3D-Printed Biocompatible Scaffolds with Built-In Nanoplasmonic Sensors

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3D printing strategies have acquired great relevance toward the design of 3D scaffolds with precise macroporous structures, for supported mammalian cell growth. Despite advances in 3D model designs, there is still a shortage of detection tools to precisely monitor in situ cell behavior in 3D, thereby allowing a better understanding of the progression of diseases or to test the efficacy of drugs in a more realistic microenvironment. Even if the number of available inks has exponentially increased, they do not necessarily offer the required functionalities to be used as internal sensors. Herein the potential of surface-enhanced Raman scattering (SERS) spectroscopy for the detection of biorelevant analytes within a plasmonic hydrogel-based, 3D-printed scaffold is demonstrated. Such SERS-active scaffolds allow for the 3D detection of model molecules, such as 4-mercaptobenzoic acid. Flexibility in the choice of plasmonic nanoparticles is demonstrated through the use of gold nanoparticles with different morphologies, gold nanorods showing the best balance between SERS enhancement and scaffold transparency. Detection of the biomarker adenosine is also demonstrated as a proof-of-concept toward the use of these plasmonic scaffolds for SERS sensing of cell-secreted molecules over extended periods of time.

1. Introduction

3D cell culture models have emerged as an alternative to more traditional 2D cell cultures, the latter being unable to recreate high cell densities or cell spheroids.[19,20] Accordingly, hydrogel-based inks with high viscosity enable printing of controlled deposition of printed hydrogel filaments.[18] Interestingly, hydrogel-based inks with high viscosity enable printing of high cell densities or cell spheroids.[19,20] The complex in vivo cellular microenvironment.[1] The biological importance of 3D cell culture models is highlighted by the ever increasing number of publications that draw attention to differences in cellular behavior as compared to standard 2D cell cultures, including cell morphology, cell–cell or cell–matrix interactions, as well as cell differentiation, when grown in a 3D model.[2–4] Among the most common materials employed to replicate the 3D environment found in vivo situations, hydrogels mimicking the extracellular matrix are particularly appealing because of their biocompatibility and provision of a porous structural matrix.[5] Hydrogels are typically defined as hydrophilic polymer networks that can retain large amounts of water.[6] Due to their high water content and porous network, hydrogels resemble living tissues and favor nutrient and oxygen transport within the matrix.[5] Moreover, their rich structural chemistry opens the way to diverse synthetic strategies focused on creating adhesion sites to promote cell proliferation, tunable degradation properties to promote tissue growth, or stimuli-sensitive features to modulate their network.[7–10]

From the various methods that can produce 3D structures composed of hydrogels, direct ink writing (DIW) offers an appealing alternative to traditional hydrogel casting techniques, since it allows for a precise geometric control in scaffold design, including the introduction of macropores to create more realistic 3D supports for cell growth.[3,5,11] Recent efforts have focused on the development of hydrogel-based inks for 3D printing, thereby meeting the requirements for reproducible production of self-standing scaffolds, cell-laden vascularized tissues or stimuli-sensitive matrices.[12–17] From a printing compatibility perspective, hydrogels are excellent candidates for the development of biocompatible inks, as they display suitable viscoelastic and non-Newtonian properties, to be extruded out from a nozzle under specific conditions, and to achieve a controlled deposition of printed hydrogel filaments.[18] Interestingly, hydrogel-based inks with high viscosity enable printing of high cell densities or cell spheroids.[19,20]

To truly understand cellular processes taking place in a 3D model, which has been devised to mimic the cellular microenvironment in vivo during disease development or tissue growth, high resolution sensing and imaging techniques are...
also needed.[21] Studying cell-material or cell-cell interactions in a particular environment is crucial to better understand the progression of a disease, such as cancer metastasis,[6,22] while sensing of cell-secreted metabolites could assist in the early detection and monitoring of various diseases.[23] However, spatial and temporal monitoring of released biomarkers remains challenging and requires analytical tools that offer both high penetration depth and high selectivity.

In this context, surface-enhanced Raman scattering (SERS) spectroscopy is an ultrasensitive analytical tool that allows for the label-free detection and imaging of a wide variety of analytes with high specificity and sensitivity.[24] This non-destructive technique provides characteristic vibrational fingerprint information, which is amplified by the interaction of the target molecules with plasmonic metal nanoparticles.[25] The incorporation of plasmonic metal nanoparticles into hydrogel-based materials has been extensively used for the design of photonic hydrogel sensors, following a variety of nano/microfabrication approaches.[26] Regarding 3D printing, plasmonic hydrogel-based inks composed of poly(ethylene glycol diacrylate) and pluronic including Ag nanoparticles have been prepared to create materials with 3D-graded plasmonic properties.[27] 3D-printed gelatin-based bioinks containing gold nanorods were used to prepare functional cardiac tissue constructs.[28] Therefore, we hypothesized that the compatibility of plasmonic nanoparticles with hydrogel matrices possessing suitable printing properties should allow the creation of 3D-printed scaffolds with SERS sensing capability. Since the laser excitation wavelength in SERS can be tuned within the so-called near infrared (NIR) biological transparency window (650–950 nm), light penetration should be favored through biological tissue.[29] SERS analysis can be performed in situ, reaching extremely low detection limits and with multiplexing provided by the characteristic Raman fingerprint of each molecule.[30,31] In view of all these advantages, SERS spectroscopy has become a powerful sensing and imaging technique for biomedical applications.[31–33] Multiplex sensing of targeted cancer biomarkers has been recently demonstrated in vivo, using bioconjugated SERS nanotags and the limitations of spatial and temporal control in animal studies have been circumvented by the development of small-animal Raman imaging instruments.[34,35]

