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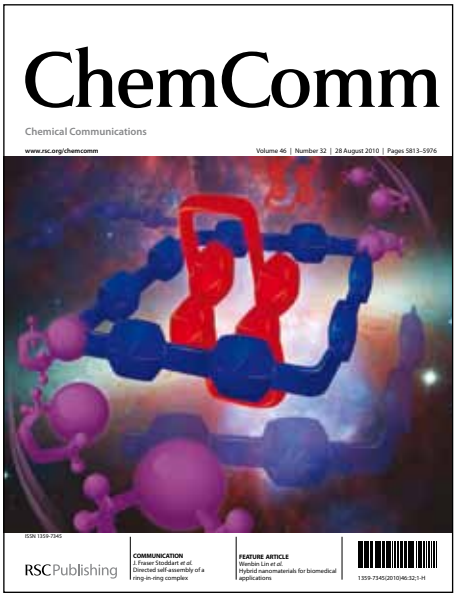

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Femtogram Cytokine Detection in a Direct Dot-Blot Assay Using SERS Microspectroscopy and Hydrophilically Stabilized Au/Ag Nanoshells

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Rapid parallel detection of two cytokines (IL-6 and IL-8) with femtogram sensitivity in a simple direct dot-blot assay is demonstrated. The corresponding microspectroscopic SERS acquisition scheme employs rationally designed, hydrophilically stabilized Au/Ag nanoshells as SERS labels optimized for signal enhancement upon red laser excitation.

Cytokines are a prominent and diverse group of signaling proteins, which regulate a broad range of biological functions including immunological processes, inflammatory responses, and the interaction between different biological pathways.[1] Typically, a whole group of cytokines is involved in the corresponding biological or disease process, which requires a comprehensive analysis of multiple cytokines down to very low concentrations for investigating the underlying mechanism of the disease state. Therefore, the development of highly sensitive and quantitative bioanalytical approaches for multiplexed cytokine detection may contribute to a more detailed monitoring and understanding of cytokine biology, biochemistry and their possible involvement in pathology. Traditional approaches for the detection of cytokines include bioassays in general and immunoassays in particular. The standard approach, enzyme-linked immunosorbent assay (ELISA), has a detection limit of about 10~20 pg/mL for the cytokine interleukin-6 (IL-6).[2]

Surface-enhanced Raman scattering (SERS) immunoassays are an attractive alternative to ELISA- and fluorescence-based approaches, particularly with respect to the parallel detection of multiple analytes (multiplexing) due to the narrow line width of vibrational Raman bands in combination with sensitivity and photostability.[4] Sandwich immunoassays are already widely used for protein detection by SERS.[5] However, this strategy usually involves long incubation times and multiple washing steps, which is time consuming and labor-intensive.[6] Direct immunoassays, which have the advantages of being simple and fast, are the most often applied platform in fluorescent and colorimetric detection schemes.[7] Depending on the affinity of the label to the target and a particular type of nanoparticles, the detection limit ranges between femtomole to attomole as reported.[8]Recently, Keiderling and co-workers introduced a direct SERS immunoassay for protein detection on nitrocellulose (NC) membrane using gold-silica nanoshells as SERS labels with a detection limit of ca. 1.25 ng.[9]

In this contribution, we demonstrate rapid cytokine detection with femtogram sensitivity in a simple and direct dot-blot assay by using rationally designed, hydrophically stabilized Au/Ag nanoshells with optimized optical properties for maximum Raman enhancement. Fig.1A shows the principle of this direct dot-blot SERS immunoassay for duplex cytokine detection on a NC membrane. Very small volumes (1 µL) of the solutions containing the cytokines (recombinant IL-6 and interleukin-8 (IL-8), respectively) were deposited on the NC membrane to form an antigen dot with a diameter of ~1-2 mm.[10] After blocking with bovine serum albumin (BSA), the SERS-labeled antibodies (anti IL-6 and antiIL-8, respectively) bind to their corresponding antigen. At larger antigen concentrations (10^3 and 10^4 pg/mL) the immune-complex can be detected even by the naked eye (Fig. 2). Au/Ag nanoshells with tunable localized surface plasmon resonances (LSPR) have been shown to provide strong Raman signal enhancement for Raman reporter molecules chemisorbed on their surface.[11] In our previous work the benefit of hydrophilically stabilized Au/Ag nanoshells for controlled bioconjugation[12] and their high stability under physiologically relevant conditions were demonstrated.[13] Herein, hydrophilically stabilized Au/Ag nanoshells were further employed for rapid duplex cytokine detection in a direct dot-blot assay with very high sensitivity requiring only very small sample volumes (femtogram level with 1µL sample volume). Fig. 1B shows the rationally designed Au/Ag nanoshells with a complete self-assembled monolayer (SAM) of hydrophilically stabilized Raman reporters (Ra) on their surface. The actual Raman reporter moieties (aryl thiols: 2-nitro-5-thiobenzoate, NTB, and 4-mercaptobenzoic acid, 4-MBA) are covalently conjugated to a short monoethylene glycol spacer with a terminal OH group (MEG-OH) and a longer tri-

