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Paper-based plasmonic substrates as surface-enhanced Raman scattering spectroscopy platforms for cell culture applications

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ABSTRACT

The engineering of advanced materials capable of mimicking the cellular micro-environment while providing cells with physicochemical cues is central for cell culture applications. In this regard, paper meets key requirements in terms of biocompatibility, hydrophilicity, porosity, mechanical strength, ease of physicochemical modifications, cost, and ease of large-scale production, to be used as a scaffold material for biomedical applications. Most notably, paper has demonstrated the potential to become an attractive alternative to conventional biomaterials for creating two-dimensional (2D) and three-dimensional (3D) biomimetic cell culture models that mimic the features of *in vivo* tissue environments for improving our understanding of cell behavior (e.g. growth, cell migration, proliferation, differentiation and tumor metastasis) in their natural state. On the other hand, integration of plasmonic nanomaterials (e.g. gold nanoparticles) within the fibrous structure of paper opens the possibility to generate multifunctional scaffolds equipped with biosensing tools for monitoring different cell cues through physicochemical signals. Among different plasmonic based detection techniques, surface-enhanced Raman scattering (SERS) spectroscopy emerged as a highly specific and sensitive optical tool for its extraordinary sensitivity and the ability for multidimensional and accurate molecular identification. Thus, paper-based plasmonic substrates in combination with SERS optical detection represent a powerful future platform for monitoring cell cues during cell culture processes. To this end, in this review, we will describe the different methods for fabricating hybrid paper-plasmonic nanoparticle substrates and their use in combination with SERS spectroscopy for biosensing and, more specifically, in cell culture applications.

1. Introduction

The development of new materials as scaffolds for mimicking the cellular micro-environment that can provide cells with physicochemical cues is of paramount importance for cell culture applications. This involves, for example, three-dimensional (3D) co-culture systems of several different types of cells such as the one reported by Lehmann et al. that comprise lung epithelial cells, monocyte-derived macrophages, and monocyte-derived dendritic cells to simulate the human epithelial airway barrier of the respiratory system [1]. The use of gold nanoparticles (Au NPs) has caught great interest mainly due to their optical properties, the so-called localized surface plasmon resonance (LSPR). This LSPR effect can be harnessed to provide great enhancement of the Raman signal when using surface-enhanced Raman scattering (SERS) spectroscopy [2].

Paper is a very versatile and common material that finds many uses in consumer-oriented products, coming from a very abundant source (cellulose) in nature, inexpensively produced and recycled. It is also biodegradable, biocompatible and can absorb fluids easily [3,4]. Remarkably, paper has been employed as a cell culture material due to its flexibility, ease of manufacturing, ability to shape into 3D free-standing structures,

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low cost, wide availability and accessibility [5,6]. The integration of Au NPs within the fibrous structure of paper can be used to generate nanosensors for monitoring different cells' cues when combined with a highly specific and sensitive optical tool such as SERS.

The attractive intrinsic properties of paper, such as biocompatibility, hydrophilicity, porosity, mechanical strength provided by the cellulose fibers, ease of chemical and physical modifications, cost efficiency, eco-friendliness, and ease of large scale manufacturing, makes it a suitable substrate for biomedical applications like paper-based biosensors or cell culture platforms [7,8]. Paper-based biosensors avoid the need for equipped centers/facilities, being an excellent example of affordable, sensitive, specific, user-friendly, rapid, and robust devices developed with the synergy between nanotechnology and biosensing technology [9]. When combined with SERS spectroscopy, paper-based plasmonic substrates can be very useful since the liquid samples can be quickly loaded into the paper-based plasmonic substrate by capillary forces (wicking), simply by dipping the paper into the sample [10]. Thus, analytes loaded into the paper device can be concentrated into a small SERS sensing region by lateral flow paper fluidics. Moreover, the intrinsic 3D structure of the fibers composing the paper may, in principle, mimic the native cell micro-environment, and its architecture, which can create physiologically relevant fluid flow. Furthermore, it can also allow controlling the presence and concentration of oxygen and nutrients gradients. *In vitro* 3D cultures are valuable models for *in vivo* tissues since enable to study cells in different locations. In fact, cells display different physiology depending on their location in the 3D tissues since they are exposed to different environments (e.g. oxygen concentration, nutrients, signaling molecules, temperature, mechanical stress, etc.) [6]. Paper inherent ability to absorb fluids through capillary action, attributed to its porous structure and large void volume ratio, makes possible the cell migration inside this type of scaffolds [11] while enabling the isolation and study of cells cultured on the different stacked layers forming the 3D culture [6].

There are various types of paper with different physical and chemical properties that are suitable to study cell behavior such as growth, cell migration, proliferation, differentiation and tumor metastasis. To mimic the features of *in vivo* environments of tissues such as porosity, stiffness, and flexibility, it is essential to work with physiologically relevant 3D models [5]. Paper has the potential to become an attractive alternative to conventional biomaterials for cell culture in both 2D and 3D, for creating biomimetic cell culture environments.

Paper offers mechanical integrity to soft hydrogels that are laden with cells, thus offering structural robustness and support to the scaffolds. The use of paper in combination with hydrogels renders practical handling of hydrogel constructs. Cell-laden hydrogels in paper sheets can be easily stacked to form flexible and robust 3D scaffolds [5,12]. To mimic tumor micro-environments, layer-by-layer paper stacking approaches have been developed by combining paper with cell-laden hydrogels [12]. Remarkably, the highly porous nature of paper assists in the transfer of oxygen, nutrients and signaling molecules to and from cells upon their culture [5,11]. The mass transport of oxygen and nutrients is particularly important because it limits cell proliferation in 3D cultures [12].

The scaffold materials, in addition to providing physical support, can closely interact with the cells to trigger essential physiological processes [13]. Then, Au NPs represent multimodal tools, not only enhancing scaffold properties but also acting as nanosensors [14,15]. Noble-metal NPs, especially Au and Ag, have raised great attention due to their fascinating optical properties [16,17]. They are originated when a beam of light hits the metal NPs, causing that the metal NPs' free electrons to be driven by the alternating electric field, collectively oscillating with the incident resonant light, known as the LSPRs [18]. The LSPR properties of metal NPs are determined by several factors such as the size and shape of the plasmonic NPs [19,20], and the dielectric properties of the surrounding medium [16]. Moreover, nowadays the plasmonic NPs can be prepared with a very narrow distribution of sizes and morphologies by easily implementable techniques based on colloidal chemistry methods [18,19,21,22]. The excitation of the LSPR can result in the intense absorption of light of a specific wavelength and the enhancement of the local electromagnetic field around the plasmonic NPs. This phenomenon is very useful for sensing techniques such as Raman spectroscopy by increasing the corresponding signal by many orders of magnitude, becoming thereby the highly specific and sensitive SERS technique [2,15,23,24]. Therefore, the integration of plasmonic NPs within the fibrous structure of paper not only can alter the cell-material interface and its affinity, but it can play the role of nanosensors for monitoring different cells' cues through physicochemical signals when combined with the highly specific and sensitive optical tool of SERS spectroscopy, enabling an extraordinary limit of detection and accurate identification of analyte molecules [25,26].

Herein, we review recent results around paper as a substrate when combined with the presence of plasmonic NPs along with its fibrous structure, becoming a paper-based plasmonic substrate. This novel material can be applied as an innovative platform for cell culture and biosensors. We start with a brief reminder of the pioneering works impregnating plasmonic NPs in paper for SERS purposes. In the following, we describe several reports on the *in-situ* synthesis of plasmonic NPs within the paper substrate. Then, we address several methods for the impregnation of plasmonic NPs previously synthesized by colloidal means, to follow with some representative biomolecular detection applications. Afterward, the SERS signal as a tool is explored in cell culture applications. This is continued with a description of the usage of paper-based substrates as scaffolds for cell culture. We end with some conclusions and a future outlook of the paper-based plasmonic substrates as SERS platforms for cell culture applications. Thus, paper-based plasmonic substrates when complemented with SERS spectroscopy represent a powerful future platform for monitoring cell's cues during cell culture processes.

2. Pioneering works on paper-based plasmonic substrates

More than three decades ago a couple of pioneering works appeared reporting the usage of paper-based substrates combined with plasmonic properties for SERS detection of analyte molecules. In 1984, Tran reported impregnating Ag NPs from colloidal hydrosols into filter paper as support to harness the enhanced Raman scattering signal of dyes molecules adsorbed on them [27]. He used colloidal dispersions with an absorption peak at 400 nm wavelength from Ag NPs within 10–30 nm size, which were applied onto the filter paper by a microsyringe [27]. Conversely, Vo-Dinh et al. used a different strategy to harness the flexible and wicking properties of paper-based substrates for the detection of organic compounds. They first deposited a uniform layer of Teflon or polystyrene latex spheres on the surface of the filter paper and, subsequently, coat the sphere-covered paper surface with silver by thermal evaporation to promote *'hot-spots'* generation [28]. Both works were able to detect sub-nanogram amounts either of dye molecules [27] or non-resonant organic compounds [28] as analytes.

Years later, Berthod et al. were the first to synthesize plasmonic NPs directly on paper-based substrates for SERS detection applications. They immersed the filter paper (Whatman No.1) in a silver nitrate solution and allowed it to drain for a few seconds; then, the wet filter paper containing Ag ions was sprayed with sodium borohydride solution, turning the white paper into black color. They obtained high polydispersity of Ag NP aggregates along the cellulose fibers, as can be seen in Fig. 1a–b. They outstand as the filter paper advantages, the fact that it was possible to obtain SERS spectra from compounds of poor water solubility using very small volumes of analyte solution, showing the detection of several compounds of biomedical interest as model analytes [29]. More than a decade afterward, Fang et al. reported on dried filter paper impregnated with silver [30] or gold NPs [31] as a substrate for SERS analysis. They used either aqueous solutions of Ag NPs or Au NPs to be added dropwise onto two layers of filter papers; then, the papers were dried and the process could be repeated to modify the amount of plasmonic NPs

impregnated into the paper-based substrate. They analyzed the intensity of the SERS spectra bands as a function of the amount of plasmonic NPs deposited when applying different numbers of depositing steps of the aqueous colloid dropwise. Fig. 1c shows the achieved distribution of Au NPs onto the filter paper [31]. These were the first efforts to obtain SERS active flexible substrates with paper, showing that it was possible to obtain SERS spectra of poorly water-soluble compounds even from very small volumes of analyte solution.

3. *In-situ* synthesis of plasmonic NPs into paper-based substrates

The advantages of paper as a substrate in terms of hydrophilicity, porosity, flexibility, ease of manufacturing, low cost, availability, and accessibility worldwide, have pointed out paper as a very promising material to confer it plasmonic properties to be harness as a substrate for SERS detection applications. The *in-situ* synthesis of plasmonic NPs into paper-based substrates has been explored by several groups [32–37].

He et al. synthesized noble metal NPs, under ambient conditions in porous cellulose fibers, using the paper-based scaffold as a unique nanoreactor and NP stabilizer. They were able to obtain NPs of less than 10 nm in diameter with narrow size distribution when the concentration of the metal precursor was optimized. The porosity of the paper material is essential for the incorporation of metal ions and reductants into the cellulose fibers as well as for the removal of unnecessary byproducts. The high oxygen (ether and hydroxyl) density of the cellulose fibers constitutes an effective nanoreactor for *in-situ* synthesis of metal NPs, not only anchoring metal ions tightly but also stabilize metal NPs by strong bonding interaction with their surface atoms [32]. The color of the cellulose-based paper changed from blank (Fig. 2Aa) to either yellow (Ag NPs) (Fig. 2Ab) or pink (Au NPs) as shown in Fig. 2Ac, and the tonality of the color could be modified when using different concentrations of the metallic precursor solution [33]. Using a different approach, Tankhiwale et al. proposed graft copolymerization of acrylamide onto cellulose-based filter paper followed by the synthesis and entrapment of Ag NPs on the filter paper. The Ag NPs were synthesized and loaded into the grafted filter paper by equilibration in $AgNO_3$ solution followed by citrate reduction. The cross-linked 3D networks serve as stabilizers for Ag NPs and prevent them from aggregation [33]. The obtained nano silver-loaded filter paper was analyzed for its antimicrobial properties against *Escherichia coli* [33]. Cheng et al. were able to obtain highly sensitive SERS substrates based on Ag NPs on commercially available filter paper. They used commercial filter paper as substrates with a silver mirror reaction. The Ag NPs were synthesized on the paper substrates by immersing the filter paper into a plating solution (Tollen's reagent), which contained $AgNO_3$, ammonia and glucose (as reducing agent) [34]. However, the results indicated that Ag NPs oxidize rapidly, registering a two-fold decrease in sensitivity when compared to freshly prepared substrates [34]. They were able to achieve detection limits within hundreds of nanomolar. Interestingly, they remark how the soaking properties of the paper-based plasmonic substrates can be harnessed for detection in a very simple fashion without sacrificing the analytical signal [34]. Gottesman et al. [35] proposed a different method for the synthesis and deposition of Ag NPs on the surface of the paper by using an ultrasonication process. Their approach involved a polyol reduction method with the reduction of soluble silver species by ethylene glycol. The paper was immersed into the solution which was irradiated with 30 or 60 min of high-intensity ultrasonication with a sonication horn. They optimized the precursor concentration and ultrasonication time as experimental parameters. Fig. 2Ba shows a photograph with the appearance of the Ag NPs coated papers, together with SEM images showing the Ag NPs distribution on the paper surface when using different experimental conditions (Fig. 2Bb–d). It should be noted that the NPs' sizes correspond to the Ag NPs synthesized on the surface of the paper only. The Ag NPs coated paper was explored for efficient antibacterial activity against Gram-positive and Gram-negative bacteria [35].

