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2010 Nanotechnology 21 255101

(http://iopscience.iop.org/0957-4484/21/25/255101)

Nanotechnology **21** (2010) 255101 (5pp) doi:10.1088/0957-4484/21/25/255101

Gold–silver-alloy nanoprobes for one-pot multiplex DNA detection

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Received 17 March 2010, in final form 21 April 2010
Published 28 May 2010
Online at stacks.iop.org/Nano/21/255101

Abstract
A specific colorimetric DNA detection method based on oligonucleotide functionalized gold–silver-alloy nanoparticles (AuAg-alloy-nanoprobes) is presented. The AuAg-alloy-nanoprobes were then used for the specific detection of a DNA sequence from TP53—a gene involved in cancer development. The AuAg-alloy-nanoprobes were then used in combination with Au-nanoprobes for a one-pot dual-colour detection strategy that allowed for the simultaneous differential detection of two distinct target sequences. This system poses an unprecedented opportunity to explore the combined use of metal nanoparticles with different composition towards the development of a multiplex one-pot colorimetric assay for DNA detection.

1. Introduction

Noble metal nanoparticles, in particular gold nanoparticles (AuNPs), have been extensively used for the development of new molecular diagnostic assays and biomedical applications due to their unique optical properties [1–4]. These optical properties are derived from the characteristic surface plasmon resonance (SPR) band that can be modulated—enhanced and easily tailored—through the synthesis of NPs with different sizes [5] or metal composition, either in an alloy or core–shell structure, e.g. different gold:silver ratios [6, 7].

AuNPs can be derivatized with thiol-modified ssDNA oligonucleotides harbouring a sequence of interest. These resulting Au-nanoprobes have been extensively applied in nucleic acid detection strategies [1–4, 8]. Following a non-cross-linking approach, our group has successfully presented a method for molecular detection of specific sequences [9–13], where detection is achieved by colour comparison upon salt addition, between solutions containing the Au-nanoprobe with either a complementary or a non-complementary/mismatched target sequence—the presence of a complementary target prevents aggregation and the solution remains red (SPR band centred around 526 nm); non-complementary/mismatched targets do not prevent Au-nanoprobe aggregation, resulting in a visible change of colour from red to blue (red-shift of the SPR band). The increasing ionic strength of the solution, due to the addition of salt (e.g. NaCl or $MgCl_2$), provides a screening effect to the electrostatic repulsion between NPs derived from the charges of the exposed bases of ssDNA, leading to the aggregation of nanoprobes [9–13]. Silver nanoparticles show similar optical properties to those of AuNPs but present a higher extinction coefficient [14]. However, functionalization of silver NPs with oligonucleotides constitutes an arduous task and the resulting nanoprobes present limited stability [14–17]. Increased functionalization has been attained by means of silver–gold core–shell nanoparticles, which present the advantage of ease of derivatization of the gold surface and the enhanced SPR extinction coefficient of Ag, allowing the application of the common strategies used in Au-nanoprobe assembly [18, 19]. Nonetheless, these nanoparticles require cumbersome synthesis, since the controlled deposition of gold over silver is hard to achieve due to galvanic displacement [20] and can suffer from inter-layer metal diffusion, which is potentiated by high temperatures and can alter the optical

properties of the nanoparticles [6, 21–23]. Nanoparticles in an alloy structure (AuAg-alloy-NPs), unlike their core–shell counterparts, can be more easily synthesized via a simple single-step citrate co-reduction method [24] and still exhibit remarkable functional properties: intense SPR bands as for silver NPs and easiness of thiol functionalization provided by gold. Additionally, AuAg-alloy-NPs present a single plasmon absorption band in the visible spectrum, while core–shell nanoparticles generally present a double plasmon band [21, 25, 26].

Molecular nanodiagnostics applied to cancer may provide rapid and sensitive detection of cancer related molecular alterations, which would enable early detection even when those alterations occur only in a small percentage of cells [27]. Two of the most relevant targets for cancer diagnostics are the tumour suppressor gene TP53 and c-myc oncogene that can, alone or in association, be responsible for cancer outcome [28–30]. The use of metal NPs for simple colorimetric sequence detection has been hampered by the need of multiple parallel assays to assess homo- and heterozygosity.

