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FTIR spectroscopic studies of bacterial cellular responses to environmental factors, plant-bacterial interactions and signalling

Alexander A. Kamnev
Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Prosp. Entuziastov 13, Saratov 410049, Russia
E-mail: aakamnev@ibppm.sgu.ru

Abstract. Modern spectroscopic techniques are highly useful in studying diverse processes in microbial cells related to or incited by environmental factors. Spectroscopic data for whole cells, supramolecular structures or isolated cellular constituents can reflect structural and/or compositional changes occurring in the course of cellular metabolic responses to the effects of pollutants, environmental conditions (stress factors); nutrients, signalling molecules (communication factors), etc. This information on the molecular level, often obtained using non-destructive techniques with minimal sample preparation, is of importance for basic studies on mechanisms of bacterial tolerance to stresses and their impact on bacterial metabolism, as well as for applied multidisciplinary research in the fields related to biotechnology, bioremediation, agriculture, biogeochemistry, etc. In the present communication, some recent examples are reviewed and discussed which illustrate the applicability of Fourier transform infrared (FTIR) spectroscopy for monitoring metabolic changes in soil bacteria, using the examples of intensively studied plant-growth-promoting rhizobacteria of the genus *Azospirillum*. Compositional and structural changes occurring in whole bacterial cells in response to different stress factors and plant signals, as well as spectroscopic images of relevant biospecific interactions are considered from the viewpoint of the possibilities provided by this versatile and easy-to-use technique and its methodology.

Keywords: Fourier transform infrared spectroscopy (FTIR), bacterial cells, stress response, environmental factors, plant–microbe interactions, plant-growth-promoting rhizobacteria (PGPR)

Abbreviations

ATR, attenuated total reflectance;
DRIFT, diffuse reflectance infrared Fourier transform;
FGNP, functionalised gold nanoparticles;
FTIR, Fourier transform infrared;
GlcNAc, N-acetyl-D-glucosamine;
$(GlcNAc)_3$, N', N'', N'''-triacetylchitotriose;
hIgG, human immunoglobulin;
PGPR, plant-growth-promoting rhizobacteria;
PHA, polyhydroxyalkanoates;
PHB, poly-3-hydroxybutyrate;
SEIRA, surface-enhanced infrared absorption;
UFA, unsaturated fatty acid;
WGA, wheat germ agglutinin.

1. Introduction

It has only recently begun to be realised that a variety of physicochemical factors play a fundamental role in diverse and as yet poorly understood processes of microbial intercellular and microbe–host organism communication and signalling [1–4]. The research field of plant-growth-promoting rhizobacteria (PGPR) and their interactions with plants is highly promising for possible applications to contribute to eco-friendly sustainable agriculture and environmental biotechnology (see, e.g. recent reviews [5–9]). However, there are still a large number of pending problems related to the basic mechanisms of the underlying biological and chemical processes that occur both in the rhizosphere soil and *in vivo* (in plants and PGPR), which require systematic investigations at the molecular level using modern instrumental techniques [3].

Bacteria are well known to be capable of responding to environmental factors, stresses or external chemical effectors (pheromones, signalling molecules, chemical 'cues', etc. [1]) by appropriately changing their metabolism [1,3,4,7–10]. In many cases, if such induced changes involve any substantial alterations in cell composition and/or structure of cellular constituents, they can be monitored using a range of selectively sensitive and informative spectroscopic techniques. In this communication, some representative recent examples are reviewed and discussed where Fourier transform infrared (FTIR) spectroscopy in various modes (transmission, DRIFT, ATR) was used to analyse compositional and structural changes in whole bacterial cells in response to different stress factors and plant signals. FTIR spectroscopy can be efficiently used also to detect relevant biospecific interactions of the type "recognising molecule – target molecule" (e.g., using antigen–antibody or lectin–carbohydrate biospecific pairs) at the molecular level. The experimental results considered herein illustrate some possibilities of FTIR spectroscopy complementary to those discussed in previous reviews on its applications in biology (see, e.g. [11–15] and references therein) and, in particular, in microbiology [16–19].

