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Abstract. Identification of tumor and normal cells is a promising application of Raman spectroscopy. The throughput of Raman-assisted cell sorting is limited by low sensitivity. Surface-enhanced Raman spectroscopy (SERS) is a well-recognized candidate to increase the intensity of Raman signals of cells. First, different strategies are summarized to detect tumor cells using targeted SERS probes. Then, a protocol is described to prepare multicore-SERS-labels (MSLs) by aggregating gold nanoparticles, coating with a reporter molecule and a thin silver shell to further boost enhancement, encapsulating with a stable silica layer, and functionalizing by epithelial cell adhesion molecule (EpCAM) antibodies. Raman, dark field and fluorescence microscopy proved the specific and nonspecific binding of functionalized and nonfunctionalized MSLs to MCF-7 tumor cells, leukocytes from blood, and nontransformed human foreskin fibroblasts. Raman imaging and dark field microscopy indicated no uptake of MSLs, yet binding to the cellular membrane. Viability tests were performed with living tumor cells to demonstrate the low toxicity of MSL-EpCAM. The SERS signatures were detected from cells with exposure times down to 25 ms at 785-nm laser excitation. The prospects of these MSLs in multiplex assays, for enumeration and sorting of circulating tumor cells in microfluidic chips, are discussed. © 2015 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.20.5.055002]

Keywords: Raman spectroscopy; SERS; nanoparticles; tumor cells.

1 Introduction

Circulating tumor cells (CTCs) are cells that have separated from a tumor mass and circulate in bodily fluids such as blood. These cells can contribute to the spread of tumors, i.e., secondary tumors or metastases distant from the originating organ. The number of CTCs in peripheral blood of patients and its time-dependent evolution are important markers to diagnose disease, to assess the success of therapy, and to estimate the prognosis. CTCs are extremely rare. Even in patients with advanced tumors there are only a few CTCs for every billion normal blood cells. This makes detection and isolation challenging. The technologies that are currently available for CTC isolation, the utility of CTCs as a diagnostic, and prognostic marker in various cancers and molecular properties of these rare cells have been reviewed.1 Current detection technologies are based on (1) free DNA or RNA circulating in plasma from patients with cancer; (2) physical properties of CTCs that include the larger size of most epithelial cells and differences in density, charge, or migratory properties; and (3) antibodies against specific cell surface antigens such as epithelial cell adhesion molecule (EpCAM, CD326). The CellSearch system (Veridex) is the only FDA (U.S. Food and Drug Administration)-approved technique that uses ferrofluids loaded with an EpCAM antibody to capture CTCs that are subsequently visualized by staining with a cocktail of antibodies against cytoplasmic epithelial cytokeratins.5 Even after the labels are applied, the acquired CTC samples are only 50% pure.1 In other words, only 50% of the cells identified as tumor cells are actually tumor cells. It is unclear how many tumor cells are misclassified as normal.

Our group developed Raman spectroscopic approaches to identify cells isolated with optical tweezers4 and in microfluidic chips comprised of glass with 514-nm excitation4 and quartz with 785-nm excitation.6 Although correct classification rates were above 95%, implementation of Raman-activated cell sorting (RACS) suffers from relatively long exposure times of seconds per cell and low throughput. Surface-enhanced Raman spectroscopy (SERS) is a variant to improve the sensitivity and throughput of Raman-based detection schemes by several orders of magnitude. Together with enrichment or preselection of suspicious cells by rapid, yet low-specificity screening tools, SERS may become a powerful tool for detection of CTCs in blood.

The SERS effect can be achieved by designing substrates (such as Ag nanorod assays7 and other microfabrication procedures8) or nanoparticles (NPs) that are enzymatically formed9 or chemically prepared (this work and the following citations). The

