# Development of anti-TNFR antibody-conjugated nanoparticles

**Ahmed Aido [1,2,*], Harald Wajant [2], Matej Buzgo[1] and Aiva Simaite [1]**

[1]  InoCure s.r.o, R&d Lab, Prumyslová 1960, 250 88 Celákovice, Czech Republic; aiva@inocure.cz

[2]  Division of Molecular Internal Medicine, Department of Internal Medicine II, University Hospital Würzburg, Auverahaus, Grombühlstrasse 12, 97080 Würzburg, Germany; harald.wajant@mail.uni-wuerzburg.de

*  Correspondence: ahmed@inocure.cz; Tel.: +49-174-913-6442

**Abstract:** Immunotherapy is considered as a new pillar of cancer treatment. However, the application of some promising immunotherapeutic antibodies, such as antibodies against certain immune-stimulatory receptors of the TNF receptor superfamily (TNFRs) including CD40, 41BB, CD27 and anti-fibroblast growth factor-inducible 14 (anti-Fn14) are limited due to their low bioactivity. It has been previously shown that the bioactivity of such anti-TNFR antibodies could be improved by crosslinking or attachment to the plasma membrane by interaction with Fcγ receptors (FcγR). Both result in proximity of multiple antibody-bound TNFR molecules what allows activation of proinflammatory signaling pathways. In this work, we have grafted antibodies on gold nanoparticles to simulate the "activating" effect of FcγR-bound and thus plasma membrane-presented anti-TNFR antibodies. We have developed and optimized the method for the preparation of gold nanoparticles, their functionalization with poly-ethylene glycol (PEG) linkers, and grafting of antibodies on the surface. We showed here that antibodies, including the anti-Fn14 antibody PDL192, can be successfully attached to nanoparticles without affecting antigen binding. We hypothesize that conjugation of monoclonal anti-TNFR antibodies to the inorganic nanoparticles is a promising technique to boost the efficacy of these immunotherapeutic antibodies.

**Keywords:** Nanoparticles; Surface modification; Drug-delivery, agonistic anti TNFRSF receptor (TNFR) antibody

## 1.    Introduction

Cancer immunotherapy is a very attractive field with high promise to provide cures for difficult cancers. Immunotherapy relies on the stimulation or silencing of signaling pathways of relevance for tumour development. Some of the promising immunotherapeutics target tumor necrosis factor receptor superfamily (TNFRSF) receptors (TNFRs) which are naturally involved in the regulation or even the inhibition of tumor growth [1]. The interactions between the ligands of the TNF superfamily (TNFSF) and their TNFRs regulate innate and adaptive immune responses including natural killer cell activation, T cell co-stimulation, and control of B cell homeostasis [1]. However, there are many obstacles in the production of recombinant soluble TNFSF ligands and they show poor pharmacokinetics (low serum half-life of only around 10–30 min) [2]–[4]. Thus, agonistic antibodies targeting TNFRs such as CD40-, 41BB, CD27 and Fn14 are considered as alternative TNFR agoists to soluble TNFSF ligand molecules. Unfortunately, anti-TNFR antibodies targeting a subgroup of TNFRs including CD40, 41BB, CD27 and Fn14 typically lack agonistic activity as free molecules. Instead, FcγR binding is required for these antibodies in order stimulate receptor signaling [5].

44  Medler *et al.* have shown that "activating" FcγR-dependent cell surface anchoring of IgG
45  antibodies can be replaced by anchoring domains genetically fused to antibodies that recognized cell
46  surface exposed structures distinct from FcγRs [4]. We hypothesized that the activating effects of
47  plasma membrane associated presentation of antibodies can be simulated by grafting of antibodies
48  to a solid support. In this work, we have developed and optimized a method for attaching antibodies
49  to gold nanoparticles with the expectation to increase their activity.
50  Gold nanoparticles (AuNPs) are widely used in different biomedical applications and are used
51  as a platform for nanobiological conjugates, such as oligonucleotides [6], antibodies [7] and proteins
52  [8]. In addition, the physicochemical and optoelectronic properties of the spherical AuNPs such as
53  surface plasmon resonance, conductivity, large surface-to-volume ratio, excellent biocompatibility,
54  and low toxicity extend the possibilities to exploit them as new generation of drug delivery systems
55  [9]. All these properties combined, make gold nanoparticles a promising tool to deliver the
56  therapeutic agents to the targeted cells. In this work, we hypothesize that gold nanoparticles can be
57  exploited as a platform to immobilize antibodies against TNFRs, to enhance their agonistic activity.
58  More speficially, we used a known gold nanoparticle synthesis protocol and optimized for grafting
59  of antibodies, including the anti-Fn14 antibody PDL192, under preservation of their antigen binding
60  abilities.

