Nanofluidic tools for bioanalysis : the large advantages of the nano-scale

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CHAPTER 5

From SERS to SERSOR: Investigation of PEG-thiol coatings to make a dynamic Surface-Enhanced Raman Scattering sensOR[a]

5.1 Abstract

A surface enhanced Raman spectroscopy (SERS) substrate, when improved with a coating inert towards the analyte but thin enough to allow surface enhancement of analytes in solution, can operate without irreversible adsorption taking place and can therefore be responsive to changes in analyte concentration as opposed to conventional SERS substrates. The range of use of this powerful detection technique can then be significantly expanded to include re-use, cleaning and calibration. Ultimately this concept of dynamic SERS can provide a chemical sensor, or SERSOR, that not only detects but also identifies unlabeled (bio)analytes in applications such as process monitoring or as a non-invasive detector in-line with separations such as HPLC, or especially, in Lab-on-a-Chip and µTAS systems. Described in this chapter are first measurements of a SERSOR, a SERS active substrate coated with self-assembled monolayers (SAM) of oligo(ethylene glycol) terminated thiol (PEG-SH). Consecutive measurements of water and adenine solutions, a molecule known for its irreversible adhesion, demonstrated no reversibility of adenine signal for the uncoated SERS substrates whereas the coated substrate demonstrated an initial adenine signal reversibility of 90%. Although adequate robustness of the sensor was not yet obtained, the device represents a first step towards the envisioned SERSOR concept.

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5.2 Introduction

5.2.1 Surface-enhanced Raman scattering (SERS)

SERS is a detection technique based on Raman scattering, where detection limits of analytes are improved by the proximity of a metal surface, typically silver or gold. The surface features of the metal, typically in the order of tens of nanometers such as for a rough metal surface or for separate nanoparticles or metal colloids[175], supports surface plasmons that when exited by an incident Raman photon induce a very strong localized field. This increases the Raman signal from molecules near the surface, typically within 10 nm[176]. Several recent books and reviews dedicated to SERS are available focusing on practical applications as well as the theoretical understanding of the phenomenon[176–183]. SERS provides a combination between molecular structural information from vibrational spectroscopy, with sensitive label-free detection. The ability of SERS to measure and identify many unlabeled biomolecules including for example glucose, enkaphalin, thrombin and adenine at excellent detection limits allows in principle its application in the biomedical field[182,184,185]. SERS was also successfully applied for off-line detection after various analytical separation methods[186].

Silver and gold irreversibly adsorb many molecules due to their inherent negative surface charge[187,188], particularly the molecules with thiol and amine groups. This adsorption ensures the close proximity of these compounds to the enhancing surface, improving detection limits as molecules accumulate onto the surface over time. However, such irreversible adsorption also has important disadvantages:

1) Substrates cannot be re-used. Therefore calibration is not possible with the same substrate, obstructing quantitative measurements if the substrate characteristics vary.

2) Dynamic measurements in solution are not possible. The signal increases over time until the surface is saturated, and the analytes can be only detected as an integrative signal. Upon a change of the solution composition the signal does not , as opposed to what would be desirable in e.g. chemical process monitoring or for an in-line detection. A measurement therefore does not reflect the current composition of SERS-active compounds in the solution but depends on the whole history of the substrate.

3) Simultaneous detection and identification of multiple analytes is in principle possible with SERS when yielding vibrational spectra that allows them to be differentiated, supported possibly by chemometrics. Irreversible adsorption may obstruct this, due to surface competition between analytes or between analytes and non-SERS active components of the sample matrix.

4) The measurements are invasive. Adsorption implies the extraction of compounds from the measurement solution so that the sample cannot be recovered for a further analysis. In-vivo applications will be invasive as well (even if the adsorption would be reversible).

These four aspects limit the current application of SERS substrates for single-

use invasive measurements, and cause it to be at most as an integrative detector, which may be quantitative only if the substrate can be reproducibly manufactured (but used as disposables) and there are no competition effects due to a complex sample composition.