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Raman bands of molecules are enhanced after adsorption on nano-sized metals and excitation of surface plasmon polariton modes by appropriate laser wavelengths. Progress and trends of SERS for sensing molecules in trace amounts within the field of chemical and biochemical analytics have been described. The high potential of SERS in cancer detection and imaging has recently been discussed. Challenges attributed to the application of SERS in the context of cell detection are nonspecific binding and low reproducibility. These issues have been overcome by surface functionalization with cell-specific antibodies and the introduction of reporter molecules. Successful implementations have been reported by several authors. SERS-labels were functionalized with an epidermal growth factor (EGF) antibody to quantify CTCs in blood samples. HER2 is another frequently used antigen for SERS-based differentiation of tumor cells by hollow gold nanospheres, gold nanorods, silver NP-embedded silica spheres, gold nanocorals, and in combination with EpCAM-labeled magnetic particles. Another combination of trapping by magnetic NP and SERS-active NP was proposed for CTC detection. Both NPs were targeted to folate receptor which is overexpressed on many cancer cells yet absent on most normal blood cells. Advantages compared to fluorescence labels include that SERS labels show a very low spectral width of the Raman bands. The narrow spectral features of the SERS signal enable more distinct probes to be measured in a smaller region of the optical spectrum with a single laser and detector, allowing for higher levels of multiplexing and multiparameter analysis, which means 10 or more SERS labels can be demixed with low crosstalk. The excellent multiplexing capacity can be further increased using a mixtures of SERS-active molecules. For the detection of CTCs, this enables a strategy to apply multiple SERS labels and simultaneously identify multiple tumor-specific antigens. As a proof of concept, the actively targeted multiplex in vitro detection of three intrinsic cancer biomarkers—EGFR, CD44, and TGFβRII—was demonstrated in a breast cancer model using three multiplexing-capable, biocompatible SERS nanoparticles/nanotags. Another work described the expression of EGF, ErbB2, and insulin-like growth factor-1 (IGF-1) receptors in human breast cancer cell lines by SERS. Silver-gold nanorods were functionalized with four Raman-active molecules and four antibodies specific to breast cancer markers, i.e., EpCAM, IGF-1 receptor β, CD44, and keratin 18. SERS NPs show also photothermal (PT) properties that were used for multimodal SERS-PT diagnosis and as PT therapeutic agents for thermo-surgery. In the latter study, nanorattles were functionalized by estrogen receptor-alpha antibody that targets MCF-7 cell surfaces. Beside single cells, immuno-SERS microscopy has been applied to tissue imaging with single-particle sensitivity. Coherent anti-Stokes Raman scattering (CARS) is a nonlinear variant of Raman spectroscopy that has been coupled with surface enhancement (SE) using antibody-labeled metallic nanoshells. The paper employed this so-called SECARS approach for selective, sensitive, and rapid localization of the basal cell protein p63 in normal prostate tissue.

The current paper addresses the application and describes further developed multicore SERS labels (MSLs) that were designed for 785-nm excitation. First, successful conjugation of the antibody EpCAM is shown by dark field and fluorescence microscopy. EpCAM antigen is a transmembrane glycoprotein involved in cell signaling, migration, proliferation, and differentiation. EpCAM is known to be highly expressed in epithelial carcinoma and is frequently used as a diagnostic marker. Second, Raman spectroscopy proved the specific and nonspecific binding of functionalized and nonfunctionalized MSLs to MCF-7 tumor cells, leukocytes from blood, and nontransformed human foreskin fibroblasts. Furthermore, the subcellular distribution of MSLs was visualized by Raman imaging and dark field microscopy. Finally, viability tests were performed with living tumor cells to demonstrate the low toxicity of MSL-EpCAM at 785-nm laser excitation.

2 Experimental

2.1 Chemicals

Tetraethyl orthosilicate (TEOS) was ordered from Wako Pure Chemical Industries (Germany). Poly(vinylpyrrolidone) (Mw 55,000) (PVP), 3-mercaptopbenzoic acid, 4-mercaptophenol, 4-mercaptopbenzoic acid, thiophenol, 6-mercaptopyrindine-3-carboxylic acid, 2-mercaptop-4-methylthiazol-5-acetic acid, (3-aminopropyl)triethoxysilane (APTES), 4,7,10-trioxo-1,13-tridecanediamine, n-hydroxysulfosuccinimide sodium salt (sulfos-NHS), PAMAM dendrimer generation 4.5 polyamidoamine, bovine serum albumin (BSA), and propidium iodide solution (1 mg/mL in water) were purchased from Sigma–Aldrich (Germany). EDC/hydrochloride was supplied from AppliChem GmbH (Germany). A silver-enhancing kit was received from BBI international, Plano GmbH (Germany). Polyvinyl alcohol (Mw 70,000) (PV A) was obtained from VWR (Germany). EpCAM (VU1D9) mouse mAb (Alexa Fluor® 488) was supplied from New England BioLabs GmbH (Germany). Sterile syringe filter (220-nm pore size) made of polyethersulfone from Rotilabo® was used. Deionized water was used in all procedures. For cell culture DMEM (Dulbecco’s Modified Eagle Medium), pen/strep (penicillin/streptomycin), EDTA (ethylenediaminetetraacetic acid), and erythrocytes lysing buffer were purchased from Invitrogen, and FCS (fetal bovine serum) was ordered from Biochrom AG (Germany).

