Rational design of thiolated polyenes as trifunctional Raman reporter molecules in surface-enhanced Raman scattering nanotags for cytokine detection in a lateral flow assay

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Abstract
The characteristic vibrational spectroscopic fingerprint of Raman reporter molecules adsorbed on noble metal nanoparticles is employed for the identification of target proteins by the corresponding surface-enhanced Raman scattering (SERS) nanotag-labeled antibodies. Here, we present the modular synthesis of thiolated polyenes with two to five C=C double bonds introduced via stepwise Wittig reactions. The experimental characterization of their electronic and vibrational properties is complemented by density functional theory calculations. Highly SERS-active nanotags are generated by using the thiolated polyenes as Raman reporter molecules in Au/Au core/satellite supraparticles with multiple hot spots. The cytokines IL-1β and IFN-γ are detected in a duplex SERS-based lateral flow assay on a nitrocellulose test strip by Raman microscopy. The thiolated polyenes are suitable for use in immuno-SERS applications such as point-of-care testing as well as cellular and tissue imaging.

KEYWORDS
POCT, Raman reporters, rational design, SERS

1 | INTRODUCTION

Quantum dots and molecular fluorophores are commonly used as labels for optical detection in bioanalytical chemistry and life sciences [1–3]. Besides labeling via established fluorescence techniques, various alternative labeling possibilities exist. One of them is the use of gold nanoparticles, exploiting their size- and shape-dependent optical properties, extremely high photostability and chemical robustness. Molecularly functionalized noble
metal nanoparticles offer the readout by surface-enhanced Raman scattering (SERS). The spectral identification of such a SERS label or SERS nanotag [4–8] is based on the characteristic vibrational fingerprint of the Raman reporter molecule, which is typically chemisorbed on the metal surface via a surface-seeking group [9]. Highly sensitive Raman reporters possess highly polarizable moieties like conjugated π-systems.

While many different fluorescent dyes are commercially available, examples for rationally designed Raman reporter molecules—either obtained by a simple single-step addition of a linker to an existing reporter or prepared in a time-consuming multi-step synthesis—are rare. For example, Graham and coworkers designed benzotriazole derivatives as Raman reporter dyes for use in surface-enhanced resonance Raman scattering (SERRS) on silver colloids with visible laser excitation [10, 11]. Kneipp and coworkers employed a thiolated derivative of β-carotene for SERS and surface-enhanced hyper Raman scattering [12]. Rationally designed thiolated olefins and alkynes, which form a self-assembled monolayer on gold surfaces, were obtained by a multi-step synthesis by Lambert and coworkers [13]. Similarly, Zhang and coworkers used an alkyne-based thiolated Raman reporter made in a single-step reaction employing cysteamine as a linker [14]. Thiolated Rhodamines as Raman dyes for visible excitation were designed and prepared in a multi-step synthesis by Brem and Schlücker [15]. For clinical applications in vivo, the use of near-infrared (NIR) laser excitation is required and NIR-active Raman dyes provide signal enhancement via NIR-SERRS. To this end, Chang, Olivo and coworkers introduced linker-modified triphenylmethane and cyanine dyes [16, 17]. More recently, Detty, Kircher and coworkers presented chalcogenopyrylium dyes for ultrasensitive NIR-SERRS [18].

In this study, we present the design and synthesis of trifunctional phenyl/polyene-based Raman reporter molecules with variable chain length from two to five double bonds (Figure 1). These polyenes consist of a surface-seeking group (thioester), a reactive carboxylate ester terminus for bioconjugation and a phenyl-substituted polyene chain as the Raman reporter moiety. The phenyl ring fulfills three separate functions: it chemically stabilizes the polyene chain against oxidation, it leads to a bathochromic shift and an increased polarizability due to conjugation with the π-system of the polyene chain. Controlling the length of the polyene chain can be utilized for signal enhancement via molecular electronic resonances and the wavenumber position of the C=C stretch vibration [19]. In contrast to natural polyenes such as β-carotene, there is no substitution of the olefinic carbons in order to maximize the number of olefinic hydrogen atoms (C=C–H) for future potential isotopic substitution by deuterium atoms (C=C–D). This isotopologue approach [20–22] could generate multiple equally/similarly bright Raman reporters (same/similar Raman cross section due to the same chain length) with distinct spectral barcodes [23–26]. A potential drawback of the employed phenyl substitution at the left terminus of the polyene chain (Figure 1), however, is the occurrence of additional vibrational bands, which may limit the maximum multiplexing capability.

