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A Multifunctional Nanoplatfom Made of Gold Nanoparticles and Peptides Mimicking the Vascular Endothelial Growth Factor

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Abstract: In this work, nanobiohybrids of plasmonic gold nanoparticles (AuNP, anti-angiogenic) and a peptide mimicking the vascular endothelial growth factor (VEGF, pro-angiogenic) were assembled and scrutinized in terms of physicochemical characterization, including optical properties, surface charge, surface chemical structure and morphology of the bioengineered metal nanoparticles, for their potential application as multifunctional theranostic (i.e., therapy + sensing) nanoplatfom (AuNP/VEGF). Specifically, a peptide sequence encompassing the VEGF cellular receptor domain 73–101 (VEGF73–101) and its single point cysteine mutated were immobilized onto AuNP by physi- and chemi-sorption, respectively. The new hybrid systems were characterized by means of a multitechnique approach, including dynamic light scattering (DLS) analyses, zeta potential (ZP), spectroscopic (UV-Vis, FT-IR, XPS), spectrometric (TOF-SIMS) and microscopic (AFM, SEM) techniques. Proof-of-work cellular experiments in human umbilical vein endothelial cells (HUVEC) upon the treatment with AuNP/VEGF samples, demonstrated no toxicity up to 24 h (MTT assay) as well an effective internalization (laser confocal microscopy, LSM).

Keywords: plasmonics; theranostics; TOF-SIMS; XPS; FT-IR; TEM; DLS; zeta potential; HUVEC; theranostics



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1. Introduction

Angiogenesis is the process by means new blood vessels are formed from pre-existing vasculature. It is strictly controlled by an equilibrium between a huge number of pro and anti-angiogenic factors [1]. Excessive or insufficient angiogenesis contributes to many and different pathologies from cancer to neurodegenerative diseases [2–4]. The proliferation step in angiogenesis has a central role in many pathological conditions including neoplasia, rheumatoid arthritis, wound healing, and chronic inflammation.

Vascular endothelial growth factor (VEGF-A) was discovered more than a decade ago and it is known as one of main key regulator of physiological angiogenesis [5,6]. VEGF includes a family of polypeptides belonging to the cysteine-knot superfamily of signalling proteins, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, placenta growth factor (PLGF) and platelet-derived growth factor (PDGF).

All VEGFs bind to and activate different VEGF receptors, i.e., VEGFR-1, VEGFR-2 and VEGFR-3, mainly expressed by endothelial cells, but displaying different and overlapping binding patterns [7].

Four isoforms of VEGF-A, having 121, 165, 189, and 206 amino acids, are produced from a single gene because of alternate splicing [8,9]. The most abundantly expressed splice variant is VEGF165, a heparin binding glycoprotein cleaved by plasmin to yield VEGF110, which is equipotent to VEGF121 with respect to mitogenic activity on endothelial cells [10]. The 121 amino acid form of VEGF induces the proliferation of endothelial cells but, in contrast to VEGF165, lacks the heparin binding ability. Only a small number of VEGF residues are important for the binding to VEGF receptors. Particularly, the positively charged domain of VEGF encompassing Arg82, Lys84 and His86, located in a hairpin loop, and a negatively charged residues Asp63, Glu64 and Glu67 resulted responsible for the respectively binding of VEGFR-1 and VEGFR-2 receptors [11], and then for the angiogenic response [12].

Different antiangiogenic approaches have been developed to block the interaction of VEGFs with their receptors to inhibit angiogenesis and tumour growth. Small molecules and peptides have been synthesized as agonist or antagonist agents for potential application in many pathologies [13]. It is to note that many VEGF mimetic peptides with antiangiogenic activity have been described [14], while very few molecules with pro-angiogenic activity have been reported [15].

A relevant issue in the application of anti- or pro-angiogenic molecules is the large amount that generally has to be administered, which could determine unspecific binding, side effects and toxicity [16].

To overcome these shortcomings, nanoparticles (NPs) have been utilized to delivery angiogenic modulators. An advantage is the requirement for low doses due to increased reactivity that derives from the large concentration of active molecules confined in the small volume of NPs [16]. In addition to acting as a delivery system, NPs can protect molecules and in particular peptides from degradation due to enzymatic action. Furthermore, NPs display pro- or anti-angiogenic properties based on their nature [17,18].

Different sizes and surface charges influenced the intracellular signalling events. Particularly, gold and silver nanoparticles seemed to inhibit the function of pro-angiogenic heparin-binding growth factors (HB-GFs), such as VEGF165 and basic fibroblast growth factor (bFGF) [19–21].

Recently, AuNP functionalized with VEGF165 and (11-mercaptopundecyl)-*N,N,N*-trimethyl-ammonium bromide showed pro-angiogenic and anti-microbial activity in wound healing process [22].

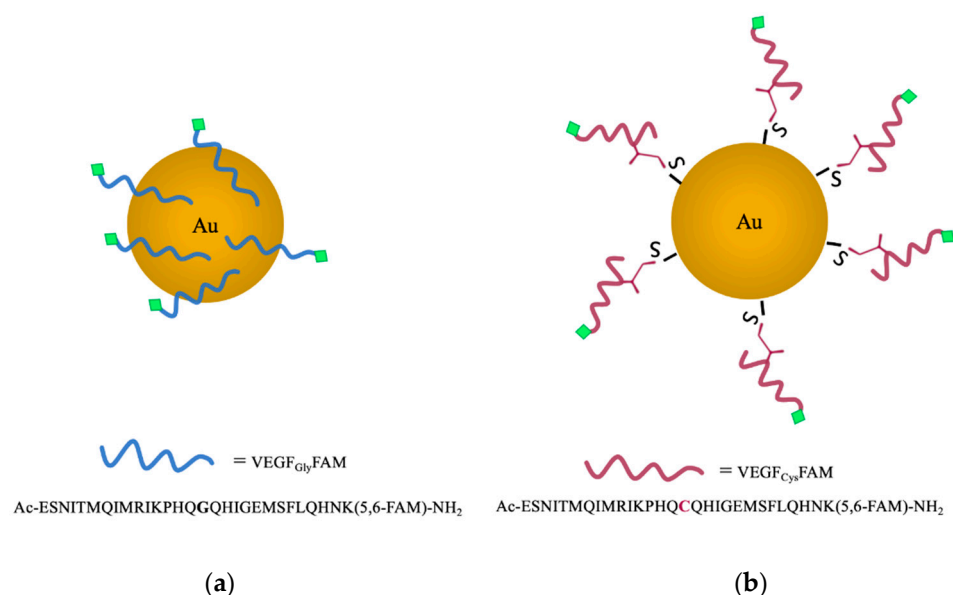
VEGF121, which do not have a heparin binding domain, was not inactivated by gold nanoparticles, so AuNP selectively inhibited VEGF165-induced proliferation of HUVEC cells [23]. Gold NPs may act as an effective inhibitor of VEGF-induced choroid-retina endothelial cells migration without affecting cells viability and their adhesion to fibronectin [24]. Moreover, gold nanoparticles conjugated with VEGF exhibit a surface carrying negative charges, which is ideal for VEGF transdermal delivery efficacy in wound repair [25]. Recently, AuNP functionalized with VEGF165 and (11-mercaptopundecyl)-*N,N,N*-trimethyl-ammonium bromide showed pro-angiogenic and anti-microbial activity in wound healing process [22].

The use of the whole protein can have drawbacks in the immune response and in the higher costs [26], for this reason new nanoparticles were synthesized and functionalized using biomimetic peptide fragments [27,28].

A relevant issue in the use of AuNPs for peptide delivery is to avoid toxicity in normal cells. NPs functionalized with peptides containing RGD sequence showed to increase uptake by HUVEC cells, while the nanoparticles were not toxic for these cells [29].

In the present work, we studied the immobilization of a peptide, VEGF_{73–101}, encompassing a recognition domain of VEGF [30,31], labelled with 5,6-carboxyfluorescein (VEGF-FAM) on AuNPs through (i) indirect physisorption of the VEGF_{73–101} peptide (VEGF-FAM/Au) and (ii) a direct chemisorption mediated by the mutated residue 88 with a cysteine (Cys) instead of the Glycine (Gly) in the VEGF_{73–101} peptide (VEGF_{Cys}FAM/Au)

(Scheme 1). The promising potentialities in the nanoparticle-driven cellular uptake of the peptide were tested on human umbilical vein endothelial cells (HUVEC).



Scheme 1. A pictorial representation of AuNPs functionalized with VEGF_{Gly}FAM (a) and VEGF_{Cys}FAM (b).

2. Materials and Methods

2.1. Chemicals

Ultrapure Milli-Q water (resistivity > 18 MΩ cm⁻¹) was used for all experiments. Glassware was cleaned immediately before use by immersing in aqua regia (HCl:HNO₃, 3:1 volume ratio), followed by rinsing with copious amount of water. Hydrogen tetrachloroaurate (HAuCl₄) and trisodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O, TSC) were purchased from Sigma-Aldrich.

2.2. Peptide Synthesis

The peptide fragments VEGF_{73–101} Ac-ESNITMQIMRIKPHQGQHIGEMSFLQHKNK-NH₂ (hereafter named VEGF_{Gly}) and VEGF_{73–101}-G88C Ac-ESNITMQIMRIKPHQCQHIGEMSFLQHKNK-NH₂ (hereafter named VEGF_{Cys}) were purchased from Genecust (Dudelange, Luxembourg). The peptide fragments were dye-labelled with 6-carboxyfluorescein (FAM) and blocked at their N- and C-terminal domains, Ac-ESNITMQIMRIKPHQGQHIGEMSFLQHKNK(5,6-FAM)-NH₂ (hereafter named VEGF-GlyFAM and Ac-ESNITMQIMRIKPHQCQHIGEMSFLQHKNK(5,6-FAM)-NH₂ (hereafter named VEGF-CysFAM).

