Nanoscale Reaction Systems

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Abstract

The work presented in this thesis describes the use of polystyrene nanoparticles as model surfaces for bioanalytical work. Nanoparticles constitute convenient platforms for the attachment of bioactive agents, and receptor coated particles offer high local concentration of binding sites for specific ligands with minimal steric hindrance. However, it is not only the amount of bound protein that matters, the proteins must also be immobilized at the surface in such ways that they fully retain their activity, while at the same time protecting the surface from unspecific uptake of undesired components. The present work relates to the controlled immobilization of multiple types of active biomolecules onto nanoparticle surfaces to make them multifunctional. The surface expansion offered by the nanoparticles, in combination with the closeness between the reactants co-immobilized on the same particle, enables coupled reactions to be carried at a higher rate than otherwise possible. Thus, particle-decorated surfaces of this kind are highly suitable for miniaturized bioanalytical systems. Sensitive microarray systems are under development, including lectin-coated nanoparticles for glycoprotein mapping and a diagnostic device for Point-of-Care testing with a nanoparticle-based detection system.

The full evaluation of protein attachment to nanoparticles requires precise analytical techniques for particle characterization, both in bare and coated form. The mass-sensitive SdFFF technique occupies a prominent position for particle characterization, as it offers both accurate determination of particle size and a quantification of adsorbed layers on small particles, whether of synthetic or biopolymeric nature. Here, this analytical technique is developed to precisely characterize nanoparticles that are sequentially coated with different layers, each rendering the particles a specific functionality. The thesis demonstrates how precise mass uptakes can be determined for each specific layer, and how control over the exact surface composition of the modified particles can be established for optimization of biological activity.

Keywords: nanoparticles, Sedimentation Field-Flow Fractionation, bioaffinity, multilayered functionalization, protein attachment, diagnostics, bioluminescence

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To my family
List of papers

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. **Shifts in polystyrene particle surface charge upon adsorption of the Pluronic F108 surfactant**. Rik ter Veen, Karin Fromell and Karin D. Caldwell, *J. Colloid Interface Sci.* **2005**, 288, 124-128


Web release date September 21.
My contribution to the papers included in this thesis was:

Paper I: I planned, performed the SdFFF parts of the experiments, and wrote the corresponding parts of the paper. Paper II: some of the experiments were planned, performed and evaluated by me. I wrote the article with the co-authors. Paper III: I planned, performed all experiments except the SEM, evaluated the results and wrote the article. Paper IV: I planned, performed and evaluated all SdFFF characterizations of the particles and wrote parts of the paper. Paper V: was experimentally planned by me, the experiments, except the preparation of antibodies, were mainly performed by me and Greta Hulting. I did the evaluation and most of the writing. All work included in these papers were performed under supervision of Karin Caldwell.

Papers not included in this thesis:

**Scanning calorimetry in probing the structural stability of proteins at interfaces** Karin Fromell, Shao-Chie Huang and Karin D. Caldwell In *Biopolymers at Interfaces*, M. Malmsten ed., Marcel Dekker, New York, 2003, ch. 20, 517-

**Poloxamer-mediated functionalization of bioanalytical surfaces – The role of nanoparticles as model surfaces** Parke M. Byron, Karin Fromell and Karin D. Caldwell *Curr. Organic Chem.* 2005, 9, 1085-1098

**Comparison between chicken and rabbit antibody based particle enhanced Cystatin C reagents for immunoturbidimetry** Lars-Olof Hansson, Mats Flodin, Tom Nilsen, Karin Caldwell, Karin Fromell, Kathrin Sunde and Anders Larsson *submitted* 2007
Abbreviations

ADP Adenosine diphosphate
ATP Adenosine triphosphate
BSA Bovine serum albumin
CCD Charge-coupled device
ConA Concanavalin A
CRP C-reactive protein
DNA Deoxyribonucleic acid
DSC Differential scanning calorimetry
EIFFF Electric field-flow fractionation
LOA Lathorus odorathus lectin
mRNA Messenger Ribonucleic acid
NTA Nitrilotriacetic acid
PCR Polymerase chain reaction
PDPH 3-(2-pyridyldithio)propionyl hydrazide
PDS Pyridyldisulfide
PK Pyruvate kinase
POCT Point-of-care testing
PS Polystyrene
SdFFF Sedimentation field-flow fractionation
SPR Surface plasmon resonance
SPDP N-succinimidyl3-(2-pyridyldithio)propionate
1. Introduction

Nanoscale particles are becoming increasingly important in such fields as medicine and biomaterials science [1-3] where they are widely used for applications ranging from diagnostic tests, drug carriers, dyes and labels, to calibration standards, just to mention a few. These nanoparticles can be of organic origin e.g. polymeric particles, liposomes and vesicles or inorganic origin e.g. colloidal gold, platinum, palladium, silica and magnetic beads. The choice of particle type depends on the field of application. For instance, biocompatibility is an important factor if the particles are to be used in diagnostic or pharmaceutical contexts and be subjected to biological environments.

One important characteristic of the particles broadly referred to as “nanoparticles” is their very large surface-to-volume ratio, which means that even small amounts of particulate matter present large surface areas to which e.g. biologically interesting substances can be bound [4]. Specifically for diagnostic applications, where small amounts of analyte are to be captured in an efficient process, this makes them suited to serve as platforms for presenting very high local concentrations of attachment sites for specific antigens, as illustrated in Figure 1.

![Figure 1. Schematic picture of functionalized particles presenting a very high local concentration of active ConA-proteins at their surface.](image_url)
Here a 240 nm Polystyrene particle has been pre-coated with a layer of the functionalized polymeric surfactant Pluronic® F108. To the polymeric side chains we have attached the lectin Concanavalin A (ConA) which has the ability to trap glycoproteins exposing the sugar mannose. From previous studies we can estimate the thickness of the two layers to be 14 and 10 nm respectively [5], resulting in a total diameter of 280 nm for the coated particle. This arrangement provides an active layer volume of $5.25 \times 10^{-18}$ L. Since the estimated amount of ConA amounts to around 700 molecules per particle this implies that the local concentration of active protein on the surface is 0.2 mM. In addition, the high local concentrations offered by such surface modified particles make them especially valuable as test surfaces for the development of various surface modification protocols.

When the capturing molecules are immobilized on a curved surface their affinity partner will be collected from a conical volume element above the surface, thereby increasing the probability of collision and binding compared to the situation in the corresponding cylindrical volume element above a flat surface [6], see Figure 2.

![Figure 2](image_url)  
**Figure 2. Illustration of the curvature effects on protein-ligand binding. (a) When the proteins are immobilized on a flat surface the ligands are captured from a cylindrical volume above the surface, while (b) a much larger conical volume is accessible for mass transport of ligands to the curved surface.**

For a given mass of particles a decrease in size per particle translates into an increase in total area of the particle population. In many cases, this size-area relationship makes a strong argument for performing analytical work with small particles. Even beyond this gain, previous studies in our laboratory have demonstrated a tenfold increase in equilibrium binding constant upon a tenfold decrease in particle diameter for the formation of Streptavidin-Biotin
complex determined for Streptavidin on different particles [6-7]. The increased binding was associated with a significant increase in the rate of binding, suggesting an additional advantage in working with curved substrates.

At times a given application might require rapid settling of particulate carriers e.g. during a washing procedure. In such cases small particles are of no use since their sedimentation under earth’s gravity is extremely slow, and very large particles are the only possible substrates. However, not to miss the benefits from the small particles, these can be coupled to the larger surface. A schematic illustration of one such arrangement is shown in Figure 3a. As seen from Figure 3b, there is a significant difference (ten-fold increase) in bioluminescence signal intensity between the light generating antibody conjugates bound to large (19 µm )PS particles covered with fines (0.24 µm particles) compared to those bound to the corresponding plain 19 µm particles.

Figure 3. (a) Schematic view of the proteins tethered to small particles in turn coupled to a larger surface and proteins linked directly to the larger surface. The small particles offer a significant expansion of the surface area available for specific binding of ligands. (b) Comparison of light intensities resulting from particle-ELISA using 19 µm particles and 19 µm particles to which 0.24 µm particles have been attached. The number of 19 µm particles in each sample was 4.2×10⁵.
1.1 Nanoparticles as reaction systems

Large numbers of coupled reactions are carried out regularly in all biological organisms. These reactions are made efficient through high local concentrations of reactants, enabled by intricate spatial organizations. The outcome of these reactions depends on the efficient transformation of reactants to products, which requires closeness between the components responsible for the reactions. One prominent example of such an efficient biological reactor is the mitochondrion, a 1-10 μm organelle responsible for cellular respiration. It is enclosed in two membranes, a smooth outer membrane and a folded inner membrane. The enzymes of the respiratory chain, generating most of the animal cell’s ATP, are embedded in the inner membrane. The many folds of the membrane greatly increase its total surface area, which is necessary for efficient cellular respiration [8]. Here, products of one reaction are readily presented as reactants for the next in the long series of reactions that ultimately convert our oxygen intake into metabolic energy. In order to mimic the efficiency of these systems one might attach capture molecules or enzymes, participating in coupled reactions, to nanometer sized particles. One will thereby get high local concentration of reactants and at the same time gain the closeness of the reactants in sequential reactions, which will promote fast conversions even at low bulk concentrations. Such arrangements would be ideal for biosensor systems where fast and sensitive detection of small amounts of analyte is essential.

1.1.1 Biosensor design

Most biosensors are based on two parts: a recognition unit and a signal generating unit. The challenge is to link these two parts in a way that creates a sensor. The first step is the development of a bioactive surface equipped with some ligand for capture or recognition of an analyte of interest. This analyte can be a cell, virus, protein, peptide, carbohydrate, or a small chemical entity. The most important properties for the choice of ligands are their specificity and selectivity. Antibodies are frequently used as capturing agents in sensor systems (immunosensors) [9-11] as they bind strongly to their antigen and can be made highly specific. Other commonly used ligands are lectins, enzymes and aptamers. The ligands are attached to some analytical surface for read-out. This read-out surface must satisfy some requirements, including easy modification to provide good quality deposition of ligands or ligand containing substrates. It must also show low non-specific signal generation, and be compatible with some detection system. [12-13].
Most detection strategies require some sort of label for signal generation. One exception is exemplified by measurements of surface plasmon resonance (SPR). The SPR technique is typically used for monitoring biomolecular interactions [14-16]. By using SPR there is no risk of affecting the molecules by addition of a label, but it requires very advanced equipment, not always available in the general laboratory.

