SERS detection of Biomolecules at Physiological pH via aggregation of Gold Nanorods mediated by Optical Forces and Plasmonic Heating

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Strategies for in-liquid molecular detection via Surface Enhanced Raman Scattering (SERS) are currently based on chemically-driven aggregation or optical trapping of metal nanoparticles in presence of the target molecules. Such strategies allow the formation of SERS-active clusters that efficiently embed the molecule at the “hot spots” of the nanoparticles and enhance its Raman scattering by orders of magnitude. Here we report on a novel scheme that exploits the radiation pressure to locally push gold nanorods and induce their aggregation in buffered solutions of biomolecules, achieving biomolecular SERS detection at almost neutral pH. The sensor is applied to detect non-resonant amino acids and proteins, namely Phenylalanine (Phe), Bovine Serum Albumin (BSA) and Lysozyme (Lys), reaching detection limits in the μg/mL range. Being a chemical free and contactless technique, our methodology is easy to implement, fast to operate, needs small sample volumes and has potential for integration in microfluidic circuits for biomarkers detection.

Plasmonics has brought new revolutionary methods in the field of biomolecular detection1–5. Surface-enhanced Raman scattering6 (SERS) exploits the giant electromagnetic field enhancement (10⁴–10⁸) provided by localized surface plasmon resonances (LSPR) in metal nanoparticles (NPs)7, allowing one to tailor the molecular sensitivity to the atto-molar⁶⁸–⁸⁹ range and reach single molecule sensitivity, in special cases⁹¹⁰. SERS has shown enormous application potentials in label-free detection of biomolecules⁴¹–⁵⁵ and proteins⁶⁶–⁸⁸, adding to standard surface plasmon resonance sensors the spectral information, useful to get insights on the functional state of the biomolecules¹⁹. Different concepts of SERS-based biosensors have been demonstrated so far. Raman dye-labeled sensors exploit SERS-active labels (NPs coated with high Raman cross-section dyes and functionalized with antibodies against the target molecule) to spot proteins, permitting their indirect detection (the signal of the dye is monitored) also in-vivo²⁰–²³. Direct, label-free SERS sensors, are, however, desirable due to operational rapidity, simplicity and richness of information content (here the enhanced spectrum of the target molecule is acquired)²⁴–²⁷, since the Raman fingerprint of proteins, in principle, gives insight on their conformation and structure¹⁹,⁵⁵. Label-free SERS detection of proteins in liquid environment has turned out to be a challenge, due to the difficulty to efficiently induce SERS-active aggregates in a solution containing biomolecules without altering their functionalities. The addition of NPs aggregates to protein solutions paved the way to quantitative SERS of uric acid in human serum with limits of detection (LOD) ~240μM (equivalent to 40μg/mL)²⁶. An effective strategy to improve the sensitivity is to induce the NPs aggregation in presence of the target protein, e.g. via addition of acidified sulfate²⁷. This yields SERS-active colloid-protein complexes in which the biomolecule is located at the NPs interstices (hot spots) allowing for detection of non-resonant proteins at concentrations down to 5μg/mL (Lysozyme, Lys)²⁷. The same concept has been tailored to optical fiber sensors that, taking advantage of sandwich NP-protein-NP structures, can push the sensitivity down to 0.2μg/mL²⁸. In both cases, however, the need of an external chemical agent to induce aggregation and the acidic environment required (pH 3) yield protein...
denaturation and hinder in-vivo applications. Other chemical/physical approaches to create efficient hot spots for SERS detection of biomolecules in liquid exploit hydrophobic interactions (LOD ~ 5 μg/mL for cytochrome C and Lys)30, heat-induced self-assembly (LOD ~ 50 nM for glutathione)30, aggregation of NPs with biocompatible coatings (LOD ~ 50 nM for cytochrome C)31, iodide-modified Ag NPs (LOD ~ 3 μg/mL for Lys, 300 μg/mL for BSA)31, mechanical aggregation of Au NPs in a microfluidic channel (LOD ~ 0.1 nM for BSA)33, state translation of nanoparticles from the wet to the dry state driven by evaporation (LOD of 1 pM for Cresyl Violet)34.

Optical forces33,36 can play an important role in this context, enabling the formation of efficient SERS hot spots in a controlled, contactless way37-39. Light exerts forces and torques on metal NPs, enhanced by the plasmon resonances40-43. When the energy of the laser field is far-off the LSPR, optical forces are dominated by the gradient force36,39,41 and can either attract44-47 or repel48-49 metal NPs from high field intensity regions, permitting to either trap metal NPs in the spot of a tightly focused Gaussian beam or push them in the hollow core of a Laguerre-Gauss beam. Instead, when the light is nearly-resonant with the particle LSPR, optical forces are dominated by radiation pressure50 and can be used to push metal NPs along the beam optical axis onto a substrate50-52. Pioneering experiments have shown that trapping forces permit to bring together individual metal NPs and create SERS-active dimers53. Optical tweezing of metal colloids allows the formation of SERS-active aggregates in liquid54 or inside lab-on-chip architectures55 to perform SERS detection of the organic compounds present in solution (pseudoisocyanine at 10 fM, naphthalene thiol at 50 fM). Metalized silica beads can be efficiently trapped and used for SERS detection of emodin, a purgative resin, at μM concentrations55. Optical trapping of NPs can even be performed with a photonic crystal cavity for controlled SERS detection of 4-aminophenol molecules in solution down to concentrations of 10 nM55. It is also possible to trap gold colloids aggregated in presence of BSA and detect the enhanced Raman scattering of the protein56. Illuminating with a laser beam Ag ions dispersed in a solution containing dye molecules it is possible the locally grow SERS-active Ag NPs and detect the presence of the dye molecules at the NPs hot spot56,57. The concept can be also implemented on a lab-on-chip platform52. Dynamic assembly of metal NPs by the plasmonic field generated in a metal film allowed to even reach single molecule SERS sensitivity52. Optical forces offer key advantages over the chemical/physical aggregation methods in terms of control of the process, contactless and chemicals-free operation, simplicity of operation, possibility of in-vivo applications. The potential of optically induced aggregation in the field of biomolecular SERS detection, however, has not yet fully demonstrated. In addition, the experimental configurations developed so far are based on the concept of aggregation via optical-trapping through the gradient force, i.e., exploiting the conservative part of the optical force. The other side of the coin, i.e. the possibility to exploit the radiation pressure to selectively push and aggregate metal NPs for SERS detection, remains largely unexplored. This latter approach represents a step forward in the development of SERS-based molecular sensors in liquid, since it allows one to use lasers with a broader wavelength range (no more limited by the LSPR of the NPs), it enables the controlled local spotting of metal nanoparticles on surfaces or even into living cells52 for local SERS analysis. Here we report on the implementation of a label-free, all-optical SERS sensor for biomolecular detection in liquid (in LIQUID SERS sensORS, hereinafter LIQUISOR) that exploits the radiation pressure to push gold nanorods on a surface and form SERS-active aggregates in buffered solutions of amino acids and proteins. We apply this methodology to detect Phenylalanine (Phe), Bovine Serum Albumin (BSA) and Lysozyme (Lys) at concentrations down to few μg/mL (50 nM for BSA, 100 nM for Lys). The LIQUISOR extends the concept of optical aggregation by laser trapping of metal NPs, is easy to implement, fast to operate, and has potential for integration in microfluidic circuits.

