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Surface-enhanced Raman scattering detection of wild-type and mutant p53 proteins at very low concentration in human serum

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ABSTRACT

The development of ultrasensitive and rapid approaches to detect tumor markers at very low concentrations even in a physiological environment represents a challenge in nano-medicine. The p53 protein is at the center of the cellular network that protects organisms against the insurgence of tumors, most of which are related to alteration of p53 expression. Therefore p53 is regarded as a valuable prognostic marker whose detection at high sensitivity may considerably contribute to early diagnosis of cancers. In this work we have applied an analytical method based on surface enhanced Raman spectroscopy with high sensitivity and rapidity to improve traditional bioaffinity techniques. The Raman reporter bifunctional linker 4-aminothiophenol (4-ATP) first assembled onto 50 nm gold nanoparticles (Nps) has then been azotated to bind low concentration wild-type and two mutated forms of p53 proteins. The Raman signal enhancement of the resulting p53-(4-ATP-Np) systems has been used to identify the p53 molecules captured on a recognition substrate constituted by the azurin (Az) protein monolayer. Az has shown a strong association for both wild-type and mutated p53 proteins, allowing us to selectively detect these proteins at concentrations as low as 500 fM, in a human serum environment.

The p53 protein is directly involved in the chain of biochemical events that follow genotoxic damage, playing a pivotal role in tumor prevention [1–3]. Accordingly, it has great promise as a diagnostic marker for monitoring the molecular machinery whose alteration results in early preneoplastic transformation. The p53 tumor suppression pathway is inactivated in almost all human cancers. In about 50% of them, this inactivation is a direct result of mutations in the p53 gene whereas the alteration of regulators of p53 occurs in many of the remainder [3–5].

An ever-growing number of publications have also reported an overexpressed level of alteration of the p53 protein, wild-type forms as well as structural mutants (non-null), i.e., the arginine 249 to serine (R249S) and cysteine 135 to valine (C135V) point mutations, as a consequence of neoplastic cell formation, tumor invasiveness, and genotoxic stresses [6–11]. These reports indicate that the level of p53 in human serum can be reasonably accurate in reflecting tissue alterations in p53 at the gene and/or protein level, thus leading to a potential convenient and noninvasive tumor screening approach.

On such a basis, much attention has been devoted to developing procedures to detect the presence of p53 protein at very low concentrations in a physiological environment. Among these procedures, the most common ones are traditional fluorescence-labeled immunological methods, such as the enzyme-linked immunosorbent assay (ELISA)[1] [12,13], which require multiple steps, and rather long incubation periods.

Beside these more traditional methods, novel label-free detection techniques have paved the way for the realization of protein chip-based immunoassays with enhanced sensitivity and specificity [14–16]. In the last few years, applications involving nanoparticles (Nps) have received much attention in clinical diagnosis [15,17–19]. In particular, gold nanocolloids have been widely used to design immunoassay tests for tumor markers, based on their peculiar properties, such as a high surface-to-volume ratio, the possibility of suitable biomolecular conjugation, the rewarding chemical stability, and the collective electronic behavior at their surface. Indeed, their optical and electrochemical signal enhancement capabilities, combined to their ability to form hybrid assemblies with biomolecules [2,15], provide the basis for ultrasensitive and molecular specific detection.

Within this context, the exploitation of the surface-enhanced Raman scattering (SERS) methodology [18–23] offers a great promise for simplified, sensitive detection of biomolecular interactions and several advantages in early diagnostic over the previously

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[1] *Abbreviations used:* AFS, atomic force spectroscopy; 4-ATP, 4-aminothiophenol; Az, azurin; CT, charge transfer; DBD, DNA-binding domain; ELISA, enzyme-linked immunosorbent assay; EM, electromagnetic; Nps, nanoparticles; PBS, phosphate-buffered saline; SERS, surface-enhanced Raman scattering; SPR, surface plasmon resonance.

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noted assay methodologies [24–26]. SERS is based on the huge enhancement of the Raman cross section of molecules when they are placed in the proximity of a nanostructured metal surface, due to the contribution of an electromagnetic (EM) and chemical or charge transfer (CT) effect [26–28]. Depending on both the chemical nature of the adsorbed molecules and the metal surface features, SERS may reach a 10^{10} fold increase of conventional Raman detection sensitivity [21].

