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ABSTRACT

Photothermal tumor ablation might be carried out with multibranched gold nanoparticles (MBAuNPs) having maximum absorbance ($A_{\text{max}}$) in the infrared region and functionalized with ligands that would bind them to the target tumor markers. However, in nanomedicine applications, the nanostructures must reach their target tissues to be effective, but the corona of serum proteins they instantaneously acquire when administered by intravenous injection may affect their activity; for this reason, we decided to analyze the effect that exposing MBAuNPs to bovine serum albumin (BSA) and human serum (HS) have on their protein corona and physical properties. The synthesized spherical Au seeds stoichiometrically generate piñata-like MBAuNPs of 8–20 peaks potentially useful for photothermal tumor ablation since they induce hyperthermia of more than 4°C in phantom gels mimicking the skin irradiated with an 808 nm laser at 0.75 W/cm$^2$. The calculated surface area of MBAuNPs ranges from 24 984 nm$^2$ to 40 669 nm$^2$, depending on the number of peaks we use for modeling the NPs. When MBAuNPs are exposed to BSA, they acquire a protein corona with an internal “hard” portion composed by one or two layers of BSA containing $\sim 1000–4000$ molecules covalently bound to their surface, and an external “soft” portion formed by agglomerated BSA molecules linked by non-covalent bonds. Functionalization with BSA decreases the tendency of MBAuNPs to agglomerate and increases their size dispersion. MBAuNPs and MBAuNPs–BSA exposed to HS bind HS albumin and other HS proteins ranging from 25 kDa to 180 kDa that increase their hydrodynamic diameter and decrease their stability. We conclude that MBAuNPs exposed to serum albumin and HS instantaneously acquire a hard and soft protein corona that may affect prior or subsequent functionalization aiming to direct them to specific cell or tissue targets.

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INTRODUCTION

Malignant tumors are usually treated by surgical resection, chemotherapy, and radiotherapy. Chemotherapy and radiotherapy are often ineffective and have undesirable side effects because they do not discriminate between healthy and cancerous cells. Although hyperthermia for tumor ablation is an alternative to surgical resection, it is rarely used because the temperature in the target tissue is not homogeneous. Since radiation in the near-infrared region (NIR) penetrates deeply but is hardly absorbed by tissues, attempts have been made to develop tumor ablation procedures based on nanoparticles (NPs) that convert NIR radiation into heat. Gold NPs (AuNPs) are non-toxic, and since spherical AuNPs absorb NIR waves much less efficiently, nowadays the use of multi-branched AuNPs (MBAuNPs) for hyperthermal phototherapy is being intensively explored.

Depending on the tumor and its anatomical location, the potential efficacy of photothermal nanotherapy would depend on both the photothermal conversion efficiency and the ability of NPs to reach and penetrate the desired tumor targets. NPs are functionalized with ligands that direct them to specific cell receptors, but when they enter the bloodstream they are instantly modified through their interaction with blood proteins attaching to their surface and forming a structure known as protein corona (PC). The PC is formed by the hard corona (HC) consisting of proteins strongly associated with the NP surface, and by the soft corona (SC) that is an outer layer of weakly bound proteins. The PC is also complex and unique for each nanomaterial and changes the physicochemical properties of NPs, such as size, surface charge, and state of aggregation. These changes may interfere with the functionalities provided in biological microenvironments. The development of NPs suitable for in vivo photothermal therapy thus depends on controlling PC formation and structure and its effects on the physical and biological properties of NPs.

In this report, we describe the synthesis of MBAuNPs, their characterization, and the features of the hard and soft protein corona they acquire after being exposed to bovine serum albumin (BSA), to human serum (HS), or BSA followed by HS. The photothermal tumor ablation potential of our MBAuNPs is supported by their high photothermal conversion efficiency leading to a net temperature increase of ~5°C in phantom gels irradiated at low power (<1 W/cm²) with an 808 nm near-infrared laser.

