X-ray Photoelectron Spectroscopic and Transmission Electron Microscopic Characterizations of Bacteriophage–Nanoparticle Complexes for Pathogen Detection

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ABSTRACT: We report the synthesis and characterization of gold–bacteriophage hybrids for biodetection purposes. The physical and optical properties of gold nanoparticles (AuNPs) and the biological features of the phages offer a multifunctional scaffold with great potential for nanotechnologically based biomedical applications. AuNPs, stabilized (PEGylated) using heterobifunctional polyethylene glycol (PEG), were coupled to methicillin-resistant S. aureus-specific phages. The PEG ligands contain a thiol group for stable anchoring to the gold surface and a terminal carboxylic acid group for further coupling to the outside of the PEG shell by carbodiimide chemistry. Transmission electron microscopic analysis showed that the NP–phage bioconjugates are highly stable, with a median diameter of 90 nm. X-ray photoelectron spectroscopy was used to chemically characterize the surfaces of the PEG-functionalized AuNPs, the bacteriophages, and the gold–phage hybrids. The role of the interface and the covalent coupling chemistry employed to attach the phages to the AuNPs have been delineated. Successful attachment of phages to AuNPs was confirmed by the presence of amide between the primary amines of the phages and the carboxylic acid terminal groups of the NPs and by the presence of carboxyl and amine species, which form hydrogen bonds.

INTRODUCTION

Conventional microbiological techniques for the detection of harmful bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA), often require up to 2 days to obtain results. Many groups have devoted significant efforts toward the development of rapid, sensitive, and specific methods for the detection of such pathogens. The past few decades have seen the advent of biosensors, based on surface plasmon resonance (SPR), for the study of the chemical and biological reactions that occur at the interface of a metal and its biochemical environment. A surface plasmon (SP) is a charge density oscillation, propagating along the boundary between a dielectric and a metal. However, the requirement for detection by attenuated total reflectance requires a rather complex optical setup. Here we use localized surface plasmon resonance (LSPR), where the plasmon is produced by metal nanoparticles (NPs). In accordance with the Mie theory, the movement of electrons through the internal metal framework of the NPs is confined in three dimensions. When the frequency of the incident light matches that of the resonance of the collectively oscillating electrons, the energy of the incident photons is absorbed, corresponding to a peak in the absorbance spectrum. The use of localized SPs permits increased resolution and sensitivity of the optical probes and therefore the amplification of the detection signal.

In the case of LSPR for bacterial detection, recent findings show that the contact area between a bacterial cell and a single NP may be too small to result in a detectable plasmon peak shift. This prompted us to explore the possibility of attaching numerous NPs to a single bacterial cell by exploiting the specificity of bacteriophage–bacteria interactions. Bacteriophages are among the most promising candidates for biosensing scaffolds due to their specificity, robustness and unique display system. Au–phage fractal networks have previously been shown to effectively integrate the unique signal-reporting properties of AuNPs while preserving the biological properties of phages. Moreover, the use of these NP–phage hybrids can be extended to the targeted separation of specific bacteria from heterogeneous samples as well as a wide range of biotechnological applications, such as labels for enhanced fluorescence and dark-field microscopy and surface-enhanced Raman scattering detection.
In a previous paper, using bacteriophages immobilized on planar gold surfaces, we studied the bacteriophage-MRSA specificity.\textsuperscript{14} The phages were covalently attached to the gold through amide bonds via a mixed self-assembled monolayer of L-cysteine and 11-mercaptoundecanoic acid.\textsuperscript{15} We present here a novel method to immobilize phages on AuNPs that are functionalized with heterobifunctional PEG molecules, consisting of a thiol anchor group on one end and a carboxylic acid group on the other end, for further reaction.

The use of these PEG linkers has the dual functions of readily substituting weakly adsorbed surface molecules and stabilizing the NPs during synthesis. The linker is there to provide a high density of docking sites for bacteriophages through carbodiimide amidization reactions between the carboxy terminal of PEG and the amine groups at the phage surface.