2.3. AuNP Synthesis and Their Biofunctionalization with VEGF-FAM Peptides

Gold nanoparticles were synthesized by a modified Turkevich's method of chemical reduction of the chloroauric acid by the reducing agent trisodium citrate [32,33]. AuNPs were synthesized by chemical reduction of HAuCl₄ (1 mM) with TSC (1 wt %). The plasmonic band centered at 518 nm and the full width at half-maximum of about 50 nm confirmed the formation of monodisperse spherical gold colloids with a diameter of 12 nm. The actual concentration of the gold nanoparticles synthesized was estimated of 18 nM, with $\epsilon = 1.9 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$, calculated according to the equation: $\ln \epsilon = k \ln D + a$, where ϵ is the extinction coefficient, in M⁻¹cm⁻¹, D is the core diameter of the nanoparticles, and $k = 3.32111$, $a = 10.80505$ [34].

To prepare the peptide-coated nanoparticles, 173 nM VEGF_{Cys}FAM or 520 nM VEGF_{Gly}FAM were added to 9 nM Au colloidal dispersion under stirring; then, the unbound peptide molecules were removed by centrifugation step (8000 r.p.m., 15 min) and the collected pellets were redissolved in MilliQ water.

2.4. UV-Visible and FT-IR Spectroscopies

UV/Vis spectroscopy characterization of the aqueous dispersions was performed on a Perkin Elmer spectrometer by using quartz cuvettes with optical path lengths of 1 cm. For vibrational spectra analyses, solid samples were deposited by drop casting of the solutions onto silica wafer, then blow-dried under gentle nitrogen flow. Infrared spectra were obtained on a Bruker IFS 66 v/s system, in the wavenumber range from 500 cm^{-1} to 4000 cm^{-1} , with a resolution of 1 cm^{-1} .

2.5. Electron Transmission Microscopy (TEM)

TEM images were acquired by using a TEM Philips CM200 with a high voltage of 120. Samples were deposited on 300 mesh carbon-coated copper grids from Agar and directly imaged.

2.6. Dynamic Light Scattering (DLS) and ζ -Potential (ZP)

A NanoPartica SZ-100 apparatus equipped with a 514 nm 'green' laser from HORIBA-Scientific was used. Reproducibility was verified by collection and comparison of sequential measurements in at least three separate series of experiments. The samples were not filtered before measurements. At least five measurements were made for each sample and data averaged.

2.7. X-ray Photoelectron Spectroscopy (XPS) and Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) Analysis

After centrifugation step, the obtained pellets were deposited on silica wafer and dried at the air for the XPS and ToF-SIMS analyses. XPS measurements were performed using a PHI 5000 VersaProbe apparatus equipped with a monochromatic Al Ka line source (1486.7 eV). Typically, the pressure in the analysis chamber was 5×10^{-9} Torr. For high resolution scans, pass energy and energy step of the analyser were fixed to 23.5 eV and 0.2 eV, respectively. Dual beam charge neutralization from an electron gun (1 eV) and the Ar ion gun (<10 eV) were used for charge compensation on the sample surface during the measurements. From all spectra, a Shirley background was subtracted before the peak fitting and the binding energy (BE) component of the resolved C 1s peak corresponding to carbon in a hydrocarbon environment was set at 285.0 eV.

Positive ion ToF-SIMS spectra were acquired with a ToF-SIMS IV (IONTOF) instrument from ION-TOF GmbH. A pulsed 10 keV argon ion beam was used as analysis beam at a current of 1 pA (positive ion mode detection), and rastered over a scan area of $300 \times 300\text{ }\mu\text{m}^2$ for an acquisition time of 3 min. These conditions ensure static SIMS acquisitions (ion fluence maintained lower than 1×10^{12} ions/ cm^2). Five different locations on the surface were accounted for the ToF-SIMS analysis of the samples. For each spectrum, the mass scale was calibrated by using well-identified ions, namely H^+ , H_2^+ , H_3^+ , C^+ , CH^+ , CH_2^+ , CH_3^+ , C_2H_3^+ . For surface spectroscopy operated in the negative ion mode detection, a pulsed Ar^+ 10 keV ion beam at a current of 2 pA was rastered over a scan area of $300 \times 300\text{ }\mu\text{m}^2$, and used as analysis beam during ≈ 6 min [35].

ToF-SIMS spectra were interpreted with the help of the PCA method, by using SIMCA-P13 software (Umetrics, Sweden). Before PCA, the peak intensity was normalized to the total ion count, to correct for the differences in total secondary ion yield, then the normalized spectra were exported from the acquisition software (Surface Lab 6.3) to the SIMCA-P13 analysis software that mean-centres and scales the variables. The scaling of all the peaks inside the ToF-SIMS spectra gives them the same statistical weight, independently of their relative intensity in the spectra. From a geometric point of view, the PCA is a projection method that turns the initial swarm of points (the samples) of the multidimensional system with n axes (n is the number of ToF-SIMS peaks considered) into a highly lowered dimensional system (two to three), the new axes being the principal components (PCi). These new PCi components display, inside the initial n -dimensional space, the main directions along which the swarm of points is the most extended (i.e.,

maximum variance). Additionally, from an algebraic point of view, the PCA calculation decomposes the initial matrix, constituted from the intensities (“the variables”) and the samples (“the observations”), into a new set of three matrices, the *scores*, coordinates of the samples onto the new principal components, the *loadings*, statistical weights of contribution of the ToF-SIMS peaks to the principal components, and the *residuals*, random and unexplained variation, respectively. This procedure ensures that the variance in the data is related to chemical differences between samples and not to artefacts in peak intensity [36].

2.8. Cell Viability Assay (MTT)

The MTT assay was performed to evaluate the viability of cells treated with 2.5 nM bare AuNP and in combination with 75 nM VEGF_{Gly}FAM and 25 nM VEGF_{Cys}FAM. HUVEC cells were seeded in 96-well plates at a density of 3×10^3 cell/well (cells treated in growth medium, EGM[®]-2 BulletKit[®] without VEGF supplementation).

After 24 h of cells treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT, Invitrogen) was used following the manufacturer’s instruction. The results were reported as the percentage of viable cells with respect to the control set to 100% (data not shown).

2.9. Laser Scanning Confocal Microscopy (LSM) Analyses

HUVEC cells were cultured on coverslips at 2×10^4 cell/cm² in EGM[®]-2 BulletKit[®] without VEGF supplementation and treated with 2.5 nM AuNPs, 75 nM AuNP/VEGF_{Gly}FAM, 25 nM AuNP/VEGF_{Cys}FAM. Measurements were carried out after 24 h.

After the incubation time, cells were washed with phosphate buffer saline solution (10 mM PBS, 37 °C, pH = 7.4), fixed and stained with the nuclear dye HOECHST.

Confocal imaging was performed with an Olympus FV1000 confocal laser scanning microscope (LSM), equipped with diode UV (405 nm, 50 mW), multiline Argon (457 nm, 488 nm, 515 nm, total 30 mW), HeNe(G) (543 nm, 1 mW) and HeNe(R) (633 nm, 1 mW) lasers. An oil immersion objective (60 × O PLAPO) and spectral filtering system was used. The detector gain was fixed at a constant value and images were taken, in sequential mode, for all the samples at random locations throughout the area of the well.

3. Results

3.1. Synthesis and Physicochemical Characterization

The synthesis of gold nanoparticles was carried out by the chemical reduction method with citrate acting both as reducing and stabiliser agent and resulted, at the used experimental conditions, in a monodisperse colloidal solution of spherical nanoparticles having diameter of 12 nm [37] and a plasmonic band centered at 518 nm (Figure 1). The strong absorption in the visible region spectra of AuNPs is the characteristic feature of gold plasmonic resonance. A red shift and broadening of this band upon the addition of a ligand can be related to the surface coverage and/or aggregation of nanoparticles [38]. Indeed, Figure 1 displays noticeable red shifts of the plasmonic band upon the addition of the peptide in the 25 nM–125 nM concentration range, with total shifts measured of 10 nm for VEGF_{Gly}FAM and 6 nm for VEGF_{Cys}FAM, respectively.

As the concentration of the added peptide increases, in the range between 125 nM and 175 nM, the width of the plasmonic bands increase up to FWHM of 130 nm compared to 81 nm for the bare AuNPs. Such findings are explained in terms of two main contributions, i.e., a predominant particle coverage by the peptide molecules in the lower concentration range, and a predominant colloidal aggregation in the higher concentration range, respectively. To confirm this hypothesis, the flocculation assay by addition of an electrolyte solution (56 µM NaCl) has been performed for the gold nanoparticle-peptide assemblies formed in the 25–100 nM range of peptide concentration. Figure 1c, d show that the addition of 75 nM VEGF_{Gly}FAM and 25 nM VEGF_{Cys}FAM were enough to stabilise the gold colloids, which exhibit a decrease in the intensity of the plasmonic intensity respectively of 30%, 26%, and 9% for bare, VEGF_{Gly}FAM- and VEGF_{Cys}FAM-coated nanoparticles. It

was, therefore, inferred that the cysteine residue was more effective as anchoring site to the metal nanoparticle surface than the glycine residue, thus owing the chemisorption forces that complement the energy balance of the peptide adsorption at the nanoparticle surface.

