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Detection of immune reaction by surface-enhanced Raman Spectroscopy: A mini-review

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This paper presents the recent progress in surface-enhanced Raman spectroscopy combined with immunochemical strategy in the detection of disease markers. The design and fabrication of immunoSERS labels are discussed in terms of their bioanalytical applications in *ex vivo* and *in vitro* screening of immune markers. We concisely discuss the sensing capability of immunoSERS for clinical-based translation.

Keywords: Surface-enhanced Raman scattering (SERS), Immunochemistry technologies, Inflammation, cancer

1 Introduction

Immunochemistry is a bunch of rapid, robust, and sensitive methods widely used in clinical and biological laboratories. In principle, it offers the detection of proteins associated with diseases by a highly specific and sensitive reaction between an antigen and an antibody [1,2]. Antigens are exclusively present or overexpressed due to the disease and are recognized by noncovalent interactions with an antibody epitope. In most methods, a primary antibody binding the antigen of interest is labeled, but this direct method of detection is less sensitive than the indirect method. In the latter, the primary antibody is firstly conjugated to the antigen, and then a labeled secondary antibody is applied to recognize the complex. Commercially available antibodies are usually polyclonal due to their relatively simple and cost-effective production compared to monoclonal antibodies. But the latters are more homogenous in terms of specificity and affinity. This approach is widely used in clinics to quantify targeted markers in bodily fluids (immunoassays) and to visualize them in cells (immunocytochemistry) and tissues (immunohistochemistry, immunoblotting). The antigen-antibody conjugate is usually identified and quantified (up to a femtomolar level) in the color reaction, the fluorescence intensity of fluorophores, and chemiluminescence [1,2]. Despite the wide applicability of the immunochemical tests and staining in clinical laboratories, the specificity and accuracy of these techniques suffer from some problems. Non-specific antigen-antibody cross-reactions increase the possibility of false-positive and false-negative results, while autofluorescence, a high background of the signal, and photobleaching of fluorescent dyes limit multiplexing quantitative analysis. However, high analytical performance, portability, fast throughput, and the wide range of detected analytes make immunochemical analysis a powerful tool in biomedical laboratories and as the point-of-care (POC) tests [1,2].

Further development of the immunochemical techniques must include standardization of the sample preparation (particularly for staining of tissue excisions), performance evaluation samples, and the development of novel ultrasensitive, bright and stable labels. Within this context, exploitation of surface-enhanced Raman scattering (SERS) spectroscopy offers several advantages for the improvement of immunochemical analysis. In particular, the synergic combination of Raman signal enhancement in the close

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proximity of the metal surface and the immobilization strategies to capture the target analyte opens a new route for advances in sandwich assays, microfluidic arrays, staining cells, and tissues [3]. Designing SERS nanoprobes is aimed to improve sensitivity, chemico-physical stability, targeting, and multiplexing ability. This review presents the design and structure of SERS immunoprobes and their biomedical applications in analyzing various targets which are important for the recognition of inflammation and cancer diseases.

2 Construction of immunoSERS probes

The immunoSERS probes are a critical key for specific and quantitative detection and should be carefully designed to interact with the target molecule in its environment. In principle, the probe is constituted by the following main parts – a metal substrate of high enhancement factor, a Raman reporter of specific spectral signature, a protection layer/shell for maintaining stability, a linker to facilitate the conjugation of targeting molecules recognizing an analyte, see Fig 1.

