 100 µg/ml) immunoconjugated and PEGylated NRs would be required. Since the average volume of blood in mice is ~1.58 ml, the final concentration of NRs in vivo would be 31.25 µM. A 6 µg/ml solution of gold NRs is 100000 times more concentrated and yet the results show no toxicity at 24 hours after treatment. Moreover, the viability assay was performed with approximately 20000 cells. The amount of NRs exposed to cultured cells is vastly

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**Figure 17.** Cytotoxicity assessment. Cell viability of CSML-100 cells after incubation with increasing concentration of immunoconjugated and PEGylated NRs and CTAB-covered NRs. **A:** cell viability obtained after 24 hours of cell incubation with the NRs. **B:** cell viability obtained after 40 hours of cell incubation with the NRs. **C:** Cell viability increment between 24 and 40 hours. **UT:** untreated cells. **Ab:** Immunoconjugated and PEGylated NRs. **CTAB:** CTAB covered NRs.
exceeding the amount of NRs per cells when applied in vivo. However, further in vitro experiments are required before preclinical studies in mice. It also needs to be taken into consideration that the assessment of the cytotoxicity in vitro might not be accurate or reflect the in vivo cytotoxicity\textsuperscript{73}. In vitro assays only allow short term testing and do not contain the effects of chronic exposure or morbidity. Health indicators such as weight loss, behavioral abnormality, mortality percentage and average life span can only be determined in vivo.

So far, scientists have not been able to reach a conclusion on wheatear or not the nanoparticles/nanorods are toxic since the conducted studies use highly variable parameters such as physical and chemical properties of the gold nanostructures, cell types, dosing parameters, and used assays. Due to the controversy and the obtained results, more cytotoxicity studies are needed before gold NRs potential for biomedical applications can be fully exploited\textsuperscript{74}.

4.5. Thermal ablation

4.5.1. Thermal ablation of cells in suspension by NR induced laser treatment

To determine if the functionalized NRs were able to thermal ablate cells in suspension, immunoconjugated and PEGylated NRs (0.25mM) were added to a MEF\textsuperscript{+/−} and CSML-100 cells in solution (4x10\textsuperscript{5} cell/50 µl). Afterwards the cells were subjected to near infrared light (808 nm) and the amount of cell death quantified. To measure the cell death a LDH assay was performed. During cell death the cytoplasm enzyme lactate dehydrogenase (LDH) will be released to the conditioned medium, which activity could be assessed by a simple colorimetric test. The results were analyzed using a two-way ANOVA test (supplementary data, Table 6).

When CSML-100 or MEFs\textsuperscript{+/−} cells were subjected to laser treatment for two minutes at 2 Ampere no effect on cell death was observed when compared to non-treated cells. However when cells were treated with NRs prior laser treatment, 60-80\% of cells died (Figure 18). The sensitivity for both cell lines towards the treatment was similar suggesting that fibroblasts and aggressive cancer cells are both affected by heat in a similar way. As positive control, cells were treated with triton-X100, a detergents which is disrupting the cytoplasm membrane.

A limitation of this assay is that it relays on enzymatic activity. However, during thermal ablation, the nanorods produce heat, which can lead to a temperature rise above 45\(^\circ\)C. This could denature the enzyme causing it to lose its activity and
thereby interfering with the results. Therefore, the temperature increase in the solution during the laser treatment need to be controlled, or alternatively this assessment should be repeated using a different assay.

Figure 18. NR-dependent induction of cell death caused by NIR laser light exposure. Cell death of MEF +/− and CSML-100 cells with immunoconjugated and PEGylated NRs after being subjected to a NIR light (808nm) at 2 Ampere for 2 minutes. Triton NL: A 1% triton solution was added to each cell type causing 100% cell death. The samples were not lasered. Cells NL: cells that we not subjected to NIR light. Cells L 2 min: cells that were subjected to NIR light for 2 minutes. Cells+NRs L 2min: cells with immunoconjugated and PEGylated nanorods ([Au]=0.125mM) subjected to NIR light for 2 minutes.

