Geometry-induced protein reorientation on the spikes of plasmonic gold nanostars: Supplementary Information Appendix

Rosália Lopes Rodrigues, Fang Xie, Alexandra E. Porter, and Mary P. Ryan

S1 Material and Methods

S1.1 Nanoparticle Synthesis

Standard procedures for seed-mediated synthesis were used to prepare the nanoparticles[1,2]. Briefly, 100 mL of a 1 mM $HAuCl_4$ (Sigma) aqueous solution was boiled under stirring followed by the rapid addition of 10 mL of a 38.8 mM solution of sodium trihydrate (Sigma). Following a series of colour changes the solution stabilised with a deed ruby red colour. This synthesis yielded monodispersed 12 nm gold nanoparticles here on referred to as seed solution. For the synthesis of gold nanostars, different volumes of seed (6, 12, 50, 100 and 400 μL) were allowed to react in the presence of 0.2mM $HAuCl_4$ and $AgNO_3$ at Au:Ag molar ratios of 3, 10, 20, 40 and 80 and 0.1 mM ascorbic acid (Sigma) under constant shaking. The solution stabilised at deep blue colour when the gold nanostars were formed.

S1.2 Protein Pegylation for Au Attachment

To conjugate Tf to the surface of the nanostructures, human holo-transferrin (Sigma) was directly modified with the heterobifunctional PEG linker alpha-Pyridyl-2-disulfid-omega-carboxy succinimidyl ester poly(ethylene glycol) (NHS-PEG_{5K}-OPSS)(Creative PEGworks) with a molecular weight of 5 kDa, in a 1:8 ratio in borate buffer, pH=9.0. Initially, PEG was attached to the protein via the reaction of the transferrin's primary amines on lysine groups with the PEG's N-hydroxysuccinimide (NHS) ester group[3,4]. The remaining PEG terminal group, orthopyridyl disulphide (OPSS), attaches to the nanoparticles surface via gold thiol dative bonding. Tf and PEG were allowed to react for 1.5h following which the excess unreacted PEG was removed by three centrifugation cycles (20000g) using a 50kDa centrifugal filter. For quality control, prior to reacting with nanostructures, the outcome of the protein PEGylation reaction was confirmed and characterised by high performance liquid chromatography analysis (Figure S3). The modified transferrin was run against an unmodified transferrin reference under the same operating conditions and the signal was recorded at 280 nm using a UV-Vis detector. The elution times of the control and reference samples are expected to differ when the PEGylation reaction is successful. Conversely, if the modification procedure is unsuccessful the retention time of the samples should coincide[4]. Figure S3 demonstrates that the elution time of the modified protein increased by 0.6 min comparatively to the unmodified control. The modified protein was stored at 4°C in sodium bicarbonate buffer (50mM, pH 8.0).

S1.3 Monitoring and quantification of Tf attachment to the gold surface

To prepare the bioconstructs, the optimal transferrin concentration was determined by performing a colorimetric salt aggregation test. The particles optical response were further characterised using UV-Vis spectroscopy (Perkin Elmers Lambda 25). Briefly, a range of transferrin amounts (0-5μg) in total volumes of 10μL of sodium bicarbonate was added to 200μL of colloid. To this 100μL of 0.1M of NaCl was added and the colour changes and the measured Uv-Vis spectra were used to determine the lowest amount of transferrin to stabilise the gold particles. The λ_{max} of the different Tf-AuNP bioconstructs dispersed in sodium bicarbonate was taken as a reference and subtracted from the λ_{max} of Tf-AuNP biconstructs reacted in the presence of NaCl to obtain the $\Delta\lambda_{max}$. The optimal amount of Tf was found at 3μg.

Following this preliminary optimisation step, Tf was incubated with AuNP or AuStr (pH 8) for 2 hours under constant stirring. A non-destructive approach to quantify protein attachment to Au nanostructures was explored (Figure S1). The prepared bioconstructs were pelleted at 20,000g (30 min, 4°C) to remove excess protein and the supernatant recovered for spectrophotometric measurement. The centrifuged pellets were resuspended in buffer, and vortexed for 10 min to loosen the pellet. Subsequently, the nanostructures were centrifuged through a 0.7 M sucrose cushion to achieve differential centrifugation of the bioconstructs from the unbound protein. This washing was repeated three more times. After washing, the unreacted Tf in the recovered supernatant was diluted in water and concentrated in centrifugal filters (50 KDa MWCO, Millipore) to remove sucrose and excess water volume. To quantify the Tf binding to the gold surface, the unreacted protein in the supernatant was spectrophotometrically measured against a protein calibration curve (0-8 μg/mL,R>0.98)

