CHEMICAL AND BIOCHEMICAL MODIFICATION OF MESOPOROUS SILICON FOR IN VIVO ANALYSIS

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ABSTRACT

In the past decades much research has been done on the use of nanocarriers as vehicles to deliver therapeutics including chemotherapeutic agents to specific parts of the body e.g. cancerous sites. Chemotherapy is the most used treatment together with surgery in cancer therapy. It involves the administration of toxic chemicals to the body in order to neutralize cancer cells over normal cells. For effective chemotherapy with minimal site effects for these chemicals the optimal treatment is the release of these compounds at cancerous sites. There are several types of nanocarriers like liposomes, polymeric nanoparticles, inorganic nanoparticles.

Inorganic mesoporous silicon nanoparticles (PSi-NP) have shown great potential as delivery vehicles. They can be modified to possess properties for cellular delivery, including wide availability, rich functionality, biocompatibility, potential capability of targeted delivery, high pay load, and biodegradability. However, when administered to the body PSi may interact with each other and become aggregated. Furthermore, PSi are rapidly removed from circulation by macrophages of the Mononuclear phagocytic system (MPS).

To avoid formation of aggregates, and fast removal of PSi-NP from blood circulation, layer by layer (lbl) method using biodegradable and nonimunogenic molecules like chitosan, alginate, and inert polyethylene glycol (PEG) were used to functionalize the surface of PSi-NP. PSi-NP surfaces were successfully functionalized using Lbl, and PEG. However lbl did not prevent opsonization of PSi-NP whereas PEG did.
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List of Abbreviations

Alg       Alginate
CC        Carboxdiimide Chemistry
Chi       Chitosan
DLS       Dynamic Light Scattering
DMF       N,N-Dimethylformamide
DNA       Deoxyribonucleic acid
EDC       1-Ethyl-3-[3-dimethylamino-propyl]carbodiimide
EDTA      Ethylenediaminetetraacetic acid
FT-IR     Fourier transform infrared spectroscopy
KDa       Kilo Dalton
LBL       Layer By Layer
M         Molar
MES       2-(N-morpholino)ethanesulfonic acid
mg        Milligram
ml        Milliliter
mV        Mili Volt
NaCl      Sodium Chloride
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>NH₂</td>
<td>Primary amine</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>nM</td>
<td>Nano molar</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PEM</td>
<td>Polyelectrolyte Multilayer</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen ion concentration</td>
</tr>
<tr>
<td>PSi</td>
<td>Mesoporous Silicon</td>
</tr>
<tr>
<td>PSi-NPs</td>
<td>Mesoporous Silicon nanoparticle</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TCPSi</td>
<td>Thermally carbonized Mesoporous Silicon</td>
</tr>
<tr>
<td>THCPSi</td>
<td>Thermally Hydrocarbonized Mesoporous Silicon</td>
</tr>
<tr>
<td>TOPSi</td>
<td>Thermaally Oxidized Mesoporous Silicon</td>
</tr>
<tr>
<td>UnTHCPSi</td>
<td>Undecyclic acid derivatized Thermally Hydrocarbonized Mesoporous Silicon</td>
</tr>
<tr>
<td>µg</td>
<td>Micro gram</td>
</tr>
<tr>
<td>µl</td>
<td>Micro liter</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta Potential</td>
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1. INTRODUCTION

Nanoparticles are routinely defined as particles with sizes between 10 and 1000 nm that show different properties compared to larger samples of the same material [Auffan et al. 2009].

In medical application, nanoparticles are used for imaging and as vehicles for drug delivery. The use of nanoparticles (NPs) as vehicles for targeted drug delivery is a burgeoning area of research that had garnered the attention of researchers in the past two decades. For drug delivery, therapeutic agents are either entrapped, adsorbed, or attached to the NPs [Muthu MS et al. 2009]. The small size of NPs enables them to penetrate barriers involved in systemic targeted drug delivery [Li W et al. 2007]. The overall size of an NP affects its degradation, tissue penetration and cellular uptake, and the degree to which it is toxic to cells (cytotoxicity) [Jiang W et al. 2008, and Rejman et al. 2004]. Also, the pharmacokinetic properties of NPs such as absorption, and distribution are influenced by the size, shape, surface charge and chemical composition of NPs [Alexis et al. 2008].

Ideal vehicles for targeted drug delivery are biocompatible, biodegradable, nonimmunogenic, stable, and specific for their target [Chenag et al. 2012].

Several types of nanoparticles (table 1) have been used as vehicles to deliver drugs to specific parts of the body [Faraji et al. 2009].
### Table 1. Types of nanoparticles used for drug delivery

<table>
<thead>
<tr>
<th>Number</th>
<th>Nanoparticle types</th>
<th>Brief description</th>
<th>Short coming or advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inorganic nanoparticles</td>
<td>Nanoparticles formed from various inorganic materials such as metals</td>
<td>Widely used</td>
</tr>
<tr>
<td>2</td>
<td>Liposomes</td>
<td>Formed from two spherical lipid layers</td>
<td>No good medical application due to instability in biological milieu</td>
</tr>
<tr>
<td>3</td>
<td>Nanocrystals</td>
<td>It is a crystalline form of the drug surrendered by a thin surfactant layer</td>
<td>High amounts of drugs can be administered with nanocrystals. However not all drugs can be crystalline. Also nanocrystals are not very stable</td>
</tr>
<tr>
<td>4</td>
<td>Nanotubes</td>
<td>Self assembled sheet(s) of atoms arranged in tubes e.g. Fullerene (C_{60})</td>
<td>Toxic - causes cell and tissue death.</td>
</tr>
<tr>
<td>5</td>
<td>Dendrimers</td>
<td>Polymer based nanoparticles formed from mono or oligomeric units</td>
<td>They are easily prepared. They are unstable in serum, and toxic</td>
</tr>
<tr>
<td>6</td>
<td>Polymeric nanoparticles</td>
<td>Formed from polymers such as alginate, polyethylene glycol</td>
<td>They are biodegradable, biocompatible. They are used to modify surface properties of other NP types. Large scale production is difficult- since it involve processes such as double emulsion solvent evaporation</td>
</tr>
<tr>
<td>7</td>
<td>Solid lipid nanoparticles</td>
<td>These are lipid-based colloidal carriers.</td>
<td>They are biodegradable, less toxic, and stable compared to polymeric nanoparticles</td>
</tr>
</tbody>
</table>
Inorganic nanoparticles which are formed from various inorganic materials such as metals, alloys, oxides chalcogenides and pnictides [C. N. R. Rao. 2012]. They are biocompatible, biodegradable, stable, and less toxic [Allen TM et al. 2004].

