Multifunctionalized Gold Nanoparticles with Peptides Targeted to Gastrin-Releasing Peptide Receptor of a Tumor Cell Line

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Functionalization of gold nanoparticles (AuNPs) with both a targeting peptide (an analogue of the peptide Bombesin) and a drug peptide ligand (an analogue of the RAF peptide) with the aim of improving selectivity in the delivery of the conjugates as well as the antitumor activity is described. Studies on the internalization mechanism of peptide—AuNP conjugates and viability of cells were carried out. An enhancement of the activity and selectivity of the peptide multifunctionalized conjugates was observed.

INTRODUCTION

Gold nanoparticles (AuNPs) have been extensively used in biological applications because of their biocompatibility, dimension, ease of characterization (1, 2), and rich history of surface chemistry. These features make these structures easily exploitable to meet the requirements of biomedical applications (3).

The size of AuNPs can be controlled from a few nanometers up to tens of nanometers, thereby placing them at dimensions that are smaller than or comparable to those of a cell (10−100 μm), a virus (20−450 nm), a protein (5−50 nm), or a gene (2 nm wide and 10−100 nm long). These sizes imply that they could get close to a biological target of interest (4). For example, AuNPs can be used to deliver a cargo, such as an anticancer drug, or a cohort of radionuclide atoms to a targeted region of the body, such as a tumor (4). On the other hand, AuNPs have been used in photothermal therapy for the destruction or molecular surgery of cancer cells or tumors. When irradiated with a focused laser in the near-infrared region (NIR) of suitable wavelength, targeted aggregates of AuNPs, nanorods, or nanoshells can kill bacteria (5) and cancer cells (6).

Thus, AuNPs provide an excellent intracellular targeting vector for two reasons: they can be synthesized routinely in appropriated sizes adequate for delivery varying from 0.8 to 200 nm with <10% size dispersity and can be modified with a large collection of small molecules, peptides, proteins, DNA, and polymers (7).

Surface modifications of AuNPs are often used to increase the functionality of nanoconjugates. These nanoparticle modifiers/conjugants include various peptides which can specifically recognize a target, thus improving cell type uptake (8) and ensuring that the AuNPs reach the desired target. Peptides attached to a single nanoparticle make the individual targeting signals more accessible to cell receptors (9), thus allowing them to participate in ligand−receptor interactions (4, 10).

The design and development of tumor-specific nanoparticles could significantly amplify the delivery capacity to a specific target of interest without affecting healthy cells. The target specificity of nanoparticles could be imparted by tagging with certain biovectors, which navigate them to particular organs or sites under in vivo conditions. The targeting vectors most commonly used are monoclonal antibodies and receptor-specific peptides. Monoclonal antibodies, due to their larger sizes, show poor in vivo mobility and thus delayed and reduced uptake over the desired target (11–13). Moreover, these antibodies are highly immunogenic and as such produce harmful side effects. In sharp contrast, peptides, which are smaller, offer various advantages, namely, rapid blood clearance, ease in the penetration of the tumor’s vascular endothelium, increased diffusion rates in tissue, and low immunogenicity. Receptors for peptides are highly expressed on a variety of neoplastic and non-neoplastic cells. Furthermore, receptor-targeting peptides have shown a high level of internalization within tumor cells via receptor-mediated endocytosis. The capacity to internalize probes within tumor cells is crucial for delivering maximum payloads to tumor cells (14−16). These attractive physical properties coupled with their smaller size make peptides ideal candidates for developing new target-specific nanoparticles. In a recent paper, Chanda et al. conjugated a bombesin/gastrin-releasing peptide (BN/GRP) analogue to gold nanorods, which are internalized in cancer cells for the development of in vivo molecular imaging agents for cancer (17).

In the present study, we functionalized AuNPs with both a targeting peptide and a drug peptide ligand with the aim to improve not only the selectivity of the conjugates, but also their antitumor activity over the free drug. Since AuNPs conjugated to a targeting ligand have a higher capacity to enter cells, carrying the antitumor peptide and thus increasing its local concentration will, in turn, enhance the antitumor activity. BN was chosen as a targeting ligand for bombesin gastrin releasing
peptide receptor (GRP r), which has been found to be overexpressed in prostate, breast, small cell lung, and cervical cancer (14–20). Furthermore, AuNPs were functionalized with the drug peptide ligand RAF analogue which inhibits Rb-Raf-1 binding in vivo and therefore inhibits tumor growth and angiogenesis (21). In addition, AuNPs were multifunctionalized with a mixture of both peptides with the goal of implementing the targeting vehicle with antitumor activity.