More recently, Kim et al. and their research group have reported a fabrication method to obtain paper-based SERS substrates using *in-situ* synthesis of Ag [36] or Au NPs [37] into the paper substrate by the successive ionic layer absorption and reaction (SILAR) method when floating the paper on aqueous solutions, as depicted in Fig. 3Aa. The roughness and porous properties of the paper allows them to directly synthesize Ag NPs within the paper without external processing, due to capillary effects [36]. The capillary effects allow the wicking properties of the paper to lead the chemicals into the interior of the fibrous matrix. This can be observed in Fig. 3Ab with Ag NPs anchored on the surface layer of the paper, or within the middle (in-depth) of the paper possessing a homogenous distribution of the Ag NPs as well (Fig. 3Ac). The Ag NPs' size can be controlled by the concentration of the reactive solutions and the number of SILAR cycles. The plasmonic properties of the SERS substrates obtained allowed an enhancement factor of $1.1{\cdot}10^9$ when detecting rhodamine B as probe analyte [36]. Subsequently, the same research group reported the synthesis and simultaneous anchoring of Au NPs into paper strips capable of label-free sensing of biofluids for the early detection of infectious eye diseases by SERS [37]. The paper strips were previously printed with a wax-containing toner to delimit hydrophilic circular spots with hydrophobic zones, as illustrated in the photograph shown in Fig. 3Ba. The Au NPs' synthesis was achieved within the permeable porous zones through the SILAR technique: as schematically illustrated in Fig. 3Bb The sizes of the obtained anchored Au NPs can be controlled by multiple repetitions of the SILAR cycle. They were able to register a SERS enhancement factor of $7.8{\cdot}10^8$ when the experimental parameters (chemical reaction concentrations and number of SILAR cycles) were optimized. Representative SEM images (Fig. 3Bc) and SERS spectra (Fig. 3Bd) are shown, comparing the fibrous morphology of the bare paper and the homogenous distribution of the Au NPs along the paper with the optimized experimental parameters.

This *in-situ* synthesis of the Au or Ag NPs was shown to be effective and a considerable SERS enhancement factor was achieved. However, the homogeneity of the NPs coverage and size distribution on the cellulose fibers from the surface was not the same as on the in-depth cellulose fibers. However, a disadvantage of this approach is the lack of control on the morphology of the NPs for tuning the final optical properties of the paper-based substrate. Moreover, when comparing SERS enhancement

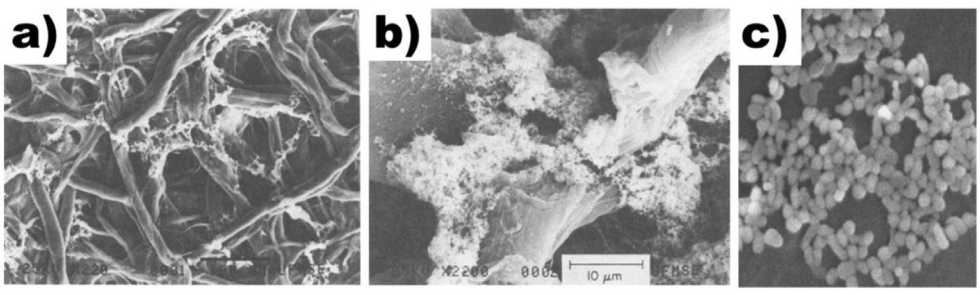

Fig. 1. Pioneering results showing the impregnation of plasmonic NPs in paper-based substrates. a) Scanning electron microscopy (SEM) image showing a filter paper coated by Ag NPs (magnification 220×); b) SEM image displaying the porous structure of cellulose fibers with Ag NPs aggregates. a) and b) reproduced with permission from Ref. [29]. Copyright 1988, Elsevier. c) SEM image with the distribution of Au NPs on filter paper (magnification 30,000×). c) is reproduced with permission from Ref. [31]. Copyright 2005, Elsevier.

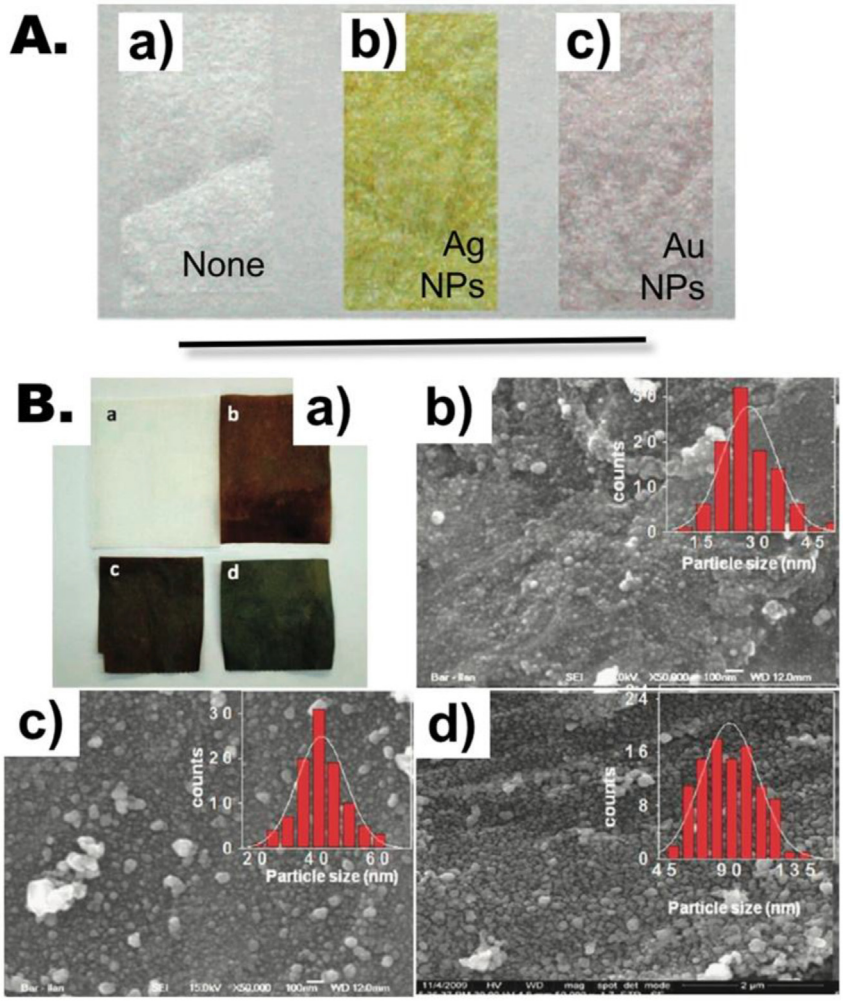

Fig. 2. *In-situ* synthesis of plasmonic NPs on paper-based substrates. A) A photograph of cellulose-based paper substrates with a) none; b) Ag NPs; and c) Au NPs growth in a paper-based scaffold. A) reproduced with permission from Ref. [32]. Copyright 2003, American Chemical Society. B) Shows the differences in paper coating by Ag NPs synthesized by a Polyol Reduction Method with the aid of sonochemistry: a) shows a photograph with the appearance of the coated paper under different precursor ($AgNO_3$) concentration and/or ultrasonication time; b) displays a SEM image and Ag NPs size distribution on the coated paper for 25 mM of $AgNO_3$ and 30 min sonication time conditions; c) 25 mM of $AgNO_3$ and 60 min sonication time conditions; and d) 100 mM of $AgNO_3$ and 30 min sonication time conditions. B) is reproduced with permission from Ref. [35]. Copyright 2011, American Chemical Society.

factors, Ag NPs showed higher values than Au NPs under similar methodologies; although the Ag NPs oxidize rapidly registering a decrease in sensitivity, therefore requiring freshly prepared substrates for analysis. It should be remarked the soaking properties of the paper-based SERS substrates once dried with the anchored NPs since this allows different liquid solutions to be analyzed by this type of flexible substrates.

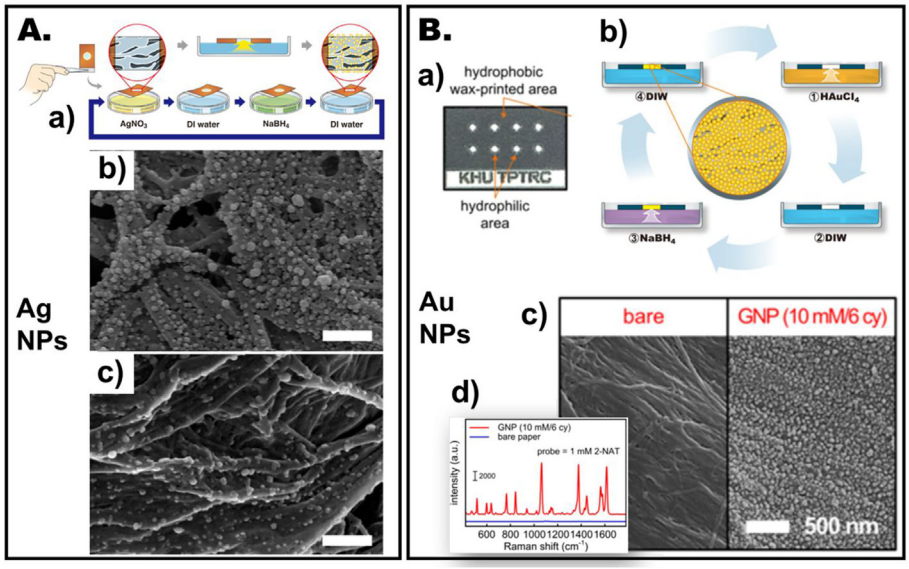

Fig. 3. Synthesis of plasmonic NPs directly into paper using the SILAR [36,37] method for SERS detection applications. A) The Ag NPs' synthesis directly into paper: a) schematically describes the SILAR approach [36]; while b) has a SEM image showing the Ag NPs' distribution along the cellulose fibers at the surface of the paper (Scale bar = 250 nm); and c) shows a representative SEM image within the middle (in-depth) of the paper (Scale bar = 250 nm). A) reproduced with permission from Ref. [36]. Copyright 2015, American Chemical Society. B) The Au NPs' synthesis directly in paper: a) shows a photograph of the paper to be impregnated with the Au NPs by their synthesis with hydrophobic and hydrophilic zones; b) contains the schematic description of the SILAR approach; c) display SEM images of the bare paper before applying the SILAR approach and after the Au NPs were synthesized in the fibrous paper; d) it has SERS spectra from the bare paper and the paper containing Au NPs. B) reproduced with permission from Ref. [37]. Copyright 2016, American Chemical Society.

4. Paper-based plasmonic substrates by impregnation with colloidal nanoparticles

A different approach could be the direct impregnation of colloidal nanoparticles (NPs), once previously synthesized, directly into the paper. This type of techniques allows harnessing the well-developed research line of the colloidal synthesis of plasmonic NPs with a wide variety of morphologies and size control [18,38], making use of the hydrophilic and absorbing properties of the paper. This strategy is robust enough to confer and allow for tuning the final plasmonic properties that the paper-based substrate could acquire.