Herein, we report a highly selective colorimetric detection method for DNA sequences based on AuAg-alloy-NPs functionalized with thiol-modified oligonucleotides, i.e. AuAg-alloy-nanoprobes, and the use of these alloy-nanoprobes in a one-pot colorimetric assay for the simultaneous differential detection of two gene sequences. The detection approach we present here is different both conceptually and in experimental details from previous systems. First, it uses AuAg-alloy-nanoprobes, showing equivalent detection capability when compared to the single metal counterparts. Second, sensitive detection is based on the optical changes of the alloy-nanoprobe solution governed only by the presence or absence of a specific target sequence that can be easily followed by conventional ultraviolet–visible (UV–vis) spectroscopy. Third, this system poses an unprecedented opportunity to explore the combined use of these new nanoprobes with the more common Au-nanoprobes for multiple target detection in a one-pot format (i.e. multicolour–multiplexed analysis), which may circumvent the need for parallel assays to characterize homo- and/or heterozygous sequence events (e.g. gene copies, mutations and single nucleotide polymorphisms).

2. Experimental procedure

All synthesized unmodified and thiol-modified oligonucleotides were purchased from STAB Vida, Lda. (Portugal). All chemicals were from Sigma-Aldrich and of the highest purity available.

2.1. AuAg-alloy- and Au-nanoparticle syntheses

AuNPs were prepared by the citrate reduction method described by Lee and Meisel [32]. The AuAg-alloy-NPs were prepared following a similar method by co-reducing $AgNO_3$ and $HAuCl_4$ with sodium citrate. Briefly, 12.5 mg of $HAuCl_4{\cdot}3H_2O$ and 5.4 mg of $AgNO_3$ were dissolved in 250 ml of milli-Q H_2O and brought to the boil while stirring in a 500 ml round-bottom flask. Twenty-five millilitres of sodium citrate 34 mM were quickly added and the mixture was refluxed for 15 min with continuous stirring. The flask was left to cool down to room temperature and the solution was later centrifuged at 233g for 20 min to remove any silver precipitate and further filtered using 0.2 μm nylon filters (Whatman, UK). Both Au- and AuAg-alloy-NPs were characterized by transmission electron microscopy (TEM) and UV–vis spectroscopy. The elemental composition (i.e. Au:Ag ratio) of AuAg-alloy-NPs was determined by inductively coupled plasma (ICP) upon dissolving the nanoparticles in freshly prepared *aqua regia*. Solutions containing NPs were kept in the dark at room temperature until further use.

2.2. AuAg-alloy- and Au-nanoprobe syntheses

The synthesized nanoparticles were derivatized with thiol-modified ssDNA oligonucleotides following a well-established salt ageing procedure [33], using a final concentration of 0.3 M NaCl and a NP/thiolated oligonucleotide ratio of 1:34 000 and 1:150 for the AuAg-alloy- and Au-nanoprobes, respectively. Functionalization was assessed by evaluation of the UV spectra upon increasing concentrations of $MgCl_2$, and further confirmed using an oligonucleotide intercalating dye—Oligreen (Invitrogen, USA), after displacement of the immobilized thiolated oligonucleotides with 100 mM dithiothreitol (DTT).

For the AuAg-alloy-nanoprobe a sequence derived from gene TP53 (GenBank accession no. NC_000017) was used (probe sequence X: 5′-thiol-$(CH_2)_6$-GCGCACAG AGGAAGAGAATC); for the Au-nanoprobe a sequence derived from gene MYC (GenBank accession no. NG_007161) was used (probe sequence Y: 5′-thiol-$(CH_2)_6$-GATTGCTCAGGACATTTCTG).