RESULTS

Size, shape, and concentration of nanoparticles

Auric seeds

Scanning electron microscope (SEM) images showed that seeds were spherical, with a hydrodynamic diameter (D_H) of 18 nm...
TABLE I. Dynamic light scattering analyses of auric seeds, MBAuNPs, and MBAuNPs-BSA suspensions. ND: No determinate.

| Sample          | DH (nm) | PDI  | ζ-potential (mV) | DH (nm) | PDI  | ζ-potential (mV) |
|-----------------|---------|------|-----------------|---------|------|-----------------|
| In PBS          |         |      |                 |         |      |                 |
| Seeds           | 18      | 0.602| −36.8           | ND      | ND   | ND              |
| MBAuNPs         | 125     | 0.455| −24.4           | 148     | 0.2048| −18             |
| MBAuNPs-BSA     | 146     | 0.929| −72.7           | 185     | 0.1672| −31             |

The ζ-potential of the seeds was −36.8 mV, with a polydispersity index (PDI) of 0.602 (Table I). The concentration determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES) was 60 mg/l, corresponding to $1 \times 10^{15}$ seeds/l.

**MBAuNPs**

SEM and transmission electron microscopy (TEM) images showed anisotropic multibranched nanoparticles with 8–20 peaks,

D$_H$ of 125 nm [Figs. 1(d) and 1(e); Table I], maximum absorbance at 775 nm, and null absorbance at 520 nm [Fig. 1(f)]. The surface areas calculated for MBAuNPs containing 8, 12, and 20 peaks were 24 984 nm$^2$, 30 212 nm$^2$, and 40 669 nm$^2$, respectively.

The ζ-potential of MBAuNPs was −24.4 mV, with a PDI of 0.455 (Table I). The MBAuNP concentration determined by ICP-OES was 23.5 mg/l, corresponding to $1 \times 10^{13}$ MBAuNPs/l.

**Hyperthermia induced by irradiation of auric seeds and MBAuNPs in aqueous and phantom gel suspensions**

The temperature was monitored in aqueous solutions containing auric seeds (60 mg/l), or MBAuNPs (23.5 mg/l), and in phantom...
gels containing MBAuNPs (2 mg/l or 3 mg/l). All samples were irradiated continuously for 10 min with an 808 nm laser at irradiation powers of 1 W/cm$^2$, 0.75 W/cm$^2$, and 0.45 W/cm$^2$.

The increase of temperature in water and aqueous suspensions irradiated with fixed 0.75 W/cm$^2$ power was: water, 1.3 °C; auric seeds, 1.7 °C; MBAuNPs, 14.3 °C [Fig. 2(a)]. The increase of temperature in aqueous MBAuNP suspensions irradiated with increasing powers (subtracting the increase in water) was: 0.45 W/cm$^2$, 4.2 °C; 0.75 W/cm$^2$, 13 °C; 1 W/cm$^2$, 10.7 °C [Fig. 2(b)].

The increase of temperature in phantom gels containing MBAuNPs (2 mg/l) irradiated with increasing powers was: 0.45 W/cm$^2$, 0.1 °C; 0.75 W/cm$^2$, 3.7 °C; 1 W/cm$^2$, 3.9 °C [Fig. 3(a)]. The increase of temperature in phantom gels with MBAuNPs (3 mg/l of Au) irradiated with increasing powers was: 0.45 W/cm$^2$, 0.7 °C; 0.75 W/cm$^2$, 4.8 °C; 1 W/cm$^2$, 5.2 °C [Fig. 3(b)].

Surface area and BSA corona in MBAuNPs

TEM and high-resolution transmission electron microscope (HRTEM) images of functionalized MBAuNPs showed a 10 nm–15 nm thick layer covering their surface that was absent in pure MBAuNPs (Fig. 4). D$_H$ values increased from 125 nm for MBAuNPs to 146 nm for MBAuNPs-BSA. The ζ-potential of MBAuNPs was –72.7 mV, with a PDI of 0.929 (Table I).

The UV–VIS spectra of pure BSA in solution and MBAuNP suspensions functionalized with BSA were determined using
phosphate-buffered saline (PBS) as a vehicle. The absorbance peaks observed were as follows: pure BSA, a single peak at 280 nm; pure MBAuNPs, a single peak at 775 nm; MBAuNPs-BSA, two peaks, a major one at 280 nm and a minor one at 825 nm (Fig. 5).

The molar extinction coefficient of BSA in solution was $43.377 \text{M}^{-1}\text{cm}^{-1}$, and that of BSA bound to MBAuNPs was $41.866 \text{M}^{-1}\text{cm}^{-1}$ (Fig. 6).

The average number of BSA molecules in a suspension containing $7.6 \times 10^{11}$ MBAuNPs/ml was calculated by UV spectroscopy. The BSA concentration in MBAuNP-BSA sedimented by centrifugation and resuspended in PBS was $4.5 \times 10^{16}$ molecules/ml, corresponding to 59,210 BSA molecules per MBAuNP. MBAuNP-BSA suspensions washed six times with PBS contained $1.14 \times 10^{15}$ BSA molecules/ml, corresponding to 1500 BSA molecules per MBAuNP (2.5% of the total BSA molecules initially bound).

