

Antibody–gold quantum dot–PAMAM dendrimer complex as an immunoglobulin immunoassay†

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Gold quantum dots (AuQDs) were synthesized and electrostatically conjugated to goat-derived anti-human IgG for the purpose of detecting human IgG in solution over a broad range of concentrations. The system is able to detect human IgG by linear fluorescence quenching over a micromolar to nanomolar concentration range. We have demonstrated the specificity and a wide dynamic range of the proposed immunoassay. The quenching is a result of competitive surface quenching of the AuQDs. Characterization, details of the immunoassay, and the quenching mechanism, are discussed.

Introduction

During the last decade, fluorescence-based assays are becoming the premier choice for immunochemistry-based *in vitro* diagnostic (IVD) tests and *in vivo* imaging assays. When compared to chromophores and radio chemistry labels, fluorescent labels, *i.e.* fluorophores, have several advantages such as higher sensitivity, better dynamic range, and potential for easy multiplexing.^{1–4} Of the available fluorophores, it is expected that semiconductor quantum dots (QDs) will become common replacements for traditional organic or organometallic fluorophores due to their optical characteristics including narrow bandwidth and size-dependent controllable emission spectra, high extinction coefficient, broad excitation, and, most importantly, virtually an infinite stability against photobleaching.^{5,6} Owing to all of the above, QDs allow for greater sensitivity compared with organic fluorophores, which in turn lowers the detection limit, leading in the near future to the ultimate goal of reaching reliable single cellular and single molecular diagnostics.^{7–13} However, to ensure solubility and stability, a thin shell or capping layer is needed and must be added through a separate chemical step in the synthesis of semiconductor-based quantum dots based on elements from periods III and V, such as CdSe. While various capping strategies have been used to minimize potential toxicity, the safe use of III–V element-based semiconductor quantum dots in living systems is still questionable. Recently, small, metallic, water-soluble,

gold quantum dots (AuQDs)^{14,15} have been synthesized using poly(amidoamine) (PAMAM) dendrimers as capping ligands through a simple one-step synthetic procedure. These AuQDs exhibit size-dependent narrow emission, narrow excitation, and it is expected that they will have an excellent biocompatibility.

While gold has been used for decades in biological applications such as staining and contrast agents,¹⁶ the luminescent properties and applications of gold nanoclusters which exhibit quantum confinement has not been thoroughly investigated and few references of gold-based QDs exist in the literature,^{17–19} although there is a large potential for AuQDs to replace, or more likely augment, semiconductor QDs.

AuQDs synthesized with PAMAM dendrimer capping provide an easily modifiable system that is well suited for biological applications: the dendrimers may be covalently or electrostatically conjugated to DNA,^{20,21} RNA,²² carbohydrates,²³ and proteins^{24,25} since the end groups of the PAMAM dendrimer most typically consist of (but are, however, not limited to), hydroxyl or amine groups. PAMAM dendrimers have also proven to be excellent cell transfection agents.^{26–30} The novel properties arising from such a well-studied biologically inert material as gold, along with the biological compatibility, the ability to penetrate cell membranes and the plurality of conjugation strategies of the PAMAM dendrimer provide the basis of a system that could lead to many life sciences applications, both *in vitro* and *in vivo*.

In this study, as a proof of concept of the use of gold quantum dots in the construction of an immunoassay, a human IgG immunoassay has been formed with PAMAM-encapsulated AuQDs electrostatically conjugated to goat-derived anti-human IgG polyclonal antibody, as is schematically illustrated in Fig. 1. As a complex of the AuQD–PAMAM dendrimer is only electrostatically bonded to an antibody ligand it was necessary to test the assay selectivity against other antigens, and to establish the influence, if any, of non-specific protein binding on the assay performance. In order to test the assay selectivity and non-specific binding interference, the assay has been tested on

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† The HTML version of this article has been enhanced with colour images.