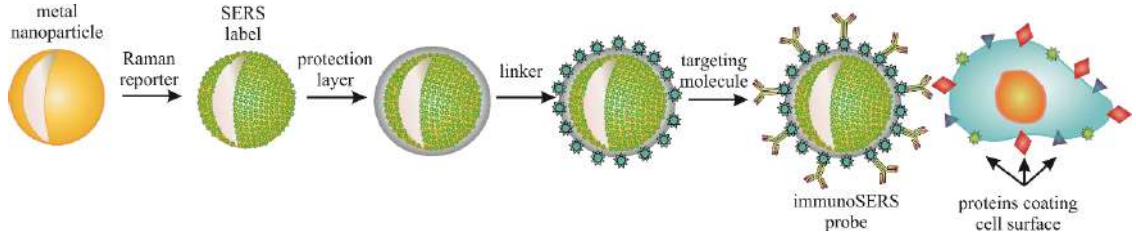

Fig 1. A schematic of the immunoSERS probe and its conjugation to protein markers at the surface of cells

2.1 Plasmonic nanostructures as the core of immunoSERS probe

The selection of the plasmonic nanostructures giving amplification of the Raman signal is crucial for achieving ultrasensitive detection. Gold and silver show the desired localized surface plasmon resonance (LSPR) in the visible and near-infrared region of electromagnetic radiation matching to commonly available laser excitations. Despite the high enhancement factor (EF) of silver nanostructures, gold is mainly used in the biomedical field because of its good stability and biocompatibility [4-6]. The optical properties of metallic nanoparticles (NP) are governed by the position of LSPR and the magnitude of the electromagnetic field generated at the hot spots. Both entities are tuned by controlling geometric parameters such as shape, size, and dimensionality of plasmonic nanostructures [7-10]. Although the simple synthesis of spherical AuNPs, proposed by Turkevich, allows tuning the size of the colloidal particles, the generation of efficient hot spots requires the aggregation of nanospheres [11]. Clusters of dimers and trimers of gold nanospherical particles were encapsulated in silica shells [12] or induced by pre-aggregation of the colloids with the use of inorganic salts [13]. The latter method does not provide unfortunately the reproducibility of the dimeric structures. The sub-nanometer distance between the Au cores results in the EF up 10^{10} [12]. The advantage of built-in hot spots in non-spherical and multi-shaped AuNPS was firstly shown for nanorods. Their synthetic route is more complex than for spherical NPs and uses a seed-mediated protocol with spherical seeds of diameter 2-5 nm added to a solution with surfactant (cetyltrimethylammonium bromide, CTAB), reducing (ascorbic acid) and shape-inducing ($AgNO_3$) agents [14]. Gold nanorods show the transverse and longitudinal plasmon resonance bands which could be manimulated. For example, the increasing ratio of the length and the width of the nanorod shifts the longitudinal band from ca. 600 nm into the NIR. This size of the nanoparticle and LSPR tunability was proven to be useful in several biomedical applications, including photothermal therapy [15]. The engineering of non-spherical plasmonic nanostructures was directed to the fabrication of particles with multiple hot spots with sharp edges like in nanocages, nanostars, nanoflowers, and nanoassemblies [16]. The synthesis of the most widely used gold nanostars with EF of 10^4 mainly relies on seed-mediated growth in the presence of reducing and capping agents which leads to stable nanostructures with extremely sharp tips

[17]. The increase of EF (ca. 10^5) was observed in metal assemblies with well-defined intermetallic junctions [18,19]. Here, the gold sphere and nanorod are surrounded by satellite nanostructures separated by the very thin silica layer. The glass shell is functionalized with silanes enabling adsorption of the negatively charged small gold spheres. Tuning the thickness of the silica layer generates the plasmonic coupling between the core and satellites and between the satellites in the single 3D supraparticles [18]. Unfortunately, these metal superstructures are not widely used due to the complex and challenging protocol of their fabrication, and, as a consequence, the long time of preparation and cost demands.

2.2 Raman tags

Raman tags (reporters) are the signal source in the SERS spectrum and they usually constitute the first coating of the plasmonic core. In principle, their key properties are:

1. the ability to conjugate with the metal surface,
2. high scattering cross-section to record intense Raman spectrum, and
3. characteristic bands to be recognized in multiplex detection,
4. high photostability with low or no fluorescence features.