Through mathematical estimation of the surface area of MBAuNPs and taking into account the area of individual BSA molecules, we calculated $3.5 \times 10^{12}$ molecules/cm$^2$, assuming that the hard protein corona formed by a molecular monolayer would amount to $\sim$900, $\sim$1000, and $\sim$1500 BSA molecules per MBAuNP with 8, 12, and 20 tips, respectively. Assuming a protein bilayer, the number of BSA molecules per MBAuNP would be $\sim$1900, $\sim$2500, and $\sim$3880 (Table II).

**XPS spectra of MBAuNPs-BSA.** The high-resolution x-ray photoelectron spectroscopy (XPS) spectrum of gold in pure MBAuNPs had three peaks (including their doublets) at 82.37 eV, 82.87 eV, and 85.06 eV, corresponding to Au$^0$, Au$^{+1}$, and Au$^{+3}$, respectively. In contrast, the high-resolution spectrum of gold in MBAuNPs-BSA had two peaks: the first at 81.31 eV identified as Au$^0$ and the second at 82.11 eV identified as a C–S–Au (I) bond [Figs. 7(a) and 7(b) and Table III]. The high-resolution spectrum of sulfur had the main peak at 166.21 eV, corresponding to the S 2p$^{3/2}$ position identified as an S–Au bond [Table III and Fig. 7(c)].

**Circular dichroism of BSA in solution and MBAuNPs-BSA**

BSA in solution and MBAuNPs-BSA suspensions had the 208 nm and 222 nm absorbance peaks characteristic of $\alpha$-helix structures. The $\alpha$-helix contents of solutions with 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml of BSA were 31.82%, 44.72%, and 48.71%, respectively, whereas in MBAuNPs-BSA suspensions with equivalent BSA concentrations, the $\alpha$-helix contents were 22.13%, 40.32%, and 43.2%, respectively (Fig. 8).

| Peaks | MBAuNP surface (nm$^2$) | BSA molecules | MBAuNP surface (nm$^2$) | BSA molecules |
|-------|------------------------|--------------|------------------------|--------------|
| 8     | 24,983                 | 900          | 52,193                 | 1,900        |
| 12    | 30,212                 | 1,000        | 71,000                 | 2,500        |
| 20    | 40,668                 | 1,500        | 108,652                | 3,880        |

**TABLE II.** Calculation of the surface area of MBAuNPs containing 8, 12, and 20 peaks, and the number of BSA molecules bound per nanoparticle in the hard and soft corona.
TABLE III. Chemical Au and S bonds identified by XPS in MBAuNPs and MBAuNPs-BSA.

| Sample               | Peak index | Position (eV) | ID                |
|----------------------|------------|---------------|-------------------|
| MBAuNPs (Au)         | Au⁰        | 82.37         | Au                |
|                      | Au⁰        | 86.00         | Au doublet        |
|                      | Au¹⁺       | 82.87         | N–Au (I)          |
|                      | Au¹⁺       | 86.53         | Au doublet        |
|                      | Au³⁺       | 85.06         | Au (III)          |
| MBAuNPs-BSA (Au)     | Au⁰        | 81.31         | Au                |
|                      | Au⁰        | 84.96         | Au doublet        |
|                      | Au¹⁺       | 82.11         | C–S–Au (I)        |
|                      | Au¹⁺       | 85.55         | Au doublet        |
| MBAuNPs-BSA (S)      | S 2p₃/₂    | 166.21        | S–Au              |
|                      | S 2p₁/₂    | 164.12        | S Doublet         |

BSA and human serum proteins bound to MBAuNPs and MBAuNPs-BSA

After exposing MBAuNPs or MBAuNPs-BSA to human serum for 24 h and washing them by repeated centrifugation with PBS,
the ζ-potential was −18 for MBAuNPs and −31 for MBAuNPs-BSA, 
PDI was 0.205 for MBAuNPs and 0.167 for MBAuNPs-BSA, and 
D_H was 148 nm for MBAuNPs and 185 nm for MBAuNPs-BSA.