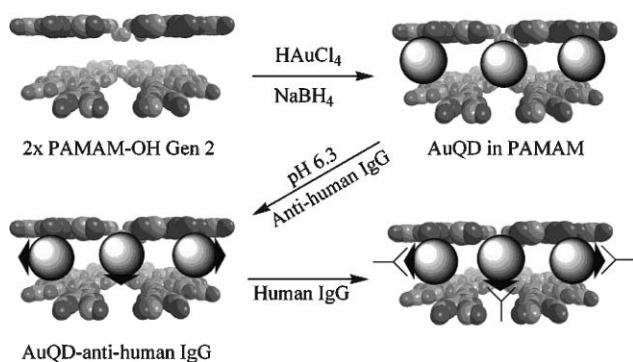


Fig. 1 Schematic representation of PAMAM-OH generation 2 dendrimer, AuQD–PAMAM complex, AuQD–PAMAM–anti-human IgG (▲) complex, and formation of the quenching complex upon addition of the human IgG (Y).

samples of known concentrations of human IgG and the results compared with results obtained when tested with rabbit IgG, and also with completely unrelated proteins, bovine serum albumin (BSA) and β -casein. Herein we have demonstrated that the system provides a linear luminescence quenching response over a micromolar to nanomolar human IgG concentration range with little to no interference from BSA or β -casein, and a slight interference from rabbit IgG, which is explainable in the terms of the specificity of the anti-human IgG antibody used.

Experimental

Synthesis and conjugation of gold QDs

Gold QDs with an emission maximum at 450 nm ($\lambda_{\text{exc}} = 387$ nm) were synthesized according to a method previously reported in the literature:^{14,15} in brief, 0.5 μmol G2-OH (Sigma-Aldrich, St. Louis, MO) and 1.5 μmol $\text{HAuCl}_4 \cdot n\text{H}_2\text{O}$ (MP Biomedicals, Solon, OH) were co-dissolved in 2 mL of distilled water (resistivity 18 $\text{M}\Omega\cdot\text{cm}$ and surface tension of 72.6 mN m^{-1} at 20.0 $^\circ\text{C}$; Continental Modulab 2020, San Antonio, TX). The gold ions were sequestered within the dendrimer prior to the slow addition of one equivalent of the reductant NaBH_4 (MP Biomedicals, Solon, OH) by allowing 30 min of stirring. The solution was stirred for 2 days so that reaction and aggregation processes could complete. Purification of the resulting solution was performed through ultracentrifugation at 16 000 g for 45 min in the L7 Ultracentrifuge (Beckmann-Coulter, Fullerton, CA) resulting in size separation of the larger red fraction of Au nano- and micro-particles from the smaller AuQDs, leaving behind a transparent solution of AuQDs. Then, the AuQDs were suspended in 2 mL phosphate buffer saline solution (PBS, pH 6.3) and mixed with 1 mg of polyclonal antibody specific to the human IgG (goat affinity purified antibody to human IgG; MP Biomedicals, Solon, OH). The antibody–dendrimer–AuQD complex was electrostatically conjugated at the above-mentioned pH. The solution was then vortexed for 12 h. All of the work involving immuno-complexation of the antibody–QD complex with the recombinant human IgG antigen was performed in the presence of PBS at a pH of 6.3. It was previously reported,¹⁵ as stated above, that AuQDs of varying sizes, and thus emission maxima, are able to be synthesized using this

method. However, we were unable to reproduce this finding: only AuQDs with an emission at 450 nm were able to be synthesized.

Nanoscale imaging of particles, antibody–particle conjugates, and antigen–conjugate complex

For the purpose of visualizing the structure and topography of the gold nanoparticles, Au nanoparticle–anti-human IgG polyclonal antibody conjugates, and the immuno-complex formed after the addition of the recombinant human IgG antigen, high-resolution transmission electron microscopy (HR-TEM) was utilized. All of the high-resolution TEM imaging was performed on the Philips CM 20 TEM with LaB6 electron gun and EDS (EDAX/4pi) system. The system has an accelerating voltage of 200 keV with an image point resolution of 0.278 nm, line resolution of 0.14 nm and maximum magnification of 750 000 $\times$ in the high-resolution mode. Diluted samples were drop-coated onto the surface of lacey carbon grids, allowed to dry and then introduced into the vacuum chamber.

For the purpose of topographic characterization, atomic force microscopy (AFM) measurements were conducted on the Dimension 5000 AFM microscope (Veeco Instruments Inc, Santa Barbara, CA) in tapping mode. Imaging was performed with a sharp silicon cantilever and under a moderately-slow scan, with a scanning frequency of 1.508 Hz.

Photoluminescence characterization was performed on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). Absorption measurements were performed on a Lambda 900 UV/Vis/NIR spectrometer (Perkin Elmer, Waltham, MA). Sample cells used for both measurements were composed of quartz and had an optical pathlength of 1 cm.