61  **2. Experiments**

62  *2.1. Materials*
63  Gold (III) chloride acid trihydrate was obtained from VWR International. mPEG-SH / mPEG-
64  Thiol (5kDa) and SH-PEG-COOH / Thiol-PEG-Acetic Acid (5 kDa ) were obtained from Biochempeg.
65  38.8 mM, trisodium citrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride  (EDC)
66  were obtained from Thermo Scientific™, N-Hydroxysuccinimide(1-hydroxy-2,5 pyrrolidinedione)
67  (NHS) was obtained from Sigma-Aldrich, β-(N-Morpholino)ethansulfonsaure (MES), water free
68  ≥99% and was obtained from VWR chemicals. 50 mM TRIS with 0.33 mg/ml mPEG-SH was used as
69  a blocking buffer.
70  The following antibodies were used: Humira (anti TNF alpha), Cosentyx® (IL-17A monoclonal
71  antibody) and anti-Fn14 PDL192. GpL-TNC-TNF has been described elsehere (Lang et al., 2016) [10]
72  Fn14ed-GpL was generated by cloning the Gaussia princeps luciferase w/o leader to the C-terminus
73  of the extracellular domain of Fn14.

74  *2.2. Methods*
75  2.2.1. AuNPs synthesis:
76  The protocol for AuNP synthesis was adapted from [11], [12] with slight modifications. 100 ml
77  of 0.4 mM chloroauric acid solution was boiled in a clean 300-ml glass flask with stir bar. A reflux
78  column was attached on top of the flask to prevent the decrease of the solution´s volume. The entire
79  apparatus was placed on a hot plate and boiled while stirring. One ml of 38.8 mM trisodium citrate
80  solution was added to the solution to produce 60 nm spherical monodisperse gold nanoparticles.
81  Other sizes were created based on the amount of added trisodium citrate and/or the concentration of
82  auric salt within a range between 15 and 100 nm. Upon addition of the trisodium citrate, the colour
83  of the solution change to blue in about 30 s and then to red in another 150 s. The colour change during
84  synthesis is attributed to the increase in size of gold nanoparticles as the citrate ions reduce the gold
85  ions [13]. The boiling was continued for another 10 min and then cooled to room temperature.

86  2.2.2. AuNPs functionalization:
87  To modify the surface of the produced gold nanoparticles with functional carboxyl groups, a
88  volume of HS-PEG-COOH solution with a selected concentration was added to the whole amount of
89  the produced colloidal of AuNPs to get 100 ug/ml of HS-PEG-COOH in the solution and was left to
90  be mixed for one hour. The whole amount of carboxyl-modified AuNPs was centrifuged in a big
91  centrifuging device (10000 RPM for 10 min) then the pellet was collected in an Eppendorf tube and

92    washed twice with mPEG (0.33 mg/ml) (in order to fill the unmodified places on the surface of
93    HOOC-PEG-AuNPs and to compensate the washed stabilizer trisodium citrate).

94           2.2.3. AuNPs grafting:
95           The carboxyl-modified AuNPs were conjugated with the protein of interest according to the
96    EDC-NHS covalent binding procedure adapted from [14]–[18]. Briefly, an amount of purified C-
97    AuNPs were resuspended in a volume of the activation/coupling buffer (50 mM MES, pH 6.0) and
98    washed with it for 3 times. Then, 24 μL of EDC (200 mM) and 240 μL of NHS (200 mM) were added
99    to 1 ml of the previous solution of AuNPs, and incubated for 30 min in RT. After washing the particles
100   from the EDC and NHS reagents for 3 times with the activation/coupling buffer, 500 μl of the
101   activated C-AuNPs were incubated with 500 μl of a selected concentration of the protein of interest
102   (anti-TNF, anti-Fn14, anti-IL17A). The antibody-grafted AuNPs were washed from the exces of the
103   unconjugated protein for 3 times with the blocking buffer (Tris 50 mM in mPEG-SH (0.33 mg/ml)) to
104   block the free activated carboxyl sites on the surface of gold nanoparticles. Finally, the antibody-
105   grafted AuNPs were resuspended in a volume of the blocking buffer to be ready for later on
106   application.