2. Bacterial responses to environmental factors, as revealed using FTIR spectroscopy of whole cells

2.1. Heavy metal stress

As a representative example, the diazotrophic rhizobacterium *Azospirillum brasilense*, a ubiquitous PGPR of the genus *Azospirillum* with phytostimulating capabilities extensively studied within the past decades [20,21], is considered. Its versatile metabolic behaviour, related to broad adaptational capabilities, is of significant fundamental interest both from the viewpoint of its specific responses to various stress factors and with regard to ecologically related differences between its endophytic (capable of penetrating into plant tissues) and epiphytic strains (colonising the plant root surface only) comprised within this bacterial species [22].

FTIR spectroscopic analyses of whole bacterial cells, grown in nutritionally rich medium supplemented with NH_4^+ as a source of bound nitrogen, allowed specific metabolic differences between epiphytic and endophytic strains of the *A. brasilense* species to be revealed in response to heavy-metal stress (Fig. 1) [23–25]. Under the aforementioned conditions, some specific metabolic changes induced by heavy metals at submillimolar concentrations (0.2 mM; moderate heavy-metal stress) were noticeable for the epiphytic strain only, the most pronounced effect being an enhanced accumulation of polyester storage compound, poly-3-hydroxybutyrate (PHB), represented by its well-resolved ν(C=O) absorption

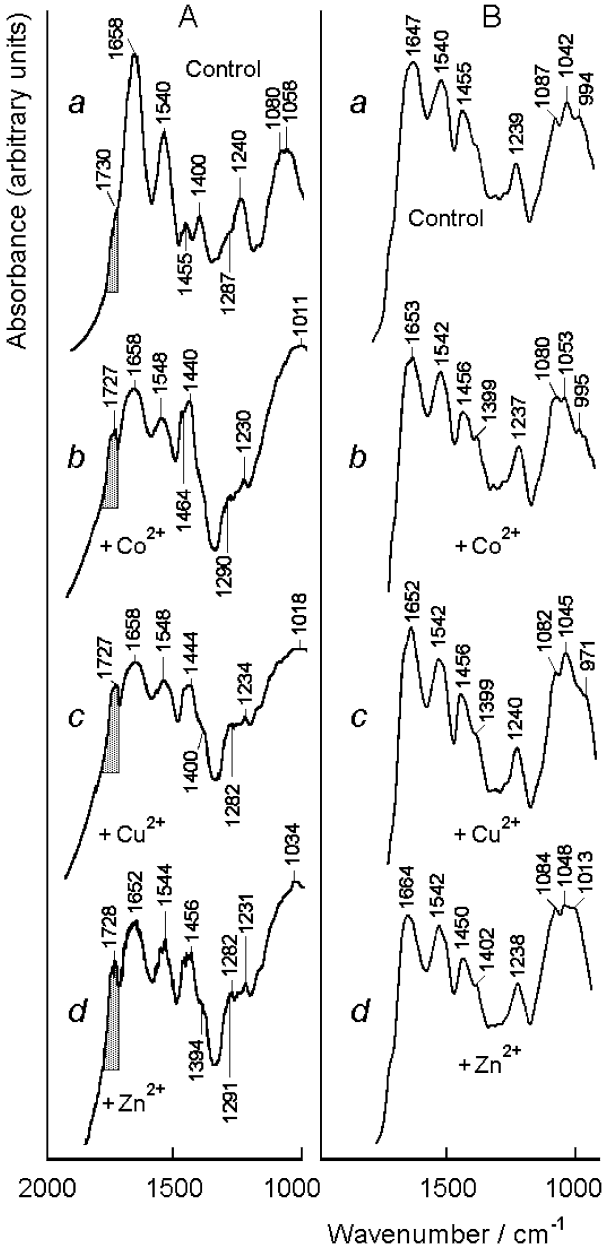

Fig. 1. FTIR spectra of dried biomass of *Azospirillum brasilense* epiphytic strain Sp7 (A) and facultatively endophytic strain Sp245 (B) grown (a) in a standard phosphate–malate medium supplemented with NH_4^+ as a source of bound nitrogen (control), as well as in the same medium in the presence of 0.2 mM (b) Co^{2+}, (c) Cu^{2+} and (d) Zn^{2+}. For (A), shaded areas indicate the region of ν(C=O) vibrations of polyester moieties at ca. 1730 cm^{-1}.