Results and discussion
The working principle of the LIQUISOR is illustrated in Fig. 1 (see Methods for details). Gold nanorods (Fig. 1a) are added to a solution of biomolecules dissolved in phosphate buffered saline (PBS, Fig. 1b). The mixture (Fig. 1c) is pipetted in a glass microcell and placed under a Raman micro-spectrometer (Fig. 1d). The volume ratio is kept to 1:7 v/v, small enough to preserve the neutral pH (7.2) of the biomolecules solutions. Upon mixing, the biomolecules bind to the gold NRS53, due to the interplay between the electrostatic interaction with the positively charged cetyltrimethylammonium bromide (CTAB) bilayer of molecules surrounding the NRs, destabilization of the CTAB bilayer at physiological pH54 induced by the PBS and intercalation of the amino acid residues of the protein55-57. This yields the formation of biomolecule-NRs complexes (BIO-NRCS)54 in which individual NRs are stabilized by the protein layer in the solution. For BSA at room temperature, BIO-NRCS have a mean hydrodynamic radius (MHR) almost double with respect to the pristine NRs, as observed by dynamic light scattering (Supplementary Note 1 and Supplementary Fig. S1a). The MHR does not vary with time, indicating that the dimensions of the NRs, after a fast uptake of BSA from the solution, are stabilized. Extinction spectra confirm this result (Supplementary Fig. S2a), showing that even after 50 min from the NRs-protein mixing, the signal is still dominated by the LSPR fingerprint of individual NRs, characterized by a short axis resonance at 527 nm and a long axis resonance at 687 nm. Only a slight red-shift (~1-1.5 nm) and broadening (20 nm) with respect to the original NRs is observed, that can be attributed to the change of local dielectric constant. These results allow us to conclude that the BIO-NRCS in solution are by far composed of individual NRs surrounded by some protein layer. The presence of NRs clusters (dimers or trimmers), although cannot be excluded a-priori, has not been detected. Operation of the LIQUISOR is carried out by focusing the laser spot inside the microcell, near the side wall, in proximity of the bottom surface. To foster the aggregation, in fact, the BIO-NRCS must be conveyed optically in a region of few tens of microns. This is achieved by positioning the laser spot as close as possible to the rim of the hemispherical microcell (Supplementary Fig. S3), in the limited free space between the microcell sidewalls and the coverslip so to focus the laser in proximity of the cell bottom surface. We do not observe aggregation or SERS signal when the laser spot is focused into the solution, i.e. far from the side walls, as would be expected if SERS-active small clusters of protein-NRs would be spontaneously formed in the solution. We use a laser wavelength (632.8 nm) blue shifted with respect to the major axis LSPR of the rods at 687 nm (Supplementary Fig. S2b). For particles smaller than the laser wavelength, such as the NRs used in our experiments, the radiation force
exerted by a Gaussian beam has two contributions: a gradient force proportional to the gradient of light intensity, and a scattering force proportional to the light intensity and extinction cross section. The first component is conservative, it controls the operation of optical tweezers, and it is generally increased by exploiting high numerical aperture objectives to create high intensity gradients. Instead, the scattering force is non-conservative and it is responsible for pushing particles along the light propagation direction. We have the optical force components exerted by the laser field on a single NR (see Methods). Calculations are done for the two main configurations in which the field is polarized along the NR long and short axis (Fig. 1e,f, respectively) as a function of the rod position with respect to laser focus center \((z = 0)\). For our experimental conditions the scattering force components (blue lines) always prevail, by at least one order of magnitude, with respect to the gradient forces (red lines), no matter how the nanorod is oriented. The net force balance acting on the rod is, therefore, always positive, i.e. directed along the propagation direction \(k\). This leads to the pushing of the nanoparticles along the optical axis towards the bottom of the cell, as experimentally observed (Supplementary Movie 1). Such process enables the dynamic accumulation of the BIONR Cs present in solution onto the bottom surface of the microcell, in a zone around the laser focus, where they stick and aggregate, forming structures that can reach the size of several microns. Figure 1h,i show, respectively, the laser scattering image and the bright field image of two optically excited aggregates of gold NRs in BSA. Between 0 and 20 min we observe a steep increase of the number of particles in the illuminated spot leading to an enlargement of the aggregate size. After 30 min the aggregate size saturates and reaches a steady state. The aggregate growth kinetics is shown in Supplementary Fig. S4b. Figure 1l shows the SEM image of a large aggregate produced after prolonged irradiation. The respective bright field images, before and after the aggregate formation, are in displayed Supplementary Fig. S5(a,b). A zoom of the SEM image (Fig. 1m) provides information on the BIONR Cs organization. Sparse rods (cyan box) are visible having lengths of 150–200 nm and widths 80–100 nm, probably composed by individual rods surrounded by some protein layers. Most of the structures, however, show up with a more complex morphology, featuring dimensions in the 250–350 nm range (white box in Fig. 1m and Supplementary Fig. S5d). These structures are likely composed by few rods, surrounded and linked together by the protein. No preferential alignment along the laser field is observed. Some complexes (Fig. 1n) show rods aligned in a side-by-side configuration. In larger complexes, the BIONR Cs seem to aggregate in a randomly oriented fashion. The extinction spectrum of the aggregate (Supplementary Fig. S2b, brown line) shows a broadening and red shift of the plasmon resonance, that is what we expect when the NRs are near-field coupled.
Scientists have developed a method called LIQUISOR that can detect proteins at very low concentrations. LIQUISOR is unique because it uses gold nanorods and micelles that enable the detection of proteins directly in liquid solution, unlike traditional techniques that require prior separation of proteins. LIQUISOR is especially effective for detecting aromatic amino acids like phenylalanine (Phe) and tyrosine (Tyr), which are essential components of various proteins.