107          2.2.4. Characterization
108          UV-Vis: AuNPs samples were collected at each stage immediately after synthesis and their
109   optical properties were evaluated by UV–vis spectrophotometry (SpectraMax.). The absorption
110   spectra were acquired in the range of 450–650 nm with a step of 5/10 nm.
111          DLS: The size of the obtained AuNPs (unPEGylated, PEGylated and grafted particles) were
112   analyzed, using the Nanophox 123 Dynamic Light Scattering (DLS) with photon cross-correlation
113   spectroscopy from Sympatec. The particles were purified by centrifugation at 22,000 g for 10 min,
114   diluted 100 time in distelled water, then analyzed. All DLS experiments were carried out at a
115   temperature of 25°C.
116          Zetasizer : The effective surface charges on the gold nanoparticles were measured using zeta-
117   potential (Malvern Instruments Zetasizer). Reported zeta potential measurements were collected on
118   aqueous solution in wich AuNPs were diluted 10-100 times dependings on their concentration .

119   **3.  Results**
120    *3.1. Optimization of gold nanoparticles synthesis protocol*

121          3.1.1.  Controling the size and concentration of gold nanoparticles

122          The size of AuNPs can be adjusted by controlling the concentration of the auric salt ($HAuCL_4$
123   .$3H_2O$) [12]. To optimize the procedure for the preparation of AuNPs needed for our work, that is in
124   order to obtain AuNPs with the size bellow 200 nm, we have set trisodium citrate concentration to
125   38.8 mM and varied the concentration of gold chloride and boiling duration. UV-vis absorption was
126   used to characterize the particles size (the wavelength of the maximum absorbance of the plasmon
127   band of the spherical particles AuNPs are in dependence of the particles size [19]. As shown in **Figure**
128   **1a,** increasing the concentration of the gold chloride leads to the lambda max shift from 520 nm to 550
129   nm, indicating the increase of particle size from 15 nm to 80 nm. On the orther hand, different results
130   were noted for the influence of the boiling duration. In this case the $\lambda_{max}$ absorbance of the colloidal
131   solution was measured after 5, 20 and 40 min of boiling. As shown in **Figure 1b**, the optical density
132   increased with fixed $\lambda_{max}$ absorbance indicating that the total amount of the produced gold
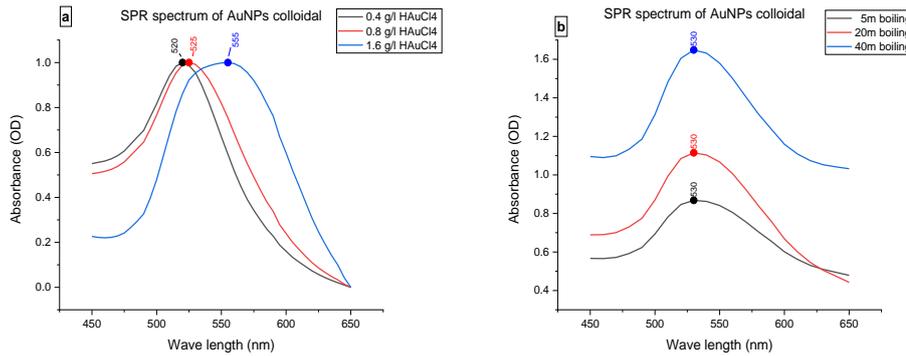133   nanoparticles increased with the continuing boiling [13].

**Figure 1: λmax absorbance of the (SPR) of AuNPs colloidal**. Comparasion between spectrum of the absorbance of AuNPs colloidals resulted from a)different concentration of HAuCL4 .3H2O. or b) different boiling duration.

