Targeting the Surface of the Protein 14-3-3 by Ultrasmall (1.5 nm) Gold Nanoparticles Carrying the Specific Peptide CRaf

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The surface of ultrasmall gold nanoparticles with an average diameter of 1.55 nm was conjugated with a 14-3-3 protein-binding peptide derived from CRaf. Each particle carries 18 CRaf peptides, leading to an overall stoichiometry of Au(115)Craf(18). The binding to the protein 14-3-3 was probed by isothermal titration calorimetry (ITC) and fluorescence polarization spectroscopy (FP). The dissociation constant \( K_d \) was measured as 5.0 \( \mu \)M by ITC and 0.9 \( \mu \)M by FP, which was close to the affinity of dissolved CRaf to 14-3-3. In contrast to dissolved CRaf, which alone did not enter HeLa cells, CRAF-conjugated gold nanoparticles were well taken up by HeLa cells, opening the opportunity to target the protein inside a cell.

The potential of supramolecular binding molecules can be enhanced by conjugating them to an ultrasmall nanoparticle (1–2 nm). Such particles have a diameter even below the size of a protein. The possibility to attach more than one ligand to a single nanoparticle permits multiavid targeting of the protein surface.

Chemically, ultrasmall nanoparticles are at the borderline between atom-sharp metal clusters (ca. 1 nm) and “classical” metallic nanoparticles with a diameter of 10 nm or more. Due to their small size, they have been explored in the last years for biological and biomedical applications. However, for instance, such nanoparticles can serve as carriers of surface-conjugated cargo molecules into cells and (in some cases) even into the cell nucleus. They can be surface-conjugated by thiol-containing ligands that lead to a strong Au–S covalent bond. In the case of biomolecules, cysteine and cysteine-containing peptides and ligands are of particular value for nanoparticle conjugation.

The diphosphorylated (pS233pS259) CRaf peptide is a strong binder for 14-3-3 proteins. Here we demonstrate how ultrasmall gold nanoparticles can be surface-conjugated with the protein-targeting peptide CRaf (residues 229–264, subsequently termed as CRaf) and show their interaction with the protein 14-3-3 and also their uptake by eukaryotic cells.

2. Results and Discussion

We have chosen the peptide CRaf in a cysteine-modified form for its conjugation to ultrasmall gold nanoparticles to target epitopes on the surface of the protein 14-3-3. Equidistant between the two phosphorylated residues pS233 and pS259, a cysteine was introduced at position 246, leaving the peptide binding arms unchanged. The peptide was attached to the ultrasmall gold nanoparticles via the thiol group of this cysteine.

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The size of the metallic nanoparticle core was probed by aberration-corrected transmission electron microscopy. It should be noted that the image contrast of the particles with respect to the background is weak, even with an aberration-corrected electron microscope, because of the small size of the particles with respect to the coating by a considerable amount of organic peptide shell (Figure 1) and the thickness of the carbon support. However, individual particles could be well distinguished with an average diameter of 1.55 nm. This corresponds to about 115 gold atoms under the assumption of a spherical particle.

The attachment of the CRaf peptide was shown by $^1$H NMR spectroscopy (Figure 2). Clearly, the ligand was attached in unchanged form, with only small changes in the spectrum. This is due to the facts that the peptide is rather big, and that most amino acids (and protons) are in a considerable distance from the metal core and also likely retained most of their flexibility. Thus, peak broadening as it is usually observed with ligands on ultrasmall nanoparticles was mostly avoided.[$^{11,13}$]

The peptide was firmly attached to the nanoparticles as demonstrated by $^1$H DOSY NMR spectroscopy (Figure 3). Clearly, all $^1$H NMR signals were associated with the same diffusion coefficient. As hydrodynamic diameter, 2.6 and 3.6 nm were obtained for peptide and nanoparticles, respectively. No free (unbound) CRaf was present in the nanoparticle dispersion.

The particle size distribution of the dispersed nanoparticles was also analyzed by differential centrifugal sedimentation (DCS), giving a hydrodynamic diameter of 1.5 nm (Figure 4). However, the diameters obtained by this method are systematically too small for functionalized ultrasmall nanoparticles because it is probing the hydrodynamic diameter by sedimentation, assuming a uniform density of the particle. Thus, this
diameter is clearly too small. If the solid particle carries a peptide shell, it sediments more slowly than expected because its effective density is lower than that of the gold core (19.3 g cm\(^{-3}\)). Nevertheless, the DCS results clearly show that the nanoparticles are present in a well-dispersed state without major agglomerations. It is also noteworthy we worked at the borderline of the method with a centrifugation time of about 5 h, that is, the sedimentation rate was low due to the ultrasmall particle size. This is enhanced by the long peptide chain of CRaf that is attached to the nanoparticles, increasing the hydrodynamic diameter.