5.2.2 Strategies so far for SERS implementation

Although many processes can already be monitored with Raman spectroscopy, an enhanced detection limit in dynamic process monitoring would be of great value[189]. SERS can provide enhanced detection limits but since the desirable strongest SERS enhancements are achieved by binding to the surface, at the same time this imposes a large constraint on their applicability. Adhesion leads to fouling, restricting the sensor to single use, preventing calibration, in-line use and dynamic measurements. Secondly SERS substrates in a complex mixture are subject to surface competition between analytes and/or matrix compounds, making SERS enhancement strongly susceptible to matrix effects. These challenges have been recognized from the beginning of SERS, and development of a means for dynamic quantitative SERS measurements has been a dominant driving force in SERS substrate development. Several strategies attempt to enable quantitative measurements as well as their limitations are listed below.

The most popular strategy is the reproducible manufacturing of substrates, as this allows measurements with separate but identically performing substrates. A multitude of methods has been applied in manufacturing substrates[190], from colloid deposition[191–200], roughening a flat metal surface electrochemically[201,202], using direct nanofabrication[203] and microfabrication techniques to create an appropriate scaffold for the metal[204–209] in combination with e.g. sputtering and vapor deposition techniques. One SERS substrate yielding reproducible enhancements is commercially available (Klarite ™, D3 Technologies Ltd. UK). Re-use of substrates or dynamic measurements are not possible with this strategy, nor does it offer a solution for the surface competition between analyte and sample matrix and/or other analytes.

A strategy to allow SERS detection in the presence of a complex matrix has been to use surface coatings that have a strong binding preference with the analyte of interest. Such coatings selectively enrich the analyte near the surface as opposed to matrix compounds. This principle was applied in e.g. qualitative protein measurements[210,211] immuno-assays[212] and anthrax markers[213]. A rarely reported coating type which however is of interest for our approach is what can be called unselective enrichment coatings. These coatings have an interaction similar to retention mechanisms as used in reversed phase high performance liquid chromatography (RP-HPLC), and may therefore be potentially reversible and interact with a range of compounds. One work using such a coating reports measurements on benzene and tert-butylbenzene aqueous solutions with n-alkanethiols, on substrates coated with 1-propanethiol (C3) up to octadecanethiol (C18)[214] , although rinsing or reuse was not reported. The only use

as a reversible coating in an actual separation was demonstrated with a coating of 1-propanethiol, in GC analysis[215] and as the stationary face in LC for BTEX (benzene, toluene, ethylbenzene, and o-, m-, and p-xylene)[216]. The only downside of this coating approach is that since it may interact reversibly with multiple analytes the affinity and hence signal enhancement is very dependent on the matrix. Hence surface competition occurs when used during a separation, or after. Another coating type uses a coating of reporter molecules, which have a strong inherent SERS signal and high affinity for the analyte of interest. Analyte-induced conformational changes on the coating are measured, allowing indirect detection of the analyte. Reporter molecule coatings have been applied to e.g. Cu^{2+} and Pb^{2+}[217], pH[218–220], lactate[221] and viruses[222]. *In-vivo* experiments have been performed with this approach and include measurements of glucose in rats[200,223] and intracellular pH[224–226]. A review on SERS in cells is also available[227]. With the appropriate coating the binding between analyte and reporter molecule may be reversible, allowing dynamic measurements. In practice this may solve the challenge for a dynamic quantitative SERS substrate, but for one or a few selected compound(s) only. Unfortunately thus, the high selectivity of enrichment when using reporter coatings excludes the capability for the measurement of multiple constituents of a complex mixture. This capability is needed e.g. for a generic detector for SERS active analytes after a separation of unknown compounds. Furthermore the requirement for binding makes these coatings invasive *in-vivo*.

5.2.3 SERS as a sensOR: the SERSOR concept.

We propose to overcome the main constraint on the broad application of SERS by means of a dense coating that is inert towards the analyte(s). This prevents adsorption of compounds and endows the SERS substrate with the ability for dynamic detection with the signal reflecting the concentration. By default this prevents bias from surface competition. Also, this changes the substrate from a disposable into a re-usable one. Most importantly, such a coated substrate may be tested and evaluated before use, or even calibrated. For such a SERS-sensOR device we propose the name SERSOR.