4.5.2. Thermal ablation of adherent cells by NIR light is dependent on the presence of NRs

Thermal ablation of adherent cells allows to determine if the damage to the cells, caused by the NRs when subjected to a NIR light, affects their morphology.

For this experiment MDA-MB-231 human breast cancer cells expressing the fluorescent marker mCherry were exposed to NIR light (808 nm) with and without NRs to determine the thermal effects. The damage inflicted on the cells could be visualized morphologically, trough alterations on mCherry fluorescence and trough the DNA-specific dye DAPI, which was added to the cell culture medium. mCherry protein, can provide an indication of cell damage. It can lose its fluorescence once denatured or due to its release into the cultured medium upon loss of membrane integrity. DAPI, on the other hand, enters the cell nuclei once the membrane integrity is lost, which can be visualized trough a strong blue staining.

As it is shown in figure 19A cells subjected to NIR light exposure without NRs present no significant damage since no morphological changes, no mCherry
denaturation and no DAPI staining, in the area where the light was focused on, was observed. However, when NRs were preset in the cell culture medium, there was protein denaturation indicating that the medium temperature rose above ~45ºC (Figure 19 B). Also, the DAPI staining is clearly visible indicating loss of membrane integrity caused by the temperature achieved. However, morphological changes were not detected. Figure 20 is a time sequence of pictures taken while thermal ablating cells with immunoconjugated and PEGylated NRs, visualizing the effect of the therapy in time on living cells. The first DAPI stained cells arise after 30 seconds. As the treatment continues, the staining of the affected cells spreads out in a circular manner. The experiment clearly showed that NRs were only able to mediate thermal effects when hit directly by the laser beam.

Further experiments need to be done to determine the lowest laser power that can thermally ablate cells to avoid that locally generated heat will effect adjacent healthy tissues.

To test the immunoconjugated NRs specificity toward S100A4, cells were incubated with S100A4 and immunoconjugated and PEGylated NRs, then subjected to several washing steps. However using this experimental setup no thermal ablation effect was detected which was quite unexpected (supplementary data, Figure 22). A possible explanation for this result could be, that the NRs were washed away during rigorous washing steps. S100A4 binding to the surface receptors could also mask the epitope for the antibody recognition, preventing the immunoconjugated NRs binding to the cell surface. However, S100A4 is an oligomeric protein with several binding sites for the antibody and it is rather unlikely that all antibody binding sites at the same time are mask. Another explanation could be that the cells have no receptor to bind S100A4. In a general, thermal ablation is a form of therapy whose best characteristic is the non-invasiveness yet, the maximal depth of treatment was found to be only 10 mm\textsuperscript{75}. Consequently to treat tumors deeply seated, fiber optic laser probes are required to deliver the near infrared light, thus increasing the invasiveness of the treatment\textsuperscript{76}. Despite this fact, off target effects are still minimal as the light is delivered to specific locations, and will not harm the tissue lacking NRs\textsuperscript{77}.
Figure 19. Thermal ablation of adherent cells. A. MDA-MB-231 expressing mCherry cells were subjected to a NIR light (808 nm) with 3.4 Ampere for 2 minutes. B. MDA-MB-231 expressing mCherry cells were incubated with immunoconjugated and PEGylated NRs ([Au]=0.25mM) and without washing the cells, they were subjected to a NIR light (808 nm) at 2 Ampere for 2 minutes.
**Figure 20.** Thermal ablation sequence. MDA-MB-231 expressing mCherry cells were incubated with immunoconjugates and PEGylated NRs ([Au]=0.25 mM) and without washing, they were subjected to a NIR light for 2 minutes at 2 Ampere. Pictures of DAPI staining were taken every 10 seconds showing cell death progression.
5. Conclusion

In this project, nanorods were used to develop a protocol for the modification of their gold surface, creating immunoconjugated nanorods. The established protocol allowed anti-S100A4 monoclonal antibodies to be successfully coupled to the nanorods and coated with polyethylene glycol, increasing their biocompatibility and reducing their cytotoxicity.