The surface loading of the nanoparticles with peptides was probed by UV spectroscopy with the FAM-labeled CRaf peptide (Figure 5). There was only little quenching (if at all) because the nanoparticles are ultrasmall, in accordance with earlier observations with similar nanoparticles.\(^{[9c]}\) From the determination of the gold concentration by atomic absorption spectroscopy (AAS) which was converted to nanoparticles (1.55 nm, 115 gold atoms), we obtained 18 CRaf molecules on the surface of each gold particle (see the Experimental Section for a detailed calculation). As the molecular weight of each CRaf peptide is 4 193 g mol\(^{-1}\), the composition of each nanoparticle is about 115·197 g mol\(^{-1}\) = 22 654 g mol\(^{-1}\) CRaf, giving a weight ratio of about 22 654:75 474 = 1:3.3 = m(Au)/m(CRaf). Consequently, each particle consists of about 23 wt% Au and 77 wt% peptide. In terms of volume, the metallic core (1.55 nm) occupies 1.95 nm\(^3\), that is, only about 8% of the total hydrodynamic volume (24.4 nm\(^3\), based on a hydrodynamic diameter of 3.6 nm). Each CRaf molecule has a molecular footprint of 0.41 nm\(^2\). For cysteine on 1.78 nm gold nanoparticles, we found a smaller footprint of 0.15 nm\(^2\) which makes sense due to the bigger peptide CRaf and its “two-armed” attachment to the nanoparticle surface. Figure 6 visualizes the structure of a gold nanoparticle with a CRaf ligand, including its interaction with 14-3-3. The homodimeric protein 14-3-3\(\sigma\) has a molecular weight of 57.62 kDa and a diameter of about 6.8 nm (PDB ID: 4FJ3).\(^{[15]}\)

The specific interaction of CRaf with 14-3-3\(\sigma\) was probed by isothermal titration calorimetry (Figure 7). The interaction of

![Figure 6](image)

Figure 6. Top: Schematic view of the modified sequence of CRaf, conjugated to a gold nanoparticle (1.55 nm in diameter), visualized by PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). Bottom: Schematic view of CRaf on a gold nanoparticle interacting with the homodimeric protein 14-3-3\(\sigma\). Only the amino acids that interact with the protein binding region are shown; the other part of the chain is shown as a dashed line. The structural data were taken from the protein database (PDB ID: 4FJ3) and visualized by PyMOL.

![Figure 7](image)

Figure 7. Interaction of dissolved CRaf peptide with 14-3-3\(\sigma\) as probed by ITC. The differential heat flow (top) and the Wiseman plot (bottom) are shown.
dissolved CRaf gave a dissociation constant $K_D$ of 1.3 ± 0.1 μM, i.e. a rather strong interaction. The binding ratio CRaf/14-3-3σ was 0.84 ± 0.01, that is, each peptide was bound by one protein. The interaction was exothermic with 67 ± 1 kJ mol$^{-1}$ (15.7 ± 0.14 kcal mol$^{-1}$). However, the binding is weaker than reported earlier by Molzan et al. who found a $K_D$ of 0.08 μM for CRaf and the isomer 14-3-3ζ.$^{[16]}$ This likely is an artifact of the fitting procedure because, in contrast to the free peptide in solution, not all peptides on a given nanoparticle can possibly be bound by protein due to steric reasons, which results in overestimating the $K_D$.

For nanoparticle-conjugated CRaf and 14-3-3σ, we measured a $K_D$ of 5.0 ± 0.1 μM and a binding enthalpy of 19 ± 2 kJ mol$^{-1}$ (4.57 ± 0.35 kcal mol$^{-1}$). The binding ratio CRaf/14-3-3σ dimer was 0.75 ± 0.03, that is, comparable to the free peptide (Figure 8).