2.2 Multicore Surface-Enhanced Raman Spectroscopy Labels

The preparation of MSLs was previously reported. In brief, aggregation of gold NPs around 40 nm in diameter was controlled with a linear water-soluble diamine. Raman reporter molecules were covalently bound at the metal surface. In our approach, a reporter molecule concentration of 1·10⁻⁶ (‰vol.) in water was applied. MSLs with the reporter molecule 3-mercaptopbenzoic acid, 4-mercaptopbenzoic acid, and thiophenol were used for cell experiments. Different charged polymers such as PVP and PVA prevent further aggregation and support the growth of a silica shell. Before preparation of the silica shell, silver was deposited at the surface of gold aggregates with reporter molecules. Then, 600 µL MSL was filtered with a syringe filter (220-nm pore size) to remove larger clusters. After that the silica shell was completed by the use of TEOS and ammonia.

2.3 Surface Functionalization

The silica shell was functionalized in an improved way with APTES as follows. After 20-min incubation with TEOS, 1 µL of APTES was added under vigorous stirring to the mixture for
further 10 min. The mixture was then washed twice with ethanol and once with water. Sonication was used between the washing steps to suspend the MSL. After a further washing step, water was removed and the pellet was suspended in a mixture of 50 μL PAMAM dendrimer, 3.7 mg EDC, and 4.2 mg sulfo-NHS aliquot with MBS buffer (pH = 4.5) to complete the final volume to 1 mL and activated for 30 min by shaking before using. The reaction mixture with the MSL was shaken for another 30 min. To remove all unreacted dendrimer solutions, the suspension was washed with MBS buffer by centrifugation (8000 rpm, 5 min) two times. 20 μL of EpCAM (alexa®fluor 488) antibody was added to the activated MSL and was carefully shaken for 3 h. Finally, all unreacted proteins were removed from the solution (6000 rpm, 5 min) and the sample was washed by centrifugation three times with block buffer (pH = 7.4, PBS + 0.1% BSA).

2.4 Cell Cultivation and Reagents

For cell experiments, the cell lines MCF-7 (breast adenocarcinoma) and human foreskin fibroblasts (HFFs) as well as primary leukocytes from healthy volunteers were used. MCF-7 cells and HFFs were cultured in DMEM + 10% FCS + 1% pen/strep at 37°C under a 5% CO2 atmosphere. For splitting the confluent culture of MCF-7 cells, trypsin and EDTA were used. Leukocytes from whole blood samples were prepared by lysis of 8-μL patient blood with 40-μL erythrocyte lysis buffer and incubated for 10 min. White blood cells were collected by centrifugation (10 min, 1200 rpm). In a second step, the leukocyte pellet was resuspended with 15-mL erythrocyte lysis buffer and incubated for 10 min. After centrifugation, the cell pellet containing leukocytes was suspended in PBS + 2 mM EDTA buffer.

