Environmental compatibility bioconjugated gold nanoparticles as efficient contrast agents for colorectal cancer cell imaging

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Abstract

In this study we show, for the first time, that gold nanoparticles (AuNPs) synthesized by a simple, inexpensive, and environmentally-correct method can be easily conjugated with the antibodies anti-β-catenin and anti-E-cadherin to specifically target colorectal carcinoma cells. The antibody/AuNPs conjugates were then successfully applied for imaging cancerous cells with fluorescence confocal microscopy. The AuNPs as well as the conjugates were very stable in high-salinity medium, a pre-requisite for application in physiological-like environments. Fluorescence results suggest that conjugation was achieved by direct adsorption of antibodies on the AuNPs surface. Finally, compared with a standard method of cell staining, our method is less laborious and the preparation time (from immobilization of cells onto glass cover slips until observation by confocal microscopy) decreased from 27 h to about 1 h, which makes the method eligible for colorectal cancer diagnostic.

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1. Introduction

Current cancer diagnostic methodologies include optical imaging techniques such as coherence tomography and fluorescence confocal microscopy. The image is generated by the backscattered light from the endogenous chromophores present in the tissue. The weak optical signal results in poor intracellular contrast and subtle spectral differences between malignant and benign cells hamper a proper diagnostic [1]. In order to overcome these problems, exogenous chemicals (mainly organic dyes) that function as optical contrast agent have been used to enhance the visibility of both precancerous and cancerous cells. However, organic dyes are subjected to rapid photobleaching [2], therefore becoming inefficient as the analysis proceeds. Other disadvantages of conventional dyes include poor hydrophilicity, low quantum yield, and low detection sensitivity in biological environment [2].

Recent advances of nanotechnology have allowed the development of new functional nanomaterials with enhanced specificity for molecular imaging. In particular, colloidal Au nanoparticles (AuNPs) are extremely advantageous as contrast agents in biomedicine [1,3–6] as they can be easily combined with a recognition agent. First, AuNPs present a size-tunable surface plasmon resonance (SPR) that leads to strong absorption and scattering in the visible-to-near-infrared region, a characteristic which makes them considerably superior to the conventional dyes applied in biomedical imaging. For instance, the plasmon resonance absorption of spherical gold nanoparticles has an absorption coefficient orders of magnitude larger than those of regular dyes. As an example the absorption coefficient (ε) for 40-nm gold nanoparticles [7] at a wavelength of 530 nm was calculated to be ~7.7 × 10² M⁻¹ cm⁻¹, while the ε of rhodamine 6G is 1.16 × 10⁵ M⁻¹ cm⁻¹ [8]. Second, AuNPs do not suffer photobleaching and seem to be biocompatible [9]. Third, the chemistry of gold is quite rich. The AuNPs surface binds strongly with amines, thiols and disulfides, allowing the surface bioconjugation with a great variety of peptides, DNA and proteins, enabling selective targeting of cancer cells. Furthermore, in opposition to dyes and organic molecules, several functional
groups can be attached onto the same nanoparticle, making them also eligible for drug delivery [10].