Among organic compounds, commercially available aromatic dyes (rhodamine 6G, crystal violet, malachite green, nile blue, phthalocyanines, etc.) are employed as the tags. Since they exhibit electronic absorption bands in the visible region of light, it is easy to match their electronic transitions with the commonly used lasers at 532 and 632 nm. This provides additional amplification of the Raman signal through resonant Raman scattering giving rise to SERRS [15]. The careful engineering of the distance between the organic dye and the metal surface should eliminate surface-enhanced fluorescence (SEF) [20]. For multiplexing, several efforts were made to design and synthesize a library of tricarbocyanines and triphenylmethines giving a set of reporters with unique Raman features [21,22].

Other groups of the Raman tags are molecules having the ability to form self-assembly monolayers (SAM) on the Ag and Au surfaces [23]. In particular, small aromatic compounds with the thiolated termini became the dominant Raman reporters in the SERS bioassays. Typical aryl thiols are thiophenol, aminohexanethiol, naphthalene dithiol, mercaptobenzoic acid, 1,4-biphenyldithiol, and many others [8,24]. Due to Au-S and Ag-S covalent bonding, these compounds cover the metal surface with a uniform orientation within the SAM and protect against co-adsorption of unwanted molecules. In that way, a non-fluorescent and clean SERS readout is achieved. Manipulating with the substituents in the aromatic ring gives distinct and non-overlapped Raman bands enabling the straightforward multiplex analysis.

Quantification of the SERS signal response relies on the determination of integral intensity since it is proportional to the concentration of the SERS tag [25]. A similar approach is employed in the case of Raman imaging performed for visualization of targeting molecules in stained cells and tissues in a form of distribution maps. A more complex strategy is required in the determination of multiplex signals. Results of Raman imaging can be analyzed by the use of multivariate analysis (e.g. cluster analysis) which separates the signal of the Raman tags from undesired background fluctuation or fluorescence emitted by native biocomponents of the sample [26]. Several encoding protocols were already proposed based on possible combinations of the signal positions characteristic reporters and bands' intensities. The components and structures of different codes are reviewed in [3].

2.3 Protection layers and conjugation with antibodies

Stabilization of the SERS nanoprobes is generally recommended to prevent their aggregation and to extend the shelf-life. In addition, the protection layer minimizes the dissociation of molecules of the Raman reporters and so it eliminates spectral interferences from the environment. This can also serve as the surface for anchoring recognizing molecules, improving water solubility and biocompatibility. There are

three approaches to accomplish these requirements. Firstly, the SAM of the Raman tags itself can maintain the stability of the SERS immunoprobe, and their terminal COOH and NH groups are employed in further bioconjugation. The most widely used encapsulation methods include polymers (thiolated PEG, MEG, and TEG, proteins) and silica shells [27-31]. The popular heterobifunctional polyethylene glycol (PEG with -SH, -COOH, or -NH_2) coats the metal surface with the Raman reporter protecting the probe against aggregation enabling further functionalization. Water-soluble SERS probes are obtained by using MEG/TEG mixture. Hydrophilic mono-ethylene glycol (MEG) acts as the hydrophilic spacer while tri-ethylene glycol (TEG) with the terminal carboxylic group is employed for bioconjugation and increases sterical accessibility for detection antibodies. In turn, proteins like bovine serum albumin (BSA) easily link with the SERS probe upon physical interactions and improve biocompatibility. However, weak BSA – nanotag interaction can lead to particles aggregation. Encapsulating the Raman reporter and metal nanoparticle in silica shell led to a technique named SHINERS (shell-isolated nanoparticle-enhanced Raman spectroscopy). SiO_2 is biocompatible, and, therefore, is ideal in biomedical applications. Silica shells are easily grown with variable thickness according to modifications of the Stöber method [32]. Changes in the concentration of tetraethyl orthosilicate (TEOS) affect the thickness of the layer and this must be controlled to save the brightness of the SERS probe.