band at about 1730 cm^{-1} (cf. Fig. 1A, spectra a–d). A more detailed list of vibrational (FTIR and FT-Raman) spectroscopic differences reflecting the metabolic changes, incited by the presence of heavy metals in the growth medium of *A. brasilense* strain Sp7 [23,24], is presented in Table 1. Note that the induction of PHB biosynthesis and accumulation by heavy-metal stress only (i.e., in an otherwise

Table 1

Characteristic changes in vibrational spectra of whole cells of *Azospirillum brasilense* (epiphytic strain Sp7) induced by heavy metals (Co^{2+}, Cu^{2+}, Zn^{2+})

Spectroscopic changes induced by heavy metals	Possible assignment, interpretation and comments
FTIR spectra [23]	
Enhanced very broad absorption in the region 3400–2700 cm^{-1}	ν(OH); enhanced hydration of bacterial cells (bound water; see also FT-Raman)
The band centred at about 3300 cm^{-1} shifted to lower frequencies (by 15–40 cm^{-1})	ν(NH); H-bonding (due to water) and possible metal binding of amide groups in proteins
The amide II band (1540 cm^{-1}) shifted to higher frequencies (by up to 8 cm^{-1})	δ(NH); H-bonding and/or possible metal binding of amide groups in cellular proteins
The band at 1240 cm^{-1} shifted to lower frequencies (by 6 to 10 cm^{-1})	$\nu_{as}(PO_2^-)$; increased hydration of phosphate moieties (may be due to metal binding)
Appearance of a strong well-resolved band centred at ca. 1727 cm^{-1} (present as a shoulder in control cells)	ν(C=O) in polyesters; accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids (PLs))
Increased absorption in the region about 1460–1440 cm^{-1}	Various δ(C–H) modes (accumulation of polyesters; see above)
Increased absorption in the region about 1150–1000 cm^{-1}	Various C–O–C and C–C–O vibrations (accumulation of polyesters; see above)
Increased absorption in the region about 750–700 cm^{-1}	Rocking CH_2 mode (accumulation of polyesters; see above)
FT-Raman spectra [24]	
Increased very broad non-specific "humps" in the regions around 3400–2700, 1800–1200, under 600 cm^{-1}	Typical regions of water vibrations (weak and non-specific in Raman); enhanced overall hydration of bacterial cells
Decreased intensity of a weak shoulder at 3010–3000 cm^{-1} (induced by Co^{2+} and Cu^{2+} but not by Zn^{2+})	ν(=CH–) in unsaturated fatty acid (UFA) residues (may reflect a decrease in UFA content in bacterial membrane PLs)
Appearance of a new medium or weak band at 945–943 cm^{-1}	C–C–O vibrations (accumulation of polyesters; see above)

nutritionally rich medium), a novel feature for bacteria, was detected for the first time in *A. brasilense* Sp7 [23].

In contrast, the endophytic strain under the same conditions did not exhibit any clearly noticeable metabolic changes (cf. Fig. 1B, spectra a–d). Interestingly, however, both the endophytic and epiphytic strains were shown to accumulate comparable amounts of each of the heavy metals from the medium [25]. It should be emphasized that under nitrogen deficiency, the endophytic strain had yet been shown earlier to exhibit the tendency, common to many bacteria under nutritional stress [21,26,27], to accumulate PHB (and/or other polyhydroxyalkanoates, PHA) both under and without heavy-metal stress, as revealed in FTIR spectra of whole cells [28] and cell membranes [29] by the appearance of the typical strong ν(C=O) absorption band.

Thus, while each of the heavy metals was similarly accumulated by cells of both strains, clear evidence for its metabolic effect was noted by FTIR spectroscopy in epiphytic strain only. Whereas for strain Sp7, that showed clear metabolic responses to heavy metals, their involvement in metabolism was undoubted, for the endophytic strain it had to be directly proved, as metal cations at moderate concentrations could in principle be accumulated by purely chemical binding at the cell surface, thus showing no clearly detectable metabolic response. Nevertheless, the direct involvement of one of the heavy metals (Co^{2+}) in cellular metabolism of the endophytic strain was proved using an alternative technique, ^{57}Co emission Mössbauer spectroscopy [25,30]. For Cu^{2+}, its specific effects on the metal content of whole cells [28]

and cell membranes [29] in *A. brasilense* Sp245 also evidenced for the involvement of Cu^{2+} in metabolic processes, in line with the reports on other bacteria [7,31,32].