In previous works, we have demonstrated the remarkable potential of this approach in revealing single molecules and in nanobiodiagnostics [21,22,29]. Proceeding from these studies, here we report a novel SERS-based detection method arising from the synergic combination of the Raman signal enhancement and bioaffinity assays, by which it has been possible to considerably lower the current threshold sensitivity in the detection of the p53 protein. In particular, we have exploited the specific interaction of p53 with the bacterial blue–copper protein azurin (Az). Indeed, cellular [30,31] and molecular studies [32,33] have demonstrated that Az is an exogenous vector able to preferentially enter cancer cells (such as in melanoma UISO-Mel-2 and breast cancer MCF-7 cells) and to form a specific and stable complex with the human p53 protein, thereby increasing its tumor suppressor activity. It is believed that their interaction involves a portion of the hydrophobic patch surrounding the copper-containing active site of Az, and the DNA-binding domain (DBD) of p53 [30,33], in which the majority of the p53 tumor-derived mutations reside. In our laboratory, the binding and kinetic features of the Az–p53 complex have been recently investigated by single molecule atomic force spectroscopy (AFS) and by surface plasmon resonance (SPR) [32,34]. These studies have demonstrated that this complex is significantly stable and that the Az–p53 interaction is not affected by the immobilization strategies used or by the binding to p53 of the Mdm2 protein, with the latter playing a key physiological role in lowering the intracellular stability of p53 [34].

In this work we have used the 4-ATP linker, having on one side a thiol group able to bind to a gold Np, and on the other side a diazonium moiety capable of reacting with the electron-rich aromatic lateral chains of p53. Then, p53, conjugated to a gold Np, has been transferred on a capture substrate composed of an Az monolayer, and the strong intense SERS bands, characteristic of the p53-(4-ATP-Np) system, have been followed to identify the p53 molecules recognized by the Az partner molecules. We have demonstrated that this approach can reveal the wild-type p53 protein initially present in a human serum solution at 500 fM. Furthermore, our proposed Az-assisted SERS detection strategy has been demonstrated to be able to detect the presence of important oncologic mutant p53 proteins (mp53), such as the hepatocellular and lung carcinoma biomarkers R249S and C135V p53 [7,35,36]. Notably, the approach has been extended to reveal p53 even in a serum environment. In particular we have shown that such a strategy is effective in revealing the presence of R249S mp53 initially introduced in human serum at different concentrations of actual diagnostic interest. Accordingly, the proposed methodology is extremely promising for revealing with high sensitivity important tumor markers in a physiological environment.

Experimental

Materials

Gold NP colloidal solution, 50 nm diameter (distribution ~20%), with 4.5×10^{10} particles/ml (corresponding to a concentration of 75 pM) was purchased from Ted Pella. Solid 4-ATP (assay 97%; absorptivity $\epsilon = 9600\ M^{-1}\ cm^{-1}$), Az, and healthy human serum were purchased from Sigma-Aldrich (St. Louis, MO). The proteins were dissolved in PBS 50 mM buffer (pH 7.2). A characteristic spectral absorption ratio of 0.48 at 630 and 280 nm was found for Az solution, indicating a good degree of purity of the protein.

High quality wild-type and R249S mutant p53 (human) recombinant proteins (purity >95%) were purchased from Thermo Scientific, dissolved in PBS 50 mM buffer (pH 7.2) obtaining mother solutions at 1 μM concentration, and used without further purifications. Recombinant human p53-Val135 mutant (C135V) was purchased from Bioworld, dissolved in PBS 50 mM buffer (pH 7.2) obtaining mother solutions at 1 μM, and stored at 4 °C. Water used for the experiments was purified by the MilliQ Reagent water system (Millipore, Billerica, MA).

Nanoparticle-based SERS probe

The procedure used to functionalize gold Nps is briefly described in Fig. 1A. In the first step, gold Np mother solution was mixed with 4-ATP (0.5 mg/ml in absolute ethanol) in a volume ratio of 1:1. The obtained solution was incubated at 20 °C for 3 h. The formation of a covalent bond between 4-ATP and gold Nps was followed by Raman spectroscopy (see also the Results and discussion). An additional investigation was performed by absorbance spectroscopy and the results are shown in the Supplementary Material, paragraph S.1. According to the literature [21], we have estimated that about 10^4 4-ATP molecules can fully cover a single gold Np.

To remove the excess of unbound 4-ATP and promote the ethanol to MilliQ water solvent exchange, the solution was dialyzed by a membrane (purchased from SPECTRA/POR), having a MW cutoff of 100 kDa. Dialysis was stopped when no more 4-ATP was detected by UV–Vis spectroscopy in the fluid beyond the membrane.

In the second step, diazo-coupling chemistry [21,37] was employed for the covalent capture of p53 (or Az, in the alternative configuration) onto gold Nps exposing the aromatic amino compound 4-ATP.