The size of AuNPs may affect the cell permeability as well as toxicity. Taking into account these relevant points in this study, we chose 20-nm-diameter gold nanoparticles for three reasons: (i) they possess a widespread organ distribution (22–24) which is very relevant for future drug delivery applications, (ii) they have higher penetration in cells with respect to bigger AuNP (>70 nm) (25), and last (iii) AuNPs of this size were reported to pose no inherent toxicity to different cell lines (26, 27) and in zebrafish embryos (28), in contrast to AuNPs of 1–2 nm that were found to be highly toxic in cell lines (29, 30).

The multifunctionalized AuNPs with RAF peptide analogues (Ac-Cys-Ahx-RAF) and BN (Ac-Cys-Ahx-BN) (Scheme 1) were evaluated for their cell internalization properties and in vitro viability in HeLa cells, which overexpress GRPr, and in neuroblastoma SHSY-5Y cells, which do not overexpress these receptors.

RESULTS AND DISCUSSION

BN and RAF Peptide Analogues. A very promising group of small targeting ligands comprises a number of regulatory peptides. Among these, the BN peptide, originally isolated from the skin of a frog, has also shown antitumor activity (31). BN-like peptides are known to bind to GRPrs with high affinity and specificity. A high number of GRPrs are overexpressed in various human tumors (32) like glioblastoma, small cell lung, gastric, pancreatic, prostate, breast, cervical, and colon cancer (16, 33–36), as well as in premalignant cells in the case of prostate, gastric, and lung cancers (37–39). These receptors are therefore promising targets for targeted therapy of cancer because of their location on the plasma membrane, which promotes the internalization of the receptor—ligand complex upon binding of a ligand. These findings open up the possibility of applying BN-like peptides as vehicles to deliver cytotoxic drugs (40, 41) into tumor cells (42). On the basis of studies by Dasgupta et al. (21), we selected the RAF peptide as the drug antitumor ligand. The retinoblastoma tumor suppressor protein (Rb) plays a vital role in regulating mammalian cell cycle progression and inactivation of this protein is required for entry into the S phase. Rb is inactivated by phosphorylation upon growth factor stimulation of quiescent cells, thereby facilitating the transition from G1 to S phase (43). Previous studies have shown that the signaling kinase c-Raf (Raf-1) physically and functionally interacts with Rb and contributes to its inactivation, thereby facilitating cell proliferation. Disruption of the Rb-Raf-1 interaction by the 9-amino-acid peptide significantly inhibits Rb phosphorylation and thus cell proliferation (21).

The peptides were synthesized using a Fmoc/Bu solid-phase synthetic strategy (Materials and Methods section) and a free thiol containing Cys was introduced at the end of a spacer to facilitate attachment onto the gold surface (Table 1). As the C-terminus of BN is required for high-affinity binding (44), the N-terminus of the peptide was modified through an aliphatic linker to allow labeling with the cargo (45). The spacers have to position the gold conjugate sufficiently far from the GRPr-binding region of BN to prevent hindrance of the binding (affinity) of the BN motif with GRPr (46, 47). The optimal hydrocarbon spacer length should vary from 3 to 8 carbon atoms without adversely affecting the resultant binding affinity (48). As reported by Hoffman et al. (49), an increase in the hydrophobicity of the linker group to excessive levels reduces the receptor-binding affinity (47, 49, 50).