Together with the NP shape and size and the specificity of the bacteriophage, the immobilization chemistry of the nanohybrid defines the performance and stability of the system. Although self-assembled monolayer (SAM) characterization on gold platforms has been extensively studied,\textsuperscript{22–25} a detailed quantitative characterization of PEG-functionalized AuNPs is lacking.\textsuperscript{26} Furthermore, a detailed physicochemical analysis for the reliable interpretation of observed biointerfacial interactions of NPs and lytic phage conjugates has not been published. The characterization of such intentionally designed nanohybrids is a crucial step in the development of novel nanomaterials.

X-ray photoelectron spectroscopy (XPS) and transmission electron microscopy (TEM) are widely used techniques for the characterization of nanobiomaterials.\textsuperscript{37} TEM was used to determine the morphology of PEG–AuNPs, and XPS was used to determine surface chemistries of the PEG–AuNPs, MRSA-specific bacteriophages, and phage–AuNPs nanohybrids.

### MATERIALS AND METHODS

1.1. Chemicals. 1-(3-Dimethylaminopropyl) ethylcarbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), glutaraldehyde, sodium chloride, magnesium sulfate, and gelatin were purchased from Sigma-Aldrich. Luria–Bertani (LB) medium was purchased from Quelabs (Montréal, Québec, Canada). Phosphate-buffered saline (PBS) was purchased from Fisher Scientific (Nepean, Ontario, Canada). Sixty nanometer diameter heterobifunctional PEG–AuNPs (cat no. CGC3K-60–25) were provided by Cytodiagnostics (Burlington, Ontario, Canada). MRSA bacteria and bacteriophages were provided by Biophage Pharma (Montréal, Québec, Canada). Uranyl acetate (UA) was purchased from Canemco (Lakefield, Québec, Canada).

1.2. Bacterial Culture. MRSA bacteria were isolated by Biophage Pharma and were grown in an incubator-shaker at 37 °C in 4 mL of LB medium for 3 h. The bacteria were then centrifuged at 2500 g (Sorvall RT7, 3500 rpm) for 20 min. The supernatant was discarded, and the bacteria were washed twice and resuspended in water. Their concentration was determined by plate count technique and expressed in colony forming units per milliliter (CFU/mL).

1.3. Bacteriophage Preparation. Bacteriophages were isolated by Biophage Pharma and amplified by pipetting 100 μL of a suspension of 10\textsuperscript{6} pfu/mL of bacteriophages into a solution containing 1 mL of 10\textsuperscript{6} cfu/mL of freshly prepared bacteria. The bacteriophage concentration was determined by plate count technique and expressed in plaque forming units per milliliter (PFU/mL). After 15 min of incubation at room temperature, the infection mix was added to an Erlenmeyer flask containing 250 mL of LB medium and incubated for 6 h, at 37 °C, in an incubator-shaker. The infected culture was then centrifuged at 2500g for 20 min, filtered (0.22 μm), and titrated. For XPS and TEM, the bacteriophages were further
centrifuged for 60 min, the supernatant was removed, and the phages were washed three times and resuspended in water.

1.4. Preparation of Phage–AuNPs Hybrids. One milliliter of NP stock solution ($9.80 \times 10^{11}$ particles/mL) was mixed with 49 mL of a solution consisting of 1 mol L$^{-1}$ EDC and 0.1 mol L$^{-1}$ NHS for 15 min. The PEGylated NPs were then added to the phage solution ($1.2 \times 10^6$ PFU/mL) and mixed overnight in an incubator shaker at 37°C. Gold-phage hybrids slowly precipitated, sank to the bottom of the tube, and were centrifuged at 10 000 g for 1 min. The pellet was resuspended in 1 mL of water.

1.5. Transmission Electron Microscopy. The TEM employed was a Hitachi H-7100, operating at 75 kV. Copper grids (200 mesh), coated with a layer of Formvar and an overlayer of evaporated amorphous carbon, were used. A 10 μL drop of sample was deposited onto the grid by the reverse drop method: a drop of sample was deposited on Parafilm for 5 min, then the grid was deposited on top of the Parafilm, and excess liquid was removed with filter paper. The grid was then dipped, for 5 min, in a drop of 2% gluteraldehyde. The excess solution was again wicked from the edge of the grid with filter paper, and the specimen was washed twice with water. A drop of 1% UA was applied to the grid for 1 min, and excess was removed with the edge of a filter paper. The grid was allowed to dry before the sample was used for TEM analysis.