Mesoporous silicon nanoparticles (PSi-NPs) have been shown to be an effective vehicle for target delivery of drugs [Vallet-Regi M et al. 2007]. PSi-NPs contains mesopores in its microstructure which increase its effective surface area [Miia Kovalainen et al. 2011]. Figure 1 shows a cross section of nanostructured PSi-NPs.

![Cross section of PSi-NPs](image)

**Figure 1.** Cross section of PSi-NPs [Kilpeläinen et al. 2011]

PSi-NPs have high number of tunable pores with a total volume of 0.6–1 cm3/g, which allow the PSi-NPs to be loaded with disparate, large and controllable amounts of drugs. Mesopores are having diameters in the range of 2- 50nm [Joakim Riikonen. 2012]. In addition, PSi-NPs have large surface area relative to size (over 700 m2/g) due to large number of pores. Furthermore, the silanol groups on the surface of PSi-NPs allow further functionalization of the surface with groups like hydroxyl, amino, thiol, carboxyl [Walcarius A et al. 2006, Han L et al. 2009, Rosenholm JM et al. 2010]. One of the most important properties of PSi-NPs is the ability to undergo several surface modifications. The
surface can be stabilized via thermal oxidation (TOPSi), thermal hydrocarbonization (THCPSi), thermal carbonization (TCPSi), and silylation (UnTHCPSi) [Kovalainen et al. 2012]. Also, the surface of PSi-NPs can be modified by grafting with molecules such as polyethylene glycol [Sudeep. P et al. 2008], folate [Erogbogbo.F et al. 2008], chitosan, and alginate. Moreover, functional groups on the surface allow the conjugation of biotargeting molecules such as antibodies [Walcarius A et al. 2006, Han L et al. 2009]. This enables targeting to specific cells or tissues [Barandeh F et al. 2012]. The functional groups also interact with loaded cargo and release them either through a diffusion dependent or in stimulus responsive manner [Luo Z et al. 2011, Bermardos A et al. 2010] allowing the release of the cargo in a controlled pattern.

Drugs, enzymes, and DNA loaded into the pores of PSi-NPs are protected from unwanted metabolism in areas other than the target site since the pores are not exposed to the immediate biological surrounding containing metabolic enzymes and chemicals [Botella P et al. 2009]. Specific targeting, and protection from unwanted metabolism of loaded drugs, increase their efficacy, bioavailability and reduce side effects [Riehemann K et al. 2009, Moorthi C et al. 2011]. In addition, the bioavailability of poorly soluble drugs is improved [Kaukonen A et al. 2007, Limnell T et al. 2007, and Wang F et al. 2010].

In addition to being promised vehicles for target delivery of drugs, PSi-NPs are very good imaging probes for diagnostic purposes when labeled with tracers like fluorophores. They protect the fluorophores from photobleaching, and interacting with the immediate biological milieu [Ow H et al. 2005]. Also, oxidized PSi-NPs are used as noninvasive imaging probes since they inherently produce luminescence [Heinrich et al. 1992].
Until now many cancer therapeutics such as siRNAs [Hom C et al. 2010, Li X et al. 2011], proteins [Slowin II et al. 2007], and chemotherapeutics [Hom C et al. 2010 and Li X 2011], have been successfully delivered using PSi-NPs. For example paclitaxel [Win and Feng, 2006], and γ-secretase inhibitors (GSIs) [Mol Ther, 2011] have been delivered to cancer cells using Psi-NPs.

However, when administered into the human system, the silanol groups of unmodified PSi-NPs interact nonspecifically with cellular membranes causing toxicity to some extent [Chang JS et al. 2007]. They also interact with each other via hydrogen bonding and become aggregated [Neetu Singh et al. 2011]. The aggregated PSi may become entrapped in the lungs, or any part of the body due to capillary obstruction [Knop K et al. 2006]. Once administered, NPs in general and PSi-NPs in particular are rapidly removed from blood circulation by macrophages of the Mononuclear Phagocytic System (MPS) [Gref et al. 1994.]. Macrophages recognize NPs through plasma proteins call opsonins bound on to the surface of NPs [Frank and Fries. 1991]. Opsonins are antibodies (IgM, IgG), products of complement system (C3b, C4b). They mark foreign bodies in blood for subsequent destruction by monocytes and phagocytes. Opsonization is a phenomenon where plasma proteins bind foreign particles in the body [Van Vlerken Le et al. 2007].

To overcome the problems associated with the use of NPs as vehicles for targeted delivery of drugs, the surface of NPs is functionalized using biodegradable and nonimmunogenic polymers such as chitosan, alginate, polyethylene glycol, folate, polystyrene [R. Gref et al. 1994]. Surface functionalisation reduces aggregation, nonselective interactions with cells and tissues, and increases the bioavailability of NPs [Alexis et al. 2008]
1.1. POLYETHYLENE GLYCOL (PEG)

The use of PEG (PEgylation) had its first description in the 1970s by Davies and Abuchowsky [Abuchowski, A. et al. 1977]. It is a process in which proteins, peptides, or NPs are modified by linking them with one or more units of polyethylene glycol (PEG) chain(s) [Fracesco.M et al. 2005]. PEG is a polymer of ethylene oxide, which can exist as a linear or branched structure [Ben-Shabat et al. 2006].

PEG has two termini designated $R_1$ and $R_2$. $R_1$ interacts with the surface of NPs, while $R_2$ interacts with the solvent. For different PEG types, a certain number of ethylene glycol units connect the two termini as shown in figure 2 [Lee H et al. 2009].

![Figure 2](image.png)

**Figure 2.** Presents the structure of ethylene glycol, PEG, and PEGylated NP [Jesse V Jokerst et al. 2011]
1.2. Effects of PEG:

PEG molecules are nontoxic, non-immunogenic, biodegradable, neutral and very soluble in water and organic solvents [Francesco.M et al. 2005].