Thus, for conjugating both BN and the RAF peptides, a 6-carbon-atom spacer (aminohexanoic acid, Ahx) was selected to be attached to a free thiol containing Cys in order to facilitate anchoring onto the gold surface (Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF) (Table 1). To check whether the free analogue peptides—unconjugated to AuNPs—BN and RAF have the capacity to penetrate the cell, we labeled peptides with a carboxyfluorescein (CF) fluorophore. The CF was introduced into the ε-amino of an additional residue of Lys, which was incorporated at the N-terminal of the peptide, before the incorporation of the Ahx residue. Peptides Ac-Cys-Ahx-Lys(CF)-BN and Ac-Cys-Ahx-Lys(CF)-RAF were also produced using a Fmoc/Bu/Alloc solid-phase strategy (Supporting Information Figure S1, Table S1). The Alloc group was used to block the Lys side chain and was removed once the elongation of the peptide sequence was done to incorporate the CF before the global deprotection and cleavage.

Conjugation of Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF with AuNPs. Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF were conjugated separately and also in an equimolar mixture with a 20 nM AuNP solution to obtain the following conjugates: Ac-Cys-Ahx-BN@AuNPs, Ac-Cys-Ahx-RAF@AuNPs, and Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs (Note: From a peptide point of view, a more correct nomenclature should be Ac-Cys(AuNPs)-Ahx-BN, Ac-Cys(AuNPs)-Ahx-RAF, and Ac-Cys(AuNPs)-Ahx-BN/Ac-Cys(AuNPs)-Ahx-RAF) (Table 1). We then tested the influence of both the targeting and drug ligands on the cell penetration and antitumor activity of these conjugates. AuNPs were obtained via the sodium citrate reduction method (51–53), which allows the synthesis of clusters of particles between 6 and 60 nm by adding various amounts of the reducing agent. The AuNPs were characterized by UV–VIS (Figure 1) and transmission electron microscopy (TEM)
used for the conjugation, the bounded peptide concentration was found to be about 50 µM and the AuNP solution was about 2 nM (determined spectrophotometrically), which results in a ratio of around 25 000 peptide molecules per AuNP. The large number of peptide molecules/nanoparticle could be attributed to a self-aggregation process that occurs in amphipathic peptides (58).

Amino Acid Analysis (AAA). The relationship among the concentrations of the amino acids His, Ile, and Phe give the relative concentration of these two peptides on the AuNPs. The relationship found, 1 for His/Ile and 2 for Phe/His, showed that both peptides were incorporated equimolorly onto the Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs (Supporting Information Table S3).

Cellular Internalization of the Carboxyfluorescinated Peptides as Shown by Confocal Laser Scanning Microscopy (CLSM). Peptides Ac-Cys-Ahx-Lys(CF)-BN and Ac-Cys-Ahx-Lys(CF)-RAF were incubated with HeLa (cervical tumoral cell line which overexpresses GRP receptors) and SHSY-5Y cells for 24 h. As expected, Ac-Cys-Ahx-Lys(CF)-BN was efficiently internalized in HeLa cells and not in SHSY-5Y, while Ac-Cys-Ahx-Lys(CF)-RAF entered both HeLa and SHSY-5Y cell lines, hence showing its lack of specificity (Figure 3).

Accumulation of Peptide—AuNP Conjugates and AuNPs as Shown by CLSM. In order to prove whether the functionalization of AuNPs with BN and RAF—or with both together—favors selectivity in the delivery of the conjugates, the reflection of the nanoparticles was exploited. The red coloration of the nanconjugates (9) of Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF inside cells was observed due to the accumulation and reflection of the AuNP.

An internalization study of the conjugates after 24 h of incubation was performed in the tumoral cell line HeLa and in the SHSY-5Y cell line. The cell membrane was marked in green by WGA, and the nucleus was stained in blue with DRAQ5. The degree of accumulation inside the HeLa cells occurred in the following order Ac-Cys-Ahx-BN@AuNPs > Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs > Ac-Cys-Ahx-RAF@AuNPs (Figure 4). Ac-Cys-Ahx-BN@AuNPs showed the greatest accumulation, suggesting a receptor-mediated internalization mechanism, most likely via endocytosis, as reported in the literature (17); however, in this paper we did not study the internalization mechanism. On the contrary, Ac-Cys-Ahx-RAF@AuNPs presented low internalization in HeLa cells due to the fact that the receptor-mediated internalization mechanism is not favored by target peptide. Finally, the conjugates Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs showed an intermediate cell penetration capacity. As expected, gold conjugates did not accumulate in the SHSY-5Y cell line because of the low concentration of GRPr for this cell line.