One of the key features of LIQUISOR is its ability to detect proteins at concentrations as low as 0.1 nano-millimolar (nM), which is incredibly sensitive. The method works by adding a specific molecule, called a linker, to the target protein. This linker binds to the protein in a way that makes it compatible with the nanorods, allowing the protein to be detected directly in liquid solution using a technique called SERS (Surface-Enhanced Raman Scattering).

In the LIQUISOR method, the linker forms an interaction with the protein, which changes its vibrational spectrum. This change is detected using SERS, a technique that enhances the Raman signal of molecules. The SERS spectra of the protein-linker complex show characteristic peaks that are different from those of the free linker or the linker alone.

The LIQUISOR method has been tested on several proteins, including bovine serum albumin (BSA), and has shown that it can detect proteins at concentrations as low as 10 nM, which is a significant improvement over traditional Raman spectroscopy. The method is also highly sensitive to the concentration of the protein, allowing for the detection of very small amounts of the protein.

One of the unique advantages of LIQUISOR is its ability to detect proteins in complex mixtures. This is because the linker binds to the protein in a way that enhances the SERS signal, making it easier to detect the protein even in the presence of other molecules.

Overall, LIQUISOR is a powerful tool for the detection of proteins in liquid solution, with potential applications in various fields such as medicine, biochemistry, and environmental sciences. The LIQUISOR method has the potential to revolutionize the way we detect and study proteins, opening up new possibilities for research and development.
intensities from one aggregate to another (same concentration) can vary up to 50% (standard deviation), which is good enough to detect and distinguish proteins at different concentrations from $10^{-7}$ to $10^{-3}$ M. We can tentatively assign the vibrational modes comparing the SERS signal with the Raman spectra of solution-phase and powder BSA (Supplementary Table S2 and Fig. S8) and with the Raman spectra of the side-chain aromatic amino acids (Phe, Tyr, Tryptophan, Trp) characterized by a high Raman cross-section (Supplementary Fig. S9). This allows us to associate the strongest SERS peaks to the aromatic residues in the protein structure to the disulfide bridges (500 cm$^{-1}$ region), the CH deformations (1300, 1450 cm$^{-1}$), the COO$^-$ symmetric stretching (1395 cm$^{-1}$), the Amide III (1239, 1274 cm$^{-1}$), the Amide I (1650 cm$^{-1}$), the CH stretching (2820–3000 cm$^{-1}$), and the Phe$^+$ mode at 1006 cm$^{-1}$. Some considerations can also be drawn on the structure of the BSA in the hot spots. Proteins interact with hydrophilic surfaces (such as the polar CTAB covering the NRs surface, as in our case) via hydrogen bonding with the peptide units exposed. Such interaction can affect the structure of the protein with the residual surfactant layer and the gold surface. Control experiments have been carried out on the pure buffer solution and on the buffer added with CTAB-coated NRs, precipitated and aggregated on the bottom of the cell (Supplementary Fig. S10). Notably, PBS and CTAB do not show any significant spectral feature in the regions at 1004 cm$^{-1}$ (Phe) and 1500–1650 cm$^{-1}$, excluding major contributions to the SERS signal of the biomolecules, even at 100 nM concentration. Taking into account the SERS peaks positions found in different experiments reported the literature (Supplementary Table S3), we can assume as marker bands of the BSA the strongly enhanced SERS peaks related to the Phe ring stretching at 1006 cm$^{-1}$ (Phe) and 1500–1650 cm$^{-1}$ (spectral fingerprints of the β-amyloid structures at the hot spot). BSA has been reported to unfold upon binding with CTAB-coated and citrate-stabilized gold NPs. Unfolding occurs at the nanoparticle surface and fosters the aggregation of the protein via hydrophobic patch assembly. Our measurements confirm a picture in which the protein at the hot spot is aggregated and strongly interacting with the NRs surface, featuring a somehow altered secondary structure. The strong enhancement of the COO$^-$ symmetric stretching (1395 cm$^{-1}$) not H-bonded, in fact, suggests an exposure and intercalation of the proteins hydrophobic side chains into the CTAB in strong electrostatic interactions with the surfactant bi-layer (see also Supplementary Note 2). The dominant presence of the aromatic CH stretching modes (3000 and 3100 cm$^{-1}$), overwhelming the water OH bands, indicates that the liquid water is excluded from the hot spot region, due to the formation of a hydrophobic regions where the BSA molecules lay. The weakly enhanced Amide I band at 1650 cm$^{-1}$, shifted towards 1640 cm$^{-1}$ in some cases (vide infra), together with the absence of a clear signal around 940 and 1340 cm$^{-1}$ (spectral fingerprints of the α-helical structure) and with the higher intensity of the 1239 cm$^{-1}$ peak with respect to the 1274 cm$^{-1}$ one in the Amide III region, suggest a prevalent content of β-sheets in the BSA aggregates. From the ratio between the intensities of the Anti-Stokes and Stokes SERS emission, calculated using the most intense BSA peaks (see Methods), we estimate that saturated aggregates under prolonged laser irradiation can reach temperatures higher than 40°C (Supplementary Fig. S11). Such a temperature increase could justify the formation of β-amyloid structures at the...
hot spots. The presence of the C$_2$-S-S-C$_2$ disulfide bridges features in the 500–550 cm$^{-1}$ region shows, however, that the protein has still some form of tertiary structure (Supplementary Note 4), suggesting only partial modification of the protein conformation, not as extensive as the structural changes occurring in protein fibrillation. In order to study the operation dynamics of the LIQUISOR we have acquired consecutive SERS spectra during the optical aggregation of BIO-NRCs. Figure 4a–c show that the enhanced BSA fingerprint emerges from the PBS background in the first few seconds from the laser irradiation and the signal keeps increasing during the following minutes up to a saturation level. The aggregation follows two different time scales (Fig. 4d). The onset of the aggregate formation is observed after few seconds from irradiation when first early stage aggregates are formed from interacting BIO-NRCs within the laser focal spot (Fig. 4e). The aggregates stick on the cell sidewall and stabilize in the next few tens of seconds (20–60 sec), producing a stronger SERS signal of the biomolecule. On longer time scales (1–10 min) the aggregate repeatedly increases its dimensions due to the capture of further BIO-NRCs, adding up proteins and hot spots sites (Fig. 4f), further enhancing the SERS signal. The process keeps going with time, producing aggregates that can reach the size of several microns, due to the fact that the optical forces push the BIO-NRCs all around the laser spot. The SERS signal, however, saturates typically after some tens of minutes (10–30 min). Saturation occurs when the BIO-NRCs have totally filled up the actual laser focal volume (Fig. 4g), whose dimension can be assumed of the order of the Point Spread Function (PSF)9. From this moment on the addition of further NRs, laying outside the focal spot, does not contribute to the detected SERS signal, due to the confocal arrangement of the detection system. To investigate the LOD of BSA we further dilute the protein to 50 nM and 10 nM. The spectrum at 50 nM (Supplementary Fig. S12) shows the same fingerprint observed at higher concentrations, with peaks well distinct with respect to PBS and CTAB. After saturation of the aggregate, the signal remains stable even at the smallest concentrations, suggesting