To conclude, the dissimilarities in heavy-metal-induced effects for the two *A. brasilense* strains (epiphyte and endophyte) can obviously be attributed to their different adaptational strategies which reflect the different ecological niches occupied by the strains in the rhizosphere [21,22].

2.2. Nutritional stress

A common type of nutritional stress (i.e., bound nitrogen deficiency) was shown to induce specific FTIR spectroscopic changes in *A. brasilense* cells (Fig. 2). These involve, along with the 'traditional' accumulation of polymeric storage materials (PHB typical for azospirilla, playing a role in bacterial stress endurance [26,27]), also alterations in the amide I band profile of cellular proteins [33]. It has

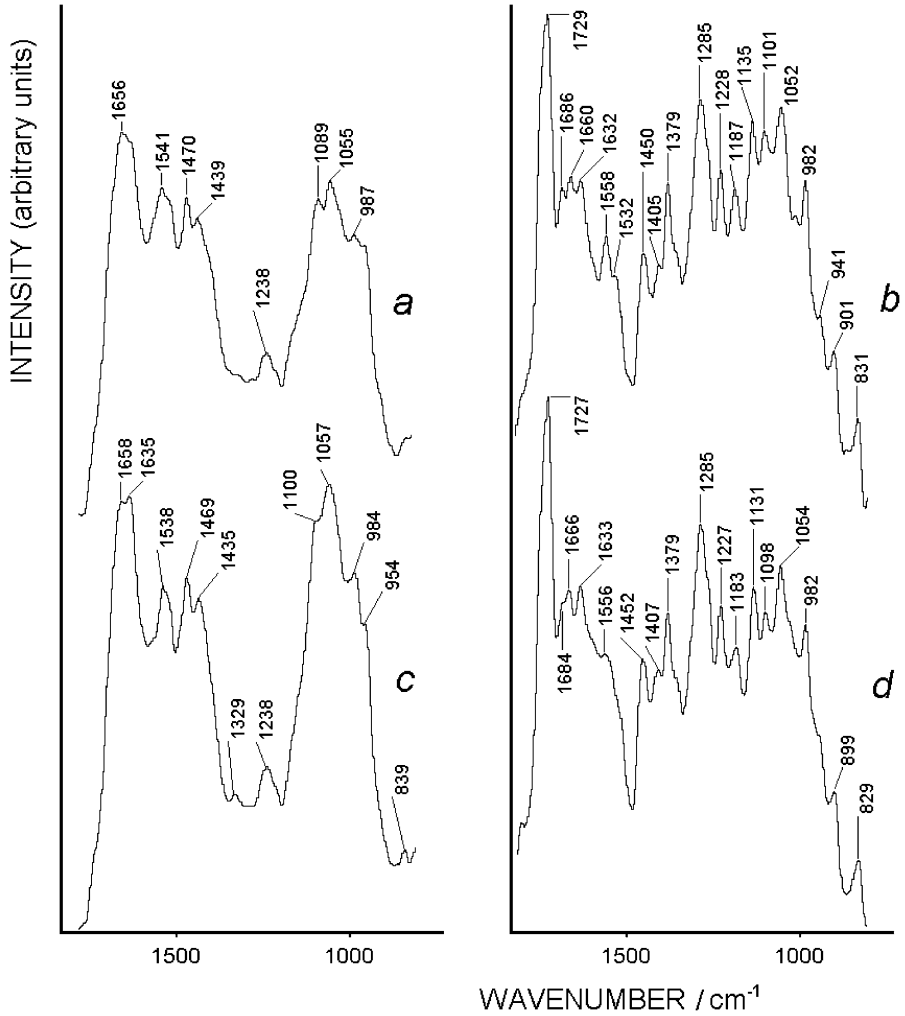

Fig. 2. DRIFT spectra of dry biomass of *A. brasilense* Sp245 cells grown in standard phosphate–malate medium [33] in the presence of 3 g/l NH_4Cl (a, c), in the same medium without the ammonium salt (b, d), and in the presence of 0.2 mg/l wheat germ agglutinin (c, d).

to be noted that in these experiments, FTIR spectroscopy in the diffuse reflectance mode (DRIFT) was applied to pure dried biomass of bacterial cells (without mixing with KBr), as special measurements had shown that grinding with KBr resulted in noticeable shifts of some important bands related to functional groups involved in H-bonding [34].