These results provide an illustration of the power of our strategy. Thus, while the RAE conjugate internalizes poorly into HeLa cells, the AuNPs containing RAF and BN did because of the presence of the BN moiety, which has the capacity to bind to the GRP or cells. Furthermore, the presence of the BN moiety provides selectivity for the drug—ligand conjugate as shown by the finding that none of the AuNPs penetrated the SHSY-5Y cells. This selectivity is potentially useful in the development of ligand-targeted therapeutics.

Colocalization of the Fluorescence and Gold Reflection Signal Caused by the Conjugate-Labeled Peptides as Shown by CLSM Imaging. The carboxyfluorescinated conjugates, Ac-Cys-Ahx-Lys(CF)-BN@AuNPs, Ac-Cys-Ahx-Lys(CF)-RAF@AuNPs, and Ac-Cys-Ahx-Lys(CF)-BN/Ac-Cys-Ahx-Lys(CF)-RAF@AuNPs, were incubated with the tumor cell line HeLa for 24 h at 37 °C to colocalize the fluorescence signal caused by the peptide—AuNP conjugate and the reflection of the AuNP.
The membrane of the cells was stained green and the internalization of the fluorinated gold complexes was visualized using CLSM. These observations revealed that nearly all the AuNPs were complexed to the fluorescently labeled peptides (Figure 5) when overlapping the red reflection of the AuNPs with the blue fluorescence of the carboxyfluorinated peptides. These results indicate that the labeled peptides do not detach from the AuNP surface. However, a partial hydrolysis of the peptide in cells may occur. This possibility could not be discarded in the present study because the fluorescence label was found in the N-terminal extreme of the peptide, which was bound to the AuNP surface.

Viability Studies. The degree of viability and effects of the free peptides (Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF), gold conjugates Ac-Cys-Ahx-BN@AuNPs and Ac-Cys-Ahx-RAF@AuNPs, and the equimolar mixture Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs and AuNPs on cell proliferation was determined by MTT assay in HeLa and SHSY-5Y cells following 24 h of incubation (Figure 6). AuNPs did not show any significant effects on HeLa cell viability, while Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF at concentrations of 1 × 10^{-5} M reduced the viability of cells in 33% and 52%, respectively, with respect to the controls. As expected, the RAF peptide possesses a higher antitumor activity, since it is reported to suppress tumor growth and angiogenesis (59, 60). However, to the best of our knowledge, the antitumor activity of both peptides has never been compared in the same conditions. In the case of BN, the conjugation with AuNPs increases the toxicity in cells possibly due to a synergistic effect in the penetration between the AuNP and the peptide, which helps to increase the local concentration which in turn enhances the antitumor activity and is in accordance with the increased delivery of Ac-Cys-Ahx-BN-AuNP to HeLa cells. The conjugates Ac-Cys-Ahx-RAF-AuNP produce similar effects on cell viability with respect to the peptide Ac-Cys-Ahx-RAF-AuNP.

Interestingly, conjugates of the peptides with AuNPs did not show any effect on cell viability in neuroblastoma SHSY-5Y cells. The lack of toxicity could be attributed to the low penetration that the conjugates present in this cell line due to the low expression of GRPr receptors. Remarkably, Ac-Cys-Ahx-RAF is toxic against this cell line which evidences the low selectivity of this peptide against cells in general (as shown by the CLSM of the CF peptide, Figure 3); however, conjugation with AuNP results in increased selectivity (toxicity in HeLa cells but not in SHSY-5Y). The conjugation of this peptide with AuNPs could induce a change in the conformation of the peptide molecules that leads to a reduction of toxicity in neuroblastoma cells. This is an interesting point which we are currently studying.