Label-based methods can either be of direct or sandwich type. The direct assay utilizes either a labeled capture agent (i.e. antibody) to bind its complementary ligand, immobilized on the surface or an immobilized capture agent for baiting the labeled target molecule from the solution. The sandwich assay, in turn, requires two capturing agents with affinity for two different epitopes on the target molecule, unless the target molecule has several identical epitopes available for binding. A schematic picture of the labeled methods is presented in Figure 4.

The label can for instance be a fluorophore, a radioactive isotope or an enzyme. Fluorescent molecules absorb photons from a light source with a certain wavelength, which excites the electrons within the molecule and cause an emission of light at a different wavelength as these electrons return to their ground state. There is a wide range of different fluorophores available for biochemical use, each with defined excitation/emission spectra [17-19]. Autofluorescent material is obviously not suited for fluorescence detection and one must be aware that photobleaching and quenching of the fluorophore might result in lower signals than anticipated.

Radioactive labeling is performed by introduction of a radioactive isotope, such as $^3$H, $^{14}$C, $^{32}$P or $^{125}$I to the protein [9]. The signal is detected by autoradiography for $\gamma$-emitting isotopes and fluorography for $\beta$-emitting isotopes. Although radioactive labeling offers sensitive detection it is not so commonly used because of health and safety issues.
In immunoassays enzymes are the most frequently used labels [20]. The enzyme acts as a catalyst, generating a colored, luminescent or conducting product. In chromogenic reactions the enzymes are acting on a colorless substrate to generate a colored product, which is readily measured. Bio- and chemiluminescence are other popular detection principles using enzyme labels. Chemiluminescence represents emission of light as the result of a chemical reaction in which chemically excited molecules decay to lower energy levels and emit photons. The number of emitted photons is directly related to the number of processed reactant molecules, allowing sensitive quantitative analysis to be performed. A typical example of a chemiluminescent reaction catalysed by enzymes, such as horseradish peroxidase (HRP) [21-22], is the oxidation of luminol by H₂O₂ giving light emission. The presence of enhancers in the form of phenols, naphthols and amines can intensify and increase the duration of the emitted light signal. Bioluminescence is a type of chemiluminescence that originates from living organisms [23-26]. The well-known firefly is one such example. The enzyme firefly luciferase catalyses the oxidation of D-luciferin, in the presence of ATP and Mg²⁺, and generates light in the process. Having ATP as a substrate makes this reaction suitable for measurement of ATP concentrations or the presence and activity of any enzyme coupled to ATP formation or degradation [27-28].

However, immobilization of luciferase by conventional modification methods has generally resulted in inactivation of the enzyme [29-31]. Therefore an enzyme that readily can be manipulated and immobilized, such as pyruvate kinase (PK) can be fused instead of the luciferase with the protein to be labeled [32-33]. Hence, an attached kinase may produces the necessary ATP, while the soluble luciferase in turn uses the ATP in the catalysis of the light producing reaction, as described in the scheme below.

\[
\begin{align*}
\text{ADP} + \text{phospho(enol)pyruvate} & \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate} \\
\text{ATP} + \text{luciferin} + \text{O}_2 & \xrightarrow{\text{luciferase}} \text{AMP} + \text{oxyluciferin} + \text{CO}_2 + \text{pyrophosphate} + \text{light}
\end{align*}
\]

The ability to relate a given quantity of ATP to a recorded amount of emitted light in the presence of a given reaction mixture can be determined using a CCD camera, if the camera previously has been calibrated with a photodiode to ensure proportionality between diode emitted light and camera current. Preferably, the camera is mounted in a light-tight box and positioned a fixed distance from a platform with a 96-well microtiter plate made of white opaque plastic for minimal cross-talk between wells. Light emissions from the wells of interest should first be measured prior to the addition of ATP (or PEP, as appropriate) and subsequently during an actual reaction. The readings can then be corrected by subtraction of the background. The oxidation
of luciferin starts immediately after addition of ATP, and the reaction proceeds with the emission of light until all ATP has been consumed.

In our laboratory a series of validation measurements has been performed by adding a luciferase/luciferin mixture to each of 5 concentrations of ATP. The reactions were followed until no detectable light was emitted. In case of an initial ATP concentration of 0.5 μM the emission reached its maximum value after 80s, and had decayed to zero after 3x10^4 s. At this point a new aliquot of ATP was added and the emission curve was faithfully reproduced. The integrated photon output curve was therefore considered to be proportional to the amount of ATP in the well. Figure 5 shows the cumulative number of photons as a function of ATP concentration. The diagram indicates that this system is faithfully reporting the amount of ATP added to the wells. We assume that the ATP produced through phosphorylation of ADP, using PEP with the catalytic assistance of pyruvate kinase, is consumed in an identical manner to ATP added directly.

![Diagram](image)

Figure 5. Cumulative photon emission from the consumption of 200μL portions of ATP by the luciferin/luciferase system. The data, in relative response units (r.u.), is produced by integration of the emission curve over the time interval 0 - 3x10^4 s. The sample concentration is given relative to the value for an ATP concentration of 5x10^-7 M.

Critical factors for the creation of all kinds of bioactive particle surfaces for use in biosensor systems are first to enable specific immobilization of one or more ligands, with retained activity, on the same particle and at the same time protecting the surface from unspecific adsorption of proteins, viruses, cells or other undesired components.
1.1.2 Passivation of the surface

In general surfaces for biological applications have to be provided with some type of shield to suppress unspecific adsorption or to prevent loss of sample components due to adsorption onto walls of tubings or labware [34-38]. For biosensor applications this shield must possess certain qualities, such as: reproducibly form a complete and uniform surface coverage, be stable in biological environments and be biocompatible. Pluronic surfactants are a group of triblock copolymers with the general composition of \((\text{PEO})_m(\text{PPO})_n(\text{PEO})_m\) also known as poloxamers [39-41]. They have proven to be very useful for many biotechnological and pharmaceutical applications as they have extremely low toxicity and immunogenic response. They are also one of few types of surfactants approved by the U.S. Food and Drug administration for use in medical applications and as food additives [42].

The hydrophobic Polypropylene oxide (PPO) block readily adsorbs to most common plastic surfaces such as Polystyrene (PS), Polycarbonate (PC) and Polymethyl metacrylate (PMMA), while the less hydrophobic Polyethylene oxide (PEO) tails will be extended outward from the surface (Figure 6). However from the adsorption study in Paper I, where different concentrations of Pluronic F108 is adsorbed to PS latex particles, it is evident that the PEO tails only will extend out in the aqueous phase if there are no other options. In case there is free space available on the hydrophobic surface also these tails will contact and cover the surface. It is therefore important to not only have a large excess of Pluronic over the surface area available, but a sufficiently high concentration to ensure close-packing of the polymers at the surface.

![Figure 6](image_url)

*Figure 6. (a) Schematic representation of the Pluronic F108 triblock co-polymer. (b) At high surface concentration the hydrophobic PPO part is adsorbed to the hydrophobic surface, while the less hydrophobic PEO tails are forced to extend out in the aqueous environment. (c) At low surface concentration not only the PPO block is adsorbed to the surface, also the PEO side-chains are spread out at the surface.*
Over the years Pluronic coated surfaces have proven to efficiently reduce
unspecific protein adsorption as well as bacterial and platelet adhesion [43-
46]. Although it is the hydrophobic PPO center block that determines the
surface concentration of poloxamer, the protection of the surface appears to
be more effective the longer the PEO blocks [5, 47-48]. Out of many differ-
ent triblock copolymers tested in our laboratory the Pluronic F108 (m=129,
n=56) was shown to be the best for suppression of protein adsorption to the
surface, deriving the repellant properties from its high content of PEO. The
effects of the Pluronic coating and the PEO length on the suppression of
HSA adsorption to the particles surface is illustrated in Figure 7 [48].

Figure 7. HSA uptake from solutions with different concentrations by untreated
polystyrene particles (signified by bars and relate to the left hand scale in the dia-
gram) or blocked with either Pluronic F108 or P105 (solid lines and relate to the
right hand scale in the diagram).

1.1.3 Protein attachment to the particle surface

In order to design reliable and reproducible sensor systems for protein detec-
tion the capture agent or ligand must be stably accommodated on a solid
support in such a way that its functionality is retained. There are several
methods for immobilizing proteins on a solid phase. A common way is to
simply let the proteins adsorb directly onto the hydrophobic surfaces, such as
those presented by polystyrene nanoparticles or microtiter plates. The prob-
lem is that proteins have a tendency to denature through contact with the
hydrophobic surface, thus loosing both their structure and function [48-51].
Since the effectiveness of most applications is based on the retention of a specific biological activity of the attached protein, adsorption is of limited applicability as an immobilization method. Instead the proteins can be linked to the surface through a spacer that allows specific attachment of the desired proteins and at the same time protects the proteins from destructive interaction with the surface. Preferably this linker is of some hydrophilic material that is compatible with biological samples such as dextran, polyethylene glycol (PEG/PEO) polyvinyl alcohol (PVA) or even another protein.

Streptavidin (SA) is a protein that has been widely used for attachment of biotinylated components to the surface. It readily adsorbs to hydrophobic substrates, but unlike most other proteins, SA retains its conformation and activity after interaction with the surface. Many commercial products have been developed using this method [52-53]. However, like many proteins, we and others have found that SA can have some avidity to other proteins in high concentrations which would be a drawback for use in applications involving complex samples containing high loads of proteins such as blood or saliva.

Another strategy is to pre-coat the surface with the Pluronic F108 poloxamers described above, known to suppress unintended protein adsorption. These molecules can be functionalized through the introduction of reactive groups to their PEO-side-chains. This way the intended proteins can be specifically attached to the surface, in a desired orientation, secured from close contact with the destructive surface. At the same time the surface is protected from unspecific protein adsorption. By that means several important criteria for successful protein immobilization are fulfilled.

