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Synthesis and characterisation of human transferrin-stabilized gold nanoclusters

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Abstract

Human transferrin has been biolabelled with gold nanoclusters (Au NCs) using a simple, fast and non-toxic method. These nanocrystals (< 2 nm) are stabilized in the protein via sulfur groups and have a high fluorescence emission in the near infrared region (QY= 4.3%; λ_{em} = 695 nm). Structural investigation and photophysical measurements show a high population of clusters formed of 22-33 gold atoms covalently bond to the transferrin. In solutions with pH ranging from 5 to 10 and in buffer solutions (PBS, HEPES), those biolabelled protein exhibit a good stability. No significant quenching effect of the fluorescent transferrin has been detected after iron loading of iron-free transferrin (apoTf) and in the presence of a specific polyclonal antibody. Additionally, antibody-induced agglomeration demonstrates no alteration in the protein activity and the receptor target ability. MTT and Vialight® Plus tests show no cytotoxicity of these labelled proteins in cells (1 μ g/mL-1 mg/mL). Cell line experiments (A549) indicate also an uptake of the iron loaded fluorescent proteins inside cells. These remarkable data highlight the potential of a new type of non-toxic fluorescent transferrin for imaging and targeting.

MSC : 92DXX or 82DXX

Keywords: Gold nanoclusters, transferrin, fluorescence, protein activity, specific recognition, cell uptake.

Short title:

Transferrin-stabilized gold nanoclusters

1. Introduction

With the emergence of nanomedicines, the use of specific fluorescent-labelled proteins for clinical diagnostics and therapeutic perspectives is highly appreciated [1-5]. Besides the physiological functionality specific cellular uptake is a key parameter. In this context, human transferrin is one of the most promising multitasking proteins for biomedical applications. Human transferrin (Tf) is a plasma protein mainly produced by the liver and its primary activity serves to control the binding and transporting of iron ions in the body fluid [6]. This protein proves to be a suitable ligand for active targeting of drugs to target cells. Indeed, transferrin allows a high efficiency of site-specific targeting due to the over-expression of their receptors on cancer cell surfaces [7, 8]. McCann et al. [9] gave a brief overview of various clinical applications using iron-free human apo-transferrin (apoTf) such as hemochromatosis diagnosis [10], cardiovascular disease [11], radiotherapy [12], targeted drug delivery [13] or cancer therapy [14]. For all these reasons, human transferrin was conjugated to many particulate structures: liposomes [15], gold nanoparticles [16], quantum dots [13] or even organic dyes [17].

Biological studies are often limited by existing fluorophores which suffer from inherent deficiencies. For example, organic fluorophores, which are most commonly used in fluorescence spectroscopy, are easily photobleached during the time scale of observation. Large fluorescent tags can also perturb the labelled biomolecules thus causing artificial movement within cells and altered protein-protein interactions. Quantum dots, as another fluorophore, show great promise in biolabelling due to their unique optical properties which cause them to emit light of different colours depending on their size [18]. Unfortunately, quantum dots are commonly synthesized using harsh conditions and toxic precursors, are difficult to surface passivate, have large physical size comparable to proteins after biocompatible surface modification, and tend to photoblink.

Research on highly fluorescent noble metal (Au, Ag) nanoclusters (NCs) has gained tremendous interest in the last five years [19-22]. Nanoclusters formed by few atoms strongly fluoresce in contrast to gold or silver nanoparticles, do not support a surface plasmon, and do not have typical metallic and bulk-like properties. This fluorescence is likely due to the transition of molecule-like electronic levels when subnanometre sizes are smaller than the Fermi wavelength (i.e. < 1 nm) [19]. Recent advances in the template-based synthesis of metal nanoclusters have led to highly fluorescent and water-soluble metal nanoclusters. Using dendrimers and DNA both as templates and stabilizers, Dickson and co-workers prepared photostable and water-soluble Ag or Au nanoclusters with discrete size, emission, and much increased quantum yields (0.1 for Au₃₁ to 0.7 for Au₅) [23-25]. More recently, Ying et al. [26] developed a new method to obtain fluorescent gold nanoclusters capped in bovine serum albumin (BSA). These Au NCs are highly fluorescent (QY = $\sim 6\%$) with a red emission and show good stability for a wide range of pH values. This breakthrough opens a promising field for *in vitro* and *in vivo* labelling and imaging applications.

