Nanomedicine has great potential to revolutionize cancer therapy by improving the therapeutic efficacy and reducing side effects. However, nanocarriers need to overcome multiple biological barriers in the human body before achieving effective therapy outcomes. The present thesis describes development of porous silicon nanoparticles for cancer therapy and imaging by investigating several relevant issues: nanoparticle stability and prolonged blood circulation time, imaging possibilities, controlled drug release, compound safety as well as the exploitation of active targeting.
Simo Näkki

FUNCTIONALIZATION OF POROUS SILICON NANOVECTORS FOR TARGETED CANCER THERAPY

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Publications of the University of Eastern Finland
Dissertations in Forestry and Natural Sciences

ABSTRACT

Non-specific drug distribution often limits the use of cancer chemotherapeutic agents. Currently, administered drugs become distributed all over the body; this means that not only can they induce adverse side effects but also that only a small fraction of the total dose reaches the malignancy, limiting the therapeutic efficacy. Ideally, the chemotherapeutic drug should specifically accumulate within cancer tissue, kill only malignant cells and not affect the functions of vital organs. These are the aims of modern nanomedicine where different types of carriers can be exploited to enhance drug delivery to the target. However, there are biological obstacles, such as the immune system which rapidly eliminates the particles from the bloodstream, thus restricting the use of many potential carriers. Furthermore, premature drug leakage may reduce the therapeutic dose reaching the cancer and this can also evoke side effects.

Porous silicon is an inorganic material that possesses several favorable features for biomedical applications, such as a large pore volume and a well-known surface chemistry. In the present thesis, porous silicon nanovectors for cancer therapy and diagnosis were developed. The designed nanovectors included features to improve colloid stability and to prolong the blood circulation time as well as having traceability for medical imaging, controlled drug release, active cancer targeting and site-specific therapy. The colloidal stability was improved by shielding the nanovectors with polyethylene glycol which also prolonged the systemic circulation time in vivo. Superparamagnetic iron oxide nanoparticles and fluorescent labelling were utilized to image the particles both in vitro and in vivo. A pH sensitive coating was applied to control the release of the drug, thereby diminishing its in vivo side effects. Furthermore, mesenchymal stem cells demonstrated an ability to carry and target porous silicon nanovectors into cancerous tissue where photodynamic therapy could be accurately utilized to induce cancer cell death in vivo. The present thesis and its results indicate that porous silicon is a potentially advantageous therapeutic agent for nanomedicine since it is capable of undertaking multiple functions which can improve the therapeutic outcome.
National Library of Medicine Classification: QT 36.5, QV 786.5.C7, QV 785, QZ 267

Medical Subject Headings: Theranostic Nanomedicine; Nanoparticles; Silicon; Porosity; Colloids; Surface Properties; Polyethylene Glycols; Drug Carriers; Drug Delivery Systems; Half-Life; Magnetic Resonance Imaging; Mesenchymal Stromal Cells; Neoplasms/drug therapy; Doxorubicin; Photodynamic therapy

Yleinen suomalainen asiasanasto: nanolääketiede; nanohiukkaset; pii; huokoisuus; kolloidit; pintailmiöt; pinnoitus; lääkeaineet; kohdentaminen; vapautuminen; puoliintumisaika; magneettikuvaus; syöpätaudit; syöpähoidot; lääkehoito; fotodynaaminen hoito
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Kuopio
August 3, 2018

Simo Nääkkä

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>APTES</td>
<td>3-(aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Typically used mouse strain</td>
</tr>
<tr>
<td>Ce6</td>
<td>Chlorin E6</td>
</tr>
<tr>
<td>Cy7.5</td>
<td>Cyanine7.5 fluorescent dye</td>
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<tr>
<td>DiO</td>
<td>3', 3'-Diocctadecylacarbocyanine perchlorate</td>
</tr>
<tr>
<td>DiR</td>
<td>1', 1'-Dioctadecyl-3, 3', 3'-tetramethylindotricarbocyanine iodide</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPEG</td>
<td>Dual PEGylation</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>ELS</td>
<td>Electrophoretic light scattering</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
</tr>
<tr>
<td>H$_2$DCFDA</td>
<td>2', 7'-dichlorodihydrofluoroscein diacetate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IPA</td>
<td>2-propanol</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVIS</td>
<td>In vivo imaging system</td>
</tr>
<tr>
<td>LTO</td>
<td>Low temperature silicon oxide</td>
</tr>
<tr>
<td>MaPSi</td>
<td>Magnetic porous silicon</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MSV</td>
<td>Multistage nanovector</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccimide</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD scid gamma mouse strain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PSi</td>
<td>Porous silicon</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>Theranostics</td>
<td>Combination of therapy and diagnostics</td>
</tr>
<tr>
<td>TMOS</td>
<td>Tetramethoxysilane</td>
</tr>
<tr>
<td>TOPSi</td>
<td>Thermally oxidized porous silicon</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible spectroscopy</td>
</tr>
<tr>
<td>VSM</td>
<td>Vibrating sample magnetometer</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis consists of a review of the author’s work in the field of utilizing nanotechnology for cancer therapy and the following selection of the author’s publications:


Throughout this thesis, these papers will be referred to by Roman numerals. The above publications have been included at the end of this thesis with their copyright holders’ permission.
AUTHOR’S CONTRIBUTION

The publications in this dissertation are original research papers of experimental studies developing porous silicon for cancer theranostics. The author was involved in planning, design and writing of each paper.

I The author was responsible for material manufacture and development of the surface coating used, conducted most of the experiments and was the principal author of the manuscript.

II The author was responsible for the preparation a part of the samples, conducted all of the cell viability experiments and some of the in vitro MRI procedures, and took part in the manuscript writing.

III The author was responsible for material manufacture, conducted part of the experiments and was the principal author of the manuscript.

IV The author was responsible for all the surface modifications, conducted most of the experiments and was the principal author of the manuscript.
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1 Introduction

Cancers are the second leading cause of death in the world [1]. Every year there are over 17 million new cases, and a total of eight million lives are lost due to various cancers. Cancers are formed by the mutation of healthy cells that then start to multiply abnormally. Normal cells have signaling pathways which allow them to control their proliferation but these features are defective in cancer cells, leading to rapid growth. Eventually the cancer cells escape from their origin and start to form metastatic cancers in new locations [2]. Cancers are a major threat to human life because they disturb the normal functions of the healthy organs and ultimately the entire organism.

Breast cancer and prostate cancer are the most frequently found cancers in women and men, respectively. Breast cancer is also the second deadliest cancer among women, with tracheal, bronchus and lung cancer leading the charts in both women and men. As is the case in many other cancers, surgery is still the leading treatment option to treat breast cancers since other options are not as effective: 58% of early stage breast cancers and 14% of late stage breast cancers are treated with partial, i.e., breast-conserving surgery [3]. The statistics for mastectomy (removal of the whole breast) are disturbing: 36% of the early stage and 58% of the late stage breast cancers are treated with mastectomy. In addition, usually other therapeutic options such as chemotherapy are still required to fully remove the malignant cancerous tissue. It is redundant to say that such invasive surgical methods for malignancy removal are not the optimal route when considering patient comfort. Therefore, either chemotherapy or other minimally invasive treatment options should be considered as the first choice without compromising the treatment efficacy.

Chemotherapy is a treatment option where anti-cancer drugs, cytostatic agents, are usually administered intravenously (i.v.) into the patient’s body and in some cases, orally [4]. The administered cytostatic agents are distributed around the body via the blood circulation so that chemotherapy should be effective against metastatic cancers which have spread throughout the body. On the other hand, the homogeneous bodily distribution is the reason why in many cases chemotherapy is not the primary choice as the treatment modality: While cytostatic agents are effective killers of the malignant cells, they do not discriminate between healthy and cancerous tissue. In fact, it has been estimated that less than 0.01% of administered therapeutic moieties actually reach the cancer site [5]. Thus, most of the anti-cancer drugs are distributed in healthy tissues, causing unwanted side effects [6].