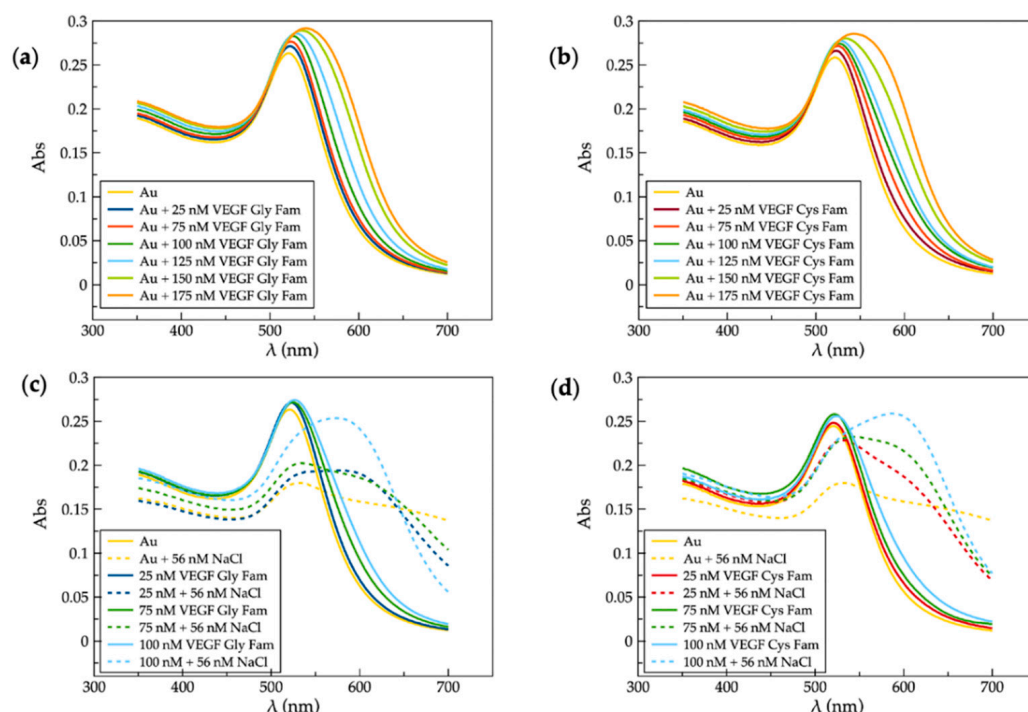


Figure 1. Changes in the AuNP (1.3 nM) plasmonic band by addition of: VEGF_{Gly}FAM (a) and VEGF_{Cys}FAM (b) at increasing concentrations (from 2.5×10^{-8} M to 17.5×10^{-8} M). In (c,d) are shown the UV-visible spectra of bare and peptide-coated AuNPs upon the addition of 56 μ M NaCl.

To remove the loosely bound and/or free peptide molecules from the peptide-coated NPs, a centrifugation step was performed. The supernatant and the pellet obtained were collected. Figure 2 shows the UV-vis spectra of recovered supernatants and re-suspended pellets and demonstrate a good stability of the prepared systems.

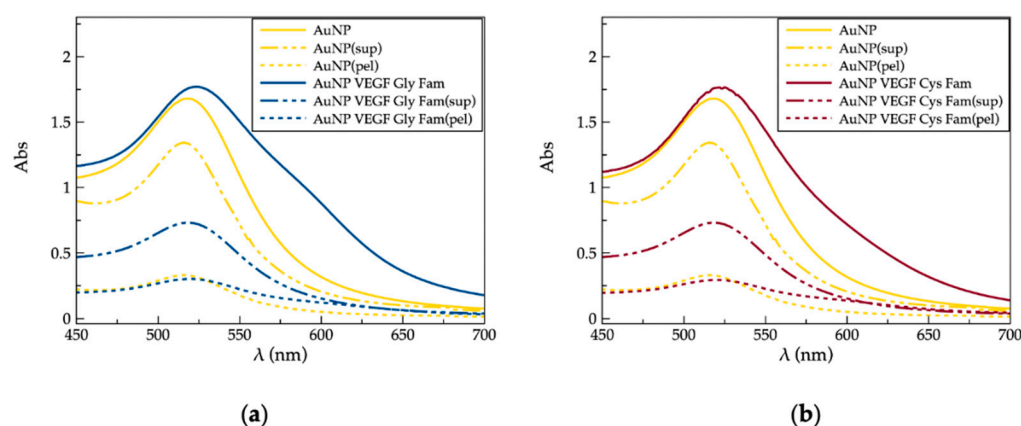


Figure 2. UV-visible spectra of AuNP/VEGF_{Gly}FAM (a) and AuNP/VEGF_{Cys}FAM (b) dispersions before (solid line) and after (dot line: pellet; dash-dot line: supernatant) centrifugation. The spectra for bare AuNP are shown for comparison.

Figure 3 shows the TEM images of the re-suspended samples. The nanoparticles appear well-dispersed with an average dimension of 30 nm, as dynamic light scattering analysis confirmed. Instead, the average size of hydrodynamic diameter for functionalized

AuNPs/VEGF_{Cys}FAM reached $\sim 159.3 \pm 11.9$ nm in comparison to AuNPs/VEGF_{Gly}FAM ($\sim 67.4 \pm 0.8$ nm). It was also possible to note a shell around the grouped nanoparticles suggesting the effectively coating of the peptide at the nanoparticles surface. On the other case, AuNPs/VEGF_{Gly}FAM image did not clearly evidence the same coating, but only a slight increasing of the average dimensions.

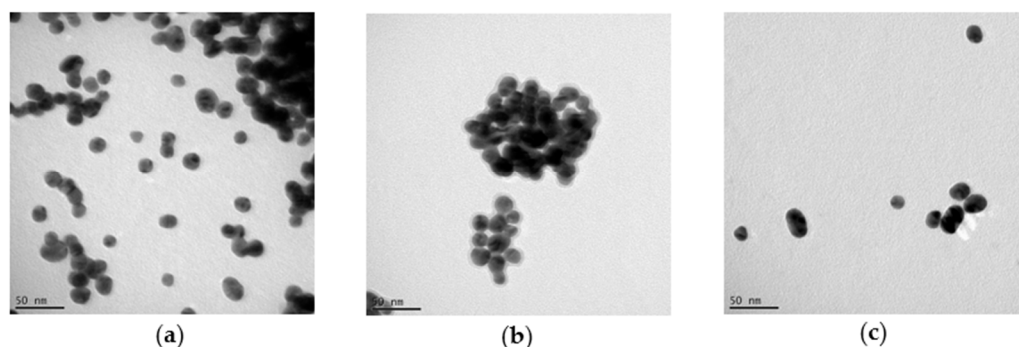


Figure 3. TEM images (scale bar = 50 nm) of bare AuNPs (a), AuNPs/VEGF_{Cys}FAM (b) and AuNPs/VEGF_{Gly}FAM (c).

The change of ζ -potential values further confirms the effective functionalization of AuNP (Figure 4). Indeed, for bare NPs we found an average ZP of ~ -60.6 mV, while the surface charge was almost neutralized after the peptide immobilization (ZP ~ -7.7 mV and 4.4 mV for AuNPs/VEGF_{Gly}FAM and AuNPs/VEGF_{Cys}FAM, respectively).

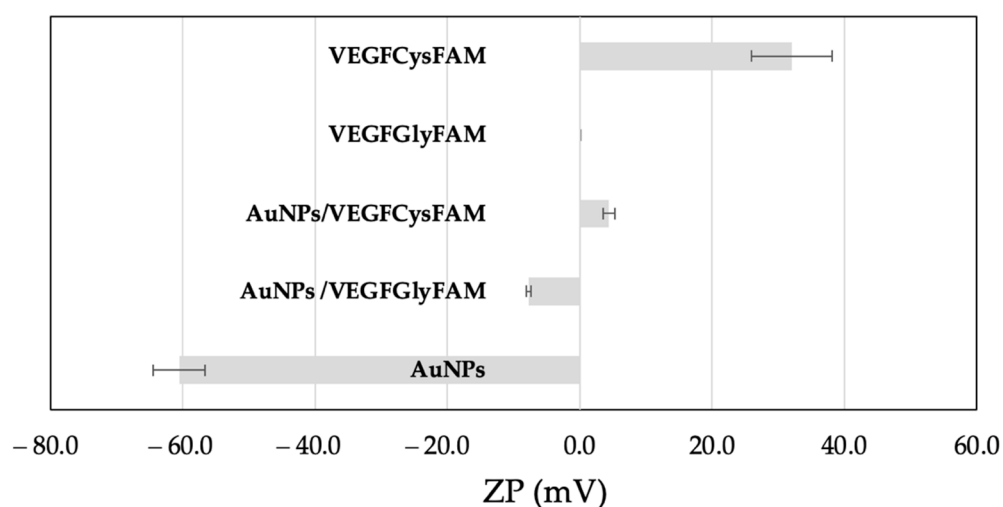


Figure 4. ζ -potential values of bare AuNPs, AuNPs/VEGF_{Cys}FAM, AuNPs/VEGF_{Gly}FAM and free peptides.

FT-IR spectra were utilized to study the characteristic bands of AuNPs before and after the peptide conjugations (Figure 5). Both VEGF_{Cys}FAM and VEGF_{Gly}FAM peptides exhibited very similar spectral features, with the characteristic vibrational modes of amide I and II ($1666\text{--}1450\text{ cm}^{-1}$), N–H (a sharp band around 3276 cm^{-1}), C = O (1737 cm^{-1}), C–O (1245 cm^{-1}), aliphatic CH₂ (1313 cm^{-1}) and C–H (1278 cm^{-1}), respectively. However, the spectrum of VEGF_{Cys}FAM did not evidence the characteristic peak of thiol group related to cysteine residue as the inset shown. FT-IR spectra were strongly affected after AuNP conjugation. Particularly, the characteristic features were still visible but less evidence; also, a shift in the position of COO[−] (asymmetric $\sim 1629\text{ cm}^{-1}$ and symmetric $\sim 1405\text{ cm}^{-1}$) and NH₃⁺ stretching was likely due to a change in their dipole moment when peptides physisorption or chemisorption on metal surface with high electron density [39].

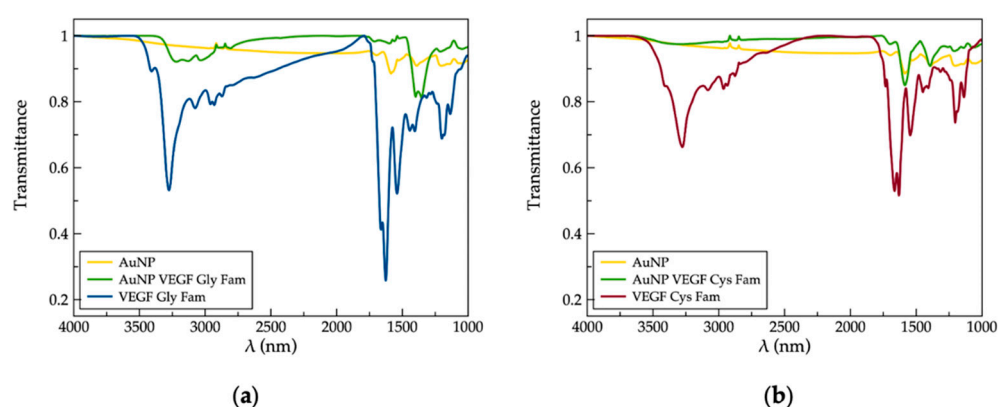


Figure 5. FT-IR spectra of the bare gold nanoparticles (yellow line) and the hybrid (green line) AuNP/VEGF_{Gly}FAM (a) or AuNP/VEGF_{Cys}FAM (b). The spectra of the free, unbound peptides are included for comparison.