PEG is the most common polymer used for surface modification of classical drug delivery systems. PEG stealth the systems from uptake by the mononuclear phagocytic system there by increasing the bioavailability, residence time, and biodistribution of the drug delivery systems. MPS is the greatest hindrance to targeted drug delivery using NPs [Immordino ML et al. 2006].

For surface modification of NPs, PEG molecules are either covalently bound or adsorbed on to the surface of NPs [Alexis et al. 2008, Rytkönen et al. 2012]. On the surface of NPs, PEG shield the high surface charge of NPs and provides an interbilayer repulsive force that over comes the Van der Waals forces of attraction. Also, PEG being hydrophilic increases the suspension stability of the NPs in serum and buffers- avoiding the aggregation of the NPs [Needham D et al. 1992].

Surface modification with PEG stabilizes the particles sterically and hides them from opsonins- preventing aggregation and rapid clearance of NPs from blood circulation by mononuclear phagocytic system (MPS) [Avgoustakis K et al. 2004, Ostsuka et al. 2003] and increases their bioavailability and plasma residence time [Moghimi et al. 2001]. The extents to which PEG sterically repels opsonins and prevents aggregation of NPs vary with chain length, shape of PEG, and the density of PEG molecules on the surface of NPs [Alexis et al. 2008]. PEGylation increases the hydrodynamic size of NPs- this decreases renal clearance and increases the circulation time of NPs [Alexis et al. 2008]. Moreover,
pegylation makes NPs flexible, which facilitates their passage into tissues and cells [Jesse V Jokerrst et al. 2011]. PEGylation also facilitates attachment of targeting ligands to NPs promoting their accumulation at specific tissues [Nagasaki Y et al. 2007, Farokhzad OC et al. 2006, Winter P et al. 2003].

1.3. Orientations of PEG on the surface of nanoparticles.

The density and conformation of PEG on the surface of NPs are important features which determine the extent to which PEG avoids aggregation of NPs, and stealths the NPs from mononuclear phagocytic system [Mikyung Yu et al. 2012].

With different densities on the surface of nanoparticle, PEGs assume different conformations on the surface of NPs. When the NP surface coverage levels of PEG are low, PEGs assume a “mushroom” conformation on the surface of NPs (figure 3 A). On the other hand, when the NP surface coverage levels of PEG are high, the lateral pressure between the overcrowded PEG molecules forces them to assume a “brushborder” conformation on the surface of NPs [Degennes PG. 1987]. The “Brushborder” conformation (figure 3 B) provides greater protein repulsion, as thus enhances the bioavailability and plasma residence time of NPs [Szleifer. I1997, Owens DE. 2006, Gbadamosi JK et al. 2002]. However, “brushborder” conformation may decrease the mobility of PEG chains. This reduces the ability of PEGs to effectively provide stealth to avoid uptake of NPs by MPS [Storm G et al. 1995].
PEG has only one active terminal functional group. The group present at the other terminal is inert, as such the capacity to conjugate PEGs on the surface of NPs is limited [Choe YH et al. 2002, Schiavon O et al. 2004].

Figure 3. The two different conformations PEG assumes on the surface of NP. A)“ muchroom” conformation. B) represents the “brushborder” conformation [Wang M, Thanou M. 2010, Owens DE. 2006, Jokerst et al. 2011]

Although PEG as a shielding moiety can have several advantages, PEGylation still faces some shortcomings: toxicity and immunogenicity due to the use of PEG have been reported, although they are less severe compared to the toxicity and immunogenicity of the plain NPs [Calieti P et al. 2003].

PEGs are resistant to serum degradation-this would lead to prolonged circulation time of PEGylated NPs in plasma. However, very long circulation time may cause side effects. It is required that after the therapeutic effect PEGylated NPs should be degraded and removed from the body [Knop K et al. 2006].

PEG is normally degraded and excreted in urine and feces. However, at high molecular weight, PEG accumulates in the liver causing macromolecular syndrome. Moreover, PEG has high water
coordination, which increases its hydrodynamic size. This reduces PEG kidney clearance rate [Francesco M. Veronese and Gianfranco Pasut. 2005].

1.4. Layer by layer (lbl) technique:

Lbl is a technique that involves electrostatic interactions between oppositely charged polyelectrolytes (natural or synthetic). The polyelectrolytes, are assembled on a charged surface in a series of steps forming a thin polymer film (polyelectrolyte multilayer) [S.T. Dubas et al. 1999, 2001, X. Arys. 2001, B. Schoeler. 2002]. Polyelectrolyte multilayer (PEMs) has been deployed as capsules for targeted delivery of drugs [J. Alumdena et al. 2008]. To form the capsule, the PEM is assembled on a colloid such as Calcium carbonate (CaCO3). The colloid is dissolved subsequent to the assembly [X. Su et al. 2009, T. Mauser. 2005]. This creates an empty space within the PEM, which can now be loaded with therapeutic agents [C. Gao et al. 2002, A.N Zelikin. 2007]. PEMs can also be formed on the surface of nanoparticles (for example mesoporous silicon nanoparticles), in which case capsules are not formed [J. Zhou et al. 2010,].

The PEM can be used to prevent aggregation of NPs, provides additional functional groups which are used to further modify the physiochemical properties of NPs and/or to attach targeting molecules [Zhou J et al. 2010]. As such, PEM is used as an alternative to PEGylation for surface functionalisation of NPs [S.-W. Choi, 2007]. For application in drug delivery, PEMs are formed using natural polyelectrolytes such as Chitosan (Chi) and Alginate (Alg). Since these polyelectrolytes are biocompatible and biodegradable [G.B. Sukhorukov et al. 2001, G. Berth et al. 2002].
1.5. Advantages of lbl:

The procedure is not complicated; it requires no organic solvents and the number of purification steps are reduced. The amount of polymer used in the procedure is small [S Tiwari and B Mishra. 2010]. It is a versatile tool for non-covalent engineering of surfaces [Zhou J et al. 2010]. It is reproducible and drugs to be delivered are not affected by the harsh- reaction conditions encountered in covalent conjugation. PEMs formed from lbl can deliver a high drug payload, and also increase the pharmacokinetics of the loaded drug [Szarpak et al. 2008].