using a BCA assay kit (Thermofisher Scientific) performed according to manufacturer's instructions. The amount of protein successfully attached to the nanoparticles was obtained by subtracting the amount of unreacted protein recovered from the sample, from that of a control sample reacted under identical conditions to the sample but without any particles. The latter procedure was performed to account for possible protein binding to the walls of the centrifuge tubes. To minimize these events low binding Eppendorf tubes (LoBind, Sigma-Aldrich) and glass coated stirring bars were used for these conjugation reactions. Finally, the recovered bioconstructs were diluted in Milli-Q water to determine the hydrodynamic diameters by dynamic light scattering measurements. Control nanostructures were modified with mPEG (5KDa, PEGworks) at large molar excess (12000 molar excess) for 2 hours and washed three time with the use of centrifuge filter. PEGylation was confirmed by TGA (Figure S2).

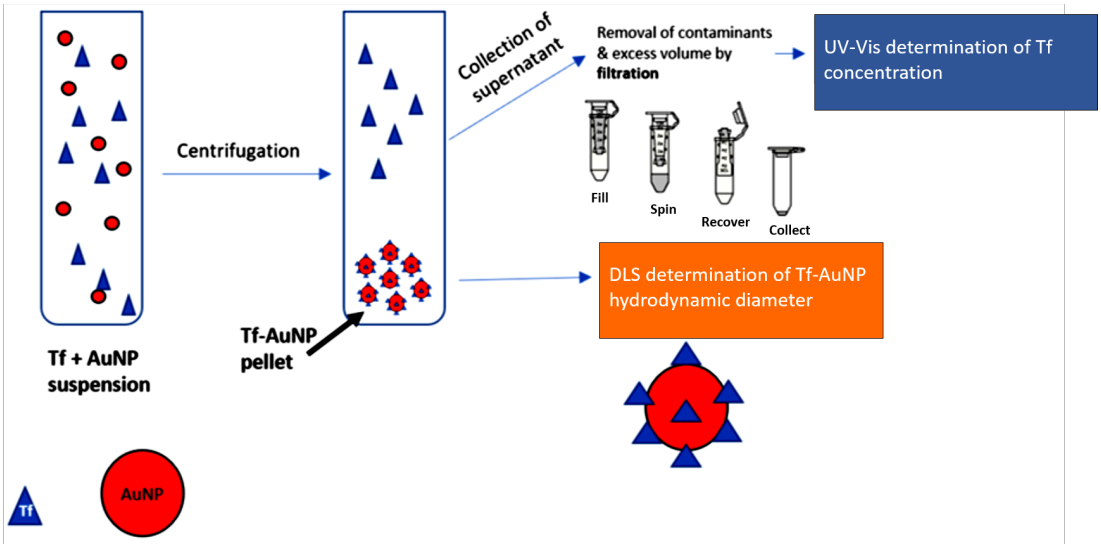

Figure S1 Schematic illustration of the procedure used quantify Tf binding to the surface of the AuNPs. Tf (blue triangles) react with AuNPs (red spheres) for 2 hours following which the solution is centrifuged to separate the unreacted protein from the Tf-AuNPs pellet. The unreacted Tf in the supernatant is concentrated in spin columns and the recovered volume is measured by UV-VIS spectroscopy to determine the protein concentration in solution. The Tf-AuNPs are further characterised by dynamic light scattering (DLS).

S1.4 Nanoparticle plate assay

S1.4.1 Cell Culture

Immortalised human brain microvascular endothelial cells (hCMEC/D3) derived from human temporal lobe microvessels were cultured in 75 cm^2 CellBIND culture flasks (Corning Inc.). The cells were cultured in EBM-2 medium (Lonza) supplemented as per manufacturer's instructions with 2.5% (V/V) foetal bovine serum, ascorbic acid, gentamicin, EGF, hydrocortisone, basic fibroblast growth factor, insulin-like growth factor-1 and vascular endothelial growth factor (VEGF). The cells were cultured in a humidified chamber at 37 C in 5% CO2 and the media replaced every 3 days.