134    3.1.2.  Functionalization of the gold nanoparticles
135    Trisodium citrate plays a role as reducing agent and a stabilizer of the produced gold nanoparticles
136    [20]. The abundance of the negative charges of the citrate structure surrounding the surface of AuNPs
137    is known to prevent their aggregation. However, the stabilization effect of citrate is not significant
138    enough for storing the particles long term and can be lost after the purification. To increase the long
139    term stability of the AuNPs and to introduce chemical groups for the subsequent fuctionalization,
140    particles were functionalized with a layer of SH- and carboxyl- (5 kDa HOOC-PEG-SH) or methoxy-
141    (5 kDa H$_3$C-O-PEG-SH) containg polymers. Grafted particles were purified by washing with distilled
142    water to discard the exces of trisodium citrate and free polymer molecules. As indicated in **Figure 2,**
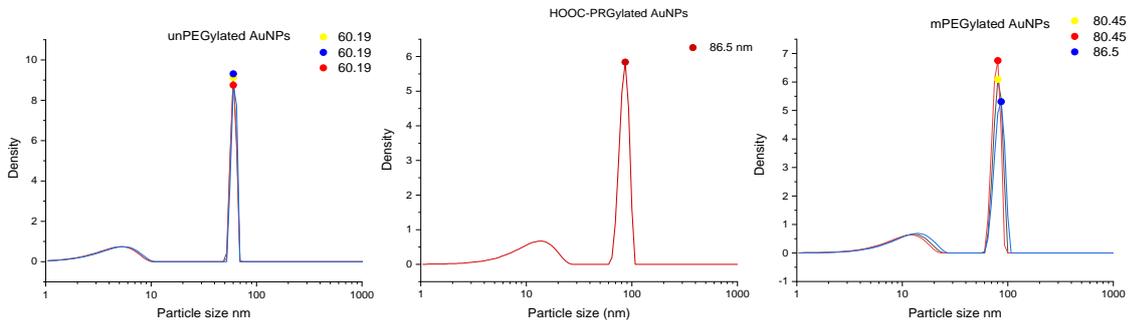


**Figure 2: Diameter distribution graphs from DLS** show the increase of particles size after PEGylation with carboxyl-PEG-SH and mPEG-SH

143    the increase of particles size after the PEGylation grafted with two types of polymers is similar, most
144    likely due to the identical molecular weight. To confirm the PEGylation, zeta potential measurements
145    were performed. As shown in **Table 1,** the PEGylation leads to a change in ζ potential values. In
146    comparison to the citrate stabilization, the dominant charge of the particles after PEGylation is even
147    more negative after PEGylation with the carboxyl- terminated polymer (-20 mV), and less negative
148    when methoxy-PEG was used (-7 mV).

149    **Table 1:** Comparation of ζ potential values and particles size between the un-PEGylated and PEGylated gold
150    nanoparticles.

| Sample structure | Particles Size | ζ potential |
|---|---|---|
| **Trisodium citrate - AuNPs** | 60.19 nm | -14 mv |
| **mPEG-AuNPs** | 80.45 nm | -7 mv |
| **HOOC-PEG-AuNPs** | 86.5 nm | -20 mv |

151     *3.2. Characterization the conjugation of C-AuNPs with different thersputics antibodies*
152     Carboxyl-modified gold nanoparticles C-AuNPs (ca. 60 nm, 25 mg/ml) were conjugated with the
153     following proteins: anti-Fn14 antibody PDL192, anti-TNF or anti-IL17A. The conjugation process was
154     performed by following the EDC/NHS covalent coupling procedure (described in the methods). To
155     confirm not only the conjugation of C-AuNPs with the antibodies but also their post-conjugation
156     functionality, binding studies using the antigens TNF and Fn14 fused to the Gaussia princeps
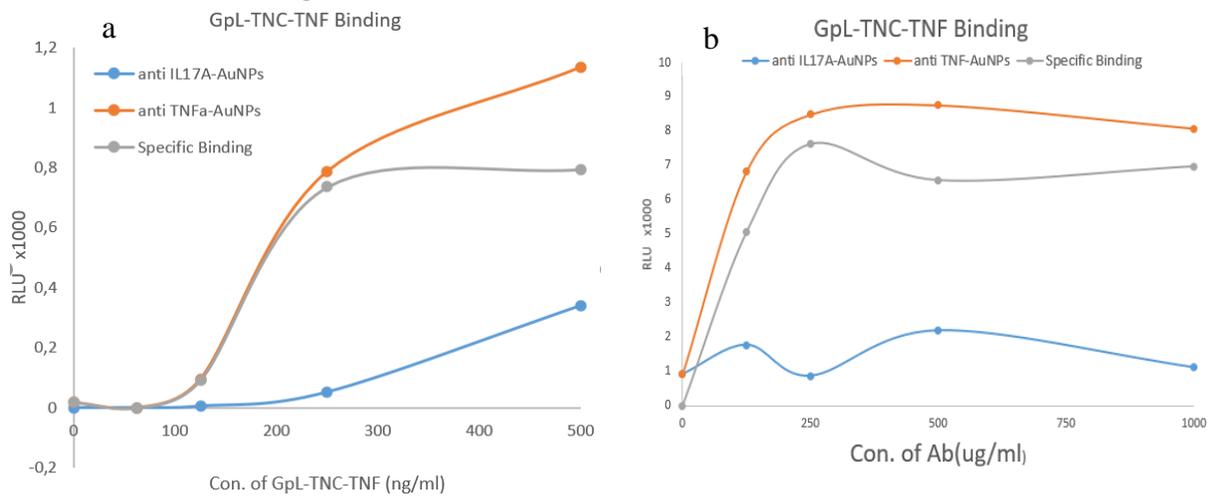