CONCLUSIONS

In summary, we report herein a strategy to increase the activity and cell selectivity of AuNP—peptide conjugates by...
Materials and Methods

Materials. Rink amide MBHA resin and Fmoc-N\textsuperscript{α}-protected amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany). 7-Aza-1-hydroxy-1\textsubscript{H}-benzotriazole (HOAt) was obtained from GL Biochem (Shanghai, China); 1-benzotriazolyloxytris(pyrrolidino)-phosohonium hexafluorophosphate (PyBOP) was purchased from Novabiochem (Lau\sselfelfingen, Switzerland); 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosohonium hexafluorophosphate (PyAOP) was obtained from GL Biochem (Shanghai, China); 1-benzotriazolyloxytris(pyrrolidino)-phosohonium hexafluorophosphate (PyAOP) was purchased from Applied Biosystems (Foster City, CA); and N,N\textsuperscript{′}-diisopropylethylamine (DIEA) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Tetrakis(triphenylphosphine)palladium (0) [Pd(PPh\textsubscript{3})\textsubscript{4}], phenyl silane (PhSiH\textsubscript{3}), triisopropylsilane (TIS), and other chemicals used were obtained from Aldrich (Milwaukee, WI, USA) and were of the highest purity commercially available.

Peptide Synthesis and Chromatography. (All reported solvent ratios are expressed as v/v, unless otherwise stated). The four peptides were synthesized manually on Fmoc Rink amide MBHA resin (100 mg, 0.68 mmol/g) using a Fmoc/Bu strategy in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings, and subsequent deprotection steps were carried out with DMF and DCM using around 10 mL solvent per gram of resin each time. The Fmoc group was removed by treatment with a mixture of piperidine–DMF (1:4 v/v) (2 × 10 min). After Fmoc removal, coupling reactions were carried out with Fmoc-amino acids (4 equiv), HOAt (4 equiv), and DCM (4 equiv) in DCM for 1.5 h. Couplings were monitored using the Kaiser test (6/1). When necessary, recouplings were done with PyBOP (4 equiv), HOAt (4 equiv), and DIEA (12 equiv) for 1.5 h. After completing the synthesis, we performed an acetylation step with a mixture of AcOH (10 equiv) and DCM (5 equiv) for 10 min. For fluorocently labeled peptides [Ac-Cys-Ahx-Lys(CF)-BN] and Ac-Cys-Ahx-Lys(CF)-RAF, after completion of the peptide sequence, the Alloc (Allyloxycarbonyl) group was removed with Pd(PPh\textsubscript{3})\textsubscript{4} (0.1 equiv) in the presence of PhSiH\textsubscript{3} (10 equiv) (3 × 15 min) under Ar atmosphere followed by several washes (5 × 1 min) with sodium N,N\textsuperscript{′}-diethyldithiocarbamate (0.02 M in DMF) and DMF. CF (5 equiv), PyAOP (5 equiv), HOAt (5 equiv), and DIEA (10 equiv) were dissolved in a mixture of DMF–DCM, preactivated for 10 min, and added to the peptide resin. The reaction mixture was stirred for 1.5 h. For the deprotection of side chain groups and concomitant cleavage of the peptides from the support, the resin was washed with DCM (3 × 1 min), dried, and treated with a TFA–H\textsubscript{2}O–TIS (95:2.5:2.5) cocktail for 1 h. TFA was then removed by evaporation with a N\textsubscript{2} stream and the peptides were precipitated with a mixture of cold anhydrous tert-butyl methyl ether (TBME) and hexane (2:1), dissolved in H\textsubscript{2}O–MeCN (1:1) and freeze–dried. The crude peptides were purified using a semipreparative RP-HPLC (Waters 2487 Dual \textlambda; Absorbance Detector (Waters, MA, USA) equipped with a Waters 2700 Sample Manager, a Waters 600 controller, a Waters Fraction Collector, a Symmetry column (C\textsubscript{18}, 5 μm, 30 × 100 mm), and Millennium software; flow rate = 10 mL/min, gradient = 0–20% B for 5 min, 20–40% B for 35 min, 40–100% B
for 5 min (A = 0.1% TFA in H₂O, B = 0.05% TFA in MeCN)). The purified peptides were identified (Supporting Information Figure S1 ai, bi, ci, di) at λ = 220 nm by analytical RP-HPLC [Waters 996 photodiode array detector equipped with Waters 2695 separation module, a Symmetry column (C₁₈, 5 µm, 4.6 × 150 mm) and Millennium software; flow rate = 1 mL/min, gradient = 20–60% D for 15 min for Ac-Cys-Ahx-BN, Ac-Cys-Ahx-RAF, Ac-Cys-Ahx-Lys(CF)-RAF, and 20–60% D for