We recently reported the use of SERS to monitor relevant tumor metabolites in microfluidic cell cultures, using nanostuctured plasmonic gold substrates, providing overall information about tumor evolution, but lacking spatial resolution.[36] Aiming at spatial resolution in three dimensions, we propose the use of DIW-3D printing to fabricate SERS-active scaffolds from a composite ink containing plasmonic gold nanoparticles (Scheme 1). We analyzed the influence of nanoparticle shape and surface functionalization on the SERS signal intensity from the scaffolds, by investigating the limits of detection of a model Raman molecule, 4-mercaptobenzoic acid (MBA), in both the lateral (xy) and axial (z) planes. Finally, we evaluated the biocompatibility of the 3D-printed scaffolds and their performance as sensing platforms for target biomolecules such as adenosine, as a proof-of-concept structure for monitoring of cancer biomarkers during tumor growth or metastasis, within 3D models.

Scheme 1. Schematic representation of scaffold preparation including 3D printing of nanoparticle inks, UV crosslinking of methacrylate precursors from hyaluronic acid and physical crosslinking in a CaCl₂ bath.

2. Results and Discussion

To create SERS-active 3D scaffolds, we incorporated gold nanoparticles (NPs) within a mixture of biopolymers that are commonly used in 3D printing, including alginate, methacrylated hyaluronic acid (HAMA) and κ-carrageenan. Specifically, we focused on gold nanorods (AuNRs) and gold nanostars (AuNSs), which were synthesized with different surfactants, so we could also analyze the effect of the coating molecules on the printing parameters, scaffold stability and SERS properties. AuNRs and AuNSs were chosen because of their excellent biostability and SERS enhancing properties under NIR irradiation.

AuNRs and AuNSs were synthesized using hexadecyltrimethylammonium bromide (CTAB) and thiolated polyethylene glycol (PEG-SH) as surface stabilizing molecules, through slight modifications of previously described protocols (see Methods for details). In brief, AuNRs were synthesized by seed-mediated growth, using CTAB as surfactant and 5-bromo salicylic acid and ascorbic acid (AA) as reducing agents, in the presence of AgNO₃.[37] For the preparation of PEG-coated AuNRs, CTAB was exchanged with PEG-SH.[38] AuNSs were prepared using citrate-stabilized seeds in the presence of AgNO₃ and AA, followed by stabilization with PEG-SH.[31] The same procedure was used for the preparation of CTAB-stabilized AuNSs, by simply dispersing them in a CTAB solution.

Considering the well-known cytotoxic properties of CTAB,[39] all NPs were extensively washed to remove excess CTAB, prior to stabilization within the ink formulation. Therefore, four different kinds of NPs were obtained, namely AuNRs and AuNSs in CTAB as well as their PEG-stabilized homologues AuNRs@PEG and AuNSs@PEG. The localized surface plasmon resonances (LSMR) of both AuNRs and AuNSs were tuned through NP morphology, to closely match the excitation wavelength of a 785 nm laser, i.e., within the NIR transparency window typically used for biomedical applications (Figure S1A, Supporting Information). TEM micrographs confirmed the expected morphology and size for both NP types (Figure S1B, Supporting Information).

2.1. Rheology of Composite Inks

Polymer-AuNP composite inks were prepared by dissolving HAMA, alginate and κ-carrageenan (a mixture collectively known as HAMCA) in a dispersion of AuNPs at the selected Au NP concentration. The selection of hydrogel-forming
polysaccharides for ink formulation was based on their well-known printability and biocompatibility. Other factors such as the rapid gelation of alginates upon CaCl₂ cross-linking, the strong elasticity of κ-carrageenan and the rapid photocrosslinking of HAMA, coupled with the ability of hyaluronic acid to regulate various cellular behaviors, also played a decisive role in the selected formulation of the inks.[40,41] Upon complete dissolution of all polymers, ≈0.25 μm fumed silica (4 wt%) was added as a rheology modifier to produce inks with suitable printing properties.[42] HAMCA bioinks were produced, containing AuNRs, AuNSs, AuNRs@PEG, and AuNSs@PEG. In order to print 3D scaffolds with high resolution, the selected inks must ensure continuous ink flow through a small nozzle (0.25 mm) and quick shape recovery, so that filament shape is maintained after printing.[13,43] Hydrogel-based inks were expected to feature shear-thinning behavior during printing, well-defined yield stress, and quick viscosity recovery immediately after printing.[13,43] Prior to UV-light crosslinking, we assessed the rheological properties of all ink formulations, including shear thinning, yield point and viscoelastic modulus at 25 °C. Figure 1A shows flow curves for HAMCA inks with different AuNR concentrations, as the shear rate increases from 1 to 1000 s⁻¹, i.e., within the shear rate range used in DIW. All selected compositions show a pronounced shear-thinning viscosity, decreasing 4–5 orders of magnitude from high to low shear rates, and similar profiles were observed regardless of AuNRs concentration.