S1.4.2 Plate Assay

For the In Vitro plate uptake study, hCMEC/D3 cells were cultured in full supplemented EBM-2 media (Lonza) in a 24 well plate lined with extracellular matrix fibres collagen and fibronectin (Sigma). Once the cells reached a confluent state, the culture media was replaced to one without the VEGF supplement to promote cell differentiation into a BBB phenotype whilst avoiding cell overgrowth. For the uptake studies 0.5 mL of the test gold nanostructures solution at a concentration of 50 μg/mL were administered apically and incubated overnight. The entire apical media was recovered, the cells washed three times to recover loosely bound mass of gold. The cells were trypsinised and lysed with 10% SDS. The amount of gold in the apical and cellular fractions was dissolved using *Aqua-Regia* and quantified by ICP-OES.

S2 Supplemental Figures

To test whether the surface of the AuStrs was conjugated with PEG, thermogravimetric analysis (TGA) was performed for the optimum 100 nm AuStrs (Figure S2). In this procedure, a known mass of dry AuStr sample (1.35 mg) was placed in a controlled atmosphere (Nitrogen and oxygen) and the sample mass was monitored as the temperature was ramped

up to 800^{c}*irc*C at a heating rate of 10^{c}*irc*C. A 12% mass change over the temperature range of 100^{c}*irc*C to 500^{c}*irc*C was measured that could be associated to the degradation of the PEG polymer. PEGylation was therefore deemed to be successful.

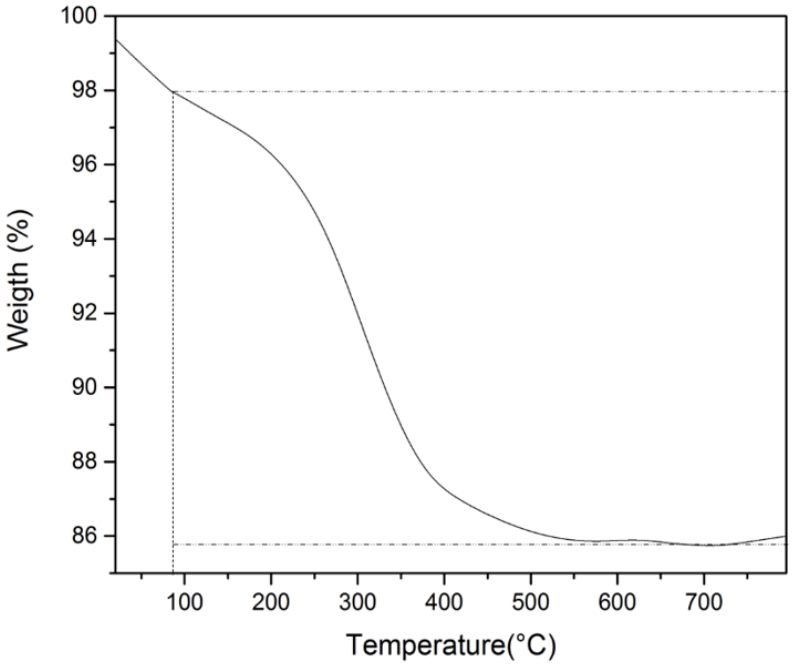

Figure S2 TGA curve of 100 nm AuStrs conjugated with 5 KDa PEG. The graph shows two mass loss regions, the first (20-90°C) corresponding to solvent loss and the second (90-650°C) corresponding to PEG degradation, followed by the plateau of the curve, indicating no further changes mass changes.

S2.1 Protein PEGylation

The AuStrs were modified with transferrin through a thiol based covalent attachment to their surface. A covalent approach was selected because proteins adsorbed solely by electrostatic interactions can be easily displaced from the Au surface. PEGylated TF was analysed by HPLC (Figure S3) as described above.

S2.2 Confirmation of protein corona on AuNP surface

Figure S4 shows cryo micrographs of unconjugated AuNPs and 100nm-AuStrs. The presence of transferrin on the surface of the AuNPs was confirmed by negative staining of Tf-AuNPs drop cast on TEM carbon grids. Negative staining was performed because the amorphous protein layer is relatively ineffective in scattering electrons and hence provides insufficient contrast to detect its presence at the particle surface. Uranyl acetate is known to strongly stain proteins, nucleic acids and phospholipids[5].

