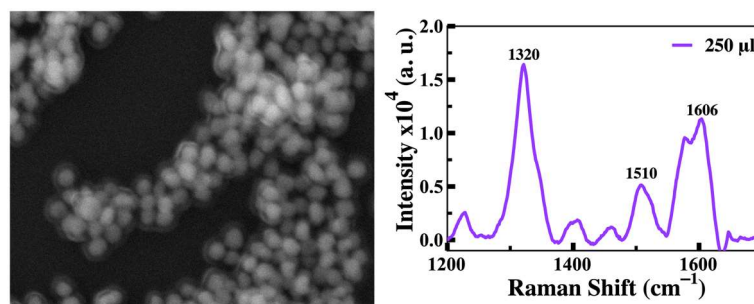


**Abstract:** Functionalized Au and Ag nanoparticles (NPs) with ascorbic and tannic acid, respectively, were used as SERS substrates (SS). Several SS were fabricated with different loads of metal NPs deposited on silicon wafers. We focused on the thyroxine (T4) band at  $1044\text{ cm}^{-1}$  and tracked its intensity and position at concentrations from 10 pM to 1 mM. For all SS, the band intensity decreased as the T4 concentration decreased. Additionally, the band shifted to larger wavenumbers as the NP loads increased. In the case of Au, the SS with the highest load of NPs, the minimum concentration detected was  $1\text{ }\mu\text{M}$ . The same load of the Ag NP SS showed a better performance detecting a concentration of 10 pM, an outcome from a SERS-EF of  $10^9$ . The NP spatial distribution includes mainly isolated NPs, quasi-spherical clusters, and semi-linear arrays of NPs in random orientations. From the numerical simulations, we conclude that the hot spots at the interparticle gaps in a linear array of three NPs are the most intense. The Ag NP SS demonstrated good sensitivity, to allow the detection of pM concentrations. Therefore, its complementation to any immunoassay technique provides an interesting alternative for point-of-care implementations, such as test strips.

**Keywords:** thyroxine quantification; functionalized nanoparticles; load of nanoparticles; wavenumber shift in SERS; hot spots

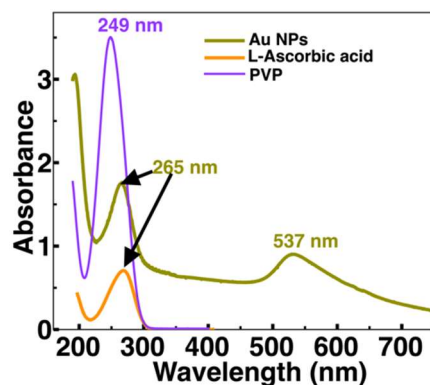
### Supplementary Materials:

The right panel of Figure S1 shows the Raman spectrum of Ag NPs, the bands located at 1320, 1510, and  $1606\text{ cm}^{-1}$  are assigned to the vibrational modes of the C-H, O-H, C-O bonds of the tannic acid [52]. The presence of these bands in the Ag NPs spectrum corroborates its functionalization with the acid.

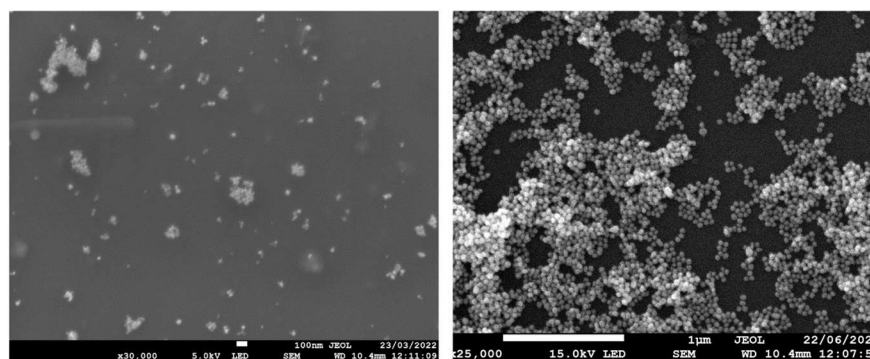


**Figure S1.** The left panel shows a SEM image of Ag NPs with a volume concentration ( $V_{NP}$ ) of  $250\text{ }\mu\text{l}$  at  $\times 160,000$  magnification. The presence of the functionalizing agent (tannic acid) surrounding the Ag NPs is seen as a transparent layer. The right panel shows the Raman spectrum of Ag NPs.