After having been exposed to human serum, MBAuNPs and 
MBAuNPs-BSA that were extensively washed by centrifugation and 
then subjected to denaturing conditions released protein bands with 
apparent molecular weights of 180 kDa, 156 kDa, 120 kDa, 84 kDa, 
75 kDa, and 25 kDa (Fig. 9).

**DISCUSSION**

The MBAuNPs described in this paper are potentially useful 
for photothermal ablation of tumors since they induce hyperthermia by 
irradiation with a near-infrared laser at minimum power both in 
aqueous suspensions and in phantom gels mimicking the human 
skin.

The auric seeds, synthesized as precursors of MBAuNPs, are spherical and stable as expected, they present maximum 
absorbance at 520 nm and have excellent stability and dispersion as 
shown by dynamic light scattering (DLS) analysis. Their concentra-
tion was determined by the ICP-MS method of Alabashi et al.,31 
used also to control the amount of seeds used for the synthesis 
of anisotropic MBAuNPs, that was carried out with a modific-
tion of the method of Maiorano et al.22 The resulting MBAuNPs 
had maximum absorbance at 775 nm, indicating an increased effi-
ciency to produce hyperthermia by NIR irradiation, whereas the 
loss of the 520 nm absorbance peak suggests that each seed gives 
rise to a single MBAuNP. The larger shift of the absorption peak 
toward the infrared by our MBAuNPs may be due to the rela-
tively high HEPES concentration (50 mM) used for their synthesis, 
leading to an increased length of the cones attached to the seed 
surface.

The highest photothermal efficiency was attained with sus-
pensions containing 3 mg/l of MBAuNPs in phantom gels irriga-
ted with the 808 nm laser at 0.75 W/cm², whose temperature was 
increased by 4.8 °C, well above the results obtained in previous studies.23–25 Our finding that the hyperthermia of phantom gels depends 
on both the MBAuNP concentration and irradiation power may be 
further tested in cell cultures and animal models.

Functionalization with specific ligands to direct MBAuNPs to 
tissues is expected to improve their therapeutic efficacy 
when administered systemically through intravenous injection. 
However, such functionalization may be affected and even nullified 
by the protein corona that instantaneously forms on their surface, 
even in MBAuNPs previously functionalized with BSA to prevent 
their agglomeration and to direct them to the albumin receptors that 
abound on the surface of the endothelial cells forming the inner wall 
of blood vessels.27

HRTEM images of extensively washed MBAuNPs-BSA have a 
10 nm–15 nm thick halo absent from naked MBAuNPs and 
resolved individual BSA molecules appearing to form a tapestry of one or two molecular layers. The thickness of the protein layer is consistent with the $8 \times 8 \times 3.5 \text{ nm}^3$ dimensions of the native 
BSA molecule.28 The 50 nm displacement of the absorbance peak 
from 775 nm in MBAuNPs to 825 nm in MBAuNPs-BSA is 
higher than the 7 nm displacement reported by Nghiem et al.27 
This difference may be due to a difference in the number of 
BSA molecules bound per nanoparticle since the magnitude of the 
displacement depends on the initial BSA concentration used for 
functionalization.

Our mathematical estimation of the surface area of MBAuNPs and 
the number of BSA molecules bound to their surface matches 
the number of BSA molecules determined by UV spectrophotom-
etry. Taking into account the HRTEM images, the increase of the 
hydrodynamic diameter, the mathematical estimation of the sur-
face area, and the experimental quantification results, it appears that 
the PC of MBAuNPs-BSA has two parts: an internal film of one or 
two layers of BSA molecules covalently bound to the MBAuNP sur-
face, and a lax external layer formed by agglomerated BSA molecules 
linked by non-covalent bonds that are released by extensive washing.

This model is consistent with that of Kokkinopoulou et al.29 who 
proposed that the protein corona consists not only of multiple layers 
covering each NP but also a three-dimensional network of proteins 
linked to the NP surface.

Brewer et al.26 estimated that the number of BSA molecules 
bound to spherical AuNPs ranges from $2.0 \times 10^{12} \text{ cm}^{-2}$ to 
$3.3 \times 10^{12} \text{ cm}^{-2}$ depending on the orientation in which BSA is bound. Assuming that the area of each BSA molecule to be 28 nm² and applying it to auric seeds and MBAuNPs, we obtained a similar 
range: $3.5 \times 10^{12}$ BSA molecules per cm².