2.3. DNA detection assay

DNA detection was carried out via the non-cross-linking method following a well-established protocol [9–13]. Hybridizations were carried out in 10 mM phosphate buffer (pH 8), 0.1 M NaCl by mixing up to a 200 nM target with either a 1.5 nM Au-nanoprobe or a 19 pM AuAg-alloy-nanoprobe. Forty-mer ssDNA oligonucleotides were used as targets: complementary to the AuAg-alloy-nanoprobe—target X′: 5′AGG CTCCCCTTTCTTGCGGAGATTCTCTTCCTCTGTGCG3′, derived from the TP53 gene; complementary to the Au-nanoprobe—target Y′: 5′AGGAAAACGATTCCTTCTAACA GAAATGTCCTGAGCAATC3′, derived from the MYC gene; unrelated sequence, non-complementary to both nanoprobes—target Z′: 5′TGGATTTAAGCAGAGTTCAAAAGCCCTTCA GCGGCCAGTA3′. For each probe assay, three solutions were prepared: one with the complementary target (Comp), one with the unrelated sequence (non-comp) and a blank to confirm aggregation. Following 10 min of denaturation at 95 °C, solutions were allowed to stand for 30 min at room temperature and then $MgCl_2$ was added to a final concentration of 35 mM. UV–vis spectra of solutions were registered 15 min after salt addition using a UV–vis Spectrophotometer Cary

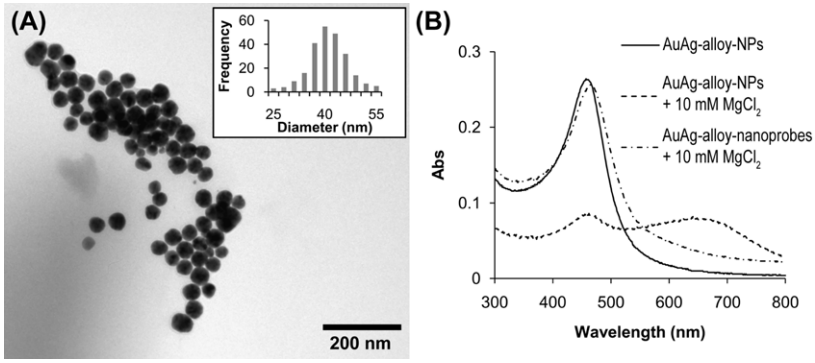

Figure 1. AuAg-alloy-NPs and AuAg-alloy-nanoprobe characterization. (A) TEM image and size histogram (inset) of AuAg-alloy-NPs determined from the analysis of >100 NPs. (B) UV–visible absorption spectrum of AuAg-alloy-NPs alone (black full line) and AuAg-alloy-NPs (black broken line) or AuAg-alloy-nanoprobes (black chain line) in the presence of 10 mM $MgCl_2$.

50 (Varian, USA) and an Ultra-Micro quartz cell (Hëllma, Germany). Digital photographs were also taken using a Canon 450D SLR digital camera (Canon, USA).

For the multiplex assay, both nanoprobes were mixed—19 pM AuAg-alloy-nanoprobe and 2.5 nM Au-nanoprobe—and the assay carried out as above using either complementary target sequences, or just the unrelated sequence. The final total DNA concentration was kept unaltered in all assays, as the specific target was substituted by an equal amount of the unrelated target sequence.

3. Results and discussion

Metal nanoparticles that differ in size and composition can be designed to scatter light of different wavelengths according to their distinct surface plasmon resonances [5, 7, 24]. The optical properties from noble metal nanoparticles with different sizes, shapes and composition has been extensively explored in biomolecular detection [1–4, 8], but these different nanoparticle labels have never been combined to develop multicolour detection platforms for homogeneous multiplex nucleic acid detection in a one-pot reaction. The synthesis of AuAg-alloy-NPs (50%Au:50%Ag) allowed us to modulate the characteristic SPR band of AgNPs, while retaining the ease of functionalization via thiol-modified oligonucleotides conferred by the gold surface. These nanoprobes could then be used to specifically detect a sequence from the TP53 gene, commonly associated with cancer development [28].