1.6. Natural polyelectrolytes used in lbl technique:

1.6.1. Chitosan (Chi)

Chi is a linear polycationic polysaccharide made up of repeating units of 
\( \beta-(1\rightarrow 4)-2\)-amino-2-deoxy-D-glucopyranose (figure 4) [Ravi-Kumar et al. 2000]. Chitosan is formed from the deacetylation of chitin- the structural element in the exoskeleton of crustaceans (crabs, crawfish, etc.) [R.A.A Muzzarel. 1997]

\[ R = H \text{ or } \text{COCH}_3 \]

Figure 4. Structure of Chitosan [Anna Lens et al. 1997]
1.6.2. Advantages of Chitosan in nanoparticle drug delivery system:

Chitosan (Chi) is nontoxic, biocompatible, and biodegradable. It is soluble in aqueous solvents, as such avoids the use of organic solvents.

In nanoparticle formation, the positively charged free amine groups present allow the ionic interaction with anions. This process is called ionic gelation. Ionic gelation prevents the use of chemical cross linkers such as 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide, likewise the toxic side effects associated with the use of such chemical cross linkers is also avoided [Shah S et al. 2009].

Moreover, the primary amine groups present in chitosan help the transportation of polar drugs across the epithelial tissues. They also make chitosan an excellent cell transfectant [Cho YW et al. 2003].

Furthermore, chitosan is physiologically inert and has high mechanical strength [Orrego C et al. 2010].

However, at pH higher than 6, aqueous solubility of chitosan, and positive charge density decrease. These respectively lead to decrease absorption rate at the target site, and formation of aggregates [Wang X et al. 2009].
1.6.4. Alginate.

Alginate is a natural polysaccharide extracted from the cell walls of marine brown algae [Holtan S et al. 2006]. It is a polysaccharide made of \( \alpha \)-l-guluronic acid (G) and \( \beta \)-d-mannuronic acid (M) units (figure 5). These units are arranged in two patterns: The homomeric pattern in which the alginate is made up of either G units only or M units only, and the heteromeric pattern in which alginate is built from both G and M units [George M et al. 2006].

![Figure 5. Structure of sodium alginate](image)

1.6.5. Advantages of alginate in nanoparticle drug delivery system:

Alginate is nontoxic, biocompatible, biodegradable. In addition it is inert, inexpensive, and its encapsulation of therapeutic agents can be performed in mild conditions [Liu JW et al. 2009].

In nanoparticle formation, the free carboxyl groups allow ionic interaction with bivalent cations such as \( \text{Ca}^{2+} \).
Alginate has high enteric and stabilization properties. These prevent aggregation of nanoparticles [Rastogi R et al. 2007].

At low pH, alginate shrinks- this prevents the release of the encapsulated agent. On the other hand, at high pH alginate expands and becomes viscous- this allows the release of the encapsulated agent. This pH depended behavior of alginate is being exploited in targeted drug delivery [Lee BJ et al. 1998].

The cis diols and carboxylates groups in alginate provide sites for further modification of nanoparticles. For example, they are used in covalent cross linking of alginate chains to increase the mechanical stability of the particles. Also, they are used to couple targeting molecules to NPs [Kerim M et al. 2009].

However, in physiological conditions alginate is unstable over time. This is due to the slow exchange of calcium and sodium ions causing swelling and subsequent rupture [Thu B et al. 1996].

1.7. Mononuclear phagocytic system (MPS) and opsonization:

The mononuclear phagocytic system is a part of the immune system. MPS includes; liver, spleen, lymphatic vessels, kupffer cells, circulating monocytes, and phagocytes. MPS has as a role:- inactivation and the removal of foreign bodies such as viruses, bacteria, intravenously administered NPs from the body [Saba TM et al. 1970].

Once foreign bodies reach the blood circulation, they are bound by plasma proteins call opsonins causing opsonization [Van Vlerken Le et al. 2007]. Examples of opsonins include antibodies (IgM, IgG), components of the complement system (C3b, C4b).
Circulating monocytes, and phagocytes recognize these foreign bodies via the bound opsonins on their surface [M.M. Frank et al. 1991]. After this, they engulf the foreign bodies and transport them to the liver and spleen for eventual degradation and excretion. Kupffer cells located in the liver are the major filter for NPs [Decker K et al. 1990].

1.8. Zeta Potential (ZP):

For NPs, zeta potential refers to the electrostatic potential between the NP’s surface and the solvent. ZP depends on the nanoparticle surface charge, solvent, pH of the solvent, salt type, and concentration of the salt.

Zeta potential values provide a way to measure the colloidal stability of NPs. With zeta potential values greater than +10mV or lower than -10mV, there exists interparticle repulsion. This provides greater stability and avoids aggregation. The ultimate goal of this is increased circulation time of the NPs [Jesse V Jokerst et al. 2011]. However, in drug delivery NPs with zeta potential values greater than +30Mv or lower than -30mV are rapidly and nonspecifically taken up by macrophages in vivo and in vitro. This leads to high accumulation of NPs in the liver after intravenous administration [Kai Xiao. 2011]. It has been presented that NPs with neutral or negative zeta potentials have reduced rate of non-specific cellular uptake and plasma protein adsorption compared to positively charged NPs of the same magnitude [Alexis et al. 2008].
Also, zeta potential values provide information on surface modifications, for example thiols and carboxyl groups have negative zeta potentials, while amines have positive zeta potentials [Jesse V Jokerst et al. 2011].

1.10. Carbodiimide chemistry

Carbodiimide chemistry involves the activation of carboxylic acids (−COOH) for direct conjugation to primary amines (−NH₂) via amide bonds. The most frequently used carbodiimides (figure 6) are EDC (for aqueous cross-linking), and N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-Diisopropylcarbodiimide (DIC) which we developed for non-aqueous cross-linking. In the cross-linking process carbodiimides do not become part of the amide bond. For this reason they are called zero length cross-linkers.

N-hydroxysuccinimide (NHS) or its water-soluble analog (Sulfo-NHS) is added in the cross-linking process to form stable amides. [Updegrove T. B. et al. (2011)].

Figure 6. Most commonly used carbodiimides. A) N,N'-dicyclohexylcarbodiimide, (B) Diisopropylcarbodiimide, (C) 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide
1.10.1. Mechanism of reaction of carbodiimides:

1. The acid (PEG) will react with the carbodiimide (EDC) to produce the key intermediate called O-acylisourea. O-acylisourea is an unstable carboxylic ester which hydrolyses in aqueous media to regenerate carboxyl groups (regenerated methoxyl- PEG).