In order to obtain information about the functionalization and the chemistry of modified nanoparticles, XPS and ToF-SIMS measurements were performed.

The atomic percentage composition of AuNPs before and after the coating with peptides showed in Table 1, demonstrate that the bare AuNPs a relatively high amount of C 1s and O 1s that hide the Au 4f signal. This finding was attributed to a thick adlayer of citrate ions coating the metal core of the nanoparticles.

Table 1. Surface atomic composition of AuNPs before and after the functionalization with peptides.

Samples	C 1s (at.%)	O 1s (at.%)	S 2p (at.%)	N 1s (at.%)	Au 4f (at.%)
Bare AuNPs	54.6	34.3	-	-	11.1
AuNPs/VEGF _{Cys} FAM	8.0	3.6	1.0	-	87.4
VEGF _{Cys} FAM	66.7	18.5	2.0	12.9	-
AuNPs/VEGF _{Gly} FAM	9.5	4.5	0.9	1.5	83.7
VEGF _{Gly} FAM	67.3	15.7	1.6	15.3	-

Accordingly, the atomic concentration for Au 4f increases due to the replacement of the citrate shell by the peptide molecules adsorbing at the interface. It must be noted that no traces of N 1s and S 2p were detected at the surface of bare AuNPs, whereas, after the coating by the peptides, comparable amounts of sulphur were found for both AuNPs/VEGF_{Gly}FAM and AuNPs/VEGF_{Cys}FAM. The S 2p high-resolution spectra of the two peptides, both free and bound to the gold nanoparticles are shown in Figure 6. The peak deconvolution has been performed according to the presence of a doublet corresponding to the S 2p_{3/2} and S 2p_{1/2} components, at a relative distance each from the other of 1.2 eV and with an intensity peak ratio, S 2p_{3/2}/S 2p_{1/2}, of about 2:1. The higher binding energy (BE) component S 2p_{3/2} has been assigned to the following chemical states of sulphur: S-Au bonds, at BE = 161.3 ± 0.2 eV [40,41] (S0 component); S-C bonds, e.g., in cysteine and methionine residues, at BE=163.5 ± 0.2 eV [39,42] (S1 component); S-O bonds, at increasing oxidation levels, respectively at BE = 166.3 ± 0.2 eV [37] (S2 component); 168.4 ± 0.2 eV (S3 component) and 170.7 ± 0.2 eV (S4 component) [43].

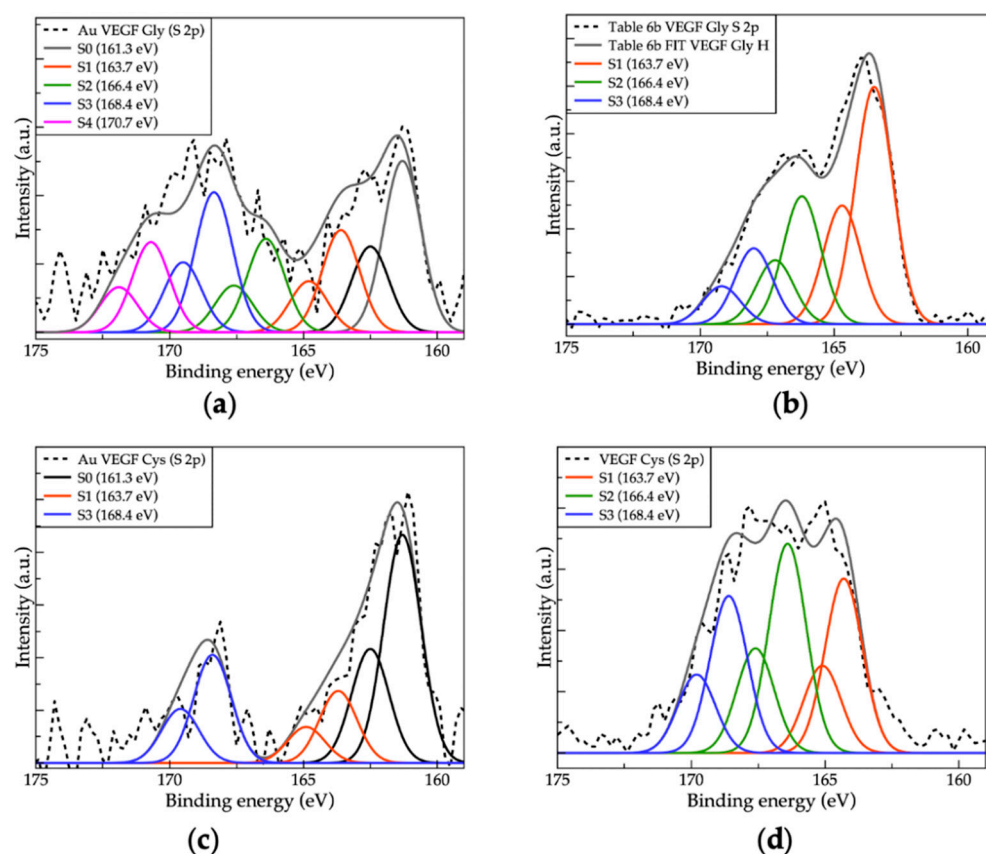


Figure 6. Representative high-resolution spectra and peak fitting curves of S 2p in AuNPs/VEGF_{Gly}FAM (a) and AuNPs/VEGF_{Cys}FAM (c) hybrids, as well as in VEGF_{Gly}FAM (b) and VEGF_{Cys}FAM (d) peptides, respectively.

Interestingly, although the free peptides exhibit different peak shapes, the peak fitting analysis resulted in the same components of the S 2p peak, i.e., S1, S2 and S3, at different relative intensity ratio. Specifically, the S1/(S2 + S3) ratio is 1.2 for VEGF_{Gly}FAM and 0.3 for VEGF_{Cys}FAM. This finding can be explained by the tendency of cysteine groups to oxidise in a non-reducing environment [44]. As to the peptides bound to the AuNPs, for both AuNP/VEGF_{Gly}FAM and AuNP/VEGF_{Cys}FAM the component S0 of chemisorbed sulphur is found. In the case of the peptide containing the cysteine such a component is expected owing to the high binding affinity of thiol groups towards metal surfaces [37,39]. On the other hand, in the methionine residue, the presence of electron pairs of the sulphur 2p orbitals is likely to prompt, e.g., by coordinative binding, the formation of S-Au bonds corresponding to S0 component. It must be noted that the relative intensity ratio between the components assigned respectively to chemisorbed sulphur (S-Au) and S-C bonds, correspond to the S0/S1 values of 3.1 for AuNP/VEGF_{Cys}FAM and 1.7 for AuNP/VEGF_{Gly}FAM, thus confirming the higher chemisorption process occurred in the presence of the cysteine residue. As to the overall oxidation state of sulphur in the AuNP/peptide assemblies, the values of the intensity ratio S1/(S2 + S3 + S4) of 0.7 for AuNP/VEGF_{Cys}FAM and 0.3 for AuNP/VEGF_{Gly}FAM point to a less extent of oxidation of the sulphur moieties for the nanoparticle-bound peptide in comparison to the free peptide in the case of VEGF_{Cys}FAM.

The spectra of Au 4f region exhibit symmetric peaks with comparable full widths at half maximum (FWHM) of about 0.9 eV for all samples (Figure 7). For the bare AuNP, the distinctive doublet peaks can be observed at the BE of 86.9 ± 0.2 eV (Au 4f_{5/2}) and 83.2 ± 0.2 eV (Au 4f_{7/2}), respectively. The peak separation of 3.7 eV and an area ratio of 3:4 are in good agreement with literature XPS data of gold nanoparticles [45]. For both AuNPs/VEGF_{Cys}FAM and AuNPs/VEGF_{Gly}FAM, the Au 4f signals display a shift of 0.4 eV and 0.5 eV respectively towards lower binding energies with respect to the bare

AuNP sample located at 83.2 eV. Such a shift can be attributed to charge-transfer effects from core electrons of gold to the peptide molecules [37,39,46]. The spectra of Au 4f region exhibit symmetric peaks with comparable full widths at half maximum (FWHM) of about 0.9 eV for all samples (Figure 7). For the bare AuNP, the distinctive doublet peaks can be observed at the BE of 86.9 ± 0.2 eV (Au 4f_{5/2}) and 83.2 ± 0.2 eV (Au 4f_{7/2}), respectively.

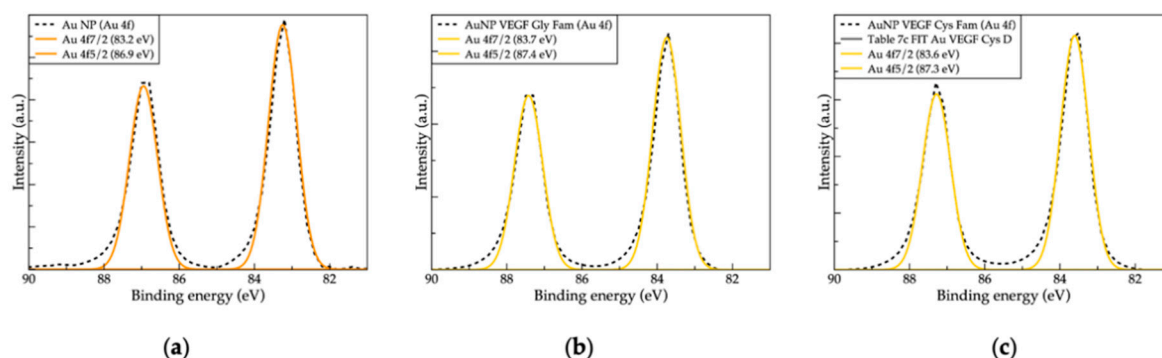


Figure 7. Representative high-resolution spectra of Au 4f of bare AuNPs (a), AuNPs/VEGF_{Gly}FAM (b) and AuNPs/VEGF_{Cys}FAM (c).