The next step in the fabrication of the SERS immunoprobe is the binding of the targeting moiety – the detection antibody. The quality of the antibodies, including the purity and affinity, significantly affects the targeting performance. Very often, a few commercially available antibodies must be tested to check their detection efficiency after conjugation with the SERS tag. The binding method should also preserve protein conformation and orientation of the fragment crystallizable (Fc) domain of the antibody to support the antigen recognition sites. The simplest method is the direct adsorption of proteins due to electrostatic interactions with the oppositely charged plasmonic nanostructures. But this procedure leads to the non-specific binding with other biomolecules in the sample, including cross-reactions between different SERS immunoprobes in multiplexing [24]. Thus the covalent binding is required and this is achieved by the amide bond formation, binding biotin-modified tags to avidin or streptavidin, and silane chemistry [6]. The amide formation is possible when the polymer brush from the protection layer possesses the terminal groups (-COOH, -NH_2) enabling their activation by N-hydroxysucinimide (NHS) and the resulting active NHS esters are then conjugated to primary amines such as lysine residues in proteins [26]. SHINERS probes were successfully modified with the coupling agents with succinimide, isothiocyanate, and hydrazine (with -NH_2), carbodiimide (with COOH), or azide (click chemistry) [33].

3 *in vitro* immunoSERS staining

Fluorescence microscopy and flow cytometry are widely used in the detection of specific or overexpressed proteins present at the cell membrane and are employed in immunology, molecular biology, bacteriology, cancer biology, and infectious disease monitoring [2,34]. Both techniques, according to standardized protocols, can investigate hundreds and thousands of cells. Advances in instrumentation, in particular, multiple laser systems, detectors, and new fluorochromes increase sensitivity and provide the identification of up to 20 parameters. Despite these advantages, the separation of the signals is still complex and subjective and requires sophisticated software for color analysis [2,34]. The combination of Raman microscopy and the SERS immunoprobes brings enormous brightness of the signal readout and the objective multiple target analysis. Still many aspects need to be examined before translation immunoSERS microscopy into biomedical laboratories. The most important issue is the affinity and avidity of antibodies after their conjugation to the immunoSERS tag.

Choo and co-workers showed a proof-of-principle of the dual targeting CD24 and CD44 proteins in breast cancer cells [35]. Phenotyping CD24 and CD44 cancer cells is the indicator of the cancer stem

cells dictating invasion and metastasis of the primary tumor. Au–core/SiO_2–shell functionalized with malachite green isothiocyanate, tris(2,20–bipyridyl)ruthenium(II) chloride hexahydrate as well as with anti–CD24 and anti–CD44 antibodies. In addition, the silica shell was covered with fluorescent dyes (fluorescein isothiocyanate and ruthenium red isothiocyanate) for multimodal imaging of the cells. Co-localisation of CD24 and CD44 was determined by the quantification of SERS band intensities characteristic for the Raman reporters and was validated by SERS spectra of different ratios of the tags. In the next experiment, the group of Choo phenotyping of breast cancer cell lines with the use of the same SERS labels determining the expression of epidermal growth factor (EGF), tyrosine-protein kinase ErbB2, and insulin-like growth factor–1 (IGF–1) receptors [36]. Further improvement of SERS tag sensitivity was achieved by coating the Au nanospheres functionalized with R6G with a silver layer decorated with PEG [37], Au@Ag nanorods [38], and core-satellites structured gold-silver nanocomposites [39]. These nanotags were conjugated with antibodies detecting cancer PLCγ1, MICA, and EGFR receptors, respectively.