Moreover, in negative staining, the sample background is stained, with the stain surrounding the sample but being however excluded from the volume occupied by the sample itself. Figure S5 shows the negatively stained unconjugated nanoparticles. As expected, there was no clear formation of a bright halo around the AuNPs due to the absence of Tf. It is therefore expected that in the presence of a Tf corona, a brighter region forms between the stain and the AuNP surface. In fact, this can be observed in Figure S6. In Figure S6 a), AuNPs are found at the edge of a stain patch on the carbon film thus facilitating the identification of the protein at the AuNP surface due to the frontier of non-interaction between the stain and these nanoparticles. Consequently, the stain boundary line becomes irregular only in the proximity to the AuNPs (Figure S6 a). For particles fully surrounded by the stain, a Tf corona halo around the nanoparticles is observed (Figure S6 b).

Interestingly, the thickness of the protein layer at the tip of the nanostar's spikes remained undisturbed, irrespective of protein concentration (Figures S7 and S8). In fact, this was consistent with the hydrodynamic size measurements of the of the Tf-AuStrs showing that both biconstructs share the same hydrodynamic diameter of about 100 nm (Figure S9).

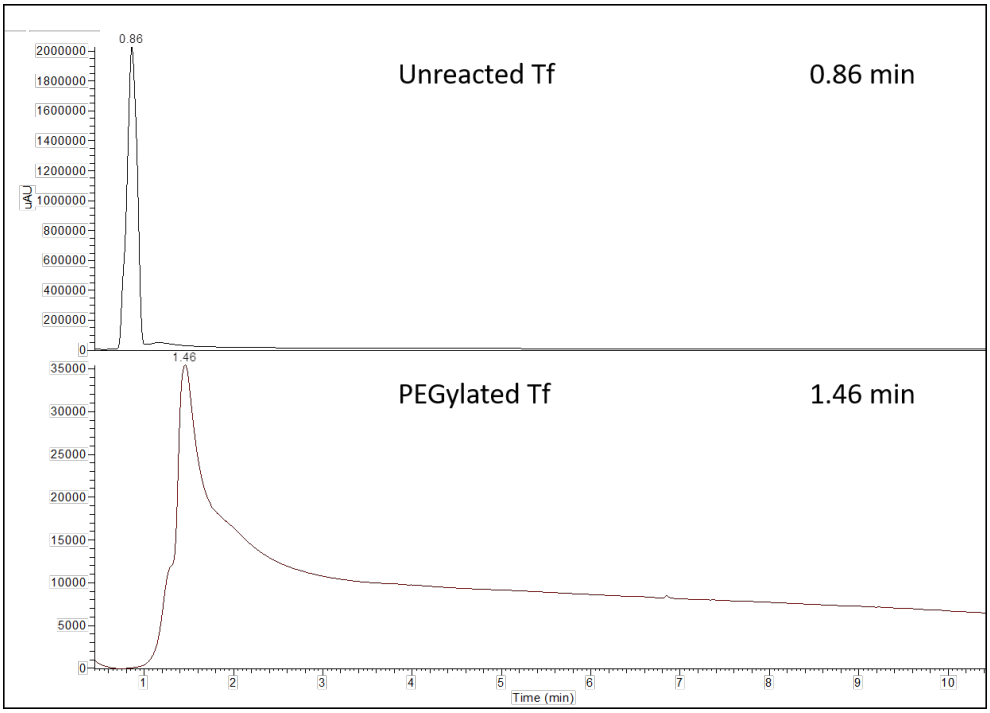

Figure S3 High performance liquid chromatography (HPLC) analysis showing successful Tf- PEGylation. The top graph shows the elution time of unreacted (non PEGylated) transferrin control and the bottom graph shows the elution time of PEGylated Tf samples. The signal was recorded at 280 nm using a UV-Vis detector.

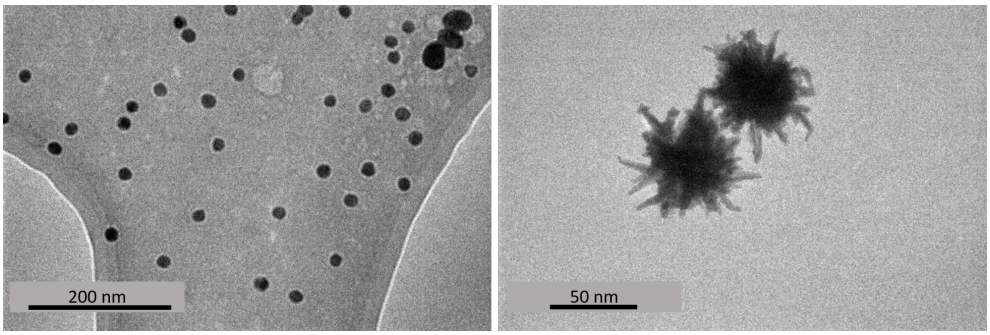

Figure S4 Cryo-TEM images of pre-functionalised AuNPs (a) and AuStrs (b).