The synthesis of water soluble AuAg-alloy-NPs with different molar ratios that can be adjusted to tune for the desired SPR band has been described [24]. However, previous attempts on surface modification of ∼20 nm AuAg-alloy-NPs (16% Au:84% Ag) with thiol-modified oligonucleotides have not been able to deliver stable conjugates that could be used for biodetection [18]. To overcome this difficulty of functionalization, we hypothesized that an increase of the Au proportion and reduction of the citrate/metal ratio could slightly enlarge the resulting NPs while increasing the gold availability at the surface and thus the available surface for easy derivatization with the thiol-modified oligonucleotides. This approach yielded stable AuAg-alloy-NPs with an average

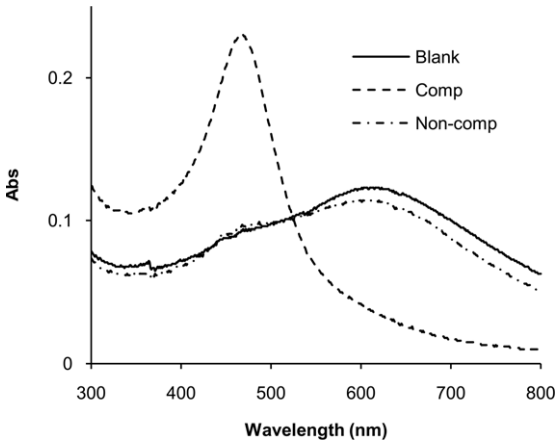

Figure 2. DNA detection with AuAg-alloy-nanoprobes. UV–vis absorbance spectrum of AuAg-alloy-nanoprobes in the absence (blank; black full line) or presence of complementary (comp; black broken line) and non-complementary (non-comp; black chain line) targets, after salt addition (final concentration 35 mM $MgCl_2$).

diameter (±STD) of 42.1 ± 5.7 nm and a Au:Ag molar ratio of 47:53 (mol%), with a single SPR band indicating formation of alloy NPs—see figures 1(A) and (B) [21, 25, 26]. Functionalization of the AuAg-alloy-NPs with thiol-modified oligonucleotides was achieved via a similar procedure to that described for Au-nanoprobe synthesis. The resulting AuAg-alloy-nanoprobes (90.3 ± 0.3 pmol nm^{-2} of thiol-modified oligonucleotides per AuAg-alloy-NP) presented an increased stability to salt induced aggregation when compared to the AuAg-alloy-NPs alone, withstanding a saline concentration up to 20 mM $MgCl_2$. The fact that the resistance to salt induced aggregation has increased, even after heating in a buffer solution, constitutes an indication of the success of functionalization (figure 1(B)). Additionally, upon derivatization with thiol-modified oligonucleotides, the initial SPR peak of AuAg-alloy-NPs shifted from 462 to ∼470 nm, analogous to what has been observed for pure AuNP functionalization [9]. Upon AuAg-alloy-NP aggregation, the initial faint yellow colour changes to blue due to a red-shift from 462 to >600 nm of the SPR absorbance band.