2. The O-acylisourea will react with primary amines present in PSi-NP to give the desired amide.

3. Addition of NHS or Sulfo-NHS stabilizes and converts the reactive O-acylisourea to an amine-reactive Sulfo-NHS ester. This increases the yield of the reaction (figure 7)
Figure 7. Carbodiimide reaction mechanism
1.11. TCPSi-NH$_2$ nanoparticles

Amine modified thermally carbonized mesoporous silicon nanoparticles are made by covalently grafting aminosilanes like 3-triethoxysilylpropylamine(APTES) or 3-(2-aminoethylamino) propyldimethoxymethylsilane(AEAPMS) on thermally carbonized mesoporous silicon [Wujun Xu et al. 2012]. Aminosilanes have primary and secondary amino groups. The primary amino groups are very reactive and act as sites for further modification of TCPSi-NH$_2$ nanoparticles. Figure 8 shows the chemical structure of TOPSi-NH$_2$ nanoparticle.

![Figure 8. Structure TOPSi-NH$_2$.](image-url)
2. MATERIALS AND METHODS

2.0. Materials

All reagents were used as supplied with no further purification. Sodium chloride (Fisher Scientific, UK), monosodium hydrogen phosphate, and disodium hydrogen phosphate (MERCK, Germany), MES (2-[morpholino] ethanesulfonic acid), N,N'-Diisopropylcarbodiimide (Perseptive Biosystems GmbH, Hamburg, Germany), N-hydroxysulfosuccinimide (Fluka), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Aldrich), Methoxyl poethylene glycol (10 KDa Iris Biotech Gmbh, Germany), chiotsan (medium molecular weight, 190-310KDa, Aldrich), alginate (medium molecular weight Alginic acid, 80-120KDa, Aldrich), TCPSi-NH₂ nanoparticles (Department of applied Physics, University of Eastern Finland, Kuopio by prof. Vesa-Pekka Lehto), Eppendorf tubes, glass pipettes, centrifuge, sonicator (Biotrans), end over end mixer (Bio rotator rs-multi, biosan, Labema oy, finland), DLS, FT-IR, pH meter, parafilm (Laboratory film, pechiney plastic packaging), Falcon tube (Sarstedt). All plasticware was rinsed with ethanol to reduce the amount of plastic softeners which interact with the nanoparticles.

Plasma was produces as follows: Blood was collected from voluntary donors into Ethylenediaminetetraacetic acid (EDTA) containing vacutainer tubes (Sarstedt). The tubes were inverted ten times to mix the anticoagulant (EDTA) and blood. Immediately, the tubes were centrifuged for ten minutes at 1300 rpm at room temperature using Rotina 46R (Hettich). Supernatant (plasma) from all the tubes was collected in to a 200ml beaker. The plasma was later put in to 2ml Eppendorf tubes and stored at -4°C.
2.1. METHODS

Layer by layer method exploits the electrostatic interactions between oppositely charged molecules. In this work, positively charged amino derivatized TCPSi-NH$_2$ nanoparticles, and the polyelectrolytes-alginate, and chitosan having negative and positive charges were used respectively. Starting with alginate and subsequently alternating between the polyelectrolytes, different layers were assembled on the positively charged TCPSi-NH$_2$ nanoparticles. After the electrostatic layering, positive and negative charged polyelectrolytes were cross linked creating a covalent bond between the two polymers. Covalent bonds were formed between different layers on the nanoparticles by cross linking COOH and NH$_2$ groups of alginate and chitosan respectively using EDC and NHS as chemical cross linkers.

Polyethylene glycol (PEG) was grafted on the surface of TCPSi-NH$_2$ nanoparticles through carbodiimide chemistry. EDC and NHS were used to activate the COOH groups on PEG to form an amine reactive ester. EDC activates the COOH groups on PEG to form O-acylisourea- which is unstable in aqueous media and hydrolysis back to PEG-COOH and EDC over the time. In the presence of NHS, O-acylisourea is converted into a stable amine reactive NHS-ester, thus NHS increases the efficiency of coupling PEG on the surface of TCPSi-NH$_2$ nanoparticles. NHS and EDC are called zero length carbodiimides since they are not part of the amide bond formed between PEG-COOH and TCPSi-NH$_2$ nanoparticles.
2.1.1. **Zeta potential and size measurements.**

For every TCPSi-NH$_2$ nanoparticles sample, 25 µg of the sample was diluted with 1.5ml of distilled water and its size and zeta potential measured three times using Zeta sizer Nano-ZS (Malvern instruments). All measurements were made at 23°C.

While measuring size, the polydispersity index (PDI) was also noted. PDI is a measure of the width of particle size distribution. Higher PDI values indicate that the sample has broad size distribution - which could be due to aggregation of the sample.

**GENERAL PROCEDURE:**

2.2. **LBL procedures.**

240µL (312µg) of amino derivatized TCPSi-NH$_2$ nanoparticles were pipetted into four 2ml Eppendorf tubes. The particles were centrifuged for twenty minutes at 13200 rpm using Eppendorf centrifuge 5415D, supernatant was removed and discarded using a glass pipette. The particles were washed three times by adding 1mL of distilled water, followed by sonication until all the particles were completely dispersed in water. Finally, the particles were centrifuged, supernatant removed and discarded.

After washing, 500µL of a 10mM acetic acid solution (pH 4.46) was added to the tubes to suspend the nanoparticles. To form the first layer, while sonicating, 500µL of alginate solution (1.5mg/1mL in10Mm acetic acid) was added drop wise to the particles. The tubes were wrapped with parafilm (Laboratory film, pechiney plastic packaging) to avoid spillage during mixing and mixed for fifteen minutes using end over end mixer (Bio rotator rs-multi, biosan, Labema oy, finland). After mixing the
particles were centrifuged and washed three times as described above and their sizes and zeta potentials were measured. The chitosan (1.5mg/1ml in 10mM acetic acid) layer was added after alginate using same procedure. This procedure was repeated for every polyelectrolyte layer.