All thiolated polyenes in Figure 1 are characterized by UV/vis absorption and Raman spectroscopy for determining their electronic and vibrational properties. Results from density functional theory (DFT) calculations enable the assignment of the experimentally observed Raman peaks. Then, the polyenes are employed in colloidal SERS experiments for highlighting their suitability as Raman reporters in SERS nanotags. After bioconjugation to antibodies directed against the cytokines IL-1β and IFN-γ, the successful application in a duplex SERS-based lateral flow assay (LFA) on a functionalized nitrocellulose (NC) membrane serving as a test strip is demonstrated.

2 MATERIALS AND METHODS

2.1 Experimental details

The starting materials were obtained from commercial suppliers and used without further purification. Ascorbic acid (99%, Sigma-Aldrich), cetyltrimethylammonium bromide (CTAB, 96%, Fluka), cetyltrimethylammonium chloride (CTAC, 95%, TCI), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 98%, Sigma-Aldrich), glycercoll (99%, Sigma-Aldrich), hydroquinone (99%, Sigma-Aldrich), α-mercaptoproxy polyethylene glycol (99%, Sigma-Aldrich), and (99%, Sigma-Aldrich), hydroquinone (99%, Sigma-Aldrich), α-mercaptoproxy polyethylene glycol (99%, Sigma-Aldrich), and 2,2-dimethoxy-2-phenylacetophenone (99%, Sigma-Aldrich), 2,2-dimethoxy-2-phenylacetophenone (99%, Sigma-Aldrich), 2,2-dimethoxy-2-phenylacetophenone (99%, Sigma-Aldrich), 2,2-dimethoxy-2-phenylacetophenone (99%, Sigma-Aldrich), 2,2-dimethoxy-2-phenylacetophenone (99%, Sigma-Aldrich). 

**FIGURE 1** Molecular structures of the trifunctional Raman reporter molecules comprising a polyene chain with two to five C=C double bonds, a thioester as a surface-seeking moiety and a reactive carboxylate ester terminus for potential bioconjugation.
glycol (PEG) (3 kDa, Rapp Polymere), (11-mercaptop-
undecyl)-N,N,N-trimethylammonium bromide (MUTAB, 95%, Sigma-Aldrich), 2-morpholin-4-yl ethane-
sulfonic acid (MES, 99.5%, Sigma-Aldrich), N-hydroxysuccinimide (NHS, 98%, Sigma-Aldrich), silver nitrate (99.9%, Carl Roth), sodium bromide (99.5%, Sigma-Aldrich), sodium citrate (99%, Sigma-Aldrich), sodium borohydride (96%, Sigma-Aldrich) and poly(sodium 4-styrenesulfonate) (PSS, Sigma-Aldrich).

Cyclohexane (99.8%, Fisher Scientific), dichloromethane (99.8%, Fisher Scientific), dimethylformamide (99.8%, VWR Chemicals), ethyl acetate (99.8%, Fisher Scientific), methanol (99.9%, Fisher Scientific), 4-bromobenzyl alcohol (98%, abcr), tert-butyl mercaptan (98%, TCI), ethyl bromoacetate (98%, abcr), triphenylphosphine (99%, Alfa Aesar), 4-(methylthio)benzaldehyde (95%, TCI), diisobutylaluminimum hydride (DIBAL-H) solution 1 mM in methylene chloride (Sigma-Aldrich), potassium permanganate (98%, Alfa Aesar), Manganese(II) sulfate monohydrate (99%, Alfa Aesar), sodium borohydride (99.5%, Sigma-Aldrich), potassium carbonate (food grade, AppliChem), potassium sodium tartrate tetrahydrate (99%, Sigma-Aldrich) and sodium sulfate (pa, Bernd Kraft).