As some problems arose, there was the need to enhance the coupling efficiency by increase the pH and resort to ultrasonic treatments. As the purpose of the immunoconjugated and PEGylated nanorods was to mediate a photothermal ablation therapy eliminating the tumor stroma, the nanorods were subjected to several functional tests to assess if they possessed the needed therapeutic properties. After the coupling of the antibodies, their ability to recognize and bind S100A4 was tested through a S100A4 pull-down that indicated that the antibodies retained their biological function. The protocol optimization resulted in less nanorods loss during the washes and they were proven to be stable for 30 days, in water at 4°C. They also proved to produce a photothermal effect, when irradiated with near infrared light, which is dependent on the gold concentration present in the solution. The cytotoxicity assay, indicated a dose dependent toxicity associated with PEGylated immunoconjugated nanorods. Still, the lowest concentration used didn’t affected cell growth for 24 hours. The thermal ablation conferred by the PEGylated immunoconjugated nanorods was able to cause cell damage to cells in suspension and in adherent cells shortly after 30 seconds of exposure to near infrared light.

Despite the exhibited toxicity caused by the PEGylated immunoconjugated nanorods, the concentration that should be used in vivo is much lower thus probably not causing adverse effects. This associated with their capability of conferring thermal ablation demonstrates a promising therapeutic potential although the immunoconjugated and PEGylated nanorods have not proven yet to be able to recognize cell bound S100A4.

As mentioned in the discussion further optimization and examination is required concerning the cytotoxicity studies and the thermal ablation process, before initiating in vivo experiments, to determine their biodistribution and potential toxicity.
6. Supplementary data

NR calculations:

Name: Gold
Symbol: Au
Type: Transition metal
Atomic weight: 196.97
Density@ 293K: 19.32 g/cm$^3$
Atomic volume: 10.2 cm$^3$/mol $\rightarrow$ 1.02x10$^{22}$ nm$^3$/mol
Average dimensions: 41nm x 11.5 nm
Concentration of [Au]: 0.25mM
Concentration of [CTAB]: 0.05M
Approximate particle concentration: 5x10$^{11}$ p/ml

$N = 0.00025 \, M \times 0.001 \, L: \quad 0.25 \times 10^{-6} \, moles$

$m = 196.97 \, g/mol \times 0.25 \times 10^{-6} \, moles = 49 \, \mu g/ml \, Au.$

Approximately surface:

Surface of a cylinder: $2(\pi \times r^2) + (2 \times \pi \times r)h$
Volume of a cylinder: $\pi \times r^2 \times h$
Surface: 1689 nm$^2$
Volume: 4258.63 nm$^3$
Moles of gold/NR: $4.17 \times 10^{-10} \, moles/NR$
Atoms of gold/NR: 251424.57 atoms/NR
gold atoms in 1 ml: $1.51 \times 10^{17}$
NR/ml: $5.99 \times 10^{11} \, NR/ml$
Concentration of NR: 0.99 nM
**Figure 21.** Sandwich ELISA standard curve. The results were analyzed using a linear regression. ☣ S100A4 obtained absorbance values.

**Table 4.** Statistic values obtained using GraphPad Prism to perform a linear regression. The provided equation was used to determine the S100A4 concentration in the TIF samples.

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**Goodness of Fit**

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**Deviation from zero?**

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**Data**

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**Equation**

\[ Y = 0,1168^*X + 0,3152 \]
Table 5. Two-way ANOVA analysis of the cytotoxicity results.

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Dunnett's multiple comparisons test

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24 hour plate

| UT vs. Ab6 | 65,33 | -89,61 to 220,3 | No | ns |
| UT vs. Ab12 | 119,1 | -35,89 to 274,0 | No | ns |
| UT vs. Ab25 | 165,5 | 10,51 to 320,4 | Yes | * |
| UT vs. Ab50 | 258,9 | 103,9 to 413,8 | Yes | *** |
| UT vs. Ab100 | 660,5 | 505,5 to 815,4 | Yes | **** |
| UT vs. CTAB6 | 1309 | 1154 to 1463 | Yes | **** |
| UT vs. CTAB12 | 1273 | 1118 to 1428 | Yes | **** |
| UT vs. CTAB25 | 1386 | 1231 to 1541 | Yes | **** |
| UT vs. CTAB50 | 1433 | 1278 to 1588 | Yes | **** |
| UT vs. CTAB100 | 1544 | 1389 to 1699 | Yes | **** |