Since SERS enhancement greatly diminishes with distance from the metal surface[177–181], the coating must be as thin as possible, preferably thinner than 10 nm[176]. Since we will measure compounds in solution instead of enriched on the surface of the uncoated substrate this will result in lower detection limits. The properties of the various SERS strategies so far compared to each other and the proposed SERSOR are summarized in Table 5.1.

A SERSOR will have a broad potential in applications such as process monitoring or as a non-invasive detector in-line coupled with separations such as HPLC. As the signal originates from within a few tens of nanometers from the surface, applications include the monitoring of highly localized process in (bio)-chemical reactions[228], relevant for *in-vivo* measurements.

Table 5.1 Properties of the various types of coated SERS substrates (see section 5.2.2). **Analyte** Indicates selectivity of the substrate. **Detection limit** General/average indication, or in case of selectivity vs. intended analyte only. *Depends on the coating thickness. **No surface competition** "No" indicates that the coating does not disable competition for the surface (either temporarily or permanent) **Dynamic** Whether the SERS response may respond to changes in solution over time, e.g. composition and concentration. †May be dynamic if the affinity is reversible: e.g. pH sensitive reporte coating[224–226]. **Quantitative** Wether the SERS response can be calibrated to reflect concentration. ‡Potentially if the total amount adhering is fixed by selecting the volume of solution e.g. dried (non-dynamic) or: if the accumulation over time from solution can be calibrated.

	Uncoated	Reporter coating	Selective Enrichement coating	Unselective enrichement coating	SERSOR
Analyte	All SERS active	Target compound only	Target compound only	Potentially multiple	All SERS active
Detection limit	Excellent	(Very) good	Good*	Average*	Average*
No Surface competition	No	Yes (Target compound)	Yes (Target compound)	No	Yes
Dynamic	No	Potentially†	Potentially†	Yes	Yes
Quantitative	Potentially‡	Potentially‡	Potentially‡	No	Yes

As SERS is an inherent nanoscale effect, it is well suited for downscaling of analytical systems. A coating, such as for a SERSOR, is not expected to increase the difficulty of downscaling SERS. Therefore, downscaling a SERSOR is primarily a microfabrication challenge for the substrate itself. Of particular interest is that a SERSOR can be implemented in lab-on-a-chip (LOC) and µTAS systems, a field in which the potential for SERS has already been recognized[34].

5.2.4 SERSOR development

Substrates based on silver colloid deposition provide the strongest SERS enhancement[184]. Since it is the target of our research to evaluate a coating for a SERSOR, and not to develop a SERS substrate, an established method of colloid deposition on glass was selected to prepare the SERS substrate[195].

A good candidate for a SERSOR coating, reported to be inert towards biological molecules such as proteins, is a self assembled monolayer (SAM) of oligo(ethylene glycol) terminated thiols (PEG-SH)[229] on gold and silver[230]. Coverage results of coating protocols using PEG-SH have been studied as a function of metal surface roughness[231] and for different solvents[232]. Interestingly, two types of thickness and surface coverage for PEG-SH coatings have been reported in relation with SERS, a dense 25 nm, PEG-SH coating (5 kDA) to block out the environment[233], and a very disperse coverage with a different PEG-SH

on colloids to stabilize the particles in solution while explicitly keeping the surface accessible[234]. Neither method is suited for a SERSOR as an optimum thickness and coverage is required in between. However, this research did establish that the complete PEG-SH coating did not appear to influence normal biological behavior in-vivo[233], making a SERSOR compatible with *in-vivo* measurements. In a different field, particular PEG-SH coatings with low molecular weights ($<$750 Da) have been reported to provide a surface coverage dense enough to inhibit the etching of flat gold in an aqueous cyanide etch bath[235]. Notably, thiol based coatings have been reported to adhere more slowly to silver than to gold[236]. In conclusion, PEG-SH may prevent molecules from adsorbing to the surface but can be made thin enough to allow SERS of molecules in solution. It however needs to be evaluated on our non-flat silver substrate.