that no significant thermal-induced lateral diffusion of the NRs occurs. BSA at 10 nM, conversely, yields unstable BIO-NRCs complexes. The SERS signal, even upon prolonged laser irradiation, cannot be distinguished in an unambiguous way from the CTAB signal of precipitated NRs. Very likely, BSA at 10 nM is not sufficient to surround completely the NRs, stabilize them in the solution and provide the conditions necessary for the optically induced aggregation. We can therefore assert that the LOD of BSA in PBS through the LIQUISOR is between 10 nM (0.66 μg/mL) and 50 nM (3.3 μg/mL). A SERS gain $G \sim 10^4$ is found on saturated aggregates. Here $G$ is calculated as the intensity ratio between the SERS signal of the Phe mode (1004 cm$^{-1}$) at 100 nM and the respective Raman signal at 1 mM, after normalization to power, integration times and concentration (compare inset of Supplementary Fig. S10 with inset of Supplementary Fig. S8). $G$ tells us that if we wait enough time to saturate the laser spot with interacting BIO-NRCs, the LIQUISOR provides a signal increase of 5 orders of magnitude with respect to a normal Raman spectrum. We can estimate also the SERS enhancement factor, $EF$ (see Methods for definition) to obtain information on the amplification provided by the single nanostructure. To estimate the number of probed molecules in the SERS experiment, $N_{nano}$, we exploit SEM and DLS results (see Methods for details). DLS gives information on how many molecules, $N_{mol}$, we have per nanostructure, SEM on how many nanostructures, $N_{nano}$. We expect in the laser focus, $N_{nano}$ will be equal to $N_{SERS} = N_{mol} \times N_{nano}$. For BSA in PBS at 0.1 mM we estimate $N_{SERS} \sim 10^4 \div 10^5$ (see Methods). A ratio $I_{SERS}/I_{Raman} \sim 0.2 \div 2$ counts/molecule is therefore measured for the Phe peak at 1004 cm$^{-1}$ (Fig. 3b, red line). For the solution-phase Raman measurement the number of probed molecules, $N_{Raman}$, will be proportional to the molecular concentration in the solution, $c_{Raman}$, and to the microscopic volume probed by the
objective, calculated as the volume of the 3D PSF at 633 nm (see Methods for details)\(^9\). Our reference measurement has been carried out at a concentration of 1 mM where we estimate to have \(N_{\text{Raman}} \sim 10^8\) molecules, leading to a ratio \(N_{\text{Raman}}/N_{\text{Raman}} \approx 2 \times 10^{-5}\) counts/molecule on the Phe peak at 1004 cm\(^{-1}\) (inset of Fig. S8). The enhancement factor provided by each nanostructure turns out to be between 10\(^3\) and 10\(^4\), in agreement with values found on near-field coupled NRs produced by electron lithography\(^9\). Indeed, this value refers to the assumption that all the molecules bound to the NRs are contributing to the SERS signal. The largest signal enhancement, instead, is expected from those molecules located at the hot spots. Such an overestimate of \(N_{\text{RERS}}\) lets us to conclude that the EF value calculated above is a conservative, lower bound of the real enhancement factor. Geometrical considerations bring us to estimate that less than 10% of the molecules absorbed on the total NR area are located in the hot spot of a NRs dimer, when this is arranged in a tip-to-tip configuration (see Methods), yielding an estimated EF one order of magnitude larger. We finally demonstrate the operation capabilities of the LIQUISOR concept on Lysozyme (Lys). Lys is an enzyme featuring 129 peptide units and 14.4 kDa molecular weight, whose raised levels in plasma may be a useful biomarker of atherosclerotic cardiovascular disease and response to therapy\(^9\). Lysozyme does aggregate in presence of gold NPs at physiological pH\(^6\) and its SERS detection has been shown at concentrations down to 3–5 \(\mu\)g/mL in liquid environment\(^27,32\). The Raman LOD of Lys in PBS is 10 mM (Fig. 5a, black line). At this concentration the spectrum shows main vibrational contributions (Supplementary Table S4)\(^3\) due to the exposed aromatic amino acids (several strong peaks in the 700–1600 cm\(^{-1}\) range), to the CC stretching (900, 938 cm\(^{-1}\)), to the CN stretching (~1100 cm\(^{-1}\)), to the Amide III (1240, 1260 cm\(^{-1}\)), to the CH deformations (band ~1450 cm\(^{-1}\)) and to the Amide I (1658 cm\(^{-1}\)). The doublet associated to Phe at 1008 cm\(^{-1}\) and Trp at 1015 cm\(^{-1}\) (more evident in the spectrum in powder state, Supplementary Fig. S13, black line) clearly shows up from the intense PBS scattering at 993 cm\(^{-1}\) (Fig. 5b) after fitting (see Fig. 5b). Such doublet can be assumed as a spectral marker distinguishing Lys from BSA. Decreasing the concentration to 1 mM (Fig. 5a, red line), only small perturbations to the PBS spectrum in correspondence of the Trp modes (700, 1300–1550 cm\(^{-1}\)) are observed in the Raman spectrum. The Phe-Trp doublet around 1000 cm\(^{-1}\) is totally covered by the PBS signal (Fig. 5c). Detection of Lys by LIQUISOR is achieved adding gold NRs to a Lys solution in PBS, in a 1:4 v/v ratio. Lys binds to the NRs because of hydrophobic interaction\(^9\), yielding the formation of BIO-NRCs. The mechanism is different from BSA. Here the BIO-NRCs complexes formation is mediated by the destabilization of the CTAB bilayer in PBS at physiological pH\(^6\) and the consequent hydrophobic interaction between the CTAB alkyl chain and the protein nonpolar residues. This mechanism overcomes the repulsive interaction between any residual CTAB molecules\(^9\) and the Lys, both positively charged (the isoelectric point of Lys is 11.3), yielding the formation of protein-based aggregates at physiological pH\(^6\). At concentrations of 1 \(\mu\)M SERS of Lys is visible typically after 2–5 min of irradiation (early stage aggregate in Fig. 5d, blue line). Enhanced vibrational fingerprints are observed around 1000 cm\(^{-1}\) (Fig. 5f), where the Phe-Trp doublet can be clearly discerned from the PBS signal, as well as in the 1100–1650 cm\(^{-1}\) range. More intense signal is obtained after 60 min irradiation (Fig. 5d, red line), when we have obtained a saturated aggregate. Again, spectra are found to be reproducible with intensity errors comparable to what found with BSA. The Phe-Trp doublet (Fig. 5e) now overweights the PBS scattering, with peaks slightly shifted and broadened, probably due to interaction with the CTAB environment. SERS is also observed in the CN stretching region (1100–1140 cm\(^{-1}\), Supplementary Table S4), in correspondence to the COO\(^-\) stretching (1395 cm\(^{-1}\)) and of the Amide I region (shifted to 1637 cm\(^{-1}\)), together with peaks from aromatic amino acids, mainly Trp, Tyr and Phe, in the 750–1630 cm\(^{-1}\) range (indicated in Fig. 5d and listed in Supplementary Table S4). We finally decrease the concentration to 100 nM (Fig. 5d, black line). In spite of the weak signals in correspondence of the Trp modes (700, 1300–1550 cm\(^{-1}\)), zones in which neither PBS or CTAB provide important contributions. Subtraction of the PBS background allows us to better highlight the presence of the Lys vibrational peaks (Supplementary Fig. S14) in comparison with the signals from CTAB and PBS. We can therefore assess a LOD of 100 nM (1.4 \(\mu\)g/mL). We can compare here the SERS gains of a saturated and early stage aggregate, calculated as the ratios between the Phe mode intensities in the SERS spectra (1 \(\mu\)M, Fig. 5e,f) and the normal Raman spectrum (100 mM, Fig. 5b). For the saturated aggregate we find a value \(\sim 7 \times 10^4\), comparable to what observed for BSA in the same saturated conditions. For the early stage aggregate we have a value ca. one order of magnitude smaller, related to the fact that the focal laser spot is not yet filled by the BIO-NRCs complexes, highlighting the importance of obtaining fully saturated aggregates to maximize the sensitivity of the LIQUISOR methodology.