These results indicate that the shear-thinning ability of as-prepared inks is mainly determined by the polymer composition of the matrix. Apparent viscosity values measured at both low and high shear rates were within the same order of magnitude for all AuNR concentrations, and within the typical viscosity range for DIW.[44] Strain amplitude sweeps were conducted for all ink formulations, to determine the linear viscoelastic region, where both the storage (G') and the loss moduli (G'') are independent of the applied strain amplitude (Figure S2A, Supporting Information). As can be observed, the (dynamic) yield point increases with AuNR concentration up to a certain limit, and then decreases at AuNR concentrations above 0.5 × 10⁻³ m. This behavior suggests that the incorporation of higher AuNR concentrations does not result in a reinforcement of the matrix. We then performed frequency sweeps at a fixed strain amplitude, previously assessed from a strain amplitude sweep, to study G' and G'' for the different inks, as well as the influence of AuNR loading (Figure 1B). All of the studied ink formulations invariably showed a solid viscoelastic behavior at rest, characteristic of hydrogel-based materials, with G' consistently larger than G'' and both moduli being independent of the applied frequency.[45] G' was found to increase for higher AuNR concentrations, up to 0.5 × 10⁻³ m, and to decrease for higher concentrations (Figure 1C). This behavior is commonly observed in polymer-based nanocomposites, where electrostatic interactions between the polymer and the NPs surface result in a reinforcement of the hydrogel network, as previously reported for gelatin-based hydrogels containing AuNRs.[28,46,47] For lower AuNR loadings, the presence of AuNRs appears to cause a reinforcement of the matrix, but as AuNR concentration is further increased the G' value decreases, being similar to that of AuNR-free controls when AuNR concentration is 1.5 × 10⁻³ m. The observed trend is similar to that previously observed for shear strain measurements, suggesting that a further increase in AuNR content does not lead to reinforcement of the polymer matrix.

In view of these results, we prepared inks in which both nanoparticle morphology and surface coating were varied, incorporating AuNRs and AuNSs, both with CTAB and PEG coatings. In all cases, a fixed concentration of gold (1 × 10⁻³ m) was used. Figure S2B (Supporting Information) displays the flow curves for the different inks, again showing a shear thinning behavior for all samples, regardless of NP shape and coating. Interestingly, the obtained viscosity values were of the same order of magnitude as those recorded for AuNR-containing inks. Strain and frequency sweeps were performed for all formulations, to determine the G’ modulus as a function of NP type (Figure S2C, Supporting Information). We found that, higher G’ values were recorded for ink formulations containing AuNRs compared to AuNSs, whereas the effect of surface coating was not significant. When comparing tan δ values, a measure of hydrogel elasticity obtained from the ratio of the loss modulus (G'’) over the storage modulus (G’), similar values were obtained for different AuNR loadings, indicating a typical gel-like behavior (Table S1, Supporting Information).[48] However, slightly lower values of tan δ (below 0.2) were recorded when the nanoparticle concentration in the ink formulation was 1 × 10⁻³ m. These results indicate that ink elasticity is more strongly affected by the concentration of AuNPs than by their surface functionality. Finally, we studied the recoverability of all NP-containing formulations, aiming at simulating the printing conditions while monitoring the viscosity through alternated
oscillatory and rotational rheology measurements (Figure 2). In a first step, an oscillatory sweep at constant amplitude and frequency was applied to measure the initial viscoelasticity of the ink. Subsequently, a rotational shear step at an applied shear of 10 s\(^{-1}\) was applied to simulate the shear stress experienced by the ink during printing. As a final step, another oscillatory measurement was performed, to test the recovery of the viscoelastic properties after intense shear. All inks showed a rapid recovery, thus demonstrating their ability to maintain a high shape fidelity (Table S1, Supporting Information) by avoiding defects induced by unwanted flows of material. The recovery capacity of the different formulations varied with NP concentration, as inks containing \(1 \times 10^{-3} \text{ M} \) Au NPs showed the highest recovery of the viscoelastic properties (≈80% or higher) after 300 s.