2.5 Cell Detection

After the cells were grown for 1 day, cells were transferred into block buffer (PBS + 0.1% BSA) for 30 min. Anti-EpCAM antibody functionalized and nonfunctionalized MSL were added to the cells in a defined concentration, volume, and time. In detail, 100-μL nonfunctionalized MSL in two different concentrations (OD = 0.11 and 0.15 at 534 nm) were added to 500 μL of MCF-7 cells or leukocytes in block buffer for 1 h. Incubation was carried out during mixing of cells. Cells were washed by centrifugation (5 min, 300 rpm) with block buffer three times. MCF-7 cells and leukocytes in suspension were also treated with EpCAM functionalized MSL (MSL-EpCAM) in three different concentrations (OD = 0.11, 0.13, and 0.15 at 534 nm) in the same manner. Adherent MCF-7 cells and HFF grown on CaF2 slides were transferred into 2.5-μL block buffer and treated with 500-μL MSL-EpCAM (OD = 0.13 at 534 nm) for 1 h. Furthermore, MCF-7 cells were incubated with MSL-EpCAM for 30 min, 1, and 2 h in the same manner. To test successful removal of free MSL by centrifugation, buffer without cells was measured.

2.6 Spectroscopy and Microscopy

Raman spectra were collected using two different microscopic Raman systems. Single Raman spectra of whole single cells (40x objective, P = 40 mW) and NP suspensions (10x objective, P = 40 mW) were collected with the RXN1 microprobe (Kaiser Optical Systems). The transmissive holographic grating covers the spectral range from 0 to 3550 cm⁻¹ at a spectral resolution of 4 cm⁻¹. The instrument contains a built-in multimode laser at 785 nm (Invictus). The laser wavelength was calibrated using cyclohexane, the intensity axis was calibrated using a white light source, and the wavenumber axis was calibrated using a helium neon source according to the manufacturer’s instructions. Cell maps were measured using an Alpha 300R Plus confocal Raman microscope with a single-mode diode laser at 785 nm (WITec, Ulm, Germany) and a 60 × /NA = 1 water-immersion objective (Nikon, Japan). Spectra were collected with a 0.5 cm⁻¹ step size, an exposure time of 0.5 s using a 300/μm grating, and 25-mW laser power. The spectral resolution was about 6 cm⁻¹ and the spectral window ranged from 300 to 3200 cm⁻¹. For collection of Raman spectra CaF2 slides and quartz glass cuvettes were used. Raman spectra were baseline-corrected and intensity-normalized (vector normalization) using the Opus software (Bruker). Raman maps were analyzed with WITec Project software.

3 Results and Discussion

3.1 Structural and Optical Characterization of MSL

The final structure of MSLs is shown in Fig. 1(a) and consists of aggregated gold NPs, a monolayer of reporter molecules, a thin silver shell, a silica shell, and antibodies. The optimized procedure included to encapsulate MSL with APTES instead of APTMS. Because of the slower reaction times of APTES, a more controlled growth of the silica shell is possible.

A thin silver shell further enhances the SERS intensities of Raman reporter 3-mercaptopbenzoic acid bound to the gold NPs [Fig. 1(b)]. The decrease of the SERS intensities with increasing thickness of the silver shell might be due to the lower penetration of radiation. A silica shell prevents adsorption and enhancement of interfering molecules, and provides stability of MSLs against further aggregation for more than 3 months. After storage for 1 year in the dark at ambient temperature, MSLs gave SERS signals of similar intensities (data not shown). The aggregation was controlled with the aim that between two and four gold NPs were present in the silica shell [Fig. 1(c)]. The silica thickness is around 12 nm after a 30-min incubation time [Fig. 1(d)]. Adding APTES to the mixture for the last 10 min gave a significant increase to overall 22 nm [Fig. 1(e)]. This is attributed to the higher reactivity of APTES compared to TEOS. In TEM images, the transition between the two silanes is visible [Fig. 1(e), blue arrow] silica provides an effective binding site for conjugation of the amine-containing APTES. The hydrolysis of APTES to silanols and the further condensation generates a surface with organo-functional alkoxysilane molecules. APTES-functionalized surfaces have been shown to be non-toxic,28 and therefore constitute a good and efficient substrate for conjugation of biomolecules. NP aggregates show good SERS enhancement with near-infrared (NIR) excitation that is consistent with their plasmonic properties with absorption maxima near 534 and 783 nm [Fig. 1(f)]. The slope of the latter
band is typical for aggregated gold NPs and enables efficient excitation by NIR lasers at 785 nm. Gaps in gold NP clusters show an enhanced electromagnetic field called “hot spots” and enable short measurement times down to milliseconds for adsorbed molecules.