1.6. X-ray Photoelectron Spectroscopy. The XPS apparatus was a VG ESCALAB 3 Mark II using a nonmonochromated Mg Kα X-ray source (1253.6 eV) at a base pressure below 10$^{-10}$ Torr. For sample preparation, drops of the colloidal solution were placed on undoped, undiced, polished, Piranha-cleaned silicon wafers and dried overnight in a pumped desiccator. High-resolution spectra were obtained at a perpendicular takeoff angle using a pass energy of 20 eV and 0.05 eV steps. Spectral peaks were separated using the VG Avantage software; the peak widths used were those established in our laboratory and are held constant for any element. The components were separated after a Shirley background was subtracted. The energy was calibrated by setting the major C1s peak, attributed to hydrocarbon, to 285.0 eV.

1.7. DNA Sequence Analysis and Bioinformatics. Open reading frame identification was performed using GeneMark.hmm. Similarity searches, for nucleotide sequences and for the deduced amino acid sequences, were performed using the FASTA, BLAST, and PARALIGN programs available on the Online Analysis Tools web site (http://molbiol-tools.ca/).

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Figure 2. XPS spectra of MRSA-specific bacteriophage. (a) XPS survey spectrum of bacteriophages showing carbon, nitrogen, and oxygen species. (b) C1s high-resolution spectrum. (c) N1s high-resolution spectrum. (d) O1s high-resolution spectrum.

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Silicon Wafer Characterization. All samples were deposited onto a silicon wafer. As can be seen from Figure 1, where the XPS survey scan of a silicon wafer is presented, the surface has, in addition to silicon (22.55%), the expected presence of natural oxide (67.33%) and lower but significant amounts of carbon (8.92%) and fluorine (1.20%). The presence of carbon indicates the existence of organic contaminants, arising from the ambient atmosphere and perhaps human handling. Fluorine contamination is certainly due to the etching process used in the original cleaning of the silicon wafer.
surface. The presence of silicon was not detected in further experiments due to the thicknesses of the phages, NPs, and phage–NP hybrid layers and will not be further discussed.

**Bacteriophage Characterization.** Taking into account that a typical layer thickness measured by XPS ranges from around 4 to 5 nm (three times the attenuation length) for C, N and O, we expected to see the chemical constituents of the outer layer of the phage capsid, primarily composed of amino acids. Figure 2a shows a bacteriophage survey spectrum. A molecular characterization\(^{14}\) of the podoviral bacteriophages virulent for *S. aureus* revealed that the open reading frame six (ORF 6) codes for the major coat proteins containing 30 amino acids (MEKIYTAVLLNYSENIYEHEIEQFEKNK). Major XPS peaks account for the presence of C1s, O1s, and N1s at 62.22, 23.26, and 14.45%, respectively. A minor S2p peak (163.66 eV) is found, at 0.06%, corresponding to sulfur present in methionine. No Si was detected, indicating that the wafer was thickly covered with bacteriophages.

Table 1 presents the binding energies of the component peaks. The high-resolution C1s spectrum was separated into five peaks (Figure 2b). The first, at 284.99 eV, is attributed to aliphatic carbon present in all amino acids, bound only to carbon or hydrogen.\(^{33–35}\) The second, at 285.91 eV, is attributed to carbon singly bound to nitrogen (C–N)\(^{33–36}\) present in all amino acids, as well as carbon singly bound to sulfur (C–S)\(^{37}\) present in methionine. The third, at 286.98 eV, is attributed to C–OH\(^{36,38}\) present in serine and threonine residues. The fourth, at 288.14 eV, is attributed to carbonyl carbon (C=O)\(^{34,36}\) present in amides and asparagine and glutamine, at 401.55 eV.\(^{41}\) The peak at 402.67 eV is attributed to protonated amino acid residues.\(^{40}\) This is in agreement with Helgstrand et al.,\(^{42}\) where some amino acids of the major coat proteins involved in the icosahedral capsid of phages are in a highly polar region and are protonated, forming hydrogen bonds with carboxylic groups. The fourth peak, at 403.85 eV, is due to a higher oxidation state, such as an amine N-oxide.\(^{59}\)

Finally, the O1s spectrum was separated into three peaks (Figure 2d). The first, at 531.69 eV, is attributed to oxidized nitrogen. The second (532.92 eV) is attributed to carbonyl groups, and the third, at 534.26 eV, is attributed to hydroxyl groups.