2.2. 3D-Printed Scaffolds

Once the rheological properties of the inks were evaluated, we used the selected inks for 3D printing of composite scaffolds. A RenGenHu Discovery bioprinter with an integrated UV lamp source was used to create a square-shaped design with an inter-linear distance of 0.8 mm and 8 layers (see the Experimental Section for details on scaffold design). After the deposition of each layer, HAMA chains were photo-crosslinked via UV irradiation to provide structural stability during printing. To achieve complete crosslinking, the fully printed scaffolds were finally immersed in a 5% w/v CaCl\(_2\) solution to ensure gelation of alginate and κ-carrageenan (Scheme 1). Shown in Figure 3A are examples of scaffolds printed using HAMCA inks, containing increasing AuNR concentrations. We observed that all such scaffolds showed a concentration-dependent, homogenous purple/brown color, characteristic of nonaggregated AuNRs (which would show a grey color when aggregated inside the scaffolds). Scaffolds printed with AuNS- and AuNS@PEG-loaded inks likewise showed a characteristic blue color, with a similarly homogenous distribution throughout the scaffold (Figure S3A, Supporting Information). All tested inks could be used to print high resolution scaffolds, with an inter-linear spacing of 250 \(\mu\)m in the hydrated state (Figure 3B), which expanded up to 400–500 \(\mu\)m after freeze-drying (Figure 3C and Figure S3, Supporting Information). The presence of nanoparticles within the scaffold was confirmed by TEM analysis of ultramicrotomed sections (Figure 3D), clearly revealing that AuNRs retained their shape within the matrix. Considering that one of our final objectives is biosensing of cell-derived metabolites, we also studied the stability of the scaffolds in complete cell media containing 10% v/v fetal bovine serum (cDMEM), to confirm their suitability for subsequent cell culture studies (Figure 3E). High swelling ratios (SR) were observed for all samples, with a trend of increased swelling when increasing AuNR concentration. The observed reduction in SR at incubation times in cDMEM longer than 24 h was attributed to slow diffusion of divalent ions into surrounding media, which leads to partial dissolution of physically cross-linked polymers within the scaffold. Notwithstanding, significant swelling ratios were recorded after 21 days of incubation, demonstrating high stability in cell media for long incubation periods.

2.3. 2D and 3D SERS Mapping

We initially evaluated the SERS sensing efficiency of the plasmonic scaffolds, using 4-mercapto benzoic acid (MBA) as a model Raman-active molecule. MBA is a thiolated molecule that readily adsorbs onto gold NP surfaces, thereby revealing its characteristic SERS fingerprint.[29] The scaffolds were therefore immersed in a solution of MBA (1 \(\times 10^{-3} \text{ M}\)) and 2D maps of the top surface were recorded, to study the effect of AuNR concentration on the SERS signal intensity. Representative maps are shown in Figure 4A, based on the integration of the 1083 cm\(^{-1}\) peak, characteristic of the ring breathing deformation mode of MBA.[29] As expected, higher SERS signal intensity was obtained for inks with increasing AuNR concentrations, in all cases revealing the grid-like structure of the scaffold.
when low-magnification tile-like maps were scanned. The homogeneous distribution of MBA within the scaffolds was confirmed, with higher SERS signals observed at the edges of the vertical holes, likely due to favored absorption of MBA molecules by the hydrogel at those areas in closer contact with the solution. Although this trend was maintained up to a AuNR concentration of $1 \times 10^{-3} \text{ M}$, a further increase in AuNR loading ($1.5 \times 10^{-3} \text{ M}$) was found to hinder the SERS signal intensity, presumably due to the corresponding decrease in the transparency of the scaffold. For determination of the detection limit for MBA, we selected a constant AuNR concentration ($1 \times 10^{-3} \text{ M}$), while gradually reducing MBA concentration (Figure 4B and Figure S4, Supporting Information). The lowest concentration that could be reliably distinguished using these scaffolds was

![Figure 3](image_url)

**Figure 3.** A) Photographs of 3D printed scaffolds (1 cm x 1 cm) containing AuNRs at different concentrations. B) Epifluorescence microscopy image of a hydrated 3D-printed scaffold. C) SEM image of a freeze-dried scaffold. D) Representative TEM image of an ultramicrotomed slice, showing the presence of isolated AuNRs within the interior structure of the scaffold (arrows point to the location of AuNRs). E) Swelling studies in cDMEM cell media at 37 °C.

![Figure 4](image_url)

**Figure 4.** A) 2D maps of the SERS intensity at 1083 cm$^{-1}$, recorded from printed scaffolds with varying AuNR concentrations: i) $0.1 \times 10^{-3} \text{ M}$; ii) $0.5 \times 10^{-3} \text{ M}$; iii) $1 \times 10^{-3} \text{ M}$; iv) $1.5 \times 10^{-3} \text{ M}$ (scale bars = 200 µm). B) Average SERS spectra obtained from maps of the scaffolds incubated with different concentrations of MBA. C) Average SERS spectra from maps of scaffolds containing AuNRs and AuNSs with different surface functionality, using $1 \times 10^{-3} \text{ M}$ MBA.
100 × 10⁻³ m, which lies within the range of metabolite concentrations typically observed in biological environments, such as the tumor microenvironment.