Nanotechnology has strongly emerged into the biomedical field and cancer therapy during the last few decades. Nanotechnology relies on the small size of the material used that can enclose drug molecules in a confined environment. The encapsulation of drug molecules into a confined space can alter their physico-chemical properties, i.e., crystallinity/amorphocity and drug dissolution rate [7, 8].
assisting in the permeation and bioavailability [9]. The oldest nanomedicine, Doxil®, has now been on the market for more than 20 years [10]. Similar to the majority of other U.S. Food and Drug Administration or European Medicines Agency approved nanochemotherapeutics (seven in total), this drug carrier is based on liposomes that carry the chemotherapeutic drug. Here, the drug payload is protected from the harsh blood environment by a lipid layer and the liposomes are shielded with polyethylene glycol (PEG) to minimize the uptake by the immune system, which would otherwise eliminate the nanomedicines rapidly out of the blood circulation. However, uncontrolled drug release is a major disadvantage of liposome based nanocarriers [11, 12] since it diminishes the therapeutic efficacy of these nanomedicines.

It is predicted that the next generation of cancer nanotherapeutics could be based on inorganic nanoparticles as they possess distinctive features compared to their organic counterparts. Similar to the organic nanoparticles, the inorganic nanoparticles can improve the biological activities, i.e., pathological targeting, drug loading and immune system evasion. In addition, they possess unique thermal and magnetic imaging abilities, near-infrared responsiveness, triggered drug release and vast functionalization possibilities [13] making them very interesting choice for biomedical applications.

The aim of the present thesis was to develop porous inorganic drug carriers and to investigate their feasibility for use in cancer therapy and imaging (the combination of therapy and imaging is called theranostics). Porous silicon (PSi) was chosen as the material to be examined due to its advantageous properties, including flexible surface functionalization possibilities and its relatively large surface area and pore volume enabling a high drug payload loading. Study I focused on shielding the porous silicon nanoparticles with hydrophilic polymers coated on the external surface of the particles and examining their behavior in vitro. In study II, the efficacy of the developed coating was assessed in vivo with magnetically modified particles to allow magnetic resonance imaging (MRI). In study III, in vivo toxicity of the particles was extensively evaluated after intravenous administration. Moreover, the therapeutic efficacy was verified in a proof-of-concept study after intratumoral administration in vivo. Finally, in study IV, an alternative therapy option, photodynamic therapy (PDT), was studied in conjunction with active cancer targeting with mesenchymal stem cells (MSC) in vivo.

In summary, the obtained results provide more insights into the use of porous silicon in cancer theranostics. The developed coating provides superb protection from the immune system, enabling their utilization in cancer therapy. Drug molecules were loaded into the pores and the versatile nature of PSi allowed the addition of different imaging moieties and functionalities so that it would be possible to manufacture multifunctional drug carriers. The formulated drug carriers exhibited excellent in vivo biocompatibility without any signs of toxicity and were capable of efficient cancer cell destruction in vivo. Moreover, an active targeting strategy was utilized with PDT, which can be combined with traditional chemotherapy into the same carrier in future work.
1.1 Porous silicon

Arthur Uhlir is widely considered as the founder of porous silicon research as he, with his wife Ingeborg, was the first to report on the production of porous silicon by electrochemical etching in 1956 [14]. Actually, the discovery of porous silicon was a mere accident as the Uhlirs intended to electropolish the silicon substrate they were using: “Instead of being shiny, the surfaces have a matte black, brown or red deposit”, they reported. Little did they know that after 60 years, this same method, electrochemical etching, would be by far the most extensively exploited technique in the manufacture of porous silicon.

Despite the fact that the manufacturing method has been known since the late 50s, it took nearly 40 years before porous silicon attracted the interests of the wider scientific community. In 1990, Leigh Canham was the first to describe the phenomenon of photoluminescence [15] and later in 1995, the biocompatibility [16] of PSi, which opened the field for biomedical applications. According to the Web of Science (search criteria: items with porous silicon in title or topic), before 1995 there had been less than 2 000 reports related to PSi. From 1996 until this year, there have been over 18 000 reports [17], reflecting the explosion in the interest about this material. Furthermore, since 2011, each year about 1000 publications have appeared every year. In the following sections, the production of porous silicon via electrochemical etching, its modifications and characterizations relevant to cancer theranostics utilized during this thesis will be described in more detail.

1.1.1 Electrochemical etching

Electrochemical etching of silicon is based on the use of an electrical current and chemical solution i.e. an electrolyte, to oxidize which in this case means to dissolve the bulk silicon to form holes. A silicon wafer is positioned on top of a metal disc inside a Teflon casing that is nonreactive to the used electrolyte. The metal disc is connected to an electrical source and this will allow the silicon wafer to act as an anode in the process. The Teflon shell is filled with the electrolyte consisting of a mixture of hydrofluoric acid (HF) and ethanol that is used to improve the homogeneity of produced PSi [18]. A platinum electrode, which is placed in the medium, acts as a cathode in the process (Figure 1).

![Figure 1. Schematic illustration of electrochemical etching cell used to form porous silicon.](image)
Depending on the used current, the silicon surface can be electropolished or a PSi film can be formed at the top of the silicon wafer. During the etching, the used current is applied to a certain area and the term current density (A/cm$^2$) is employed to describe the effect of the etching current to the process. While many other factors (e.g. wafer doping, electrolyte composition) influence the process and the final PSi (e.g. pore size, porosity), in general it can be stated that porous silicon is formed when the current density is between 1 to 100 mA/cm$^2$, and electropolished with current densities higher than 100 mA/cm$^2$ [18]. During the etching process, the current density can be kept constant to form a uniform PSi film or it can be altered to aid in the particle formation later during the manufacturing process. In general, increasing the current density leads to higher porosity [19, 20] meaning also the loss of the structural support. Therefore by increasing the current, a fragile high porosity layer between the desired porous silicon layers is formed and the film will break more easily [21]. At the end of the etching process, the current density is increased to the electropolishing region to allow lift-off of the formed PSi film from the wafer to be collected for further use. There are also other manufacturing methods such as metal-assisted etching [22] and chemical reduction from silicon dioxide [23] to produce porous silicon but these are outside the scope of this thesis and will not be discussed any further here.

1.1.2 Particle formation

The collected PSi film is fractionated to obtain particles of the desired size for biomedical applications. Mechanical ball milling is a robust method for grinding the films into smaller micro- and nanoparticles. Porous films are easily fractionated into smaller pieces with a planetary ball mill but the resulting particle distribution is quite wide [21]. After the ball milling, separation methods, typically sieving and centrifugation, are utilized to collect nanoparticles, leading to fact that the nanoparticle yield of desired size is less than 100%. Typically, with ball milling, the yield for 122 nm sized nanoparticles from a porous silicon film prepared by pulsed etching is ~ 25% [21] and the yield increases somewhat if larger particles are desired.

On the other hand, photolithographic patterning is a more sophisticated process to obtain monodispersed particles with exactly determined dimensions. There is a downside to obtaining particles with precise dimensions, i.e. the yield is typically very limited (ca. < 5%). Photolithographic patterning can be made before the PSi formation [24] or on a porous silicon film [25]. A typical photolithographic process is demonstrated in Figure 2 [25]. First, the porous film (Figure 2a) is prepared as explained in the previous section. A low temperature silicon oxide (LTO) is deposited on top of the PSi film and a negative photoresist is added for patterning the particles (Figure 2b). Reactive ion etching is performed in order to dissolve the PSi elsewhere than below the photoresist, resulting in PSi particles of clearly defined dimensions (Figure 2c). The LTO and photoresist are stripped off (Figure 2d&e) and finally the monodispersed PSi particles are detached with sonication (Figure 2f).
Figure 2. Schematic figure describing the formation of PSi particles with photolithographic patterning. A) PSi film. B) Low temperature silicon oxide (LTO, blue) is deposited and photoresist patterns (green) are laid on top of the PSi. C) Reactive Ion Etching is performed to produce the particles and D) LTO and the photoresist are removed. SEM images showing E) formed PSi particles still attached to the PSi film and F) the released particles [20].