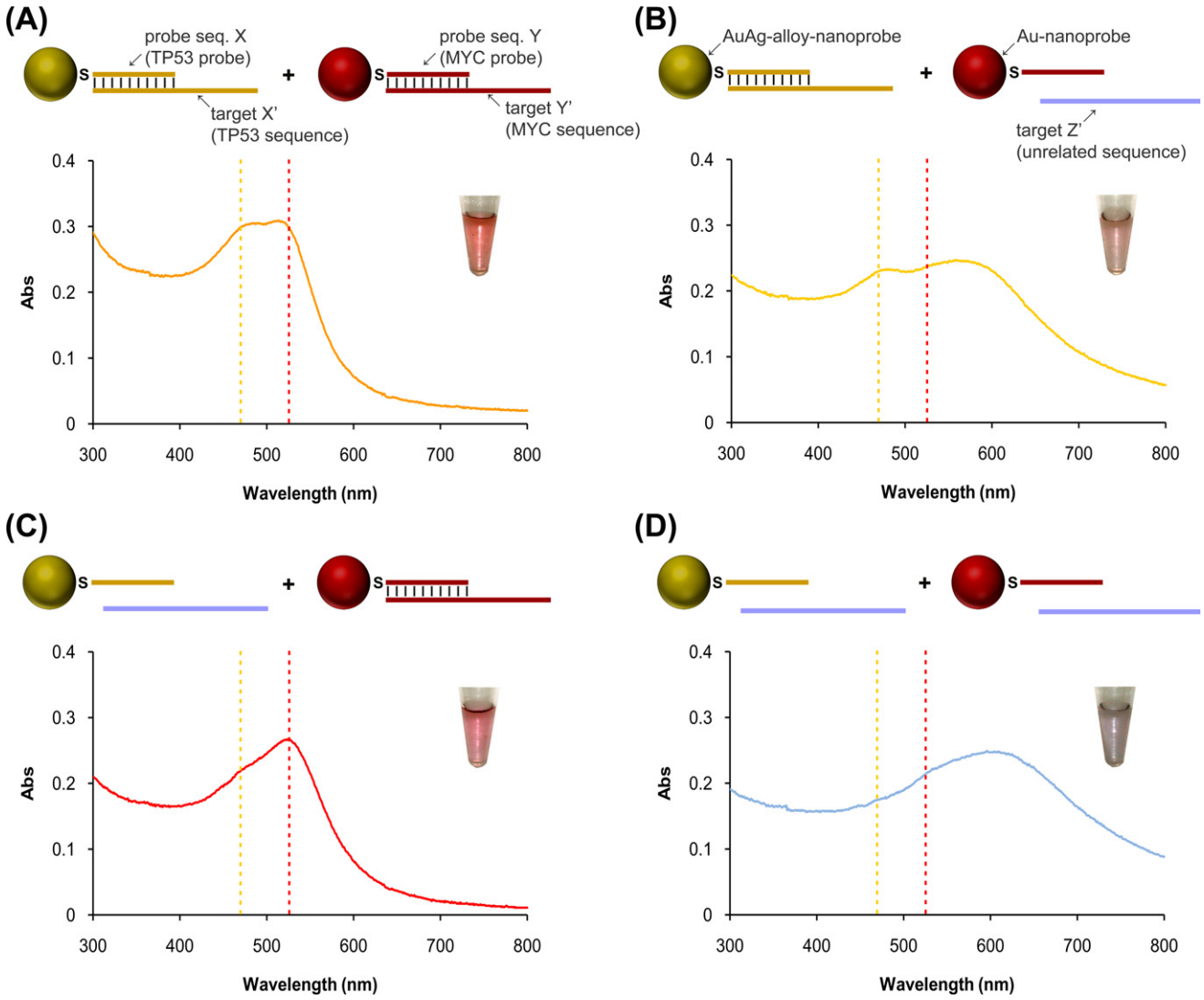

Figure 3. One-pot colorimetric multi-target detection. UV–visible spectrum and digital photography of AuAg-alloy- and Au-nanoprobe mix in the presence of: (A) complementary targets to both the AuAg-alloy- and Au-nanoprobe; (B) a complementary target to the AuAg-alloy-nanoprobe; (C) a complementary target to the Au-nanoprobe; (D) a non-complementary target to both AuAg-alloy- and Au-nanoprobes. Vertical dashed lines represent the absorption peaks of AuAg-alloy-nanoprobes (orange broken line; 470 nm) and Au-nanoprobes (red broken line; 526 nm) when dispersed in solution.

To test the nucleic acid detection capability, the AuAg-alloy-nanoprobes were used in hybridization reactions following a non-cross-linking approach previously described for Au-nanoprobes [9] where, after salt addition, the presence of a complementary target to the nanoprobe sequence prevents aggregation. The AuAg-alloy-nanoprobes allowed for the detection of a fully complementary target at a concentration as low as 12.5 nM, i.e. 750 fmol of target per assay, and the solution retained its initial yellow colour with an SPR absorption band peak located at 470 nm (figure 2). In the absence of any target or in the presence of a non-complementary target, the solution changed colour with a concomitant shift in the SPR absorption band from 470 to ∼600 nm. AuAg-alloy-nanoprobes presented comparable target sensitivity to Au-nanoprobes. These results show that AuAg-alloy- and pure Au-nanoprobes present near identical colorimetric properties as labels for hybridization-based DNA detection, but account for different colour changes. This equivalent detection capacity is a prerequisite for using AuAg-alloy- and Au-nanoprobes for the simultaneous DNA detection in a homogeneous one-pot reaction.