There are four main techniques related to this type of impregnation strategy: inkjet printing [39,40], screen printing [41,42], direct calligraphy [43,44], and paper dipping into the colloidal NPs dispersion [45–55]. The following lines will describe some of the most representative published results for obtaining paper-based substrates with tunable plasmonic properties.

4.1. Inkjet printing

Inkjet-printed SERS spectroscopy arrays on cellulose paper were reported by Yu and White [39]. They developed a novel, ultralow-cost SERS substrate when modifying the surface chemistry of cellulose paper and patterning silver nanoparticle (Ag NP) arrays with a commercial inkjet printer. Prior to the printing of Ag NPs onto paper, the substrate was made hydrophobic by inkjet printing hexadecenyl succinic anhydride (ASA), a common paper-sizing agent, onto the sensing region to control the posterior delivery of the aqueous Ag NPs sample. To form the Ag NP ink, the NP colloid was concentrated (98% of the supernatant was removed) and glycerol in a volume ratio of 2:5 (glycerol: colloid dispersion) was added to the remaining colloid to adjust the viscosity and surface tension of the ink for optimal printing; the prepared ink was then injected into refillable printing cartridges. The synthesized Ag NPs can be printed onto the hydrophobic paper to form the sensing arrays. Fig. 4a shows an optical microscope image from one of the 1 mm diameter printed circles, while Fig. 4b presents a scanning electron microscopic (SEM) image from the printed region with several Ag NPs aggregates distributed along the cellulose fibers of the paper. Subsequently, the same authors demonstrated a paper-based surface swab and lateral-flow dipsticks using inkjet-printed SERS substrates for analyte detection [40]. They harnessed the capillary action wicking of cellulose to enable the simple loading of liquid samples into the detection device (an Ag NP inkjet-printed region) and, in addition, it provides inherent analyte concentration within the detection volume by lateral flow operation

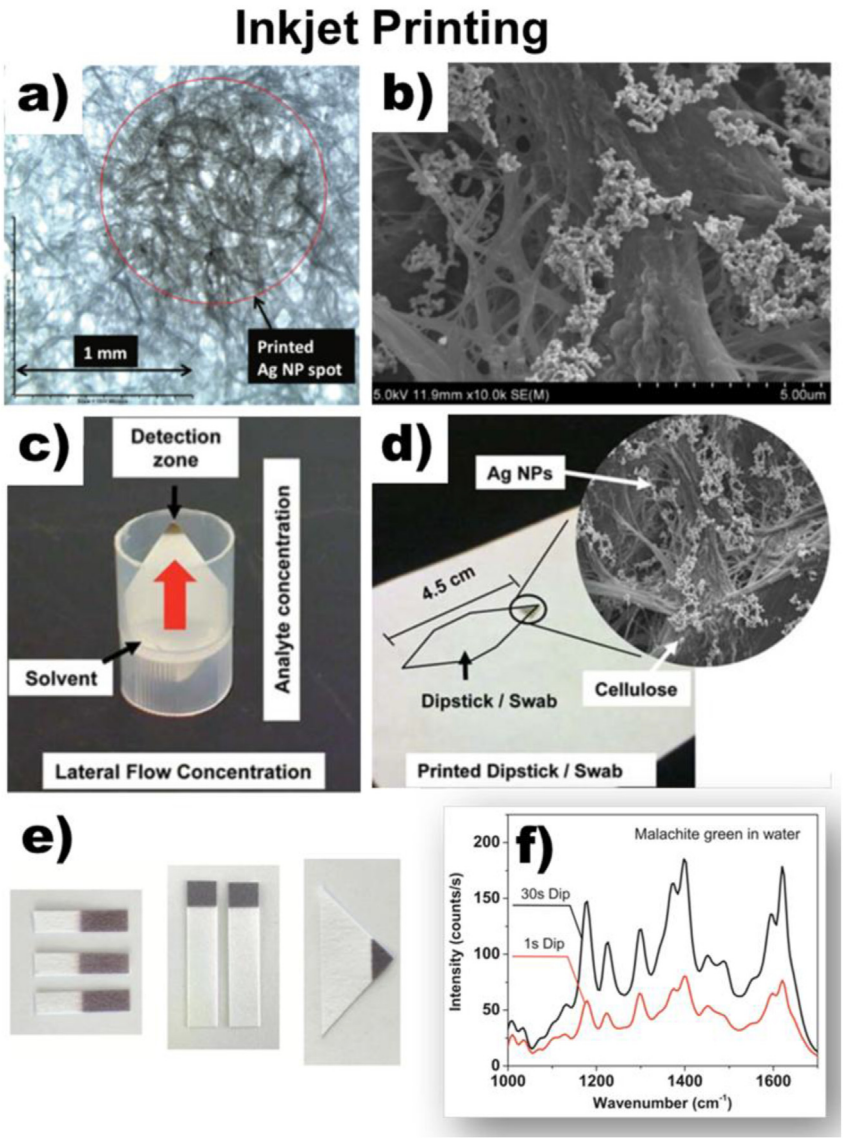

Fig. 4. Paper-based plasmonic substrates by inkjet printing. a) Optical micrograph of one inkjet printed spot on paper with Ag NPs [39]; b) SEM image of one inkjet printed zone with Ag NPs aggregates within the printed spot on the cellulose fibers [39]; c) Photograph illustrating the lateral flow concentration by the wicking properties of the paper substrate into the tip, where the Ag NPs were inkjet printed for the detection zone [56]; d) Photograph showing the inkjet printing of Ag NPs on paper [56]; e) A series of photographs illustrating how the inkjet printed paper with Au NPs can be cut as demanded [40]; and f) SERS spectra showing the detection of Malachite green in water by a paper-based plasmonic substrate, it can be seen how the signal increases as a function of the time that the paper-based substrate is submerged in the analyte sample due to its absorbing properties [40]. a) and b) reproduced with permission from Ref. [39]. Copyright 2010, American Chemical Society. c) and d) reproduced with permission from Ref. [56]. Copyright 2013, Royal Society of Chemistry. e) and f) reproduced with permission from Ref. [40]. Copyright 2013, Elsevier.

when the paper ending corresponds to a tip type morphology (see illustration in Fig. 4c). As shown in Fig. 4c, the dipstick is placed in a container with a volatile solvent, which transports the analyte molecules to the tip as it is wicked up the paper; moreover, this lateral flow step helps to improve the SERS signal intensity by concentrating the analyte to a very small sensing area. They demonstrated a detection limit of 95 fg of Rhodamine 6G, without any complex nanofabrication technique, using only the microcapillary action of the paper. Fig. 4d presents a photograph with the inkjet printed pattern on filter paper, which afterward can be just cut by conventional scissors [40]. As illustrated in Fig. 4e, the methodology to obtain trace chemical detection substrates using inkjet-printed plasmonic NPs on paper can be adapted by cutting the paper into different morphologies as demanded [40]. Fig. 4f shows SERS spectra from the detection of Malachite green in water, it can be seen how the signal increases as a function of the time (1 s or 30 s) that the paper-based substrate is submerged in the analyte sample due to its absorbing properties [40].

The ink-jet printing allows for a fast and low-cost approach to obtain several specific domains with great potential as SERS substrates for sensing in biomedical applications, harnessing the swabbing or/and lateral-flow properties of the flexible paper-based substrates. Moreover, the wicking properties of the cellulose paper can be used to increase/modulate the amount of analyte sample loaded into the substrate as a function of the immersion time of the paper-based substrate in the analyte solution.

4.2. Screen printing

A different approach was proposed by Qu et al., by using a screen printing ink containing Ag NPs, they were able to propose a large-batch method to fabricate disposable SERS arrays [41]. They prepared the screen-printing ink by concentrating the Ag NPs colloid (removing 99% of the supernatant) and adding sodium carboxymethylcellulose (CMC) solution in a volume ratio 1:3 (CMC: concentrated colloid) to adjust the correct viscosity of the ink. After spreading the screen-printing ink for the stamping process, the paper substrate was left to air-dry. Fig. 5a has a schematic illustration of the fabrication process for the screen-printing of SERS arrays on paper substrates, including a photograph with the array of circles printed on paper (Fig. 5aA) and a representative SEM image from one of the printed circles (Fig. 5aB) [41]. Using a similar screen printing ink, Kim et al. fabricated SERS substrates using Au NPs based inks with the screen printing technique [42]. The design of their paper-based SERS devices, as illustrated in Fig. 5bA, contains a 2 mm hydrophilic circular reservoir surrounded by a hydrophobic zone previously printed with wax by a commercial printer. The cross-sectional view of the SERS active region on the paper is shown in Fig. 5bB, while Fig. 5bC presents a photograph of the paper-based SERS device before and after printing with the CMC-Au NPs ink [42].

The main advantage of this screen printing approach is its capability for a large-batch fabrication process of low cost and disposable SERS arrays. However, the ink viscosity needed for this type of printing technique results in a very high density of plasmonic NPs coverage mainly on the paper surface, which would not allow the use of the 3D cellulose fibers network along the paper substrate efficiently for cell culture applications.

4.3. Direct calligraphy

A very simple but powerful approach corresponds to the plasmonic calligraphy, which involves the usage of a conventional pen filled with ink containing the plasmonic NPs, that allows to directly writing test domains on paper substrates [43,44]. The realization of multiplexed label-free bio-assays was reported by Tian et al. using plasmonic calligraphy [43]. The main advantage with respect to inkjet printing, besides simplicity, corresponds to the avoidance of temperature, which can result in loss of recognition functionality of biomolecules. The basic implementation of this approach is schematically illustrated in Fig. 6aA and demonstrated in the photograph shown in Fig. 6aB. The plasmonic ink is prepared by concentrating the plasmonic NPs colloid 100 times and then the resulting concentrated dispersion is used as the ink injecting it into an empty refillable ballpoint pen [43]. The authors used Au nanorods (Au NRs) as plasmonic transducers considering the high refractive index sensitivity of their longitudinal LSPR. Fig. 6aC has an image of a paper strip with the written pattern at the bottom of the strip as the test domain, which harnesses the wicking properties of the paper to absorb the analyte from a liquid solution passing through the plasmonic pattern. A SEM image showing the homogenous distribution of the Au NRs along the cellulose fibers composing the paper substrate is presented in Fig. 6aD. The authors were able to detect a specific target biomolecule (anti-rabbit IgG protein) by functionalizing the Au NRs with rabbit immunoglobulin G (IgG) [43]. In a subsequent report by the same research group, they use plasmonic calligraphy for the formation of chemically selective test domains on paper substrates using functionalized Au NRs with polyelectrolytes either of positive (PAH: poly(allylamine hydrochloride)) or negative (PSS: poly(styrene sulfonate)) charge [43]. The charged plasmonic patterns enable them to make multiplexed SERS detection of two or more target analytes from a complex chemical mixture. The schematic illustration in Fig. 6bA describes the charge-selective multiplexed SERS approach, using either PSS-Au NRs to trap and detect positively charged molecules or PAH-Au NRs to trap and detect negatively charged molecules. Fig. 6bB contains a series of SERS spectra demonstrating the detection of a negatively charged analyte (MO: methyl orange), while Fig. 6bC has a series of SERS spectra detecting a positively charged analyte (R6G: rhodamine 6G) [43]. Plasmonic calligraphy using an ordinary fountain pen filled with plasmonic inks was also developed by Polavarapu et al. for making SERS substrates on paper [44]. They were able to implement plasmonic inks with metal NPs of arbitrary shape and size, including Au NPs, Ag NPs, and Au NRs which allows for the tuning of different excitation wavelengths for SERS detection [44].

One of the main advantages of this approach corresponds to the

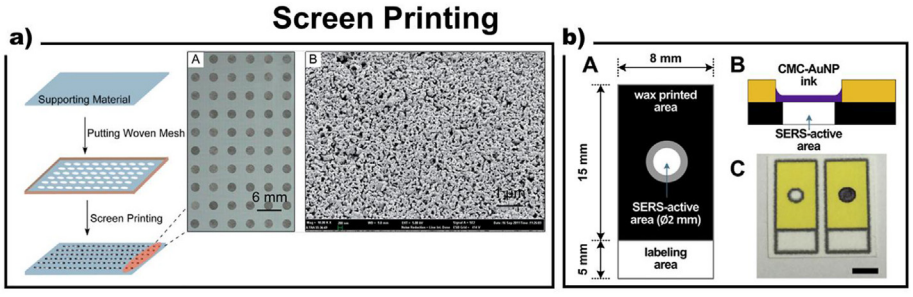

Fig. 5. Paper-based plasmonic substrates by screen printing. a) Schematic illustration of the fabrication process for screen-printing SERS arrays on paper substrates, including A) a photograph with the array of circles printed on paper and B) a representative SEM image from one of the printed circles [41]. a) reproduced with permission from Ref. [41]. Copyright 2012, Royal Society of Chemistry. b) Scheme of the paper-based plasmonic substrate as SERS platform obtained by screen-printing [42]: A) Design of the paper-based plasmonic device; B) Cross-sectional view of the SERS active area on the paper-based device; C) Photograph of the paper-based devices before and after printing the CMC-AuNP ink (Scale bar = 5 mm). b) reproduced with permission from Ref. [42]. Copyright 2016, Elsevier.