In this paper, we describe a simple route to obtain fluorescent human transferrin using gold nanoclusters. The ability of the modified protein to load iron ions was not influenced allowing application of the transferrin labelled with such an improved biolabel. Furthermore, the *in vitro* toxicity was investigated as well as first results regarding cellular interaction are presented to highlight the particles' potential for biological usage.

2. Experimental: materials and methods

2.1. Synthesis

All of the chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and used

without further purification. Ultrapure water (18.2 M Ω) was used in all experiments. Since the first protocol reported by Ying et al. in 2009, few groups have optimized the synthesis of protein-stabilized gold nanoclusters. Briefly, 1 mL aqueous solution of HAuCl₄ (5 mM) was reacted to 2 mL of human apo-transferrin (apoTf > 98%; 10 mg/mL) under vigorous stirring at 37°C. Then, 10 μ L of ascorbic acid (0.35 mg/mL) was added dropwise. The molar ratio Au/Tf = 1/20 was found to be the optimal value to promote the formation of a high concentration of gold clusters in transferrin. The solution of ascorbic acid allows for the triggering of gold nanoclusters formation without using a large amount of apoTf. After 5 min, 0.2 mL of NaOH solution (1M) was introduced and the reaction was preceded for 3 hours at 37°C. The solution changed colour, from colourless to pale brown after 1 hour. Two additional hours were necessary to complete the reduction of Tf-encapsulated Au precursor. The new solution **Au-apoTf** (~ 6 mg/mL) was washed twice using a viva spin column (Sartorius AG, Göttingen, Germany/ 5,000 Da) and kept in the fridge. The labelled proteins were stable for at least 5 months. The same protocol was used with human transferrin (Tf > 95%; 10 mg/mL) to obtain **Au-Tf** (~ 6 mg/mL).

2.2. Cell experiments

A549 cells (CCL-185; ATCC, Manassas, VA, USA) were cultivated in RPMI 1640 with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FCS. A549 were grown at 37°C, 5% CO₂ and 95% humidity until usage. One day prior to experiments, A549 cells were seeded at a density of 100,000 cells/ml in an imaging plate FC (PAA Laboratories GmbH, Pasching, Austria) for microscopic investigations or at a density of 50,000 cells/cm² in 96 well plates (Greiner Bio One, Frickenhausen, Germany) to test the acute toxicity of the clusters. On the day of experiment, cells were washed with PBS pH 7.4 and incubated for 4 h with the fluorescent transferrin in respective buffer.

Staining procedures. For microscopic analysis, A549 were again washed with PBS followed by application of WGA-fluorescein to bind to the cell membrane (Wheat germ agglutinin, 1:500 in HBSS, Vector Laboratories, Burlingame, CA, USA) for 15 minutes at 37°C. Nuclei were stained with DAPI (1: 15,000 in PBS, Sigma-Aldrich, Schnellendorf, Germany) for 10 minutes at 37°C. The staining procedures were followed by fixation of the samples with 4% formalin. The samples were stored at 4°C until microscopic investigation.

MTT assay. This test is a quantitative colorimetric method to determine cell viability. It utilizes the yellow tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] (Sigma-Aldrich, Schnellendorf, Germany) which is metabolized by mitochondrial succinic dehydrogenase activity of cells to yield a purple formazan reaction product. A549 cells were incubated with 100 μ L of Au-apoTf at different concentrations (1 μ g/mL-1 mg/mL in Krebs Ringer buffer, KRB) for 4 hours in 96 well plates. Three replicates for each concentration were prepared. Negative control without protein and positive control with 1% Triton X were set up at the same time. Cells were washed twice with buffer and 100 μ L of MTT (5 mg/mL) was added to each sample. After 4 h at 37°C, measurements were performed by checking the absorbance for all samples at λ = 550 nm.

Vialight® plus assay. The kit (Lonza, Verviers, Belgium) is based upon the bioluminescent measurement of ATP that is present in all metabolically active cells. A549 cells were incubated with 100 μ L of Au-apoTf at different concentration (1 μ g/mL-1 mg/mL in KRB) for 4 hours in 96 well plates. Three replicates for each concentration were prepared. Negative control without protein and positive control with 1% Triton X were set up at the same time. 50 μ L of cell lysis reagent was added to each well and left for 10 minutes at room temperature to extract ATP from cells. Then, 100 μ L of ATP monitoring reagent plus was added to generate the luminescent signal. Two minutes later, luminescence was checked on a microplate reader.