From the TEM image in Figure 3, the MRSA-specific bacteriophage is seen to be very similar to other *S. aureus* phages, such as the 44AHDJ virus, and is most probably from the *Podoviridae* family. As seen in Figure 3, it possesses a small isometric head, measuring 42 nm, and at its lower left a short, noncontractile tail, measuring 25 nm; the tail is responsible for its ability to adhere to and infect its bacterial host. The length of the phage (recognition element) is of critical importance to LSPR and will be discussed later.

**Heterobifunctional PEG-Functionalized Gold Nanoparticles.** Previous studies showed that the chemical synthesis of the gold NPs, through the reduction of a precursor salt by a reducing agent, often leads to surface contamination.\(^{43}\) An XPS survey of the 60 nm PEGylated NPs shows, as expected, the presence of gold, sulfur, carbon, and oxygen (Figure 4a). The relative concentrations were, respectively, 26.04, 2.46, 37.87, and 23.26%. Silicon was not detected, indicating a thick, homogeneous coverage of the wafer. No other contaminants, such as chlorine, fluorine, or sodium were detected.

Our XPS results show that the PEG linker is composed of seven repeat units, which is not in accordance with the manufacturer,\(^{44}\) who claims that the PEG functional group has a molecular weight of 3000 Da (≈70 repeat units). We can still see the gold through the PEG surface layer; because XPS can probe up to 4.5 nm (three times the attenuation length of Au, 15 Å), the PEG surface layer must be <4.5 nm in thickness. For S–(O–C–C)n–COOH with *n* = 7, we should have 15 times the amount of carbon relative to that of sulfur (i.e., 36.9%) and 9 times the amount of oxygen (i.e., 22.14%). A comparison with what we found experimentally leads us to believe that there is very little, if any, carbon and oxygen contamination.

A detailed chemical analysis of the high-resolution Au4f spectrum showed that the NPs may be partially oxidized by the oxygen present in solution (Figure 4b). High-resolution gold spectra are characterized by three pairs of peaks contained in the Au4f\(^{7/2}\) and Au4f\(^{5/2}\) doublet. The positions of the first and most important pair (BEs of 83.83 and 87.5 eV) are due to elemental gold (Au\(^0\)); those of the other pairs are due to Au–S (BE 84.22 and 87.89 eV), and oxidized gold (Au–O) (BEs of

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**Table 1. C1s, N1s, and O1s Peaks Detected for Bacteriophage Samples**

<table>
<thead>
<tr>
<th>Peak</th>
<th>BE (eV)</th>
<th>FWHM (eV)</th>
<th>at (%)</th>
<th>Suggested attributions</th>
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<td>534.26</td>
<td>3.45</td>
<td>3.45</td>
<td>Hydroxyl</td>
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**Figure 3.** TEM photomicrograph of an MRSA bacteriophage.
85.63 and 89.30 eV) (Table 2). Our results clearly indicate that gold is present at the interface in several oxidation states, explaining the low-energy O1s components of O\textsuperscript{−}Au species. On the basis of relative peak areas, their respective atomic percentages were estimated as 18.94\% for Au\textsuperscript{0}, 7.04\% for Au\textsuperscript{−}S, and 0.52\% for Au\textsuperscript{−}O.

The S2p high-resolution spectrum (Figure 4c) consists of two pair of peaks, attributed to S\textsuperscript{−}Au (BEs 162.24 and 163.42 eV) and S\textsuperscript{−}O\textsuperscript{−}C (BEs 165.71 and 166.89 eV), with respective atomic percentages of 4.15 and 1.14.

The high-resolution C1s spectrum (Figure 4d) contains four peaks attributed to hydrocarbon (285.02 eV), (O\textsuperscript{−}C\textsuperscript{−}C) (286.42 eV), S\textsuperscript{−}O\textsuperscript{−}C (287.86 eV), and COOH (289.53 eV). Respective atomic percentages were found to be 5.67, 21.01, 3.12, and 7.87 of the total.