The goal of this paper is to prove the concept of a SERSOR using an PEG-SH coating on a SERS-active substrate. The investigation will focus on the questions whether this particular coating applied on silver surfaces is sufficiently inert, and dense to prevent small biomolecules from adsorbing while at the same time thin enough to allow sensitive SERS.

5.3 Experimental

5.3.1 Chemicals

Silver nitrate 99.9999%, Sodium Citrate, 3-aminopropyltrimethoxysilane (APTMS), adenine, sulphuric acid and hydrogen peroxide were acquired from Sigma Aldrich (Sigma Aldrich, Zwijndrecht, The Netherlands). Deionized water (DI-water) used was generated by a Milli-Q ®Gradient A10 ®(Millipore, Amsterdam, The Netherlands). When very pure solvent was required, water and MeOH were of UL-CMS grade (Biosolve, Westford, USA). Isopropanol and acetone were obtained from VWR (BASF, VLSI Selectipur grade). Oligo(ethylene glycol) terminated thiol ($CH_3-O-[CH_2-CH_2-O]_3-[CH_2]_5-SH$) was a kind gift from Dr. Nicole Botterhuis(department of supramolecular polymer chemistry, Eindhoven University, The Netherlands). Standard microscope slides were obtained from Menzel (Menzel GmbH & Co. KG, Braunschweig, Germany). SERS measurements were performed with an aqueous solution of 10 mmol/L adenine.

5.3.2 Raman instrumentation

Measurements were performed using a RAMANRXN1® Microprobe (Kaiser Optical Systems Inc., Ecully, France) controlled with the included Holograms®software, equipped with a solid state laser, 785 nm at 200 mW. The Raman system was integrated with an Olympus BX51 microscope with a 60x water immersion lens (Olympus Corporation, Zoeterwoude, The Netherlands). The microscope was equipped with a high-accuracy xyz positioning stage (SCAN IM 120x100, 1 mm

lead screw pitch, linear encoders, Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar, Germany) driven by a Corvus high-resolution positioning controller and the included software Winpos (miCos GmbH, Eschbach, Germany). Spectra were acquired by summation of three 1 second acquisitions, with the immersion lens in the analyte solution, focused on the surface of the SERS substrates. Data (pre)processing and analysis of spectra was performed using Matlab (The MathWorks Inc., Natick, USA). A scanning electron microscope (SEM) (Nova 200 NanoSEM, FEI Company™, Hillsboro, USA) was employed to image the colloid distribution on the surface.