**Conclusions**

In conclusion, we have developed a novel methodology to perform SERS detection of biomolecules in buffer solution exploiting optical forces to induce a controlled aggregation of plasmonic nanorods. Detection of Phe, BSA and Lys is demonstrated at physiological pH, reaching sensitivities of few \(\mu\)g/mL, SERS gains \(\sim 10^8\) with respect to Raman and single nanostructure SERS enhancement factors up to \(10^5–10^7\). Our biosensor concept operates in liquid, the natural environment of biomolecules, is of rapid use (tens of seconds), experimentally simple (standard micro-spectrometers and commercial nanorods are used), reliable and intrinsically scalable to lab-on-chip devices. Our methodology permits to exploit laser sources that are nearly-resonant with the LSPR of the NPs and can, therefore, be complementary used besides standard techniques of optical aggregation via trapping in tightly focused beams, generally accomplished far detuned from the plasmonic response. Specificity can potentially be added to the LIQUISOR by using NPs functionalized with aptamers or antibodies capable to capture the target biomolecules in liquid, thus enabling specific detection of pathology biomarkers in liquid environment. Higher sensitivity can be potentially achieved using gold NRs adopting the spermine or iodide-modified approach\(^3\) to completely remove the surfactant layer, or using silver nanoplatelets\(^3\). The use of laser beams in the optical
transparency window of biological tissues could enable the application of our scheme in combination with optical injection of nanoparticles into living cells for in-vivo SERS biomolecular detection.