Although elasticity measurements (see above) indicated that the surface coating on AuNPs has a negligible effect on the rheological properties of HAMCA inks, the accessibility of the analyte molecules to the plasmonic gold NP surface is likely to hinder the SERS efficiency when NPs are stabilized with PEG. We therefore recorded 2D SERS maps for scaffolds containing AuNRs, AuNSs, AuNRs@PEG and AuNS@PEG. In all cases, the Au concentration was set at 1 × 10⁻⁵ m AuNRs, AuNSs, AuNRs@PEG and AuNS@PEG. In all cases, the Au concentration was set at 1 × 10⁻⁵ m, which resulted in higher SERS signals for AuNRs (Figure 4A). As shown in Figure 4C, the MBA SERS signal intensity varied considerably when changing both nanoparticle shape and surface functionalization. In particular, the SERS signal from scaffolds prepared with PEG-coated particles was insufficient to reveal the macrostructure of the scaffold (Figure 4C and Figure S5, Supporting Information). The obtained maps revealed the internal structure of the scaffold, with black areas indicating the location of vertical holes, while a continuous signal was obtained when following the horizontal line along the printed scaffold. We were thus able to distinguish between different parts of the scaffold, as well as to study the SERS signal intensity at specific regions of interest. A higher resolution map was finally obtained from a smaller area of the scaffold at different heights, by recording a point spectrum every 50 µm (z) for 400 µm (x) (Figure 5D), to better estimate the depth of the signal. Reliable SERS signals were detected up to a depth of 1500 µm, confirming the results shown in Figure 5A.

After successful demonstration of the sensing properties of our composite scaffolds for a standard SERS analyte, we investigated a biologically relevant molecule, namely adenosine. Adenosine has been reported to play an important role in the development of primary tumors and metastases, and is currently being used as a cancer biomarker. Compared to MBA, we noted two important aspects with regard to SERS detection. First, a longer incubation time was required, most likely due to the lack of thiol groups in adenosine or other functional groups with high affinity for gold surfaces. Second, the diffusion of adenosine within the scaffold was slower and longer incubation times were needed to detect a SERS signal from the scaffold, likely due to its higher molecular weight compared to MBA. The scaffolds were incubated in aqueous solutions with three different concentrations of adenosine: 10 × 10⁻⁶ m, 100 × 10⁻⁶ m, and 1 × 10⁻³ m, for increased incubation times up to 1 week (Figure 6 and Figure S8, Supporting Information). The corresponding
SERS maps were obtained by monitoring the intensity of the 735 cm⁻¹ band, which is characteristic of the adenine base. We observed that, after 12 h of incubation, the SERS signal intensity was similar for all adenosine concentrations. However, after longer incubation times the scaffold incubated with 100 × 10⁻⁶ M adenosine showed the highest SERS signal. The dependence of the SERS signal on analyte concentration can be understood on the basis of previous reports. Whereas at low adenosine concentrations not enough molecules are present to cover the surface of AuNRs, at 100 × 10⁻⁶ M adenosine concentration, more scattering molecules are present and an increased signal is registered. The SERS intensity increases with adenosine concentration up to a saturation point, where a maximum is achieved, due to maximum contribution of both electromagnetic and chemical enhancements. A further increase of adenosine concentration up to 1 × 10⁻³ M, above the saturation point, causes a decrease of the SERS signal intensity due to the formation of additional layers of adenosine on the particles. In view of the application of these scaffolds for in situ SERS sensing of cell-secreted metabolites in 3D cell culture, control experiments were performed after incubating the scaffolds, both in the presence of MBA and adenosine, in cDMEM (Figure S8, Supporting Information). Scaffold incubation in cell culture media was found to reduce the SERS signal by roughly 7-fold in the case of MBA, and 5- and 3-fold in the case of adenosine incubated for 24 and 48 h, respectively. The presence of proteins in cDMEM is likely to hinder the contact of the analyte with the gold surface and, thus, lower SERS signals were recorded when compared with the same incubation times in aqueous solution, but still sufficiently high to allow detection. The intensity of the SERS signals was observed to increase with incubation time in the case of adenosine, indicating that longer incubation times may be required for analyte detection under realistic conditions.

2.4. Cell Growth within 3D Scaffolds

Application of the hybrid plasmonic scaffolds to the detection of biologically relevant analytes over time requires biocompatibility with mammalian cells. We thus tested the scaffolds with the cervical cancer cell line HeLa, through evaluation of cell viability after incubation, as well as investigation of cell growth over time inside the 3D scaffold. As all materials comprising the HAMCA inks are biocompatible, we assumed that cytotoxicity could only be possibly observed due to the incorporation of AuNRs coated with CTAB, since residues may remain even after extensive washing. Our results however, do not show any significant effects on cell viability from scaffolds printed with bioinks, regardless of the presence of AuNRs in their composition (Figure S9A,B, Supporting Information). We also performed a growth test with bacterial agar plates, to ensure that scaffolds remained sterile after incubation in cDMEM for 96 h (Figure S9C, Supporting Information).