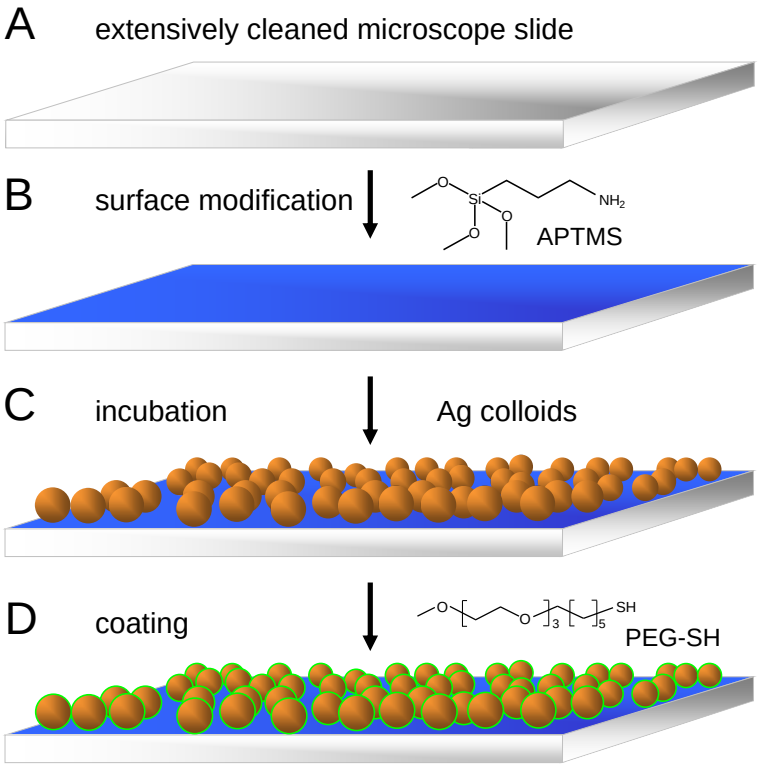

Figure 5.1 SERSOR manufacturing steps. (A) An extensively cleaned microscope slide (B) was modified with 3-aminopropyltrimethoxysilane (APTMS), prior to (C) incubation with a Ag hydrosol to ensure a strong adhesion of the colloids. (D) This SERS-active substrate was then coated with PEG-SH.

5.3.3 SERSOR manufacturing.

A citrate-reduced silver hydrosol was made in ULCMS grade water[237] and its SERS activity confirmed by measurement of adenine (results not shown). These

silver colloids were then immobilized onto a microscope slide using the procedure as detailed by Park et al.[195], shown schematically in Figure 5.1A-C. Briefly, microscope slides were cleaned thoroughly by wiping with a tissue and household dish washing soap solution followed by extensive rinsing with DI-water. For further cleaning the slides were then placed in an ultrasonic bath in acetone and then MeOH for 10 minutes each and dried for 20 min at 110 ℃ in an oven to remove organic solvent. After evaporation of all solvent the slides were submerged in piranha solution (H_2O_2:H_2SO_4 1:3 v:v for several days (Caution! Piranha acid is very corrosive and potentially explosive upon contact with organic substances. Appropriate safety measures should be taken while handling) to both clean and activate the surface. Afterwards the slides were rinsed with water and stored in MeOH (both ULCMS grade) awaiting further processing. The slides were rinsed with isopropanol and placed in a solution of 8% APTMS in isopropanol (v:v) for 21h. After rinsing with isopropanol the APTMS was annealed on the slides at 110 ℃ in an oven. The trimethoxy silyl group of APTMS reacts with the activated glass surface, and the amine group then present a positive surface charge towards the solution at neutral pH. This positive surface charge facilitates a strong adhesion between the inherently negative Ag colloids[187,188] and the slides. The slides were incubated in undiluted silver hydrosol for 1 day on an orbital shaker, followed by rinsing with water (ULCMS grade). SERS substrates were coated by submersion in an aqueous 1 mmol/L PEG-SH solution for 1 day. Afterwards the coated substrates were rinsed with and stored in water (ULCMS grade), prior to measurements. Uncoated SERS substrates were retained in water (ULCMS grade) for reference measurements.

5.4 Results & Discussion

5.4.1 SERSOR manufacturing

SERS active substrates were successfully produced and coated with PEG-SH. The colloid density on the microscope slides was studied with scanning electron microscopy (SEM) (Figure 5.2). The manufacturing method was relatively quick and simple but the colloid density was found to be inhomogeneous over the microscope slide. The colloid density was found to correlate positively with the SERS signal strength and observed scattering of the laser from the surface. In practice, the laser scattering was successfully used to locate strongly enhancing sites rapidly without analyte, by moving the SERS substrate under the laser spot and observing the reflection intensity by eye.

5.4.2 SERSOR measurements

A SERSOR needs to demonstrate reversibility in analyte signal, in order to measure concentration changes in a dynamic manner. To evaluate the effectiveness

of the coating for this purpose, the biomolecule adenine was selected as a test analyte. Adenine has a strong SERS signal and normally adheres irreversible to a silver surface[238], and is therefore a challenging analyte for a SERSOR.

The PEG-SH coated SERS substrate successfully demonstrated surface enhancement of Raman detection of adenine. This was established by placing a solution of 10 mmol/L of adenine on the SERS substrate and measuring the signal with the Raman microscope focused on the surface through the solution using an immersion objective. While with a bare glass slide only, adenine could not be detected, while it was clearly detectable with the SERS substrate.