The ToF-SIMS technique has been extensively used to study the interaction of biomolecules with surfaces including nanofilms and nanoparticles, often as complementary tool of XPS studies [47,48].

The principal component analysis (PCA) method [49] used to thoughtfully analyse the ToF-SIMS spectra, and the corresponding results are shown in Figure 8. In this plot, each individual point represents the ToF-SIMS spectrum acquired at a specific location of the sample. PC1, represented on the vertical axis, from the PCA study of positive ToF-SIMS spectra for AuNP, AuNP/VEGF peptide and VEGF peptide samples reveals that the bare AuNP and the peptide-coated AuNP samples are clearly discriminated, i.e., located in distinct regions (i.e., PC1 < 0) with respect to the free peptide one (PC1 > 0). This result is found for both VEGF_{Gly}FAM (left hand side panel) and VEGF_{Cys}FAM (right hand side panel), respectively.

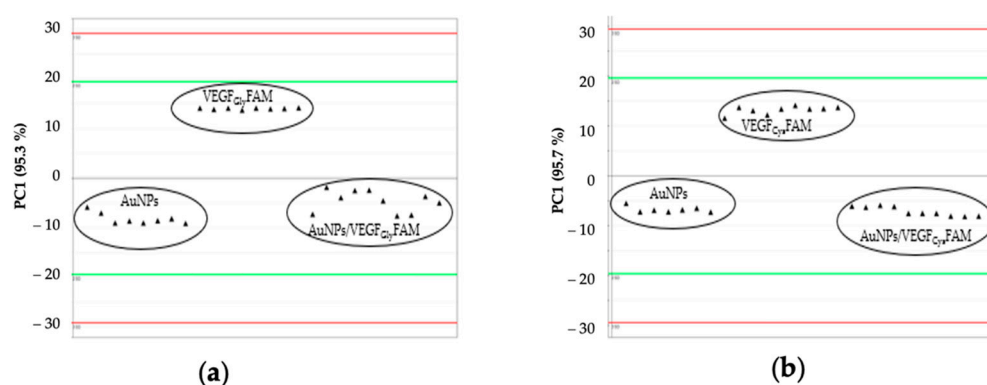


Figure 8. PC1 score plot obtained from PCA analysis of positive ToF-SIMS spectra (at least five spectra for each sample) of functionalized AuNPs with VEGF_{Gly}FAM (a) and VEGF_{Cys}FAM (b) peptides.

In Figure 9 is displayed a second PCA model, which takes into account only the spectra recorded from bare and peptide functionalised AuNP samples.

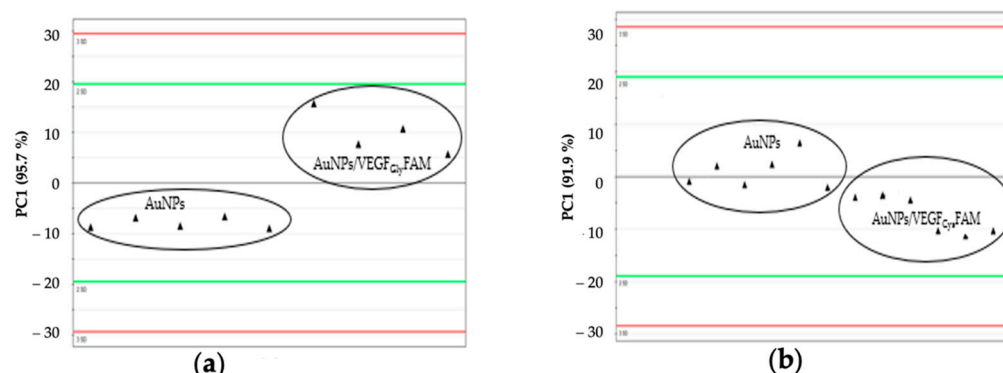


Figure 9. PC1 Score Plot obtained from PCA analysis of positive ToF-SIMS spectra (at least five spectra for each sample) of bare and functionalized AuNPs with VEGF_{Gly}FAM peptide (a) and VEGF_{Cys}FAM (b).

The PC1 score plots well discriminate between two samples, and a higher separation is obtained for the ensembles of PC1 values of AuNP vs. AuNP/VEGF_{Gly}FAM than those of AuNP vs. AuNP/VEGF_{Cys}FAM. According to the picture figured out from UV-visible and XPS analyses, this finding is explained in terms of the higher peptide coverage of the gold nanoparticles by VEGF_{Gly}FAM with respect to VEGF_{Cys}FAM.

The loadings plots related to the PC1 score plots above-described focus on the most significant peaks of the considered samples. The PC1 loadings list for the AuNPs/VEGF_{Gly}FAM, reported in Table 2, distinguishes a main Na⁺ fragment located a negative PC1 value, which can be considered characteristic of naked AuNPs due to the layer of citrate used during the reduction reaction. Instead, positive PC1 values are observed for all characteristic fragments related to the functionalized peptide at the surface of AuNPs. Most of them can be correlated to aminoacidic residues (selected in bold in the table) as Wagner et al. have reported [50], specific of the VEGF_{Gly}FAM sequence. Among them, also a fragment related to Au⁺ was found.

Table 2. PC1 loadings list related to the functionalized AuNP/VEGF_{Gly}FAM.

PC1 < 0			PC1 > 0				
Na ⁺	C ₇ H ₇ ⁺	C ₆ H ₆ ⁺	C ₆ H ₈ ⁺	C ₄ H ₆ ⁺	C ₄ H ₄ ⁺	C ₅ H ₉ O ⁺	C ₅ H ₁₂ N ⁺
	C ₈ H ₁₁ ⁺	C ₃ H ₅ O ⁺	C ₅ H ₉ ⁺	C ₂ H ₃ ⁺	C ₄ H ₇ O ⁺	C ₇ H ₅ ⁺	C ₂ H ₆ NS ⁺
	C ₆ H ₇ ⁺	C ₅ H ₇ O ⁺	C ₄ H ₆ N ⁺	C ₇ H ₆ ⁺	C ₉ H ₈ N ⁺	C ₅ H ₁₀ N ⁺	CH ₂ ⁺
	CHO ⁺	C ₁₀ H ₈ ⁺	C ₇ H ₇ O ⁺	C ₄ H ₈ N ⁺	C ₉ H ₈ N ⁺	C ₅ H ₁₀ N ⁺	CH ₂ ⁺
	C ₅ H ₇ N ₂ ⁺	C ₈ H ₉ O ⁺	C ₃ H ₄ NO ⁺	C ₃ H ₂ ⁺	C ₃ H ₆ ⁺	C ₂ H ₆ N ⁺	CH ⁺
	C ₆ H ₉ ⁺	C ₄ H ₆ O ⁺	C ₂ H ₄ ⁺	C ₃ H ₃ ⁺	C ₃ H ₅ ⁺	NH ₄ ⁺	CH ₃ ⁺
	C ₄ H ₅ O ⁺	C ₅ H ₅ ⁺	C ₈ H ₉ ⁺	C ₄ H ₅ ⁺	C ₅ H ₄ ⁺	C ₈ H ₆ O ⁺	C ₂ H ₅ ⁺
	C ₄ H ₈ ⁺	CH ₂ N ⁺	C ₇ H ₁₁ ⁺	C ₂ H ₄ N ⁺	C ₅ H ₂ ⁺	C ₈ H ₁₀ NO ⁺	Au ⁺
	C ₈ H ₈ ⁺	C ₆ H ₁₀ ⁺	C ₄ H ₆ NO ⁺	C ₃ H ₃ O ⁺	C ₆ H ₁₁ ⁺	C ₁₂ H ₉ O ⁺	H ⁺
	C ₃ H ₄ O ⁺	C ₇ H ₉ ⁺	NH ₃ ⁺	C ₉ H ₈ ⁺	C ₂ H ₆ NO ⁺	C ₆ H ₁₀ O ⁺	C ₃ H ₅ O ₂ ⁺
	C ₇ H ₈ ⁺	C ₉ H ₇ ⁺	C ₅ H ₇ ⁺	C ₈ H ₁₀ N ⁺	C ₄ H ₇ ⁺	C ₂ H ₃ O ⁺	C ₂ H ₅ O ₂ ⁺
	C ₅ H ₈ O ⁺	C ₅ H ₈ ⁺	CH ₄ N ⁺	C ₅ H ₃ ⁺	CH ₃ O ⁺	C ₇ H ₁₃ ⁺	C ₂ H ₅ O ⁺
	C ₉ H ₉ ⁺	C ₁₀ H ₉ ⁺	C ₂ H ₄ O ⁺	C ₄ H ₃ ⁺	CH ₃ N ₂ ⁺	C ₇ H ₅ O ⁺	C ₄ H ₉ ⁺
	C ⁺	C ₃ H ₇ ⁺	C ₅ H ₁₁ ⁺	C ₈ H ₇ O ⁺	C ₈ H ₇ ⁺	C ₆ H ₅ ⁺	C ₄ H ₂ ⁺

Table 3 shows the PC1 loadings list for the characteristic fragments related to AuNPs/VEGF_{Cys}FAM sample. However, as discussed, the loadings list displays only positive fragments suggesting a low peptide coating at the nanoparticle surface. In any case, it is possible to recognize characteristic amino acid fragments. The results obtained from ToF-SIMS analysis confirm what was observed through the XPS study.

Table 3. PC1 loadings list related to the functionalized AuNP/VEGF_{Cys}FAM.