We subsequently studied the growth of live HeLa cells within the scaffold, by means of confocal microscopy. HeLa cells were visualized by fluorescent labeling with a commercial Cell Tracker fluorophore, prior to seeding in the scaffolds. Figure 7

![Figure 6. A) Average SERS spectra from SERS maps of scaffolds containing 1 × 10⁻³ M AuNRs and incubated in solutions containing increasing concentrations of adenosine. B) SERS maps (intensity at 735 cm⁻¹) of scaffolds containing 1 × 10⁻³ M of AuNRs incubated in 100 × 10⁻⁶ M adenosine for different incubation times (scale bars: 500 µm).](image-url)
shows z-stacks of the scaffolds (in green, imaged with reflection microscopy) and cells (in red, imaged by fluorescence microscopy), as well as a 3D projection of the z-stacks. We could clearly observe that cells stick along the scaffold, preferably around macropores resulting from the 3D printed pattern. 2D tiles of the scaffolds were also performed to obtain a more general view of the whole scaffold (Figure S10, Supporting Information).

3. Conclusions

Plasmonic composite hydrogel-based scaffolds were obtained by incorporating AuNRs within a combination of natural polymers, showing a great potential for 3D SERS biosensing. The hybrid inks meet the basic requirements for the preparation of 3D scaffolds for cell culture, such as high biocompatibility, suitable rheological properties and stability in cell culture media. Using MBA as a model Raman-active molecule, we analyzed the SERS efficiency of scaffolds containing different nanoparticle concentrations, with varying shape and surface coating. Scaffolds containing AuNRs without PEG coating produced the highest SERS signal and their performance was better than AuNSs or AuNS@PEG-containing scaffolds. Both lateral (xy) and axial (z) SERS maps revealed the characteristic grid-like pattern of the 3D printed scaffolds, capable of sensitive detection of Raman-active molecules. Spatially controlled SERS detection was dependent on both AuNR and analyte concentrations, with detection limits for MBA and adenosine down to 100 × 10⁻⁹ m and 10 × 10⁻⁶ m respectively. Finally, cell studies confirmed scaffold biocompatibility and their ability to support cell-growth. We demonstrated the potential of these plasmonic hybrid scaffolds for 3D SERS sensing of biorelevant analytes and we expect that these results will be extended for the detection of a vast array of relevant biomarkers in more complex in vitro 3D cell models designed toward tumor or tissue growth.

4. Experimental Section

Materials: Hexadecyltrimethylammonium bromide (CTAB, ≥99.0%), 5-bromosalicylic acid (5-BrSA, technical grade, 90%), hydrogen tetrachloroaurate trihydrate (HAuCl₄·3H₂O, ≥99.9%), silver nitrate (AgNO₃, ≥99.9%), L-ascorbic acid (AA, ≥99%), sodium borohydride (NaBH₄, ≥99%), hyaluronic acid sodium salt from Streptococcus equi (MW ~1.5–1.8 × 10⁶ Da), alginic acid sodium salt, fumed silica (0.2–0.3 µm average particle size), (2-hydroxy-4′-(2-hydroxyethoxy)2-methylpropionophenone) (98%), methacrylic anhydride (MA, 94%), LB broth, and agar, were all purchased from Sigma Aldrich. κ-carrageenan was purchased from Fisher. α-Mercapto-ω-aminopropyl polyethylene glycol hydrochloride (HS-PEG-NH₂, MW 5000 Dalton) was purchased from Rapp Polymere. Cell Tracker Deep Red, DMEM, fetal bovine serum (FBS), and penicillin-streptomycin (PS) were purchased from Invitrogen. FBS and PS were used at 10% and 1% respectively to prepare complete DMEM (CDMEM). The lactate dehydrogenase (LDH) kit was purchased from Pierce (Thermo). The MTT kit was purchased from Roche. All chemicals were used as received. Milli-Q water was used in all experiments. All glassware used for AuNRs synthesis was washed with aqua regia, rinsed with water and dried before use.

Nanoparticle Synthesis: AuNRs were prepared following a well-established procedure. First, seeds were prepared following the standard CTAB/NaBH₄ procedure at 30 °C. 25 µL of a 50 × 10⁻⁹ m HAuCl₄ solution was added to 4.7 mL of 0.1 m CTAB solution and the mixture was stirred for 5 min. Then, 300 µL of a freshly prepared 10 × 10⁻³ m NaBH₄ solution was injected under vigorous stirring. The seeds were kept for 30 min at room temperature until excess borohydride was consumed. For the preparation of AuNRs, 45 mg of 5-BrSA was added to 25 mL of 0.1 m CTAB. After complete dissolution of 5-BrSA, 480 µL of 0.01 m AgNO₃ was added and the solution stirred for 15 min at room temperature. Then, to perform the pre-reduction of Au (III) to Au (I), 500 µL of 50 × 10⁻³ m HAuCl₄ solution was added to the mixture. Pre-reduction was monitored by UV–vis spectroscopy until the value of the absorbance at 396 nm was 0.8–0.85, to obtain AuNRs with a longitudinal plasmon band around 800 nm. At this time, 130 µL of 100 × 10⁻³ m AA solution was added and the solution turned colorless within a few seconds. After 30 s, 80 µL of seed solution was added under vigorous stirring. After 30 s, the stirring was stopped and the mixture was left undisturbed for 4 h. The resulting solution was centrifuged at 8000 rpm for 30 min to purify the AuNRs. The precipitate containing the
particles was collected and dissolved in hot concentrated CTAB solution (300 × 10^{-3} m). The solution was then placed in a glass tube and the AuNRs precipitated upon cooling. The precipitate was collected and the AuNRs were redispersed in 1 × 10^{-3} m CTAB solution. Following this procedure, AuNRs with a LSPR of 788 nm, 56.5 ± 4.5 nm length and 17.3 ± 3.8 nm width were obtained.