The main FTIR absorption bands characteristic of PHB, besides that at about 1730 cm^{-1} (ν(C=O) of the ester moieties), appear at around 1450–1452 and 1379 cm^{-1} (antisymmetric and symmetric bending vibrations of methyl (CH_3–) and methylene (–CH_2–) groups, respectively), as well as at about 1285, 1135 and 1055 cm^{-1} (polyester C–C–O fragment vibrations), etc. [16–19,35]. Some clearly noticeable enhancement in the corresponding regions can be seen in the DRIFT spectrum of *A. brasilense* cells under the nutritional stress, as compared to those of control cells (cf. Fig. 2a–b).

As is known [19,36], the α-helix type of protein secondary structure is featured by a single relatively narrow band at about 1655–1658 cm^{-1}; different types of β-sheets (differing in the strength of interaction between the chains, from weak to strong) exhibit bands with a maximum from 1637 to 1623 cm^{-1}, respectively, usually with an accompanying band at 1680–1690 cm^{-1} (β-antiparallel pleated sheets); β-turns give a band at 1660–1680 cm^{-1}; random coil is commonly characterised by a relatively broad band around 1645–1648 cm^{-1}. In view of that, under normal growth conditions, the maximum of the amide I band, commonly observed at about 1650–1660 cm^{-1}, reflects the predominance of α-helices among the secondary structure components of bacterial cellular proteins. However, for cells grown under nitrogen deficiency (cf. Fig. 2a, b), the amide I band appeared to be split, with the additional bands at 1632 and 1686 cm^{-1} corresponding to an enhanced proportion of β-structure components. Note that, while some changes in the protein content in azospirilla under nutritional stress have been reported earlier [37,38], nothing has been known so far about conformational structural changes in their cellular proteins induced by external factors.

2.3. The effect of plant stress-response protein as a molecular signal

The aforementioned effect of amide I band splitting was spectroscopically detected also in the presence of nanomolar plant molecular signal, wheat lectin, which is historically known as wheat germ agglutinin (WGA). This stress-response protein of the host plant, from which the endophytic *A. brasilense* strain has been isolated, is also considered to be a molecular signal in plant-microbe interactions (see [39] and references reported therein).

In particular, both under normal conditions (Fig. 2c) and under nutritional stress (Fig. 2d), the addition of 0.2 mg/l WGA ($\sim$5.6 nM) to the culture medium under aerobic conditions [33] resulted in a similarly expressed splitting of the amide I band, thus featuring a correspondingly increased proportion of β-structures in cellular polypeptides.

The observed spectroscopic changes may be related to alterations in bacterial cell-surface proteins (glycoproteins) for the following reasons. Bacteria are well known to be capable of rapidly and adequately altering their cell surface, thereby adapting to the varying conditions of the environment. A substantial role in this process is played by cell-surface biopolymers including proteins, glycoproteins, glycoproteolipids, as well as other macromolecular metabolites. For some of the azospirillum cell-surface proteins characterised so far, including lectin (hemagglutinin) and the major outer membrane protein (MOMP, an adhesin homologous to bacterial porins) [21,40–42], their involvement in stress resistance and/or colonisation of plant roots has been proved (note that plant-root colonisation by rhizobacteria also contributes in part to their stress resistance). Thus, the enhanced biosynthesis of proteins rich in β-structures under nutritional stress and in the presence of WGA traces, revealed in DRIFT spectra (see above), can be related to cell-surface hemagglutinin and/or porin [33].

It is also worth noting that the DRIFT spectroscopic technique applied to dried bacterial cells is sensitive not only to changes in their composition, but also to fine conformational changes in cellular proteinaceous constituents. This can be useful in revealing the molecular mechanisms underlying diverse responses of bacteria to varying ecological conditions.