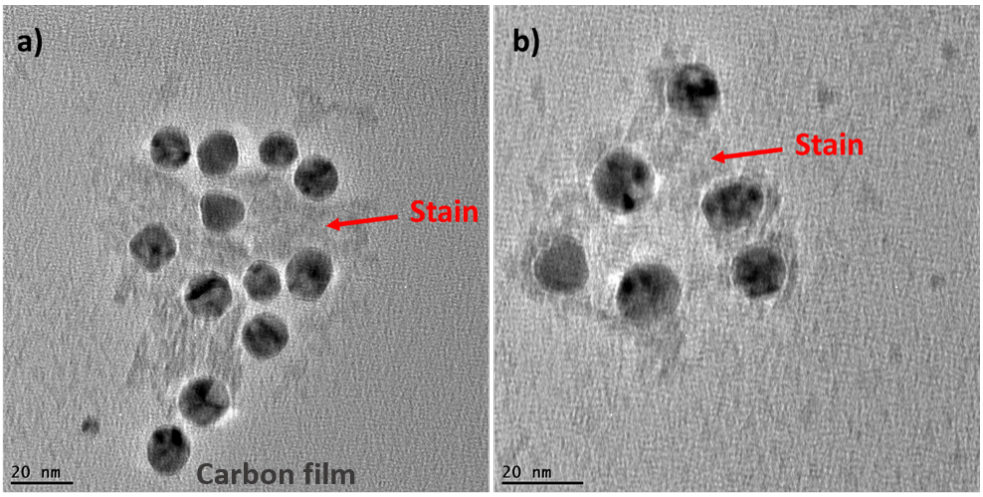

Figure S5 Bright field TEM images (a) and (b) showing negatively stained unconjugated AuNP.

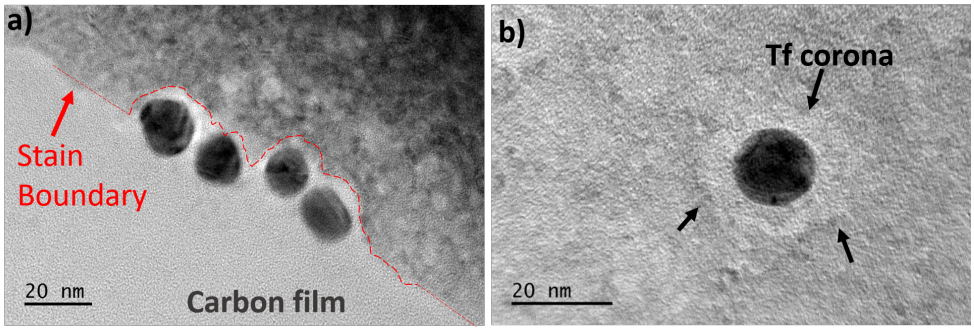

Figure S6 Bright field TEM images (a-d) showing negatively stained Tf-AuNPs with black arrows pointing to Tf coronas.

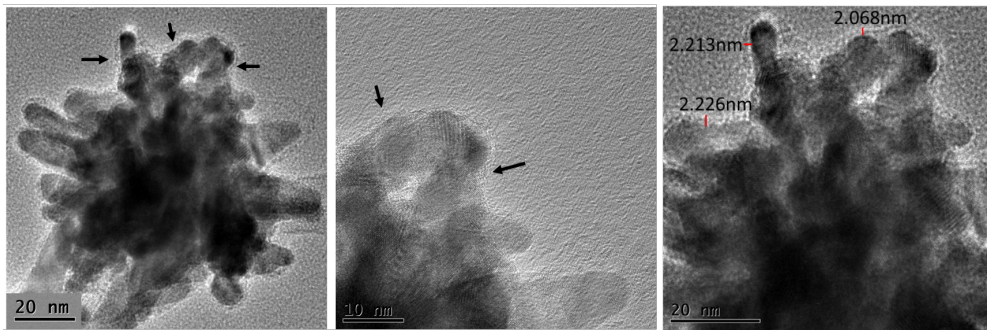

Figure S7 Bright field TEM micrographs of negatively stained 100 nm Tf-AuStrs[1x] with black arrows showing the presence of a surface protein layer with an average thickness of 2.28 nm.