In this paper we synthesized AuNPs bioconjugated with β-catenin antibody (anti-β-catenin) and E-cadherin antibody (anti-E-cadherin) and applied them as novel contrast agents for the detection of colorectal cancerous cells with fluorescence confocal microscopy. E-cadherin is localized on the surface of epithelial cells in regions of cell–cell contact known as adherens junctions and is implicated in cell–cell adhesion in epithelial tissues [11–13]. β-Catenin interacts with cadherins through its cytoplasmic domain. α-catenin connects the E-cadherin and β-catenin complex to actin filaments. The dissociation of E-cadherin-catenin complex from cell membrane is important in malignant progression. In many epithelial cancers, membranous E-cadherin is lost and β-catenin dissociates in the cytoplasm and accumulates in the nucleus as a transcription factor, concomitantly with tumor progression [12]. Down-regulation of membranous E-cadherin and β-catenin, and cytoplasmic/nuclear accumulation of β-catenin have been previously reported in several cancers and hold promise as prognostic markers [14]. In humans β-catenin and E-cadherin are encoded by the adenomatous polyposis coli (APC) and CDH1 genes, respectively, and their mutation may result in colorectal cancer leading to an overexpression of β-catenin and E-cadherin by the cell [11–16]. The strategy was therefore to combine the specificities of anti-β-catenin and anti-E-cadherin with the fluorescent properties of the AuNPs to produce a selective contrast agent for targeting cancer. In this system, the AuNPs function as the signaling part and the anti-β-catenin and anti-E-cadherin act as recognition units for specific binding with the overexpressed β-catenin and E-cadherin in the cell. The confocal microscopy revealed that the antibody-conjugated AuNPs were very efficient in marking cancerous cells. On the other hand, no specific interaction between normal cells and conjugated AuNPs was observed. Another interesting feature of the present work is the use of an environmentally friendly route to produce the AuNPs based on the reduction of gold ions by glycerol in alkaline medium. Glycerol’s non-toxicity and biodegradability make it an excellent alternative to commonly used reducing agents. The conjugations with anti-β-catenin and anti-E-cadherin are achieved by simple incubation after reducing the pH of the nanoparticle colloidal solution to around 7. In comparison to current methods for production and conjugation of AuNPs, our method is quite simple, low-cost and environmentally compatible, with a potential to be routinely applied in cancer diagnostics.

2. Experimental

2.1. Chemicals and reagents

Gold trichloride (30 wt% in HCl), polyvinylpyrrolidone (PVP, \(M_W = 10.000\)), sodium hydroxide, dialysis tubing cellulose membrane, and glycerol were products of Sigma-Aldrich Chemical Co. Sulphuric acid and hydrogen peroxide were purchased from Vetec. Phosphate-buffered saline (PBS) solution and bovine serum albumine (BSA, 5%) were purchased from Life Technologies Corporation©. β-Catenin antibodies (anti-β-catenin) and E-cadherin antibodies (anti-E-cadherin) were acquired from ABCAM© at the concentration of 200 μg L\(^{-1}\) in PBS buffer. Dulbecco’s modified Eagle’s medium (DMEM) and heat-inactivated bovine serum were purchased from Life Technologies Corporation© and Cultilab Ltda/Brasil, respectively.

2.2. Production and characterization of AuNPs

AuNPs were produced using a previously reported method [17]. Briefly, all glassware was kept overnight in \(\text{KMnO}_4 + \text{NaOH}\) solution, rinsed with deionized water, kept for 10 min in \(\text{H}_2\text{O}_2 + \text{H}_2\text{SO}_4\) solution (1:1 v/v), again rinsed with deionized water and dried prior to use. Known amounts of PVP (\(M_W = 10.000\)) and gold chloride were dissolved in 10 mL of water. In a separate beaker, determined quantities of glycerol and NaOH were dissolved in 10 mL of water. The glycerol–NaOH solution was then added to the \(\text{AuCl}_3\)-PVP solution to yield the following final concentrations: 1.0 mM Au\(^{3+}\), 0.10 M NaOH, 0.10 M glycerol and 10 g L\(^{-1}\) PVP. The final mixture had a deep-red color due to the formed AuNPs. The AuNPs colloidal

![Fig. 1. (A) UV–vis spectra of the colloidal AuNPs, (B) high-resolution TEM images of a AuNP and (C) size distribution of the AuNPs.](image-url)
solution was allowed to rest for 1 week to guarantee reaction completion. Considering a quantitative transformation of gold ions into nanoparticles, the concentration of AuNPs was estimated to be 197 μg mL⁻¹. The whole procedure for estimating the nanoparticle concentration can be found in [18]. The AuNPs colloidal solution had then its pH adjusted to 7 by addition of diluted HCl. Afterwards, the AuNPs colloidal solution was subjected to dialysis for purification and to remove extra free small molecules. The dialysis process is as follows: the outside and inside of the dialysis bag were washed with deionized water and then placed into a beaker with pure water to boil for 5 min. The bag was then washed with water, filled with the AuNPs colloidal solution and allowed to boil in a large beaker for 6 h. During the boiling water was changed three times.