PC1 > 0						
C ₅ H ₇ ⁺	C ₂ H ₄ ⁺	C ₇ H ₇ O ⁺	C ₈ H ₁₀ NO ⁺	CH ₂ N ⁺	C ₁₂ H ₉ O ⁺	C ₅ H ₁₂ N ⁺
C ₆ H ₉ ⁺	C ₂ H ₃ ⁺	C ₇ H ₆ ⁺	C ₅ H ₉ ⁺	C ₆ H ₅ ⁺	CHO ⁺	C ₃ H ₇ O ₂ ⁺
C ₈ H ₉ ⁺	C ₈ H ₁₁ ⁺	C ₄ H ₇ ⁺	C ₃ H ₂ ⁺	C ₈ H ₈ ⁺	C ₅ H ₁₁ ⁺	CH ⁺
C ₆ H ₈ ⁺	C ₄ H ₁₀ N ⁺	CH ₃ O ⁺	C ₂ H ₅ ⁺	C ₈ H ₁₀ N ⁺	C ₄ H ₄ ⁺	C ₁₀ H ₉ ⁺
C ₄ H ₅ ⁺	C ₅ H ₈ O ⁺	C ₈ H ₇ O ⁺	C ₄ H ₈ ⁺	C ₇ H ₇ ⁺	C ₇ H ₅ ⁺	C ₉ H ₈ ⁺
C ₅ H ₇ N ₂ ⁺	C ₃ H ₅ O ⁺	C ₃ H ₆ ⁺	C ₃ H ₅ O ₂ ⁺	C ₇ H ₁₃ ⁺	NH ₄ ⁺	CH ₄ N ⁺
C ₄ H ₅ O ⁺	C ₃ H ₅ ⁺	C ₄ H ₇ O ⁺	C ₇ H ₈ ⁺	C ₃ H ₇ O ⁺	CH ₃ N ₂ ⁺	C ₄ H ₃ ⁺
C ₃ H ₃ ⁺	C ₅ H ₇ O ⁺	C ₆ H ₁₀ O ⁺	C ₄ H ₆ O ⁺	C ₄ H ₉ ⁺	C ₅ H ₁₀ N ⁺	C ₄ H ₈ N ⁺
C ₆ H ₇ ⁺	C ₃ H ₃ O ⁺	C ₄ H ₆ N ⁺	C ₂ H ₄ O ⁺	C ₂ H ₄ O ₂ ⁺	C ₄ H ₆ NO ⁺	C ₂ H ₆ N ⁺
C ₈ H ₇ ⁺	C ₄ H ₆ ⁺	C ₈ H ₉ O ⁺	C ₂ H ₅ O ⁺	C ₂ H ₆ NO ⁺	C ₂ H ₅ O ₂ ⁺	C ₄ H ₂ ⁺
C ₇ H ₉ ⁺	C ₅ H ₈ ⁺	C ₃ H ₄ O ⁺	C ₂ H ₄ N ⁺	C ₃ H ₇ ⁺	C ₅ H ₅ ⁺	C ₅ H ₄ ⁺
C ₆ H ₁₀ ⁺	C ₃ H ₄ NO ⁺	C ₉ H ₉ ⁺	C ₆ H ₁₁ ⁺	H ⁺	CH ₂ ⁺	C ₅ H ₃ ⁺
C ₇ H ₁₁ ⁺	C ₅ H ₉ O ⁺	C ₈ H ₆ O ⁺	C ₂ H ₃ O ⁺	CH ₃ ⁺	C ₆ H ₆ ⁺	C ₂ H ₆ NS ⁺
C ⁺	C ₇ H ₅ O ⁺	C ₅ H ₂ ⁺	C ₁₀ H ₈ ⁺			

3.2. Cytotoxicity and Cellular Uptake

Cells incubated for 24 h (37 °C, 5% CO₂) with 2.5 nM AuNP, 25 nM VEGF_{Cys}FAM, 75 nM VEGF_{Gly}FAM and the corresponding hybrid composites did not exhibit any toxicity. Moreover, experiments of cellular uptake of AuNPs, both bare and peptide-functionalised, were performed in HUVECs by confocal microscopy (LSM) to investigate the potential application of these smart nanosystems as potential theranostic nanoplatform. Figure 10 shows the highest cellular internalisation of peptide for AuNP/VEGF_{Cys}FAM, as visible by the green emission of the FAM moiety covalently bound to the peptide sequence; it must be noted that this fluorescence mostly co-localises with the dark spots in the optical image (due to metal nanoparticles aggregates), thus confirming the strong chemisorption of the peptide containing the cysteine residue onto the AuNPs. On the other hand, a lower green fluorescence but still co-localised with AuNPs, is detected for AuNP/VEGF_{Gly}FAM, according to the weaker binding of this peptide to the nanoparticle surface. No significant peptide uptake could be detected for the free VEGF_{Gly}FAM (75 nM) nor VEGF_{Cys}FAM (25 nM and 75 nM).

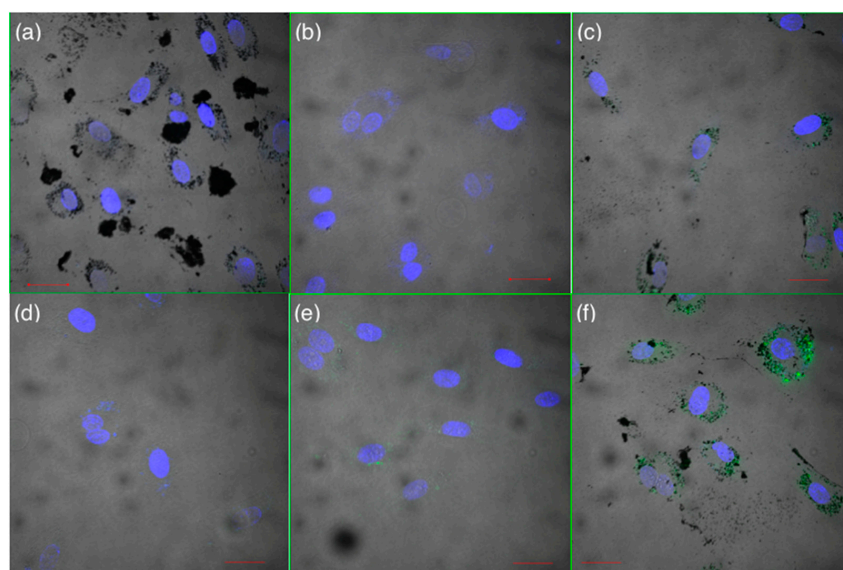


Figure 10. LSM merged micrographs of fluorescence (blue: DAPI; green: FAM) and optical bright field of HUVECs 24-h treated with (a) 2.5 nM bare AuNP; (b) 75 nM VEGFGlyFAM; (c) AuNP/VEGFGlyFAM; (d) 25 nM VEGFCysFAM; (e) 75 nM VEGFCysFAM; (f) AuNP/VEGFCysFAM.

4. Discussion

The angiogenic factor VEGF is the most studied growth factor, due to its specificity and important role in the activation of all steps of angiogenesis in the endothelium vasculature [51]. The splicing variant VEGF165 is the predominant form and has been shown to be up-regulated in tumour microenvironment by hypoxia or activation of oncogenes [52]. VEGF is a glycoprotein and consists of an antiparallel homodimer structure containing inter- and intra-disulphide bonds, and it has been shown to bind to three receptor types as follows: VEGFR-1 (flt-1), VEGFR-2 (flk-1 or KDR), and neuropilin-1 (NR-1). In the endothelial cells, most of the angiogenesis signaling (proliferation, migration, and survival) proceeds via the interaction between VEGF and VEGFR-2. The latter has been identified and includes residues at a loop region formed by the anti-parallel β -sheets $\beta 5$ – $\beta 6$ in the VEGF protein [53].

Only a small number of VEGF residues are important for binding to VEGF receptors [54], and, therefore, several molecules able to modulate VEGF biological activities have been reported [55]. Some VEGF mimetic peptides with antiangiogenic activity have been described [30]. It was reported that the peptide fragment encompassing the residues 73–101 blocked at N- and C-terminus (Ac-ESNITMQIMRIKPHQGQHIGEMSFLQHKNH₂), included in the loop $\beta 5$ – $\beta 6$ region involved with VEGFR2 interaction, showed anti-proliferative activity on HUVEC cells [30].

In recent decades, nanodrug delivery system has played an important role in depressing the toxic side effects and enhancing the therapeutic efficiency [56]. Functionalization of AuNPs with biological molecules has many applications in biomedical imaging, clinical diagnosis and therapy [19]. Recently, it was reported that gold nanoparticles exhibited potentially antiangiogenic effects by interacting with the heparin-binding domain of VEGF165. Furthermore, AuNPs seem to inhibit migration, tube formation and proliferation VEGF165-induced of endothelial cells [23,57]. It was also reported that intravenously administrated AuNPs could pass through blood-retinal barrier and induce no retinal toxicity [58]. We investigated the gold nanoparticles conjugated with VEGF fragments peptide Ac-ESNITMQIMRIKPHQGQHIGEMSFLQHKNH₂ and its mutated VEGF73–101-G88C 5,6-FAM labelled.

The obtained results showed the enhanced intracellular uptake respect to AuNPs alone and no toxicity on HUVEC cells. Indeed, the fluorescent dye conjugated to the peptide sequence allowed for the certain simultaneous localization of both the peptide molecules and the gold NPs. To note, VEGF receptors are known to use clathrin-dependent endocytosis that ultimately leads endocytosed material to lysosomes [59]. On the other hand, different physicochemical properties of gold nanoparticles, including size, clustering and surface chemistry of nanoparticles regulate their cellular uptake and transport [60,61]. Among the most common internalization mechanisms of gold nanoparticles, there is autophagosome accumulation through size-dependent nanoparticle uptake and lysosome impairment [62,63]. Specifically, AuNPs are known to reduce lysosomal activity by alkalization of the lysosomal lumen [64,65].