1.2 Surface modifications

The surface of freshly prepared porous silicon is covered with hydrogen bonds (SiH$_x$, $x$ = 1 - 3) which are not stable and prone to be replaced by other elements such as fluorine, carbon or oxygen; this happens mostly during storage [26, 27]. Thus, the surface needs to be passivated in order to ensure good chemical stability. The main three passivation processes are hydrosilylation, thermal carbonization and oxidation [26] (Figure 3). The physico-chemical properties of PSi are considerably affected by the chosen passivation method; after oxidation in air or thermal carbonization in acetylene gas above 700 °C, the particles become hydrophilic whereas after hydrosilylation or thermal carbonization below 700 °C, the particles acquire a hydrophobic nature [26]. Thermal and wet chemical oxidations were used throughout this thesis and will be discussed in more detail next.
Figure 3. Schematic figure of the three most common surface stabilization methods for porous silicon: a) hydrosilylation, b) thermal carbonization and c) oxidation (thermal).

1.2.1 Oxidation

Thermal oxidation can be conducted at various temperatures although normally a range between 300 – 1000 °C is used depending on how the sample needs to be oxidized. At lower temperatures (~300 °C), the oxygen will penetrate between the first layers of Si-atoms and create oxygen bridges (i.e. back-bond oxidation) (Figure 3c) [26, 28]. At the same time, the surface is partially oxidized and some hydroxyl groups are formed. At higher temperatures, the oxidation is much more thorough so that at temperatures closer to 900 °C the whole silicon structure will be oxidized forming a uniform Si-O-Si structure [26, 29]. Throughout this thesis, thermal oxidation at 300 °C was utilized and the material was denoted as thermally oxidized porous silicon (TOPSi).

The PSi surface chemistry is extremely conducive to further modifications so that one can achieve the desired properties suitable for biomedical applications. Wet chemical oxidation can be conducted separately in an aqueous solution or after thermal oxidation to increase the surface hydroxyl group density prior to later modifications [30]. Although water is capable of oxidizing the PSi surface and covering it with hydroxyl groups [30], nonetheless commonly other chemical solutions are applied. Adjusting the pH will help in this process, as pH values above 7 will allow hydroxide ions to attack both Si-O and Si-H bonds but a too high pH (~10) will result in the total dissolution of the PSi [29]. Furthermore, the use of different oxidants and elevated temperature can speed up the oxidation process. Hydrogen peroxide (H$_2$O$_2$) is a widely used oxidant [31-33]. Normally, it is used in combination with other chemicals such as HCl or NH$_4$OH in an aqueous solution [34]. Commonly, a two-step chemical oxidation is used by combining oxidations in H$_2$O$_2$:NH$_4$OH:H$_2$O and H$_2$O$_2$:HCl:H$_2$O to produce highly hydroxyl terminated surfaces that enable
efficient conjugation platform via silanol chemistry to add crucial functional groups onto the surface of PSi [35-37]. The material initially thermally oxidized at 300 °C and subsequently chemically oxidized with the two-step method mentioned above will be denoted as TOPSi-OH.

1.2.2 PEGylation

Polyethylene glycol (PEG) is a homo- or heterobifunctional hydrophilic polymer, which is used to improve colloidal stability [37-39], diminish protein adsorption [37, 40, 41] and confer a “stealth” effect on the nanoparticles to protect them from the mononuclear phagocyte system (MPS) [42-45] which is a major component of the immune system and acts to remove foreign objects from the bloodstream. The main structure of PEG is composed of ethylene glycol units (Figure 4a), which are repeated n-times depending on the size of the molecule. Commonly PEGs are heterobifunctional; one end is suitable for covalent conjugation to nanoparticles whereas the distal end possesses the desired property to interact with the surrounding environment or to allow further modification (Figure 4b).

Figure 4. Schematic figure presenting a) ethylene glycol unit b) general structure for silane-PEG-R, where R represents functional group, c) the covalent conjugation to PSi with silanol chemistry assuring the formation of either a mushroom, brush or combined PEG conformation.
The colloidal stability, or as simplified, the particle size stability in the medium, is explained by the DLVO-theory, named after the scientists who devised it (Derjaquin-Landau-Verwey-Overbeek). The DLVO-theory explains particle colloidal stability, or in other words, the aggregation/agglomeration through the means of attractive forces (van der Waals forces), repulsive forces (ζ-potential), particle size and distance to each other [46-48]. As stated by Derjaquin [46], a particle ζ-potential above |30| mV will create a repulsion force strong enough to avoid particle aggregation if the particles are small (of the order of 100 nm). However, TOPSi nanoparticles (100 - 200 nm) do possess a ζ-potential ca. -30 mV, but still intense aggregation can occur instantly in phosphate buffered saline (PBS, pH 7). In fact, every situation should be evaluated individually as a huge number of factors will eventually affect the colloidal stability [49], for example, proteins adsorbing onto the surface might cause changes in ζ-potential and lead in aggregation of the nanoparticles [50].

PEG molecules can be covalently conjugated to TOPSi-OH particles via silanol chemistry (Figure 4c). Conjugated PEG will form a protective coating layer to the surface of PSi particles which will increase the colloidal stability. The shape of the formed layer depends on the size of the used PEG(s) and the surface density of PEG molecules. Short chain PEGs or a high amount of PEG will generally form a brush-like layer whereas long chain PEGs or a low PEG amount will result in a mushroom-like layer [51-53]. Logically thinking, the layer will presumably have some sort of mixture of these PEG conformations. Furthermore, a mixture of different sized PEG molecules can lead to particles with both PEG conformations (Figure 4c) [37].

The conjugation of PEG molecules can also decrease protein adsorption on the particle surfaces. Avoiding protein adsorption is a crucial feature for therapeutic nanoparticles as the MPS relies on the insertion of adsorbing proteins onto the foreign particles so that they can be recognized and thus removed from the bloodstream. In this process, opsonins (usually blood proteins) are bound onto the surface of foreign particles and subsequently they are recognized by phagocytic cells which accumulate them to liver and spleen to be degraded. There are reports that the PEG properties, such as size, grafting density and conformation affect drastically the adsorption of proteins onto the particles. Generally, high grafting density and long PEGs are favorable [40, 52, 54] but also short PEGs have been shown to decrease significantly the protein adsorption onto the particles [51].

The reason for decreased protein adsorption is the steric repulsion that PEG creates on the particle surface. The repulsive force is produced when an object pushes against the PEG and is affected by the PEG conformation and flexibility; these are ultimately influenced by the PEG size and grafting density [52, 55, 56]. One could argue that the brush-formation is always the best option for inhibiting adsorption of proteins onto the particle as in these situations the grafting density is high and the steric hindrance could be expected to be greatest. However, if one has too high a PEG density, then this can lead to impaired flexibility and poor chain mobility, leading to a loss of the repulsive forces [52, 56, 57]. Furthermore, it has been shown that a similar
inhibition of the adsorption of proteins onto the particles can be achieved with both PEG confirmations [51].

Finally, the addition of PEG will also alter the ζ-potential of the coated particles since it will shift the charge into the neutral region which is beneficial with regards to the avoidance of opsonization [37, 52, 56]. According to DLVO theory, this should decrease the colloidal stability. Nevertheless, because of the steric hindrance from the PEG chain, the colloidal stability is increased, further highlighting the fact that every situation needs to be assessed on an individual basis.

1.2.3 Other surface modifications

Like PEG molecules, amino-groups can be conjugated via silanol chemistry. The addition of amino-groups will change the ζ-potential to a more positive direction and might decrease the colloidal stability [36, 58]. However, it provides functional groups (-NH₂) that are widely used in covalent conjugation of other useful molecules.