Most frequently the PEO side chain has been activated through introduction of a pyridyldisulfide (PDS) group to which thiol-containing molecules can be covalently attached [54]. Many proteins do not have any accessible thiols for coupling. In that case, extra thiols can be provided through reaction with the heterobifunctional reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), followed by reductive cleavage with DTT [55]. This procedure uses the available ε-amino groups of one or more lysine residues or the amino terminals of the protein for coupling, which possibly can result in a random orientation of the attached protein. A scheme for attaching a protein (here IgG) to particles via PDS derivatized Pluronic F108 is shown in Figure 8.
A possibility for more directed coupling of for example antibodies goes via the carbohydrate groups located on their Fc portions, well separated from the sensitive binding site at the Fab region. The 3-(2-pyridyldithio)propionyl Hydrazide (PDP-Hydrazide) reagent can be used to add a sulfhydryl-group to the carbohydrate containing macromolecule [56-58]. The introduced 2-pyridyldisulfide can then be cleaved off with DTT to yield the thiolated protein. However, this method is of limited applicability, as some proteins lack carbohydrate moieties or have their carbohydrates in more delicate positions less suitable for coupling. This method also includes an extra step and is thus a little more labor intensive. However, an advantage of both coupling methods described above is that the degree of substitution easily can be determined from the concentration of released pyridine-2-thione after cleavage with DTT. This concentration can be determined by measuring the absorbance at 343 nm, using 8080 M⁻¹cm⁻¹ as the extinction coefficient and is equivalent to the concentration of pyridyl disulfide groups in the protein [55].
Instead of the PDS group, other reactive groups can be introduced to the Pluronic F108, one example being the chelating nitrilotriacetic acid (NTA). When this structure has been loaded with chelating metal ions (i.e. Ni$^{2+}$ or Cu$^{2+}$) it will readily bind to proteins with available residues for metal coordination, predominantly histidine [59]. Proteins without such residues can easily gain affinity for chelating metal ions by genetic modification or covalent attachment of a histidine-tag. The interaction between the His-tagged protein and the chelating metal ions is highly stable (Kd = 10$^{-13}$ M). An advantage is that this bond is reversible under nondenaturing conditions and can easily be made to release the immobilized proteins through lowered pH alternatively addition of imidazole or free chelators [60].

There are a number of occasions when it is extremely useful to enable co-immobilization of two or more functions, whose relative concentrations can be varied independently, on the same nanoscale entity. This can be achieved by adsorption of a predetermined mixture of derivatized Pluronic F108 providing different coupling chemistries. This is for example of outmost importance for diagnostic devices were close proximity between the donor and acceptor molecules are essential for fast signal generation (see above). In one such approach we have co-immobilized the capture agent in the form of a synthetic binder and a signal generating enzyme (luciferase) on the same 240 nm PS particle. Their relative concentrations were regulated by coating the particle surface with a predetermined mixture of F108-PDS, for attachment of the binder and F108-NTA, for subsequent attachment of the His-tagged luciferase enzyme. This complete multicomponent arrangement showed activity in a proof-of-principle demonstration for the VINNOVA-supported biosensor development project under the name of “Loc & Poc” (see figure 16 below).

Another approach is coupling through hybridization of complementary oligonucleotides. The four base pairs (adenine, thymine, cytosine and guanine) can be combined in $4^n$ ways, where n is the chain length of the oligonucleotide, providing the possibility to an almost infinite number of specific binding sites. In Paper 3 ConA lectin and a small oligonucleotide are made to coexist on the same particle, where the oligonuclotide is used for specific coupling of the particle to the surface. This coupling has proven to be robust as it withstands shear forces of up to 0.65 pN (Paper II).

By a combination of the coupling strategies discussed above, it is further possible to achieve an even larger number of components on the same nanoscale carrier. In one such approach we have explored the use of 240 nm PS particles equipped with tree different functionalities, namely oligonucleotides for attachment of the particle to the surface, PDS-groups for covalent attachment of thiol containing capture probes, in this case antibodies, and
nitrilotriacetic acid groups for chelate coupling of His-tagged enzymes for signal generation. A schematic illustration of the multiparticle composition is seen in Figure 9. The composition of the multifunctional particles were carefully analysed by SdFFF (see below) and the surface concentration of each component is presented in Table 1.

Table 1. *Determination of surface composition of the multifunctionalized nanoparticles by SdFFF.*

<table>
<thead>
<tr>
<th>Surface conc of:</th>
<th>g/PS</th>
<th>Molecules/PS</th>
<th>mg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>F108-PDS/NTA</td>
<td>3.94×10⁻¹⁶</td>
<td>16250</td>
<td>2.18</td>
</tr>
<tr>
<td>dG</td>
<td>8.48×10⁻¹⁸</td>
<td>1000</td>
<td>0.05</td>
</tr>
<tr>
<td>IgY</td>
<td>4.32×10⁻¹⁷</td>
<td>145</td>
<td>0.24</td>
</tr>
<tr>
<td>Luciferase</td>
<td>2.66×10⁻¹⁷</td>
<td>240</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Figure 9. Schematic view of the multifunctionalized nanoparticles. These particles have been provided with three different functionalities, i.e. oligonucleotides for attachment to the surface, PDS-groups for covalent binding of antibodies and NTA-groups for chelate-coupling of His.tagged enzymes responsible for signal generation.
1.2 Characterization of nanoparticles

The development of bioactive surfaces, whether for analytical work or pharmaceutical purposes, requires exact knowledge of the whole surface composition from the bare surface to each added component. One important parameter is the surface concentration of capturing agents (molecules per unit area). A low surface concentration offers fewer binding sites for the analytes, which may lead to modest detection limits. On the other hand, too high surface concentrations can potentially provide interference between the proteins on the surface affecting the binding to their analytes or give raised levels of unspecific binding. However, interference between neighboring proteins can be significantly reduced by positioning the desired capturing ligands on a highly curved surface, as illustrated in Figure 2 above. In addition, if the ligands are tethered to the surface using modified Pluronic F108 (“F108” in the following text) it is possible to fine-tune the surface concentration of immobilized protein. This can be accomplished by varying the relative amount of functionalized F108 (e.g. PDS derivatized) and unmodified F108.

Analyses of flat surfaces are often problematic. Many times quantification of the substrates immobilized on the flat surface is performed with molecules equipped with some kind of label. However, introduction of label may alter the molecular conformation of the substrate or block its active site. Moreover, the commonly used flat surfaces, such as a 96-hole well of a microtiterplate or a 8 cm Petri dish, expose a relatively small area which will carry minute amounts of attached substrates requiring very sensitive techniques for analysis. Instead the analysis and optimization of the chemical properties of the surface can be carried out on model platforms using nanoparticles [4, 61]. The difference in total surface area of some commonly used surfaces is illustrated in Figure 10. As evident from this picture, the small nanoparticles present a several-fold larger surface area for binding of substrates, which facilitates quantification while at the same time yielding higher analytical accuracy.
There are several ways to determine the surface concentration of material attached to the analytical nanoparticle surface. In general, these methods can be classified in two groups; i.e. as either “direct” or “indirect”. The “indirect” methods determine the amount of attached material from the difference in solute concentration measured before and after the attachment of the molecules to the surface, which often means the small difference between two large numbers and thereby connected considerable uncertainties. Another problem with these methods is that not only the concentration of the firmly attached layer is determined, also loosely adherent material and material adsorbed to the walls of the tubes etc will be included, which will give unrealistically high values of the surface concentration. The “direct” methods estimate the amount of material taken up by the particles. This in turn requires the knowledge of the exact amount of particles in the sample and their surface area available for attachment. However, it is important to keep in mind that the best information is gained through a combination of several independent analysis methods.

1.2.1 Sedimentation Field-Flow Fractionation

The mass-sensitive Sedimentation Field-Flow Fractionation (SdFFF) technique is a highly informative tool for analysis of particles with sizes ranging from 20 nm – 1 μm. It is also a very suitable technique for characterization of the particles’ adlayers as it allows a direct determination of the mass increase from the observed differences in retention between the bare and coated particles [62-63] with high precision and few error sources [64]. In
addition, the mass of the coating is determined on a *per particle* basis, which is advantageous to many other “direct” methods.

In SdFFF are the samples separated according to differences in buoyant mass. The separation takes place under the influence of a centrifugal field in a very thin channel, which is curved to fit inside a rotor basket. The field is applied perpendicular to the channel walls and forces the particles in the sample towards one of the channel walls. This effect is opposed by the particles’ Brownian motion. At equilibrium, the particles will be concentrated in thin layers, whose distance to the wall depends on the mass of each particle. When the system has reached equilibrium a laminar flow of carrier liquid is initiated; it establishes a parabolic flow profile, which means that the velocity near the wall is lower than at the center of the channel. The various components in the sample will thus be eluted according to their susceptibility to the applied field and their diffusion coefficient [65-69]. An SdFFF system is shown in Figure 11.
Figure 11. (a) Picture of SdFFF system operated by the author. (b) Schematic illustration of the sample separation in the curved channel.

The retention ratio, $R$, is experimentally determined as the ratio of the channel void volume ($V_0$) to the observed retention volume ($V_r$).

$$R = \frac{V_0}{V_r} = 6\lambda \left[ \coth \left( \frac{1}{2\lambda} \right) - 2\lambda \right]$$  \hspace{1cm} (1)

From the $\lambda$-value obtained through Eq. 1 the mass of the particles can be calculated according to:

$$\lambda = \frac{kT}{m_a(1 - \frac{\rho}{\rho_c})Gw}$$  \hspace{1cm} (2)

where $k$ and $T$ are the Boltzmann constant and absolute temperature respectively, $\rho_c$ is the density of the carrier liquid, $\rho_a$ the density of the particle, $G$ the strength of the applied field, $w$ the channel thickness and $m_a$ is the mass of the particle. For spherical particles of uniform density $m_a$ can be replaced by the product of volume and density so that $\lambda$ becomes an explicit function of the particle diameter

$$\lambda = \frac{6kT}{d^3 \pi \Delta \rho Gw}$$  \hspace{1cm} (3)
In this equation $\Delta \rho$ is the difference in density between the particle and the carrier liquid. When the mass of a bare particle has been determined, the mass of added layers can be determined through a series of sequential analyses, where for each step:

$$\lambda = \frac{kT}{\sum_{i} m_i \left(1 - \frac{\rho_i}{\rho_p}\right) G_w}$$

Here the subscript $i$ in the sum is allowed to run from 1 for the core particle to as many coatings as the analysis series include.

As evident from these equations the evaluation of particle size or mass requires the exact knowledge of the densities for both the particles and suspension medium. Density measurements can be performed using an oscillating U-tube density meter; such as PAAR’s model DMA60 + DMA602. The measuring principle of this instrument is based on a U-tube which has a resonant frequency that is inversely proportional to the square root of its mass. The introduction of a liquid or a gas causes a change in the frequency due to the mass change of the oscillator. Since the volume of the tube is given; the density of the liquid sample filled into the U-tube can be calculated from its resonant frequency. The density of the PS particles, Pluronic F108-PDS, oligonucleotides and proteins were determined to be 1.053, 1.186, 1.65 and 1.35 g/cm$^3$ respectively.