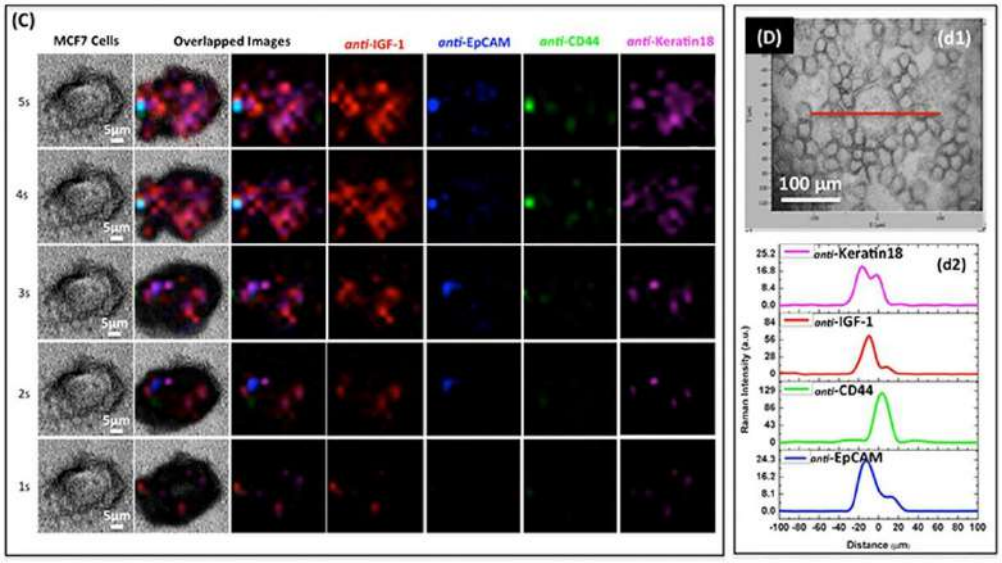

Fig 2. Transmission images and multicolor SERS maps of MCF-7 cells showed the co-localization of four cancer markers. In (C), SERS maps indicated the acquisition time of 3s required to detect the targeted proteins. (D) SERS line scan across the single MCF-7 cell among blood cells (d1) with the spectral profile of employed SERS nanoprobes (d2). The figure was adapted from [41] under the terms of the Creative Commons Attribution License

In another work, the surface of gold nanoparticles was covered with the SAM of azide derivatives conjugated with folate cyclooctynes in the copper-free click chemistry [40]. These SERS nanotags were then employed for bioimaging of the folate receptor (FR) in typical cancer cells. SERS images indicated FR-positive and FR-negative cells showing also a different level of the FR expression. The detection of circulating tumor cells (CTC) with the 4-color immunoSERS imaging was offered by Nima and co-workers [41]. The SERS nanotags consisted of silver-decorated Au nanorods, four Raman reporters (4-mercaptobenzoic acid, *p*-aminothiophenol, *p*-nitrothiophenol, and 4-(methylsulfanyl)thiophenol), and four antibodies (anti-EpCAM, anti-IGF-1 Receptor β, anti-CD44, and anti-Keratin18) and were stabilized by PEG. Characteristic SERS signals were observed only in images of MCF cells spiked into ca. 7×10^6 WBCs and whole blood after the 30 min incubation with the tags. This work showed the high specificity of the constructed probes in multiple targeting and confirmed the affinity of the modified antibodies to the surface cell receptors. Similarly, the successful detection of CTCs from peripheral blood of patients with squamous cell carcinoma of the head and neck was reported by Wand *et al* [42]. Here, epidermal growth factor peptide and PEG were

anchored on the surface of spherical AuNPs. This SERS probe identified 1-720 CTCs in a milliliter of whole blood. Since the molecular phenotype of CTCs changes dynamically with the progression of the cancer disease and upon treatment, this evolution should be monitored to guide clinical therapies. A potential of SERS immunochemistry to accomplish this task was recently reported by Tsao *et al* [43]. Four melanoma CTC surface markers, including melanoma-chondroitin sulfate proteoglycan and melanoma cell adhesion molecule expressed in over 70% of the melanoma lesions, erythroblastic leukemia viral oncogene homolog 3 involved in therapy resistance, and low-affinity nerve growth factor receptor - a stem-cell biomarker, were targeted. The detection of these proteins were achieved by using simple spherical AuNPs decorated with four Raman reporters (4-mercaptobenzoic acid, 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid, 4-mercapto-3-nitro benzoic acid, and 4-mercaptopyridine) and the corresponding antibodies. Detection specificity and sensitivity were validated on multiple cell lines and stage-IV melanoma patients. Data analysis was carried out by plotting the frequency vs. Raman intensity distribution of each SERS marker and showed signal distribution within cell populations. In the case of the successful treatment, this SERS distribution of cell phenotypes was narrowed in contrast to treatment resistance. This approach was able to detect 10 tumor cells in 10 mL of blood. Bifunctional (magnetic and SERS) nanoparticles and core-shell AuNPs with an interior Raman tag-encoded nanogap were also efficient in the detection of CTCs [44,45].