3. FTIR spectroscopic studies related to molecular plant–bacterial biospecific interactions

As a plant molecular signal, wheat lectin (WGA) has been reported to induce multiple metabolic effects in azospirilla under appropriate conditions [21,39,43–46]. The development of such effects is commonly initiated by biospecific interactions, which generally involve a biospecific pair "receptor molecule–target molecule". In particular, WGA is well known to specifically bind *N*-acetyl-D-glucosamine (GlcNAc) residues, which are present in some of bacterial cell-surface biomacromolecules, thus bringing about the aforementioned multiple metabolic effects in azospirilla under appropriate conditions [21, 39,43–46].

Bonnin et al. [47] have shown using FTIR spectroscopy that WGA interaction with GlcNAc oligomers as well as with GlcNAc-bearing liposomes is accompanied by conformational changes in the lectin molecules reflected, in particular, in the amide I band profile. However, there are few reports related to lectin–carbohydrate specific biosensors (see, e.g. [48] and references therein).

An FTIR spectroscopic approach, based on the surface-enhanced IR absorption (SEIRA) effect (for recent reviews on SEIRA see, e.g. [49–51], including its applications in biochemistry [51]) on functionalised gold nanoparticles (FGNP), has been proposed in order to detect immunochemical interactions ("antigen–antibody") [52] (see the scheme in Fig. 3, upper left-hand panel). Note that both FGNP and FGNP-based biocomplexes (see below) can easily be separated from the solution by centrifugation [52]. Thus, staphylococcal protein A (antigen) adsorbed on 30-nm-diameter gold nanospheres (see Fig. 3, middle left-hand panel) was found to give FTIR spectra of dried films on a ZnSe support (in the absorption [53] or attenuated total reflectance (ATR) [52] modes; see Fig. 3, middle right-hand panel, sample 2) differing from those of the pure protein (see Fig. 3, upper right-hand panel, sample 1).

When the gold nanoparticles functionalised by protein A were mixed in aqueous suspension with traces of human immunoglobulin (hIgG; antibody) dissolved in water (see Fig. 3, lower left-hand panel), the resulting biospecific complex on FGNP (see Fig. 3, lower right-hand panel, sample 3) exhibited further FTIR spectroscopic alterations, in particular, in the amide I–amide II region ($\sim$1700–1500 cm^{-1}). In Fig. 4, the FTIR-ATR spectra of dried films on a ZnSe support [52] are shown for pure protein A (spectrum a) as well as its conjugate with 30-nm gold nanospheres before (spectrum b) and after biospecific interaction with hIgG (spectrum c). The spectroscopic changes make it possible to detect such immunochemical interactions using the SEIRA effect with an enhancement factor found [52] to be 10–15, typical for SEIRA of adsorbed biomolecules. Note also that smaller FGNP (e.g., 15 nm in diameter) are more stable in solution and, although giving a somewhat less clear-cut FTIR-SEIRA spectroscopic changes than 30-nm particles [54], can also be used for the SEIRA spectroscopic detection of biospecific interactions.

This approach was applied to SEIRA-based detection of lectin–carbohydrate biospecific interactions [55]. Gold nanospheres (mean diameter 15 nm) functionalised by WGA were found to give SEIRA spectroscopic changes, as compared to pure WGA (cf. Fig. 5a, b), similar to those for the protein A-FGNP system (see Fig. 4a, b). For WGA, the region of amide I and amide II bands is represented by maxima at