### 3.2 Antibody Conjugation

The conjugation of the antibody to MSL is shown in Fig. 2. Dark field (c, d) and fluorescence images (a, b) of dried MSL are compared after conjugation of anti-EpCAM antibodies labeled with a fluorescent dye. A polyamidoamine (PAMAM) dendrimer was used to enlarge the surface for covalently binding of antibodies at the silica surface. PAMAM has a specific size and shape and a highly functionalized terminal surface. The term “dendrimer” was derived from its tree-like branching structure. The G4.5 dendrimer contains 128 terminal carboxyl groups on the outer surface. For cross linking of secondary amines (APTES with IgG antibody) with carboxylates (PAMAM dendrimer), EDC and sulfo-NHS enable formation of amide bonds.

![Fig. 1](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics/)

**Fig. 1** (a) Structure of multicore surface-enhanced Raman spectroscopy (SERS) labels (MSL). (b) SERS spectra of aggregated gold nanoparticles with reporter molecule 3-mercaptopbenzoic acid without (black), thin (green), medium (red), and thick silver shell (blue). (c) Transmission electron microscope (TEM) images of MSLs, (d) silica coated MSL after incubation with tetraethyl orthosilicate (TEOS) for 30 min (e) and after incubation with TEOS for 20 min followed by APTES for 10 min. The blue arrow marks the transition between both silanes. (f) Absorbance spectrum of MSL.

![Fig. 2](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics/)

**Fig. 2** Fluorescence images (a, b) and dark field images (c, d) of anti-EpCAM antibody (Alexa®488) conjugated MSL. Every dot consists of MSL clusters. Little dots may be single MSL. In images (a) and (c) the sample margin is marked. The sample shows the same greenish and bluish backgrounds as the glass slide, respectively.

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of a stable amide bond. The efficiency of EDC-mediated coupling is increased in the presence of sulfo-NHS. Fluorescence images indicate the success of antibody conjugation at the MSL surface after several washing steps. The positions of MSL agree well in the dark field and fluorescence image. Virtually all MSLs are conjugated with a high number of antibodies (Fig. 2).

### 3.3 Cell Detection by Nonfunctionalized MSL

The specificity of nonfunctionalized MSLs (MSL-non) for tumor cell detection was tested by incubating MSLs-non at two different concentrations with living MCF-7 cells in suspension. Raman spectra of about 20 single cells were measured in 1 s with 785-nm excitation. MSLs-non are not expected to bind specifically to cells because of the missing antibody, and subsequently they should not show SERS spectra of the Raman reporter 3-mercaptobenzoic acid. The percentage values indicate the fraction of cells with a SERS spectrum from MSL (Table 1). At low concentration of MSL-non (OD$_{534 \text{ nm}} = 0.11$), MSL-non did not bind to MCF-7 cells. By increasing the MSL-non concentration (OD$_{534 \text{ nm}} = 0.15$), an unspecific interaction was detected in 70% of cells, this means 30% of measured cells show no SERS signal. Forthcoming experiments will investigate the conditions (such as incubation time and concentration) in more detail if MSLs-non constitutes an alternative route for 100% tumor cell detection.

### 3.4 Cell Detection by Functionalized MSL

The specificity of anti-EpCAM antibody-functionalized MSL (MSL-EpCAM) for tumor cell detection was tested in an analogous manner. As EpCAM is over-expressed in MCF-7 cells, the MSL-EpCAM is expected to bind specifically. At low concentration of MSL-EpCAM (OD$_{534 \text{ nm}} = 0.11$), SERS spectra of the reporter molecule were detected for 75% of the MCF-7 cells in suspension after antibody-antigen binding (Table 1). Compared with the results of MSL-non, this observation suggests true-positive cell detection. After increasing the concentration to OD$_{534 \text{ nm}} = 0.15$, SERS spectra were obtained for 100% of the MCF-7 cells, probably due to a mix of specific and nonspecific binding of MSL-EpCAM to MCF-7 cells. As described above, already 70% of cells bound MSL-non at this concentration. On the one hand, only 75% of MCF-7 cells were specifically detected at OD$_{534 \text{ nm}} = 0.11$ concentration; therefore, the MSL-EpCAM concentration needs to be increased for 100% specificity. On the other hand, the concentration should be below OD$_{534 \text{ nm}} = 0.15$ to avoid nonspecific interactions. Consequently, the MSL concentration was adjusted. In further experiments, the concentration of MSL-EpCAM was set to an optical density near OD$_{534 \text{ nm}} = 0.13$. After incubation of MCF-7 cells with MSL-EpCAM for 1 h, approximately 100% of $10^6$ MCF-7 cells were detected (Table 1).