4 immunoSERS histology

Immunohistochemical (IHC) staining with the SERS nanoprobes was firstly reported by the Schlücker group [46]. Biopsied prostate tissue excision was stained with Au nanoshells conjugated with 5,50-dithiobis(succinimidyl-2-nitrobenzoate) and the corresponding antibody for prostate-specific antigen (PSA). A distribution map constructed for the NO_2 band of the Raman reporter showed the presence of prostate cancer cells in the epithelium and this result was in concordance with high expression PSA in these cells of the prostate gland. A similar result was obtained for Au nanoshells stabilized with MEG and TEG to cover completely the metal surface with the hydrophilic SAM enabling bioconjugation. In that way, the SAM layer increases the sterical accessibility of the terminal COOH group to the anti-PSA antibody [27]. Au nanostars stabilized by captured ethylene glycol-modified Raman reporter molecules also visualized the tumor suppressor p63 in prostate biopsies [47]. Next Sun and co-workers synthesized composite organic-inorganic nanoparticles by coating Ag nanospheres (COINs) with BSA, two Raman reporters (basic fushin and rhodamine 6G), and anti-PSA and anti-CK18 antibodies [48]. The characteristic Raman bands of the reporters were appropriately found in the epithelium of prostate tissue showing the duplex immunoSERS staining for the first time. Next, the COINS were used in triplex immunoSERS probing with the simultaneous fluorescence detection of nuclei in the tissue [49]. The further comparison of the COIN-antibody conjugate and the well-established reference IHC method with fluorescent Alexa Fluor in PSA-antigen imaging showed comparable brightness of both tags but the immunoSERS nanoprobe exhibited increased spot-to-spot variability of its signal. Very likely this effect was caused by the aggregation of the COIN-Ab complex or by poor conjugation of the COIN particle with the antibody [50]. Wand et al reported the immunoSERS microscopy based on Au nanostars for the detection of human epidermal growth factor receptor 2 (HER2) in breast tissue [51]. High plasmonic activity on sharp edges of the nanostars gave a very intense signal of a 4-nitrothiobenzoate derivative and localization of the antigen was confirmed by chromogenic IHC. A similar Raman reporter molecule was linked to gold nanoparticles with a 2-5 nm size and conjugated with skin carcinoma cell reporter BerEP4 [51]. The reliability and feasibility of immunoSERS staining for clinical diagnosis were shown by Chen and co-workers. They fabricated immunoSERS nanoprobes composed of Au/Ag-core shells, 4-mercaptobenzoic acid as the reporter, and the antigen for targeting latent membrane protein 1 (LMP1) associated with nasopharyngeal carcinoma [52]. 34 patients with carcinoma and 20 healthy subjects were analyzed simultaneously with the SERS probe, conventional immunohistochemistry, and in

situ hybridization (ISH). Based on the number of the true-positive cases, immunoSERS staining results were consistent with extremely sensitive ISH contrary to IHC. The authors pointed out that the critical step ensuring stability, activity, and immune specificity of the SERS probes is the optimal reporter – SERS probes ratio. In turn, Li and others proposed a universal one-pot preparation of SERS nanotags incorporating Raman reports in the reaction of dopamine polymerization on the surface of Ag nanospherical particles [53]. This method enables easy preparation of a large library of SERS probes (ca. 80) with alkynes (C≡C), nitrile (C≡N), azide (N3), and deuterium (C-D) showing single narrow band in the biological Raman-silent spectral region (1800-2800 cm^{-1}). Four of these tags were conjugated with the corresponding antibodies for multiplexed staining of HER2, ER, PR, and EGFR biomarkers in breast cancer biopsies and their affinity to the antigen was confirmed by traditional IHC. It should be stressed here that IHC accessed the presence of one marker at a time, whereas immunoSERS microscopy identified all proteins simultaneously shortening the time of analysis.