Fluorescence polarization spectroscopy is an alternative method to study the interaction between a protein and a binding ligand. The protein 14-3-3σ was titrated to a solution of FAM-CRaf or a dispersion of FAM-CRaf on gold nanoparticles, respectively (Figure 9). For the dissolved ligand, we determined a $K_D$ of 1.5 ± 0.1 μM, in good agreement with Molzan et al. for 14-3-3ζ.$^{[16]}$ For CRaf on gold nanoparticles, we found 0.9 ± 0.1 μM with 18 CRaf molecules on each gold nanoparticle. The binding strength was comparable or even slightly higher than that of CRaf alone.

For a practical application, it is important to target proteins inside cells. Therefore, we investigated the uptake of CRaf-conjugated gold nanoparticles by HeLa cells (Figure 10). The particles were easily taken up whereas dissolved CRaf could enter the cells only to a very small extent (Figure 11). This illustrates that a carrier is needed to transport functional peptides across the cell membrane. However, additional studies would be required to prove that the CRaf-functionalized nanoparticles are actually targeting the protein 14-3-3 inside the cell. The fact that we observed only few agglomerations by confocal microscopy indicates that the particles are well dispersed in cell culture medium. Unfortunately, dynamic light scattering is not applicable because the particles are too small.

Table 1 summarizes all measured properties of dissolved CRaf and of CRaf, conjugated to gold nanoparticles.
proteins, its function during protein–protein interaction (PPI) should be controllable. This is demonstrated here for 14-3-3, but easily transposable to other proteins of biological or medical relevance. Future studies should address the selectivity of CRaf-functionalized nanoparticles by competitive binding studies in protein mixtures, ideally in living cells.

**Experimental Section**

**Chemicals:** An aqueous solution of tetrachloroaurotriocetic acid (TETA) sodium salt (NaAuCl₃, prepared by dissolving sodium tetrachloroaurate(III) dihydrate (NaAuCl₃·2H₂O, Sigma-Aldrich; 99 %) in hydrochloric acid (37 %) and ultrapure water) was used as gold source. Sodium borohydride (NaBH₄, Sigma-Aldrich; ≥ 96 %) in aqueous solution was used as reducing agent. For the protein-interaction studies, the gold nanoparticles were conjugated either with the modified diprophosphorylated CRaf peptide QHRD(pS)TPHAFTNTSSPGCRFQHPFTTTFSSG (residues 229–264; 95–96 %, 4192.63 g mol⁻¹), harboring a cysteine replacing serine 246 at the indicated site (mutant S246C), or with its FAM-labeled derivative QHRD(5,6-FAM)-pS)TPHAFTNTSSPGCRFQHPFTTTFSSG (residues 229–264; 95–96 %, 5451.34 g mol⁻¹). It was important to minimize the formation of peptide disulfides during the synthesis (requiring work under inert gas atmosphere to avoid thiol oxidation) because disulfides are very difficult to remove from the nanoparticle dispersion after they have formed.

The peptides were obtained from Caslo ApS (Denmark) as lyophilized chloride salts and used as received without further purification. In all experiments, ultrapure water with a specific resistivity of 18.2 MΩ (Purelab ultra instrument from ELGA) was used as solvent unless otherwise stated. All glassware was cleaned with boiling aqua regia and ultrapure water before all reactions involving nanoparticles. The functionalized gold nanoparticles were stored in HEPES buffer (25 mM HEPES, 100 mM NaCl, 2 mM MgCl₂, 1 mM L-mercaptoethanol, pH 7.4) in potassium phosphate buffer (47 mM K₂HPO₃, 3 mM KH₂PO₃, pH 8) after the synthesis.

**Nanoparticle synthesis:** For the syntheses of peptide-functionalized gold nanoparticles, we dissolved 0.1–1.4 μmol peptide in 0.13–1.87 mL degassed water and adjusted the pH to 5.5–5.7 with 0.1 M sodium hydroxide solution. The amounts of NaAuCl₃ and NaBH₄ were adapted according to the molar amount of peptide. It was necessary to work at low pH to minimize the thiol oxidation to disulfide is difficult to remove. Then, 1–19.5 μL NaAuCl₃ (corresponding to 33–467 nmol gold) was added to the peptide solution under stirring. The color of the peptide/gold mixture rapidly changed from yellow to colorless, indicating that the gold ions were reduced from Au³⁺ to Au₀. After 10 min of cooling the mixture in an ice bath, 1–10 μL of 0.2 M NaBH₄ solution (freshly prepared with 4 °C cold water; corresponding to 0.1–2 μmol) was added and stirred for another hour to reduce Au⁰ to Au²⁺. We carried out all syntheses under inert gas atmosphere (argon, Schlenk technique).