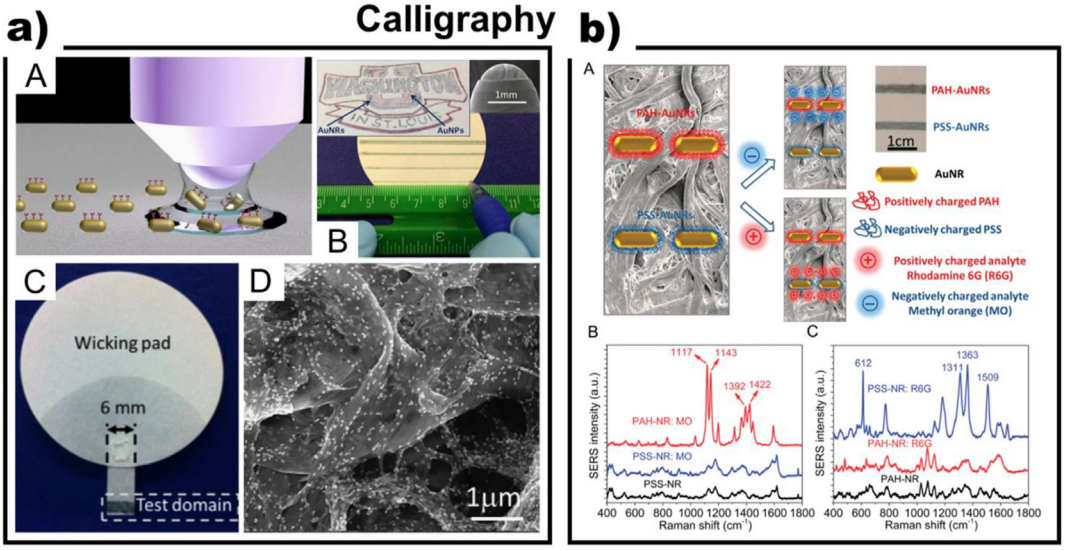

Fig. 6. Paper-based plasmonic substrates by direct calligraphy. a) A general description of plasmonic calligraphy [57]: A) Schematic illustration of the concept of plasmonic calligraphy; B) Photograph demonstrating the writing process on paper with a convention ball-point pen filled with Au NR ink; C) Image of a paper strip with the written pattern at the bottom of the strip (test domain); D) SEM image showing the homogenous distribution of the Au NRs along the cellulose fibers composing the paper substrate. a) reproduced with permission from Ref. [57]. Copyright 2014, Elsevier. b) The concept of charge-selective SERS on paper using plasmonic calligraphy is presented [43]: A) Schematic illustration of the charge-selective multiplexed SERS detection using either PSS-Au NRs or PAH-Au NRs; B) SERS spectra from PAH-Au NRs and PSS-Au NRs upon exposure to MO aqueous solution and the PSS-Au NRs without exposure as a control signal; C) SERS spectra from PSS-Au NRs and PAH-Au NRs upon exposure to R6G aqueous solution and the PAH-Au NRs without exposure as a control signal. b) reproduced with permission from Ref. [43]. Copyright 2014, Royal Society of Chemistry.

simple and easy-to-use technique by directly writing the test domains. The avoidance of higher temperature allows having functionalized plasmonic NPs with biorecognition functionalities. Moreover, different morphology NPs with different LSPR properties can be written and alternated easily by changing pens in the same paper-based substrate for multiplexed SERS detection of two or more target analytes from a complex mixture.

4.4. Paper dipping

The most general but versatile approach corresponds to simply dipping the paper substrate into a colloid dispersion, harnessing the hydrophilicity and absorbing properties of paper, which allows the homogenous anchoring of the plasmonic properties into the cellulose fibers composing the paper. Following this approach, Lee et al. demonstrated the fabrication of a simple but highly efficient paper-based SERS substrate by loading Au NRs in commercially available filter paper after dipping it into a colloid dispersion [45,52]. Fig. 7aA shows how the filter paper color changed from white to purple after being exposed to Au NR colloid capped with cetyl trimethyl ammonium bromide (CTAB), while the intensity of the purple color of the Au NRs colloid decreased after removing the paper from the aqueous dispersion. Fig. 7aB shows a SEM image with the uniform decoration of the Au NRs along the cellulose fibers of the paper. Even vigorous rinsing with water or alcohol did not noticeably alter the Au NRs density. The SERS substrates exhibited excellent homogeneity with a relative standard deviation of ~15% [52]. Swabbing their paper-based plasmonic substrates with a contaminated surface, they were able to demonstrate the detection of trace amounts of analyte (140 pg spread over 4 cm^2) [45]. A similar approach was explored by Ngo et al. using Au NPs colloidal dispersions of different concentrations [51]. It can be seen in Fig. 7bA how the initial pink tonality of the paper substrate obtained when dipped into the lowest concentration colloid was increased as the concentration of the employed colloid was higher. The size distribution of the Au NPs along the cellulose fibers of the paper remained constant for the different substrates dipped into different concentrations colloids; however, the concentration of Au NPs on the paper increased monotonically with the colloid concentration in which the papers were dipped (see SEM images in Fig. 7bB) [51]. A posterior work by the same research group showed that by treating paper beforehand with a cationic polyelectrolyte, they could control the extent of Au NPs aggregation on paper, increasing the SERS enhancement factor of their plasmonic substrates by the generation of SERS hot-spots [49]. Using aqueous solutions of cationic polyacrylamide (CPAM) they were able to obtain uniform distribution of Au NPs aggregates into the paper to achieve a high degree of reproducibility in the SERS measurements [49]. Following this dipping strategy for impregnating the paper-based substrates with plasmonic NPs, multibranched gold NPs were successfully anchored into filter paper for dipsticks and swabs for SERS sensing [55]. Similarly, Schumucker et al. were able to incorporate Au NPs of different shapes such as concave nanocubes, nanorods, and spheres, into paper-based substrates as demonstrated by the SEM images shown in Fig. 7c [53]. These paper-based plasmonic scaffolds add stability to the NPs on the paper for exposure to chemical environments that would otherwise disrupt a NPs colloid.

Following the dipping approach for the impregnation of plasmonic NPs into paper-based substrates, Zheng et al. reported Au NPs loaded filter paper when using highly concentrated Au NPs suspensions in toluene [50]. The main advantages of the toluene suspensions over the aqueous plasmonic NPs colloids are the NPs content and the faster drying time. Dipping the filter paper into the concentrated colloid in toluene and followed by drying using a hair-dryer, it was possible to achieve a close-packed Au NPs assembly after repeating the loading process five times within a time of some minutes. The highly concentrated Au NPs paper was used to monitor in real-time the catalytic reaction progress for the conversion of 4-nitrophenol into 4-aminophenol by SERS [50]. These types of highly concentrated NPs solutions or inks in organic solvents can be prepared by inexpensive, versatile, and very reproducible methods [46,54]. Hiramatsu et al. have reported a large-scale synthesis of organoamine-protected Au NPs or Ag NPs with polydispersities as low as 6.9%, using only three reagents: tetrachloroauric acid or silver acetate, oleylamine, and toluene as the solvent [54]. Polavarapu et al. reported a facile method for the synthesis of alkylamine capped metal (Au or Ag)

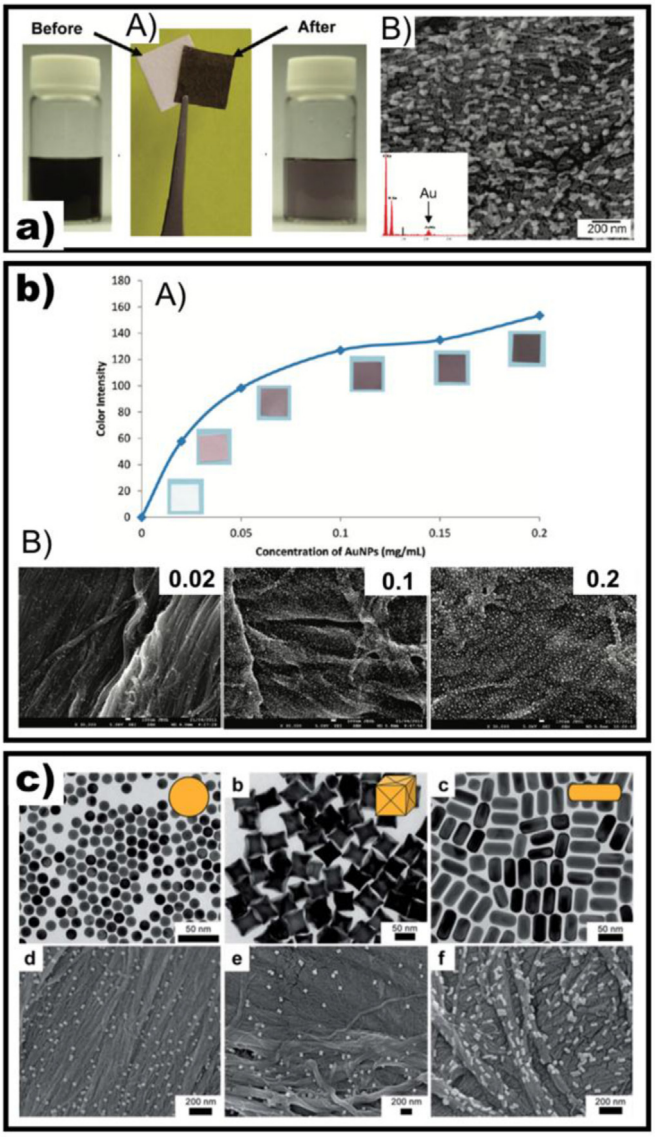

Fig. 7. Paper-based plasmonic substrates obtained by dipping the paper substrates in plasmonic NPs colloids. a) A) Includes photographs of the Au NR dispersions and the filter paper before and after being immersed into the colloid; B) corresponds to a SEM image showing the homogenous distribution of the Au NRs anchored on the cellulose fibers of the paper substrate [45]. a) reproduced with permission from Ref. [45]. Copyright 2010, American Chemical Society. b) A) The color intensity of filter papers treated in Au NPs colloids of different concentrations can be observed; B) has three SEM images of the filter papers treated in colloids with different concentrations, showing that the color intensity corresponds to a higher density of Au NPs homogenously anchored on the cellulose fibers of the paper [51]. b) reproduced with permission from Ref. [51]. Copyright 2012, American Chemical Society. c) Illustrates the versatility of the dipping method to impregnate the paper with plasmonic NPs of different morphologies; the upper panel corresponds to TEM images of the NPs, while the bottom panel shows SEM images of the different type of plasmonic NPs homogenously anchored into the cellulose fibers of the paper substrate [53]. c) reproduced with permission from Ref. [53]. Copyright 2016, Royal Society of Chemistry.

NPs in toluene, using either $AgNO_3$ or $HAuCl_4$ with oleylamine in a toluene solvent, followed by reduction with aqueous $NaBH_4$ [46].

The two main advantages of the impregnation of plasmonic NPs by paper dipping for cell culture applications are the following: (1) NPs of the selected shape can be easily incorporated homogeneously along the 3D cellulose fiber network; and (2) the coverage density can be easily tuned by modifying the concentration of the NPs colloid or the dipping time. Moreover, the paper-based plasmonic scaffold adds stability to the NPs on the paper for exposure to chemical environments that would otherwise disrupt a colloidal dispersion.

The comparison of the four abovementioned approaches for the impregnation of plasmonic NPs within paper-based substrates highlights key differences. In the case of ink-jet printing, although easy to implement in a large batch manner, the temperature factor can hinder the possibility of functionalizing the plasmonic NPs with biorecognition compounds. On the other hand, while the screen printing permits large batch disposable SERS sensors arrays, the ink viscosity required does not allow a homogenous impregnation of the NPs all along the 3D cellulose fiber network. This leaves us with direct calligraphy and paper dipping as the best choices for cell culture applications. Yet, these techniques retain important differences that make them optimally suitable for different applications. In fact, while direct calligraphy allows to easily alternating domains with different NPs properties (LSPR or coating functionality) for multiplex SERS detection of two or more target analytes from a complex chemical mixture; the paper dipping permits obtaining a very homogenous impregnation with tunable coverage density of the selected NP all along the 3D cellulose fiber network.