At this point, the reader will be introduced to the complex tissue preparation in immunohistochemical staining, since these steps significantly affect the affinity of the detecting antibody to the antigen [54]. In clinics, biopsy samples are firstly fixed in formalin and embedded in paraffin (FFPE, formalin-fixed paraffin-embedded tissue) and then sectioned into thin (4-5 μm) sections. The staining process includes:

1. antigen retrieval to recover the antigens,
2. blocking endogenous enzymes,
3. applying a primary antibody to bind specifically the antigen of interest (labeled in the direct method),
4. applying a labeled secondary antibody to bind the primary antibody in the indirect method,
5. counterstaining to visualize nuclei and overall tissue architecture, and
6. staining of negative control or other reference samples.

Despite the clinical importance of IHC, there is still a minimal number of IHC protocols standardized for use. Each of these key steps requires the control and optimization of the whole procedure to identify the antigen in cells and structures with both low and high expression [54]. In particular, a variety of sources of reagents and a strong impact of the tissue on the antigenicity of the target molecules contribute to a lack of IHC standardization and poor reproducibility. Additional issues in IHC are the quantification of the targeting molecules and multiplexing analysis. IHC staining of more than four antigens is rarely performed due to a small number of readout channels in routine fluorescence microscopes [54]. The limitations in the tissue preparation and staining protocols in IHC affect the development of immunoSERS histological staining. Investigators should not only propose novel SERS nanoprobes suitable for this purpose but first of all, they need to examine the impact of the staining procedure on the detection accuracy.

The published reports so far about the potential of immunoSERS staining showed that each group employed various methods of antigen retrieval and blocking of non-specific interactions but only the direct staining was tested even though it is less sensitive than the indirect technique mostly used in classical IHC due to its higher sensitivity. Each protocol requires the optimization of antibody concentration conjugated to the SERS probe, incubation time, the order of staining in multiplexing. For instance, protease-induced epitope antigen retrieval (PIER) was employed in [49,50] while protease-induced epitope antigen retrieval (HIER) in works [26,27,46,47,52]. All the studies regarded the detection of the PSA antigen in the prostate tissue. To prevent false-positive signals from cross-reactions, PBS/BSA [49,50,51,52], BSA [47,53], and mixture of normal goat serum and non-fat milk in PBS were applied in the staining procedure [26]. Some experiments did not include this step [27,47,52].

A few works investigated the effect of these issues on the efficacy of immunoSERS staining [26,55,56]. Quality of immunoSERS images obtained after HIER and PIER pre-treatment were evaluated in

PSA and HER staining in prostate and breast cancer biopsies, respectively [55]. In addition, Au nanospheres, nanostars, and Au/Ag nanoshells were tested. In all cases, selective adsorption of the nanoprobes onto epithelial cells was observed, but non-specific binding occurred in sections treated according to the HIER unmasking of antigens. So PIER is recommended for SERS-based immunostaining unless PEGylation of nanoparticles is employed. EDTA–Tris buffer was also recommended for dual staining [56]. Molecular recognition of the target antigen is another important process for obtaining high specificity in IHC [54]. Traditional bioconjugation methods do not ensure controlling targeting lysine residues to the Fc part of the receptor in the case of relatively big SERS probes compared to small molecules of chromophores. Therefore, Salehi and co-workers proposed coating of the SERS tag by chimeric protein A/G which possesses multiple bindings to the Fc domain of the antibody [56]. Silica-coated trimers of 60 nm AuNPs with this protein were used in single and duplex immunoSERS imaging for the co-localization of p63 and PSA in the neoplastic prostate. This work concluded that protein A/G coating can prevent non-specific binding when large and heavy SERS probes are used. Another study investigated the efficiency of Au nanostars functionalized with α-mercapto-ω-carboxy PEG and 4-nitrothiobenzoic acid (4-NTB) to label antibodies against α-smooth muscle actin (SMA) in the atherosclerotic aorta according to in/direct procedures established for IHC and coating with protein A/G [26]. Commercially available fluorescent immunodetection of SMA relies on the conjugation of the primary antibody labeled with the cy3 dye (direct method), whereas multiplexing localization of more than two biomarkers of atherosclerosis requires the use of the indirect method [57]. The immunoSERS staining protocols were modified accordingly and showed localization of α-actin positive area in the aortic wall as well as within the atherosclerotic plaque area like in reference fluorescence images [26]. This result indicated that the construction of the SERS nanotags does not affect the staining sensitivity giving flexibility in the future choice of antibodies (Fig 3). Whilst the role of the chimeric protein A/G was minor in that case. In addition, quantification of SMA-related pixels in SERS and fluorescence images led to a very good agreement between the methods showing high robustness of the SERS-based procedure that could overcome issues met in IHC.