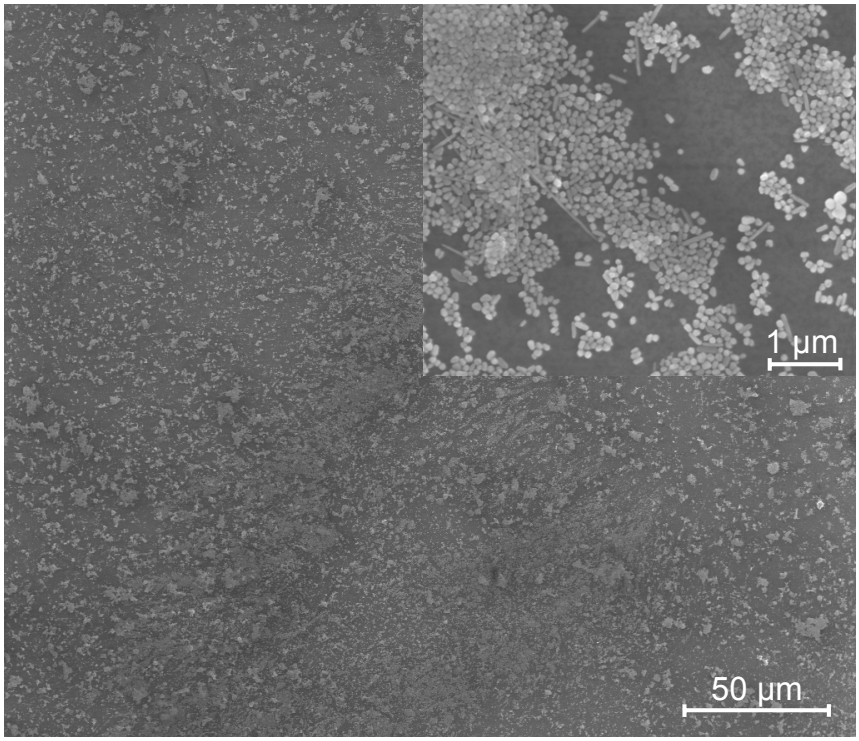

Figure 5.2 SEM image of a SERS substrate coated with PEG-SH. The image shows an area of 300 x 300 µm with the surface covered with silver colloid clusters, associated with the strong enhancements from these locations. The insert shows that clusters are on average 1-2 layers of colloids thick and several microns in size. Also present are silver nanowires several microns in length and tens of nanometers wide.

Subsequent SERS measurements of water, a solution of 10 mmol/L adenine, and after intermediate rinsing with water, again water, were acquired for coated and uncoated SERS substrates. The uncoated SERS substrate showed no significant reduction in signal of adenine after even very extensive rinsing with DI-water (data not shown), as has been previously reported in literature[238]. The spectra

of a set of consecutive measurements from an identical position for the PEG-SH coated SERS substrate are shown in Figure 5.3.