One of the most widely used conjugation strategies involves utilizing carbodiimide chemistry to connect primary amines either with NHS esters modified molecules or with carboxylic acids (functional group -COOH) by utilizing crosslinker mediated EDC/NHS chemistry. In direct linking with NHS, the desired molecule is crosslinked to an NHS ester, which will spontaneously react with the amino-group to form a stable bond (Figure 5). For example, this is one of the most straightforward methods for conjugating fluorescent molecules to PSi particles to enable fluorescence imaging. Fluorescence imaging is based on materials that absorb light at certain wavelengths and emit it back at a longer wavelength. The method is commonly exploited in many scientific disciplines due to its straightforward nature even though the penetration of light through tissues is rather limited. In the linking between an amine and a carboxyl group, the EDC molecule is a crosslinker that works most efficiently under acidic conditions (pH 4.5) where it reacts with carboxyl groups. However, this product is unstable in aqueous solutions and susceptible to hydrolysis. Thus, NHS (or sulfo-NHS, a water-soluble counterpart) is conjugated to create a stable NHS ester which can react with primary amines (Figure 5) [59]. Overall, countless numbers of different molecules and nanoparticles have been bonded together utilizing amine groups with different chemistries and crosslinkers including PSi and photosensitizers [60] or fluorescent molecules [36].
1.3 Toxicity and degradation of PSi

The potential toxicity of any treatment modality is a very important aspect to consider before moving a product to clinical use. As mentioned in chapter 1.2.2, MPS is a defense mechanism which clears foreign objects from the body and degrades them. Furthermore, if MPS is bypassed and the material becomes accumulated in other organs, for example in the lungs, the decomposition of the material will become crucial with regards to the avoidance of toxic and chronic effects. This will require that the particles need to be biodegradable within a reasonable time scale. Fortunately, porous silicon is a biocompatible [16] and biodegradable [26] material, which is an essential prerequisite for therapeutic in vivo applications.

Porous silicon decomposes in aqueous solutions into monomeric silicic acid via reactions (1) and (2) [61]. The human body can eliminate this degradation product by excretion through the kidneys into urine [62], avoiding all toxic and harmful effects. Furthermore, silicon is needed in a vast array of biological functions although perhaps its main site is in the bone where it is needed to maintain optimal bone growth and elasticity throughout the human lifespan [63, 64]. Currently, the exact need of silicon is not well defined but the average daily intake in the Western world ranges from 20 to 50 mg/day [65], with beer being one of the major sources of silicon, since there is approximately 30 mg of Si in a liter of beer [66].
\[ Si + O_2 \rightarrow SiO_2 \] \hspace{1cm} (1)

\[ SiO_2 + H_2O \rightarrow Si(OH)_4 \] \hspace{1cm} (2)

The PSi degradation rate is affected by e.g. the different surface modifications, particle size and morphology, porosity and pore size [26, 37, 67-70]. Generally, all the surface modifications passivate the surface and prolong their lifetime compared to plain PSi. Furthermore, the PSi dissolution rate increases with increasing pore size but other factors such as a medium pH also influence this process [68, 69]. Thus, the degradation of the porous silicon can be tuned to range from hours to months [67] and an optimal lifetime for different applications can be achieved by proper design of the carrier system.

### 1.4 Cancer

In everyday use, the terms tumor and cancer are often mixed or their true meaning is not understood. In 2000, six hallmarks of cancer were introduced to define better the malignancy process: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis [71]. However, this classification has been criticized since actually five out of these six hallmarks are also present in benign tumors [72]. The only unique factor of these hallmarks that applies exclusively to malignant cancers, is their capacity to invade tissues and form metastatic cancers far away from their original location. This feature truly defines malignant cancers; they are extremely serious as they start to interfere with organs that are vital for sustaining human life.

#### 1.4.1 Cancer microenvironment

Although representing a serious threat to human life, there are positive aspects in cancer characteristics regarding their treatment with nanotechnology. Cancers grow at a much more rapid rate than normal tissues and this demands that they have access to a good supply of nutrients and oxygen. Thus, the formation of new blood vessels is needed to provide sufficient nourishment for the exponentially growing cancer. Cancers are capable of angiogenesis (formation of blood vessels), this process is regulated by several signaling agents [73]. Due to the unbalanced amount of these agents, the vasculature growth is often abnormal, resulting in gaps between the endothelial cells and the leaky vasculature [74]. Whereas normal blood vessels are tightly bound with endothelial cells next to each other, the veins reaching into the cancer can have holes as large as 3 μm in size [75] allowing objects from the bloodstream to flow freely into the tissue. Nanotechnology can exploit this phenomenon to passively target nanoparticles into cancer tissues as particles smaller than the fenestrations in the endothelial cells possess the capability of passing with the blood into cancer tissues and accumulating there (Figure 6).
Cancers are also capable of lymphangiogenesis (the formation of lymphatic channels) [73] but similarly to the vascular blood vessels, their formation has been found to be imperfect, leading to the impaired removal of medium from the cancer tissue and elevated pressure [73, 76]. In summary, the phenomenon of a leaky vasculature and impaired removal by the lymphatic system is referred to as an enhanced permeation and retention (EPR) effect. Currently, the majority of cancer nanotechnological products rely on passive targeting by EPR although this phenomenon would require more extensive research.

Cancer cells exhibit unique surface moieties that have been studied for active targeting. Indeed, cancer cells have been shown to overexpress a variety of receptors useful for targeting [77, 78]. Molecules that interact with the cancer surface receptors; for example, antibodies and peptides [79] can enhance the uptake inside the cancer cell [80]. However, in order to take advantage of receptor-mediated delivery, the nanoparticles are required to enter into the cancer tissue first to the vicinity of cancer cells via passive targeting so that they can interact with cell surfaces. Furthermore, the addition of targeting ligands may promote opsonization and removal by the immune system.

Nanoparticles can also take advantage of other “active targeting” mechanisms such as magnetic targeting [81]. In magnetic targeting, the particles are driven to the target area via a directed magnetic field. This method does not directly provide a mechanism for the particles to gain access to the cancer cells but will increase this probability, as the particle concentration in the cancer tissue is increased. Moreover,
magnetic particles can be utilized for other purposes such as their tracking with magnetic resonance imaging [42, 82].

The extracellular environment in cancer is different from that of healthy cells. Since they have elevated rates of glycolysis and membrane proton pump activity [83], many cancerous tissues have acidic surroundings with pH ranging from 6.2 to 6.9 [84]. This confers on nanotechnology the beneficial possibility to control the drug release with the use of pH responsive particles; the drug release can be triggered by lowering the pH, minimizing drug release into the bloodstream and healthy tissues (~ pH 7.4) and maximizing drug delivery to the cancerous tissue and cells. This is a highly desirable feature in decreasing the chemotherapeutic side effects, which are attributable to poor drug distribution.
### 1.5 Mesenchymal stem cells

Mesenchymal stem cells (MSC) can be isolated from a variety of sources including bone marrow and placenta. MSCs have the potential to differentiate into a variety of cell lines such as myocytes and osteoblasts. Thus, they have been studied for regenerative purposes [85], and it has also been noted that their differentiation can be triggered by external stimulation [86].

These features alone make MSC very interesting agents for biomedical applications. However, with regard to nanotechnology based therapy, their most intriguing feature (arguably) is their ability to migrate towards inflammation areas such as cancer [87]. The overall mechanisms are still unclear but several factors have been proposed to be at least partially responsible for this phenomenon [88]. Interestingly, whereas nanoparticles seem to be eliminated from cancer tissue after a short period of time [89], the MSCs have shown excellent retention in the cancer tissue, still being detected still 60 days after their i.v. administration [90]. Thus, they represent a very promising tool to target the nanotherapeutics inside the cancer, diminish their removal and potentially increase the therapeutic effect (Figure 7).