UV–vis absorption spectra of the AuNPs were acquired with an Evolution 60S UV–visible spectrophotometer (Thermo Scientific) spectrophotometer. Fluorescence spectroscopy was carried out with a Perkin Elmer LS45 fluorometer. Transmission electron microscopy (TEM) images were acquired with a JEOL 2100F microscope operating at 200 kV.

2.3. Conjugation of antibodies with AuNPs

Conjugation of AuNPs with anti-β-catenin and anti-E-cadherin was achieved by simple incubation. Both antibodies were acquired at the concentration of 200 μg mL⁻¹ in PBS buffer. PBS and BSA were used as medium to yield the following solutions of conjugates: (1) 3 μL antibody + 1000 μL PBS/BSA + 500 μL AuNPs; (2) 3 μL antibody + 750 μL PBS/BSA + 750 μL AuNPs; (3) 3 μL antibody + 500 μL PBS/BSA + 1000 μL AuNPs. The conjugate solutions were allowed to rest for 1 h prior incubation with cells. The conjugates (1), (2), and (3) had antibody/AuNPs ratios of 6.1 × 10⁻³, 4.1 × 10⁻³ and 3.0 × 10⁻³, respectively.

2.4. Cell culture

Non-cancerous cells (HKE) and cancer cells HT29 (colorectal adenocarcinoma) were used as a model for cell imaging. Both types of cells were purchased from the Culture Collection of the Bio-science Centre, Federal University of Rio Grande do Norte. HT29 cells are known to overexpress E-cadherin and catenin. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% v/v heat-inactivate fetal bovine serum. Cultured cells were then plated onto glass coverslips in 24 well plates (5 × 10⁶ cells/well) and allowed to grow for 24 h. Afterwards, the cells were washed, fixed with paraformaldehyde, permeabilized with Triton-X and incubated with 100 μL of either the catenin–AuNPs conjugate or catenin–AuNPs conjugate for one hour in a humid atmosphere at room temperature. Control experiments were performed under the same conditions but without addition of the conjugates. Instead, cells were incubated with Alexa Fluor® 488 goat anti-rabbit secondary antibody (Abcam) for 2 h. The glass coverslips were then directly observed with the confocal microscope.

2.5. Confocal microscopy

Healthy and cancerous cells were examined under a LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a condenser using laser excitation from 442 to 520 nm. In this technique the specimen is pointed-illuminated and the light produced by fluorescence very close to the focal plane is detected. In control experiments, cell images were acquired in the

![Fig. 2. Normalized UV–vis spectra of AuNPs in different conditions distinguished with differently-colored curves shown in the legends. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)

![Fig. 3. Fluorescence emission spectra of AuNPs excited at 330 nm in the presence of (a) β-catenin and (b) E-cadherin. Excitation and emission slits were 10 nm. The concentrations of both antibodies are: 2.0 μg mL⁻¹ (red curve), 3.0 μg mL⁻¹ (green curve) and 5.0 μg mL⁻¹ (blue curve). The concentration of AuNPs was held constant at 3.94 μg mL⁻¹. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
same conditions using Alexa Fluor® 488 goat anti-rabbit secondary antibody instead the antibody–AuNPs conjugates.

3. Results and discussion

The AuNPs used in this work were synthesized by reducing Au$^{3+}$ with glycerol in alkaline medium. Glycerol is an inexpensive chemical and readily biodegradable under aerobic conditions, therefore a greener option when compared to current reducing chemicals such as formamide, sodium borohydride and hydrazine. Sodium citrate [19] is efficient in generating gold nanoparticles, however they are formed only upon heating the solution (80–100°C). Fig. 1A and B shows a UV–vis spectrum and a high-resolution TEM image of as-prepared AuNPs produced by simple addition of NaOH-glycerol to AuCl$_3$-PVP at room temperature. The colloidal AuNPs spectrum had a maximum absorbance ($\lambda_{\text{Max}}$) at 520 nm, a value typical for spherical gold nanoparticles [20–22]. The symmetry of the band implies a fair similarity in the shape of the nanoparticles and low degree of aggregation in the solution [23]. The HR-TEM image illustrated that the as-prepared AuNPs were spherical in shape, thus corroborating the UV–vis results. The mean particle size calculated from the distribution of Fig. 1C was 6.8 ± 1.6 nm.