5. Paper-based substrates for biomolecules detection

This section describes some representative works aimed at tailoring the properties of paper-based plasmonic substrates for biomedical applications using SERS spectroscopy. The main goal is to show how the integration of plasmonic NPs within the fibrous structure of the paper-based substrates can be harnessed for monitoring different biomolecules to outstand the capabilities of these substrates for measuring different cells' cues in a cell culture environment when combined with a highly specific and sensitive tool such as SERS. For a more extended review on ease-of-use flexible SERS sensors for chemical and biomolecular compounds at the point-of-sample, the reader can consult related published works [58,59].

The dipping approach has been employed for obtaining paper-based plasmonic substrates for biomolecules detection. Tian et al. designed a paper-based plasmonic substrate with biofunctionalized Au NRs as a highly sensitive transduction platform for rapid detection of trace bioanalytes in physiological fluids [48]. They make use of the very sensitive spectral shift of the LSPR wavelength of Au NRs due to the increase in the refractive index around the Au NRs when the bio-analyte is bound. As schematically shown in Fig. 8aA, the Au NR-IgG conjugates were prepared by functionalizing Au NRs with SH-PEG-IgG molecules; subsequently, Au NR-IgG conjugates were adsorbed onto paper substrate by dipping the paper into Au NR-IgG conjugate solution, followed by rinsing with water to remove the weakly adsorbed Au NRs; then, the paper substrate was exposed to SH-polyethylene glycol (PEG) solution to block non-specific binding; finally, it was exposed to anti-IgG solution resulting in its specific binding to IgG, which can be detected as a spectral shift of the LSPR wavelength of Au NRs. Fig. 8aB has a SEM image showing the homogenous distribution of the Au NRs along the cellulose fibers of the paper achieved after dipping impregnation [47]. A similar approach used peptide molecules as recognition elements for the selective capture of target chemical species from a complex chemical mixture [47]. Trinitrotoluene (TNT) was detected down to 100 pM concentration by SERS, even in a complex organic chemical mixture by this biomimetic approach enhancing the selectivity of plasmonic Au NRs [47]. A bioplasmonic paper device was also designed for LSPR-based bio-assay with Au NRs functionalized with a peptide recognition element with high affinity for a biomarker (cardiac biomarker troponin I) [60]. The general concept is schematically illustrated in Fig. 8bA, showing how the presence of the bound biomarker is observed by a spectral shift of the LSPR wavelength of the Au NRs. Using this bioplasmonic paper device, the sensitivity of LSPR biosensors based on natural antibodies and short peptides was compared. It was demonstrated that peptides provide significantly higher

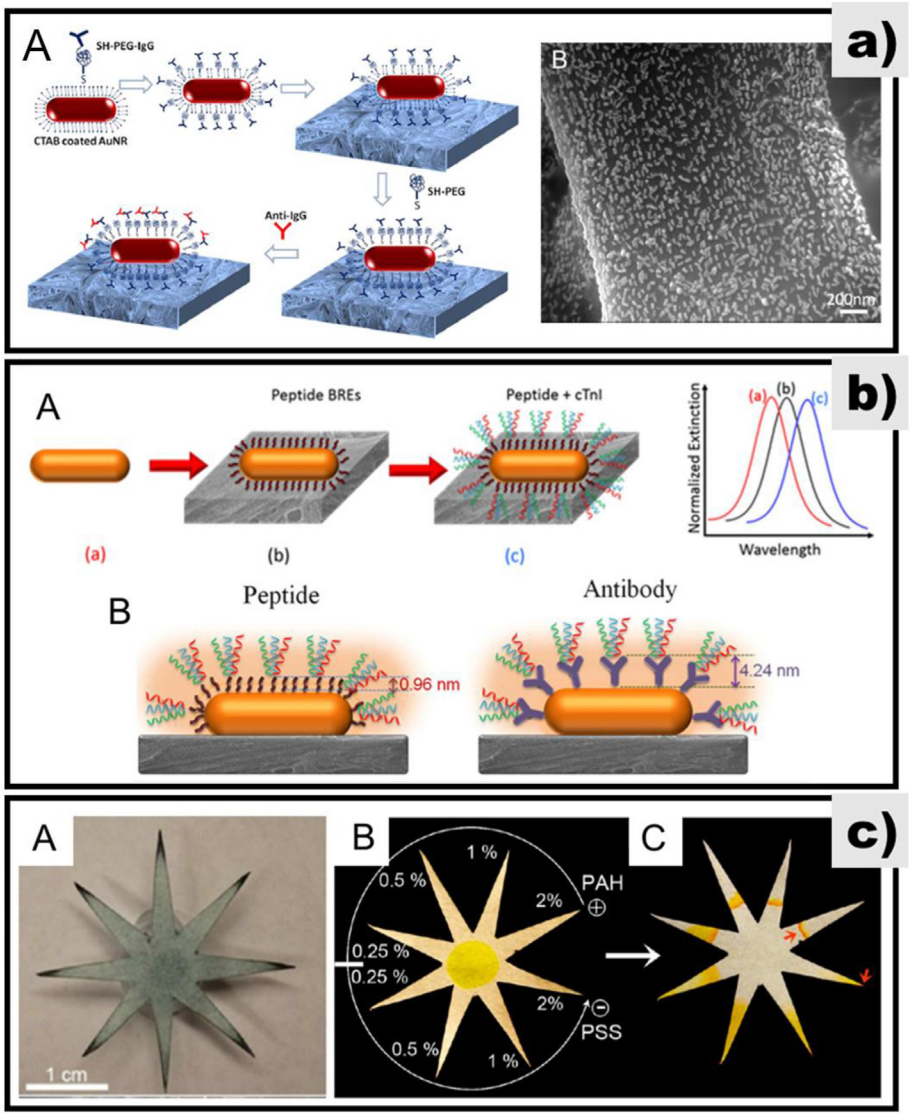

Fig. 8. Biomolecules detection with paper-based plasmonic substrates obtained by the dipping strategy. a) A) Schematic illustration representing the steps to design the paper-based plasmonic substrate for biomarkers detection (anti-IgG); B) the right panel shows a SEM image showing the homogenous distribution of the Au NRs along the cellulose fibers of the paper [48]. a) reproduced with permission from Ref. [48]. Copyright 2012, American Chemical Society. b) General approach used with a plasmonic paper device for the detection of biomarkers with peptide-functionalized Au NRs using their refractive index sensitivity [60]. A) Schematic illustrating the design of the biosensor with peptide recognition elements; and B) shows the effect of the distance of the peptide or antibody recognition element from the surface of the nanotransducers (i.e., Au NRs) for the refractive index sensitivity. b) reproduced with permission from Ref. [60]. Copyright 2015, Nature Publishing Group. c) Multifunctional analytical platform on a paper-based device for separation, concentration beforehand and detection [61]. A) Microfluidic paper-based analytical device (μPAD) designed with eight fingers showing the accumulations of Au NRs at the tips (dark spots); B) Charge gradient designed in the μPAD by different concentrations of PAH and PSS polyelectrolytes, while the yellow spot at the center corresponds to a fluorescein droplet as deposited; C) Migration of fluorescein into the different fingers with different spreading properties due to the charge gradient adjusted with the polyelectrolytes. c) reproduced with permission from Ref. [61]. Copyright 2013, American Chemical Society.

sensitivity and lower limit of detection compared to antibodies as a recognition element, which is ascribed to the differences in the thickness of the adsorbed peptide and antibody layer, as illustrated in Fig. 8bB [60]. A more complex but robust plasmonic paper-based analytical platform with functional versatility and subattomolar detection limit using SERS as a transduction method was reported [61]. This paper-based platform corresponds to a microfluidic paper-based analytical device (μPAD) made with a lithography-free process by a simple cut and drop method. A star-like geometry with eight branches or fingers was designed by cutting the filter paper (see Fig. 8cA). This shape enhances the capillary forces, resulting in much faster drying of the solvent at the tips compared to the rest of the paper substrate, thus causing a rapid flow from the wetted surface to the dry area concentrating the analytes into the tiny test regions (the tips). Moreover, the plasmonic μPAD can be designed with a chemical gradient at their different fingers, which can be created by differential polyelectrolyte (PAH with positive charge or PSS with negative charge) coating of the different paper fingers (this is described in Fig. 8cB). Then, complex samples can be separated by this surface chemical gradient, giving separation abilities into the paper-based plasmonic device as demonstrated in Fig. 8cC with fluorescein as probe analyte. Therefore, different molecules can migrate into different fingers (depending on the electrostatically compatible surface) and at the same time they can be concentrated at the tip providing an extremely high concentration in a very small surface area where the plasmonic NPs are impregnated, making this type of paper-based plasmonic devices a very robust detection platform when combined with SERS [61].

The works just described illustrate several key concepts towards the design of paper-based substrates for cell culture applications. First of all, it is shown how plasmonic NPs can be coated or biofunctionalized with compounds for biorecognition and, subsequently, homogeneously impregnated on the 3D cellulose fiber network. Second, the comparison between using peptides or antibodies as recognition elements to biofunctionalize the plasmonic NPs highlights the difference in the intrinsic thickness of the biomolecular adsorbed layers, a parameter which is very important for SERS purposes since the signal increase is closely related to the analyte separation with respect to the plasmonic NPs (the intensity of electric field rapidly declines with distance) [62–64]. Third, paper-based substrates allow for the design of microfluidic paper-based analytical devices (μPAD) by a simple cut method with conventional scissors. A star-shaped paper-based substrate as the one illustrated in Fig. 8cA could allow monitoring a cell culture system as a function of time for cell-secreted analytes if the cells are cultured within the middle circle zone while making use of the capillary forces for the lateral flow of the secreted medium towards the detection zones at the finger tips. Moreover, a similar star-shaped paper-based substrate could be designed for

multiplex SERS detection such as via modification of each star finger with differently charged polyelectrolytes or with plasmonic NPs bio-functionalized with distinct recognition molecules.

6. SERS platforms for cell culture applications

The development of new materials as scaffolds for mimicking the 3D cellular micro-environment that can provide cells with physicochemical cues is of paramount importance for cell culture applications. The intrinsic 3D structure of the fibers composing the paper in principle could indeed mimic the native cell micro-environment and its architecture, allowing physiologically relevant fluid flow, as well as oxygen and nutrients gradients. Then, the integration of plasmonic NPs within the fibrous structure of paper can enable the generation of nanosensors for monitoring different cells signals when combined with a highly specific and sensitive optical tool such as SERS. However, the use of flexible substrates based on cellulose paper with anchored plasmonic NPs for cell culture applications as a full system has been underexplored. Nonetheless, several recent works are using alternative substrates with plasmonic properties showing the great potential of SERS spectroscopy for accurate surveying of cell culture analytes.