Figure S2 shows the absorbance spectra of Au NPs, L-ascorbic acid, and PVP. The Au NP colloidal solution spectrum shows the surface plasmon band at 537 nm and other optical bands at 265 nm and below 200 nm. The L-ascorbic acid spectrum shows one band in 265 nm. Besides, the spectrum of PVP shows one band in 249 nm. Both, L-ascorbic acid and PVP, were used during the synthesis of Au NPs. After four washes the band at 265 nm remains in the spectrum of Au NPs NP colloidal solution. Therefore, we conclude that the L-ascorbic acid is functionalizing the Au NPs.



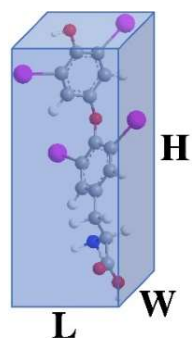
**Figure S2.** Absorbance spectra of Au NPs, L-ascorbic acid, and PVP colloidal solutions.



**Figure S3.** The left panel shows the amount of Ag NPs deposited on a section of Si wafer with a  $V_{NP}=50\ \mu\text{l}$  and the right panel with  $V_{NP}=250\ \mu\text{l}$ . For comparative purposes, an area of the wafer of the same size ( $2.7\ \mu\text{m} \times 3.6\ \mu\text{m}$ ) is shown in both panels. The area shown is smaller than the laser spot area ( $12.56\ \mu\text{m}^2$ ).

#### **$N_{\text{Raman}}$ and $N_{\text{SERS}}$ Determination**

For  $N_{\text{Raman}}$  determination, we assumed a uniform deposit of T4 on the Si wafer (SW) surface. We also assume each molecule occupies a volume of a rectangular prism (Figure S4). For the determination of the rectangular prism volume, ChemDraw ultra 12.0 software was used. A height (H) of  $11.47\ \text{\AA}$  (1.147 nm), a width (W) of  $8.37\ \text{\AA}$  (0.837 nm), and a length (L) of  $8.06\ \text{\AA}$  (0.806 nm), were obtained. The base area ( $L \times W$ ) occupied by one T4 molecule in a vertical position is  $L \times W = 67.46\ \text{\AA}^2$  ( $0.6746\ \text{nm}^2$ ).



**Figure S4.** Thyroxine molecule occupying a volume of a rectangular prism, with  $H=11.47 \text{ \AA}$ ,  $W=8.37 \text{ \AA}$ , and  $L=8.06 \text{ \AA}$ . The carbon, hydrogen, nitrogen, oxygen, and iodine atoms are distinguished using the CPK coloration convention.

The SW area used to fabricate the SERS substrate is  $1 \text{ cm}^2$  ( $1 \times 10^{14} \text{ nm}^2$ ), therefore the maximum number of T4 molecules in the vertical position covering the Si area is  $N_{T4, Si} = 1.48 \times 10^{14}$ . For each T4 concentration the number of molecules,  $N_c$ , can be larger or smaller than  $N_{T4, Si}$ . Then, we assume that layers of vertical-aligned molecules are stacked on the SW surface. Each complete layer has  $N_{T4, Si}$  T4 molecules and a thickness equal to  $H=11.47 \text{ \AA}$ . The number of layers ( $N_L$ ) for each T4 concentration was estimated simply dividing  $N_c$  by  $N_{T4, Si}$ . Therefore, an array of  $N_L$  layers has a thickness given by  $N_L$  times  $H$ .

Table S1 contains the studied T4 concentrations from 10 pM to 1 mM,  $N_c$ ,  $N_L$ , thickness of the molecule-layer array and  $N_{Raman}$ . For  $N_{Raman}$ , only the molecules of T4 under the laser excitation (spot area of  $12.5 \times 10^6 \text{ nm}^2$ ) have been taken into account. The  $N_{Raman}$  value was calculated by using the next expression:

$$N_{Raman} = \frac{N_L \times (\text{laser spot area})}{L \times W} = \frac{(N_c)(\text{laser spot area})}{\text{area of Si wafer}} \quad (1)$$