To calculate the surface area of MBAuNPs, we used Eq. (3) to 
estimate the area that would bind the hard corona and a slightly 
modified Eq. (4) for the area available for the soft corona. Our results 
were similar to the values of the area for MBAuNPs calculated by 
Tsoulos et al.,21 and for the number of molecules attached to a given 
Au surface published by Brewer et al.31

NPs with ζ-potential values ≥30 or ≤−30 are considered stable.32 Our spheric seeds (ζ-potential = −36.8 mV) are quite stable 
and do not agglomerate for months, whereas our MBAuNPs (ζ-
potential = −24.4 mV) tend to agglomerate. Since our MBAuNPs- 
BSA have a ζ-potential = −72.7 mV, we confirmed that BSA 
decreases their tendency to agglomerate and turn them into a 
stable system.

High-resolution XPS analyses allowed us to unambiguously 
determine the presence of C–S–Au bonds between BSA molecules 
and gold atoms on the MBAuNP surface, they are probably Au–S 
cystine disulphide coordination bonds as those described by Wang 
et al.33 Each BSA molecule contains 34 cysteine residues and a free 
thiol at Cys34 that most likely is involved in the covalent binding 
of BSA to the gold surface, making MBAuNPs-BSA stable.

Assessment of the secondary BSA structure in solution and 
that bound to MBAuNPs revealed a decrease in the proportion of α-helices on the functionalized protein. Disulphide bonds are the 
main form of sulfur in BSA that stabilizes α-helices to maintain 
the protein structure. XPS analysis indicates that BSA binding 
to the MBAuNP surface through S–Au bonds may decrease 
the proportion of α-helices. Similar changes in the secondary pro-
tein structure have already been reported,36,37 and it is not known 
if these changes affect the affinity of BSA to the receptor-binding 
domain located on the surface of endothelial cells and some cancer cells.27

We also found that exposure of MBAuNPs and MBAuNPs- 
BSA to human serum increased their ζ-potential values close to 
those of uncoated MBAuNPs, and also increased D_H values of 
23 nm for MBAuNPs and 39 nm for MBAuNPs-BSA. These effects 
may be explained by the presence of human serum molecules other
than BSA or HSA and led us to characterize the electrophoretic patterns of the proteins bound to MBAuNPs and MBAuNPs-BSA exposed to human serum. HSA and other unidentified proteins with apparent molecular weights of 180 kDa, 156 kDa, 120 kDa, 84 kDa, 75 kDa, and 25 kDa bound both to MBAuNPs and MBAuNPs-BSA were associated with the higher increase of ζ-potential and D₃ in MBAuNPs-BSA, suggesting that previous functionalization with BSA affects the binding of human serum proteins.

This study reinforces the potential of MBAuNPs for photothermal tumor ablation. Their use in medical application, however, depends greatly on the successful delivery to their biological targets through the fine control and tuning of the protein corona, a process that requires research by oncologist/molecular biologist/physicist teams.

**Experimental**

**Materials**

Chloroauric acid (HAuCl₄) (USA), Heps sodium salt (99.5%) (USA), hydroxyamine (NH₂OH) (China), 99% sodium citrate (USA), bovine serum albumin (BSA) (96%) (USA), agar (Portugal), and polyethylene powder (USA) were purchased from Sigma-Aldrich; TX-151 was purchased from Balmar, L.L.C. (USA), Nonidet P40 (ultrapure) from Thermo Scientific (USA), hydrochloric acid (HCl) from J. T. Baker (USA), and nitric acid (HNO₃) from CTR Scientific (USA).

**Synthesis of auric seeds**

Synthesis of spherical seeds was performed using the Turkevich method in mixtures containing 25 mM HAuCl₄ and 0.75 mM sodium citrate, pH 7.4, as a reducing agent. Briefly, 19.8 ml of 0.75 mM sodium citrate pH 7.4 was added to a 30 ml beaker with a magnetic bar, and the mix was stirred on a magnetic hot plate at 70 °C; 0.2 ml of 25 mM HAuCl₄ was immediately added, and the mixture was kept under the same conditions for 10 min until the color was changed from light gray to red, with the appearance of red wine. When the reaction ended, the UV–VIS spectrum was recorded in a Cary model 60 Agilent spectrophotometer. To determine the shape and size of NPs, the samples were also analyzed by dynamic light scattering (DLS) with a zetameter (Zetasizer). HAuCl₄ was reduced in aqueous suspensions and phantom gels with hydroxylamine (NH₂OH) and 50 mM Hepes pH 7.4. The spectrophotometer probe (A₁₀₀₀–A₃⁹⁰⁰, average reading speed) was kept immersed in a 30 ml beaker containing a magnetic bar and placed in a container with ice to keep the mixture at 4 °C, and the absorbance measurements were recorded at the start and every 2 min while the reaction occurred; 12.5 ml of 50 mM Heps pH 7.4 µl, 150 µl of seed suspension, 400 µl of 100 mM hydroxyamine, and 2.2 ml of 0.8 mM HAuCl₄ (one drop every 8 s) were successively added.