It can be seen in Fig. 9Aa how an Au quasi 3D plasmonic nanostructure array (Q3D-PNA) was used to map the extracellular pH of living cells based on SERS. This was achieved by immobilizing a probe molecule (4-mercaptobenzoic acid, 4-MBA) characterized by a pH-dependent SERS spectrum to enable sensitive and reproducible pH measurements [65]. This was an important proof-of-concept study that used a normal fibroblasts cell line (NIH/3T3) and a liver tumor cell line (HepG2) cultured on the 4-MBA modified plasmonic substrate, allowing for mapping the extracellular pH with good spatial resolution and pH sensitivity. The Q3D-PNA was fabricated by electron beam lithography. As shown in Fig. 9Ab-c, the SERS peak of 4-MBA at ~1420 cm^{-1} increases with the rising of pH, being an accurate pH indicator. Nevertheless, it is pointed out that such peak not only responds to the variation of solution pH due to protonation/deprotonation, but also changes with the presence of cations due to chelation [66]. As a result, possible

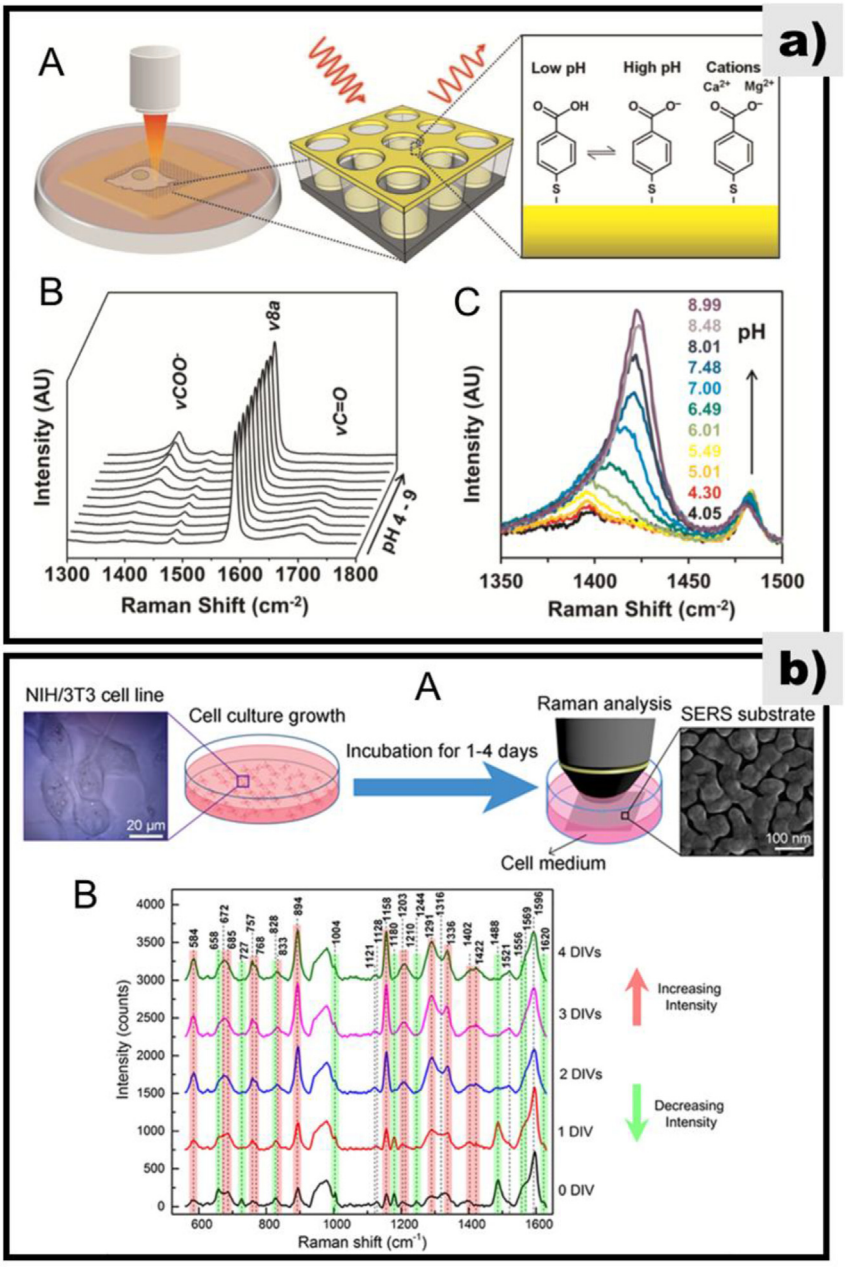

Fig. 9. SERS platforms for cell culture applications. a) (A) Schematic illustration of the process for mapping the extracellular pH of a single living cell cultured on a 4-MBA modified Q3D-PNA SERS substrate in the culture media. (B) SERS spectra of 4-MBA modified Q3D-PNA substrates in PBS at different pH (from ~4.0 to ~9.0). (C) A closer look at the intensity of the SERS peak at ~1420 cm-1 to highlight its variation as a result of a different degree of protoation/deprotonation of the 4-MBA molecule [65]. a) reproduced with permission from Reference [65]. Copyright 2015, Elsevier. b) (A) Schematic illustration of the experimental approach followed for monitoring the extracellular metabolites. (B) Raman spectra of the 3T3 conditioned medium collected from one to four DIVs (days *in vitro*) [68]. b) reproduced with permission from Reference [68]. Copyright 2017, PLoS ONE.

interference of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) was considered for the calibration curves [65]. Since the extracellular pH of living cells is one of the major factors influencing cell behaviors (e.g. cycle progression, migration, proliferation, differentiation, and tumor progression), its accurate sensing and mapping are of high importance. It is worth noting that collecting SERS mapping of large areas (e.g. > micrometric ranges) with high spectral typically requires relatively long acquisition processes. To tackle this issue, novel methodological approaches, such as SERS holography, are currently developed to enable single-shot three-dimensional mapping, including live cell [67].

More recently, a simple approach for fabricating cell-compatible SERS substrates was reported by Milewska et al. [69]. A plasmonic material was used to carry out Raman imaging of fixed mesenchymal stromal cells derived from bone marrow (BM-MSCs), which were cell-cultured directly on the substrates by standard protocols. Since BM-MSCs are multipotent progenitors that are plastic-adherent under standard cell culture conditions *in vitro*, the study of their proliferation, differentiation and interactions with the external environment by non-invasive approaches represents an important task. The SERS substrates were obtained on glass cover slips by repeated gold deposition with an electron beam evaporator and thermal annealing. The results of viability assays confirmed that the substrates were highly biocompatible while displaying high SERS signal uniformity and SERS enhancement factor up to 6 orders of magnitude. The SERS imaging of attached cells was able to detect an increase of cellular components. Nonetheless, the volume of the study was limited to the parts of the cell adjacent to the substrate, being able to probe the outer membrane of the adhering cells. The authors were able to report and co-localize multiple chemical and molecular fingerprints of cells related to the lipids, proteins and carbohydrates presumably as cell membrane components [69].

A different plasmonic substrate was prepared through electroless deposition of Ag on silicon substrates obtaining Ag nanoislands (see SEM image in Fig. 9bA). These substrates were used to monitor extracellular metabolites as a function of time by a label-free and non-destructive SERS methodology. The type and levels of metabolites produced by living cells can change, depending not only on the cell type, but also on their nutritional state, their functional state and/or their phase of differentiation. The SERS analysis was done by first cell culturing '3-day transfer, inoculum 3×10^5 (NIH/3T3) cells' *in vitro*, then the extracellular medium was collected at different time points over a period of 4 days to be analyzed with the SERS substrates, as depicted in Fig. 9bA [68]. By analyzing the intensity of individual Raman peaks (Fig. 9bB), they were able to assign them to specific metabolites, which allow them to detect changes over time for several cell medium components. In particular, the consumption of different cell media nutrients such as L-tyrosine during the conditioning time, with the corresponding production of acetoacetate and fumarate, was detected [68].

A different work proposed the usage of nanostructured plasmonic substrates comprising Au NPs, self-assembled as ordered superlattices, for the precise label-free SERS detection of selected tumor metabolites in the extracellular media [70]. The authors employed a hydrogel-based 3D cancer model to recreate the tumor micro-environment. As a proof-of-concept, they focus on the analysis of kynurenine (a secreted immunomodulatory derivative of the tumor metabolism) and the related molecules of tryptophan and purine derivatives. The SERS technique facilitates the unambiguous identification of trace metabolites (down to 10^{-6} M) and allows their multiplex detection in the extracellular medium to monitor their fluctuations. The measurements were performed by culturing a collagen bio-ink laden with HeLa cervical cancer cells inside chambers filled with cell media. At selected times, the chamber was stacked with the plasmonic substrate and illuminated with the laser for SERS analysis [70].

As shown with these reports, integrating plasmonic properties to substrates where the cells can be cultured, affords SERS platforms capable of monitoring multiple extracellular cues by SERS signal not only as a function of time, but even with the capability of obtaining spatial mapping. Nonetheless, the flexibility of cellulose paper substrates together with their 3D cellulose fibers network may add a closer mimicking of the 3D extracellular micro-environment. Moreover, an advantage of these SERS platform systems for cell culture applications is their versatility to enable the non-invasive chemical characterization of cell cultures and biological samples with high clinical value.

7. Paper-based substrates as scaffolds for cell culture

Paper inherent ability to absorb fluids through capillary action, attributed to its porous structure and large void volume ratio, makes possible the cell migration inside this type of scaffolds [11]. This, added to the layered nature of paper, enables it to be stacked in multiple paper layer forming a 3D cell culture scaffold [6]. Moreover, the intrinsic structure of the fibers composing the paper could, in principle, mimic the native cell architecture and micro-environment allowing physiologically relevant fluid flow, and oxygen and nutrients gradients.

Tissue engineering combines active biological molecules with scaffolds and cells to assemble functional constructs that help to replace, improve, and repair damaged tissues, and promote endogenous tissue regeneration [71–73]. It implies a precise understanding of the cellular and molecular mechanism involved in cell growth, division, death, and the cell communication with its micro-environment. Thus, biomaterials play a key role in this field. This research area has evolved into the use of tissues as biosensors to detect biological and chemical threat agents, second metabolites, drug's by-products, or even to test the toxicity of a given experimental medication. Tissue engineering aims to overcome the limits imposed by organ transplantation, graft rejection, and complete restoration of functional tissue. Therefore, nanotechnology paves the way of this area by allowing the fabrication of complex and sensitive devices to design organ- and trials-on a chip and microfluidic scaffolds for boosting biocompatibility [74].

Cellulose papers can adsorb and be functionalized with different surface-active compounds or polymers such as collagen, attached via succinic acid in cellulose [75] or mannosylated surfaces on papers scaffolds to elicit specific cellular responses such as fibroblast adherence and growth [76]. The physicochemical properties of cellulose papers, such as porosity, mean channel size, hydrophobicity/hydrophilicity, filtration speed, among others, can be modified to suit the intended tissue engineering applications' requirements.

Due to its properties, cellulose has been used to generate paper-based analytical devices, sensor arrays, and microfluidic analytical devices designed for non-invasive detection of pathogens such as viruses and biomarkers of various diseases such as cancer [8]. The challenges to overcome in developing new biosensing devices based on paper mostly focus on selecting papers with good purity, size porosity, density, surface hydrophilicity, organic polymer modification, filtration speed, and other physicochemical properties. Cellulose papers on the market, such as Whatman filter papers No. 1 and No. 4, are often selected due to the best combination of porosity, diffusion properties, lateral fluid flow rate, stiffness, mechanical strength and hydrophilicity that are suitable for the development of paper-based biosensors. Among other papers used are nitrocellulose membrane, polymer fiber papers, weighing paper, Janus paper, Kimwipes, and print paper [11,8].

7.1. Bioplasmonic paper and biosensors

Paper-based biosensors function as a scaffold for immobilized reagents in the dry state for the future analysis of a liquid sample by adding a given analyte. Those reactions can be detected as a color change, emitted fluorescence, or other analytical signals. This technology plays an essential role in diagnosing enzymatic reactions and essential biomarkers present in body fluids (e.g. saliva, blood, sweat, urine, exhalation, etc.) related to a disease state. Importantly, biosensors must be able to sense impairment in protein-protein interactions and extracellular matrix-protein interactions to improve their efficiency to regenerate

tissue. Some of their advantages are their low cost, easy to cut or shape, and easily disposable features. Another important fact is that the paper's porous structure allows a larger surface area for a high amount of NPs adsorption compared to a non-porous surface. Also, by solvent evaporation, analytes can be concentrated in a specific area of the paper to function in microfluidics (Fig. 10c) [78].

The extensive fabrication of diagnosis microfluidic devices based on paper is due to the low cost of materials, biocompatibility, easy-handle, and complete accessibility [79]. Besides, paper is a very cost-effective disposable platform that can preserve strong capillarity action and improved biocompatibility. Both features are desirable to fabricate point-of-care applications and paper-based microfluidic diagnostic devices that exploit the ability to transport liquid by capillarity into the precise patterns made with hydrophobic materials like wax, permanent marker ink, polydimethylsiloxane, and alkyl ketene dimer, on the surface of the paper. This creates at the same time a path and a barrier where the analytes present in the fluid are stored and then analyzed avoiding the use of complex equipment [78,80].

There have been improvements to paper-based biosensors, especially those provided by incorporating nanomaterials to them that offered unique properties such as the high surface area to volume ratio, electrochemical properties, and ability to manifest biological signaling and transduction mechanisms. It allows an enhancement of sensitivity, limit of detection, and selectivity against interfering species [64,81]. Moreover, NPs and other nanostructures offer a new detection and transduction paradigm and increase the sensing layer's entire surface. Optical biosensing is the most common type of biosensor, due to its advantage of measuring in a real-time manner a wide variety of molecules contained in samples from cells, enzymes, and other proteins [64]. Apart from fluorescence read-outs [82–84], the analytes' detection can then be transduced to Localized Surface Plasmon Resonance (LSPR) NPs, which amplifies the signal, thus showing an improvement of sensitivity and accuracy.

