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Gold nanoparticles functionalization notably decreases radiosensitization through hydroxyl radical production under ionizing radiation

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ABSTRACT

The purpose of this work was to study the influence of gold nanoparticles (GNP) coating on hydroxyl radical ($HO^{\bullet}$) production under ionizing radiation. Though radiosensitizing mechanisms are still unknown, radical oxygen species are likely to be involved, especially $HO^{\bullet}$. We synthesized six different types of GNP, choosing relevant ligands such as polyethylene glycol or human serum albumin. A great attention was paid to characterize these GNP in terms of size, charge and number of atoms in the coating. Our results show that functionalization dramatically decreases $HO^{\bullet}$ production, which is correlated to reduced plasmidic DNA damages. These findings are of high importance as GNP translation from fundamental research to applied medicine requires their functionalization to increase blood circulation time and specific cancerous cells addressing. We suggest that to keep GNP efficient for radiotherapy, a wispy coating is required.

1. Introduction

Cancer is one of the leading causes of mortality with an estimated 14.1 million cancer cases around the world in 2012, number expected to increase to 24 million by 2035 according to the World Cancer Research Fund International. Radiotherapy is a major treatment as for the most prevalent cancers among males and females, namely prostate and breast, 35% and 50% of patients undergo radiotherapy [1]. Still, it is limited by potential radiotoxicity to essential normal tissues in the path of the radiation beam. In the search of new concepts to achieve better treatment outcomes, radiosensitizers, that could increase the dose specifically to the tumor cells, constitute a promising strategy: chemical agents were first developed [2,3] but interest for nanomaterials is now rising [4]. Hainfeld et al. pioneered the use of gold nanoparticles (GNP) as radiosensitizer in a mice model [5]. Since then, studies on animals [5,6], cells [4] and biomolecules [7–9] have confirmed this effect with sometimes contradictory results. Nevertheless, nanoparticle (NP) biological applications cannot be considered unless they have a sufficient blood circulation time which demands functionalization [10]. Targeting cancerous cells also requires the modification of NP surface [11,12].

In an attempt to propose the most efficient nanoparticle, we and others got interested in the mechanisms of GNP radiosensitization [7,13,14]. As hydroxyl radicals ($HO^{\bullet}$) were proposed to play a key role in this process [9], we recently developed a 'reference' protocol to quantify these radicals in the presence of GNP [15]. Given the high amount of $HO^{\bullet}$ measured with bare GNP, the aim of this work was to evaluate how their coating could impact this production and to correlate it with the radiosensitization of a relevant biological molecule, DNA. We synthesized and thoroughly characterized different types of GNP and compared their capacity to produce $HO^{\bullet}$, which should help us in designing the most efficient GNP for radiotherapy applications.

2. Materials and methods

2.1. GNP synthesis and functionalization

Whatever the following functionalization, GNP were prepared through the Turkevich procedure [16]. In a typical process, 4.6 mL of tri-sodium citrate (1% w/v) were added to 100 mL of an aqueous solution of $KAuCl_4$ (10^{-3}M) and heated under stirring until the red color apparition. Non-functionalized GNP were washed with water as in Brun et al. [7] by three centrifugation cycles of 3000 × *g*

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for respectively 20, 18 and 15 min in order to remove most of free citrate.

2.2. GNP@PEG$_{1000}$ and GNP@PEG$_{4000}$

GNP were first PEGylated with non thiolated-PEG. Pellets of concentrated non-functionalized GNP were diluted in a 1 mM solution of PEG (MW = 1000 g mol^{-1} or 4000 g mol^{-1}, from Sigma–Aldrich and Fluka respectively) with molar PEG:GNP ratio of *ca.* 40,000. 3000 × *g* centrifugation for 15 min was repeated twice in order to remove the excess of PEG, then a final spin cycle was performed after addition of water.

2.3. GNP@S-PEG$_{3500}$-NH$_2$ and GNP@S-PEG$_{3500}$-COOH

Two thiolated PEG of 3500 g mol^{-1} (Interchim) with different charges, as they ended with NH_2 or COOH, were used to functionalize GNP. PEGylation was performed as in Xia et al. [17] by incubating overnight GNP with thiolated PEG solutions of 0.25 mM corresponding to a final concentration of thiolated PEG of 50 μM and a molar PEG:GNP ratio of *ca.* 10,000. Then three centrifugations at 18,000 × *g* for 7 min were performed to remove the excess of reactants.

2.4. GNP@HSA

In a similar manner, GNP covered with albumin were prepared from pellets of concentrated GNP resuspended in 0.5 μM aqueous solution of HSA (corresponding to a ratio HSA: GNP of 5000:1) and centrifuged to remove the excess of protein.