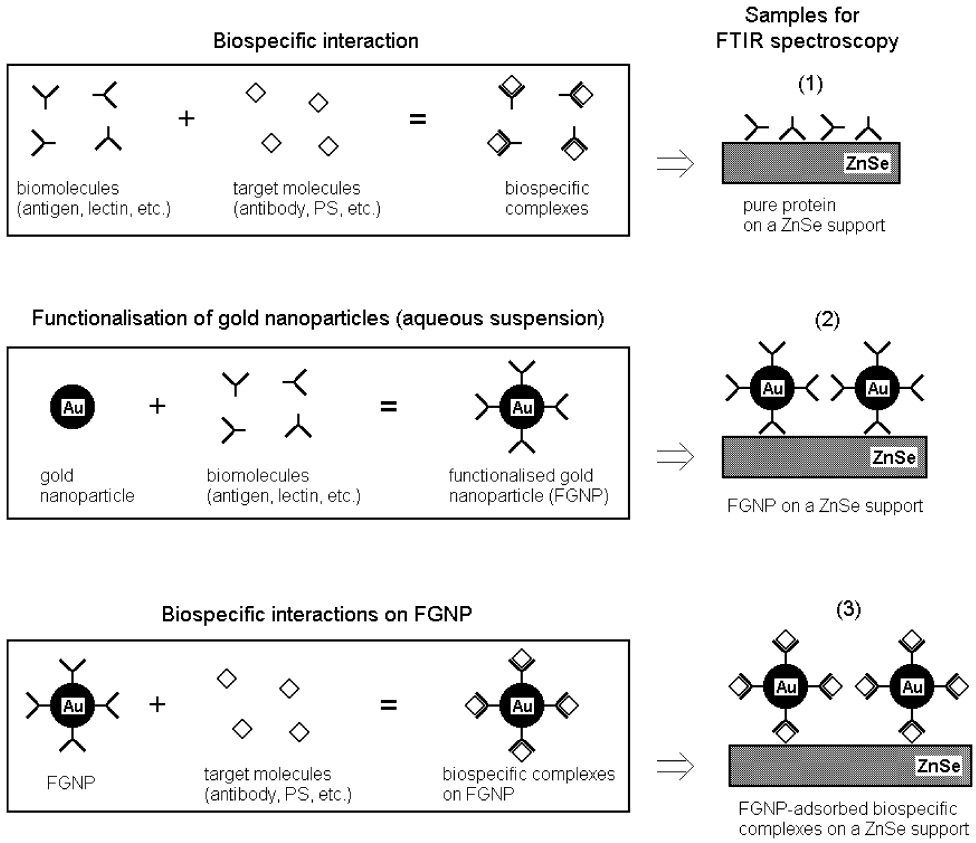

Fig. 3. Schematic presentation of the processes of biospecific interactions between pure biomolecules in solution (upper left-hand panel), functionalisation of gold nanoparticles by biomacromolecules (middle left-hand panel) and biospecific interactions on functionalised gold nanoparticles (FGNP; lower left-hand panel), as well as of the corresponding samples for FTIR spectroscopic measurements (right-hand panels 1–3, respectively).

1651 and 1545 cm^{-1}, respectively, in agreement with the FTIR data reported in [47]. These bands comprise the main contributions from the stretching vibrations of the carbonyl group (amide I) and bending N–H vibrations coupled to stretching C–N (amide II) of peptide moieties.

In the FTIR absorption spectrum of the WGA-FGNP dried film (Fig. 5b), instead of typical well-resolved amide I and amide II bands, there is an intermediate strong band centred at 1571 cm^{-1} superimposed on the amide I and II band residues. In addition, there appears a strong band at 1398 cm^{-1}. These changes are analogous to those observed in the FTIR spectrum of protein A conjugate with gold nanospheres taken in the transmission geometry [53], reflecting the interactions of amide moieties and amino groups with the gold nanoparticle surface, in agreement with the conclusions drawn by other authors [56–59]. Note that proteins adsorbing onto the gold nanoparticle surface have been shown [59] to displace citrate ions weakly bound at colloidal gold particles formed upon citrate reduction of $(AuCl_4)^-$ ions. The SEIRA enhancement factors for the WGA-FGNP system (Fig. 5b) were estimated to be also around 10–20 for different bands [55], depending on the orientations of transition dipole moment (TDM) components relative to the metal surface.

After biospecific interactions of the WGA-FGNP system in aqueous suspension with the GlcNAc trimer, $(GlcNAc)_3$ (N, N', N''-triacetylchitotriose), further spectroscopic changes are observed