The SdFFF system used in the work presented in this thesis is a prototype of the commercially available SdFFF system from Postnova (Salt Lake City, USA). The separation takes place under the influence of a centrifugal field in a very thin channel (dimensions $940 \times 20 \times 0.254$ mm) curved to fit inside a rotor basket and positioned 155 mm from the axis of rotation. A PC controls the engine driving the rotor by feedback control. Carrier liquid is fed to the system by a peristaltic pump of type Gilson Minipulse 3, which is controlled by the computer with a predetermined voltage-to-flow relation to keep a constant flow rate. A Sartorious Electronic Precision balance is connected to the computer for continuous measurements of the elution volume (elution weight divided by density of carrier liquid). The signal is monitored by an UV-detector (Pharmacia LKB VWM 2141) held at a fixed wavelength of 254 nm. The output signal from the detector is transferred to the PC. A digital thermometer gives the temperature at the time of start of the experiment.

The choice of carrier liquid is an important factor for precise SdFFF analysis [65]. Particulate samples, such as the core PS particles, are commonly analyzed in an aqueous solution containing a small amount of anionic or non-
ionic detergents (e.g. FL-70), to maintain dispersion of the particles during the separation process. The detergent solutions can generally not be used as carrier liquids for the coated PS particles, as the detergent may affect the attached layers. Instead the analyses of coated particles are performed in aqueous buffer solutions with optimized ionic strength. An increase in ionic strength will reduce the repulsion forces between particles and between the particles and the accumulation wall. These repulsion forces will lead to early elution of the sample particles whose mass will appear smaller than their actual value. Such unwanted ionic effects on the particles in the channel can be determined by varying the ionic strength of the carrier liquid and registering the effect on retention. On the other hand, a high ionic strength may lead to aggregation of the particles in the channel. By adding a small amount of Pluronic F108 to the buffer solution, this type of aggregation may be avoided. For each new sample it is therefore essential to establish that the particles behave ideally, i.e. that determined masses are independent of carrier composition.

1.2.2 Electrical Field-Flow Fractionation

Electrical Field-Flow Fractionation (ElFFF) is based on the same general concept as SdFFF, albeit with an electrical field instead of a centrifugal field driving the particles towards the accumulation wall [65]. The field is applied across a narrow channel sandwiched between two flat (polished) electrodes consisting of two titanium electrodes (395x35x11 mm) spaced by a mylar film (thickness w=140 μm). In this case the expression for \( \lambda \) can therefore be written as:

\[
\lambda = \frac{6kT}{E_{\text{eff}}\mu d}
\]

where \( E_{\text{eff}} \) is the effective electric field strength, \( \mu \) is the electrophoretic mobility of the particle and \( d \) is its diameter. A large part of the voltage drop over the channel is concentrated to the electrode surface, which makes the absolute effective field difficult to specify. To avoid this dilemma the electric field strength can be factored out by comparing the retention of an unknown particle to that of a standard with known diameter \( d_0 \) and mobility \( \mu_0 \):

\[
\frac{\lambda}{\lambda_0} = \frac{\mu d}{\mu_0 d_0}
\]

The ElFFF technique can be used for separation of particles according to their size if their mobility is known, but separation of particles or macro-
molecules can also be based on differences in electrical charge on particles or macromolecules. This difference can be numerically evaluated if the sizes of the particles are known. Therefore a combination of the mass-sensitive SdFFF and the surface charge sensitive E1FFF can provide valuable information for characterization of particles and their adlayer structures.

1.2.2 Differential Scanning Calorimetry

It is of utmost importance to have control over the structural stability of a protein in the different environments where it will be used. Differential Scanning Calorimetry (DSC) is a suitable technique for determination of the thermal stability of proteins [48,50]. It gives a direct measure of the transition enthalpy, ΔH, which is associated with the heat-induced change from native to denatured form. It is therefore a sensitive measure of environmental effects on the protein structure and reflects how the protein is affected by changes in pH, ionic strength and solvent composition, including the presence of structure stabilizing or decomposing components. Structural changes of the protein can thereby easily be quantified by DSC. In a typical DSC experiment the sample is heated over a given temperature range. At some point the sample material starts to undergo a chemical or physical change that releases or absorbs heat. The temperature increases and the process continues to its completion. During the DSC-run the excess heat capacity (C_p) is measured as a function of temperature for a protein- (or other macromolecule-) containing solution, relative to the same amount of buffer solution. Integration of the C_p versus T curve yields the transition enthalpy (ΔH), which is associated with the heat induced shift from the protein’s native to its denatured state, as illustrated in Figure 12.

![Figure 12. Thermal transition of a single domain protein. The area A under the excess heat capacity curve represents the unfolding of the entire protein. The difference in heat capacity between native and unfolded (denatured) states is indicated as ΔC_p in the diagram.](image)
When proteins are attached to solid surfaces, by adsorption or other attachment modes, this may more or less affect their structure and stability. The surfaces’ impact on the protein’s stability can easily be analyzed by microcalorimetry, if the protein under investigation is immobilized on nanoparticles. A conventional microcalorimeter needs at least 0.5 mg protein per measurement and has a cuvette volume of about 1 mL, while the surface concentration of adsorbed protein usually is in the 1-5 mg/m$^2$ range. This means that a minimum of 0.1 m$^2$ of protein coated surface is required for measurement, which apparently only is possible if small particles are used presenting a large surface area per a small volume.

In the work presented in this thesis the ligands have been immobilized on nanoparticulate carriers through end-group activated Pluronic F108. This form of ligand attachment has earlier proven to protect the protein structure from denaturation upon attachment to the solid surface [dsc].

The lack of a measurable transition enthalpy in the thermogram after adsorption could either indicate a complete denaturation of the protein upon contact with the hydrophobic surface or a stabilization of the protein by the surface so that melting takes place at a temperature above 100°C. This has been evaluated by DSC studies in combination with SdFFF of the lectin LOA (*Lathurus odoratus*) from sweet pea [48]. The thermogram in Figure 13a shows LOA free in solution with a well defined transition at 74.4°C and adsorbed to 240 nm Polystyrene particles where no transition occurs in the interval 30-90°C. The presence of adsorbed LOA on the particles was detected by SdFFF and the surface concentration was determined to be 0.997 mg/m$^2$ from the difference in retention volume between the samples with and without adsorbed lectin, as seen in Figure 13b. The LOA lectin has four binding sites for glucose-containing carbohydrates, such as dextran 40 000. If it had retained its activity upon adsorption to the 240 nm PS particles it should at least bind one dextran molecule per lectin. However, as evident in Figure 14b, there is no difference in retention between the LOA coated particles alone and those incubated with dextran, which means that no dextran has been taken up by the adsorbed lectin. The arrow in the same figure indicates where the particles would have eluted if they had bound one dextran molecule per lectin. It can therefore be concluded that the LOA lectin lost all of its native structure and activity in the adsorption process.
Figure 13. (a) DSC analysis of LOA lectin, free in solution (upper trace) and adsorbed to PS particles (lower trace). The absence of thermal transition in the lower trace representing the LOA adsorbed to the particles is indicative of significant loss of protein structure upon adsorption. (b) SdFFF fractograms with and without adsorbed layer of LOA. The mass uptake of adsorbed LOA is calculated from the difference in elution volume. The third line in the fractogram represents the LOA particles after exposure to dextran (Mw: 40 000) to which the LOA is known to have high affinity. If the LOA lectin was active after adsorption to the surface, the complex would have eluted at a point marked with an arrow, assuming specific binding of one dextran per lectin molecule.

1.3 Characterization of nanoparticles on surfaces

1.3.1 Measurement of resistance to shear

In order to evaluate the robustness of the coupling of small particles to a planar surface, the particle-decorated surface can be mounted in a laminar flow cell, as illustrated in Figure 14.
When the particles accommodated in the flow cell are subjected to a laminar flow, the hydrodynamic forces acting on them will mainly be drag forces. Hence, the force $F$ can be expressed by Stoke’s law:

$$ F = 6\pi \eta r U $$

(7)

assuming that the particles are spherical with the radius $r$ and the liquid with the viscosity $\eta$ has the flow velocity $U$. In a thin channel the initiated flow will have a parabolic flow-profile, which means that the flow rate will be higher in the center of the channel than closer to the walls, as illustrated in Figure 15b. The flow velocity at a certain level $h$ from the center of the flow-channel can be determined as

$$ U_h = \frac{3Q}{wb} \left( 1 - \frac{h^2}{b^2} \right) $$

(8)

where $Q$ is the volumetric flow rate, $w$ and $b$ are the width and thickness of the channel. To determine the shear forces acting on the particles at a distance $d$ from the channel wall the following equation can be used [70-71]

$$ F_d = F_d^* (6\pi \eta r U_c) $$

(9)

$F_d^*$ is a function of the ratio of the distance between the center of the particle to the wall and the particle radius. This value is tabulated in reference [71]. $U_c$ is the flow velocity at the level of the particle center calculated according to equation 8.
1.3.2 Microscopy

Light microscopy is a useful method to in real-time monitor the presence of particles remaining on the surface after mounting in the flow-cell and subsequent initiation of flow. Although the resolution of an ordinary light microscope is too low to get a close up view of sub micron particles, the difference between a clean surface and a surface covered with nanoparticles is clearly distinguishable.

On the other hand, to get a high resolution picture of the particle-coated surface, scanning electron microscopy (SEM) is a more suitable method. From the SEM micrograph the number of particles attached to a given surface area after careful washing can be determined. In addition, information about how the particles are distributed at the surface after deposition and subsequent washing can be obtained.

1.3.3 Evaluation of fluorescence-based arrays

After deposition of the nanoparticles equipped with capture agents in a predetermined array-pattern, they can be exposed to their labeled target molecules. In case the label is a fluorophore, the signal from the array.scanner is received by photo-exciting the fluorophore and then collecting the emitted photons by a photon-detecting device. Most laser-based scanners use photomultiplier tube (PMT) for detection. In coming photons are converted into electrons at the photo emissive cathode by the photoelectric effect. The electrons are then accelerated towards a series of electrodes having increasingly positive potential. Thus, an additional number of electrons are generated at each electrode resulting in amplification of the signal as the electrons are traveling through the detector. This makes the PMT useful for detection of low light intensities, such as that generated by fluorescence [72]. Since the signals in the nanoparticles array are generated by a large number of small particles accommodated at the surface, the total signal for each spot is received by integrating the intensity over the spot area.