40 hour plate

<p>| UT vs. Ab6 | 219,1 | 64,19 to 374,1 | Yes | ** |
| UT vs. Ab12 | 287,6 | 132,7 to 442,5 | Yes | **** |</p>
<table>
<thead>
<tr>
<th></th>
<th>Mean Diff,</th>
<th>95% CI of diff,</th>
<th>Significant?</th>
<th>Summary</th>
</tr>
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<tbody>
<tr>
<td><strong>24 hour plate</strong>&lt;br&gt;versus&lt;br&gt;<strong>40 hour plate</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>UT</td>
<td>-439.5</td>
<td>-602.5 to -276.5</td>
<td>Yes</td>
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<tr>
<td>Ab6</td>
<td>-285.7</td>
<td>-448.7 to -122.7</td>
<td>Yes</td>
<td>****</td>
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<tr>
<td>Ab12</td>
<td>-271</td>
<td>-433.9 to -108.0</td>
<td>Yes</td>
<td>****</td>
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<tr>
<td>Ab25</td>
<td>-372.4</td>
<td>-535.3 to -209.4</td>
<td>Yes</td>
<td>****</td>
</tr>
<tr>
<td>Ab50</td>
<td>-325.8</td>
<td>-488.8 to -162.9</td>
<td>Yes</td>
<td>****</td>
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<tr>
<td>Ab100</td>
<td>-206.9</td>
<td>-369.9 to -43.98</td>
<td>Yes</td>
<td>**</td>
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<tr>
<td>CTAB6</td>
<td>46.88</td>
<td>-116.1 to 209.8</td>
<td>No</td>
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<tr>
<td>CTAB12</td>
<td>117.5</td>
<td>-45.5 to 280.4</td>
<td>No</td>
<td>ns</td>
</tr>
<tr>
<td>CTAB25</td>
<td>73.31</td>
<td>-89.65 to 236.3</td>
<td>No</td>
<td>ns</td>
</tr>
<tr>
<td>CTAB50</td>
<td>114.9</td>
<td>-48.10 to 277.8</td>
<td>No</td>
<td>ns</td>
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<tr>
<td>CTAB100</td>
<td>54.09</td>
<td>-108.9 to 217.1</td>
<td>No</td>
<td>ns</td>
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Bonferroni's multiple comparisons test

<table>
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<tr>
<th></th>
<th>Mean Diff,</th>
<th>95% CI of diff,</th>
<th>Significant?</th>
<th>Summary</th>
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<tbody>
<tr>
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<td>232.6</td>
<td>77.66 to 387.5</td>
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<td>217.6 to 527.5</td>
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<tr>
<td>UT vs. Ab100</td>
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<td>738.1 to 1048</td>
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<td>UT vs. CTAB6</td>
<td>1795</td>
<td>1640 to 1950</td>
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<td>UT vs. CTAB12</td>
<td>1830</td>
<td>1675 to 1985</td>
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<td>1744 to 2054</td>
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<td>1987</td>
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<tr>
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<td>-433.9 to -108.0</td>
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<td>****</td>
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<td>Ab25 vs. Ab50</td>
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<td>-488.8 to -162.9</td>
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<td>Ab100 vs. CTAB6</td>
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<td>CTAB12 vs. CTAB25</td>
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<td>-45.5 to 280.4</td>
<td>No</td>
<td>ns</td>
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<tr>
<td>CTAB25 vs. CTAB50</td>
<td>73.31</td>
<td>-89.65 to 236.3</td>
<td>No</td>
<td>ns</td>
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<tr>
<td>CTAB50 vs. CTAB100</td>
<td>114.9</td>
<td>-48.10 to 277.8</td>
<td>No</td>
<td>ns</td>
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<tr>
<td>CTAB100 vs. UT</td>
<td>54.09</td>
<td>-108.9 to 217.1</td>
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Table 6. Two-way ANOVA analysis of the LDH assay results.