Note that for T4 concentrations lower or equal than  $1 \text{ }\mu\text{M}$ , the number of T4 molecules is insufficient to create a uniform monolayer on the Si wafer, so an extreme case was assumed: all molecules are under the laser spot and they are vertically oriented. Then, the  $N_{Raman}$  was determined by

$$N_{Raman} = \frac{N_c \times (L \times W)}{\text{laser spot area}} \quad (2)$$

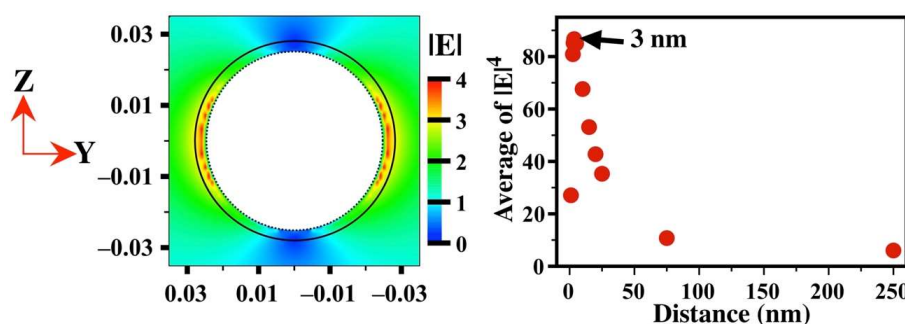
**Table S1.** T4 concentration, number of molecules in each T4 solution concentration ( $N_c$ ), number of molecule-layers on the Si wafer ( $N_L$ ), thickness of the multi-layer array, and  $N_{Raman}$  value calculated by using eq. (1) or (2).

T4 Concentration	$N_c$ = number of molecules of T4	$N_L$ =Number of molecule-layers	Thickness (nm)	$N_{Raman}$
10 pm	$3.01 \times 10^8$	$2 \times 10^{-6}$	-	16.2
1 nM	$3.01 \times 10^{10}$	$2 \times 10^{-4}$	-	$1.62 \times 10^3$
10 nM	$3.01 \times 10^{11}$	$2 \times 10^{-3}$	-	$1.62 \times 10^4$
20 nM	$6.02 \times 10^{11}$	$4 \times 10^{-3}$	-	$3.24 \times 10^4$

1 $\mu$ M	$3.01 \times 10^{13}$	$2 \times 10^{-1}$	-	$1.62 \times 10^6$
10 $\mu$ M	$3.00 \times 10^{14}$	2	2.3	$3.78 \times 10^7$
0.05 mM	$1.50 \times 10^{15}$	10	11.7	$1.88 \times 10^8$
0.10 mM	$3.00 \times 10^{15}$	20	23.4	$3.78 \times 10^8$
0.30 mM	$9.00 \times 10^{15}$	61	70.1	$1.13 \times 10^9$
1.00 mM	$3.01 \times 10^{16}$	203	233.7	$3.78 \times 10^9$

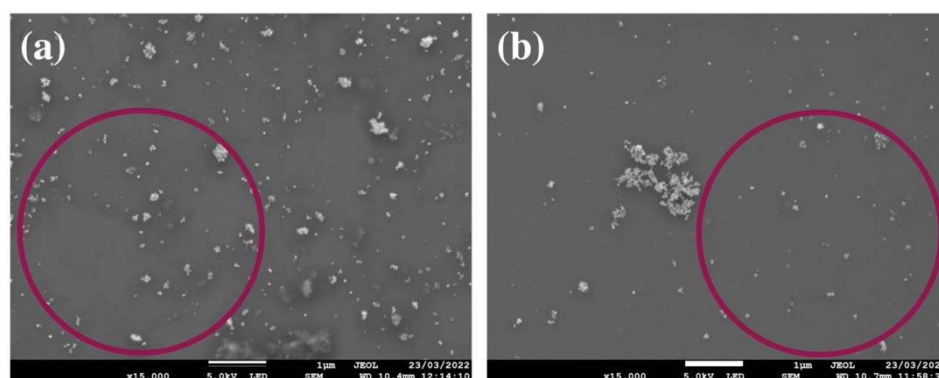
To calculate NSERS, we assumed a homogeneous deposit of T4 molecules on the NP surfaces. Assuming that molecules that are in the close vicinity of the metal surface will be excited more efficiently by the electric field of the NP, the next question arises What is the distance that defines “in the close vicinity”? To answer this, we have calculated the electric field at several distances away from the surface of a NP. On the left of Figure S5, the electric field intensity of a spherical Au NP is shown. In the numerical simulation, we included an incident plane wave with a wavelength of 632 nm, with a polarization state in the Y-axis, and propagating direction along the X-axis. As we can see, the field intensity decreases as we move away from the surface. Besides, due to the dipole configuration, the electric field intensity is not uniform over the whole spherical surface at a particular distance. To highlight this fact, we have drawn a circle (solid line) a few nm away from the NP surface (dotted line). The annular area enclosed between those circumferences contains hot spots and spots with lower intensities. We have calculated the average intensity for annular regions with different thicknesses (representing distances away from the metal surface). Plot shown on the right of Figure S5 indicates a decrement in average intensity as the annular region becomes thicker. The annular region with a thickness of 3 nm gives the maximum average value.