To this aim, 200 μl of acidified nitrosating agent $NaNO_2$ (0.1 M, pH 3), were added to 200 μl of dialyzed 4-ATP-Np solution, very

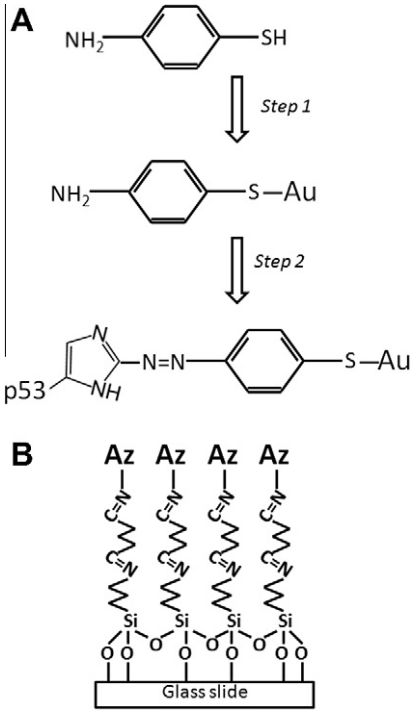

Fig.1. Chemical reactions describing the functionalization of gold nanoparticles with p53 (A) and of glass slides with Az (B).

slowly and under a constant stirring, for 15 min at about 2 °C. Only rare bubbles of nitrogen were observed, confirming the stability of the diazotized 4-ATP-Np system. The reaction was monitored by adding phenol to the mixture, since it confers a characteristic red color to the solution when the product appears in the reaction. Excesses of nitrous and hydrochloric acid were removed by dialysis against MilliQ-ice water at 2 °C and the pH of the resulting solution was stabilized at 7.2 by PBS buffer, avoiding the hazard of deamination and the unfolding of proteins during the coupling reaction.

The obtained diazonium compound was slowly added to 200 μl of p53 (from to 0.1 pM to 1 nM, pH 7.2), or alternatively of Az (10 μM, pH 7.2) and kept under a gentle stirring at 2 °C for 2 h. The final solution was further dialyzed to remove unbound proteins. The success of the diazo-coupling reaction was assessed by Raman and ultraviolet–visible (UV–Vis) spectroscopy (see Results and discussion) and the efficiency of the coupling reaction was estimated to be about 0.5.

Under the described conditions, proteins are expected to be involved in the diazo-coupling with 4-ATP-Np mainly by means of their exposed histidyl and tyrosyl groups [21,37] (see Fig. 1A).

Capture substrate

Glass substrates were cleaned by sonication in acetone for 2 min and subjected to Piranha Etch (H_2O_2:H_2SO_4, 1:3 v/v) which strongly increases the number of silanol groups on the surface. Successively, they were treated with 3-aminopropyltriethoxy-silane (APTES) dissolved in 2-propanol (8% solution) for 4 h to form a self-assembled monolayer [38]. The modified plates were thoroughly rinsed with 2-propanol, baked at 110 °C for 10 min and then reacted with 1% glutaraldehyde solution for 30 min at room temperature [38]. After thoroughly rinsing with milliQ water, the aldehyde-modified surfaces were incubated with Az (1×10^{-5} M) to form a self-assembled protein monolayer through their solvent exposed amino groups [34] (see Fig. 1B).

Selectivity experiments were performed by incubating the functionalized substrate with wild-type and mutant p53 solutions (1 μM, in PBS solution, pH 7.2), human serum, and human serum/p53 mixtures at different p53 concentrations (ranging from 5 to 500 pM).

Each step of the substrate preparation was followed using tapping-mode atomic force microscopy (AFM) in PBS solution (pH 7.2) (see Supplementary Material, paragraph S.2).

Protein sensing experiments

The protein-covered substrates were incubated for 3 h at room temperature with the corresponding protein partner previously conjugated to (4-ATP-Np) in PBS buffer solution (pH 7.2), allowing the Az–p53 interaction. Then the substrates were washed with PBS buffer to remove the unbound p53-(4-ATP-Np).

Raman and SERS spectra were recorded in air at 20 °C by a Labram confocal setup (Jobin-Yvon) equipped with a Peltier-cooled CCD detector and a single-grating spectrograph (1800 grooves/mm) allowing a resolution of 5 cm^{-1} and a 16 bit dynamic range. The microscope objective was a 100× with a numerical aperture of 0.9 producing a laser spot size of about 1 μm in diameter. The source was a He–Ne laser (Melles Griot) providing a 632.8 nm radiation with a power emerging from the objective of 6.5 ± 0.5 mW.