Further studies on the peptide-functionalized hybrid nanosystems can focus to map the trafficking process of the gold-nanoparticle bound peptides as well as to test them in cancer cells as a potentially anti-cancer drug or on retinal cells to regulate retinal neovascularization. Another potential follow-up of this study can involve cell migration and tubulogenesis studies in the HUVEC treated by VEGF peptide-modified AuNPs. Indeed, we recently demonstrated that different physi- vs. chemi-sorption processes by mutated angiogenin isoforms at the AuNP surface may offer a new strategy to modulate the angiogenesis processes in wound care [66]. Moreover, the plasmonic properties of the metallic nanoparticles, characterized in silico, add a further potential application of the hybrid system as multifaceted theranostic platform, to be exploited, e.g., in micro-Raman microscopy and hyperthermia studies.

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References

1. Kazerounian, S.; Lawler, J. Integration of pro- and anti-angiogenic signals by endothelial cells. *J. Cell Commun. Signal.* **2017**, *12*, 171–179. [[CrossRef](#)] [[PubMed](#)]
2. La Mendola, D.; Giacomelli, C.; Rizzarelli, E. Intracellular Bioinorganic Chemistry and Cross Talk among Different -Omics. *Curr. Top. Med. Chem.* **2016**, *16*, 3103–3130. [[CrossRef](#)] [[PubMed](#)]
3. Naumov, G.N.; Akslen, L.A.; Folkman, J. Role of Angiogenesis in Human Tumor Dormancy: Animal Models of the Angiogenic Switch. *Cell Cycle* **2006**, *5*, 1779–1787. [[CrossRef](#)] [[PubMed](#)]
4. Vallon, M.; Chang, J.; Zhang, H.; Kuo, C.J. Developmental and pathological angiogenesis in the central nervous system. *Cell. Mol. Life Sci.* **2014**, *71*, 3489–3506. [[CrossRef](#)]
5. Ferrara, N. Vascular Endothelial Growth Factor: Molecular and Biological Aspects. In *Vascular Growth Factors and Angiogenesis (Current Topics in Microbiology and Immunology)*; Springer: Berlin/Heidelberg, Germany, 1999; pp. 1–30.
6. Ferrara, N.; Henzel, W.J. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 851–858. [[CrossRef](#)]
7. Li, X. Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *Int. J. Biochem. Cell Biol.* **2001**, *33*, 421–426. [[CrossRef](#)]
8. Houck, K.A.; Ferrara, N.; Winer, J.; Cachianes, G.; Li, B.; Leung, D.W. The Vascular Endothelial Growth Factor Family: Identification of a Fourth Molecular Species and Characterization of Alternative Splicing of RNA. *Mol. Endocrinol.* **1991**, *5*, 1806–1814. [[CrossRef](#)]
9. Tischer, E.; Mitchell, R.; Hartman, T.; Silva, M.; Gospodarowicz, D.; Fiddes, J.C.; Abraham, J.A. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem.* **1991**, *266*, 11947–11954. [[CrossRef](#)]
10. Houck, K.A.; Leung, D.W.; Rowland, A.M.; Winer, J.; Ferrara, N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J. Biol. Chem.* **1992**, *267*, 26031–26037. [[CrossRef](#)]
11. de Vries, C.; Escobedo, J.; Ueno, H.; Houck, K.; Ferrara, N.; Williams, L. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **1992**, *255*, 989–991. [[CrossRef](#)]
12. Waltenberger, J.; Claesson-Welsh, L.; Siegbahn, A.; Shibuya, M.; Heldin, C.H. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J. Biol. Chem.* **1994**, *269*, 26988–26995. [[CrossRef](#)]
13. Rosca, E.V.; Koskimaki, J.E.; Rivera, C.G.; Pandey, N.B.; Tamiz, A.P.; Popel, A.S. Anti-Angiogenic Peptides for Cancer Therapeutics. *Curr. Pharm. Biotechnol.* **2011**, *12*, 1101–1116. [[CrossRef](#)] [[PubMed](#)]
14. Zhong, H.; Phillip Bowen, J. Recent Advances in Small Molecule Inhibitors of VEGFR and EGFR Signaling Pathways. *Curr. Top. Med. Chem.* **2011**, *11*, 1571–1590. [[CrossRef](#)] [[PubMed](#)]
15. Finetti, F.; Basile, A.; Capasso, D.; Di Gaetano, S.; Di Stasi, R.; Pascale, M.; Turco, C.M.; Ziche, M.; Morbidelli, L.; D’Andrea, L.D. Functional and pharmacological characterization of a VEGF mimetic peptide on reparative angiogenesis. *Biochem. Pharmacol.* **2012**, *84*, 303–311. [[CrossRef](#)]
16. Neves, K.B.; Montezano, A.C.; Lang, N.N.; Touyz, R.M. Vascular toxicity associated with anti-angiogenic drugs. *Clin. Sci.* **2020**, *134*, 2503–2520. [[CrossRef](#)]
17. Bartczak, D.; Muskens, O.L.; Sanchez-Elsner, T.; Kanaras, A.G.; Millar, T.M. Manipulation of in Vitro Angiogenesis Using Peptide-Coated Gold Nanoparticles. *ACS Nano* **2013**, *7*, 5628–5636. [[CrossRef](#)]

18. Bhattacharya, R.; Mukherjee, P. Biological properties of “naked” metal nanoparticles. *Adv. Drug Deliv. Rev.* **2008**, *60*, 1289–1306. [\[CrossRef\]](#)
19. Arvizo, R.R.; Rana, S.; Miranda, O.R.; Bhattacharya, R.; Rotello, V.M.; Mukherjee, P. Mechanism of anti-angiogenic property of gold nanoparticles: Role of nanoparticle size and surface charge. *Nanomed. Nanotechnol. Biol. Med.* **2011**, *7*, 580–587. [\[CrossRef\]](#)
20. Gurunathan, S.; Lee, K.-J.; Kalishwaralal, K.; Sheikpranbabu, S.; Vaidyanathan, R.; Eom, S.H. Antiangiogenic properties of silver nanoparticles. *Biomaterials* **2009**, *30*, 6341–6350. [\[CrossRef\]](#)
21. Sangiliyandi, G. Antitumor activity of silver nanoparticles in Dalton’s lymphoma ascites tumor model. *Int. J. Nanomed.* **2010**. [\[CrossRef\]](#)
22. Wei, S.C.; Chang, L.; Huang, C.C.; Chang, H.T. Dual-functional gold nanoparticles with antimicrobial and proangiogenic activities improve the healing of multidrug-resistant bacteria-infected wounds in diabetic mice. *Biomater. Sci.* **2019**, *7*, 4482–4490. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Bhattacharya, R.; Mukherjee, P.; Xiong, Z.; Atala, A.; Soker, S.; Mukhopadhyay, D. Gold Nanoparticles Inhibit VEGF165-Induced Proliferation of HUVEC Cells. *Nano Lett.* **2004**, *4*, 2479–2481. [\[CrossRef\]](#)
24. Chan, C.-M.; Hsiao, C.-Y.; Li, H.-J.; Fang, J.-Y.; Chang, D.-C.; Hung, C.-F. The Inhibitory Effects of Gold Nanoparticles on VEGF-A-Induced Cell Migration in Choroid-Retina Endothelial Cells. *Int. J. Mol. Sci.* **2019**, *21*, 109. [\[CrossRef\]](#)
25. Chen, Y.; Wu, Y.; Gao, J.; Zhang, Z.; Wang, L.; Chen, X.; Mi, J.; Yao, Y.; Guan, D.; Chen, B.; et al. Transdermal Vascular Endothelial Growth Factor Delivery with Surface Engineered Gold Nanoparticles. *ACS Appl. Mater. Interfaces* **2017**, *9*, 5173–5180. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Trapani, G.; Satriano, C.; La Mendola, D. Peptides and their Metal Complexes in Neurodegenerative Diseases: From Structural Studies to Nanomedicine Prospects. *Curr. Med. Chem.* **2018**, *25*, 715–747. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Lin, W.; Ma, G.; Yuan, Z.; Qian, H.; Xu, L.; Sidransky, E.; Chen, S. Development of Zwitterionic Polypeptide Nanoformulation with High Doxorubicin Loading Content for Targeted Drug Delivery. *Langmuir* **2018**, *35*, 1273–1283. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Arriortua, O.K.; Insausti, M.; Lezama, L.; Gil de Muro, I.; Garaio, E.; de la Fuente, J.M.; Fratila, R.M.; Morales, M.P.; Costa, R.; Eceiza, M.; et al. RGD-Functionalized Fe₃O₄ nanoparticles for magnetic hyperthermia. *Colloids Surf. B Biointerfaces* **2018**, *165*, 315–324. [\[CrossRef\]](#)
29. Paris, J.L.; Villaverde, G.; Gómez-Graña, S.; Vallet-Regí, M. Nanoparticles for multimodal antivasular therapeutics: Dual drug release, photothermal and photodynamic therapy. *Acta Biomater.* **2020**, *101*, 459–468. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Grasso, G.; Santoro, A.M.; Magri, A.; La Mendola, D.; Tomasello, M.F.; Zimbone, S.; Rizzarelli, E. The Inorganic Perspective of VEGF: Interactions of Cu²⁺ with Peptides Encompassing a Recognition Domain of the VEGF Receptor. *J. Inorg. Biochem.* **2016**, *159*, 149–158. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Zimbone, S.; Santoro, A.M.; La Mendola, D.; Giacomelli, C.; Trincavelli, M.L.; Tomasello, M.F.; Milardi, D.; García-Viñuales, S.; Sciacca, M.F.M.; Martini, C.; et al. The Ionophoric Activity of a Pro-Apoptotic VEGF165 Fragment on HUVEC Cells. *Int. J. Mol. Sci.* **2020**, *21*, 2866. [\[CrossRef\]](#)
32. Frens, G. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nat. Phys. Sci.* **1973**, *241*, 20–22. [\[CrossRef\]](#)
33. Turkevich, J.; Stevenson, P.C.; Hillier, J. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discuss. Faraday Soc.* **1951**, *11*. [\[CrossRef\]](#)
34. Liu, X.; Atwater, M.; Wang, J.; Huo, Q. Extinction coefficient of gold nanoparticles with different sizes and different capping ligands. *Colloids Surf. B Biointerfaces* **2007**, *58*, 3–7. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Briggs, D.; Hearn, M.J. Interaction of ion beams with polymers, with particular reference to SIMS. *Vacuum* **1986**, *36*, 1005–1010. [\[CrossRef\]](#)
36. Denis, L.; Cossement, D.; Godfroid, T.; Renaux, F.; Bittencourt, C.; Snyders, R.; Hecq, M. Synthesis of Allylamine Plasma Polymer Films: Correlation between Plasma Diagnostic and Film Characteristics. *Plasma Process. Polym.* **2009**, *6*, 199–208. [\[CrossRef\]](#)
37. Di Pietro, P.; Caporarello, N.; Anfuso, C.D.; Lupo, G.; Magri, A.; La Mendola, D.; Satriano, C. Immobilization of Neurotrophin Peptides on Gold Nanoparticles by Direct and Lipid-Mediated Interaction: A New Multipotential Therapeutic Nanoplatfor for CNS Disorders. *ACS Omega* **2017**, *2*, 4071–4079. [\[CrossRef\]](#)
38. Burda, C.; Chen, X.; Narayanan, R.; El-Sayed, M.A. Chemistry and Properties of Nanocrystals of Different Shapes. *Chem. Rev.* **2005**, *105*, 1025–1102. [\[CrossRef\]](#)
39. Di Pietro, P.; Zaccaro, L.; Comegna, D.; Del Gatto, A.; Saviano, M.; Snyders, R.; Cossement, D.; Satriano, C.; Rizzarelli, E. Silver nanoparticles functionalized with a fluorescent cyclic RGD peptide: A versatile integrin targeting platform for cells and bacteria. *RSC Adv.* **2016**, *6*, 112381–112392. [\[CrossRef\]](#)
40. Hu, Z.; Ritzdorf, T. Superconformal Electrochemical Deposition of Gold for Metallization in Microelectronic Devices. *J. Electrochem. Soc.* **2006**, *153*. [\[CrossRef\]](#)
41. Semenov, A.; Spatz, J.P.; Möller, M.; Lehn, J.-M.; Sell, B.; Schubert, D.; Weidl, C.H.; Schubert, U.S. Controlled Arrangement of Supramolecular Metal Coordination Arrays on Surfaces. *Angew. Chem. Int. Ed.* **1999**, *38*, 2547–2550. [\[CrossRef\]](#)
42. Zhong, C.-J.; Porter, M.D. Evidence for Carbon-Sulfur Bond Cleavage in Spontaneously Adsorbed Organosulfide-Based Monolayers at Gold. *J. Am. Chem. Soc.* **2002**, *116*, 11616–11617. [\[CrossRef\]](#)
43. Castner, D.G.; Hinds, K.; Grainger, D.W. X-ray Photoelectron Spectroscopy Sulfur 2p Study of Organic Thiol and Disulfide Binding Interactions with Gold Surfaces. *Langmuir* **1996**, *12*, 5083–5086. [\[CrossRef\]](#)