Figure 5. Localization of carboxyfluorescinated peptides attached to AuNPs in HeLa cells incubated for 24 h. (a) Ac-Cys-Ahx-Lys(CF)-BN@AuNPs, (b) Ac-Cys-Ahx-Lys(CF)-RAF@AuNPs, (c) Ac-Cys-Ahx-Lys(CF)-BN/Ac-Cys-Ahx-Lys(CF)-RAF@AuNPs. Membranes (green) were stained with a fluorescence marker (WGA). Reflection of the AuNPs in red (i), carboxyfluorinated peptides in blue (ii), and the overlay of both channels in purple (iii). The conjugated peptides were added at a final concentration of 1 × 10⁻⁵ M of peptides and 0.4 nM of AuNPs.

Figure 6. Cell viability assay after incubation of cells with Ac-Cys-Ahx-BN, Ac-Cys-Ahx-RAF, and their gold conjugates for 24 h: (a) HeLa cell line and (b) SHSY-5Y cell line. The conjugated and nonconjugated peptides were added at a final concentration of 1 × 10⁻⁵ M of peptides and 0.4 nM of AuNPs. The results are expressed as percentages compared to untreated cells and represent the mean ± standard deviation of three independent experiments (** P < 0.001).
suspensions were seeded at a concentration of 3.5
enediaminetetraacetic acid (EDTA) solution, and the cell
mycin. For experiments, exponentially growing cells were
HeLa cell line and high glucose medium for SHSY-5Y
and ATCC no CRL-2266, respectively (Manasas, VA). Both
and SHSY-5Y cell lines were obtained from ATCC no CCL-2
pellet was removed, the free peptide containing solution was
concentration of grafted peptide was determined by interpolation
which was determined spectrophotometrically considering the
tration of grafted peptide by the number of AuNPs in solution,
HAuCl4
aii, bii, cii, dii).
Preparation of AuNPs. AuNPs were produced by reduction of
H2O (8.7 mg) was added to a sodium citrate
the area of the HPLC peak of the solution resulting after
concentration of 13 500 rpm for 30 min. Once the AuNP
the agitation was then maintained for 15 min. The conjugates
were then purified by dialysis over three days in a Spectra/Por
62
62
CM2 for the HeLa line and 1
mm microwell, 1.5 mm cover glass) (Nalge Nunc International,
biomolecules (Supporting Information Figure S1
Ac-Cys-Ahx-BN and Ac-Cys-Ahx-RAF or their fluorescently
solution was refreshed six times to remove the excess peptide
were then purified by dialysis over three days in a Spectra/Por
solution was refreshed six times to remove the excess peptide
Ac-Cys-Ahx-RAF@AuNPs) and for the carboxyfluorinated peptides