2.3. Functionality of the modified protein

Iron loading protocol. To form iron loaded apoTransferrin (apoTf) (i.e., to saturate apoTf with iron) we followed the protocol detailed in the product information of apoTf by Sigma-Aldrich. Briefly, 2 mg of apoTf is mixed with 2% of its mass in ferrous ammonium sulfate hexahydrate with sodium carbonate buffer, pH 5.9, for 1.5 hours. The pH is then raised to 8.5 with sodium carbonate and the solution is mixed for an additional 1.5-2 hours. The sample is then dialysed against water to remove the buffer salts. The same protocol is also applied for Au-apoTf sample to form iron loaded Au-apoTf. Iron concentration is determined for all samples by ferrozine assay.

Iron determination protocol (ferrozine assay). In order to determine the total iron binding capacity (TIBC) of the labelled transferrin which corresponds to the sum of all iron binding sites, a ferrozine assay was performed. The protocol is an optimized procedure described by Stookey et al. [27]. First, stock solutions of saturated hydroxylamin in 1M HCl and 0.05% wt/wt ferrozine in 50 mM HEPES buffer pH 7.4 are prepared. These solutions are stable for a week in the dark at room temperature. 100 μ L of the sample was mixed with the hydroxylamin in an eppendorf cup and then incubated for 1 hour at 40°C to reduce the iron Fe^{3+} to Fe^{2+} . Following this, 50 μ L of the incubated solution was transferred to a new eppendorf cup containing 200 μ L ferrozine reagent and 0.6 mL of deionized water was added. After a rapid stirring the absorbance at 562 nm was checked. Using this protocol, a calibration curve was established with stock solutions of 0.01-2 mM ferric ammonium citrate (see Figure S8). Iron concentration is then converted by weight in iron per protein ($\mu\text{g/g}$).

Ferrozine assay was set for human transferrin (Tf), human apo-transferrin (apoTf), iron loaded human apo-transferrin (iron-loaded apoTf) and their respective gold labelled samples (see calibration curve in supporting information).

Complexation assay. Specific recognition of Au-apoTf was also investigated using a goat polyclonal transferrin antibody (abcam; ab19177). ApoTf and Au-apoTf (1 mg/mL in PBS pH 7.4) in the presence of a specific polyclonal transferrin antibody (1 $\mu\text{g/mL}$) at 37°C for 1 hour was set up to detect the agglomeration of the proteins by confocal microscopy as described earlier. Both samples are checked under transmission and fluorescence ($\lambda_{\text{ex}} = 514$ nm and the fluorescence emission was detected in the range of $\lambda \in [650-710]$ nm using a bandpass) for Au-apoTf. Experiments without antibody are used as a reference.

3. Results and discussion

3.1. Structural investigation

Over the last decade, many researchers have studied the total structural determination of gold-thiolate (Au-S) cluster compounds [28, 29]. Figure 1a shows the MALDI MS measurement of the Au-apoTf sample with the spacing of m/z 197 and m/z 32 attributed respectively to the gold and the sulphur atoms. The peak intensities related to $[\text{Au}_n\text{S}_x]^-$ species follow a large Gaussian distribution with a maximum corresponding to 22-33 gold atoms. Peaks attribution and an expanded view of these separations are given in the supporting information (Figure S1). These results are in agreement with spectrometric analysis performed on red emitting gold nanoclusters labelled to other proteins [30]. Surprisingly, MALDI MS data of the Au-Tf (iron containing) sample present the same features as Au-apoTf (iron free) indicating that iron ions in transferrin have no effect on the growth of gold clusters. Hence, the NCs do not interfere with the protein cage. The XPS spectrum (Figure 1b) indicates two distinct doublets, one with the Au $4f_{7/2}$ peak at 84.2 eV and the other at 85.2