![Fig. 3](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics on 25 Aug 2019 Terms of Use: https://www.spiedigitallibrary.org/terms-of-use)
An uptake of MSL-EpCAM (OD$_{534}$ nm $= 0.13$) into the cytoplasm of living adherently grown MCF-7 cells was not observed even after prolonged incubation for 2 h. Dark field images at three depths resolved MSLs only at the upper cell membrane [Fig. 3(a)] but not in cytoplasm (b) and at lower membrane (c). The results in Fig. 3 are consistent with fluorescence images in Fig. 4 and SERS images in Fig. 5 that confirm the localization of MSL-EpCAM near the cell membrane. The large size of MSL over 120 nm and low incubation times might explain the lack of internalization of MSL by cells and the low toxicity in viability tests (see Fig. 6). Incubation times of only half an hour lead to incomplete binding of MSL-EpCAM to MCF-7 cells, and thus give similar results as for using concentrations of MSL-EpCAM below OD$_{534}$ nm $= 0.13$.

MSL-EpCAM was also incubated with leukocytes and binding was monitored by SERS spectroscopy. It was observed that MSL-EpCAM did not bind nonspecifically to leukocytes for concentrations between OD$_{534}$ nm $= 0.11$ and 0.15 (Table 1). Although it is unlikely that the binding capacity of MSL-non to leukocytes is higher than MSL-EpCAM, the interaction of MSL-non to leukocytes will also be probed in the context of the specificity of MSL-non for tumor cell detection in forthcoming experiments.

As leukocytes are nonadherent cells in suspension, the specificity of MSL-EpCAM was further tested for HFF as an adherent cell line without EpCAM antigen. Cells were grown on CaF$_2$ slides and incubated with fluorescently labeled EpCAM [Figs. 4(a)–4(d)] and MSL-EpCAM [Figs. 4(e)–4(h)]. Whereas dark field images show a number of cells, fluorescence images prove the binding of EpCAM to MCF-7, but not to HFF [Figs. 4(a)–4(d)]. For MSL-EpCAM, dark field and fluorescence images reveal NPs bound to MCF-7 [Figs. 4(e) and 4(f)] consistent with Figs. 4(a) and 4(b) but no NPs bound to HFF [Figs. 4(g) and 4(h)].

Raman images of single cells were collected to visualize cellular distribution of MSL-EpCAM whose reporter molecules cannot be detected by fluorescence. Chemical Raman images [Figs. 5(c) and 5(d)] were generated by plotting the intensities of the C-H stretching vibrations between 2800 to 3020 cm$^{-1}$ for MCF-7 and HFF cells [Figs. 5(a) and 5(b)]. All cells are visualized at a similar intensity scale. The intensities of the reporter molecule band at 1073 cm$^{-1}$ of thiophenol are also

![Raman intensity vs Raman shift](image)

**Fig. 5** Bright field image of (a) MCF-7 and (b) HFF cells. Chemical map of the C-H deformation band at 2880 cm$^{-1}$ of (c) MCF-7 and (d) HFF cells. (e, f) Chemical map of the MSL band at 1073 cm$^{-1}$. Representative Raman spectra of MSL and cell from the Raman images (position indicated by blue and green star).

![Representative Raman spectra](image)

**Fig. 6** Viability test with propidium iodide. Fluorescence and dark field images of MCF-7 cells after incubation with (a, b) MSL-EpCAM or (c, d) pure EpCAM as reference for 1 h. Nuclei of dead cells are colored in red after 1 h incubation time.
plotted for both cell types on the same scale [Figs. 5(e) and 5(f)]. High intensities were found near the cellular membrane of MCF-7 cells that is consistent with the specific binding of MSL-EpCAM. The Raman image in Fig. 5(e) shows a nonuniform binding of MSL-EpCAM that is consistent with dark field and fluorescence images in Figs. 3 and 4, respectively. The nonuniform binding pattern might point to different affinities or potential blocking of some antigen binding sites.