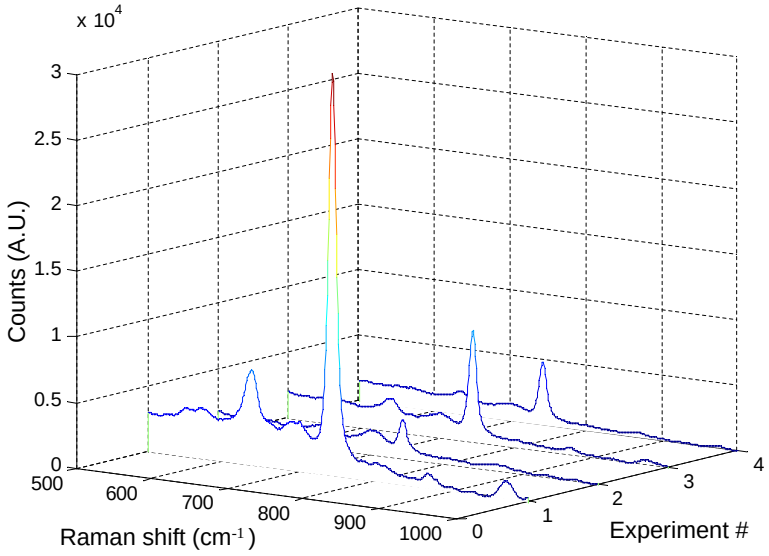

Figure 5.3 Subsequent SERS measurements of adenine and DI-water at an identical surface position of an PEG-SH coated substrate. Experiment No. 1 corresponds to adenine (10 mmol/L), No. 2 DI-water, No. 3 reapplied adenine (10 mmol/L) and No. 4 DI-water. The most intense band, characteristic for adenine, is apparent at 734 cm^{-1}

The PEG-SH coated substrate demonstrated an initial reduction of adenine signal of almost 90% after rinsing with water. When the substrate was exposed again to adenine, the signal of adenine returned (albeit weaker), and was reduced again by rinsing (albeit not entirely). It can be concluded that the coating prevented the irreversible adsorption of adenine to the SERS substrate and was thin enough to allow SERS, as opposed to other PEG-SH coatings that were intentionally thick to obstruct SERS signals from beyond the coating[233]. The positive identification of adenine, while limiting its irreversible adsorption to the SERS substrate demonstrates the feasibility of the SERSOR concept.

As can be seen from the measurement series of Figure 5.3, with consecutive measurements a reduced intensity of the adenine signal and increased carry-over was seen. Immersion in DI-water for 1h as well as extensive rinsing did not affect the carry-over signal strength. This indicates that adenine was attached to the silver and therefore the coating did not completely prevent adsorption to the SERS substrate. The increasing carry-over signal of adenine (Figure 5.3, measurements No. 2 and No. 4) may therefore be explained by decreasing coverage of the coating on the SERS substrate.

The reduced response to adenine during the experiment (Figure 3, experiment No. 1 and No. 3) is a typical result and found in other measurement series we

performed, but it is less readily explained, particularly since with uncoated SERS substrates no loss of signal was found, despite rinsing efforts. In further investigation of this observation it was found that the signal of PEG-SH itself, although unaffected by storage for extensive periods in water, is strongly affected by laser exposure for sub-second periods, much shorter than the order of the acquisition time used. We hypothesize that the apparent wear of the coating with consecutive measurements (Figure 5.3), is due to the reaction of silver with the thiol group of the coating, accelerated by irradiating with the laser. This corresponds to silver tarnishing, the well known reaction of silver with sulphur groups, such as in H_2S and SO_2, forming the characteristic black silver sulfide (Ag_2S), known to affect the surface plasmon resonance of silver[239,240]. Although this particular effect is of potential interest for tarnishing studies of silver, it is beyond the scope of this paper and the kinetics were too fast to evaluate effectively with our setup.

5.4.3 Perspectives

For the purpose of the development of a robust SERSOR, improving the present colloid coating protocol is needed in coverage and reproducibility, although colloid coatings of similar quality as we show in Figure 5.2 have been reported[241]. Notably, in a later stage we achieved much improved colloid substrate manufacturing, with a homogeneous colloid distribution and correspondingly a maximum variation in surface signal intensity by a factor of 2. A SEM image of this surface is provided in the appendix Figure 5.4. Unfortunately these new substrates could not yet be tested with the SERSOR coating. A different SERS substrate may also be used, e.g. Klarite, or one of the many other strategies to manufacture substrates (See introduction for a selection reported in literature). A reproducible substrate would enable quantification of the effect of the coating on SERS enhancement. In addition, it will be of interest to study the effect of a coating on the SERS activity of the different surface geometries provided by the various SERS substrate manufacturing methods. Also, the influence of these variations on the coating effectiveness or its application procedure may be investigated.

The particular coating used here for a SERSOR, i.e. thiol chemistry to coat silver, potentially induces unwanted effects. To solve these problems, gold substrates could be used, or a coating relying on other binding principles could be considered such as amine, which also adheres to silver.