The gold nanoparticles were purified by ultracentrifugation for 15 h at 30,000 rpm (66,000 g). After that, we obtained two phases that is, the dark brown concentrated gold nanoparticle dispersion in the lower part of the centrifugation tube and a colorless phase of unreacted parent compounds and synthesis by-products in the upper part. The colorless phase was carefully removed with a pipette. The nanoparticle dispersion was then purified by spin filtration with a Pierce Protein Concentrator (PES, MWCO 10 kDa, 20 mL, Thermo Fisher) for 60 min at 4000 rpm (2000 g). Subsequently, the nanoparticles were multiply washed with HEPES buffer.

**Table 1.** Properties of dissolved CRaf and of CRaf-conjugated ultrasmall gold nanoparticles from HDOY, DCS, and HRTEM. An error of 10 % for the diffusion coefficient and the hydrodynamic diameter measured by HDOY was assumed. Note that DCS systematically underestimates the particle diameter.

|                        | Dissolved CRaf | Dispersed <Au-CRaf nanoparticles |
|------------------------|---------------|---------------------------------|
| D(H DOY) (m² s⁻¹)      | (1.89 ± 0.19)×10⁻⁹ | (1.36 ± 0.14)×10⁻⁹ |
| d(H DOY) (nm)          | 2.6 ± 0.3     | 3.6 ± 0.4                       |
| d(DCS) (nm)            | 1.5 ± 0.5     | 1.2 ± 0.3                       |
| d(HRTEM) (nm)          | 1.5 ± 0.5     | 1.3 ± 0.2                       |
| I₆₇ from ITC (μM)      | 1.3 ± 0.1     | 5.0 ± 0.1                       |
| binding ratio from ITC | 0.84 ± 0.01   | 0.75 ± 0.03                     |
| binding enthalpy from ITC (kJ mol⁻¹) | 66 ± 1 19 ± 2 |
| δc from FP (μM)        | 1.5 ± 0.1     | 0.9 ± 0.1                       |
| uptake by HeLa cells   | poor          | good                            |

**3. Conclusions**

The targeting of the relevant protein 14-3-3 is possible with peptide-functionalized ultrasmall gold nanoparticles. About 18 CRaf molecules can be covalently attached to a 1.55 nm ultrasmall gold nanoparticle. The binding ability of CRaf for 14-3-3Δ remains unchanged as shown by isothermal titration calorimetry and fluorescence polarization spectroscopy. A particular advantage of the nanoparticles is their easy uptake by cells, opening up the possibility for protein targeting inside cells.

![Figure 11. Uptake of FAM-CRaf by HeLa cells (CRaf concentration in the well 10 μM; 10,000 cells per well) after 24 h of incubation. Actin: red, cell nucleus: blue, nanoparticles: green.](image-url)
or potassium phosphate buffer by redispersion/spin filtration. The resulting volume of the purified and concentrated nanoparticle dispersion was about 50 to 60 μL. The nanoparticle dispersion was light-brown. The absence of the typical red color due to surface plasmon resonance of larger gold nanoparticles (10 nm or more) confirms that all particles are ultrasmall.

### Protein expression and purification

14-3-3 was expressed with a His-tag in Ni2+21D3E3E3) competent cells from a pPReoxHb vector in 2YT medium. The purification was carried out by affinity chromatography on nickel columns (HisTrap HP, 5 mL). The tags were cleaved with a TEV protease. The proteins were then loaded again on nickel columns to remove any uncleaved protein. A final purification step was performed by loading the proteins on a size-exclusion chromatography column (HiLoad 26/600 Superdex 75 pg) equilibrated in 20 mM TRIS-HCl buffer at pH 7.5, 150 mM NaCl, and 2 mM dithiothreitil (DTT). Before the ITC measurements, the protein was dialyzed with HEPES buffer (25 mM, pH 7.4).

### Cell uptake studies

The uptake of peptide-functionalized ultrasmall gold nanoparticles was carried out with human cervix carcinoma cells (HeLa). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10 % fetal bovine serum (FBS), 100 μM L- penicillin, and 100 U mL- streptomycin at 37°C in 5% CO2 atmosphere. The cells were trypsinized and seeded in a glass-bottom dish (ibidi Planegg, Germany) with 10⁴ cells per well in 200 μL cell culture medium 24 h prior to the uptake studies. 20 μL of 125 μg mL⁻¹ 5,6-FAM-CRaf-functionalized gold nanoparticles in 180 μL cell medium again on nickel columns to remove any uncleaved protein. A final purification step was performed by loading the proteins on a size-exclusion chromatography column (HiLoad 26/600 Superdex 75 pg) purification step was performed by loading the proteins on a size-exclusion chromatography column (HiLoad 26/600 Superdex 75 pg) equilibrated in 20 mM TRIS-HCl buffer at pH 7.5, 150 mM NaCl, and 2 mM dithiothreitil (DTT). Before the ITC measurements, the protein was dialyzed with HEPES buffer (25 mM, pH 7.4).