SERS spectra were acquired by scanning different sample areas per time unit, at the same confocal section. This allowed us to increase the irradiated number of SERS "hot spots" and, at the same time, to reduce the bleaching effects on the irradiated sample. The typical Raman acquisition parameter was a 20 s integration time with 5 scan repetitions. Each sample was prepared three times to repeat independent experiments for checking reproducibility.

Results and discussion

Ultrasensitive SERS signaling of Az–p53 recognition

Fig. 2 shows the SERS detection approach used to detect the p53 protein at very low concentrations. In the first step the 4-ATP molecules are linked to gold Nps by means of a covalent S–Au bond, and the resulting 4-ATP-Np system is successively conjugated to p53 proteins via a diazo-coupling reaction to form the p53-(4-ATP-Np) system (Fig. 2A). In the last step the Az-modified substrate (Fig. 2B) is incubated with the p53-(4-ATP-Np) system, thus allowing p53 to be recognized (Fig. 2C).

The stepwise molecular assembly of the p53-(4-ATP-Np) system has been investigated by Raman spectroscopy in order to disentangle the contribution of each component to the total SERS spectrum. The resulting spectra are shown in Fig. 3 and the main vibrational bands together with the literature-based assignments [39,40] are listed in Table 1.

The spectra of 4-ATP linker before (Fig. 3A) and after (Fig. 3B) conjugation with gold Nps exhibit marked differences. The formation of an S–Au covalent bond, indicative of the conjugation of 4-ATP with gold Nps, is witnessed by the disappearance of the band centered at 2550 cm^{-1}, inherent to the stretching vibration of S–H (see the insets of Fig. 3A and B) [21]. Most of the peaks in the 1000–1700 cm^{-1} region of 4-ATP show a strong increasing in intensity on binding to the Nps, together with some changes in the relative intensity; an appreciable red-shift in frequency is also observed. Generally, the huge enhancement of the Raman cross section when the molecules are bound to a metal surface (SERS effect) has been attributed to two main SERS mechanisms: (i) an EM mechanism, associated with a large local field caused by surface plasmon resonance; and (ii) a CT from the metal to the adsorbed molecules [19,28]. For 4-ATP molecules bound to gold Nps, the enhancement of the band at about 1435 cm^{-1} seen in Fig. 3B has been ascribed to the CT mechanism, while the other bands reported in Table 1 for 4-ATP-Np are thought to be selectively enhanced mainly via EM mechanisms [39]. From the signal to noise intensity ratio of the

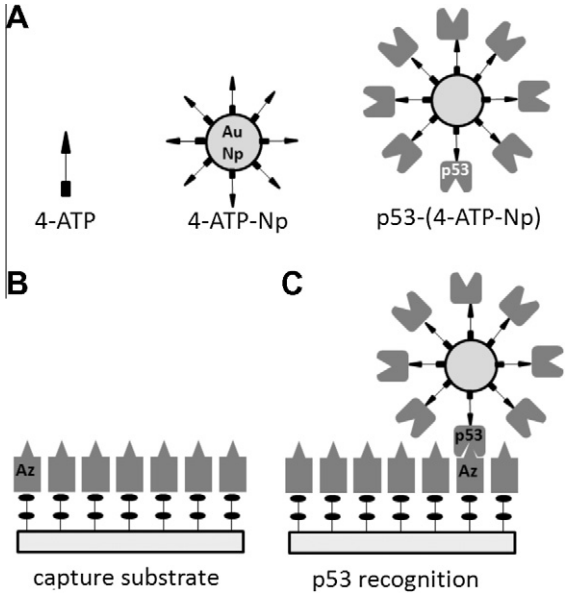

Fig.2. Schematic sketch of bioaffinity-based SERS detection of p53 proteins. Formation of p53-(4-ATP-Np) by linking the thiol group of 4-ATP with gold Np and subsequent conjugation of p53 proteins with 4-ATP-Np via a diazo-coupling reaction (A); formation of the capture substrate, exposing the Az protein partner of p53 (B); deposition of p53-(4-ATP-Np) on the Az-modified platforms to allow Az–p53 recognition (C).