To test the potential of this AuAg-alloy/Au-nanoprobe dual system for one-pot detection of two different DNA targets, a mixture of 19 pM AuAg-alloy-nanoprobes (TP53 probe sequence X), 1.5 nM Au-nanoprobes (MYC probe sequence Y) and a final concentration of 200 nM ssDNA target oligonucleotide were used for hybridization and detection. The targets included ssDNA oligonucleotides harbouring a sequence complementary to either the AuAg-alloy- (target X′ harbouring the TP53 sequence) or the Au-nanoprobe (target Y′ harbouring the MYC sequence), or non-complementary (target Z′) to both nanoprobes. In the presence of both targets X′ and Y′, the two different nanoparticle probes were successfully and independently hybridized to the corresponding complementary

targets and did not aggregate upon salt addition, retaining the initial orange colour solution (figure 3(A)). In the presence of only target Y′ or X′, only the AuAg-alloy- or the Au-nanoprobe aggregated, correspondingly, as can be seen by the respective spectral and colour change in figure 3(B) (solution exhibiting a yellow colour) and C (solution exhibiting a red colour). The presence of only the non-complementary target Z′ resulted in extensive aggregation for both types of nanoprobes and a concomitant colour change from orange to blue (figure 3(D)).

These results show that one can easily distinguish either target according to the different spectra composition. In addition, the fact that the assay is based on the non-cross-linking mechanism [9–13] allows differential target identification to be possible in a mixture of targets, i.e. simultaneous parallel detection based on the colour changes in a one-pot reaction. This capability is crucial for the detection of two allelic variants present in an individual, i.e. homo- versus heterozygous, of extreme relevance in tumour suppressor genes, such as in TP53 [28], oncogene (e.g. c-myc) [29] and others where allelic differences modulate the phenotype (e.g. CYP450 [31]).

4. Conclusions

We have demonstrated the functionalization of AuAg-alloy-NPs with ssDNA and the use of the resulting alloy-nanoprobes for the specific detection of a target of interest. The attained AuAg-alloy-nanoprobes show a combination of the optical properties of Ag nanoparticles (high extinction coefficient) with the ease of functionalization via a thiol bond provided by the gold. This approach circumvents the issues related to the functionalization of Au and Ag composite nanoparticles, while retaining the relevant properties for DNA detection.

Based on the combination of AuAg-alloy- and Au-nanoprobes, it was possible to develop a sensitive, selective, one-pot multicolour method for the simultaneous DNA detection of multiple targets in a single reaction. The simultaneous differential analysis of two sequences is of utmost importance when performing genome based screening, where there is a mandatory need to assess whether a given genotype is present in homo- or heterozygous form. Also, exploitation of the quantitative potential of nanoparticle-based strategies [12], e.g. gene expression studies, where quantitative analysis of one transcript is often performed in relation to a reference gene, could profit from this multicolour system.

This approach should be extendable to additional colours using nanoparticles of different sizes and compositions, i.e. Au:Ag ratios, and with higher scattering coefficients presented by alloy composites may provide a multicolour homogeneous system for application in array strategies and to rival fluorescence based assays.

Acknowledgments

This work was supported by FCT/MCTES (CIGMH, REQUIMTE and grants PTDC/SAU-BEB/66511/2006 and PTDC/QUI/64484/2006). G Doria was supported by FCT/MCTES (SFRH/BDE/15544/2005) and STAB Vida, Lda.