44. Stadtman, E.R.; Van Remmen, H.; Richardson, A.; Wehr, N.B.; Levine, R.L. Methionine oxidation and aging. *Biochim. Biophys. Acta (BBA) Proteins Proteomics* **2005**, *1703*, 135–140. [[CrossRef](#)] [[PubMed](#)]
45. Joseph, Y.; Besnard, I.; Rosenberger, M.; Guse, B.; Nothofer, H.-G.; Wessels, J.M.; Wild, U.; Knop-Gericke, A.; Su, D.; Schlögl, R.; et al. Self-Assembled Gold Nanoparticle/Alkanedithiol Films: Preparation, Electron Microscopy, XPS-Analysis, Charge Transport, and Vapor-Sensing Properties†. *J. Phys. Chem. B* **2003**, *107*, 7406–7413. [[CrossRef](#)]
46. Martí, A.; Costero, A.M.; Gaviña, P.; Gil, S.; Parra, M.; Brotons-Gisbert, M.; Sánchez-Royo, J.F. Functionalized Gold Nanoparticles as an Approach to the Direct Colorimetric Detection of DCNP Nerve Agent Simulant. *Eur. J. Org. Chem.* **2013**, *2013*, 4770–4779. [[CrossRef](#)]
47. Frasconi, M.; Marotta, R.; Markey, L.; Flavin, K.; Spampinato, V.; Ceccone, G.; Echegoyen, L.; Scanlan, E.M.; Giordani, S. Multi-Functionalized Carbon Nano-onions as Imaging Probes for Cancer Cells. *Chem. Eur. J.* **2015**, *21*, 19071–19080. [[CrossRef](#)]
48. Jimenez Jimenez, A.M.; Rodrigo, M.A.M.; Milosavljevic, V.; Krizkova, S.; Kopel, P.; Heger, Z.; Adam, V. Gold nanoparticles-modified nanomagnetite and quantum dots-based hybridization assay for detection of HPV. *Sens. Actuators B Chem.* **2017**, *240*, 503–510. [[CrossRef](#)]
49. Shaffer, R.E. *Multi- and Megavariable Data Analysis. Principles and Applications*; Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wold, S., Eds.; Umetrics Academy: Umeå, Sweden, 2001; 533p, ISBN 91-973730-1-X.
50. Wagner, M.S.; McArthur, S.L.; Shen, M.; Horbett, T.A.; Castner, D.G. Limits of detection for time of flight secondary ion mass spectrometry (ToF-SIMS) and X-ray photoelectron spectroscopy (XPS): Detection of low amounts of adsorbed protein. *J. Biomater. Sci. Polym. Ed.* **2012**, *13*, 407–428. [[CrossRef](#)]
51. Shibuya, M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes Cancer* **2011**, *2*, 1097–1105. [[CrossRef](#)]
52. Goel, H.L.; Mercurio, A.M. VEGF targets the tumour cell. *Nat. Rev. Cancer* **2013**, *13*, 871–882. [[CrossRef](#)]
53. Vicari, D.; Foy, K.C.; Liotta, E.M.; Kaumaya, P.T.P. Engineered Conformation-dependent VEGF Peptide Mimics Are Effective in Inhibiting VEGF Signaling Pathways. *J. Biol. Chem.* **2011**, *286*, 13612–13625. [[CrossRef](#)] [[PubMed](#)]
54. Muller, Y.A.; Li, B.; Christinger, H.W.; Wells, J.A.; Cunningham, B.C.; de Vos, A.M. Vascular endothelial growth factor: Crystal structure and functional mapping of the kinase domain receptor binding site. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7192–7197. [[CrossRef](#)] [[PubMed](#)]
55. Ho, Q.T.; Kuo, C.J. Vascular endothelial growth factor: Biology and therapeutic applications. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 1349–1357. [[CrossRef](#)]
56. Shi, M.; Ho, K.; Keating, A.; Shoichet, M.S. Doxorubicin-Conjugated Immuno-Nanoparticles for Intracellular Anticancer Drug Delivery. *Adv. Funct. Mater.* **2009**, *19*, 1689–1696. [[CrossRef](#)]
57. Pan, Y.; Wu, Q.; Qin, L.; Cai, J.; Du, B. Gold Nanoparticles Inhibit VEGF165-Induced Migration and Tube Formation of Endothelial Cells via the Akt Pathway. *BioMed Res. Int.* **2014**, *2014*, 1–11. [[CrossRef](#)]
58. Kim, J.H.; Kim, M.H.; Jo, D.H.; Yu, Y.S.; Lee, T.G.; Kim, J.H. The inhibition of retinal neovascularization by gold nanoparticles via suppression of VEGFR-2 activation. *Biomaterials* **2011**, *32*, 1865–1871. [[CrossRef](#)] [[PubMed](#)]
59. Simons, M.; Gordon, E.; Claesson-Welsh, L. Mechanisms and regulation of endothelial VEGF receptor signalling. *Nat. Rev. Mol. Cell Biol.* **2016**, *17*, 611–625. [[CrossRef](#)]
60. Liu, M.; Li, Q.; Liang, L.; Li, J.; Wang, K.; Li, J.; Lv, M.; Chen, N.; Song, H.; Lee, J.; et al. Real-time visualization of clustering and intracellular transport of gold nanoparticles by correlative imaging. *Nat. Commun.* **2017**, *8*. [[CrossRef](#)] [[PubMed](#)]
61. Di Pietro, P.; Strano, G.; Zuccarello, L.; Satriano, C. Gold and Silver Nanoparticles for Applications in Theranostics. *Curr. Top. Med. Chem.* **2016**, *16*, 3069–3102. [[CrossRef](#)] [[PubMed](#)]
62. Ma, X.; Wu, Y.; Jin, S.; Tian, Y.; Zhang, X.; Zhao, Y.; Yu, L.; Liang, X.-J. Gold Nanoparticles Induce Autophagosome Accumulation through Size-Dependent Nanoparticle Uptake and Lysosome Impairment. *ACS Nano* **2011**, *5*, 8629–8639. [[CrossRef](#)]
63. Sanfilippo, V.; Caruso, V.C.L.; Cucci, L.M.; Inturri, R.; Vaccaro, S.; Satriano, C. Hyaluronan-Metal Gold Nanoparticle Hybrids for Targeted Tumor Cell Therapy. *Int. J. Mol. Sci.* **2020**, *21*, 3085. [[CrossRef](#)]
64. Lee, D.; Hong, J.H. Nanoparticle-Mediated Therapeutic Application for Modulation of Lysosomal Ion Channels and Functions. *Pharmaceutics* **2020**, *12*, 217. [[CrossRef](#)] [[PubMed](#)]
65. Aguilar, R.C.; Woldemichael, T.; Rosania, G.R. The physiological determinants of drug-induced lysosomal stress resistance. *PLoS ONE* **2017**, *12*, e0187627. [[CrossRef](#)]
66. Cucci, L.M.; Trapani, G.; Hansson, Ö.; La Mendola, D.; Satriano, C. Gold Nanoparticles Functionalized with Angiogenin for Wound Care Application. *Nanomaterials* **2021**, *11*, 201. [[CrossRef](#)] [[PubMed](#)]