The signal-to-noise ratio is a quantitative measure of how well the system resolves the proper signal from the background noise and can be determined as

$$\text{signal} - \text{background}$$

$$\text{standard deviation of background}$$
A signal-to-noise of 3 is generally considered as the lower limit for accurate detection. Although signals lower that this value may be detectable, they will not be accurately quantified.

1.4 Array and POCT systems

1.4.1 Microarrays

Advances in genomics and proteomics have increased the demand for high through-put screening methods that allow analysis of thousands of parameters simultaneously. Microarray technology offers one such approach. DNA microarrays have been widely used over the last decade for monitoring gene expression. Yet, information from gene expressions can not be used to predict protein abundance and activity as the mRNA transcription level is not always correlated with the protein translation. [73-76]. Currently the most important indicators in for example cancer diagnostics are single biomarkers, such as prostate-specific antigen (PSA). The new trend is to profile the tumours and produce “molecular fingerprints” or signatures, based on larger numbers of variables. Obviously multiple ligands for a given cancer type will provide more reliable clinical diagnostics compared to those received from tests where only one ligand is used [77]. Such fingerprints will be very complex and require multiple ligands.

Miniaturized formats for high throughput screening of proteins are therefore rapidly evolving, but have not proven to be as straight-forward to design and operate as the corresponding DNA arrays [78-81]. This is largely because proteins have much more complex structures and functions compared to DNA, and furthermore that there are no easy PCR-amplification methods for proteins. This lack necessitates using highly sensitive signaling systems for the detection of minute amounts of analytes from complex media. One way to intensify the signal is by increasing the local concentration of analyte. This can be done through attachment of the capture molecules on small nanoparticles, which in turn are immobilized on a planar read-out surface, an approach that is described in more detail in paper III.

Moreover, the information from mRNA transcripts does not include post-translational modifications, such as glycosylation, phosphorylation and acetylation. Protein glycosylation plays a crucial role in many important biological processes such as protein folding, cellular adhesion, interaction, rec-
ognition, and signalling. Changes in the glycan structure of the protein usually have substantial effects on its activity and function and have shown to correlate with the development and progression of many diseases [82-87]. In addition, many recombinant proteins and FDA approved biopharmaceuticals are glycoproteins, with a correct glycosylation being of utmost importance for their desired function. A method for fast, systematic and qualitative analysis of glycoproteins is therefore very valuable for many applications. The currently most common techniques for glycosylation analysis, such as mass spectrometry and affinity chromatography, are often time-consuming, include elaborate sample preparations and are not always suited for complex samples. Instead an array constituted of a panel of lectins with affinity for different carbohydrates can be used for glycoprotein profiling [88-89]. Lectins are carbohydrate binding proteins with one or more binding sites for its specific sugar moiety [90-92]. They have been used in a large variety of different assays. To get the advantages of nanoparticles as platforms combined with the carbohydrate specificity of a panel of different lectins, we are aiming to develop a miniaturized array of lectin coated nanoparticles. This array will be designed for glycoprotein profiling and evaluated using pattern recognition techniques. A schematic view of the intended lectin array is illustrated in Figure 16.

Figure 15. Description of the nanoparticle microarray platform.
1.4.2 Point-of-Care Testing

An important application of biosensors is in “point of care testing” (POCT), also referred to as near patient or bedside testing. The purpose of POCTs is diagnostic testing that is performed on site and provides test results to the patient almost immediately. A reliable POCT will therefore help the physician to take appropriate action, whether this means to start a selected treatment or to send the patient home. The increased use of POCT will lead to improved clinical and economic outcomes by reduced hospital stays, faster start and optimization of treatment, fewer complications, better optimized drug treatment and less inappropriate use of drugs [93-96]. POCT is also ideal for veterinary practices, as this type of diagnostic tests is especially well-suited for field-use.

The instrumentation for POCT can be classified in two groups: small bench-top analysers and hand-held single use devices. Typical bench-top analysers for the doctor’s office are blood gas and electrolyte analysers measuring e.g. pH, pCO2, pO2, sodium, potassium, glucose, lactate, haemoglobin and haematrocit. The hand-held disposable devices are simple on the outside, but include quite complex biosensors on the inside and are developed using microfabrication techniques [97]. These low-cost diagnostic devices are particularly well suited for self-tests, such as routine monitoring of blood glucose concentrations in patients with diabetes or for osteoporotic patients taking drugs to improve bone mineral density. They can also be used in point-of-patient care for disadvantaged and under-served populations in both developed and developing countries.

In our group we are developing a prototype of a diagnostic device for POCT equipped with a nanoparticle-based detection system. At this point the sensor’s microfluidic chip is designed for the testing of CRP levels in blood as a proof of principle demonstration, but it could easily be exchanged to permit testing of other biomarkers. The proposed chip is presented in Figure 16.
Figure 16. Illustration of the proposed POCT device equipped with antibody-coated nanoparticles for capture of CRP markers from the blood sample of a patient. Specific binding of CRP is detected by a light signal generated through bioluminescence. The light signal is pictured by the mobile phone camera and is to be sent over the net to the doctor’s office for evaluation.

The intention is that a blood sample, collected from the patient, will be applied to the sample wells on the chip. Small built-in micropumps push the sample over to the analytical part, where the target molecules (i.e. CRP) are captured by antibody-coated nanoparticles immobilized on the chip surface. After washing driven by the micropumps, the specific binding of targets will generate a light by bioluminescence, which in turn can be detected by a CCD-camera or similar devices. Bioluminescence assays are capable of very low detection limits with relatively simple detection systems and have proven to be very sensitive methods for measuring enzyme labels [98]. The bioluminescence measurement is also robust and generally free of background signal, which makes it a suitable for “point of care testing” POCT techniques where it is important that the quantification is based on a reproducible signal that is generated within a few minutes after sample application.
2. Results and discussion

2.1 Paper I: Shifts in polystyrene particle surface charge upon adsorption of the Pluronic F108 surfactant

In the present study we have used Sedimentation Field-Flow Fractionation (SdFFF) and Electrical Field-Flow Fractionation (ElFFF) in combination to gain information about the close packing and structure of adsorbed layers of Pluronic F108 to PS latex spheres. In previous characterization studies of Pluronic adsorption a variety of different methods have been used including surface plasmon resonance (SPR) [99], fluorescence and isotope labelling [100], X-ray photoelectron spectroscopy (XPS) [101-102], photon correlation spectroscopy (PCS) and field-flow fractionation (FFF) techniques. The FFF is advantageous compared to many other techniques as it allows direct determination of the mass uptake on a per particle basis, without any prior labelling or washing procedures, since it leaves the particles well washed and free from loosely adherent material. A series of seven different concentrations of Pluronic solutions, ranging from 0-2 mg/mL, were allowed to adsorb to suspensions of 1% solid 200 nm PS particles. The surface concentration of adsorbed Pluronic was accurately determined from the SdFFF analyses. The same samples were also analysed by ElFFF and the values for \( \lambda_0/\lambda \), expressed by equation 6 in section 1.2.2, were determined from the observed retention times. The \( \lambda_0/\lambda \)-values from the ElFFF analysis are plotted as a function of the surface concentration of adsorbed Pluronic F108, measured by SdFFF, in Figure 17.
Figure 17. Experimental values for λ_d/λ (●) from ElFFF and μ/μ₀ (-) calculated with the proposed adsorption model plotted as a function of the surface concentration of adsorbed Pluronic F108 determined by SdFFF.

From this figure it is clear that the ElFFF retention is decreased upon increased surface concentration of adsorbed Pluronic on the particles. This is due to decreasing electrophoretic mobility, μ, as the negatively charged PS surface becomes shielded by the electroneutral surfactant. However at one point the electrophoretic mobility reaches a plateau value and does not undergo further decrease, even though the amount of adsorbed Pluronic increases. A simple model was set-up, as an effort to explain these data.

Only part of the surface is assumed to be available for adsorption of Pluronic. Initially, before maximum coverage (θ_{max}) is reached, both the PPO and PEO units will adsorb to the surface in a “loop and train conformation” as described in Figure 6c. In this condition the coverages of PPO (θ_{PO}) and PEO (θ_{EO}) can be described as:

\[ \theta_{PO} = n \frac{n_{PO} a_{PO}}{\pi d^2} \]  

(10)

\[ \theta_{EO} = 2n \frac{n_{EO} a_{EO}}{\pi d^2} \]  

(11)

\[ \theta = \theta_{PO} + \theta_{EO} \]  

(12)

Here n is the number of moles of adsorbed surfactant, \( n_{PO} \) and \( n_{EO} \) is the number of PO and EO units in a block of the polymer, \( a_{PO} \) and \( a_{EO} \) are the surface areas covered by one mole of PO and EO units respectively, and d is
the diameter of the particle. The factor 2 appearing in the $\theta_{EO}$-equation comes from the fact that there are two EO chains in the Pluronic molecule.

The electrophoretic mobility of the sample is assumed to be proportional to the amount of free surface:

$$\frac{\mu}{\mu_0} = 1 - \theta$$  \hspace{1cm} (13)

When $\theta_{\text{max}}$ is reached, i.e. the available surface area is covered, additional Pluronic can only be adsorbed through a competition between the more hydrophobic PPO units and the less hydrophobic PEO units. Hence, the total coverage remains constant, while $\theta_{PO}$ still increases according to equation 10. The remaining part of the surface area is covered with PEO units, according to:

$$\theta_{EO} = \theta_{\text{max}} - \theta_{PO}$$  \hspace{1cm} (14)

The amount of adsorbed mass of surfactant per unit of surface area, $\Gamma$, can finally be calculated as:

$$\Gamma = n \frac{n_{PO} m_{PO} + 2n_{EO} m_{EO}}{\pi d^2}$$  \hspace{1cm} (15)

where the $m_{EO}$ and $m_{PO}$ are the molar masses of the EO and PO units.