eV, assigned to Au (0) (88%) and Au (I) (12%) respectively. Moreover, the S 2p_{3/2} peak (Figure S2) at 161.9 eV is attributed to a gold thiolate [31] confirming the covalent interaction of gold nanoclusters to the sulfur groups of the cysteine (40 residues per protein). Transmission Electron Microscopy (TEM) and elemental analysis of Au-apoTf determined by EDX (energy dispersive x-ray radiation) confirm the presence of gold nanoclusters with a size smaller than 3 nm inside the protein (Figure S3). Atomic absorption spectroscopy measurements performed on the dried sample indicate a gold concentration of 3.15 wt%. Infrared measurement of apoTf and Au-apoTf (Figure 2) showed a shift of the peak corresponding to the amide I at high wavenumbers from 1634 to 1643 cm⁻¹ as expected. It is related to a modification of the secondary structure of the protein backbone predominantly due to transition dipole coupling [32, 33]. In our case, the capping of gold inside the transferrin involves a modification/distorsion of the β -sheets of apoTf. Regarding the size and especially the stability of apoTf against agglomeration after labelling, the volume distribution was checked by dynamic light scattering in water (Figure S4) given pH values ranging from 5 to 10 and in PBS and HEPES buffers (pH 7.4). No significant aggregation (at protein concentration ~ 6 mg/mL) was detected with a 9 nm monodisperse size distribution correlating to single transferrin proteins.

3.2. Photophysical properties

Absorbance spectra of apoTf and Au-apoTf show the presence of the protein with the characteristic peak at $\lambda_{\text{max}} = 280$ nm correlated to the absorbance of aromatic amino acids (see Figure 3a). This peak appears as a shoulder for the Au-apoTf sample due to the high extinction of gold in the UV region [34]. Moreover, we observe no plasmon resonance of gold at $\lambda = 520$ nm indicating the absence of gold nanoparticles ($\varnothing > 2$ nm) [24]. During the synthesis, the solution changes from colourless to pale brown indicating the formation of gold nanoclusters. The fluorescence of the Au-apoTf solution is clearly detectable with an intense red emission under UV irradiation ($\lambda_{\text{ex}} = 366$ nm) (Figure 3b). Figure 3c shows the emission and excitation spectrum of Au-apoTf with 2 excitation peaks: one intense at 390 nm and a second one at 530 nm. Au-apoTf presents a high Stokes shift (> 150 nm) with a weak band located at 450 nm and a broad red fluorescent emission with a maximum intensity located at $\lambda_{\text{max}} = 695$ nm. Many authors have described the correlation between gold-cluster size and its emission indicating towards a distribution around 25 Au atoms as described for gold nanoclusters capped in polymeric ligands and in BSA which correlates with our structural investigation [35, 36]. Theoretically, photophysical properties of Au clusters with a specific number of gold atoms should present discrete excitation-emission bands related to HOMO-LUMO electronic transitions at 530-700 nm. Nevertheless, for Au-apoTf, the number of excitation peaks and the large emission peak indicate a relative polydispersity of gold nanoclusters trapped in the protein. The strong fluorescence in the infrared region after UV irradiation could be explained by an energy transfer between the previously formed small nanoclusters (5-8 gold atoms) having an excitation-emission wavelength at 380-450 nm [25] to nanoclusters with a size of 22-33 atoms. This broad size-distribution was expected considering the difficulty in controlling the growth of metal clusters in wet chemical synthesis. We found QY $\sim 4.3\%$ for gold clusters in apoTf and in Tf (Rhodamine 6G solution in ethanol used as reference, QY = 0.95) which are in agreement with values obtained by other authors for gold clusters stabilized by BSA [36, 26]. Lifetime decay of Au-apoTf presents two different lifetime populations at 1.3 ± 0.3 ns (60%) and 199 ± 7 ns (40%). Compared to Au-apoTf, no significant variation of the lifetime was noticed for the Au-Tf sample containing iron (Tf - iron content = 300-600 $\mu\text{g/g}$) (Figures 4, S5). Thus, steady-state fluorescence and lifetime measurements suggest no significant quenching of Au NCs attached to the protein by iron in Tf.

3.3. Protein activity

Whenever working with proteins intended for biological application, the conservation of the protein structure and hence its functionality is of outmost importance. Transferrin is widely