To obtain PEG coated AuNRs, CTAB was exchanged with PEG-SH following a reported procedure.[56,57] As synthesized AuNRs were centrifuged at 8000 rpm for 40 min and redispersed in 50 mL 1 × 10^{-3} m CTAB. The sample was then centrifuged again and dispersed in water. To this mixture ([Au] = 240 μg mL^{-1}, 330 μL of 0.1 × 10^{-3} m PEG-SH solution was added dropwise, under vigorous stirring, and left for 2 h. The solution was then washed by centrifugation (8000 rpm, 40 min) and redispersed in water. The LSPR of AuNRs@PEG slightly shifted to 780 nm.

AuNSs with LSPR maximum at 820 nm were prepared following a published procedure with some modifications.[57] Seeds were prepared by adding 25 mL of 34 × 10^{-3} m citrate solution to 500 mL of boiling 0.5 × 10^{-3} m HAuCl₄ under vigorous stirring. After 15 min, the reaction was cooled down and the solution stored at 4 °C. Then, 50 μL of citrate-stabilized seed solution was added to 10 mL 0.25 × 10^{-3} m HAuCl₄ (with 10 μL HCl 1 m) at room temperature under moderate stirring. Immediately, 100 μL of 3 × 10^{-3} m AgNO₃ and 50 μL of 100 × 10^{-3} m ascorbic acid were added simultaneously. The solution turned to green-blue color, indicating AuNSs formation. Then, 150 μL of 0.1 × 10^{-3} m PEG-SH was added and the solution was stirred for 15 min. The solution was centrifuged at 2000 rpm for 10 min and redispersed in water. The size of AuNSs was 46.7 ± 6.3 nm (core diameter), 28.2 ± 6.2 nm tip length.

For the preparation of CTAB-coated AuNSs, the same procedure was followed (no PEG-SH added). After AuNSs formation, the solution was added dropwise to 30 mL of CTAB 0.1 m and stirred for 1 h at room temperature. Then, the solution was centrifuged at 2000 rpm for 10 min and redispersed in CTAB 1 × 10^{-3} m.

**Nanoparticle Characterization:** TEM images were collected using a transmission electron microscope JEOL JEM-1400PLUS operating at 120 kV. A solution drop was deposited on a carbon film copper grid and dried under ambient conditions. UV–vis optical extinction spectra were recorded using an Agilent 8453 UV–vis diode array spectrophotometer.

**Hyaluronic Acid Methacrylation:** Methacrylation of hyaluronic acid was carried out following a reported procedure.[56,57] Hyaluronic acid (500 mg) was dissolved overnight in 50 mL of Milli-Q water. After complete dissolution of the polymer, the solution was placed in an ice bath and the pH of the solution was adjusted to 8, using 0.5 m NaOH. Methacrylhydridine was then added in 20-fold molar excess under vigorous stirring and the pH was readjusted to 8, using 5 m NaOH. The reaction proceeded for 24 h at room temperature. After that time, the polymer was precipitated in 500 mL of ethanol under stirring and dialyzed against Milli-Q water for 4 days using cellulose dialysis membranes (MW cut off = 14 kDa) to remove any unreacted reagent. The product, HAMA, was recovered after freeze-drying of the dialyzed solution.

**Ink Preparation:** Different ink compositions were prepared by varying the concentration of AuNRs. Prior to using the AuNRs suspension, the particles were gently washed to remove excess CTAB. Therefore, 1 mL aliquots of AuNRs suspension were centrifuged at 5000 rpm for 10 min. The supernatant was then removed and replaced with 1 × 10^{-3} m CTAB solution. Subsequently, the AuNRs were centrifuged again under the same conditions and re-suspended in Milli-Q water for further use. As different AuNR concentrations were employed, the concentration was adjusted during this cleaning step to achieve concentrations of 0.1, 0.5, 1, and 1.5 × 10^{-3} m of AuNRs. HAMA (1 wt%), alginate (1 wt%), and κ-carrageenan (2 wt%) were dissolved in a AuNRs suspension and thoroughly mixed using a ThinMix Mixer at 3500 rpm for 1 min. Upon complete polymer dissolution, fumed silica (4 wt%) and 2-hydroxy-4′-(2-hydroxyethoxy) 2-methylpropionophenone (0.25 wt%) were added and mixed again (3500 rpm, 1 min). For inks containing AuNRs@PEG, AuNSs and AuNSs@PEG, the same procedure was followed dissolving the polymers in a 1 × 10^{-3} m nanoparticles dispersion. A control sample without AuNRs was prepared following the same procedure, but dissolving the polymer mixture in Milli-Q water. Samples were stored at 4°C before usage.