Results and discussion

Characterization of the AuQDs was performed by a series of spectroscopic and microscopic techniques. It has been shown that PAMAM dendrimers fluoresce;³¹ however, this fluorescence is only substantial at low pH levels.³¹ All of the work presented here has been conducted at physiologically relevant pH ranges, in this particular case at the pH of 6.3; therefore, the observed luminescence results from the AuQDs and not the dendrimer – we have not observed any luminescence at 450 nm in the absence of the AuQDs. Spectroscopic characterization of the PAMAM-stabilized AuQDs revealed narrow excitation and emission maxima at 387 and 450 nm, respectively (Fig. 2B), agreeing with the results published in the literature.^{14,15} Upon electrostatic coupling to goat-derived polyclonal anti-human IgG these maxima were unchanged.

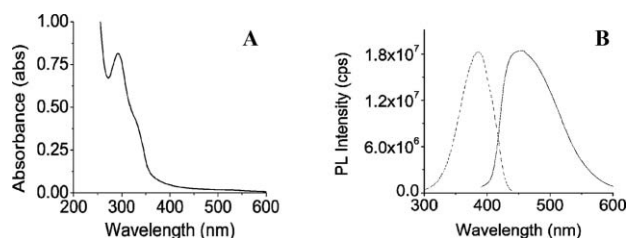


Fig. 2 (A) Absorption spectrum of AuQD; (B) excitation and emission spectra of AuQD in aqueous solution pH = 6.3 (---) excitation and (—) emission.

Absorbance measurements revealed no Mie-scattering plasmon band, which for Au is at 520 nm (Fig. 2A), indicating that the core AuQDs embedded within the dendrimer are smaller than 2 nm.^{32,33} The main absorption band at approximately 300 nm is a result of the PAMAM dendrimer.³⁴ It has been shown that AuQDs with the above-mentioned optical properties are composed of only eight gold atoms,¹⁵ and we believe that the only active fluorescence coming out of the complex is coming from that small fraction of particles. However, it must be noted that while the core AuQDs are extremely small, the dendrimer capping enlarges the system by a few nanometers allowing for microscopic investigation; the second generation PAMAM dendrimer is approximately 2.9 nm in diameter.³⁴ The size and topography of the AuQD complex including the PAMAM dendrimer stabilizer were further investigated by high-resolution transmission electron microscopy (HR-TEM) and atomic force microscopy (AFM). HR-TEM revealed an overall average complex size of 4.8 nm (sample size of 100) – this includes the core AuQDs and the second generation PAMAM dendrimer in which they are embedded. While the individual AuQDs are not visible as a result of their small size, the difference in contrast between the AuQDs and dendrimer due to vastly differing densities may be observed in Fig. 3A. While individual AuQDs are not discernable in the HR-TEM images, the contrast between the dendrimer and the Au is clearly visible. The complex is spherical in shape with AuQDs found in various localities including the center and edges of the dendrimer.

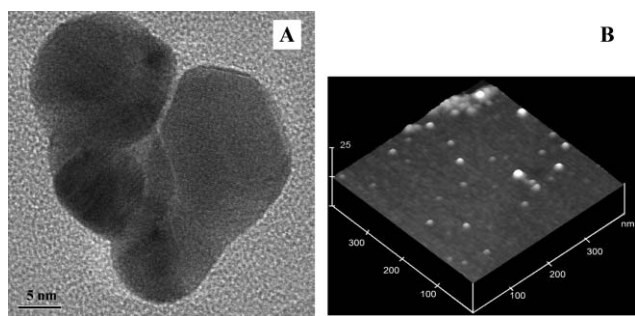


Fig. 3 (A) HR-TEM micrograph of the AuQD–antibody conjugates showing the difference in densities of the Au and PAMAM dendrimer, data bar size 5 nm; (B) AFM micrograph of the AuQD–antibody conjugates, image size 400 × 400 nm.

The AFM micrograph of the AuQD–antibody conjugates is shown in Fig. 3B. These results show that the complex is overall uniformly spherical and the size determined by measurement of the structure height is 4.9 nm, consistent with the size determined by HR-TEM, of 4.8 nm. The experimental values obtained also agree with calculations accounting for the size of the AuQD, the dendrimer, and a complex formed between these two species. In this case it is possible to discern individual AuQDs as the AFM technique allows for higher resolution imaging. The AuQDs are randomly distributed on the surface of the substrate with occasionally clustered groups. The observation of clusters could either be aggregated AuQDs or multiple AuQDs within the same dendrimer appearing as separate species.