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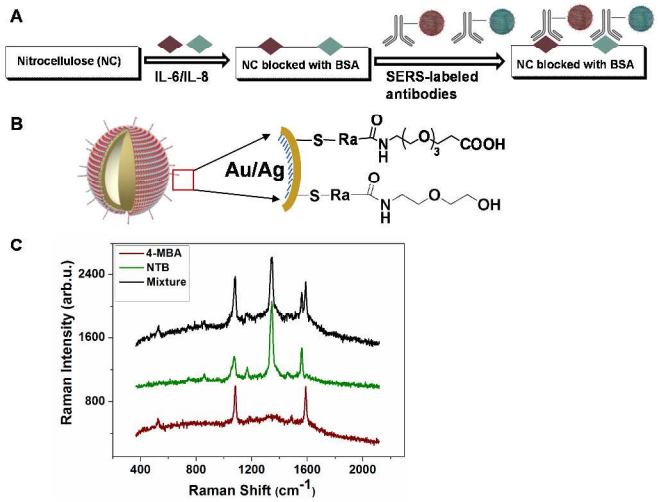
†Electronic Supplementary Information (ESI) available: [The experimental details, normal Raman spectrum from NC membrane, characterization of Au/Ag nanoshells, SEM images of NC membrane with and without antigen binding, negative controls for IL-6 detection and duplex cytokine (IL-6 and IL-8) detection. See http://dx.doi.org/ 10.1039/b000000x/

ethylene glycol spacer with a terminal CO_2H group (TEG-CO_2H) for bioconjugation, respectively.[12] Hydrophilically stabilized SERS labels were obtained by incubating the Au/Ag nanoshells with a 100:1 mixture of Ra-MEG-OH and Ra-TEG-CO_2H.[14] SERS labels with the hydrophilic spacer (MEG-OH) conjugated to the Raman reporter moiety are stable at physiologically relevant conditions, while non-stabilized SERS labels aggregate.[13] The SERS spectra of NTB and 4-MBA are shown in Fig. 1C. The characteristic Raman peak of NTB at 1340 cm^{-1} is assigned to the symmetric nitro stretching vibration,[15] while the peaks at ca. 1080 and ca. 1580 cm^{-1} for NTB and 4-MBA arise from phenyl ring modes.[16] The SERS spectrum of the binary mixture from the two Raman reporters (NTB and 4-MBA) clearly shows the distinct fingerprint from the corresponding SERS labels, which could be used for identification of the targets. Interestingly, as already reported by Keiderling and co-workers, [9] the intrinsic Raman signature of the NC membrane can be used as an internal standard since the dominant peak at 1288 cm^{-1} does not significantly interfere with the Raman peaks from the SERS labels (ESI, Fig. S1). In this contribution, we also used this characteristic peak of the NC membrane for internal referencing in order to achieve a reliable quantitative detection of cytokines (femtogram level) which is independent of "external" experimental parameters (such as laser power density, acquisition time etc.). The conjugation of antibodies to the SAM-functionalized Au/Ag



nanoshells was accomplished by methods reported in earlier work[12-13] (details in ESI, experimental part).

Fig. 1. Scheme of the direct SERS dot-blot immunoassay platform for duplex cytokine detection (A), schematic illustration of hydrophilically stabilized Au/Ag nanoshells with Ra-MEG-OH/TEG-COOH (B) together with their SERS spectra including a binary mixture (C).