cellular localization of AuNP conjugates (Ac-Cys-Ahx-BN@
photometer (Shimadzu Corporation, Kyoto, Japan).
Electron Energy Loss Spectroscopy (EELS). AuNP or
AuNP-peptide (Ac-Cys-Ahx-BN@AuNPs, Ac-Cys-Ahx-RAF@AuNPs, Ac-Cys-Ahx-RAF/Ac-Cys-Ahx-BN@AuNPs) solutions
(20 µL × 3) were placed on holley carbon Cu grids and allowed
to dry at room temperature. Electron energy loss spectra were
obtained in a Gatan Image Filter (GIF 2000) coupled to the
the JEOL 2010F microscope, with an energy resolution of 1.2 eV.
Amino Acid Analysis (AAA). Amino acid analysis was carried out by the AccQ. Tag method after acid hydrolysis with
HCl (6N) for 24 h at 110 °C was used. The analysis was performed in a Waters Delta 600 RP-LC system with UV
detection at 254 nm.
Determination of Conjugate Concentration. The number of peptides per particle was calculated by dividing the concen-
tration of grafted peptide by the number of AuNPs in solution,
which was determined spectrophotometrically considering the
molar extinction coefficients detailed in the literature (57). The concentration of grafted peptide was determined by interpolation
into a calibration curve (Supporting Information Figure S2) of
the area of the HPLC peak of the solution resulting after
centrifugation with an amicon filter (Amicon ultra-15, 3 kDa,
MiliPore Corporation, Billerica, MA, USA) of a known volume of the conjugates at 13 500 rpm for 30 min. Once the AuNP
pellet was removed, the free peptide containing solution was
freeze-dried, dissolved again, and injected into the HPLC.
Cell Culture and Incubation with the Conjugates. HeLa
and SHSY-5Y cell lines were obtained from ATCC no CCL-2
and ATCC no CRL-2266, respectively (Manasas, VA). Both
cell lines were maintained in DMEM (low glucose medium for
HeLa cell line and high glucose medium for SHSY-5Y)
(Biological Industries) containing 10% fetal calf serum (FCS),
2 mM glutamine, 50 U/mL penicillin, and 0.05 g/mL strepto-
mycin. For experiments, exponentially growing cells were
detached from the culture flasks using a trypsin—0.25% ethyl-
endiaminetetraacetic acid (EDTA) solution, and the cell
suspensions were seeded at a concentration of 3.5 × 10^3 cells/cm^2 for the HeLa line and 1 × 10^3 cells/cm^2 for the SHSY-5Y
line onto glass-bottom microwell dishes (35 mm Petri dish, 14
mm microwell, 1.5 mm cover glass) (Nalge Nunc International,
Rochester, NY). Experiments were carried out 24 h later, when
the confluence was approximately 70–80%. Nonadherent cells
were washed away and attached cells were incubated at 37 °C
in 5% CO_2 in DMEM with a known concentration of free
AuNPs, peptide–AuNP conjugates, and carboxyfluorinated peptides. The conjugated and nonconjugated peptides were
added in a 1:4 (conjugate/DMEM) ratio at a final concentration of 1 × 10^{-5} M of peptides and 0.4 nM of AuNPs.
MTT Assay. HeLa and SHSYS-5Y viability in the presence of
nonconjugated or conjugated nanoparticles and the free
peptides was tested by the reduction of 3-[4,5-dimethylthiazol-
2-yl]-2,5-diphenyltetrazolium bromide (MTT), including a control with no nanoparticles added. For each assay, 3.5 × 10^3
plates/cm^2 of the HeLa cell line and 1 × 10^3 cells/cm^2 of the
SHSY-5Y cell line were seeded onto a 96-well plate (Nalge Nunc)
and cultured for 24 h. The conjugates were added in a 1:4 (conjugate/DMEM) ratio, and the free peptides were added
at the same concentration as the peptide–gold conjugates at a
final concentration of 1 × 10^{-5} M of peptides and 0.4 nM of AuNPs. Cells were incubated for 22 h at 37 °C under a 5%
CO_2 atmosphere. After incubation, the medium was removed
and the cells were incubated with 200 µL fresh medium
containing MTT (0.5 mg/mL) for 2 h. The resulting blue
formazan was solubilized in 150 µL acidified isopropanol (0.04
M HCl) and the absorbance was measured at 560 nm using a
plate reader (Multiskan Ascent, Thermo Scientific). The values
of the untreated cells were taken as 100%, and cell viability
was expressed as a percentage of the untreated cells. The data
were obtained from three independent experiments performed
in triplicate.
Confocal Laser Scanning Microscopy (CLSM). For the
cellular localization of AuNP conjugates (Ac-Cys-Ahx-BN@AuNPs, Ac-Cys-Ahx-RAF@AuNPs, Ac-Cys-Ahx-BN/Ac-Cys-Ahx-RAF@AuNPs) and for the carboxyfluorinated peptides
(Ac-Cys-Ahx-Lys(CF)-BN and Ac-Cys-Ahx-Lys(CF)-RAF),
HeLa and SHSY-5Y cells were plated onto glass coverslips,
grown to 60% confluence, and then incubated at 37 °C
under a 5% CO_2 atmosphere with either Ac-Cys-Ahx-Lys(CF)-BN, Ac-
Cys-Ahx-Lys(CF)-RAF or nanoparticle conjugates for 24 h.
WGA as plasma membrane marker and DRAQ5 as a DNA dye
were added just before recording the image. The samples were
examined using an Olympus Fluoview 500 confocal microscope with UV
detection at 600 nm.

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Supporting Information Available: Additional information
as described in the text. This material is available free of charge
via the Internet at http://pubs.acs.org.

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