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### Analytical methods

The gold concentration in the nanoparticle dispersion was determined by atomic absorption spectroscopy (AAS) with a Thermo Electron M-Series spectrometer (graphite tube furnace according to DIN EN ISO/IEC 17025:2005) after dissolving the nanoparticles in aqua regia.

Analytical disc centrifugation (differential centrifugal sedimentation; DCS) was performed with a CPS Instruments DC 24000 disc centrifuge (24000 rpm). Two sucrose solutions (8 wt % and 24 wt %) formed a density gradient that was capped with 0.5 mL dodecan as stabilizing agent. The calibration standard was a poly(vinyl chloride) (PVC) latex in water with a particle size of 483 nm provided by CPS Instruments. A calibration was performed to be carried out prior to each run. A sample volume of 100 μL of dispersed nanoparticles was used. The recording time was about 6 h at the given centrifugation speed due to the small particle size. The density of elemental gold (19300 kg m⁻³) was used for the computations.

UV/Vis spectroscopy was performed with a Varian Cary 300 instrument from 200 to 800 nm after background solvent correction (HEPES buffer). Suprasil quartz cuvettes with a sample volume of 500 μL were used.

Fluorescence spectroscopy was performed with an Agilent Technologies Cary Eclipse Spectrophotometer in the range of 500 to 700 nm after background solvent correction (HEPES buffer). A 96-well opaque flat bottom microplate with a sample volume of 300 μL was used.

High-resolution transmission electron microscopy was performed with an aberration-corrected FEI Titan transmission electron microscope equipped with a Cs-probe corrector (CEOS Company) and a point resolution of 0.08 nm operating at 300 kV. The nanoparticle concentrations were computed as follows (given here for a nanoparticle diameter of 1.55 nm and a gold concentration of 0.22 g L⁻¹):

$$V_{np} = \frac{4}{3} \pi \left(\frac{d}{2}\right)^3 = \frac{4}{3} \cdot 3.14 \cdot \left(\frac{1.55 \cdot 10^{-9} \text{ m}^3}{2}\right)$$

1.95 × 10⁻²⁷ m³

$$m_{np} = V_{np} \cdot \rho_{Au} = 1.95 \cdot 10^{-27} \text{ m}^3 \cdot 19300 \cdot 1000 \frac{g}{m^3} = 3.76 \cdot 10^{-26} g$$

$$c_{np} = \frac{c_{Au}}{m_{np}} = \frac{0.22 \frac{g}{L}}{8.09 \cdot 10^{-26} g} = 5.85 \cdot 10^{18} \text{ L}^{-1}$$

At a FAM-CRAF concentration of 179 μM as obtained by UV/Vis spectroscopy, this corresponds to 1.08 × 10²⁰ FAM-Raf molecules per L, giving 1.08 × 10²⁰/5.85 × 10¹⁸ = 18 FAM-CRAF molecules per gold nanoparticle.

NMR spectra were recorded in a 3 mm sample tube at 25°C with a Bruker Avance III 700 MHz spectrometer, equipped with a 5 mm TCI h/1h/1h/C/N/D cryoprobe with z-gradient. All gold nanoparticle dispersions and protein solutions were prepared in 200 μL in potassium phosphate buffer (47 mM K₂HPO₄ and 3 mM KH₂PO₄, pH 8) with 10 % D₂O. The 1H DOSY pulse program from the Bruker library was equipped with a presaturation pulse to suppress the water signal. For the DOSY experiments, a diffusion time of 100 ms was used, and the pulsed gradient duration δ was 3 ms for the free peptide and 4 ms for the peptide conjugated to the gold nanoparticles. The gradient strength was incrementally increased from 5 to 95% of the maximum gradient strength (50.4 G cm⁻¹ as the signal intensity without gradient, γ as the gyromagnetic ratio of H, δ as the diffusion gradient pulse length, Δ as the diffusion delay, G as the gradient strength, and D as the translational diffusion coefficient.