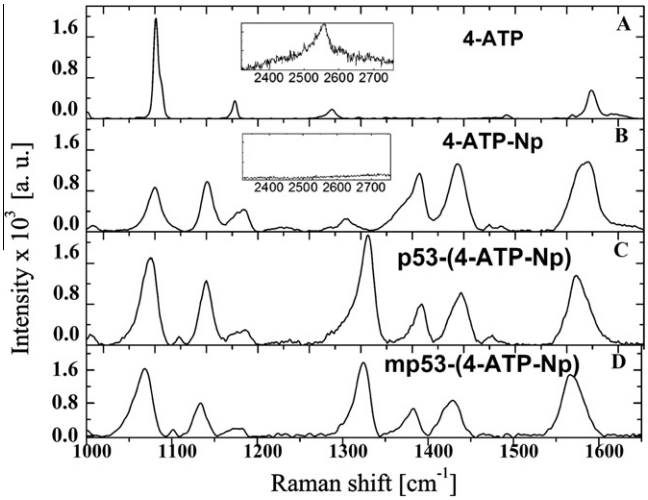

Fig.3. Raman and SERS spectra of water solutions of 4-ATP (A); 4-ATP self-assembled on 50 nm gold Nps (B) wild-type p53 conjugated via diazotization to 4-ATP-functionalized gold Nps (C); mp53 (R249S or C135V mutants) conjugated via diazotization to 4-ATP-functionalized gold Nps (D). The spectral range corresponding to the S–H stretching mode is shown in the insets. The measurements were performed by a 633 nm laser line, obj. 100× and 50× (no smoothed spectra).

Table 1
Selected Raman and SERS bands of 4-ATP, 4-ATP-Np, and p53-4-ATP-Np with their vibrational assignments.

Vibrational assignment[a]	4-ATP Raman (cm^{-1})	4-ATP-Np SERS (cm^{-1})	p53-(4-ATP-Np) SERS (cm^{-1})
SC str + NH_2 rock	1089s[b]	1079s	1076s
CH bend	1176w[b]	1141m[b]	1140w
CN bend	1211vw[b]	1190w	1182w
CH str	1286w	1302w	
NN str			1328s
CC str + CH rock + NH_2 rock		1388m	1390m
CC str + NH_2 rock		1433m	1436m
CC str + CH bend	1492w	1470;1483w	1470;1483w
CC str + NH_2 bend	1593s	1580s	1572s
SH str	2555w		

[a] Refs. [39–40].
[b] Intensity: s (strong); m (medium); w (weak); vw (very weak).

C–S stretching mode (around 1089 and 1078 cm^{-1} for the solid 4-ATP and 4-ATP-Np system, respectively), an enhancement due to the SERS effect of about seven orders of magnitude has been estimated. After the diazotization reaction, which involves the conjugation of p53 through its exposed histidine and/or tyrosine residues, the SERS spectrum of the whole p53-(4-ATP-Np) system displays an additional band centered at about 1328 cm^{-1} (Fig. 3C). The new band is assigned to the stretching vibration modes of the diazo bond (–N=N–) which tightly keeps linked the protein to the 4-ATP-Np system [40]. Accordingly, the band at 1328 cm^{-1} together with the other Raman bands characteristic of the 4-ATP-Np system exhibits a great enhancement of the fingerprint Raman signal, with vibrational features well distinguishable and stable in time.

The intensity of this band increases with the p53 concentration used for the diazotization reaction (not shown), indicating that each 4-ATP-Np system is able to conjugate a higher and higher number of p53 molecules when the concentration of the latter is increased. By assuming that p53 can be represented as a sphere with a diameter of about 6 nm [32], about 250 of p53 can be, at maximum, closely packed around each Np whose diameter is about 50 nm.

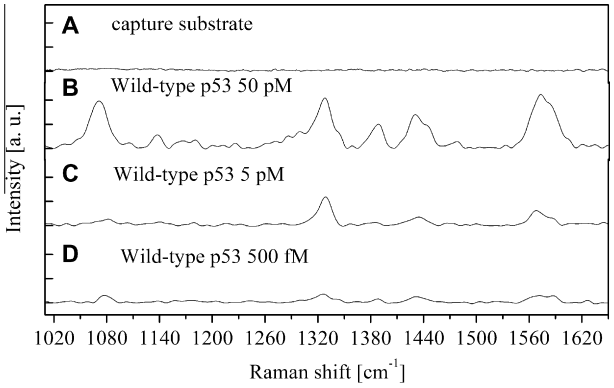

Fig.4. SERS spectra of the Az-coated substrate before (A) and after the incubation with the p53-(4-ATP-Np) system, at different wild-type p53 concentrations, 50 pM (B), 5 pM (C), and 500 fM (D). Spectra were collected by an obj. 100× immersed in PBS.