![Figure 7. Schematic figure representing the possible therapeutic MSC use. a) Drug loaded PSi, b) MSC, c) Therapeutic MSC, d) MSC migration and retention in cancer and e) reduced cancer volume.](image)

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2 Aims of the study

If one wishes to develop effective cancer nanotheranostics, then it will be essential to overcome multiple obstacles within the human body such as the immune system and the complex tumor environment. In this thesis, a few of these issues were tackled in order to develop a nanovector capable of being used for efficient cancer therapy and imaging. The aims of the present thesis can be divided into the following sections:

1. To improve the biocompatibility of nanocarriers with hydrophilic polyethylene glycol shielding.
2. To develop a nanocarrier with a long-term blood circulation time and MRI-imageability.
3. To combine multiple functionalities in the nanovectors to diminish adverse drug side effects, and to induce efficient cancer therapy.
4. To utilize stem cells to carry and target nanovectors to induce cancer cell death with photodynamic therapy.

Each of the aims have their role in the logical development of the material towards a final, multifunctional drug nanovector capable of overcoming all of the obstacles within the human body. Improvements in the properties of PSi can help to avoid aggregation and uptake by the immune system; this is of the utmost importance when striving to achieve a sufficient blood circulation time to enable passive cancer accumulation. The introduction of diagnostic features will allow real-time monitoring of the biodistribution to evaluate the success of the therapy. Furthermore, the use of targeting mechanisms will improve the accumulation of the nanotherapeutic agent into the cancer. Finally, the use of controlled drug release or a triggered cell killing mechanism minimizes the side effects and increases the therapeutic response.
3 Materials and methods

3.1 Production of porous silicon

Single crystal p⁺-type wafers (100) with resistivity of 0.01-0.02 Ωcm were anodized in a 1:1 electrolyte of HF (38%) and ethanol (99.5%) to form PSi films. A pulsed etching sequence was utilized with current densities of 50 mA/cm² (2200 ms) and 200 mA/cm² (350 ms) to form a porous layer and a fractionating layer to ease the particle formation, respectively. A total etching time of 20 min was used, followed by a lift-off pulse in the electropolishing region to detach the formed PSi film. The PSi film washed subtly with ethanol and dried at 65 °C before fractionating into particles with high-energetic ball mill. The desired fraction of nanoparticles was separated with sieving and centrifugation. The described protocol was utilized in studies I-III.

In study IV, p-type (100) silicon wafer (0.005 Ωcm) was anodized with a current density of 10 mA/cm² in 1:3 HF/ethanol solution. Underneath the porous layer, a high porosity release layer was formed with 76 mA/cm² pulse. A 80 nm LTO layer was added in a low pressure chemical vapor deposition oven and photolithographic method was used to make 1000 nm diameter patterns with aligner and photoresist. Reactive ion etching was utilized to form the particles before stripping of the LTO layer and releasing the particles with sonication to achieve uniform discoidal multistage nanovectors (MSV) with dimensions of 1000x400 nm [25].

3.2 Surface functionalization

In studies I-III, the porous silicon nanoparticles were thermally oxidized in 300 °C for 2 h followed by chemical wet oxidations in H₂O₂:NH₄OH:H₂O (1:1:5) and H₂O₂:HCl:H₂O (1:1:5) at 85 °C for 5 and 15 min, respectively. The particles after these oxidation steps are referred to as TOPSi-OH. In study IV, the oxidation of multistage nanovectors was conducted in H₂O₂ at 100 °C for 2h.

In study I, the TOPSi-OH particles were PEGylated simultaneously with 0.5 kDa and 2 kDa mPEG-silane in toluene at 120 °C overnight under reflux. Throughout the thesis, this method is referred to as Dual PEGylation (DPEG) and the particles are called DPEG-TOPSi.

In study II, superparamagnetic iron oxide nanoparticles (SPIONs) were precipitated into the pores of TOPSi-OH in n-hexane with FeCl₃ · 6 H₂O (2 M), FeCl₂ · 4 H₂O (1 M) and NH₄OH (24.5%). The particles were denoted as magnetic porous silicon (MaPSi). Similar DPEGylation as conducted in study I was performed after SPION incorporation and the particles were named DPEG-MaPSi.

In study III, the particles were coated with SPIONs by the thermal decomposition method. TOPSi-OH and iron (III) acetylacetonate (Fe(acac)₃) were dispersed in triethylene glycol and heated at 300 °C for 45 min (Fe-TOPSi). Fe-TOPSi were loaded
with doxorubicin (DOX) and encapsulated with pH sensitive calcium carbonate (CaCO$_3$) layer by microemulsion method (DOX-CaFe-TOPSi). Briefly, DOX-Fe-TOPSi was dispersed in cyclohexane and Igepal Co-520 solution with CaCl$_2$ and Na$_2$CO$_3$. After an overnight incubation at RT, the DOX-CaFe-TOPSi particles were reacted with APTES and TMOS for 5 h prior to DPEGylation at 70 °C in toluene (DPEG-DOX-CaFe-TOPSi). Nanoparticles for fluorescence imaging were prepared without DOX with minor modifications. In addition to the omission of DOX, additional PEGylation with amine-PEG-silane in IPA at 70 °C was performed before normal DPEGylation. Fluorescent labelling was conducted by incubating the nanoparticles with Cy7.5 NHS ester overnight at RT. These particles were denoted as Cy7.5-TPEG-CaFe-TOPSi.

In study IV, the particles were modified with APTES (8 % v/v) in 5 % H$_2$O/IPA solution for 2 h at 35 °C to make NH$_2$ modified multistage nanovectors (NH$_2$-MSV). Chlorin e6 (Ce6) (1 mg/ml) was activated with EDC (0.1 M) and NHS (0.3 M) in DMSO at RT for 4 h. NH$_2$-MSV were reacted with an activated Ce6 solution in MES buffer (pH 5) at RT overnight and the final product was denoted as Ce6@MSV. These particles were internalized into mesenchymal stem cells by incubating overnight Ce6@MSV with the MSC (200:1 Ce6@MSV to MSC ratio).

### 3.3 Characterization methods

#### 3.3.1 Physico-chemical modifications

Nanoparticle size and the effect of PEGylation on colloidal stability were quantified with dynamic light scattering (DLS) in various mediums (PBS and plasma) by detecting backscattered light at 173° angle (I-III). The nanoparticle ζ-potential was measured with electrophoretic light scattering in water (ELS) (I, II, IV). Porous properties were analyzed with N$_2$-gas sorption (I-III) at -196 °C. Scanning electron microscopy (SEM) with 20 kV was utilized to measure particle morphology and pore sizes (IV). SEM with 1 and 5 kV was used to evaluate the PEG layer thickness (I). Nanoparticle morphology was evaluated with transmission electron microscopy (TEM) at 200 kV (II, III). The organic content of the nanoparticles was assessed by thermogravimetric analysis (TGA) (I-III). In TGA, the sample was heated up to 800 °C (20 °C/min) in an N$_2$ atmosphere and the weight change was used to analyze the amount of organic material.

Fourier-transform infrared spectroscopy (FTIR) curves with transmission or attenuated total reflectance was measured to evaluate variations in the surface chemistry to verify the successful conjugation of molecules (I-IV). Ultraviolet visible spectroscopy (UV-Vis) was used to evaluate the amount of loaded drug after its release in buffer (III) and the successful addition of a Ce6 photosensitizer from a solution of Ce6@MSV dispersed in ethanol (IV). Atomic absorption spectroscopy was used to measure the iron (II, III) and calcium carbonate contents (III). MRI was utilized to assess the T$_2$-relaxivity from nanoparticles dispersed in water (II, III). The degradation rate of PSi in PBS was measured with inductively coupled plasma mass
spectrometry (ICP-MS) (I). Elemental analysis (C, N and H) was used to calculate the amount of different PEG molecules (I). The crystalline size of iron and calcium carbonate were calculated from X-ray powder diffraction data with the Scherrer equation after full profile fitting (III). The particle magnetization was studied with a vibrating sample magnetometer (VSM) (III). Confocal microscopy was used to evaluate the quantum dot and doxorubicin loading capacity of MSV by measuring the fluorescent intensity (IV).