Based on the above analysis, we consider that only molecules that are at most 3 nm away from the metal surface contribute the most to the SERS signal.



**Figure S5.** Plot on the left is the calculated electric field intensity of an Au NP of 50 nm in diameter, excited with a wavelength of 632 nm and a polarization state along the Y-axis. The circumference with a dotted line highlights the surface of the NP, the solid line surrounds the sphere at a distance of 5 nm away from its surface. We calculated the average value of  $|E|$  in the annular region between the two circumferences. On the right, the average of  $|E|^4$  decreases as one moves away from the Au NP surface (the annular region becomes thicker). The maximum average value is obtained from the field intensity values in an annular region of thickness 3 nm.

We consider that only the excited T4 molecules are the ones on the metallic NPs located in the laser spot area. Figure S6 shows representative SEM images of Au and Ag NPs for 50  $\mu$ l SERS substrates; the laser spot area is limited by the red circle. From several SEM, a count of the number of particles inside the laser spot area was made. The average number of Au NPs is 150 and 70 Ag.



**Figure S6.** SEM micrograph at a magnification of x15,000 of the for 50  $\mu\text{l}$  SERS substrate of (a) Au NPs and (b) Ag NPs.

On the other hand, the main feature of the SERS substrates with  $V_{NP}=250\ \mu\text{l}$  is its large load of NPs on the silicon wafer. On the silicon surface, large areas with a great agglomeration of NPs, isolated NPs, clusters with a few numbers of NPs, and quasi-linear arrays of NPs, are observed (see right panel of Figure S3). The silicon surface is not completely covered. A count of the average number of particles inside the laser spot area was made in a similar way as above, but in this case, for regions where NPs are accumulated or stacked on top of each other, only the ones at the top were taken into account. The average number of NPs in the laser spot area is 2000 and 1378, respectively, for Au and Ag substrates.

We assume that when the droplets of the molecules in solution are dropped on the plasmonic substrate, the molecules that will be excited by the SERS phenomenon are the ones that fall on the upper hemisphere of each metallic NP. Then, the optically active metal surface is determined by multiplying the average number of metal NPs by the area of the top hemisphere. We show in Table S2 the average diameter of an Au or Ag NP, the area of the hemisphere, the average number of NPs in the laser spot, and the optical-active metal surface; for four different SERS substrates. The occupied area is larger as the volume concentration increases.

**Table S2.** Values used to estimate the total metal area occupied by 50 and 250  $\mu\text{l}$  of Au and Ag NPs that is under the laser spot.

SERS substrate	Average Diameter (nm)	Hemisphere area of one NP ( $\text{nm}^2$ )	Average number of NPs under the laser spot	Optical-active metal surface ( $\text{nm}^2$ )
Au NP (50 $\mu\text{l}$ )	53	$4.41 \times 10^3$	150	$6.62 \times 10^5$
Au NP(250 $\mu\text{l}$ )	53	$4.41 \times 10^3$	2000	$8.82 \times 10^6$
Ag NP (50 $\mu\text{l}$ )	46	$3.32 \times 10^3$	70	$2.33 \times 10^5$
Ag NP (250 $\mu\text{l}$ )	46	$3.32 \times 10^3$	1378	$4.41 \times 10^6$

For the Au and Ag SERS substrates (50  $\mu\text{l}$ ) the  $N_{\text{SERS}}$  was estimated as follows. We considered that the vertical-aligned T4 molecules cover totally the upper hemisphere of each NP. Therefore,  $9.81 \times 10^5$  molecules of T4 are adsorbed on the Au surface, and  $3.45 \times 10^5$  on the Ag surface. For each T4 molecule concentration, again, we assume that molecule layers were formed and the first layers on

the surfaces forming a 3 nm thickness contributed the most to the SERS-EF. Then, for the determination of the  $N_{\text{SERS}}$ , we follow the next equation:

$$N_{\text{SERS}} = \frac{(\text{number of molecules assembling a single layer on the metal surface}) (3 \text{ nm-thickness})}{H}$$

Finally, the  $N_{\text{SERS}}$  for the Au NP substrate is  $2.56 \times 10^6$ , and for the Ag NP substrate is  $8.99 \times 10^5$ . It is noteworthy to mention that these values are the same for all T4 concentrations, this is because we restrict the excited molecule layers to a thickness of 3 nm.