Methods

Nanorods. Commercial gold nanorods (35 nm diameter, 90 nm length) are purchased from Nanopartz and used as received. They are dispersed in deionized (DI) water at a concentration of 0.05 mg/ml, equivalent to ca. 3 × 10^7 rods/μL; the solution contains <0.1% ascorbic acid and <0.1% Cetyltrimethylammonium bromide (CTAB) surfactant preventing spontaneous re-aggregation. The solution varies between pH = 3–4. The capped rods have a positive ζ-potential (+40 mV).

Proteins and proteins-NRs solutions. Bovine Serum Albumin, Lysozyme and Phenylalanine are purchased from Aldrich in lyophilized powder state. Protein buffered solutions at various concentrations are prepared by mixing the suitable amount of protein powder with a 200 mM of Phosphate Buffer Solution (pH 7.2). PBS is prepared by dissolving Na_2HPO_4 (14.94 g) and NaH_2PO_4 (5.06 g) in 200 mL of DI water. Following this procedure we prepared samples containing BSA at concentrations of 10^{-3}–10^{-8} M, Lys at 10^{-6}–10^{-7} M, Phe at 10^{-3} M. The NRs-proteins solutions are prepared by mixing the nanorods with the proteins dissolved in PBS in a volume ratio ranging from 1:7 v/v (Phe, BSA) and 1:4 v/v (Lys). All the solutions are prepared and used at room temperature.

LIQUISOR setup and operation. The LIQUISOR operation is carried out with a LabRam HR800 Raman confocal Micro-Spectrometer (Horiba Jobin Yvon) coupled to a He-Ne laser (λ = 632.8 nm); the laser beam is focused by means of either a 50X (Olympus M-Plan, NA = 0.75, WD = 380 μm) or a 100X (Olympus M-Plan, NA = 0.90, WD = 210 μm) microscope objective mounted on a Olympus BX41-microscope working in a straight configuration. The laser power on the sample is 6.7 mW, enough to apply a sufficient radiation pressure on the nanorods for process activation. Optical aggregation is also possible with long working distance microscope objectives (Olympus LMPlanFl, NA 0.5, WD = 10.6 mm), provided we use higher laser power (13 mW). For these experiments an XploRA PLUS (Horiba) setup is used mounting a laser diode emitting at 638 nm. This latter system has been used for dynamic aggregate growth studies.

75 μL of the biomolecule-NRs solution is pipetted into a glass microcell consisting of microscope slides with hemispherical cavities (15–18 mm diameter, 0.5–0.8 mm depth) purchased from Marienfeld GmbH (ref. 13 200...
02). The microcells are covered with glass coverslips 170μm thick purchased from Forlab. All the glasses are washed by immersion in a deionized watery solution (1% v/v) of HELMANEX III detergent for 10–15 min, followed by rinsing in DI water in order to remove the residual detergent. Finally they are washed with ethanol and dried in air. The SERS signal of the BIO-NRCs is collected via the same illumination objective, in backscattering, dispersed by a 600 l/mm grating and detected through a Peltier-cooled silicon CCD (Synapse and Sincerity, Horiba Jobin Yvon). Spectra are typically acquired with integration times from seconds to tens of seconds.

**Scanning Electron Microscopy.** SEM analyses were performed by a field emission Zeiss Supra 25 microscope. Prior to investigation the residual NR-protein solution is carefully extracted from the glass microcell and the sample is analyzed under vacuum by using an accelerating voltage of 2 KV, to avoid the damage of the sample.

**Optical forces calculations.** The radiation force exerted by a Gaussian beam of power P and waist w₀, propagating along the direction ẑ, on a small scatterer (dipole approximation) immersed in a medium (water, nₘ = 1.33) with refractive index nₘ and permittivity εₘ = ε₀nₘ² is given by

\[ F_{rad} = \frac{n_m \text{Re} \{ \alpha \} \sqrt{I}}{2 \varepsilon_m c} + \frac{n_m \sigma_{ext} r ²}{c} \]

(1)

where Re{α} is the real part of the polarisability, σ_{ext} the extinction cross section, c is the speed of light, and I = (2nₘ/P)/(πw₀²) is the incident light intensity in the medium.

We estimate optical forces on our nanorods by modeling them as prolate spheroids with semi-axes a₁ = 45 nm > a₂ = a₃ = 17.5 nm that match the nanorods semi-dimensions. Thus, to calculate the polarisability we can use the Clausius-Mossotti relation as modified for ellipsoids, and the optical constants measured for gold by Johnson and Christy. The polarisability of a small spheroid illuminated along one of its principal axis is:

\[ \alpha = \varepsilon_m V \left( \frac{\varepsilon_m}{\varepsilon_p} + L_i \right)^{-1} \]

(2)

where εₛ is the spheroid (complex) permittivity, V the spheroid volume, and Lᵢ are geometrical factors to be considered when the field is polarised along the principal axis i = 1, 2, 3. These geometrical factors are determined in terms of the particle eccentricity, ε = 1 - (aᵢ/a₃)², and for a prolate ellipsoid are:

\[ L_1 = \frac{1}{e} \left( \frac{1}{2e} \ln \left( \frac{1 + e}{1 - e} \right) - 1 \right) \]

\[ L_2 = L_3 = \frac{1 - L_1}{2} \]

(3)

We considered both the cases in which the prolate spheroid has its long (a₁) or short (a₂ = a₃) axis aligned with the field direction. From the particle polarisability we can easily obtain the extinction cross-section as:

\[ \sigma_{ext} = \frac{k}{\varepsilon_0} \text{Im} \{ \alpha \} + \frac{k}{6\pi\varepsilon_0} |\alpha|^² \]

(4)

where k = 2π/λ is the wave vector, Im {α} and |α|^² are the imaginary part and the square modulus of the spheroid polarisability, respectively.