2.2 Synthesis and functionalization of gold nanoparticles for the SERS-based LFA

2.2.1 Au/Au core/satellite NP

Citrate-stabilized gold nanoparticles (AuNP) were prepared via the Turkevich method according to Bastús et al [27]. In order to grow gold seeds, 1 mL of a 0.2 M citrate solution is rapidly injected to a mixture of 318 μL of 0.425 M HAuCl₄ and 248 mL water (100°C). After 30 minutes, the temperature was reduced and the growth was stopped resulting in AuNP with 20 nm in size.

Spherical CTAB-stabilized AuNP were synthesized using the method of Ruan et al [28], which can be divided in four steps. The first step of this route is the synthesis of nanoclusters. 250 μL of a 10 mM HAuCl₄ solution was added to 10 mL of a 100 mM CTAB solution. The injection of 600 μL of an ice-cold 10 mM NaBH₄ solution started the cluster growth. The mixture was stored for 2 hours at 30°C.

The second step is the growth of Au seeds. The clusters (0.12 mL) were injected into 190 mL DI water. A mixture of 10 mL of a 100 mM CTAB solution, 4 mL of a 10 mM HAuCl₄ solution and 15 mL of 100 mM ascorbic acid was added to the cluster dispersion. The mixture was stored for 2 hours at 30°C and then washed (i.e., centrifugation followed by redispersion).

The resulting seeds with a diameter of 30 nm were further grown by adding 30 mL of a 25 mM CTAC solution, 0.75 mL of 100 mM ascorbic acid solution and 1.5 mL of 10 mM HAuCl₄ solution. The mixture was stored for 2 hours at 30°C, washed and redispersed in 30 mL 20 mM CTAB solution. The last step, an oxidation, was carried out by adding 1 mL of 10 mM HAuCl₄ solution. The mixture was diluted with 150 mL of 20 mM CTAB solution and then stored for 2 hours at 40°C and washed.

The core particles were incubated with 2 μL (10 mM) of the corresponding Raman reporter molecules and washed. Citrate particles were added to the positively charged core particles and washed afterwards.

2.2.2 Au nanostars

Gold nanostars were prepared in a CTAB-free approach [29]. In order to synthesize gold nanoparticles as seeds with a diameter of ~17 nm, 900 μL of a 1% sodium citrate solution, 300 μL of a 1% HAuCl₄ solution and 42.5 μL of 0.1% AgNO₃ solution were added into 30 mL of boiling water [30, 31]. 300 μL seed solution was suspended (under stirring at 700 rpm) in 9.8 mL DI water and 1.0 mL glycerol. A mixture of 22 μL 1% sodium citrate solution, 100 μL 1% HAuCl₄ solution and 42.5 μL 0.1% AgNO₃ solution was rapidly added after 5 minutes, immediately followed by the addition of 100 μL 1% hydroquinone solution. The red color changes to deep blue within a few minutes.

2.5 μL of the polyene solution in ethanol (10 mM) was added to 1 mL of the nanostar suspension. The mixture was shaken for 1 hour and washed twice via centrifugation. The resulting precipitate was resuspended in 1 mL DI water and mixed with 25 μL PEG solution (10 mM, H₂O). Hydrophilic ethylene glycol (EG) moieties are known to significantly increase the colloidal stability of SERS nanotags [32]. The particles were incubated for 1 hour, washed twice and resuspended in 300 μL of MES buffer (10 mM). 5 μL NHS solution (100 mM) and 5 μL EDC solution (100 mM) were added to the PEGylated NP and incubated for 2 hours at rt with the corresponding monoclonal anti-IL-1ß and anti-IFN-γ antibodies (each 1 μg). In order to wash the NP, 20 μL of a 1% BSA/PBS buffer solution was added and the NP were centrifuged (590 g, 30 minutes). The precipitate was resuspended in 300 μL of a 1% BSA/PBS solution, incubated for 1 hour at rt and washed twice with the same procedure.