The complex was also analyzed using Bio-attenuated total reflectance Fourier transform infrared spectroscopy (Bio-ATR

FTIR). While the dendrimer and the anti-human IgG can be identified by Bio-ATR FTIR, the AuQDs cannot. Fig. 4 presents the Bio-ATR FTIR results of reference solutions of dendrimer and anti-human IgG along with the PAMAM-encapsulated anti-human IgG-conjugated AuQD complex. The spectrum of the dendrimer (Fig. 4A) is characterized by a large amide absorption at 3590 cm⁻¹ and a band at 3385 cm⁻¹ resulting from the terminal hydroxyl groups of the dendrimer, along with an amide I band at 1610 cm⁻¹. Other less characteristic bands found in the spectrum are –NH bending at 1560 cm⁻¹ and methylene bending at 1407 cm⁻¹. In contrast, the spectrum of the anti-human IgG (Fig. 4B) is characterized by amine stretching at 3729 and 3625 cm⁻¹, hydroxyl stretching at 3150 cm⁻¹, a band at 2050 cm⁻¹ that could result from C, N, O stretching or stretching of a carbon–carbon double bond, and an amide I band at 1637 cm⁻¹. Less characteristic bands are found at 2929 and 2856 cm⁻¹ resulting from methylene stretching, 1552 cm⁻¹ resulting from –NH bending, and 1448 cm⁻¹ resulting from methylene bending. It is worth noting that the sign of the absorbance bands of the sample is related to the orientation of the sample to the crystal surface, resulting in both positive and negative bands.

Fig. 4C presents the Bio-ATR spectrum of the AuQD–PAMAM–anti-human IgG complex (dendrimer-encapsulated Au quantum dot and anti-human IgG conjugated to it). The resulting spectrum is a convolution of the Bio-ATR spectra of the PAMAM dendrimer and the anti-human IgG. An amide absorption resulting from the dendrimer can be seen at 3590 cm⁻¹, while the band resulting from the terminal hydroxyl groups of the dendrimer is at 3385 cm⁻¹; both are found in the same position as in the spectrum of the dendrimer alone. Methylene stretches can be seen at 2964, 2923, and 2854 cm⁻¹. The band at 2050 cm⁻¹ found in the spectrum of the anti-human IgG along with the amide I at 1637 cm⁻¹ are also present in the spectrum of the AuQD complex. The results of the luminescence, TEM, AFM, and IR indicate that, indeed, the AuQD is stabilized by the dendrimer and that the antigen is conjugated to the system through the specific binding of the antibody-variable chains.

The purpose of designing this complex was for the proof of the concept fluorescence-based immunoassay. As our particular implementation of such an assay, we chose detection of human IgG in aqueous solution. The mechanism of action is a well-known, direct-binding immunoassay principle, and its implementation with the AuQD–PAMAM-encapsulated reporting label, and initial results have been previously reported in a brief communication.¹⁹ Immunoglobulins may have various sites available for binding; however, in this case of human IgG there are two sites available for binding of the human IgG antigen. As can be seen from the results presented in Fig. 5, the introduction of antigen into the system results in nano-sized agglomeration, and additionally, luminescence quenching of the AuQD results. The luminescence can be effected in two ways: first, as QDs are sensitive to surface effects, the introduction of antigen and selective binding of an antibody–PAMAM dendrimer–Au QD complex with an introduced antigen such as human IgG results in interaction with the portion of the chain of the antigen anti-human IgG and the surface of the AuQD. Another possibility is that the resulting agglomeration that occurs after antigen