3.3.2 In vitro experiments

Particle cytotoxicity was tested with several cell lines utilizing the CellTiter-Glo-(I-III), FDA- (I), MTT- (IV) and alamarBlue (IV) assays. The used cell lines included HepG2 (I, II), Ea.Hy926 (I), RAW 264.7 (I, II), CT26 (III), 4T1 (III), A549 (III) and MSC (IV). In vitro opsonization experiments were performed by incubating particles with 50% plasma to form protein corona on the particles. The protein corona extracted with dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). First the proteins were denaturated in PAGE buffer and ran on a SDS-PAGE-gel before staining with Coomassie brilliant blue. Excised protein bands were excised in trypsin and analyzed with liquid chromatography-mass spectrometry (I, II). Nanoparticle uptake in macrophages was studied with TEM imaging after 6 h incubation with RAW 264.7 cells (I). Confocal microscopy was utilized to evaluate internalization of nanovectors in RAW 264.7 (III), CT26 (III) and MSC (IV) cells after 4 or 24 h incubation with the corresponding cells. RAW 264.7 and CT26 cell membranes were labeled with Deep Red CellMask and nuclei with Hoechst staining (III). The MSC cells were labelled with DiO-dye (IV). The reactive oxygen species (ROS) production was evaluated with confocal microscopy after laser stimulation (100 mW, 405 nm) with H2DCFDA staining (IV). Photodynamic therapy in vitro was tested with MSC internalized with Ce6@MSV that were stimulated with a laser (100 mW, 405 nm), subsequently labelled with the LIVE/DEAD assay and imaged with confocal microscopy (IV).

3.3.3 In vivo animal experiments

The blood circulation time and biodistribution of magnetic nanoparticles were evaluated after i.v. injection in healthy Wistar rats (II). Blood was collected at predefined time points and the relaxivity was measured to estimate the iron and nanoparticle content. After 3 h, the mice were sacrificed and liver, spleen and kidneys were collected for analysis. Liver slices were prepared and the location of the nanoparticles was imaged with TEM. The organs were digested and measured with ICP-MS to evaluate silicon content in each organ. Furthermore, the nanoparticle accumulation in liver was assessed with live MRI imaging before and at 40 min after injection.

Biodistribution and tumor homing of Ce6@MSV internalized MSC were studied with BALB/c mice bearing subcutaneous 4T1 luciferase expressing tumors (IV). MSC were labelled with DiR-dye and administered intravenously. The MSC location was
measured daily with *In vivo* imaging system (IVIS) up to the 3rd day when the mice were sacrificed and the organs were collected for *ex vivo* imaging to evaluate the biodistribution and tumor migration.

The biodistribution of Cy7.5 labelled nanoparticles was studied after their i.v. administration to healthy NSG mice (**III**). Mice were sacrificed 4 h after injection, organs were collected and imaged with IVIS. The nanovector safety was evaluated with orthotopic A549 lung cancer bearing NSG mice. Mice were injected via the tail vein once a week with either PBS (control), DOX (5 mg/kg), DPEG-CaFe-TOPSi (50 mg/kg) or DPEG-DOX-CaFe-TOPSi (50 mg/kg, 5 mg/kg of DOX). The first injection was given at 7 days after tumor inoculation and the experiment lasted for a total of 45 days. The well-being of the mice was evaluated by monitoring the appearance, behavior and weight of the mice. Furthermore, the organs and blood from sacrificed mice were collected for histology and blood biochemical analysis.

The *in vivo* therapeutic effect was studied after intratumoral injection of DPEG-DOX-CaFe-TOPSi (**III**) or MSC internalized with Ce6@MSV (**IV**) nanovectors in subcutaneous tumor model bearing mice. DPEG-DOX-CaFe-TOPSi (50 mg/kg, 5 mg/kg of DOX) were delivered via a single injection and tumor growth was monitored for 10 days after which the mice were sacrificed and tumors were collected to be subjected to weight and histopathological analyses (**III**). MSC+Ce6@MSV were injected at three different locations in the tumor and excited with a laser (100 mW, 405 nm) (**IV**). The mice were injected with luciferin, sacrificed and imaged with IVIS 30 min after the PDT to detect tumor viability around the treatment areas.
4 Results and discussion

4.1 Improving colloidal stability and biocompatibility

In study I, PSi was coated with PEG molecules to evaluate the effects on the colloidal stability, protein adsorption and macrophage uptake. According to the colloidal stability, the DPEGylation was discovered to be superior to the mono-PEGylation, and thus DPEGylation was chosen for further testing. The DPEGylated particles maintained their original size (~200 nm) throughout the experiment (9 days) whereas uncoated or mono-PEGylated samples were seen to aggregate (Figure 8a). This was concluded to be a result of their high-density coating with the mushroom and brush conformation (Figure 8b) which increased the $\zeta$-potential of the nanoparticles from approx. -50 mV to approx. -10 mV. This assumption was supported by the decrease in size of DPEG-TOPSi within the first time points (Figure 8c), presumably from longer PEG folding into the mushroom conformation. Furthermore, the thickness of the DPEG layer (~10 nm) visualized with SEM imaging corroborated this hypothesis. TGA revealed that a substantial amount of PEG (>20%) had been grafted onto the particle surface (Figure 8d) which was also seen in the FTIR spectrum as additional vibration bands. The gas sorption revealed a decrease in the particle surface area, pore size and volume after addition of shielding DPEG layer possibly due to partial blocking of the pores.

Figure 8. a) The colloidal stability of differently PEGylated samples in PBS. b) Schematic figure of Dual PEGylated PSi with brush and mushroom conformation. c) Colloidal stability of Dual PEG-TOPSi and d) Thermogravimetric analysis to evaluate the PEG content. The figure is adapted from figures 1-3 and 5 from study I, and utilized with the permission of the publisher.
The DPEGylation affected also the degradation rate of the particles. The DPEGylated particles had more than a doubled half-life in PBS in comparison with their non-coated counterparts (62 h versus 19 h, respectively). The DPEG-TOPSi nanoparticles showed good biocompatibility in the tested cell lines and did not induce any toxic effects. Moreover, *in vitro* opsonisation was studied in 50% plasma to evaluate the real shielding effect of the DPEG. Compared to non-coated TOPSi particles, the DPEG significantly decreased the amount of adsorbed proteins, which could be detected from the strength of visible bands and from the absence of others that were clearly evident in the non-PEGylated sample (Figure 9). Finally, the uptake by macrophages was evaluated to simulate the uptake by the phagocytic cells which are present in the blood. TOPSi particles were taken up by the RAW 264.7 cells and aggregated into large lumps, whereas the DPEG-TOPSi particles avoided the uptake. These findings highlight the probability also to have a stealth effect from the immune system *in vivo*.

![Figure 9. Plasma protein adsorption of TOPSi-OH and Dual PEG-TOPSi particles. The right-hand column “plasma” indicates the proteins present in the plasma. The figure is reprinted from study I with permission of the publisher.](image-url)
4.2 Long term circulating diagnostic nanoparticles

In study II, the circulation life of DPEG-MaPSi nanoparticles was evaluated with MRI. Superparamagnetic iron oxide (14.5 wt%) was precipitated in the pores of PSi to introduce magnetic properties for imaging purposes and the particles were shielded with DPEGylation. Iron loading did not affect the particle morphology as evaluated with TEM but slightly increased the particle toxicity as revealed in the cell viability studies. The iron loaded nanoparticles displayed good relaxivity in vitro, reflecting their MRI suitability. The circulation time, defined from blood with MRI experiments, was found to be significantly increased by DPEGylation (Figure 10a). In fact, the DPEG particles (half-life = 241 min) were detected in the bloodstream still at the last time point (3 h after i.v. injection) whereas the non-coated MaPSi particles (half-life = 1 min) were removed within the first 10 min.