2.2.3 Assay preparation and performance

A duplex assay was designed for the simultaneous detection of the human cytokines IL-1ß and IFN-γ on the same test strip. A NC membrane (CN140, Sartorius) was
blotted as dots with protein A (1 μg) at the control point (CP) and the polyclonal anti-IL-1β (1 μg) and anti-IFN-γ (1 μg) capture antibodies together at the same test point (TP). The NC was dried at 40°C for 1 hour. After immersing the NC into the blocking buffer (1% BSA/PBS), the NC was dried again at 40°C for 30 minutes.

15 μL of each of the SERS-labeled antibodies (OD 0.5) against IL-1β and IFN-γ was incubated with the two antigens IL-1β and IFN-γ (each 0.2 μg) in a purified system (PBS buffer) for 5 minutes. Afterwards, the mixture was dropped onto the test strip and finally washed with PBS buffer.

For the detection of IL-1β and IFN-γ in a spatially separated duplex SERS LFA, protein A (2 mg/mL) was immobilized as control line on a NC membrane (CN140, Sartorius) via a dispenser (ClaremontBio) for capturing SERS-labeled antibodies. Polyclonal antibody IL-1β (1 μg) was blotted as dot at test point 1 (TP-1) and polyclonal antibody IFN-γ (1 μg) at test point 2 (TP-2), respectively.

2.3 | UV/vis absorption spectroscopy

All UV/vis absorption spectra were recorded with a two-beam JASCO V-630 spectrometer using a 2 mm quartz cuvette and 1 × 10⁻⁶ M solutions in ethanol. After collecting the baseline without inserted samples, the measurements were performed in the range of 200 to 600 nm. All measurements were performed with a 2 mm cuvette filled with ethanol as reference.

2.4 | Raman instrumentation

The Raman experiments with 632.8 nm laser excitation were performed on a home-built setup comprising an inverted microscope (Nikon Eclipse Ti) and a spectrometer with 55 cm focal length (HORIBA Scientific iHR550, 600 grooves/mm grating, Peltier-cooled CCD). The 632.8 nm radiation from a He-Ne laser was focused on the sample by a 20x microscope objective (Olympus, NA 0.4).

The Raman experiments with 785 nm laser excitation were performed with a commercially available Raman microscope (Bruker Senterra) with an integrated spectrometer with 20 cm focal length (400 grooves/mm). The 785 nm radiation from a diode laser was employed for excitation of the Raman scattering using a 4x microscope objective (Olympus, NA 0.1).

2.5 | Data processing of UV/vis absorption and SERS spectra

Baseline corrections were applied by using concave rubber-band correction method [33], using 50 iterations and 100 baseline points. A non-negative least-square fit was performed with routines available in MATLAB.

2.6 | DFT calculations

DFT calculations of the vibrational and UV/vis absorption spectra of the polyenes were performed at the B3LYP/6-311++G (d, p) level of theory with the Gaussian 09 program package [34]. Fully optimized geometries were obtained since no imaginary/negative frequencies were observed.

3 | RESULTS AND DISCUSSION

3.1 | Synthesis of polyene-based Raman molecules

The class of novel mono-thiolated polyenes was rationally designed for use as Raman reporters in SERS nanoparticles. The synthesis route is briefly summarized in Scheme 1. The starting material is the commercially available 4-bromobenzyl alcohol, which is converted to
4-(tert-butylthio)benzaldehyde (1) by using manganese dioxide for oxidation [35] followed by a nucleophilic aromatic substitution for introducing the tert-butyl-protected sulfur (2) [36]. The introduction of a C=C double bond is achieved by the reaction of the aldehyde 1 with the Wittig reagent 2 to form the corresponding phenyl acrylate 3. The reduction of the ester group in the phenyl acrylate 3 by DIBAL-H yields the corresponding phenyl allyl alcohol 4 [35]. Similar to the starting material 4-bromobenzylalcohol, compound 4 is also a primary alcohol. The latter can be oxidized to the resulting aldehyde by MnO2 for a subsequent chain elongation, again by a Wittig reaction [37] to the polyene 5 with two C=C double bonds. Polyenes with three and more C=C double bonds 6 are obtained by repeating the same reaction sequence (oxidation/Wittig) [38].