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The high-resolution O1s spectrum (Figure 4e) contains three peaks attributed to Au\textsuperscript{−}O (532.87 eV), carbonyl (BE 533.95 eV), and hydroxyl components of PEG (534.92 eV). Respective atomic percentages were found to be 5.86, 22.85, and 7.87.

From Figure 5, it can be seen that the Au NPs are approximately cubic and reasonably monodisperse. From the TEM images, we obtain an average particle diameter and standard deviation of 62 ± 6 nm. The size of the NPs is of
critical importance for biodetection purposes using LSPR and will be further discussed in the following section.

**Bacteriophage−Gold Nanoparticle Nanohybrids.** We recently investigated several coupling strategies aimed at attaching lytic bacteriophages to gold surfaces. It is of paramount importance that phages retain their native conformation and binding activity while being stably immobilized to a sensor surface. Thus, an understanding of the interface between the gold surface and the systems to be studied is fundamental to all surface-sensitive sensor systems.

Phages were coupled to heterobifunctional PEGylated NPs using carbodiimide chemistry through reaction of the phage amino groups with EDC/NHS and the carboxylic acid terminations of the PEG deposited onto the gold NP surfaces. Chemical alterations of the sample surfaces, leading to the formation of an amide bond between the bacteriophages and the AuNPs, were studied by XPS. An elemental survey of the samples shows the presence of carbon, oxygen, and nitrogen, contributed by the presence of phages as well as small but detectable amounts of gold and sulfur, contributed by the PEGylated NPs (Figure 6a). The low atomic percentages of gold (0.32) and sulfur (0.77) indicate that Au NPs were densely covered by phages. Thus, we expect relatively similar carbon, nitrogen, and oxygen percentages for phage−NPs hybrids when compared with phages. The atomic percentages of carbon were 60.72 for phage−NPs hybrids compared with 60.18 for phages. Similarly, the nitrogen and oxygen contributions remained very similar, at 15.4 and 22.82%, respectively, for phage−NPs hybrids, compared with 15.60 and 24.16% for phage species. There was, however, an increase in sulfur components (0.77%) for phage−NP when compared with phages alone (0.06%) due to the presence of thiolated heterobifunctional PEG NPs.

A detailed chemical analysis of the high resolution Au4f spectrum showed a component composition pattern similar to...
Table 3. Au4f, S2p, C1s, N1s, and O1s Peaks Detected for Bacteriophage–NP Hybrids

<table>
<thead>
<tr>
<th>name</th>
<th>peak BE</th>
<th>fwhm eV</th>
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that of the PEGylated NPs (Figure 6b). Gold was characterized by three Au4f1/2 and 4f3/2 doublets. The relative atomic percentage of elemental gold (Au5) (0.10%, BEs 83.88 and 87.55 eV) as well as Au–S (0.12%, BEs 84.86 and 88.53 eV) diminished when compared with PEGylated NPs. This is due to the high-density coverage of the NPs with phages, lowering the relative atomic percentage of the gold species compared with the C, N, and O contributions of the phages. The presence of Au–O species (BEs of 85.71 and 89.38 eV) was also detectable (0.08%). The S2p high-resolution spectra again consisted of two pairs of S2p1/2 and S2p3/2 doublets (Figure 6c). The first pair, attributed to S−Au (0.53% BEs 162.56 and 163.74 eV), is derived from PEG attachment to NPs; the second peak, less electronnegative than that reported for S−O−C component of PEGylated AuNPs, is be attributed to oxidized sulfur at 0.28% with BEs of 163.72 and 164.90 eV.

High-resolution C1s spectra (Figure 6d) showed a decrease in the relative atomic percentage of aliphatic carbons in phage–NP hybrids (6.38%, BE 284.99 eV) compared with phages (9.87%). This decrease was also noted for the peak attributed to C−N and C−S (15.77%, BE 285.98 eV) compared with 19.63% in phages. There was an increase in the relative atomic percentage of C−OH (16.82%, BE 286.94 eV) when compared with phages (13.68%). The peak attributed to the amide contribution (288.79 eV) showed a slight increase (12.52%, compared with 10.20% for phages) due to bond formation between the NP and the phage surface amine. The peak corresponding to the carboxylic acid components of phages remained stable (6.85%, 289.31 eV). A sixth peak appeared at BE 290.54 eV at 2.37%. This peak may be due to the EDC/NHS complex used to form active ester functionalities with carboxylate groups.