**Auric seed and MBAuNP concentration analyses**

**Gold content in auric seeds and MBAuNPs**

To determine the amount of gold present in auric seeds and MBAuNPs, an aliquot from each sample was digested with HCl and HNO₃ in a water bath for 1 h. Inductively coupled plasma atomic emission spectroscopy (ICP-OES) of samples was performed in a Varian, model 730-ES instrument.

**Concentration of auric seeds**

The Au concentration obtained by ICP-OES was used to determine the number of auric seeds per liter, dividing the total number of gold atoms in the sample by the number of atoms per NP, with the following formulas:

\[ N_{at} = N_A \pi a^2 D^3 / 6M, \]

\[ N = A_i / N_{at}, \]

where \( N_{at} \) = Average number of Au atoms per nanoparticle, \( N_A \) (Avogadro’s number) = \( 6.022 \times 10^{23} \) atoms/mol, \( p \) (fcc density of the seeds) = \( 1.93 \times 10^{-20} \) g/nm³, \( D \) (diameter of the nanoparticles) = 18 nm, \( M \) (gold atomic weight) = 197 g/mol, and \( A_i \) (total number of atoms in the sample) = 1.834 \( 11 \times 10^{20} \) atoms/l, obtained from the ICP-OES analysis.

**Mathematical approach to estimate the surface area of MBAuNPs**

To determine the surface area of MBAuNPs, their shape was modeled as a “piñata,” i.e., a structure formed by a spherical core to whose surface are attached the bases of cones with blunt tips. The total surface area of each MBAuNP would be given by the following contributions: (i) the total area of the spherical core, minus (ii) the area of the sphere caps that occupy the N bases of the cones, plus (iii) the area of N cones, plus (iv) N times the area of the half-sphere that forms the tip of the cones. The terms appear in the same order in the following equation for the total area:

\[ A(1) = 4\pi a^2 - 2\pi a \sum_{i=1}^{N} \left( \sqrt{a^2 - R_i^2} \right) \]

\[ + \sum_{i=1}^{N} \left( \pi (R_i + R_i) \sqrt{ (R_i - r_i)^2 + h_i^2 + 2\pi r_i^2} \right), \]

where core average radius: \( a = 34 \) nm, average tip radius: \( r_i = 4.67 \) nm, average tip length: \( h_i = 29.16 \) nm, and average radius at the base of the tip: \( R_i = 15.1 \) nm.

The values assigned to these variables originate from the statistics of the SEM micrographs of our MBAuNPs. Figure 10 illustrates “piñata-like” NPs with 8 and 20 tips, where \( N \) is the number of peaks per NP. Considering that MBAuNPs have different numbers of tips, a first geometric approximation was used with \( N \) values of 8, 12, and 20.

**Hyperthermia assays with auric seeds or MBAuNPs in aqueous suspensions and phantom gels**

These assays were performed with MBAuNP aqueous suspensions in 500-µl microcentrifuge tubes or MBAuNP suspensions
in phantom gels, both of which were irradiated for 10 min with an 808 nm laser (Lasermate model IML-808) at different powers (0.45 W/cm², 0.75 W/cm², and 1 W/cm²). The temperature of each sample was measured with an infrared thermal camera (Flexcam R2 model IR) from time zero until the end of the irradiation period. To prepare phantom gels resembling human tissues, we prepared a suspension containing MBAuNPs (2 mg/l or 3 mg/l of Au), 100 ml of ultrapure water, 1.5 g of agar, and 2.5 g of TX-151, all added to a vacuum flask. The mixture was heated in a microwave oven for 15 s periods until complete agar and TX-151 dissolution was reached. To prepare phantom gels resembling human tissues, we prepared an MBAuNP suspension (containing 2 μg or 3 μg of gold, equivalent to 1.3 × 10⁹ or 1.9 × 10⁹ MBAuNPs per ml, respectively), 2 g of polyethylene powder was added. After polyethylene dissolution, air bubbles were eliminated by aspiration with a vacuum pump (Welch, Model 2522). The mixtures were poured onto 35 mm Petri dishes and left to solidify overnight at room temperature.