The polyenes with shorter chain length (n = 1-3 DB) can be obtained in high yields and with high purity; in the case of longer polyene chain lengths (n = 4-5 DB), reaction yields are decreasing. The Wittig reaction dominantly gives the E (trans) configuration isomer. In all cases (n = 1-5), the all-trans isomers are isolated by fractionated chromatographic separation. Detailed protocols of the synthesis of the polyenes together with their characterization can be found in the supporting information.

3.2 | Electronic absorption spectroscopy

Figure 2 depicts the experimental UV/vis absorption spectra of the polyenes with two to five C=C double bonds (1 μM solutions in ethanol). As expected, the absorption maximum $A_{max}$ shifts linearly from 318 nm for two double bonds to 391 nm for five double bonds. Our DFT calculations confirm that the most intense absorption peak is due to the corresponding HOMO→LUMO transition. The fine structure is attributed to the breakdown of the Born–Oppenheimer approximation, that is, the coupling between electronic and nuclear motion, in the low-lying excited states [39]. For more details on electronic structure and spectroscopy of linear polyenes, the reader is referred to the literature [40, 41].

3.3 | Raman spectroscopy of the polyene-based Raman reporters

Figure 3 shows the baseline-corrected and normalized Raman spectra of the conjugated polyenes with two to five C=C double bonds obtained with 632.8 nm laser excitation (10 mM solutions in chloroform). The Raman spectra are normalized to the solvent peak of chloroform at 667 cm$^{-1}$ for comparing the relative intensities. The Raman spectrum of the polyene with two double bonds (2 DB) in Figure 3 (bottom) is multiplied by a factor of 10 and exhibits three dominant Raman peaks: at 1134 cm$^{-1}$ due to the C=C stretching vibration of the polyene chain as well as at 1586 and 1624 cm$^{-1}$ due to coupled C=C stretching and phenyl ring vibrations. According to the corresponding DFT-computed vibrational eigenvectors (Figure S1), the peak at 1586 cm$^{-1}$ has dominant stretching contributions from the phenyl ring, whereas the peak at 1624 cm$^{-1}$ has dominant stretching contributions from the polyene chain. The C=C stretching peak undergoes a minimal shift to higher wavenumbers from 1134 cm$^{-1}$ (2 DB) to 1140 cm$^{-1}$ (5 DB) and a >20-fold increase in intensity due to the significantly increased polarizability of the polyene chain.
With increasing chain length, the wavenumber difference between the two spectrally resolved peaks at 1586 and 1624 cm$^{-1}$ (2 DB) decreases to 1588 and 1605 cm$^{-1}$ (3 DB), as predicted by the DFT calculations (Figure 4). The peak at 1588 cm$^{-1}$ (3 DB) is shifted to 1591 cm$^{-1}$ in the Raman spectrum of the polyene with four double bonds (4 DB), while the peak at 1605 cm$^{-1}$ (3 DB) is shifted to 1578 cm$^{-1}$ (4 DB). Again, this spectral assignment is based on our DFT calculations and the corresponding eigenvectors of the normal modes (Figure S1). In the Raman spectrum of the polyene with five C= C double bonds (5 DB) only a single very intense peak at 1565 cm$^{-1}$ is observed. This is in agreement with theory, which predicts only a single normal mode with high Raman activity in this spectral region.

Figure 4 shows a comparison of the experimental and computed wavenumber positions of the normal mode with dominant C= C double bond stretching contributions as a function of chain length (1-5 DB). Both experimental and theoretical values exhibit an almost linear decrease as expected from the vibrational properties of conventional polyenes without substitution by thiophenol and/or a carboxylic ester [19].

The Raman spectrum of the compound with a single C= C double bond (1 DB) exhibits two dominant bands at 1586 and 1631 cm$^{-1}$, which are assigned to the phenyl ring and the olefinic C= C stretching vibration, respectively (Figure S3). The Raman intensity of this olefin (1 DB) is ca. 10 times lower compared to the first polyene (2 DB). The nonlinear increase in Raman intensity with chain length (1-4 DB) is experimentally observed both for 632.8 nm (Figure 3 and Figure S3) and for 785 nm laser excitation (Figure S4). Since both laser excitation wavelengths are far from any detectable electronic resonances (cf. UV/Vis spectra in Figure 2), we tend to exclude the role of pre-resonance enhancement. Our DFT calculations (gas phase) also predict a significant increase in Raman activity (ca. threefold for each additional C=C bond; results not shown) due to the increased transition polarizability; however, only a linear trend is theoretically predicted. A detailed analysis of this discrepancy between experiment and theory is beyond the scope of this paper, which is focused on the application of these novel Raman reporters in SERS nanotags.