Although the relative percentages of the N1s, O1s, and C1s components remained the same in phage–NP samples compared with phages, high-resolution N1s spectra were different, with only three peaks for phage–NPs compared with four for phages (Table 3, Figure 6e). Moreover, we see an increase in amine concentration (6.28%, 400.20 eV) and amide groups (6.86%, 401.18 eV) when compared with phages (1.88 and 4.16%, respectively), reflecting the formation of a peptide bond between the PEGylated gold and the phage. Our N1s XPS results reveal the presence of both amine (−NH2) and ammonium ion (−NH3+) in phage samples. However, following the covalent attachment of phages to AuNPs, N1s spectra show a marked decrease in protonated amine (2.27%, 402.10 eV, compared with 6.00% in phage samples) and a complete disappearance of the peak attributed to N-oxides compared with 3.62% for the phage samples. This indicates that the −NH2 covalently binds to the AuNPs, while the −NH3+ electrostatically binds to the negatively charged silicon surface. These results are consistent with previously published work:43 although amines do not generally react with bulk Au, they do react with nanosized Au, in solution, to form a weak covalent bond.45,46 Moreover, amine oxides are very reactive and can be readily converted to amine in the presence of reducing agents, such as NHS. We note the presence of −NH3 species at 400.20 eV, while −NH3+ species are present at 402.10 eV; −COO− are found at lower binding energy (531.93 eV) than −COOH groups (533.99 eV). These are consistent with recent findings, where core-level shifts larger than 1 eV in the N1s and O1s spectra are induced by the formation of strong intermolecular H bonds with opposite sign for the proton donor and the proton acceptor.47 Positions and atomic percentages for the high-resolution peaks of the O1s spectrum remained essentially the same (Figure 6f).

TEM was used to directly visualize the nanohybrid complex at high resolution (Figure 7). Negative staining was required to verify the presence of bacteria and bacteriophages. To date, very few studies have investigated negative staining factors affecting these types of complexes. Recent studies show that phosphotungstic acid (PTA) solution interrupts the interactions between Au NPs and phage particles, resulting in the detachment of the NPs from phages.48 To avoid this, we used a 2% UA solution. Although the NP–phage hybrids were darkened, lytic bacteriophages are still recognizable. Moreover, phage–NP hybrids can be seen to still adhere to their host bacterium, implying that the phage remains active. Further studies are needed to confirm this.

We have used a finite-element model that solves for Maxwell’s equations in the time-harmonic regime48 to support our views and to estimate the optimum size of NPs to be used for the field enhancement of LSPR in biodetection. The distribution of the amplification of the electric field in space was obtained by solving the Helmholtz equation and using the
Figure 8a presents the near-field enhancement ($|E|^2$) around our 60 nm AuNPs suspended in a PBS solution (refractive index $\approx 1.334$), following linearly polarized irradiation at 680 nm. Calculations (Figure 8b) show that the enhanced field intensity reaches well beyond the size of an average phage ($\approx 42$ nm$^{14}$), confirming the possibility of phage–NP hybrid-based biodetection. Varying the AuNP diameter, Figure 8c also reveals the dependence of the enhanced near-field intensity, at the phage–bacterium interface, relative to the size of the NPs and shows that the greatest intensity is predicted for 150 nm NPs; unfortunately, this could not be verified because PEGylated Au NPs of this dimension were not available at the time. However, those NPs would be, as a consequence, the most promising candidates for the development of efficient phage–NP hybrids.

### CONCLUSIONS

AuNPs, functionalized with a heterobifunctional PEG, were successfully coupled to lytic bacteriophages specific to MRSA, via carbodiimide chemistry, for biodetection purposes. XPS was used to study the interface between the metal and the bacteriophage. XPS characterization of the lytic phage showed the presence of protonated nitrogen species attributed to the major capsid proteins. PEGylated gold NPs were shown to efficiently bind to phages through the formation of amide bonds between the primary amines of the phage and the carboxylic acid terminal groups of the NP platforms. Our results also showed the formation of strong intermolecular hydrogen bonds between carboxyl and amine species, as shown by N1s and O1s core-level shifts.

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**Notes**

The authors declare no competing financial interest.

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