SERS spectra similar to those recorded for the wild-type p53 protein have been obtained for mp53-(4-ATP-Np) systems, i.e., when R249S or C135V p53 mutant proteins were conjugated to the 4-ATP-Np (compare Fig. 3C and D). As expected, the punctual mutation does not affect the binding to the functionalized gold Nps. Hence, we have exploited the enhanced SERS bands to investigate if wild-type or mutant p53 proteins, previously conjugated with 4-ATP-Np, can be ultrasensitively and easily revealed as specifically captured by Az. More specifically, for the detection of p53 or mp53 proteins we have flowed the p53-(4-ATP-Np) or mp53-(4-ATP-Np) solutions onto the Az-coated platforms. After a short incubation, the system has been rinsed several times to remove the unbound p53-(4-ATP-Np) molecules and then interrogated by SERS spectroscopy.

Fig. 4 shows the SERS spectra of the Az substrate, before (Fig. 4A) and after the treatment with different concentrations of wild-type p53 proteins bound to (4-ATP-Nps) (Fig. 4B–D). A control experiment in which we have dropped 4-ATP-Np molecules (1 pM gold Nps) without p53 on the Az-coated platform resulted in a spectrum similar to that of Fig. 4A, thus revealing no recognition events. The characteristic Raman fingerprint can be instead clearly observed in Fig. 4B–D, confirming the presence of wild-type p53 molecules, linked to 4-ATP-NPs, recognized and captured by Az. Since the signal to noise ratio (S/N) is reduced on decreasing the p53 concentration, below 500 fM (Fig. 4D), S/N becomes too low (<3) and therefore we can assume such a concentration as the detection limit for the p53 protein by our method.

The high sensitivity reached by our biorecognition-assisted SERS method is based on the considerable strength of the specific interaction between Az and the p53 protein. Indeed, in our group, it was found by AFS for the Az–p53 complex an unbinding force of about 75 pN which is close to those measured for antigen–antibody interactions of immunodiagnostic relevance [41]. All these features ensure that an elevated number of p53-(4-ATP-Np) systems can be found onto the Az platform even at picomolar levels of p53.

The capture capability from the Az substrate even of mutated p53 proteins has been checked by taking into consideration two different mutants. First, we have analyzed the single point mutant R249S, which is highly frequent in hepatocellular carcinoma and it is considered one of the six most common missense mutations associated with cancer [4]. The inability of this “hot spot” R249S mutant to bind DNA has been ascribed to substantial structural distortion around the mutation site located in the L3 loop (residues 243–249) of DBD [42]. Since such a DNA-binding site of p53 has been demonstrated to not be involved in the Az binding, this mutation is expected not to interfere with Az interaction [33].

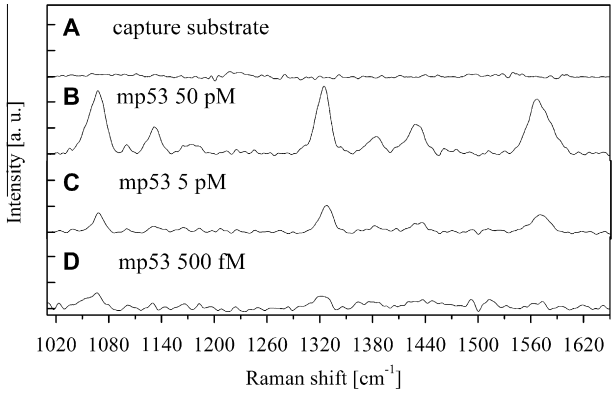

Fig.5. SERS spectra of the Az-coated substrate before (A) and after the incubation with the mp53-(4-ATP-Np) system, at different mp53 concentrations, 50 pM (B), 5 pM (C), and 500 fM (D). Spectra were collected by an obj. 100× immersed in PBS.

Additionally, we have taken into consideration the C135V p53 mutant which is known as a temperature-sensitive mutant [36]. At low temperatures it behaved as the wild-type p53; when the temperature rises to 37 °C, the C135V p53 conformation switches, converting it from wild-type to mutant phenotype. From the above analysis on wild-type p53, we can thereby infer that at temperatures largely below 37 °C also such a mutant would exhibit a remarkable ability to bind Az.

Fig. 5 shows the SERS spectra of the Az substrate, before (Fig. 5A) and after the treatment with different concentrations of R249S p53 proteins bound to (4-ATP-Nps) (Fig. 5B–D), in analogy with what has been done for wild-type p53 (see Fig. 4); similar results having been obtained for the C135V p53 mutant. These results indicate that this methodology is able to easily detect with the same high sensitivity different forms of p53 proteins, whose presence in the human body may be related in early prognosis and invasiveness of different and frequent malignant neoplasms.