For quantitative multiplexing applications, each polyene in Figure 3 could serve as a starting point for the synthesis of isotopologues, that is, a set of Raman reporters with similar Raman activities due to same polyene chain length and the same surface affinity. Figure 5 shows the normalized, DFT-computed Raman spectra of the undeuterated and perdeuterated polyene with five C= C double bonds (molecular structures, see Figure S5). The coupled C= C/phenyl stretching peak is shifted approximately by 60 cm$^{-1}$ due to C-deuteration from 1605 to 1545 cm$^{-1}$. Interestingly, the computed absolute Raman activity of the perdeuterated isotopologue is ca. 30% higher compared to the nondeuterated species (see Figure S5).

Figure 1 shows the all-trans configuration for all polyenes. We employed conventional 1H-NMR spectroscopy for unambiguously determining the chain length based on the ratio of the integrated signals from the olefinic protons and the methylene protons of the ethyl ester. However, the broad multiplet structure in the region of the olefinic protons does not permit us to resolve the corresponding vicinal coupling constants, which are necessary to confirm the presence of cis/trans configurations. We therefore employed Raman spectroscopy since this vibrational spectroscopic technique is also able to discriminate between trans and cis isomers. Figure 6 top shows the recorded experimental Raman spectrum (solid green line). Figure 6 bottom depicts the computed Raman spectra of the polyene with five double bonds: the all-trans form (dashed black line) and the 2/3-cis-isomer (dashed red line). The latter has cis configurations at the
second and third C═C double bond (counted from the thiophenol) and exhibits two equally intense Raman peaks well separated by ca. 20 cm\(^{-1}\). Overall, the computed spectrum of the all-trans form agrees much better with the experimental spectrum than the computed spectrum of the isomer with two cis configurations. Please note that for comparison purposes, the wavenumber values at the top (experiment) and at the bottom (theory, unscaled harmonic values) are shifted relative to each other.

### 3.4 SERS spectra with polyenes as Raman reporters

For the SERS experiments, the polyenes were co-adsorbed on 50 nm Au cores together with mercapto undecanoic acid trimethylammonium bromide (MUTAB) in order to obtain Au/Au core/satellite [42, 43] supraparticles via electrostatic assembly with negatively charged citrate-capped 17 nm Au satellites (Figure S6). The plasmonic coupling (hot spots) between Au core and Au satellites leads to a high Raman signal enhancement and a strong plasmon resonance around 630 nm (Figure S7). Figure 7 shows the SERS spectra of the polyenes with two to five double bonds. The intensities are normalized to the dominant Raman peak of ethanol at ca. 882 cm\(^{-1}\). Solvent peaks are indicated by an asterisk (*). Two asterisks (**) indicate an overlapping peak with contributions from the C-S vibration and ethanol. Laser excitation wavelength: 632.8 nm

![FIGURE 6](image1.png)

**FIGURE 6** Experimental Raman spectrum of the trifunctional polyene Raman reporter molecule with five double bonds (green continuous line, top) compared to the simulated Raman spectra (bottom) for the polyene species with five double bonds in all-trans (black dashed line) and 2/3-cis (red dashed line) configuration.

![FIGURE 7](image2.png)

**FIGURE 7** Surface-enhanced Raman scattering spectra of the trifunctional polyene Raman reporter molecules with two to five C═C double bonds. Suspension medium: water and 15% ethanol as internal standard. Solvent peaks are marked with an asterisk (*). Two asterisks (**) indicate an overlapping peak with contributions from the C-S vibration and ethanol. Laser excitation wavelength: 632.8 nm

For the polyenes, the position of the coupled polyene/C═C peak exhibits only a minimal shift to higher wavenumbers from 1139 cm\(^{-1}\) (2 DB) to 1146 cm\(^{-1}\) (5 DB). The constraint of employing relatively small reporter molecules is not given for gold nanostars since the hot spots occur at the tips, which are spatially easily accessible. We therefore
decided to test gold nanostars as bright SERS tags because they also can be detected at the single-particle level [46, 47]. Figure S8 shows a TEM image together with the UV/vis extinction spectrum of the gold nanostars employed for the bioanalytical SERS experiments described in the following section.