References

[1] Boisselier E and Astruc D 2009 *Chem. Soc. Rev.* **38** 1759–82
[2] Radwan S H and Azzazy H M 2009 *Expert Rev. Mol. Diagn.* **9** 511–24
[3] Shim S Y, Lim D K and Nam J M 2008 *Nanomedicine* **3** 215–32
[4] Huang X, Jain P K, El-Sayed I H and El-Sayed M A 2007 *Nanomedicine* **2** 681–93
[5] Link S and El-Sayed M A 1999 *J. Phys. Chem.* B **103** 4212–7
[6] Wilcoxon J 2009 *J. Phys. Chem.* B **113** 2647–56
[7] Liz-Marzan L M 2006 *Langmuir* **22** 32–41
[8] Baptista P, Pereira E, Eaton P, Doria G, Miranda A, Gomes I, Quaresma P and Franco R 2008 *Anal. Bioanal. Chem.* **391** 943–50
[9] Baptista P, Doria G, Henriques D, Pereira E and Franco R 2005 *J. Biotechnol.* **119** 111–7
[10] Baptista P V, Koziol-Montewka M, Paluch-Oles J, Doria G and Franco R 2006 *Clin. Chem.* **52** 1433–4
[11] Doria G, Franco R and Baptista P 2007 *IET Nanobiotechnol.* **1** 53–7
[12] Conde J, de la Fuente J M and Baptista P V 2010 *J. Nanobiotechnol.* **8** 5
[13] Costa P, Amaro A, Botelho A, Inácio J and Baptista P V 2010 *Clin. Microbiol. Infect* doi:10.1111/j.1469-0691.2009.03120.x
[14] Thompson D G, Enright A, Faulds K, Smith E W and Graham D 2008 *Anal. Chem.* **80** 2805–10
[15] Tokareva I and Hutter E 2004 *J. Am. Chem. Soc.* **126** 15784–9
[16] Vidal B C Jr, Deivaraj T C, Yang J, Too H, Chow G, Gan L M and Lee J Y 2005 *New J. Chem.* **29** 812–6
[17] Lee J-S, Lytton-Jean A K R, Hurst S J and Mirkin C A 2007 *Nano Lett.* **7** 2112–5
[18] Cao Y, Jin R and Mirkin C A 2001 *J. Am. Chem. Soc.* **123** 7961–2
[19] Cao Y C, Jin R, Thaxton C S and Mirkin C A 2005 *Talanta* **67** 449–55
[20] Ung T, Liz-Marzan L M and Mulvaney P 1998 *Langmuir* **14** 3740–8
[21] Mulvaney P, Giersig M and Henglein A 1993 *J. Phys. Chem.* **97** 7061–4
[22] Hodak J H, Henglein A, Giersig M and Hartland G V 2000 *J. Phys. Chem.* B **104** 11708–18
[23] Abid J-P, Girault H H and Brevet P F 2001 *Chem. Commun.* 829–30
[24] Link S, Wang Z L and El-Sayed M A 1999 *J. Phys. Chem.* B **103** 3529–33
[25] Sinzig J, Radtke U, Quinten M and Kreibig U 1993 *Z. Phys.* D **26** 242
[26] Mulvaney P 1996 *Langmuir* **12** 788–800
[27] Farrell D, Alper J, Ptak K, Panaro N J, Grodzinski P and Barker A D 2010 *ACS Nano* **4** 589–94
[28] Whibley C, Pharoah P D and Hollstein M 2009 *Nat. Rev. Cancer* **9** 95–107
[29] Meyer N and Penn L Z 2008 *Nat. Rev. Cancer* **8** 976–90
[30] Ho J S, Ma W, Mao D Y and Benchimol S 2005 *Mol. Cell. Biol.* **25** 7423–31
[31] Rodriguez-Antona C and Ingelman-Sundberg M 2006 *Oncogene* **25** 1679–91
[32] Lee P C and Meisel D 1982 *J. Phys. Chem.* **86** 3391–5
[33] Hurst S J, Lytton-Jean A K R and Mirkin C A 2006 *Anal. Chem.* **78** 8313–8