The solid line in Figure 17 shows $\mu/\mu_0$ versus the surface concentration of adsorbed Pluronic, as calculated with this model, which compares well with the experimentally determined $\lambda_0/\lambda$. Hence, both the model and experimental data show that the PEO chains of the Pluronic molecule only will extend into the aqueous phase if there is no free space available on the surface. When the Pluronic surfactant is adsorbed from a solution with a low concentration, the EO units will contact and shield the hydrophobic PS surface as long as there is some space available. However, at higher concentrations of the Pluronic solution the strongly hydrophobic PPO units will competitively displace the weakly hydrophobic PEO chains at the PS surface and force them to extend outwardly until the surface is fully saturated. Through this exchange mechanism more Pluronic molecules can be packed in to the surface without causing further shielding of the surface charge.
2.2 Paper II: Surface attachment of nanoparticles using oligonucleotides

In most protein arrays recognition and detection depends on the amount of analyte available. One approach is therefore to use nanoparticles as solid support and thereby increase the surface area available for binding and at the same time gain the positive effects of the curved surface with regard to steric hindrance. For many applications the possibility to position reactive centers, such as particulate bioreactors, at pre-determined locations on a readout surface is highly desirable. In these cases it is important that the attached substrates remain at their position during washing and handling. This work is a demonstration of the possibility to use oligonucleotides for the attachment of polystyrene nanoparticles to a planar polystyrene read-out surface. There are $4^n$ possible $n$-meric oligonucleotides, which readily will hybridize with their respective complementary sequence, each constituting a unique spot for binding. Hence, coupling through oligonucleotide hybridization offers large possibilities for specific deposition of different components to the surface.

Thiolated decamers of guanine and cytosine oligonucleotides were covalently attached to PDS-derivatized Pluronic F108 adsorbed to the surface of the nanoparticles and to the planar surface, respectively. Oligomers of G-C have the advantage of providing three hydrogen bonds compared to only two bonds between adenine and thymine, which was decisive for this study. The size of the bare particles as well as their coating layers were analysed by SdFFF, and the fractograms obtained after injection of bare, F108-coated and F108-oligonucleotide coated particles are displayed in Figure 18.

Table 2. Uptake of F108 and F108-dG to 239 nm PS particles quantified by SdFFF and UV-spectroscopy.

<table>
<thead>
<tr>
<th>Bare particles (239 nm)</th>
<th>Mass/particle (g/particle)</th>
<th>No of molecules/particle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$SdFFF$</td>
<td>$UV$</td>
</tr>
<tr>
<td>F108</td>
<td>$4.5 \times 10^{16}$</td>
<td>18600</td>
</tr>
<tr>
<td>dG</td>
<td>$7.9 \times 10^{17}$</td>
<td>13100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14900</td>
</tr>
</tbody>
</table>
Figure 18. SedFFF fractograms obtained after injection of uncoated 239 nm PS particles, F108-PDS coated particles and F108-PDS coated particles to which dG oligonucleotides had been attached.

The surface concentration of each component was determined from the observed shift in retention due to the mass increase caused by the adsorption of Pluronic F108 and the subsequent attachment of oligonucleotides. When thiolated oligonucleotides are coupled to PDS-groups pyridine-2-thiones will be released. The concentration of cleaved off pyridine-2-thione in the supernatant (after washing the particles by means of centrifugation) is thereby equivalent to the concentration of oligonucleotides coupled to the particle. This amount can be quantified by UV-absorbance at 343 nm using 8080 M$^{-1}$ cm$^{-1}$ as the extinction coefficient. As seen from the results compiled in Table 4 there is a reasonable agreement between the SedFFF measurements and the values obtained from UV-spectroscopy. In addition, from this table it can be concluded that about 75 % of all the adsorbed F108 molecules have taken up an oligonucleotide, which in turn will be available for coupling to its complementary sequence.

Flat surfaces were similarly coated with oligonucleotide-modified Pluronic F108, only this time the tag was complementary to that on the particle surface which allowed hybridization to take place and lock the particle to the surface. In order to evaluate the robustness of this coupling, the surfaces with their particle coating were assembled in a parallel plate flow cell, as described in section 1.3.1 above. A laminar flow of aqueous buffer was applied and gradually increased from 2-6 mL/min while the process was followed in real time using an optical microscope. When the flow was initiated some loose particles immediately left the surface, but subsequently there was
no further discharge of particles, even at the highest flow rate. After the flow-cell experiment the presence of particles coupled to the surface was confirmed by electron microscopy, as seen from the SEM micrograph in Figure 19.

Figure 19. SEM micrograph of the particles attached to the surface after shear wash in the flow cell experiment.

The shear forces acting on the particles at 6 mL/min. were estimated to be between 0.44 and 0.65 pN, assuming that the particles’ position in the channel lies between 120 nm (one particle radius) and 220 nm from the wall. However, this shear is far beyond what is practically used in the thin, flow-based array systems used today.

2.3 Paper III: Nanoparticle decorated surfaces with potential use in glycosylation analysis

In this work we describe a method with potential use in glycoprotein profiling, based on lectins as capture probes immobilized on nanoparticles. The lectin-coated particles, in turn, are coupled through oligonucleotide hybridization to a planar analytical surface for read out. As a proof-of-principle demonstration the mannose-binding lectin Concanavalin A (Con A) has been used as a model, immobilized at the particle surface through Pluronic F108-PDS. The particles are further functionalized by co-attachment of pentamers of dG oligonucleotides, while the complementary dC oligonucleotides are attached to the planar surface to allow coupling of the particles to the surface. This method, described in Paper II, has proven to be a robust way to firmly attach particles to a surface.
In the development of bioactive surfaces it is important to have control of the exact surface composition, from the size of the bare particles to the surface concentration of each added component. The particles were therefore carefully characterized by SdFFF. As seen from the fractograms in Figure 21 the successive attachment of each component (F108-PDS, dG and ConA) causes significant shifts in retention from which the mass of the adsorbed or attached components were calculated. The precision in the surface concentration measurements is $9.4 \times 10^{-18}$ g/PS particle, which in this case is equivalent to 55 ConA molecules. The uptake of F108-PDS and dG oligonucleotides were also verified by UV-spectroscopy from the concentration of released pyridine-2-thione after cleavage with DTT, as well as from the coupling of dG to the F108-PDS at the surface, and is in good agreement with the SdFFF analysis. All mass determinations are summarized in Table 3. For evaluation of the activity of the ConA immobilized at the particle surface, a suspension of ConA-coated particles was allowed to bind a glycoprotein ligand (ovalbumin), for which it is known to have high affinity. The mass increase due to specific binding of ovalbumin was determined by SdFFF, as seen from the fifth trace in the fractogram in Figure 20. Since ConA has four sites per molecule for binding of specific ligands, and since almost three of these have captured an ovalbumin molecule, it is evident that the immobilized ConA has retained its activity.

Table 3. Mass determination of bare particles and their coating layers.

<table>
<thead>
<tr>
<th>Bare PS: 239.4 ±1 nm</th>
<th>Surface conc (g/particle)</th>
<th>No of molecules/particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>F108-PDS</td>
<td>$3.5 \times 10^{-16}$</td>
<td>14400 ± 400</td>
</tr>
<tr>
<td>dG</td>
<td>$5.2 \times 10^{-17}$</td>
<td>6200 ± 1000</td>
</tr>
<tr>
<td>ConA</td>
<td>$1.2 \times 10^{-16}$</td>
<td>700 ± 55</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>$1.5 \times 10^{-16}$</td>
<td>2000 ± 125</td>
</tr>
</tbody>
</table>
Figure 20. SdFFF analysis of bare and coated 239 nm PS particles.

A planar polystyrene surface in the form of a microscope slide was prepared with dC oligonucleotides tethered to the surface via the F108 linker, before deposition of the particles coated with both ConA and complementary dG oligonucleotides. A controlled accommodation of the particles at the surface was accomplished using a GeSIM nanoplotter. It allowed a fast and systematic deposition of equally sized droplets of 6.4 nL in a 5×5 spot pattern. Scanning electron microscopy evaluation of ten sample images showed that the particles were deposited in spots of equal size with an average diameter of 309 ± 13 μm. The number of particles coupled to the surface was estimated to 15 per μm², which translates to about 1.06 × 10⁶ particles in each spot. Since the number of ConA molecules on each particle was determined by SdFFF analysis to be 700, one can assume that there will be about 7.4 × 10⁸ available ConA capture probes per spot of 0.07 mm², each capable of binding 2.8 ligands of the same size as ovalbumin.

For assessment of the selectivity of the immobilized ConA-coated particles a series of four different well-characterized glycoproteins, i.e. ovalbumin, fetuin, thyroglobulin, and an artificially mannosylated albumin (Man-BSA) [103-105], were selected together with an unglycosylated protein, HSA, as a negative control. All five analytes were labelled with Alexa Fluor 680 fluorescent dye for detection. Each row of 5 spots with particles was then exposed to one of the labelled analyte solutions (1.3–5 ng analyte/sample) and incubated for 20 min. followed by repeated washing. The slides were placed in a GenePix scanner for measurement of the signal intensity from the five spots in each row.

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ConA is known to have high affinity for mannose-containing carbohydrates and preferentially binds to mono and bi-antennary structures, while it has less affinity for branched structures of the tri- and tetra- antennary types [106]. This is in good agreement with the results presented in Figure 22 and Table 4. The artificially glycosylated albumin (Man-BSA) has several well-exposed mannose residues. As expected, this analyte shows the highest signal intensity, which is indicative of the strongest binding. Ovalbumin that has carbohydrates of either high-mannose or hybrid type, and thyroglobulin with its c:a 10 % carbohydrates of which 50 % are of bi-antennary high-mannose type, both show strong bindings. Fetuin, on the other hand, with glycans primarily of the tri-antennary type shows significantly lower fluorescent signals, while HSA, used as a negative control, does not appear to bind to judge from its much weaker signal.

Table 4. Average background corrected signal intensity from five spots in each row for the five analytes in Figure 21.

<table>
<thead>
<tr>
<th>Row</th>
<th>Sample</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HSA</td>
<td>171 ± 108</td>
</tr>
<tr>
<td>2</td>
<td>Ovalbumin</td>
<td>1301 ± 540</td>
</tr>
<tr>
<td>3</td>
<td>Fetuin</td>
<td>714 ± 144</td>
</tr>
<tr>
<td>4</td>
<td>Thyroglobulin</td>
<td>1311 ± 468</td>
</tr>
<tr>
<td>5</td>
<td>Man-BSA</td>
<td>3765 ± 1157</td>
</tr>
</tbody>
</table>
Figure 21. Binding of immobilized ConA particles to four different glycoproteins (ovalbumin: row 2, fetuin: row 3, thyroglobulin: row 4 and mannosylated BSA: row 5) and one unglycosylated protein used as negative control (HSA: row 1).

The sensitivity of the ConA array was determined by incubation of the immobilized ConA particles with four different concentrations of Ovalbumin (17 ng/mL - 17 μg/mL), this time labelled with Cy5 and an analyte-free sample as negative control. As seen from the results presented in Figure 22, the lowest concentration of 17 ng/mL has a noticeably higher signal intensity compared to the negative control. The relatively small standard deviation in intensity of the ten replicates measured for each concentration of ovalbumin is pointing to a good reproducibility of particle attachment within each spot.