known as an iron binding protein in the presence of a carbonate anion [37]. The total iron binding capacity (TIBC) of Au-apoTf was determined by ferrozine assay and compared to apoTf after an iron loading process. Iron content of Au-apoTf and apoTf after iron loading was 1418 $\mu\text{g/g}$ and 1450 $\mu\text{g/g}$, respectively, which is in the range of the iron amount in commercial human holo-transferrin (1100-1600 $\mu\text{g/g}$). Size measurement and fluorescence values (steady-state, lifetime) indicate no aggregation of the labelled transferrin and show no quenching effect in the presence of iron (Figures 4, 5a and S6). However, a 15 nm blue-shift of the fluorescence emission band is noticed after iron uptake from 695 nm to 680 nm (Figure 5b). This behaviour demonstrates the fact that the presence of iron ions influence the photophysical properties of the gold nanoclusters [38]. Even if the relation between the fluorescence of Au NCs and the iron uptake still remains unknown, the sensitivity of the maximum peak of gold clusters to the presence of the ferric ion is of interest for analytical purpose. Ferrozine assays on Tf and Au-Tf gave almost the same iron concentration in the range 150-350 $\mu\text{g/g}$ which is expected considering that under normal circumstances, approximately one-third of transferrin iron-binding pockets are filled. This indicates no significant iron loss of human transferrin after incorporation of Au NCs and a preserved functionality of the protein for iron transport.

The other biological peculiarity of transferrin is the ability to recognize receptors on the cell membrane. First of all, to demonstrate the activity of the protein without cells, a simple experiment was set up to demonstrate that transferrin can still interact with its antibody. Therefore, recognition of Au-apoTf was investigated using a goat polyclonal transferrin antibody. Au-apoTf and apoTf (1 mg/mL in PBS pH 7.4) were incubated in the presence of the antibody (1 $\mu\text{g/mL}$) at 37°C for 1 hour and confocal microscopy was used to detect the aggregation of the proteins as described earlier [13]. Figure 5c shows clearly an aggregation of Au-apoTf induced by the presence of the antibody. Thus, this aggregation is caused by the antibody recognition indicating the protein activity. Only a few aggregates could be detected without the antibody confirming the specific recognition of Au-apoTf. Experiments performed with apoTf, Tf, and Au-Tf showed the same trend as Au-apoTf (Figure S7). At the early stage of the experiment, when labelled proteins (Au-apoTf and Au-Tf) react with specific antibodies, only a slight decrease of the relative fluorescence intensities of the gold nanoclusters was observed suggesting no strong quenching effect by the interaction transferrin-antibody (Figure S6). This specific agglomeration behaviour reveals a preserved binding and recognition activity of transferrin after labelling with respect to antibody binding. This underlines the high potential of Au-apoTf as a multitasking protein for molecular-receptor-targeted imaging.

3.4. Cell study

Several studies have demonstrated the highest affinity of an iron loaded transferrin called holoTf for the receptor TfR which is 10- to 100 fold greater than that of apoTf at physiological pH [39, 6]. Therefore, iron loaded Au-apoTf named Au-holoTf was selected for cell study along with A549 lung tumour cells which are well known to over-express transferrin receptor to internalize transferrin. Cytotoxicity assays MTT and Vialight[®] plus confirmed the viability of A549 cells in the presence of the fluorescent transferrin even at high concentrations (1 $\mu\text{g/mL}$ -1 mg/mL) after 24 hours of incubation time (data not shown). As illustrated in Figure 6, tracking of Au-holoTf in A549 cells by confocal microscope shows a strong membrane association (yellow signal) but also cellular uptake of the labelled transferrin after 4 hours incubation. Fluorescence detected inside the cells could be only attributed to the proteins and not to some free Au NCs because these clusters are unstable without capping agents. A ligand exchange in the cell (e.g., transferrin against glutathione) is very unlikely and can be excluded. Another critical issue remains on the specific uptake of the labelled transferrin, especially if we consider the modification of the secondary structure proved by infrared spectroscopy. This problem inherent in the metal clusters synthesis in presence of proteins still needs to be solved. Furthermore, no photobleaching happened during the measurement indicating the potential for imaging applications. Those first results show that the AuNC-labelled transferrin is a promising

biolabel for biological applications.