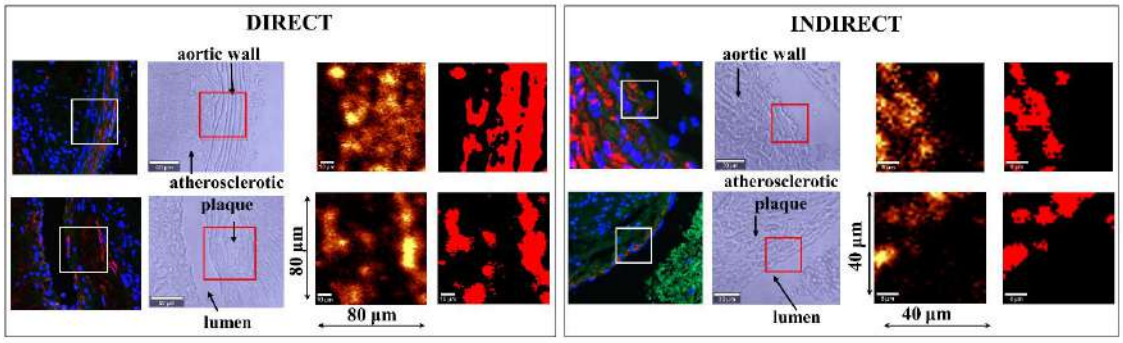

Fig 3. The comparison of IHC and immunoSERS staining of the atherosclerotic plaque in a murine aorta with the use of direct and indirect methods (adapted from Ref. [26])

6 Conclusion and outlook

To summarise, immunoSERS technology offers high sensitivity and multiplexing detection of immune reaction on cellular membranes of single cells and cells in tissues. Since SERS-based immunodetection is applicable for a plethora of samples, its potential application in the biomedical and clinical fields is promising. The main advantage of immunoSERS compared to absorption and fluorescence readout is the photostability of the SERS labels, easier quantification of the signal, and multiplexing capability with only one laser excitation. The development of rapid-scanning, high-resolution, and automated Raman

instruments and their combination with the brightness of the SERS nanotags should overcome the limitations of the currently available Raman microscopes in large-area scanning of tissues and big cell populations. At this point, optical and fluorescence microscopy and cytometry remain unbeaten in terms of devices with high throughput in protein biomarkers detection. Although SERS studies demonstrated in the literature are the proof-of-concept of *in vitro* cell and *ex vivo* tissue imaging, more efforts should be made to show the reproducibility of the offered staining protocols. This must include their universal use, the rational synthesis and functionalization of nanoparticles, and well-established protocols confirmed by inter-laboratory study, reference methods, and appropriate control samples. Moreover, the library of Raman reporters, similarly to chromophores, would provide a flexible choice of molecules in the multiplexing analysis. Despite the obvious fact that immunoSERS methods will not replace traditional methods of the detection of protein biomarkers, they will undoubtedly complement them at the level of the single-cell detection crucial for early disease diagnostics. In addition, the rapidly growing development of multifunctional tags will boost multimodal imaging toward clinical applications.

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