Figure 19. Evaluation of the sensitivity of the ConA-array using different concentration of ovalbumin. A series of 10 replicates was made for each concentration of ovalbumin and the intensities were corrected for background fluorescence.
The results presented in this work clearly show that ConA immobilized on nanoparticles has retained activity and the ConA-coated particles can after coupling to a planar read-out surface selectively bind to a variety of glycoproteins. We therefore propose that it would be possible to use the same strategy with a panel of different lectins for analysis of more complex samples.

2.4 Paper IV: Characterization of surface-modified nanoparticles for in vivo biointeraction. A Sedimentation Field-Flow Fractionation study

This study demonstrates how SdFFF can be used for characterization of nanoparticles prepared for in vivo applications to obtain information about size distribution, charge-related effects and mass-uptake of added components. The particles were intended to be parts of a model system for drug delivery to so called Payer’s patches and were therefore modified to allow crossing of the intestinal epithelium via interaction with specific β-integrin receptors presented at the surface of the epithelium. The transport of a polystyrene particle across the epithelium can be increased several-fold by the presence of RGD peptides (arginine-glycine-aspartic acid) at the particle surface [107-109]. Therefore the polystyrene particles used in this work were equipped with thiolated RGD-motifs coupled to the particle surface via pyridyl disulfide derivatized Pluronic F108 (F108-PDS) preadsorbed to the surface. Three types of fluorescent particles, with a nominal diameter of about 200 nm, were selected as possible candidates for drug delivery vehicles. All three samples were analysed by SdFFF with respect to size and size distribution prior to surface modification. Particles of type 1 and 2 were determined to be 226.3 and 242.3 nm in diameter respectively, as seen in Table 6. These samples also showed relatively narrow size distributions, while the third sample displayed significant deviations from uniformity as evident from the SdFFF fractogram in Figure 23a. Instead of being uniform, this sample appeared to contain four different populations with sizes ranging from 201 nm – 320 nm, listed as 3.1, 3.2, .3.3 and 3.4 in Table 5. A SEM image (Figure 23b) confirmed the polydispersity of the third sample.
Table 5. Size and mass uptake of Pluronic-F108 by three types of commercially available fluorescent latex particles determined by SdFFF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (bare PS)</th>
<th>Surface conc. F108-PDS (mg/m²)</th>
<th>No F108-PDS molecules/particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fluospheres, carboxylated</td>
<td>226.8</td>
<td>1.3</td>
<td>8900</td>
</tr>
<tr>
<td>2. Fluoresbrite, plain PS</td>
<td>242.3</td>
<td>2.2</td>
<td>14700</td>
</tr>
<tr>
<td>3-1. Fluospheres, sulphated</td>
<td>201.1</td>
<td>2.3</td>
<td>11800</td>
</tr>
<tr>
<td>3-2.</td>
<td>254.6</td>
<td>2.4</td>
<td>20500</td>
</tr>
<tr>
<td>3-3.</td>
<td>290</td>
<td>2.6</td>
<td>28400</td>
</tr>
<tr>
<td>3-4.</td>
<td>320</td>
<td>3.0</td>
<td>39500</td>
</tr>
</tbody>
</table>

Figure 23. (a) SdFFF analysis of the bare and F108-PDS coated sulfate-modified Fluosphere particles. The four different peaks represent four different particle distributions. (b) SEM micrograph of the same (unfractionated) particles verifying the polydispersity of the sample.

All three particle samples were prepared with Pluronic F108-PDS to allow coupling of thiolated RGD-peptides while at the same time protecting the hydrophobic surfaces from unspecific adsorption of undesired material during the in-vivo experiments. The surface concentration of adsorbed F108 was determined by SdFFF from the shift in elution volume towards stronger retention, as illustrated in Figure 23a. To judge from the mass uptake determinations compiled in Table 6, the carboxylated particles in sample 1 are less prone to adsorb the F108 compared to the plain PS particles in sample 2. In addition, from these data it appears that F108 adsorbs to different degrees to the four size populations in sample 3, as the smaller particles present a lower surface concentration than the larger ones. This observation is in accordance with previous studies where smaller particles adsorbed Pluronic
F108 in a less crowded arrangement than the larger particles [63]. However, the polydispersity of particle sample 3 made them unsuitable as model substrates in the continuing study.

Samples 1 and 2 were subjected to further peptide decoration to allow passage through the intestinal epithelium after specific interaction with the surface-exposed integrins. Control particles were also prepared, which were identical to the test particles except that they carried an RGE peptide instead of the RGD sequence recognized by the integrins. The mass uptake of both types of peptides were analysed by SdFFF and verified by UV-spectroscopy for the two particle types. As seen in Table 6, equal amounts of the two different peptides RGD and RGE are attached to the respective particle type, which was expected as the peptides are very similar in both size and charge. However, what was not expected was the observed difference in the amount of peptide attached to the two particle types. Since there are a maximum of one PDS-group per F108 molecule, the number of peptides that can be attached to the particles is less than the number of Pluronic molecules. The plain PS particles, Fluoresbrite, in sample 2 had about one peptide attached to every 2nd F108 molecule, which can be considered as a normal degree of coupling for peptides of this size. On the other hand, the 1st sample, with carboxylated particles called Fluospheres, had taken up far more peptides than available PDS-groups at the surface.

Table 6. Mass determinations of the bare particles and their coating layers.

<table>
<thead>
<tr>
<th></th>
<th>Diam. (nm)</th>
<th>Amount ads (g/particle)</th>
<th>Surface con. (mg/m²)</th>
<th>No of molecules/particle</th>
<th>SdFFF</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluospheres (carboxylated)</td>
<td>226.8</td>
<td>2.17×10⁻¹⁶</td>
<td>1.3</td>
<td>8900±400</td>
<td>9700</td>
<td></td>
</tr>
<tr>
<td>+F108-PDS</td>
<td></td>
<td>4.64×10⁻¹⁷</td>
<td>0.28</td>
<td>36700±2850</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+RGD</td>
<td></td>
<td>4.51×10⁻¹⁷</td>
<td>0.28</td>
<td>35200±2800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoresbrite (plain PS)</td>
<td>242.3</td>
<td>3.6×10⁻¹⁶</td>
<td>2.2</td>
<td>14700±400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+F108-PDS</td>
<td></td>
<td>1.0×10⁻¹⁷</td>
<td>0.05</td>
<td>7900±2850</td>
<td>8100</td>
<td></td>
</tr>
<tr>
<td>+RGD</td>
<td></td>
<td>1.0×10⁻¹⁷</td>
<td>0.06</td>
<td>8600±2800</td>
<td>8100</td>
<td></td>
</tr>
</tbody>
</table>

These particles also showed an abnormal behaviour in the in vivo experiments, as seen in Figure 24. The left diagram (A) illustrates the transport of bare PS particles (sample 2) with and without the RGD-motif across the Peyer’s patches epithelium compared to the surrounding Villus epithelium. As evident from this picture, the plain PS particles with RGD-peptides ex-
posed at their surface are crossing the Payer’s patches epithelium to a much larger extent compared to those without. This is in contrast to the carboxylated particles (sample 1) that had taken up relatively large amounts of RGD-peptides, but did not show an increased ability to cross the epithelium compared to the particles without RGD, as seen in the right diagram (B) in Figure 24.

Figure 24. Particle transport in cell culture models of the Payer’s patches epithelium (PP) and Villius epithelium(VE) for bare PS particles (A) and carboxylated particles (B) with and without RGD peptides. (Note the different scales on the y-axes)

Most likely the carboxylated particles’ abnormal behaviour in vivo can be related to the unusually low surface concentration of protective Pluronic coating and the resulting large uptake of peptides. For example, the low surface density of Pluronic F108 indicates that the particles only are partially shielded by surfactants. Then a large number of negative carboxylic groups will be available to form relatively weak hydrogen and ionic bonds with the peptides. Hence, a possible explanation to the low degree of particles passing over the membrane could be that the loosely adherent RGD-peptides were competitively displaced by serum components from the environment of the epithelial cells in the cell culture. Due to their size the released RGD-peptides would thereby be free to compete favourably with the particle-bound ones. Consequently, very few particles passed the epithelium.

This study clearly illustrates the importance of careful characterization of both the starting material and the functionalized surface before using particles in biological applications. In the characterization of this type of complex particle samples the SdFFF showed itself as an invaluable tool.
2.5 Paper V: Bioluminescence-based ELISA for sensitive analyte quantification

The present study is part of a feasibility study aiming at developing a robust point-of-care device for sensitive diagnostics. Here we explore a sandwich ELISA strategy in which the “second antibody” is conjugated to pyruvate kinase generating the necessary ATP to be used by the subsequent luciferin-luciferase reaction responsible for the light production. First the light emission as a measure of the ATP concentration was validated by adding a mixture of luciferin/luciferase to five different concentrations of ATP. These experiments proved linearity between the cumulative number of photons emitted and the ATP concentration. Hence, the system was shown to accurately report the concentration of ATP in the sample.

To examine the use of a bioluminescence-based ELISA, a test antibody and its antigen were selected; in the present case the pair consisted of an egg antibody (IgY) [110-112] with high affinity for bovine serum albumin (BSA). In this type of immunoassays the stability and activity of the conjugates between antibody and signal generating enzyme is crucial. Before preparation of the conjugates, the kinase was reacted with different concentrations of SPDP-reagent to elucidate potential effects on the kinase activity. As seen from Figure 25, the enzymatic activity was fairly well retained after interaction with 3 mM SPDP, while a 10-fold increase of the reagent completely abolished the activity. Pyruvate kinase has four lysyl ε-amines and four sulfhydryl groups that are essential for its activity [113]. Since the bifunctional SPDP reagent has reactivity towards both groups it must be added with caution, so that the derivatization primarily involves the kinase amines. The preparation of conjugates also included activating the IgY antibody with SPDP followed by a reductive cleavage with DTT to set free the thiols and enable coupling of kinase to antibody. Under the chosen reaction conditions the coupling only had minor effects on the kinase activity, and the conjugates could be stored in a refrigerator (+4°C) for more than a week without significant loss of activity.
Figure 25. Activity of the SPDP-modified pyruvate kinase resulting from reaction with the coupling reagent in the indicated concentrations.