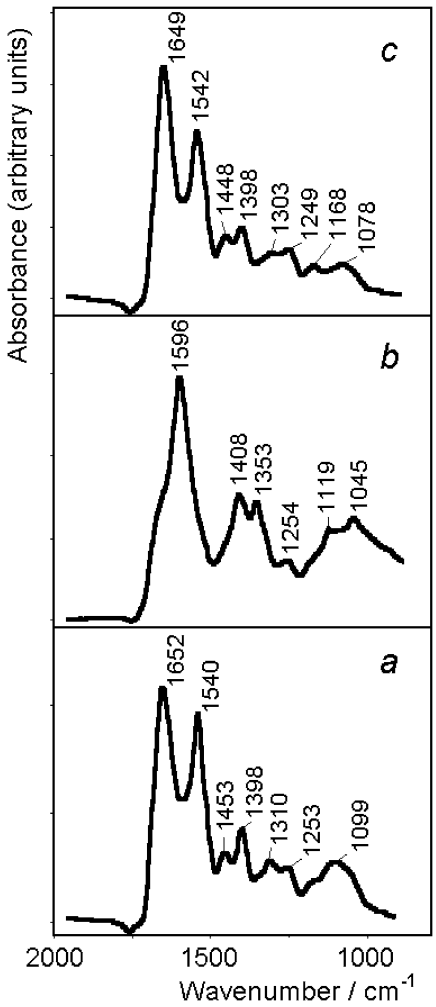

Fig. 4. FTIR-ATR spectra of dried films of (a) pure protein A (100 μg of protein), (b) protein A conjugated with 30-nm gold nanospheres (20 μg of protein), (c) the same as (b) after interaction with human IgG in aqueous solution. All spectra were measured on a standard ZnSe 45° flat-plate ATR contact sampler (12 reflections) [52].

(Fig. 5c). In particular, separate amide I and amide II bands have restored, in line with the spectroscopic changes observed for the protein A–hIgG system (cf. Fig. 4b, c). Such changes imply that upon biospecific interactions of the proteins with their target molecules, the configuration of the protein attached to the gold surface is modified. This conclusion is in agreement with the observations reported earlier for an immune reaction on colloidal gold using surface-enhanced Raman scattering [60]. As noted above, FTIR spectroscopic evidence was also provided for conformational changes in pure WGA upon its interaction with GlcNAc oligomers including $(GlcNAc)_3$ [47].

Thus, the FTIR-SEIRA spectroscopic methodology has been shown to be applicable to the detection of lectin–carbohydrate biospecific interactions, using the example of WGA-FGNP interacting with GlcNAc-containing haptens [55] and, recently, also with GlcNAc-containing bacterial cell-surface macromolecules ([61], Kamnev et al., in preparation), suggesting their involvement in plant-bacterial interactions via WGA-mediated signalling.

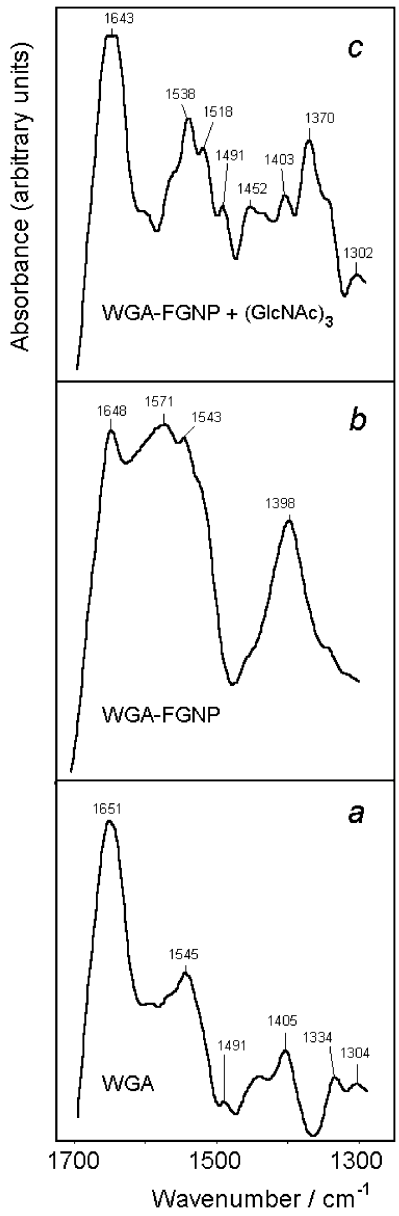

Fig. 5. FTIR spectra of dried films of (a) pure wheat germ agglutinin (ca. 20 μg of protein), (b) its conjugate with 15-nm gold nanoparticles (ca. 10 μg of protein), as well as (c) the same as (b) after interaction with 5 μg $(GlcNAc)_3$ in aqueous solution. All spectra were measured in the transmission geometry on a standard ZnSe crystal disc (∅13 mm, 2 mm thick) [55].

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