4. Conclusion

In summary, we use a simple and low cost protocol to produce fluorescent human transferrin. These labelled proteins have an intense fluorescence emission ($QY = 4.3\%$) in the near infrared region ($\lambda_{\max} = 695 \text{ nm}$) with a high Stokes shift making them good candidates for application (detection) in biological systems. Their photo-physical properties, such as quenching, are not significantly altered due to the presence of iron or conjugation to antibodies. The gold nanoclusters ($< 2 \text{ nm}$) are covalently bound to the protein and do not affect the stability of the protein in solutions with a wide range of pH and in different buffers (PBS, HEPES) used during the *in vitro* experiments. Colorimetric assay and antibody-induced aggregation confirmed the preserved activity of the labelled transferrin regarding iron binding capacity and transferrin-antibody interaction. Cell viability tests verified the non-toxic nature of the labelled transferrin and cellular uptake of the fluorescent transferrin could be demonstrated. Therefore, this new biomolecule presents all the properties necessary to make it a promising candidate for drug targeting and clinical applications such as hemochromatosis diagnosis or cancer therapy. Nevertheless, some critical issues still need to be investigated such as the labelling procedure which requires less harsh condition in order to reduce the modification of the protein (basic condition) and the determination of the specific cellular uptake of the labelled proteins.

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Supporting Information Available: instrumentation, additional characterization of labelled transferrin, calibration of ferrozine assay.

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Figure 1. MALDI-TOF MS in a positive mode (a) show a broad distribution of gold nanoclusters predominantly 22-30 gold atoms (maximum of Gauss distribution) covalently bond to the thiol-bearing cysteine residues of the transferrin. Major spacing between the adjacent peaks of m/z 197 and minor m/z 32 are attributed respectively to the gold and the sulphur atoms. (b) XPS spectra of Au 4f spectra were fitted (red line) and confirm the presence of two distinct doublet Au 4f_{7/2} peaks at 84.2 eV and the other at 85.2 eV, assigned to Au (0) (88%) and Au (I) (12%) respectively.

Figure 2. FTIR spectra of apoTf, Au-apoTf, and apoTf at pH 9. All samples were freeze-dried before characterization. The peak corresponding to amide I is shifted to higher wavenumbers from 1634 to 1643 cm⁻¹ after labelling. The increase of the peak intensity at 1420 cm⁻¹ is related to a deprotonation of carboxyl groups of Au-apoTf due to the basic condition of the synthesis (pH 9).

Figure 3. Absorbance spectra of apoTf (dashed line) and Au-apoTf (solid line) showing the presence of the transferrin with a characteristic peak at 280 nm (shoulder for the labelled protein (a); pictures of Au-apoTf solution under natural light and UV irradiation ($\lambda = 366$ nm) (b); excitation (dashed line) and fluorescence (solid line) emission spectra of Au-apoTf (c).

Figure 4. Lifetime decays of Au-apoTf, Au-Tf, and iron loaded Au-apoTf samples at 405 nm excitation and with an emission filter 685-70. Lifetime values were evaluated with a biexponential fit.

(a) Samples		Lifetime τ			
Samples	Relative Fluorescence Intensity	τ_1 (ns)	A1 (%) Size (nm)	τ_2 (ns)	A2 (%) Iron content ($\mu\text{g/g}$)
Au-Tf	1.2 $\pm$ 0.3	193 $\pm$ 1	64	196 $\pm$ 7	36
Au-apoTf	104.3 $\pm$ 0.3	193 $\pm$ 1	60 8	199 $\pm$ 7	40 0
Iron loaded apoTf	151 $\pm$ 0.3	193 $\pm$ 1	72 8	209 $\pm$ 10	28 1418
Iron loaded Au-apoTf	9791		10		1450

(b)

(c)

Figure 5. Relative fluorescence intensity ($\lambda_{em.} = 690\text{nm}$; gain=100), size measurement and iron content of apoTf, and Au-apoTf before and after iron loading (a); 20 nm blue shift of the fluorescence emission peak of Au-apoTf (dashed line) after iron uptake (solid line) (b); aggregation of iron loaded Au-apoTf (c) (1 mg/mL in PBS pH 7.4) induced by the presence of a specific polyclonal goat Tf antibody (1 $\mu\text{g/mL}$). Measurements performed by transmission and by fluorescence detection using a confocal laser scanning microscope show strong interaction after 4 hours incubation of Au-apoTf with antibody.

Figure 6. iron loaded Au-apoTf (*i.e* Au-holoTf) uptake in A549 lung tumour cells after 4h incubation with HBSS: nuclei stained with DAPI (in blue, images A, B, C), membrane stained with fluorescein-WGA (in green, image C) and fluorescent proteins (1 mg/mL) are visible in red. The yellow signal is due to a co-localisation of membrane and labelled protein (A). Red fluorescence signals clearly demonstrating the presence of labelled protein inside the cells suggesting an uptake into the cytoplasm (images A, B). Bar 21 μm .