For future SERSORs, no single coating can be expected to block the interactions in all applications as the interactions depend on the analyte(s) of interest and its matrix. Therefore, as each application field imposes a different condition on inertness, different customized coatings need to be developed. These can be based on polymers, metals or oxides. A successful SERSOR coating will require optimization of the surface coverage, stability and thinness of the coating on a SERS-active substrate. Lastly the potential of a SERSOR for downscaling is not limited by the coating but manufacturing of the substrate. With state of the art

technology we expect that a SERSOR can be integrated in μTAS and Lab-on-a-Chip systems.

5.5 Conclusions

A proof of concept for a dynamic surface enhanced Raman sensor, a so-called SERSOR, was demonstrated. An initial reversibility of the signal of 90% was achieved by coating a SERS active substrate with a non fouling coating that was inert and thin. The coating, PEG-SH, was evaluated with the biologically relevant molecule adenine, a normally strong binding molecule to silver whose signal could not be reduced by rinsing with water. Although further optimization of the coating is needed, this proof-of-concept opens the way for the development of appropriate coatings for applications requiring the measuring of unlabeled (bio)molecules in process monitoring in large reaction vessels, as well as detection in-line with separations in systems, potentially as small as μTAS or lab-on-a-chip.

5.6 Acknowledgements

We gratefully acknowledge prof. Age K. Smilde (University of Amsterdam) for access to the Raman spectrometer. We acknowledge Ing. Marcel Hesselberth from the Department of Condensed Matter Physics, Leiden University, The Netherlands, for measuring the SEM images. We also would like to thank Dr. Nicole Botterhuis, department of supramolecular polymer chemistry, Eindhoven University, The Netherlands, for her kind gift of the PEG-thiol. We acknowledge support from NanoNed and from the Netherlands Metabolomics Centre (NMC) which is a part of The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

5.7 Appendix

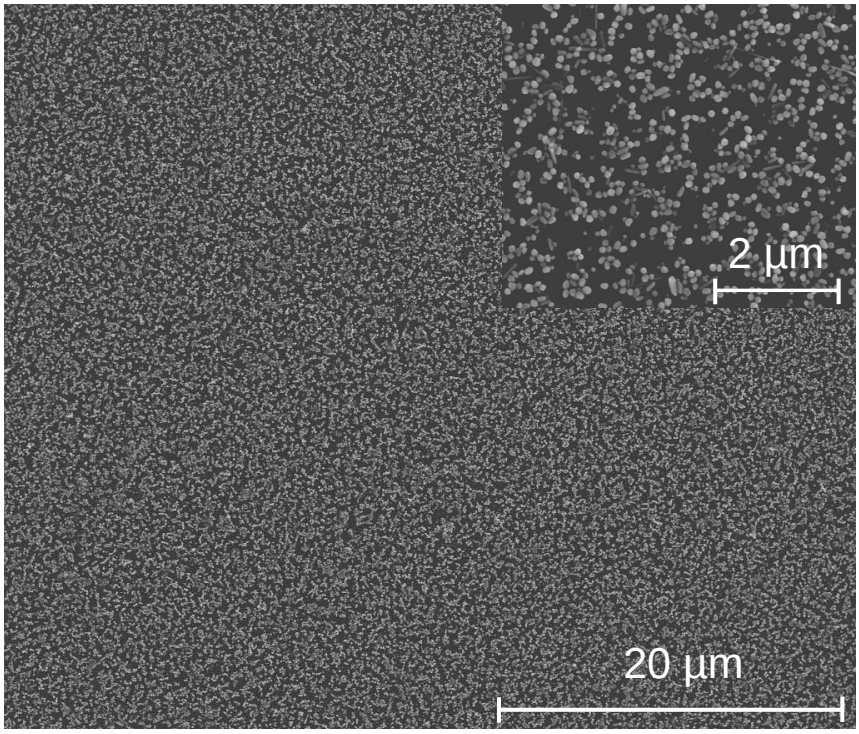

Figure 5.4 SEM image of a SERS substrate. The image shows an area of 50 x 50 µm with the surface covered with silver colloids and fused silica. A much larger area, $\approx$ 1x1mm showed the same coating state.