After adding the fifth layer on to the particles, half of the particles were further layered with chitosan solution to produce six layered particles and their sizes and zeta potentials were measured. To cross link five and six layered, nanoparticles were suspended in DMF and 22µl of EDC (100mg/mL in DMF) and16µL of NHS (100mg/mL in DMF) were added to the nanoparticles. The particles were mixed overnight using end over end mixer. Subsequent to overnight mixing, the particles were centrifuged and washed twice as described above and their sizes and zeta potentials were measured.

To evaluate the stability and opsonization of the particles, both five and six layered cross linked nanoparticles were divided into three equal aliquots. These aliquots were incubated in distilled water, human EDTA plasma, phosphate buffered saline (PBS) respectively. The sizes and zeta potentials of nanoparticles in these media were measured after every 24 hours.

2.3. PEGylation procedures

0.5ml (320µg) of the TCPSi-NH$_2$ nanoparticles in ethanol was placed into a 2ml eppendorf tube, centrifuged for twenty minutes, supernatant was removed and discarded. The particles were washed twice using 1ml of distilled water as described above.

PEGylation of TCPSi-NH$_2$ nanoparticles using NHS and EDC was carried out in a two-step procedure. In the first step; 5mg 10KDa PEG, 50mg NHS, and 50mg of EDC were weighted and dissolved in 0.5ml of MES buffer (pH 6) in a 2ml eppendorf tube. The tube was sonicated for two minutes to effectively mix the reagents. After sonication the tube was wrapped with parafilm to avoid spillage and
mixed for twenty minutes to form amine reactive NHS-ester using end over end mixer. Subsequent to end over end mixing (second step) 750µl of PBS (pH 7.6) was added to the tube to raise the pH to 7. This mixture was immediately added to the washed TCPSi-NH₂ nanoparticles. The tube was sonicated for two minutes to mix the TCPSi-NH₂ nanoparticles with the formed amine reactive NHS-ester. The tube was wrapped with parafilm and mixed for two hours using end over end mixer. After the mixing the tube was centrifuged and washed two times as mentioned above to remove excess of reagents and unbound amine reactive NHS-ester. The sizes and zeta potentials of these particles (PEGylated TCPSi-NH₂ nanoparticles) were measured in water. Both PEGylated TCPSi-NH₂ nanoparticles and plain TCPSi-NH₂ nanoparticles were placed in human EDTA plasma and incubated for 30 minutes at 37°C to see if PEG reduces the binding of plasma proteins to PEGylated TCPSi-NH₂ nanoparticles compared to the plain TCPSi-NH₂ nanoparticles. The incubated particles were centrifuged, washed twice, and their sizes and zeta potentials measured in water.

2.4. LBL – PEGylation procedure

240µL (312µg) of amino derivatized TCPSi-NH₂ nanoparticles were placed into a 2mL Eppendorf tube. The particles were centrifuged for twenty minutes at 13200 rpm and supernatant was removed and discarded using a glass pipette. The particles were washed twice using 1mL of distilled water. After washing, the nanoparticles were suspended into 500µL of a 0.5M NaCl solution. While sonicating, 500µL of alginate (1.5mg/1mL in 0.5M NaCl pH 4) solution was added drop wise to the particles. The tube was wrapped with parafilm to avoid spillage during mixing and mixed using end over end mixer for fifteen minutes to add the first layer (alginate) on the surface of nanoparticles. Subsequent to end over end mixing the particles were centrifuged and washed as described above and their sizes and zeta potentials were measured. This procedure was repeated for every polyelectrolyte
layer added on the nanoparticles, but using alginate or chitosan (1.5mg/1mL of 0.5M NaCl solution pH 4) solution in alternate layers. This procedure was repeated until six poly electrolyte layers were formed on TCPSi-NH$_2$ nanoparticles.

5mg 10KDa PEG, 50mg NHS, and 50mg of EDC were weighted and placed in to a 2ml Eppendorf tube. 1.5ml of 0.5M NaCl solution (pH 4) was added to the tube. The tube was sonicated for two minutes to effectively mix the reagents. After sonication the tube was wrapped with parafilm to avoid spillage and mixed using end over end mixer for twenty minutes to form the amine reactive NHS-ester. This mixture was immediately added to the washed six layered TCPSi-NH$_2$ nanoparticles. The tube was sonicated for two minutes to thoroughly mix the layered TCPSi-NH$_2$ nanoparticles with the formed amine reactive NHS-ester. The tube was wrapped with parafilm and mixed overnight using end over end mixer. After overnight mixing the tube was centrifuged and washed twice to remove excess reagents. The sizes and zeta potentials of the particles were measured in water.
3. RESULTS AND DISCUSSIONS

3.1 layer-by-layer

Using the layer by layer method, alternating layers of alginate and chitosan polyelectrolytes dissolved in a 10mM acetic acid solution (pH 4.46) were added to TCPSi-NH$_2$ nanoparticles. Once a layer was added to the nanoparticles, their size and zeta potential were measured using dynamic light scattering (Table 2).

<table>
<thead>
<tr>
<th>Outer layer</th>
<th>Number of layers</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plain TCPSi-NH$_2$</td>
<td>0</td>
<td>186</td>
<td>0.1</td>
<td>76</td>
</tr>
<tr>
<td>alginate</td>
<td>1</td>
<td>200</td>
<td>0.08</td>
<td>-35</td>
</tr>
<tr>
<td>chitosan</td>
<td>2</td>
<td>220</td>
<td>0.06</td>
<td>40</td>
</tr>
<tr>
<td>alginate</td>
<td>3</td>
<td>200</td>
<td>0.1</td>
<td>-21</td>
</tr>
<tr>
<td>chitosan</td>
<td>4</td>
<td>250</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>alginate</td>
<td>5</td>
<td>311</td>
<td>0.1</td>
<td>-35</td>
</tr>
<tr>
<td>chitosan</td>
<td>6</td>
<td>350</td>
<td>0.1</td>
<td>40</td>
</tr>
<tr>
<td>alginate</td>
<td>5 cross linked</td>
<td>290</td>
<td>0.09</td>
<td>-58</td>
</tr>
<tr>
<td>chitosan</td>
<td>6 cross linked</td>
<td>242</td>
<td>0.07</td>
<td>-54</td>
</tr>
</tbody>
</table>

Table 2. Size and zeta potentials as a function of polyelectrolyte layers on TCPSi-NH$_2$ nanoparticles.
As illustrated in table 2, the size and zeta potential of the particles changed from 186 to 200nm and from +70mV to -35mV respectively during the lbl suggesting that a layer of aginate was formed on the surface of the particles. The size of the particles increased after every layering step except after the third layering with alginate. As expected, the zeta potential changed to negative when an alginate layer was added and to positive when a chitosan layer was added. This increases in size and changes from negative to positive zeta potential suggest successful addition of the polyelectrolytes. Moreover, the gradual increase in size of the layered particles suggests that single and not aggregated particles were layered. Furthermore the PDI values (range from 0.08 to 0.1) also suggest that the particles were not aggregated.