**3D Printing of Scaffolds:** A multi-headed 3D Discovery bioprinter (RegenHU, Switzerland) was used. For all compositions, a high precision plunger dispenser was used at a constant volume flow rate of 2 μL s^{-1} and a stainless steel needle with an inner diameter of 0.25 mm. All samples were printed at room temperature. The G-code for square scaffolds was produced using BIOCAD software (RegenHU, Switzerland) with 0.8 mm spacing between fibers and an initial height of 300 μm. The collector plate speed was set at 20 mm s^{-1} and after each layer was deposited, HAMA was cross-linked in situ using the light curing kit cartridge at 365 nm (output power of 500 mW). Once printing was completed, the scaffolds were immersed in a 5% w/v calcium chloride (CaCl₂) solution for 5 min.

**Scaffold Characterization:** Rheological properties were characterized using a Physica MCR 302 rheometer (Anton Paar, Spain). All tests were carried out in triplicate at 25 °C using 25 mm parallel plate geometry and a solvent trap to prevent water evaporation. First, the inks were subjected to amplitude sweeps to determine the linear viscoelastic region of each composition. To determine the storage and loss moduli of the different samples, frequency sweeps were carried out from 0.1 to 100 rad s^{-1}, at a fixed strain determined from the amplitude sweeps. Shear stress and viscosity were measured for shear rates from 0.01 to 1500 s^{-1} of 100 × 1 mm. Oscillatory-rotational-oscillatory tests were performed to characterize the materials recovery behavior by monitoring the material at rest for 120 s, followed by a shear rate of 10 s^{-1} for another 120 s and finally monitor materials at rest for another 300 s. The shear rate for these experiments was estimated by applying the power law model, used for non-Newtonian fluids, to the data obtained from the flow curve as shown in Equation (1)

\[ n = K \gamma^{m-1} \]  

(1)

where \( n \) is the viscosity of the material, \( \gamma \) corresponds to shear rate, \( K \) is the consistency index and \( n \) is the flow index.[56] From the obtained \( n \) value, the shear rate was calculated using Equation (2) proposed by Li et al.[59]

\[ \gamma \text{m} = \left( \frac{n}{3n+1} \right)^{\frac{1}{m-1}} -1 \]  

(2)

where \( V \) is the flow rate of the extruded hydrogel in the nozzle, \( R \) is the inner radius of the nozzle, and \( r \) is a radial position between 0 and \( R \).

Structural characterization was carried out to study the dispersion of AuNRs within the polymer matrix and the microstructure of the printed scaffolds. Scanning Electron Microscopy (SEM) experiments were performed by a JEOL JSM-6490LV operating at an accelerated voltage of 15 kV and at a working distance of 15 mm. Wet scaffolds were also imaged using an inverted epifluorescence microscope (Axio Observer, Zeiss). TEM images of the scaffolds were collected using a transmission electron microscope JEOL JEM-2100F UHR. Samples were prepared by embedding the scaffolds in an epoxy resin and 100–500 nm samples were cut with the help of an ultramicrotome.

The swelling capacity and stability of the as-prepared hydrogels was studied by a general gravimetric method. Samples (n = 3) were incubated at 37 °C in cell culture media (cDMEM) and at selected time intervals the swollen hydrogels were removed, the excess of liquid absorbed with filter paper and the scaffold weighed. The swelling ratio (SR) was calculated using Equation (3)

\[ SR = \frac{(W_s - W_i) / W_i}{100} \]  

(3)

where \( W_i \) is the weight of the swollen sample and \( W_s \) is the weight of the dried hydrogel sample.

**SERS Measurements:** Raman spectra were acquired using an inVia Renishaw Raman microscope, excited through a 50x Leica objective with a 785 nm diode laser at 10% power for 10 s. Each scaffold was swollen in a 1 × 10^{-3} m 4-mercaptopentobenzoic acid (MBA, Sigma Aldrich) for 30 min.

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The scaffold was then placed on a quartz slide and covered with a drop of water to prevent drying during the measurement. Scaffolds were also incubated in MBA and adenosine solutions of varying concentrations, from $100 \times 10^{-9}$ M to $1 \times 10^{-3}$ M for MBA; $10 \times 10^{-6}$ M, $100 \times 10^{-6}$ M, and $1 \times 10^{-3}$ M for adenosine. In the case of adenosine, the incubation time was increased to 12 h. Control experiments were conducted by incubating the scaffolds in cDMEM. For MBA detection, the scaffolds were first incubated in cell media for 12 h and subsequently incubated in $1 \times 10^{-3}$ M MBA solution for 30 min. For adenosine detection, the incubation with the analyte was performed for 24 and 48 h ($1 \times 10^{-3}$ M adenosine in cDMEM).

For z measurement analysis, scaffolds prepared with $1 \times 10^{-3}$ M AuNRs were excited through a 40x Leica immersion objective with a 758 nm diode laser at a 50% power. Raman spectra were taken in reflection imaging (561 nm excitation, shown in green) and cells with 633 nm excitation (shown in red). A Plan-Apochromat x10 objective (0.45 NA) was used for z-stacks and tile scans. Post image processing included a median 3-pixel filter and contrast and brightness changes.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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