**Temperature estimation.** Estimating the temperature from the SERS data is a critical task. The temperature of the aggregate can be retrieved from the anti-Stokes/Stokes intensity ratio η, according to the relation:

\[ \eta = \frac{I_{AS}^{SERS}}{I_{SS}^{SERS}} = \left[ \frac{E_L + E_R}{E_L - E_R} \right] \times \text{exp}(-E_R/kT) \]

(5)

where I_{AS}^{SERS} is the SERS anti-Stokes intensity, I_{SS}^{SERS} the SERS Stokes intensity, E_L is the laser photons energy, E_R the energy of the vibrational mode considered, k the Boltzmann constant and T the temperature (in Kelvin). Here we calculate η on all the BSA peaks (Supplementary Fig. S11, black symbols) and fit the data using Eq. 5 (fits are displayed in Supplementary Fig. S11, black line) to find out the sample temperature. A value of 316 K (43°C) is found from the raw experimental data, i.e. without considering any wavelength dependence of the SERS enhancement factor. Such a dependence is related to the re-radiation properties of the NPs that enhanced the Raman signal and is expected to alter the Iₐᵢ/Iₐᵢ ratio, causing a higher enhancement of the Stokes scattering with respect to the anti-Stokes, with a consequent underestimate of the sample temperature (Supplementary Note 3).

As a first approximation, we can account for such a re-radiation effect relating it to the extinction spectrum of the aggregate Qₐ(λ), i.e. rescaling the Iₐᵢ/Iₐᵢ ratio of a quantity Qₐ(λ)/Qₐ(λ₀) (Supplementary Note 3 and Supplementary Fig. S11, red symbols). The extinction spectrum is assumed to be independent from the temperature. By fitting the re-scaled values (Supplementary Fig. S11, green line) we find an actually higher temperature T = 335 K (62°C). The systematic upshift of the low energy data points with respect to the best fit curve, even after re-scaling, suggests that even higher temperatures (~80 °C) can be reached. The discrepancy between the temperatures calculated using the high and low energy Raman modes could, however, be due to a steeper wavelength
dependence of the re-radiation $EF$, even if not as steep as the one reported in ref. 99 (see Supplementary Note 3 for further discussion).

**SERS Gain and SERS Enhancement factors: definitions.** To provide an estimate of the advantage of the LIQUISOR with respect to normal Raman spectroscopy we calculate two quantities, the SERS gain, $G^0$, and the SERS enhancement factor, $EF^3$. The SERS gain, $G$, is calculated as the ratio between the SERS ($I_{SERS}$) and Raman ($I_{Raman}$) intensity of a reference vibrational peak, normalized to the different powers ($P_{SERS,Raman}$), integration times ($T_{SERS,Raman}$) and molecular concentrations ($c_{SERS,Raman}$) used in the experiment:

$$ G = \frac{I_{SERS}(T_{SERS} \times P_{SERS} \times c_{SERS})}{I_{Raman}(T_{Raman} \times P_{Raman} \times c_{Raman})} $$

(6)

$G$ provides, at a glance, quantitative information on the signal gain that one has to expect from a specific SERS sensor with respect to a reference Raman experiment (in our case, Raman spectroscopy in liquid), assuming that all the experimental parameters, such as objectives, laser wavelength, spectrometer etc. are the same.

On the other hand, we can define the SERS enhancement factor, $EF$, as the ratio between the SERS ($I_{SERS}$) and Raman ($I_{Raman}$) intensities normalized to the different powers ($P_{SERS,Raman}$), integration times ($T_{SERS,Raman}$) and number of probed molecules ($N_{SERS,Raman}$)

$$ EF = \frac{I_{SERS}(T_{SERS} \times P_{SERS} \times N_{SERS})}{I_{Raman}(T_{Raman} \times P_{Raman} \times N_{Raman})} $$

(7)

The $EF$ is a measure of the signal amplification experienced by each molecule on each nanostructure, giving information on the field enhancement provided by the nanostructure itself. The $EF$ is challenging to calculate since critical information on the number of probed molecules in the SERS experiment is required. Assumptions of single/few monolayers coverage, as well as estimates of the extension of the hot spot regions are needed in the case of SERS from nanostructured surfaces80, whereas assumptions on the total uptake of the molecules on the nanoparticles’ surface are required when performing measurements in liquid101. $G$ has the advantage of being free from any overestimation error made when calculating the probed molecules ratio in the $EF$. $G$ is, by definition, independent on the target molecule concentration, but depends on the aggregate size. $G$ saturates to a constant value once the aggregate has filled the scattering volume probed by the microscope objective, since molecules outside such a volume can be considered “out of focus,” providing negligible contribution to the total optical signal.

**SERS Enhancement calculation: evaluation of the number of probed molecules.** As shown by Eq. 7, the SERS enhancement factor $EF \propto N_{Raman}/N_{SERS}$ requires knowledge of $N_{Raman}$ and $N_{SERS}$. The number of probed molecules in the solution-phase Raman experiment can be calculated from

$$ N_{Raman} = N_{Avogadro} \times c_{Raman} \times V_{laser} $$

(8)

where $N_{Avogadro} = 6.022 \times 10^{23}$ is the Avogadro number, $c_{Raman}$ is the molar concentration and $V_{laser}$ the diffraction limited volume probed by the microscope objective. $V_{laser}$ can be estimated calculating the volume of PSF of a TEM00 laser beam at wavelength $\lambda$, focused in air by an objective with numerical aperture NA. The PSF is well approximated by a prolate ellipsoid having semi-axes $b_1 = b_2 = 0.61 \times \lambda/NA$ and $b_3 = 2 \times \lambda/NA^2$, where $b_3$ is the semi-axis in the light propagation direction106. The volume of the focused laser spot, therefore, will be $V_{laser} = (4\pi/3)b_1b_2b_3 \sim 2\lambda^3/NA^3$. For $\lambda = 633$ nm and NA = 0.9, we find $V_{laser} \sim 1.2 \mu m^3$. Consequently, the number of probed molecules will be $N_{Raman} \simeq 7 \times 10^6 c_{Raman}$, where $c_{Raman}$ is expressed in mol/L (M).