**Functionalization of MBAuNPs with BSA**

For functionalization, MBAuNPs (7.6 × 10¹¹ MBAuNP/ml) were washed five times by centrifugation with 0.1% Nonidet P40 aqueous solution. For the last wash, phosphate-buffered saline (PBS: 137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 18 mM KH₂PO₄, pH 7.4) was used, and the UV–VIS spectrum was recorded before and after functionalization. After the sixth wash, the supernatant was removed by aspiration, and the pellet was sonicated in an ultrasonic bath (Branson, Model 2800) for 4 min, and vortexed for 15 s; 1 ml of BSA (10 μg/μl) was added, and the mixture was vortexed immediately to prevent MBAuNP agglomeration. The MBAuNPs-BSA suspension was kept at 4°C for 24 h and then washed four times by centrifugation with PBS to eliminate the BSA in solution.

**BSA quantitation**

*Calculation using UV–VIS spectra*

Since the maximum absorption peak of BSA is at 280 nm ($A_{280}$), the BSA concentration in solution and MBAuNPs was determined by measuring $A_{280}$. The molar extinction coefficients of BSA in solution and MBAuNP-BSA suspensions were calculated by comparing the slopes of standard curves of BSA in solution and MBAuNP-BSA suspensions, using a 0.2 cm optical path quartz cell. To determine the average number of BSA molecules per MBAuNP, seven consecutive washes by centrifugation with PBS were carried out after 24 h of exposure to BSA, by dividing the number of MBAuNPs by the number of BSA molecules (estimated from $A_{280}$ and the molar extinction coefficient).

**Mathematical estimation of the number of BSA molecules per MBAuNP**

The mathematical approximation to calculate the number of BSA molecules per MBAuNP was performed by dividing the total available surface area of the MBAuNP (calculated as explained above) by the area of native BSA molecules. Each native BSA molecule has a tridimensional structure with an area of 28 nm² (Fig. 11).²⁴,⁴²

Assuming a bimolecular BSA layer, the total area of the protein corona on the surface of each MBAuNP-BSA would be

$$A(2) = A(1) + \sum_{i=1}^{N} \left\{ \pi (r_i^2 + R_i) \sqrt{(R_i - r_i)^2 + h_i^2} + 2\pi r_i^2 \right\}. \quad (4)$$

For a uniform 4-nm-thick protein hard corona, the following values were used: average tip radius: $r_i = 8.67$ nm, average tip length: $h_i = 33.16$ nm, and average radius of the tip base: $R_i = 18.1$ nm. The new total area available will then be divided by the area of native BSA molecules.

**FIG. 11.** Diagram of the native BSA tridimensional structure. The image shows the 3D structure of BSA as an equilateral triangular prism. Cysteine 34 may be involved in the binding of BSA to gold. Reproduced with permission from Yu et al., Nanoscale 8, 14393 (2016). Copyright 2016 Author(s), licensed under a Creative Commons Attribution 4.0 License.
Characterization of MBAuNPs and MBAuNPs-BSA

**Scanning electron microscopy (SEM)**

To a 1.5 ml microcentrifuge tube containing 500 μl of ethanol, 1 ml of the MBAuNP reaction mixture was added. The tube was immersed in an ultrasonic bath for 5 min, vortexed for 20 s, and centrifuged for 10 min at 13 000 rpm (15 304 × g) and 4°C. The supernatant was removed by careful aspiration, and 1 ml of ultrapure water was added to the pellet that was further washed by centrifugation six more times. On an aluminum sample holder (pin), 75 μl-samples were deposited and left to dry overnight before observing them in a scanning electron microscope (Dual Beam FEI-Helios Nanolab 600) at 150 000× magnification, 5 kV, 88 pA, and 4 mm working distance.

**Transmission electron microscopy (TEM, HRTEM)**

Pure and functionalized MBAuNP samples were stained with uranyl acetate in Petri dishes covered with Parafilm. On a grid (Lacey Carbon), a drop of a concentrated sample was applied and allowed to sediment for 2 h at room temperature. After adding uranyl acetate (10 μg/μl), the samples were left to dry for 15 min and then were observed with a JEOl 200 CX 100 keV transmission electron microscope (TEM) and a JEOl ARM200F high-resolution transmission electron microscope (HRTEM).