### 3.5 | LFA with polyene-based SERS nanotag/antibody conjugates

LFA are the most commonly employed tests in point-of-care testing (POCT) since they are cheap, fast and reliable. Conventional LFAs are based on a NC membrane with immobilized antibodies for capturing the target protein and optical readout typically based on the reddish color of the gold nanoparticles in the corresponding immuno-sandwich complex. In a SERS-based LFA, the conventional citrate-capped gold nanoparticles are exchanged by SERS nanotags for lowering the limit of detection [43, 48–50]. Figure 8A shows the basic principle of the custom-made duplex LFA with SERS readout at the TP. The two target proteins in the duplex assay for parallel detection are the cytokines interleukin (IL)-1β and interferon (IFN)-γ. They are captured by anti-IL-1β and anti-IFN-γ polyclonal antibodies (pAB), respectively, immobilized at the TP. For the SERS detection of IL-1β and IFN-γ, the corresponding detection antibodies are conjugated to SERS nanotags with two different polyenes as Raman reporter molecules. The detection antibodies are monoclonal antibodies (mAB), which selectively bind to a second epitope of IL-1β and IFN-γ, respectively. The validity of the test is visually confirmed at the CP, which contains Protein A (PrA) for capturing excess SERS nanotag/mAB conjugates. A photo of the NC membrane after performing the test is shown in Figure 8B. The intense blue color at the CP confirms the validity of the test: the excess SERS nanostar-labeled detection antibodies are captured by PrA and the blue color of the SERS nanostars can be detected by the naked eye. At the TP only a pale bluish color can be seen since the number of the SERS nanotags in the corresponding immuno-sandwich complex is significantly lower compared to the CP. Figure 8C (bottom) depicts the SERS spectrum recorded from the TP (hollow circles) together with the relative contributions of the SERS nanotags with 2 and 5 DB, respectively, obtained by a least-square fit. Laser excitation wavelength: 785 nm, laser power at the sample ca. 8 mW, 30 seconds integration time, three accumulations. Spectra were recorded at 11 separate spatial positions.

### 4 | CONCLUSION

SERS nanotags offer the unique combination of ultrasensitive, quantitative and multiplexed detection of target molecules. In this contribution, we have presented...
the design and synthesis of polyene-based trifunctional Raman reporters, together with their application in a SERS LFA for cytokine detection. The polyenes are phenyl-substituted for chemical stabilization and the absorption maximum is shifted to longer wavelengths. DFT calculations reveal that the vibrational coupling between the phenyl ring and the polyene chain depends on the chain length. For short polyenes with 2 and 3 DB, two spectrally separated Raman peaks in the region 1550 to 1625 cm$^{-1}$ are observed, which spectrally overlap for longer polyenes with 4 and 5 DB. All polyenes with two to five C=C double bonds were employed as Raman reporter molecules in Au/Au core/satellite supraparticles. Finally, the successful use of two spectrally distinct SERS nanotag/antibody conjugates in a duplex SERS LFA was demonstrated. Since the Raman scattering cross sections for the two polyene-based Raman reporters with 2 and 5 DB are significantly different (cf. Figure 1) due to the different polyene chain length (cf. Figure 1), the use of C-D isotopologues (cf. Figure 6) in future SERS experiments could be a promising approach. The modular organic synthesis based on repetitive Wittig reactions (cf. Scheme 1) enables the introduction of C-D-labeled C$_2$ building blocks at selected positions of the polyene chain. Preliminary DFT calculations of the Raman spectra of undeuterated and perdeuterated polyenes (5 DB) clearly demonstrate the feasibility of spectrally distinguishing the two isotopologues.

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