<table>
<thead>
<tr>
<th>Two-way ANOVA</th>
<th>% of total variation</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant?</th>
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</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0,5976</td>
<td>0,5427</td>
<td>ns</td>
<td>No</td>
</tr>
<tr>
<td>Row Factor</td>
<td>0,8772</td>
<td>0,0815</td>
<td>ns</td>
<td>No</td>
</tr>
<tr>
<td>Column Factor</td>
<td>87,54</td>
<td>&lt; 0,0001</td>
<td>****</td>
<td>Yes</td>
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<table>
<thead>
<tr>
<th>Sum-of-squares</th>
<th>DF</th>
<th>Mean square</th>
<th>F (DFn, DFd)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>0,1619</td>
<td>3</td>
<td>0,05396</td>
<td>F (3, 40) = 0,7255</td>
</tr>
<tr>
<td>Row Factor</td>
<td>0,2376</td>
<td>1</td>
<td>0,2376</td>
<td>F (1, 40) = 3,195</td>
</tr>
<tr>
<td>Column Factor</td>
<td>23,71</td>
<td>3</td>
<td>7,904</td>
<td>F (3, 40) = 106,3</td>
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<tr>
<td>Residual</td>
<td>2,975</td>
<td>40</td>
<td>0,07437</td>
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Bonferroni’s multiple comparisons test

<table>
<thead>
<tr>
<th>Mean Diff, 95% CI of diff, Significant?</th>
<th>Summary</th>
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<tbody>
<tr>
<td>MEF +/- CSML</td>
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<tr>
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<td>-0,2981</td>
</tr>
<tr>
<td>Cells NL</td>
<td>-0,04095</td>
</tr>
<tr>
<td>Cells L 2min</td>
<td>-0,01817</td>
</tr>
<tr>
<td>Cells+NRs+L 2min</td>
<td>-0,2056</td>
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</tbody>
</table>

Bonferroni’s multiple comparisons test

<table>
<thead>
<tr>
<th>Mean Diff, 95% CI of diff, Significant?</th>
<th>Summary</th>
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</thead>
<tbody>
<tr>
<td>MEF +/-</td>
<td></td>
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<tr>
<td>Triton NL vs. Cells NL</td>
<td>1,463</td>
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<tr>
<td>Triton NL vs. Cells L 2min</td>
<td>1,476</td>
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<tr>
<td>Triton NL vs. Cells+Nrs+L 2min</td>
<td>0,4337</td>
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<tr>
<td>Cells NL vs. Cells L 2min</td>
<td>0,0133</td>
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<tr>
<td>Cells NL vs. Cells+Nrs+L 2min</td>
<td>-1,029</td>
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<tr>
<td>Cells L 2min vs. Cells+Nrs+L 2min</td>
<td>-1,043</td>
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</tbody>
</table>
### Table 1

<table>
<thead>
<tr>
<th>CSML</th>
<th>Triton NL vs. Cells NL</th>
<th>1,72</th>
<th>1,283 to 2,157</th>
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</thead>
<tbody>
<tr>
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<td>1,756</td>
<td>1,319 to 2,193</td>
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<td></td>
<td>Triton NL vs. Cells+NRs+L 2min</td>
<td>0,5262</td>
<td>0,08917 to 0,9633</td>
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<tr>
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<td>Cells NL vs. Cells L 2min</td>
<td>0,03608</td>
<td>-0,4010 to 0,4731</td>
<td>No</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Cells NL vs. Cells+NRs+L 2min</td>
<td>-1,194</td>
<td>-1,631 to -0,7569</td>
<td>Yes</td>
<td>****</td>
</tr>
<tr>
<td></td>
<td>Cells L 2min vs. Cells+NRs+L 2min</td>
<td>-1,23</td>
<td>-1,667 to -0,7930</td>
<td>Yes</td>
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</tr>
</tbody>
</table>

**Figure 22.** Thermal ablation of adherent cells. MDA-MB-231 expressing mCherry cells were incubated with S100A4 for 30 minutes at RT. Subjected to two washes with 1xPBS +Ca\(^{2+}\)/Mg\(^{2+}\) and incubated with immunoconjugated and PEGylated NRs for 30 minutes at RT. The sample was then subjected to a NIR light (808 nm) with 3.4 Ampere for 2 minutes.
7. References