The SERS nanoparticles were characterized by different physical techniques, in particular TEM and UV/Vis extinction spectroscopy, for characterizing their size and monodispersity as well as their optical properties, respectively. The TEM image in Fig. S2A (ESI) reveals monodisperse hollow structure of Au/Ag nanoparticles with a diameter of about 60 nm. The LSPR peak of the Au/Ag nanoshells occurs at ca. 630 nm (Fig. S2B). Upon formation of the SAM, the LSPR peak of the Au/Ag nanoshells exhibits a red shift to 650 and 657 nm, respectively, depending on the particular Raman reporter. This LSPR position is ideal for maximizing SERS with red laser excitation since both the incident light as well as the Stokes-scattered light areenhanced.[11]The SERS-labeled antibodies then selectively bind to the corresponding antigen on the NC membrane (Fig. 1A). IL-6 was first used as the target to determine the sensitivity and specificity of this platform. As evident from the bright field optical images in Fig. 2(top), the binding of the SERS-labeled antibodies to the antigen can already be observed by the naked eye and the dots became much brighter with an increasing concentration of IL-6. This is in agreement with results obtained by other groups for the colorimetric detection of different targets.[8] Fig. 2 (bottom) shows the corresponding SERS false color images obtained from the microspectroscopic imaging acquisition scheme employed for signal averaging in order to achieve a higher reproducibility since highly representative SERS spectra based on spatial averaging over larger areas including many pixels can be obtained. With an increasing concentration of IL-6, successively more SERS labels are selectively bound onto the NC membrane, which leads to the formation of "SERS dots" with high Raman intensities. For covering a larger surface area and signal averaging, two different areas from the same dot were chosen for the Raman mapping experiments with point illumination.

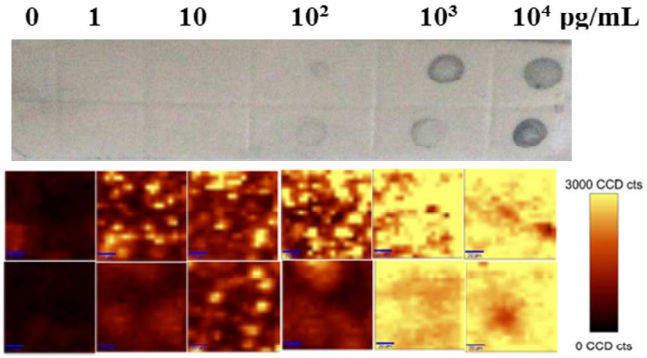


Fig. 2. Wide-field optical images of the SERS dots (top) and representative false-color SERS images for IL-6 detection at different antigen concentrations (bottom).

One option for further increasing the reproducibility in future experiments is SERS microspectroscopic mapping with line illumination[17]in combination with scanning perpendicular to the line focus, employing a high power laser source for maintaining the same laser power density at each location/pixel of the sample.As an additional control, we also employed scanning electron microscopy (SEM) to investigate the loading of the NC membrane with SERS nanoparticles (Fig. S3, ESI). In the absence of antigen, only pore structures from the NC membrane itself were observed. SERS particles could only be observed after upon immobilization of the antigen (100 pg/mL) on the NC membrane (SEM images in Fig. S3B and C, ESI). We note that for relatively high packing densities with SERS nanoparticles on the NC surface, also the plasmonic coupling as an additional factor for increased SERS intensities at high antigen concentrations should be considered.

The corresponding average SERS spectra for each antigen concentration are shown in Fig. 3A. The intensity of the characteristic peak (1340 cm^{-1}) from NTB-SERS labels increases with an increasing concentration of IL-6. According to the normalization of the SERS intensity with the internal peak from NC (1288 cm^{-1}), we were able to establish a normalized concentration-dependent SERS response curve (from 1 pg/mL to 10 ng/mL, Fig. 3B). The lowest detectable concentration for IL-6 is ca. 1 pg/mL (corresponding to ca. 1 fg due to the low volume of 1 µL. It should be noted that the SERS intensityobtained here on a NC membrane is higher than that obtained on glass at the same concentration in our previous report[13] because of the accumulation of the antigens within a very small area, leading to the concentrated NPs under the same laser beam. Toclearly demonstrate the specific detection of cytokine with this experimental approach, negative control experiments with this SERS-based platform were also performed, using other two different proteins (BSA and casein) at a high concentration (2%) and

PBS buffer as the control indicated in Fig. S4 (ESI). No dots were observed and almost no Raman signals were detected in these negative controls. In comparison, dot and SERS intensity are stronger for the IL-6 even at the low concentration of 100 pg/mL.