Fig. 4. Confocal images of healthy cells acquired at an excitation laser of 520 nm. Left column: images acquired with the conjugate of β-catenin. The conjugates had β-catenin/AuNPs ratios of (A1) $6.1 \times 10^{-3}$, (A2) $4.1 \times 10^{-3}$ and (A3) $3.0 \times 10^{-3}$. Right column: images acquired with the conjugate of E-cadherin. The conjugates had E-cadherin/AuNPs ratios of (B1) $6.1 \times 10^{-3}$, (B2) $4.1 \times 10^{-3}$ and (B3) $3.0 \times 10^{-3}$. Magnification: 10×. Scale bar = 100 μm.
β-catenin [24] and E-cadherin [25] are proteins playing important roles as cell–cell receptors, forming adherens junctions to bind cells within tissues together. While normal cells do not overexpress β-catenin and E-cadherin, enlarged amount of these proteins indicates cancer cell aggressiveness [13]. Due to their proteic nature, β-catenin and E-cadherin might undergo denaturation when subjected to pH other than the physiological one. Thus, before bioconjugation, the pH of the AuNPs colloidal solution had to be adjusted to near 7.0 and tested against aggregation under different conditions. Results of this study are shown in Fig. 2. Results revealed that the AuNPs are extremely stable in aqueous solution at different pH and salinity. The spectra of the as-prepared and pH-adjusted AuNPs are practically overlapped, which denotes excellent stability under different pH. Small molecules such as glycerol’s oxidation
products were then removed via dialysis and this process had no influence of the shape of the spectra. The AuNPs were then 1:3 v/v diluted in the PBS/BSA buffer. Under this high salinity AuNPs are expected to be unstable, however in our case the steric hindrance provided by PVP resulted in increased stability. Upon introduction of the antibodies the spectra of AuNPs had no significant change, implying that the bioconjugation did not provoke obvious aggregation of AuNPs.

Fig. 3 shows fluorescence emission spectra for purified AuNPs in absence and presence of β-catenin (Fig. 3A) and E-cadherin (Fig. 3B). The excitation wavelength was fixed at 330 nm and the emission recorded in the range of 700–900 nm. The emission band at that range did not shift with the excitation wavelength (data not shown), indicating that the band was not due to any scattering process. The fluorescence emissions were slightly enhanced upon absorption of both β-catenin (increase of 30 a.u. for the highest antibody concentration) and E-cadherin (increase of 39 a.u. for the highest antibody concentration) onto the AuNPs. Such enhancement has been rationalized in terms of competition between adsorbing species and molecular oxygen dissolved in solution. Alqudami and Annapoorni [26] showed that fluorescence enhancement takes place when BSA adsorbs on the surface of silver nanoparticles. As the surface plasmon resonance (SPR) (responsible for the fluorescence signal) is a function of the surface free electrons, more free electrons led to a more intense SPR signal. The authors argued that the adsorbed BSA prevented the surface free electrons from binding with oxygen molecules, hence the fluorescence signal was somewhat increased. In our case β-catenin and E-cadherin may have adsorbed on AuNPs impeding O2 to reach the nanoparticle surface, an explanation supported by Fig. 3, where a fluorescence signal increment occurred only for the lowest antibody concentration, remaining constant at the higher ones. This means that a small antibody concentration is sufficient to insulate the AuNPs from oxygen.

The antibody–AuNPs conjugates were then applied as labeling probes for cell imaging. In the experiments we first produced the conjugates as explained in Section 2 and then inoculated them into healthy and cancerous cells. The strategy to combine the specificities of anti-β-catenin and anti-E-cadherin with the fluorescent properties of the AuNPs to produce a selective contrast agent for targeting cancer was proven efficient as shown below. Fig. 4 shows confocal microscopy images of healthy cells in contact with AuNPs conjugated with different concentrations of antibodies. One can see that the nanoparticles are inhomogeneously spread over the cover slab surface regardless of the antibody/AuNPs ratio in the conjugates. The lack of specific binding of the conjugates with the cells is a consequence of normal levels of β-catenin and E-cadherin produced by non-cancerous cells. We should mention that during the confocal microscopy experiments with non-cancerous cells it was extremely difficult finding a region with good fluorescence intensity. As there are no specific interactions with the cells, the nanoparticles are spread over the glass coverslips and the high-local fluorescence intensities are due to non-specific AuNPs agglomeration.