The incorporation of plasmon-related NPs such as gold nanoparticles (Au NPs) and silver nanoparticles (Ag NPs) into paper-based biosensors, so-called plasmonic papers, are effective SERS substrates resulted in fluorescence quenching and color changes (Fig. 10a) [85,86]. By including reduced graphene oxide (GO) nanosheets, it has been possible to increase the active surface area by immobilizing a large number of biorecognition molecules. Also, NPs labeled with enzymes or other ligands, enable multistage chemical amplification of the sensor analytical signal to achieve ultra-high sensitivity. Moreover, the importance of NPs incorporation into a paper-based biosensor confers them with high pseudocapacitance for charge storage [87]. Therefore, the functionalization of paper-based biosensors with plasmonic nanoparticles, especially Au NPs also further allows for easy conjugation with other active ligands on their surface and their application for cell metabolites detection, especially for disease diagnosis [88].

7.2. Biosensors for early disease diagnosis

The physicochemical properties of paper enable the storage of immobilized reagents in a dry state for further analysis. This approach has transformed detection tools such as the paper-based biosensors into easier to handle and cost-effective point-of-care devices, significantly improving the early diagnosis of chronic diseases.

Paper-based biosensors can detect different biological molecules, for example, the 8-hydroxy-2′-deoxyguanosine (8-OhdG), an oxidative stress biomarker that is used as early diagnosis carcinogenesis, Alzheimer's

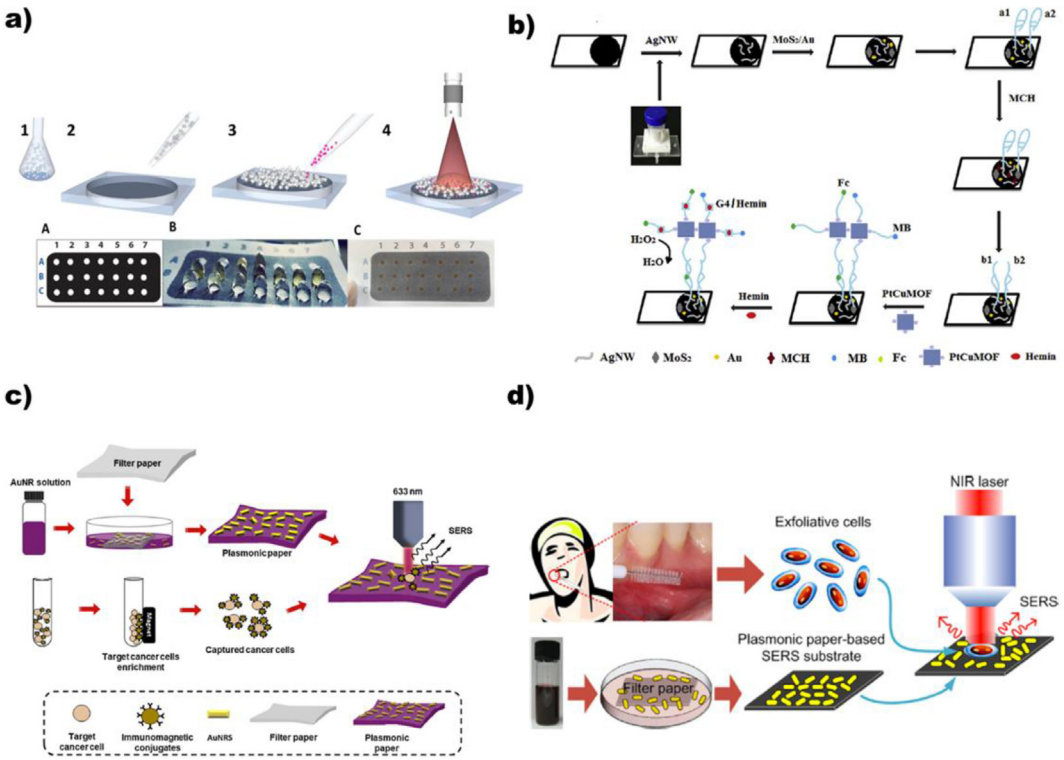

Fig. 10. Bioplasmonic papers using as biosensors. a) Steps of a plasmonic SERS paper fabrication that includes 1) synthesis of Ag NPs; 2) pattern of hydrophobic barriers to form the wells; 3) addition of rhodamine 6 G; and 4) SERS signal analysis by a 633 nm laser. A) Schematic view of the 2 mm wells; B) photograph of the plasmonic paper after drop-casting of Ag NPs; and C) paper view after drops drying [86]. b) Schematic representation of a paper biosensor with a hierarchically assembling of nanocomposites to detect two different miRNAs [92]. c) Schematic representation of plasmonic paper fabrication using Au NRs and immunomagnetic conjugate enrichment for the detection of cancer cells [78]. d) Schematic representation of the fabrication of paper-based plasmonic paper for a sensitive, non-invasive and rapid device for cancer screening using Au NRs [93]. a) Reprinted with permission from Ref. [86]. Copyright 2017, Nature. b) Reprinted with permission from Ref. [92]. Copyright 2019, Elsevier. c) Reprinted with permission from Ref. [78]. Copyright 2019, Elsevier. d) Reprinted with permission from Ref. [93]. Copyright 2014, Elsevier.

disease, and autism. A biosensor made of cellulose paper coated with a conductive carbon-based ink was manufactured; this forms a catalytically active biosensing electrode in a differential pulse voltammetry setup that allows the detection of 8-OhdG, a biomarker of DNA damage by oxidative stress and a marker of prior stage to mutagenesis, especially in cancer cells. The biosensor is able to detect up to 50–1000 ng/mL with a limit detection of 14.4 ng/mL in diluted samples [89].

Small non-coding RNAs, also called micro- RNAs, are associated with different diseases such as cancer, diabetes, cardiovascular, and nervous system disorders [90]. Thus, they constitute a novel target for biomarker detection. For example, in the case of -RNA-141 (mir-141) and micro--RNA-21 (mir-21), both are recognized as earlier markers for cancer detection [91]. A paper-based electrode biosensor was made of a hierarchical assembling of Ag nanowires (NWs), and MoS_2/Au NPs for simultaneously electrochemical detection of those two clinical relevant micro-RNAs. Under optimal conditions, the nanomaterials' hierarchical assembly enabled the biosensor to have a large surface area for capturing the micro-RNAs. The device has a detection limit of up to 0.1 fM, thus being a promising platform for an easy access diagnosis of cancer by detecting micro-RNAs in real serum samples (Fig. 10b) [92].

Tumor cells detection and purification are also possible, as demonstrated by the use of a filter dipped into Au NRs dispersion to create a paper-based SERS substrate conjugated for magnetic separation of cells. The device allowed the separation and enrichment of HT-29 colorectal cancer cells among other cell samples that do not overexpress on their cell membrane the adhesion molecule EpCAM, showing with this the potential of the plasmonic paper for screening cancer cells biomarkers [78]. Cancer screening through SERS has been proved- to be highly sensitive, non-invasive, and easy to handle. Au NRs are adsorbed into filter papers to create a flexible and 3D scaffold for cancer screening (Fig. 10d) [93].

Standard laboratory filter paper has been modified with plasmonic Au NRs as a three-dimensional scaffold for cancer detection using the aquaporin 1 (AQP1), a protein biomarker found in urine for the early and non-invasive detection of renal cancer carcinoma. This plasmonic paper has a limit detection of 10 ng/mL (0.35 nM) that matches the range of AQP1 in patients with renal cancer [48]. Circulating tumor cells are found in the bloodstream and are shed from the tumor and migrate to distant organs to form metastases. Therefore, their detection provides a powerful tool for early diagnosis of severe cancer progression and for evaluating the patient's responses to a given anticancer treatment. However, due to the small amounts of circulating tumor cancer cells, it is scarce to detect them. To overcome this, microfluidic devices have been exploited such as reported by Zhang et al., where they employed a nitrocellulose membrane as a substrate to trap a smaller amount (34 cells) of circulating tumor cells present in human peripheral blood, then facile SERS probes conjugated with antibodies and Raman reporters recognized the cells specifically [94].

Thus, besides their cancer detection application, a novel revolution has emerged by the smart combinatorial of biosensors and nanotechnology, which exploits chemical, electrical, optical, and magnetic features of nanomaterials to monitor cell growth, damaged tissue repair, and wound healing. It can be identified and measured through biomarkers, such as producing specialized proteins such as enzymes, cytokines, or peptides related to biological activities such as cell proliferation (Fig. 11b) [74,95]. Moreover, during a disease condition, the detection of biomolecules can be possible by using high-sensitive and accurate scaffolds based on the addition of plasmonic surface NPs and SERS technology. Indeed, the ability of plasmonic papers to detect samples containing biological analytes in trace amounts combined with their portability, storage, handling, and low cost make these systems a feasible detection tools for difficult-to-reach laboratory sites or faster analysis without compromising the sensitivity of the tests.

7.3. *Paper-based substrates for cell culture*

The stacking and reshaping properties of paper are favorable for cell culture procedures; moreover, the paper's porosity allows the flow of cell culture media across it, becoming beneficial for nutrient, gas and, waste transportation [74]. However, the paper is not suitable for cell cultures directly due to the lack of cell adhesion moieties. Cell culture improvement relies on the functionalization of paper with peptides, nucleotides, and other biomolecules. For example, extracellular matrix proteins such as albumin, vitronectin, collagen, and fibronectin, have been printed onto the paper to enhance cell adhesion (Fig. 11a) [5,11,96,97]. Cell

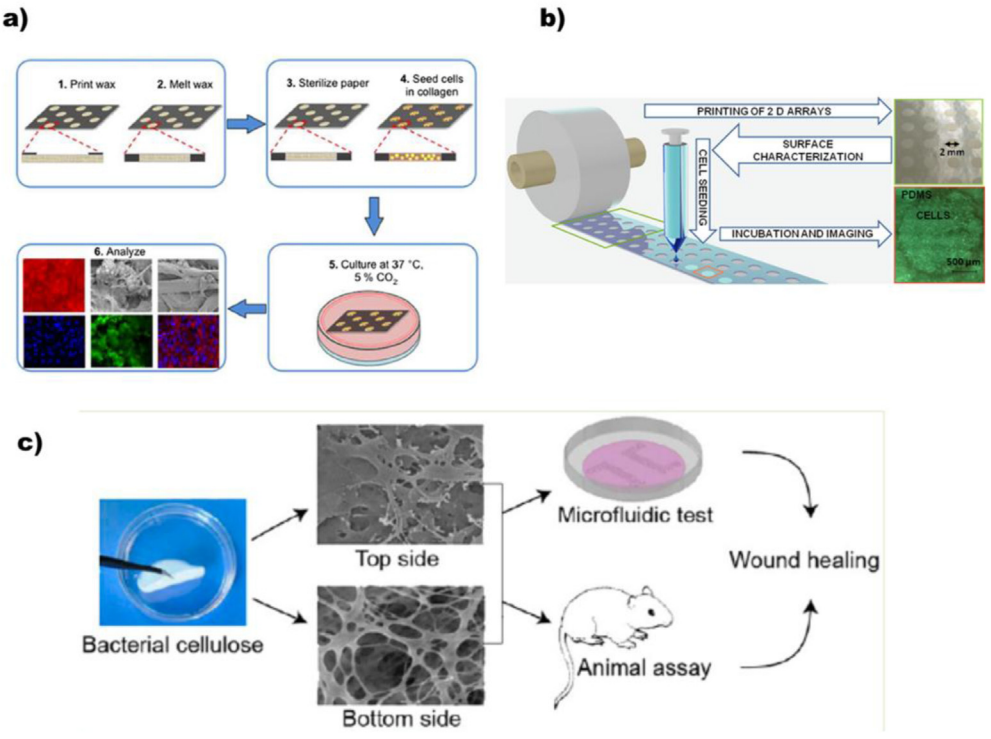

Fig. 11. Cellulose-based scaffolds for cell culture. a) Filter paper was patterned using wax printing, and osteoblasts cells were seeded on the wells of the paper scaffolds in a collagen matrix. After culturing the samples for 21 days, SEM, colorimetric assays, and immunostaining were performed to evaluate the deposition of minerals in the paper scaffolds [5]. b) Arrays for 2D cell cultures using printer paper showed suitable biocompatibility for cell culture of ARPE-19 human retinal pigmented epithelium cells [95]. c) Hierarchical structure of bacterial cellulose films for skin wound healing in Wistar rats [99]. a) Reprinted with permission from Ref. [5]. Copyright 2018, MRS Communications. b) Reprinted with permission from Ref. [95]. Copyright 2013, Elsevier. c) Reprinted with permission from Ref. [99]. Copyright 2015, ACS Publications.

proliferation, migration, alignment, and differentiation can be influenced by the physical cell micro-environment and also by the nanostructure of cellulose-based biomaterials [3]. For example, it has been reported that the paper stiffness can significantly affect the anchorage-dependent cell attachment in terms of cellular behavior, stem cell proliferation, adhesion, locomotion, spreading, morphology, striation, and even differentiation. It has been reported that paper with smoother surfaces have shown improved attachment and growth of epithelial cells, whereas those with a rougher surface are more favorable for the growth, spreading and attachment of osteoblast cells (Fig. 11b) [11,95,98]. These coatings altered the surface properties of paper, including surface roughness, for example, paper with low roughness promotes cell growth of human arising retinal pigment epithelial cell. However, it was demonstrated that hydrophobic materials such as wax and Teflon, have low surface energy, which inhibits cell attachment, acting as a barrier to the direct contact between cells and the desired growth area on paper.