2.5. GNP Characterization

2.5.1. TEM analysis

1 μL of GNP stock solution was deposited onto a formvar/carbon coated copper grid (400 meshes) for 1 min and imaged with a JEOL 100CXII TEM instrument (JEOL, Tokyo, Japan) operating at 120 keV. GNP size was determined from recorded images with ImageJ 1.41 software (at least 300 counted particles) [18].

2.5.2. UV–Visible spectroscopy

Absorption of GNP solutions was measured from 400 to 850 nm in 500 μL disposable polystyrene micro cuvettes (Brand) using a Thermo Electron corporation Evolution 500 spectrometer, absorbance value providing GNP concentration and wavelength plasmon resonance an estimation of GNP size [19]. In addition, plasmon resonance main peak revealed the absence of any GNP aggregation with time.

2.5.3. Dynamic light scattering (DLS)

DLS measurements were performed on a Malvern instrument ZetaSizer ZEN3600 equipped with a 633 nm laser at 25 °C on 0.8 nM GNP solutions in 100 μL disposable micro cuvettes (UV cuvette, Brand) at a fixed lens position of 4.65 mm.

2.5.4. Fourier transform infrared (FTIR) spectroscopy

FTIR Spectroscopy was performed on a Bruker FTIR Vertex 70 equipped with an ATR (attenuated total reflectance) accessory. Spectra were collected by averaging 128 scans with a resolution of 4 cm^{-1}. GNP samples were deposited on the ATR and dried before recording spectra.

2.5.5. Zeta potential determination

All zeta potential measurements were performed on a Malvern instrument ZetaSizer ZEN3600 in 1 mL folded capillary cells on 1 nM GNP samples with a fixed voltage at 150 mV at 25 °C.

2.5.6. X-Ray irradiation and dosimetry

X-rays of 17.5 keV effective energy were generated with a Diffractis 583 X-ray generator (Enraf Nonius, Mo cathod). Coumarin irradiations were performed at dose rates of 20 and 3.4 Gy min^{-1}. All samples were irradiated less than 40 s to free us from the fluorescence quenching phenomenon of 7-hydroxycoumarin due to the presence of GNP [15]. DNA irradiations were performed at a dose rate of 3.4 Gy min^{-1}.

In order to quantify exactly the irradiation dose, Fricke dosimetry was performed [20]. In addition, dose rates were verified for each experiment with Gafchromic® film HB-810 analyzed as in Brun et al. [21].

2.5.7. Hydroxyl radicals quantification

Hydroxyl radicals were quantified by measuring coumarin hydroxylation. 0.5 mM coumarin solution was irradiated in the presence of NP in a concentration range from 0 to 1 nM. Quantification of 7-hydroxycoumarin fluorescence was performed on a Synergy H1 microplate reader at 25 °C. Excitation was set at 326 nm and emission maximum detected at 450 nm. Before any analysis, NP were removed from the samples by addition of 10% (w/v) NaCl solution to induce GNP aggregation and by centrifugation to remove gold aggregates.

2.5.8. DNA preparation and irradiation damage quantification

pBlueScript (2958 base pairs) plasmid was extracted from *E. coli* and purified with the QIAfilter Plasmid Giga Kit (Qiagen). Any protein contamination was excluded by comparing DNA absorption at 260 and 280 nm; a ratio of 1.98 indicated a good DNA purity. DNA concentrations were determined by measuring its absorbance at 260 nm, using a molar absorption coefficient of 5.3×10^7 L mol^{-1} cm^{-1} at pH 7. Stock water solutions of DNA and of concentrated GNP were mixed just before X-rays exposure, resulting in solutions with final concentrations of 1 nM for GNP and 10 nM for DNA. DNA damages were quantified by agarose gel electrophoresis stained with SYBR® Gold (Invitrogen). The gels were imaged with a Syngene Bio-imaging system. Resulting images were analyzed using ImageJ software to determine the relative amounts of supercoiled, circular and linear DNA forms [18].

2.5.9. Determination of gold content in GNP

Gold content was determined based on a spectrophotometric assay, after coordination of gold ions by rhodamine B [22]. For each NP type, digestion was performed on three different GNP volumes in triplicate to ensure that gold atoms oxidation by bromide was complete.

The expected number of gold atoms (N_{exp}) in a NP solution is determined by Eq. (1):

$$N_{exp}[GNP] \times N_A \times 59 \times \frac{4}{3}\pi r^3 \times V_{GNP} \quad (1)$$

With [GNP] the GNP concentration in mol L^{-1}, N_A the Avogadro number (6.02×10^{23} mol^{-1}), 59 the number of gold atoms per nm^3 [23], r the GNP average radius determined by TEM in nm and V_{GNP} the volume of concentrated GNP used for the assay in L. Assuming 59 atoms per nm^3, 1 nM of 32 nm GNP corresponds to 1.05 mM gold concentration or 2.1×10^{-2} wt%.