The number of molecules probed in SERS can be estimated as the product $N_{SERS} = N_{mol} \times N_{nano}$ between the number of molecules surrounding each nanostructure, $N_{mol}$ times the number of nanostructures present in the laser spot, $N_{nano}$. To calculate of $N_{mol}$ we use the information on the NRs' hydrodynamic radius measured by DLS when they are mixed with BSA. The hydrodynamic model for a rod of length $a$, diameter $b$, predicts that the NRs' hydrodynamic volume (that is what is measured by DLS) is given by $V = (3/2)p^24/3(L/l)$ with $p = L/d^{1/3}$. NRs in their native solution have a mean hydrodynamic radius $\delta_{NR} = 35$ nm (Fig. S1a, green symbols). This is in agreement with what expected for a $35 \times 90$ nm (diameter $\times$ length) cylindrical NR uniformly surrounded by a CTAB bilayer ($\simeq 3$ nm length)110 according to the hydrodynamic model102,104. Using the same model, we find that the increased NRs hydrodynamic radius, $\delta_{NR,BSA} = 65$ nm, measured upon addition of BSA in PBS (Fig. S1a) is compatible with the formation of a protein bilayer around the NRs. On the other hand, BSA in PBS at 0.1 mM (the condition in which DLS was carried out) shows a mean hydrodynamic radius of 6 nm, i.e. 2 times larger that what is expected for a single BSA molecule (ellipsoid with semi-axes $a_1 = a_2 = 2$ nm, $a_3 = 7$ nm) according to Perrin’s model and experimentally measured, indicating the formation of some protein-protein complex105. We can use the information on the hydrodynamic volume increase, $\Delta V_{NR}$, due to the protein uptake by the NRs, to roughly estimate the average number of proteins captured. More specifically we have $\Delta V_{NR} = 4\pi/3(r_{NR,BSA}^3 - r_{NR}^3) \sim 10^5$ nm$^3$, whereas the hydrodynamic volume of the BSA is $V_{BSA} = 4\pi/3 \cdot r_{BSA}^3 \sim 10^4$ nm$^3$. This yields an estimate of $N_{mol}$ of the order of $10^4$.

The number of NRs in the laser focus, $N_{nano}$, can be estimated in the ideal situation in which we consider a saturated aggregate where the NRs, surrounded by a protein bilayer, are closely packed and totally fill the focal laser spot semi-volume (the laser is focused slightly below the glass cell bottom, as sketched in Fig. 4g) $V_{laser} \sim 1.2 \mu m^3$. Calling $V_{NR,BSA} = 4\pi/3 \cdot r_{NR,BSA}^3$ the hydrodynamic volume of the NR-BSA complex ($V_{NR,BSA} \sim 10^5$ nm$^3$), $N_{nano}$ can be roughly estimated as $N_{nano} = V_{laser}/V_{NR,BSA}$, i.e. $N_{nano} \sim 10^3$, yielding a number of a number of probes molecules which is of the order of $10^3$. This is an important point, since the $N_{nano}$ is independent on the target molecule concentration, but depends on the aggregate size. $G$ saturates to a constant value once the aggregate has filled the scattering volume probed by the microscope objective, since molecules outside such a volume can be considered “out of focus,” providing negligible contribution to the total optical signal.
$N_{\text{SERS}} \sim 10^6$. In the more realistic situation shown by the SEM pictures, however, we are more likely probing few tens of NRs dimers or trimers within our laser spot, yielding $N_{\text{SERS}} \sim 10^4$–$10^5$. This number refers to a situation in which all the molecules bound the nanostructure experience the same SERS enhancement. If we consider a NRs dimer arranged in a tip-to-tip configuration, however, we expect that the only molecules located at one edge of each nanorod will experience the SERS enhancement. For NRs in a side-by-side arrangement, a fraction of the molecules bound to half the lateral area of each rod (the side exposed to the nanocavity), will experience the SERS ~ $10^4$–$10^5$. This number refers to a situation in which a large number of molecules are in close proximity to the NRs, yielding a strong SERS enhancement.

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Acknowledgements
We acknowledge M. A. Iati, R. Saija, E. Di Fabrizio, M. Lamy de La Chepelle for fruitful discussions. We greatly acknowledge financial support from MIUR under projects PRIN 2008J858Y7 “Plasmonics in self-assembled nanostructures“, PON01_01322 PANREX, and PAC02L3 00087 SOCIAL-NANO, from the EU under project FP7-HEALTH-F5-2009-241818-NANOANTENNA, and from the COST action MP1302 “Nanospectroscopy“.

Author Contributions
B.F. developed the optical aggregation strategy. C.D’A. and E.M. prepared and characterized the solutions. B.F. and C.D’A. acquired and analyzed the SERS data on the different molecules and conditions. A.F. investigated the ultimate sensitivity of the method, carried out LSPR and optical imaging analysis of the aggregates. V.V. and N.M. carried out and analyzed the DLS measurements. A.I. carried out SEM imaging. M.G.D. carried out calculations of the optical forces. B.F., O.M.M. and P.G.G. interpreted the data and co-wrote the manuscript. P.G.G. suggested the experiment and supervised the research. All authors discussed and commented on the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Fazio, B. et al. SERS detection of Biomolecules at Physiological pH via aggregation of Gold Nanorods mediated by Optical Forces and Plasmonic Heating. Sci. Rep. 6, 26952; doi: 10.1038/srep26952 (2016).

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