Recent works report on novel bioaffinity-based methods, employing consensus DNA sequences and monoclonal antibodies adsorbed on SPR sensor disks, gold electrodes, and metal nanostructures, able to improve the tumor marker detection limits of the ELISA test. These new techniques can reveal the p53 protein in the order of picomolar concentrations [43], witnessing that our detection value is comparable with the best results obtained by these biosensors. Nevertheless, our method has the advantage that low concentrations can be easily detected with a relatively fast assay time, and it does not require multiple steps as the classical "sandwich enzyme immunoassay." Concerning the latter method, we stress that once an antibody has recognized a given antigen, a second antibody labeled with an enzyme is added and thereby is activated to produce an observable signal, like a color change in the medium, which allows the compound identification. The total assay time for the detection of the p53 protein via ELISA is usually about 2 days, while about 6 h are needed when using our Az-assisted SERS procedure.

It is worth also noting that the choice of Az as a biorecognition partner for the detection of p53 provides some advantages connected with its stable structure within a wide range of chemical–physical conditions [34]. In particular, its demonstrated capability to form specific interactions with p53 molecules *in vitro* and *in vivo* [30,31] can open the possibility of extending the present method to detect the p53 protein also in heterogeneous cell extracts.

SERS detection of highly specific Az–p53 recognition in human serum

It is known that an accumulation and stabilization of mp53 proteins occurs in the nuclei of several p53-positive tumor cells (i.e., lung, colon, ovarian, breast, bladder, pancreatic and hepatocellular carcinomas) [1,4]. Overexpression of wild-type p53 has been also reported [3], likely due to p53 nonmutational events, such as specific oncogenic stresses, or Mdm2 gene alterations. Under these critical conditions, concomitant alterations of cell-free protein levels of p53 in blood serum, together with its immune response-mediated antibodies, may be expected as reported in several publications [6–11,44,45]. The concentration of p53 protein in the plasma is found at lower levels than within cell lysates of patients, such an extent (usually from 1 to 100 pM) varying in carcinogenesis of different organs [6–9,44,45], and under particular stress conditions [10,11]. In bladder cancer, the p53 protein level in human serum can have a 5-fold increase, moving from across the three grades of the superficial form [44]. It has been also reported that detection of p53 in human serum can increase the frequency of hepatocellular carcinoma prediction from 79.5% to 86.3%, and a significant positive correlation between p53 and tumor size (cm) for tumor grade II and III was identified [9].

These results suggest that the serum levels of p53, which may reasonably reflect tissue alterations in p53 at the gene and/or protein level, can be used as a convenient and noninvasive tumor screening approach. Such a detection can be also useful for the selection of treatment regimens, in evaluating the response to chemo- and radio-therapies in various malignant diseases and even to relate their insurgence to genotoxic environmental carcinogen exposure. To evaluate the ability of our method in selectively revealing the tumor marker p53 in human serum, we have used an alternative configuration in which Az has been conjugated with 4-ATP-Np by diazotization, and used as the SERS probe to screen the various samples made up of p53/serum mixtures. The linking of Az to the Raman marker, if compared to the previous configuration, results in a more intense SERS signal, due the higher number of Az molecules surrounding each Np (about 980 Az molecules against 250 p53 ones), this providing a more sensitive quantification of the binding events. To investigate the selectivity of this system, first we need to compare the SERS signaling corresponding to its association for p53 molecules alone, with respect to that for the proteins, such as albumin and globulins, constituting a healthy human serum solution. Then, the ability of the Az-assisted SERS probe in detecting p53 proteins when they are present in human serum at concentrations high enough to be considered for the cancer risk assessment should be analyzed [45]. Toward this ends, we have

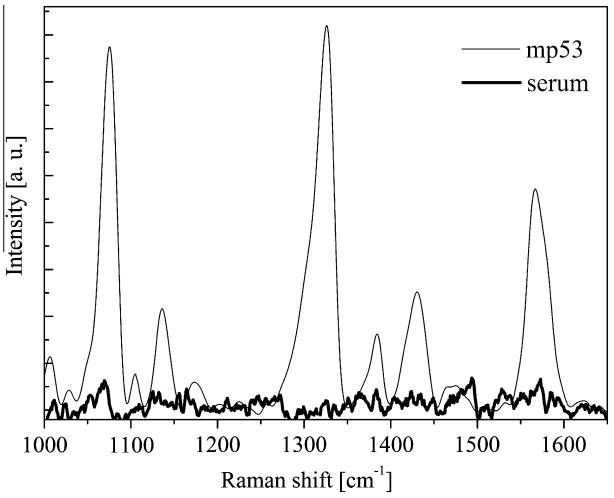

Fig.6. SERS spectra of the capture substrates functionalized with p53 alone (light line) and healthy human serum (heavy line); after incubation with the Az-(4-ATP-Np) system (5 nM Az). SERS spectra were acquired with a laser source at 633 nm in air by a 100× obj.