The five and six layered TCPSi-NH₂ nanoparticles were cross linked using EDC and NHS. The sizes and zeta potentials of the cross linked particles were measured. (Table2).

Both five and six layered cross linked TCPSi-NH₂ nanoparticles were smaller than five and six layered TCPSi-NH₂ nanoparticles before cross linking. This suggests that cross linking produced compact polyelectrolyte layers with reduced spaces between them. In the un-cross linked layered TCPSi-NH₂ nanoparticles water molecules penetrate spaces between the layers and cause swelling of the polyelectrolyte layers. PDI values from 0.07 to 0.09 indicates that cross linked TCPSi-NH₂ nanoparticles were highly dispersed in water compared to uncross linked particles (table 2). Interestingly, the charge on six layered cross linked particles with chitosan as outermost layer was negative.

The sizes and zeta potential of the cross linked TCPSi-NH₂ nanoparticles placed in three different media (water, PBS, plasma) were measured after every 24 hours for five days with dynamic light scattering (Figure 9, and 10).
Figure 9. Changes in the size of five layered cross linked TCPSi-NH$_2$ nanoparticles placed in water, PBS, and plasma respectively.

There were small changes in the size of five layered cross linked nanoparticles placed in water, indicating that five layered cross linked nanoparticles were fairly stable in water. There were greater size changes (290 to 600nm) for particles placed in PBS relative to size changes for particles in water. This suggests that the particles were aggregating in PBS. Particles placed in plasma had largest increase in size (290 to 1200nm). This also suggests that plasma proteins were bound to the particles and the particles were aggregating in plasma.
Figure 10. Changes in the size of six layered cross linked nanoparticles placed in water, PBS, and plasma respectively.

Up to five days in water, the size of six layered cross linked TCPSi-NH$_2$ nanoparticles remained unchanged - suggesting that they were very stable in water. This could be due to the steric repulsion provided by the six layers. Six layered cross linked TCPSi-NH$_2$ nanoparticles placed in PBS, and plasma had increased sizes. This suggests that the particles were not stable in PBS, and plasma, and that plasma proteins were bound to the particles in plasma.
3.2 PEG conjugation

EDC and NHS were used in a two-step procedure to couple PEG-COOH to the primary amino groups (-NH₂) of TCPSi-NH₂ nanoparticles. In the first step a reactive amine NHS-ester was formed and in the second step this ester was coupled to the primary amines present in TCPSi-NH₂ nanoparticles through an amide bond. After washing, the size and zeta potential of the PEGylated TCPSi-NH₂ nanoparticles were measured (Table 3)

Table 3. Sizes and zeta potentials of PEGylated TCPSi-NH₂ nanoparticles compared with that of the plain TCPSi-NH₂ nanoparticles

<table>
<thead>
<tr>
<th>TCPSi-NH₂ Type</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAIN</td>
<td>257</td>
<td>0.2</td>
<td>70</td>
</tr>
<tr>
<td>PEGylated</td>
<td>214</td>
<td>0.1</td>
<td>26</td>
</tr>
</tbody>
</table>

The reduced zeta potential (from 70 to 26 mV) of the PEGylated- nanoparticles suggest that Me-PEG-COOH (methoxyl polyethylene glycol which is neutral) was bound to some NH₃⁺ groups responsible for the positive zeta potential of TCPSi-NH₂ nanoparticles. Moreover, the low PDI and size values (0.1, 214 nm) of the PEGylated- nanoparticles compared to the plain TCPSi-NH₂ (0.2, 257 nm) suggest that PEG on the surface of nanoparticles reduced aggregation of the particles.

Also, the sizes and zeta potentials of plain and PEGylated TCPSi-NH₂ nanoparticles incubated in plasma for thirty minutes were measured (Table 4).
Table 4. Sizes and zeta potentials of plain and PEGylated TCPSi-NH$_2$ nanoparticles incubated in plasma for thirty minutes at 37°C.

<table>
<thead>
<tr>
<th>TCPSi-NH$_2$ Type</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAIN</td>
<td>548</td>
<td>0.2</td>
<td>-20</td>
</tr>
<tr>
<td>PEGylated</td>
<td>254</td>
<td>0.09</td>
<td>-18</td>
</tr>
</tbody>
</table>

The smaller sizes (254nm) of PEGylated- nanoparticles compared to the size (548nm) of plain nanoparticles suggests that PEG reduced the amount of plasma opsonins binding to the PEGylated-nanoparticles. Also, the large size (548nm) of plain particles after incubation suggest that binding of opsonins promotes aggregation of nanoparticles. Moreover, comparison of PDI values suggest that in plasma plain nanoparticles aggregated more than the PEGylated- nanoparticles. However, the negative zeta potential values suggests that plasma opsonins were bound on both plain and PEGylated nanoparticles.

3.3. LBL – PEGylation procedure

Using the layer by layer method, alternating layers of alginate and chitosan polyelectrolytes dissolved in 0.5M NaCl solution (pH 4.00) were added to TCPSi-NH$_2$ nanoparticles. Once a layer was added to the nanoparticles, its size and zeta potential were measured using DLS (Table 5).