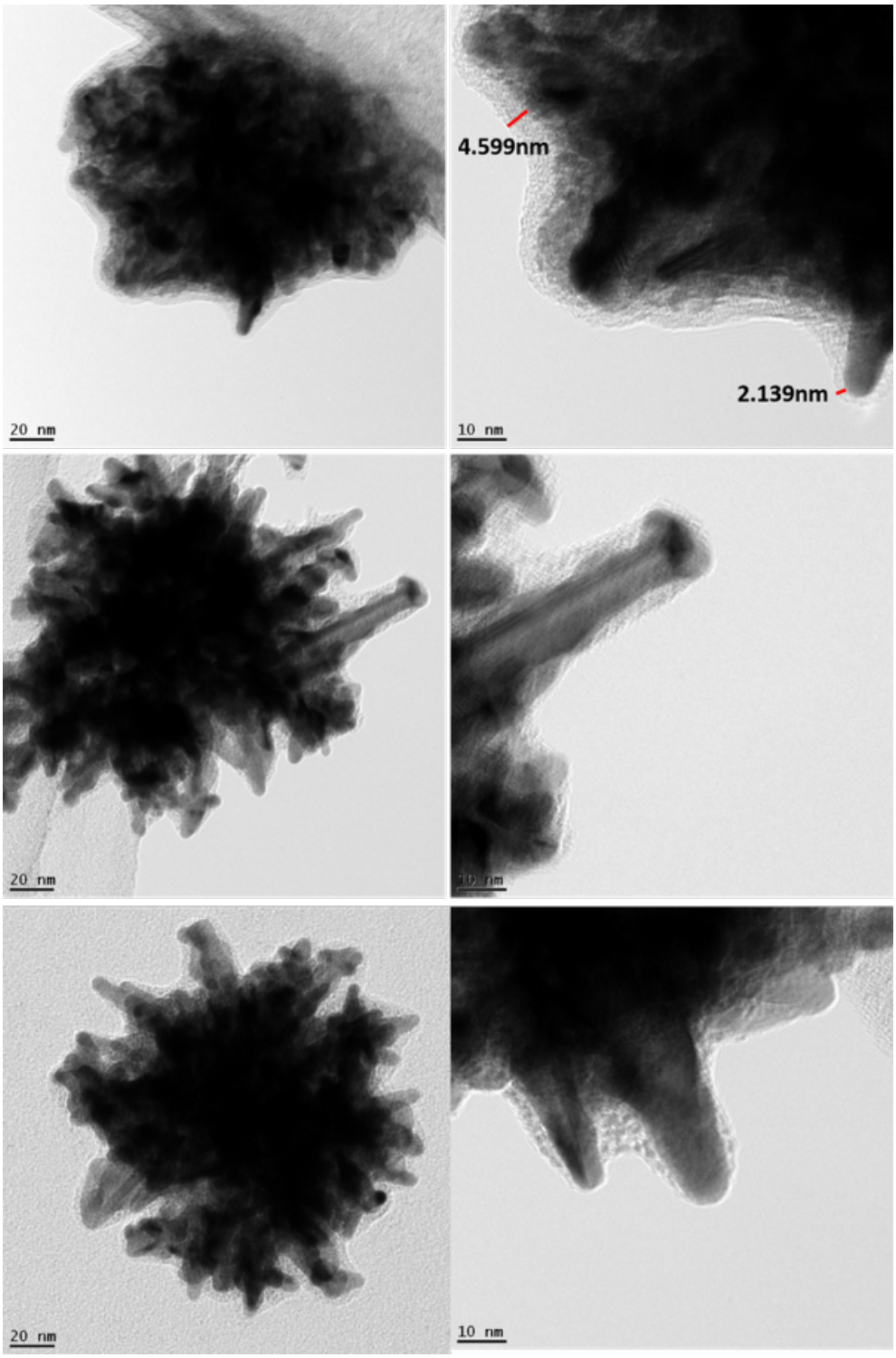

Figure S8 Bright field TEM micrographs of negatively stained 100 nm Tf-AuStrs conjugated at high Tf to AuStr ratios [10x].A thick biofilm is observable around the AuStrs whilst the coating remains thin at the tip of the spikes.