![Figure 10](image)

**Figure 10.** Long circulating magnetic nanoparticles. a) Blood relaxivity values obtained with MRI and b) Biodistribution of silicon in MPS organs after i.v. injection. The figure is adapted from figures 3 & 4 from the study II, and reused with permission from the publisher.

After 3 h, the rats were sacrificed and major MPS organs (liver, spleen and kidney) were collected for analysis. The particle biodistribution within these organs was evaluated with ICP-MS. Interestingly, the bio-fate of the particles had been extensively altered. The non-coated particles were taken up by the liver, whereas DPEGylated nanoparticles were detected mainly in the spleen (Figure 10b). Altered biofate was hypothesized to be caused by the difference in the adsorbed proteins which was examined in an in vitro protein adsorption study. Indeed, the DPEGylation had altered the amount and the nature of adsorbed proteins significantly, which may have been responsible for the altered in vivo behavior (Figure 11).

Finally, the in vivo diagnostic features were evaluated by static imaging of a rat liver before and 40 min after the particle injection. The liver was selected as the monitored organ as this is the most probable accumulation site for the nanoparticles. Alongside with the previous data, the non-coated particles were observed to
accumulate in the liver, which was detected as contrast changes in MRI. It further confirmed that these particles were taken up by the MPS rapidly and accumulated in the liver. On other hand, no visible effect was observed with DPEGylated particles (Figure 12) highlighting the stealth effect and the altered location of particle accumulation. Therefore, the results confirmed the DPEG-MaPSi particles are effective in escaping from the initial uptake by the MPS, which will increase their blood circulation time and enable accumulation to other locations.

Figure 11. Bioinformatic analysis of nanoparticle corona proteins revealing the functions of the adsorbed proteins. The blue circles represent proteins enriched in DPEGylated nanoparticles and red circles indicate proteins enriched in the non-coated nanoparticles. The gray circles represent the proteins that were not specifically classified on either of the samples. The circles at the top of the lines belong to several categories and are closer to the function to which they are more related. The figure is reprinted from study II with permission of the publisher.
4.3 Multifunctional nanocarriers for safe cancer therapy

In study III, the nanocarrier was prepared with multiple functionalities. Iron oxide was included to achieve magnetic properties and the Fe-TOPSi particles exhibited a high saturation magnetization value of 100.7 emu/g Fe (Figure 13a). T2-relaxivity values of approx. 520 ml/(mg·s) before and approx. 190 ml/(mg·s) after DPEGylation were obtained. These values reveal the suitability for MRI imaging and especially the saturation magnetization is one of the highest ever reported for magnetic PSi [91, 92]. The addition of CaCO₃ coating blocked the pores effectively as seen from the N₂-gas sorption measurement (Figure 13b) as the pore volume decreased by 86% in comparison with the plain particles. The nanocarriers exhibited clearly pH dependent DOX release (Figure 13c) as an evidence of the inhibitory effect of the calcium carbonate coating at neutral pH. Moreover, the DOX loaded nanocarriers exhibited a concentration dependent toxicity to cells, exerting a similar effect as the free drug (Figure 13d).
Figure 13. a) Saturation magnetization of Fe-TOPSi was evaluated with VSM, and value of 100.7 emu/g was obtained. b) Nitrogen sorption isotherms reveal the pore blocking induced of CaCO$_3$ layer. c) pH dependent drug release from DPEG-DOX-CaFe-TOPSi and d) in vitro therapeutic response of CT26 cancer cells. The figure is adapted from figures 1 & 2 from study III, and reused with permission from the publisher.

The in vivo biodistribution was evaluated by monitoring the fluorescence emerging from conjugated Cy7.5 dye with IVIS (Figure 14a). The uncoated TOPSi particles accumulated highly in lungs, liver and kidneys due to aggregation, MPS uptake and degradation, which led to the renal excretion of the small dye molecule, respectively. However, the multifunctional TPEG-CaFe-TOPSi avoided entrapment by the lung, evaded from the uptake by the liver and accumulated mainly in spleen. Studies of long circulating nanoparticles and a high spleen uptake have been reported [93, 94] which indicates that there might be a correlation between these two factors.

The safety of the nanocarrier was evaluated after i.v. injection and compared with the free drug molecules. In this experiment, free DOX dose proved to be highly toxic, evoking a significant weight loss (Figure 14b). Due to the weight loss, the mice in DOX group had to be sacrificed before the intended experimental end-point. On other hand, the DPEG-CaFe-TOPSi nanocarrier or the drug loaded DPEG-DOX-CaFe-TOPSi did not cause any weight loss when compared with the control group. At the end of the experiment, blood was sampled and liver biomarkers were analyzed showing significantly increased ALT and AST levels in the DOX treated mice (Figure 14c). Furthermore, the histopathological analysis revealed toxic lesions in the liver and spleen in the DOX group whereas organs in the other groups did not
display any differences from the control group. These results highlight the high toxicity of DOX and that the multifunctional nanocarrier was capable of sealing the drug safely inside the pore matrix and thus reducing its side effects.

The therapeutic response in vivo was evaluated after intratumoral injection to subcutaneous CT26 bearing mice. Both free DOX and DPEG-DOX-CaFe induced effective inhibition of the tumor growth (Figure 14d) compared to the control group. After excising and weighing the tumors, a clear size reduction could be observed (Figure 14f). The free drug (81%) and drug loaded nanocarrier (77%) exerted a comparable size reduction of the tumor, highlighting good therapeutic efficacy but a further histopathological analysis revealed that DOX had also induced toxic lesions in liver and spleen. This indicates that the free DOX had diffused out of the tumor and affected vital organs whereas the nanoparticles were able to retain the payload better inside the tumor [95, 96] and minimize the adverse side effects of the chemotherapeutic drug.
Figure 14. a) Biodistribution of bare TOPSi and shielded TPEG-CaFe-TOPSi nanoparticles. b) Mice average weight after systemic i.v. injection of PBS, DOX, DPEG-CaFe-TOPSi and DPEG-DOX-CaFe-TOPSi. c) Liver biomarkers evaluated from the blood collected after sacrifice of the mice. d) Relative tumor volume after intratumoral injection PBS, DOX, DPEG-CaFe-TOPSi and DPEG-DOX-CaFe-TOPSi. e) The size of excised tumors in the therapy experiment. The image is adapted from figures 3 & 4 from study III, and reused with permission from the publisher.
4.4 Stem cell targeted PSi for photodynamic therapy

In study IV, the interesting properties of mesenchymal stem cells were utilized for targeting of the multistage nanovectors. Moreover, the therapeutic effect was performed with photodynamic therapy that can be precisely triggered at the target and subsequently avoid side effects. With respect to PDT, the photosensitizer Chlorin e6 (Ce6) was conjugated to the surface of MSV (Ce6@MSV) resulting in ~ 7 wt% conjugation, and thus affecting the product’s absorption and fluorescence properties (Figure 15a). The conjugation of Ce6 did not block the pore matrix, which was confirmed by loading of therapeutic (DOX) and diagnostic (QD) payloads (Figure 15b).

Figure 15. a) Characteristic absorption and fluorescence of MSV, Ce6@MSV and Ce6. b) Confocal imaging of MSV and Ce6@MSV loaded with DOX micelles and quantum dots. The figure is adapted from figure 2 from study IV, and reused with permission of the publisher.
The Ce6@MSV particles without DOX or QDs were successfully internalized in MSC without causing a cytotoxic effect in the absence of external stimulation. (Figure 16a&b). However, the particle stimulation (405 nm laser) triggered ROS production and subsequently cell death was observed. Interestingly, these events were strictly limited within the stimulation area (Figure 16c&d). Furthermore, the stimulation was seen to cause ~ 60% cell death within the stimulation area with Ce6@MSV whereas no significant effect was observed in MSV or control groups.