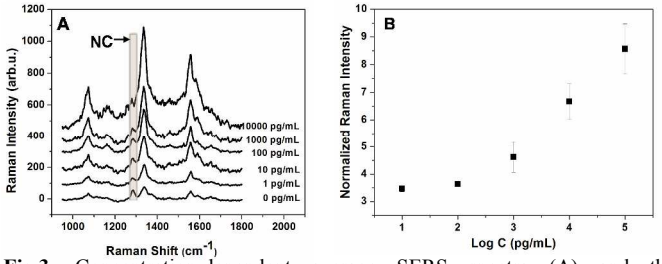


Fig.3. Concentration-dependent average SERS spectra (A) and the corresponding concentration-dependent response function (from 1 pg/mL to 10 ng/mL) based on normalized SERS intensities (B) for the detection of IL-6.

Due to the unique multiplexing capability of SERS, duplex cytokine detection was further tested by choosing IL-8 as another distinct target markers because IL-6 and IL-8 are important proinflammatory proteins and involve with recruitment of inflammatory cells.[1, 18] Duplex SERS labels were first optimized to give comparable SERS intensities.[19] After incubation of the SERS-labeled antibodies onto the NC membrane comprising dots of the two antigens, the spectral signatures of the two SERS-labels could be simultaneously identified (Fig. 4). Figure 4A shows the corresponding SERS false-color images. The total amount of protein was kept constant, but the ratio of the two cytokines (IL-6:IL-8) was varied from 0:1 (left), 1:10, 1:5, to 0:1 (right). With an increasing ratio IL-6:IL-8 the SERS intensity of NTB (SERS label for IL-6) at 1340 cm^{-1}increases, while the SERS intensity of MBA (SERS label for IL-8) at 1589 cm^{-1}decreases. Average SERS spectra determined from the SERS microspectroscopic data are displayed in Fig. 4B and C. Overall, these results indicate that the SERS label-antibody conjugates selectively bind to IL-6 and IL-8, respectively.

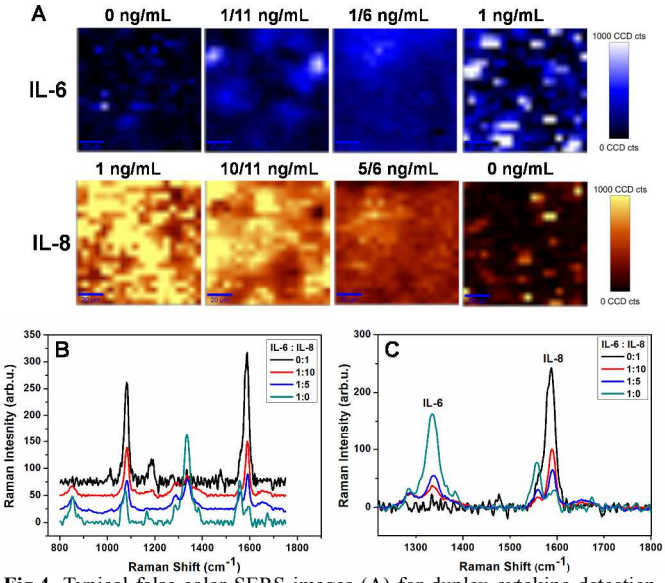


Fig.4. Typical false-color SERS images (A) for duplex cytokine detection. The total protein amount was kept constant, but the IL-6/IL-8 ratio was varied from 0:1, 1:10, 1:5 to 1:0. Average SERS spectra (B and C) were determined from the corresponding SERS microspectroscopic data.

In summary, a simple and rapid platform for duplex cytokine detection was developed, which could significantly increase the sensitivity (down to 1 pg/mL), employs very low sample volumes (1 μL), and simplifies the assay preparation process without a labor-intensive procedure. This SERS platform is achieved by using Au/Agnanoshells as the enhancing plasmonic material and the dot-blot membrane as the supporting substrate. Hydrophilically stabilized Au/Ag nanoshells provide terminal COOH moieties for controllable bioconjugation of the antibody on the nanoshells surface and high stability under physiologically relevant conditions. Using the Raman peak from NC at 1288 cm^{-1} as an internal standard peak, a quantitative detection of two cytokines with high biological relevance was achieved. Finally, we have also demonstrated the multiplexing potential of this simple, fast and sensitive bioanalytical detection platform.

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