**Figure 3: Binding of GpL-TNC-TNF fusion protein to either anti-TNF-AuNPs or anti-IL17A-AuNPs:**
Carboxyl-modified gold nanoparticles C-AuNPs (ca. 60 nm, 25 mg/ml) were conjugated with either fixed
concentration of anti-TNF or anti-IL17A. Then binding studies have been carried out with serial dilutions of
GpL-TNC-TNF (a). Alternatively, different concentrations of the two antibodies were used for gold
nanoparticle conjugation and GpL-TNF-TNC binding with a fixed concentration was analyzed (b). The curve
of the specific binding was obtained by subtraction of the binding to anti-IL17A from the values of the binding
to anti-TNF.

157     Luciferase (GpL-TNF-TNC, Fn14ed-GpL) [10]. The GpL domain allows the quantification of the
158     binding to the corresponding gold nanoparticle associated antibodies by measurement of the
159     luminescence upon removal of the free GpL fusion protein molecules. The binding curves between
160     GpL-TNF-TNC and anti-TNF-AuNPs (total biding) or anti-IL17A-AuNPs (unspecific binding) are
161     shown in **Figure 3a**. A serial dilution of the GpL-linked antigen (from 0 ng/ml to 500 ng/ml) were
162     mixed with the fixed concentration (25 mg/ml) of the AuNPs conjugated with antibody (1mg/ml conc
163     in linking solution). As show in the **Figure 3a**, in all cases, the luminescence increased with the
164     increasing antigen concentration. However, notably higher binding was observed with anti-TNF than
165     with anti IL17A AuNPs. This indicates high specific binding of GpL-TNC-TNF to gold nanoparticle
166     immobilized anti-TNF. Thus the conjugation of the antibody did not notably affected the interaction
167     between the conjugated Ab and its antigen.
168         In order to evaluate the maximum amount of the antibody that can be conjugated to the
169     nanoparticles, increasing concentrations of anti-TNF or anti-IL17A were used for conjugation. The
170     binding of a GpL-TNF-TNC solution with constant concentration was then determined as shown in
171     **Figure 3b**. With a concentration of 250 ug/ml of anti-TNF in the coupling reaction the maximum
172     amount of antibody could be immobilized on the particles. Increasing the antibody concentration did
173     not lead to an further increase in antigen binding. Similar binding studies with anti-Fn14 PDL192-
174     AuNPs and their GpL-fused antigen (Fn14ed-GpL) were performed. As shown in **Figure 4**, maximum
175     conjugation capacity was again reached at the antibody concentration of 250 ug/ml – similar to the
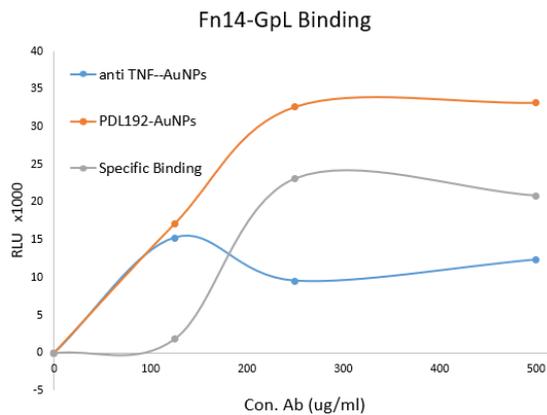176     results shown in **Figure 3b.**

**Figure 4: Binding of Fn14ed-GpL fusion protein to either antiTNF-AuNPs or PDL192 -AuNPs:** Carboxyl-modified gold nanoparticles C-AuNPs (ca. 60 nm, 25 mg/ml) were conjugated with different concentration of the both antibodies and then the signals of the emmited light resulted from the binding with fixed concentration of Fn14ed-GpL were measured and plotted. The curve of the specific binding was obtained by subtraction of the binding to anti-TNF from the values of the binding to PDL192.