**X-ray photoelectron spectroscopy (XPS)**

One drop of either MBAuNPs or MBAuNPs-BSA suspension was placed on 1 × 1 cm² silicon plates. The plates were left to dry at room temperature for 24 h and analyzed in an ultra-high vacuum with an XPS PHI-5000 spectrometer (Physical Electronics, VersaProbe II Model), and the results were processed with the XPS Multipak software.

**Human serum**

Human blood obtained with informed consent from healthy donors following the Declaration of Helsinki was left to clot spontaneously. Clotted blood was centrifuged for 10 min at 13 000 rpm, and the supernatant serum was transferred to 15 ml plastic tubes. Pooled serum from four volunteers was stored at -80°C until it was used.

**Dynamic light scattering (DLS)**

The 2θ-potential and size of MBAuNPs and MBAuNPs-BSA were determined by DLS at 22°C and 90° dispersion angles with a zetameter (Zetantrac, Microtrac). 0.1% Nonidet P40, PBS, BSA dissolved in PBS, and human serum were used as vehicles to prepare MBAuNPs and MBAuNPs-BSA suspensions.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

The interaction of MBAuNPs with BSA and with human serum was assessed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) of suspension samples washed three times with centrifugation after a 24 h exposure at 37°C. 10-μl from each sample was mixed with 10 μl of Laemmli buffer (10% 2-mercaptoethanol, 0.004% Bromphenol blue, 20% glycerol, 4% sodium dodecyl sulfate, 125 mM Tris–HCl, pH 6.8), and the mixtures were boiled for 5 min at 100°C. 20-μl volumes were applied to 12% polyacrylamide slab gels, run for 2 h at 120 V, stained with Coomassie Blue for 30 min, and destained overnight in a 5:4:1 methanol–water–glacial acetic acid mixture.

**Circular dichroism (CD)**

To determine the proportion of α-helices from BSA both in solution and bound to MBAuNPs, circular dichroism (CD) measurements were performed at 4°C in a 0.2 cm quartz cell with a 205 nm–330 nm reading range in a MOS-500 spectropolarimeter (BioLogic Science Instruments). In mixtures with a 7.6 × 10^11 MBAuNPs/ml fixed concentration dissolved in PBS, the BSA concentrations tested were 1.0 mg/ml, 0.5 mg/ml, and 0.25 mg/ml. The proportion of α-helices from BSA in solution or bound to MBAuNPs was calculated with the following formulas:

\[
\text{MRE} = \frac{\text{Observed DC (mdeg)}}{10C_p\mu l},
\]

\[
\alpha - \text{Helix}(\%) = \frac{\text{MRE}_{4000} - 4000/33 000 - 4000 \times 100},
\]

where MRE = mean residual ellipticity, Observed DC (mdeg) = ellipticity obtained directly from DC, C_p = molarity of the protein, n = number of amino acid residues in BSA, and l = path length of the cell in centimeters.

The 4000 and 33 000 values denote the total ellipticity values of the β-sheet form and the pure α-helix form of the protein at 208 nm and 222 nm.

**CONCLUSIONS**

From spherical Au seeds (DH = 18 nm, surface = 1020 nm², \(A_{\text{max}}\) at 520 nm, \(\zeta\)-potential = -36.8 mV, and PDI = 0.602), piñata-like MBAuNPs (8–20 peaks, \(D_H\) = 125 nm, \(A_{\text{max}}\) at 775 nm, \(\zeta\)-potential = -24.4 mV, and PDI = 0.455) are stoichiometrically synthesized. The surface area of MBAuNPs ranges from 24 984 nm² to 40 669 nm². MBAuNPs are potentially useful for photothermal tumor ablation since they induce hyperthermia of more than 4°C in phantom gels irradiated with an 808 nm laser source with a power of 0.75 W/cm². MBAuNPs exposed to BSA acquire a protein corona with an internal "hard" portion composed by one or two layers of BSA molecules covalently bound to the surface of each nanoparticles (1500 molecules per MBAuNP), and an external "soft" portion formed by non-covalent bound agglomerated BSA molecules. BSA functionalization decreases the tendency of MBAuNPs to aggregate but increases the polydispersity index, indicative of greater size dispersion. MBAuNPs and MBAuNPs-BSA exposed to human serum bind HSA and other human serum proteins ranging from 25 kDa to 180 kDa, which decrease their stability and increase their DH, especially in MBAuNPs-BSA.

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