The conjugates’ ability to bind its antigen was determined by preparing a test material with the model antigen (BSA) tethered to a particulate solid phase via a Pluronic linker. Prior to exposure to the antibody conjugates, the protein surface coatings of the particle suspension were precisely quantified, to ensure the necessary sensitivity criteria. From the SdFFF analyses in Figure 26a each particle with a size of 240 ± 1 nm, was found to carry 700 ± 50 BSA molecules. Similarly sized aliquots of the well-characterized BSA-particles were added to a concentration series of the conjugates and allowed to react. After careful washing, the particles were assayed for enzymatic activity by addition of a reaction cocktail containing the luciferin/luciferase reagent. The results, presented in Figure 26b, reveals that 700 nmol of conjugate is needed to fully saturate the surfaces of $7.5 \times 10^{10}$ BSA containing particles. This is substantially more than originally expected. Presumably, the conjugates formed large complexes of more than two protein molecules, which also were indicated by SEC-MALS analysis of the same conjugates. These large complexes are quite bulky and therefore a bit restricted in finding their antigen.
Figure 26. (a) SdFFF analysis of bare PS particles (---), F108-PDS coated particles (---) and particles with immobilized BSA (---). (b) Particle ELISA: $3.75 \times 10^{10}$ particles with the composition PS-F108-BSA to which additions of the antibody conjugate PK-IgY (anti BSA) have been made. The formed complex is assayed after careful wash.

A dilution series of the completely conjugate-saturated particle suspension was prepared in order to test the detection limit of the bioluminescence signal provided by the conjugates. As anticipated the bioluminescence signal decreased with decreasing number of particles, eventually reaching a level indistinguishable from the background. The results from this assay, seen in Figure 27a and b, showed that the limit of detection for the system was 2.5 pmol particle-bound BSA and 13.5 fmol of PK-IgY conjugate. Although the conjugates are only six times larger than the BSA molecules, there were 22 BSA molecules per conjugate. Most likely, spacious conjugate complexes are occupying large parts of the surface making it less accessible for further binding and thereby reducing the number of conjugates bound to the surface. Yet, the particles are producing a strong and stable signal that begins immediately after substrate addition, suggesting that this arrangement will be suitable for immnosensing.

Figure 27. (a) BSA coated particles in different amounts; assayed following additions of one and the same amount of antibody conjugate (700 nmol). (b) Light emission curves representative of the amounts of conjugate associated with the data points in (a).
In addition, the selectivity of the IgY antibody was tested in a particle ELISA. In this set-up, the capture antibody was immobilized on the PS particle via the Pluronic F108 linker, as previously described for the BSA-antigen. The particles were then exposed to either BSA analytes, for which they have high affinity or to another serum protein, CRP, used as a negative control. After addition of PK-IgY conjugates, and careful washing, the particles were assayed for enzymatic activity in a coupled reaction with the luciferin/luciferase reagent. A clear difference in light intensity was seen between the particles that specifically had captured BSA and those that had been in contact with the negative control, as seen in Figure 28.

![Figure 28. Testing the selectivity of the particle ELISA using the conjugates. The dark line represents the BSA sample and the light grey line is the negative control. The intensity is in arbitrary units.](image)

However, there is a minor unspecific uptake of the negative control, as antibodies (like most other proteins) have some avidity to other proteins in high concentrations.
Nanoparticles offer some attractive advantages as platforms for ligand binding, as they increase the surface area available for binding compared to a flat surface, they present very high local concentration of specific binding sites for analytes and the curved surface facilitates capture analyte. Another important factor is that the particles can be prepared with desired functionalities and accurately characterized prior to use so that surface composition of the particles is readily controlled. This makes them highly suitable as model surfaces for many bioanalytical applications.

From the characterizations of a variety of different particles presented in this thesis, it is clear that the SdFFF technique works as a very sensitive microbalance. In these studies the SdFFF has provided accurate information about the size distribution, mass uptake and charge-related effects associated with surface modification of polystyrene particles for diagnostic as well as in vivo use. The surface concentration of multi-layered particles is determined with a precision of the measurements at the attogram level. Furthermore, not only the exact amount of attached protein can be determined, but also activity of the immobilized proteins can be evaluated.

In Paper III ConA was used as a model to examine the interaction with a series of well characterized glycoproteins. The same strategy can in future work be used with an array of nanoparticles loaded with different sugar-specific lectins positioned at pre-determined spots at the read-out surface. Variations in glycosylation will be detected as different patterns of specific binding to the lectin panel. Since a large number of particles, each presenting a high local concentration of lectins, can be accommodated in small spots, this type of array will be suitable for capture of low-abundant targets from complex samples, while at the same time allowing a large number of samples to be run in parallel. It therefore has the potential to become a useful tool both for screening of large libraries of glycoproteins and in diagnostic work.

Today there is a demand for faster and more sensitive devices in a portable format for point-of-care testing. With this purpose in focus bioluminescence is found to be a suitable detection technique as it allows fast and sensitive detection of small amounts of analytes without the requirement of compli-
icated instrumentation. The results presented in Paper V show that pyruvate kinase conjugated antibodies readily can be used in bioluminescence-based particle ELISA tests. Here an IgY antibody with affinity for BSA was selected in a proof-of-principle arrangement. However, in the proposed POCT device this antibody can be replaced by an antibody with affinity for any other antigen of diagnostic interest.

A limiting factor in the development of high throughput screening detection systems is the lack of easily synthesized high affinity ligands. Therefore new types of ligands such as synthetic peptides, aptamers or protein fragments could be interesting alternatives for future work.
4. Acknowledgements

There are many people to acknowledge that have supported me in the work presented in this thesis and I would like to express my sincere gratitude to all of you.

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eration within this project. I would also like to thank Dr. Nigel Tooke at Biotage AB for helpful discussion regarding bioluminescing particles, large and small.

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Finally, to Jonas, my beloved husband and best friend, and to my son Tor, mammas prins, I am so happy and so proud to have a family like you.
5. Swedish Summary

Reaktionssystem i nanostorlek


I alla biologiska organismer sker regelbundet ett stort antal kopplade reaktioner där produkterna av en reaktion genast presenteras som reaktanter till nästa i en lång serie av reaktioner. De här reaktionerna har gjorts effektiva genom att skapa höga lokala koncentrationer av reaktanterna med hjälp av avancerad spatial organisation, eftersom lyckad överföring av reaktanter till produkt kräver korta avstånd mellan de inblandade komponenterna. För att efterlikna effektiviteten på dessa system kan receptor molekyler eller enzymer (biologiska katalysatorer) som ska delta i kopplade reaktioner fästas på nanometer stora partiklar. Därigenom får man både hög lokal koncentration och samtidigt närhet mellan reaktanterna, vilket kan resultera i snabba överföringar även vid låga analyt koncentrationer. Ett sådant arrangemang skulle vara idealiskt som biosensor system där det är väsentligt med snabb och känslig detektion av små mängar analyt.

För att skapa pålitliga och reproducerbara sensor system för detektion av exempelvis viktiga biologiska markörer är det viktigt att infängstmolekylerna (vanligtvis proteiner) kan fästas på en fast fas på ett sådant sätt att de bi-
behåller sin funktion och aktivitet samtidigt som ospecifik adsorption av proteiner, virus, bakterier eller andra oönskade komponenter förhindras. De allra flesta ytor som är avsedda för biologiska tillämpningar måste skyddas på något sätt för att undvika att de belarmeras av de olika substanser som förekommer i alla biologiska miljöer. Ett effektivt sätt att skydda ytan är att adsortera s.k. Poloxamerer, d.v.s. en sorts polymera tensider med den generella sammansättningen (PEO)_m(PPO)_n(PEO)_m, som brukar gå under varumärket Pluronic. Mittdelen består av polypropylenoxid (PPO) som lätt kan adsorberas till de flesta hydrofoba plastytor, medan de relativt hydrofila polyetylenoxid (PEO) sidokedjorna kommer sträcka ut sig i den omkringliggande vattenfasen. Tidigare studier har visat att ytan skyddas bättre ju längre sidokedjorna är. Av de många olika Pluronic polymerer som testats i vårt laboratorium har Pluronic F108 (m=129, n=56) visat sig vara den mest effektiva för att minimera ospecifik protein adsorption till plastytor, vilket beror på dess höga innehåll av PEO.


Det finns mängd tillfällen då det är mycket användbart att kunna sätta fast två eller flera funktioner, vars relativa koncentration oberoende kan varieras, på samma nanopartikel. Ett typiskt exempel är i diagnostisk utrustning där korta avstånd mellan givar- och mottagarmolekylerna är avgörande för snabb signalalstring. Det kan åstadkommas genom att partiklarna täcks med en förutbestämd blandning av Pluronic F108 utrustad med olika kopplingsmekanismer till vilka de olika önskade funktionerna kan kopplas in. Vi har i en sådan ansats utrustat en 240 nm polystyren partikel med tre olika funktionaliteter. De olika funktionerna var: en kort oligonukleotid för hybridisering med motsvarande komplementär sekvens på en plan yta vilket möjliggör fastsättning av nanopartiklarna för avläsning, pyridyl disulfid (PDS) för inkoppling
av infångstmolekyler och nitrilotriättiksyra (NTA) för kelatkoppling av histidin-märkt enzym som kan generera en detekterbar signal.


Protein microarray är av betydande intresse för att kunna kartlägga protein uttryck, förändringar eller interaktioner och på så sätt t ex kunna identifiera viktiga markörer för diagnostik av olika cancerformer på ett tidigt stadium. Därför är miniatyriserade format för att avsöka stora mängder proteiner sam-tidigt under snabb utveckling, men har visat sig vara svårare att åstadkomma än de motsvarande befintliga arrayer för DNA analys. Det beror på att prote-iner är avsevärt svårare att arbeta med jämfört med nukleinsyror, bland annat
p.g.a. att det inte finns samma möjlighet att öka mängden protein markörer på samma sätt som PCR amplifierar DNA. Istället måste detekterbarheten ökas genom att öka den lokala koncentrationen av eftersökt markör. Vår idé är att det kan göras genom att igenkänningsmolekylera sätts fast på små nanopartiklar, som i sin tur kopplas på en plan avläsningsbar yta. I en modelluppsättning av sådan tillämpning har ett kolhydratsspecifikt lektin, ConA, satts fast på 240 nm nanopartiklar. Samma partiklar har dessutom utrustats med korta sekvenser av dG oligonukleotider som genom att hybridisera med en komplementär dC oligonukleotidsekvens effektivt kopplar fast kulorna på den plana ytan. De fastsatta partiklarna exponerades sedan för en serie om fyra kolhydratsinnehållande proteiner med känd struktur samt ett kolhydratsfritt protein som negativ kontroll för att demonstrera värdet av det föreslagna nanopartikel-array konceptet.

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A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)