Fig. 5 presents images of cancerous cells labeled with the conjugates. These images are clearly distinguished from those of Fig. 4. In this case the antibody–AuNPs conjugates could specifically bind on the cell surfaces due to the overexpressed β-catenin and E-cadherin. Even nuances of the cell membrane could be nicely revealed, as for example in Fig. 5A2, where a group of cells was
spotted. Note that the gold nanoparticles are no longer spread around the cells, but rather concentrated on them. It is interesting to notice that in Fig. 5A1 the boarders of the cells are brighter than the centers. Since β-catenin and E-cadherin are mainly membrane proteins, their overexpression led to an increased concentration of the conjugates in that area. Another important feature is that even the conjugates with the lowest antibody/AuNPs ratio (Fig. 5A3 and B3) were able to specifically bind with the cells, which has a positive implication on the cost as antibodies are not inexpensive.

Finally, we compare the herein proposed method with a standard one for labeling cancerous cells. The standard method employed consisted of inoculating the Alexa Fluor® 488 – which is an antibody bound to a dye – with cancerous cells for subsequent cell imaging. Fig. 6A shows results for this study, in which the cells in all images were stained with both β-catenin–AuNPs conjugate and Alexa Fluor® 488. One can see that both labeling agents reveal the same sites, which is a further proof of the specificity of our conjugate. Furthermore, the signal intensities of the β-catenin–AuNPs and Alexa were not that different, with that for the Alexa being slightly more intense. The right picture is a signal overlap from β-catenin–AuNPs and Alexa. The brighter areas are the intensity combinations of both labeling agents, showing that the β-catenin–AuNPs conjugate indeed binds cancerous cells as both signaling agents populate the same areas. Fig. 6B presents the same comparison but now with the E-cadherin–AuNPs conjugate. Unlike with the β-catenin–AuNPs, the signal intensities of E-cadherin–AuNPs and Alexa were practically the same, which suggests that the cancerous cells expressed more E-cadherin than β-catenin, existing therefore sufficient of sites for equal adsorption of E-cadherin–AuNPs and Alexa. From these results one concludes that the antibody/AuNPs could efficiently replace the commonly used secondary antibody for identification of colorectal cancerous cells.

Another point worth to mention is the reduced overall preparation time with the antibody–AuNPs conjugates, as presented in Scheme 1. The standard procedure requires incubation of primary antibody (β-catenin or E-cadherin) with Alexa Fluor® 488 for 24 h, followed by washing and fixation steps prior observation under the microscope. The entire procedure takes about 27 h. On the other hand, our procedure requires only a 1-h incubation followed by a single 10-min rinsing step, which is a clear advantage in terms of labor and time-saving.

4. Conclusions

Anti-β-catenin/AuNPs and anti-E-cadherin/AuNPs conjugates were successfully applied for imaging colorectal cancerous cells. UV–vis results showed that the conjugates are stable in solutions that resemble the physiological medium. Based on fluorescence results, low antibody concentrations are sufficient to achieve maximum adsorption of antibodies onto the surface of the AuNPs. Furthermore, the herein proposed conjugates had similar performance compared with a standard procedure, with the advantage of having reduced the overall analysis time from 27 h to only 1 h. Based on these results, we envisage the application of the conjugates for routine imaging of colorectal cancerous cells.

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References

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Currently Luiz Henrique da Silva Gasparotto holds a position of assistant professor at University of Rio Grande do Norte, Brazil. He obtained his Ph.D. in Sciences in 2009, followed by a post-doc stage at TU Clausthal, Germany, under the supervision of Prof. Frank Endres. Gasparotto’s interests comprise the synthesis and application of nanomaterials obtained through chemical and electrochemical routes.