Surface-enhanced Raman scattering with silver nanostructures generated in situ in a sporopollenin biopolymer matrix†

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Silver nanoparticles were generated based on citrate reduction in the ultrastructure of the sporopollenin biopolymer of Ambrosia artemisiifolia (ragweed) and Secale cereale (rye). The nanoparticles enable the acquisition of SERS spectra and thereby a vibrational characterization of the local molecular structure of sporopollenin.

Sporopollenin, the biopolymer shell of pollen grains of higher plants, is a very stable and versatile yet so far greatly ill-defined material. It has been proposed as a drug carrier,1 a template for morphosynthesis of catalytically active materials,2 for taste masking in food technology,3 as well as for purification of waste water.4,5 Its ultrastructure provides interesting physical properties for high-stick surfaces6 and immobilization of enzymes for catalysis.8 However, so far, access to the molecular structure of the intact pollen outer layer, the exine, has proven extremely difficult. Sporopollenin of most pollen species has escaped analysis by Raman spectroscopy because of a high fluorescence background. Here we report SERS data obtained with silver nanostructures that were generated in situ in the intact sporopollenin biopolymer matrix.

To generate nanostructures in close proximity of the sporopollenin matrix, exine from two species, rye and ragweed, were purified using a protocol by Dominguez et al.9 After washing and drying, silver nanoparticles were synthesized based on ref. 10 in the presence of both sporopollenin materials silver nanostructures were generated. The position of the plasmon band was at 436 nm and 423 nm in the case of the nanoparticles generated with the rye pollen sporopollenin and ragweed pollen exine, respectively. A shoulder at ~500 nm in the plasmon spectrum of the silver nanoparticles generated with the rye pollen sporopollenin suggests the presence of small nanoaggregates in the nanoparticle solutions. Fig. 1b displays a transmission electron micrograph of typical silver nanoparticles in the size range from 10–40 nm obtained in the presence of ragweed pollen exine.

The pollen outer shells were separated from the nanoparticle solution by filtering, washed in water, ethanol and methanol, and stored in deionized water. They were analyzed by environmental scanning electron microscopy (ESEM).† In Fig. 2, a and (b) without nanoparticles; (c) and (d) with silver nanoparticles. The pollen outer shells were separated from the nanoparticle solution by filtering, washed in water, ethanol and methanol, and stored in deionized water. They were analyzed by environmental scanning electron microscopy (ESEM).†
ESEM images of purified exine particles (Fig. 2a and b) and of the exine particles after synthesis of the silver nanoparticles (Fig. 2c and d) are displayed. A change in the appearance of the exine with respect to the control samples can be observed for both ragweed and rye sporopollenin (compare Fig. 2a and c and Fig. 2b and d, respectively). On the microscopic scale, both species display distinct patterns of metal deposition. The typical protrusions of the star-shaped ragweed pollen shell show a very pronounced contrast due to the presence of silver (Fig. 2c). In the rye pollen exine, the edges of the exine, as well as the thickened shell in the proximity of the pollen aperture also show higher contrast (Fig. 2d). The presence of silver in both species was also verified by EDX data obtained from the same exine particle with the ESEM experiment (data not shown here).

The resolution of ESEM is not sufficient for providing information on the properties of the silver generated in/at the exine biopolymer matrix. Furthermore, it was unclear on whether there existed larger silver deposits inside the lumen of the empty exine particles. Therefore, focused ion beam (FIB) preparation followed by transmission electron microscopy (TEM) was applied to study the silver nanoparticles in the context of the sporopollenin ultrastructure. Fig. 3a shows an overview TEM-image of the lamella containing a cross-section through the pollen after FIB-preparation. Before cutting, the pollen was embedded in glue and stabilized by ion-induced platinum deposition within the FIB-instrument. The TEM micrograph displayed in Fig. 3b suggests the presence of silver nanoparticles of 10–40 nm in size distributed over the entire cross-section of the exine layer. The presence of metallic silver nanoparticles was corroborated by electron diffraction. The faint spotty rings visible in Fig. 3c correspond to the silver nanocrystals in different orientations. The high particle density leads to a positioning of the individual nanoparticles next to one another. This, in principle, can result in very favourable conditions for high SERS enhancement, as very high local fields can occur in the gaps between the particles.\(^{11-14}\)

The normal Raman spectra of both species, in particular that of rye sporopollenin, displayed a high fluorescence background at various visible and near-infrared excitation wavelengths. Fig. 4a shows the normal Raman spectra of the exine of two pollen species obtained at an excitation wavelength of 633 nm. While an excitation irradiance of \(\sim 10^2\) W cm\(^{-2}\) and an accumulation time of 100 \(\times\) 10 seconds were needed to acquire the normal Raman spectra (Fig. 4a), the data shown in Fig. 4b were obtained from the silver-containing exine particles at \(\sim 10^3\) W cm\(^{-2}\) within 1 second. Taking into account the lower irradiance and shorter time needed for acquisition, and comparing the intensities of the strong bands in the spectra for both cases (Fig. 4a and b), we can roughly estimate an enhancement due to SERS of 4–6 orders of magnitude in the samples containing the silver nanoparticles. Due to the presence of the silver, efficient decay channels for the fluorescence are created, and no background is observed (Fig. 4b). Both samples display a characteristic SERS spectrum which differs remarkably from the normal Raman spectrum not only regarding signal strength but moreover regarding positions, number and width of the bands. Among the differences between the two species is a pronounced band of ester carbonyls at 1745 cm\(^{-1}\) in the rye sporopollenin (Fig. 4b, bottom trace) that is absent from the spectrum of ragweed sporopollenin (Fig. 4b, top trace). The spectra also give an idea of the different contributions of aliphatic and aromatic compounds in both exine species. In rye sporopollenin, the CH deformation is much less pronounced and up-shifted in frequency to 1469 cm\(^{-1}\) compared to the ragweed exine. In ragweed exine, a strong CH\(_2\) scissoring at 1447 cm\(^{-1}\) indicates the presence of long aliphatic chains in accord with other work concluding that sporopollenin may be derived from the polymerization of polyunsaturated fatty acids.\(^{15}\)