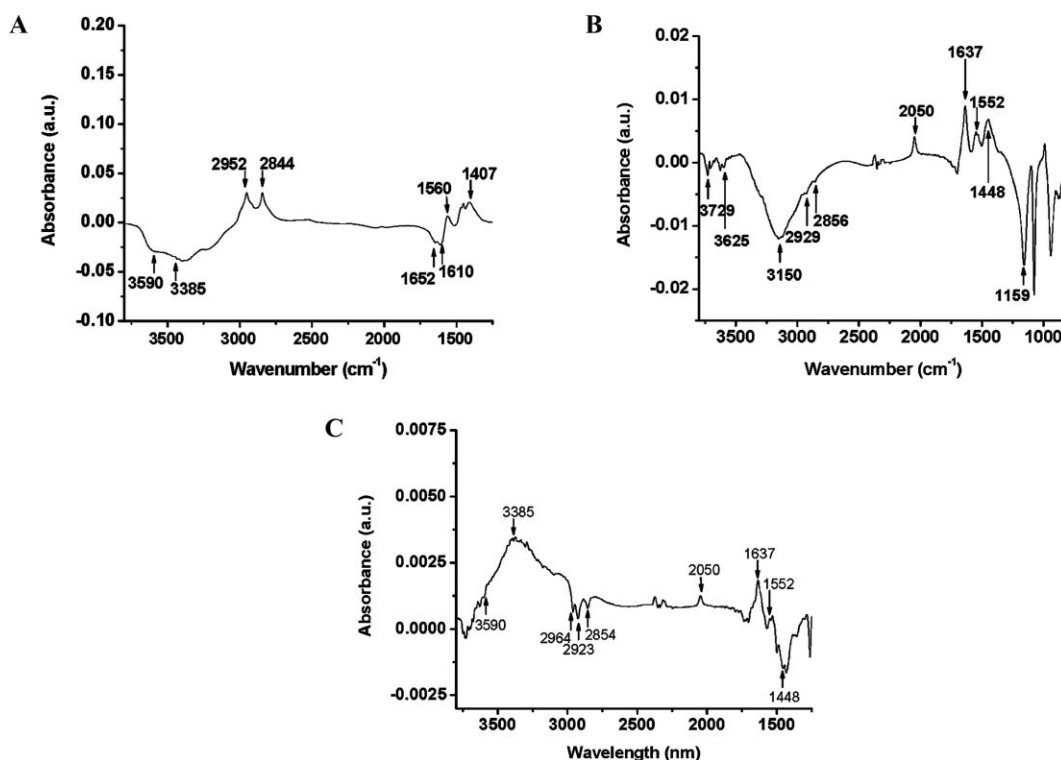


Fig. 4 Bio-ATR FTIR spectra of aqueous (A) PAMAM-OH generation 2 dendrimer; (B) anti-human IgG; (C) PAMAM-encapsulated anti-human IgG-conjugated AuQDs.

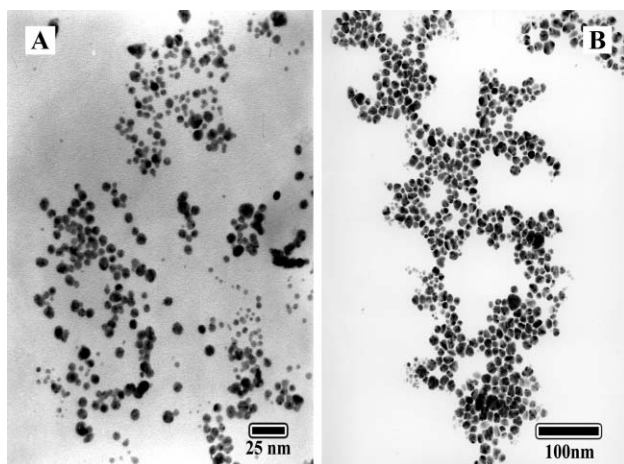


Fig. 5 (A) HR-TEM micrograph of AuQD-antibody conjugates, data bar size 25 nm; (B) HR-TEM of the formation of the complex agglomerate formed upon the addition of human IgG, data bar size 100 nm.

introduction, as visualized in Fig. 5, can act as a filter decreasing the luminescence intensity of emission.

As can be seen, prior to the addition of human IgG (Fig. 5A), the AuQD anti-human IgG complex is found virtually free of clusters. However, after the introduction of human IgG antigen (Fig. 5B), agglomeration occurs to an extent that could indeed filter out or scatter the emission of the AuQDs.

To determine the mechanism of quenching, selectivity, stability, and possible interference, various quenching studies were

performed with numerous complexes. Human and rabbit IgG were used to compare the specificity of the system towards immunoglobulins, while bovine serum albumin (BSA) and β -casein were used as model proteins that are found in high concentration in biological systems to probe sources of interference and to demonstrate assay selectivity against non-specific protein binding. As the antibody used did not show a good selectivity between rabbit and human IgG antigen, the quenching of fluorescence occurred with the addition of rabbit IgG also. However, the system does not respond to non-specific binding, as the addition of both BSA and β -casein resulted in minor luminescence quenching effects, as depicted in the Fig. 6A.