Figure 16. a) Ce6@MSV internalized to MSC and imaged with confocal imaging. Red indicates Ce6@MSV and green indicates the cells b) MSV proliferation after internalizing 0, 50, 100 or 200 MSV per each cell. c) ROS production after stimulation (white circle indicates the stimulation area) detected with a green H$_2$DCFDA-dye. d) LIVE/DEAD analysis after PDT. Green indicates live and red refers to dead cells. The figure is adapted from figure 3 from study IV and reused with permission of the publisher.

The tumor targeting capacity was evaluated with a 3-day experimental setup where the stem cell (w/ and w/o Ce6@MSV) location was monitored by fluorescence imaging. Both the plain MSC and Ce6@MSV internalized MSC were first observed in the lungs but they started to gradually migrate towards the cancer site after 1 d. During the 3-day span, the signal increased by 20% in the tumor while a similar reduction was seen at lungs (Figure 17a) as an evidence of migration of the MSC.
After 3 days, the mice were sacrificed and organs were collected for analysis, which confirmed that a significant amount of MSC had migrated to cancer (Figure 17a).

Finally, the therapeutic effect in vivo was evaluated after intratumoral injections of stem cells (w/ or w/o Ce6@MSV). Intratumoral injections were selected to mimic the situation where a sufficient number of MSC had migrated to the cancer and to further demonstrate pin-pointing of the therapeutic effect in an accurate manner. The injection sites were localized with fluorescence imaging and stimulated with a 405 nm laser. Breast cancer with luciferase was utilized to make it possible to study cell viability with bioluminescent imaging after luciferin administration. Based on the bioluminescent signals (Figure 17b), the bare MSC did not cause any cell death, underlining the safety of the laser treatment on its own (i.e. no photothermic effect). Interestingly, the bioluminescence signal arising from Ce6@MSV treated cancers was clearly decreased in the vicinity of the injection areas. Moreover, further validation revealed a significant decrease in the signal (approx. 70%) as a result from cancer cell death. Therefore, the photodynamic efficacy of Ce6@MSV was clearly demonstrated which could be combined with the characteristic migration of MSC.

Figure 17. a) Empty and Ce6@MSV internalized MSC amount at lungs and cancer regions during 3-day experiment and biodistribution after 3-day migration. b) Bioluminescent imaging of the cancer cell viability after PDT. White circles represent the location of empty MSC or Ce6@MSV internalized MSC. Statistical analysis of cancer cell viability after PDT, demonstrating the effective therapeutic effect with Ce6@MSV loaded stem cells. The figure is adopted from figure 4 from study IV, and reused with permission of the publisher.
5 Summary and conclusions

Since it was decided to study the potential of PSi-based nanocarriers for cancer therapy, the first aim of this thesis was to improve the biocompatibility of the nanocarrier by incorporating PEG molecules. This was implemented with the novel Dual PEGylation, which remarkably increased the colloidal stability and decreased the \textit{in vitro} toxicity of the nanoparticles. The high-density PEG coating reduced the degradation rate in PBS, diminished the adsorption of proteins and minimized the uptake by phagocytic cells. Therefore, Dual PEGylation conferred suitable properties for the nanocarrier to be then further evaluated for therapeutic applications.

The second aim was to achieve a long circulating nanocarrier with MRI suitability. SPIONs were successfully incorporated into the porous matrix, allowing MRI monitoring, and Dual PEGylation was utilized to prolong the \textit{in vivo} blood circulation time of the nanoparticles. DPEGylated nanoparticles were observed to possess a superior blood circulation time as compared to the plain particles. Furthermore, the protein corona adsorbed onto the particles was considerably different after PEGylation, which altered the particles' biodistribution. MRI was utilized to verify the liver accumulation of bare nanoparticles, which was visualized as a clear change in the contrast. A similar effect was not seen with DPEGylated nanoparticles, highlighting the avoidance of the first-pass uptake by the liver, reflecting the altered biodistribution. The results demonstrate explicitly that the experiment's aim had been achieved.

The third aim was to develop a nanovector with multiple functionalities capable of efficient therapy without inducing adverse side effects. This aim was fulfilled by designing a nanovector with magnetic properties for MRI, pH responsive drug release to reduce premature leakage and Dual PEG layer to increase the blood circulation time and EPR properties. The developed pH sensitive capping was effective in reducing unwanted drug release at neutral pH and it readily dissolved in an acidic environment, releasing the encapsulated drugs. These multifunctional nanoparticles were tested \textit{in vivo} and showed excellent biocompatibility whereas the free drug caused excessive toxicity, leading to the premature death of the animals. Furthermore, the nanoparticles had a comparable therapeutic effect to the free drug, indicating their suitability for clinical applications.

Finally, the mesenchymal stem cells were found to be capable of carrying PSi particles and migrating towards the cancerous tissue, making it possible to accurately target the porous silicon particles. The photosensitizer loaded particles were biocompatible without laser stimulation and after the appropriate stimulus, they triggered the release of reactive oxygen species, leading to cell death. Stimulated cell death (\textit{in vitro} and \textit{in vivo}) was found to be very well defined in the stimulation area, underlining the pin-point accuracy of the treatment as well as minimizing possible side effects. These findings emphasize that this study aim had been successfully achieved.
6 Future perspectives

The development of new tools for cancer theranostics is on the verge of major breakthroughs. Scientists all around the world are attempting to resolve the related challenges in the best possible way with all sorts of innovations. This will eventually lead to a new era in cancer therapy and it seems likely that porous nanoparticles will be part of this due to their desirable features.

After the theranostic nanoparticles are intravenously administered into the bloodstream, the initial contact will be made with the blood proteins and the MPS with phagocytic cells trying to remove these foreign objects from the body. In the present thesis, this hurdle was successfully overcome by devising a novel coating strategy that significantly altered the material behaviour both \textit{in vitro} and \textit{in vivo}, resulting in increased blood circulation times. Thus, the first problem has been resolved which makes it possible for the particles to accumulate to cancers via EPR. However, the uptake by MPS cannot be totally avoided as seen in the present thesis. Therefore, either triggered drug release or alternative focused therapy is essential for localizing the treatment into the target area. Thus, even with irregular accumulation, the side effects can be minimized if drug leakage is minimal at off-target locations or if the compound is only toxic after it has been activated with an appropriate stimulus. Furthermore, the ability to monitor the particle location is not only crucial in applying the stimulation at the correct locations but also in evaluating the treatment efficacy to determine the optimal therapy for each patient, as each subject will react individually to different treatments.

This will eventually lead to personalized medicine where the treatment is tailored to the individual. During this thesis, we scratched this surface with MSC. Although, the MSC already are a biological reality and induce a minimal immunological response, the best situation eventually will be isolating the used cells from the patient him/herself to prepare the compound and minimize all complications. Similarly, particles can be coated with cell membranes \cite{97} to convert them into biomimetic materials.

During this thesis, new insights were obtained into cancer theranostics by attacking existing problems and developing answers to them. However, the PSi nanoparticles require more consistent work and innovative developments to be used for efficient cancer therapy in the future. This thesis provides the solid first steps in devising a PSi-based theranostic solution which in the future will be used in enhanced personalized cancer therapy - I have no doubt in that prediction. I can make this statement with confidence, due to the experience that I gained while completing this thesis project investigating the versatile properties and possibilities of PSi nanoparticles.
7 References


Nanomedicine has great potential to revolutionize cancer therapy by improving the therapeutic efficacy and reducing side effects. However, nanocarriers need to overcome multiple biological barriers in the human body before achieving effective therapy outcomes. The present thesis describes development of porous silicon nanoparticles for cancer therapy and imaging by investigating several relevant issues: nanoparticle stability and prolonged blood circulation time, imaging possibilities, controlled drug release, compound safety as well as the exploitation of active targeting.