In contrast, the SERS spectrum of rye sporopollenin primarily shows contributions from aromatic rings with characteristic bands of isoquinolin at 1554 cm\(^{-1}\), 1257 cm\(^{-1}\), 1088 cm\(^{-1}\) and

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**Fig. 3** (a) TEM overview micrograph of a thin cross-section of a rye pollen exine embedded in glue and fixed by platinum cap layer, (b) TEM-image of the pollen exine at higher magnification showing distribution of nanoparticles, (c) electron diffraction pattern showing diffraction rings corresponding to silver nanocrystals.

**Fig. 4** (a) and (b) Raman spectra from exine of ragweed (top) and rye pollen (bottom), measured at 633 nm excitation. (a) Normal Raman spectra (acquisition time 100 \(\times\) 10 s, intensity \(2.5 \times 10^2\) W cm\(^{-2}\)). (b) SERS spectra (acquisition time 1 s, intensity \(2.5 \times 10^3\) W cm\(^{-2}\)).
920 cm\(^{-1}\) as well as plant-derived flavonoids such as quercetin at 1597 cm\(^{-1}\) and 578 cm\(^{-1}\).\(^{16,17}\)

The qualitative differences between the SERS and normal Raman spectra are due to the extreme restriction of the SERS signal to the immediate proximity of the silver nanoparticles that are embedded in the sporopollenin ultrastructure. In contrast, in the normal Raman experiment, the signal is an average of the signals obtained from molecules along the whole depth of the exine particle. In addition, the SERS signals may differ from the normal Raman signals due to the direct interaction of some exine constituents with the metal and also due to large field gradients of the local optical fields of the silver nanostructures. The SERS spectra were observed to be very similar for different exine particles of the same species. Together with the TEM micrograph (Fig. 3c) this confirms that the SERS signal obtained for each exine particle is a sporopollenin ‘bulk’ spectrum that comes from silver nanoparticles distributed all over the sporopollenin matrix, that is, an ‘average SERS’ signal similar to that discussed in the literature before.\(^{18}\) The difference between the SERS spectra of the exine samples from the two plant species in Fig. 4b directly reflects a different composition of the two types of sporopollenin. Such differences between the composition of the exine of different plant species have been suggested in other studies before.\(^{19}\) In addition to leading to a different vibrational spectrum \textit{per se}, the differences in chemical composition and/or structure of the two sporopollenin types can also lead to a difference in the generation and/or incorporation of the silver nanoparticles into the ultrastructure of the pollen capsules. This would have an influence on the enhancement of vibrational modes of specific parts of the sporopollenin polymer. Separating the extent of each of these two proximal causes is not possible at this stage. Species-specific sporopollenin microarchitecture has been reported recently.\(^{20}\)

The distribution of the nanoparticles across the whole exine (Fig. 3b) suggests that the reduction of the silver salt takes place directly in the sporopollenin material. This conclusion is also supported by ESEM data of exines that had been incubated with silver nanoparticles grown prior to incubation, which show a more superficial distribution of the silver with respect to the exine micromorphology compared to those with silver nanoparticles produced in their presence (data not shown). Furthermore, EDX-spectra taken at different points of Fig. 3b, all revealed silver. It should be noted that, different from other \textit{in situ} synthesis approaches of plasmonic nanoparticles in biopolymers and complex biomaterials,\(^{21–24}\) the sporopollenin biopolymer in this work did not serve as the reducing agent, but sodium citrate was added for reduction. For SERS this has the advantage that the surface of the silver nanoparticles is the same as in other experiments where solutions of nanoparticles are used for probing \textit{e.g.}, other constituents of pollen.

In summary, it was possible to synthesize silver nanoparticles in the presence of purified pollen shells. The silver nanoparticles are embedded in the ultrastructure of the sporopollenin biomatrix. The presence of silver nanoparticles in the pollen exine ultrastructure has enabled vibrational spectroscopic access to the sporopollenin biopolymer based on SERS. Due to the extreme localization of the SERS signals to the immediate proximity of the silver nanoparticles, the SERS spectra provide a new perspective on the so far unknown local molecular structure of sporopollenin.

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Notes and references

† ESEM images were taken using an ESEM XL 30 (Philips) equipped with a backscattered electron (BSE) detector. The acceleration voltage was 15 kV for each sample and the water vapor pressure varied between 0.3 Torr and 0.4 Torr for the different samples. § For FIB preparation (FEI Strata 200 X), the exine particles were first fixed on a glass slide, and furthermore embedded within a glue (M-Bond 610). Then, the whole assembly was sputter-coated with gold to avoid charging during further FIB preparation. Next, a platinum bar \(20 \times 2 \times 2 \mu\text{m}\) was deposited at the site of interest by ion beam induced chemical vapour deposition in the FIB instrument. After these pre-preparation steps, a micro cross section was prepared and imaged with the FIB. A thin lamella, approximately 100 nm thick, was cut with the Ga\(^{+}\)ion-beam and investigated with TEM (Fig. 3b). TEM images were taken using a TEM of type JEOL 4000FX and investigated at an acceleration voltage of 400 kV.