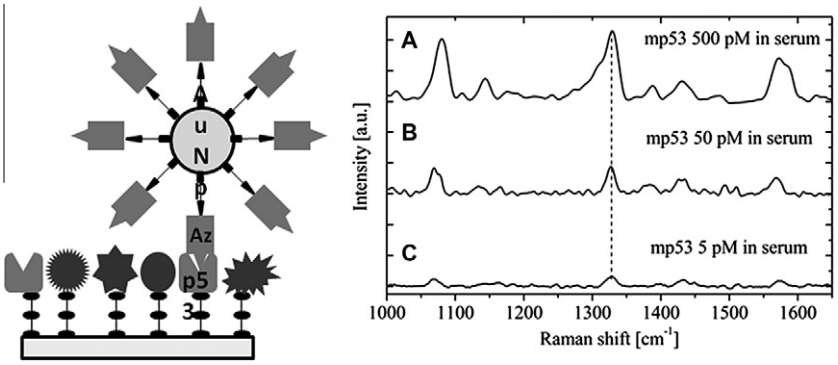

Fig.7. Sketch of a mp53-Az recognition event in the presence of an excess of human serum proteins (left) and SERS spectra (right) of the capture substrates functionalized with serum/R249S mixtures: at mp53 concentration of 500 pM (A), 50 pM (B), 5 pM (C); after incubation with the Az-(4-ATP-Np) system (5 nM Az). SERS spectra were acquired with a laser source at 633 nm in air by a 100× obj.

incubated different solutions of p53 alone (wild-type or mp53), healthy serum, and p53 introduced in human serum at 5, 50, and 500 pM, on glass slides bearing aldehyde moieties on their surface, to allow the self-assembling of the proteins [36]. Successively we have flowed on these substrates the Az-(4-ATP-Np) systems and performed SERS measurements after extensive rinsing to remove molecules probe that had not been captured by the protein-coated substrates. As shown in Fig. 6 on neat R249S p53 samples, we have measured a reproducible strong SERS signal which is representative for both wild-type and mp53 samples examined; on the contrary, for the sample corresponding to pure human serum no significant SERS signal over the noise has been detected (compare light and heavy lines of Fig. 6). Based on the literature, it can be expected that the concentration of wild-type p53 in healthy serum is lower than the detection limit of our SERS approach [11]. Therefore we can reasonably infer that such an absence of the SERS signal for the serum sample may reflect the lack of interaction of its most abundant components for the Az-(4-ATP-Np) probe. In this respect the presence of heterophilic antibodies interference (i.e., cross-linking) in immunometric assays can strongly affect detection accuracy within human serum. This means that our method can be effectively used for specifically recognizing anomalous levels of both wild-type p53 and mp53 proteins within serum-like environments.

As a benchmark to test the effectiveness of our method in identifying p53 in human serum, we have mainly focused our attention on the mp53 R249S, which is a valid bioindicator of rapid aggressive tumor liver progression [45]. Fig. 7 shows the SERS spectra corresponding to the serum coatings, containing mp53 R249S at 5 (panel A), 50 (panel B), and 500 pM (panel C), all of them being treated with our Az-modified Nps. It is worth noting that the intensity of the SERS bands characteristic of the Az-(4-ATP-Np) system undergoes a sensitive increase with the increasing of the R249S p53 concentration (Fig. 7). As previously shown in Fig. 6 (heavy line), these bands are instead completely absent on the substrate containing only healthy human serum. It can thereby be deduced that the fingerprint SERS signal arises only from the Az-(4-ATP-Np) molecules which have formed a complex with p53 immobilized onto the substrate, thus indicating that this method is potentially able to specifically identify low levels of p53 in the presence of human serum molecules.

Conclusions

The SERS-based detection method, exploiting the covalent binding of proteins to 4-ATP functionalized Nps and the strong enhancement of Raman fingerprints, is able to reveal the recognition events between the tumor suppressor p53 protein and the Az, used as receptor. Such an approach has allowed us to detect, with high sensitivity and selectivity, both wild-type and mutant p53 proteins, initially present in a solution at 500 fM. The proposed Az-assisted SERS detection strategy could represent an easy, rapid ultrasensitive way to monitor p53 levels in human serum for a noninvasive and early tumor screening. Moreover, due to the non-specific linking strategy, the presented approach can be easily extended to the detection of other specific markers and implemented into a multiplex assay, thus opening new prospects for high-throughput screening of biomolecules with very high sensitivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.10.010.

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