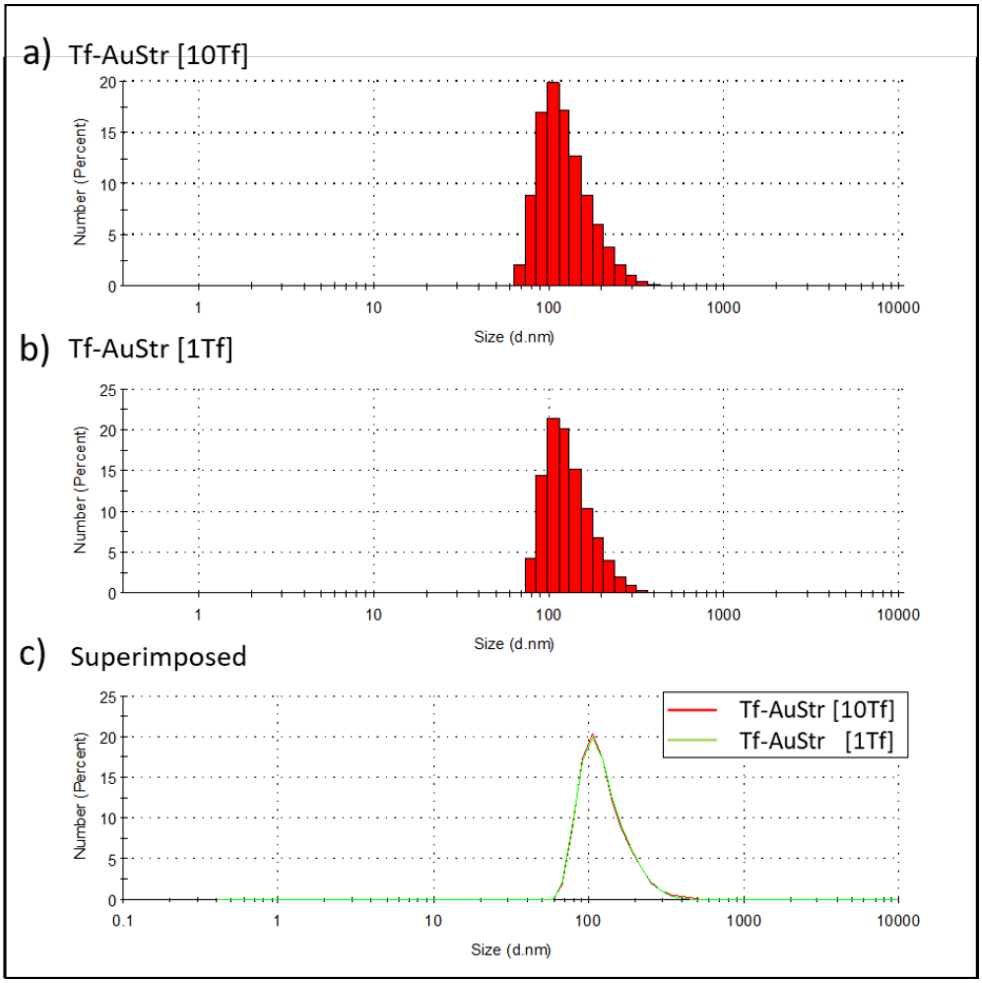

Figure S9 Hydrodynamic diameter measured through Dynamic Light scattering (DLS) for AuStrs reacted at high tf concentrations Tf-AuStr[10Tf] (b) and standards tf concentrations Tf-AuStr [1Tf]. Figure (c) shows the superimposed size distribution curve of the two AuStr formulations. The graphs show that changing the Tf to AuStr ratio did not affect bioconstruct size.

Notes and references

[1] G. Frens, *Nature*, 1973, **241**, 20–22.

[2] J. D. Ramsey, L. Zhou, C. K. Almlie, J. D. Lange and S. M. Burrows, *New Journal of Chemistry*, 2015, **39**, 9098–9108.

[3] N. C. Bellocq, S. H. Pun, G. S. Jensen and M. E. Davis, *Bioconjugate Chemistry*, 2003, **14**, 1122–1132.

[4] D. T. Wiley, P. Webster, A. Gale and M. E. Davis, *Proceedings of the National Academy of Sciences*, 2013, **110**, 8662–8667.

[5] M. A. Hayat, *Principles and Techniques of Electron Microscopy: Biological Applications*, Cambridge University Press, 2000.