NOTE: table 2 represents lbl results in which all reagents were dissolved in a 10mM acetic acid solution (pH 4.46), and table 5 represents lbl results in which all reagents were dissolved in 0.5M NaCl solution (pH 4.00). This was to avoid activating the COOH groups of acetic acid during PEGylation.
Table 5. Changes in size and zeta potential as a function of polyelectrolyte layers on TCPSi-NH₂ nanoparticles

<table>
<thead>
<tr>
<th>Number of polyelectrolyte layer</th>
<th>Size(nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>184</td>
<td>0.06</td>
<td>+64</td>
</tr>
<tr>
<td>1 layer</td>
<td>212</td>
<td>0.09</td>
<td>-47</td>
</tr>
<tr>
<td>2 layers</td>
<td>325</td>
<td>0.1</td>
<td>+73</td>
</tr>
<tr>
<td>3 layers</td>
<td>299</td>
<td>0.2</td>
<td>-37</td>
</tr>
<tr>
<td>4 layers</td>
<td>316</td>
<td>0.08</td>
<td>+59</td>
</tr>
<tr>
<td>5 layers</td>
<td>387</td>
<td>0.1</td>
<td>-77</td>
</tr>
<tr>
<td>6 layers</td>
<td>405</td>
<td>0.1</td>
<td>+67</td>
</tr>
</tbody>
</table>

The change in zeta potential from +64 to -47mV, and the size increase from 184nm to 212nm following the addition of alginate indicates that an alginate layer was formed on the surface of TCPSi-NH₂ nanoparticles. As shown in table 5, the size of the TCPSi-NH₂ nanoparticles kept increasing whenever an electrolyte layer was added, and the zeta potential kept alternating from negative when alginate was added to positive when chitosan was added. These suggest that alternating layers of alginate and chitosan were formed on the surface of TCPSi-NH₂ nanoparticles. Like in table 2, the third layer was again smaller than the preceding layer.

After the sixth layer, EDC and NHS dissolved in 0.5M NaCl solution (pH 4.00) were used in a two-step procedure to couple PEG-COOH to the primary amino groups (-NH₂) of chitosan on the outermost layer of six layered TCPSi-NH₂ nanoparticles in table 6 and also to form covalent bonds between different layers on the nanoparticles by cross linking COOH and NH₂ groups of alginate and chitosan respectively (table 6).
Table 6. Size and zeta potential of six layers cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles in 0.5M NaCl solution (pH 4.00)

<table>
<thead>
<tr>
<th>NP Type</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential(mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six layers cross-linked and PEGylated</td>
<td>326</td>
<td>0.1</td>
<td>+26</td>
</tr>
<tr>
<td>1.5 HOURS LATER</td>
<td>326</td>
<td>0.1</td>
<td>+19</td>
</tr>
<tr>
<td>3 HOURS LATER</td>
<td>318</td>
<td>0.1</td>
<td>+15</td>
</tr>
<tr>
<td>4 HOURS LATER</td>
<td>338</td>
<td>0.1</td>
<td>+15</td>
</tr>
</tbody>
</table>

The size of six layered cross-linked and PEGylated nanoparticles was smaller (326nm) compared to six layered nanoparticle (405nm) in table 5, suggesting that covalent bonds were formed between the polyelectrolyte layers on the surface of TCPSi-NH$_2$ nanoparticles-this led to the formation of compact layers on TCPSi-NH$_2$ nanoparticles. Also, the reduced zeta potential from +67 in the six layered TCPSi-NH$_2$ nanoparticles to +26mV in the six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles suggests that PEG was bound to some NH$_2$ groups present on the sixth layer. Also, the fact that the six layered cross-linked and PEGylated particles with a mild zeta potential of +26mV were stable in water for at least four hours suggests that PEG was bound on the surface of the layered particles. Also the size and zeta potential of six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles and plain TCPSi-NH$_2$ nanoparticles incubated in plasma for fifteen minutes at 37°C were measured (table 7).
Table 7. Size and zeta potential of plain and six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles before and after incubation in plasma.

<table>
<thead>
<tr>
<th>Number of layers</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six layers cross-linked and PEGylated</td>
<td>350</td>
<td>0.07</td>
<td>+15</td>
</tr>
<tr>
<td>0 layer</td>
<td>200</td>
<td>0.1</td>
<td>+60</td>
</tr>
<tr>
<td>Six layers cross-linked and PEGylated incubated in plasma</td>
<td>789</td>
<td>0.2</td>
<td>-0.4</td>
</tr>
<tr>
<td>Plain TCPSi-NH$_2$ incubated in plasma</td>
<td>2837</td>
<td>0.3</td>
<td>-15</td>
</tr>
</tbody>
</table>

Both plain (0 layer) and six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles were aggregated in plasma. However the smaller size of six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles (789nm) compared to the plain TCPSi-NH$_2$ nanoparticles (2837nm) and the smaller change in zeta potential (from +15 to -0.4mV) for six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles compared to the zeta potential change (from +60 to -15mV) for plain TCPSi-NH$_2$ nanoparticles suggest that more opsonins bound to the plain particles than to six layered cross-linked and PEGylated TCPSi-NH$_2$ nanoparticles.
4. Conclusion

In this project TCPSi-NH$_2$ nanoparticles was successfully modified using lbl-technique, PEGylation, and lbl-technique combined with PEGylation.

With the lbl-technique different polyelectrolyte layers were formed on TCPSi-NH$_2$ nanoparticles. TCPSi-NH$_2$ nanoparticles with cross linked polyelectrolyte layers on their surfaces were stable in distilled water for up to five days. This indicates that the polyelectrolyte layers provided electrosteric stability to the TCPSi-NH$_2$ nanoparticles. However the particles were not stable in PBS, and plasma. This could be due to high ionic concentration in PBS, and hydrophobic interaction in plasma.

The two-steps carbodiimide chemistry procedure was an effective procedure to graft PEG-COOH to primary amine. Grafted PEG-COOH on the surface of TCPSi-NH$_2$ nanoparticles reduced the amount of opsonins bound to the particles. However, the grafted PEG-COOH did not completely prevent opsonins from binding to the particles. This could be partly due to the fact that PEGylated TCPSi-NH$_2$ nanoparticles were still carrying a positive charge. This positive charge attracted negatively charged opsonins.

5. DEDICATION

This work is dedicated to my late mum, Ma Christina Enowma. She made me understand that success is easy to come by through the strength of the brain than the strength of the muscles.
6. ACKNOWLEDGEMENTS

I owe sincere and earnest thanks to my head of department Prof Tapani Pakkanen, and coordinator Mari Heiskanen for an offer of a place in the Research Chemists program and financial support throughout this program.

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I am obliged to my colleagues in medicinal chemistry for their words of courage whenever lab work became a nightmare.

I am sincerely and heartily grateful to my elder brother, Beteck Samuel Tabot for the study abroad orientation he gave me. With this I knew I have hurdles and not leisure times while studying in Europe.

7. REFERENCES