## 4. Discussion

Since the early work of Turkevich and Frens,  method to produced gold nanoparticles in the scale, from 9 to 120 nm and with defined size distribution, have been optimized for various applications [13], [21]. Gold NPs can be coated with a ligand shell, which provides colloidal stability, or conjugated with (biological) molecules via thiols [22, Rivera-Gil *et al*]. However attaching Abs directly to the surface of NPs has drawbacks, that is, the process may affect their activity by blocking the active side. To overcome this problem, a method depending on directional covelant binding to a functional polymer on the surface of NPs has been investigated in the litretures [23]. One effective method is the use of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) chemistry [24].

Antibodies bear amine groups on some of their amino acid side chains including residues in the the antigen binding site. To detect the probablitliy of the binding to antigen binding-irrelevant sites of the antibody, we functionalized AuNPs with carboxyl-containing polymer and then conjugated them with the therapeutic Abs using EDC/NHS chemistry, and carried out binding studies. The performed binding studies revealed that, even though some antibodies might have coupled through their antigen binding sites, other coupled through antigen biding irrelevant amino acids and remian active.

Recent studies found that specific antibodies to the TNF receptor Fn14, can mimic some effects of the soluble TWEAK, Fn14 specific TNF ligand, by triggering the related signaling pathways. However, the soluble anti-Fn14 antibody failed to activate all of the needed signaling pathways of the soluble TWEAK. This drawback of the soluble anti-Fn14 can be overcome by the oligomerization usinig protein G or by the anchoring to Fc$\gamma$ receptors of the effector cells, which provides the antibody with agonistic activity [4], [25]. In the future work, we will exploite the gold nanoparticles as a platform to immobilize the therapeutic antibody (PDL192) as a model. We hypothesize that the AuNPs-conjugated anti Fn14 monoclonal IgG1 antibody will posses the agonistic activity which resembles the Fc$\gamma$ receptors anchoring-dependent efficiency. Such anchored antibodies should then be able to trigger the associated proinflammatory signaling pathways. If successful, this approach will enable promising applications of nanocomposites with antitumor antibodies. Furthermore we will work on the nanoencapsualtion of these nanoparticles to target tumor tissues specifically and prevent the systemic side effects associated with such antibodies.

## 5. Conclusions

Gold nanoparticles of diameter ca. 60 nm have been synthesized by sodium citrate reduction

208  of gold chloride, then functionalized with COOH-PEG-SH to stabilize the colloidal solution of the
209  gold nanoparticles. We have also demonstrated that the carboxyl-modified gold nanoparticles can be
210  coupled with the antibodies of interest using the EDC/NHS coupling procedure. The binding studies
211  of the Ab-grafted AuNPs against their labeled specific antigen confirmed the conjugation of C-
212  AuNPs. We showed that our coupling protocol allows the conjugation of antibody so that its activity
213  is maintained. Our future work will focus on the *in vitro* assays needed to compare the activity of the
214  conjugated antibodies and their soluble variants.

217  **Author Contributions:** A.A. designed the study, performed the experiments, data processing, analysis of the
218  results and wrote the paper; A.Si. and H.W. helped with method development, supervised the work and
219  contributed with insights and discussions;; A.Si. and M.B. guided the design of work and experiments, helped
220  with the writing of the manuscript and reviewed the paper.

221  **Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design
222  of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the
223  decision to publish the results.

## 224  Abbreviations
225  The following abbreviations are used in this manuscript:

| Abs: | Anti bodies | PEG: | Poly-ethylene glycol |
|------|-------------|------|----------------------|
| AuNPs: | Gold nanoparticles | PDL192 : | Anti Fn14 monoclonal IgG1 antibody |
| C-AuNPs: | Carboxyl-modified gold nanoparticles | SPR: | Surface Plasmon Resonance |
| DLS: | Dynamic light scattering | TNF : | Tumor Necrosis Factor |
| GpL: | Gaussia princeps luciferase | TWEAK: | TNF-related weak inducer of apoptosis |
| IgG: | Immunoglobulin G | | |

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