In order to determine how the various non-specific immunoglobulins and proteins interact with the surface of the AuQDs, the AuQD-PAMAM complex (without anti-human IgG) was used in the titrations, the results of which can be seen in Fig. 6. Both BSA and β -casein resulted in an increase in the luminescence intensity with increasing concentration. With increasing concentrations of the IgGs the luminescence intensity decreased; interestingly, however, they caused a decrease of the same magnitude, such that they were indistinguishable based on the quenching curves. This would indicate a strong non-specific interaction between the AuQD surface and the immunoglobulin regardless of its source. Based on the observed results, the luminescence quenching when antigen is present in the vicinity of the complex is a result of multiple antibody binding leading to agglomeration and surface quenching of the AuQDs. In the proof-of-concept case of the human IgG detection, the system is linearly sensitive and linear over a broad range from micromolar

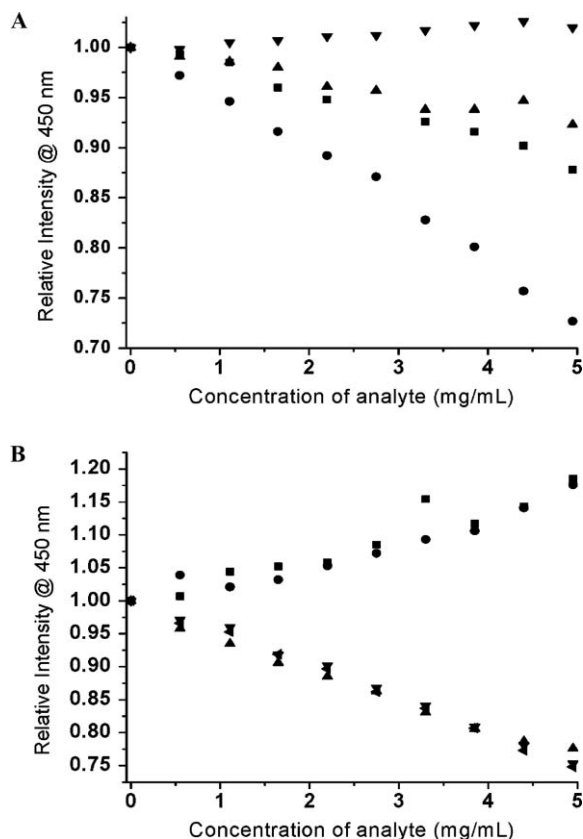


Fig. 6 Relative intensity at 450 nm of the photoluminescence versus concentration of analyte added in aqueous solution for (A) AuQD-PAMAM-anti-human IgG complex titrated with β -casein (▼), rabbit IgG (●), BSA (▲), and human IgG (■); and (B) AuQD-PAMAM complex titrated with BSA (■), β -casein (●), human IgG (▲), rabbit IgG (▼), and goat IgG (◄).

to nanomolar concentrations, as demonstrated on a dynamic curve.

The other proteins tested, namely BSA and β -casein, did not cause a large change in the fluorescence signal. BSA resulted in a minor decrease in the intensity of the luminescence, while β -casein resulted in a minor increase in the luminescence intensity, which can be attributed to some of the non-specific binding of those proteins to PAMAM or directly to gold QDs. Thereby, it is to be expected that with a higher saturation of the antibody-PAMAM-AuQD complex with antibodies, the statistically available surface of PAMAM or AuQD for non-selective binding will decrease, and the assay specificity will further increase.

Conclusions

AuQDs encapsulated within second generation PAMAM dendrimers were electrostatically conjugated to anti-human IgG for the purpose of detecting human IgG in solution. The complex formed exhibits excellent stability. A linear decrease in the luminescence of the AuQDs was found with increasing concentrations of human IgG over a micromolar to nanomolar concentration range. Neither BSA nor β -casein caused significant interference. The results teach us that the mechanism of antibody-induced selectivity in competitive surface quenching,

coupled with the inherent property of quantum dots of size-controlled fluorescence spectra, has a great promise for the use and design of a variety of ligand-specific assays for diagnostics and imaging applications.

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