It has been reported that bacterial nanocellulose fibers have good biocompatibility to human cells in 3D cell culture systems [11]. Three-dimensional cell culture models acquire more importance as more reliable *in vitro* model to mimic cell responses [100]. Moreover, 3D cell cultures, for example spheroids, are essential tools of engineering complex tissue constructs and are fundamental in implementing organoids [101]. However, the key limiting of 3D systems is their lack of spatial distribution of oxygen, metabolites, and signaling molecules normally secreted by living tissues. Thus, the size with which they can reasonably be cultured is limited.

To overcome this, a paper support for 3D cell cultures was designed by stacking layers of chromatography paper impregnated with a hydrogel of extracellular matrix and cells. This construct promoted the deposition of cells and allowed oxygen and nutrients diffusion in a 3D cell model [12].

Notably, the paper stacks mimic the layers of tissue, and their disassembling enables its analysis. Moreover, it was reported that human bone marrow HS-5 cells stable transfected with mCherry fluorescent protein could diffuse into the dry paper by capillarity wicking. Confocal microscopy of the cells inside the paper localizes them only in space where the hydrogel drop was spotted. The authors were also able to study the 3D cell arrangement responses to oxygen and nutrient distribution and compared them within the multilayered 3D constructs. The *in vivo* implantation of this hydrogel-based multilayered paper containing Lewis lung carcinoma cells into C57BL mice demonstrated that the paper layers were invaded from cells of surrounding tissues that were mainly hematopoietic (CD45) and endothelial (CD31) cells. Also, neovascularization of the tumor implants was reported. Thus, cancer cells were seeded on this paper-supported hydrogel; their growth resembles the same as that found in both, *in vitro* and *in vivo*. With this it was demonstrated that these multilayer stacked papers are suitable to support 3D cell models to study cells' responses and molecular gradients *in vivo* [12].

Polymeric plasmonic scaffolds are also used to sense metabolites for 3D cell cultures. A composite material made of plasmonic Au NPs embedded in a poly-2-hydroxyethyl methacrylate matrix was used as a framework for osteoblast cell growth and as a plasmonic biosensor to measure *in situ* the metabolic activity of living cells (Fig. 12a) [102]. Specific metabolites associated with the culture's confluence (e.g. gluconolactone, malate, fumarate, histidine, and phenylalanine) were measured by SERS. The data obtained by the spectroscopic studies indicate osteoconductive conditions in the osteoblasts cells seeded on the scaffold. An advantage of this plasmonic embedded NPs system is that it avoids forming a protein corona around the Au NPs [102].

7.4. Paper-based substrates for tissue engineering

Cellulose-based biomaterials are used for artificial skin and wound healing dressings; the ability to tune their mechanical properties allows them to obtain high biocompatibility and improve cell infiltration, proliferation, and angiogenesis activities [3]. It has been reported that the size of the pore in bacterial cellulose allows fabricating looser films with a rougher surface. In fact, cell migration and diffusion into the scaffold is permissible. Using a microfluidics-based *in vitro* wound-healing model demonstrated that the bottom of the bacterial cellulose film promotes the migration of the cells. This improved the wound-healing rate and

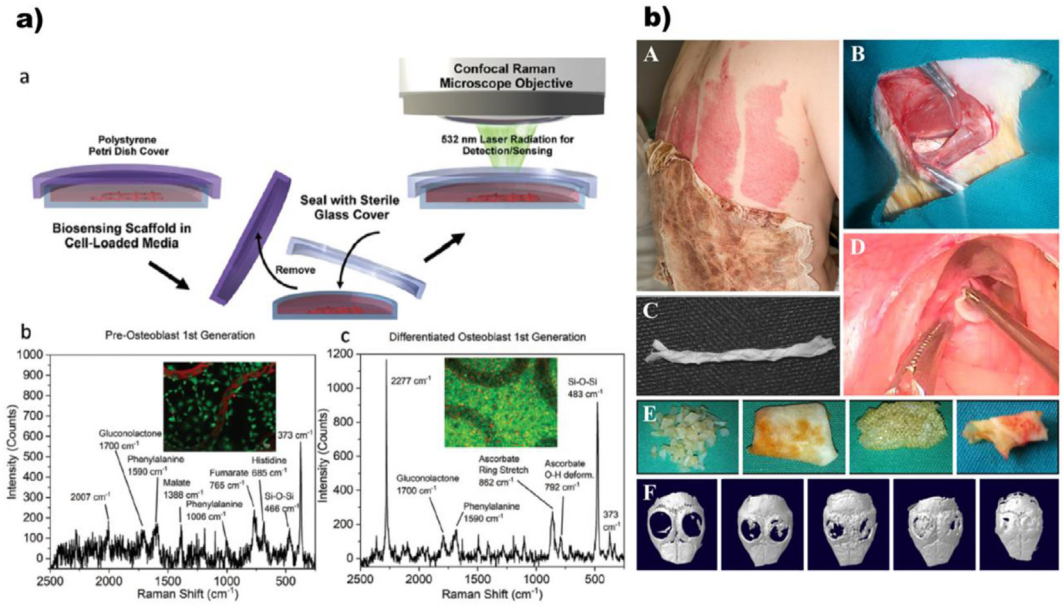

Fig. 12. Applications of cellulose-based scaffolds in tissue engineering. a) (a) Schematic representation of biosensing scaffold in cell-loaded media for SERS, the scattered photons are collected during microscopic sensing by the optical objective. (b)–(c) Differentiation monitoring of prior stage to occurrence of osteoblasts by *in situ* SERS cultured in the plasmonic scaffolds. The Raman peaks of metabolites related to the growth and differentiation process of prior stage to occurrence of osteoblasts are presented. Inset images represent live (green) and dead (red) osteoblasts cells [102]. b) Different applications of cellulose biomaterials for engineering, and tissue regeneration (A) Skin; (B) nerve; (C) tendon/ligament; D) larynx; E) cartilage, and F) bone [3]. a) Reprinted with permission from Ref. [102]. Copyright 2020, Wiley Online Libraries. b) Reprinted with permission from Ref. [3]. Copyright 2019, Frontiers.

reduced inflammatory responses compared with gauze in a skin wound model in Wistar rats [99]. It was also reported that bacterial cellulose film has different properties on distinct sides, demonstrating the feasibility of creating heterogeneous cellulose-based biomaterials for tissue engineering. Notably, the microfluidic chip platform represents a reliable and rapid method to follow wound dressing healing effects (Fig. 11c) [99].

Moreover, the applications of cellulose-based biomaterials for tissue engineering have been reported in skin, nerve, ligament, cartilage and bone replacement (Fig. 12b). Nerve tissue engineering is also taking advantage of the tunable properties of cellulose-based scaffolds, especially for the 3D cell proliferation and differentiation of neurons. A cationized nanocellulose scaffold was used to investigate the effect of surface charges in the cell-cell interactions of PC12 cells. The existence of neurites of approximately 50 μm connected into clusters and individual cells was demonstrated, promoting the initial cell adhesion on the scaffold, starting to form a 3D neuronal network [103].

The potential role of nanocellulose or cellulose nanocrystals in the hydrogel-based paper is known to exhibit low toxicity and ecotoxicological risk [104]. Therefore, the trends in tissue engineering and cell culture scaffolds are led by low-cost biocompatible materials such as cellulose, that favors the formation of hierarchical scaffolds and allow the measurement of metabolites to monitor cellular metabolism, growth, differentiation, and motility.

Finally, it should be remarked the need to wisely design frameworks made of paper together with plasmonic nanoparticles for SERS technology multidetection of metabolites. It is crucial to detect low production of by-products, enzymes, growth factors, cytokines, and other metabolites such as amino acids, oxygen, and nitrogen reactive species. They are crucial to monitor tissue repair and the facile low-cost detection of chronic and metabolic diseases, making the highly specific and sensitive optical SERS spectroscopy an extraordinary tool for their accurate identification.

8. Conclusions and perspectives

The studies performed so far have shown the capability of paper-based substrates for obtaining SERS signal when appropriately combined with plasmonic NPs, either by the *in situ* synthesis or the impregnation of the NPs once synthesized. This additional key property combines with the inherent ability for absorbing fluids through capillary forces of the paper. The 3D network of the cellulose fibers, with its porous structure and large void volume, results very attractive for using paper-based substrates for cell culture applications since it can mimic the cellular architecture allowing cell adhesion, migration, proliferation, and differentiation on this type of scaffolds. Besides, it also allows for physiologically relevant fluid flow, and transfer of oxygen, nutrients and signaling molecules. Moreover, the layered morphology of paper permits its stacking to form 3D scaffolds for cells as it has been previously demonstrated with cells laden in hydrogels within this 3D stacked paper array offering mechanical integrity. Therefore, the paper-based substrate can be considered as a very versatile material and scaffold for mimicking the 3D cellular micro-environment.

These characteristics of the paper would be even more harnessed with the integration of plasmonic NPs within the fibrous structure of paper, taking full advantage as nanosensors for monitoring different cells' cues when combined with a highly specific and sensitive optical tool such as SERS.

Among the methodologies reviewed for obtaining paper-based plasmonic substrates, the impregnation approaches give higher versatility to choose the final plasmonic properties to be conferred to the paper-based substrates. In particular, the paper dipping permits obtaining a very homogenous impregnation with tunable coverage density of the selected NP all along the 3D cellulose fiber network; while the direct calligraphy offers the possibility to easily alternate detection domains with different NPs properties (LSPR or coating functionality) for multiplex SERS detection of two or more target analytes from a complex chemical mixture.

Some of the reviewed examples shed light on how to design these paper-based plasmonic substrates, including the biofunctionalization of NPs with specific recognitions. However, these illustrative cases are not intended to be exhaustive but, on the contrary, to motivate creative designs to be proposed. Proof of it is the huge field that microfluidic paper-based analytical devices (μPADs) could offer by a simple cut and folding when used for cell culture applications.

Similarly, the SERS platforms described for cell culture applications reflect the large field that remains to be explored, showing the possibility of mapping chemical analytes in space, including extracellular metabolites, when combined with appropriate plasmonic substrates that allow the generation of SERS signal. However, not under all scenarios the paper is suitable for cell culture directly due to the lack of cell adhesion moieties. Nonetheless, alternatives such as hydrogels laden with the cells are available; or the difference in smoothness or roughness of various types of papers with different physical and chemical properties can be explored. Additionally, the biofunctionalization of paper with adhesion molecules, growth factors, and peptides is a suitable approach to increase the ability of paper-based cell scaffolds to promote biocompatibility and cell proliferation and differentiation.

This review intends to show the potential that plasmonic paper-based substrates have to become an attractive alternative to conventional biomaterials for cell culture in both 2D and 3D, recreating extracellular environments and allowing to study cell behavior such as growth, cell migration, proliferation, differentiation or tumor metastasis. Therefore, it is necessary to wisely design systems made of paper with plasmonic NPs for detection of metabolites and/or low production of by-products harnessing the optical tool of SERS spectroscopy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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