The Stejskal–Tanner plots of the well-discriminable proton signals of the peptides and of the peptide-functionalized gold nanoparticles.
were first analyzed separately. Upon giving the same diffusion coefficient within the error margin, the relative intensities \( I/I_0 \) of all signals were averaged for dissolved peptide and nanoparticle-conjugated peptide, respectively. Error bars of the averaged data points represent the standard deviation of these proton signals. The accuracy of the diffusion coefficient was determined by averaging the errors obtained from the 2D \(^1\)H DOSY spectrum.

The hydrodynamic diameter was calculated by the Stokes-Einstein equation

\[
d_\text{h} = \frac{k \cdot T}{3 \pi \eta \cdot D}
\]

with \( d_\text{h} \) the hydrodynamic diameter, \( k \) the Boltzmann constant, \( T \) the temperature [K], \( \eta \) the dynamic viscosity of H\(_2\)O at 25 °C, and \( D \) the translational diffusion coefficient.

Isothermal titration calorimetry (ITC) was performed with a MicroCAL ITC2000 (Malvern Panalytical) instrument in HEPES buffer (25 mM, pH 7.4) at 25 °C. In the sample cell, 300 \( \mu \)L of 14-3-3-\( \sigma \) (50 \( \mu \)M) was titrated with dissolved CRaf (1 mM), and 300 \( \mu \)L of 14-3-3-\( \sigma \) (10 \( \mu \)M) was titrated with a dispersion of CRaf-functionalized gold nanoparticles (gold nanoparticle concentration 13.9 \( \mu \)M; CRaf concentration 0.25 \( \mu \)M), respectively. The first injection volume was 0.4 \( \mu \)L with a mixing time of 0.8 s and a time interval of 180 s. Then, 17 injections of 2 \( \mu \)L aliquots of the nanoparticle dispersion followed with a mixing time of 4 s and a time interval of 200 s, respectively. The measurements were carried out with an initial delay of 180 s, a reference power of 5 \( \mu \)Watt, a stirring power of 750 rpm, and a filter period of 3 s. The dissociation constant \( K_D = 1/K_0 \), the molar binding stoichiometry \((N)\), and the molar binding enthalpy \((\Delta H^0)\) were calculated by integrating the peaks obtained from enthalpy changes and presenting them in a Wilman plot.[20] A one-set-of-sites specific-binding model was assumed. The points 2 to 18 were fitted by the Hill Equation

\[
H = H_0 + (H_{\text{max}} - H_0) \left(1 + \frac{(N)}{N} K_0\right)^{-1}
\]

with \( H \) the molar enthalpy change per injection volume, \( H_0 \) the molar enthalpy change at the beginning of the measurement, \( H_{\text{max}} \) the maximum measured molar enthalpy change, \( n \) the molar ratio of peptide to protein, and \( N \) the molar binding stoichiometry. Here, the point of inflection gives the molar binding stoichiometry, and the slope at the point of inflection gives \( K_0 \). All data analyses were done with ITCOR Pro.

Fluorescence polarization spectroscopy (FP) was performed with a JASCO FP-8300 fluorescence spectrometer in HEPES buffer (25 mM, pH 7.4) at 20 °C in Suprasil® quartz glass cuvettes with a sample volume of 60 \( \mu \)L, 14-3-3-\( \sigma \) (50 \( \mu \)M) was added stepwise either to the FAM-labeled peptide (20 \( \mu \)M) or to the CRaf-functionalized gold nanoparticles (gold nanoparticle concentration 31 \( \mu \)M; CRaf concentration 0.56 \( \mu \)M). The resulting anisotropy at the emission wavelength of 520 nm was measured by exciting the sample with linearly polarized light at 500 nm. Fitting for the K\(_{0}\) determination was done with IGTOR Pro with the following quadratic binding equation for a one-site specific binding model

\[
\frac{[FL]}{r} = r_0 + r_{\text{max}} \cdot \sqrt{\frac{[FL] + [P] + K_0^2}{2 \cdot [FL]}}
\]

with \( r \) the anisotropy, \( r_0 \) the anisotropy without protein, \( r_{\text{max}} \) the maximum anisotropy, \([FL]\) the concentration of the titrant (fluorescence-labeled CRaf), \([P]\) the concentration of the added titrant (14-3-3-\( \sigma \)), and \( K_0 \) the dissociation constant.

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**Conflict of Interest**

The authors declare no conflict of interest.

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