Low intensities in the Raman image of HFF cells demonstrate the absence of MSL-EpCAM binding. A typical spectrum of MSL-EpCAM from the Raman image of MCF-7 cells is dominated by spectral contributions of the reporter molecule (Fig. 5, blue star). For comparison, a typical Raman spectrum of HFF cells is shown with spectral contributions of proteins and lipids, but without the reporter molecule (Fig. 5, green star).

### 3.5 Cell Viability

Viability tests were carried out with MCF-7 cells using propidium iodide during incubation with MSL-EpCAM in a suspension of living cells for 1 h. Propidium iodide stains necrotic and apoptotic cells in red. Figure 6 shows that the majority of cells are unstained confirming their viability and low toxicity of MSL-EpCAM at the concentration OD$_{534 \text{ nm}}$ = 0.13. Only a few cells were stained, which appears to be independent of the incubation with MSL-EpCAM [Figs. 6(a) and 6(b)] or pure EpCAM [Figs. 6(c) and 6(d)]. A few dead cells can always be expected in cell cultures because of occasional necrosis and apoptosis. The low toxicity of MSL-EpCAM is probably related to the large size of more than 120 μm and the lack of internalization as written above. This observation is consistent with a report that amorphous silica NPs below 100 μm induced cytotoxicity. The authors suggested that the size is decisive to produce biological effects. This hypothesis was confirmed in a murine toxicology study by administration of SERS-active NPs measuring 100 nm in diameter.

The shape of cells in Figs. 4–6 is slightly different, because of the applied preparation protocol. The cells in Figs. 4 and 5 grew adherently onto CaF$_2$ slides, whereas the cells in Fig. 6 were treated with trypsin to detach them from slides and transfer them in suspension. Such treatment is known to change the shape of cells. Gold NPs are also used in PT therapies due to their known PT effects by laser irradiation. By using short measurement times of 1 s or less, exciting with a NIR laser and 40-mW laser power phototoxic effects of cells in buffer is expected to be small. Heat-induced cell damage would cause spectral changes in protein vibrational modes that have not been observed so far.

### 4 Conclusions

As stated in the Introduction, the CellSearch system (Veridex) gives only 50% pure CTC samples and fails in a considerable number of cases to identify CTCs in a patient’s blood. Possible explanations are that EpCAM is the only antibody that is used to capture CTCs and the expression of antigens varies remarkably between single tumor cells. Therefore, the application of multiple antibodies is a well-accepted route to improve identification and sorting of tumor cells. However, widely used optical and spectroscopic tools in cytometers are based on elastic light scattering and fluorescence that only allow simultaneous detection of a few tags. Another limitation is the stability of fluorescent tags due to photobleaching. SERS tags offer (1) higher stability against photobleaching, (2) high multiplex capability due to enhancement of fingerprint-like signatures, and (3) comparable or even higher intensities than fluorescent tags. The authors used the height of one SERS band and the height at the fluorescence maximum to determine a lower detection limit for a SERS-labeled antibody than for a fluorescence labeled antibody using the same instrument with the same laser excitation source. This paper described preparation and properties of SERS-tags against the antigen EpCAM. The protocol can easily be adapted to other reporter molecules and antibodies for multicolor detection of multiple antigens as summarized in the Introduction. Specific detection of MCF-7 tumor cells and no nonspecific binding of MSL to leukocytes and HFF were demonstrated. No toxicity of MSL for cells makes them very interesting for detection of living cells with the option of separation for further investigations. Excitation of localized surface plasmon polariton modes using NIR laser at 785 nm induces low phototoxicity, which depends on the lower energy input at longer wavelengths. Fast measurement times as low as 25 ms per cell render MSL ideal for high-throughput RACS in microfluidic chips. This time is significantly lower than exposure times of 1 s and more in a similar SERS study describing a different protocol. SERS-based cytometry has already been recognized as a powerful complement to conventional fluorescence-based cytometry and has been demonstrated to detect leukemia and lymphoma cells.

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### References


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